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Forensic MicroRNA Analysis of Body Fluids Relating to Sexual Assaults

Kimberley Jane Bexon MSc ACSFS



*A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for
the degree of Doctor of Philosophy*

The University of Huddersfield
Department of Biological Sciences

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Table of Contents

Copyright Statement.....	2
Table of Contents	3
List of Tables.....	9
List of Figures.....	10
List of Case Studies	14
Dedications and Acknowledgments.....	15
Abstract.....	16
List of abbreviations	17
1. Sexual Assaults and Offences	20
1.1. Sexual Assault	21
1.1.1. High profile reports into sexual assault recording.....	26
1.1.2. Large scale investigations into historical allegations of sexual abuse	26
1.1.3. Creation of independent inquiries for sexual abuse	27
1.2. Vulnerable and High Risk Groups	28
1.3. Common Perpetrators of Sexual Assault.....	30
1.4. Prosecution and Defence of a Sexual Assault.....	32
1.5. Under-reporting and low convictions rate	33
1.5.1. Reporting rates within the UK.....	34
1.5.2. Increases in reporting rates.....	36
1.5.3. Conviction rates within the UK.....	38
1.6. Rape Myths	38
1.7. Sexual Assault Forensic Medical Examination	40
1.8. The Aftermath of Sexual Assault	41
1.8.1. Physical trauma and medical health.....	41
1.8.2. Emotional and psychological trauma.....	42
1.8.3. Social stigma	42
1.8.4. Re-victimisation	43

1.8.5. Crime reported effects of serious sexual assault	43
1.9. Consent.....	43
1.9.1. Verbalised non-consent	45
1.9.2. Automatically void consent.....	46
1.9.3. Age of consent	48
1.9.4. Revoked consent.....	50
1.9.5. Drunken consent.....	51
1.9.6. Conditional consent.....	53
1.10. Drug Related Sexual Assaults.....	54
1.11. Domestic Sexual Assaults.....	57
1.12. Sexual Fantasies of Rape.....	58
1.13. False and Baseless Sexual Assault Reports	60
1.14. Summary of Sexual Assault.....	64
2. Forensic Applications for Body Fluid Identification	66
2.1. Forensic Scientists	67
2.2. Components of Body Fluids Often Located at Crime Scenes	67
2.2.1. Whole blood.....	67
2.2.2. Vaginal material.....	69
2.2.3. Menstrual blood	69
2.2.4. Saliva.....	76
2.2.5. Semen and seminal fluid	77
2.3. Current Body Fluid Identification Techniques.....	81
2.3.1. Catalytic presumptive tests for body fluid identification	82
2.3.2. Protein analysis/ Immunological techniques for confirmatory body fluid identification	84
2.4. Limitations of Current Body Fluid Identification Techniques.....	85
2.4.1. Absence of semen.....	85
2.4.2. Menstrual blood vs. venous blood	86

2.4.3. Differentiating vaginal material from saliva.....	87
2.5. Current and Emerging Molecular Based Techniques for Body Fluid Identification...	87
2.5.1. Analysis of DNA methylation patterns.....	88
2.5.2. Messenger RNA.....	91
2.5.3. MicroRNA:	94
2.5.4. Advantages of miRNA analysis	97
2.5.5. Comparison of current mRNA and miRNA techniques	98
2.5.6. miRNA Suitability for Identifying Body Fluid Components.....	102
2.5.7. Marker Selection	107
2.6. Summary of Current and Emerging Body Fluid Identification Techniques.....	111
3. Research Aims and Objectives	113
3.1. Aims and Objectives.....	114
4. Methods and Materials	117
4.1. Ethics Statement.....	118
4.2. General Reagents and Consumables.....	118
4.3. Volunteer Selection and Sample Collection.....	118
4.3.1. Vaginal secretions	119
4.3.2. Menstrual blood	119
4.3.3. Semen and seminal fluid	119
4.3.4. Blood and saliva.....	120
4.3.5. Skin.....	120
4.4. Nucleotide Extraction	120
4.4.1. Standard total DNA extraction	120
4.4.2. Total RNA extraction	121
4.4.3. Differential extraction.....	122
4.5. Quantification of Samples	124
4.5.1. DNA quantification utilising absolute qPCR	125

4.5.2. RNA quantification using a UV-visible spectrophotometer	127
4.6. Determination of Semen through Haematoxylin & Eosin Staining.....	129
4.7. Stem-loop Reverse Transcription alongside qPCR for miRNA Analysis	130
4.7.1. Hydrolysis probe and Oligonucleotide RT and PCR primer synthesis	131
4.8. Stem-loop Reverse Transcription.....	136
4.9. miRNA Expression through Relative qPCR.....	137
4.9.1. Negative controls	139
4.10. Analysis.....	139
4.10.1. Normalisation.....	141
4.11. Data Representation and Statistical Analysis	141
4.12. MIQE Guideline Compliance	142
4.13. Experimental Modification to Standard Protocols	142
4.13.1. Identification of miRNA markers.....	142
4.13.2. Characterisation of markers over a 31-day period	142
4.13.3. Detection of semen markers within vaginal material following unprotected sexual intercourse	142
5. The classification of MicroRNA for Body Fluid Identification	144
5.1. Rationale.....	145
5.2. Sample Collection	146
5.3. Results	147
5.3.1. Expression comparison of TaqMan® and custom assays in undiluted sample..	147
5.3.2. Limits of Detection.....	156
5.3.3. Identification of body fluid specific markers and endogenous controls.....	158
5.4. Conclusion	177
5.5. Summary	177
6. Characterisation of Markers over a 31-Day Period	179
6.1. Rationale.....	180
6.1.1. Sample Collection	181
6.1.2. Marker selection	183

6.2. Results	184
6.2.1. Proposed Endogenous Controls	184
6.2.2. Body Fluid Specific miRNA	189
6.2.3. DNA v RNA Extraction Methods	211
6.3. Conclusion	217
6.4. Summary	218
7. Detection of Semen Markers within Vaginal Material Following Unprotected Sexual Intercourse	220
7.1. Rationale.....	221
7.2. Experimental Design	223
7.3. Results	226
7.3.1. Identification of Background Expression of Specific Markers.	226
7.3.2. Identification of Proposed Semen Markers within Vaginal Material Samples across Various Extraction Methods.	227
7.3.3. DTT Addition to Total DNA Extraction.	231
7.3.4. Discussion	233
7.4. Conclusion	236
7.5. Summary	237
8. Discussion	239
8.1. Total DNA extraction coupled with stem-loop RT-qPCR as a robust and efficient method for body fluid identification	241
8.2. MicroRNA expression fluctuates across the menstrual cycle, with the exception of hsa-mir-124	247
8.3. The Technique is capable of detecting instances of UPSI from vaginal swabs up to 96 hours after penetration following standard DNA extraction.	249
8.4. The expression of hsa-mir-205 within vaginal material is regulated by contraceptive methods	250
8.5. Applications to case work.....	250
8.6. Comparisons of miRNA and mRNA for BFID.....	254
8.7. Future Perspectives	255
8.8. Concluding Remarks	256

9. References.....	258
10. Appendix.....	277
A) Female Sampling Questionnaire	278
B) Male Sampling Questionnaire.....	280
C) Full Primer Sequences	281
D) R - Heat Map Code	283
E) R - Box and Whisker Plot Code	284
F) Volunteer Information	285
G) Limit of Detection of hsa-mir-888 in Vaginal Material.....	288

Word Count: 61,433

List of Tables

Table 1.1: Results of self-completion module completed by female victims (aged 16-59) of a serious assault.	31
Table 1.2: Proportion of victims of sexual assault by rape or penetration (including attempts) while under the age of 16 informed anyone about the abuse at the time, by disability, year ending March 2016 [25]	36
Table 2.1: Persistency of semen found from various intimate swabs [110].....	80
Table 4.1: Dilution series for creation of standards for quantifying vaginal material.	126
Table 4.2: Primer sequences utilised within screening study.	135
Table 4.3: Categories for levels of expression observed throughout analysis by all qPCR experiments	140
Table 4.4: Various mixing ratios of body fluids containing semen samples treated and untreated with DTT.	143
Table 5.1: The average DNA concentrations (ng/µl) for all tested body fluids observed via qPCR.	147
Table 6.1: Volunteer provided information on menstrual cycle, contraceptive, age and ethnicity.....	182
Table 6.2: Markers selected for analysis over a 31-day period.....	183
Table 7.1: Samples information of UPSI volunteers, including contraception, days without UPSI and OI, and if samples were provided for previous studies.....	224
Table 8.1: List of conditions and factors for RT-qPCR analysis with equivalent advantages and disadvantages for each technique.	254

List of Figures

Figure 1.1: Trends in victims of serious and standard sexual assaults from 2002 to 2013 observed as a percentage of total sample base.	24
Figure 1.2: Volume of sexual offences reported and recorded to 44 police forces over the financial year of April – March, recorded from 2002 to 2016.	25
Figure 1.3: Proportion of rape offences, categorised by age and sex of the victim.....	29
Figure 1.4: Proportion of sexual assault offences, by age and sex of the victim.....	29
Figure 1.5: Percentage of female victim based sexual assaults, categorised by severity and relationship to victim [19].	31
Figure 1.6: Prevalence of sexual assaults against males and females with severe mental illness.....	47
Figure 1.7: Prevalence of sexual assault cases involving alcohol [18].	55
Figure 1.8: Breakdown of reported domestic abuse cases occurring from April 2015 to March 2016.	58
Figure 2.1: Mechanisms and hormonal fluctuations over a standard menstrual cycle in a healthy female [88].....	72
Figure 2.2: mRNA work flow protocol for mRNA expression analysis and DNA profiling. Adapted from [143].....	92
Figure 2.3: The pathways of miRNA within the cell created from [159, 161-164].	96
Figure 4.1 Stages of DNA extraction following the QIAamp DNA Mini Kit protocol [202].	121
Figure 4.2 Processes involved in total RNA extraction	122
Figure 4.3 A micrograph of a typical semen stain using H&E (1) [215] and a micrograph displaying the complete absence of spermatozoa in the vasectomised sample	130
Figure 4.4: Sequences incorporated into the custom-built stem-loop primer.	132
Figure 4.5: Process of stem-loop reverse transcription.	136
Figure 4.6: Processes involved within each cycle of qPCR.....	138
Figure 5.1: Venn diagram displaying the number of volunteers who provided multiple body fluid samples.....	146

Figure 5.2: Expression of Applied Biosystems TaqMan® assays, measured using $\Delta^1\text{Cq}$ ($\text{Cq}_{\text{max}} - \text{Cq}_{\text{target}}$) in all body fluids.....	148
Figure 5.3: Expression of custom markers, measured using $\Delta^1\text{Cq}$ in all body fluids.....	149
Figure 5.4: Expression of custom markers, measured using Δ^1 in all body fluids.	150
Figure 5.5: Expression of custom markers, measured using $\Delta^1\text{Cq}$ in all body fluids.....	151
Figure 5.6: Individual $\Delta^1\text{Cq}$ ($\text{max Cq} - (\Delta\text{Cq of target})$) value of each body fluid	153
Figure 5.7: $\Delta^1\text{Cq}$ values ($\text{max Ct} - \text{marker Ct}$) + SEM of hsa-mir-124 within vaginal material samples across serial dilutions.....	156
Figure 5.8: $\Delta^1\text{Cq}$ values ($\text{max Ct} - \text{marker Ct}$) + SEM of SNORD 47 within vaginal material samples across serial dilutions.....	157
Figure 5.9: Expression of custom made miRNA and SNORD markers, measured using $\Delta^1\text{Cq}$ ($\text{Max Cq} - \text{Cq of marker}$) in all body fluids.	159
Figure 5.10: Expression of custom made miRNA markers, measured using $\Delta^1\text{Cq}$ in all body fluids..	160
Figure 5.11: Expression of custom made miRNA markers, measured using $\Delta^1\text{Cq}$ ($\text{Max Cq} - \text{Cq of marker}$) in all body fluids..	161
Figure 5.12: Expression of custom made miRNA markers, measured using $\Delta^1\text{Cq}$ ($\text{Max Cq} - \text{Cq of marker}$) in all body fluids.	162
Figure 5.13: $\Delta^1\text{Cq}$ values of custom assays across all body fluids.	163
Figure 5.14: $\Delta^1\text{Cq}$ values of custom assays across all body fluids.	164
Figure 5.15: $\Delta^1\text{Cq}$ values of custom assays across all body fluids.	165
Figure 5.16: $\Delta^1\text{Cq}$ values of custom assays across all body fluids.	166
Figure 5.17: $\Delta^1\text{Cq}$ values of custom assays across all body fluids.	167
Figure 5.18: Individual ΔCq value of each body fluid sample across all markers used in the screening of the 0.7ng/ μl samples..	169
Figure 5.19: Individual ΔCq value of each body fluid sample across all markers used in the screening of the 0.7ng/ μl samples.	171
Figure 5.20: Individual $\Delta^1\text{Cq}$ value of each body fluid sample across all endogenous control markers used in the screening of the 0.7ng/ μl samples..	172

Figure 5.21: Expression of hsa-mir-205 observed in vaginal material samples, categorised by contraceptive method.....	176
Figure 6.1: $\Delta^1\text{Cq}$ + SEM for proposed endogenous controls hsa-mir-93 (DNA (A) RNA (B)) and hsa-mir-508 (DNA (C) RNA (D)) of all volunteers across 31 days.	184
Figure 6.2: $\Delta^1\text{Cq}$ + SEM for proposed endogenous controls SNORD 47 (DNA (A) RNA (B)) and hsa-mir-1260b (DNA (C) RNA (D)) of all volunteers across 31 days.	185
Figure 6.3: $\Delta^1\text{Cq}$ + SEM of the average or all endogenous controls tested within vaginal material over 31 days, following DNA and RNA extraction..	186
Figure 6.4: Differences in expression within a hypothetical sampler when utilising a good and poor collection technique..	188
Figure 6.5: $\Delta^1\text{Cq}$ + SEM of hsa-mir-124 in vaginal material over 31 days, following DNA (A) and RNA extraction (B).	190
Figure 6.6: $\Delta^1\text{Cq}$ + SEM of hsa-mir-205 in vaginal material over 31 days, following DNA (A) and RNA extraction (B).	192
Figure 6.7: $\Delta^1\text{Cq}$ + SEM of hsa-mir-203a in vaginal material over 31 days, following DNA (A) and RNA extraction (B).	194
Figure 6.8: $\Delta^1\text{Cq}$ + SEM of hsa-mir-412 in vaginal material over 31 days, following DNA (A) and RNA extraction (B).	196
Figure 6.9: $\Delta^1\text{Cq}$ + SEM of hsa-mir-507 in vaginal material over 31 days, following DNA (A) and RNA extraction (B).	198
Figure 6.10: $\Delta^1\text{Cq}$ + SEM of hsa-mir-144-5 in vaginal material over 31 days, following DNA and RNA extraction.....	200
Figure 6.11: $\Delta^1\text{Cq}$ + SEM of hsa-mir-142 in vaginal material over 31 days, following DNA and RNA extraction.	202
Figure 6.12: $\Delta^1\text{Cq}$ + SEM of hsa-mir-144-3 in vaginal material over 31 days, following DNA (A) and RNA (B) extraction.....	204
Figure 6.13: $\Delta^1\text{Cq}$ + SEM of proposed semen markers hsa-mir-10a (DNA (A) RNA (B), 135a (DNA (C) RNA (D) and 888 (DNA (E) RNA (F) in vaginal material over 31 days.....	206
Figure 6.14 : $\Delta^1\text{Cq}$ + SEM of hsa-mir-205 in two volunteer's vaginal material samples.	209

Figure 6.15 Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers SNORD 47, hsa-mir-508, hsa-mir-93 and hsa-mir-1260b.	211
Figure 6.16: Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers hsa-mir-124, hsa-mir-144-3, hsa-mir-412, hsa-mir—142 and hsa-mir-144-5.	213
Figure 6.17: Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers hsa-mir-10a, hsa-mir-135a, hsa-mir-507, hsa-mir—888, hsa-mir-203a and hsa-mir- 205.	216
Figure 7.1: $\Delta^1 Cq$ values of expression of endogenous control and semen markers within vaginal material samples before UPSI occurred.	226
Figure 7.2: $\Delta^1 Cq$ values for markers hsa-mir-888 (A) and endogenous control SNORD 47 (B) within UPSI samples across various time points using different extraction methods.....	229
Figure 7.3: $\Delta^1 Cq$ values for markers hsa-mir-135a (C) and hsa-mir-10a (D) within UPSI samples across various time points using different extraction methods.	230
Figure 7.4 $\Delta^1 Cq$ values of hsa-mir-10a across various mixing ratios in samples with and without DTT. N= 6 (P<0.001).	231
Figure 7.5: $\Delta^1 Cq$ values of hsa-mir-888 across various mixing ratios of vaginal material, semen and venous blood in samples with and without DTT.....	232
Figure 7.6: proposed additional workflow stage for differential extraction.	235
Figure 8.1: flow chart for the Identification of a full range of body fluids.....	243
Figure 8.2: Example flow chart for the identification of a range of body fluids when present in a two body fluid mixture.....	246
Figure 8.3: Example flow chart for the identification of three body fluid mixtures in crime scene stains.	247
Figure 10.1: $\Delta^1 Cq$ values + SEM obtained from hsa-mir-888 within six vaginal material samples.....	288

List of Case Studies

Case Study 1-1: Pandora's survivors stories 2015:.....	45
Case Study 1-2: Supreme Court Case ID: UKSC 2016/0083:	49
Case Study 1-3: March 23, 2000:.....	50
Case Study 1-4: R v Bree [2007]:	51
Case Study 1-5 R. v. Dougal, 2005:.....	52
Case Study 1-6: EWHC 945, 2013:	53
Case Study 1-7: B-Heard survivor's story: 55	
Case Study 1-8: R v Weller [2010] EWCA 1085:	56
Case Study 1-9: DPP v Morgan (1976).	59
Case Study 1-10:	60
Case Study 1-11:	62
Case Study 1-12:	62
Case Study 1-13: R v Ashley [2010] EWCA Crim 2913:	63
Case study 2-1: LGC reporting officer 2015:.....	86

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Abstract

DNA profiling has become a universal technique for identifying individuals for evidential use in courts of law. In more complex cases such as sexual assaults, identifying the origin of a stain or sample offers valuable information as to the events that occurred. Currently, many 'in service' body fluid identification (BFID) techniques are presumptive, require significant sample volumes and generate false positives. As such, the development of a highly specific and reliable BFID technique would be highly beneficial to forensic scientists in provide more informative and reliable evidence.

MicroRNAs (miRNA) are short, stable, non-coding RNA's which modulate gene expression. Expression of some of these miRNA are body fluid specific, making them a potentially robust tool for BFID. The possibility for the integration of a robust, miRNA based BFID technology for forensic casework employing stem-loop reverse transcription and qPCR analysis was the theme of the research presented here. To be incorporated into the workflow of current forensic laboratories, the protocol must be able to be carried out alongside current techniques with limited addition of cost, equipment, analysts and time.

A range of custom designed miRNA markers were analysed on vaginal material, menstrual blood, saliva, venous blood, semen, seminal fluid and skin. Screening indicated specificity of hsa-mir-124 to vaginal material, hsa-mir-10a, 135a and 888 to semen, hsa-mir-412 and 507 to menstrual blood, hsa-mir-144-3, 144-5, 142 and 451 to blood and although highly expressed in saliva, hsa-mir-205 was also observed in vaginal material. Universal expression was observed in hsa-mir-93, 508, 1260b and SNORD 47 providing a means of normalisation through the designation of these markers as endogenous controls. A combined panel of markers are presented which were capable of identifying all body fluids, excluding skin from single stains. The panel was successful at identifying certain mixtures such as semen within vaginal material but was unable to confirm saliva presence within vaginal material. Screening of hsa-mir-205 within vaginal material uncovered the observation that hsa-mir-205 was impacted by the use of female contraception.

Once a full BFID panel was generated the robustness of the markers was further analysed across the menstrual cycle. No significant difference ($p > 0.001$) was seen in markers highly expressed in vaginal material during screening (hsa-mir-124, 203a, 205). Expression of non-specific markers highlighted the importance of the optimisation of input miRNA. Differential extraction of genetic material was found to be detrimental to miRNA sample integrity. As such, total DNA extraction was employed for vaginal swabs obtained from volunteers following unprotected sexual intercourse, markers hsa-mir-10a, 135a and 888 were able to successfully detect semen presence for up to 96 hours.

The data generated to date has highlighted a number of miRNA markers that provide a platform for BFID. The developed protocol is reliable and robust; requiring minimal optimisation and is capable of integration with current laboratory workflow with minimum implications to time and cost. The markers identified for BFID have been implemented within studies that are representative of real case scenarios, and have demonstrated their ability to be stable, specific and successful in the identification of certain body fluids. Overall, this research showcases a reliable and body fluid specific protocol capable of being performed alongside DNA profiling.

List of abbreviations

ACPO - Association of Chief Police Officers
AP - Acid Phosphatase
BFID - Body Fluid Identification
BPA - Blood pattern analysis
cDNA – Complementary deoxyribonuclease acid
CPS - Crown Prosecution Service
CSEW - Crime Survey for England and Wales
Depot – Contraceptive Injection
DNA – Deoxyribonuclease acid
DTT – Dithiothreitol
EDTA - Ethylenediaminetetraacetic acid
EWHC - High Court of England and Wales
H&E stain - Hematoxylin and eosin stain
HMCPS - HM Crown Prosecution Service Inspectorate
HMIC - Her Majesty's Inspectorate of Constabulary
HPSF – High purity salt free
HRM – High resolution melt curve
HVS – High vaginal swab
IUD - Intrauterine device
KM - Kastle–Meyer
LOD – Limit of detection
LMG – Leucomalachite Green
ItDNA – low template DNA
LVS – Low Vaginal Swab
MBG - Molecular Biology Grade
mRNA – Messenger RNA

MIQE - The Minimum Information for Publication of Quantitative Real-Time PCR Experiments

miRNA – microRNA

MM – Master Mix

MSRE–PCR - methylation-sensitive restriction enzyme-PCR

NaCl – Sodium Chloride

ONS – Office of National Statistics

PBS buffer - Phosphate Buffered Saline buffer

PBMC - Peripheral Blood Mononuclear Cells

PRC - Police Recorded Crime

Pro K – Proteinase K

PSA - Prostate specific antigen

qPCR – Quantitative polymerase chain reaction

RASSO – Rape and Serious Sexual Offences

RBC - Red blood cells

RISC - RNA-induced silencing complex

RNA - Ribonucleic acid

rRNA - Ribosomal RNA

RT – Reverse Transcription

rt-PCR – real time polymerase chain reaction

RT-qPCR - Quantitative reverse transcription PCR

RTS – Rape Trauma Syndrome

SARC - Sexual Assault Referral Centre

SDS - Sodium Dodecyl Sulphate

SEM – Standard Error of Mean

siRNA – Small interfering RNA

SNORD RNA – Small nucleolar RNA

SOA - Sexual Offences Act

SOCO - Scene of Crime Officer

Tris HCl - Tris(hydroxymethyl)aminomethane hydrochloride

tDMRs - tissue-specific differentially methylated regions

tRNA - Small transfer RNA

UPSI – Unprotected sexual intercourse

WBC – White blood cells

W/B – White British

1. Sexual Assaults and Offences

1.1. Sexual Assault

Sexual assault is a crime that occurs worldwide, despite prevalence of this crime reporting and conviction rates are universally low [1]. Although many individuals experience an incident that meets the legal definition of sexual assault, many do not label their experience as such; these individuals are typically described as unacknowledged victims. Research shows that a large proportion of rape victims are unacknowledged [2]. The Sexual Offences Act of 2003 (SOA) relates to sexual offences that were perpetrated within England and Wales later than May the 1st, 2004; If an offence occurred earlier the SOA of 1956 would apply. The SOA 2003 defines various levels of sexual assault. The statutory definition states that an individual has committed the offence of rape if he deliberately penetrates an orifice, such as the vagina, mouth or anus with his penis without the consent of the other individual, usually with force or a threat of force [3, 4]. Life imprisonment is the maximum sentence for rape cases however the majority of sentences administered in a court of law are around 5 years [5]. Cases where aggressive or forceful aspects are involved can increase the custodial sentence imposed by the Judge.

The SOA 2003 states that consent is only valid if the individual has the freedom and capacity to consent, therefore if an individual feels pressurised, threatened, incapacitated by drugs/excessive alcohol or a certain disability they cannot legally consent to sexual intercourse [4]. The SOA 2003 states that penile penetration would lead to a charge of rape, whereas penetration of an orifice using a foreign object or another part of their body, such as a digit or tongue should face charges of assault by penetration. Therefore under UK law only a male can commit the act of rape [4]. Sexual assault encompasses the full range of sexual acts, the majority of which are forceful or controlling in nature. Sexual assault includes acts of rape but is broadened to sexual acts such as unwanted sexual touching, kissing, cunnilingus and grooming [6].

The correct case level for sexual assault is difficult to define due to various factors surrounding case details that can alter the severity of the sexual offence. The Metropolitan Police define sexual assault on two levels; standard and serious. Standard sexual assaults

involve occurrences such as the intentional sexual touching of another individual without their consent, while a serious sexual assault involves penetration of the vagina or anus of another individual with an object or any part of the body without consent [7]. The physical action of a sexual assault, known as the *actus reus*, occurs when an individual performs sexual actions against a man or woman when consent has not been provided. The mental aspect of criminal activities, known as the *mens rea* or 'guilty mind', was re-defined by the SOA 2003. The SOA requires a negative answer to two questions; whether the defendant held a belief that the individual provided consent, and if that belief was reasonable. Prior to SOA 2003 a lack of *mens rea* could be argued if the suspect provided an entirely unbelievable reasoning for why they believed consent was provided [4, 5]. The prosecution must prove the *actus reus*, a standard for most crimes as well as *mens rea*.

The difficulties that courts of law face when dealing with cases of sexual assault is that a large range of actions; from verbal or light touching to full penetrative intercourse are categorised as a sexual offence. It can therefore be difficult to identify case level, prove that consent was absent, or for a judge to assign a correct sentence. Within sexual assault cases the burden of proof usually lies on the defendant, as opposed to other crimes such as theft or murder where burden of proof must come from the prosecution [5]. The defence have only to provide reasonable doubt the sexual assault did not occur or the complainant consented for a jury to likely provide a non-guilty verdict.

The creation of the rebuttable presumption in the SOA 2003, supports victims of sexual assault and states that consent was absent and the suspect had *mens rea* if; violence or the threat of violence was carried out against the victim or another party, the victim was being held against their will, they were asleep, had a disability or were incapacitated by an administered substance [5]. Despite the changes within the SOA 2003, victims can often feel very ashamed of their traumatic event or feel that will not be believed or supported by authorities. It has also been seen in many sexual assault cases that assailants go unpunished even when a case reaches the courts. Victims facing such uncertainty and public attention may choose not to report their traumatic experience [8].

Measurement of crime within England and Wales is constructed from two main statistical sources: the Crime Survey for England and Wales (CSEW), associated with the Office for National Statistics (ONS) and the Police Recorded Crime (PRC) data, collected from 44 forces across England and Wales. These two data sets in combination provide an informative perspective on crime rates and actual reporting of crime. The CSEW is conducted via face-to-face interviews, due to the sensitivity and discomfort of discussing instances of sexual assault the CSEW excludes sensitive cases such as murder and sexual assault. To obtain the most reliable overview of sexual assault based crime rates, self-administered electronic surveys are performed by adults aged 16-59. Participants are asked to describe any experiences involving domestic abuse, sexual abuse or severe violent crime. Reports for crime statistics are gathered through the PRC for each financial year of April to March. Crime statistics have the benefit of displaying actual crime figures but do not provide information on sexual assaults where the victim did not inform the police [9].

Within both reports, sexual offences include instances of rape, sexual assault, grooming, indecent exposure and any illegal sexual act such as touching involving adults and children. The self-administer section of the CSEW observed no significant difference in the estimated percentage of individuals who were victims of sexual assault or attempted sexual assault within that period of April to March. Current data suggests that instances of sexual assault have fallen over the last 10 years (Figure 1.1). This reduction however, appears to be as a result of fewer instances of less serious sexual assault, where the instances of rape including attempts has remained consistent.

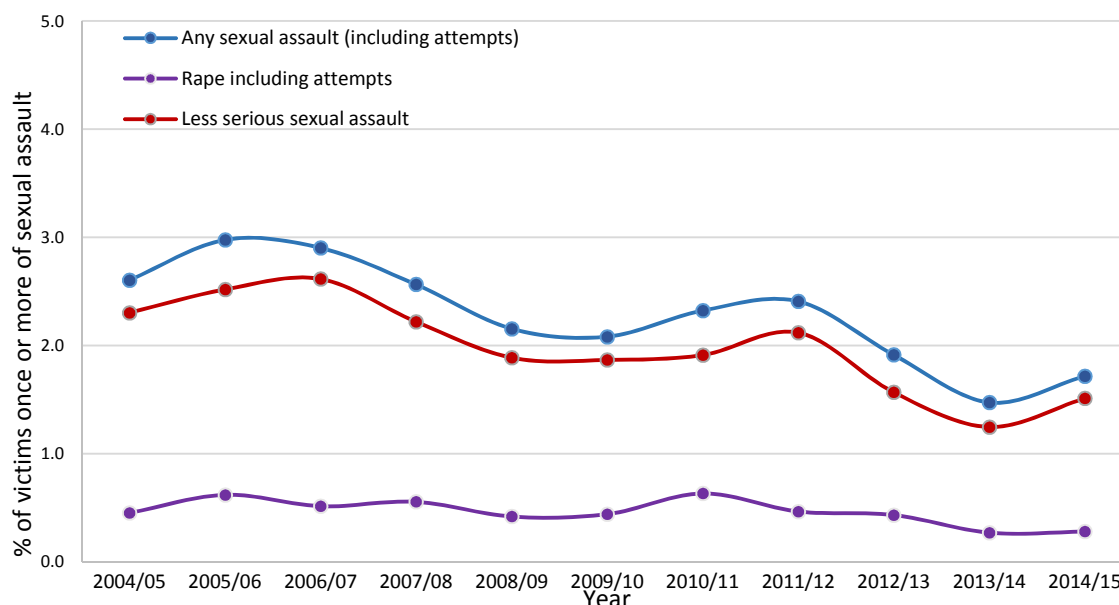


Figure 1.1: Trends in victims of serious and standard sexual assaults from 2002 to 2013 observed as a percentage of total sample base. Data was collected throughout each year with crime data published in April obtained from the CSEW self-completed crime survey [9]

PRC statistics include data from all reported cases of sexual offence, the data is however only representative of cases where individuals have reported the crime and it has been recorded. Total values of sexual offences recorded can be observed in figure 1.2. In March 2016 the total recorded number of sexual offences was 106,378, consisting of 35,798 reports of rape and 70,508 other sexual offences. This was an increase of 21% from the recorded crime figures of 2015 (increase 22% rape and 20% sexual offenses) and a significant increase from 2011/2012 figures of 52,760 total sexual offences, 16,038 rape cases and 36,722 other (an increase of 97%, 125% and 85% respectively [10-12]).

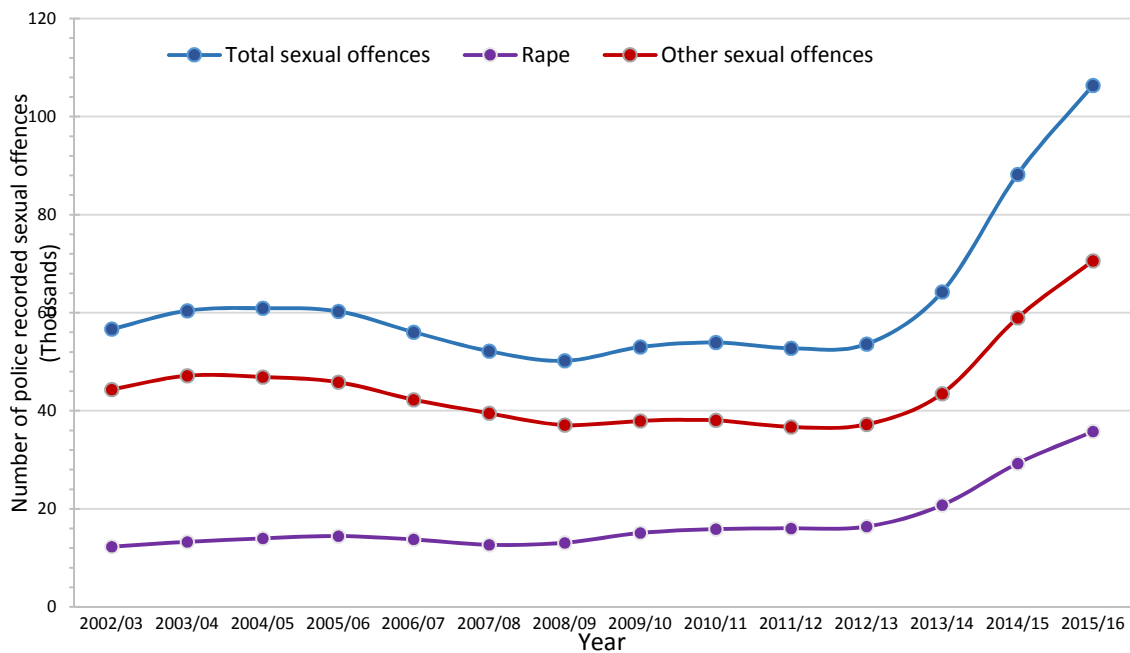


Figure 1.2: Volume of sexual offences reported and recorded to 44 police forces over the financial year of April – March, recorded from 2002 to 2016. Data is represented in thousands and obtained from yearly PRC figures [11, 12].

The CSEW results display opposite trends to PRC figures. The CSEW includes residents that at the time of the survey were dwelling in households in England and Wales. The range of sexual offences included in the survey is significantly less than those recorded by the police and participants are not included if they reside in large residencies, such as care homes or campus residences or if they are only visiting the country as a tourist. The most at risk group of sexual assaults are adolescents in environments such as universities and the elderly or mentally ill therefore if sexual assaults occurred within these higher risk groups it would not be included within the survey [8].

The significantly increased observation of all sexual offences including rape within the PRC statistics over the past 5 years is not necessarily representative of the true number of crimes reported. A number of factors have shown a positive impact on the willingness of sexual assault victims to come forward and report their experience to the police.

1.1.1. High profile reports into sexual assault recording

In 2014 Her Majesty's Inspectorate of Constabulary (HMIC) and the HM Crown Prosecution Service Inspectorate (HMCPS) published investigative findings from 2012 that analysed the various police forces management and recording of sexual assault reports [13]. The report stated there was a significant nationwide problem of under reporting of many types of crime. A large scale audit of available records determined that across England and Wales, 19% of cases that should have been recorded as a criminal offences were not, with a national under reporting of sexual assaults at 26% (1 in 4 reports). The extensive investigation discovered the procedures of specialist rape and sexual assault units for recording were highly unsuitable, especially when charged with underage or vulnerable individual's cases. The investigation uncovered a multitude of cases that had not be properly transferred to the appropriate crime recording system and therefore were not used within the national official statistics. This report highlighted the need for improvements within reporting and recording of sexual assault cases and set a high precedent for police forces. This increase in recording and care of transfer of information will undoubtedly increase the national statistics for sexual offence occurrence.

1.1.2. Large scale investigations into historical allegations of sexual abuse

The active investigation into large scale sexual abuse cases such as 'Operation Yewtree' saw a prolific number of sexual offences being uncovered [14]. Operation Yewtree was an extensive investigation of alleged sexual abuse mainly against children, predominantly focusing on the sexual acts performed by Jimmy Savile and other well-known public figures. By July 2014 the investigation had uncovered a large volume of sexual assault allegation against a further 16 public figures, with individuals such as Gary Glitter, Max Clifford and Rolf Harris being convicted of multiple accounts of sexual assault [15]. When these alleged sexual assaults occurred, victim's stated they felt they could not come forward and therefore their cases were not included in the recorded crime of that year, the significant

volume of occurrences uncovered by Operation Yewtree would cause a significant increase in reported figures for that year [14].

Current investigations into historic allegations of sexual abuse of youth football players by coaches have been launched by 15 police forces into 148 football clubs¹. 429 alleged victims have been identified, the majority of which ranged in age between four and twenty years old. Such a large-scale investigation would therefore contribute a significant increase in recorded sexual assault for 2016/17 when these cases occurred years beforehand [16, 17].

1.1.3. Creation of independent inquiries for sexual abuse

The report of the HMIC and the extensive investigation of Yewtree saw an increased need to determine if individuals in positions of authority had shown true duty or care or abused their power. The 'Independent Panel Inquiry into Child Sexual Abuse' was produced to investigate public authoritative bodies and their ability to protect children and higher at-risk groups [14].

The CSEW collected feedback from all police forces which stated that both current and historical sexual offences continued to rise in the year ending March 2015 when compared with March 2014. Police forces did however indicate that the major contribution to this increase was believed to be caused from present-day sexual offences. The lack in significant change over CSEW data compared to the continual increased in reported cases of police recorded data supports the hypothesis that victims of sexual abuse are more willing to come forward about their experiences. This increase in willingness is likely due to the high-profile coverage when investigations occur, the observed serious response that the police have shown in dealing with historic cases of abuse and the significantly improved recording and reporting procedures of all police forces [12, 14].

¹ Correct as of December 2016

1.2. Vulnerable and High Risk Groups

The PRC provided case details for all reported cases of sexual assault from April 2014 to March 2015. Within this year there was a reported rate of 29,265 rape offences, of these 90% (26,339 cases) were females of varying ages. Across the 29,265 rape offences almost 25% were aged between 15 and 19 years old, with 16% aged ten to fourteen and 14% aged 20-24 [18]. The predominance of reported rape in young adults and children was clearly observed when 73% of all recorded rape cases involving women were aged below 30 years old with a third of those females being children under the age of 16. Although rape offences involving males accounted for 10% of all rape cases 88% of these males were under 30 years with 60% falling under the age of 16. The data represented highlights that young adolescents are more at risk of experiencing sexual assault than individuals over 30 [18]. The overall statistics for all rape victims, regardless of age group displayed women in a higher risk category than males. This could be due to accurate representation of data or that males who experience rape may be less likely to report their experience. Figure 1.3 displays the proportion of rape offences categorised by the gender and age of each victim. The trends that can be observed are that the highest incidents occur in individuals under the age of 30 with a significant majority being female, however when a sexual offence involves a male they will likely be under the age of 16 [18].

In cases of sexual assault the age of victims decreased with individuals more frequently aged between 10 and 14 [18]. This age group, as seen in Figure 1.4, made up 28% of all sexual assault cases, compared to the 16% in all rape cases, while 15-19 years old comprised 19% of all sexual assaults and 23% of all reported rapes. Of the 28% aged 10-14, 83% were female and 17% male further supporting females as the highest risk group of sexual assaults. Approximately half all reported sexual assault cases involving children aged 10-14 were recorded as sexual activity involving children such as indecent exposure or pornographic images with the remaining involving a physical form of sexual assault. The data showed female victims under the age of 30 made up 78% of all female sexual assault cases while male victims under the age of 30 made up 84% of male sexual assault offences.

Combined over 50% of all male and female offences occurred when the victim was under 16 [18].

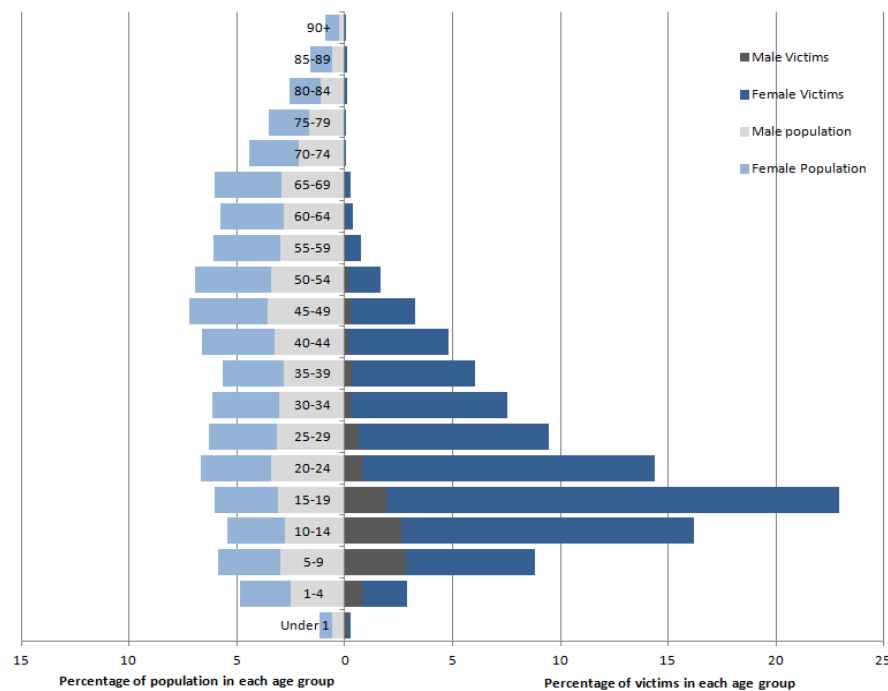


Figure 1.3: Proportion of rape offences, categorised by age and sex of the victim, provided by the Home Office Data Hub (13 forces), April 2014 to Mar 15. The highest volume of rape occurred in females aged 15-19 [18]

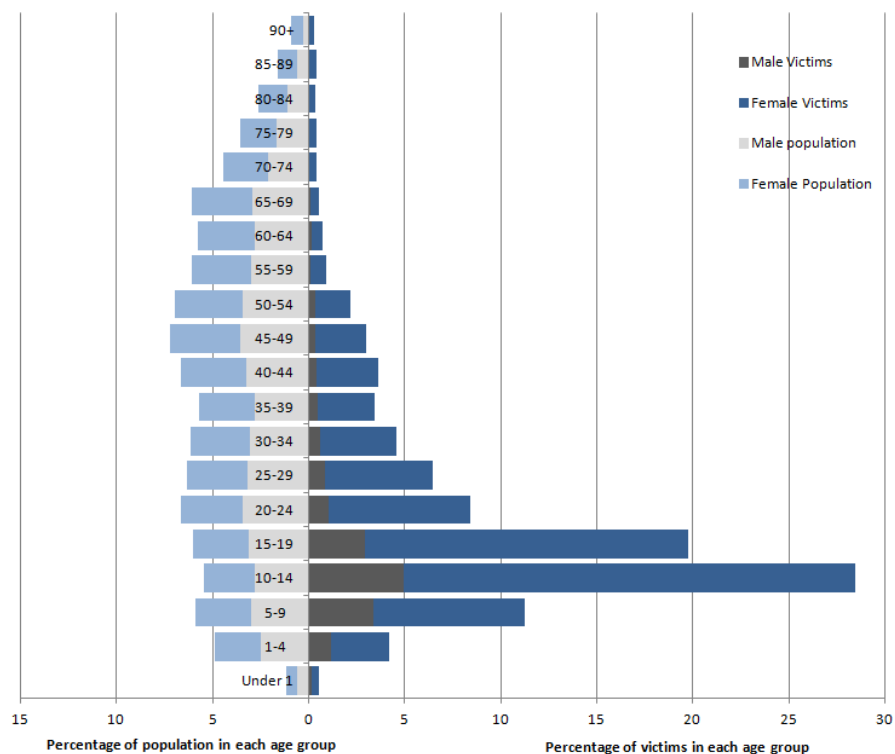


Figure 1.4: Proportion of sexual assault offences, by age and sex of the victim, provided by the Home Office Data Hub (13 forces), April 2014 to Mar 15. The highest volume of sexual assault occurred in females aged 10-14 [18].

The PRC data for both reported rape and other sexual assault offences display a clear trend of individuals at higher risk, females under the age of 30 comprised the complainant in a significant volume of cases compared to all other incidences in both reported rape and sexual offences. With both males and female being categorised as high risk of sexual assault below the age of 16 [18].

1.3. Common Perpetrators of Sexual Assaults

In 2013, a collaborative review on sexual offending was performed by the Ministry of Justice, Home Office and the ONS. The review gathered all available statistics and reported an extensive summary on sexual offending throughout England and Wales [19]. The data reported that approximately 90% of individuals who experienced a serious sexual assault throughout the previous year knew who committed the assault. In cases of other sexual offences, for example voyeurism or exposure, the percentage of victims who knew their attacker decreased to below 50% [19].

The review combined recorded data from 2009-2012 to assess the female based victim-offender connection within serious and less serious sexual assault cases (Figure 1.5). The observable trend throughout 2009-2012 is that in cases of serious sexual assault only 10% of cases were committed by a stranger, while 56% of cases displayed a current or ex-partner as the perpetrator. The majority of cases (52%) of other sexual assaults were perpetrated by someone unknown to the victim [19].

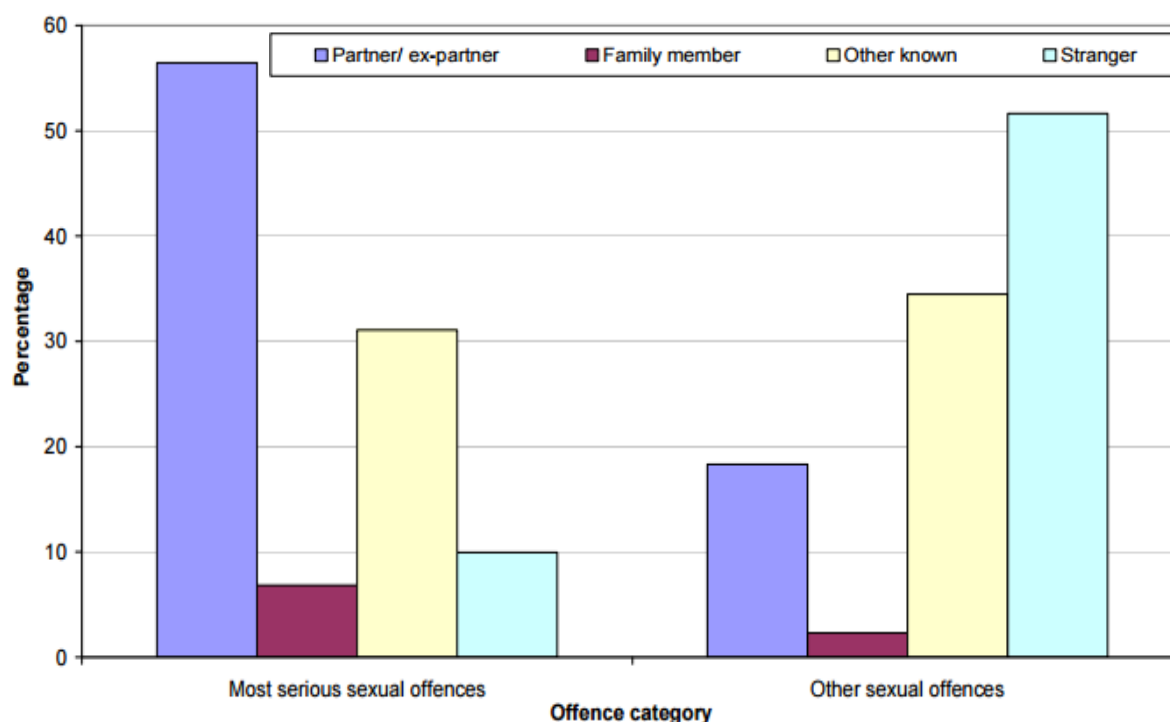


Figure 1.5: Percentage of female victim based sexual assaults, categorised by severity and relationship to victim [19].

The reviews analysis of the self-completion survey, completed by females aged 16-59 from 2009-2012 also showed the most frequently reported perpetrators were males aged 20-39. Table 1.1 shows that in no incidents were women the perpetrator with the highest number of sexual assault being committed by young to middle aged adults. This data is not entirely comprehensive as cases involving multiple perpetrators were not included however a distinct trend in the perpetrators of female victim-based sexual assaults is clear [19].

Age	Male perpetrator	Female perpetrator
<16	1	0
16-19	29	0
20-39	47	0
40-59	20	0
>60	2	0

Table 1.1: Results of self-completion module completed by female victims (aged 16-59) of a serious assault. Results of 2009/10- 2011-2012 were combined with a sample base of 62. Incidents involving more than one perpetrator are not included in the results [19].

As previously discussed, the most at risk group of sexual assaults are young females. Of female victim based crime the perpetrator is highly likely to be male and aged below 40 years old. In cases of rape and serious sexual assaults the victim is highly likely to have a known connection with the perpetrator, either through an intimate relationship or another acquaintance. In instances of less serious sexual offences, where no penetration occurred the perpetrator is more likely to be unknown to the victim.

1.4. Prosecution and Defence of a Sexual Assault

Once the CPS has been provided with evidence from forensic scientists and medical examiners and have been provided the key data such as age and vulnerability of the victim, the sexual assault can progress to a court of law. The prosecution must then decide the offence level that they wish to assign to the case. The SOA 2003 states that a serious sexual assault includes rape and even the unclothed touching of genital organs whereas the prosecution can assign a lower level in instances where touching was performed through clothing or where the vagina or anus were not penetrated. Case level can be heightened however in cases where factors that have previously been discussed are involved, such as alcohol or violence [4]

Once case level is assigned the prosecution would provide a prosecution hypothesis that that the complainant performed these sexual acts without the consent of the victim while the defence would provide an alternate hypothesis in which the complainant's testimony would be taken into account. The outcome would be either the sexual assault never occurred or that the events described by the victim happened but they had provided consent for every action. Depending on the defence hypothesis the forensic evidence can be vital. If the former defence is provided any DNA or BFID evidence would show strong support for the prosecution however if the latter is utilised the same evidence would support both hypotheses as consent cannot be addressed by any DNA and BFID forensic evidence.

1.5. Under-reporting and low convictions rate

Figures for the UK show it has one of the lowest conviction rates for rape in Europe [1]. A report by Amnesty International suggested that these low conviction rates could be due to “rape myth” ideology held by members of the jury or judges. Over a third of their study population (1,000 people) believed that *‘women may be partially or wholly accountable for being raped if her actions included flirtatious behaviour and therefore they cannot be declared a victim’*. 250 of the 1,000 interview participants also believed that wearing inappropriate clothing or becoming intoxicated meant partial or complete blame fell on the victim. The majority of the individuals surveyed had no awareness of the prevalence of sexual offences within the UK [20]. If these views are held by a large percentage of the population, victims are less likely to receive justice in a court of law.

Research has however been performed to show low conviction rates are not solely due to victim blaming and rape myths but due to high attrition rates and lack of belief shown by police forces [14]. Rape cases are complex and views held by people in positions of power will affect how a sexual offence is reported. If a rape occurs where a young female has been drinking at a university party, police officers may have doubts as to whether consent was provided, due to intoxication and possible regret when sober. If, however, rape occurs by a stranger in a public area, the police are more likely to believe the victim [14]. In 2007, 14% of reported cases resulted in trial. Approximately 9%, all involving those aged 16-25, were categorised as false allegations. Over 33% of these reported that they did not proceed to trial as a result of victim credibility, or issues with evidence. A large proportion of the remaining reports were retracted due to the stigma they would face, or fear of being disbelieved [21].

Research into attrition of police forces in England and Wales propose that only a marginal number of rapes are reported to the police [1]. Of those that are reported, the bulk do not result in prosecution of the suspect, or conviction. Retraction of statements from victims is highly likely during the early stages of case construction, highlighting that the way police officers approach and interact with victims of sexual assault may not be suitable and likely

to cause withdrawal of statements. The belief from an authority figure after a traumatic ordeal will have a significant impact on whether a victim of sexual assault is likely to pursue the prosecution of the offender [1]. Research into rape allegations reported to the London Metropolitan police suggested attrition was influenced by rape myths but also heavily by ethnicity of the victims and offenders, if the offender was a stranger, discrepancies within the victim's statement, the opinion of the police and prosecutors on the level of significance the evidence will provide or any prior notification of false accusations by the victim. Overall the research determined that a range of factors influence the level of importance that police deal with some sexual assault cases. These include the relationship of the victim and offender, the resistance shown by the victim and the injuries they obtained, the length of times that elapsed before reporting, their mental health and one of the largest influences involved alcohol consumption [21].

The in-depth review of the HMIC 2013 inspection into how England's and Wales police forces dealt with sexual assaults showed 26% of sexual assaults were not recorded by the police, therefore victims reported their experiences to the police and were incorrectly informed it did not meet the criteria to be recorded as a sexual assault offence. The inspection also investigated the police protocol of recording a sexual offence and subsequently cancelling the report and classifying it a 'no-crime'. The data from the report highlighted 664 reports that were incorrectly voided due to labelling as 'no-crime', of these 200 were rape charges. Overall the statistics for a 'no-crime' cancellation was stated at 3.1%, however for crimes recorded as rape the level of 'no-crime' was 7.3% suggesting that police forces are more likely to 'no-crime' a case if the charge involves rape [13].

1.5.1. Reporting rates within the UK

The Department of Justice produced data that states approximately 344 of every 1,000 cases of sexual assault results in the police not being informed, leaving a large proportion of victims as unacknowledged. The data showed that only 28% of elderly patients and 20% of young females reported sexual abuse to the police. The reports questions victims who did report their experiences and discovered the majority did so to protect a loved one from

further attacks, to prevent reoccurrence of abuse or they believed it was their duty to protect their community from the offender. The majority of victims who did not report their experiences stated they did so out of panic of retaliation, to protect the perpetrator, they believed the police would do nothing or it was a personal issue that should be dealt with without police intervention [22, 23].

There are a multitude of additional factors that could prevent victims from reporting sexual assaults [8]

- Fear of social stigma: The fear of what the surrounding community or family may think of them if their experiences were known. If their surrounding communities were receptive to rape myths there is a possibility the victim may be blamed for causing their own sexual assault.
- Acceptance: Some victim may feel so traumatised they feel they cannot accept the events that happened to them and thus do not share their experience with anyone.
- Fear of retribution or repeat offences: If the assault is reported and not taken seriously the fear that the offender may punish them in a manner far worse than the original offence may silence some victims.
- Pressure from offender not to speak out: In situations where the offender is a close family member or friend of the family, they may tell the victim that it will hurt the family if the assault was revealed or threaten to hurt someone they care about if they speak to anyone.
- Previous trouble with authority: Individuals who may have had negative interaction with the police prior to the sexual assault may feel they would not be shown due care if they reported their experiences.
- Disability: Individuals may lack the physical or mental capacity to explain that they were the victims of sexual assault or feel ashamed they were unable to prevent the act from occurring.
- Domestic sexual abuse: Partners may feel it is their duty to have sexual interactions with their partners regardless of their wishes and thus not deem their experience rape.

- Fear of honour killing or being disowned: In certain regions around the world and within the UK, the loss of virginity caused by sexual acts with an individual who is not their married partner is considered a great dis-honour to their family. The fear that severe familial consequences may occur from revealing their assault would lead them to not reporting their offence [24].

The ONS looked at abled and disabled individuals under the age of 16 who experienced rape or penetration and whether they felt like they could inform anyone of their experience, as seen in table 1.2. The results showed that disabled individuals only told someone, not necessarily the police in 32% of occurrences, with this number decreasing to 23% in individuals with no disability. In all individuals the majority heavily favoured keeping their experiences to themselves.

<i>Within England and Wales</i>	Individuals aged 16 to 59		
	Informed	Did not inform	number of adults
	Percentages		
<i>Long-standing illness or disability</i>	32	68	180
<i>Limits activities</i>	34	66	156
<i>Does not limit activities</i>	-	-	24
<i>No long-standing illness or disability</i>	23	77	298
<i>Total</i>	26	74	478

Table 1.2: Proportion of victims of sexual assault by rape or penetration (including attempts) while under the age of 16 informed anyone about the abuse at the time, by disability, year ending March 2016 [25]

1.5.2. Increases in reporting rates

There has been significant efforts through many governing bodies and law reforms to increase reporting and conviction rates with the goal of protecting sexual assault victims. Prior to 2003, the acts involved to be concluded as sexual assault were very vague. The enhanced definition of rape, including factors such as consent, domestic rape and smaller sexual offence improved the police and prosecutions ability to assign case level. The construction of Sexual Assault Referral Centres (SARC) allowed for dedicated and specially trained staff to provide the appropriate medical care and support toward victims of sexual

assault, prioritising their emotional and physical needs to support them after experiences traumatic events such as rape [1, 4]

In 2014 the CPS created a National Scrutiny Panel, involving a large scale discussion between police forces, prosecutors, respected academics and victims of sexual assault. In 2015 the CPS then went on to collaborate with the Association of Chief Police Officers (ACPO) The result of both of these events was strict new guidelines and a 'rape action plan' being implemented within police protocols that improved the management and recording practices as well as the attitudes towards rape [1, 26, 27]. The new protocols demonstrated dramatic improvements were required from every police force with regards to every reported sexual assault case.

- Section 3.1: "The Police will appoint a Rape Champion and the CPS will appoint a Rape and Serious Sexual Offences (RASSO) Unit Head who will ensure that the investigation and prosecution of rape is co-ordinated between the agencies"
- Section 6.1: "Any forensic medical examination of the victim should take place in a dedicated examination suite to maximise victim care and the integrity of the evidence. Special requirements for victims with special needs must be considered and may involve assistance with transportation or the use of hospital facilities for the medical examination"
- Section 9.3: "Rape specialists will assist in making a provisional assessment of the case, and participate in discussion around lines of further enquiry, identification of the likely charges and of the evidence required to support them. They will be proactive in identifying, and where possible rectifying evidential deficiencies and in bringing to an early conclusion those cases that cannot be strengthened by further evidence. In addition the investigating officer and rape prosecutor can discuss questions to be put to suspects in interview, any pre-charge court procedures and any strategy for a likely prosecution"[27].

These improvements are all aimed at providing support to victims so they feel they will be properly represented if the case is carried forward. The attitudes of the police forces are also

required to be supportive and non-judgemental when assessing cases of alleged sexual assault. It is likely these improvements as well as large scale investigation such as Operation Yewtree are the biggest influences in the increase in reporting rates.

1.5.3. Conviction rates within the UK

The PRC data for 2005/2006 displayed over 60,000 reported sexual offences, consisting of nearly 15,000 instances of rape. Although a steady increase in reporting was observed in Figure 1.2, the conviction rates for rape were constantly low with successful conviction observed in 6% of cases in England and Wales and 4% in Scotland [9, 21]. Improvements in conviction rates have been observed, the CPS released a report in 2016 stating the current conviction rates against violent crimes against women. The results state a 57.9% increase in the number of convictions for cases involving rape. Within that year the CPS prosecuted over 4,600 individuals for charges of rape with conviction being successful in over 2,500. Although these figures are promising when compared to the 5.7% observed in the 2005 Home Office report [28], the volume of reported sexual assaults reported by police forces surpassed 100,000 with the CPS only prosecuting 4,600 cases. Of those cases only 60% achieved a successful conviction [29]. This low conviction rate, although steadily increasing discourages victims of sexual assault from reporting. The criminal justice process can be long, humiliating and invasive for a victim. If the chance of conviction is approximately 5% many victims may view the process not worthwhile.

1.6. Rape Myths

The societal views surrounding rape and sexual assault can have a serious effect on the awareness, reporting and the investigation of sexual assault claims. Rape myths are narrow and descriptive views about sexual assault such as the cause, context and people involved that could provide justification for the act [30]. Rape myths involve false beliefs about the victim, perpetrator and the act itself revolving around a stereotypical view of a likely victim that shows disbelief and reduced credibility to victims that do not fit that ideal [31]. Barnett *et al.* undertook a study to determine how aspects such as religion and gender effects

societal views towards rape. Participation by 653 University students aged 18-30 was fulfilled through completion of a questionnaire. The results suggested that people who viewed themselves as atheist or agnostic endorsed rape myth acceptance at a significantly lower level than individuals within the Roman Catholic or Protestant faith. Individuals of these faiths may hold a stronger belief on views such as appropriate clothing and the protection provided from their religion. Males were the most likely to endorse rape myth ideology however no significant link was determined between gender and religion. Due to the high volume of female victims in sexual assault offences it is likely males would be more open to the ideology that females put themselves in bad situations than females who may have friends that have suffered some form of sexual assault [32].

The most commonly circulated rape myth is that the majority of sexual assaults and rapes involve an unknown individual that violently attacks, usually with a weapon, a woman. These attacks cause serious injuries to the victim and occur when the victim is in a remote, open area [33]. The common factor within all rape myths is that the victim is to blame for the act occurring, such as provocative clothing, travelling alone, visiting unsafe places or that the victim is after revenge and thus accuses an alleged suspect with rape [30].

Previous studies have determined these myths are acknowledged by individuals of all sex, age and race, McGee *et al.* reported that above 40% of their sample population believed that a claim of rape was invented by the alleged victim [34]. It is therefore simple to understand why victims of rape may not wish to share details to police or the people surrounding them. Clay-Warner and McMahon-Howard observed victim reporting patterns and discovered a two fold increase in victim report rates if the attack occurred in a public or communal area or if the perpetrator invaded the victim's dwellings. Their results also indicated an increase in reporting rates in attacks where an unknown individual was involved as opposed to someone familiar to the victim, however the rate of an outside party reporting the act increased if the perpetrator was known to the victim. The main cause for an influx in reporting rates was however linked to whether a weapon was involved/ serious injuries were caused to the victim [35].

1.7. Sexual Assault Forensic Medical Examination

Within the United Kingdom a specialist unit was created to deal with the intricate and complex process of sexual assault examination. The sexual assault referral centre (SARC) comprises of highly trained and committed individuals, with the knowledge and experience to identify possible injuries or consequences of a sexual assault. The examination is performed using a rape kit and must place the medical, physical and psychological requirements of the victim as paramount while identifying the key forensic features that would be required by the CPS to form a case for the courts [3].

The SARC examination involves a thorough examination of the individual's psychological and physical condition. Forensic evidence is obtained from examining the entire body for trauma as well as a comprehensive genito-anal examination. The intimate exam starts with a visual examination of the external areas surrounding the genitals followed by an exterior swab. A speculum is then utilised to cause the least discomfort to the patient while obtaining an endocervical swab, if a speculum is too traumatising for the victim a blind vaginal swab can be obtained. During the examination it is important to collect swabs from the vagina and cervix, with high vaginal swabs (HVS) being taken before the low vaginal swabs (LVS). The final swab is obtained from the individual's anal canal [36, 37]

If unprotected penile penetration followed by ejaculation occurred, a SARC team would utilise various tests that would assess whether spermatozoa were present, if the results were positive it would support the prosecution hypothesis that penile penetration occurred. As previously discussed the reporting rates of sexual assaults are low, and victims are rarely examined immediately after the assault occurred [3]. Currently there is no defined time period for utilising a rape kit after an assault however collection of evidence becomes challenging after 48 – 72 hours [38]. The likelihood of finding identifiable spermatozoa decreases when the length of time or reporting increases. A medical exam taken two weeks after an assault may struggle to identify any wound that occurred or identify the presence of spermatozoa and thus provide support for the defence hypothesis that intercourse did not occur.

1.8. The Aftermath of Sexual Assault

Sexual assault can cause immediate and long term damage to the victim, the immediate damage usually encompasses physical trauma that requires treatments such as provision of emergency contraception, medical care to injuries and tests for sexually transmitted infections (STI). As time progresses more emotional and psychological effects tend to emerge [8, 38].

1.8.1. Physical trauma and medical health

Family general practitioners and doctors care for survivors of sexual offences immediately after the event and for many years to come. Sexual assaults involving penetration of the vagina can, dependant on the force used and resistance displayed cause significant injuries to genitalia as well other areas of the body. Since the highest risk group for sexual assault is young females Baker *et al.* investigated the relationship between age and the genital injuries that were observed following rape [39]. They reviewed 234 medical records obtained from the emergency department of a special rape unit, all cases involved women ages 14 – 29, including multiple ethnicities. Their findings showed genital damage among 62.8% of cases, higher instances (65.7%) were observed in 14-21 year olds compared to the 58.5% observed in young adults (22-29). The volume of injuries within each case was also assessed, with one survivor suffering 24 distinctive injuries in various locations. The average number of genital injuries was approximately 1.8 with the highest frequencies seen in 14-21 year olds. Overall the study indicated that although younger age groups did not display significantly more injuries than slightly older age groups, the frequency of injuries to the thighs, labia and vagina were higher per younger child compared to young adults.

The injuries survivors display are not always genital, if force such as strangling or a knife is used to make the victim comply then significant bruising or laceration may also be present requiring further medical care. In addition to physical injuries there are immediate and long-term medical issues that could arise from sexual assaults, if a condom was not worn by the offender tests for STIs will need to be administered and treated if positive. In the months following a sexual assault wounds could become infected or the victim may fall pregnant

and have the associated medical issues surrounding pregnancy. In certain cases, such as a sexual assault between two men there will be a higher risk of being infected with hepatitis B or HIV [40].

1.8.2. Emotional and psychological trauma

Research into the emotional state of survivors of sexual assault has shown a severe negative impact of psychological well-being following the experience, with the most damage seen in young adolescents. Survivors often suffer from depression, self-harm, anxiety, post-traumatic stress syndrome (PTSD) and can become more submissive to further oppressive behaviour from partners or strangers [41]. Victims can also feel self-loathing due to guilt or shame of the event. Mental disorders such as PTSD and Rape Trauma Syndrome (RTS) are common within survivors of sexual assaults, where RTS is the intense fear and distress that they will be killed during a sexual assault. Burgess *et al.* interviewed more than 600 victims of sexual assault and identified RTS as prevalent in the majority of case. The recommended requirement of care was crisis intervention counselling with psychiatrist evaluation if improvements was not observed [42]. A number of interviews with survivors of sexual assault disclosed that they were physically repulsed by the idea of engaging with sexual activity with new or current partners and they became withdrawn in situations where intimacy and physical interaction occurred [43].

1.8.3. Social stigma

Despite the internal feelings victims may deal with there is a high possibility of stereotypical judgements and negative reactions caused by the prevalence of rape myths and victim blaming [44]. If survivors are struggling emotionally and physically with the aftermath of sexual assaults, the effects will only be heightened if their surrounding community believe that the individual is at least partially to blame for its occurrence. The fear of these judgements may lead to victims not informing the police of their action and further escalating their negative emotional and psychological behaviour.

1.8.4. Re-victimisation

Survivors of sexual assault are at a higher risk of re-victimisation during their life. Research by Najdowski *et al.* observed that women suffering from PTSD or RTS were more likely to use destructive coping mechanisms such as alcohol, drugs or sexual activities and therefore are at a higher risk of being involved in a subsequent sexual assault. Results from a one year survey of 555 women who had experienced childhood sexual assault, showed that approximately 45% suffered re-victimisation within that year [45].

1.8.5. Crime reported effects of serious sexual assault

Data from 2015 PRC and crime survey indicated that approximately 45% of victims of a serious sexual assault suffered physical trauma and injuries, with 30% displaying minor bruising. Data showed 2 out of 5 victims reported severe trust issues and problems developing personal relationships while 3 of 5 victims reported mental and/or emotional disorders. Of all incidents of serious sexual assault 9% of victims attempted suicide, 3% contracted an STI and 5% fell pregnant [8]. As previously discussed physical trauma and mental health influenced attrition and rape myth belief. If the 25% of victims who did not show physical signs of trauma displayed psychological conditions while dealing with the experience they may experience a lack of belief and assistance from the police and their community.

In summary, the implications of sexual assault are numerous. The physical effects are immediate but when coupled to emotional impacts the harm can last throughout the victim's life, with devastating effects on their health and emotional wellbeing.

1.9. Consent

Forensic science involves the application of scientific principles to provide evidence that aids with queries associated with the law. The issue that forensic scientists face is that consent is not a factor that can be addressed, the definitions within the SOA 2003 means that the scientific evidence provided cannot determine rape, only the likelihood that an

event such as sexual intercourse occurred [46]. The theme of any definition for sexual assault is that of consent and can change the verdict of a sexual assault case dramatically.

Consent is however, very difficult to prove and falls into many categories; it can be revoked at any time, it can be conditional, and alcohol and drugs have an effect on the ability of an individual to provide consent. Prior to the introduction of the SOA 2003, the SOA 1956 did not state a definition for consent. Here, the members of a jury were simply reminded at the start of a trial that submission and consent were different and should be taken into consideration. Consent was however readdressed in the SOA 2003 and states that consent has only been given when a person agrees to participate in a sexual act while having the capability and free-will to make that decision [4, 47].

Numerous factors have an effect on whether it is deemed consent has been provided.

- An Individual may feel they are mature enough for sexual intercourse however may change their mind during the activity and withdraw their consent. If the activity continues after this withdrawal then according to SOA 2003 an offence has been committed [4].
- An individual may state they only feel comfortable with vaginal intercourse, if consensual vaginal intercourse occurred followed by anal intercourse. The anal intercourse would be considered a sexual offence.
- Individuals may willingly consume a large volume of alcohol or drugs that could inhibit their mental and physical capacity to consent. It would then be up to a court of law to decide if the individual consented or was assaulted while in a vulnerable state.
- A sexual partner or spouse of the victim may force sexual intercourse on an unwillingly partner, the defence used could suggest that since they are in a relationship consent has always been given for sexual acts.
- Two adolescents under the legal age of consent may both state they provided consent to sexual intercourse with each other [4]

1.9.1. Verbalised non-consent

In many case studies of sexual assault involving adolescents, individuals wish to engage in a relationship with another individual. In these cases, one of the individuals may feel the other party is willing and consenting to performing intimate acts. When told they do not wish to perform them the situation can escalate and victims may feel powerless to stop what is happening, if they have made their feelings clear and they are ignored and forced into sexual acts they may feel there is nothing they can do to stop the situation. This however falls into the SOA 2003 definition of rape, if consent is known to not be provided the individual continuing the act is committing a sexual offence fulfilling *actus reus* and *mens rea*.

Case Study 1-1: Pandora's survivors stories 2015: Elizabeth was always considered a "full of life individual" with a happy outlook on life, this part of Elizabeth was ripped away from her when she was sexually assaulted. She described the event as splintering her world and feeling broken and alone, she wasn't sure she would ever feel happiness again. During her first week at University she met a young male she described as witty, clever and admired by his peers, she described how excited she felt that he would want to kiss her while at a party. Elizabeth agreed to enter his room but explicitly said she did not want sex, this was however not acknowledged and she felt she had lost control of the situation. He asked Elizabeth if he should find a condom to which her reply was a firm no. He refused to acknowledge this and proceeded to sexual assault her, thus taking her virginity. As with many victims Elizabeth didn't want to acknowledge what had happened and therefore tried to convince herself and friends it had been a loving, wonderful experience. She managed to convince herself for three months before realising how much this trauma had affected her life [48].

The fact that consent was explicitly not given before any sexual interaction occurred means that under the law, this case would be deemed as rape. However in a court of law consent within this case is hard to prove, the defence would state she consented while the prosecution would state she did not. The fact that she willingly entered his room, stated to friends that it had been consensual and the time that lapsed before reporting the assault

would all support the defence hypothesis leaving the prosecution with limited viable evidence. Body Fluid Identification (BFID) would likely not be useful in a case such as this, after three months the likelihood of finding external body fluids present from a penile swab or vaginal swab would be very low. If the defence stated that sexual intercourse did occur however it was consensual BFID would provide no additional support to either case hypothesis.

The application of BFID would be extremely useful in sexual offences where the prosecution state the victim verbalised non-consent or the suspect understood consent was not provided while the defence state sexual assault did not occur. Semen identification within the victim's vaginal material samples or the identification of vaginal material from the suspect's penile swab would provide substantial support to the prosecution.

1.9.2. Automatically void consent

Under certain circumstances the prosecution is not required to prove absence of consent, the SOA 2003 states strict regulations on scenarios and individuals where consent is automatically void. The criteria needed for such cases focuses mainly on individuals that are deemed vulnerable or at risk or on perpetrators in a position of power [4].

- Any child below the age of 13: Any sexual activity has voided consent.
- Individuals with a mental disability. For example, if that disorder debilitates choice, if they engage in a sexual activity with their carer or if they are mental manipulated or threatened into sexual activity.
- Individuals with a physical disability: If that disability removes their ability to stop or defend themselves from a sexual assault: If a carer takes advantage of a patient in a paraplegic state.
- Children below the age of 18: If sexual activity occurred with a member of their family who is above the age of 13 or who is in a position of authority or trust [4].

Khalifeh *et al.* performed a study to identify the prevalence of sexual offences against patients with severe mental illness. 303 randomly selected patents diagnosed with severe

mental illness who had been in contact with community services at any point in 51 years provided information using the British Crime Survey domestic/sexual violence questionnaire. Data was compared to 22,606 people in a control group consisting of the general population participating in the 2012 national crime survey. As observed in Figure 1.6, a serious sexual assault happened to 40% of females with mental illness compared to 7.1% of the general population, the figures were still high for men where prevalence was 12.5% compared to 0.5% of the general population. 61.2% of all females stated a form of sexual assault had occurred since the age of 16, compared to 21.1% of the general population. The conclusions from this study are that individuals with severe mental illness are at a higher risk of sexual assaults relative to the general public and therefore more observance and support must be provided to protect patients with mental illnesses [49].

	Prevalence			
	Patients		Controls	
	Total N	N victims (%)	Total N ^a	N victims (%)
Women				
Any SA since age 16	129	79 (61.2)	12289	2587 (21.1)
Indecent exposure	129	45 (34.9)	12289	1316 (10.7)
Unwanted sexual touching	129	56 (43.4)	12289	1567 (12.8)
Serious sexual assaults	129	52 (40.3)	12289	871 (7.1)
Domestic SA	129	37 (28.7)	6117	396 (6.5)
Non-domestic SA	129	61 (47.3)	6117	907 (14.8)
Any SA in past year	129	13 (10.1)	12288	245 (2.0)
Men				
Any SA since age 16	157	36 (22.9)	10317	321 (3.1)
Indecent exposure	157	12 (7.6)	10317	129 (1.3)
Unwanted sexual touching	157	26 (16.6)	10317	193 (1.9)
Serious sexual assaults	157	19 (12.1)	10317	56 (0.5)
Domestic SA ^e	157	7 (4.5)	5195	18 (0.35)
Non-domestic SA	157	32 (20.4)	5195	107 (2.1)
Any SA in past year ^e	157	5 (3.2)	10317	33 (0.32)

Figure 1.6: Prevalence of sexual assaults against males and females with severe mental illness. Data shows a high prevalence of serious sexual assaults in women suffering mental illness compared to the control group [49].

BFID would be an essential tool in cases where consent is automatically void. In cases where a careers DNA is found on a patient the defence could state that the DNA was transfer during routine care of the patient, possibly washing or restraining if the patient became agitated. DNA evidence alone would therefore not be substantial enough for the prosecution, if

however BFID ran alongside DNA profiling, and the DNA present could be linked with semen the prosecution hypothesis that the patient had been involved in a sexual would be proven and due to void consent could not be argued.

1.9.3. Age of consent

Sexual abuse of children includes any act that the law defines as sexual with a child who is below the current legal age of consent, these acts could be a lone occurrence or be recurrent over many years. Sexual abuse of children can occur from strangers, adults or peers however the offender in most cases is an individual who has obtained a position of trust or authority with the child.

Current UK legislation identifies two classifications of offence which contain different stipulations dependent upon the age of the child: the categories classify sexual offences against children that are below the age of 13 and 16. Worldwide statutory rape is defined as sexual intercourse with an unmarried individual who is below the legal age of consent of that country. Force is not a pre-requisite; the crime lies exclusively with the age of the victim. The law presumes that adolescents lack the maturity and are included in the vulnerable category and therefore cannot provide valid consent to sexual intercourse. These laws attempt to safeguard young individuals by discouraging those who might take advantage of the lack of maturity. The laws governing statutory rape punish those who have committed sexual abuse against young people, however they also act as a deterrent for consensual sexual intercourse amongst young individuals. The law surrounding statutory rape provides no support for a defence hypothesis, a defendant's claim of being unaware of their sexual partner's age will not be acknowledged in a court of law [4, 50].

1.9.3.1. Offences against children below 13

In case where sexual activity has occurred with a person over the age of 18 and a child below the age of 13 the prosecution must only prove that sexual activity occurred and the age of the individual on the date the event occurred. The SOA 2003 states that a child below 13 years old cannot legally consent to any sexual activity, regardless of any surrounding circumstances [4]. Upon conviction a prison term not exceeding 14 years can be imposed.

BFID of semen/saliva within or surrounding intimate areas of a child below 13 years old would provide highly supportive evidence that sexual assault occurred and therefore the suspect would be charged with rape.

1.9.3.2. Offences against children below 16

Within the UK the lawful age of consent is 16, this applies to both sexes and sexual activity between different or matching genders. The SOA 2003 states that a sexual offence has occurred if the victim is under 16 and the suspect does not reasonably believe the victim is 16 or over. Upon conviction a prison term not exceeding 10 years can be imposed. The law applies in a similar fashion to prosecution of assaults of children under 13 in that consent cannot be provided, however in cases such as two 15 year olds engaging in consensual sexual intercourse the courts are unlikely to deliver a custodial sentence.

1.9.3.3. Offences against children below 16 when perpetrated by an individual below 16

Regardless of consensual sexual activities occurring between two young adolescents the SOA 2003 states that sexual activity with an individual under 16 is still a sexual offence, however leniency dependent on the situation is more likely. When determining the legislation for the sexual assault act 2003 key priority was to protect victims of sexual assault. During the passage of the Sexual Offences act, Lord Falconer stated *"Our overriding concern is to protect children, not to punish them unnecessarily. Where sexual relationships between minors are not abusive, prosecuting either or both children is highly unlikely to be in the public interest. Nor would it be in the best interests of the child ..."*[51]. If however one individual is below the age of 13, statutory rape would still be applied regardless whether the other party was below 16.

Case Study 1-2: Supreme Court Case ID: UKSC 2016/0083: At the age of 14, an individual was charged with showing pornography and indecently exposing himself to other children his age. The Scottish police did not carry forward with any charges. At the age of 19 however the individual was arrested and charged with engaging in sexual intercourse with a female

under the age of 16. In certain cases the defence could state that the individual reasonably believed that the female was over the age of 16. However section 39 of the Scottish SOA 2009 state that this defences cannot be applied to individuals who have previously been charged with a relevant sexual offence [52]. This case is still be deliberated in the courts as to whether the defendant can plead ignorance of knowledge of age considering he was not charged with a sexual offence at the age of 14 [53].

In cases involving individuals under the age of 16 the law surrounding consent can become convoluted. In certain cases, a defence can be made that there was no reasonable knowledge the individual was under 16, however every case may have implicating factors that will change the prosecution's defence hypothesis meaning all evidence can alter the outcome of a case within a court room. In cases where the individual is below the age of 13 years old, or sexual intercourse occurs when the suspect is aware their partner is below 16 years old, the only thing that must be proven by the prosecution is that sexual assault happened. BFID would be essential to providing this evidence, if saliva or semen is identified on intimate areas of a 13-year-old child it would indicate sexual activity occurred.

1.9.4. Revoked consent

Penetration is an on-going act and as such consent can be withdrawn at any time after initial penetration has begun. Individuals may feel they are ready to take their relationship to a more intimate level and thus consent to sexual intercourse, if however they regret this decision and verbally or physically make their partner aware they wish the action to cease and the act does not, it transforms from consensual intercourse to rape [4, 54]. A qualitative study performed by the NSPCC stated that approximately 40% of young people engage in sexual texting with peers [55]. In these cases individuals may feel safe and confident with expressing sexual thoughts or ideas, however these feelings may change when faced with physically performing them with a partner who believes they are willing.

Case Study 1-3: March 23, 2000: Laura T, a 17 year old female finished her job at a supermarket. At approximately 6.30pm she picked up a gentleman she had met two weeks prior called Juan and drove them to a small gathering at a friend's house. Everyone present

consumed alcohol with the exception of Laura. Laura states that at 8.10pm she declared that she wished to leave but was persuaded to join Juan and his 16 year old friend John in the bedroom. After they entered the dark bedroom Laura, Juan and John engaged in consensual sexual interaction, this included the removal of Laura's garments, the fondling of her breasts and digital penetration. Laura was then asked if she would like to "have two guys at the same time" to which Laura said she did not. Following this John left the room while Juan opened and put on a condom. Noticing this Laura verbally and physical objected and resisted to sexual intercourse with Juan, however despite this Juan used force to have sexual intercourse with Laura. Laura's consistent resistance resulted in Juan ceasing intercourse and leaving the room to which Laura then got up to find her clothes. During this time John entered the room naked, he complimented and kissed Laura and asked if she would be his girlfriend. Laura then kissed John, to which he responded by climbing on top of her and engaging in sexual intercourse. Sexual intercourse lasted from approximately 10 minutes, during this time Laura physically struggled and repeated three times that she "needed to go home", John's response was always "just give me a minute". Following the termination of sexual intercourse John turned to Laura and stated "Well, I didn't rape you so you cannot call the cops" indicating that he felt no criminal action had been committed [54].

In this case study BFID would only prove sexual intercourse occurred, since the victim accepted consensual intercourse occurred but then revoked her consent the BFID of his semen would provide no support to the prosecution. This case would rely on DNA profiling and prosecution to provide evidence that consent was not provided.

1.9.5. Drunken consent

The matter of capacity to consent in cases of sexual assault where a large volume of alcohol has been voluntarily consumed by the victim is difficult to prove in court.

Case Study 1-4: R v Bree [2007]: Bree visited his brother at University and consumed a large volume of alcohol over the course of the evening. Bree heavily drank with another female he had met that night and they proceeded back to Bree's home. She recalled having sexual

intercourse with Bree and that although she had not stated no, she did not provide consent. Bree's defence states that he held reasonable belief that they were undertaking consensual sexual intercourse due to her taking her own clothes off and appearing to be willing and awake throughout the event. Bree was sentenced to a custodial sentence for rape, however the court of appeals overturned this decision stating drunken consent is still consent and that the evidence provided was not substantial enough to prove consent was not provided [56].

In this case the Court of Appeal addressed capacity to consent and stated that if a complainant, due to intoxication lost her capacity to decide to willingly engage in sexual intercourse then consent was not provided and the act would be charged as rape. If intercourse occurred where a complainant had voluntarily consumed alcohol but remained capable of making decision and provided consent it would not be rape [57]. In cases where alcohol is involved the capacity to consent of the victim is difficult to prove and must be properly assessed with each individual case. The court of appeal suggested that if the victim was incapable of making decisions, as would be the case if unconscious then rape had occurred, however the recollection of events due to unconsciousness could have a negative impact on prosecution support.

Case Study 1-5 R. v. Dougal, 2005: A 21-year-old student drank vodka with her friends before attending a University party on campus where she continued to drink with friends. Due to intoxication, she began to feel ill and ask a nearby security guard to take her back to her dorm. The female recalled very little from the evening except briefly emerging from unconsciousness to become aware she was lying in the hallway outside her door and something was happening to her. When police interviewed Mr Dougal he stated intercourse did occur however it was consensual, the victim stated she was adamant she would not have provided consent and if that were the case they would have entered her flat. The High Court Judge in this case directed the jury to reach a verdict of not guilty, stating drunken consent is still equivalent to consent. The case was subsequently abandoned [58].

Since the victim has no recollection of providing consent there is reasonable cause that it was provided, be it in an intoxication state and therefore sexual assault is incredibly difficult to prove. BFID would only be required in cases where the defence stated that no sexual activity occurred. If the defence of Bree or Dougal had stated they did not have intercourse with these women but intimate swabs detected the presence of semen the support would be towards the prosecution.

The impact of alcohol consumption and the recollection of events while intoxicated varies between individuals and research into the effect alcohol has on memory loss is ongoing. A study by Hagsand *et al.* analysed the accuracy of testimony under various alcohol consumption levels, their results suggested that alcohol does not always have a negative impact on memory and that although intoxicated witnesses may recall fewer details there were many who displayed recall as accurate as sober witnesses [58]. Current research however does not take into account the context of the traumatic event, such as a sexual assault that occurred and how mentally processing while intoxicated could impact memory recall. This lack of recollection by a victim may provide stronger evidence to the defence, as observed in case study 1-5 however BFID can add an additional aspect to the evidence that may, in cases where the suspect denies intercourse occurred be essential to supporting the victims account.

1.9.6. Conditional consent

In certain cases, the capacity to consent is not addressed but the conditions stated before sexual activities occurred are. If an individual states they wish to engage in vaginal intercourse however do not feel comfortable with anal intercourse they are conditionally consenting to sexual intercourse. If the partner decided to penetrate the anus then a sexual act without consent has occurred. In other cases oral intercourse may be consensual between partners but not vaginal penetration. If this is known and penetration occurs then consent is removed and a sexual offence has been committed.

Case Study 1-6: EWHC 945, 2013: The complainant stated that following their Islamic marriage her husband exerted substantial control over her, he regularly demanded sexual

intercourse which often included physical aggression. The complainant was submissive to these demands as she felt it was her duty and therefore provided consent. Throughout a year of marriage the court heard a pattern of sexual aggressiveness had become established, and although her husband knew it was reluctant acceptance he concluded consent was given. In February 2010, her husband demanded sexual intercourse with his wife. The complainant knew she did not want any further children and due to medical reasons preventing hormonal contraception and her husband's aversion to condoms, withdrawal was their accepted method of contraception. The complainant stated clearly before intercourse occurred that she would only be happy if he withdrew and did not ejaculate. After intercourse began her husband told her that he would ejaculate inside her "because you are my wife and I'll do it if I want". The results of these events were that complainant became pregnant [60].

If conditional consent is provided on the basis that ejaculation does not occur or a condom will be worn the application of BFID would be beneficial to the prosecution. The identification of semen, where in both situations it should not be present would conclude that the conditions of consent have been breached and therefore was not provided by the victim.

1.10. Drug Related Sexual Assaults

The voluntary consumption of alcohol and drugs has previously been described however drug facilitated sexual assaults (DFSA) are defined as sexual activity that occurred due to the administration of drugs by the suspect. DFSA are classed as pro-active or opportunistic, relating to whether an individual purposefully administers substances such as sedatives to victims with the intention of committing a sexual assault or if while an individual is incapacitated they take advantage of this debilitation and perform sexual acts [61].

The ONS CSEW collected data from 26 forces where alcohol was involved in a sexual offence claim, the results for alcohol related sexual offences can be observed in Figure 1.7. Across all the cases observed over 20% of cases recorded rape of a female over the age of 16

involved alcohol. Values were not much greater than the percentage of male victims observed. It was observed that as the victim age decreased, the less alcohol was involved. This could be due to adolescents over the age of 16 being present in an environment where alcohol is abundant and that children do not have access to alcohol [18].

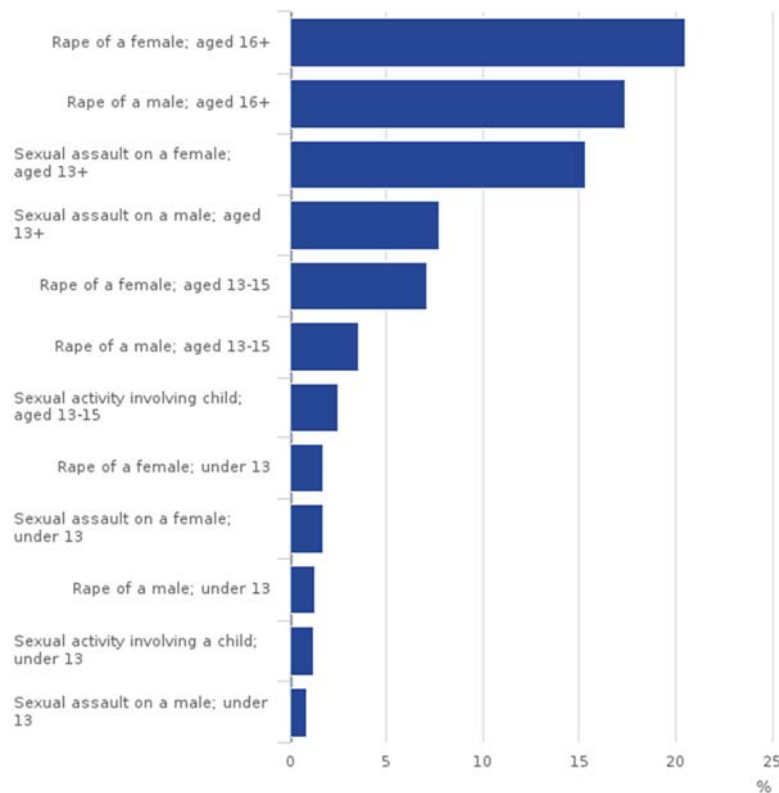


Figure 1.7: Prevalence of sexual assault cases involving alcohol [18].

Many reports of sexual assault involve females that recall being out with other people for a night out and their next memory involves waking up somewhere unfamiliar.

Case Study 1-7: B-Heard survivor's story: A young female, between the age of 18 -25 went out for her best friend's birthday with a group of friends. A space was rented and invites were sent out to a large number of individuals from different academic institutions. The female recalls having one glass of champagne and then states 'everything went black'. She woke up in the morning in an unrecognised apartment with no recollection of the previous evening. She was completely naked and had no idea where she was. She recalled that she was menstruating and was aware that her tampon had been removed and felt general pain

and discomfort especially around her vagina and anus. She woke up to a note wishing she had a good night and to help herself for breakfast. The female felt too scared and ashamed to tell anybody but felt extreme discomfort around her genitals so went to the hospital. An intimate exam confirmed that she had been penetrated in the anus and vagina – with her tampon being removed prior. To all of this the female has no recollection [43].

The lack of any recollection from the victim would mean that support and case severity would be hard to determine. The defence may state that an object was used to penetrate the female, that she penetrated herself while masturbating or that no sexual activity occurred at all. Due to the victim's lack of recollection, although examination showed penetration occurred a jury would have reasonable doubt that rape didn't occur. If BFID could be utilised on her intimate samples that indicated semen presence, then ideally alongside the suspects DNA profile a clear description of the events could be provided to the jury.

In the case of Regina v Weller, self-administer intoxication led to the suspected opportunistic sexual assault of a young female.

Case Study 1-8: R v Weller [2010] EWCA 1085: Emma was a 16 year old female who attended a party with her friend. Emma knew the appellant who lived at the residence of the party. She drank steadily through the night and after realising her friend had gone home and she was unable to contact anyone to take her safely home, decided to stay the night. Emma felt drunk and dizzy and vomited throughout the evening. The appellant was tentative and took care of Emma, after a few visits to her bedroom he began stroking her body and breasts and then inserted his finger into her vagina in such a way that caused a lot of pain. She later managed to leave the house via a friend who took her to be examined upon hearing about the events of the evening. The defendant admitted to taking care of Emma but denied the charges of sexual assault. Fingernail clippings were taken which showed a minor profile contribution matching Emma's reference profile was found on the underside of the fingernail. The defendant, although charged with sexual assault stated there was a number of way the DNA evidence could have got there, including, contact with

the victims vomit, moving/holding her hair while she was vomiting, transferring her into her bed or while placing her in the recovery position and well as picking up her clothes [62].

In this case, the likelihood of the defence's causes for the victims DNA being found was accounted for and deemed unlikely, resulting in a conviction for sexual assault by penetration. This evidence within this case could however have been improved with BFID. If the material under the fingernail was shown to be vaginal material the defence would not be able to suggest the possible reasons they did, with the only reason being penetration of the vagina. It could have been further implemented on the vaginal swabs of the complainant; if a full or partial DNA profile is obtained along with an identification for skin it would further support the prosecution that digital penetration occurred.

1.11. Domestic Sexual Assaults

When a sexual assault occurs within a marriage or a long term relationship the victim may feel they are obligated to have sexual interactions with their partners and therefore not determine the experience as rape. This obligation or feeling that they should serve their partner regardless of their feelings makes it unlikely they will report the incident to police. The home office collected data of all domestic abuse cases that were reported and identified the percentage involving sexual assault, observed in Figure 1.8. This data is based solely on reported crimes and therefore the value is likely higher due to lack of reporting. Domestic violence made up 11% of all recorded cases. Violence against a partner was observed to be the most prevalent offence (33%) however 12% of all domestic violence cases were reported to include sexual offences [63]. Women may not feel an obligation to engage in sexual behaviour with their partner but rather suffer sexual abuse and feel they have no way to remove themselves from the situation. In cases such as this the women may feel they cannot report their abuse and therefore are subjected to re-victimisation.

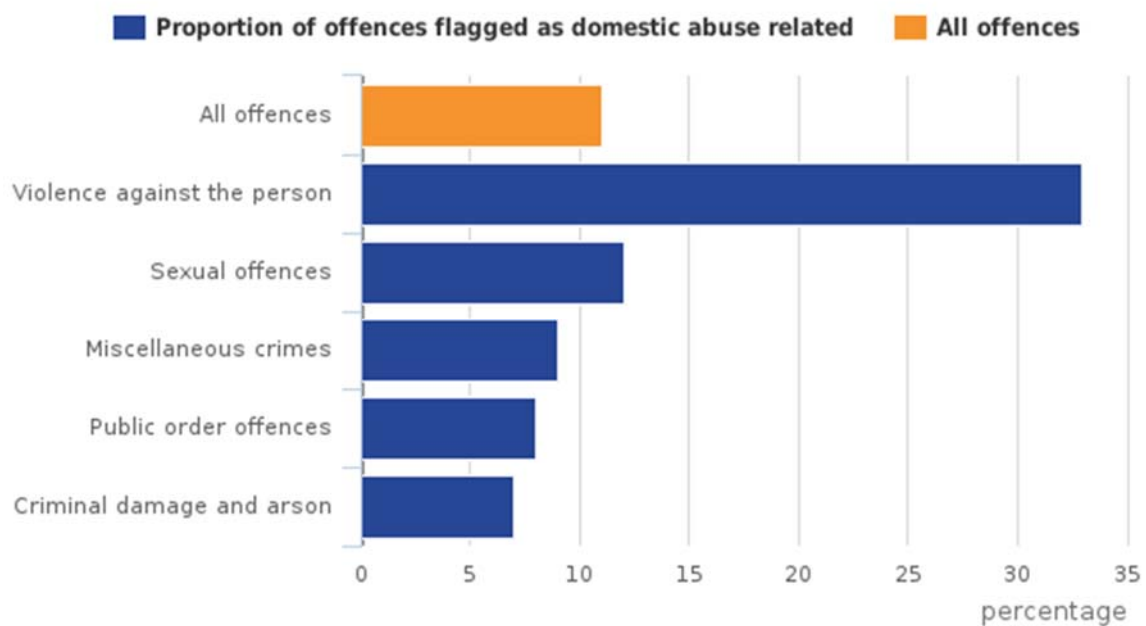


Figure 1.8: Breakdown of reported domestic abuse cases occurring from April 2015 to March 2016. Data reports that 11% of all recorded offenses within that year involved domestic violence (Orange). Of that 11%, 12% involved an aspect of sexual assault [63].

1.12. Sexual Fantasies of Rape

The area of sexual fantasies is extremely diverse; however the area that has proven to be most intangible is that of 'rape fantasies'. The idea that women find pleasure in fantasising about an experience that would be viewed unpleasant by the majority of public opinion has been an interesting topic of research for many psychologists since the 1940s [64, 65]. It is understood in some research groups that fantasies operate in terms of wish fulfilment and therefore on a certain level, females desire to experience this, this has however been disputed in other publications [66-68]. Studies are however deficient due to a multitude of factors; if awareness is made that some woman have erotic rape fantasies it might reinforce an individual's notion that women want to be forced into having sexual intercourse. There is also a stigma attached in revealing these fantasies within a public setting, such that they could be viewed as having something wrong with them [66-68].

Critelli's research group suggested that 31-57% of women have fantasies, on some level in which they are forced into sex, with 9-17% of those women affirming these are frequent

fantasies. Critelli' *et al* correlated data from many studies and found that almost all included the words "overpowered", "forced" and "surrender" in their description of their fantasies [69]. Research by Laumann *et al.* indicated that over 99% of women specified that they no desire to be genuinely raped, saying the idea would repulse and traumatise them [70]. However, women who find themselves frequently fantasising about rape or divulging these fantasies to others, could unintentionally find themselves in situations where they are at a higher risk.

Case Study 1-9: DPP v Morgan (1976). Morgan was a married man with a senior role in the Air Force. He began an evening of drinking with three junior officers, he explained that his wife had rape fantasies and that the men should join him at his home and all have sex with his wife. She would protest and try to resist but this greatly heightened her sexual pleasure and therefore they should go along with the act. The junior officers agreed and all travelled to Morgan's house where they dragged Morgan's wife into a spare bedroom and all proceeded to have sexual intercourse with her. Morgan's wife resisted and struggled throughout the assault and was later admitted to hospital for her injuries [5].

Under the SOA active in 1976 a husband could not rape his wife and therefore he was only charged as an accomplice, the junior officers were however all charged with rape. The junior officers argued they lacked *mens rea*, which at the time was stated as 'at the time the perpetrator is aware that the person does not consent', due to the their senior officers comments on his wife's rape fantasies and that she was consenting even though she displayed physical signs of resistance. The House of Lords accepted that there was a lack of *mens rea* due to their genuine belief however the convictions were maintained on the basis that a correctly directed jury would never have accepted that Morgan's wife had provided consent.

A current case such as this would be addressed differently under the current SOA, a lack of *mens rea* is only acceptable if their mistake is deemed reasonable. In a case such as this the SOA states although the junior officers truly believed they had consent they had not taken the due care to determine if the victim was providing consent [5]. If in cases such as DPP v

Morgan the victim had voiced her excitement over rape fantasies to her husband or her friends it could have provided support to the defence even though she may only have wished them to be fantasies. BFID and DNA profiling would have identified the individuals and the presence of semen but the issue of consent could not be addressed.

1.13. False and Baseless Sexual Assault Reports

When a criminal investigation is launched into an accusation of rape the police can label the case 'unfounded' for either of two reasons, if they believe the claim is false, or if they believe it is baseless [71]. The problem with this system is that, although occasionally interchangeable they carry significantly different definitions. A deliberate fabrication of events by an individual describing themselves as the victim of rape will be reported as false. It is defined as baseless when the complainant's account is truthful but surrounding factors do not meet the legal definition of rape or other sexual offences [72]. In a case where a sexual assault is committed against an unconscious female that results in no forensic evidence that the event occurred, the complainant's allegation would be determined baseless but not deliberately false. Both these issues need addressing; false reports must be assessed correctly to ensure innocent people are not prosecuted and baseless reports must have more efficient techniques for identifying factors of sexual assault.

Case Study 1-10: A woman contacted police about a white van that pulled up beside her and asked if she required a lift, after she entered the vehicle the suspect parked under an overpass, brandished a knife and threatened her with violence if she was not compliant. When the complainant next visited her therapist she spoke of her ordeal and was advised to report her claim. Upon visiting the alleged crime scene with an investigating officer, she was made aware that a security camera was present and would be able to identify the vehicle involved in her complaint. It was at this moment that the complainant admitted to fabricating the incident, stating that when depressed she occasionally initiates sexual encounters with older men and that all of the stated event was consensual [71].

In this case DNA profiling and BFID techniques would have identified semen that matched the profile of the suspect, providing substantial support to the prosecution. The prosecution of this case may have led to an innocent man facing a custodial sentence.

Although it is important to increase the conviction rate of sexual assault cases there is also a need to protect victims of false accusations. In 2012, a report was conducted by the CPS equality and diversity unit, into charges made against false reports of sexual assault and domestic violence. The Director of Public Prosecutions understood the controversies and sensitivities that surround false allegation charges and wished to assess all charging decisions. The Director analysed 159 cases throughout January 2011 to May 2012, 121 of which included an allegedly false rape or sexual assault claim. Of these 121 cases, 105 claimed rape, ten claimed sexual assault and five claimed other sexual offences. The results of these allegations resulted in twenty five charges for perverting the course of justice and ten for wasting police time. 75 cases received no further actions with the remaining cases being dealt with using out of court disposal methods [73].

The report also analysed the age and sex of the suspects, stating that 92% of all the alleged rape and domestic violence cases were female. The report showed that 51% of the 121 rape cases were made by individuals under the age of 21, 8% of those being below the age of 16. In these alleged rape and domestic abuse claims 98% of the victim's attackers were male, with the majority of victims being above the age of 21 [73].

The final factor the report analysed was the relationship between suspects and the individuals they had accused. From the 121 rape allegations 96 suspects had identified a specific individual as the alleged attacker, of these cases, 25 were currently or previously within an intimate relationship, 11 were a family member and 32 were a simple acquaintance. The report's conclusions showed that over half the suspects, who at the time were under 18 did not make the initial allegations, but through a third party. It also states that in the majority of these cases the suspect felt like the investigation had spiralled out of control and they were unable to cease the investigation [73].

The report highlighted many reasons that may cause an individual to feel like they should make an allegation of rape, these include family pressure, mental disability, and embarrassment and/or resentment against another individual, selected case studies from this report are displayed below [73].

Case Study 1-11: A 14-year-old female was in an intimate relationship with a 17-year-old male, the father became aware of this relationship and forbade them from continuing. The male and female had penetrative intercourse, when questioned by her father she stated she did not want to have sex and that he forced himself on her. Her father contacted police to which the female proceeded to give a statement, the young male was arrested and prosecuted for sexual offences against a child. Throughout the investigation police found evidence supporting the defence and therefore re-questioned the young female. She admitted to making a false allegation as to not upset her father however felt powerless to stop the investigation once police were involved [73].

Case Study 1-12: A 20-year-old male, known as Mr W was in a sexual relationship with a female, during a conversation with his mother he claimed he had been raped by another man. A report was made with police through the mother and the individual was arrested and interviewed. He claimed sexual intercourse had occurred however it was consensual. As the investigation continued evidence supporting the defence was found and Mr W was re-interviewed. Mr W then admitted he had been struggling to reconcile with his homosexuality and made the allegations out of depression and guilt over his sexuality. Within this case, Mr W was prosecuted with perverting the course of justice [73].

The cases and data from this report seems to suggest that younger women are more likely than men to make a false accusation, especially against an older male, however in context only 29% were prosecuted for false allegations. The report also compared their data with CPS data for rape prosecutions, during the same time period 5,651 claims of rape were prosecuted in a court of law throughout England and Wales whereas only 35 individuals were prosecuted for making a false accusation of rape. This data therefore suggests that over this time period approximately 0.6% of prosecuted sexual assault cases were false,

highlighting that although false allegations are a serious and sensitive issue, they are minimal in number compared to genuine sexual assaults [73].

The issues surrounding the investigation and prosecution of false reports of sexual assault become even more convoluted when placed in common circumstances such as marriage or family settings

Case Study 1-13: R v Ashley [2010] EWCA Crim 2913: In 2010, the Court of Appeal addressed a difficult case. Mrs Ashley contacted the police and reported she was regularly abused and had been raped by her husband on multiple occasions. Mr Ashley was subsequently arrested and charged with sexual offences. A short period after Mrs Ashley states that although her statement was true, she and her husband had resolved their problems and she no longer wished for him to be prosecuted. The CPS determined that cases of rape were not considered a private matter between domestic partners and therefore continued prosecution. This prompted Mrs Ashley to declare that her initial statement had been fabricated and therefore removed any evidence from the case. Due to Mr Ashley spending time in custody over these false allegations the CPS prosecuted Mrs Ashley for perverting the course of justice, after being charged Mrs Ashley retracted her previous statement and stated the initial allegations of rape were true. The result of her case was a sentence of 8months imprisonment for dishonestly withdrawing a real allegation, known as a double retraction [74].

In cases such as this the victims of sexual assault are further punished for trying to speak out about their experiences. In domestic situations victims can feel loyalty or obligations to their family / partner or face pressure or stigma from others and therefore refuse to report their ordeal or refuse to acknowledge the severity of what happened. Mrs A describes her husband and father of her children as a man who felt he must exert dominance and control over his family, women in these situations become particularly vulnerable and need to be protected by a court of law. The appeal for this case saw Mrs Ashely's custodial sentence replaced with a community sentence.

The cases described above highlight the need for improved techniques in forensic examination and the CPS's processing of false allegation. Ensuring cases that are reported false or are retracted out of fear or pressure can be accurately assessed and victims of rape or false accusations are fully protected. BFID techniques may provide substantial support in correctly identifying false allegation: If an individual states they were sexually assaulted by a male and supporting evidence causes suspicion on the truthfulness of the events, the lack of DNA profile and absence of markers used for identification of body fluids would increase the support of the defence hypothesis that sexual interaction did not occur.

1.14. Summary of Sexual Assault

Sexual assault is a highly prevalent act with seriously damaging effects on the victims. Despite the associated trauma, victims frequently do not report their experiences to the police and justice is not provided. Although reporting rates have dramatically increased there is still a high proportion of victims that are not supported. Sexual assault cases are seen across a widespread population, however the most at risk groups are young females, the mentally and physical disabled and the elderly.

Overall sexual assault can be very difficult to prosecute in a court of law, there are many contributing factors that affect whether a case will even be heard in a court room. In many cases additional information would provide essential support to a victim, be it eye witness accounts or the identification of body fluids present, to fully understand what events occurred without the need to rely on the statements of those involved. The research and development of BFID techniques could provide this additional evidence, resulting in an improvement in conviction rate. An increase in conviction rates may lead to an increase in reporting rates as victims can physically observe this trend and could think they will be successful as well. As previously described, a tenable link was suggested from the investigations into high profile cases and an increase in reporting rates, therefore this would likely be seen again if conviction rates were to also increase [12,14].

DNA evidence has been available for use in the courts since 1986. Its first implementation was to exonerate a 17 year old male accused of a double rape-murder charge. The first conviction using DNA evidence was in America, following a DNA match from a semen sample and the suspect's blood sample in a rape case [75, 76]. Although the technique has improved it is still the same evidence and even though a suspect can be identified, it only places them at the scene. A simple defence argument can transform the weight of DNA evidence, for example, the suspects DNA found in the vaginal canal is from epithelial cells from digital penetration not from semen. This would be hard to prove without BFID. If offenders as well as the prosecution and defence were aware of the existence of BFID it may limit the variability of the defence hypothesis but ideally make potential perpetrators aware of the increased capabilities for building evidence and thus not commit the offence.

2. Forensic Applications for Body Fluid Identification

2.1. Forensic Scientists

It is the obligation of the forensic scientist to interpret data objectively and to form and state a hypothesis based on the results obtained from crime scene stains. Many forensic scientists may analyse samples for a case and it is important they remain unbiased and impartial. It is their role to provide evidence that can support either the defence or prosecution; they cannot however address consent and therefore stating whether charges of rape or sexual assault should be applied is impossible. Any information such as DNA profiling or BFID obtained from samples can only be presented to support a hypothesis, be it defence or prosecution [77]. Forensic evidence is therefore essential in determining the *actus reus* but not the *mens rea*.

2.2. Components of Body Fluids Often Located at Crime Scenes

2.2.1. Whole blood

Analysis of bloodstains is a key component to many crime scenes, utilising techniques including DNA profiling and blood pattern analysis (BPA). These techniques are constantly evolving with continuous research into age estimation, degradation or obtaining valuable information about the individual who deposited the stain [78, 79]. Human whole blood is composed of four major components, red blood cells (RBCs), platelets, plasma and white blood cells (WBC). The normal presence of these components in 1 microlitre of blood is approximately, 5,000,000 RBCs, 150,000 -350,000 platelets and 5,000 - 10,000 WBCs [80, 81].

2.2.1.1. Plasma

Plasma is the largest component of whole blood, making up approximately 55%, its major component is water (92%) with the remaining 8% composing of dissolved proteins and <1% solutes. The plasma has many roles including maintenance of blood pressure and body temperature as well as transport of RBCs, WBCs, platelets and dissolved resources such as glucose, minerals, salts, vitamins, CO₂ and hormones around the body. The dissolved

proteins consist of albumin – involved in blood cleansing and maintaining fluid levels, coagulation factors – proteins involved in the immediate clotting and immunoglobins – antibodies to target infectious foreign material [81, 82].

2.2.1.2. Red blood cells and platelets

RBCs known as erythrocytes comprise approximately 45% of whole blood, they are distinguishable by their biconcave disc shape and their absence of nucleus and mitochondria. This absence means that processes involved with DNA replication do not occur within these cells and therefore different maintenance mechanisms are employed [81]. Platelets known as thrombocytes are small fragments of bone marrow cells responsible for the transportation of specific clotting chemicals. They compose <4% of total whole blood volume and due to their fragmented nature do not contain a nucleus or genetic material [81, 82].

2.2.1.3. White blood cells

WBCs known as leukocytes are the smallest contribution within whole blood, making up <1% of total volume [82,83]. WBCs are large cells that house a nucleus but no haemoglobin. Despite their limited presence their role within blood is essential for healthy biological processes. Three classes of WBC exist all responsible for defending the human body from pathogens through various immune responses.

Granulocytes are the most abundant WBCs composing 50-80% of all WBCs, and are the primary cells to arrive at an infected site, the defence mechanism involves phagocytosis, in which the pathogen is engulfed by the WBC and destroyed. The second immune response is controlled by Lymphocytes which secrete antibodies to defend against foreign material and contribute 25-33% of all WBC with the final class of WBCs, composing 4-8% of total WBCS being monocytes. Despite their low abundance they are the largest of the WBCs and can identify antigens that need to be destroyed by lymphocytes or perform phagocytosis on foreign material [81, 83].

2.2.2. Vaginal material

The human vagina has a beneficial relationship with many microbes such as *Lactobacillus* however it is continually prepared for processes involved in reproduction and the defence from a large range of microbial pathogens. Invading pathogens can lead to infections such as bacterial vaginosis or thrush, heightened susceptibility to STI's, various cancers and even fertility problems [84, 85]. Regardless of this continual battle with microbial pathogens the occurrences of these symptoms is reasonably infrequent due to the efficiency of the vaginal tract's defence mechanisms. The most efficient mechanism to deter colonisation by external microbes is a mixture of intense sloughing of squamous epithelial cells that may have adhering bacteria, the competition for nutrients and reduction in pH provided by the already colonised commensal microbes and the hydration and cleansing of the cervix and vaginal areas by cervical-vaginal fluid (CVF) secreted from the glands within the cervix and vagina. The volume of CVF present within a sexually un-stimulated vagina is reported at around 4mL [84, 85].

Submucosal glands neighbouring the cervico-vaginal mucosa and plasma cells control the immune response within the vagina, secreting antimicrobial proteins/peptides (AMPs) such as defensins and lysozymes, immunoglobulin A and G (IgA, IgG) as well as neutrophils and eosinophils. All of which are essential in the immunological defences that protect the human body. Antimicrobial proteins derived from epithelial cells also provide additional defence to destroying foreign pathogens [84, 85]. Forensic vaginal swabs will therefore have a high abundance of epithelial cells and proteins involved in the immunological response. The issue that saliva is very similar in appearance to vaginal material is also apparent on a cellular level due to both body fluids requiring immunological defence of foreign microbes.

2.2.3. Menstrual blood

The human female undergoes a continuous 28 day reproductive cycle in which an ovum is prepared for fertilisation. Following ovulation at day 14 the uterus forms a protective lining known as the endometrium in which a fertilised ovum can be implanted. The endometrium

consists of a collection of compact epithelial cells, connective stroma and various glands, these align to form a basal layer, a transitional spongiosa layer and a dense epithelial layer. The spongiosa and epithelial layer are connected via fibrous tissue and small blood vessels that would provide a supportive environment for fertilised ovum growth [86, 87].

When an ovum is released and not fertilised there is a decline in progesterone production, this causes vasoconstriction of the blood vessel which results in apoptosis. The destruction of the blood vessels causes blood to enter under the compact epithelial top layer causing inflammation and further rupturing of the endometrium. Anticoagulant factors such as plasmin are activated by protease to prevent clotting while matrix metalloproteases (MMP) breakdown the stroma for release during menstruation. This is followed by immediate construction of extracellular material and stroma for building the endometrium for the next cycle. Menstrual blood therefore originates in the uterus and consists of three main components; the single celled unfertilised ovum, the endometrium and blood. The endometrium is reasonably fibrous and therefore is the bulkiest component however is in the minor component when compared to blood. As the endometrium sheds from the uterus wall the small blood vessels travelling across the endometrium are broken and release the contained blood into the uterus. Menstrual blood then travels through the vaginal canal and combines with all the components of CVF [86, 87].

The proteins involved in menstruation have protective and destructive roles and are therefore possess the most dynamic mechanisms compared to other body fluids. A proportion of proteins are responsible for the preparation of implantation by a fertilised ovum followed by supporting its development. If implantation does not occur enzymes responsible for apoptosis and immunological responses are activated to ensure the destruction of all material so that fresh material can be prepared for the following cycle [86, 87]. The expression of protein in all tissues and organs is a result of the current environment and functional needs, since the menstrual cycle involves structural and hormonal changes almost daily, the regulation of gene expression must be tightly regulated.

2.2.3.1. Comparison of venous and menstrual blood

Venous blood is comprised of RBCs, WBCs, platelets, haemoglobin as well proteins such as fibrin and molecules such as glucose. Since blood fills the endometrium these same components will be present in menstrual blood, however menstrual blood will have the addition of a large volume of epithelial cells, the stromal basal lamina biomaterial and various glands. The primary function of venous blood when outside the blood vessel is to form a protective clot, whereas as high number of anti- coagulation factors are present within menstrual blood to ensure removal from the uterus. Overall although similar in appearance the genetic material content within menstrual blood is significantly higher than in venous blood with an abundance of protein regulated processes [81, 82, 86, 87]

2.2.3.2. Factors that affect the menstrual cycle

A regular menstrual cycle is a dynamic and continuous change within a female's body controlled by multiple hormones. It involves dynamic changes in the immune cell population to ensure simultaneous destruction and remodelling of cells and blood vessels. There are however many internal and external factors that can cause physiological changes to the frequency, composition and flow rate. The main processes of the menstrual cycle can be seen in Figure 2.1. The cycle is controlled by 4 main hormones; luteinising hormone, follicle-stimulating hormone, oestrogen and progesterone [88].

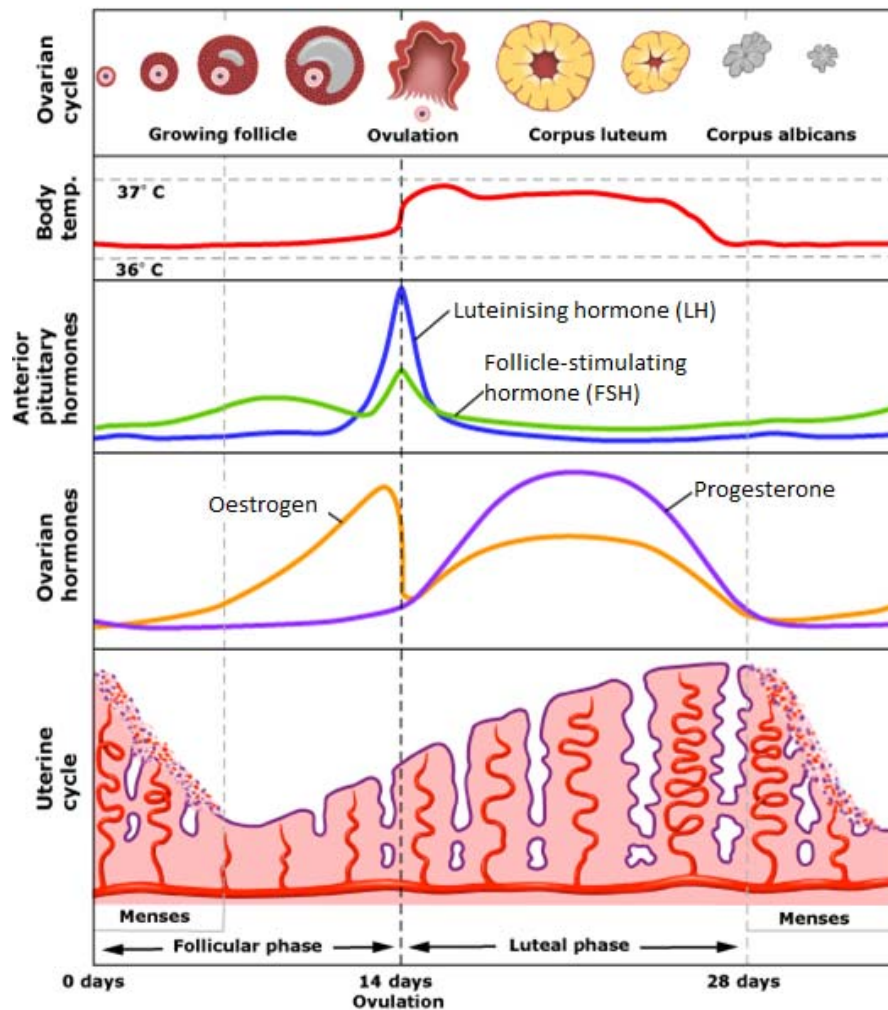


Figure 2.1: Mechanisms and hormonal fluctuations over a standard menstrual cycle in a healthy female [88].

2.2.3.2.1. Age, weight, ethnicity and lifestyle

The menstrual cycle is not a synchronised event that occurs with all females, variations in regularity, flow rate and duration occur across all individuals. It can therefore be suggested that internal and external factors have a strong role in the regulation of the menstrual cycle. Liu *et al.* performed a study analysing the menstrual cycles of 309 women in the age range of 20-44. On average the females provided data for five menstrual cycles, with mean cycle length (MCL) analysis being performed on the metabolites of menstrual hormones present in urine. The results discovered that on average the MCL of women over 35 was reduced by

approximately 1 day (usually observed in the follicular phase). They also discovered that Asian females had a MCL that was approximately two days longer than those observed in Caucasian females. The data also indicated that alcohol (a minimum of 1 drink per week) had a reducing effect on MCL in younger women while physical exercise minimally increased follicular phase and therefore mean cycle length in the majority of women. The only factors where no observable difference in MCL occurred were BMI and smoking habits [89].

2.2.3.2.2. Current available contraception

Research states that in 2012, approximately 645 million females in developing countries were utilising some form of contraception [90]. There are many reasons females may wish to take contraception such as pregnancy prevention, aiding skin issues such as acne, reduce/regulate heavy or painful menstruation and manage conditions such as iron deficiency anaemia by reducing menstrual flow. Various contraceptive methods have also been linked with considerable decreases in likelihood of ovarian and endometrial cancer [91, 92]. The type of contraception available are dependent on a female's health and family history as well as her personal preference.

2.2.3.2.3. Hormonal contraception

The most popular form of current contraception is the combined oral contraceptive pill (the pill), with current use estimated at above 60 million females worldwide [92]. Ingested daily it releases artificial forms of oestrogen and progesterone into the body [93, 94]. Oestrogen inhibits FSH, which is responsible for releasing of an ovum, the increase of this hormone therefore inhibits the body from releasing an ovum that has the potential to become fertilised. Progesterone is responsible for the thickening of the endometrium however an increased presence will not trigger this regulation and a thinner endometrium will be produced thus reducing menstrual volume, progesterone also causes thickening of the mucus within the cervix and therefore create a barrier in which spermatozoa cannot pass.

The full advantages and disadvantages of hormonal contraceptive are not comprehensively understood with contradicting research on the increased risk of cancers, largely focused on

oestrogen and the link to breast cancer [92, 95, 96]. For women categorised as high risks for breast cancer a progesterone only pill or non-hormonal contraceptive is available. The two other widely used contraceptive methods are the contraceptive implant, a thin tube inserted into the arm to release hormones and the contraceptive injection (Depot) a large dose of hormones that circulate the body for approximately 12 weeks. Both forms releases progesterone and therefore manage the menstrual cycle as the progesterone only pill would [93, 94].

The menstrual flow and heaviness will vary between individuals and different contraception. From a survey of 50 females on various forms of hormonal contraceptive the menstrual cycle can completely cease, become continuous but light, reduce from heavy flow to light bleeding, become extremely irregular (bleeding once every 2 or 3 months compared to every 28 days) or become regular but very heavy. Since these contraceptives contain the same hormones it is the individuals body that causes these varied changes and therefore expression of regulatory mechanism may change. Fluctuations in expression would also be expected due to introduction of artificial hormones which may cause suppression of the regulatory mechanisms employed by the body for a 'normal' menstrual cycle. Overall menstrual blood from females using hormonal contraception will likely not contain an ovum and have a more fluid viscosity due to a reduction in endometrium.

2.2.3.2.4. Non-hormonal contraception

Females who do not wish to use methods involving artificial hormones are offered hormonal free alternatives. An intrauterine device (IUD) can be inserted into the female's uterus, IUDs utilise the spermicidal effects of copper and is manufactured as a copper coil or wrapped around T-shaped plastic object. The IUD contains copper which increases abundance of copper ions, prostaglandins and WBCs within the uterus and fallopian tube fluid, this causes an immunological response that destroys any semen that are present. The uterus also detects the IUD as a foreign body and therefore increased phagocytic WBC activity is present within the stroma and epithelium of the endometrium. This continual destruction of the endometrium ensures if a fertilised ovum is created it cannot be

implanted. Menstrual blood within females using IUDs tend to be heavier and darker in colour, this is likely caused by the high abundance of WBC but also the increased RBC and cellular debris caused by apoptosis of the cells within the endometrium [97].

2.2.3.2.5. Diseases

Endometriosis is a reasonably common benign gynaecological condition affecting around 1 in 5 women. It is present during the reproductive periods of the female's life and involves endometrium-like tissue forming on the outside of the uterus and fallopian tubes [98]. The disease is recorded to increase the 'heaviness' of menstrual flow and bleeding in between normal menstruation. Blood obtained from the vaginal canal when menstruation is not actually occurring would need to be analysed to assess whether the additional components of menstrual blood are present. Research suggests that immunological response cells present within regular endometrium are highly dysregulated in individuals suffering from endometriosis [98].

2.2.3.2.6. Cessation of menstrual cycle

Many factors cause a cessation of the menstrual cycle, as described some hormonal contraceptives are responsible; however, the process of human pregnancy also removes the requirement for preparation as the uterus adapts to protecting and providing nutrition to the developing baby. The menstrual cycle can also be affected by mental and physical conditions, eating disorders are highly prevalent in adolescent females, with approximately 40% of cases consisting of females aged 15-19 [99]. Eating disorders can have very harmful effects not only on mental health but regularly cause severe problems with physical health. Vale *et al.* analysed 62 adolescent females with diagnosed eating disorders. Their results showed 21 females (34%), the majority comprising of bulimia and anorexia disorders displayed irregularities or disruption of the menstrual cycle; seven participants experienced irregularity where menstruation was still present but unpredictable and varying flow rate while secondary amenorrhea, the complete lack of menstruation for a period longer than three months was observed in the other 14 females. 22% of the females diagnosed with

eating disorders experienced complete cessation of their menstrual cycle with uncertainty that a regular cycle would return [100].

In summary, a 'normal' menstrual cycle is dynamic and involves many processes that require strict regulation. Flow rates and frequency vary between individuals however the introductions of factors such as disease, contraception and pregnancy can have massive effects on the composition, duration and flow rate of menstrual blood and therefore expression of regulatory factors must be taken into account when validating a BFID marker for menstrual blood.

2.2.4. Saliva

Salivary fluid is secreted from the salivary or oral mucous glands with the objective of lubricating the mouth and ingested food for swallowing. Saliva is composed of around 99% water, within this solution are many enzymes, immunoglobulins, antimicrobial factors, glycoproteins and traces of albumin there is also an abundance of minerals such as potassium, sodium, calcium, magnesium and phosphates. All the proteins and minerals present ensure a healthy oral cavity, capable of defending against infection and foreign antigens [101]. There are many salivary glands within the oral cavity, all with various roles and differing enzyme abundance and activity. Some salivary glands such as the parotid and submandibular glands secrete a higher volume of saliva when stimulated by food ingestion and mastication therefore their role is not focused on lubrication or oral health but the initial digestion of food. This is performed by the secretion of α -amylase, an enzyme responsible for breaking down starch within food products. α -amylase makes up around 50% of all proteins synthesised by the salivary glands, enzyme activity is inhibited by the acidic conditions throughout the gastrointestinal tract and therefore its presence is only located within the mouth making it an ideal protein in salivary identification techniques [101, 102].

Whole saliva is a mix of all fluids secreted by the various salivary glands, mucous, food debris, epithelial cells and oral bacteria. Buccal swabs obtained from individuals will contain a combination of all these fluids and factors however the proteins being secreted at the time of collection will fluctuate dependant on if the participant has a healthy mouth, an

infection or has been eating. If eating has recently occurred there will be a higher volume of α -amylase in the sample however if the participant has a mouth infection a range of immunologic proteins may be present. Immunoglobulin A (IgA) is the most abundant immunologic protein secreted by the gingival fold salivary gland, this protein along with the less abundant IgG and IgM destroy harmful bacteria and viruses to preserve oral health. Medication such as antidepressants and antihistamines have also been shown to alter the composition of salivary fluid [101, 102].

2.2.5. Semen and seminal fluid

Post pubertal males create sperm cells within the testes which are then matured in the epididymis. The epididymis secretes potassium and sodium to supply energy to the spermatozoa. Once matured they travel through the vas deferens where they are mixed with components to form semen. Human semen is a combination of components that have been synthesised by various glands such as the vas deferens, seminal vesicle, the prostate and the epididymis, the major components are proteins, lipids, carbohydrates and peptides. During ejaculation the components in the initial ejaculate are not thoroughly mixed and are therefore not a completely homogenous mixture [103, 104].

Up to 75% of semen is secreted by the seminal vesicles, which incorporates a large volume of fructose and antioxidants. Fructose ensures the spermatozoa have a sufficient energy supply while the antioxidants provide protection from oxidative substrates. Approximately 20% is secreted by the prostate gland, consisting of the protein acid phosphatase, calcium, magnesium and coagulation/liquefaction enzymes. The remaining components consist of spermatozoa and proteins secreted from the bulbourethral gland when sexually stimulated [103, 105].

Research by Fung *et al.* aimed to identify the peptide and proteins that made up seminal fluid, they analysed 5 human semen samples using two-dimensional electrophoresis (2DE). Their conclusions stated identification of over 100 different proteins and peptides in a healthy human semen sample [104]. Research by Oliva *et al.* attempted to characterise the proteins involved with in the spermatozoa and seminal fluid, their research observed that

approximately 50% of the proteins identified were responsible for providing the spermatozoa with energy. This is expected as the spermatozoa is required to travel a large distance through hostile environments to reach the ovum, if the energy source is depleted reproduction cannot occur. Oliva *et al.* research also observed there was very little proteome variation between participants that were noted as highly fertile, whereas in participants known to have low spermatozoa count, many of the proteins were either increased or completely absent [106].

2.2.5.1. Semen persistency

The survival on semen within the vaginal canal following sexual intercourse is dependent on many factors. These include the stage of the menstrual cycle, the fertility of the spermatozoa, and the health of the environment within the vagina [107].

Following sexual intercourse the spermatozoa quickly move throughout the vaginal canal with some of the spermatozoa reaching the site of fertilisation in approximately 5 minutes. A female's cervix contains a dense mucous plug which the spermatozoa must pass, this plug varies in composition throughout the menstrual cycle. Spermatozoa will pass through the mucous plug with least resistance when the female is ovulating. Cervical mucus is comprised of a long chain of mucoproteins that have a highly variable level of cross-linkage between the chains. The thickness of the mucus is altered by the number of cross links which in turn is controlled by hormonal presence. Mucus in the follicular phase is sparse but very thick, the increase in Oestrogen then reduce the cross-linkage and reduces the viscosity [107].

Secreted by glands on the cervical canal wall, the cervical mucus forms strands which direct the majority of spermatozoa towards the canal wall. Once incapacitated on the wall the flow of CVS ejects the spermatozoa from the vagina. Regardless of the capability of the vaginal canal to eject spermatozoa, fertility and viability spermatozoa can persist in the vagina with some research suggest motile spermatozoa can be identified up to 8 days after ejaculation [107]. The degradation of spermatozoa in the female reproductive tract is also due to phagocytic activity from neutrophilic leucocytes and mononuclear cells. Phagocytosis of

spermatozoa occurs in both the vaginal and cervical fluids, with components such as the sperm head and tail being observed within the cytoplasm of these cells [110].

Wilson *et al.* studied the forensic benefit to ascertaining the persistency of spermatozoa within the vagina. The results showed every vaginal swab obtained prior to 9 hours following UPSI were positive for spermatozoa. A steady decrease in positive results as well as number of spermatozoa present was then observed as the time interval increased. Their results did indicate that the location of sampling could have an effect on positive identification [108].

The quantity of spermatozoa identified on vaginal swabs was much higher than those identified on cervical swabs on fresh swabs. The quantities did not become equal until approximately 24 hours had passed. When 48 hours had passed their results showed an increased chance of identifying spermatozoa on the cervical swab compared to vaginal swabs. They concluded their study by showing positive identification in the cervix and the vagina up to a period of 10 days following UPSI, with a more complete spermatozoa usually being found on the cervical swabs [108].

A study performed by Davies *et al.* obtained samples from female donors within their laboratory. Cotton-wool swabs were inserted as high as possible at pre-determined intervals following unprotected sexual intercourse (UPSI). Their sample number was limited and obtaining samples passed 96 hours proved difficult however their results showed a positive identification of spermatozoa on all samples prior to 30 hours, limited negative results prior to 48 hours and a frequent trend in negative results up to 96 hours. They noted their last positive results was at 144 hours. From time points 90 to 156 hours, only 34% of swabs gave a positive identification for spermatozoa. Motile spermatozoa were commonly found in samples up to 16 hours but decreased rapidly after this time point [109].

One of the more comprehensive semen persistency studies was performed by Willott *et al.* They assessed the presence of semen on 2410 casework swabs, of which 1332 were internal vaginal swab. Murder victims were not included in the study therefore all samples were from living victims. The method for identification was visual, utilising haematoxylin and

eosin staining on microscope slides. The classified the quantity of spermatozoa present into many in every field (++++), many in most fields (+++), some in some fields (++) hard to find (+) few and zero. The presence of tails was also noted. 6% of swabs came back +++++, 29% between +++ and ++, 22% between few and + and 43% with zero. The longest duration a spermatozoa was identified was 120 hours, this fell to 26 hours when recording presence of tails [110].

Willott *et al.* found discrepancies in results when comparing to studies by Davies and Wilson. The results obtained from this study showed a large proportion of case work swabs did not show any spermatozoa when taken three hours after the alleged UPSI compared to the 100% of positive samples identified in Davies and Wilson's studies. Willott was however studies case work samples, some of which may not always contain semen. Despite the variation to other literature positive identifications were made on a number of swabs from different areas, the longest time duration after UPSI for positive identification can be seen below in table 2.1 [110].

Location	Persistence
<i>Internal vaginal swabs</i>	120 hours
<i>External vaginal swabs</i>	120 hours
<i>Rectal swabs</i>	65 hours
<i>Anal swabs</i>	46 hours
<i>Oral swabs</i>	6 hours

Table 2.1: Persistency of semen found from various intimate swabs [110].

Overall the literature shows various time durations following UPSI for positive semen identification, with no research showing 100% positive identification passed the initial 48 hours. Positive identification was however seen following 120-156 hours suggesting that likelihood of spermatozoa survival will vary in each sexual assault case. An improved technique for the identification of spermatozoa may improve the number of positive identifications made as well as the length of time they can be detected.

2.2.5.2. Azoospermia

Azoospermia is a condition in which spermatozoa are not present in ejaculate [111]. It can be caused by an obstruction in the vas deferens, such as inflammation or a failure of spermatogenesis. Spermatogenesis is strictly regulated by a large number of genes which control spermatozoa development and transport, with research showing that around 29% of cases of azoospermia involve genetic abnormalities such as chromosomal/ gene deficiencies or epigenetic modifications [111].

2.3. Current Body Fluid Identification Techniques

Biological samples of various body fluids collected and then analysed from crime scenes are the foundation for forensic investigations. The samples provide vital evidence through the technique of DNA profiling. Profiling analyses the number of short tandem repeats (STRs) are specific loci to provide a DNA fingerprint that, if excluding monozygotic twins has an extremely low likelihood of completely matching another individual. Close genetic relatives such as siblings and parents will share many common alleles however the large number of loci analysed in current DNA profiling techniques ensures the differences between closely shared profiles can be identified [112]. The profiling of the biological samples can identify individuals that participated in a crime but can also be used to exonerate and thus is often key evidence in a court of law. DNA profiling cannot however provide information as to the events that occurred during a crime and thus a robust technique is needed that could provide this data [113, 114].

The determination of the type and source of body fluids located at crime scenes allows for forensic scientists to reconstruct the actions that took place by providing a connection between the body fluids identified and the statements of the complainant and alleged perpetrator. The presence of certain body fluids can be used as indicators of the sequence of events which occurred. For example, venous blood stains can indicate a violent or forceful altercation that could be supportive of a prosecution hypothesis in a murder case; just as

the identification of semen or vaginal material would be suggestive of a sexual act occurring [115].

The most frequent body fluids identified by scene of crime officers (SOCO) are venous blood, semen, saliva, vaginal material, urine, and sweat [116]. In cases involving sexual assaults the likelihood of vaginal material and semen presence is very high. When a SOCO locates a possible body fluid stain, chemical or visual methods are utilised to determine which body fluids are present. Presumptive tests are performed to provide a preliminary identification of the source of the body fluid stain with further confirmatory tests, if available, conducted by scene of crime DNA analysts in a forensic laboratory [114].

Certain BFID techniques have been utilised since the beginning of the 1900's with many of the same presumptive techniques and protocols still being used in current forensic laboratories [114]. Development of techniques for BFID include catalytic activity of specific enzymes, visual and chemical test, immunological testing and spectroscopy. The problem with the majority of these techniques are they are only presumptive and therefore only provide an indication of the body fluid source, confirmation of BFID can only be achieved in tests such as the physical observation of spermatozoa under a microscope [114]. Presumptive techniques tend to become unreliable in degraded or low volume samples suggesting low specificity and sensitivity. Samples obtained from crime scenes are valuable and in most cases cannot be obtained again if needed, it is therefore imperative that identification techniques do not waste value sample during analysis, this is however the case in many presumptive BFID techniques.

2.3.1. Catalytic presumptive tests for body fluid identification

2.3.1.1. Identification of blood: Leucomalachite Green and Kastle-Meyer

Catalytic methods are founded on enzymatic activity which catalyse reactions of numerous substrates which create observable changes in colour. A variety of catalytic tests are frequently utilised to screen blood samples providing a presumptive identification. This is achieved by analysing the peroxidase-like activity of the haem group [113, 114]. Red blood

cells are saturated with iron rich haemoglobin which binds to oxygen and transports it around the body. The haem group present on the haemoglobin molecule catalyses the degradation of hydrogen peroxide (H_2O_2). This process produces an oxidising molecule that causes a colour altering reaction when interactions occur with other substrates. Two common substrates used are leucomalachite green (LMG) and phenolphthalein (Kastle-Meyer) which produce a green and pink colour change respectively. If the sample contained haemoglobin the addition of the leucomalachite or phenolphthalin reagent and H_2O_2 will result in a colour change and a positive identification for blood. Although very popular it only provides a presumptive identification and is known for displaying false positives when substances such as rust, grass or vegetable peroxidases are also present within a sample. The advantages of LMG and Kastle-Meyer is that analysis is instantaneous, LOD is 1: 10,000 and although requiring part of the sample to test it does not damage the remaining sample so DNA profiling can be performed. This technique cannot however differentiate between venous and menstrual blood [113, 114].

2.3.1.2. Identification of semen: Acid phosphatase

The most frequently used identification technique for semen is the acid phosphatase (AP) test. This test focuses on the activity of enzyme AP due to its high abundance within semen. Secreted by the prostate gland the activity of AP is approximately 1,000 greater in semen compared to other body fluids. The catalytic activity of AP results in colour changes by the hydrolysis of phosphates which react with reagents such as Brentamine Fast Blue to produce a violet colour. The limitations to this method include cross reactivity due to presence of AP within body fluids including vaginal material, low sensitivity and sample usage [114].

2.3.1.3. Identification of saliva: alpha-amylase and Phadebas® test

Identification of saliva focuses on alpha amylase, an enzyme that is highly abundant within saliva. This test involves the addition of iodine which reacts with any starch present in the saliva to create an intense violet colour, the enzyme hydrolyses polysaccharides such as starch into many sugar molecules. The result is the reduction of intensity of any colour

observed due to the hydrolysis of the starch. Another catalytic method for identification of alpha amylase is the Phadebas® test, instead of iodine this test incorporates amylopectin-procion red to cause a colour change due to starch hydrolysis. The advantages to these methods are a relative high sensitivity, low cost and quick analysis time. There are limitations in that as with most catalytic tests, cross-reactivity to substances such as urea, faecal matter or moisturisers create false positive [114, 117].

2.3.2. Protein analysis/ Immunological techniques for confirmatory body fluid identification

2.3.2.1. Identification of blood: HemaTrace test strip

The specific reaction between antibody and antigen is the foundation for the majority of immunological testing. The detection of blood using the HemaTrace test strip is based on the same haemoglobin molecule used in LMG and KM. If present within a sample, the haemoglobin will react and combine with anti-human haemoglobin antibody that resides within the HemaTrace test strip. The test strip contains a membrane which is permeable to the antibody-antigen complex, once permeated it is collected. The more antibody-antigen complex that travel through the membrane and accumulate the more intense the colour change will become resulting in an intense pink colour observable within the test site. The HemaTrace test strip has shown popular due to its lack of cross reactivity, almost immediate analysis, its portability for use by SOCO or DNA analysts and its high sensitivity [114].

2.3.2.2. Identification of semen: prostate specific antigen testing

Identification of semen can be performed through staining and visualisation through a microscope, a more frequently utilised confirmatory protocol focuses on the identification of prostate specific antigen (PSA) [118, 119]. As with AP it is abundantly produced and secreted by the prostate gland. The detection is shown by an observable line along the test strip in which the anti-human PSA antibody has adhered to the PSA within the sample and travelled the length of the test stripe. It has the same advantages as the HemaTrace test strip however it has the added advantage of semen identification that does not rely on the presence of spermatozoa [11, 119].

2.3.2.3. Spectroscopy techniques for body fluid identification

Current research into the use of attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy has shown promising data into the identification of number of body fluids. The technique produces a spectrum for each sample, within this spectra are peaks which are characteristic of vibrations within structural bond of biological samples. Comparing the spectra to reference body fluids allow for confirmatory identifications to be made [116].

Orphanou *et al.* analysed samples of venous blood, semen, saliva and vaginal material using ATR FT-IR. Five samples per body fluid were analysed with a reference spectra being obtained for all four body fluids. Each spectra displayed dominant peaks that could be correlated to known proteins within that fluid, these peaks were significantly different enough to distinguish from the other three body fluids. Although body fluids such as menstrual blood, azoospermic semen and skin were not analysed this method proposes a very promising non-molecular technique for the identification of body fluid. Its application into a forensic lab would require new equipment and lab space, training of new technicians and analysts and a completely separate protocol to DNA profiling however if validated on a full panel of body fluids could be a useful technique to forensic scientists [116].

2.4. Limitations of Current Body Fluid Identification Techniques

2.4.1. Absence of semen

The inability to determine the presence of spermatozoa in samples taken from victims of sexual assault can be explained by a number of factors; a prolonged post coital interval, use of spermicidal solutions, digital penetration or use of a condom. In these cases semen is destroyed or was never present. In cases where the male is azoospermic or has been vasectomised and in some instances where penetration but not ejaculation occurred, semen will be present but the spermatozoa will be absent [111]. In these instances DNA profiling is highly unlikely to provide a useable profile and testing for spermatozoa will

come back negative thus providing support to the defence. In cases such as these the identification of seminal fluid would support the statements that declare sexual intercourse occurred and provide the courts with clearer evidence on the event that occurred. It is important that a technique is available that can identify the presence of seminal fluid without the need for spermatozoa being present.

2.4.2. Menstrual blood vs. venous blood

Sexual assaults can often be violent in nature, such that bleeding injuries occur. In cases where penile swabs from a suspect show the victim's blood, the origin is very important to assigning case level. The aforementioned sections show just how easily sexual assaults can occur and how important it is to obtain correct evidence. In cases where a female victim states sexual assault has occurred, an intimate exam will be performed on her as well as any possible suspects. If blood is found it may show support that a violent assault occurred and thus support the prosecution, however the suspect may state that consensual intercourse occurred while the female was menstruating. These contradicting reports would not be useful in a court of law. The utilisation of a technique that could differentiate these two fluids within the sample would show an increase in support to either the defence or prosecution hypothesis. Alternatively, if a female is assaulted while menstruating, identifying venous blood within a mixture of menstrual blood would support the prosecution hypothesis.

Case study 2-1: LGC reporting officer 2015: An individual was arrested on suspicion of murder. Analysis of his car showed a blood stain in the front seat that matched the victim's DNA profile. The defendant claimed the victim had been sat in his front seat and started menstruating, thus leaving the blood stain when she left the defendant to go home

In this case the differentiation of venous blood from menstrual blood would provide vital evidence into the validity of the suspect's statement. A BFID technique that confirmed an absence of menstrual blood would show the blood within the car came from an open wound on the victim,

2.4.3. Differentiating vaginal material from saliva

As stated, sexual assault is not solely defined as vaginal penetration from a penis. It is important to determine body fluid origin to assign correct case level. In cases where the complainant's DNA is found on a suspect's penile swab, the DNA evidence from that would indicate that the suspect's penis came into contact with the victim. Identification of body fluids would allow for a greater description of the events to be determined. The complainant in this case may suggest she was vaginally sexually assaulted, while the suspect may claim he only received consensual oral intercourse. Both hypotheses would result in the victim's DNA being found on a penile swab. If a technique could be used to differentiate vaginal material and saliva the origin of the body fluid present on the penile swab could be identified and thus show strong support to either the complainant's or the suspect's version of events.

Alternatively, in a case where non-consensual oral intercourse occurred, the defence may state it never occurred or if the complainant's DNA profile from a buccal swab is identified suggest it was consensual or from her saliva while kissing. The use of a BFID technique that could identify vaginal material from a buccal swab would confirm the suspect gave false information and provide support to the prosecution.

2.5. Current and Emerging Molecular Based Techniques for Body Fluid Identification

The human body is composed of approximately 210 different cell types, each with a specialised function that is varied from other cell types [120, 121]. Despite their differing regulatory roles, nucleus containing cells all contain an identical genome within an individual. The identical genome within cells is the foundation for DNA profiling, enabling vaginal material samples obtained from penile swabs to be profiled against a reference buccal swab obtained from a potential victim. Current molecular based techniques however focus on the factors that cause regulatory changes within cells which when

identified could be used to determine the same molecular changes within cells found at crimes scenes [120].

Throughout embryonic growth the determination of the type of cell is tightly regulated by gene expression. Gene expression is controlled through the process of transcription of DNA into single stranded complimentary RNA followed by the translation of messenger RNA (mRNA) to produce proteins. The human genome consists of over 22,000 coding genes, of which only a small proportion are expressed per cell type [120]. The identification of the regulating factors over expression could therefore be essential to forensic BFID. RNA molecule analysis is continually being researched and is becoming an encouraging novel technique for many forensic applications. They are currently used in post-mortem interval (PMI) and wound age estimation [122], blood stain age estimation [123] and human body fluid identification [124-127].

The majority of the presumptive and confirmatory techniques described include many limiting factors, however they are also performed as a precursor for DNA analysis and are not run in parallel to it. The development of molecular techniques have identified a number of methods that can analyse BFID alongside DNA profiling. These techniques focus on tissue specific mRNA and miRNA and the patterns within DNA methylation. These methods are more suitable for BFID due to high sensitivity and specificity [120, 126].

2.5.1. Analysis of DNA methylation patterns

Since recent whole-epigenome analyses indicated that DNA carries tissue-specific methylation patterns, the potential of tissue-specific differential DNA methylation for BFID has been examined within the forensic field [115, 120, 128-131]. DNA methylation is an epigenetic mechanism that controls the precise expression or silencing of genes. Unique sequences throughout the human genome encode for exclusive patterns of DNA methylation and are referred to as tissue-specific differentially methylated regions (tDMRs) [132]. Methylation alters as human develop due to ageing as well as environmental factors such as smoking and therefore may provide a solution to the problem of identical twins with matching genomes [129], Stewart *et al.* performed a study on a selection of identical

twins, samples were obtained from both blood and saliva and underwent bisulfite treatments followed by qPCR and high resolution melt curve (HRM) analysis. Results showed a difference in methylation in one set of 53-year-old twins, with no significant success in twins aged around 20. If patterns change with age it would be expected that a more observable difference could be made in older adults [131].

DNA methylation occurs at the 5'-position of the pyrimidine ring of cytosine in large clusters of repetitive CpG dinucleotide sequences known as CpG islands. Methyl groups are adhered to alter the activity of the DNA sequence without modifying its nucleotide sequence. These are heavily found in the promoter regions so repression of transcription can occur. Within the human genome up to 80% of CpG sites are methylated, with the remaining unmethylated CpG sites being responsible for housekeeping genes [132, 133]. Current research has therefore analysed DNA methylation at certain CpG site of tDMRs to identify a new BFID technique.

Frumkin *et al.* identified 15 genomic loci which display different methylated patterns among blood (venous/menstrual), vaginal material, semen, saliva, skin epidermis and urine. In this research, they applied an assay for certain markers using methylation-sensitive restriction enzyme-PCR (MSRE-PCR) made up of methylation-sensitive restriction enzyme digestion of sample DNA followed by multiplex PCR of specific genomic loci with fluorescence-labelled primers, capillary electrophoresis of amplification products and automatic signal detection. The assay could easily be assimilated into standardised procedures of forensic laboratories like short tandem repeat (STR) analysis, and could successfully identify source tissues in 50 DNA samples from blood, semen, saliva, and skin epidermis [134].

Later, Wasserstrom *et al.* advanced Frumkin's approach by developing the kit, DNA source identifier (DSI)-Semen™, which aimed to replace microscopic examination of sperm cells for forensic semen identification in casework samples. This assay is based on the recognition of semen-specific DNA methylation patterns in five genomic loci using MSRE-PCR. The kit was validated with 135 samples of various body fluids and 33 samples from casework from the

forensic biological laboratory. It suggested it was robust and reliable by showing a positive result for semen given as little as 31pg of template DNA input [135].

Lee *et al.* examined the potential of tDMRs for forensic body fluid identification using a bisulfite sequencing method. Bisulfite sequencing determines the DNA methylation status by detection of nucleotide base change due to sodium bisulfite treatment. Bisulfite treatment has no influence on methylated cytosine, but converts free or un-methylated cytosine of CpG to uracil, which becomes thymine during subsequent PCR. Using this method, they produced methylation profiles for five tDMRs in pooled DNA samples from blood, saliva, semen, menstrual blood, and vaginal fluid. The tDMRs for DACT1 and USP49 were chosen as a semen-specific marker by showing semen-specific hypomethylation, and the PFN3 tDMR was suggested to be used for vaginal fluid identification [115].

An *et al.* further investigated age-related methylation changes in semen-specific tDMRs using body fluids from young and elderly men, since DNA methylation patterns are known to be susceptible to change by aging. After confirming the stability of the body fluid specific DNA methylation profile, they suggested two multiplex systems to analyse the methylation status of the USP49, DACT1, PRMT2 and PFN3 tDMRs. The two multiplex systems were created using MSRE-PCR and methylation SNaPshot, and both successfully identified semen with sperm cells and could distinguish menstrual blood and vaginal fluids from other body fluids in a test with 144 DNA samples. Unlike MSRE PCR, which shows only the quantity of methylated CpGs, methylation SNaPshot has the advantage that it can measure the proportion of the methylated and/or un-methylated cytosine of the target CpG site simultaneously, because this assay is carried out by the amplification of bisulfite-converted DNA and subsequent single base extension reaction [136].

Alternatively, since genomic DNA can be degraded during bisulfite treatment, a bisulfite-based methylation SNaPshot assay may consume more samples than MSRE-PCR. Therefore, a sensitivity test was performed for the multiplex methylation SNaPshot. The result showed that a minimum of 500pg of starting genomic DNA, or 125pg of bisulfite-converted DNA, was sufficient for successful DNA methylation profiling of the selected tDMRs, which

demonstrates the possible practical application of the multiplex system to forensic casework [136].

A recent paper by Madi *et al.* also described tissue-specific DNA methylation in forensically relevant biological samples including blood, saliva, semen and epithelial cells. They examined various genomic loci using bisulfite modification and pyrosequencing to find that the methylation patterns at the ZC3H12D and FGF7 loci can differentiate sperm from other biological samples while the C20orf117 locus and the BCAS4 locus can differentiate blood and saliva from other samples, respectively. These results also suggest that the DNA methylation-based methods could be a valuable analysis tools for the characterisation of forensically relevant biological fluids, but further validation studies including more markers will be required for actual casework applications [130].

2.5.2. Messenger RNA

Messenger RNA (mRNA) are single stranded RNA molecules that convert the sequences of triplicate nucleotides into amino acids for proteins synthesis during translation. When DNA is transcribed into RNA by RNA polymerase it contains vital coding information as well as non-coding regions [120, 137]. The RNA molecules are spliced, with approximately 9 exon sequences retained and 8 intron sequences being cut out to produce an informative mRNA molecule that will code for a particular protein synthesis for gene regulation. mRNA in humans have an approximate half-life of 10 hours, ensuring the cell can immediately change its protein synthesis based on the cells current requirements [120, 137, 138].

RNA is well known for its instability due to universally present ribonucleases that degrade these nucleotide chains [114, 125]. However, research has reported that RNA isolated from some forensic stains showed unexpectedly high stability. Using whole-genome gene expression analysis on aged blood and saliva stains, Zubakov *et al.* identified blood and saliva specific mRNA markers which showed stable expression patterns in stains following 180 days of storage. Some of these markers showed successful and consistent amplification in much older stains, such as 16 year-old blood stains [139]. Setzer *et al.* lead a more comprehensive study on mRNA stability in forensic samples. They exposed various

biological stains to a collection of environmental conditions and performed mRNA profiling analysis using eight different mRNA transcripts of selected housekeeping and tissue-specific genes. The results established that RNA is detectable in some samples stored at room temperature, even after 547 days, with heat and humidity appearing to be most detrimental to RNA stability [140].

2.5.2.1. Advantages to mRNA analysis

One of the key advantages of body fluid identification by mRNA profiling is the possibility of simultaneous extraction of mRNA and DNA from the same body fluid stain. In forensic investigations, where sample material is often limited and highly valuable this offers a major advantage. Multiple optimised methods have been developed for simultaneously isolating mRNA and DNA from the same crime scene stain, this then allowed identification of the stain as well as confirming the assailant's identity through DNA profiling [125, 141, 142]. Although this has the advantage of allowing parallel analyses currently used protocols require modification to the standard DNA extraction protocol (Figure 2.2) [141]. RNAs are eluted in the lysis buffer fraction and require its recovery and storage (-80°C) for RNA analysis. Not only does this require a separate storage space to archived DNA extracts, it also means there is no cold case capability on samples that have already been extracted.

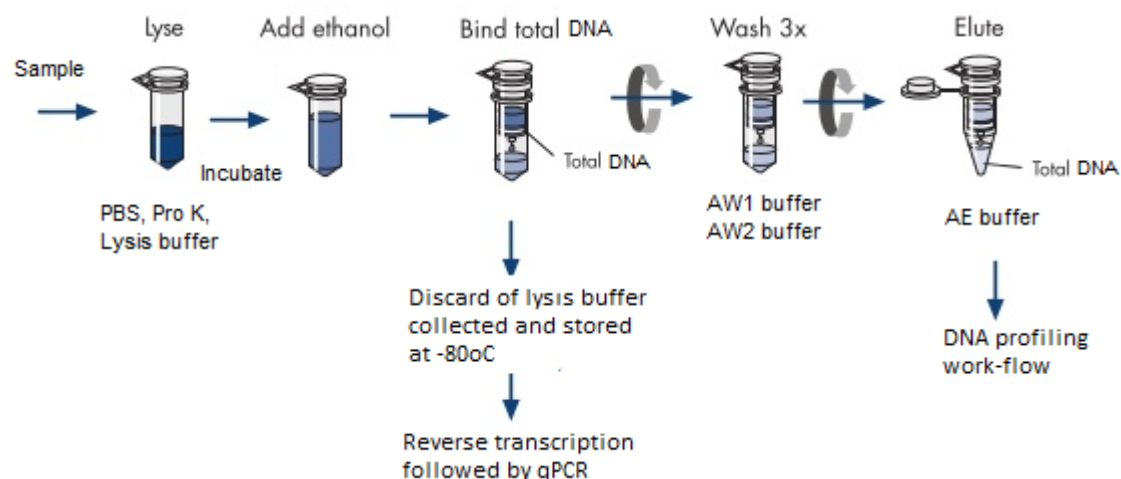


Figure 2.2: mRNA work flow protocol for mRNA expression analysis and DNA profiling. Adapted from [143]

Another important advantage of mRNA profiling is the possibility of detecting several body fluids in one multiplex reaction, providing data on the expression of multiple genes simultaneously. Various multiplexes have been reported, using reverse transcription endpoint polymerase chain reaction (RT-PCR) methods and real-time quantitative reverse transcriptase-PCR (RT-qPCR) assays [126, 142, 144]. Many transcripts are not completely tissue specific, exhibiting differences in expression level. This allows for RT-qPCR to be more appropriate for the detection of relative gene expression levels in different samples, meaning endpoint PCR might be suitable for the detection of certain transcripts with highly tissue-specific expression. Xu *et al.* proposed a multiplex RT-PCR method for the identification of body fluids that are commonly encountered in forensic casework. They evaluated using 16 selected body fluid-specific genes, i.e., Glycophorin A (GLY) and porphobilinogen deaminase (PBGD) for blood, histatin 3 (HTN3) for saliva, transglutaminase 4 (TGM4) and protamine 2 (PRM2) for semen, and human beta-defensin 1 (HBD-1) and mucin 4 (MUC4) for vaginal secretions for BFID in a multiplex reaction. Their results demonstrated a highly sensitive and specific multiplex assay capable of differentiating a number of body fluids which would not only bring down the cost of analysis but reduce the workload of the analyst [145].

To validate mRNAs incorporation into forensic casework the European Forensic Genetics Network of Excellence (EUROFORGEN-NoE) performed a collaborative research study in which a multiplex mRNA technique regularly implemented at the Netherlands Forensic Institute (NFI) was provided to a variety of laboratories with the aim of analysing the reproducibility of the results. With the exception of a skin and a vaginal material marker the multiplex consistently performed well in all laboratories and successfully identified challenging crime scene stains such as seminal fluid from a vasectomised or azoospermic male. Overall the collaborative exercise demonstrated the importance of providing both DNA and RNA analysis to ensure robust evidence is delivered in a court of law [146].

2.5.3. MicroRNA:

Multiple classes of small RNA molecules exist, each with various and essential roles in the regulation of genes:

- Small nucleolar RNA (snordRNA) – non-coding RNA which manage and control ribosomal RNA assembly and function
- Small interfering RNA (siRNA) – Around 25nt in length, siRNA bind to complementary sequences within mRNA and initiate cleavage.
- Transfer RNA (tRNA) – Aides at specific sites in the ribosome when decoding mRNA sequences into proteins.
- Ribosomal RNA (rRNA) – RNA component within ribosomes.
- microRNA – non-coding RNA which regulate gene expression through translation repression of mRNA [147-149].

MicroRNA (miRNA) were first discovered in the nematode *Caenorhabditis elegans* by Victor Ambros *et al.* in 1993 [150]. Further research showed miRNA presence in the majority of eukaryotes and their high abundance in human cells [151]. Since their discovery, the study of miRNA has progressively increased with research into its many applications being performed. The vast interest in miRNA revolves around their central role in gene-expression regulation which controls various metabolic and cellular pathways and the implication of miRNA-specific expression in the pathogenesis of cancer, cardiac, immune-related and other diseases [152-156]. If regulation is controlled by the varied processes within the cell then the likelihood of cells within different body fluids containing different miRNA is high.

MicroRNAs are naturally occurring, non-coding, single stranded RNA molecules with an approximate length of 18-22 nucleotides (nt) [157-159]. MicroRNA control gene regulation by silencing mRNA molecules; miRNA bind to target specific mRNA sequences to negatively control expression and thus inhibit protein production. Silencing of mRNA and therefore regulation of vital cellular process occurs through two processes, translational repression or cleavage of a specific mRNA [151, 158, 159]. The guide miRNA strand interacts and is integrated into the RNA-induced silencing complex (RISC), the complimentary sequence of

miRNA then binds to the target complementary sequence of the mRNA that the cell wishes to silence. Proteins within the RISC complex will either cleave the mRNA molecule and thus inhibit translation or ensure that the ribosomal sub-unit responsible for translation cannot bind and cause translational repression. The choice of repression is presumed to be caused by the level of complementarity between the miRNA and target mRNA [147, 15].

Research estimates that miRNA contribute up to 5% of the entire human genome, which control over approximately 30% of protein-coding genes suggesting that abundance of various miRNA within a cell is very high [147]. Currently miRBase states that 2588 mature *Homo sapiens* miRNA sequences have been identified² [160] many of which have been used in clinical, medicinal and forensic research. With only around 30% of gene not being silenced due to miRNA and the distinct variation in cell types over different body fluids it is highly likely that tissue-specific miRNA are present within all cells.

2.5.3.1. Biogenesis

Within the human genome, miRNA genes are present. They contain the genomic information of a number of miRNAs and exist as distinct transcriptional units. Research groups have proposed that roughly 50% of determined mammalian miRNA are located in non-protein coding RNA or the intron of protein coding genes, as opposed to them being present in their own unique transcriptional unit [151, 159]. Intronic miRNA are usually located in the equivalent orientation and thus are co-ordinately processed with the precursor mRNA where they reside, thus sharing a single primary transcript. The miRNA genes are transcribed by RNA polymerase II, a eukaryotic enzyme that targets the promoters of protein-coding genes in living cells and a double stranded RNA-binding domain (dsRBD) protein partner, as seen in Figure 2.3. The transcription produces long 'hair-pin' primary transcripts known as pri-miRNA. A nuclear RNase III type enzyme known as Drosha then processes the pri-miRNA, by cleaving the RNA approximately 11nt from the hair pin base to produce a stem loop, ~ 70nt in length known as precursor miRNA (pre-miRNA). Drosha leaves an overhang of two or three nucleotides on the 3' end of the pre-miRNA, this

² Correct as of 04/12/2016

overhang is recognised by Exportin 5, a RanGTP-dependent dsRNA-binding protein that causes the pre-miRNA to be exported from the nucleus into the cytoplasm. Once outside the nucleus an RNase III protein called Dicer severs the hair pin to create an unstable 19-25nt mature miRNA duplex structure. The less stable mature miRNA strand of the duplex is integrated into a multiple-protein nuclease complex known as the RISC. This combination allows for mature miRNA to target certain genes and regulate their activity by causing cleavage or translational repression of the mRNA, thus ceasing protein synthesis [159, 161-164].

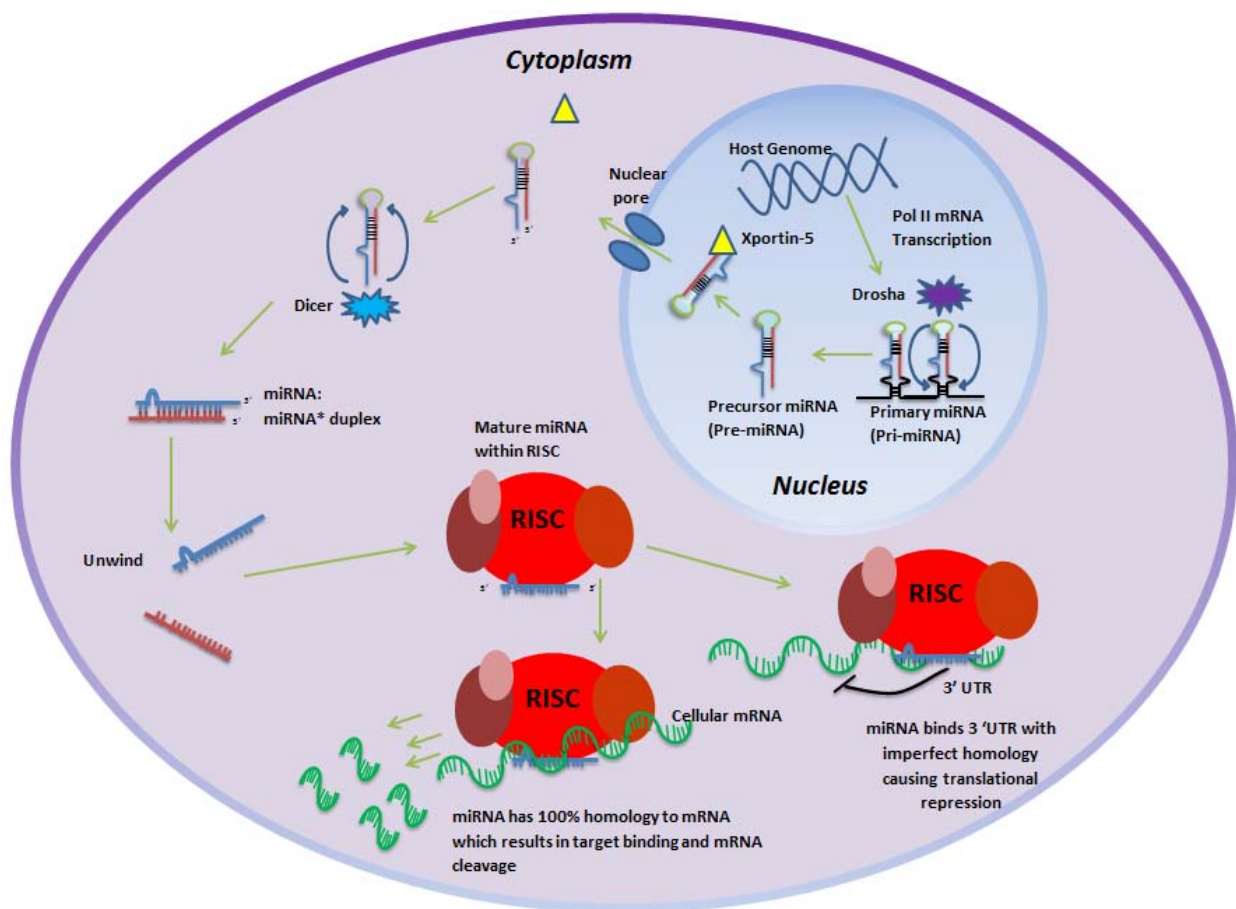


Figure 2.3: The pathways of miRNA within the cell created from [159, 161-164].

2.5.4. Advantages of miRNA analysis

The small size of miRNA makes them highly stable with strong research supporting them as a more efficient and suitable identification tool compared to their longer mRNA counterparts. Court's research group presented data that show approximately 200 miRNAs performed better than several thousand mRNAs when being used to classify highly similar tumours [165]. Their stability also means that they should be more robust and reliable in their performance as the likelihood of the small strands being damaged or denatured is considerably less. Another promising quality is that they are vastly abundant within a cell Batel *et al.* assessed that within an adult worm cell, certain miRNA contained approximately 50,000 strands of distinct miRNA [151]. Human cells have much more complex regulatory mechanism and therefore the abundance of miRNA is likely to be higher. This would be of great importance as samples found at crime scene can be degraded or have low volumes of sample. This huge presence in a cell would mean that analysis could still be performed even with small volume samples.

Due to the important role miRNA play in gene regulation it can be suggested that certain miRNA only target an individual gene, therefore their expression would only be identified in the body fluid related to the process that gene controlled. Since menstruation only occurs at the end of the menstruation cycle, the miRNA that regulates that pathway should only be present during that time, therefore if a menstrual blood specific miRNA can be identified it would be extremely valuable in determining menstrual blood and thus solve a current problem facing forensic scientists.

Wang *et al.* researched miRNA application into BFID. qPCR was utilised to analyse 7 proposed miRNAs against forensically related body fluids. Their results indicate 5 of their 7 miRNA markers could determine with statistical significance the presence of venous blood, semen and menstrual blood [166]. Sauer *et al.* also researched the effectiveness of miRNA for forensic BFID, in this study qPCR was also utilised on 36 proposed miRNAs. Their results determined that only four of their miRNAs were required to identify saliva, blood, vaginal

material, menstrual blood and semen. The study also utilised 36-year-old blood stains that were correctly identified as displaying the stability and robustness of miRNA in BFID [157].

The ideal BFID technique would run in parallel to DNA profiling, although this was achievable in mRNA techniques it required waste elute being kept and analysed. Van der Meer *et al.* analysed the capability of DNA/ miRNA co-extraction using Qiagen's total DNA extraction kit. Blood and saliva samples were extracted and underwent DNA profiling and RT-qPCR for BFID. Results showed all samples were correctly identified by body fluid as well as producing a full DNA profile [167]. This technique removes all additional steps within DNA extraction with no destruction of sample, it also provides the opportunity for previously analysed samples where BFID would have been useful to be reanalysed signifying miRNA analysis has a vast cold case capability.

2.5.5. Comparison of current mRNA and miRNA techniques

There is limited literature comparing the techniques of mRNA against miRNA. Both show very promising results for BFID and both have components that make them suitable for forensic application. The majority of comparative research has been performed on formalin-fixed paraffin-embedded (FFPE) samples. This is an important technique in many laboratories for storing and archiving tissue samples [168]. It is important to note that crime scene samples would not undergo FFPE treatment and therefore may differ to current literature. Crime scene samples are often degraded so it is useful to note the stability of miRNA and mRNA over various conditions.

Liu *et al.* performed a study to identify which provided the best molecular expression data and if the need for a fresh tissue sample was an absolute requirement. Total RNA was prepared from paired samples of FFPE and fresh frozen malignant melanoma. A microarray was then utilised to compare the expression profiles obtained from miRNA in the FFPE and fresh tissue with the mRNA expression profiles obtained from the same sample. The expression profile obtained using miRNA from the FFPE sample closely mimicked that obtained from the fresh sample. This relationship was distinctly more observable than that

obtained from the mRNA expression profiles. They concluded that a fresh sample was not necessary for successful molecular expression analysis and that miRNA displayed a most vigorous analysis tool for gene expression analysis than mRNA [169].

Klopfleisch *et al.* performed a more extensive review of the potential of identification and quantification in FFPE tissue obtained from DNA, miRNA, mRNA and proteome techniques. Their results showed that the chemical reactions involved in creating FFPE samples highly degraded mRNA and proteome samples. The poly A tail of the mRNA was almost always completely degraded meaning reverse transcription would only be suitable with random primers. Fragmentation was also observed in DNA samples, with fragments longer than 200bp becoming split and unable to amplify in PCR. The only technique that seemed relatively unaffected by the formalin fixation was miRNA [168].

A small selection of studies have been performed on mRNA stability over various conditions. Leonard *et al.* isolated mRNA from brain tissue that had been frozen following post-mortem examinations. The samples varied in factors such as time since death, length of time before freezing and the duration the samples were in cold storage. The results discovered that when kept at a temperature of -70°C the majority of RNA had lost their Poly A tails meaning oligo-dT primers could not be used for reverse transcription. It was noted however that in some of the cases there were enough partial sequences present to perform amplification by PCR if random primers were used. They concluded that although not as successful when compared to fresh tissue, gene expression analysis may still be successful following long-term storage [170].

The most comparative study of miRNA and mRNA was performed by Ge *et al.* The study assessed the effect on stability caused by various storage lengths and temperature on mRNA and miRNA stability. Healthy volunteers provide plasma samples, these were then stored at temperatures of 4 °C, -20 °C and -80 °C and underwent no freeze-thaw cycles. Exosomes (minute membrane-bound vesicles secreted by the majority of cell types) were then taken from the plasma at time points of two weeks, two months, three years and five years. RNA and miRNA were isolated for the various exosome and equivalent plasma

samples. When studying RNA derived from the exosomes, no significant difference was observed across the various storage conditions. The results were very different when looking at the RNA derived from plasma, significant degradation was observed in samples stored at 4 °C for two weeks as well as severe degradation in samples stored at –20 °C up to a period of five years [171].

When results of miRNA were compared to that of RNA a distinct increase in stability was observed. A decrease in expression was seen in samples stored at 4 °C for two weeks for some of the miRNA, however all miRNA tested were still detectable at this time point. No decrease in expression was observed across any other storage condition suggesting there is a superior stability of miRNA in plasma and exosomes than in mRNA [171].

The study went on to examine mRNA and miRNA stability when undergoing freeze-thaw cycles. Plasma and exosome samples which had been kept at –20 °C with one and two freeze-thaw cycles were analysed against the same storage conditions with no freeze-thaws. RNA from plasma samples saw a vast decrease in concentration following one cycle, which was even greatly decreased when analysing a second cycle. RNA from exosomes only displayed a vast decrease following the second cycle. When looking at miRNA a decrease was observed following one and two cycles in plasma however not to the extent observed in RNA, with no significant difference being observed in exosomes [171].

The presence of miRNA within the components of blood was discovered in 2008. The miRNA isolated from plasma showed high stability under extreme conditions such as boiling, extreme pH values, long durations stored at room temperature and a multitude of freeze-thaws. When tested against synthetic mimics a quick degradation was observed, suggesting that cell miRNA's have a protection mechanism that allows them to avoid being degraded. Although this mechanism is unknown it has been theorised that they could be packaged into micro-particles such as micro-vesicles, be linked with RNA-binding proteins or complexes such as high-density lipoproteins [172].

Current research into miRNA stability is still being performed however various studies have been performed. Balzano *et al.* became aware of the increased levels of research into miRNA

and BFID but not the stability of miRNA over time in storage. His study involved samples from clinically healthy donors which were then tested for abundance and stability following long durations in cold storage [173]. qPCR was performed on eight markers on samples that were freshly isolated from plasma and from those that had been stored at -80°C for a period of between six and 12 months. They noted that no significant degradation could be observed therefore showing the miRNA markers were stable during this length of storage. They increased their experiment to include samples stored over a period of up to 14 years. The results showed miRNA remained stable up to a period of four years, following which although still detectable they became less stable [173]. The samples used in forensic workflow would either be fresh or archived in a -80°C freezer therefore suggesting the prospect that miRNA could be used on cold case samples.

Mittchell *et al.* was aware of the high levels of miRNA stability observed in FFPE tissues which led him to further test the stability of miRNA in plasma and serum. RT-qPCR was performed using hydrolysis probes. Several miRNA were assessed on plasma samples that had been incubated at room temperature for a period of 24 hours and plasma samples that had been through eight freeze-thaw cycles. The RNase activity within blood plasma is very high thus it could be suggested that the highest rate of instability would be observed. The results for both freeze-thaw and room temperature incubation samples showed no significant change in expression was observed. Synthetic mimics were included as measured in the same way, these mimics quickly became degraded when added to the plasma which indicated that endogenous miRNA have developed a technique that provides protection from RNase activity. This study was repeated on samples obtained from serum with miRNA again showing not significant difference in stability [174].

Overall, this research currently available suggests that miRNA offer a higher level of stability over a wide range of factors, there is however some discrepancies in that Mittchell *et al.* [173] states miRNA are highly stable at room temperature while Ge *et al.* [171] showed a decrease in miRNA expression when stored at length at room temperature. Degradation rates of miRNA and mRNA are clearly linked to the protective property of the nucleic acid, with exosomal RNA continually showing high stability in both mRNA and miRNA compared

to samples obtained from pure serum. Current literature does show that although both mRNA and miRNA gene expression analysis can be performed after long term storage it is more successful and higher expression is observed when utilising miRNA.

2.5.6. miRNA Suitability for Identifying Body Fluid Components

2.5.6.1. Venous blood

As RBCs and platelets are the only cells within any body fluid without genetic material it would suggest that it is miRNA that regulate homeostasis and replication. These miRNA are likely to be specific to that regulation and would not be found in the cells containing genetic material. The cytoplasm of all RBCs is saturated in haemoglobin, an iron rich protein that binds with oxygen in oxygen rich regions such as the lungs, transports it around the circulatory system and releases oxygen in poorly oxygenated regions such as muscles. Due to the continual alteration in shape to travel through narrow blood vessels, the RBC can only survive for approximately four months with the haemoglobin from old cells utilised to recreate new RBCs within bone marrow [76]. This constant change in structure to fit through narrow vessels and the destruction and regeneration of new RBC is a highly regulated process and due to the specific requirements and lack of similar processes to other human cells the regulation is likely to be specific and thus require specific miRNA.

The plasma and WBC within whole blood contain a large number of proteins and regulate immunological responses as well as maintaining body temperature and blood pressure [76]. These factors can fluctuate rapidly and therefore would require strict regulation from miRNA. Due to no other body fluid having homeostatic responsibilities it could be suggested the miRNA responsible are highly specific.

There are many conditions that may cause fluctuation in the regulation of miRNA. Blood conditions such as anaemia involve the deficiency of haemoglobin or RBCs, while cancers, not limited to those of the blood, will cause drastic changes in the homeostatic environment of cells. Schultz *et al.* analysed the regulation of biomarkers in whole blood in

patients with and without pancreatic cancer. The study included 312 healthy participants and 434 patients with pancreatic cancer or chronic pancreatitis, their analysis resulted in the observation that 38 miRNA were down-regulated in patients with pancreatic cancer when compared to the healthy patient control group [175]. This is a large proportion of miRNA and if specific to regulation within blood, would impact on the suitability for BFID. In cases where blood residue is located the medical history and health of the source will be unknown therefore down-regulation due to diseases may cause a false identification. Bio-markers must therefore be consistently expressed regardless of factors such immunological responses to diseases.

2.5.6.1.1. Blood response to injury

If venous blood is located at a crime scene it is likely from trauma to the body, such as tearing of the labia during a sexual assault. The instant biological response is for the body to instigate physiological reactions to prevent severe blood loss. This is performed by creating a haemostatic plug around the damaged site. Haemostasis is the essential process of halting excess bleeding at a damaged location, while maintaining regulated blood flow to the rest of the circulatory system. The endothelium of blood vessels acts as an interface between circulating blood and the vessel wall, it has an anticoagulant surface which maintains blood fluidity and ensures cells do not adhere to the blood vessel wall. When blood vessels are torn or damaged, as may occur in violent and forceful crimes, fibrous components within the endothelium are exposed to the blood. Since platelets are the lightest of the cells they are pushed to the edges of the blood vessels and are in first contact. They adhere to the fibrous tissue to create a haemostatic plug and thus clot the blood. This process is under strict regulation with haemostasis occurring within seconds to ensure the minimal amount of blood is lost [176-178].

Haemostasis is a vital process for survival following traumatic events, it does however suggest that blood leaving a wound may be slightly different in composition to circulatory blood. With such tight regulation of haemostasis it would be likely that many miRNAs are designated to this essential role. Since no other body fluid requires a clotting factor these

haemostasis specific miRNA are likely to be only found in blood. The presence of miRNA within venous blood from various trauma wound may also differ; a nose bleed may have a completely different composition to venous blood from a tear in the vagina.

2.5.6.1.2. Blood response to foreign material

Severe cases of blood loss require whole blood transfusions. Blood types must be matched and screening of blood is essential. With all blood transfusions there is a risk of rejection of the foreign blood by the patient, if the components within the blood determines the foreign material is a threat an immunological response will be initiated [179]. These reactions are frequently due to plasma proteins, RBC antigens, leucocytes or additional pathogens. With such a significant and immediate response to management of foreign material within the blood, it is likely that tight regulation of processes are control by a selection of miRNA, which although may regulate an immune response may be specific to RBC and plasma control [179].

2.5.6.1.3. Stability of blood components

Zou and colleges utilised Mid-infrared spectroscopy and Raman microscopy; versatile optical analysis techniques, to determine the components of whole blood [143]. Analysis was performed on 16 blood samples, inclusive of all blood types and both genders. The peaks identified were attributed to haemoglobin, acetates, haems, lactates and tryptophan. Their results showed these components are always found in blood, regardless of different ages, genders or blood groups. These findings suggest that whole blood is very stable and therefore the regulation of genes and other biological processes should be the same between individuals and therefore so should the presence of miRNA.

2.5.6.2. Similarities between saliva and vaginal material

Whole saliva is regulated by many enzymatic processes which would suggest a high volume of various miRNA are present. These would quickly respond to the increased need of immunological proteins or digestive proteins. The specificity of α -amylase within saliva would suggest the miRNA responsible for its regulation may also be specific to saliva however the issue that saliva is similar in appearance to vaginal material is also apparent on

a cellular level due to both requiring immunological defence against foreign microbes. MicroRNA responsible for rapid changes in immune responses may be present in both fluids which would display dual expression when analysed. The miRNA required for BFID would ideally not have a regulatory role in the immune system.

Research has shown that down-regulation of genes is a key feature in a majority of cancers [164]. This would therefore lead to the assumption that miRNA are also down-regulated. Wilting *et al.* analysed the methylation levels of 3 loci encoding the mature sequence of hsa-mir-124 in patients free of cancer and those known to have cervical cancer. Methylation levels, and thus the silencing of hsa-mir-124, showed no methylation in healthy individuals but an increase of 93% in cervical carcinoma tissue [164]. Such a significant increase in the silencing of hsa-mir-124 and possible other miRNA would make ensuring a BFID technique is robust and reliable a difficult challenge.

2.5.6.3. Menstrual blood

The higher concentration of genetic material coupled with the high volume of protein regulated processes indicate that although similar in appearance, menstrual blood should contain a large number of miRNA that would not be required in venous blood. The menstrual cycle is largely regulated by oestrogen and progesterone, although these are steroid hormones, they interact with protein receptors to catalyse reactions and regulate gene expression [93]. It can therefore be suggested that all four hormones are likely controlled by miRNA regulation and since the hormone levels change so rapidly and abundantly throughout the cycle it is likely miRNA presence will follow this pattern.

As previously described there are multiple factors that change the frequency and consistency of menstrual blood. Both hormonal and non-hormonal contraception change the structure and regulation of the endometrium. The IUD causes continual destruction of the endometrium suggesting miRNA responsible for regulation of progesterone for endometrium building may also be effected as they will likely be in a continuous switched on state to repair damage. Pregnancy will initiate strict regulation of new mechanisms to prevent damage to the foetus and therefore a whole range of new miRNA may be found in

the vaginal material and potential vaginal bleedings of pregnant females. Conditions such as endometriosis display a down-regulation of immunological response cells and therefore a down-regulation of the miRNA responsible for that response. It could then be suggested that women with endometriosis may show no expression pattern of certain miRNA when compared to 'healthy' menstrual blood samples.

2.5.6.4. Semen

The large number and variation of proteins present in fertile males would require strict regulation from multiple miRNA. Spermatozoa are only produced in the testes therefore it is likely these miRNAs are specific to semen. The remaining are dedicated to providing energy to the spermatozoa, since no other body fluid requires an energy source the miRNA and their regulation mechanisms are likely to be highly specific to semen. The depletion of many proteins when spermatozoa are low or absent would mean that the miRNA responsible for regulation of those proteins would also be absent suggesting that a BFID technique utilising miRNA must be carefully validated to ensure presence is high regardless of spermatozoa presence.

The current available literature on semen persistency does not provide a definitive time frame for positive identification within vaginal material. With no research being performed on the persistency of biomarkers such as mRNA or miRNA. It is likely that these would be located within the fluid as well as the spermatozoa themselves meaning they may have an increase like hood of being identified compared to trying to identify spermatozoa as a whole component. Overall each body fluid presents a role within the body that is specific to that fluid, although many involve immunological responses they will not all be identical and therefore may not share miRNA. The majority of body fluids are susceptible to factors that would likely alter the expression pattern of regulatory miRNA, requiring any miRNA chosen for BFID to be thoroughly validated before implementation into casework.

2.5.7. Marker Selection

2.5.7.1. Current body fluid specific miRNA from literature

Markers were selected based on body fluid specificity from current literature. Park *et al.* utilised a genome-wide miRNA micro array comprising more than 1700 miRNAs. Successful markers were then screened using RT-qPCR for further analysis of specificity. Their results indicated a selection of markers showing specificity, such as hsa-mir-1260b and 654 for vaginal material, hsa-mir-223 for saliva and hsa-mir-2392, 891a and 3197 for semen. Park *et al.* research differs from the methods used in this research due to substitution of a hydrolysis probe for SYBR green [165]. SYBR is a fluorescent intercalating dye that binds itself into double stranded DNA molecules. Binding causes alterations in the dyes composition which causes fluorescence. The more DNA produced throughout amplification the higher the intensity of fluorescence. The disadvantage of intercalating dyes is the known lack of specificity when compared to probe based qPCR, this due to the dye being amplified in all double stranded DNA sequences. The likelihood of non-specific amplification is therefore high and a melt-curve is required after every analysis. [166]. Park *et al.* observed that hsa-mir-16 was highly expressed in venous blood but also displayed high expression in saliva and vaginal material. While hsa-mir-203a and 205 which were highly expressed in saliva were also highly expressed in semen.

Sauer *et al.* also performed a genome-wide miRNA micro array to highlight potential BFID miRNA. They analysed a selection of potential markers with TaqMan® based RT-qPCR, they concluded that hsa-mir-891a-5p was wholly specific for semen, with hsa-mir-10a-5p, 10b-5p and 135-5p showing significantly higher expression in semen than other body fluids. Sauer's research found markers specific to both venous blood and menstrual blood, hsa-mir-144-5p, 144-3p and 451a, however these markers could not differentiate between the two. Analysis of hsa-mir-205 and 203a showed high specificity to saliva and vaginal material; but again could not differentiate the two body fluids, with only hsa-mir-124 showing complete specificity to vaginal material [167].

Hanson *et al.* performed RT-qPCR following SYBR green protocol on 452 human microRNAs. Results showed that although true specificity was not identified significant differences between body fluids were sufficient for BFID. The markers chosen for BFID were hsa-mir-16 and 451a for venous blood, hsa-mir-135a and 10b for seminal samples, hsa-mir-205 and 658 for saliva, hsa-mir-124a and 372 for vaginal material and utilising differential expression hsa-mir-451 and 412 for menstrual blood. Of vaginal material markers, hsa-mir-124 was observed as highly expressed in vaginal material over all other body fluids, while hsa-mir-372 showed expression in all body fluids but a higher expression value was observed in vaginal material [168].

Research performed by Zubakov *et al.* which also utilised microarray screening of 718 human miRNAs, followed by TaqMan® RT-qPCR displayed body fluid specificity in a number of markers, such as hsa-mir-185 and 144 for blood identification, no differentiation from menstrual blood and venous blood, and hsa-mir-135a, 10a, 507 and 891a for semen. Their research attempted to replicate the work of Hanson *et al.* using TaqMan® instead of SYBR green, with reproducibility only seen in venous blood markers hsa-mir-16 and 451a, and semen markers hsa-mir-10b and 135b. Their data did not show reproducibility of hsa-mir-205 and 658 in saliva, hsa-mir-124a and 372 in vaginal material or hsa-mir-412 in menstrual blood. The conclusions of Zubakov *et al.* work highlight discrepancies between publications could be a small sampling size or the differences in methodology in SYBR green analysis [185]. The research also showed discrepancies between microarray data and RT-qPCR data in which hsa-mir-617 for vaginal material identification and a handful of other markers showed high specificity which was only observed in microarray screening and not RT-qPCR analysis.

Research performed by Wang *et al.* first screened a number of miRNA using the qPCR-array (TaqMan® Array Human MicroRNA Cards) which indicated hsa-mir-16 for venous blood, hsa-mir-888 and 891a for semen, hsa-mir-214 for menstrual blood, hsa-mir-124 for vaginal material and hsa-mir-138-2 for saliva. These markers were then validated through TaqMan® qPCR normalising with SNORD44. Subsequent TaqMan® qPCR analysis only supported hsa-mir-16 for venous blood, hsa-mir-888 and 891a for semen and hsa-mir-214 for menstrual

blood, with hsa-mir-138-2 not showing specificity [181]. Current research within and surrounding BFID also suggest hsa-mir-508 [187], 888-5p [186, 183] as semen specific: High expression of hsa-mir-888-5p was observed when performing in-vitro testing on the early stages of spermatozoa development indicating it may have semen specific expression [189]. Research into endometriosis has also shown a significant down regulation of hsa-mir-199a in patients suffering from the disease, indicating that it may have a significant role in the normal regulation of the endometrium [190].

Many researchers have looked at identifying the function of specific miRNA. New miRNA are constantly being identified with the majority of functionality unknown. Current literature does determine that miRNA's are responsible for the regulation of specific genes with many showing they have a large role in both oncogenic and tumour suppressive functions [191]. Martin *et al.* studied the function of miRNA in seed biology and concluded that miRNA have evolved so that their functionality is diverse, they can target multiple different genes which have a role in various processes. Therefore miRNA have evolved within each species to perform multiple functions. [192]. It would therefore be difficult to determine the exact function of the miRNA selected for this screening study. The likelihood of multiple functions increases the probability of cross reactivity across body fluids, therefore markers that are only expressed within one body fluid would be the ideal BFID candidate.

2.5.7.2. Comparison of techniques and miRNA selection

When using micro or qPCR arrays i.e. non-TaqMan® assay, each research group observed at least one marker that displayed specificity discrepancies between microarray and RT-qPCR data. This was possibly due to the microarray detecting artefacts or that qPCR arrays require a higher RNA input and therefore pre-amplification is required [185, 186]

In current literature, certain miRNA such as hsa-mir-16 for 451 for blood [124, 184, 185, 193, 194], 205 in saliva [124, 184, 175, 186, 194], hsa-mir-135b and 10b [124, 184, 185] for semen are supported by multiple publications as body fluid specific, with other publications disagreeing on hsa-mir-205 specificity [186, 193]. Research by Wang *et al.* also disagreed with Courts *et al.* findings that hsa-mir-203a was specific to saliva in that when tested

against other body fluids, it was specific against venous blood but highly expressed in vaginal material and menstrual blood [186]. Various endogenous controls were analysed throughout these publications such as SNORD 47 [196] and SNORD 44 [186]. Other studies were also performed on the reliability of certain markers as endogenous controls; Applied Biosystems performed a robust study identifying many endogenous control candidates such as RNU38B, 44 and 48 [196] while Sauer *et al.* analysed the reliability of hsa-miR-93-5p, SNORD 7, 38b, 44 and 48, stating the most reliable was SNORD 38b [197].

The majority of body fluid miRNA markers in current publications have not been independently validated by other publications, when this has occurred discrepancies have regularly arisen. Further literature from Hanson *et al.* utilising SYBR qPCR also suggested a range of specific markers, hsa-mir-658, 205 and 124* for saliva identification, hsa-mir-892a and 891b for semen, hsa-mir-4286 and 124 for vaginal material, hsa-mir-142, 144-5, 144-3 and 185 for menstrual blood, hsa-mir-455-3p, 3169 for skin cells and hsa-mir-451a and 16 for blood. The results obtained for this study support other markers found in other SYBR studies but also disagrees with other research previously described [193]. The main discrepancies observed in literature is the specificity of markers to menstrual blood or venous blood, in certain publications hsa-mir-144 is menstrual blood specific [184, 185, 198] whereas other report it as venous blood specific [183,185] This has also been observed in assigning specificity of hsa-mir-16 with some literature supporting specificity and some identifying high expression in other body fluids.

Overall the current literature surrounding miRNA specificity is not wholly reproducible across laboratories. The differences are likely due to various extraction methods, whether DNA digestion was utilised, whether SYBR or hydrolysis probe methods were used and if pre-amplification occurred. The aim of this research is to run alongside or phase into current laboratory processes therefore extraction methods will vary from current research due to current forensic laboratory protocol, with steps such as DNA digestion and pre-amplification being avoided. The variation between studies using SYBR and hydrolysis is reasonably large suggesting the selected qPCR technique is important to gaining reproducible specific expression values. This would therefore require any miRNA BFID

technique implemented into casework to have a set protocol where all laboratories performed analysis via hydrolysis probes or SYBR.

2.6. Summary of Current and Emerging Body Fluid Identification Techniques

Traditional confirmatory and presumptive tests for BFID suffer from a lack of specificity and sensitivity as well as being occasionally labour-intensive with high risks of false positives. They are also performed before DNA analysis and the majority require destruction of valuable sample to provide identification. Among emerging techniques that have been discussed, mRNA markers have been most rigorously investigated and the number of specific markers is sufficient for the identification of forensically relevant body fluids. Some of the other currently used methods have potential for the rapid and non-destructive identification of body fluids, but in most cases, tissue specificity is problematic due to cross-reaction with biological or non-biological material present in samples at crime scene. mRNA markers show high tissue-specificity and adequate sensitivity for forensic analysis and can be considered as a valuable new approach to overcome the limitations of conventional methods. BFID through mRNA profiling is not yet widely used in forensic laboratories owing to the fact that further validation is required and it is not deemed cost-effective to store waste elute for possible BFID. In addition, heat and humidity remain threats to the stability of mRNA markers.

DNA methylation profiling was proposed as a promising new tool for forensic body fluid identification which uses the same DNA samples that are used for DNA profiling. Like mRNA profiling methods, DNA methylation profiling showed high specificity and sensitivity, and also allowed for the simultaneous analysis of multiple markers, specific for various tissues in a single multiplex system. Moreover, DNA methylation profiling methods fit well with current forensic applications, and accordingly, can be easily integrated into current forensic standardised procedures. Although an extremely promising forensic technique, DNA methylation still needs to identify more markers for future practical application to casework. BFID is not the only forensic application for methylation analysis with current research

showing its potential for age determination and identifying which monozygotic twin a body fluid sample originated from [131].

Overall miRNA, although not currently validated for forensic laboratory work has displayed all the benefits of mRNA and methylation analysis. The technique displays the additional advantages of having increased stability over mRNA, parallel analysis with DNA profiling and with analysis performed on DNA elute the requirement for further storage space is removed. This also provides prosecutors of crimes committed years before BFID technology emerged to re-analyse already profiled and archived samples for information that could be essential to a conviction in a court of law.

Although miRNA has shown suitability as a BFID technique there are many factors that can alter the composition and gene regulation of body fluids. An immunological response to pathogens is an ability all body fluids possess, since this involves strict gene expression and protein production the regulatory miRNA will also be present in all body fluids. Research highlights miRNA as a reliable and tissue specific bio-marker for use in BFID however implementation into criminal casework cannot proceed until a number of validation and optimisation studies have occurred. This research therefore aims to solve the aforementioned limitations of BFID techniques by identifying miRNA that are highly tissue-specific while robustly analysing them in conditions that could inhibit expression such as the menstrual cycle, the absence of spermatozoa or the suppression by higher quantities of genetic material from other body fluids.

3. Research Aims and Objectives

3.1. Aims and Objectives

Sexual assault is an offence which suffers from very low reporting and conviction rates. Victims suffer with a wide variation of traumas from the events therefore new techniques must be developed to increase the reporting and conviction from the less serious to the major offences. When introduced, the impact of the inclusion of DNA evidence within a court room was substantial, with it currently being the most sort out piece of evidence. DNA evidence only identifies a person was at or involved in a crime scene, not the events and therefore it can be manipulated by the defence and prosecution to show varied versions of how the crime occurred. BFID can provide an outline of the events that occurred, making evidence clearer for Jury's to understand but also remove many of the statements that the defence and prosecution could use to display their version of events.

Currently, the Identification of body fluid samples found at crime scenes involve techniques that were developed over the past 100 years. These technique are rarely improved upon, consume and degrade valuable sample, often require trained experts for testing and are mostly only usable as a presumptive identification. It is therefore important that a new technique that can run alongside and support DNA profiling evidence is introduced. The aim of this research project is to develop a technique that is confirmatory, doesn't destroy valuable samples, can be run in parallel with DNA profile, does not required additional extensive training to perform and will not vastly increase the cost or time of analysis.

Current literature has contradicting opinions on the regulation and expression of miRNA in BFID. This appears to be linked to the variety in techniques used with the most contradictions being observed with different extraction techniques and when using SYBR green based chemistry against probe based chemistry. This research aims to confirm the results of current literature using probe base chemistry and to outline a simple protocol that can be reproduced by all forensic laboratories. As well as support current literature this research aims to identify a panel of markers, ideally multiple for each body fluid that can identify the major body fluids found at crimes science with no risk of false identification.

The next objective of this research is to provide a definitive identification to problems that current forensic scientists face. The differentiation of vaginal material and saliva is currently unachievable yet is arguably one of the most important differentiations in a sexual assault case. This research aims to provide markers that can not only identify vaginal material and saliva as a single body fluid but can differentiate the body fluids when in mixtures of up to three body fluids. The lack of DNA evidence in cases where perpetrators have low or absent sperm counts will have a large impact on how the case is described in court. This research aims to be able to identify ejaculate regardless of whether spermatozoa are present so not only can the differentiation between azoospermic/ vasectomised semen and regular semen be determined but the presence of either can be identified from a vaginal swab. The final identification objective is the differentiation of menstrual blood and trauma blood. The identification of trauma blood suggests that an aggressive action occurred which could provide greater support to the victim and prosecution hypothesis. It is therefore important that this research can isolate multiple markers that can determine which source of blood was obtained from the crime scene sample.

In addition to demonstrating specificity, markers must also demonstrate sensitivity. Crime scene samples are rarely found in large volumes with no degradation and in cases of sexual assault, the perpetrators contributions may be significantly lower than that from the victim. Once a panel of markers have been identified for BFID, their ability for detection at trace level will be assessed.

Once identified, validation of this technique is imperative. Expression of genes within cells is a dynamic process, and as such any marker identified as being body fluid specific must also be robust enough to deal with these changes. One of the most dynamic processes within human physiology is that of the menstrual cycle, and as such the next component of this research investigated the specificity and robustness of both target and endogenous control markers over a full 31-day screening. The objective of this study was to characterise any changes in expression that could occur due to the various stages of the menstrual cycle which may ultimately cause a false positive/negative result if analysed on various sexual assault samples.

The false identification of a body fluid could lead to the exoneration of a guilty suspect or the conviction of an innocent. The process for DNA profiling is the same in all forensic laboratories, it would therefore be highly beneficial if the technique could utilise some of these work flow stages. Although miRNA extraction kits are available all forensic laboratories utilise DNA extraction protocols. The introduction of an additional extraction method would require double the sample volume, additional personnel to perform the analysis and an increase in cost of reagents. This research aims to validate the extraction methods currently used in forensic laboratories for their ability to extract miRNA, with a comprehensive comparison of DNA and RNA specific extraction methods. The efficiency for miRNA extraction as well as the reproducibility and reliability will be fully analysed to ensure the implementation of a non-specific miRNA extraction kit would not obstruct BFID.

The identification of markers that can differentiate body fluids when found in self-collected, un-contaminated, single body fluid samples is important however they must provide the same infallible identification when utilising on 'real world' samples that contain mixtures of multiple body fluids. The final research project will analyse the markers ability to detect semen within a vaginal material mixture obtained following UPSI as well as the duration that efficient and accurate identification can be achieved. To achieve the objective of developing a technique that will fit into current forensic work flow, this study will also assess the effect DTT has on miRNA. DTT is an essential part of DNA profiling from semen, it is destructive to the spermatozoa head therefore the likelihood for damage to miRNA is high. This study aims to determine the extent, if any of damage on miRNA through various extraction techniques utilising DTT.

Overall, this research aims to identify a full BFID panel that can solve the current issues forensic scientist face as well as providing additional strong weighted evidence for the events of a crime scene. Once identified validation will include the most suitable extraction method, the identification of body fluids when found in mixtures and the duration certain body fluids such as menstrual blood and semen can be detected.

4. Methods and Materials

4.1. Ethics Statement

The study was approved by the School of Applied Sciences Ethics Committee on Human Research of the University Huddersfield and written informed consent was obtained from all participants. Full ethical approval was gained for collecting and processing all body fluid samples contained within this research.

4.2. General Reagents and Consumables

Unless stated all plastic consumables described in this chapter were purchased from Sarstedt (Germany) with all reagents purchased from either Thermo Fisher Scientific, (MA, USA), Eurofins Genomics (Ebersberg, Germany), Qiagen (Germany) and Primerdesign (Chandler's Ford, UK)

4.3. Volunteer Selection and Sample Collection

Individuals were sought out and chosen based on factors that literature and fundamental knowledge suggested may cause variation in miRNA expression: Simple factors such as age, ethnicity, lifestyle habits (smoking and drinking) and type of contraception were included, but also more in-depth factors such as pregnancy history/complications, sterilisations, diseases (especially those that target the reproductive systems) and stage of menopause (See appendix A and B). A vast range of factors were selected such that if a marker was significantly expressed in all the samples it would show its robustness as a biomarker in sexual assault cases while also highlighting a possible cause if all but one sample displayed expression.

All samples were self-collected using sterile cotton swabs. After sample collection the swab was returned to the sterile tube and dried at room temperature, thus preventing cross contamination of samples. Benches were regularly cleaned with MicroSol 3+ to remove any DNA/RNA contamination and full PPE was worn while processing the samples. Information sheets were provided, followed by completion of a comprehensive questionnaire to ensure all intimate female samples with a high likelihood of semen presence were known, proper

sampling techniques were followed and to ensure honest answers to intimate questions could be anonymously provided. All volunteers were assigned a symbol to ensure anonymity throughout processing and analysis.

4.3.1. Vaginal secretions

Women self-collected LVS using a standard sterile cotton swab, once sampled the swab was returned to its casing, labelled and extracted immediately. If storage before extraction was required the swab was kept at -20°C. Volunteers ranged in age from 18 to 65. Individuals had extremely varied lifestyles differing in age, smoking, previous pregnancies, all forms of contraception, cervical cancers, ethnicity, sterilisation, medication and varying levels of sexual activity. Volunteers were requested to provide the approximate day of their last menstruation sexual interaction (either oral or penile) on their consent sheet. This ensured that any samples that could possibly contain semen or menstrual blood could be identified.

4.3.2. Menstrual blood

All women providing vaginal material samples were asked to provide a menstrual blood sample, this was achievable in half of the vaginal material volunteers. Factors such as cessation due to hormonal contraceptives or time/ distance constraints while menstruating meant not all volunteers were able to donate both body fluids. Additional females donated menstrual blood as their only body fluid sample. Collection occurred as per vaginal secretions. Samples ranged broadly in age of volunteer from woman in variation stages of the menopause as well as younger women on different types of contraceptive and with varying pregnancy histories. Volunteers were requested to provide their approximate day of menstruation on their consent sheet.

4.3.3. Semen and seminal fluid

Semen and seminal fluid was obtained from men ranging from 18 to 52. Samples were self-collected into a Falcon™ 50mL Conical Centrifuge Tube. The Falcon tubes were stored in a -20°C freezer and nucleic acids extracted within 24 hours. All samples underwent

Haematoxylin and Eosin staining (H&E) to determine presence of spermatozoa (as observed in 4.6).

4.3.4. Blood and saliva

Samples were collected for each body fluid to allow for comparisons with previous research [199]. Saliva was obtained through self-collection using sterile cotton buccal swabs from the inside of both cheeks, taken at least 30 minutes after eating. Blood was self-collected collected using an Owen Mumford Unistik 3 Extra to pierce the tip of the thumb or index finger, a buccal swab was then used to collect the blood until saturated. Antiseptic wipes were used to clean the finger before and after to reduce contamination. Visual examinations were performed on saliva buccal swabs to reduce the risk of blood being present in the sample.

4.3.5. Skin

Skins cell samples were self-collected using a scalpel to gently remove the skin cells from the back of the hand. Antiseptic wipes were used to clean the hand before and after to decrease risk of contamination. Visual inspections of the samples were performed to ensure blood wasn't present within the sample.

4.4. Nucleotide Extraction

4.4.1. Standard total DNA extraction

Total DNA extraction was performed using the QIAamp DNA Mini Kit. DNA was isolated from the body fluids using lysis buffer which breaks down the nucleus of the cell to release the genomic material while proteinase K degrades proteins that could be damaging to DNA [201, 202]. The high chaotropic salt concentrations from the lysis buffer precipitates DNA and therefore the DNA binds to the silica-gel membranes within the spin column. The addition of ethanol solubilises organic contaminants associated with the cell which then pass through the silica membrane. Proteins, divalent cations and other PCR inhibitors are removed throughout wash steps: by eliminating cations such as Mg^{2+} DNA will not be

degraded by nucleases. An elution buffer is then used to remove the DNA from the silica membrane so they can be eluted to obtain a pure DNA extract.

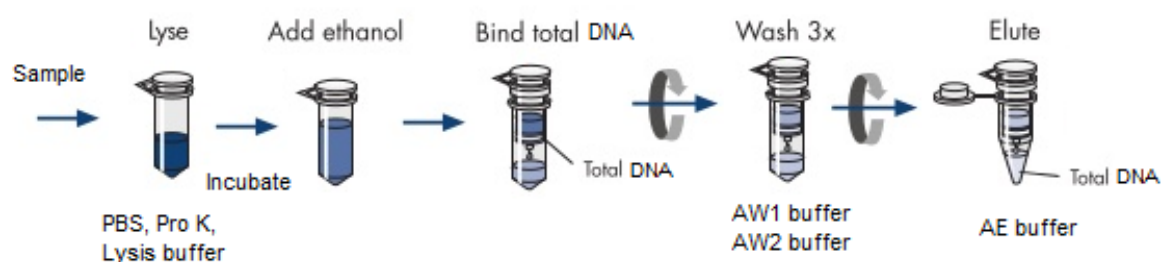


Figure 4.1 Stages of DNA extraction following the QIAamp DNA Mini Kit protocol [202].

Figure 4.1 shows the process of extraction: 400µl of PBS, 20µl proteinase K and 400µl lysis buffer were added to a sample within a sterile 2ml tube. This was then vortexed thoroughly and pulse centrifuged to retain the sample at the base of the tube. The samples were then incubated for 10m at 56°C. Absolute ethanol (400µl) was then added to the lysate and subjected to a further pulse centrifugation. 700µl of lysate was then transferred into a spin column and centrifuged at 8,000 rpm for 1 minute, the flow-through within the tube was then discarded and the remaining lysate was transferred to the spin column and the process repeated. AW1 wash buffer (500µl) was then added to the top of the spin column and centrifuged at 8,000rpm for 1 minute, with the flow through discarded. AW2 wash buffer (500µl) was then transferred to the spin column and centrifuged at 14,000rpm for three minutes. After discarding the flow through, a further centrifugation step at 14,000rpm for one minute was carried out to remove any residual wash buffer from the column. The spin column was then transferred to a fresh 2ml tube and 150µl of AE elution buffer added and left to incubate at room temperature for one minute. The DNA was then eluted from the column via centrifugation at 8,000rpm for one minute. The column was then discarded and the sample retained at -20 °C prior to downstream analysis.

4.4.2. Total RNA extraction

RNA extraction was performed with Qiagen's RNeasy Mini Kit. The kit has similar principles to the DNA extraction kit in that it utilises the selective binding properties of the silica membrane. Samples are first lysed and homogenised using a highly denaturing guanidine-

thiocyanate-containing buffer, inactivating RNases to ensure pure RNA is not degraded. 70% ethanol is then added to the lysate to create the ideal binding environment. The lysate is then transferred to an RNeasy spin column where binding of RNA occurs with contaminants being efficiently removed using wash buffers. RNase free water is then used to elute to pure RNA. [203].

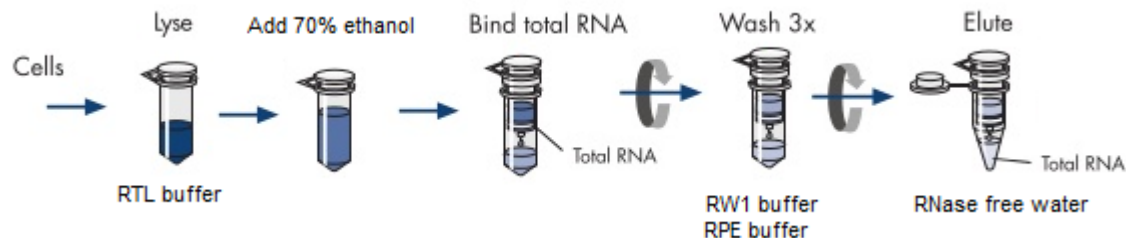


Figure 4.2 Processes involved in total RNA extraction following RNeasy Mini Kit protocol [203]

Figure 4.2 describes the process of extraction: 350µl of RTL buffer was added to a sample contained in a 2ml tube, which was then vortexed thoroughly and pulse centrifuged to collect the sample at the base of the tube. 350µl of 70% ethanol was then added to lysate. The lysate was then transferred to the QIAamp spin column and centrifuged at 10,000rpm for 15 seconds. 700µl RW1 buffer was added to the spin column and centrifuged at 10,000rpm for a further 15 seconds. The flow through was discarded and 500µl of RPE buffer added followed by centrifugation at 10,000rpm for 15 seconds. The flow through was discarded and 500µl of RPE buffer added and centrifuged at 14,000rpm for three minutes. Spin columns were then placed in a new 2ml tube and centrifuged at 14,000rpm for one minute. RNase free water (50µl) was then added and centrifuged at 8,000rpm for one minute to elute RNA.

4.4.3. Differential extraction

Differential extraction is the preferred extraction technique when dealing with samples from a sexual assault case. The process involves splitting the epithelial portion, the majority of which will obtain DNA from vaginal material from the possible spermatozoa. Both samples are then DNA profiled with the aim of obtaining two distinct profiles. DTT is added

to the seminal fraction to degrade the protein disulphide bridges that make up sperm nuclear membranes [204].

Samples were placed into a 2ml tube containing 1ml of PBS buffer. The swab head underwent agitation with a sterile stick for 60 seconds to ensure the maximum yield of spermatozoa was removed from the swab followed by vortexing at 12,000rpm for 60 seconds, this stage was then repeated. Samples were then centrifuged at 14,000rpm for five minutes to form a pellet. The swab head and supernatant were then carefully removed so the pellet was not disturbed, leaving ~50µl of supernatant surrounding the pellet.

4.4.3.1. Epithelial fraction

125µl of 1% SDS along with 125µl 2mg/ml Proteinase K (made from 1:5 dilution of Qiagen Pro K with molecular biology grade water (MBG) was then added to the 2ml tube containing the pelleted sample. This was then vortexed at 12,000rpm for 15 seconds to ensure complete disruption of the pellet. The samples were then incubated at 37°C for 30 minutes, briefly vortexed to break up any new remaining solids and incubated again at 37°C for 30 minutes. Samples were then vortexed and centrifuged at 14,000rpm for five minutes. 250µl of supernatant was transferred to a 2ml tube and combined with 250µl of Qiagen ATL buffer. These samples were then processed using the standard Qiagen DNA protocol described above in section 3.4.1, excluding the lysis stage.

4.4.3.2. Seminal fraction

500µl of wash buffer (10mM Tris HCl, 10mM EDTA, 50mM NaCl and 2% SDS, pH adjusted to 7.5 using 2M NaOH and autoclaved at 121°C for 15 minutes to remove DNase/RNase) was added to the pellet retained in section 3.4.3.1 and vortexed at 12,000rpm for 60 seconds. Samples were then centrifuged at 14,000rpm for 5 minutes to create a pellet, the supernatant was discarded with the pellet reconstituted with 50µl of wash buffer. This wash step was then repeated three times. After this third wash step, the supernatant was discarded and reconstituted in 1,000µl of MBG water by vortexing. The mixture was then centrifuged for a further 5 minutes at 14,000rpm and the supernatant discarded.

475µl of ATL Buffer, 25µl of Qiagen Proteinase K and 20µl 1M DTT were then added to the pellet and vortexed for 60 seconds at 12,000rpm. After a pulse centrifuge the samples were incubated at 56°C for 60 minutes with frequent agitation from a vortex at 900rpm. The samples were then spun at 8,000rpm for 60 seconds and then followed the wash process of the standard DNA protocol (3.4.1).

4.5. Quantification of Samples

Quantification of molecular samples is crucial for many analytical techniques, with the recommended template volume of DNA for successful human identification is 0.5-2ng [205]. An input higher than this will result in too much DNA for a viable DNA profile electropherogram, demonstrated by a higher frequency of “pull ups”, “stutters” and heterozygous peaks that are not even and/or split peaks [205, 206] In relation to sexual assault, samples obtained from intimate swabs will contain a large volume of vaginal material with, if present a small volume of seminal fluid. As a result, a seminal profile would not be obtained due to a high concentration of vaginal sample causing a failed electropherogram, where dilution would likely remove trace of the seminal fraction.

At the commencement of this research, no accepted method for quantifying miRNA was available. Therefore, for the studies performed, the DNA concentration obtained from quantification assays (3. 5.1) were used to calculate a normalised concentration for all samples. Recently, companies such as ThermoFisher scientific have developed quantification assays for miRNA (Qubit® microRNA Assay Kit released February 2015 [207]. This technique provides an overall quantification of all miRNA present within a sample however it not specific to a set marker, the technique also requires additional equipment, training, reagents and analysis. The aim of this research is to develop a viable BFID technique that can be implemented into current laboratory protocol. The addition of this technique would cause an increase to the cost and duration of analysis as well as increasing the required man power, training and equipment. All these factors, including the lack of specific marker quantification would decrease the likelihood of implementation into forensic laboratories and therefore normalisation through DNA concentration was

performed throughout the remaining studies. All DNA samples underwent qPCR quantification while RNA samples were quantified using a spectrophotometer at wavelength 260nm.

4.5.1. DNA quantification utilising absolute qPCR

Calculating qPCR results from samples can be achieved by absolute or relative quantification. Absolute quantification provides the exact copy number following conversion of the data via a standard curve of known DNA copy number. Relative quantification does not provide the total starting quantity of unknown template, instead the expression is represented relative to another gene or internal control.

DNA samples were quantified using Quantifiler® Human DNA Quantification Kit on Applied Biosystems 7500 Fast Real-Time PCR machine. The Quantifiler® Human DNA Quantification Kit quantifies the total volume of amplifiable human DNA that is present in a sample using two 5' nuclease assays, an internal control (IPC) and a human target specific assay. The target specific assay contains two primers that are specific to human DNA and a FAM™ labelled TaqMan® MGB probe. IPC contains template DNA, the sequence of which is not found in nature, two primers specific to this synthetic sequence and VIC® labelled TaqMan® MGB probe. Probes contain a non-fluorescent quencher (NFQ) at the 3' end which suppress fluorescence. Throughout qPCR, the TaqMan® MGB probe anneals specifically to a complementary sequence in the middle of the forward and reverse primer sites. If no amplification occurs the probe will stay whole keeping the reporter and quencher dyes in close proximity causing suppression of reporter fluorescence. Cycles are used to exponentially amplify any DNA present, once a threshold of total DNA has been reached the fluorescence caused by the disassociation of the quencher from the reporter will be detected by the instrument and alongside a standard curve, concentration is calculated [208].

Serial dilutions using Quantifiler® Human DNA standard and water were made to encompass the range of samples being quantified to create a standard curve. The supplied control DNA was at a concentration of 200n g/μl. To ensure the samples that contained

large volumes of DNA were accurately quantified 1.5X serial dilution from 133.33ng/μl were made for quantifying vaginal material and menstrual blood samples, as seen in table 4.1. For saliva, semen, skin and venous blood samples, the 5X serial dilutions stated in the supplier's protocol were made. The required volume of control DNA and water were transferred into a 0.2ml tube, tubes were vortexed thoroughly and pulse centrifuged, the required volume of the previous standard was then transferred into the next.

Standard	Concentration (ng/μl)	Volume (ml)	Dilution factor
1	133.333	10μL 200 ng/μL stock 15μL of water	1.5X
2	88.888	10μL Std 1 15 μL of water	1.5X
3	59.25925	10μL Std 2 15 μL of water	1.5X
4	39.506	10μL Std 3 15 μL of water	1.5X
5	17.55	10μL Std 4 15 μL of water	1.5X
6	11.705	10μL Std 5 15 μL of water	1.5X
7	7.803	10μL Std 6 15 μL of water	1.5X
8	5.202	10μL Std 7 15 μL of water	1.5X
9	3.46	10μL Std 8 15 μL of water	1.5X
10	2.3122	10μL Std 9 15 μL of water	1.5X

Table 4.1: Dilution series for creation of standards for quantifying vaginal material.

Each 12.5μL reaction contained of 5.25μL of Quantifiler® PCR Reaction Mix, 6.25μL of Quantifiler® Human Primer Mix and 1μL of either sample, standard or water. Each 0.2ml PCR contained enough MM and sample, standard or water to create three technical replicates. Each tube was vortexed, pulse centrifuged and 12.5μL was then aliquoted into 3 designated wells of a 96 well plate. All samples, standards and negatives were run in triplicate. The 96 well plate was then sealed with optical lids, pulse centrifuged and placed within the 7500 FAST real time PCR instrument. Absolute quantification (FAST – Quantitation standard curve with expert mode) was selected with enzyme activation occurring at 95°C for 2 mins followed by 40 cycles of (denaturing at 95 °C for 2 seconds and annealing and extension at 60 °C for 20 seconds).

4.5.1.1. Advantages to qPCR

The main advantage of qPCR is the ability to observe amplification of target fragments in real time, target amplification is measured during the exponential phases as opposed to regular PCR where amplification can only be measured in the plateau phase. The benefit to this is that the exponential phase provides a true starting quantity of genetic material whereas the plateau phase is not always a specific. qPCR offers advanced specificity and sensitivity due to presence of TaqMan® probes which ensures that any recorded fluorescent signal is a result of amplification from a specific target sequence. The ability to design probes and primers with various dyes alongside the qPCR instruments multiple dye channels allows for multiple TaqMan® probes and primer combinations to be utilised to distinguish between closely related sequences or for different fluorophores attachment to probes which enables the development of multiplex qPCR protocols resulting in several required targets being co-amplified. Overall the use of hydrolysis probe qPCR is highly sensitive and specific in a protocol where assay design and run methods are relatively simple, reducing the requirement for extensive training of operators [209, 210]

4.5.1.2. Disadvantages to qPCR

The advancements within qPCR development have made it one of the most popular quantification techniques with the only disadvantage being the requirement that different probes must be designed and synthesised for multiplex analysis thus increases cost of analysis. When comparing to other techniques such as UV/VIS spectrophotometry, although superior in specificity it is a much more time consuming and costly method of quantification.

4.5.2. RNA quantification using a UV-visible spectrophotometer

RNA quantification was performed on the Thermo Scientific Nanodrop 2000 spectrophotometer instrument. Spectrophotometry determines the ability of a chemical substance to absorb light; every chemical compound and molecule absorbs, reflects or transmits light particles when measured over a certain wavelength - spectrophotometry

records the samples absorbance. UV-visible spectrophotometers measure absorbance of the RNA samples over the ultraviolet range of 184-400 nm and the visible range of 400-700nm of electromagnetic radiation spectrum. This absorbance is then converted into a concentration value [211].

After calibration and measurement with a blank, 1µl of sample was placed onto the pedestal and measured at 260nm (A₂₆₀), purity values were checked and concentration values recorded. Concentration values were obtained in triplicate for each sample, with only a variation of 0.5ng/µl allowed between values.

4.5.2.1. Advantages to UV-VIS spectrophotometry

The advantage of UV-VIS spectrophotometry is that there is minimal sample wastage, concentration values are obtained quickly with minimal training required, it is cost effective due to lack of additional reagent requirement and analysis includes evaluation of the relative purity of samples [212]. Another benefit is that there is no direct manipulation of the samples being analysed therefore the integrity is not compromised.

4.5.2.2. Disadvantages UV VIS spectrophotometry

The disadvantages with this technique is that accuracy can be effected by a number of contaminants; if total RNA extraction eluted DNA, proteins, chaotropic salts or other complexes such as carbohydrates it will cause an incorrect value will be determine. UV VIS spectrophotometry cannot distinguish between the target of interest and contaminants that absorb light at an equal wavelength, since all nucleic acids display peaks at approximately 260nm all DNA or RNA molecules will contribute to the accumulated total absorbance at 260nm. Accuracy can also be effected by impurities within the samples reflecting light, since all light reaching the detectors is recorded this reflection off impurities would cause inaccuracy in concentration calculation.

Accurate light absorption is also controlled by the environment within the samples, absorbance is heavily influences by pH and temperature, which if combined with impurities will alter the absorption properties resulted in an incorrect concentration value for that sample. UV VIS spectrophotometry also displays inaccurate results at low concentration

samples, due to the high concentration with vaginal material this was not deemed a highly contributing factor to quantification [212].

4.6. Determination of Semen through Haematoxylin & Eosin Staining

When analysing semen samples the presence of spermatozoa must be confirmed, this is to ensure that any expression seen can be identified as coming from a 'standard' sample. A normal sample can be defined as having more than 20 million spermatozoa per millilitre of semen [213], this cannot be confirmed using a standard microscope however the identification of a large presence of semen is sufficient for this study. In the case of vasectomised samples the absence of spermatozoa must be confirmed; if spermatozoa are found then the miRNA expression will not distinguishable from the other semen samples collected.

The presence or absence of spermatozoa can be confirmed using the Haematoxylin and Eosin (H&E). It is a widely used staining technique and utilises two dyes. Haematoxylin is referred to as a basic dye (when mixed with a mordant such as an aluminium salt that help it bind) that stains basophilic or acidic structures, such as the nucleus a purplish-blue colour. The mordant binds to any basophilic structure which is followed by the binding of the haematoxylin to the mordant. Eosin is a negatively charged, acidic dye which stains acidophilic or basic structures, such as most of the proteins in the cytoplasm, with a reddish pink colour. The combination of these two dyes provides a stain that highlights the nucleus and the areas of the cytoplasm where RNA is present in purple with the remainder of the cytoplasm being pink [214, 215]. In the case of staining semen, the basophilic heads of the spermatozoa will stain a purplish blue and thus make them detectable under a high powered microscope as seen in Figure 4.3.

Semen samples (1µl) were placed on labelled glass slides. Each slide was then placed in haematoxylin dye for 15 seconds before being gently rinsed around the stain with tap water. The slide was then placed into the eosin stain for two minutes followed by a gentle

rinse around the stain. A cover slip was then placed over the stain and viewed under a microscope using 100x total magnification.

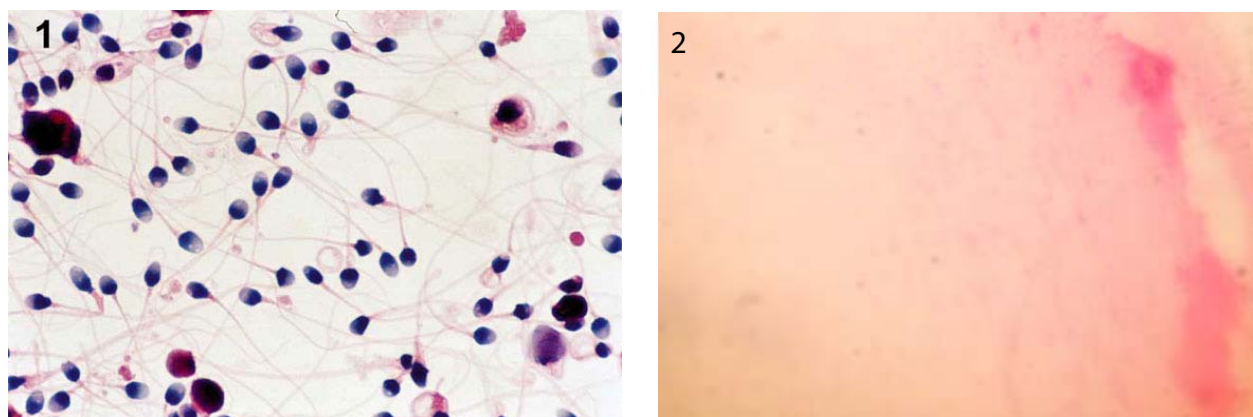


Figure 4.3 A micrograph of a typical semen stain using H&E (1) [215] and a micrograph displaying the complete absence of spermatozoa in the vasectomised sample provided by volunteer 63 following H&E staining (2).

4.7. Stem-loop Reverse Transcription alongside qPCR for miRNA Analysis

When analysing individual miRNA an extremely sensitive and reproducible method is needed. MicroRNAs range from 18 to 22nt in length with standard and quantitative PCR methods requiring a template that is a minimum of two times the length of either of the specific forward or reverse primers, each typically 20nt in length. Thus, the target minimum length is ≥ 40 nt, meaning miRNAs are too short for standard RT-qPCR methods [216]. The solution to this problem is stem-loop reverse transcription followed by qPCR (RT-qPCR).

RT-qPCR involves several components that are optimised for miRNA specificity and sensitivity. The RT-primer contains a highly stable stem-loop structure that lengthens the target miRNA to form complementary DNA (cDNA). The forward PCR primer adds additional length with nucleotides that optimize its melting temperature and enhance assay specificity. The reverse primer disrupts the probe to produce fluorescence. As previously described, specificity can be improved by placement of the probe over much of the original miRNA sequence. The disadvantage to this strategy is that multiple high cost probes would be required to analyse multiple miRNA targets. The solution is to incorporate a universal

probe sequence into the synthesised stem- loop RT primer which allows for a probe to be designed that can analyse any target marker. The T(m) of the probe is optimised by addition of a minor groove binding (MGB) complex [216].

4.7.1. Hydrolysis probe and Oligonucleotide RT and PCR primer synthesis

The oligonucleotides utilised within this research were commercially manufactured by Eurofins Genomics to the specifications of HPSF purification, 0.01µmol synthesis scale and lyophilised delivery. Primers were initially centrifuged for 3 minutes at 14,000rpm and stock solutions of 100µM prepared via the addition of MBG water as per the synthesis report. RT primers were designed to incorporate a universal hydrolysis probe sequence into the stem, a universal reverse primer sequence (GTG CAG GGT CCG AGG T) into the loop and a complimentary target miRNA sequence overhang as observed in Figure 4.4. The universal probe was synthesised by ThermoFisher Scientific to the specification of HPLC purification and TCG CAC TGG ATA CG sequence.

All stock RT primers were then made into a 1:100 dilution followed by a further dilution in MBG water to obtain a working stock of 12.5nM. Hydrolysis probe, universal reserve primer and all stock forward primers were then made into a working stock of 40µM. All stock and working stock were accurately labelled and stored at -20°C. Primer sequences utilised within this study can be found in Table 4.2 within chapter 4.9.1.1 with a full list of all screened primers being available in Appendix C.

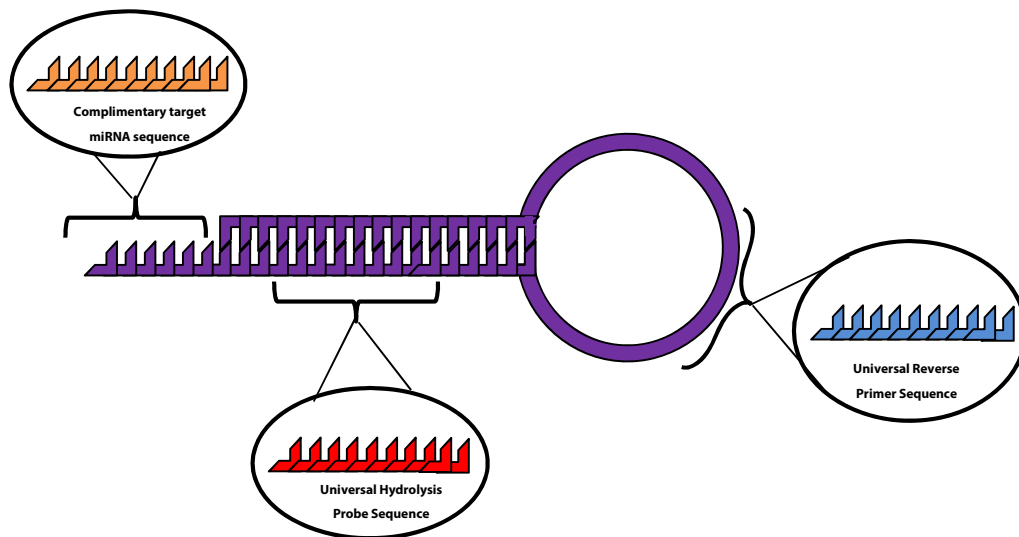


Figure 4.4: Sequences incorporated into the custom-built stem-loop primer.

4.7.1.1. miRNA selection for validation through screening via RT-qPCR

41 markers were selected from current literature: some already validated, some that had shown promising expression values but were not further replicated and some that surrounding research suggested may be a pre-cursor to identifying diseases linked to that body fluid. All 41 markers were custom designed and synthesised with TaqMan® assays ordered for 4 of those markers. Validation of custom assays was implemented and 5 of these markers and endogenous controls failed due to amplification in testing of negative samples. Markers were redesigned and failed again and were therefore removed from the study.

Target	Name	Primer	Sequence (5'-3')	Size bp	Ref
vaginal material	hsa-mir-124	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GCA TT	50	[17] [184, 185, 198]
	hsa-mir-124	PCR	TAA GGC ACG CGG TG	14	
	hsa-mir-1260b	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TGG TG	50	[181, 183]
	hsa-mir-1260b	PCR	ATC CCA CCA CTG CCA C	15	
	hsa-mir-654-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CAC AT	50	[181, 183]
	hsa-mir-654-5p	PCR	TGG TGG GCC GCA GAA C	21	
	hsa-mir-372-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CGC TC	50	[184, 183]
	hsa-mir-372-3p	PCR	AAA GTG CTG CGA C	13	
Saliva	hsa-mir-617	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CCA CC	50	[185]
	hsa-mir-617	PCR	AGA CTT CCC ATT TGA A	16	
	hsa-mir-205	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGA CT	50	[183, 184, 186, 189]
	hsa-mir-205	PCR	TCC TTC ATT CCA CC	14	
	hsa-mir-124*	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TCA AG	50	[187]
	hsa-mir-124*	PCR	CGT GTT CAC AGC GGA C	16	
	hsa-mir-138-2	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACC CT	50	[186]
	hsa-mir-138-2	PCR	GCT ATT TCA CGA CAC	15	
Menstrual blood	hsa-mir-223	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GGG GT	50	[181]
	hsa-mir-223	PCR	TGT CAG TTT GTC AAA	15	
	hsa-mir-144-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GTA CA	50	[183, 185, 198, 199]
	hsa-mir-144-3p	PCR	TAC AGT ATA GAT GAT G	16	
	hsa-mir-144-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TTA CA	50	[181, 183, 185, 199]
	hsa-mir-144-5p	PCR	GGA TAT CAT CAT ATA C	16	
	hsa-mir-412-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CGG CT	50	[184]

<i>Venous blood</i>	hsa-mir-412-3p	PCR	ACT TCA CCT GGT CCA C	16	[181]
	hsa-mir-142-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CCA TA	50	
	hsa-mir-142-3p	PCR	TGT AGT GTT TCC TAC	15	
	hsa-mir-199a-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AAC AG	50	[190]
	hsa-mir-199-5p	PCR	CCC AGT GTT CAG ACT AC	17	
	hsa-mir-214-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CTG CC	50	[183]
	hsa-mir-214-3p	PCR	ACA GCA GGC ACA GAC	15	
	hsa-mir-451	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACT CA	50	[124, 183-180]
	hsa-mir-451	PCR	AAA CCG TTA CCA TTA C	16	
	hsa-mir-16	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCC AA	50	[181, 183-185]
	hsa-mir-16	PCR	TAG CAG CAC GTA AAT	15	
<i>Semen</i>	hsa-mir-888-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GAC TG	50	[186, 188]
	hsa-mir- 888-5p	PCR	TAC TCA AAA AGC TGT C	16	
	hsa-mir-10a-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA AA	50	[183, 180]
	hsa-mir- 10a-5p	PCR	TAC CCT GTA GAT CCG	15	
	hsa-mir- 10b-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TTC CC	50	[184, 185, 190]
	hsa-mir- 10b-3p	PCR	CGC GCG ACA GAT TCG ATT CTA GGG G	25	
	hsa-mir- 10b-5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA AA	50	[183]
	hsa-mir- 10b-5p	PCR	CGC GCG TAC CCT GTA GAA CCG A	25	
	hsa-mir-135a -5p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAC AT	50	[183-185]
	hsa-mir- 135a-5p	PCR	TAT GGC TTT TTA TTC	15	
	hsa-mir-135b	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAC AT	50	[185]
	hsa-mir- 135b	PCR	TAT GGC TTT TCA TTC	15	
	hsa-mir-508	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ATG AG	50	[187]
	hsa-mir-508	PCR	GCC TGT ACT CCA GAG GGC GT	20	

<i>Skin</i>	hsa-mir-507	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT TCA CT	50	[185]
	hsa-mir-507	PCR	GCG GCG GTT TTG CAC CTT TTG G	22	
	hsa-mir-891a	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG TG	50	[183]
	hsa-mir-891a	PCR	TGC AAC GAA CCT GAG	15	
	hsa-mir-2392	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACC TC	50	[181]
	hsa-mir-2392	PCR	TAG GAT GGG GGT GAG	18	
	hsa-mir-3197	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCC TT	50	[181]
	hsa-mir-3197	PCR	GGA GGC GCA GGC TCG	15	
	hsa-mir-203a	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAG TG	50	[183]
	hsa-mir-203a	PCR	TGA AAT GTT TAG GAC	15	
	hsa-mir-455-3p	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG TGT AT	50	[199]
	hsa-mir-455-3p	PCR	CGC GCA CTT CAC CTG GTC CAC	21	
	hsa-mir-3169	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAT GT	50	[179]
	hsa-mir-3169	PCR	TAG GAC TGT GCT TGG	15	
<i>endogenous control</i>	SNORD-38b	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CTC CT	50	[183, 196, 197]
	SNORD-38b	PCR	TGA CAG TAA GTG AAG ATA AAG TGT GTC	27	
	SNORD-44	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GTC AG	50	[186, 196, 197]
	SNORD-44	PCR	ACT GAA CAT GAA GGT CTT AAT TAG CTC	27	
	hsa-mir--93	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAC CT	50	[197]
	hsa- miR-93	PCR	GCT CGC AAA GTG CTG TTC GT	20	
	SNORD- 47	RT	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CCT CA	50	[196]
	SNORD- 47	PCR	GAT ATC ACT GTA AAA CCG TTC CAT TTT G	30	

Table 4.2: Primer sequences utilised within screening study, categorised by literature proposed body fluid and corresponding publication.

4.8. Stem-loop Reverse Transcription

The result of stem-loop reverse transcription is the synthesis of cDNA that is used as a template for qPCR. This is performed by the RT enzyme MMLV, a monomeric protein obtained from the Moloney Murine Leukaemia virus. MMLV degrades RNA into a RNA-DNA hybrid because of its RNase H activity [1]. The miRNAs are reversed transcribed individually by using specific stem loop primers as seen in Figure 4.5 and are designed so that they are complementary to a short single-stranded region at the 3' of the target miRNA. They also contain a double stranded region known as the stem and a loop (optimally 4-8 bases long) which contains the universal primer and hydrolysis probe binding sequences. Once the stem-loop RT primers are hybridised to the miRNA molecule at the 3' region reverse transcription of the miRNA is initiated [218]

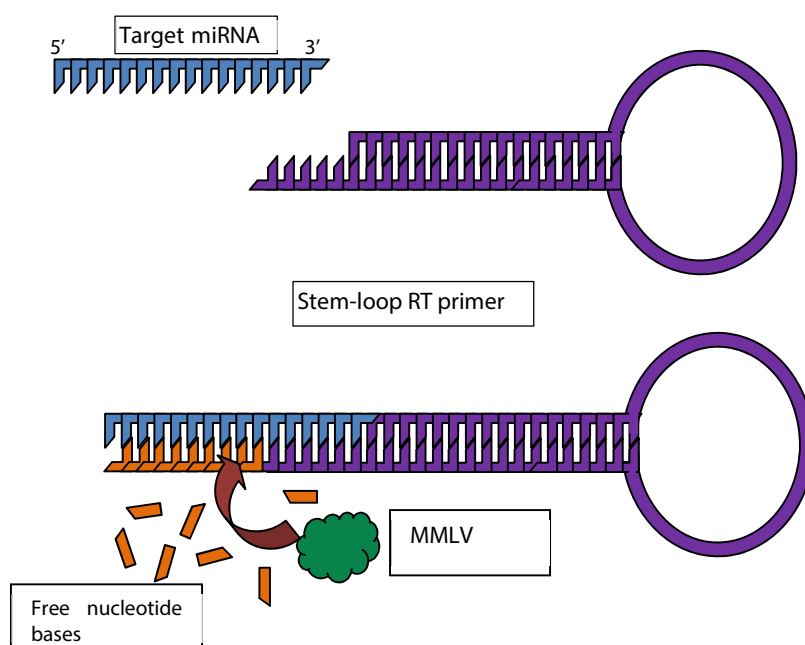


Figure 4.5: Process of stem-loop reverse transcription. The single stranded overhang binds to the complimentary 3' sequence of the target miRNA. MMLV then proceeds to bind circulating complimentary nucleotides to create cDNA.

TaqMan® MiRNA Reverse Transcription kit (Applied Biosystems) was used on each sample [219]. Each 7.5µl RT reaction contained 2.5µl of sample, 1.5µl of 12.5nM RT primer, 0.095µl of RNase Inhibitor 20U/µl, 0.75µl 10X RT Buffer, 0.075µl 10mM dNTPs, 2.08µl of nuclease-free water and 0.5µl of MultiScribe™ Reverse Transcriptase 50U/µl (MMLV). RT negatives

included a RT+ and a RT-. RT+ contained samples and no MultiScribe™ while RT – contained reverse transcriptase and no sample, this eliminates false positives as a result of genomic contamination. A MM was made per body fluid sample and aliquoted into 0.2ml tubes, the appropriate RT primer was then added. Reactions were then incubated in a 96 well Applied Biosystems Veriti® Thermal Cycler for 30 minutes at 16°C, 30 minutes at 42 °C followed by five minutes at 85 °C. This was then held at 4°C until collected and carried through to qPCR.

4.9. miRNA Expression through Relative qPCR

The second stage of analysis involves RT products undergoing relative qPCR, this is the constant collection of fluorescent signals from a polymerase chain reaction over a certain range of cycles. The detected fluorescence is then converted from each reaction into a numerical value per sample. The RT products are amplified and monitored in real time using a miRNA-specific forward primer and the universal reverse primer, resulting in every target molecule being copied once per cycle [217]. When using qPCR, the reactions are defined by the moment in time throughout cycling that amplification of a target is initially detected, not the total amount accumulated overall the cycles. The greater the starting copy number of nucleic acid target the quicker a substantial escalation in fluorescence can be observed [220]. As opposed to the absolute quantification requiring a standard curve, relative quantification is performed by analysing expression of a target against the expression of a housekeeping gene known as an endogenous control. The endogenous control is present within all samples therefore comparisons can be made within analysis of samples.

A universal RNA-based hydrolysis probe contains a fluorescent reporter dye at the 5' end and a quencher of fluorescence dye at the opposite 3' end of the probe, as seen in Figure 4.6. The close proximity of the quencher to the reporter prevents detection of its fluorescence. The hydrolysis probe can only anneal if the target sequence exists, it anneals further down from one of the primer sites and *Taq* DNA polymerase cleaves the hydrolysis probe through its 5' to 3' exonuclease activity when it is extending from the primer site. Degradation of the probe caused by the *Taq* polymerase removes the silence of

fluorescence caused by the reporter-quencher proximity and thus allows unquenched emission of fluorescence.

This is detected and provides fluorescence data that is converted into a numerical value. The breakdown of the probe also enables the primer extension to continue to the template strands end-point completing the PCR cycle. Therefore, the addition of the hydrolysis probe does not hinder the overall PCR process in any way. An increase in the product targeted by the hydrolysis probe at each PCR cycle therefore causes a proportional escalation in fluorescence intensity due to the degradation of the probe and thus discharge of the reporter [220, 221].

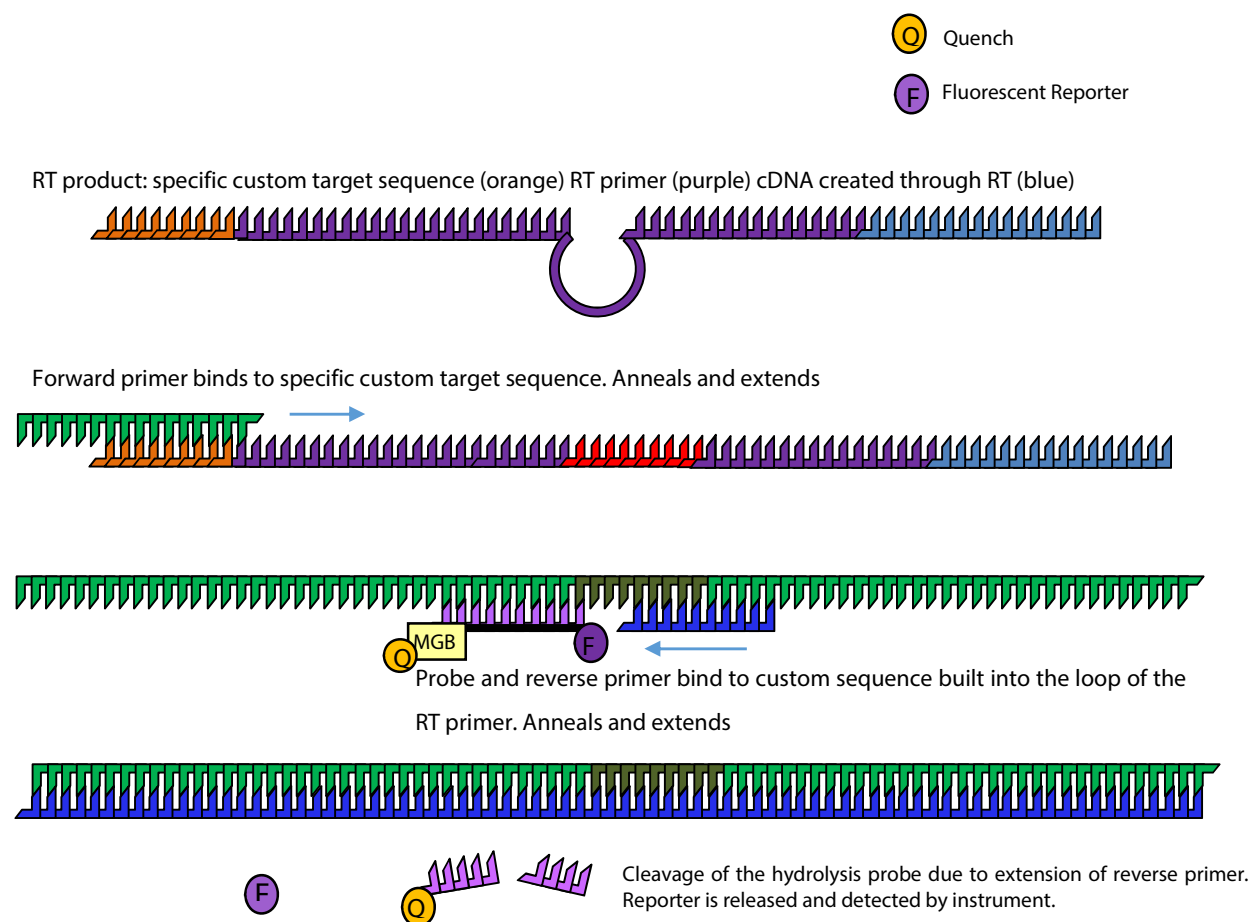


Figure 4.6: Processes involved within each cycle of qPCR: the cDNA is denatured and unravels; the custom forward primer then binds with the custom target sequence. Extension (direction indicated by arrows) by Taq polymerase creates a complimentary double strand which is subsequently denatured. The hydrolysis probe and universal reverse primer bind to the custom designed sequences within the loop of the RT primer. Extension of the reverse primer by Taq polymerase cleaves the hydrolysis probe causing the reporter to move away from the quencher and fluoresce. PCR product is determined by the 5' termini of the forward and reverse primers.

qPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. 10X concentration assays were constructed for custom made assays and 20X concentration assays were used for validated primers. Working stock solutions (40 μ M) were made from the universal probe, forward primer and universal reverse primer. This was achieved by diluting the primers as directed from Eurofins genomics to make a 100 μ M solution. 40 μ l of this was added to 60 μ l of nuclease free water to create the working stock. From the working stock 10x assays were prepared containing 3.75 μ l forward primer, 1.75 μ l reverse primer, 4.24 μ l nuclease-free water and 0.62 μ l probe per triplicate sample.

Each qPCR reaction contained 5.5 μ l of 2x Luminaris MasterMix (Primerdesign: Precision FAST MasterMix with ROX at a lower level), 3.66 μ l of nuclease-free water, 1.1 μ l of 10X assay and 0.73 μ l of RT product. For the validated assays this was altered to 0.55 μ l of 20X assay and 3.85 μ l of nuclease free water. Each reaction was made up in triplicate in a 0.2 μ l tube this was vortexed and centrifuged. 10 μ l was then pipetted into a predetermined well on a 96 well plate with the remaining reactions in consecutive wells. The 7500 Software v2.0.6 was set up in expert mode under 'quantitation, comparative Ct $\Delta\Delta$ Ct' FAST conditions for each 96 well plate. The reactions were incubated in a 96 well plate at 95°C for 2 minutes followed by 50 cycles of 95°C for 2 seconds and 60°C for 20 seconds.

4.9.1. Negative controls

All protocols were routinely performed with negative controls. MGB water was substituted for sample in all extraction, RT+ and qPCR negative controls, while MGB water was substituted for MMLV in RT- samples. Negative controls are essential for monitoring possible contamination and are required during each stage of analysis. All negative controls showed no amplification during qPCR indicating no genomic or outside contamination.

4.10. Analysis

Analysis of qPCR data is performed utilising Δ Cq (Cq target–Cq of endogenous control). Δ Cq is the normalisation of the target to a housekeeping control. Due to lack of suitable endogenous controls Δ^1 Cq values will be utilised (Max cycles (50)–Cq target). Once suitable

endogenous controls were identified ΔCq was calculated on all trace samples and displayed via heat map.

Varying opinions occur in literature surrounding cycle threshold, generally a Cq value is determined upon a positive expression result however false positives can occur and therefore an arbitrary cut off is determined by laboratory operators. It is suggested that high Cq values can be produced through probe base fluorophore degradation or as seen in most PCR reactions, non-specific amplification of background nucleic acids [222]. Caraguel *et al.* assessed a selection of cut off values for qPCR and determined that although the application of Cq cut off values might be practical, its justification and assignment should be constructed on evidence. They assessed research with cut off values of 31, 42 and 45, all of which provided justifiable evidence for cut off selection [223]. Monleau *et al.* analysed miRNA expression in PBMCs (peripheral blood mononuclear cells) and human serum, due to lower quantity of starting material human serum had no cycle cut off, this differed in PBMCs, where Cq values had a threshold of <32 [224].

To ensure all possible positive results are taken into consideration and determine all cross reactivity within body fluids, max cycles will be set at 50. Due to probe based techniques being used and the high levels of specificity required for use in a court of law the threshold for inclusion into body fluid analysis will be 40 cycles (Lower than $\Delta^1 Cq$ of 10). To display expression across all body fluids Cqs above 40 cycles will be presented in all results chapters but will not be taken forward as displaying miRNA expression. From current literature and self-determined requirements, the thresholds observed in table 4.3 were chosen to categorise strength of expression within this research.

<i>Cq Value</i>	Reaction	$\Delta^1 Cq$ value
<3	strong reliable positive reactions indicative of abundant target within sample	>21
30-37	Positive reactions indicative of moderate levels of target within sample (Expected level from hydrolysis probe qPCR)	$13 < > 20$
38-39.9	Poor reactions indicative of minimal levels of target within samples	$10.1 < > 12$
40 - 50	Weak unreliable reaction	<10

Table 4.3: Categories for levels of expression observed throughout analysis by all qPCR experiments

4.10.1. Normalisation

Normalisation is an important factor in the design of any experiment, two commonplace methods are the use of endogenous controls and the standardisation of concentration to the same value over all samples. As previously discussed normalisation was performed using DNA concentration and not miRNA concentration, the aim being that the ratio of miRNA to DNA is constant over samples and therefore will provide suitable normalisation. Due to the lack of universally accepted endogenous controls the second method of normalisation is also difficult to perform within this study. A range of SNORD and miRNA were selected from literature and analysed to find the most robust and efficient endogenous control. All samples were normalised to the same concentration as determined within study medication (4.13) and once identified, endogenous controls were used to further normalise samples.

4.11. Data Representation and Statistical Analysis

Throughout this research $\Delta^1\text{Cq}$ values will be displayed in box and whisker plots constructed in R version 3.3.2 using ggplots2 package and bar and line graphs constructed in Excel 2013. ΔCq values are displayed in heat maps created in R version 3.3.2. Heat map and box and whisker codes can be seen in appendix D-E. Each sample was analysed in triplicate, with standard error of mean (SEM) and average of all triplicates for that body fluid being shown on all bar and line graphs.

When analysing statistical significant of expression across the different body fluids and across vaginal samples within a volunteer, such as within chapter 5 and 6. A one way ANOVA with post hoc Tukey test (P threshold ranged from 0.01 to 0.001) was utilised. This determined any significant differences between the means of the six body fluids and the vaginal samples. When comparing volunteers of the same body fluid and therefore correlated, (DNA against RNA extraction, DTT against no DTT), such as within chapter 6 and 7 a paired sample t-test was utilised to compare the two population means. (P threshold ranged from 0.01 to 0.001).

4.12. MIQE Guideline Compliance

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines provide a reference check list that all data submitted for publications must comply with. All standards and requirements stated within the MIQE guidelines were observed in all experiments and analysis to ensure reliable and definitive qPCR were obtained [225].

4.13. Experimental Modification to Standard Protocols

4.13.1. Identification of miRNA markers

Samples were extracted following Qiagen total DNA protocol (4.4.1) and quantified using qPCR (4.5.1). Sample were manipulated in 3 ways; for undiluted testing eluate was directly taken as template for stem-loop RT. For analysis of the LOD, vaginal material samples were normalised to 5ng/μl and diluted through a 1 in 10 serial dilution while low template DNA samples were all normalised to 0.7ng/μl. All samples then underwent stem-loop reverse transcription (4.8) qPCR (4.9.1) and analysis (4.11)

4.13.2. Characterisation of markers over a 31-day period

Daily samples were extracted through Total DNA (4.4.1) or total RNA protocol (4.4.2). Quantification of samples from DNA extraction was performed using absolute qPCR (4.5.1), while quantification of RNA samples was performed on the Nanodrop 2000 instrument (4.5.2). Samples were then diluted to 5ng/μl and underwent stem-loop RT followed by relative qPCR (4.8 and 4.9.1). Data is displayed via bar graphs for $\Delta^1\text{Cq}$ +SEM and heat maps for ΔCq (4.11).

4.13.3. Detection of semen markers within vaginal material following unprotected sexual intercourse

Swab 1 from all sampling sets underwent total DNA protocol (4.4.1) with swab 2 from all sampling sets undergoing standard differential extraction (4.4.3). All samples were then

quantified using qPCR (4.5.1), diluted to 2ng/μl and underwent stem-loop RT followed by relative qPCR (4.8 and 4.9.1).

For DTT analysis two semen stains were obtained from the same sample, swab 1 followed total DNA protocol (4.4.1) with swab 2 followed the same protocol but with the addition of 20μl of 1M DTT during the lysis step prior to incubation. Vaginal material, venous blood and both semen samples were quantified as per (4.5.1) and normalised to 1 ng/μl. Various mixing ratios were made, observed in table 4.4, one set with semen extracted with addition of DTT, the other with the semen samples that underwent standard total DNA extraction. All mixtures then underwent stem-loop RT followed by relative qPCR (4.8 and 4.9.1). Data was analysed via box and whisker plots (4.11).

Body fluids	Semen extracted without DTT	Semen extraction with DTT
<i>vaginal material: semen</i>	1:1	1:1
<i>vaginal material: semen</i>	2:1	2:1
<i>vaginal material: semen</i>	5:1	5:1
<i>vaginal material: semen</i>	1:2	1:2
<i>vaginal material: semen</i>	1:5	1:5
<i>vaginal material: semen: venous blood</i>	1:1:1	1:1:1
<i>vaginal material: semen: venous blood</i>	2:1:1	2:1:1
<i>vaginal material: semen: venous blood</i>	5:1:1	5:1:1

Table 4.4: Various mixing ratios of body fluids containing semen samples treated and untreated with DTT.

5. The classification of MicroRNA for Body Fluid Identification

5.1. Rationale

Currently, a number of miRNA biomarkers have been identified as being body fluid specific (See section 2.6.1). The aim of the first component of this chapter was to validate the RT-qPCR method proposed in this research. TaqMan® assays were selected as these are commercially available markers that would eliminate the possibility of reactions failing due to primer specificity issues. In addition, a number of custom primers were also selected to validate body fluid specificity. To ensure expression, an undiluted extracted sample was used as the template for RT-qPCR.

In forensic casework, the amount of sample provided is likely to be low, and therefore it is important to identify the minimum template that can be used within these techniques. In a similar fashion, DNA profiling is a vital tool for forensic scientists and any techniques that could provide information on body fluid origin must not compromise the integrity of the original sample or the DNA profiling process. As such, it would be beneficial for the template used here to dovetail with other established BFID techniques. The UK Government forensics provider, LGC Forensics utilise Promega's PowerPlex® ESI 17 Pro System for STR profiling. This protocol states that 0.5ng of template DNA is recommended for obtaining a full profile [226]. Cellmark Forensic Services follow AmpFISTR® NGM SElect™ PCR protocol which suggests a higher template of 1ng of DNA is recommended to obtain a full profile [227].

Once a LOD has been determined for these markers, the specificity of these markers at the lower limits of starting template concentration can be investigated. The third section of this chapter concludes these initial investigations by defining the specificity of the complete library of body fluid specific markers, acting to validate the specificity of each marker or suitability of markers as endogenous controls as identified within the literature.

5.2. Sample Collection

Thirty women, ranging in age from 18 to 65 provided LVS. LVS were selected due to these being the easiest swabs for self-collection. HVS using require a speculum for correct sampling and it was unlikely that all participants would be comfortable with obtaining HVS samples but also that LVS would likely contain the least sample to sample variation. Individuals were selected to ensure different factors were included in the sample group. Females varied in age, smoking habits, previous pregnancies, contraception, cervical cancers, ethnicity, sterilisation, medication and varying levels of sexual activity, a list of notable factors can be found in appendix F. Eight of these females along with ten other volunteers provided menstrual blood samples on the day they believed was the middle of menstruation. One volunteer provided two menstrual blood samples; the first while on no form of contraction, the second, one month later, while on the contraceptive pill. Volunteers also provided blood (10), saliva (10) and skin (5). Twenty volunteers provided semen samples and six volunteers provided semen samples with known absence of spermatozoa. All volunteers and their body fluid samples can be seen in Figure 5.1.

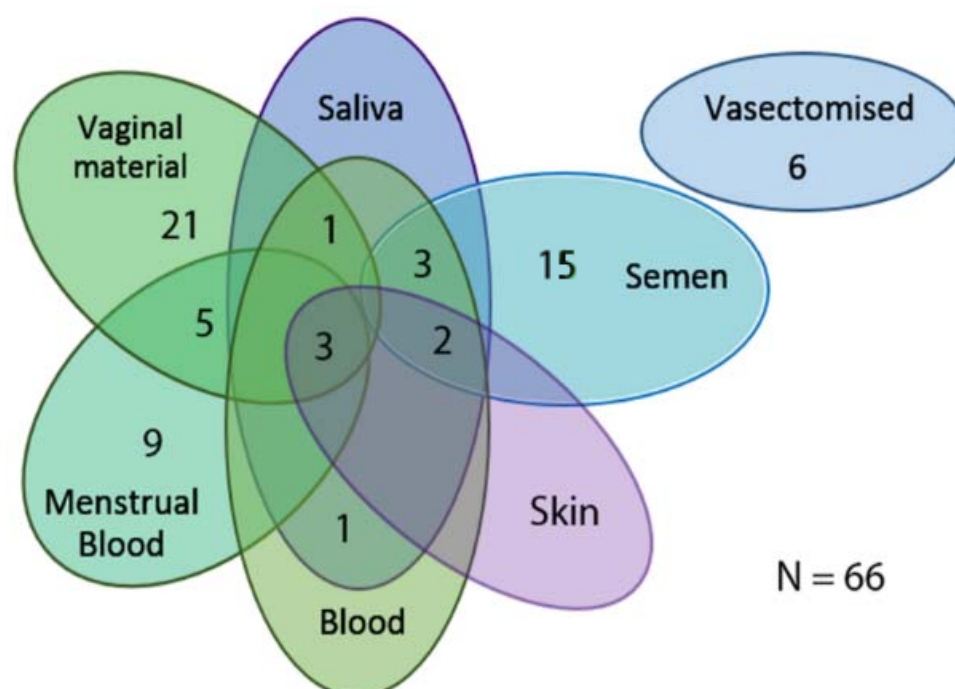


Figure 5.1: Venn diagram displaying the number of volunteers who provided multiple body fluid samples

5.3. Results

5.3.1. Expression comparison of TaqMan® and custom assays in undiluted sample

5.3.1.1. Quantification

The mean concentration (ng/μl) of DNA obtained from each sample type can be seen in table 5.1. The highest concentration observed in vaginal material and menstrual blood were 85.95 and 95.31ng/μl respectively with lowest concentrations of 18.89 and 20.10ng/μl. While venous blood and semen observed highest concentration at 4.27 and 9.28ng/μl respectively and lowest concentrations of 0.74 and 0.87ng/μl. The concentration for all seminal fluid and skin sample were below 0.006 and therefore in all applications in this research were used undiluted.

<i>Body Fluid</i>	Mean concentration (ng/μl)	SEM
<i>Saliva</i>	8.97	2.63
<i>venous blood</i>	2.43	0.39
<i>menstrual blood</i>	49.37	2.81
<i>Vaginal material</i>	41.64	2.66
<i>Semen</i>	4.12	0.53
<i>Seminal Fluid</i>	0.006	0.02
<i>Skin</i>	0.0002	0.01

Table 5.1: The average DNA concentrations (ng/μl) for all tested body fluids observed via qPCR.

5.3.1.2. R-qPCR

Markers hsa-mir-199a, 412, 135b and 507 (TaqMan® assays) were analysed on all body fluid types and the $\Delta^1\text{Cq}$ values can be seen in Figure 5.2. In the case of hsa-mir-199a, no significant difference was seen in expression across all body fluids. A similar pattern was observed when marker hsa-mir-507 was screened, with the exception of slightly higher expression within vaginal material and menstrual blood. Where markers hsa-mir-412 was screened, high expression was observed in vaginal material, menstrual blood and saliva, with weak expression (<10) in the other body fluids samples. Marker hsa-mir-135b was moderately expressed in all body fluids screened, except for venous blood and skin (<10).

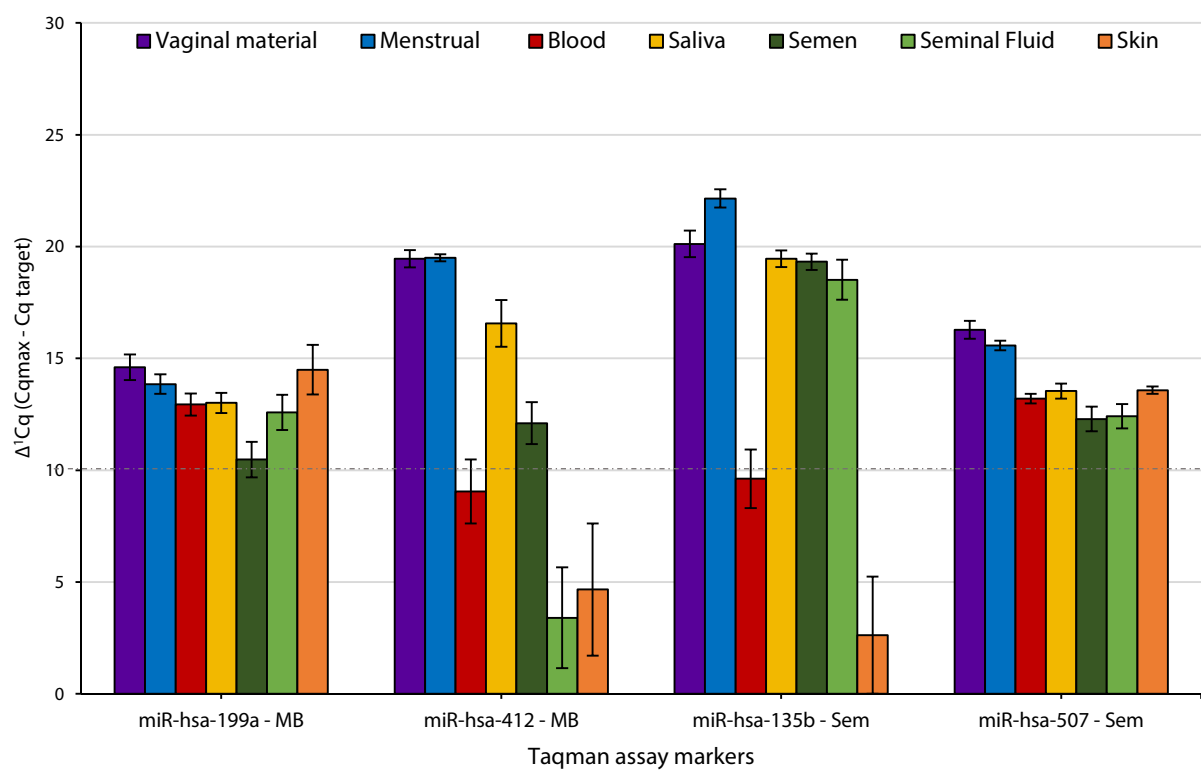


Figure 5.2: Expression of Applied Biosystems TaqMan® assays, measured using $\Delta^1\text{Cq}$ ($\text{Cq}_{\text{max}} - \text{Cq}_{\text{target}}$) in all body fluids. $\Delta^1\text{Cq}$ and SEM was calculated and plotted for menstrual blood markers hsa-mir-199a and hsa-mir-412 and semen markers hsa-mir-135b and hsa-mir-5. All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicated threshold for inclusion in analysis.

Markers hsa-mir-124a, 205 and 451 were analysed on all body fluid types and the $\Delta^1\text{Cq}$ values can be seen in Figure 5.3. Marker hsa-mir-124a showed significantly greater ($p < 0.001$) expression (16.3 and 19.3) in vaginal material and menstrual blood over all other body fluids. Saliva marker hsa-mir-205 displayed greater expression (16.8) in saliva but was found to be significantly greater ($p < 0.001$) in menstrual blood (23.2). Marker hsa-mir-205 expression was weak in vaginal material (< 10) and completely absent in blood, semen, seminal fluid and skin. Venous blood marker hsa-mir-451 was significantly expressed in both blood types (25.66 and 24.72) with absent or minimal expression in all other body fluids ($p < 0.001$).

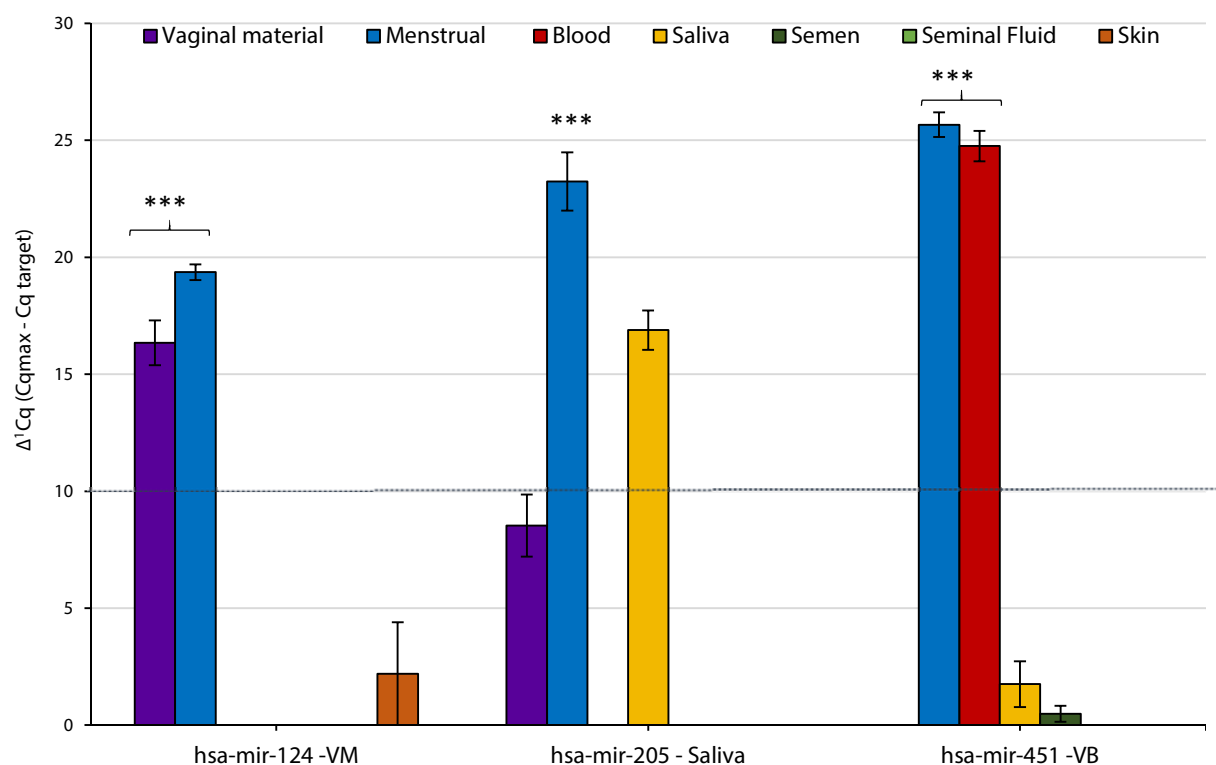


Figure 5.3: Expression of custom markers, measured using $\Delta^1\text{Cq}$ in all body fluids. $\Delta^1\text{Cq}$ and SEM was calculated and plotted for vaginal marker hsa-mir-124, saliva marker hsa-mir-205 and blood marker hsa-mir-451. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

Proposed semen markers hsa-mir-10a, 10b and 508 were analysed on all body fluid types and the $\Delta^1\text{Cq}$ values can be seen in Figure 5.4. Markers hsa-mir-10a, 10b and 508 did not display $\Delta^1\text{Cq}$ values in semen that were statistically different from any other body fluid. Markers hsa-mir-10a and 10b showed the highest expression value in menstrual blood (15.4 and 11.8 respectively) but not venous blood. Marker hsa-mir-10a showed minimal expression in all other body fluids except semen and seminal fluid, while hsa-mir-10b showed significant expression only in menstrual blood ($p<0.001$). Marker hsa-mir-508 showed universal expression >10 in all body fluids.

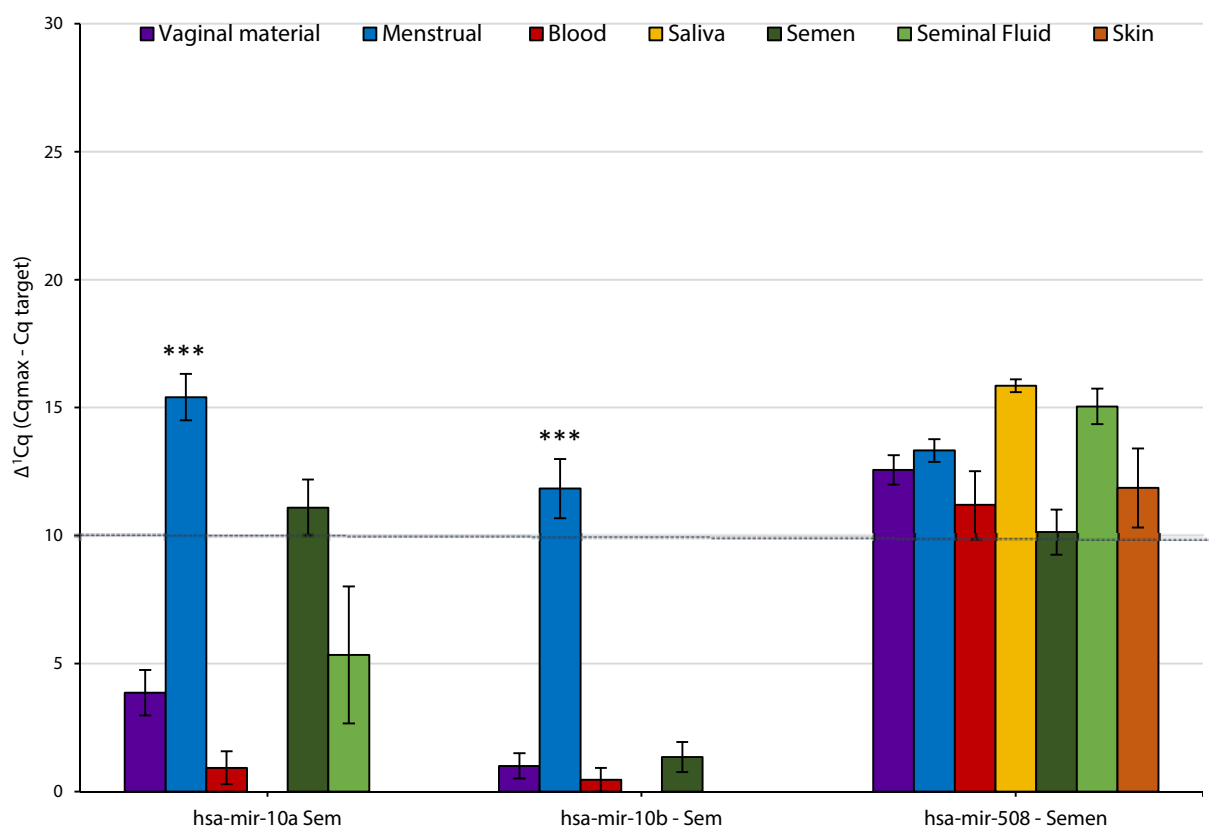


Figure 5.4: Expression of custom markers, measured using Δ^1 in all body fluids. $\Delta^1\text{Cq}$ and SEM was calculated and plotted for semen markers hsa-mir-10a and hsa-mir-10b and hsa-mir-508. ($p<0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

Proposed skin and vaginal material markers hsa-mir-203a, 372, and 617 were analysed on all body fluid types with $\Delta^1\text{Cq}$ values observed in Figure 5.5. Vaginal markers hsa-mir-372 and 617 showed limited or no expression in all body fluids (<6). Expression of skin marker hsa-mir-203a was observed in vaginal material (21.8), menstrual blood (24.8) and saliva (22.6), but was completely absent in blood, seminal fluid and skin. Minimal expression was also observed in semen samples (2.4).

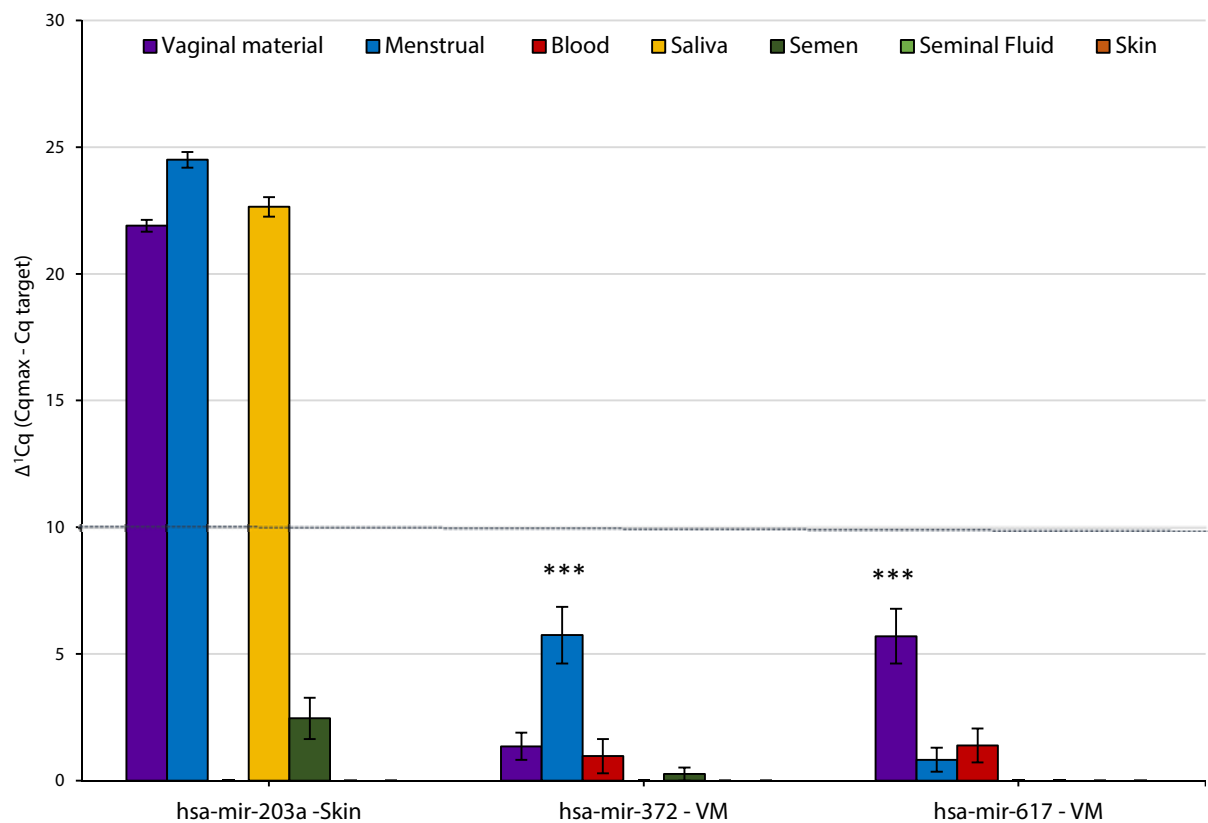


Figure 5.5: Expression of custom markers, measured using $\Delta^1\text{Cq}$ in all body fluids. $\Delta^1\text{Cq}$ and SEM was calculated and plotted for skin markers hsa-mir-203a and vaginal material markers hsa-mir-372 and hsa-mir-617. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

5.3.1.3. Heat map analysis of custom assay expression across individual undiluted samples.

A summary of the expression across this panel of markers can be seen in Figure 5.6. Marker hsa-mir-124 displayed high expression in all vaginal material and menstrual blood samples except for three vaginal material samples. One of these showed no expression while the remaining two displayed variation across triplicates. The complete absence of hsa-mir-124 expression in remaining body fluids is clear. The expression of hsa-mir-10a within each body fluid was sporadic, and absent within saliva and skin. Only one vaginal material sample displayed high expression, whilst expression was high in all but one menstrual blood sample. Semen and seminal fluid showed great variation across samples, only 50% showed high expression and only 1 of the 3 seminal fluid samples expressed hsa-mir-10a.

Although hsa-mir-10b was expressed in all menstrual blood samples, expression was varied and also expressed in two semen samples. Markers hsa-mir-372 and 617 were not consistent between body fluids and showed large variation within each body fluid. Saliva marker hsa-mir-205 was present in all saliva samples, again, with varying expression values. Expression was very high in all but one menstrual blood sample whilst moderate/weak expression was only observed in 45% of the vaginal material samples. Marker hsa-mir-451 displayed high expression in both venous and menstrual blood but was also weakly detected in one saliva and one semen sample. Expression of hsa-mir-203a was consistently high within vaginal material, menstrual blood and saliva samples with sporadic expression detected within 5 of the 22 semen samples. Marker hsa-mir-508 showed moderate to high expression in all samples, with the exception of single venous blood and semen samples, in particular, expression was high in skin and seminal fluid. Expression of TaqMan® markers hsa-mir-135b, 199a, 412 and 507 was universal across all samples; with high expression in both vaginal material and menstrual blood samples, with the exception of hsa-mir-412 displaying no expression in one sample of saliva. Expression of hsa-mir-412 and 199a was also absent in two semen samples.

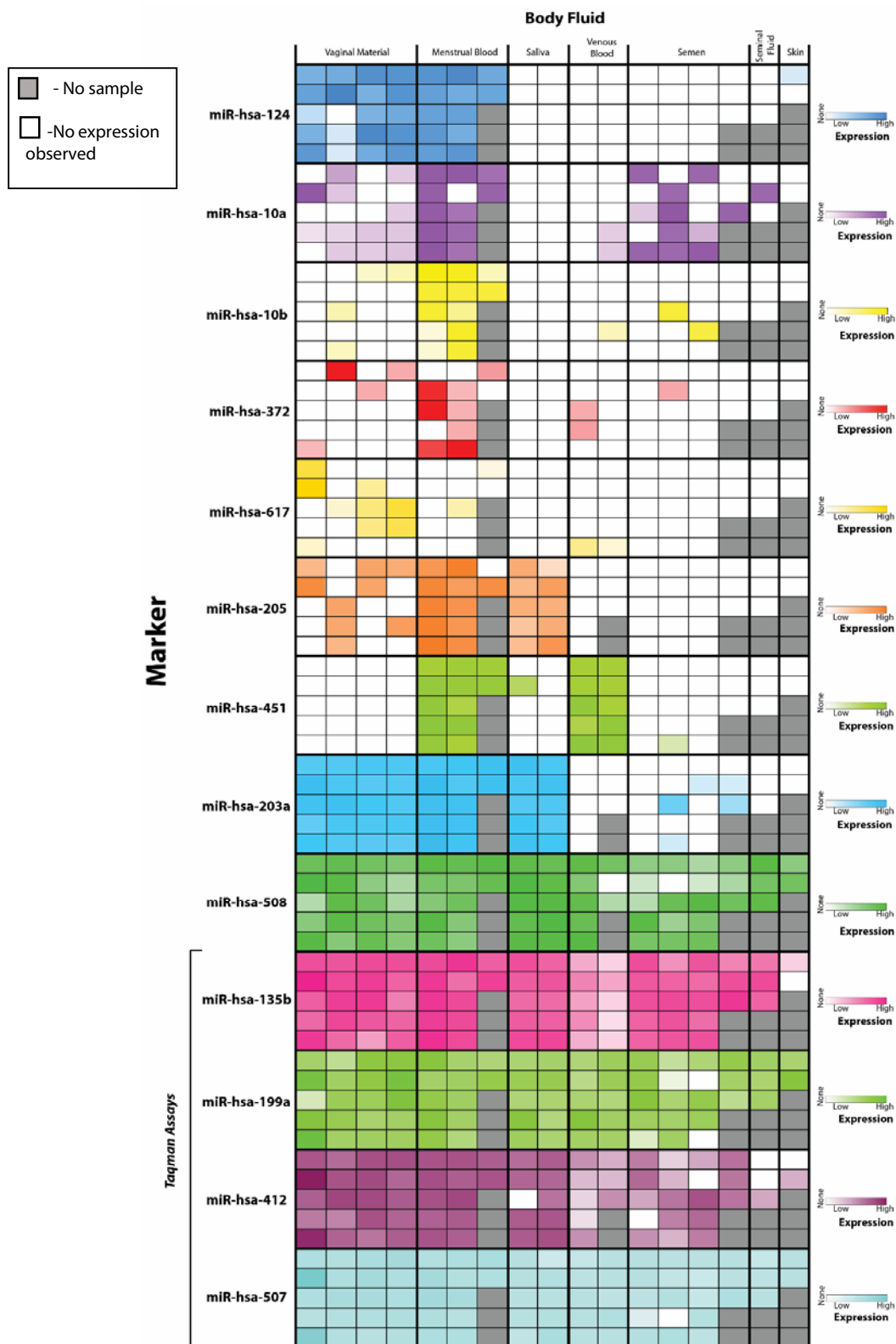


Figure 5.6: Individual Δ^1Cq (max Cq - (ΔCq of target) value of each body fluid sample across proposed BFID markers utilised within undiluted screening.

5.3.1.4. Discussion of Undiluted Samples

The results obtained within this section of the work demonstrate that the extraction and RT-qPCR methods employed are suitable for the determination of expression of a number of markers associated with BFID. Quantification of these samples showed significant differences in the volume of genetic material, where the degree of variation observed is most likely as a result of both the sampling area and technique. In many cases the use of directly extracted DNA resulted in a successful RT-qPCR reaction, thus eliminating the requirement for a quantification step within case work where time is a critical factor and quantity of sample is high.

A number of markers showed a degree of specificity to certain body fluid types. In these cases, it could be argued that the lack of expression in other body fluids are a result of the variation in miRNA concentrations obtained. The TaqMan® assays and custom marker hsa-mir-508 showed universal expression and could therefore act as positive validation that the concentration of sample was sufficient, but more broadly can act as an endogenous control for a successful reaction within case work.

Despite variations in concentration of genetic material, hsa-mir-124a displayed complete specificity to vaginal samples however $\Delta^1\text{Cq}$ values were absent or <10 in three of the samples. If a sexual assault occurred, on this data set the results would indicate no vaginal material present and therefore provide a false negative. Marker hsa-mir-124 therefore has the potential for BFID but may need another vaginal material specific marker.

The specificity of hsa-mir-451 within blood makes it an ideal candidate for BFID, one saliva sample did show a high $\Delta^1\text{Cq}$ value, thus giving a saliva sample a positive result for the presence of blood. Expression in this sample could be a true positive result; an individual having gum disease, ulcers or laceration within their mouth would cause blood contamination within the sample. This would thus alter the defence and prosecutions perspective of a case. This may aid in providing additional information about the assailant, however it is likely to cause additional analysis to be required to avoid misidentification.

The varied expression of hsa-mir-205 within vaginal material, menstrual blood and saliva would not aid in a case where oral or penile intercourse occurred. All samples contained high DNA concentrations and therefore high respective miRNA levels would be expected. The results suggest that may be a reason these individuals are not expressing that miRNA when the majority of others are, factor such as disease, contraceptive methods, pregnancy history or age could cause this change in miRNA regulation. The absence of hsa-mir-205 within venous blood and semen, even at high concentration could be useful in determining the absence of a body fluid from a crime scene stain, if suitable controls were in place.

Most of the markers showed high uniform expression across one body fluid while also displaying expression that fluctuated from absent to strong in another. This would make identification difficult and decrease the validity of a body fluid marker. If hsa-mir-10b is highly expressed it would indicate menstrual blood, however, due to some semen samples with high Δ^1Cq values that reliability of BFID is removed. For markers that were highly expressed in menstrual blood samples the same levels were not seen in vaginal material, they ranged from moderate to absent indicating a huge increase in miRNA involved within the menstrual cycle. Overall the least fluctuation can be seen in the high DNA concentration body fluids (vaginal material, menstrual blood and saliva) whereas fluctuation is greatest in the lower concentration body fluids (venous blood, semen, seminal fluid and skin).

5.3.2. Limits of Detection

5.3.2.1. Sample Dilution series RT-qPCR

As the results obtained in section 4.3.1 demonstrate, where concentration of miRNA sample is sufficient, a result can be obtained. Within the field, not all samples provided are likely to contain significant quantities of genetic material. It is therefore important to determine the lower limits for detection for the RT-qPCR method. Here, two candidate markers (one specific, one an endogenous control) were subjected to sample serial dilutions to estimate a LOD for the reaction.

hsa-mir-124 displayed specificity to vaginal material and menstrual blood. When diluted the most uniform expression was seen at 5ng/ μ L (circled, Figure 5.7), and showed no significant difference in expression data seen in undiluted samples. Expression was consistent and although $\Delta^1\text{Cq}$ values varied moderate and strong expression was observed when samples were diluted to 0.5ng/ μ L. At concentrations $\leq 0.05\text{ng}/\mu\text{L}$, expression became variable across samples and in two samples, a complete cessation of expression was observed at 0.005ng/ μ L.

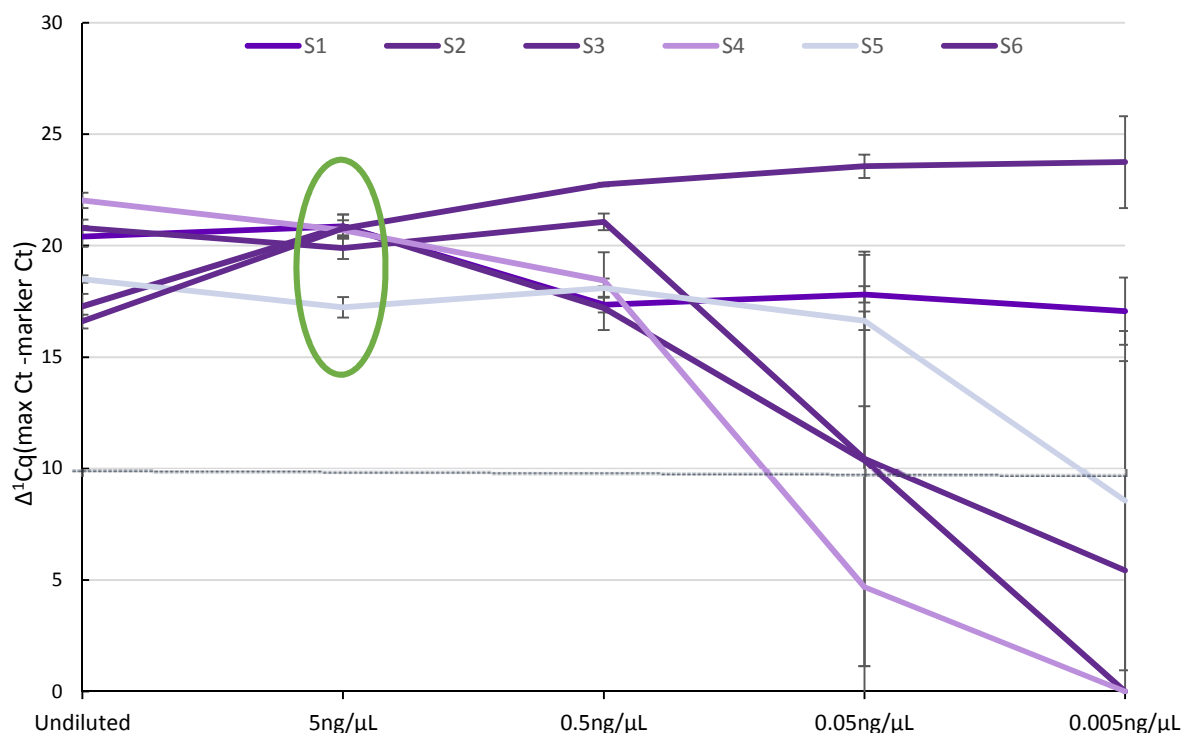


Figure 5.7: $\Delta^1\text{Cq}$ values (max Ct - marker Ct) + SEM of hsa-mir-124 within vaginal material samples across serial dilutions. Dotted line indicates threshold for inclusion in analysis.

SNORD 47 was suggested as an endogenous control [228, 229]. Figure 5.8 shows expression values within samples diluted to 5ng/μl were comparable to those obtained from undiluted sample. Expression from samples was still consistent when diluted to 0.5ng/μl. Subsequent dilutions of sample to 0.05ng/μl resulted expression being detected in only 2 of the 6 samples, with no expression detected at 0.005ng/μl.

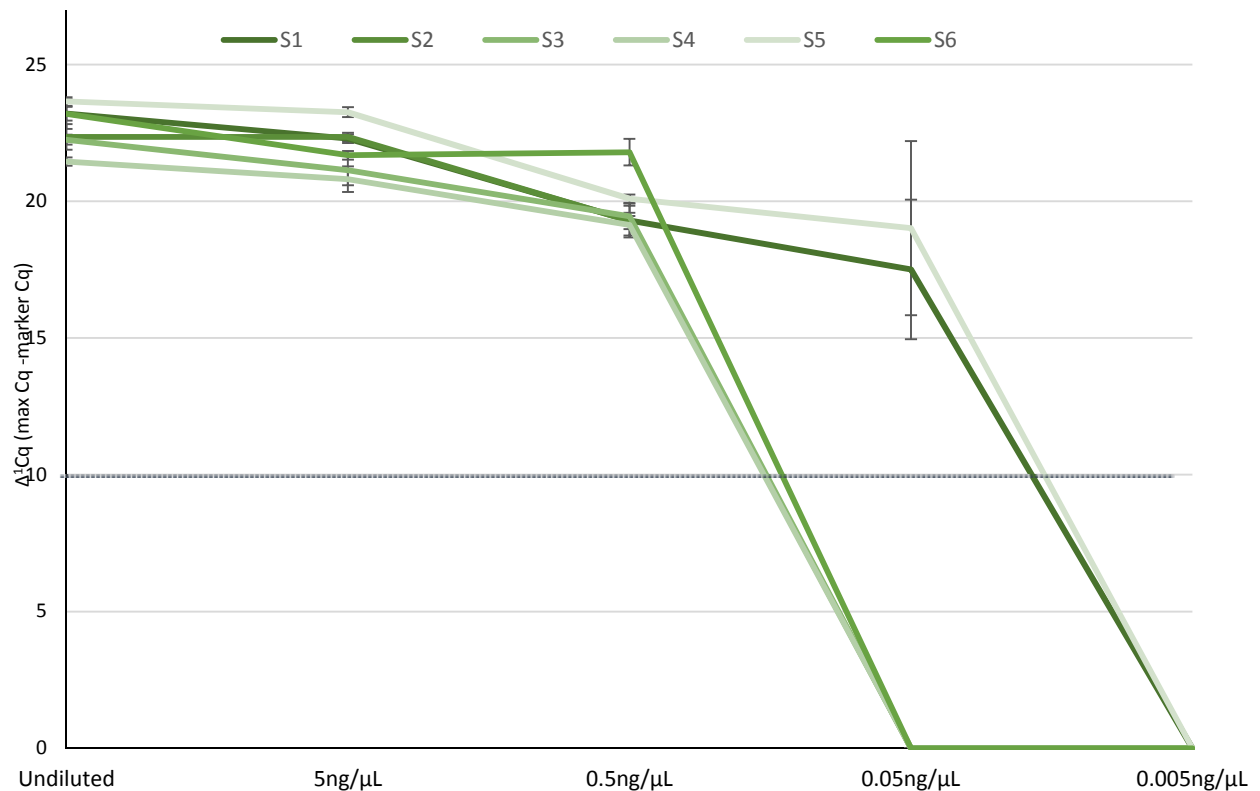


Figure 5.8: Δ^1Cq values (max Ct - marker Ct) + SEM of SNORD 47 within vaginal material samples across serial dilutions. Dotted line indicates threshold for inclusion in analysis.

5.3.2.2. Discussion of detection limits

Although variation in LOD between markers can be seen, all markers used within this component of the study displayed a strong, uniform positive reaction at concentrations $>0.5\text{ng}/\mu\text{l}$. The data generated here suggest that a working concentration for assays would be $>0.5\text{ng}/\mu\text{l}$. Marker hsa-mir-124 did however show strong expression could be detected when diluted to $0.05\text{ng}/\mu\text{l}$. To investigate the specificity of low template DNA (ItDNA) samples, subsequent analyses were carried out following dilution of samples to $0.7\text{ng}/\mu\text{l}$.

5.3.3. Identification of body fluid specific markers and endogenous controls

The aim of this section was to carry out a screening of body fluid specific and endogenous control miRNA markers described in the literature. To fully assess the robustness of a BFID marker its expression must be compared to the expression values in other body fluids. The data generated showed the volume of genetic material has a large effect on expression. A minimum of two endogenous controls must be identified as universally expressed in all body fluids to allow for comparisons across body fluids to be made. Since TaqMan® assays were carried out within section 4.3.1, the possibility of replacing these with custom assays was also investigated. In addition to screening a full panel of miRNA markers, the samples obtained in 4.3.1 were diluted to concentrations that would be confluent with other established molecular identification techniques.

hsa-mir-451 was not analysed on ItDNA samples; the significantly high expression and specificity within blood samples supports current literature [165, 185, 195, 230, 231]. The inability of this marker to differentiate between venous blood and menstrual blood would not aid in cases where the identification of the type of blood left at a crime scene is important. Marker hsa-mir-617 showed no significant expression within any body fluid and was therefore deemed unsuitable for BFID and removed from the study. All the markers within this panel of screening were identified from the literature and in all cases, had been identified as being an endogenous control or a body fluid specific marker.

5.3.3.1. Low template RT-qPCR

Expression of markers of proposed endogenous controls can be seen in Figure 5.9. Marker hsa-mir-93 and SNORD 47 showed high expression values in all body fluids, except for skin in SNORD47. Expression of SNORD 44, 38b and 7 was low or absent in all body fluids and was not consistent throughout samples of the same body fluid.

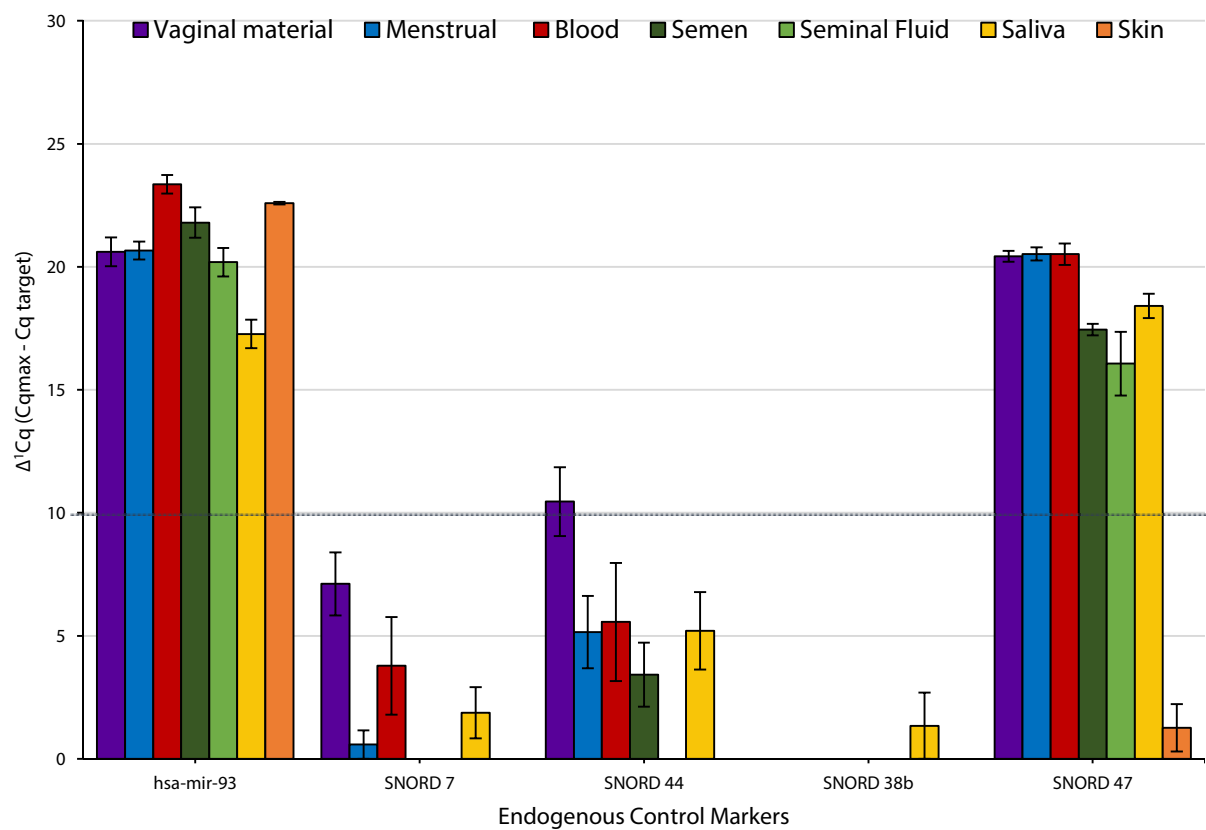


Figure 5.9: Expression of custom made miRNA and SNORD markers, measured using Δ^1Cq (Max Cq- Cq of marker) in all body fluids. Δ^1Cq was calculated and plotted for endogenous control markers hsa-mir-93a and SNORD 47, SNORD44, SNORD38b and SNORD 7. All body fluid samples were analysed, an absence of bar denotes no observable expression/ Δ^1Cq of 0. Dotted line indicates threshold for inclusion in analysis.

Proposed vaginal material markers hsa-mir-124a, 372, 654-5p and 1260b were characterised (Figure 5.10). Marker hsa-mir-1260b displayed high expression in all body fluids (>22.1), contrasting with hsa-mir-372, which showed low/negligible expression in menstrual blood and saliva samples, and hsa-mir-654-5p which showed negligible expression in vaginal material. Marker hsa-mir-124 displayed complete specificity to vaginal material and menstrual blood, with significant expression values observed that obtained within other body fluids ($p < 0.001$).

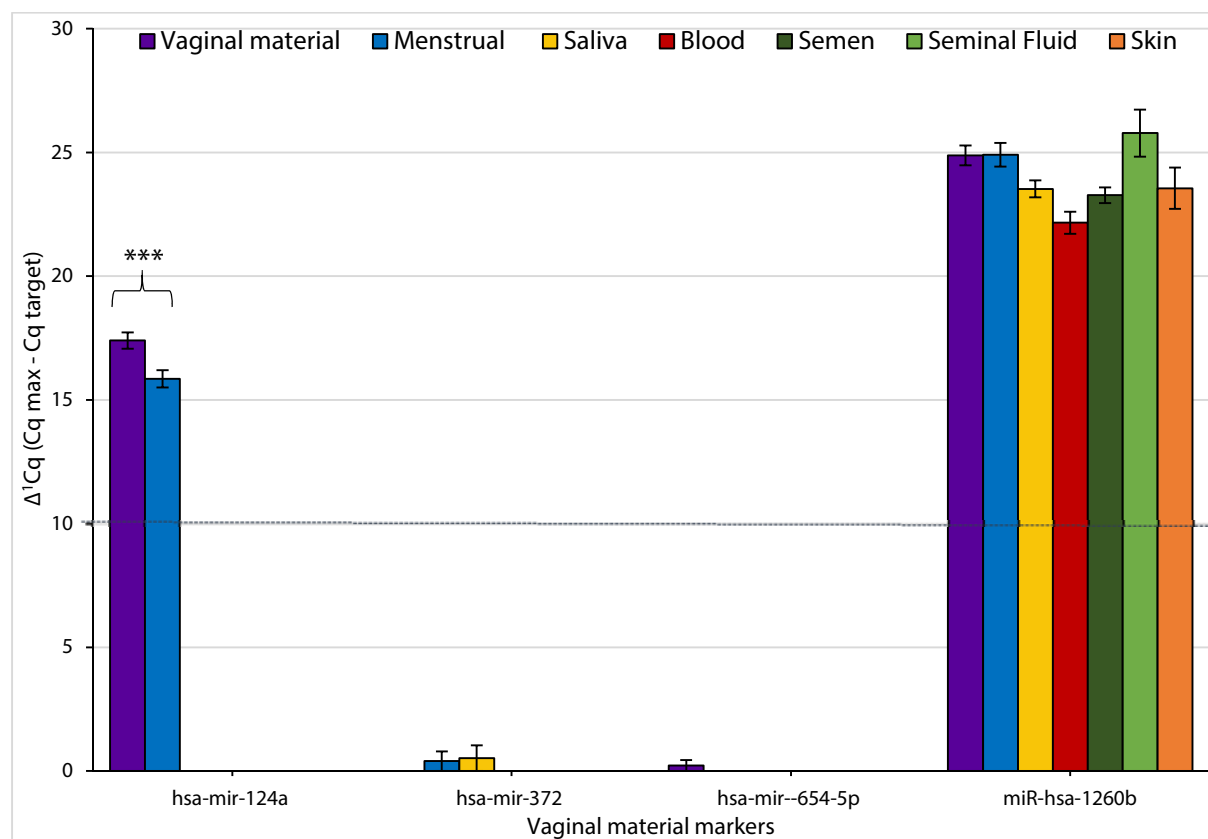


Figure 5.10: Expression of custom made miRNA markers, measured using $\Delta^1\text{Cq}$ in all body fluids. $\Delta^1\text{Cq}$ (Max Cq - Cq of marker) was calculated and plotted for vaginal markers hsa-mir-124a, hsa-mir-372, hsa-mir-654-5p and hsa-mir-1260b. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

Proposed saliva markers hsa-mir-124* did not express in any body fluid (Figure 5.11), with low expression of 138-2 observed in vaginal material, menstrual blood and semen. Marker hsa-mir-205 showed no expression in venous blood, seminal fluid or skin but showed low expression (<0.5) in semen and moderate expression in vaginal material (13.4). Marker hsa-mir-205 showed the largest expression values in saliva (19.9) and menstrual blood (19.8). High, consistent expression was observed from marker hsa-mir-223 in vaginal material, menstrual blood, venous blood and saliva (20.3 - 20.9) samples, but was low in both semen and skin and moderate in seminal fluid samples.

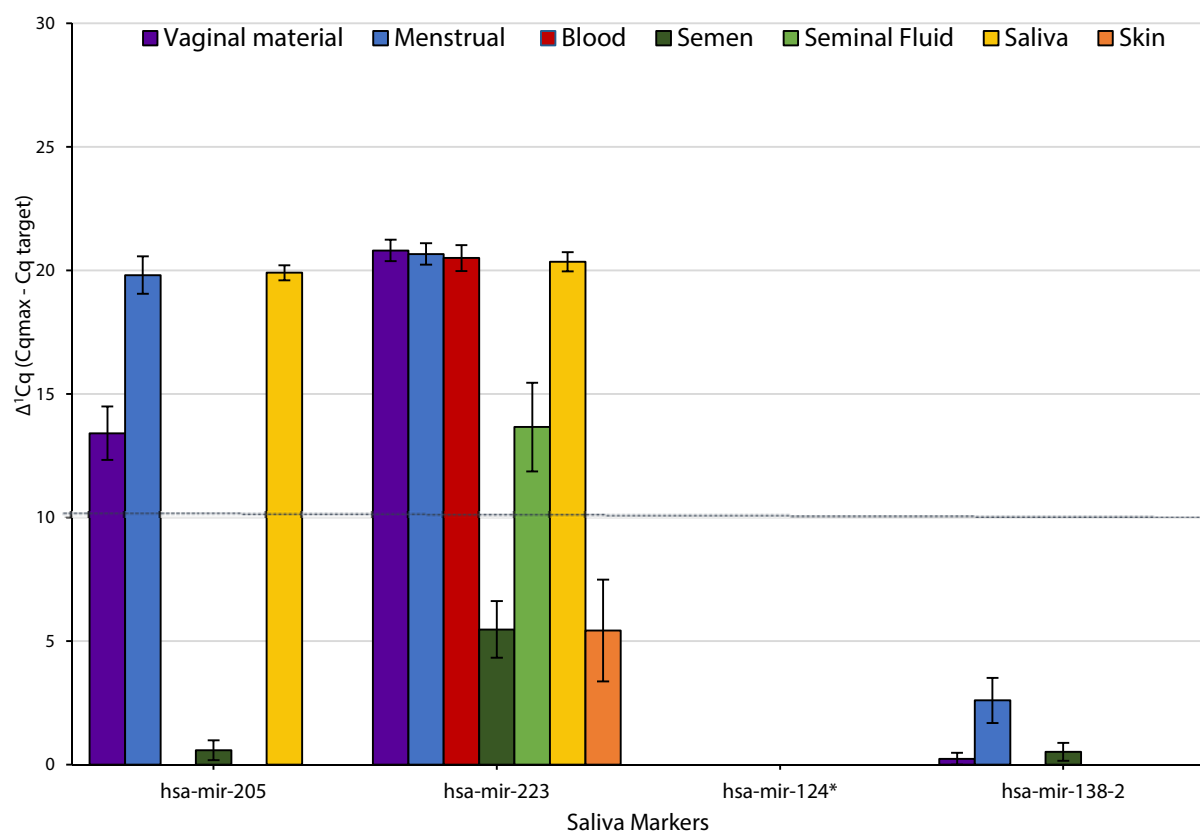


Figure 5.11: Expression of custom made miRNA markers, measured using Δ^1Cq (Max Cq- Cq of marker) in all body fluids. Δ^1Cq was calculated and plotted for saliva markers hsa-mir-205, hsa-mir-223, hsa-mir-124* and hsa-mir-138-2. All body fluid samples were analysed, an absence of bar denotes no observable expression/ Δ^1Cq of 0. Dotted line indicates threshold for inclusion in analysis.

Proposed venous blood marker hsa-mir-16 showed significantly high expression in blood samples (menstrual blood 23.06 and venous blood 27.3) ($p < 0.001$). However, expression was also present in vaginal material, semen and saliva (15.8 – 16.6) (Figure 5.12). Skin markers hsa-mir-3169 and 455 did not display high expression values in any body fluid. Marker 203a showed significantly high ($p < 0.01$) expression in menstrual blood (22.1). Expression was also high in vaginal material and saliva (20.6 and 19.9). Expression was seen in skin and semen but these were weak signals (< 10), a moderate expression was however observed in seminal fluid samples.

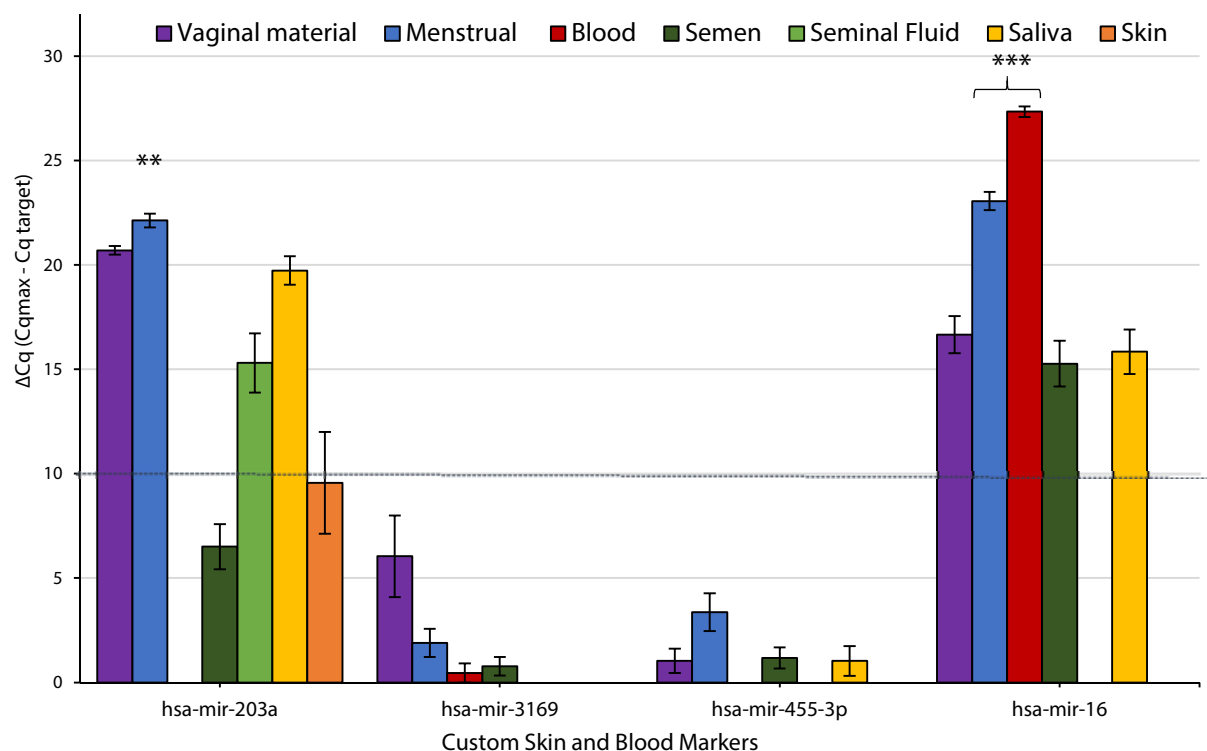


Figure 5.12: Expression of custom made miRNA markers, measured using $\Delta 1Cq$ (Max Cq - Cq of marker) in all body fluids. $\Delta 1Cq$ was calculated and plotted for skin markers hsa-mir-203a, hsa-mir-3169 and hsa-mir-455-3p and blood marker hsa-mir-16. ($p < 0.01$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta 1Cq$ of 0. Dotted line indicates threshold for inclusion in analysis.

Eleven proposed semen markers were included in the panel for ItDNA BFID analysis, $\Delta^1\text{Cq}$ values can be seen in Figures 5.13 - 5.15. Both hsa-mir-10a and 135a were moderately but significantly ($p < 0.001$) expressed in semen and seminal fluid over other body fluids, however weak expression was observed in menstrual blood in both cases (Figure 5.13). Marker hsa-mir-10b-5 showed weak expression in semen, menstrual blood and vaginal material, with weak expression seen in semen from has-mir-10b-3.

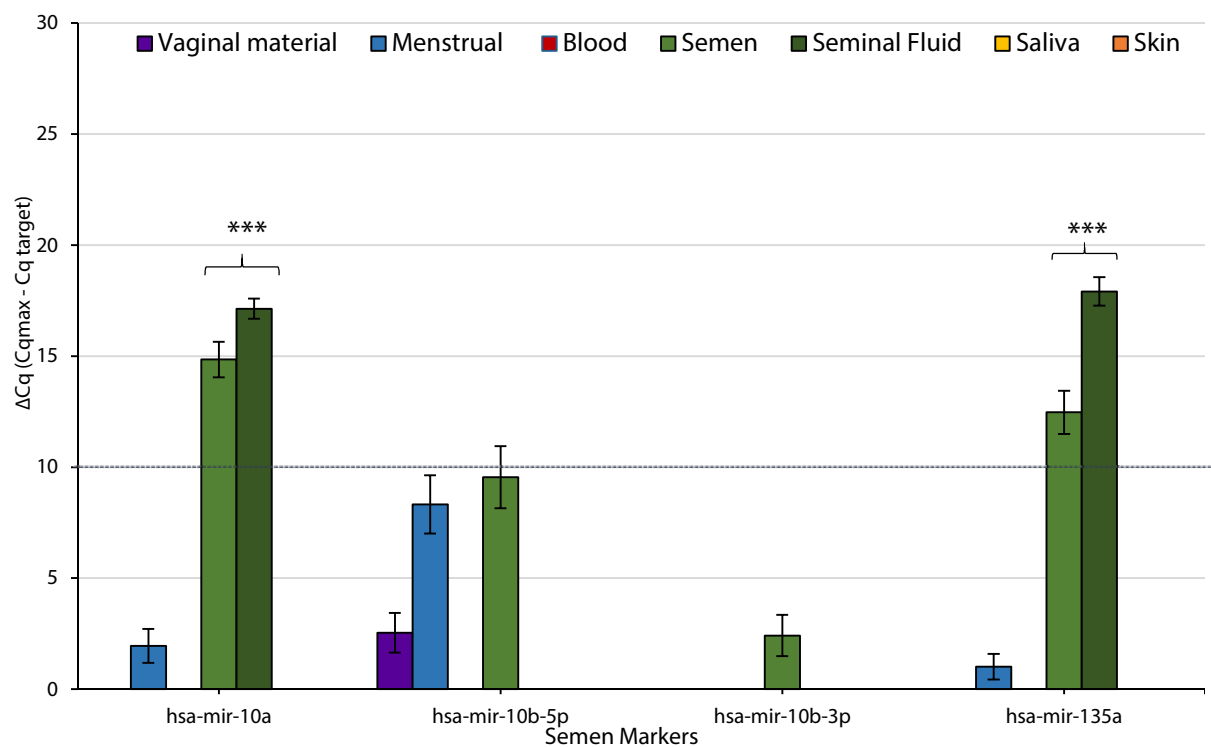


Figure 5.13: $\Delta^1\text{Cq}$ values of custom assays across all body fluids. $\Delta^1\text{Cq}$ (Max Cq- Cq of marker) was calculated and plotted for semen markers hsa-mir-10a, hsa-mir-10b-5p, hsa-mir-10b-5p and hsa-mir-135a. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

Marker hsa-mir-135b showed no expression across body fluids with the exception of weak expression in vaginal material, this contrasts with the results of hsa-mir-508 which displayed universal moderate expression across all body fluids (Figure 5.14). Custom marker hsa-mir-507 varied in expression values compared to those seen using TaqMan® assays where expression was observed in all body fluids. Expression was low/weak in blood, semen and saliva, low in vaginal material and skin (all <9) and poor in seminal fluid (10.6). The moderate expression observed in menstrual blood (15.28) was significantly higher than all other body fluids ($p < 0.001$).

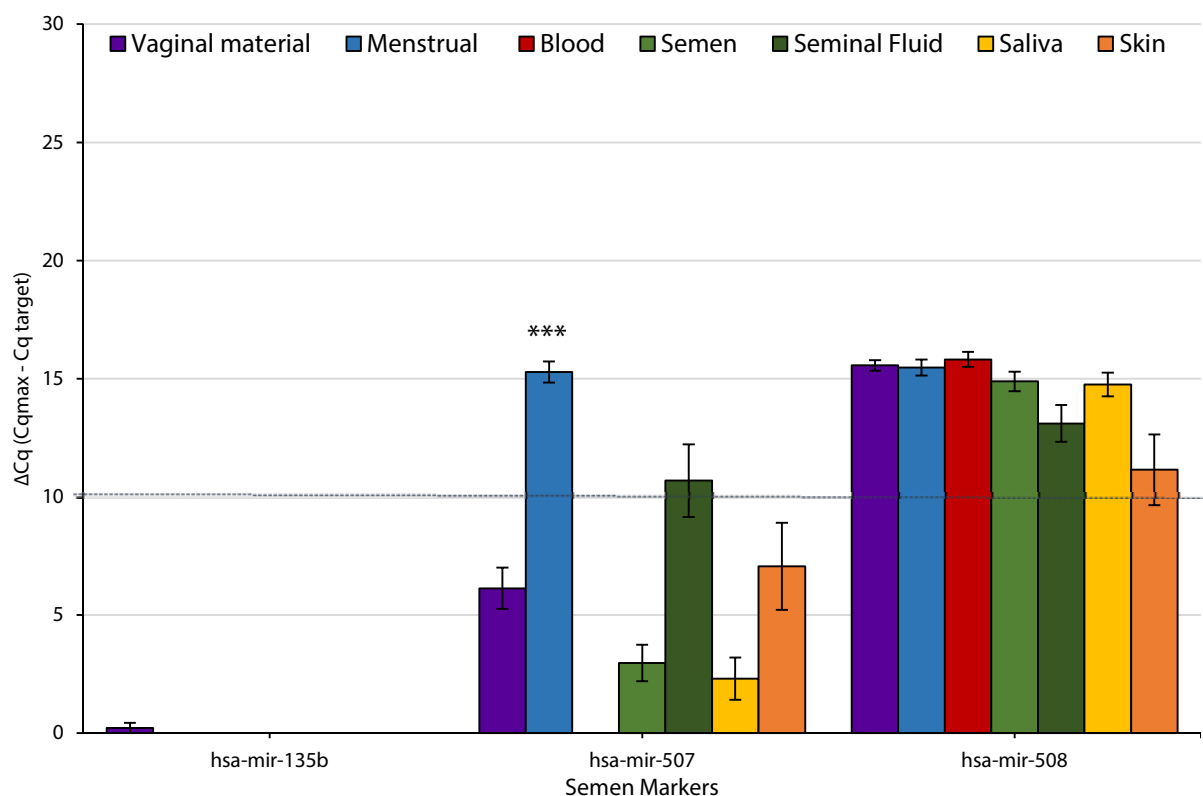


Figure 5.14: Δ^1Cq values of custom assays across all body fluids. Δ^1Cq (Max Cq - Cq of marker) and SEM was calculated and plotted for semen markers hsa-mir-135b, hsa-mir-507 and hsa-mir-508. ($P < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ Δ^1Cq of 0. Dotted line indicates threshold for inclusion in analysis.

Marker hsa-mir- 888 showed moderate, but significantly greater expression in semen and seminal fluid ($p < 0.001$), with weak expression observed in other body fluids (Figure 5.15). Marker hsa-mir-892 and hsa-mir-3197 showed weak expression in all body fluids, with the exception of a moderate but not significant expression observed within vaginal material. Marker hsa-mir-2392 showed moderate expression in all body fluids except for seminal fluid, where no expression was observed.

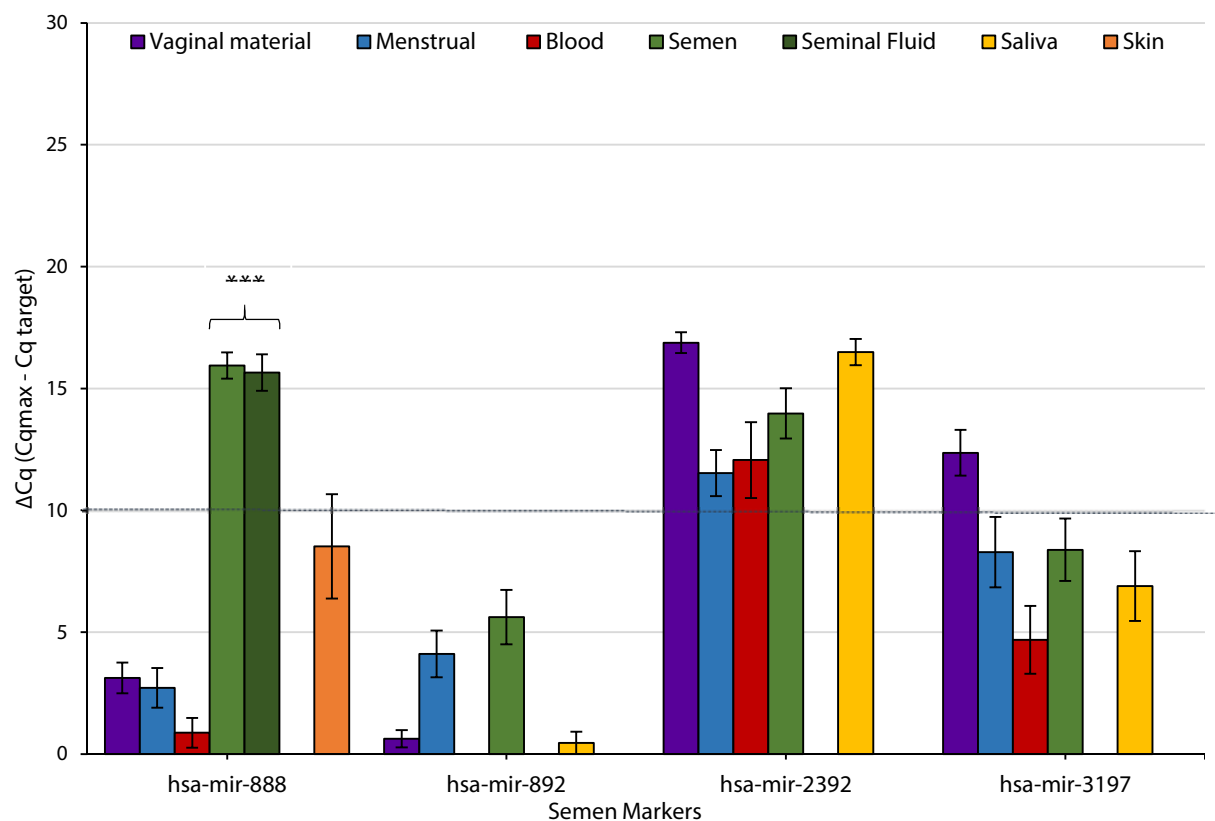


Figure 5.15: Δ^1Cq values of custom assays across all body fluids. Δ^1Cq (Max Cq- Cq of marker) and SEM was calculated and plotted for semen markers hsa-mir-888-5p, hsa-mir-892a, hsa-mir-2392 and hsa-mir-319 ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ Δ^1Cq of 0. Dotted line indicates threshold for inclusion in analysis.

Six miRNAs were proposed for menstrual blood identification, $\Delta^1\text{Cq}$ values can be seen in Figures 5.16 and 5.17. Marker hsa-mir-142, 144-3 and 144-5 showed high $\Delta^1\text{Cq}$ values in venous blood samples with significantly lower expression ($p < 0.001$), being observed in menstrual blood (< 12 , Figure 5.16). Marker hsa-mir-412 displayed moderate expression in menstrual blood (12.68) with all other body fluids having weak or no expression (Figure 5.17). Marker hsa-mir-199a and 214 were weakly or not expressed across the full range of body fluids.

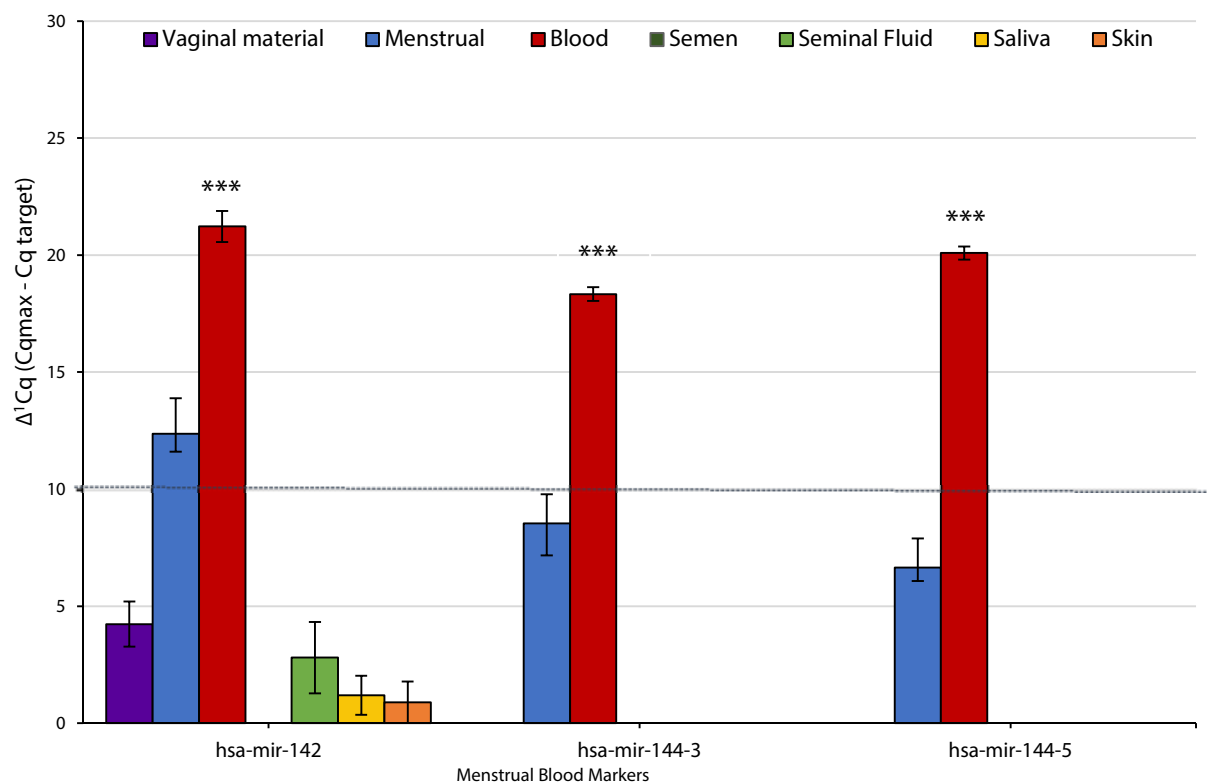


Figure 5.16: $\Delta^1\text{Cq}$ values of custom assays across all body fluids. $\Delta^1\text{Cq}$ (Max Cq- Cq of marker) and SEM was calculated and plotted for menstrual blood markers hsa-mir-142-3p, hsa-mir-144-3p and hsa-mir-144-5p. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ $\Delta^1\text{Cq}$ of 0. Dotted line indicates threshold for inclusion in analysis.

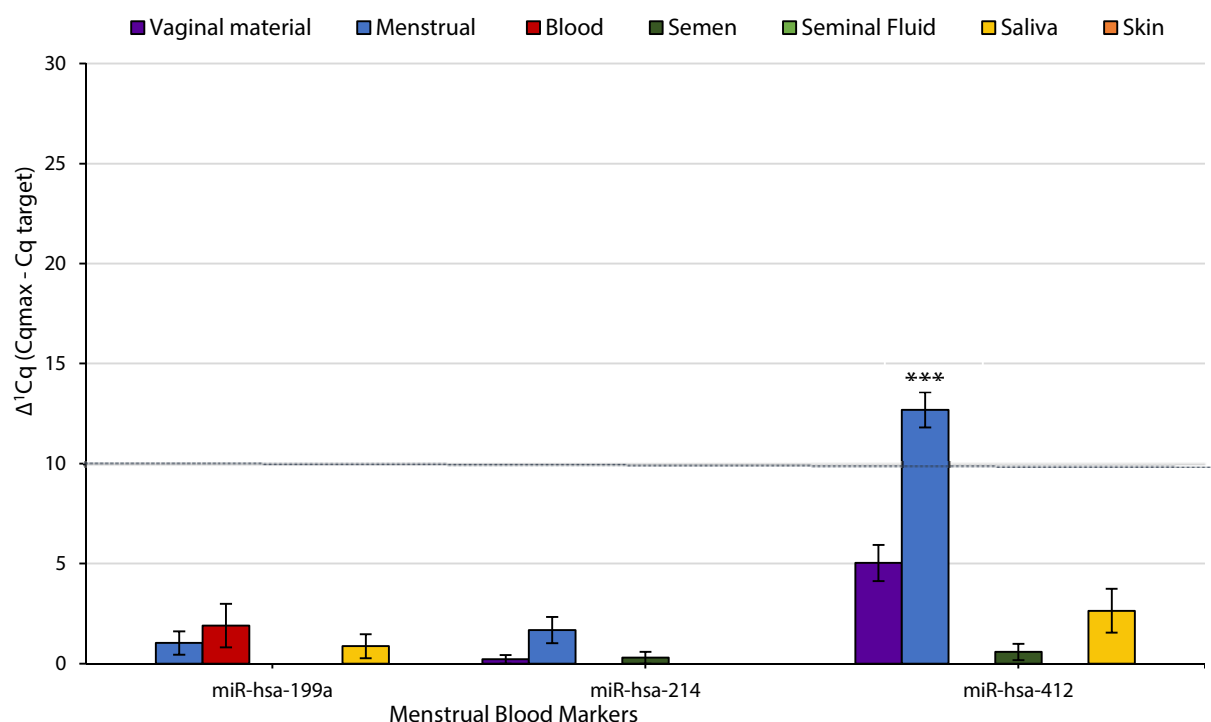


Figure 5.17: Δ^1Cq values of custom assays across all body fluids. Δ^1Cq (Max Cq- Cq of marker) and SEM was calculated and plotted for menstrual blood markers hsa-mir-199a, hsa-mir-214 and hsa-mir-412-3p. ($p < 0.001$). All body fluid samples were analysed, an absence of bar denotes no observable expression/ Δ^1Cq of 0. Dotted line indicates threshold for inclusion in analysis.

5.3.3.2. Heat map analysis of custom assay expression across individual ItDNA samples.

All BFID candidate markers were further analysed by calculating ΔCq (ΔCq of endogenous control - (ΔCq of target) on individual samples. Figure 5.18 displays expression levels for hsa-mir-124, 412, 507, 205, 16, 203 and 223. Marker hsa-mir-124 displayed strong expression across all vaginal material and menstrual blood samples with a complete absence in all other body fluids. Although hsa-mir-412 showed statistically significant expression values in menstrual blood, some menstrual blood samples display weak or no expression, while a diverse range of expression values can be seen in vaginal material. High expression was seen in all menstrual blood samples using hsa-mir-507. Weak expression was seen in a large number of vaginal material, semen, seminal fluid and skin samples with occasional high expression in a minority of samples.

Marker hsa-mir-205 was strongly expressed in all saliva. The majority of vaginal material and menstrual blood samples also displayed strong expression. Absent or weak expression was seen in venous blood, semen, seminal fluid and skin. Marker hsa-mir-16 was highly expressed in all body fluids however absent and weak expression was seen in a small selection of samples in each body fluid. Marker hsa-mir-203a showed universally high expression across all samples in vaginal material, menstrual blood and saliva, a range of weak to strong expression across semen and skin samples and no expression in venous blood. This expression pattern was almost identical in hsa-mir-223 with the inclusion of universally high venous blood expression in all samples.

Volunteers donating vaginal material and menstrual blood samples who had regular unprotected sexual intercourse (UPSI) are indicated by a circle. The duration from UPSI to sampling can be seen in sample information in appendix F. Samples obtained from volunteers from African descent are indicated by a star, neither of these volunteers demonstrated expression of markers 412 and 205 in vaginal material and menstrual blood. This may be significant but further studies with larger sample numbers would have to confirm this observation.

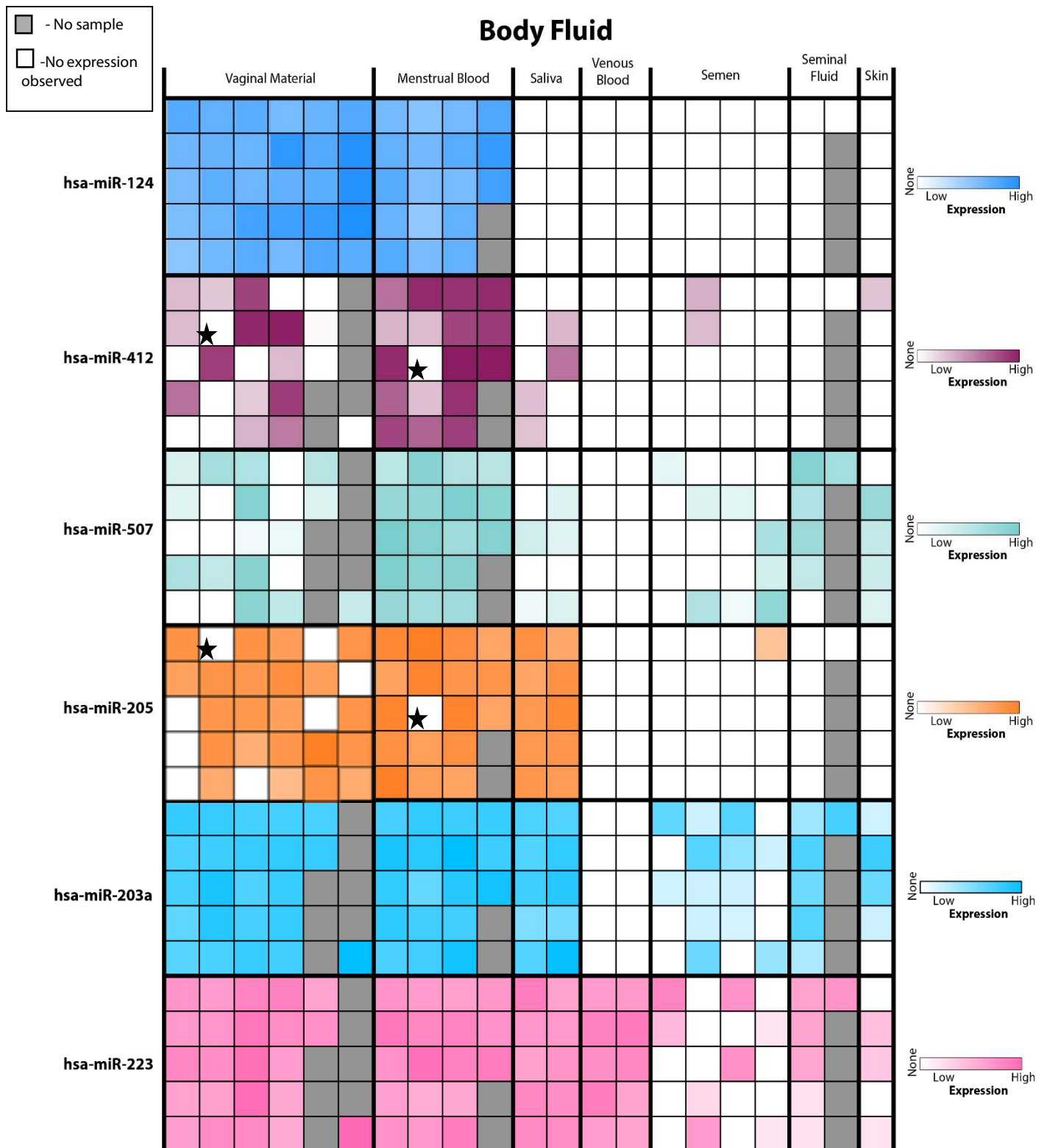


Figure 5.18: Individual ΔCq (ΔCq of endogenous control (Average of selected endogenous control's) - (ΔCq of target) value of each body fluid sample across all markers used in the screening of the 0.7ng/ μ l samples. Sample from African females are denoted by a star.

Figure 5.19 displays expression levels for hsa-mir-142, 144-5, 144-3, 135a, 10a and 888. Marker hsa-mir-142 shows high expression in all venous blood samples, weak expression was seen in a small number of vaginal material and seminal fluid samples, hsa-mir-144-3 and 144-5 displayed high expression in all venous blood samples as well as some menstrual blood samples. No expression was seen in any other fluid. Semen markers hsa-mir-10a and 135a were expressed in all seminal fluid and over 90% of semen samples. No expression was seen in vaginal material, venous blood or saliva however high expression was seen in all but one UPSI samples of vaginal material and menstrual blood. Marker hsa-mir-888 was present in a small selection of vaginal material samples and weak expression is seen in one venous blood sample.

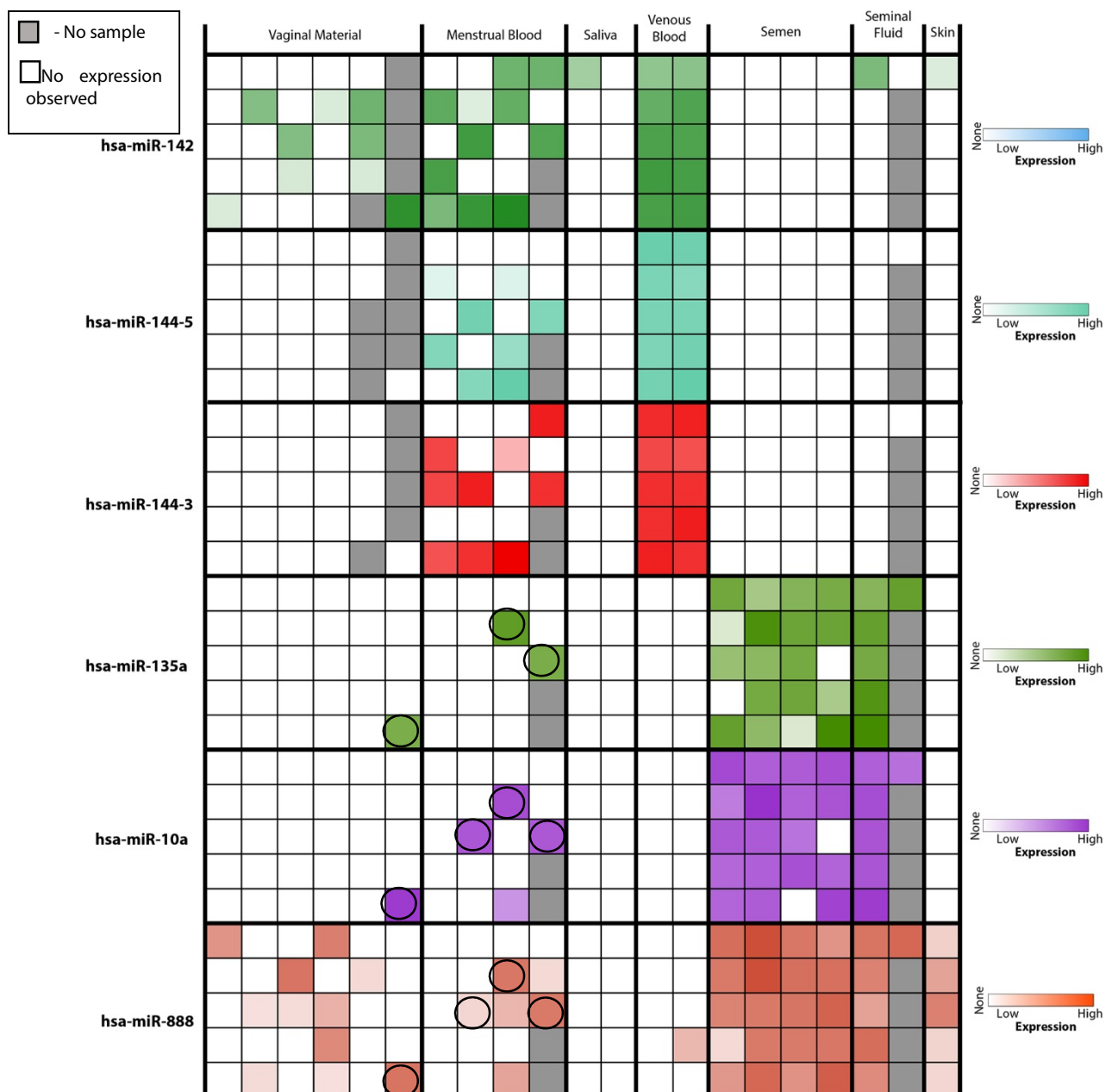


Figure 5.19: Individual ΔCq (ΔCq of endogenous control (Average of selected endogenous control's) - (ΔCq of target) value of each body fluid sample across all markers used in the screening of the 0.7ng/ μ l samples. Sample where UPSI had recently occurred are indicated by a circle. White boxes indicate a ΔCq of 0 (no observed expression), grey boxes indicate no sample tested.

The expression of the endogenous controls established within this panel of screening can be seen in Figure 5.20. SNORD47, hsa-mir- 93, 508 and 1260b show universal and strong expression in all samples. Although hsa-mir-2392 displayed universal expression across body fluids, within body fluid samples expression values fluctuated between high, low or absent.

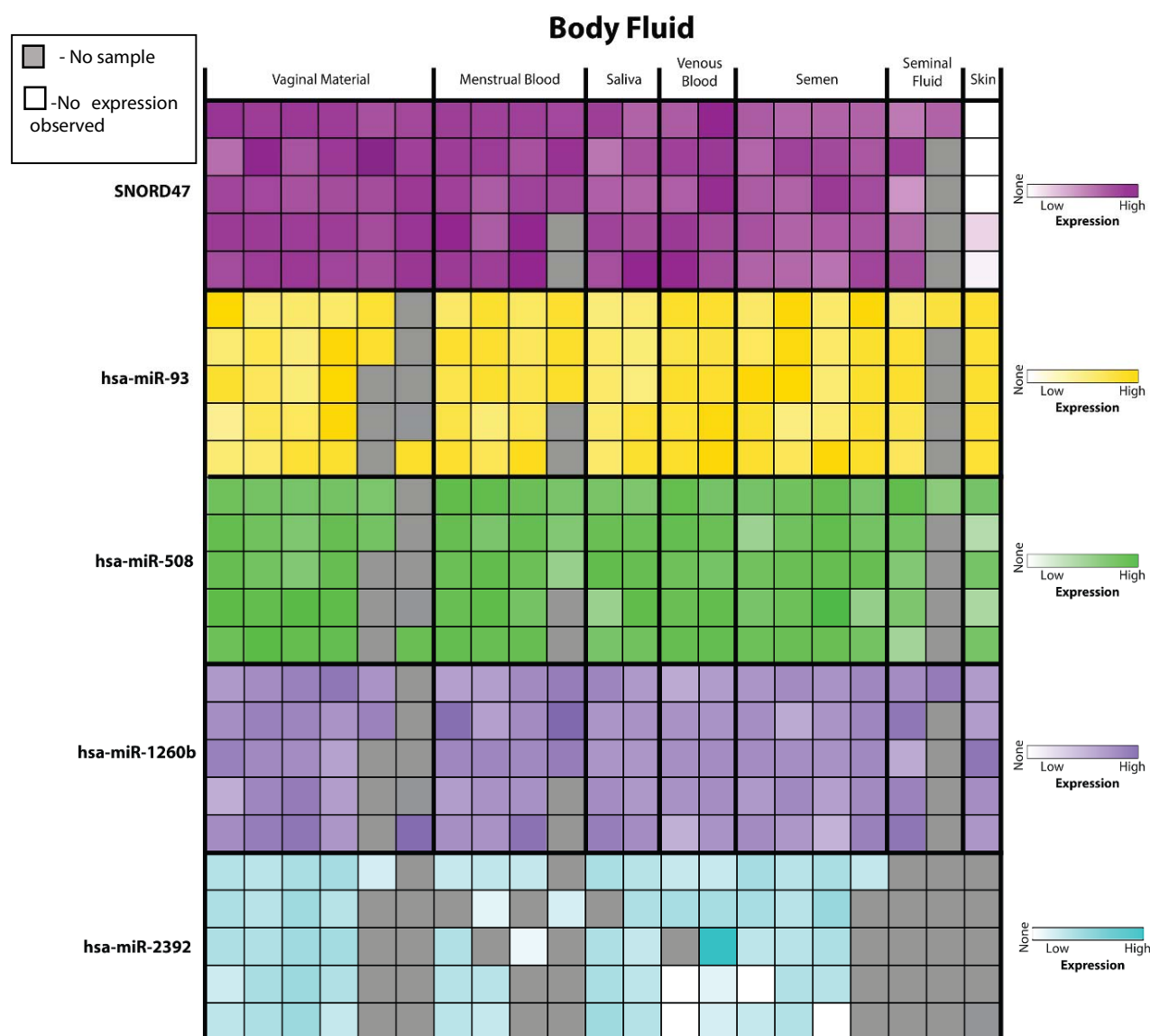


Figure 5.20: Individual $\Delta^1\text{Cq}$ (max Cq- (ΔCq of endogenous control target) value of each body fluid sample across all endogenous control markers used in the screening of the 0.7ng/ μl samples. White boxes indicate a ΔCq of 0 (no observed expression), grey boxes indicate no sample tested.

5.3.3.3. Discussion of low template analysis

Initial investigations demonstrated that custom assays modelled upon TaqMan® assays gave comparable results that could allow for custom assays to be utilised within the screening panel and other downstream experiments. The aim of this section of work was to screen for markers that could be used individually or as part of a matrix to identify specific body fluids. In particular, being able to distinguish between vaginal material, saliva, semen, seminal fluid, skin and venous and menstrual blood would be of great benefit towards forensic case work. A number of markers generated weak or no expression across samples and were therefore unsuitable for BFID.

Of the four vaginal markers tested, only one supported current literature in that the expression was specific to vaginal material. The high expression of hsa-mir-124a in vaginal material and menstrual blood coupled to a complete absence of expression in all other fluids in undiluted and 0.7ng/µl samples suggest it is an ideal candidate for vaginal material identification. At 0.7ng/µl the expression values in vaginal material and menstrual blood are high and stable in all samples. In cases where a suspect is identified and a penile swab taken, the expression of hsa-mir-124 would identify that vaginal penetration occurred and thus increase the support to the prosecution hypothesis.

Saliva and vaginal material are very similar in appearance and genetic composition therefore it could be expected that in many markers the expression values may be similar. This was evidenced within expression data for marker hsa-mir-203a, where strong expression was observed within samples of vaginal material (including menstrual blood) and saliva. In a similar fashion, strong expression of hsa-mir-205 was observed in all saliva samples but also in 95% of menstrual blood samples and 73% of vaginal material samples. Despite expression in menstrual blood, the saliva associated expression was statistically significant, but not significantly greater than vaginal material. In this case, for presumptive classification of a clear stain suspected of being saliva, a panel of 203a, 205 and 124 would confirm saliva in the absence of 124 expression. In case work, this matrix could be useful in

a situation where a victim claims the suspect forced them to provide/ receive oral intercourse.

Five markers were capable of distinguishing between menstrual and venous blood, with hsa-mir-124, 412, 507, 205 and 203a all showing expression within menstrual blood with a complete absence of expression of venous blood. The caveat to these findings is that none of these markers were entirely menstrual blood specific, with expression in other body fluids being observed. Since weak/poor expression of hsa-mir-507 and 203a was also demonstrated expression in some semen, seminal fluid and skin samples, it could provide uncertainty for application into BFID casework. Interestingly, only marker 412 was identified as being a potential menstrual blood specific marker within the literature [179]

In contrast, markers hsa-mir-142, 144-5 and 144-3 had all been identified in the literature as being specific to menstrual blood but showed significantly greater expression in venous blood. Expression of these markers in menstrual blood was sporadic across individuals, but was moderately/strongly expressed where expression was detected. Within this panel of markers therefore, a conclusive marker for venous blood could not be identified. In a case where the defence states that the suspect and victim had consensual intercourse, expression of markers hsa-mir-142, 144-3 and 144-5 with an absence of hsa-mir-412 being observed would provide increased support to the prosecution that venous blood was present within the sample. To include hsa-mir-142 in the BFID matrix increases the reliability of the analysis however extra care throughout analysis would be needed due to weak expression in certain vaginal material samples.

Markers hsa-mir-135a, 10a and 888 were all capable of detecting expression from semen and seminal samples. The high expression of hsa-mir-10a, 135a and 888 in every seminal fluid sample supports them as potential candidates for the identification of semen with or without the presence of spermatozoa. In addition to this, these markers were detected in vaginal material and menstrual blood samples. The expression of hsa-mir-135a, 888 and 10a within vaginal material and menstrual blood correlated with the incidence of recent unprotected sexual intercourse. This detection therefore highlights the proposed semen

markers ability for BFID of casework samples where semen is suspected within vaginal swabs. Expression of hsa-mir-888 within vaginal material, although only strong in a small number of samples was observed in samples where no semen was present therefore impacting the specificity of this marker.

Previously in cases where a vasectomised male sexually assaults an individual, the lack of a DNA profile would support the defence. The inclusion of markers hsa-mir-10a and 135a would now identify the presence of seminal fluid from vaginal swabs and therefore provide support to the prosecution. Where a penetrative sexual assault had occurred within a short time frame after a consensual sexual encounter, seminal fluid would be present from both individuals. When both individuals can produce semen, the use of DNA profiling would be used, however in instances of azoospermic assailants, these markers or other BFID techniques could not be used.

The screening panel was unable to identify a marker for skin. Where those identified as being specific in the literature were found to not be detectable in the skin, rather than being expressed in all samples as a result of skin cells being present in all swab samples. Five markers were identified as being potential endogenous controls for subsequent experiments. Here, SNORD 47, hsa-mir- 93, 508 and 1260b were universally expressed. Of these, only SNORD47 and hsa-mir-93 have been identified as being endogenous controls, with the others being identified as specific to a body fluid within the literature.

In current literature hsa-mir-205 is designated saliva specific in certain publication and present in multiple body fluids in others (see 2.5.6). This discrepancy could be due to the techniques used. It can be observed in Figure 5.21 that various forms of contraception could have an impact on the regulation of hsa-mir-205 within vaginal material. No expression was observed in any sample where contraception was not used by the participant, while all excluding one female on the contraceptive injection displayed moderate/ strong expression. This up-regulation could be due to the increase in hormones caused by most contraceptive methods, however the copper coil which contains no hormones and participants with cervical conditions also displayed high expression. It is therefore likely that

hsa-mir-205 has a regulatory role in maintenance and repair. The hormones within contraceptives alter the construction and destruction of the endometrium, while the IUD physically destroys it. Participants with cervical conditions included having sections of cervix removed and a hysterectomy, both involving large changes to the uterus and a need to repair and maintain healthy tissue. The only participant using contraception that did not display expression of hsa-mir-205 utilised the contraceptive injection and stated that it caused a complete cessation of her menstrual cycle. With no menstrual cycle, there would be no need for constant repair of the endometrium or an up-regulation of has-mir-205.

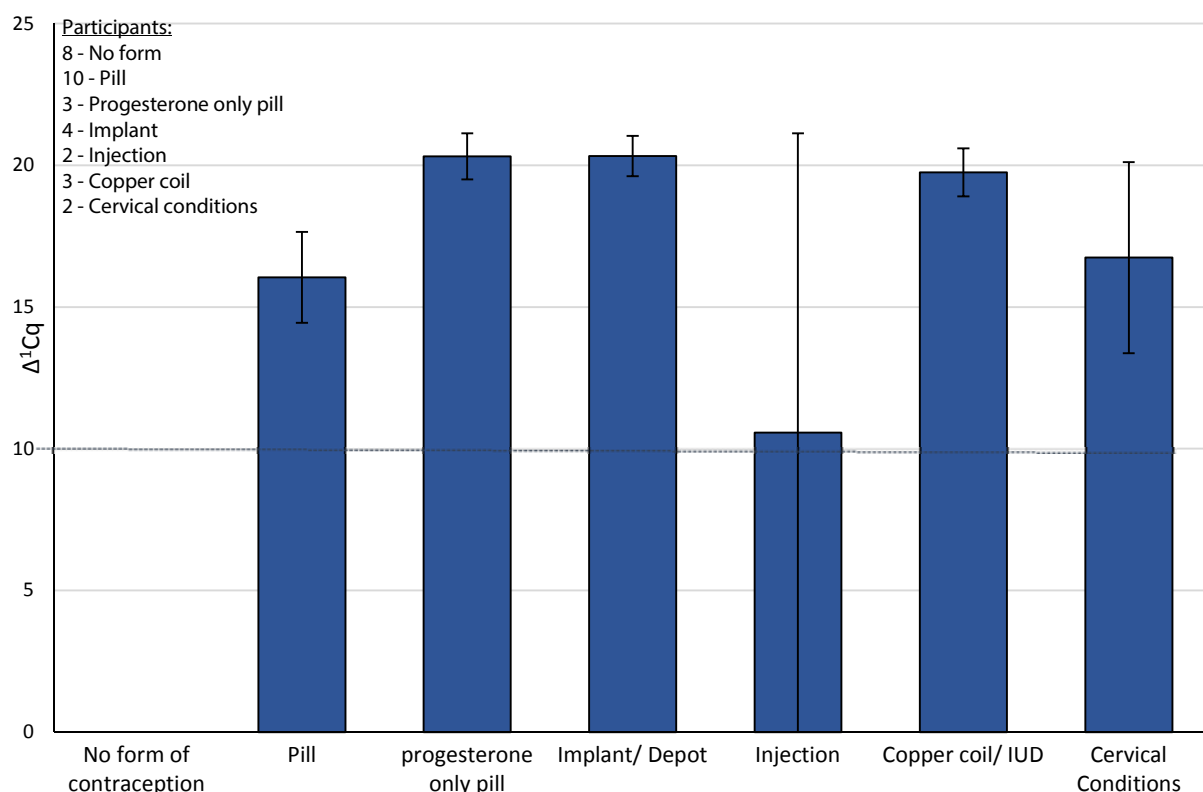


Figure 5.21: Expression of hsa-mir-205 observed in vaginal material samples, categorised by contraceptive method. Cervical conditions include volunteers who have undergone a hysterectomy or have had sections of cervix removed). Dotted line indicates threshold for inclusion in analysis.

5.4. Conclusion

The data generated has demonstrated that the use of RT-qPCR has the potential to be a robust and reproducible method that could be applied to case work. The study has shown that custom markers can be easily introduced into the assay, potentially increasing in specificity and number as new miRNA markers are being researched and identified. The assay also allows for the use of concentrations of nucleic acid template which are comparable with those used in other established techniques allowing for a parallel testing to take place. In addition, a panel of miRNA markers have been screened for specificity. A number of markers within current literature were not found to provide reproducible results in this study. The data does however show that the use of multiple markers can be used to identify vaginal material, distinguish between venous and menstrual blood, distinguish between saliva and vaginal material and identify seminal fluid in the absence of spermatozoa, suggesting penetrative intercourse associated with an azoospermic assailant could be detected. Many of these were within a screening matrix, which aids in providing the robustness required to give confidence within a real case. In addition, a number of endogenous controls have been identified to validate the methodology.

5.5. Summary

- Dilution and normalisation of samples is key to correctly identifying body fluid specificity of markers
- The LOD for this technique lies between 0.05 – 0.5ng/μl when analysing using DNA quantification values.
- A selection of BFID markers that can ascertain specific body fluids in a range of sexual assault case scenarios have been identified.
- A panel of markers can be used to distinguish, when present in single stains, vaginal material from saliva, seminal fluid with or without spermatozoa and menstrual blood from venous blood.

- Custom designed assays show higher specificity to an individual body fluid than TaqMan® assays.
- Four robust and reliable endogenous controls have been identified
- Semen can be detected in vaginal material and menstrual blood samples within 48 hours of UPSI
- Contraceptive methods had a regulatory role in the expression of proposed saliva marker hsa-mir-205
- Has the potential for cold case capability for BFID of archived samples that have undergone total DNA extraction.

6. Characterisation of Markers over a 31- Day Period

6.1. Rationale

The aim of this component of the research was to fully analyse the robustness and specificity of the markers identified in the previous chapter by analysing their expression over a full menstrual cycle. The environment within the vagina is adaptively hostile as well as constantly being prepared for the various stages of the menstrual cycle or pregnancy. The need for vigorous control over gene expression would likely involve fluctuation of many miRNAs, especially during menstruation. For example, if a miRNA marker chosen for BFID is responsible for the continual inhibition of ovum release, then over the course of the cycle there will be a minimum of one day where this miRNA must be down-regulated. This decrease in expression of a miRNA designated for BFID would provide a false negative identification and provide incorrect evidence in court.

In addition to the identification of body fluid specific markers, the previous chapter also identified candidates for endogenous control markers, which play a major role in experimental validation. If a significant change in expression is observed in a proposed body fluid specific marker, consistent expression of an endogenous control would eliminate the possibility of questioning sample integrity. Tentative links to factors such as contraceptive, ethnicity and possibly lifestyle choices affecting miRNA expression were also observed. It is therefore reasonable to hypothesise that miRNA expression over a full menstrual cycle as well as between individuals will not be universal and uniform. For a marker to be fully validated as a bio-marker or an endogenous control for BFID it must show consistent expression for that body fluid, regardless of any environment or internal factors affecting that body fluid.

The study also aims to determine the most efficient method for extraction of miRNA. Numerous miRNA may be specifically expressed within a body fluid but if the extraction technique is not effective during elution they cannot be analysed for BFI. Current DNA profiling laboratories utilise standard protocol total DNA extraction methods to obtain high volume and purity DNA for profiling. To ensure miRNA analysis could be included within the standard protocol of DNA profiling the efficiency for miRNA extraction from total DNA

extraction must be tested. Various laboratories also utilise total RNA extraction methods for RNA analysis, although rarely used within forensic laboratories it could be proposed that this method could be more efficient at extracting miRNA due to its optimisation for extracting single stranded molecules. The most frequently used DNA kits used in forensic laboratories are purchased from Qiagen, these kits use spin column technology. To keep the method as similar as possible the RNA extraction kit from Qiagen will be use on the RNA samples with the DNA extraction kit for DNA samples.

The results obtained in the previous chapter also suggested dilution had a large effect on marker specificity, especially in vaginal material and menstrual blood. Vaginal material samples obtained from crime scene samples will most like be retrieved from a LVS provided during a SARC examination or from a suspect's penile swab. The volume of genetic material from these samples is likely to be high, and therefore dilution to trace would not be necessary or representative to real case scenarios. The most uniform expression seen within section 5.3.2 was at 5ng/μl compared to other dilutions. Therefore, to analyse fluctuation of miRNA a concentration of 5ng/μl was made from all vaginal material samples.

6.1.1. Sample Collection

Five women each self-collected two LVS every morning for 31 consecutive days, labelling the first swab '1' and the second '2'. Volunteers completed questionnaires to determine current lifestyle choices, medical/ pregnancy history and contraceptive methods (Volunteers were asked to make no changes to current lifestyle/ daily habits, noting only when UPSI or vaginal oral intercourse (OI) occurred³. UPSI/ OI was noted on swab tubes and recorded for analysis, in these samples saliva and semen markers would be expected. If UPSI is not recorded and a semen marker was expressed within a vaginal material sample, it would suggest that the miRNA is present in vaginal material and therefore cannot be utilised for vaginal material and semen mixtures, instead of highlighting the specificity of the semen marker.

³ Throughout all research OI is only noted when the recipient is female.

All swabs were extracted within two hours of sampling, when this was not possible, the sample was stored at -20°C until extraction. The initial swab underwent total DNA extraction while the second underwent total RNA extraction. All eluates were stored in 1.5ml tubes at -20°C. The volunteers, seen in table 6.1, did not vary greatly in age but differed in length and flow of menstruation and also in contraception/ lifestyle and pregnancy history. Menstruation of all participants commenced on different days throughout the study, all results were therefore normalised so day 1 displayed the start of menstruation.

Volunteer	Age	Ethnicity	Menstruation	Key information
1	27	White British (W/B)	Day 20 / heavy/ 7 days of menstruation	Contraceptive pill V2 in Screening study
2	27	Asian	Day 6 / light/ 8 days of menstruation	Contraceptive pill V18 in Screening study
3	22	W/B	N/A	Contraceptive injection ceases menstrual cycle/ Smoker. UPSI and OI day 10, 17 and 25 V15 in Screening study
4	29	W/B	Day 20 / heavy / 5 days of menstruation	Previous pregnancy V5 in Screening study
5	29	W/B	Day 15 / heavy / 7 days of menstruation	Coil, Smoker, Previous pregnancies V12 in Screening study

Table 6.1: Volunteer provided information on menstrual cycle, contraceptive, age and ethnicity.

6.1.2. Marker selection

All markers displaying BFID specificity as well as miRNA displaying expression beneficial to BFID in specific crime scenarios were chosen for further validation. Markers displaying complete absence are only beneficial if that absence is permanent, just as body fluid specific expression must be permanent. Eleven markers were selected for a BFID matrix alongside four for use as endogenous controls, observed in table 6.2.

Marker (<i>hsa-mir</i> / <i>SNORD</i>)	Proposed role
124	Vaginal material specific
507, 412	Menstrual blood specific
10a, 135a, 888	Semen specific
205	Saliva specific
508, 47, 1260b, 93	Endogenous control
144-3, 144-5, 142	Blood specific
203a	BFID through absence of expression

Table 6.2: Markers selected for analysis over a 31-day period categorised by specificity.

6.2. Results

6.2.1. Proposed Endogenous Controls

6.2.1.1. Expression from DNA and RNA extracts over 31 days

The expression of proposed endogenous control markers hsa-mir-93 and hsa-mir-508, from both DNA and RNA extracts, across the 31-day study can be seen in Figure 6.1 A-D. Strong consistent expression (>20) of hsa-mir-93 was observed across all 31 days of sampling from both DNA (A) and RNA (B) from all volunteers. In addition, a consistent expression of hsa-mir-508 was observed in both DNA (C) and RNA (D) samples from all volunteers, however expression was moderate.

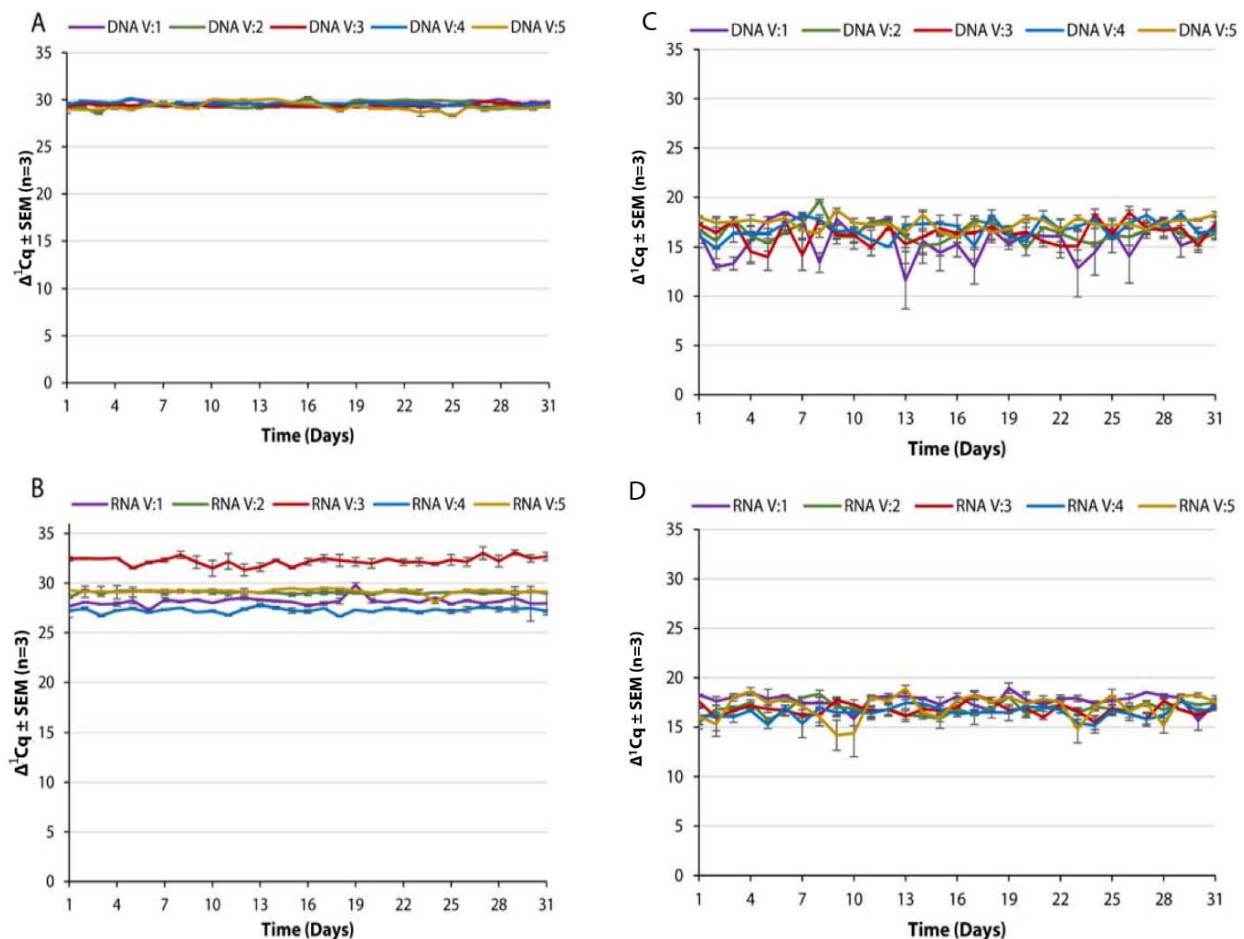


Figure 6.1: $\Delta^1\text{Cq} \pm \text{SEM}$ for proposed endogenous controls hsa-mir-93 (DNA (A) RNA (B)) and hsa-mir-508 (DNA (C) RNA (D)) of all volunteers across 31 days.

Proposed endogenous control markers hsa-mir-1260b and SNORD47 were both strongly expressed in DNA samples across all volunteers over the 31-day study (Figure 6.2 A, C). The expression observed was not as consistent between days within volunteers, however, these were not significantly different. Distinct decreases in expression was observed from these markers when RNA was used as a template from volunteer 4 (Figure 6.2 B, D). In these cases, weak-moderate expression of the marker was observed.

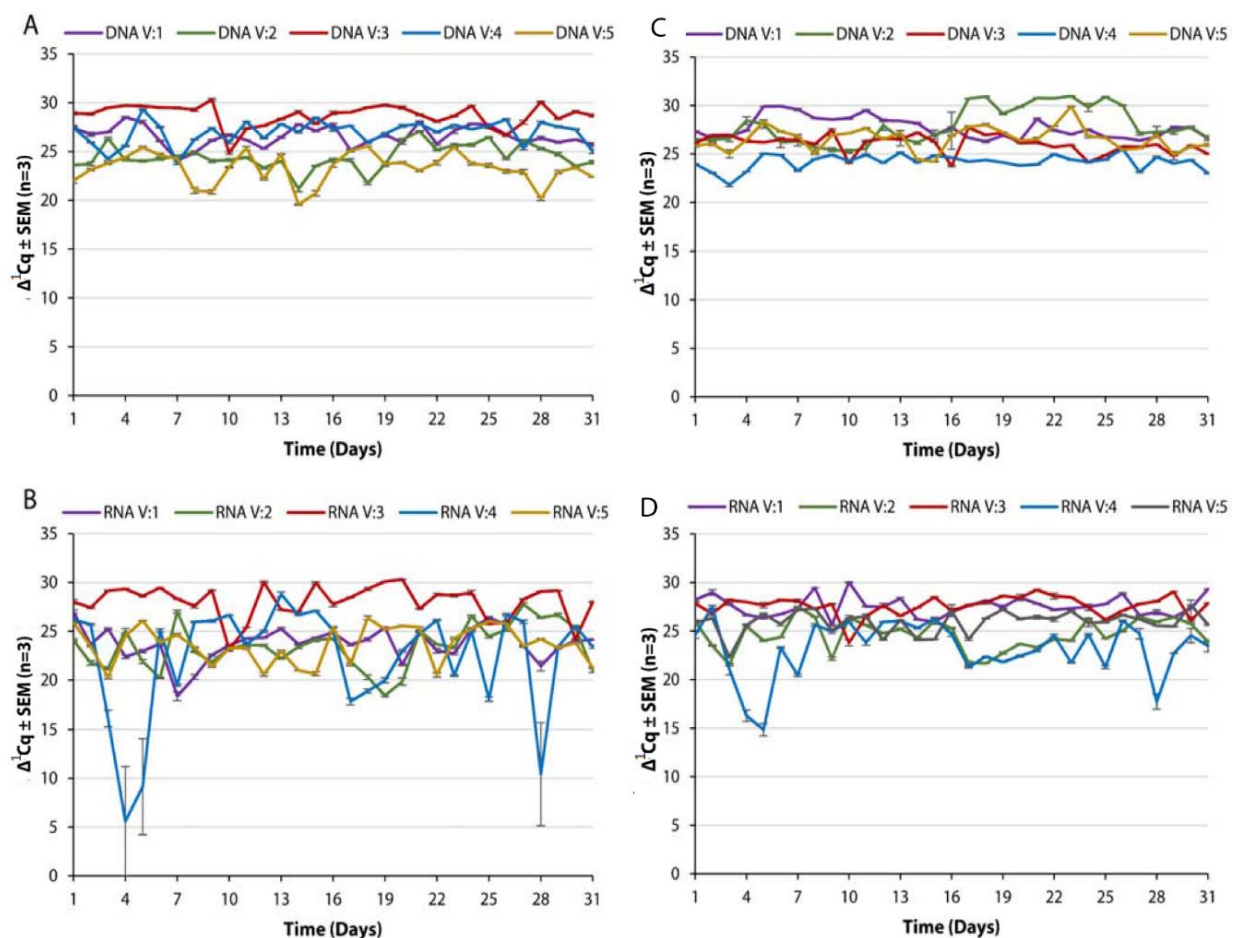


Figure 6.2: $\Delta^1Cq \pm SEM$ for proposed endogenous controls SNORD 47 (DNA (A) RNA (B)) and hsa-mir-1260b (DNA (C) RNA (D)) of all volunteers across 31 days.

6.2.1.2. Discussion of proposed endogenous controls

Within a scientific study multiple endogenous controls are required to provide accurate normalisation of samples. The $\Delta^1\text{Cq}$ values of the average of SNORD 47, hsa-mir- 508, 93 and 1260b across extraction methods can be observed in Figure 6.3. The data generated suggests that when the original sample of miRNA was obtained from DNA, all four of the markers within this study represent suitable endogenous control markers. In contrast, the expression values obtained from RNA samples, although consistent across a number of volunteers, did reduce in one of the volunteers and was primarily associated with markers SNORD47 and hsa-mir-1260b.

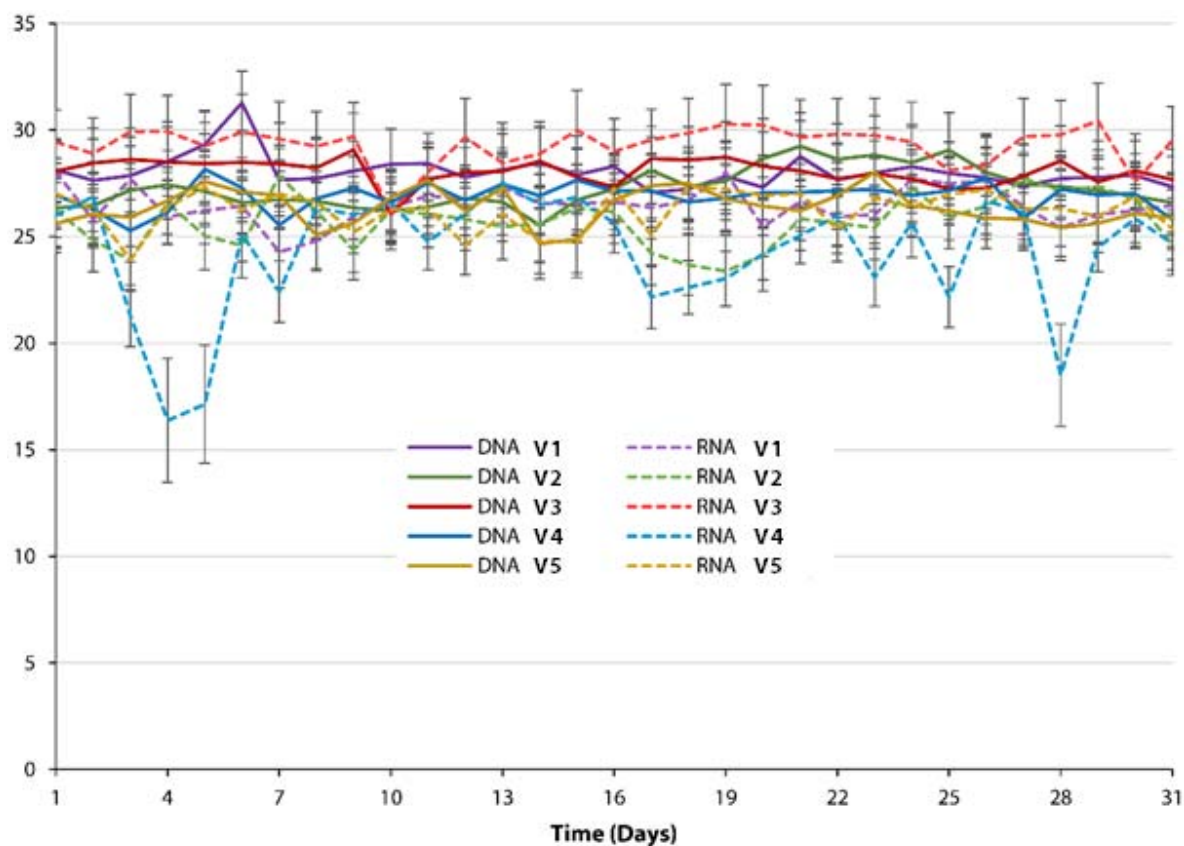


Figure 6.3: $\Delta^1\text{Cq}$ + SEM of the average of all endogenous controls tested within vaginal material over 31 days, following DNA and RNA extraction. Each data point – N=15).

Sampling by volunteers is likely to be inconsistent, it is therefore important that an endogenous control marker is capable of highlighting these inconsistencies. Expression of markers hsa-mir-508 and 93 were consistent across all samples, where variation was observed with SNORD47 and hsa-mir-93. It is therefore possible that the quantities of hsa-mir-508 and 93 are so numerous within all cell types, that expression is limited by the experimental factors (e.g. binding efficiencies, availability of probe) which mean that high Δ^1Cq values are always obtained. An experienced sampler is likely to provide a sample which will contain enough material to detect expression of a specific marker (see Figure 6.4). Whereas an inexperienced or inconsistent sampler is likely to provide a sample that may not contain enough material to detect the presence of a specific marker. In these cases, no change in expression of hsa-mir-93 and 508 would be detected and a false negative would be obtained. An endogenous control marker therefore not only be expressed, but display variance based on the quality of sample material provided as an internal control. Since markers SNORD 47 and hsa-mir-1260b were capable of highlighting these variations, they are more robust at validating the test where hsa-mir-508 and 93 are markers capable of validating the RT-qPCR reaction has been successful. A combination of these markers is therefore beneficial for case work.

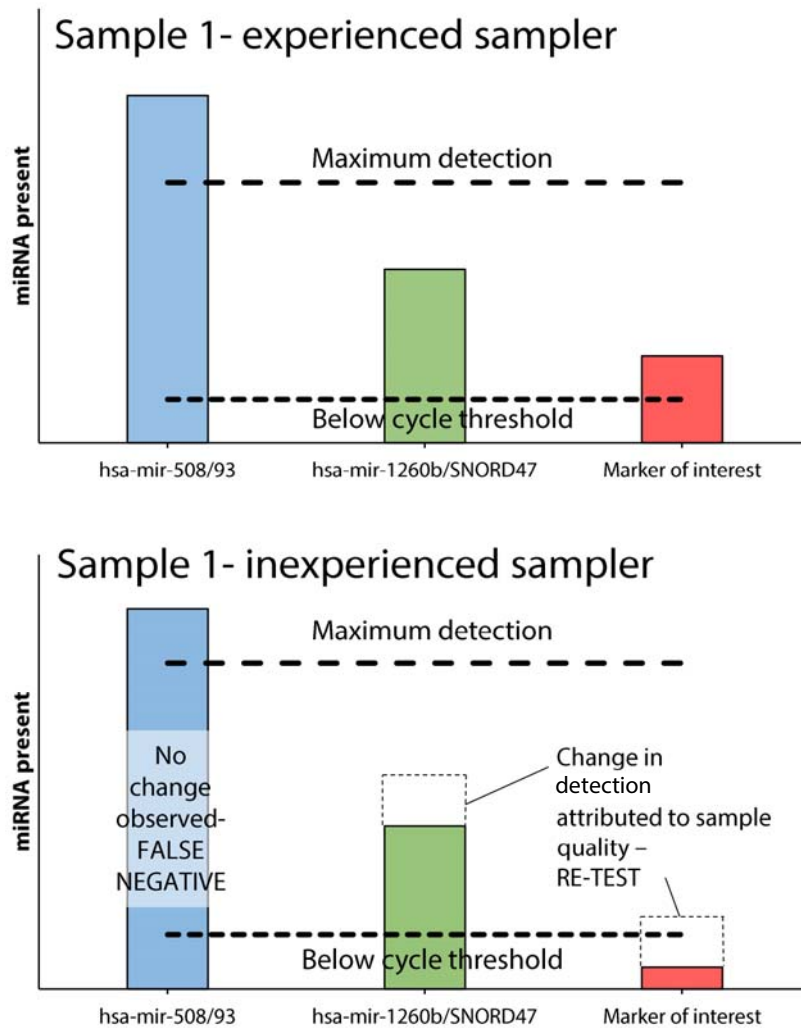


Figure 6.4: Differences in expression within a hypothetical sampler when utilising a good and poor collection technique. The sample obtained from the experienced sampler will show expression in all markers analysed. The same sample when taken by an inexperienced sampler will still display maximum expression in the most abundant miRNAs, but will display a reduced expression in the moderately abundant markers and a possible absence of miRNA that are not as abundant in the cells.

6.2.2. Body Fluid Specific miRNA

6.2.2.1. Proposed vaginal material marker

Marker hsa-mir-124 $\Delta^1\text{Cq}$ values from DNA and RNA extractions can be seen in Figure 6.5. The expression values obtained throughout the 31 days following DNA extraction were consistently high (>17) across all volunteers and days and no significant difference was seen between each data set. Although a number of $\Delta^1\text{Cq}$ values associated with RNA samples were higher than those seen from DNA extraction, a greater degree of fluctuation was observed. In five samples provided by volunteer 4, an absence of expression was observed. The absence of expression correlated with the decrease in expression of hsa-mir-1260b and SNORD 47 suggesting an issue within the sample. With the exception of these samples, no sample displayed weak or absent expression leading to an incorrect identification of vaginal material.

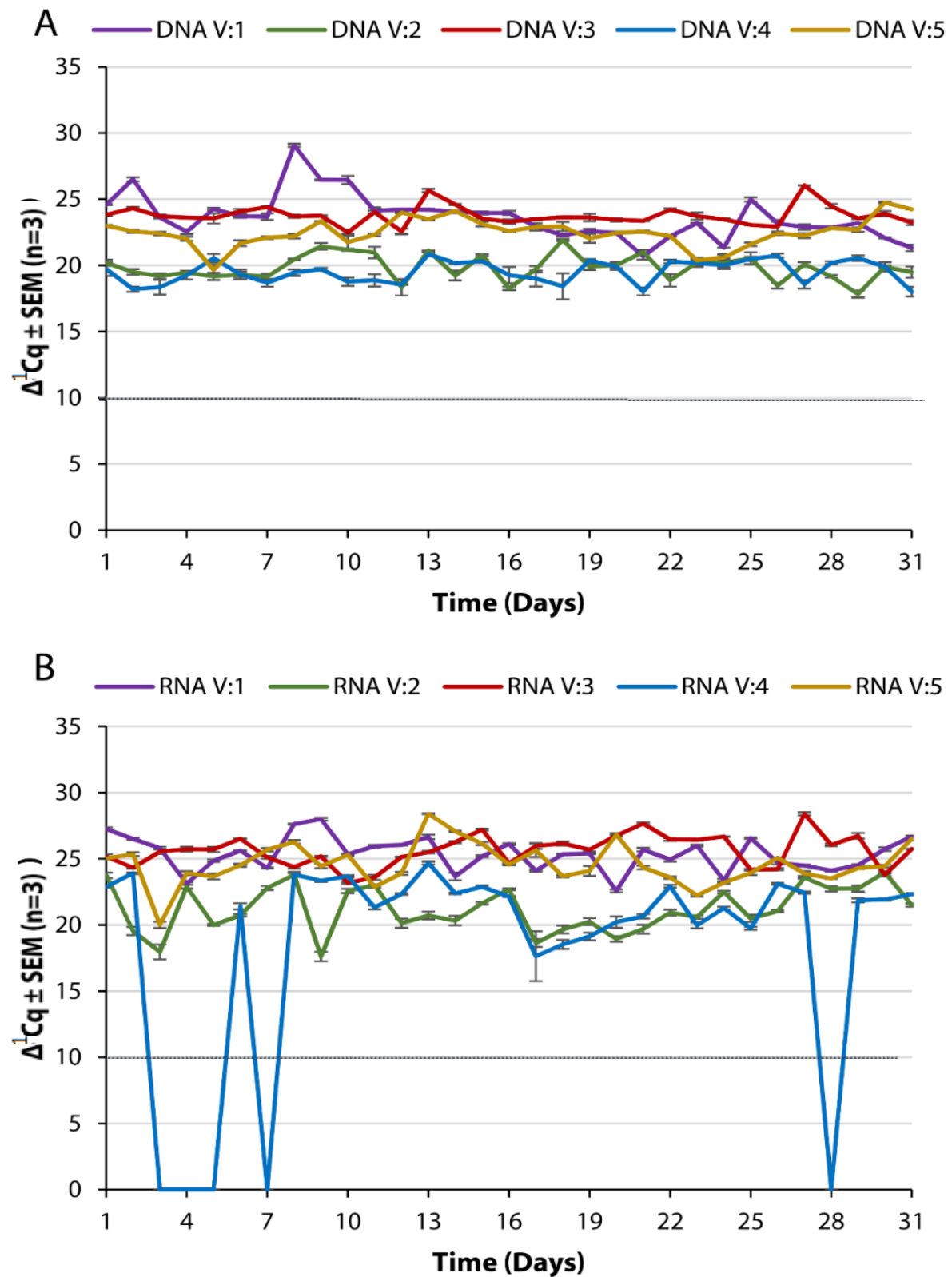


Figure 6.5: $\Delta^1Cq + SEM$ of hsa-mir-124 in vaginal material over 31 days, following DNA (A) and RNA extraction (B). No significant difference observed across samples (each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

6.2.2.2. Proposed saliva marker

In the previous screening, it was determined that the assumption of saliva within a sample would require the absence of expression of hsa-mir-124 alongside the expression of hsa-mir-205 with addition support from hsa-mir-203a. Marker hsa-mir-205 was included within the study to assess how the expression evolved over the 31 days, with focus on expression change following receipt of OI and to assess the validity of contraception's effect on hsa-mir-205 regulation. Expression values for DNA (A) and RNA (B) can be seen in Figure 6.6. The expression patterns for samples within DNA extraction were either uniformly expressed or completely absent. V2, V3 and V4 displayed no expression throughout any time point. V1 and V5 displayed very strong expression from the samples. A decrease in expression was observed at D₂₃ of the V5 dataset, however expression was still moderate.

In a similar fashion, expression of this marker from RNA samples of V1 and V5 was moderate/strong. V1 maintained a uniformly strong expression throughout the 31 days as per DNA samples, V5 displayed strong expression as with DNA samples but a complete absence of expression was seen at 4 data points. V3 was the only data set that remained absent of expression, V2 displayed two data points where a detection of expression was observed. A similar pattern was also seen within V2 however D₂₅-D₃₀ where a spike in expression was observed before returning to an absence. In the case of both of these markers, volunteer 3 received OI on 3 separate occasions, which did not result in any change in expression pattern with either marker. This lack of expression following OI is not unexpected as following the samples taking from a SARC examination, forensic scientist would have a higher expectation of detecting saliva on the external swab rather than an internal swab.

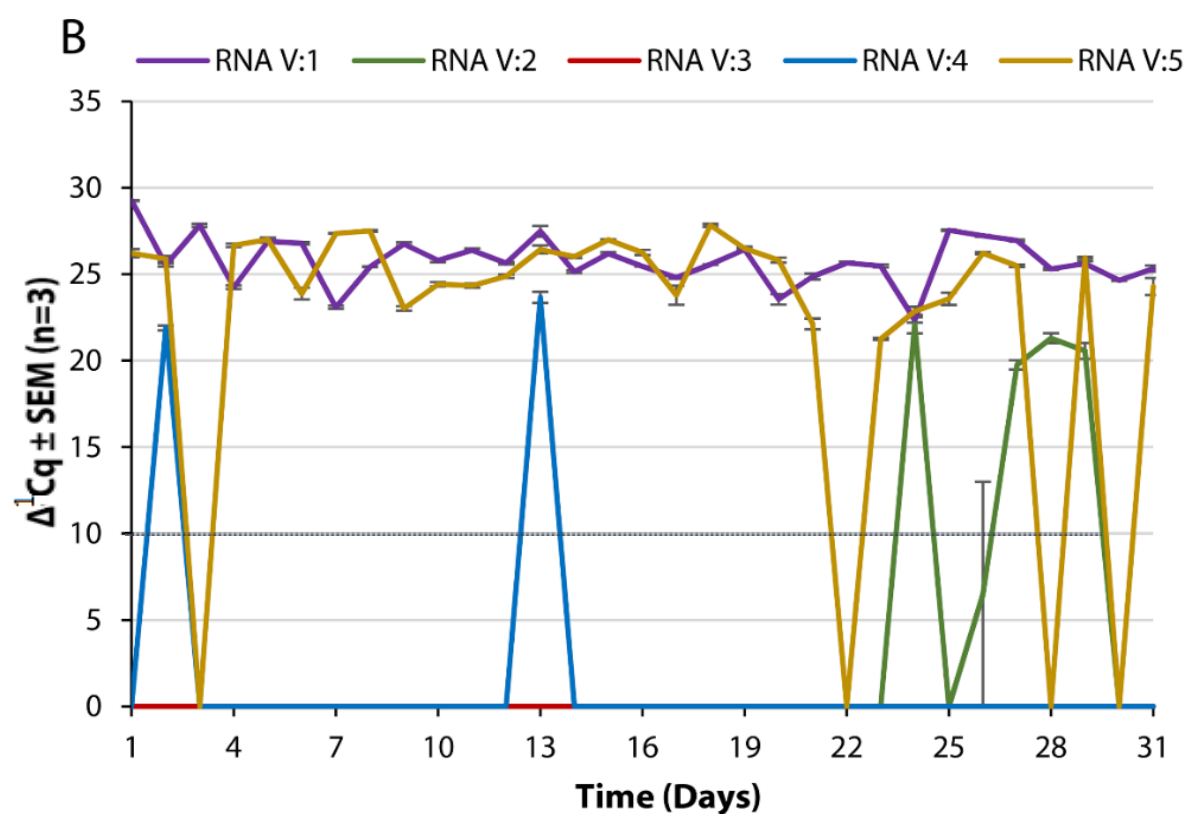
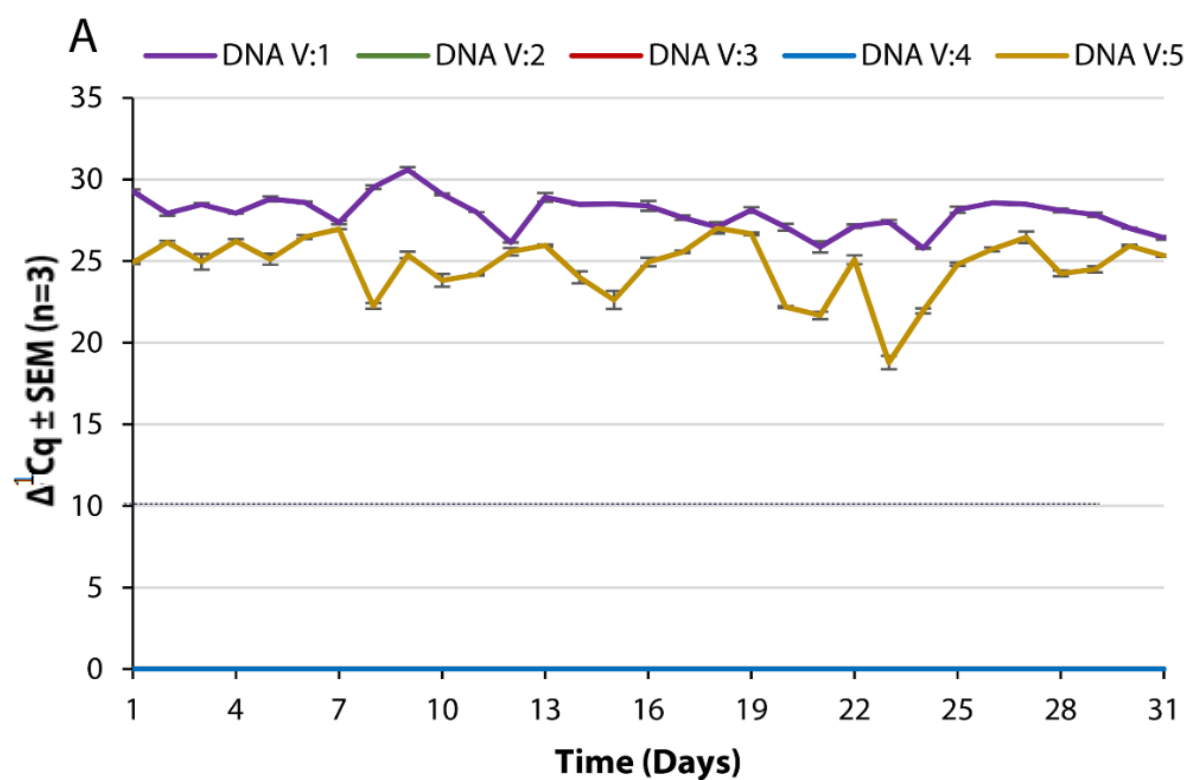


Figure 6.6: $\Delta^1Cq \pm SEM$ of hsa-mir-205 in vaginal material over 31 days, following DNA (A) and RNA extraction (B). Each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

1.1.1.1. **Proposed dual-expression saliva and vaginal material marker**

Marker hsa-mir-203a has displayed high expression values across all vaginal material and saliva samples, it has been suggested that its presence in all body fluids bar venous blood could be useful in a panel of BFID markers. It was included to assess to robustness and stability within vaginal material across the menstrual cycle. Expression of hsa-mir-203a within DNA samples can be seen in Figure 6.7: A, with RNA samples observed in Figure 6.7: B. The $\Delta^1\text{Cq}$ values across all volunteers and days in DNA and RNA samples were high (>19.7) indicating strong uniform expression across all samples. Although a degree of fluctuation within individual data sets is evident, no significant differences were observed that would indicate a false negative within vaginal material. As with hsa-mir-124, expression was, to a degree, higher in RNA compared to DNA extracted samples. Absence of expression was observed again around the same instances within the profile of volunteer 4.

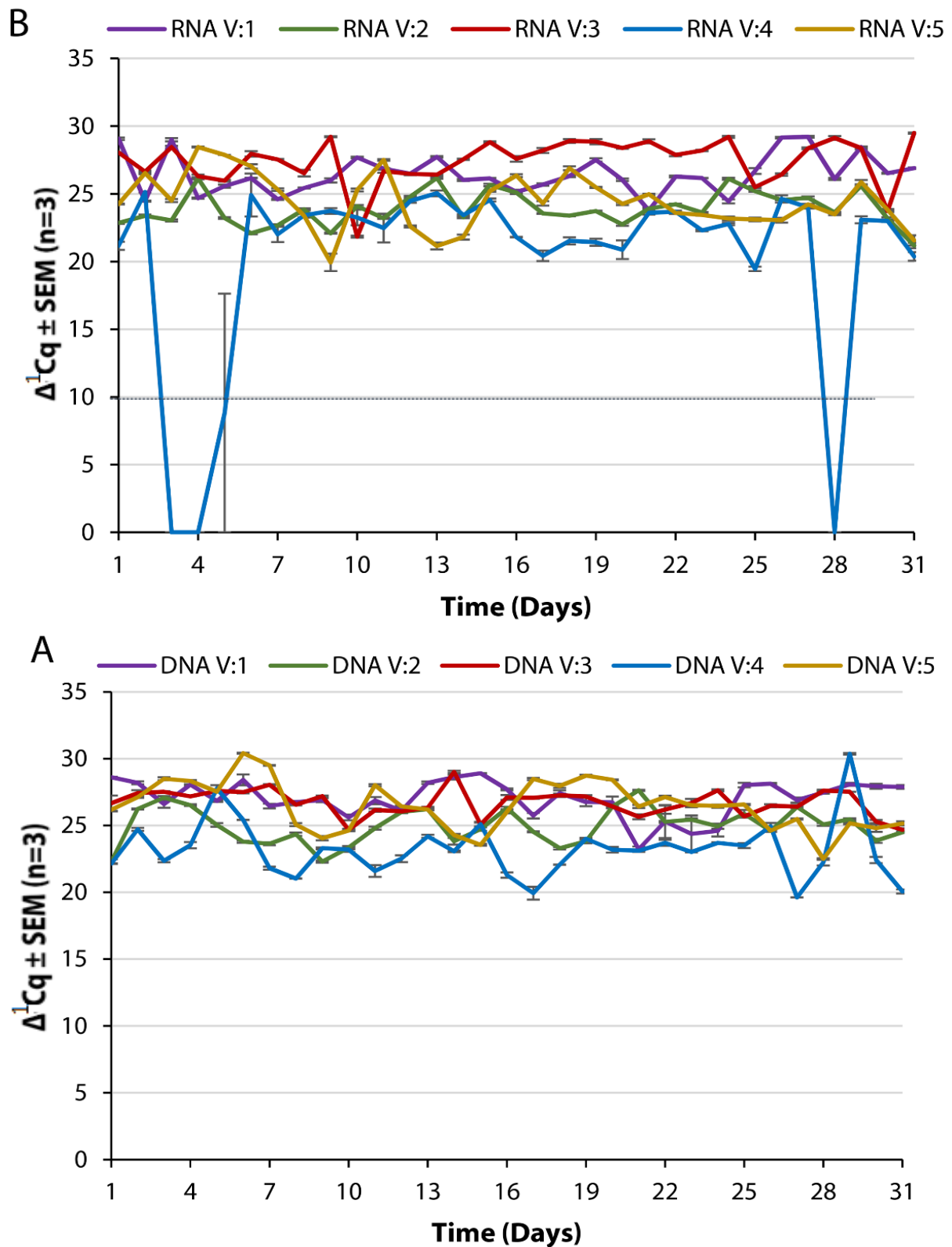


Figure 6.7: $\Delta^{13}\text{Cq} \pm \text{SEM}$ of hsa-mir-203a in vaginal material over 31 days, following DNA (A) and RNA extraction (B). Results observe no significant difference across samples (excluding outliers, each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

6.2.2.3. Proposed menstrual blood markers

Expression of marker hsa-mir-412 from both DNA and RNA can be seen in Figure 6.8. Expression values within V1, V2, V3 and V5 from DNA (A) were uniformly strong throughout the 31-day investigation, with no significant difference being observed throughout samples or these volunteers. Expression of this marker observed within the samples provided by volunteer 4 was sporadic and where expression occurred, it was moderate. Δ^1Cq values observed were significantly lower than those observed in other volunteers at all time points ($P<0.001$). Excluding days 9-10 which although moderate, were still less than the other volunteers ($P<0.01$). In addition, these spikes in expression did not correlate with menstruation. Expression values from RNA (B) samples were similar to those observed within DNA extracted samples provided from volunteers V1, V3 and V5. The data generated from both volunteers 2 and 4 displayed variability across the sampling period, again demonstrating moderate expression which did not correlate with menstruation.

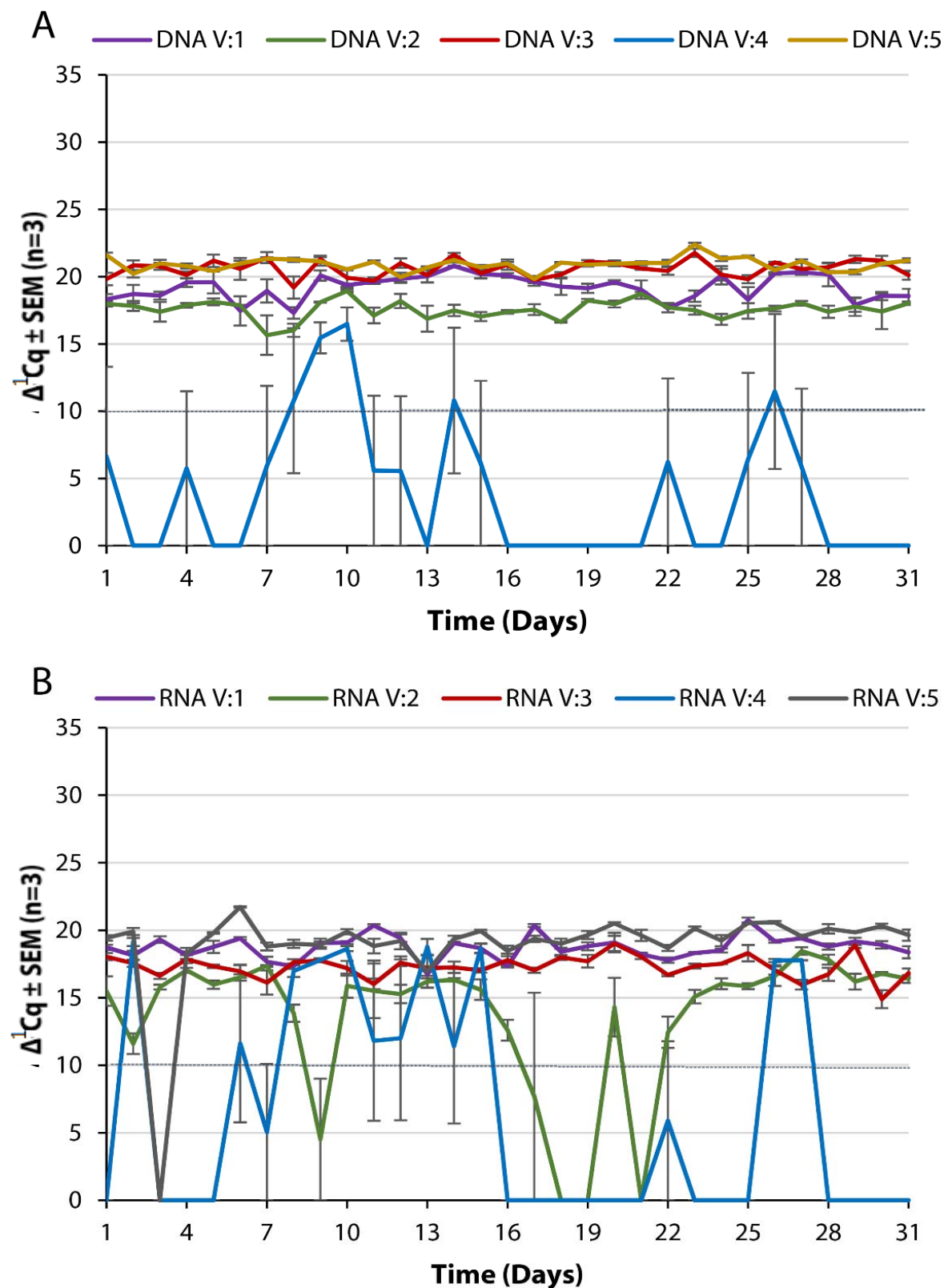


Figure 6.8: Δ^1Cq + SEM of hsa-mir-412 in vaginal material over 31 days, following DNA (A) and RNA extraction (B). No significant difference observed across samples (each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

Expression of the second proposed menstrual blood marker hsa-mir-507 from DNA was moderate across all 31 days from samples provided by volunteers 3, 4 and 5 (Figure 6.9: A). Strong expression was observed in the majority of the DNA samples provided by volunteer 1 with the remaining samples demonstrating moderate expression of the marker. The samples provided by volunteer 2 were sporadic over the course of the study with an absence, weak and moderate expression observed in samples, these however did not correlate with menstruation within this individual. The samples provided from volunteer 1 also moderately expressed marker 507 across all 31 days from RNA (Figure 6.9: B). The remaining samples provided from all volunteers demonstrated sporadic expression which ranged from absent to weak. Again, these variations in expression did not correlate with menstruation.

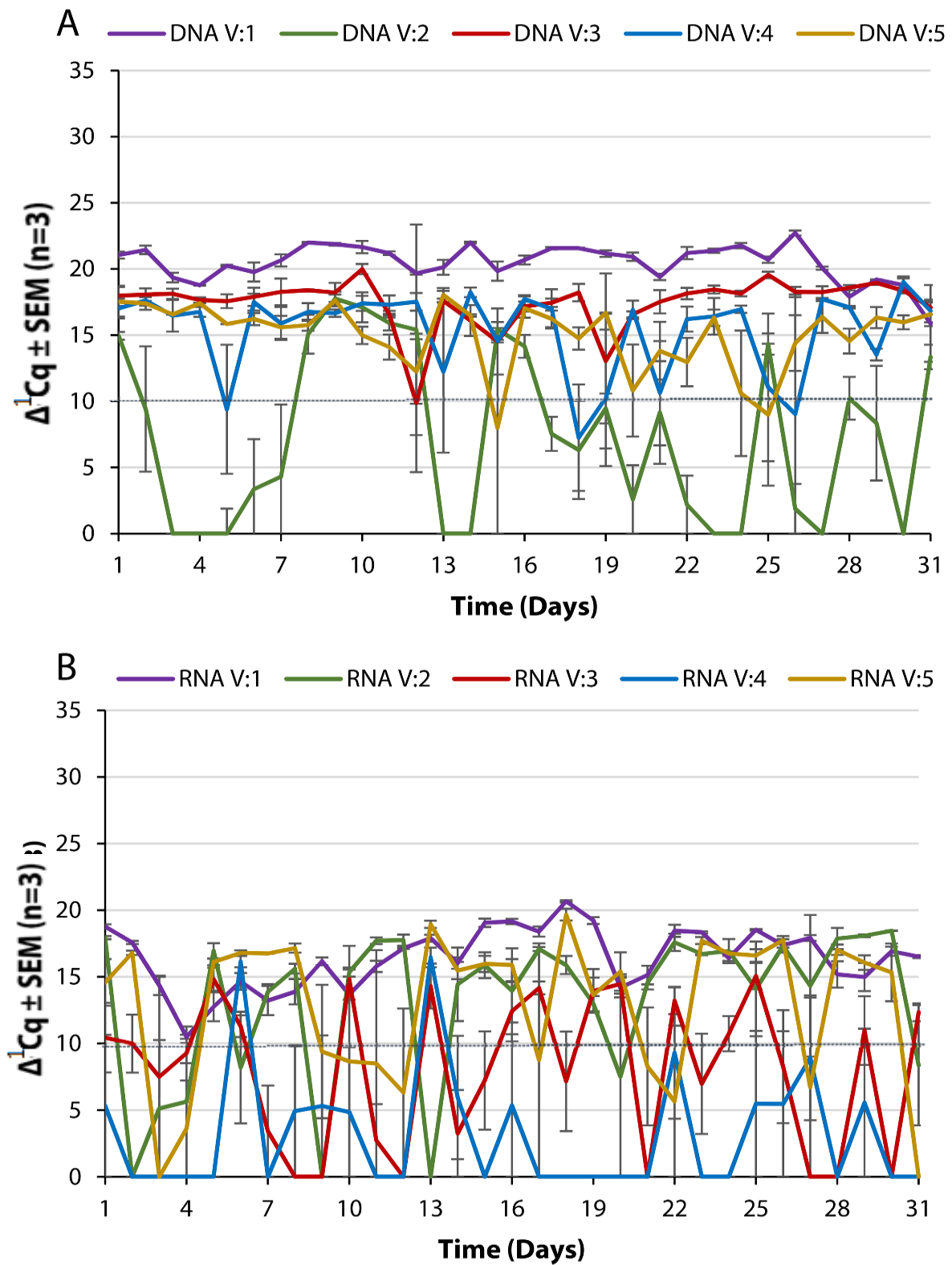


Figure 6.9: $\Delta^1Cq \pm SEM$ of hsa-mir-507 in vaginal material over 31 days, following DNA (A) and RNA extraction (B). Each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

6.2.2.4. Proposed venous blood markers

Moderate to strong expression of marker hsa-mir-144-5 was observed across the first 7-10 days of sampling from DNA samples provided by all volunteers with the exception of volunteer 3 (Figure 6.10: A). These observations correlate with menstruation occurring, and blood being present within the swabs provided by the volunteers. A similar expression pattern was observed from the RNA extracted from the samples (Figure 6.10: B), where expression was moderate rather than strong as it had been from DNA. Again, no expression was detected from samples provided by volunteer 3. All samples obtained at the start of menstruation correlated with a significant increase in expression ($P < 0.001$). The small peaks in expression from V5 observed on D18 in DNA and RNA and D10 in RNA are likely due to probe degradation or unspecific priming. These values do not reach the threshold for inclusion and therefore would be disregarded as a positive result.

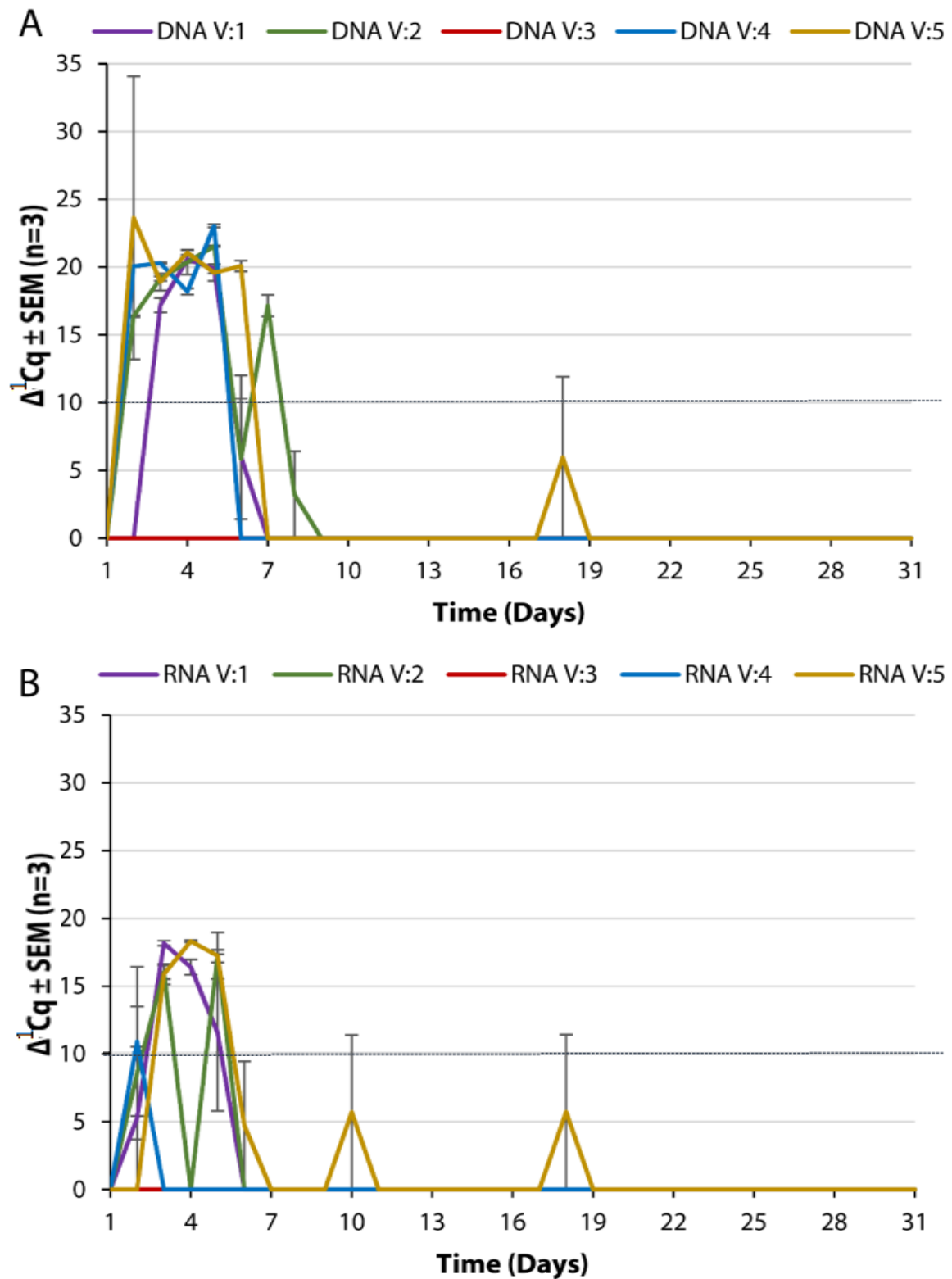


Figure 6.10: $\Delta^1Cq \pm SEM$ of hsa-mir-144-5 in vaginal material over 31 days, following DNA and RNA extraction. A significant difference was observed in expression during menstruation ($P < 0.001$). Each data point – $N=3$. Dotted line indicates threshold for inclusion in analysis.

Consistent moderate-strong expression of hsa-mir-142 was observed across the majority of both DNA (A) and RNA (B) samples provided by volunteers across the 31 days of sampling (Figure 6.11). The only fluctuation observed was seen in DNA and RNA samples provided by V3 and RNA samples provided by V4, where absent or weak expression was observed. Within DNA samples expression associated with V3 was sporadic in nature, but only in time periods around and within the menstrual cycle. The reduction of expression during menstruation was also observed in RNA samples with similar patterns from V4. The decrease in V4 does however follow the decrease in expression observed in the endogenous control markers. RNA samples for V3 did not however display the consistent expression across samples where menstruation did not occur as in corresponding DNA samples. This trend was not observed within any of the samples provided by the remaining volunteers.

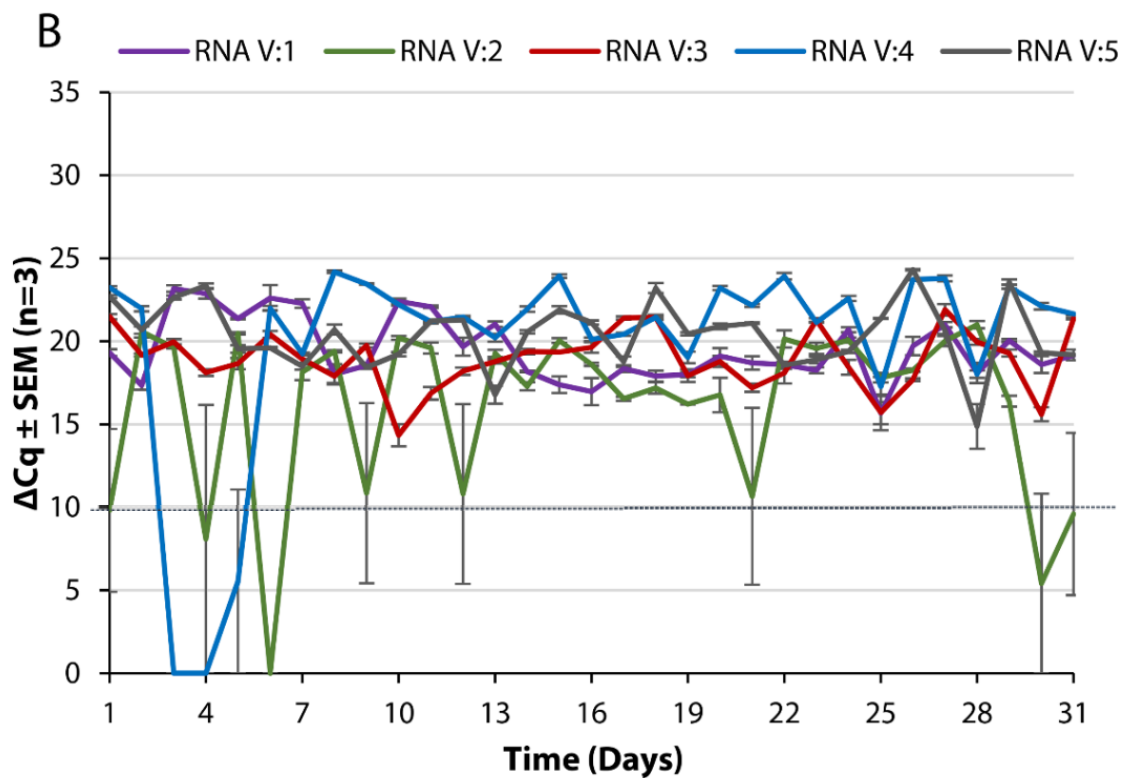
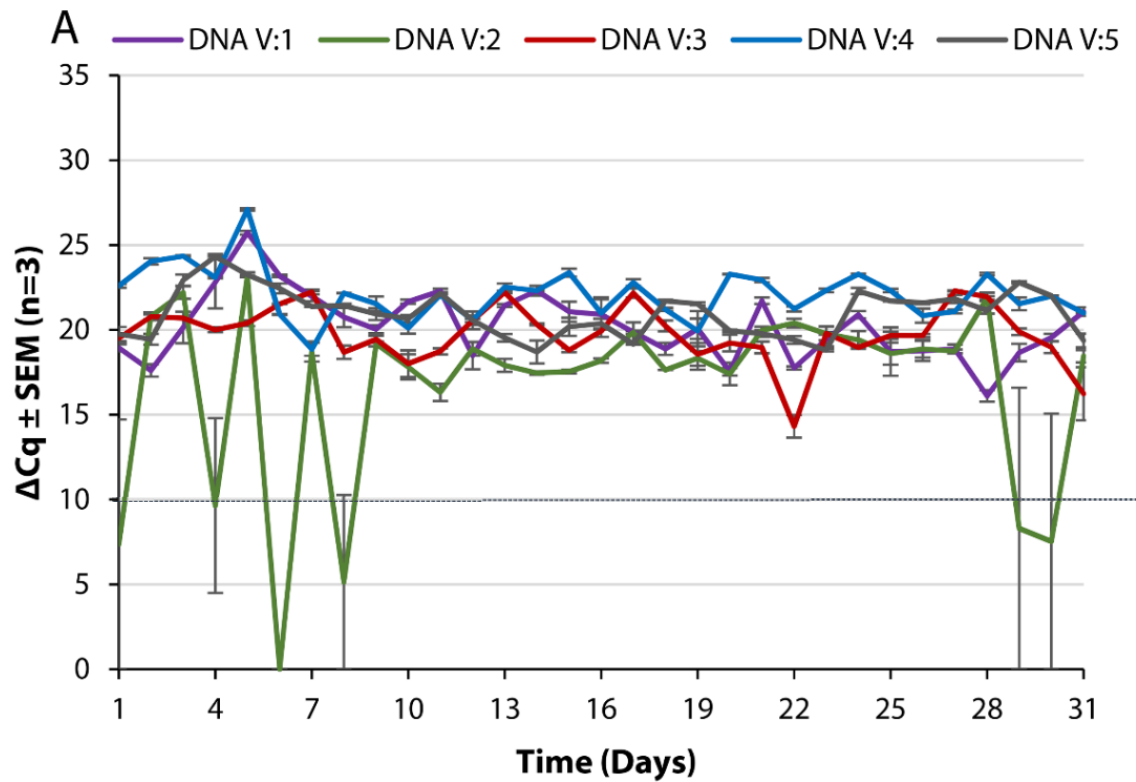


Figure 6.11: $\Delta Cq \pm SEM$ of hsa-mir-142 in vaginal material over 31 days, following DNA and RNA extraction. No significant difference observed across samples (A) (each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

$\Delta^1\text{Cq}$ values for hsa-mir-144-3 from DNA extraction can be seen in Figure 6.12: A. Expression values within DNA V3 is stable and uniform across all vaginal material samples. V1 and V4 display a significant increase in expression ($P<0.001$) during menstruation followed by high uniform expression throughout following samples. The majority of V5 DNA display positive expression, however sharp decreases and drop-outs are observed in 5 of the time points. V2 displayed high fluctuation across time points, during menstruation expression is high, with the exception of D₆ displaying a complete drop out as seen in hsa-mir-142. Expression then fluctuates from absent to around 10, lying within the weak expression category. Outside of menstruation moderately expression was only seen in D₁₃ and D₂₇.

$\Delta^1\text{Cq}$ values from RNA extraction can be seen in Figure 6.12:B. V1 displayed a significant increase in expression during menstruation however subsequent expression was not as stable with sharp decreases being observed. V4, following the sample pattern as V1 however a decrease during menstruation was observed. $\Delta^1\text{Cq}$ values within V1 all fell below 6, indicating no expression within vaginal material using RNA extraction. The expression within V2 and V5 in RNA samples was even more irregular than observed within DNA samples. V2 only displayed moderate expression the days of and immediately after menstruation, while $\Delta^1\text{Cq}$ values within V5 were significantly lower across all samples ($P<0.01$).

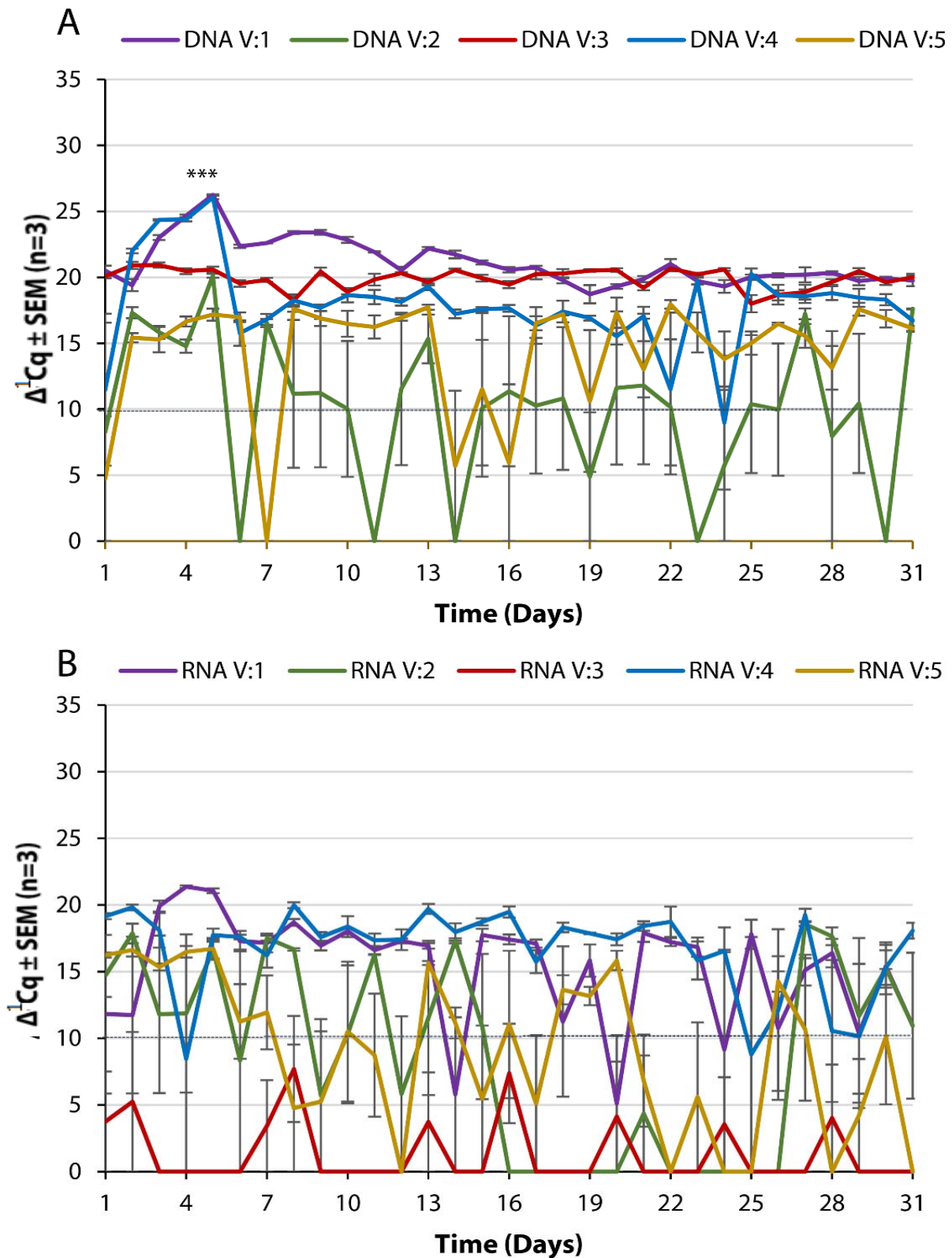


Figure 6.12: $\Delta^1\text{Cq} \pm \text{SEM}$ of hsa-mir-144-3 in vaginal material over 31 days, following DNA (A) and RNA (B) extraction. Each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

6.2.2.5. Proposed semen and seminal fluid markers

The expression values of proposed semen markers hsa-mir-10a 135a and 888 can be seen in Figure 6.13. Expression of marker hsa-mir-10a from DNA samples was moderate and consistent from DNA extracted samples originating from volunteers 1, 3 and 5, with sporadic expression that displayed no trend from volunteers 2 and 4 (Figure 6.13: A). Fluctuating expression was also observed from RNA extracted material across all volunteers, although consistently moderate expression was apparent within those samples obtained from volunteers 1 and 3 (Figure 6.13: B).

Sporadic expression of hsa-mir-135a was also observed from both DNA and RNA extracted samples over the 31-day study (Figure 6.13: C-D). The expression values obtained varied from absent-moderate with no clear trend being apparent, however an increase in expression was observed where an incidence of UPSI occurred (Volunteer 3, day D₁₇). The remaining instances of UPSI (volunteer 3, days D₁₀ and D₂₄) were however, not detected. Expression of hsa-mir-888 within samples of DNA and RNA obtained from volunteers 2 and 4 also displayed no clear pattern of expression (Figure 6.13: E-F). Expression of this marker from samples 1, 3 and 5 displayed a reasonable consistent trend from miRNA obtained through DNA and RNA extraction, where expression was moderate. Volunteer 3 did have UPSI during the 31 days of study, however this is not reflected within the data obtained for this marker.

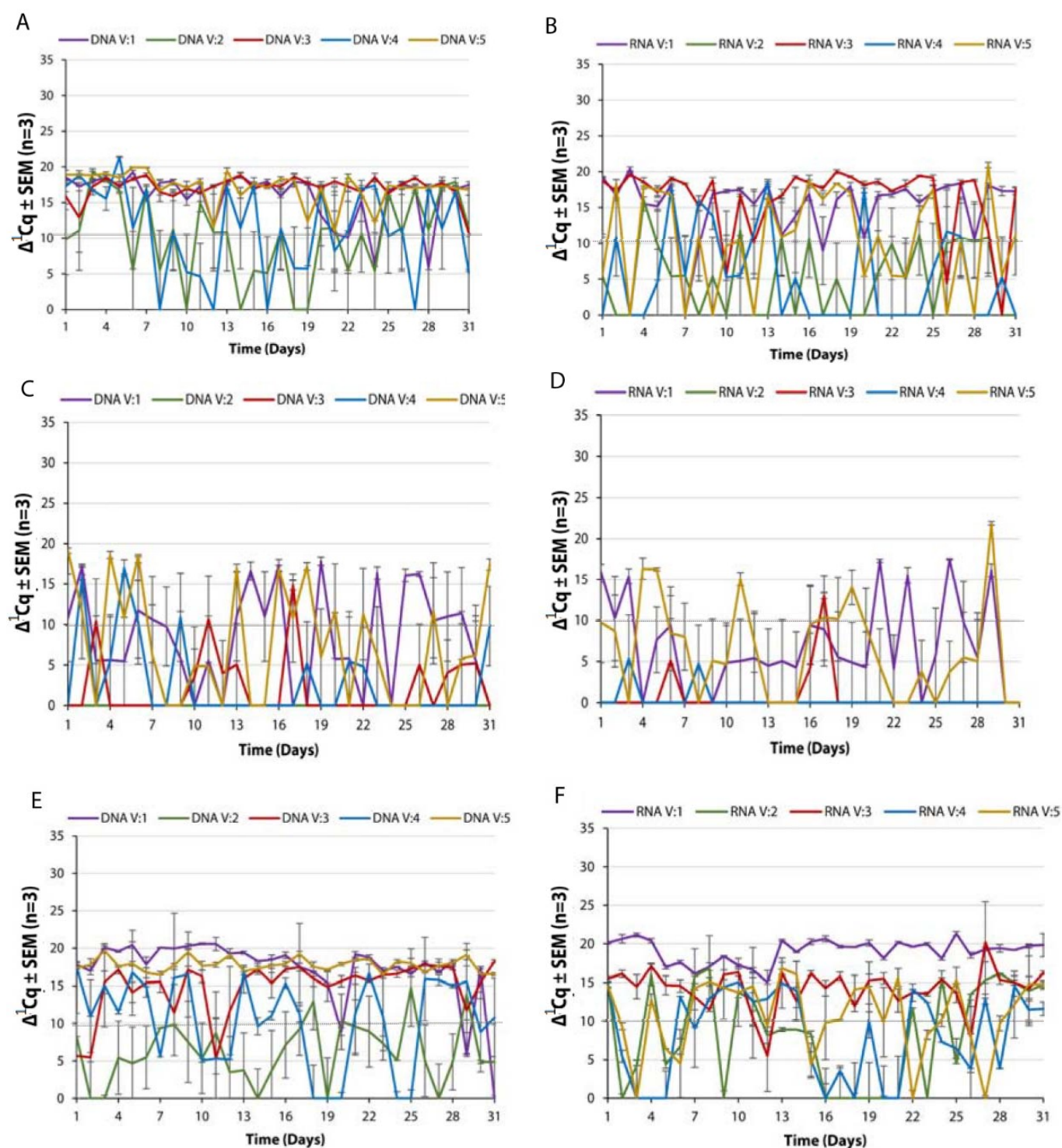


Figure 6.13: $\Delta^1Cq \pm SEM$ of proposed semen markers hsa-mir-10a (DNA (A) RNA (B), 135a (DNA (C) RNA (D) and 888 (DNA (E) RNA (F) in vaginal material over 31 days. Each data point – N=3). Dotted line indicates threshold for inclusion in analysis.

6.2.2.6. Discussion of Proposed body fluid specific miRNA

Initial screening in the previous chapter identified hsa-mir-124 as being vaginal specific regardless of dilution factor. Vaginal material will be involved in almost all sexual assault cases, the bio-marker responsible for its detection must therefore be fully reliable with no false positive or negatives observed within any analysis. Within the 31-day screening, it is evident that this miRNA marker is strongly expressed within vaginal material at all times. In cases where a suspect is arrested for suspected sexual assault a penile swab could be obtained and analysed, an expression of hsa-mir-124 could only be due to the transfer of vaginal material to the penis and therefore provide support to the prosecution.

One of the factors within any sampling is compliance with the instructions provided. The volunteers were not observed during the sampling process and training was provided via a verbal and written communication. The sampling methods were described in the information sheet of this study. If volunteers pressed deeply into the vaginal canal wall or increased the duration of sampling it may yield a larger volume of miRNA than volunteers who possibly did not sample for the required duration of time or avoided sampling the vaginal canal walls. Since expression of hsa-mir-124 was consistent, it suggests that this miRNA is abundant within vaginal material and demonstrates the robustness observed in endogenous control markers in that regardless of the quality of the sampling, expression can be detected.

The physiological similarities between vaginal material and saliva described in 2.5.5.2 were also evidenced throughout the screening. The detection of moderate to strong expression of marker hsa-mir-203a within the samples provided by all the volunteers across 31 days leads to support that this miRNA is likely to be imperative to functional cellular processes associated with both of these secretions. Within DNA samples the expression of hsa-mir-205 was consistently expressed or absent in each volunteer, with the only occurrences of OI in V3 not being detected. The strong expression of this marker within vaginal material means that when applied to forensic casework the occurrence of OI could not be detected.

The unsuccessful identification of saliva after OI does not necessarily reflect on the robustness of hsa-mir-205 to detect saliva; Swabs were taken inside the vaginal canal, unless penetration of the tongue occurred any saliva present would be deposited on external surfaces of the vagina and would therefore not be sampled. Since marker hsa-mir-124 is strongly expressed in vaginal material, this would become a discriminatory factor in stains as described in the previous chapter. This combination may also lend to the determination of vaginal material obtained from buccal swabs, but this would require further investigation.

The previous chapter highlighted a possible link between contraceptive methods and expression of hsa-mir-205. Throughout this study the contraceptive method of V1, 2, 3 and 5 remained the same as when screening samples were provided, the result was a similar expression value as observed at 0.7ng/μl. During screening V2 (V4 in screening) utilised the contraceptive pill while V18 did not use any form of contraception, throughout this study V2 ceased all contraceptive methods while V18 began taking the contraceptive pill and provided two LVS samples for screening, one a week after initial ingestion and the second 2 months after. Data from both volunteers, on and off the pill can be seen in Figure 6.14. All samples were analysed at 0.7ng/μl with the expectation of 'V18 no pill' where the average of the 31 day results were taken. The absence of expression in 'V18 no pill', even at much higher concentrations of genetic material as well as the almost immediate increase in expression of hsa-mir-205 in V4 after using the pill support the indication that some contraceptive methods upregulate expression of hsa-mir-205. This cannot be observed for all contraceptive methods as V3 utilises the contraceptive injection and as per results observed in ItDNA analysis, an absence of hsa-mir-205 was seen in all samples.

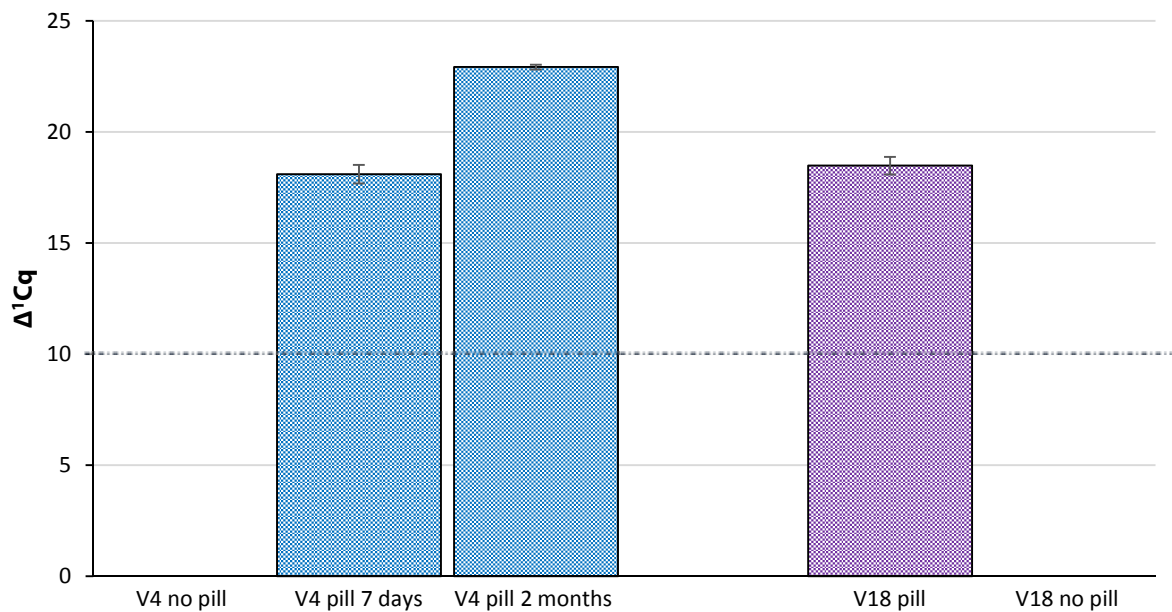


Figure 6.14 : $\Delta 1Cq$ + SEM of hsa-mir-205 in two volunteer's vaginal material samples. Samples include V4 no pill (provided during screening while on no form of contraception) and V4 pill (obtained 7 days and 2 months after commencement of the pill) as well as V18 pill (provided during screening while on the pill) and V18 no pill (obtained through the average of all data points from 31-day screening). Dotted line indicates threshold for inclusion in analysis.

Markers hsa-mir-412 and 507 were candidate indicators of menstrual blood, but only when expression of 124 was absent in the initial screening. The data here expose that these markers are likely expressed in vaginal material and menstrual blood, but lack the robustness associated with marker 124. Therefore, within case work, it would be inappropriate to use this testing scheme on swabs that are taken directly from the vagina. The use of a hsa-mir-124 in combination with these markers still has the potential for BFID where the sample was obtained from blood spots or stains present at a crime scene.

A further two markers were identified in the previous study for the identification of venous blood, distinguishing it from menstrual blood. Marker hsa-mir-144-5 was expressed during the bleeding associated with menstruation and not expressed for the remainder of the study in all volunteers that were capable of menstruation. The results obtained here suggest that hsa-mir-144-5 must be a common component of both venous and menstrual blood. The second marker used within the study, has-mir-144-3 showed expression in all volunteers across the 31-day study with fluctuations showing no apparent correlation with the menstrual cycle. It is therefore likely that this marker is present within both vaginal

material and blood independently of one another, which could lead to false positives within a forensic case study. Again, the use of marker hsa-mir-124 within a combined marker panel screening would be the key to eliminate the prospect of the sample originating from the vagina (i.e. being menstrual blood) rather than a single marker being capable of this detection. In this case, the multiplex would again not be capable of being applied to blood obtained from vaginal injuries, but would be capable of eliminating suspected menstrual blood being present on a stain or other blood residue present at a crime scene.

Proposed semen markers, hsa-mir-10a and 888 were expressed in all volunteers over the course of the 31 days, expression fluctuated, with no clear pattern over the course of the study within all volunteers. These results show that this test for semen identification is unsuccessful at this DNA concentration. If this was used in a court of law all markers would show expression, this would lead to the evidence highly supporting sexual intercourse occurred even if the reality was it had not. The consequences of this false negative could be a wrong conviction.

The results suggest that markers hsa-mir-10a and 888 are expressed in the components of both vaginal material and semen. Since the samples in the previous chapter did not exhibit expression in the ItDNA, the data indicates that this marker is perhaps expressed more in semen than vaginal material. These two markers would therefore require a greater level of template concentration optimisation prior to being considered for robust BFID. This was also evidenced with marker hsa-mir-135a, however it would appear that this marker shows variation in expression between individuals. Of the three instances of UPSI that occurred, one of these was detected through an increase in expression. This detection of UPSI, although demonstrating a low success rate for detection within this study (33%), holds further promise and will be investigated further.

6.2.3. DNA v RNA Extraction Methods

6.2.3.1. Proposed endogenous controls

ΔCq values for SNORD 47, hsa-mir-508, 93 and 1260b are displayed in Figure 6.15. No significant difference was observed between extraction methods across endogenous control makers with the exception of the significantly decreased expression observed with V4 RNA D₁₋₅, D₇ and D₂₈.

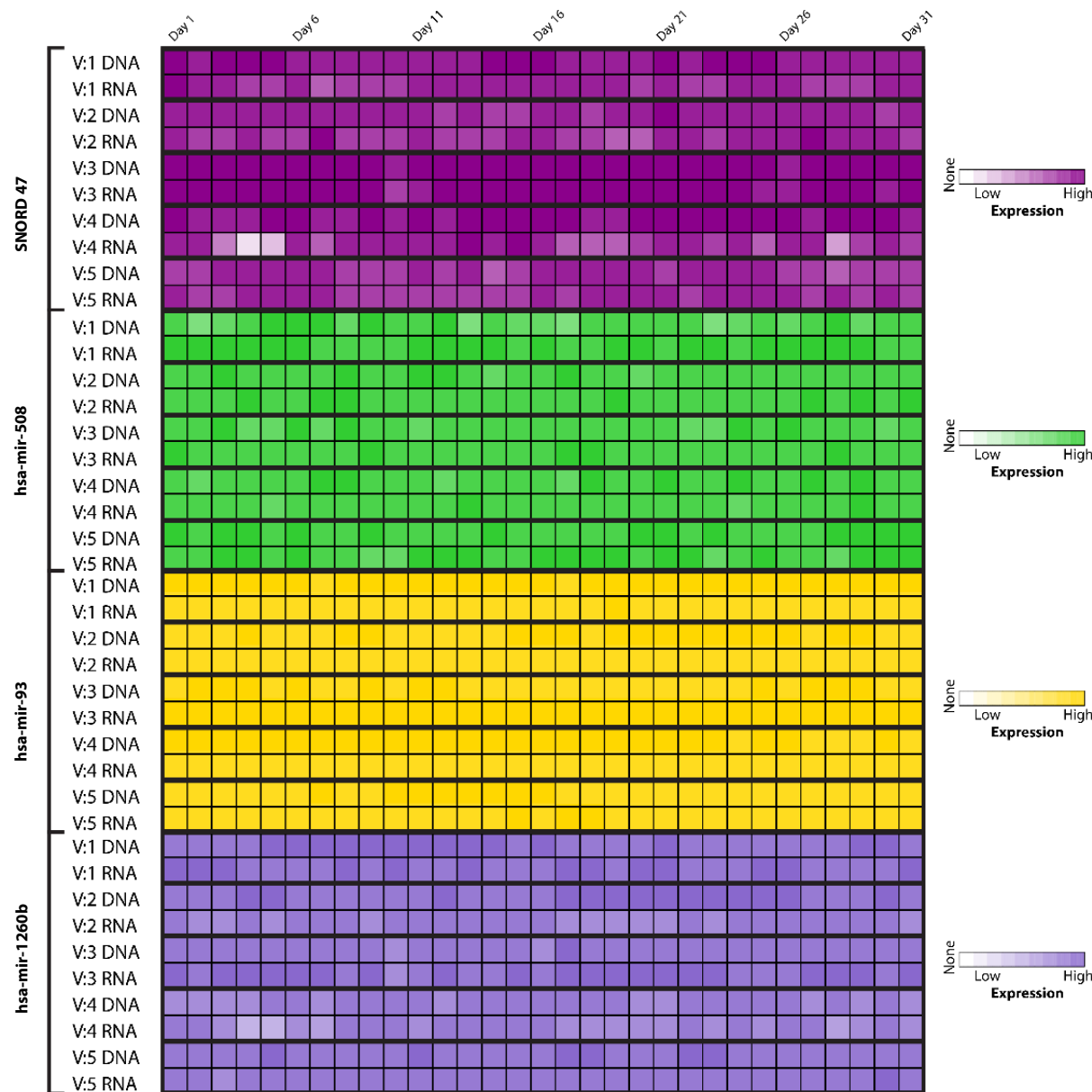


Figure 6.15 Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers SNORD 47, hsa-mir-508, hsa-mir-93 and hsa-mir-1260b.

6.2.3.2. Proposed body fluid specific markers

The ΔCq was calculated for all samples utilising the average values obtained from the four endogenous controls, values for hsa-mir-124, 144-3, 412, 142 and 144-5 are displayed in Figure 6.16. Heat maps display the normalised expression values of all samples across both extraction methods. The endogenous controls identified a lack of miRNA present in V4's RNA samples D₃₋₅, D₇ and D₂₈, these samples are identified by a '/'.

ΔCq values for vaginal material marker hsa-mir-124 showed high expression in all samples, across all volunteers and all extraction methods. Although absence of expression was observed in a small number of DNA samples. The frequency of low or absent expression within blood marker hsa-mir-144-3 is high in the majority of RNA extraction samples. The absence observed in one extraction type did not always correspond with the alternative extraction type. Within hsa-mir-412, absence of expression was seen in DNA samples from V1, V2, V3 and V5, however irregular decreases were observed in corresponding RNA samples. The expression patterns are similar in V4 in that expression is frequently absent or low in both data sets, however higher expression values are seen in RNA extracted samples over DNA extracted samples.

ΔCq values for hsa-mir-142 are fairly consistent between samples. Although the higher ΔCq tend to alternate across extraction methods all ΔCq displayed no significant difference between them. The decreases and absences observed in V2 can be seen the corresponding extraction technique. The ΔCq values for hsa-mir-144-5 indicate clear strong expression in all DNA samples when menstruation occurred, this was not observed in RNA samples. Expression was not as high as corresponding DNA samples and in some RNA samples, menstrual blood was not even detected. This would be a significant problem if used in evidence as it would give a false negative and provide incorrect support to the prosecution or defence. In a case where a suspect stated consensual intercourse while the female was menstruating occurred, a false negative for menstrual blood would strengthen the prosecutions defence that the blood originated from venous blood.

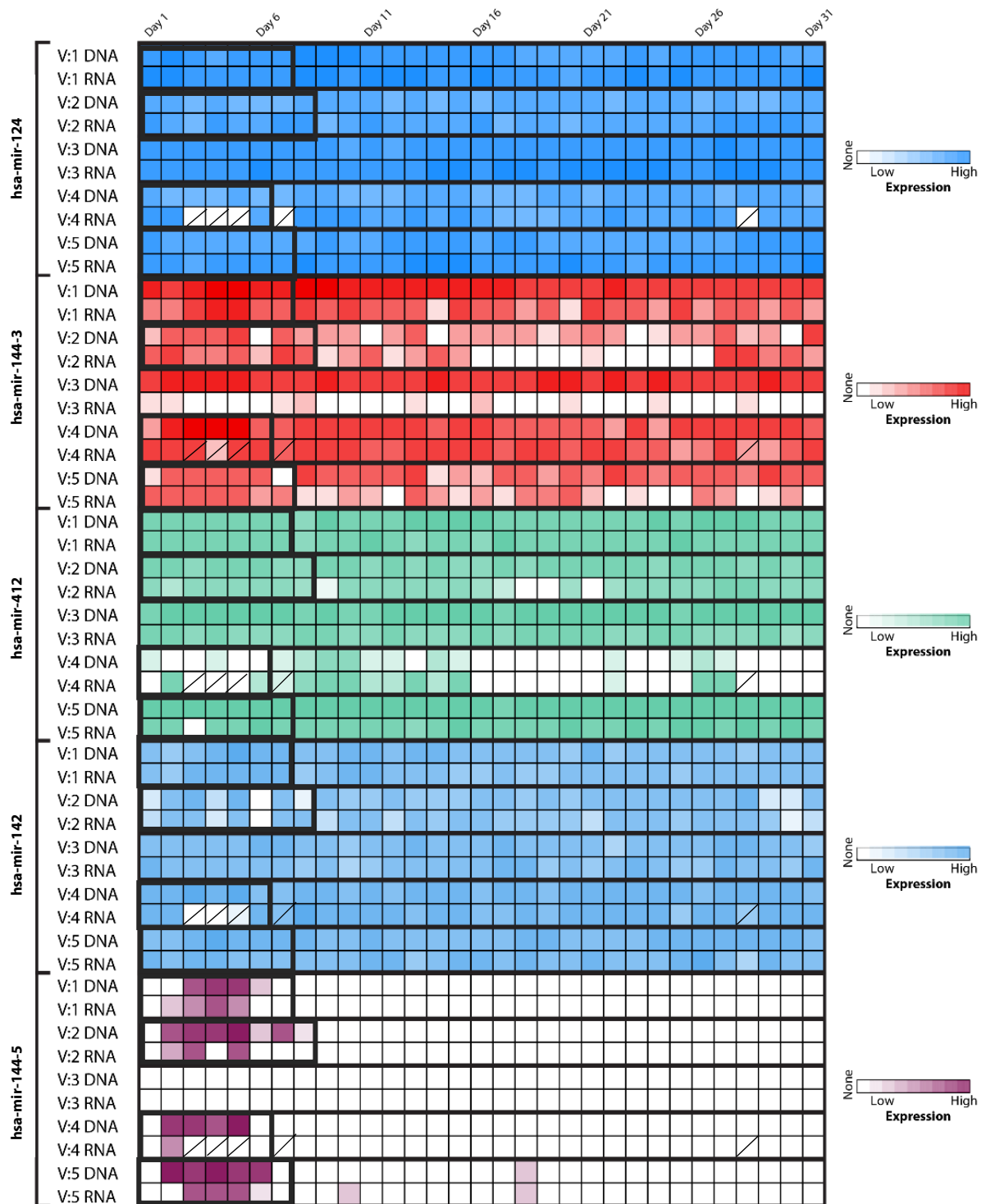


Figure 6.16: Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers hsa-mir-124, hsa-mir-144-3, hsa-mir-412, hsa-mir-142 and hsa-mir-144-5. Instances of sample integrity are indicated by /. White boxes indicate a ΔCq of 0 (no observed expression). Black box indicates days where menstruation occurred.

ΔCq values for hsa-mir-10a, 135a, 507, 888, 203a and 205 are displayed in Figure 6.17. Days where semen should be identified by semen markers hsa-mir-10a, 135a and 888 due to UPSI are identified by a circle. Expression of hsa-mir-10a was varied between volunteers and across extraction methods, DNA V1 displayed instances of high expression which were mirrored in the majority of RNA samples. At some time points, such as D₇ and D₁₇ expression was significantly higher in DNA samples, whereas in days D₂₄ and D₂₈ expression was higher within RNA samples. Limited expression was seen within V2 RNA samples whereas DNA samples display high expression on multiple days that were not observed in RNA samples. V3 displayed high expression values across all DNA samples, expression was marginally lower in RNA samples where an absence of expression was observed within certain samples. The expression pattern of V4 fluctuated throughout the 31 days. Where expression was strong it was observed in both extraction methods, however days where expression was moderate, detection only occurred from DNA samples. V5 displayed moderate to high expression in all DNA samples, but poor to weak expression was seen in many RNA samples. The expression pattern for semen markers hsa-mir-888 was very similar to hsa-mir-10a, V1 displayed strong expression in both DNA and RNA samples, with RNA exhibiting higher expression values. V2 exhibited weak expression in the majority of samples from both extraction methods with RNA presenting the higher expression values when expression was observed. Marker hsa-mir-888 was seen in all V3 samples with similar expression values across extraction types. V4 displayed weak expression across most samples, however RNA samples presented the absences of expression. V5 displayed equal expression patterns as observed in hsa-mir-10a; strong expression in both extraction methods but absent and weak expression observed in a high number of RNA samples.

Marker hsa-mir-135a was absent or weak in the majority of samples. The highest expression was observed within V1 and V5 samples, this expression was erratic and abrupt and frequently followed by absent or weak expression. In the majority of samples both extraction methods displayed absence or presence across sample days. V2 display no expression across either extraction type. No significant expression was observed in V3 with

the exception of D₁₇ which was highly expressed in both the RNA and DNA sample. High expression was only seen in 2 samples of V4 DNA with no significant expression being observed in RNA samples. The majority of absences observed in semen markers in RNA samples occur at the same time points; weak or absent expression is observed in V5 RNA D₃, D₁₂, V2 D₂, D₂₅ and V4 D₁₇₋₁₈, equivalent absences were not observed within DNA samples.

The expression of hsa-mir-507 varied greatly across volunteers and extraction methods. In V1 a strong expression is seen across all samples in both extraction methods, however higher values are observed in DNA compared to RNA. In V2 frequent occurrences of no expression are observed within DNA samples, however this was not always seen in corresponding RNA samples. Strong expression as often observed when the equivalent sample ΔCq value is 0. V3 displays uniform expression across all DNA samples except for a slight decrease in D₁₂, in RNA this sample is complete absent. RNA samples for V3 show no uniformity, only irregular drop-outs where corresponding DNA samples display strong expression.

Expression in hsa-mir-203a was consistent across all samples and extraction methods, no significant difference is seen between samples of different extractions. The ΔCq values for hsa-mir-205 within DNA extraction samples were uniform, volunteers displayed a complete absence or absolute presence across all samples. Variation was seen in equivalent RNA samples, V2 and V4 displayed expression in samples where DNA observed an absence and V5 samples exhibited absences where high expression was observed in corresponding DNA samples.

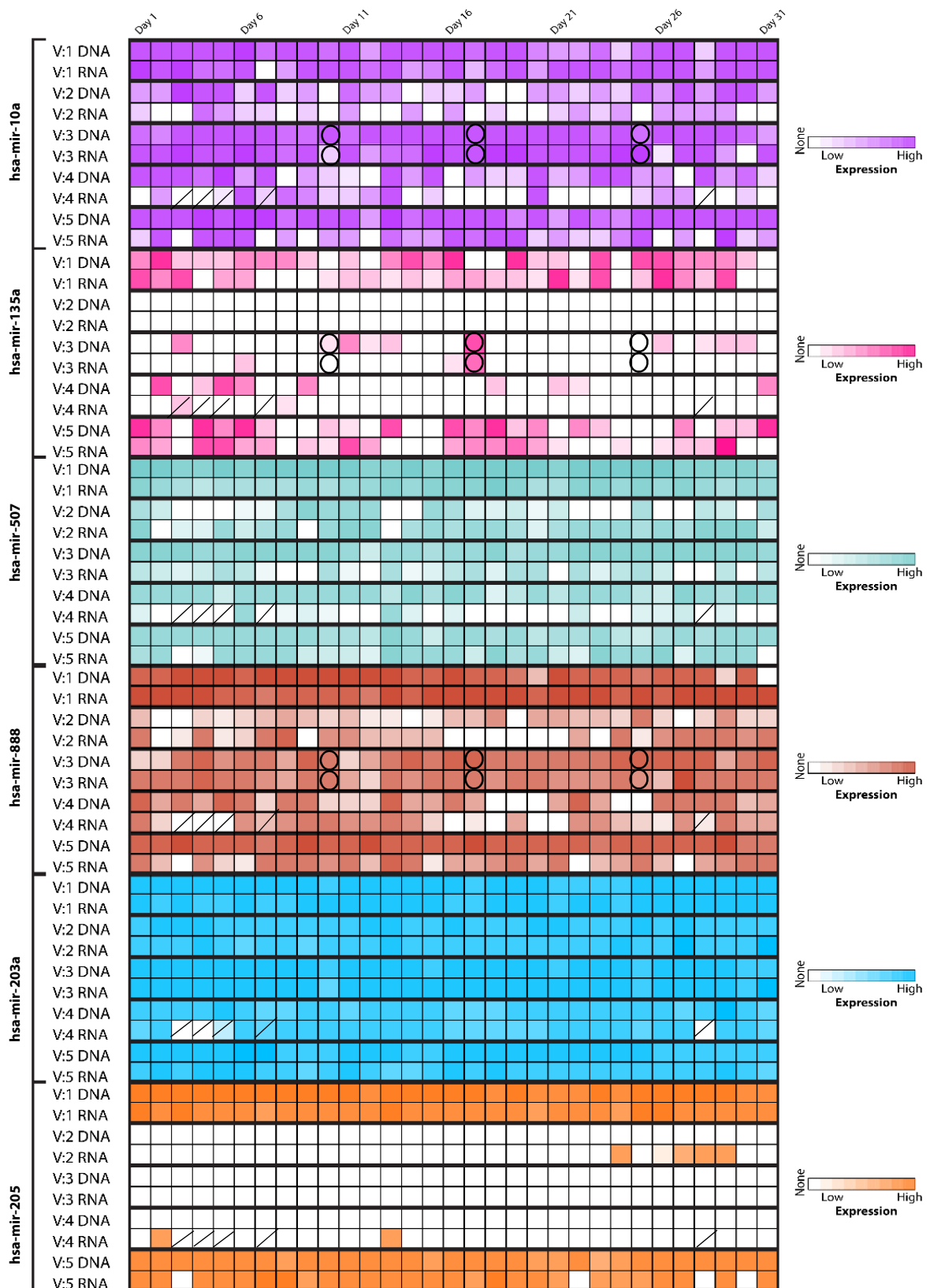


Figure 6.17: Individual ΔCq (ΔCq of marker – avg. ΔCq of endogenous controls) of each sample over a 31-day period for markers hsa-mir-10a, 135a, 507, 888, 203a and 205. Instances of UPSI are indicated by a circle, with sample integrity indicated by /.

6.2.3.3. Discussion of extraction method comparison

Comparisons of the DNA extraction and RNA extraction protocols for the obtaining of miRNA indicated that both methods were capable of extracting enough miRNA template to be detected with the RT-qPCR technique employed in the study. Despite this, the expression detected from both extraction methods were not always comparable and varied between samples and volunteers. The results obtained do however suggest that the use of the DNA extraction protocol is likely to provide the most reliable and robust method for the extraction of miRNA from tissue samples. The difference in capabilities of the kits could be due to the length of the miRNA, the difference of one nucleotide could mean the difference of adhering to the silica membrane or being removed with the wash buffers. The buffers within each kit may also have an effect on the binding of the miRNA to the silica membranes.

6.3. Conclusion

The work presented within this chapter has identified a panel of endogenous controls suitable for the validation of BFID from vaginal material within case work. The previous chapter showed that these markers were expressed across all body fluid types and the results here confirm that the expression from these markers is not influenced by the complexities of the menstrual cycle.

In a similar manner, vaginal material marker hsa-mir-124 was also confirmed as robust marker for this body fluid. This marker is therefore a key marker for any multiplex screening for the detection of the presence or absence of vaginal material within a sample. In particular, hsa-mir-144-5 used in combination with 124 would form a robust and reproducible method for discriminating between menstrual and venous blood. In addition, this marker is also the basis for the discrimination of hsa-mir-205 and 203a expression associated with saliva and vaginal secretions.

One of the three miRNA markers, hsa-mir-135a showed promise as a semen marker, detecting an instance of UPSI within one of the samples. All the proposed semen markers appear to be to some extent expressed in vaginal secretions which leads to complications

when high concentrations of template are used. Therefore, the next chapter will look to determine whether expression of these markers can be detected when input template concentration is optimised.

Finally, this study represents a comprehensive comparison of miRNA expression extracted from DNA and RNA extractions protocols. Although both methods were successful at determining expression, it would appear that DNA extraction protocols provided the most consistent data. This suggests that previously extracted DNA from past cases are likely to contain miRNA that could be used within these techniques if required.

6.4. Summary

- Total DNA extraction has shown to be more reliable and predictable than RNA extraction
- Vaginal material marker hsa-mir-124 is regulated at a consistent level throughout the entire menstrual cycle and is therefore an ideal candidate for vaginal material identification.
- Markers highly expressed in vaginal material during screening displayed high expression in all samples, with no significant difference ($P < 0.001$) across days.
- Markers that showed absent expression in low template DNA vaginal material samples displayed high expression in some samples indicating many of the selected miRNA may be present within vaginal material.
- Further dilution of samples is required to reduce the non-vaginal specific miRNA present in vaginal material beyond LOD.
- Expression of hsa-mir-205 appears to be upregulated in volunteers using contraception methods compared to those on no form of contraception.
- hsa-mir-144-5 is not specific to venous blood as ItDNA screening suggested but is highly specific to blood; expression was only seen throughout menstruation in all volunteers.

- When analysing sexual assault samples involving vaginal material, it would be highly beneficial for medical examiners to record any current form of contraception being taken.

7. Detection of Semen Markers within Vaginal Material Following Unprotected Sexual Intercourse

7.1. Rationale

The previous two results chapters have indicated that miRNA markers are capable of distinguishing between vaginal material, menstrual blood, venous blood, semen/ seminal fluid and saliva from varying sample types. The identification of robust endogenous controls has also allowed this to be carried out in a validated manner. The results of chapter 6 identified semen and seminal fluid specific markers which were, at nucleic acid concentrations of 5ng/μl also expressed within extracts from vaginal material samples. The human body is capable of making complex body fluids consisting of a number of components. The commonality of these components (for instance RBC in venous blood and menstrual blood) means that as well as miRNA being specific to a body fluid, a body fluid may be characterised by the proportion of each miRNA.

Where ItDNA samples are involved, the ability to detect proposed semen markers in vaginal material is removed or diminished (Chapter 5). Therefore, their detection in ItDNA samples would require the introduction of additional miRNA from a secondary source, i.e. the ejaculation of semen/seminal fluid into the vagina. This was observed in vaginal material and menstrual blood where UPSI had occurred within 48 hours and in one of the cases of UPSI within the 31-day study (Chapter 5 and 6), where expression of marker has-mir-10a was observed. Expression of this marker was not detected in the subsequent samples provided, raising the question as to the ability of the miRNA to be detected in the time following a penetrative sexual assault taking place. Many sexual assaults are not reported immediately, the longer reporting takes the higher the likelihood semen will not be identified.

Crime scene stains involving sexual assaults will likely contain multiple body fluids, data presented within the previous chapters suggest that swabs obtained from victim examination would consist of a small genetic material contribution from semen which is overwhelmed by the genetic material from the vaginal material contribution. Techniques for BFID must therefore be detectable not only in a 1:1 ratio mixture but mixtures where

vaginal material is the majority contributor. Semen identification must also be made in multiple body fluid mixtures in varying mixing ratios.

When samples from sexual assault cases enter a forensic laboratory, they are logged as 'major crime samples' and are processed by specialist examiners. Currently, the only aim within this forensic process is to obtain as much male DNA as possible from the spermatozoa to produce a full DNA profile. To ensure this, an alternative extraction technique is utilised to the total DNA extraction of 'scene of crime' samples. Samples are extracted in two stages; first DNA from vaginal and epithelial cells is obtained followed by the elution of the DNA from semen cells by the addition of DTT. The hostile nature of DTT is ideal for breaking open the head of a spermatozoa however it could be detrimental to miRNA that are present. As such, the comparison of RT-qPCR data from a total DNA extraction and the same protocol with the addition of a DTT step must be made. Semen markers were also highly detected in seminal fluid samples, suggesting these markers are present outside the spermatozoa and thus would be eluted with the vaginal fraction. Allowing the possibility of BFID to be performed on the vaginal fraction and DNA profiling on the semen elution treated with DTT.

The aim of this section of work was to determine the ability of the miRNA RT-qPCR technique to identify semen and seminal fluid within vaginal samples following the 'major crime' extraction methods and compare them with standard total DNA extraction. The semen markers selected from screening were also analysed to determine if they could positively identify semen in a situation where natural mixing ratios would occur, what duration of time expression could still be detected and which of the various extraction techniques with or without the addition of DTT utilised in a forensic laboratory are the most efficient at miRNA extraction.

7.2. Experimental Design

Five females ranging from 22 – 28 years old currently engaging in regular UPSI volunteered for this study. Spermatozoa survival within the vagina can range from hours to days dependent on various conditions, if the spermatozoa do not travel to the cervix the acidic conditions within the vaginal canal make survival impossible, dependant on ovulation the cervix can secrete a more fertile mucus and therefore provide a less hostile environment allowing semen to survive up to 5 days [232]. Lutz Roewer assessed the ability to perform Y-STR analysis on vaginal material and semen mixtures and his results showed profiling could be successful 2–8 days after sexual assault [233]. This study shows a large range in the detectability of spermatazoa and is likely due to the variation of spermatazoa levels of individual males. Volunteers were therefore asked to abstain from sexual intercourse for a minimum of 7 days prior to initial sampling and the subsequent 4 days of sampling.

During a SARC examination external, high/cervical and LVS will be taken, with semen expectation on the high/cervical swabs. As previously described for the comfort of participants and to maintain the same sampling technique throughout participants were asked to provide LVS swabs. LVS Control samples were obtained prior to UPSI, followed by two LVS samples at pre-determined time intervals (0 minutes, 20 minutes, 24 hours, 48 hours and 96 hours). These time points were chosen so results could be comparable with currently available literature and so the initial movements of semen within the vagina could be further researched. Sample information can be seen in table 7.1. The presence of semen obtained from the male participants within this study was assessed using a self-collected reference sample (unless previously provided in screening study) and underwent H&E staining.

	Age	Days prior to UPSI	Contraception	Key information
<i>Volunteer 1</i>	28	7 days	Implant	V2 – Screening study V1 – Menstrual cycle validation Oral intercourse occurred
<i>Volunteer 2</i>	26	+ 7 days	Contraceptive Pill	V 29 – Screening study Oral intercourse occurred
<i>Volunteer 3</i>	22	+ 14 days	IUD (no hormones)	V 28 – Screening study
<i>Volunteer 4</i>	23	+ 7 days	Contraceptive Pill	V22– Screening study Menstrual cycle started from 24hr swab Oral intercourse occurred
<i>Volunteer 5</i>	24	+ 7 days	Contraceptive Injection (Depot)	V 15 – Screening study V3 – Menstrual cycle validation Oral intercourse occurred

Table 7.1: Samples information of UPSI volunteers, including contraception, days without UPSI and OI, and if samples were provided for previous studies.

Due to the expression of the semen marker observed within vaginal material samples at concentrations of 5ng/μl, a lower concentration was used for this study. A LOD of hsa-mir-888 within vaginal material displayed expression in some samples at 5ng/μl, supporting the fluctuating expression pattern seen over 31 days, absence of expression at 0.5ng/μl indicated that the threshold fell between these points (appendix G). The reference samples that would be obtained from the SARC kit would have a high concentration of genetic material therefore ItDNA analysis would not be required. Due to ItDNA analysis displaying no expression in vaginal material but difficulties in identification in >24 hours samples an increased concentration of 2ng/μl was set. Control samples were analysed for expression of semen markers, if this concentration showed expression a further dilution to 1ng/μl would have been applied.

Six semen, vaginal material and venous blood samples were selected for comparative analysis of miRNA expression in total DNA extraction with and without the addition of DTT. All vaginal material, venous blood and semen samples underwent extraction following total DNA protocol (4.4.1)⁴. An additional extraction on each semen sample was performed

⁴ The normalisation followed by RT-qPCR of samples within the DTT vs no DTT experiment was carried out under my strict supervision by project student Ellis Fletcher-Heeley. Conception, development and all analysis was carried out by myself.

following total DNA protocol with DTT (4.13.3). Once normalised both semen samples were combined with vaginal material and venous blood over specific mixing ratios to create equivalent sample mixtures, the only differentiating factor being the presence of DTT.

7.3. Results

7.3.1. Identification of Background Expression of Specific Markers.

Expression of endogenous control SNORD47 and proposed semen/semenal fluid markers hsa-mir-10a, 135a and 888 can be seen in Figure 7.1. As with previous chapters in the study, strong expression of SNORD47 was observed. Expression of hsa-mir-10a was absent in all samples except for one outlier displaying weak expression. The upper limit of expression from both hsa-mir-135a and hsa-mir-888 also displayed weak expression. As per the 40-cycle cut off threshold outlined in 4.11, $\Delta^1\text{Cq}$ expression values below 10 would not be included in analysis and therefore the background values for all proposed semen markers display an absence of expression.

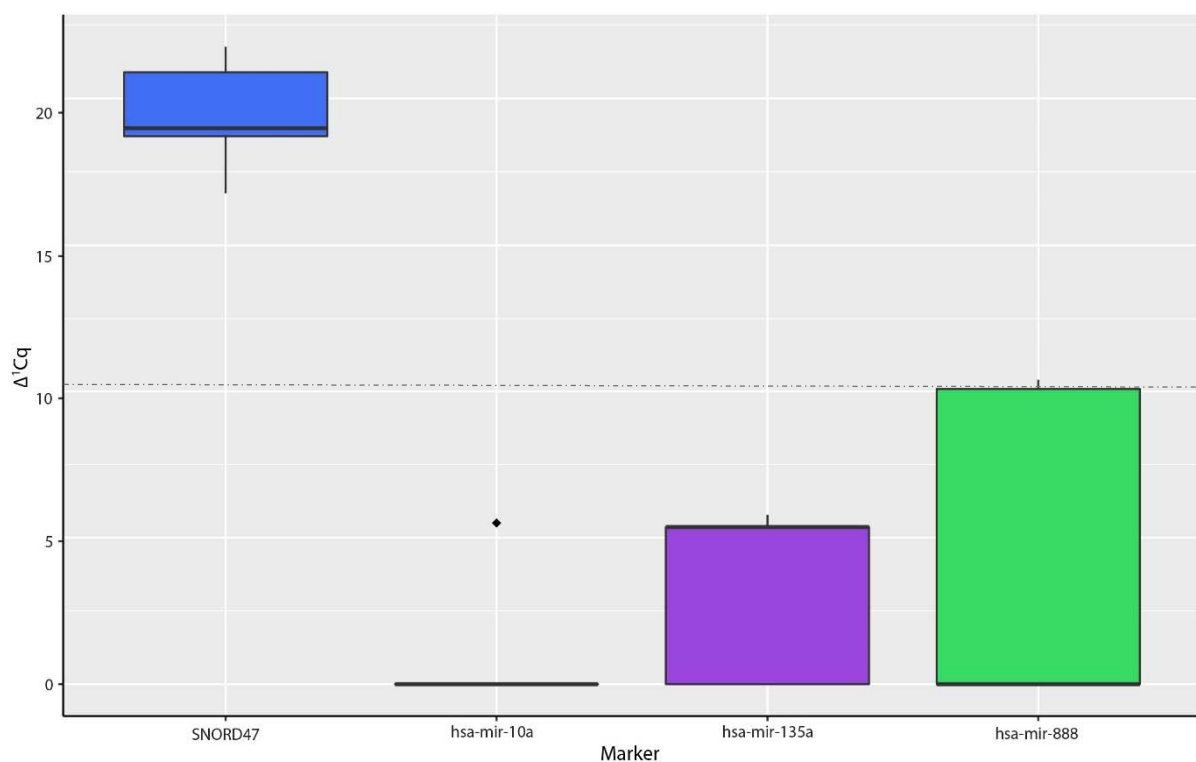


Figure 7.1: $\Delta^1\text{Cq}$ values of expression of endogenous control and semen markers within vaginal material samples before UPSI occurred. The top and bottom of the box indicate the upper and lower quartiles with the median being represented by the central black line. Whiskers display the minimum and maximum values excluding outliers which are identified by a black diamond showing results lying 1.5x of the interquartile range. Dotted line indicates threshold for inclusion in analysis.

7.3.2. Identification of Proposed Semen Markers within Vaginal Material Samples across Various Extraction Methods.

Δ^1Cq values for expression of proposed semen markers hsa-mir-10a, 135a and 888 as well as endogenous control SNORD 47 utilising total DNA extraction can be seen in Figure 7.2 and 7.3 (Purple boxes). Marker hsa-mir-888 (A) and SNORD 47 (B) displayed strong uniform expression values (>16.1) across all time points and all volunteers following UPSI. Marker hsa-mir-135a (C) displayed stable high expression across all time points with the exception of V2 showing weak expression at T_{48} . Marker hsa-mir-10a (D) displayed moderate to high expression in all samples at T_0 , T_{24} and T_{96} , except for V4 at T_{24} that displayed no expression. Expression ranged from weak to high across samples in T_{20} and was only high in one samples at T_{48} .

Expression of hsa-mir-888 (A) within vaginal elution (green boxes) displayed moderate expression (13.5-19.5) across all time points and all volunteers following UPSI. Fluctuation of SNORD 47 median expression was seen across all time points (B). No sample within T_0 displayed expression that surpassed 5.2. Expression was seen in all samples within T_{20} however only V1 and V3 surpassed the cycle threshold for positive expression while T_{24} exhibited strong expression in all but V4. Expression values were high within T_{48} with the exception of V2 which displayed moderately weak expression, this was replicated within T_{96} with the moderately weak expression seen in V1.

The majority of vaginal extraction samples showed strong expression of hsa-mir-135a (C) at T_0 , with only V4 falling below 10. Expression was similar within T_{20} with an additional decrease in expression seen from V5. Moderate to high expression was only seen in sample V2 at T_{24} and V2 and V3 at T_{96} in remaining time points. High expression of hsa-mir-10a (D) was seen in 4 samples across all times points; V1 T_0 , V2 T_{20} , V3 T_{20} and V4 T_{96} , all other Δ^1Cq fell below 10 or were 0.

The seminal elution (blue boxes) displayed strong expression of hsa-mir-888 (A) in all but one sample at T_0 . At T_{20} 3 samples display no expression while 2 samples displayed strong

$\Delta^1\text{Cq}$ values. At T_{24} all but one samples showed expression values within the moderate to strong range, V4 displayed weak expression that did not surpass the cycle threshold. 4 of the 5 T_{48} showed positive expression however much weaker than previous time points, with V4 displaying no expression. Only one sample (V1) at T_{96} showed strong positive expression while all others displayed weak $\Delta^1\text{Cq}$ values below. Overall the $\Delta^1\text{Cq}$ values in seminal extraction across all time points were lower than those seen in total DNA and vaginal extraction.

Within the seminal fraction 2 of the 5 samples displayed weak to absent expression of SNORD 47 at T_0 (B). Except for V3 at T_{24} , all expression values across T_{20} and T_{24} fell below 5. At T_{48} V3 was the only samples to not display moderate to high expression, within T_{96} expression fluctuated from absent to high across all samples. Expression of hsa-mir-135a (C) was only seen in V1 and V2 at T_0 . V1 at T_{20} was the only sample from the subsequent time points that displayed moderate expression, all other $\Delta^1\text{Cq}$ values were below 9. No sample displayed moderate or high expression of hsa-mir-10a (D) at T_0 , expression was marginally higher but similar at T_{20} , T_{24} and T_{48} with 3 of 5 samples lying on the 10 $\Delta^1\text{Cq}$ cycle threshold but none exceeding it. At T_{96} V4 displayed strong expression whereas in all other samples, weak or absent expression was observed

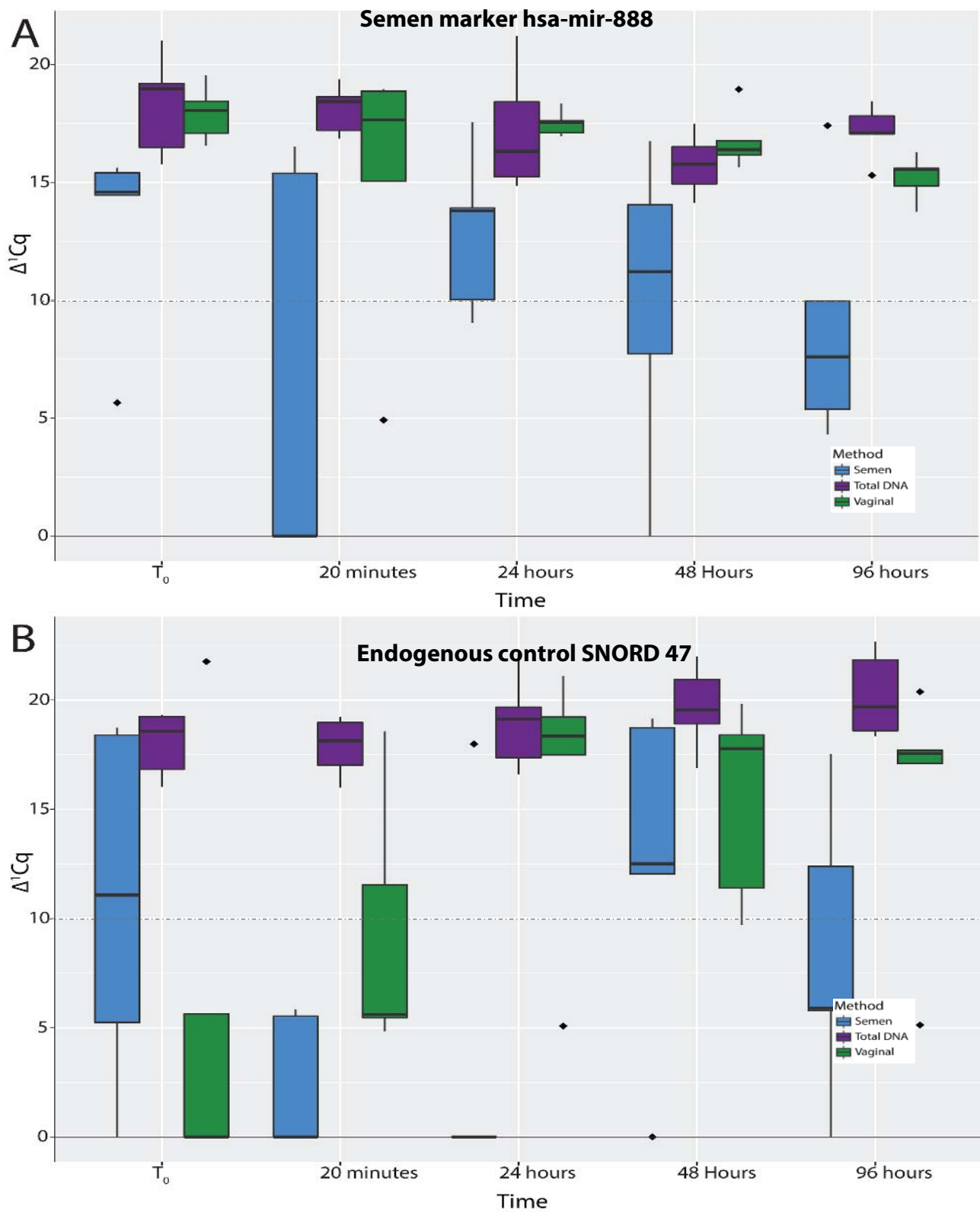


Figure 7.2: $\Delta'Cq$ values for markers hsa-mir-888 (A) and endogenous control SNORD 47 (B) within UPSI samples across various time points using different extraction methods. The top and bottom of the box indicate the upper and lower quartiles with the median being represented by the central black line. Whiskers display the minimum and maximum values excluding outliers (identified by a black diamond and show results lying 1.5x outside the interquartile range'. Dotted line indicates threshold for inclusion in analysis.

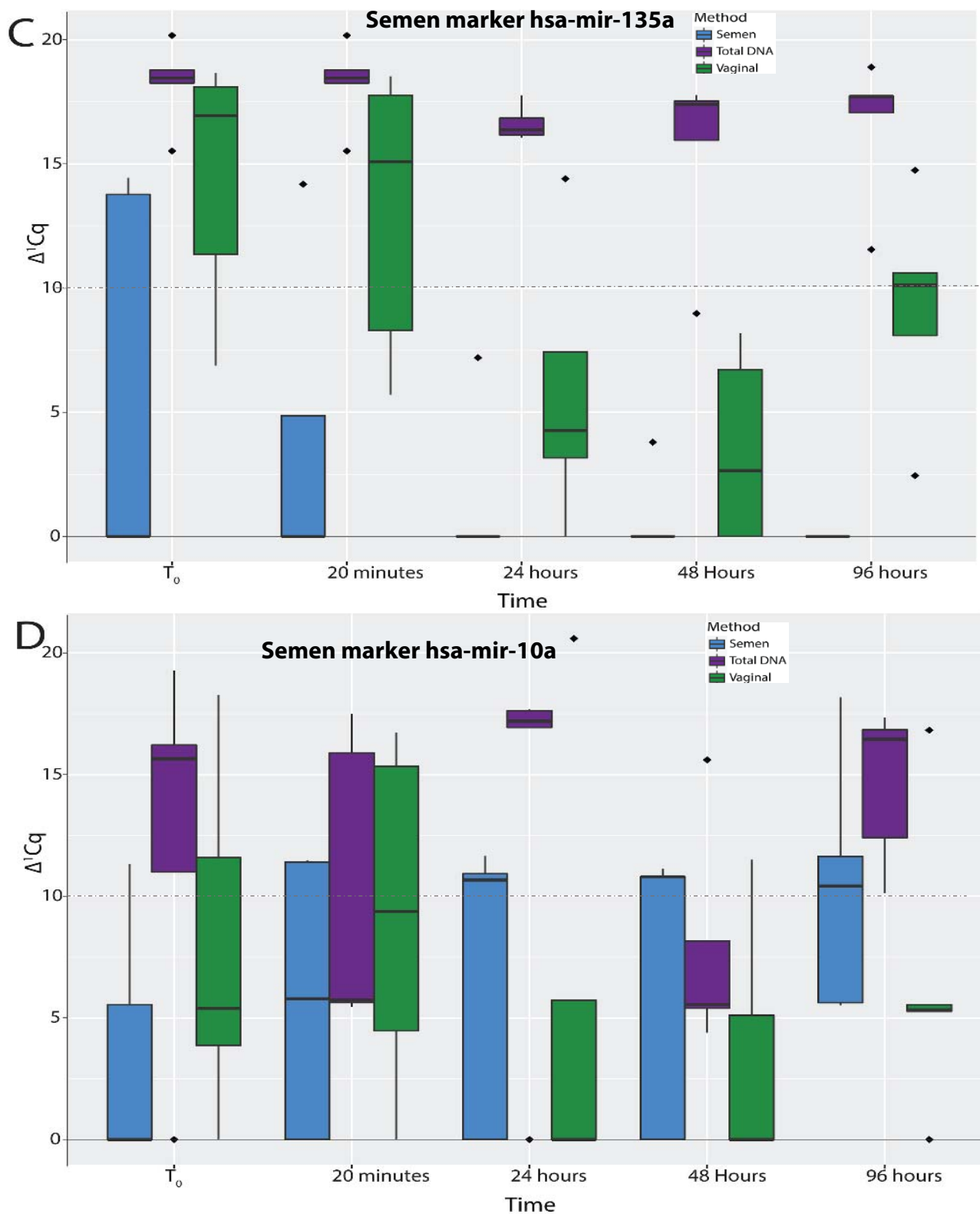


Figure 7.3: ΔCq values for markers hsa-mir-135a (C) and hsa-mir-10a (D) within UPSI samples across various time points using different extraction methods. The top and bottom of the box indicate the upper and lower quartiles with the median being represented by the central black line. Whiskers display minimum and maximum values excluding outliers (identified by a black diamond and show results lying 1.5x outside the interquartile range). Dotted line indicates threshold for inclusion in analysis.

7.3.3. DTT Addition to Total DNA Extraction.

The addition of DTT to semen samples had a detrimental effect on the expression of hsa-mir-10a in all samples (Figure 7.4). $\Delta^1\text{Cq}$ values were significantly lower than equivalent samples without DTT ($P < 0.001$). No sample displayed a $\Delta^1\text{Cq}$ value corresponding to strong expression and the majority of samples showed a complete absence of hsa-mir10a regardless of mixing ratio. Without DTT $\Delta^1\text{Cq}$ values of hsa-mir-10a were uniformly high within all samples, regardless of mixing ratio or additional body fluid presence.

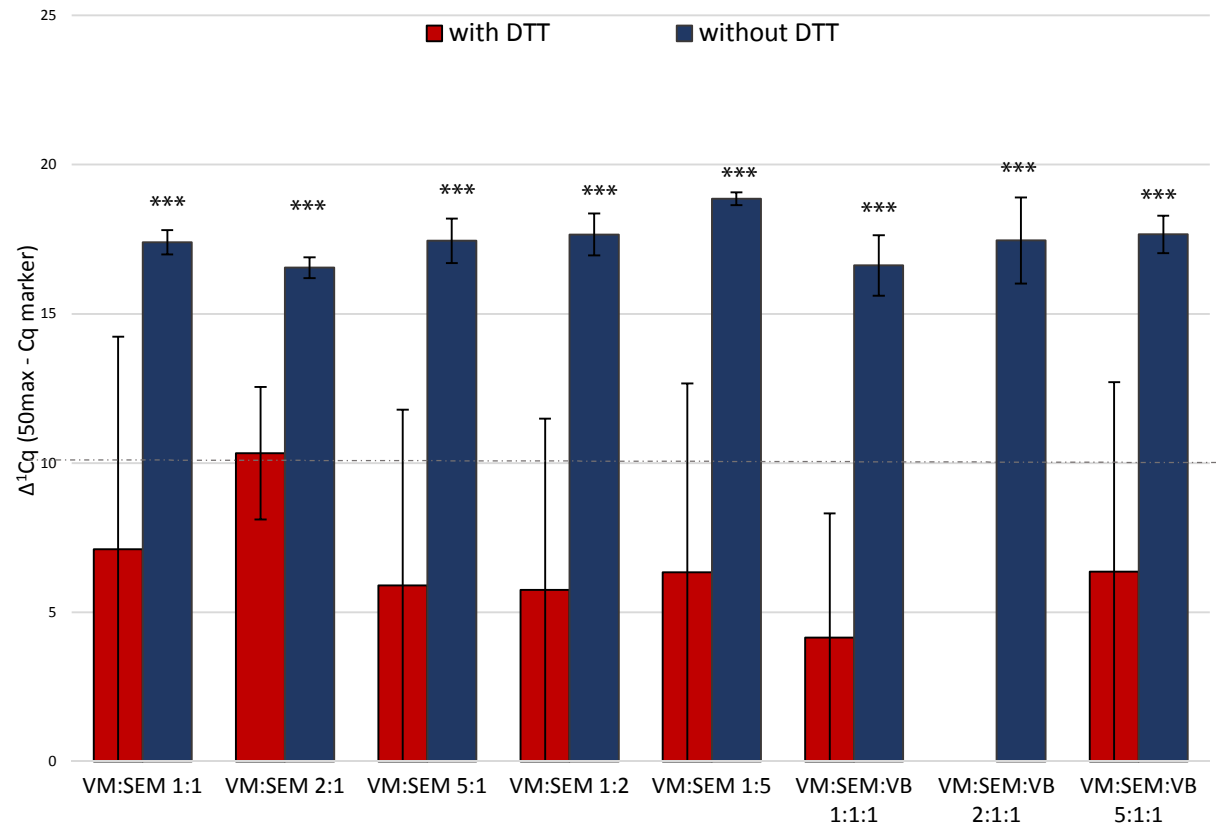


Figure 7.4 $\Delta^1\text{Cq}$ values of hsa-mir-10a across various mixing ratios in samples with and without DTT. N= 6 ($P < 0.001$). All normalised at 1ng/ μl . Dotted line indicates threshold for inclusion in analysis.

DTT had a less detrimental effect on the expression of hsa-mir-888 compared to has-mir-10a in all semen samples (Figure 7.5). All $\Delta^1\text{Cq}$ values were lower than equivalent samples without DTT with the largest variation being 5 cycles at a 2:1:1 of vaginal material: semen: venous blood. Significantly higher expression was observed in a number of samples without DTT ($p < 0.01$ - 0.001) however this did not correlate to a pattern dependant on which was the higher ratio body fluid. All samples, regardless of addition of DTT or mixing ratio displayed positive reliable $\Delta^1\text{Cq}$ values corresponding to moderate to high expression.

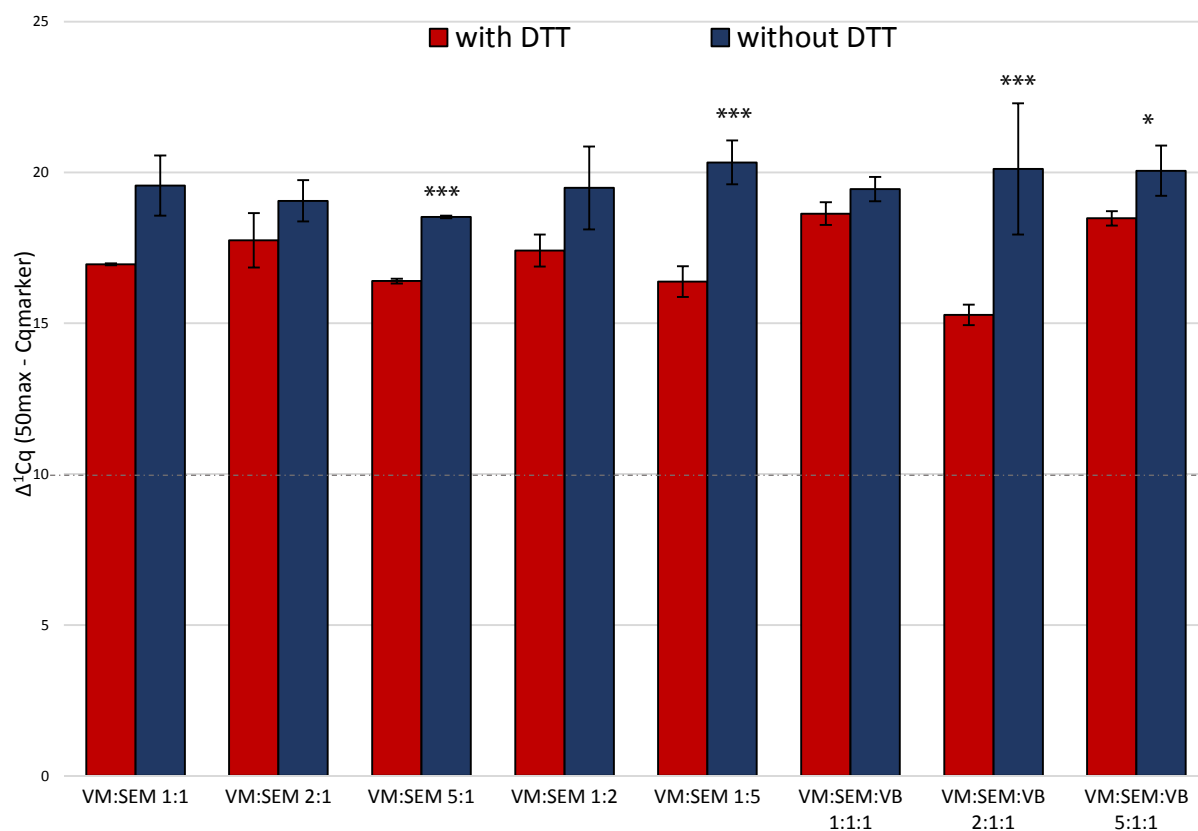


Figure 7.5: $\Delta^1\text{Cq}$ values of hsa-mir-888 across various mixing ratios of vaginal material, semen and venous blood in samples with and without DTT. N= 6, ($p < 0.001$). – All normalised at $1 \text{ ng}/\mu\text{l}$. Dotted line indicates threshold for inclusion in analysis.

7.3.4. Discussion

The minimal expression of hsa-mir-10a, 135a and 888 in controls samples indicate that these markers, at concentrations of 2ng/μl or below, will not display a false positive when analysed on vaginal material that is absent of semen. Control samples were obtained from volunteers abstaining from UPSI for a minimum of 7 days before initial sampling, due to control samples indicating no positive identification for semen it can suggested that BFID would likely be unsuccessful if samples were obtained 7 days after a sexual assault occurred.

Markers hsa-mir-888 and 135a showed consistent expression across all time points from total DNA extracted samples, reliably identifying semen presence within a vaginal material mixture. In cases where sexual assaults have occurred and samples are known to contain vaginal material and suspected semen, total DNA extraction will provide reliable identification of semen up to 96 hours after UPSI occurred. An increased expression of hsa-mir-10a was also observed compared to background expression, however variability in expression was detected across time points. This may raise potential concerns with regards to its reliability as a semen identification marker. The use of all three markers within a combined BFID panel would be necessary to provide the robustness required within casework.

The differential extraction technique currently utilised for major crime samples does not show reliable and reproducible results for BFID using miRNA. Positive identifications were made; however, the number of false negatives were greater in number. If BFID were utilised on differential extraction samples a positive result would be confirmatory however a negative result would support neither prosecution nor defence. Within the vaginal and seminal fractions SNORD 47 did not display stable high expression across all samples and therefore full analysis could not be performed using this endogenous control, compromising the validity nature of this marker.

The addition of DTT proved to have a negative effect on the expression of miRNA within the seminal fraction and from total DNA extraction utilising DTT. Analysis with hsa-mir-10a in total DNA extracted samples displayed similar expression patterns to the seminal fraction

of standard differential extraction. When expression from total DNA extraction with DTT was observed it was at least 6 cycles after expression within the same sample mixture without DTT, an approximate 64-fold reduction. No Δ^1C_q values within the strong expression classification were observed and the majority of samples displayed no expression at all. Expression of hsa-mir-888 was detected in all mixing ratios thus indicating the presence of semen, however on average expression was observed at least 3.5 cycles after their counterpart samples without DTT. This would indicate an approximate 10-fold reduction in the volume of miRNA present with samples treated with DTT. Since previous results have already determined hsa-mir-888 dual expression in vaginal material it could be suggested a proportion of the expression observed came from vaginal material and the true effect of DTT is not reflected.

The addition of DTT allows for higher yields of DNA which are subsequently used for DNA profiling, within this study samples that were extracted using the total DNA extraction method typically had a DNA concentration of 1.5ng/ μ l, the same samples when treated with DTT yielded around 45ng/ μ l. Since normalisation was based on DNA concentration it could be suggested that the large dilution factor applied to the seminal fraction of differential extraction and the DTT DNA extraction reduced the miRNA abundance to below LOD. A possible solution would be to perform analysis on undiluted semen fractions. Due to the strong consistent expression of the semen markers in vaginal material following total DNA extraction and the unreliable and weak expression observed with DTT, it can be concluded its addition into BFID techniques should be avoided. Forensic examiners then have the problem of the requirement of DTT and therefore differential extraction to increase DNA concentration for successful profiling and the need for a lack of DTT to ensure miRNA are preserved for BFID analysis.

Two solutions could be proposed; removing an aliquot of the differential extraction after agitation before undergoing vaginal and seminal protocols, as seen in Figure 7.6, or an additional swab being taken within the hospital or SARC unit during the victim's sexual assault examination.

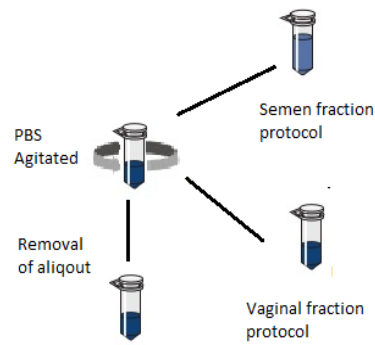


Figure 7.6: proposed additional workflow stage for differential extraction.

The advantage to removal of an aliquot of eluate would be that free circulating miRNA could be analysed without risk of damage from cell lysis. The disadvantage would be that due to lack of cell lysis it would be unlikely to obtain expression values of miRNA being regulated within the body fluid's cells.

An additional examination swab would allow for a swab to follow the usual route of differential extraction for 'major crime' samples and the other the standard total DNA protocol of 'scene of crime samples'. Once extracted the samples would then fall back into the regular workflow of analysis utilising RT-qPCR. This has the advantage of providing a more reliable miRNA expression analysis as well as providing the best sample for DNA profiling. The requirement for BFID would be case dependant and therefore the additional swab could be stored to avoid the cost of additional extraction until the requirement for BFID occurs, as well as providing a back-up sample if the original became contaminated. If the sample has already undergone total DNA extraction the semen concentration may be low but provides an additional route for analysis. If BFID is not needed it would allow for an additional differential extraction to occur. The disadvantages of this solution include additional stress and examination time of the victim as well as the additional cost and man power of processing the same samples over two extraction methods.

In comparison to current literature, the results from total DNA extraction showed positive identification could be made from all participants at 96 hours, this is a great improvement on Davies work which showed frequent negative results at 96 hours [116]. Wilsons work did

display positive results up to 10 day which would support this results however not all samples displayed positive results showing a higher level of successful identification using miRNA and RT-qPCR.

Overall all samples from differential extraction displayed variability across volunteers and time points in all markers, including endogenous control, suggesting it is not a reliable and robust technique for BFID. Despite this, the method developed here utilising total DNA extraction has reliably identified the presence of semen with at least two markers throughout every time point for each volunteer. Sample mixtures from total DNA extraction also successfully identified semen presence regardless of large volumes of genetic material from vaginal material and additional body fluids. This technique has the potential for the identification of sexual assaults where the assailant may not leave a DNA profile (i.e. those with azoospermia) and could potentially be applied to cold case work provided a) DNA extraction was carried out and b) the extraction occurred within 96 hours of the assault.

7.4. Conclusion

The work presented in this chapter shows that there is potential for miRNA based BFID techniques to be employed within casework. The markers currently available for the identification of semen and seminal fluid are also expressed within vaginal material, and therefore the identification is reliant on analysis being performed on samples below their LOD in vaginal material. Overall total DNA extraction provided the most reliable identification of semen, regardless of duration after UPSI (up to 96 hours) and presence of menstrual blood. Considering all three markers would be used, with the requirement of at least two displaying high expression values for semen identification, positive identifications were made across all time points using total DNA extraction.

Total DNA extraction has demonstrated its potential for cold case BFID. In current literature studies have shown that miRNA show little change in stability when stored up to five years [173-175] therefore it is likely these body fluid markers would also be highly expressed. Dependant on the body fluids involved, the identification of body fluid in cold case samples

is also achievable with differential extraction: All body fluids not relating to semen could be identified in the vaginal fraction, with a reduced likelihood of identifying semen within either vaginal or seminal fractions. Over most of the times points at least one semen marker showed expression, it was not observed within the same volunteer at each time point therefore it is likely expression identifying seminal presence will be observed, although it may not provide robust evidence in a court of law. The successful identification of semen markers within UPSI samples coupled with the high expression in all constructed various ratio mixtures demonstrates the ability of this BFID technique for semen identification in forensic case work.

Overall, the miRNA BFID technique is not compatible with the DTT extraction method currently applied to 'major crime' samples. Throughout both extraction methods DTT appears to play a role in the degradation of miRNAs, resulting in the observation of unreliable expression, in particular of the endogenous control markers used to validate the technique. The result being unreliable evidence in a court of law. In conclusion, the method could be implemented to casework, but does not demonstrate the potential to transition directly into the methods currently used within forensic laboratories for analysis of sexual assault samples.

7.5. Summary

- Currently semen identification by miRNA analysis would not be reliable on major crime samples that enter forensic laboratories and undergo differential extraction. Analysis can be performed and if expression is seen a positive identification can be made however if absent the presence or absence of semen cannot be confirmed.
- Dilution of the seminal fraction may have caused the lack of semen marker expression. Within these samples the DNA is unlikely to be proportional with miRNA and therefore heavy dilution may have reduced miRNA presence past the LOD.
- A concentration of 2ng/μl is more likely to correctly identify semen in samples greater than 24hr following UPSI than concentrations of 0.7ng/μl

- BFID on cold cases could be achieved from samples that have undergone differential extraction. This capability would be body fluid dependant with the likelihood of semen identification being reduced.
- The most suitable method for BFID identification is total DNA extraction which resulted in identification of semen in all semen samples, across all time points. This was also observed when combined with additional mixtures of venous blood and menstrual blood.
- DTT is very detrimental to miRNA, regardless of extraction technique. Although beneficial for DNA profiling it should be avoided within BFID techniques.
- To utilise total DNA extraction a subsequent swab would be required. This would ensure the maximum chance for a full male DNA profile to be obtained through differential extraction and BFID to be successful through total DNA extraction.

8. Discussion

Incidences of sexual assault are serious offences which are likely to cause a significant amount of stress to a victim and in the case of false allegations, someone who is accused of carrying out such an offence [39-41, 73]. Determining the exact events surrounding a sexual assault claim is a near impossible task, however it is the role of the forensic scientist to remove doubt and provide evidence that may support claims made by both parties. The determination of the presence/absence of a body fluid within a crime scene is often a key piece of evidence of any case [116]. The identification of semen or seminal fluid on a victim, or the presence of vaginal material on a penile swab may be key to determining whether penetrative sexual intercourse occurred. The ability to detect saliva and skin may also indicate the type of sexual offence committed, and where bleeding occurs the differentiation between menstrual and venous blood may add significant weight to the defence or prosecution hypothesis.

There are currently many techniques employed to provide presumptive tests for a number of these body fluids comprising of catalytic [113, 114], immunological [118, 119] and spectroscopic techniques [116]. DNA profiling is one of the most well-known techniques for the identification of individuals involved in a crime scene, frequently being the only identification evidence presented in the courts [234]. DNA profiling can now be complemented with a number of emerging molecular biology techniques. The research carried out within this body of work utilised miRNA as a potential route to body fluid identification. MicroRNA are non-coding RNA molecules which regulate gene expression, by controlling the translation and repression of RNA [151, 158, 159]. Since translation and repression profiles of genes within cells associated with body fluids are likely to be unique, miRNA represent a novel method for the discrimination between body fluid types.

The use of miRNA is an emerging technique, in that a number of authors have already explored the possibility of using this technique for body fluid identification, with varying and often contradictory findings. It was therefore important within the initial sections of this work to validate the RT-qPCR technique; not only as a methodology, but to provide comparisons with current literature and place the research within current case work. The preliminary findings within Chapter 5 showed that the RT-qPCR technique could be

validated not only through the use of negative controls, but also through the identification of endogenous controls. In this case, the endogenous controls were miRNA markers which were present universally across cell types.

8.1. Total DNA extraction coupled with stem-loop RT-qPCR as a robust and efficient method for body fluid identification

Two important considerations for a technique to be integrated into case work is that of time, and cost. The current cost for a single tube TaqMan® gene expression assay from Thermo Fisher Scientific is £230⁵ for 200 reactions [235]. For BFID analysis, the full marker panel would be required to confirm absence and presence of miRNA. The need for a minimum of one assay, particularly where a casework sample may not require a full complement of BFID, means the technique could become unnecessarily expensive. The BFID matrix was therefore redesigned to incorporate custom designed assays to minimise costs using alternative suppliers (for example: Eurofins Oligonucleotide design: £6.95 forward primer, £6.95 reverse primer, £15.95 reverse transcription primer [236]¹).

In current literature extraction techniques involve RNA isolation techniques and protocols regularly utilising reagents such as guanidine isothiocyanate-phenol/chloroform followed by precipitation by isopropanol [179], with only Omelia *et al.* displaying efficient miRNA extraction with Qiagen's total DNA protocol [190]. The majority of protocols were followed with DNA digestion to ensure pure miRNA was eluted. All data from Chapter 5, 6 and 7 shows that total DNA extraction is the more reliable miRNA extraction method compared with RNA and differential extraction. The efficiency of miRNA expression analysis throughout this research also suggests that DNA digestion is not a vital component of miRNA analysis and can therefore be removed to reduce costing, timing and work-load within the laboratory.

⁵ Prices correct as of 01/11/16

The data generated within Chapter 5 showed that RT-qPCR could be used with the custom designed assays for detecting miRNA expression. The assays could be used on undiluted samples obtained from DNA extractions to obtain positive identification of body fluids, however, dilution of the samples increased the body fluid specificity and reliability of miRNA detection. For current DNA profiling protocols the concentration for required template DNA would be 0.03ng/μl (LGC [226]) and 0.1ng/μl (Cellmark [227]). Both concentrations are below the tested 0.7ng/μl however LOD results indicate BFID would be successful with AmpFISTR® NGM SElect™ but may be unsuccessful with Promega's PowerPlex® ESI 17. The solution would be to perform a duplicate sample dilution to 0.75ng/μl, a time-consuming process or to increase the total sample input into the stem-loop RT reaction. The improved resolution of identification following dilution alongside the requirement to these levels for DNA profiling allows for implementation into current laboratory protocols.

The use of the same DNA extract as these techniques as a stem loop RT-qPCR template means that no additional time or cost is required, with the time of reaction set-up to a result (when optimized) is under 3 hours. In addition, the results of Chapter 6 also identified that the isolation of miRNA could also be achieved using RNA extraction protocols, obtaining expression profiles that were comparable to DNA extraction methods. Within the study, we were unable to identify a marker for skin, despite previous studies suggesting that this was possible [183, 199]. Despite this, the data obtained within this chapter showed that a combination of markers could be utilised for correct identification of a number of single body fluid stains within a sample (Figure 8.1).

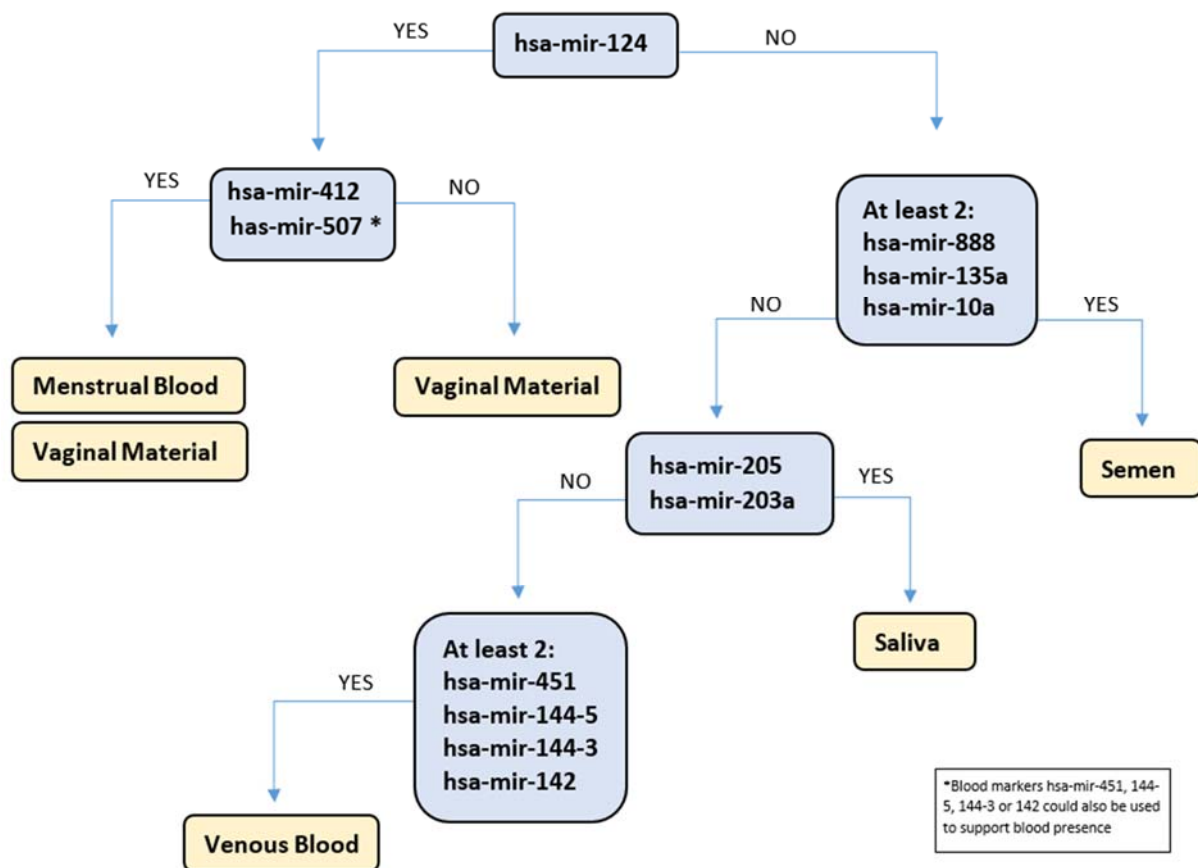


Figure 8.1: flow chart for the Identification of a full range of body fluids. For forensic application each marker would require a strict pass criteria, with thresholds for each marker and EC's Cq value. This would be determined by the various forensic bodies and criminal institutes.

Marker hsa-mir-124 presented itself as one of the key discriminatory markers within this technique. Previous research has shown that this marker is present in a number of tissue types, including the colon, breast, and lung [237], with Wilting *et al.* identifying its regulation within those affected by cervical cancer [180]. Wilting suggested that hsa-mir-124 was suppressed in cervical cancer cells. V8 provided vaginal and menstrual samples with partial removal of cervix due to stage 1A cervical cancer provided in her information. They were currently diagnosed with stage 2 cervical dysplasia, although these cells are not cancerous they are considered pre-cancerous and are closely monitored. It would therefore be expected that V8's samples would show weak or absent repression of hsa-mir-124a. Both samples displayed extremely high $\Delta^1\text{Cq}$ values disagreeing with Wilting's conclusions and further displaying the stability of hsa-mir-124. The data generated here indicates that although current research suggests this miRNA may be present within a number of tissue

types, its expression is significant within the vagina and associated material. Therefore, presence or absence of its expression provides most information as to the origin of a body fluid sample or stain.

The detection of this marker was then employed to confirm the presence of menstrual blood using markers hsa-mir-412 and 507. Here, our findings confirmed the findings of Hansen *et al.* that marker hsa-mir-412 could be used for detecting menstrual blood, but with the requirement that hsa-mir-124 detection must also be employed. Contrary to the findings of Zubakov *et al.* our findings show that marker hsa-mir-507 was not suitable for use as an endogenous control, but could be used for menstrual blood detection. The compositional similarities between menstrual and venous blood and associated expression of miRNA provided evidence throughout this screening that a number of markers were simply capable of detecting 'blood'. Therefore, it was proposed that these two aforementioned markers be utilized for detection of menstrual blood.

Four other markers (hsa-mir-451, 144-5, 144-3 and 142) were therefore proposed for the identification of venous blood. Expression of any two of these markers coupled to an absence of marker hsa-mir-124 could be integrated to achieve this. The abilities of these markers to detect the components of blood confirm previous work for the use of hsa-mir-451 and 142 [238]. In the same way that menstrual and venous blood share similar properties, so too do vaginal material and saliva, here the use of markers 203a and 205 were capable of distinguishing saliva through the absence of marker hsa-mir-124 expression. Interestingly, these markers were also confirmed to be saliva specific by Hason *et al.* [179] and non-saliva specific by Park, Zubakov and Sauer [181, 183, 185]. The detection of hsa-mir-144-5 and 144-3 contradict the findings of Sauer in that although it was highly present in menstrual blood, it displayed no ability to differentiate venous and menstrual blood. Again, coupled to the absence of marker hsa-mir-124, semen could be detected using markers hsa-mir-888, 135a and 10a, which agrees with the findings of Zubakov, Hanson, Sauer and Wang [183-185].

Many sexual assault cases involve the analysis of multiple body fluids, the most common being a mixture of semen and vaginal material. The results obtained from this chapter also demonstrate that, to an extent these markers when used in combination could be implemented to discriminate between the presence of multiple body fluids within mixtures. When two body fluids are present within a mixture, the range of detection for these mixtures can be seen in Figure 8.2. When three or more body fluids are present within a mixture the ability for discrimination becomes more limited (Figure 8.3). Although a large combination of multiple body fluids can be identified using this miRNA BFID panel it is limited in mixtures involving saliva and vaginal material. Although hsa-mir-124 is only expressed in vaginal material, miRNA that display high expression in saliva (hsa-mir-205 and 203a) often share expression with vaginal material and therefore mixtures containing both cannot be confirmed for saliva presence. In these cases, the screening would likely be supplemented by non-molecular techniques but would still act as a useful tool within the forensic scientist's arsenal.

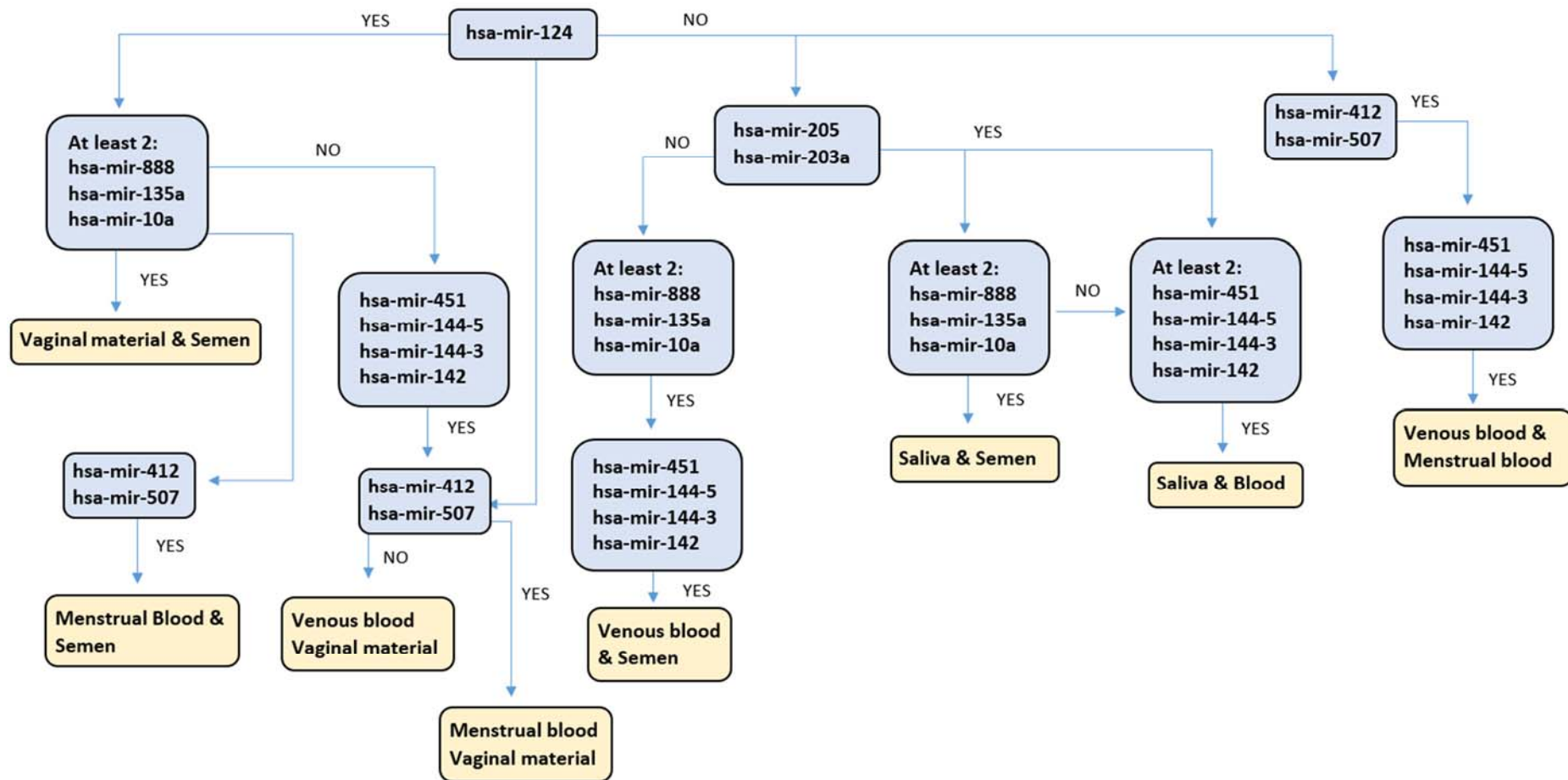


Figure 8.2: Example flow chart for the identification of a range of body fluids when present in a two body fluid mixture. For forensic application each marker would require a strict pass criteria, with thresholds for each marker and EC's Cq value. This would be determined by the various forensic bodies and criminal institutes.

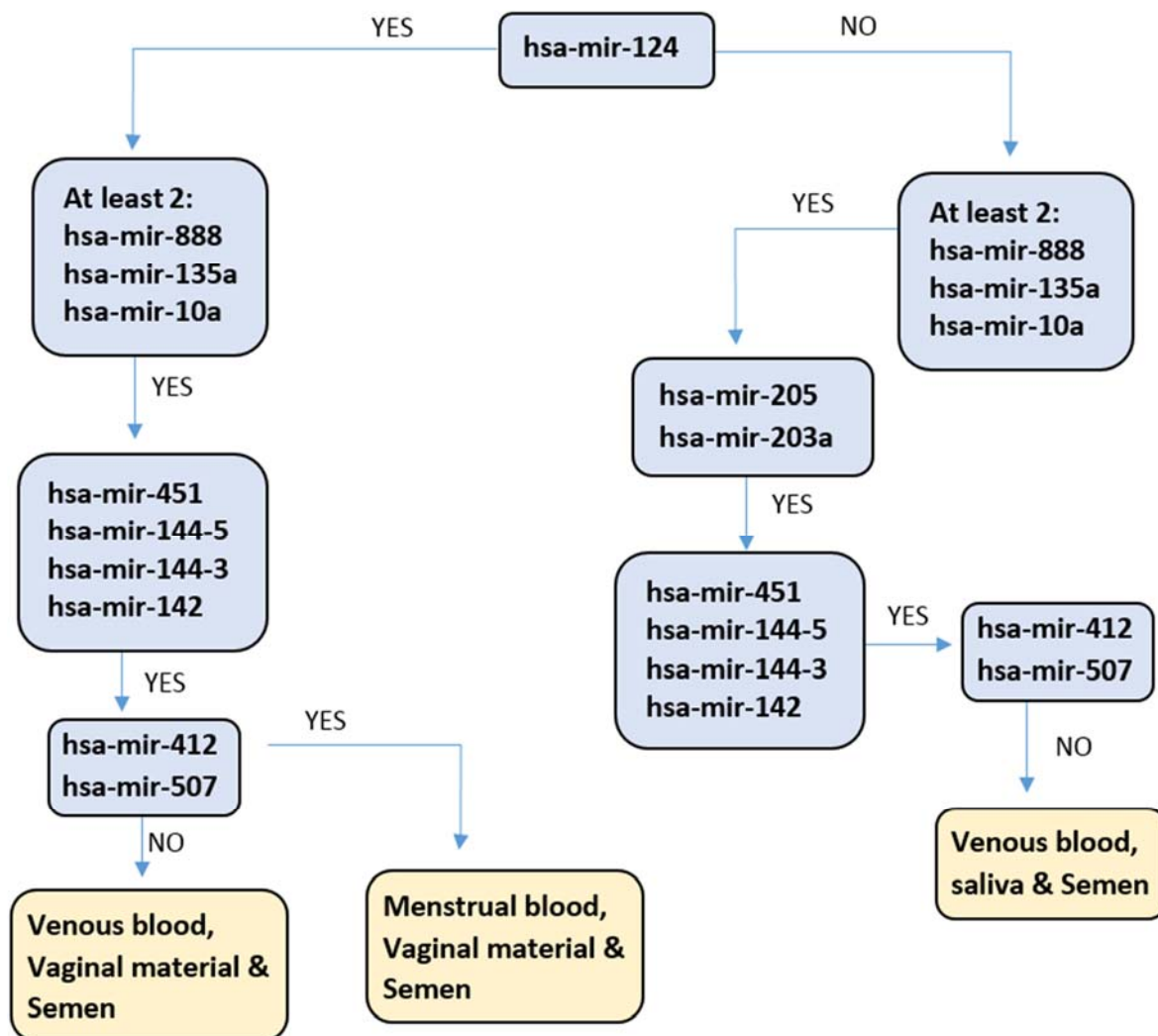


Figure 8.3: Example flow chart for the identification of three body fluid mixtures in crime scene stains. For forensic application each marker would require a strict pass criteria, with thresholds for each marker and EC's Cq value. This would be determined by the various forensic bodies and criminal institutes.

8.2. MicroRNA expression fluctuates across the menstrual cycle, with the exception of hsa-mir-124

The significant physiological changes within the vaginal environment over the course of the menstrual cycle mean that expressional change of certain miRNA is certain. Since the identification of body fluids using the stem-loop RT-qPCR technique was heavily reliant on the detection of expression of hsa-mir-124, it must show consistent expression across the menstrual cycle. Although previous research conducted by Rekker *et al.* identified a panel of markers which were expressed within plasma and blood, no expression values for hsa-

mir-124 were obtained and only two sampling points throughout the entire cycle were taken [239]. Therefore, the results described in Chapter 6 are the first to provide a comprehensive description of expression profiles across the full menstrual cycle of a range of miRNA markers, including hsa-mir-124.

The expression of this marker was relatively consistent across the full menstrual cycle regardless of the extrinsic factors associated with each volunteer (age, contraception, pregnancy etc.). Where changes in expression occurred, the data showed the importance of endogenous control selection. With SNORD47 and hsa-mir-93, the expression of these markers correlated with that of marker hsa-mir-124, revealing where sample consistency fluctuated most likely as a result of a poor sampling technique or loss of sample integrity on collection. The other candidate endogenous control markers hsa-mir-508 and hsa-mir-1260b did not demonstrate this fluctuation, most likely as a result of the high abundance of miRNA within the original sample.

The results obtained show that the use of multiple endogenous controls is key to ensuring the robustness of these test methods. Here, four markers were identified (hsa-mir-508, hsa-mir-1260b SNORD47 and hsa-mir-93). Within the literature, only markers SNORD 47 [196] and hsa-mir-93 [197] have been described as suitable endogenous controls, where hsa-mir-508 has been described as specific to semen [187] and hsa-mir-1260b as specific to vaginal material [181, 183].

The results obtained within Chapter 5 and 6 also confirmed that a number of markers (hsa-mir-203a, 507, 508, 205, 142) were expressed within both vaginal material as well as their candidate body fluid. These findings further reinforced the role of marker hsa-mir-124 in confirming the absence of vaginal material from samples to provide the correct identification of body fluids from stains. Marker hsa-mir-144-5 however showed robust specificity for blood, with no expression observed in volunteers who did not menstruate, with no background expression associated with vaginal material. The data also suggest a potential limitation to the RT-qPCR technique when samples originate from the vagina. The differentiation of venous and menstrual blood would not be possible since marker hsa-mir-124 expression will always be detected; for menstrual blood to be confirmed hsa-mir-412 and 507 must be present in the sample but absent in a reference sample of vaginal fluid.

The technique however, does still have significant potential in the identification of body fluids from stains.

8.3. The Technique is capable of detecting instances of UPSI from vaginal swabs up to 96 hours after penetration following standard DNA extraction.

The time from a sexual assault taking place to being reported to the relevant authority can vary considerably. In cases where non-consensual unprotected vaginal intercourse is being alleged, which in itself can increase a sentence following conviction from lesser sexual assault to rape [4], providing evidence to support or disprove these allegations is critical. The results obtained within the 31-day study (Chapter 6) demonstrated that the detection of the occurrence of UPSI could be possible. The results obtained here also suggested that these markers were also expressed in vaginal material, but could only be detected when template concentration was high. The initial results of Chapter 7 demonstrated that when the initial template concentration used in the technique was reduced, that the presence of these markers were well below threshold up to seven days prior to an instance of UPSI. As a result, the increased detection of these markers would have to be as a result of the introduction of material containing these markers (i.e. the ejaculation of semen).

Stem loop RT-qPCR of the miRNA extracted from vaginal swabs immediately after UPSI showed a marked increase in the detection of all three markers. In addition, these markers could be detected up to 96 hours following UPSI. Previous research has suggested that these markers were specific to semen [167-175, 177] and therefore the data generated here provide further evidence for this. Importantly, where a male produces no sperm, the markers were also capable of detecting the presence of seminal fluid. This provides the technique with a significant advantage over current methods, where a DNA profile would not be provided and a conviction would not be obtained.

Currently, the accepted method within casework is that genetic material is obtained through the use of differential extraction methodology (described in 4.4.3). Using this method, in particular the use of DTT, severely impacted on the integrity of the miRNA

present within the sample and most importantly that of the endogenous control. As a result, for use within casework, a modification would be required to the extraction process. The modification was described within Chapter 7, however, in short, an aliquot of the sample would be taken following the initial lysis stage and carried through to a standard DNA extraction or an additional swab during examination would be taken for BFID analysis following total DNA extraction.

8.4. The expression of hsa-mir-205 within vaginal material is regulated by contraceptive methods

Conflicting results explaining the specificity of hsa-mir-205 has been observed in current literature, even between methods using similar analysis techniques. The results obtained from this study suggest a reason for this discordance. All females on some form of contraception, be it hormonal or non-hormonal display high expression of hsa-mir-205 in vaginal material samples, whereas all samples obtained from females currently not utilising a contraceptive method displayed no expression of hsa-mir-205. This was further supported in samples obtained where contraceptive methods had been changed. Expression seen during screening disappeared throughout all 31-day study samples when the contraceptive pill no longer taken, while expression in another volunteer that was absent in initial sampling displayed strong expression 7 days prior to commencing the contraceptive pill. This expression increased in an additional sample taken two months later. Likely due to hsa-mir-205 role in the regulation and repair, this regulation over hsa-mir-205 would explain why certain research groups, if using hydrolysis probe qPCR obtained dual expression in saliva and vaginal material.

8.5. Applications to case work

Although the results show that BFID can be carried out using miRNA markers, it is important to apply the technique into the perspective of real case work scenarios to give the results context. The following examples show how these techniques could be applied to case work.

Example 1. Male is accused of sexual assault, states it never occurred/ was consensual. Penile swab is taken

Where a suspect claims no sexual assault took place, the detection of vaginal markers hsa-mir-124 from a penile swab would indicate that vaginal penetration occurred. DNA profiling of the material would indicate if this was a result of the complainant's material being present, eliminating the defence that the intercourse was with someone else. The identification of semen would not however be useful if the defence states that intercourse was consensual.

Example 2. Complainant is sexually assaulted by penile penetration, male ejaculates and leaves. Defence claims it never occurred.

As above the identification of hsa-mir-124 from a penile swab would indicate that vaginal penetration occurred. The identification of two of the markers hsa-mir-888, 135a and 10a from the victim internal intimate swabs would show ejaculations took place, if the suspects DNA profile can also be identified this would remove any support for the defence that the event didn't happen. Semen identification would also be reliant on the time since intercourse and sampling.

Example 3. Complainant is sexually assaulted by penile penetration, a vasectomised male ejaculates and leaves. Defence claims it never occurred.

The detection of at least two of the markers hsa-mir-888, 135a and 10a confirms that semen is present within the vaginal canal and sexual intercourse occurred. Within Example 1, the data could be combined with a DNA profile, providing significant support to the defence. With respect to Example 2, these markers have shown a higher expression value in vasectomised males, therefore if two or more markers display expression it would confirm that sexual intercourse did occur without the need for an additional DNA profile - as long as the defendant had not had UPSI with anyone else in the preceding 7 days. Semen identification would also be reliant on the time since intercourse and sampling.

Example 4. Victim claims penile penetrative sexual assault occurred while suspect claims consensual oral intercourse was the only act that happened. Vaginal swabs are taken.

As with examples 2 and 3, the expression of semen markers would indicate that penile penetration occurred and therefore provide support to the defence. If a penile swab was

taken at the time of the incident, or before the suspect had the opportunity to remove any residues (i.e. by washing) then the detection of saliva marker hsa-mir-205 may be possible. If the victim had removed any residues then an identification would be extremely unlikely. If the female was on no form of contraception, a presence of hsa-mir-205 would identify saliva presence (This could be confirmed via absence in a reference swab taken a while after the initial examination). Research was however unable to successfully identify OI from vaginal samples therefore the additional of an external vaginal swab could be more successful at sampling any saliva present. In the instance that semen markers are not detected in vaginal swabs, but hsa-mir-205 detection combined with a DNA profile the suspect there would be evidence that oral intercourse did in fact take place.

Example 5. Male is accused of sexual assault. A red stain is visible on the penis and he states consensual intercourse occurred while menstruating.

The use of a combination of markers (hsa-mir-451, 144-5, 144-3 and 142) would confirm that the red stain is in fact blood. Failure to detect menstrual blood markers hsa-mir-507 and 412 would confirm that the blood did not originate from the vagina, which would confirm that the blood was as a result of other injuries potentially sustained by the victim. The confirmation of menstrual blood is however more difficult to determine. Since hsa-mir-507 and 412 were weakly displayed in a number of vaginal material samples expression cannot be reliably stated as originating from menstrual blood and not vaginal material. A solution would be to obtain a reference sample from the victim where no menstruation occurred, absence within the reference sample and presence within the crime stain sample would be confirmatory of menstrual blood. The aim of this research was to be able to distinguish menstrual and venous blood, even when in a mixture. The markers identified within this body of work were unable to differentiate these two body fluids when in the same mixture, therefore if a trauma caused venous blood to mix with any menstrual blood that may be present during the assault it could not be identified.

Example 6. As per case study 2.1: A murder suspect is found with blood on the passenger seat of his car and states it was left after a female menstruated.

The use of blood markers hsa-mir-451, 144-5, 144-3 and 142 would confirm the presence of blood. Ultimately the absence of hsa-mir-124 would confirm the sample did not originate

in the vagina with further confirmation from the absence of hsa-mir-412. The incorporation of hsa-mir-203a into the panel would also confirm blood origin; the absence of expression would only be observed in venous blood. The confirmed blood stain must therefore have been a result of an injury potentially sustained by the victim. Reference samples would also be required from other areas of the crime scene to ascertain whether any background levels of miRNA were present.

Example 7. Victim is not vaginally penetrated but claims they were forced to receive oral intercourse.

The use of hsa-mir-205 failed to detect the presence of saliva following instances of oral intercourse. The robustness of hsa-mir-124 however highlights the potential for buccal swabs of suspects to be taken and positive identifications to be made, thus confirming oral intercourse had occurred. This would be strengthened with the addition of DNA profiling.

Example 8. Victim is non-consensually digitally penetrated. Defence state it did not occur

The results of this research were unable to identify a skin specific marker. In cases where digital penetration occurs, where DNA profiling is likely to be unsuccessful, BFID would provide no provide suitable evidence for the defence or prosecution. During digital penetration cases usual practise would be to examine the suspect's fingernail cuttings, as seen in R vs Weller [62]. If hsa-mir-124 was identified on these fingernail cuttings it would lend a higher level of support to the prosecution that digital penetration occurred, especially if a DNA profile could also be obtained.

Example 9: DNA is found on a male penile swab. Prosecution state the suspect vaginally penetrated the victim while the defence states consensual oral intercourse was given.

Due to the up-regulation of hsa-mir-205 caused by contraceptive methods in vaginal material the expression of hsa-mir-205 cannot confirm the present of saliva when vaginal material is present. If both hsa-mir-124 and 205 are expressed, saliva presence or absence cannot be confirmed. If, however hsa-mir-124 is expressed while hsa-mir-205 is absent it would confirm the absence of saliva, confirming vaginal penetration occurred and not oral intercourse. In a case where vaginal and oral penetration occurred the body fluid panel would be able to identify the presence of vaginal material but not saliva.

8.6. Comparisons of miRNA and mRNA for BFID

Current literature has shown that RT-qPCR can be performed successfully with mRNA and miRNA. Comparisons can therefore be made from this body of work and current research. The main factors involved in both techniques can be observed in Table 8.1.

	miRNA	mRNA
<i>Flow in forensic casework</i>	Standard DNA extraction, can be used on diluted eluate then moved onto RT-qPCR	Adaption to standard protocol, DNase treatment can be used on diluted sample then moved onto RT-qPCR
<i>Sensitivity</i>	Equivalent to DNA profiling	Equivalent to DNA profiling
<i>Storage</i>	Storage as per standard DNA eluate achieving system [172-175]	-80oC freezer, no long term storage unless reverse transcribed immediately [171-172] *
<i>Cold case capability</i>	Potential on total DNA extraction, limited with differential extraction [172-173]	No cold case capability as the eluate removed during standard protocol is not performed during standard extraction [171-172]
<i>Contamination by gDNA</i>	Stem loop primers are not affected by gDNA contamination therefore will not produce false positives	gDNA contamination must be removed, treatment with DNase
<i>Cost per reaction</i>	With custom primers very low	With custom primers very low (additional expense being DNase)
<i>Primer design</i>	Forward, stem loop primer with universal reverse and probe site	Forward and reverse and probe site
<i>Extraction</i>	Total DNA, Total RNA	Total RNA
<i>Degradation of crime scene samples</i>	Highly stable [172-175]	Many factors affect degradation, dependent on length of mRNA and environmental factors [171-172]
<i>Marker abundance</i>	Still in validation	Many markers that have been verified by multiple laboratories for reproducibility*
<i>BFID capability</i>	Most body fluids with many combinations unable to identify	Full marker panel current being used in case-work (still an issue with saliva)*
<i>EC</i>	Still contested in literature however many have been found to be stable and suitable	Multiple confirmed EC's selected and available – currently being used in casework*
<i>Quantification</i>	Non-specific, new technology, quant value for all miRNA present	UV-VIS spectroscopy –purity and yield, can test integrity with gel or bio analyser
<i>Specificity</i>	Likely to code for multiple mRNAs	Longer sequence so likely to be more specific
<i>Ideal usage</i>	Potential for degraded sample/ old samples/ archived DNA samples/ low and abundant samples [172-175]	Abundant fresh samples, degraded samples can be difficult and may alter cDNA primer choice (e.g. Random or Oligo dT) [171]

Table 8.1: List of conditions and factors for RT-qPCR analysis with equivalent advantages and disadvantages for each technique. *Information obtained from senior forensic scientist Tim Clayton who supervises and carries out mRNA BFID.

Overall both miRNA and mRNA have factors that make them ideal for use in RT-qPCR BFID. Although mRNA is currently being implemented in some forensic laboratories and they are likely to be more specific than miRNA the results from this research suggest that miRNA, once further markers are identified would be the more suitable method. MiRNA analysis would require the least deviation from current DNA profiling protocols to obtain a BFID and DNA result. The stability of miRNA and lack of gDNA contamination would also make it a more robust tool for BFID with less likelihood of false positives. The storage of miRNA at -20°C within the already extracted DNA samples would additionally save equipment and storage space as opposed to the -80°C required for storing an additional elute of mRNA.

8.7. Future Perspectives

The research presented here has demonstrated the viability of miRNA as a BFID technique, however further studies would increase confidence in the technique for casework applications. For miRNA to be implemented into casework a large collaborative exercise between academic and industrial laboratories must be performed following the same protocols as stated within this research. Currently, previous studies within the literature have used varied protocols, for instance, using SYBR or TaqMan® assays or various RNA extraction techniques. Reproducibility across all laboratories would validate the robustness and specificity of these miRNA. Although this research was successful in identifying a number of body fluids, it was unable to determine skin and saliva when in a mixture of vaginal material. Further screening of current and emerging miRNAs may identify a body fluid specific marker which would address the limitations of this technique. An increase in the number of miRNA within the BFID panel may also increase the capability for body fluid origin, if further investigation into blood markers is performed, the site of injury might be possible to identify.

The detection and duration of detection of hsa-mir-124 collected from penile swabs following UPSI and 'suspect' buccal swabs following vaginal oral intercourse would continue to further demonstrate the robustness of this technique. The detection of semen markers within body fluid stains found in underwear following UPSI also presents an interesting application of the technique. This research would also benefit from the

design and implementation of a custom miRNA quantification kit that would quantify specific miRNA within a sample as well as fit into current DNA profiling/ miRNA BFID protocol.

Once validated; the testing on archived crime scene samples would be imperative in concluding its complete robustness for BFID: If samples retrieved from airbags that tested positive for saliva when examiners utilised Phadebas testing also displayed positive for hsa-mir-205 (and any future propose saliva markers) in the absence of the remaining BFID panel it would show the specificity and suitability of miRNA BFID.

8.8. Concluding Remarks

The data generated in this thesis highlight that miRNA is a common component of the cells associated with body fluids. The variance in composition between forensically relevant body fluids, coupled to the ubiquitous nature of certain markers provide a stable platform from which specific body fluids can be identified with confidence. The use of the stem-loop RT-qPCR technique uses instrumentation and reagents that are common in most molecular biology laboratories, and routinely in use in forensic laboratories. The stem loop RT is reliant on custom designed primers which are fully compatible with current RT-qPCR protocols and are relatively inexpensive to purchase.

Within the study a number of miRNA markers were identified that could be used in combination with each other to successfully identify body fluids. In particular, the ability to determine whether a sample originated from the vagina and the ability to detect seminal fluid of azoospermic males provides significant support to the forensic scientist. A panel of endogenous controls are described which provide validation to the technique that could be potentially used within a court of law. The technique does not require specialist equipment and can be carried out on genetic material obtained from standard practice DNA extractions, providing cold case capability.

A number of examples in which these techniques would be relevant to casework are provided. The data also suggest that there are some instances in which the use of miRNA as a marker would not be suitable. As of January, there are 2588 mature *Homo sapien* entries

within the 21st iteration of the database of miRNAs [151]. miRBase - version 1.0 (2012) contained 218 miRNA encompassing the entire animal kingdom. Clearly as the identification of miRNAs continues, the number of potential markers that could be screened is also growing, which could eventually eliminate these uncertainties. Above all, forensic science will never be capable of addressing the issue of consent, however the work presented here provides a platform for improving the confidence in the testimony of both the defence and prosecution, which can only serve to aid the criminal justice system.

9. References

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10. Appendix

A) Female Sampling Questionnaire



The University of Huddersfield, Queensgate, Huddersfield, HD1 3DH

CONSENT FORM

Researcher: Kimberley Bexon; kimberley.bexon@hud.ac.uk – PhD researcher, School of Applied Sciences, The University of Huddersfield.

Supervisor: Dr Graham Williams; g.a.williams@hud.ac.uk – School of Applied Sciences, The University of Huddersfield.

The project aims to identify body fluids associated with sexual assault cases, such as vaginal material and menstrual blood, using short RNA markers. A selection of short RNA markers has been selected that are suggested to be highly specific to vaginal material and menstrual blood. Intimate samples are therefore required to explore and validate the short RNA markers with respect to forensic investigations.

You will be provided with two buccal swabs to take home with you. A low vaginal swab can then be collected in any manner that you are comfortable with. The swabs should then be delivered to the University of Huddersfield by the next day, or placed into freezer storage. Please do not use freezers designated for domestic usage for this purpose.

All samples collected will be anonymised. Once received at the research laboratory, all samples will undergo DNA and RNA extraction. Once the DNA and RNA have been extracted, the rest of your sample will be disposed of in accordance with Human Tissue Authority guidelines. Your DNA and RNA sample will then be retained for analysis. The analysis includes measuring the amount of the short RNA markers present using standard molecular biology techniques. Once this analysis is completed, your DNA and RNA samples will be disposed of. No further testing will be conducted such as DNA profiling.

To aid analysis certain information is required about the donated samples, all information provided is strictly confidential and will be held by myself and Dr Graham Williams in order to comply with the Data Protection Act and the Human Tissue Act.

Please enter the following information. If you are uncomfortable or unable to answer the following question, then please leave the section blank. .

1. Age and ethnicity:
2. What day of your cycle was the sample taken:
3. How regular is your cycle, how heavy:
4. Are you currently on any form of contraception:
5. Do you smoke - If so how many a day:

6. What is your pregnancy history? Are you currently or did you breast feed:
7. Have you had a medical condition that affected your menstrual cycle or uterus:
8. Do you take any medication:
9. Have you been through the menopause:
10. Have you had operations on any of your reproductive organs:
11. How many unit of alcohol per week do you consume on average:
12. Have you have unprotected sexual or oral intercourse within the past 96 hours
13. Is there any other comment you can provide that you feel may have altered your menstrual cycle?

Please initial all boxes

I confirm that I have read and understand the information for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

☐

I understand that my participation is voluntary and that I am free to withdraw my consent at any time without giving any reason. I also understand that if I withdraw my consent, I will not have my sample(s) returned to me.

☐

I agree to take part in the above study.

☐

Name of Participant

Date

Signature

Thank you again for helping with my project, it is greatly appreciated. If you have any queries please feel free to contact me or my supervisor at kimberley.bexon@hud.ac.uk or g.a.williams@hud.ac.uk

Kimberley Bexon MSc ACSFS

B) Male Sampling Questionnaire

CONSENT FORM

Researcher: Kimberley Bexon; kimberley.bexon@hud.ac.uk – PhD researcher, School of Applied Sciences, The University of Huddersfield.

Supervisor: Dr Graham Williams; g.a.williams@hud.ac.uk – School of Applied Sciences, The University of Huddersfield.

The project aims to identify body fluids associated with sexual assault cases, such as semen, using short RNA markers. A selection of short RNA markers has been selected that are suggested to be highly specific to semen. Semen samples are therefore required to explore and validate the short RNA markers with respect to forensic investigations.

You will be provided with a collection tube to take home with you. You will then deposit a semen sample in to the collection tube in any manner that you are comfortable with. The collection tube should then be delivered to the University of Huddersfield by the next day, or placed into freezer storage. Please do not use freezers designated for domestic usage for this purpose.

All samples collected will be anonymised. Once received at the research laboratory, all semen samples will undergo DNA and RNA extraction. Once the DNA and RNA has been extracted, the rest of your sample will be disposed of in accordance with Human Tissue Authority guidelines. Your DNA and RNA sample will then be retained for analysis. The analysis includes measuring the amount of the short RNA markers present using standard molecular biology techniques. Once this analysis is completed, your DNA and RNA samples will be disposed of. No further testing will be conducted such as DNA profiling.

To aid analysis certain information is required about the donated samples, all information provided is strictly confidential and will be held by myself and Dr Graham Williams in order to comply with the Data Protection Act and the Human Tissue Act.

Please enter the following information. If you are uncomfortable or unable to answer the following question, then please leave the section blank.

- a) Age:
- b) Have you had a vasectomy?
- c) Are you currently aware of your sperm count?

Please initial all boxes

1. I confirm that I have read and understand the information for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my participation is voluntary and that I am free to withdraw my consent at any time without giving any reason. I also understand that if I withdraw my consent, I will not have my semen sample returned to me.
3. I agree to take part in the above study.

☐
☐
☐

Name of Participant

Date

Signature

Thank you again for helping with my project, it is greatly appreciated. If you have any queries please feel free to contact me or my supervisor at kimberley.bexon@hud.ac.uk or g.a.williams@hud.ac.uk

Kimberley Bexon MSc ACSFS

C) Full Primer Sequences

		BF	RT Sequence	F Sequence
SNORD-	7	EC	GTC GTAT TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC	CCA GGG TAT GAT CTC TTA ATC TTC TC
hsa-miR	10a-5p	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA AA	TACCTGTAGATCCG
hsa-miR	10b-3p	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TTC CC	CGC GCG ACA GAT TCG ATT CTA GGG G
hsa-miR	10b-5p	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACA AA	CGC GCG TAC CCT GTA GAA CCG A
SNORD-	38b	EC	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CTC CT	TGA CAG TAA GTG AAG ATA AAG TGT GTC
SNORD-	44	EC	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GTC AG	ACT GAA CAT GAA GGT CTT AAT TAG CTC
SNORD-	47	EC	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CCT CA	GAT ATC ACT GTA AAA CCG TTC CAT TTT G
hsa-miR	93	EC	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAC CT	GCT CGC AAA GTG CTG TTC GT
hsa-miR	124	Vaginal Material	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GCA TT	T AAG GCC GCG GTG
hsa-miR	124*	Saliva	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATA CGACATCAAG	CGTGTTACAGCGGAC
hsa-miR	135a	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAC AT (50 bp)	TAT GGC TTT TTA TTC
hsa-miR	135b	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAC AT	TATGGCTTTTCATTC
hsa-miR	138-2	Saliva	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATA CGACAACCCT	GCTATTTACAGACAC
hsa-miR	142-3p	Menstrual Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CCA TA (50 bp)	T GTA GTG TTT CCT AC
hsa-miR	144-3p	Menstrual Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GTA CA (50 bp)	TACAGTATAGATGATG
hsa-miR	144-5p	Menstrual Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TTA CA (50 bp)	GGATATCATCATATAC
hsa-miR	185 -5p	Venous Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG GA (50 bp)	TGG AGA GAA AGG CAG
hsa-miR	199a-5	Menstrual Blood	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGACTGGATA CGACGAACAG	CCCAGTGTTCAGACTAC
hsa-miR	203a	Skin	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAG TG (50 bp)	TGA AAT GTT TAG GAC
hsa-miR	205	Saliva	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC AGA CT (50 bp)	TC CTT CAT TCC ACC
hsa-miR	214-3	Menstrual Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CTG CC	ACAGCAGGCACAGAC
hsa-miR	223	Saliva	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GGG GT	TGTCAGTTTGTCAAA
hsa-miR	372-3p	Vaginal Material	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CGC TC	AAAGTGCTGCGAC

hsa-miR	412	Menstrual Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CGG CT	ACTTCACCTGGTCCAC
hsa-miR	455-3p	Skin	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGTGTAT	CGC GCA CTT CAC CTG GTC CAC
hsa-miR	507	Semen	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTTCACT	GCGGCGGTTTTGCACCTTTTGG
hsa-miR	508	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ATG AG	GCC TGT ACT CCA GAG GGC GT
hsa-miR	654-5q	Vaginal Material	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CAC AT (50 bp)	TGGTGGGCCCGAGAAC
hsa-miR	888-5p	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT GAC TG (50 bp)	TA CTC AAA AAG CTG TC
hsa-miR	891a	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG TG (50 bp)	TGCAACGAACCTGAG
hsa-miR	891b	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAA TG	CGC GCG TGC AAC TTA CCT GAG TC
hsa-miR	892a	Semen	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCTACGC	CGC GCG CAC TGT GTC CTT TCT G
hsa-miR	940		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGGGGAG	GCGGCGGAAGGCAGGGCCCCCG
hsa-miR	1260b	Vaginal Material	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA TGG TG (50 bp)	ATCCCACCACTGCCAC
hsa-miR	2392	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC ACC TC (50 bp)	TAG GAT GGG GGT GAG
hsa-miR	3169	Skin	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCTATGT	TAGGACTGTGCTTGG
hsa-miR	3197	Semen	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCC TT (50 bp)	GGAGGCGCAGGCTCG
hsa-miR	4286	Vaginal Material	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GTA CC	ACCCCACTCTGGTAC
hsa-miR	16	Venous Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC GCC AA	T AGC AGC ACG TAAAT
Only in Neat				
hsa-miR	658	Saliva	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CCA AC	GGCGGAGGGAAGTAG
hsa-miR	617	Vaginal	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG CCA CC	A GAC TTC CCA TTT GAA
hsa-miR	451	Venous Blood	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA ACT CA	AAACCGTTACCATTAC
Universal Reverse				GTG CAG GGT CCG AGG T

D) R - Heat Map Code

```
#set working directory
setwd("K:/Kimberley/18_R_Studio/Heatmap code/05082016")
#Install required package
install.packages("gplots")
install.packages("RColorBrewer")
#load package into session
library(gplots)
library(RColorBrewer)
#import csv file from folder, if columns are labelled, header=TRUE, if not =FALSE
DNAdat<-read.csv("DNA2.csv", header=FALSE)
#view data in console
DNAdat
#View subset of data
DNAdat[1:5,]
#Subset the big data into individual markers
M1260b<-DNAdat[1:5,2:22]
M1443<-DNAdat[6:10,2:22]
M10a<-DNAdat[11:15,2:22]
M142<-DNAdat[16:20,2:22]
M124<-DNAdat[21:25,2:22]
M203a<-DNAdat[26:30,2:22]
#Set colour scale, colours taken from colour guide, n= step number
M1260pal<-colorRampPalette(c("white","purple4"))(n=256)

#Make data matrix
M1260bM<-data.matrix(M1260b)
#make heatmap code, data file,
M1260bHM <-heatmap.2(M1260bM, margins=c(15,5), Rowv=FALSE, Colv=FALSE, colsep=0:21,rowsep=0:5,
sepcolor = "black", sepwidth = c(0.01, 0.01),
col=M1260pal,tracecol =NULL, density.info="none", linecol=NULL, hline = NULL, vline=NULL,
symkey= FALSE, key=TRUE, keysize = 1.0, key.title = "", key.xlab = "Deltact")

#Make data matrix
M1443M<-data.matrix(M1443)
#Make colour scale
M1443pal<-colorRampPalette(c("white", "mediumaquamarine")) (n=256)
#make heatmap code, data file,
M1443HM <-heatmap.2(M1443M, margins=c(15,5), Rowv=FALSE, Colv=FALSE, colsep=0:21,rowsep=0:5,
sepcolor = "black", sepwidth = c(0.01, 0.01),
col=M1443pal,tracecol =NULL, density.info="none", linecol=NULL, hline = NULL, vline=NULL,
symkey= FALSE, key=TRUE, keysize = 1.0, key.title = "", key.xlab = "Deltact")

#Make data matrix
M10aM<-data.matrix(M10a)
#Make colour scale
M10apal<-colorRampPalette(c("white", "maroon4")) (n=256)
#make heatmap code, data file,
M10aHM <-heatmap.2(M10aM, margins=c(15,5), Rowv=FALSE, Colv=FALSE, colsep=0:21,rowsep=0:5,
sepcolor = "black", sepwidth = c(0.01, 0.01),
col=M10apal,tracecol =NULL, density.info="none", linecol=NULL, hline = NULL, vline=NULL,
symkey= FALSE, key=TRUE, keysize = 1.0, key.title = "", key.xlab = "Deltact")
```

E) R - Box and Whisker Plot Code

```
#Set working directory
setwd("K:/phd_data/Intercourse_study/Rstudio")
#install the plot package
install.packages("ggplot2")
#Load the package
library(ggplot2)
#Read in the data file
dataset<-read.csv("data.csv", header=TRUE)
#Name a plot 'plot1' and tell ggplot to use the dataset
#aes sets the x axis to column labelled "Time" and y axis to the data in this case column
Cq.10a
#Tell it to add the additional categories 'fill' from the column labelled method
plot1<-ggplot(dataset, aes(x=Time, y=Cq.10a, fill=Method))
#Tell ggplot to make it a box plot, and set the outliers to black, a diamond shape "18" and
size "2"
+geom_boxplot(outlier.colour="black", outlier.shape=18, outlier.size=2)
# Tell it to label the y axis "deltaCq"
+ labs(y="ΔCq")
#Tell it to add a manual scale to the fill the series, with the colours as values
plot1+scale_fill_manual(values=c("dodgerblue", "darkorchid4", "forestgreen"))

setwd("K:/phd_data/Intercourse_study/Rstudio")
install.packages("ggplot2")
library(ggplot2)
dataset<-read.csv("data.csv", header=TRUE)
plot1<-ggplot(dataset, aes(x=Time, y=Cq.10a, fill=Method))
+geom_boxplot(outlier.colour="black", outlier.shape=18, outlier.size=2)
+ labs(y="ΔCq")
plot1+scale_fill_manual(values=c("dodgerblue", "darkorchid4", "forestgreen"))
```

F) Volunteer Information

Unless stated in sample notes all female samples are NO for pregnancy, previous pregnancy, disease, contraception, smoking, drugs and have a regular cycle menopause

UPSI – Unprotected intercourse within the past 7 days

Grey boxes indicate repeat volunteer.

Body Fluid		Age	Ethnicity	Sample notes
Vaginal material	Volunteer 1	36	White / British	Contraceptive pill
	Volunteer 2	26	White / British	Contraceptive pill
	Volunteer 3	22	White / British	Condoms,
	Volunteer 4	37	White / British	
	Volunteer 5	28	White / British	Irregualr cycle, 1 child, breastfed, low alcohol consumption
	Volunteer 6	28	Afro Caribbean	
	Volunteer 7	21	White / British	Implant
	Volunteer 8	35	Mixed British	1 child
	Volunteer 9	39	White / British	2 pregnancies, implant
	Volunteer 10	23	White / British	Contraceptive pill
	Volunteer 11	34	White / British	1 child, Contraceptive injection
	Volunteer 12	28	White / British	2 children, breastfed, Copper coil injection, smoker, average alcohol consumption
	Volunteer 13	26	White / British	1 child, miscarriage, stage 1 cervical cancer
	Volunteer 14	31	White / British	
	Volunteer 15	21	White / British	No menstrual cycle, Contraceptive injection, light smoker, avg. 10unit of alcohol p/w
	Volunteer 16	20	White / British	Progesterone only pill
	Volunteer 17	22	White / British	Cerelle – progesterone only pill
	Volunteer 18	26	Japanese	Contraceptive oral pill
	Volunteer 19	22	White / British	Progesterone only pill
	Volunteer 20	40	White / British	2 children, contraceptive pill
	Volunteer 21	21	White / British	N/A, no previous sexual activity
	Volunteer 22	23	White / British	No menstrual cycle, Pill contraceptive
	Volunteer 23	27	White / British	Day 6 of cycle
	Volunteer 24	19	White / British	Day 5 of cycle
	Volunteer 25	34	White / British	No menstrual cycle, Implant contraception, Smoker, 2 previous children
	Volunteer 26	25	White / British	No period for 3 years, Implant contraception, Heavy smoker, Previous miscarriage, taking medication
	Volunteer 27	40	White / British	Full hysterectomy
	Volunteer 28	22	White / British	Irregular and very heavy cycke, using IUD
	Volunteer 29	24	White / British	Irregular light cycle, using IUD
	Volunteer 30	21	White / British	UPSI, Pill contraceptive, day 10 of cycle, contraceptive pill
Menstrual Blood	Volunteer 4			Medium stain
	Volunteer 4	38	White / British	Very light stain, Pill contraceptive

	Volunteer	31	36	White / British	Very light stain, unpredictable menstrual cycle, currently on day 12 of variable flow.
	Volunteer	8			Light stain on menstrual sample
	Volunteer	2			Very heavy stain, Pill contraceptive, day 3 of cycle
	Volunteer	12			Very heavy stain,
	Volunteer	21			Medium stain
	Volunteer	32	30	White / British	Very heavy stain, 1 child
	Volunteer	33	21	White / British	Very light stain
	Volunteer	13			Very light stain
	Volunteer	34	43	White / British	2 children, going through menopause
	Volunteer	18			Medium stain
	Volunteer	35	35	White / British	Light stain
	Volunteer	5			Medium stain
	Volunteer	36	20	Mixed: white and Asian	Very heavy staining, Pill Contraceptive – progesterone only
	Volunteer	37	21	White / British	Very light stain, UP, Rod implant
	Volunteer	38	41	White / British	3 pregnancies (miscarried first) , breastfed all. Both C section not vaginal birth. Regular although altering recently possible due to onset of menopause
	Volunteer	39	48	IC4 - African	Day 3 of cycle, regular and heavy cycle. Heavy stain. Suffered from endometriosis. On medication for anaemia.
Saliva	Volunteer	40	22	White / British	No red stains observed
	Volunteer	41	26	Italian	No red stains observed
	Volunteer	42	27	White / British	No red stains observed
	Volunteer	43	26	White British	No red stains observed
	Volunteer	2			No red stains observed
	Volunteer	44	21	Asian (Indian)	No red stains observed
	Volunteer	15			No red stains observed
	Volunteer	5			No red stains observed
	Volunteer	45			No red stains observed
	Volunteer	12			No red stains observed
Blood	Volunteer	41			
	Volunteer	44	21	Asian (Indian)	
	Volunteer	12			Heavy stain
	Volunteer	5			
	Volunteer	40			
	Volunteer	2			Heavy stain
	Volunteer	43			
	Volunteer	15			
	Volunteer	45			
	Volunteer	42			Light stain
Semen	Volunteer	42			Sperm count unknown, no vasectomy normal consistency
	Volunteer	41			Sperm count unknown, no vasectomy
	Volunteer	43	26	White British	Sperm count unknown, no vasectomy

	Volunteer	46	26	White British	Sperm count unknown, no vasectomy, very thick
	Volunteer	47	29	White British	Sperm count unknown, no vasectomy
	Volunteer	48	52	Afro Caribbean	Sperm count unknown, no vasectomy, normal consistency
	Volunteer	49	21	White / British	Sperm count unknown, no vasectomy
	Volunteer	40			Sperm count unknown, no vasectomy
	Volunteer	50	26	White British	Sperm count unknown, no vasectomy, thick and goopy
	Volunteer	51	25	White British	Sperm count unknown, no vasectomy
	Volunteer	52	21	White British	Sperm count unknown, no vasectomy normal consistency
	Volunteer	53	22	White British	Limited volume sample normal consistency
	Volunteer	54	30	White British	Sperm count unknown, no vasectomy
	Volunteer	55	24	White British	Sperm count unknown, no vasectomy
	Volunteer	56	27	Asian	Limited volume sample, quite clear and fluid
	Volunteer	57	24	White / British	Sperm count unknown, no vasectomy
	Volunteer	58	21	White / British	Sperm count unknown, no vasectomy normal consistency
	Volunteer	59	24	White / British	Large volume samples, fairly fluid
	Volunteer	45	24	White / British	Sperm count unknown, no vasectomy
	Volunteer	60	21	White / British	Sperm count unknown, no vasectomy
Vasectomised/ Seminal Fluid	Volunteer	61	51	White / British	Vasectomised
	Volunteer	62	42	White / British	Vasectomised
	Volunteer	63	55	White/ Dutch	Vasectomised
	Volunteer	64	52	White / British	Vasectomised : Known zero sperm count: Almost clear in appearance
	Volunteer	65	46	White / British	Vasectomised: Almost clear in appearance
	Volunteer	66	45	White / British	Vasectomised: very 'goopy'
Skin	Volunteer	2			
	Volunteer	41			
	Volunteer	15			
	Volunteer	5			
	Volunteer	47			

G) Limit of Detection of hsa-mir-888 in Vaginal Material

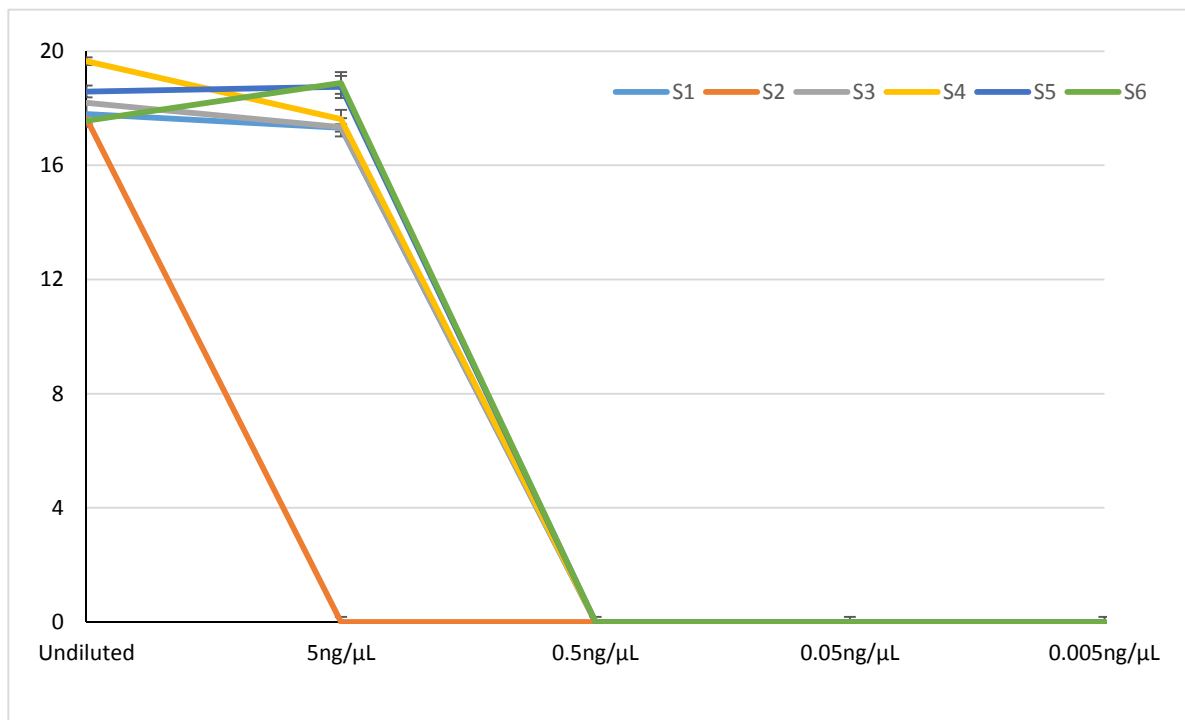


Figure 10.1: Δ^1Cq values + SEM obtained from hsa-mir-888 within six vaginal material samples