IDENTIFICATION AND RESOLUTION OF CAPABILITY GAPS IN FORENSIC SCIENCE

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Copyright statement

Author’s background

The author has five years of experience as an operational forensic biologist with the London Laboratory of the Forensic Science Service (FSS) and carried out further casework between 2007 and 2012. During this time he has processed over 400 criminal cases in which DNA, body fluid or clothing damage evidence was required. Approximately 150 of these cases involved DNA recovered from firearms. As such, elements of this project are based on his personal experiences or from the professional training provided by the FSS.

When information has come from professional training, it is referenced as personal correspondence with the Forensic Science Service (FSS). When information or strategy has come from his own personal experience, no reference has been provided.

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Abbreviations

ADI	-	Allelic drop in
ADO	-	Allelic drop out
AP	-	Acid phosphatase
BFID	-	Body fluid identification
bp	-	Base pair
CPE	-	Combined population excluded
CPI	-	Combined population included
dcDOP-PCR		A variation of DOP-PCR developed by Dawson-Cruz *et al*
DOP-PCR-		Degenerate oligonucleotide primed PCR
EDNAP	-	European DNA profiling group
ELISA	-	Enzyme linked immunosorbent assay
FSS	-	Forensic Science Service
H&E	-	Haemotoxylin and eosin
KM	-	Kastle-Meyer
LMG	-	Leucomalachite Green

LR	-	Likelihood ratio
MDA	-	Multiple displacement amplification
miPEP	-	Modified improved PEP
miRNA	-	MicroRNA
Mr	-	Mixing ratio
mRNA	-	Messenger RNA
mtDNA	-	Mitochondrial DNA
Mx	-	Mixing proportions
NGM	-	Next Generation Multiplex
NGS	-	Next Generation Sequencing
ng	-	Nanogram
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PEG	-	Poly-ethylene glycol
PEP	-	Primer extension preamplification
pg	-	Picogram
Pro-K	-	Proteinase K
PSA	-	Prostate specific antigen
rfu	-	Relative fluorescent unit
RMNE	-	Random man not excluded
STR	-	Short tandem repeats
SV	-	Stochastic variations
SYBR	-	Synergy Brands
WGA	-	Whole genome amplification

Abstract

Although forensic biology is a powerful tool in criminal investigations, there are a number of capability gaps; namely, the interpretation of low-level DNA mixtures, associating the DNA profile with a body fluid, and the issue of consent in sexual offences. A research strategy was developed that utilises whole genome amplification (WGA), messenger RNA and microRNA analysis, DNA profiling, and clothing damage analysis. An evaluation of a WGA technique - multiple displacement amplification - with and without a macromolecular crowding agent, indicated that this may be of use for DNA samples containing certain mixing ratios; however, for this to be truly of use, knowledge of the nature of the sample pre-analysis is required, which is not feasible in a forensic environment. A SYBR Green-based mRNA gene expression test was developed that was capable of distinguishing between saliva and blood by using relative quantitation on real-time PCR. However, the low specificity of the SYBR Green meant that a higher number of controls were required for this to work at forensic standard. A single channel simultaneous analytical test for DNA and microRNA was also developed, which meant that it could be possible to definitively identify the body fluid origin of a DNA profile. This represented a significant step forward in improving forensic biology capability. Reconstruction studies were carried out in response to a sexual assault case where consent was an issue. This study demonstrated that it was possible to cause significant damage to a bra without causing damage to the hook and eye fastening; thus, negating a hypothesis offered by the defence. A long term research strategy has been developed and significant progress has been made in improving the capability of the operational forensic biologist.

Commentary

Forensic biology is the use of biological techniques to address questions of interest in a court of law. In the context of this study, this includes the sub-disciplines of forensic genetics and forensic serology (there are other sub-disciplines which fall under the banner of forensic biology, but these lie outside the scope of this study).

Forensic genetics is dominated by the production of DNA profiles. Increasingly, however, in recent years this field has also included the analysis of various RNA molecules (1), single nucleotide polymorphisms (2) and mitochondrial DNA (3).

Forensic serology is the name generally given to the examination of body fluid evidence, including blood, semen, saliva, urine and faeces. However, the use of the term ‘serology’ is a misnomer, as this term refers to the study of the blood serum. Consequently, the term ‘body fluids’ is used rather than forensic serology. The use of this term reflects common practice as carried out across various forensic science providers in the United Kingdom.

Forensic biology as a discipline generally deals with violent crimes, from muggings to physical and sexual assaults to homicides and acts of terrorism. In addition to these violent crimes, forensic biology can also assist with varying degrees of success in property crimes such as theft and burglary.

The initial aim of this study is to use current literature and professional experience to identify areas in forensic biology which are relatively weak, and to investigate ways in which these areas can be strengthened.

0. Identification of capability gaps within forensic science

There are a number of capability gaps within the forensic sciences; such gaps can have a greater or lesser importance, depending on the end-user of the techniques. Identification of such capability gaps came from a variety of sources, including the author's own professional experience, personal correspondence with other forensic practitioners, and from end user organisations, such as forensic science providers and police organisations.

Low level mixtures – The author has worked on hundreds of cases, with ~150 of these being firearms cases, where it was required to obtain DNA profiles from the ridged surfaces of the firearms in order to determine who could have come into contact with them. According to personal correspondence from various police officers, such firearms can be handled by multiple people. Thus such DNA results from firearms can be characterised as low-level mixtures; where there are multiple sources of touch DNA. As discussed later on, such low level mixtures may be considered uninterpretable.

Definitive identification of body fluids – Most chemical tests for body fluids are presumptive and as such have an element of unreliability; particularly when there are low levels of the body fluid in question. As such any confidence in the identification of the body fluid is diminished (FSS).

Lack of tests for the identification of body fluids – Currently, there exists no test for the identification of vaginal material and menstrual blood. Such body fluids can be present in sexual offences and would progress the case if they could be identified. As it stands, it is not currently possible to differentiate between saliva, vaginal material and skin cells to a forensic standard. The same also applies to menstrual blood and peripheral blood; although personal correspondence with

colleagues in New Zealand have indicated that such capability has been successfully developed and applied to case work.

Association of a DNA profile with a body fluid – This is when a body fluid stain has been identified and then recovered for DNA profiling. If a DNA profile is obtained, can it be said it was obtained from the identified body fluid? This is a controversial area as, according to personal correspondence with various colleagues, different scientists have different opinions. For example, some will quite happily attribute a single source DNA profile to an abundant body fluid stain, such as blood. Others will be more cautious, arguing that there is no scientific rationale for this, given that the tests in question target different chemical components.

Issue of consent in sexual offences – It is almost the lack of consent on the part of the complainant that defines a sexual assault. This consent considers the frame of mind that the complainant has at the time of the incident. Consequently, this is not considered something that can be addresses using forensic genetics. However, there may be multi-disciplinary approaches to resolving this issue.

Turn round times – Police services are under a lot of political and social pressure to resolve cases to completion in a timely fashion. The National Police Improvement Agency (NPIA) conducted a survey of police officers and scene of crime officers and found that one of the biggest concerns was at the length of time it took for results to be reported back to the police officers (personal correspondence with NPIA). This is such a major concern that when the police services tender for forensic services, one of the major key performance indicators required is one of turn round times. The forensic science provider who can turn round larger number of samples in a faster period of time is the one most likely to be successful in their bid for tenders. There are a number of strategies in place for trying to increase turn round

times. The most common one is automation. Automation is the use of robotics to process samples. By removing the human element, large numbers of samples can be processed simultaneously and without error (thus eliminating or reducing the need for repeated analysis).

Costs – This issue is also a major issue considered by police officers, scene of crime officers and forensic science providers. However, this is an issue that can be linked in with turn round times. Past a certain point, the main reason for the expenses is the length of time it takes to progress a case, so many of the strategies in place for turn round times can also address those of cost. For example, the use of automation can use smaller amounts of liquid, reduce wastage and remove the need to employ large numbers of DNA analysts. Although the cost factor was not the primary driving force behind the selection of capability gaps, it was a factor that was being considered for Article 3 and the benefits of Article 4 included the issue of cost.

As it is not reasonable to address all of these issues within the scope of this study, a number of gaps were focussed upon and others disregarded at this stage. A number of factors were considered in selecting such capability gaps; the equipment available within the School of Applied Sciences laboratories, the relative amount of competition in that area (i.e. there is a lot of investment by commercial companies in developing automation solutions and robotics) and the opportunities for novel research. In one situation, it was a chance occurrence, in that Article 5 was published in response to a case, which led to the area of consent in sexual assaults being addressed.

Consequently, the areas being investigated are:

1. Alternative DNA amplification techniques on single source and mixed DNA results.

2. The development of more definitive body fluid identification tests.

3. Association of a DNA profile with a particular body fluid.

4. Addressing the issue of consent in sexual offences.

1. Alternative DNA amplification techniques on single source and mixed DNA results

Since its inception in 1985 by Alec Jeffreys *et al* (4), DNA profiling has undergone considerable improvement, and this progress is still continuing to the extent that it can be difficult to keep on top of progress in this area. Consequently, the use of DNA profiling is becoming an increasingly powerful forensic investigation tool. However, there are still issues relating to the DNA profiling of certain types of samples; namely, low-level samples and mixed DNA samples.

1.1. Low-level DNA

Low-level DNA - also referred to as low-trace or ultra-trace DNA - mainly relates to samples where the amount of DNA present falls below a certain threshold. This threshold has different definitions depending on the forensic science provider, but generally falls to approximately 100-200 pg of DNA material (5, 6). DNA profiles obtained from such samples represent particular challenges, mainly due to the less than optimal amount of DNA required for the PCR process. This less than efficient PCR reaction can give rise to increased stochastic variations (SVs). The most significant SV encountered in low-level DNA analysis are allelic drop outs (ADOs). These occur when the peak height of the alleles drop below the analytical threshold; thus, they are not identified or included in the profiling information (6).

The main consequence of ADOs is the removal of an eliminating allele. For example, if the suspect's genotype at TH01 is 9, 9.3 and the genotype of the crime stain is 9, 10 then the suspect is eliminated from having contributed to that DNA result. If the crime stain sample is low-level, then may indicate a DNA profile of 9, F (with F representing an unknown allele). This DNA result no longer eliminates the suspect, and a slightly diminished match probability may then be reported. The phenomenon of ADOs are also frequently observed in poor quality results.

1.1.2 Mini-STRs

When DNA profiling was first utilising in the 1980's the fragment length were considerably large (in the region of Kbs), which made them more susceptible to degradation (refs). When STRs started being utilised, it was possible to obtain full DNA profile from mildly degraded samples. However, when the samples are further degraded, even STR analysis can only produce partial DNA profiles. The susceptibility of DNA fragment to degradation is directly related to their sizes; this is one of the principles behind using microRNA over messenger RNA in Article 4.

Taking this further, miniSTR analysis has been developed which targets much smaller STR fragments (refs); thus such fragments are more resistance to degradation and according to Mulero *et al* (63) also resistant to PCR inhibition. Such analyses will be of benefit to samples which have been subject to degradation, such as old stains and deep muscle tissue from badly decayed remains. However, its application to the issue of low trace DNA samples, such as from skin cells, will be limited, but it is thought that such short fragment will be resistance to the effects of stochastic variations (63).

1.2. Mixed DNA samples

Mixed DNA samples occur where more than one person has contributed to the sample. These are predominantly samples relating to sexual offences, where two different body fluids may be mixed together. However, mixed DNA samples can be obtained from many items and cases - basically, anywhere where two or more people have come into contact with a surface. Mixtures can also be encountered where contamination has taken place (7-9).

The issue with mixtures is that it is not always possible to identify the two contributors to the DNA result. For example, if the DNA result indicates that the alleles at a locus are A, B, C and D (see Table 1), and it is assumed that only two people contributed to the DNA result, then the possible combinations are:

Contributor 1	Contributor 2
A, B	**C, D**
A, C	**B, D**
A, D	**B, C**

Table 1 - Possible combinations for a four peak loci (reciprocals not included)

Contributor 1	Contributor 2
A, A	**B, C**
B, B	**A, C**
C, C	**A, B**
A, B	**A, C**
A, B	**B, C**
A, C	**B, C**

Table 2 - Possible combination for a three peak loci (reciprocals not included)

Contributor 1	Contributor 2
A, A	B, B
A, B	B, B
A, A	A, B
A, B	A, B

Table 3 - Possible combinations for a two peak loci (reciprocals not included)

The problem, then, is the determination of the two DNA profiles. As can be seen in Table 1, there are six possible combinations (three unique combinations and three reciprocal combinations) in a four peak loci. There are more possible combinations for two peak and three peak loci (see Tables 2 and 3). Often, determining the contributors can be easy, especially when the DNA mixture is classed as a simple mixture or a 'major-minor' (7, 9); this is when one contributor has made a significant contribution to the DNA result and the second contributor has made a marginal contribution. This can be exhibited on a 4 peak loci as two large peaks and two small peaks. The two largest peaks can be considered the 'major' profile and the two smaller peaks can be considered additional 'minor' contributions (8). Different laboratories have different criteria for what constitutes a major-minor mixture, but a typical example is the use of the mixing ratio in which a ratio of greater than 5:1 is considered a simple mixture. Alternatively, a mixing ratio of less than 5:1 can be considered a complex mixture. This threshold is merely a guideline and results require expert oversight to determine whether they are complex or not (FSS).

1.2.1. The role of the mixing ratio in mixture calculations

The mixing ratio (Mr) is a key statistic when it comes to carrying out mixture calculations. One of the main purposes of mixture calculations is to separate out the

DNA profile (also referred to as ‘deconvolution’). There are two steps utilised in achieving this. The first is the use of the ‘preferential amplification’ rule (or allelic imbalance), whereby if a particular combination is proposed, then the peak heights should be broadly comparable. However, even in single source profiles there can be differences in peak heights between alleles in one locus. Consequently, a peak height difference threshold is suggested. Depending on the forensic science provider, this can range from 50% to 60%, although in practice 50% is more commonly adopted for simplicity (7).

Consequently (as per FSS procedures), if a proposed combination has a peak difference of less than 50%, then that combination is deemed acceptable at this stage. By contrast, if the difference is greater than 50%, then that combination is considered invalid.

The ‘passed’ combinations then undergo the second step, which is the calculation of the mixing ratio. The mixing ratio is calculated by using variations of the following equations (where 'Ph' refers to peak height):

$$\frac{\sum PhA \text{ and } PhB}{\sum PhC \text{ and } PhD}$$

Equation 1 - mixing ratio calculation for 4 peak loci

$$\frac{PhA}{\sum PhB \text{ and } PhC}$$

Equation 2 - mixing ratio calculation for 3 peak loci and an unshared homozygote

$$\frac{(PhA + (0.5\ PhB))}{(PhC + (0.5\ PhB))}$$

Equation 3 - mixing ratio calculation for 3 peak loci and a shared homozygote

$$\frac{2PhA}{PhB - PhA}$$

Equation 4 - mixing ratio calculation for 2 peak loci

Once a mixing ratio is obtained, this figure can then be used to assess the relative contributions of each donor. This is done by comparing the mixing ratio with the overall mixing ratio. The overall mixing ratio is obtained by applying the following equation to all four peak loci and obtaining a mean:

$$\frac{\sum Ph\ of\ the\ two\ largest\ peaks}{\sum Ph\ of\ the\ two\ smallest\ peaks}$$

Equation 5 - Overall mixing ratio

From personal experience, the mixing ratio tends to fall into one of three categories. Firstly, a mixing ratio can be obtained that is below 1 (for example 0.48:1). This will render the proposed combination invalid, but it suggests that the actual combination is the reciprocal of the proposed combination.

Secondly, the mixing ratio can be in excess of 1:1, but very different from the overall mixing ratio (for example 23:1 against 3:1). In this case the proposed combination is invalid.

Thirdly, the mixing ratio can be similar to the overall mixing ratio (for example, 2.3:1 against 3:1). In this case the proposed combination is a viable combination and must be incorporated within the match probability calculations.

This does raise questions as to how close the mixing ratio has to be in order to be considered. Currently, there is no real consensus as to how close they have to be, but in most cases these values tend to be in the extremes (certainly a difference of more than a factor of 10). If there is doubt as to how close these values can be, then that combination must be accepted.

In most cases, the mixing ratio will fall into one of these three categories; however, it can be fairly common to get two values that are close to each other (for example 2.3:1 and 2.8:1 against 3:1). In such cases, both proposed combinations should be considered in subsequent match probability calculations. However, some experts adopt a ‘best fit’ approach in which they would only consider the proposed combination with a mixing ratio closest to the overall mixing ratio. This can be a non-conservative approach, but it very much depends on the case and it is up to the expert to justify his selection.

It is always best to follow a conservative approach; one that favours the defence hypothesis. Consequently, when it comes to eliminating combinations, it should be entirely justifiable and demonstrable using the above thresholds. If it cannot be justified, then that combination must be included in the subsequent calculations.

This is one of the flaws relating to expert evidence regarding mixture calculations. If a conditioned DNA mixture is obtained and only one combination at each locus is valid, then the subsequent calculation is relatively simple, as this is the same as the single DNA profile match probability. Consequently, the expert can be put under pressure to reject potentially valid combinations, particularly by using the ‘best fit’ approach.

Some forensic science providers may prefer to use mixing proportions (Mx), which are calculated by using the following equation which shows the percentage contribution of one possible combination to the whole mixture:

$$\frac{\sum PhA \text{ and } PhB}{\sum PhA, PhB, PhC \text{ and } PhD}$$

Equation 6 - calculating the mixing proportion

For example, PENDULUM, a mixture calculation programme developed by the Forensic Science Service in 1995 (64), adopts the use of mixing proportions as part of its calculations; which differs from the practice of using mixing ratio for manual calculations (FSS).

However, the mixing ratio is more widely used (for manual calculations) and it is more logical to use mixing ratios; as this considers the relative contributions of both sources, rather than the contribution of one source into the whole mixture. Consequently, the use of the mixing ratio was adopted as a performance indicator in Article 2 as mixture calculations were carried out manually.

The study in Article 2 also focussed on 4 peak mixtures only; neglecting 2 and 3 peak calculations. The rationale behind this was that the 2 peak and 3 peak formulae made an assumption that shared alleles were shared equally; which is not necessarily the case. However, given that most of the results exhibited a reasonably large number of 4 peak loci, it was not felt to be a major issue. Any further work in this area will reconsider the use of 2-peak and 3-peak formulae and even the incorporation of using such mixture calculation software as opposed to carrying this out manually.

1.2.2. Complex mixtures

A complex mixture in this context refers to a result where it is not possible to separate out (or deconvolute) two DNA profiles with a reasonable amount of confidence due to insufficient difference in peak heights (i.e. an Mr of ~1:1) (FSS). A complex mixture can also refer to a DNA result indicating the presence of DNA from more than two individuals. In the UK, such a result is considered uninterpretable unless there is a clear single contributor (as indicated by significantly higher peaks than the additional minor contributions – i.e. a 'major' profile) (FSS). In the US, however, such mixtures are still considered and a statistic may be applied by adding together all the allelic frequencies within loci and then multiplying across the loci (10). This is referred to as CPI, or combined population included, and indicates the percentage of the population that could be included as having contributed to the result. When reporting, the percentage of the reciprocal is used, generating a CPE or combined population excluded. The more alleles present in the result, the lower the combined allelic frequency. Both CPI and CPE are variations of the Random Man Not Excluded (RMNE) (10) method of interpreting DNA mixtures, whereas in the UK the likelihood ratio (LR) (7) method is employed. However, more and more international forensic science providers are moving over to the LR model.

A complex mixture can also refer to any DNA mixture result where most of the peaks are below 150 relative fluorescent units (rfu) (or 'red' as defined by the European DNA Profiling Group (EDNAP) DNA mixtures working group). The issue with low-level mixtures relates to the phenomenon of ADOs as discussed earlier (8).

So, by combining the two main issues relating to DNA profiling results - low-level results and mixed DNA results - some very complex results can be obtained from crime scene stains which are predominantly uninterpretable and are

often reported as being ‘unsuitable for any meaningful comparison with other DNA profiles’. Thus, these samples carry no evidential value using current methodology.

1.2.3. Mixture calculation software

The use of the preferential amplification and mixing ratio rule forms the basis of a number of computer programmes which are designed to carry out this process automatically. A series of algorithms and guidelines have been developed from the above formulae. One of the first ones to be developed was the PENDULUM software developed by the FSS (64); such a system is referred to as an ‘expert system’. Since then, a large number of commercial companies and forensic science providers have developed their own; for example, the Scottish Police Services Authority have developed an Excel mixture calculation file. This is freely available as an open-source package on the International Society of Forensic Genetics website (65). Both Life Technologies and Qiagen have developed their own mixture calculation software packages; Genemapper ID-X and Investigator ID Proof, respectively. However, such commercially available mixture calculation software packages are expensive.

One possible strategy to address such samples is the use of whole genome amplification. The first article submitted in this portfolio is a literature review exploring the forensic applications of whole genome amplification, bringing together the research published by various groups, including a small number of groups working in forensic genetics. A number of different groups were working on various whole genome amplification strategies, but were publishing their own protocols - such as crowded multiple displacement amplification (MDA) (11, 12), modified

improved PEP (miPEP) (13) and dcDOP-PCR (14) - without consideration of each other's work. The purpose of the review was to compare and contrast these strategies in terms of background theory, ease and reproducibility, and forensic relevance, as well as in relation to the success of the strategies.

It was possible to compare the different published results, arrive at a number of conclusions and propose some hypotheses. One observation was that different analyses were used on mixtures, including peak height differences and mixing proportions. However, it was still possible to compare data from one study with another and arrive at some conclusions as discussed in Article 1.

Williams, G. (2011) 'Forensic Applications of Whole Genome Amplification' *International Journal of Criminal Investigation*, 1 (3);123-135

The second article submitted in this portfolio explored one of the hypotheses proposed from the literature review and investigated the effect of MDA upon a range of samples covering a variety of mixing ratios. The results were interesting in that the use of MDA was able to improve the DNA result, but only in a limited way. One outcome observed from this research was that the calculated mixing ratios bore little resemblance to the actual mixing ratio, particularly when the actual mixing ratio was in excess of 10:1. This research suggested that if the 5:1 threshold was the point at which a complex mixture became a simple mixture, then a 10:1 ratio could be the point at which the second contributor was completely eliminated thus giving a single source DNA profile. This study also indicated that it might be erroneous to assume that the major DNA profile of the result is synonymous with the major contributor to the DNA tested.

Further work could be carried out by investigating alternative whole genome amplification techniques, such as miPEP (13) and dcDOP-PCR (13, 14). Further work could also explore alternative macromolecular crowding agents, such as PEG-4000.

Bexon, K. and Williams, G. (2012) 'Evaluating the effect of multiple displacement amplification upon low-level multiple source DNA profiles' *International Journal of Criminal Investigation* 2 (2);103-110

1.2.4 Next Generation Sequencing

Molecular biology techniques are rapidly advancing in recent years; particularly in the area of sequencing. There is considerable investment from various organisations in to developing sequencing techniques that can drive down the cost of analysis per sample. A number of techniques have been developed, which falls under the umbrella term of Next Generation Sequencing (NGS) (66). It should be noted that this is different from the Next Generation Multiplex (NGM) used in Articles 2 and 4; NGM is an STR analysis kit, which is an advanced form of the Second Generation Multiplex plus (SGM+) currently used in the UK.

Such NGS techniques include pyrosequencing (67), fluorescently labelled sequencing by synthesis (68), sequencing by hybridization and ligation (69) and microchip electrophoresis-based sequencing (70).

The main thing that appears to be in common with these NGS techniques is the massively parallel sequencing strategy, where they analyse many sections of the sample simultaneously.

The role of NGS in STR analysis has also been considered. Sequencing is not usually used in the STR analysis of nuclear DNA, which measures the fragment length. However, if the area of interest is sequenced, then it is possible to actually count the number of short

tandem repeats directly. However, given that one of the grand challenges in NGS is to reduce the cost of whole genome sequencing down to ~$1000 per sample, it is unlikely that NGS will be used for STR analysis in the next few years. Especially when you consider that an STR profile can be obtained for less than £30 per sample. However, the role of NGS could revolutionise mtDNA analysis, which currently targets the hypervariable regions. The application of NGS could feasibly sequence the entire mtDNA genome for a relatively low cost and rapidly (66).

2. The development of more definitive body fluid identification tests

From professional experience, although DNA profiling is a useful technique, it is often necessary to say that the DNA was obtained from a particular body fluid. Consequently, it is important to be able to identify the body fluid, not only in order to progress the case, but also to increase the importance and value of the DNA profile.

One of the key components of forensic biology is the identification of body fluids. Identifying the presence of a particular body fluid at a crime scene or on evidential items can be crucial to the case; for example, the presence of blood or semen is of obvious significance. However, such tests are rather limited, as most of them are not conclusive.

There are a number of tests currently in use in forensic casework to identify body fluids, which are predominantly enzyme-based.

Body fluid	Test	Marker targeted	Specificity of test
Blood	Leucomalachite Green (LMG) (15)	Peroxidase activity	Any oxidants or sources of other peroxidase activity such as in plants will cause a colour change.
Saliva	Phadebas (16)	α- amylase	α- amylase is expressed in other body fluids, such as semen, sweat and vaginal material, albeit at much lower levels; the exception is faeces, which can have a higher expression.
Semen	AP test (17)	Acid Phosphatase	This is also expressed in other body fluids and in particular in vaginal material. This expression increases in post-menopausal women.
Vaginal Material	None (18, 19)	-	-

Table 4 - A selection of the more commonly used body fluids tests

2.1. Blood

This is generally considered to be one of the most commonly encountered body fluid stains at crime scenes and on evidential items. It can be encountered in a wide range of cases, from theft through to sexual offences and homicides.

The two most commonly used tests for the possible identification of blood are Leucomalachite Green (LMG) and Kastle-Meyer (KM – also known as phenolphthalein). Both of these tests utilise the same principles, in that the presence of peroxidase activity within the haemoglobin (the haem group) cleaves H_2O_2, resulting in H_2O and an oxygen radical. This causes an oxidation of LMG or KM reagent (which are in a reduced form), which, in turn, then causes a colour change from transparent to green or pink, respectively (15).

2.1.1. Kastle-Meyer test versus Leucomalachite Green test

Both the KM test and the LMG test are used in the forensic sciences in the UK. The KM test is more predominantly used at crime scenes, whereas the LMG test is predominantly used in the laboratory.

Both KM and LMG utilise the same principles and have the highest specificity of chemical tests for blood currently available (15). However, LMG does tend to revert from its reduced state over time and, thus, as it is becoming oxidised, it turns green. Consequently, it needs to be kept stored in a refrigerator and fresh solutions need to be made up on a regular basis. Thus, this test is best suited to a laboratory environment, rather than a crime scene environment or in the back of a crime scene vehicle.

The KM test is more stable and is stable at room temperature for longer periods of time without reverting from its reduced state. Although there are published studies (15) that indicate that KM is more sensitive than LMG, internal studies by the FSS contradict this. The FSS has also demonstrated that rust can be a false positive for KM, but not for the LMG test (FSS).

This information appears to be contradictory, as there is no apparent reason why one test should be more specific than the other, given that both respond to the peroxidase activity. The only reason why one test would react in the presence of rust (oxidation of iron) is if one test is sensitive enough to detect it and the other test is not. Logically, this should mean that the KM test is more sensitive than the LMG test, which contradicts the information provided by the FSS. This is also supported by the published study (15). However, this does demonstrate why LMG is the preferred test over KM. Its inability to react in the presence of rust, especially if a

stain is on a piece of metal (for example a rusty knife or firearm) is key in this respect.

The false positives can be broken down into two groups: oxidative false positives and peroxidase false positives.

2.1.2. Oxidative false positives

This is the oxidation reaction produced by the cleaving of hydrogen peroxide that causes the colour change of the reagent (15). However, this oxidation reaction is not specific; therefore, any oxidation will cause a colour change. Typical examples of oxidants encountered at crime scenes or on evidential items include bleach (encountered in bathrooms and certain types of crimes) and shoe polish (which can be encountered when examining shoes for blood in ‘kicking’ cases) (15).

The possibility of oxidative false positives can be eliminated by the simple action of adding the reagent first and then adding hydrogen peroxide last. If there is a colour change after adding the reagent and before adding the hydrogen peroxide, then an oxidant is present. If there is no colour change at this stage and then a rapid strong colour change after adding the hydrogen peroxide, then there is a high chance that the oxidation is a result of the interaction between the peroxidase activity and the hydrogen peroxide.

2.1.3. Peroxidase false positives

The last three letters of peroxidase ('ase'), indicate that it is an enzyme; however, there is no single structure associated with peroxidase. Consequently, this is better thought of as ‘peroxidase activity’, rather than as peroxidase. However, peroxidase activity is also present in other materials, particularly in plants (15). Such plants

typically encountered in casework include tea and grass stains. However, for a positive result to occur, highly concentrated samples are required.

Peroxidase activity can also occur within bacteria, which has an impact when considering the locations in which blood stains may be encountered, such as sink traps, wet items and dirty areas.

In the author's professional experience, such false positives are rarely encountered, but have included shoe polish and grass stains.

2.1.4. Common misconceptions

From interactions with undergraduate students, it is observed that there can be some misconceptions on the part of students. Firstly is the notion that haemoglobin causes the colour change. Whilst it is true that the presence of haemoglobin will subsequently lead to a colour change, it is important to clarify that it is the peroxidase activity of the haem group that leads to the colour change. By stating that it is haemoglobin, this gives the chemical test the appearance of higher specificity than it actually does.

Secondly is the idea that iron causes the colour change. This notion mostly stems from the fact that iron, when exposed to oxygen, undergoes oxidation and subsequently causes the colour change. Whilst it is true than an oxidation reaction will cause the colour change, the Fe molecules within the haemoglobin do not actually oxidise. Even if this were possible, then it can be eliminated like any other oxidative false positives.

2.1.5. ‘Presumptive’ test

The LMG/KM test is also referred to as a ‘presumptive’ test for blood. Whilst technically this is correct, in practice such a test can become confirmatory for the forensic expert in certain conditions by incorporating the visual examination of blood within a Bayesian framework.

2.1.6. Visual examination of blood

Blood can be quite distinctive when wet as it is red, shiny and rather viscous. When it is dry, it can be brown and crusty. As well as the colour and texture of the blood, the pattern and location of the blood can offer useful information; however, such blood stain pattern analyses are beyond the scope of this study. Taking all of these factors into consideration, the visual stain assessment can be summarised as ‘does the stain look like blood?’

By applying the likelihood ratio aspect of the Bayesian model (20), one can conceptually ask the two following questions:

1. What is the probability that this stain is blood, given that it looks like blood and gave a positive chemical reaction for the presence of peroxidase activity?

2. What is the probability that this stain is <u>not</u> blood, given that it looks like blood and gave a positive chemical reaction for the presence of peroxidase activity?

This is basically asking, how many substances look like blood and give a positive chemical reaction? So far, there is only one substance that satisfies both criteria.

In practical terms, when the forensic biologist examines an item that has an apparent blood stain, an assessment is made as to whether or not that stain is blood, based upon its visual appearance. Depending on the expertise and experience of the biologist, this assessment can be very reliable. At this point, the chemical test is applied. If there is a positive reaction, then this ‘presumptive’ test actually confirms the forensic biologist’s opinion. In such cases, evidence can be reported in a witness statement much more definitively than might be expected.

However, there are frequently cases where there is a trace amount of blood present which may not actually be visible, or which may not look like blood (such as old stains or diluted stains) or be potentially mixed in with another substance. In such cases, the presence of blood cannot be confirmed and it can only be reported that ‘a chemical component of blood was detected, but the presence of blood could not be confirmed’. In these situations, a more definitive test for blood may be required; this may be provided by forensic RNA analysis.

2.2. Saliva

Saliva can be encountered at crime scenes and on evidential items in a number of forms; ranging from recovered saliva deposits (‘spits’) to mouthed-areas of bottles and cigarette ends and, in particular, in cases of sexual assaults where there were oral interactions.

The most common test for saliva is the ‘phadebas’ test which utilises the presence of α-amylase present within the saliva. α-amylase is a carbohydrase which breaks down starch as part of the digestion process of foodstuff (16).

The phadebas test utilises a starch polymer/azo-dye complex which is insoluble. Exposure to a carbohydrase (such as α-amylase) will break down the starch polymer

complex, thus releasing the azo-dye, which then becomes soluble and a blue colour change becomes apparent.

However, α-amylase is also present, albeit to a lesser extent, in other body fluids, such as sweat, vaginal material and semen. There is an exception in that α-amylase can be found to a greater extent in faeces, due to the presence of pancreatic amylases (21). However, it is possible to distinguish between saliva and faeces organoleptically (i.e. using visual and olfactory senses).

The α-amylase expression in vaginal material increases in post-menopausal women (22). Professional experience has shown that it is possible for α-amylase expression in post-menopausal vaginal material to be comparable to that of saliva (FSS).

Consequently, just like the LMG/KM test for blood, the phadebas test is a 'presumptive' test. However, unlike the test for blood, there is no additional visual-based test. Therefore, given the strongest possible result, it can be reported that 'in my opinion, the stain tested is most likely to be saliva'. In addition to the test itself, in forensic casework a certain amount of logic and common sense is also applied. For example, if a balaclava is recovered from a location near the scene of an armed robbery and the phadebas test reveals a strong rapid chemical reaction on the inside of the balaclava, then this corresponds to an area which would be in contact with the mouth if the balaclava is worn normally. Therefore, the location of the staining can add to the identification of the stain; however, given that it is not generally known what the activity of the balaclava was prior to recovery and the associated false positives, it can never be definitively reported that 'saliva was found' using the phadebas test.

Although it is rare to receive a case in which it is absolutely necessary to identify the presence of saliva, it is common to receive cases where the differentiation between saliva and another body fluid is required - such as between saliva and trace amount of blood, or between saliva and vaginal material. This may now be achieved using the forensic RNA analysis test developed as part of this study.

2.3. Semen

From an evidential value perspective, semen evidence is the strongest of the body fluids; even stronger than blood. If you consider finding semen on a high vaginal swab from an under-age girl, then this is extremely strong evidence and convictions have been obtained based on this evidence in conjunction with the associated DNA evidence.

Unlike blood and saliva, the presence of semen can be definitively identified by using histological techniques. Most commonly used is the haemotoxylin and eosin (H&E) test (23) which stains the nucleus and cytoplasm of the spermatozoa blue and pink, respectively. Microscopic identification of the presence of a single sperm cell is sufficient to confirm the presence of semen.

In order to confirm the presence of semen, it first needs to be located; so, in addition to the H&E test, there is a chemical test for semen that identifies the presence of acid phosphatase (AP) within semen (17). This test can be used to screen garments for semen prior to extraction for H&E staining. It can also be used as a spot test for the range of intimate swabs in order to prioritise the swabs for subsequent H&E staining.

AP is also present in other body fluids, including vaginal material; therefore, this test is more useful as a presumptive screening test, rather than as a tool for semen identification (17).

2.3.1. Intimate swabs

One of the issues with the identification of semen on intimate swabs is that these same swabs will also contain a significant amount of vaginal epithelia. This can have the effect of obscuring any spermatozoa present, particularly if there are trace amounts of semen present.

One protocol developed in the FSS resolved this by the use of the 'Pro-K' method which utilises proteinase K. This is regularly used as part of a lysis buffer for the extraction of DNA; it works by proteolysis of the cell membranes. However, spermatozoa has a proteinaceous coating which is resistant to the effect of proteinases; therefore, the presence of proteinase K only serves to break down the epithelial cells, leaving the spermatozoa relatively intact (it will have lost the tail). Following a brief period of centrifugation, the heavier spermatozoa forms a pellet and the epithelial debris remains in the supernatant. The supernatant can then be removed and retained as an 'epithelial fraction' and the pellet is then resuspended in 20µl of phosphate buffered saline (PBS), which is then spotted onto a slide prior to H&E staining. This method not only removes the epithelial cells, but also serves to concentrate any spermatozoa from the whole swab into a 20µl solution; thus increasing the chances of finding any spermatozoa present (FSS).

2.3.2. Prostate specific antigen

One of the problems associated with semen evidence are vasectomies or medical conditions, such as azoospermia. In such cases, no spermatozoa are present in the semen; thus, the presence of semen cannot be confirmed using the H&E method.

One test is available which identifies the presence of prostate specific antigen (PSA) - also known as p30 - within the seminal fluid (24). This is a protein that is

specific to the male genito-urinary tract. There are various forms of immunology-based tests that can be used to identify the presence of PSA, such as ELISA or cross-over immuno-electrophoresis.

This particular test can be somewhat controversial as, on rare occasions, PSA has been detected in female urine and breast milk. Although, it can be said that a high presence of PSA can be confirmatory for the presence of seminal fluid, the level at which the amount of PSA becomes confirmatory is rather flexible (24).

2.3.3. Vaginal material

Vaginal material is a significant body fluid in sexual offences; it can be recovered from penile swabs, from the outside of condoms and from any other objects inserted into the vagina. Currently, there exists no reliable test for the presence of vaginal material, although it is a good source of DNA material.

One test that was used to try and identify vaginal material was Lugol's test (18, 19) which tested for the presence of glycogen within vaginal material; however, its prevalence of false positives and false negatives rendered this test unreliable for forensic purposes.

2.4. Body fluid identification tests

From this it can be seen that there is a clear need for more definitive and more widely encompassing body fluid identification (BFID) tests. However, there is also a need to be able to associate the body fluid with the DNA profile so that it is possible to say with a high degree of certainty (if not definitively) that the DNA profile was obtained from the blood or the saliva, for example.

Using current methodology, any association between the body fluid and the DNA profile that can be made is one of logic and common sense. For example, if a heavily stained blood swab is submitted for DNA profiling and a well-amplified, clean, single-source DNA profile was obtained, then it is considered reasonable to assume that the DNA material which contributed to the DNA profile was obtained from the white blood cells. Consequently, this can be reported as: 'in my opinion, this DNA profile was obtained from the blood'. As stated previously, when dealing with abundant single source DNA, it can be relatively simple to interpret the data; however, like with DNA, when there is low-level or trace amounts of body fluid, or more than one type of body fluid present, then the interpretation becomes complex and it is in this area where the capability gap truly lies - an area which forensic RNA analysis could resolve.

In addition to improving the identification of the main body fluids, there is also a need to identify vaginal material.

It is felt that the best approach to developing a BFID test that can be associated with a DNA profile is to adopt a genetic strategy. This way, two tests can be carried out on the same sample, targeting the same type of material. Thus, the use of forensic RNA analysis was adopted as a way to further enhance the capability of the forensic biologist.

At the commencement of this period of study, much of the work in this area used gene expression studies of body fluid specific mRNA markers, with the earlier studies utilising gel electrophoresis and, later on, real-time PCR with Taqman chemistry (1, 25-34).

During the author's time in the FSS, the issue of using RNA for body fluid identification was discussed and whilst it was agreed that such a test would be useful, it was considered too expensive and too subtle for the police to appreciate and, thus, fund. Consequently, one of the initial strands of research in this area was to develop a cost-effective mRNA-based BFID test. This was considered to be crucial in order for this test to be adopted by commercial forensic science providers.

After looking at the costs of various chemistry systems required for real-time PCR, it was clear that SYBR Green chemistry was cheaper than the Taqman chemistry. In addition to this, the Taqman chemistry required labelled primers, whereas SYBR Green could be used with unlabelled primers (35). This represented a significant difference in the costs of the reagents required for testing, particularly for primers.

However, a fundamental disadvantage of SYBR Green was that it bound to any double-stranded DNA (35), which meant that not only was it necessary to demonstrate that the reverse transcription was successful, but that the unlabelled primers were sufficiently specific. This was addressed by using controls.

However, this was not considered a major issue, as forensic scientists in the UK tend to adopt a likelihood ratio approach (20, 36) in interpreting forensic genetic evidence, so as long as it could be demonstrated that it was more likely to be one body fluid rather than another, then this should suffice for forensic purposes; however, the courts and the investigating officers require much more definitive answers.

The third article in this portfolio was in relation to a study that was successful in that it distinguished blood and saliva using mRNA markers and SYBR Green chemistry. However, in order for the result to be sufficiently robust for use in a court

of law, quite a substantial number of controls were required. Since SYBR Green is bound to any double-stranded DNA, multiplexing could not be carried out. This meant that the cost of carrying out a single test was significantly higher than anticipated and was at least equal to (if not more than) a test using Taqman chemistry.

Connolly, J. and Williams, G. (2011) 'Evaluating an mRNA based body fluid identification test using SYBR green fluorescent dye and real-time PCR' *International Journal of Criminal Investigation* , 1 (4);177-185

Based upon the findings of the third article, the research strategy of the group changed to focus upon using Taqman chemistry. Following the ultimate aim of developing a test that is both cost-effective and allows for the association of the DNA profile with a particular body fluid, strategies for the co-isolation of DNA and RNA were devised.

As mRNA-based BFID tests are most likely to be of great benefit in trace amounts of body fluid, co-isolation techniques are crucial in order to minimise the volume of the sample required. The probative value of a DNA profile will almost always exceed the probative value of the BFID test, so if this test destroys the sample before a DNA profile can be obtained, then this would defeat the purpose of the investigation. The importance of this strategy is recognised by several research groups across the world. However, they have all been focusing on the separation of DNA and mRNA from the cellular material in to two separate phases (25, 28, 37). This would then lead to two separate analytical processes: one for DNA and one for mRNA. Whilst this is a great step forward, this dual channel process does come with

some disadvantages; namely, increased costs as there are two separate work channels to fund, increased opportunities for contamination and, finally, the DNA profile cannot be associated with the body fluid.

Consequently, a single channel simultaneous extraction technique was devised. This strategy represented a significant departure from other research groups in that it did not seek to separate the DNA and the mRNA out into two distinct phases, but keep them together. Initially, it was considered that aliquots could be taken from the extract and could then undergo subsequent analysis separately. However, this very quickly led to the question: 'why is it necessary to separate out the DNA and mRNA at all?'

Thus, this represented a major shift in the research strategy from real-time PCR analysis of mRNA to capillary electrophoretic fragment analysis. However, consideration of this strategy quickly identified a problem. The amplicon sizes of the mRNA fragments will be within the range of 100-400 bp, which is also the range of most STR markers utilised in forensic DNA profiling kits (38).

Consequently, attention turned to microRNAs (miRNA), which are very small fragments (~22-25 bp). By carrying out stem-loop reverse transcription (39), amplicons of ~60-70 bp could be generated, which is outside the fragments size range of STR analysis.

Scrutiny of the literature relating to miRNA revealed an additional advantage over mRNA: namely, its increased stability.

2.5. Perceived instability of mRNA

One of the main objections to the use of mRNA for forensic purposes has been that mRNA is considered to be highly unstable, due to the presence of ribonucleases within the cell (1). However, this is only true in active cells. In forensic casework, most stains arrive at the laboratory dried. Whenever a biological cell is dried, all intracellular processes stop, including that of the ribonucleases. Consequently, any mRNA still active at the point of drying is protected from any intrinsic degradation process. Indeed, a couple of studies have shown that RNA is capable of surviving in dried stains for long periods of time (up to 23 years) (40).

One of the advantages of using miRNA over mRNA is that miRNA is more stable than mRNA. This is due to two factors: firstly, its small size of ~22-25 bp, which makes it resistant to degradation; and, secondly, the copy number of miRNA is considerably higher than for mRNA, with a reported estimate of ~50,000 copies of miRNA per cell (41, 42).

The fourth article in this study provides proof for the concept that a single channel simultaneous analysis of DNA and miRNA is possible. This study demonstrated that a single electropherogram can be obtained that indicates a DNA profile and the body fluid origin of the said profile. This process had no physical separation of the DNA and miRNA phase at any point. Consequently, if a single source DNA profile is obtained and only miRNA markers specific to a particular body fluid are present, then that DNA profile can be far more closely associated with the said body fluid than was previously possible.

Van der Meer, D., Uchimoto, M. and Williams, G. (2012) Simultaneous analysis of microRNA and DNA for determining the body fluid origin of DNA profiles. *Journal of Forensic Sciences* (Accepted and in proof)

As part of the research group's long term strategy, low-level mixtures using this method will also be explored. One particular area to be explored is whether it is possible, in the event of a major-minor DNA profile, to associate a particular contributor to a particular body fluid. For example, a penile swab may give a major-minor DNA profile, with the victim as the major contributor and the suspect as the minor contributor. Given the nature of the sample, the suspect's DNA is accounted for and it is the victim's DNA profile that is of probative value. If miRNA analysis is carried out and it reveals the presence of skin cells and of saliva, then it can be reasonably assumed that at least some of the skin cells came from the suspect, given the nature of the sample. However, the victim's contribution could either be saliva or it could be skin cells alone (with the saliva coming from the suspect). If the miRNA result were to indicate a much higher amount of saliva than of skin cells, then it might be possible to say that the major DNA profile came from the saliva.

Based upon case work experience, it is felt that such a situation would be the exception rather than the rule; however, such a capability would be worth exploring and will be the subject of a future study.

3. Addressing the issue of consent in sexual assaults

Until this point, a number of capability gaps in forensic biology have been identified and strategies have been developed and are in progress that should resolve these gaps, or at least enhance the capability of the operational forensic biologist. However, there is still one major capability gap in forensic biology: the issue of consent in sexual assaults.

It is not currently within the realm of body fluid evidence or forensic genetics to address the issue of consent, nor can this be feasibly envisioned. However, there is a non-biological discipline which may resolve this capability gap: clothing damage analysis (43-48).

Although clothing damage analysis is not a sub-discipline of forensic biology, there is a correlation between the two, in that clothing damage analysis can be used to address a capability gap with forensic biology; namely, the issue of consent in alleged rape cases. Consequently, this research — by adopting a multi-disciplinary approach — falls under the category of the identification and resolution of capability gaps within forensic biology.

Following an accusation of rape, there can be (at least) three main defences:

1) That the alleged incident never actually took place.
2) That the alleged incident did take place, but the offender was someone else other than the suspect.
3) That the suspect did engage in sexual intercourse with the victim, but it was consensual.

Given the firmly established technique of DNA profiling and the enzymatic and histological identification of semen evidence, the first two defences are relatively easily accommodated within interpretative frameworks such as the Bayesian model of Case Assessment and Interpretation (20, 36). This basically asks two questions which are mutually exclusive and exhaustive. For all defences, the first question, or the prosecution hypothesis, would be the same: “What is the probability of finding semen that could have come from the suspect given that the suspect had sexual intercourse with the victim?”

For each of the possible defences there are correlating questions or defence hypotheses, which are:

1) What is the probability of finding semen that could have come from the suspect, given that no sexual intercourse took place?
2) What is the probability of finding semen that could have come from the suspect, given that someone else other than the suspect had sexual intercourse with the victim?
3) What is the probability of finding semen that could have come from the suspect, given that the suspect had sexual intercourse with the victim?

The difference in the probability value (expressed as a likelihood ratio) represents the strength of the evidence. From looking at the first two defence hypotheses, it can be clearly seen that semen evidence can sufficiently differentiate between the prosecution hypothesis and the first defence hypothesis and, in conjunction with DNA evidence, can sufficiently differentiate between the prosecution hypothesis and the second defence hypothesis.

However, the prosecution hypothesis and the third defence hypothesis rests on the same question and, consequently, the probability value will be the same and thus give a likelihood ratio of 1, which represents inconclusive evidence, or no support for either hypothesis.

As a consequence of this, and the increased awareness of forensic evidence, most defences against rape allegations are now based upon issues of consent. Although the capability to distinguish between saliva and vaginal material, as discussed previously, can help to address this issue, it can only address those cases of 'withdrawn consent'.

Withdrawn consent is when there is initial consensual sexual activity; for example, kissing and heavy petting or oral intercourse. Such initial activity may then progress on to vaginal intercourse, at which point the complainant has withdrawn her consent. One common example of withdrawn consent is when consensual vaginal intercourse is taking place and then the defendant proceeds onto anal intercourse, at which point consent may no longer be forthcoming.

Clothing damage analysis is a discipline in which information about an incident can be gleaned — typically, by assessing damage in order to determine whether it is caused by normal wear and tear or by the application of force (45) — and can be useful within a Bayesian framework and within the right case circumstances.

Although the field of clothing damage analysis falls outside the discipline within this study, the fifth article explores how this discipline can resolve a capability gap within forensic biology.

This article was published in response to a case in which the author was asked to provide technical advice. The case was of a sexual assault between two parties who had previously engaged in consensual intercourse. During the alleged incident, it is

claimed that the defendant removed the complainant's bra by force, thereby causing significant damage to the garment. However, the hook and eye fastening was intact, which was presented by the defendant's legal team as counter-evidence to the claim.

The defendant's version of the events was that intercourse took place, but that it was consensual. Thus, the value of any biological evidence, such as semen and a DNA profile matching that of the defendant, was negated. The defendant then went on to state that he never caused the damage observed. Consequently, the defence postulated that the damage must have been caused by the complainant herself in an effort to support a false claim of sexual assault, pointing to the fact that the hook and eye fastening on the reverse of the bra was intact.

Williams, G. and Haider, I. (2012) 'Differentiating Between Genuine Damage and Falsified Damage to a Garment Following an Alleged Sexual Assault', *Journal of Forensic Sciences*. (Accepted)

This study empirically demonstrated that it was possible to cause damage to a bra, to the extent that the cups are separated, without causing any damage to the hook and eye fastening. This research had quite an impact as it challenged popular preconceptions and demonstrated the importance of expert input into a case.

4. Discussion

Overall, a number of capability gaps within forensic science were identified, based upon professional experience and correspondence with various practitioners and forensic and policing organisations. A number of such gaps were selected for attempted resolution; namely, the issue of low-level mixed DNA samples, the issue of body fluid identification and subsequent association with a DNA profile, and the issue of consent in sexual offences.

The first issue was a very complex issue and is the subject of many research strategies across the globe. A literature review was carried out in order to explore what strategies were being developed; the field of whole genome amplification appeared to be a promising strategy and a full literature review was carried out and published (Article 1). From this, it was possible to compare and contrast a number of WGA strategies and develop some hypotheses.

In retrospect, even though this article focused on WGA, it may have been useful to discuss recent non-WGA techniques that may address issues of low level DNA, such as mini-STRs (63, 71).

One of these hypotheses — that the use of multiple displacement amplification (MDA) in conjunction with a macro-molecular crowder may improve the mixing ratio of DNA mixtures — was explored (Article 2).

It was found that both MDA and MDA with a macro-molecular crowder could significantly improve the DNA mixtures, but only at certain mixing ratios. So whilst from an academic point of view this was an interesting result, within the context of this study these results are insufficient for the purpose of resolving capability gaps in forensic biology given the unknown nature of the sample prior to analysis.

In reviewing this article at a later stage, the value of this research is limited when considering whether it could resolve the issue of low level mixtures. Broadly speaking, it would not – at least for evidential purposes. The addition of another amplification step would complicate the result and would be subject to further challenges in a court of law. There is also the issue of appearing to modify a sample prior to analysis. This may be considered as 'tampering' with the evidence as any analysis is supposed to accurately represent the sample. Thus it remains a possibility that any results obtained post-WGA step maybe inadmissible in a court of law. Nevertheless, it may also be possible that such a strategy would be of intelligence value; in that information may be obtained that would be of interest to the investigating team. However, based on these results, it is likely that the sample extract (~150µl) would be depleted before crowded MDA would be considered.

The second issue of developing a more definitive body fluid identification test has been addressed by a large number of publications, including Article 3. This article demonstrated how a SYBR Green-based BFID test was developed in an attempt to provide a more cost-effective option.

Although the capability gap relating to cost to the police services and forensic science providers was identified, this was not the driving motivation for the development of a SYBR green based test, but rather it was the cost of doing such tests in the lab. Whilst the SYBR green test could be used to differentiate between blood and saliva, thus progressing towards the goal of addressing the capability gap of body fluid identification, ultimately, it was not a cost effective solution; due to the requirements for further controls and the inability to multiplex the primers.

This study did demonstrate that the use of SYBR green chemistry has a minimal role in further research in BFID, despite having further diagnostic capability in the

guise of melting curve analysis. As a direct consequence of this study, all future research in BFID in the forensic genetics research group will be using the Taqman chemistry.

This issue was expanded to include the association of the body fluid with a DNA profile and this was addressed by Article 4 in which the ‘proof of principle’ was demonstrated for a single channel simultaneous analysis of both DNA and microRNA.

Of the five articles included in this portfolio, this one is the most groundbreaking one. Not only does it address the issue of body fluid identification, it also addresses the issue of associating a DNA profile with a body fluid and by developing a single stream strategy it also addresses the issue of reducing costs. It also proposes a completely different tactic to carrying out forensic RNA analysis in a way that is more likely to be taken up by forensic science providers.

However, this is a proof of concept paper that exhibits some limitations. Firstly it is that the miRNA amplicons are rather close to the low molecular weight artefacts. However, this can be overcome by modifying the artificial stem-loop structure and labelling with the appropriate dye.

Secondly, there is the issue of confirming that the miRNA amplicons are indeed miRNA as opposed to genomic DNA. This has been overcome by the use of an artificial stem loop primer, which is not present in nature. Also, the gene that is transcribed into miRNA is actually larger than the miRNA molecule (41). However, this needs to be emphasised and will be addressed in a future paper.

The final issue was that of addressing consent within sexual assaults. Although clothing damage analysis is not a biological discipline, it can be used to resolve capability. The inclusion of Article 5 demonstrates the importance of a multi-

disciplinary approach to case work-based problems. This article also demonstrates the importance of carrying out experiments and reconstruction studies to support case work theories.

This article demonstrated a clear point, in that popular expectations and, indeed, those of the forensic scientists can be incorrect. In this particular case, the investigating officer, the legal team and the scientists involved all felt that it was not possible to use force to remove a bra without causing damage to the hook and eye fastening. This simple and empirical study clearly demonstrated that this was erroneous.

However, this study lacked rigour in that no real data analysis was carried out and a small sample set was utilised. Further study in this area would include the establishment of further parameters, a much wider sample set and a development of a quantifiable performance indicator. However, there are currently no plans to progress this research further.

5. Original contributions to the field

This study made, to a greater or lesser extent, a number of original contributions:

- Development of hypotheses derived from comparing and contrasting published research in whole genome amplification **(Article 1)**.

- A more meaningful analysis (using mixing ratios) of the effect of multiple displacement amplification, with and without the macromolecular crowding agent, which demonstrated a very limited improvement in the analysis of DNA mixtures **(Article 2)**.

- The development of a SYBR Green-based body fluid identification test capable of distinguishing between trace amounts of blood and saliva **(Article 3)**.

- The development of a single channel simultaneous analysis of DNA and microRNA, enabling a more definitive identification of a body fluid and a much greater association with a DNA profile than previously possible **(Article 4)**.

- Demonstration of how a multi-disciplinary approach (such as clothing damage analysis) can help resolve other capability gaps within forensic biology **(Article 5)**.

6. Contribution of author to each publication included in submission

Article 1 - The Forensic Applications of Whole Genome Amplification

Sole contributor – Graham Williams

Article 2 - Evaluating the effect of multiple displacement amplification upon low-level multiple source DNA profiles

Corresponding author – Graham Williams

Project conception – Graham Williams

Experimental Design – Graham Williams and Kimberley Bexon

Laboratory Analysis – Kimberley Bexon (under Graham Williams' supervision)

Published data analysis – Graham Williams

Production of article – Graham Williams (proofread by Kimberley Bexon)

Article 3 - Evaluating an mRNA based body fluid identification test using SYBR green fluorescent dye and real-time PCR

Corresponding author – Graham Williams

Project conception – Graham Williams

Experimental Design – Graham Williams and Jo-Ann Connolly

Laboratory Analysis – Jo-Ann Connolly (under Graham Williams' supervision)

Published data analysis – Jo-Ann Connolly (and double checked by Graham Williams)

Production of article and discussion – Graham Williams

Article 4 - Simultaneous analysis of microRNA and DNA for determining the body fluid origin of DNA profiles

Corresponding author – Graham Williams

Project conception – Graham Williams

Experimental design – Graham Williams and Donny van der Meer

Laboratory analysis – Donny van der Meer and Mari Uchimoto (under Graham Williams' supervision)

Published data analysis – Graham Williams (and double checked by Donny van der Meer)

Production of article – Graham Williams

Article 5 - Differentiating Between Genuine Damage and Falsified Damage to a Garment Following an Alleged Sexual Assault

Corresponding author – Graham Williams

Project conception – Graham Williams

Experimental design – Graham Williams

Laboratory analysis – Imran Haider (under Graham Williams' supervision)

Published data analysis – Graham Williams

Production of article – Graham Williams

7. Future work

This project represents a significant portion of a long-term research strategy designed to significantly enhance the capability of the operational forensic biologist. Such future strategy is derived from the professional experience of the author. This includes:

- Characterisation of DNA mixtures pre- and post-STR analysis, with a view to identifying strategies to maximise the information obtained. The next step is to explore an alternative WGA technique; namely, modified improved PEP-PCR.

- Improvement of the single channel simultaneous analytical technique to include a wider range of body fluids, with initial focus on skin cell specific microRNA markers and vaginal material specific microRNA markers.

- Refinement of the stem-loop reverse transcription stage in order to optimise the size of the miRNA amplicons.

- Development of a complementary strand in clothing damage analysis, with a view to addressing consent issues.

8. Summary

In summary, since the start of this project in 2008, the DNA and RNA analysis capacity of the research group has been significantly enhanced through the acquisition of various genetic analysis instruments and a long-term research strategy has been developed.

This portfolio of published articles represents the fruits of this initial work and heralds the start of a series of further publications over the next ten years which will investigate further areas, such an improving the BFID tests and the novel single channel simultaneous analytical technique developed at the University of Huddersfield.

A multi-disciplinary approach is also being adopted, such as in the use of clothing damage analysis. Future work will incorporate the high capability levels of chemical analysis, such as Raman Spectroscopy, within the School of Applied Sciences.

9. Latest update to literature

This section represents the latest publications in 2012 up to the point of the portfolio submission (Submission date: July 2012).

9.1. Whole genome amplification

A study by Tate *et al* (2012) re-explored an earlier version of MDA called rolling circle amplification (RCA). Since RCA is typically used for circular genetic structures (such as mtDNA), the authors investigated the use of ssDNA ligase, which served to form single-stranded circular genetic structures from linear templates. According to the authors, such techniques are regularly used to detect specific nucleic acid sequences for enhanced immunoassays. The authors applied this technique to degraded DNA samples (characterised by fragmentation) prior to carrying out RCA. The authors found that the use of ssDNA ligase provided a lower template (prior to amplification) than not using this method. Consequently, although an alternative technique was developed, it did not provide a superior response to linear template amplification. The term 'linear template' was used to differentiate it from ligated samples, referred to as 'circular templates' (49).

Another study by Han *et al* (2012) compared two commercially available MDA kits, namely GenomiPhi (GE Life sciences) and Repli-G (Qiagen). The aim of this study was to evaluate kits for use in medical study, namely for sequencing in cystic fibrosis patients. Whilst this does not have the forensic issue in mind, the comparison between two commercially available kits was of interest. This showed that the GenomiPhi kit was the better kit of the two, in terms of both yield and consistency. This contradicted the in-house study (which was preliminary and unpublished) conducted within the forensic genetics research group, in which higher yields were obtained using the Repli-G kit. However, in this study no STR analysis was carried out, and the real-time PCR quantification was targeting

specific markers relating to cystic fibrosis, rather than general human DNA markers as with Quantiplex or Quantifiler. Sequencing was then carried out, as opposed to fragment analysis, targeting STR markers (50). Consequently, the outcome of this study did not directly contradict our own findings, but it does warrant closer investigation, which will form part of the future strategy of the research group.

A study by Kang *et al* (2011) took a slightly different approach to adopting WGA; rather than using it for pre-amplification prior to PCR, it was used to develop a very precise and very sensitive method of detection down to as low as 400 fg (femtogram = 1 $x10^{-15}$ g). The WGA technique utilised was DOP-PCR, and the technique was referred to as real-time DOP-PCR (rtDOP-PCR). However, this technique is being promoted as a universal quantification technique as it is non-species specific; thus, at this stage, it is not yet suitable for human DNA profiling (51). If this technique could be adapted to be human specific, then this could represent a significant improvement on DNA quantification procedures. If this technique could also be harnessed for forensic RNA analysis, it could significantly strengthen body fluid identification capabilities. This will be explored as part of the future strategy for the RNA strand of the forensic genetics research group.

Quite a number of publications have been published that have utilised various WGA techniques, but other than the ones already mentioned, none of them discussed the use of WGA within the context of forensics. However, the non-forensic literature will continue to be scrutinised as it may be applicable to forensics, such as the Kang study already discussed.

9.2. DNA mixtures

Following a review of recent publications (in 2012), it seems that no-one is really exploring techniques to improve DNA mixtures. However, a small number of studies have explored the statistical interpretation of mixtures and strategies in order to make use of DNA mixtures.

For example, studies by Chung *et al* (2011) and Chung *et al* (2012) have examined the use of familial searching for two person mixtures. Familial searching using single source profiles is relatively simple, but two person mixtures can introduce an element of uncertainty. Chung *et al* (2011, 2012) proposed a strategy in which the profiles derived from a two person mixture are prioritized (53, 54).

Another study by Pfeifer *et al* (2012) investigated various mixture interpretation strategies; not just on one set of STR markers, but they also compared four different kits, including a non-commercially available kit. Both the consensus interpretation and the composite interpretation were applied, and the resulting profile was then compared with the original profile. The authors recommended that the consensus approach was more reliable, but accepted that there were times when the composite approach would be more appropriate. One observation that was interesting was the use of different kits. Even though the kits were nominally targeting the same markers, the primer designs meant that the amplicon lengths could vary (53). From this finding it may be inferred that when conducting a second run for low template analysis (as is standard), the use of a different kit may be more appropriate. Although this particular strategy is not currently one of the strands of the work of the research group, it is one that can be investigated rather quickly; however, the budgetary aspect may affect this.

9.3. Forensic RNA analysis

There are numerous publications being published relating to various aspects of messenger RNA profiling, including:

A study by Lindenbergh *et al* (2012) in which a 19 mRNA marker multiplexed RT-PCR/CE assay was developed and validated with a full body fluid panel. This method included the co-isolation of DNA and mRNA, thus DNA profiles can be obtained. The authors acknowledged that one difficulty will be the reporting of the results to criminal

justice professionals. This is a highly significant study that is strongly supported by the Netherlands scientific community (54). However, the use of such a large BFID panel will make the test rather expensive and complex.

This is significantly different from the approach taken within this submission, in that the use of microRNA is now the focus of the study, rather than mRNA. Another difference in approach is that one of the aims of this submission is to develop a single stream capability of DNA and microRNA, resulting in a single EPG. The development of the test in the Lindenbergh *et al* study will not allow for the capability proposed in Article 4, despite the inclusion of the co-isolation step.

A study by Akutsu *et al* (2012) explored the use of bacterial RNA (*Lactobaccilus* strands) to identify the presence of vaginal material. This is a preliminary study, and the authors stated that further study would be required. One area of particular interest in this study is that the authors carried out standard DNA extraction of the intimate swabs using the Qiagen DNA mini kit and found that they were still able to detect the bacterial RNA (55). This supports our findings (as in Article 4), in that RNA is retained following DNA extraction, and vice versa.

Whilst the use of bacterial RNA analysis on vaginal material is of great interest, the authors did state that they also detected the bacterial RNA in female urine, and considered that this could be a by-product of contamination prior to sample recovery (55). This is a major issue in sexual assaults, in that there will almost always be another body fluid present that could account for those findings, thus having a test just for vaginal material alone may lack the robustness required for a court of law.

There is also quite an effort to address the current incapability to identify vaginal material in a number of studies published in 2012, with Hanson *et al* (2012) exploring highly specific vaginal secretions (material) mRNA markers (56), and a number of studies taking a

similar approach to Akutsu *et al* (55), by exploring the bacterial route such as the study on the microbial signature of vaginal 'fluid' by Giampaoli *et al* (2012), and a review by Hou *et al* (2012) on microbial forensics (57, 58).

As noted above, there are different names relating to vaginal material, vaginal secretions or vaginal fluids. The use of 'vaginal material' is preferred as it is a more encompassing phrase, as vaginal material may contain non-secretory or non-fluidic elements (such as epithelial cells).

No studies relating to forensic microRNA analysis have been published in 2012 to date (other than those by the author of this submission). However, numerous studies explored the phenomenon of microRNA in a non-forensic context, particularly as there is increasingly strong evidence that microRNA have a role in cancer formation. One particularly useful review in 2012 (Pritchard *et al*) summarised the option for microRNA analysis, and offered some suggestions for testing and interpretation of results. Interestingly, however, capillary electrophoretic analysis of fragment size was not discussed. However, the topics of quantitation (qRT-PCR and microarrays) and sequencing (RNA-seq) were raised. A more easily understood explanation of stem-loop reverse transcription was also included (59).

9.4. Clothing damage analysis

Most recent publications relating to clothing damage tended to be from either a textiles engineering perspective or from a quality control perspective. As well as Article 5, there is a study by Mitchell *et al* (2012) which explores the effects of burial on fabrics (60), and another study exploring the effect clothing had on wound morphology was carried out by Ferllini *et al* (2012) (61).

In relation to sexual assaults, there is one study by Dann *et al* (2011) which explored the ease of tearing knicker fabric in response to the concept of false allegations. Although a lot of work has been done in this study, it is still at a preliminary stage, so the information

provided is rather fundamental. One strand of information that was revealed was that laundered knicker fabrics were significantly easier to tear (62).

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