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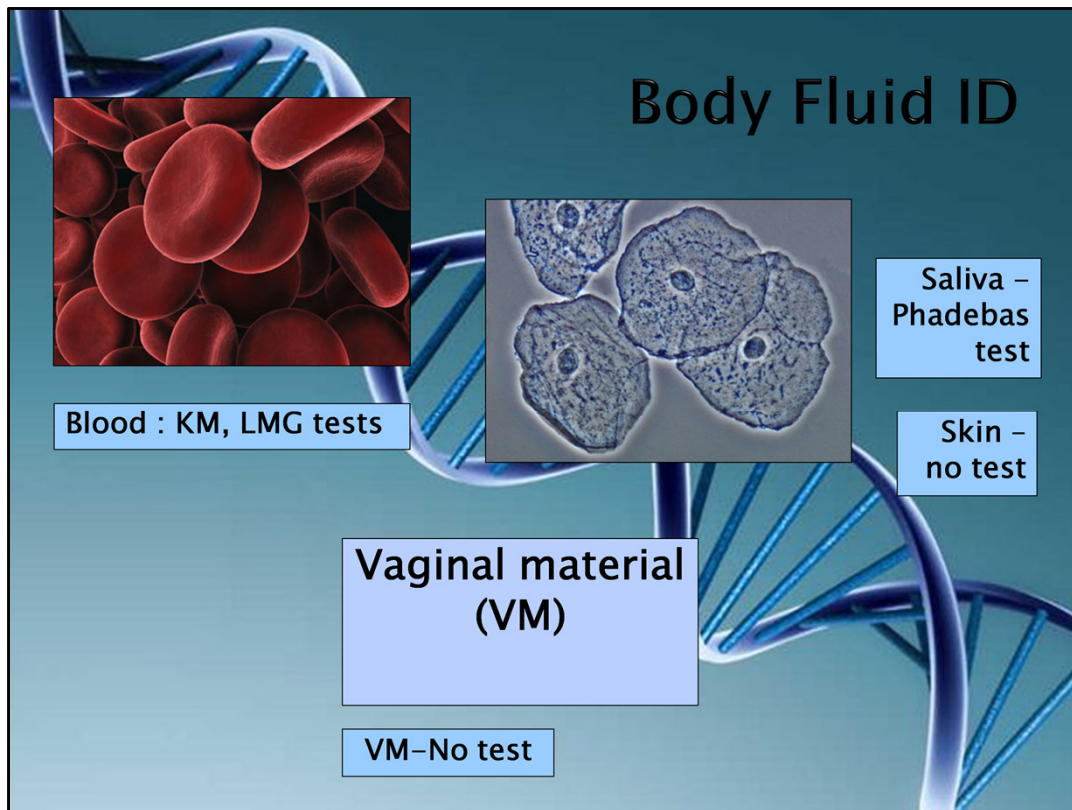
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Single channel simultaneous analysis of DNA and microRNA

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One of the main issues surrounding our area of work is the identification of body fluids and its association to a DNA profile.

At the moment the tests that are available for blood include the KM and LMG test. These tests target the peroxidase present in heme in the blood.

For saliva, there is a test called the phadebas test. This test targets α -amylase in saliva, note DNA/RNA is present in epithelial cells not saliva itself.

There is no test for distinguishing between skin cells or VM

One of the main things that all of these body fluids have in common is that there is no single test that will distinguish between them all.

This is where microRNA becomes useful.

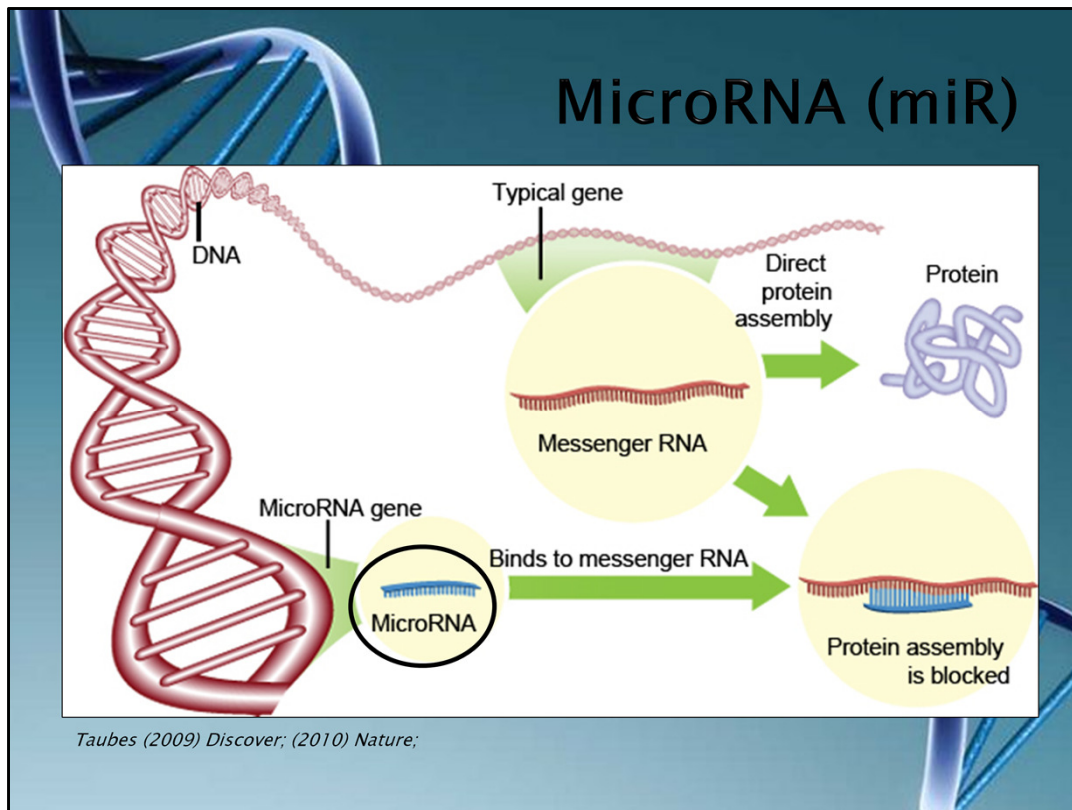
So this is where microRNA comes in.

(KM –phenolphthalian added then hydrogen peroxide. Presence of blood, phenolphthalian oxidise and cause a colour change. False positives ie. horse radish)

(LMG – leucomalachite green in place of phenolphthalian)

(Phadebas-alpha amylase cause a colour change)

(ELISA-enzyme linked immunosorbent assay – distinguish different amylase ie pancrease vs bacterial.



So what is microRNA?

MicroRNA are short sequences about 22 nucleotides in length.

They are found in non-coding regions of DNA, or areas not containing genetic information.

Here is a diagram showing a typical gene.

DNA holds information for the formation, function and maintenance of an organism.

How is this done?

DNA will be transcribed into messenger RNA.

mRNA is then translated into complementary DNA via enzymes and primers.

cDNA will then aid in the formation of proteins, which will have a specific role in the body.

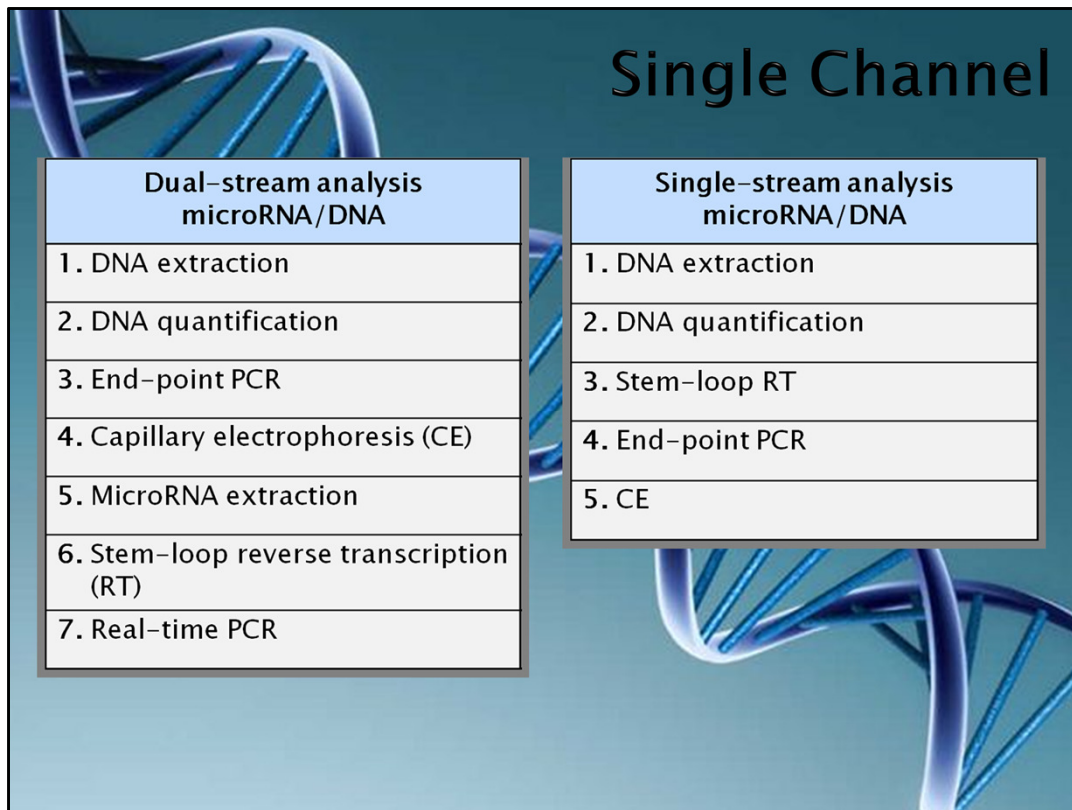
MicroRNA will come in during the post-transcription pre-translational stage.

It will bind to the complementary strand of mRNA.

Thus, blocking the formation of proteins.

How is this analyzed?

(non-coding regions – introns are spliced “cut” post-transcription)



Current research in this area work with two separate work streams – one for DNA and one for RNA.

DNA side – it goes through extraction, this is then quantified a human id kit, it is then amplified via PCR and then undergoes CE where a DNA profile is obtained.

RNA side – it is also extracted, no quant step no kit available, it then goes stem-loop reverse transcription which I'll explain later, then real-time PCR where relative miR expression is determined.

Drawbacks of this method include:

Increase time and reagent consumption

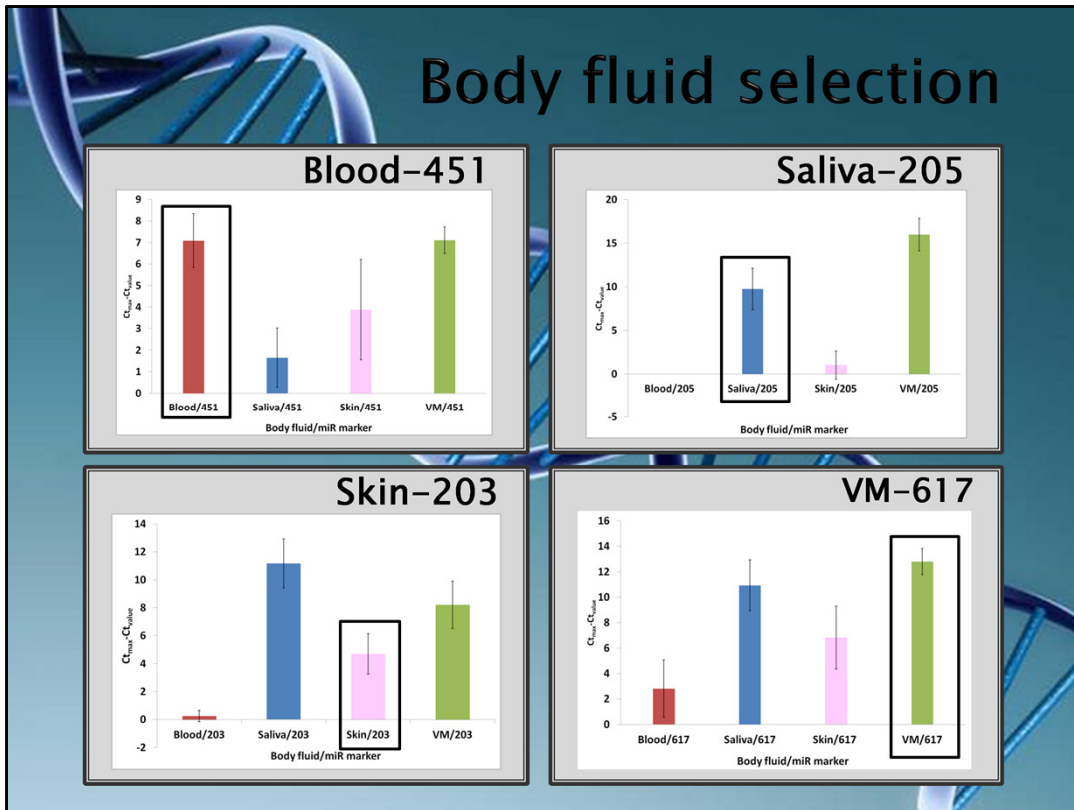
Increased opportunities of contamination

Limited association between body fluid and profile.

Our aim is to develop a single work stream during which both DNA and RNA are analysed.

DNA/RNA – DNA extraction because we wanted a method that introduced the

least amount of modification on the DNA profile, we then took a fraction out to quantify the DNA using human ID kit, performed stem-loop RT, PCR and capillary electrophoresis.



- For this procedure we needed to select some body fluids.

We chose blood, saliva, skin and VM

Blood-451, Saliva-205, Skin-203, VM-617

- Here I've shown the specificity of each marker between each body fluid.

451, blood – is significantly expressed in blood, it is significantly different to saliva and skin, but not so much VM will explain later.

205, saliva – is also significantly expressed when compared to blood and skin however not so much with VM.

203, skin – significant difference between blood and skin, but not so much saliva and VM.

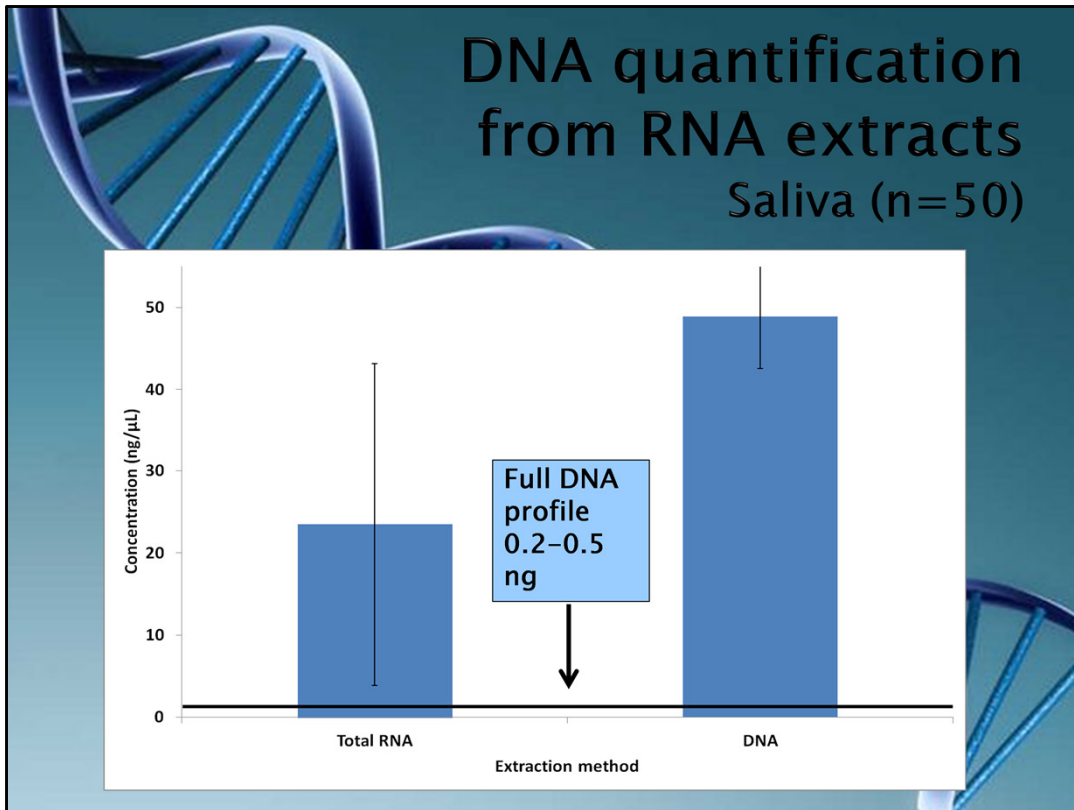
617, VM – is significantly expressed in VM, it is significantly different in blood and skin, but not so much saliva.

- Summary:

In the case of blood and VM in 451 we could not distinguish the two. However when paired with 617 this should be possible.

Saliva, skin and VM it was difficult to distinguish the three. This is possibly due to the fact they are structurally very similar ie. epithelial cells, mucous etc. So we

are working on adding additional markers for each.



We then moved onto extraction.

We see if we could get DNA from an RNA extraction.

Saliva was used for this study.

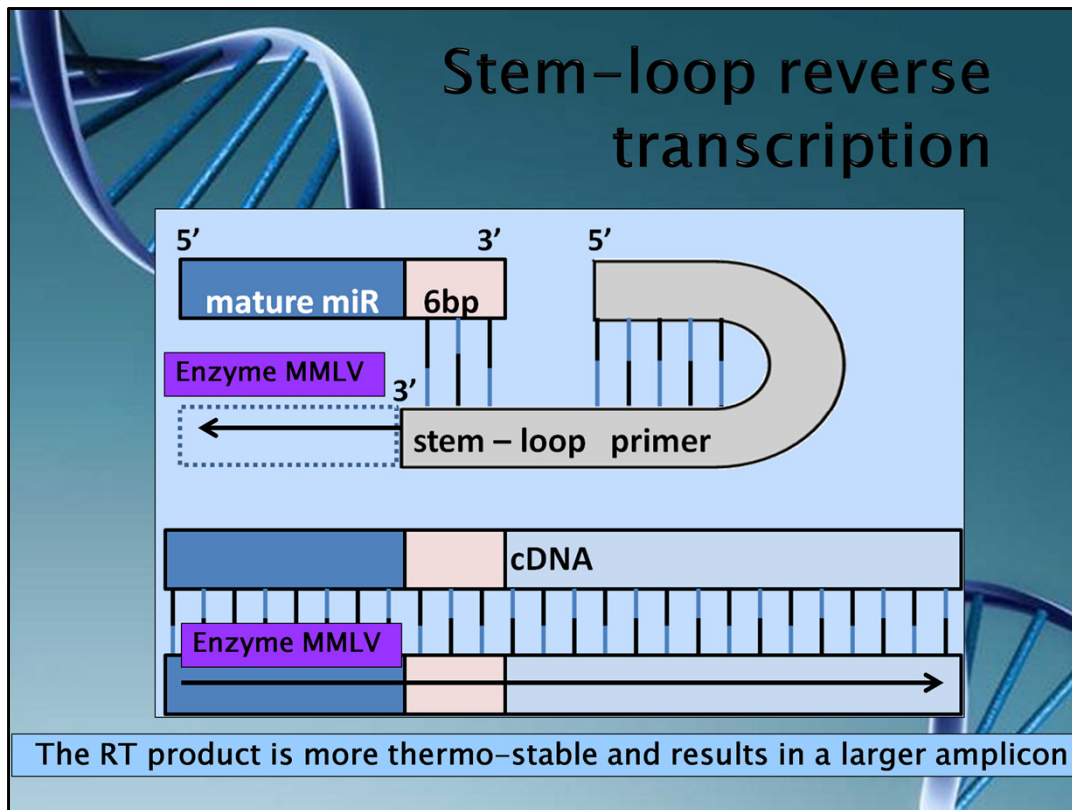
Two commercially available kits from QIAGEN

DNA extraction gave the most DNA, expected kit is optimised for this.

Total RNA also gave DNA.

Line is indicative of the amount required to obtain a full DNA profile.

Concluded we could use either.



First of all what is reverse transcription

Process by which mRNA ss converted into ds DNA via primers and MMLV

With stem-loop reverse transcription different:

Because mature miR is so small primers cannot attach to make an accurate sequence

Instead a stem-loop primer is used

This primer is artificial and designed has a 6bp overhang

When introduced to the mature miR the overhang will bind to the last 6bp mature miR

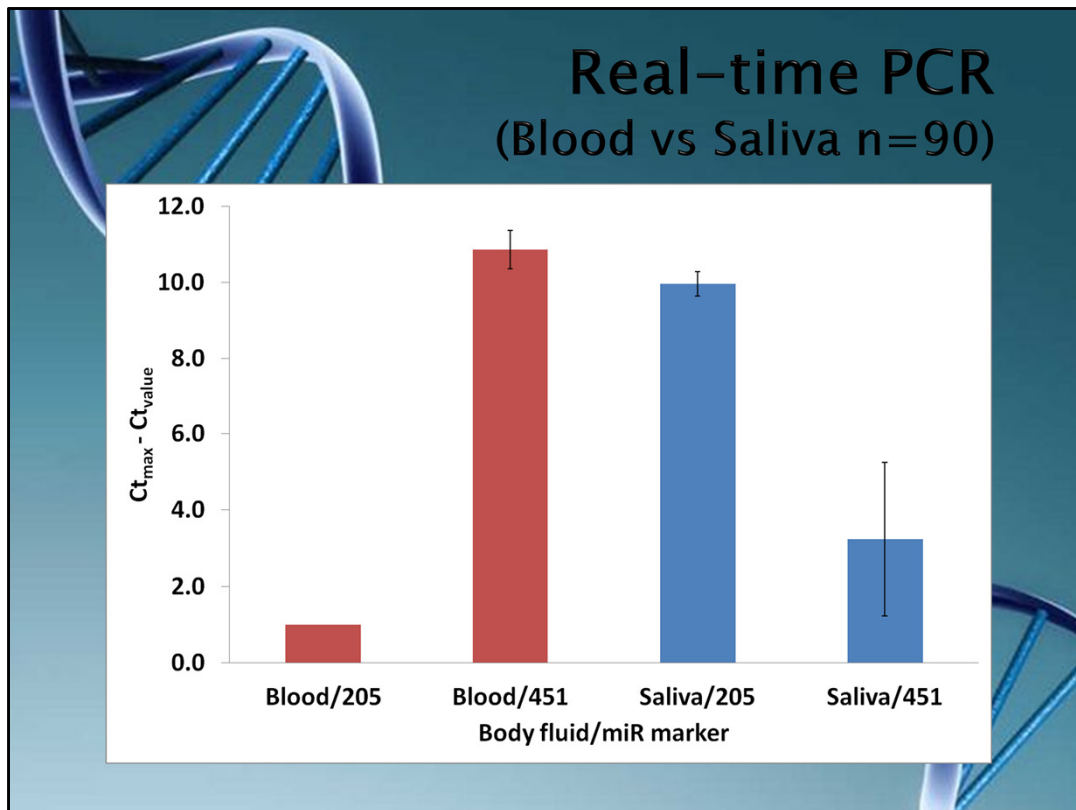
The 5' of the primer is designed to overlap onto itself forming a primer dimer

This extended using MMLV and cDNA sequence is formed.

Denatured and the stem-loop structure opens up.

Enzyme comes in again and completes the cDNA structure

The resulting product is a more stable and the amplicon is larger.



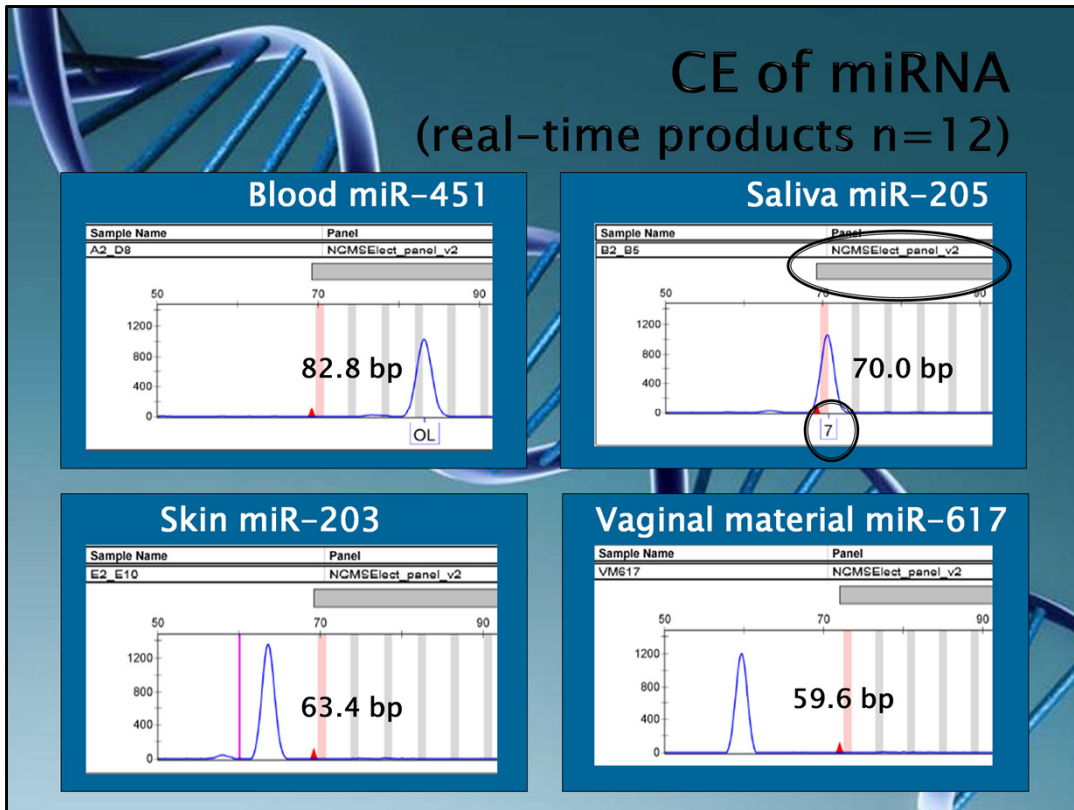
We then moved onto real-time PCR:

Determine the specificity of blood and saliva to miR markers 451 and 205

451 for blood is more significantly expressed than 205.

The same pattern was seen for 205 for saliva.

From this we could distinguish between blood and saliva



We then moved onto capillary electrophoresis miR we eventually be combining it with DNA profiling:

Used real-time PCR products for each of the body fluids, blood saliva skin and VM

Blood 451 – avg bp size 82.8, saliva 205 – bp 70, skin 203 – 63.4, vm bp 59.6

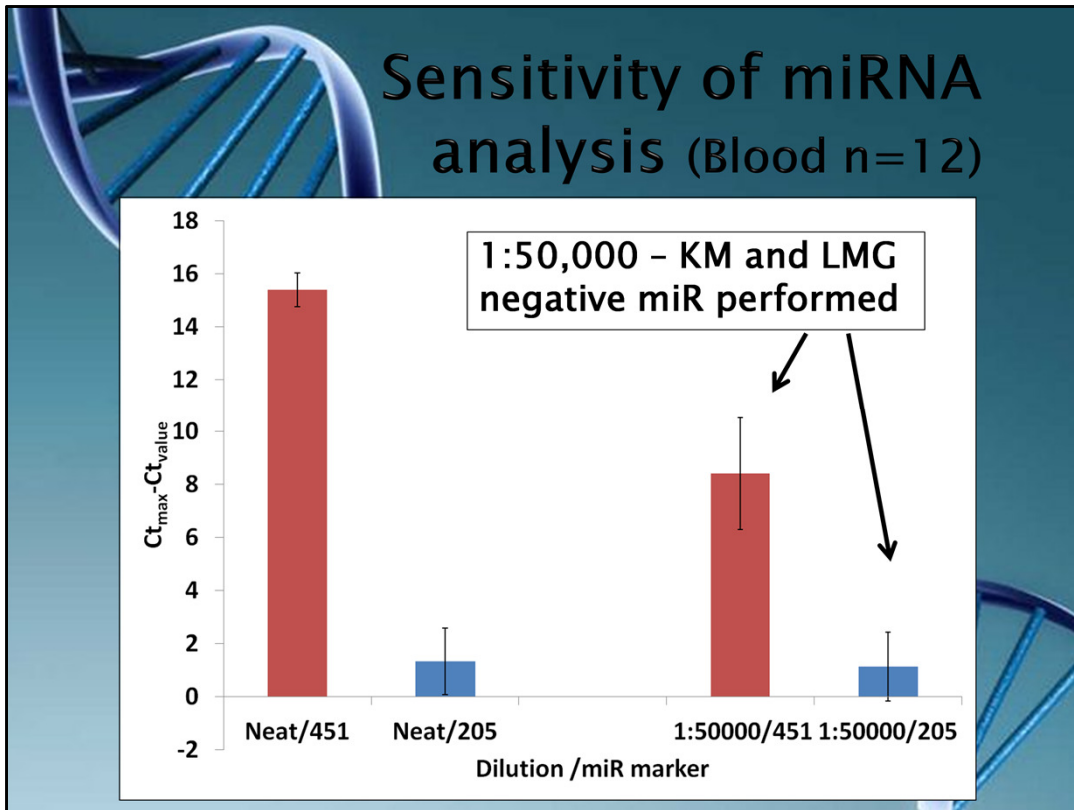
Each was very different and the variation between individuals was very small ranging between 0.2-0.4 bp

You can see a grey window above the peaks.

This is where part of the DNA profile begins.

To resolve this we can simply put the markers onto a different channel.

(CE- DNA negatively charged due to phosphate group RNA cations migrate to anions)
(Electroosmotic flow -



The sensitivity of presumptive tests can vary greatly

Compare the sensitivity of miR analysis using blood 451 and 205

1:10, 1:100, 1:1000, 1:10,000, 1:100,000

Found at 1:50:000 KM LMG negative, performed miR analysis

Fresh blood stain was included.

Results:

Fresh sample gave significant expression of 451 in blood

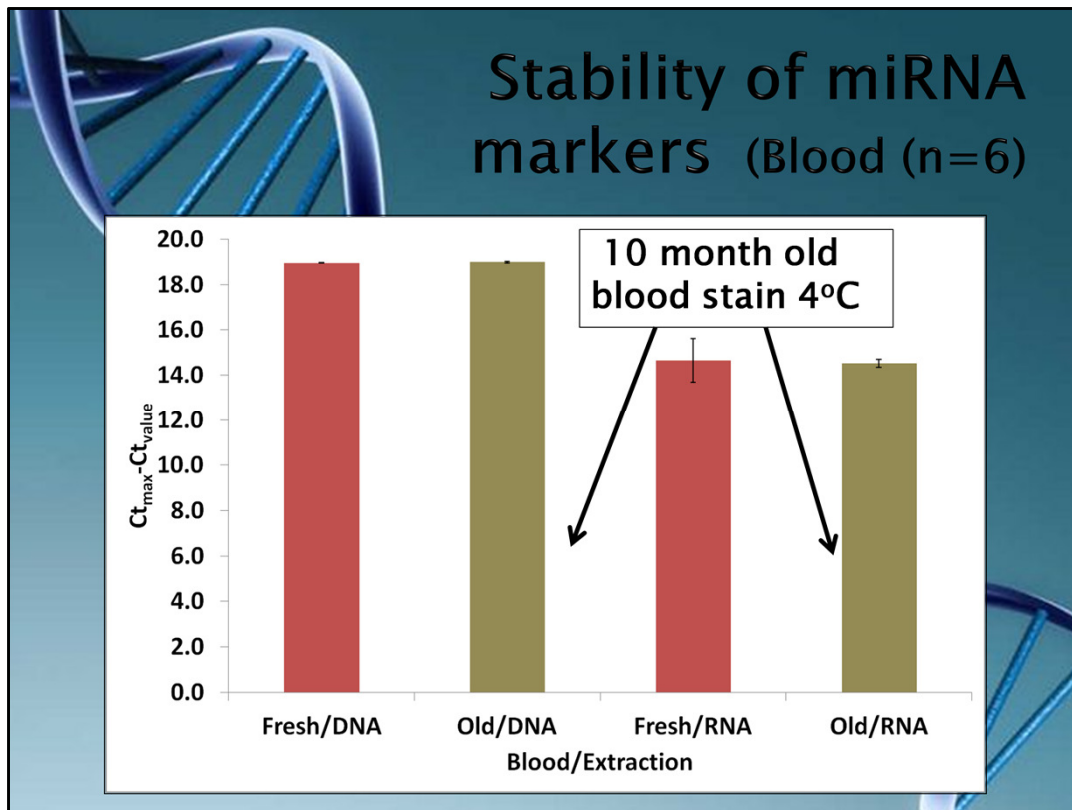
1:50,000 in blood also showed significant expression

Error is greater but that is expected with the dilution

Further work

Interesting see LOD miR analysis

(LOD KM, LMG: 1 in 100,000 to 1 in 1,000,000)



In the past RNA has not been used for BFID due to its perceived instability.

However recent research has shown its mRNA stability of up to 23 years in a blood stain.

We wanted to test the stability of microRNA.

In our fridge we found 10 month stain stored at 4 C.

Performed a DNA and total RNA extraction on this

We also performed it on a fresh blood stain

We used blood specific miR 451.

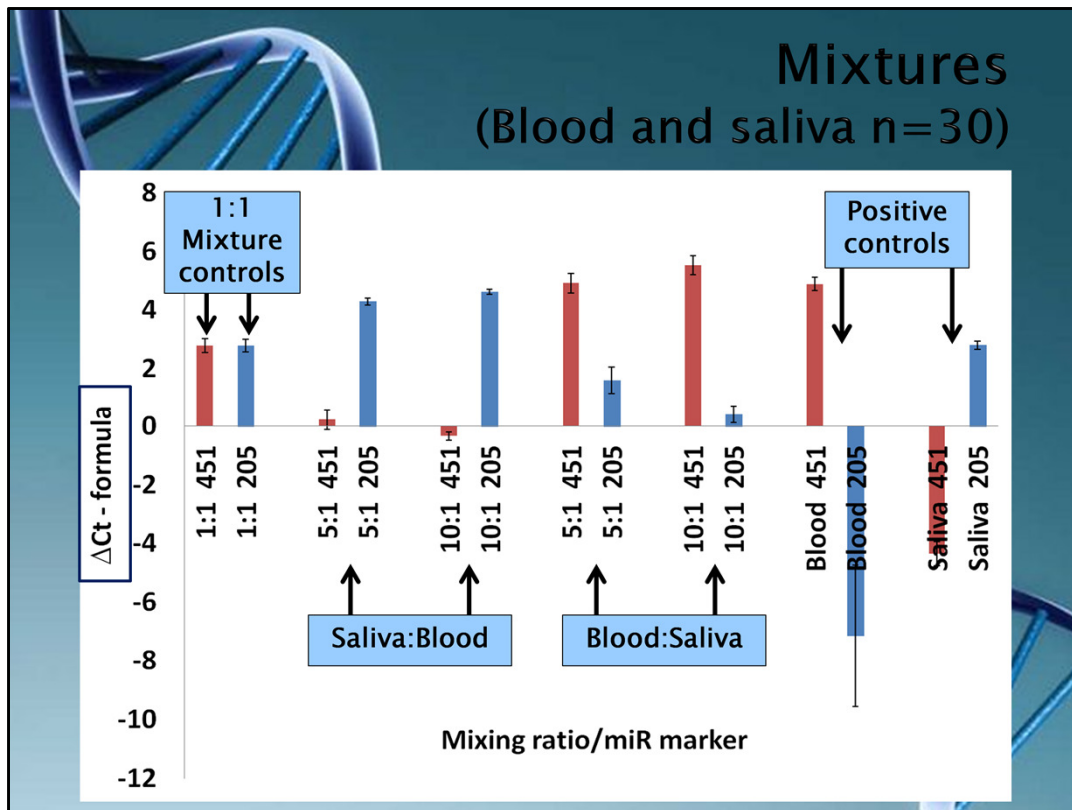
What we found:

Results showed significant expression of miR 451 in all extracts.

No difference the fresh or old extracts for either method.

Possibly due to the fact the stain was dry preserving the genetic material.

Will need to explore different conditions to see the affects.



Experiment: mixtures will often encounter more than one body fluid

Mixture of blood and saliva, F and M, ratios 1:1, 1:5, 1:10

Positive controls blood and saliva, specific expression to their respective markers

1:1 mixture controls using 451 and 205, not perfect

Applied a formula to normalize the results

Mixtures 5 saliva:1 blood – saliva is more expressed

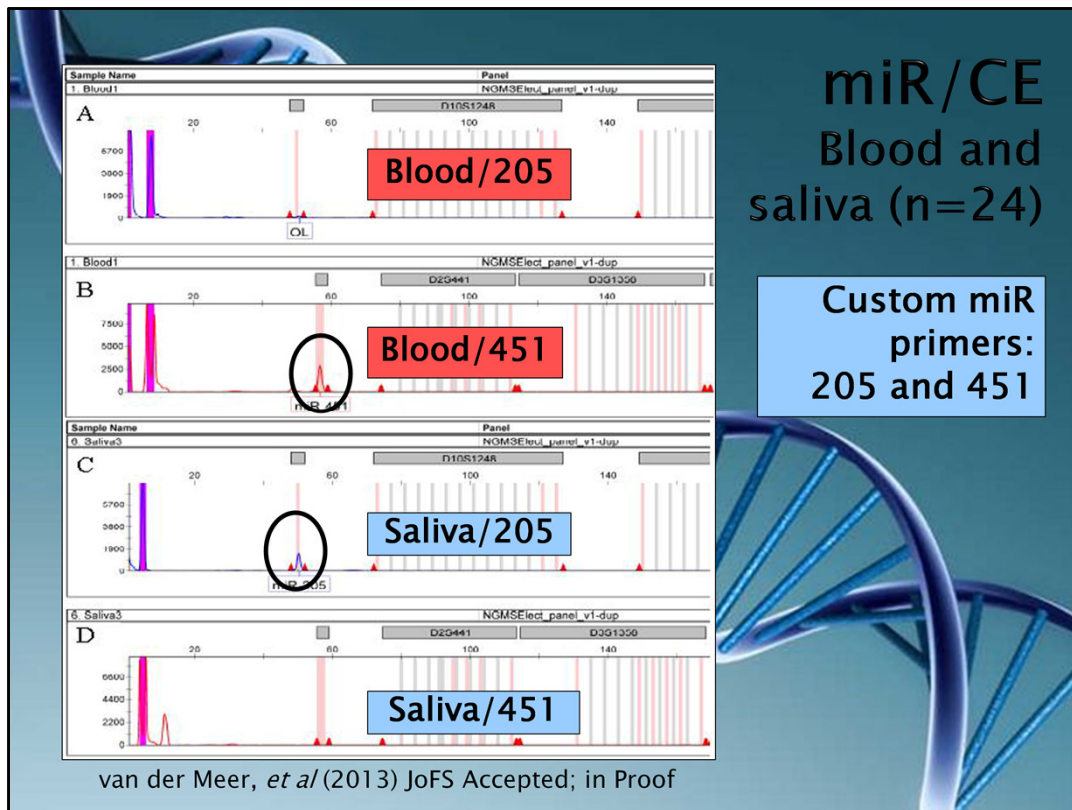
Mixtures 10 saliva:1 blood-greater gap

Similar pattern is seen for the reverse for blood to saliva

Results:

Weren't able to determine mixing ratios: 5:1 is not much different 10:1 both S:B and B:S

However determine major and minor contributors



We then performed miR of CE blood and saliva

We used custom primers for 205 and 451

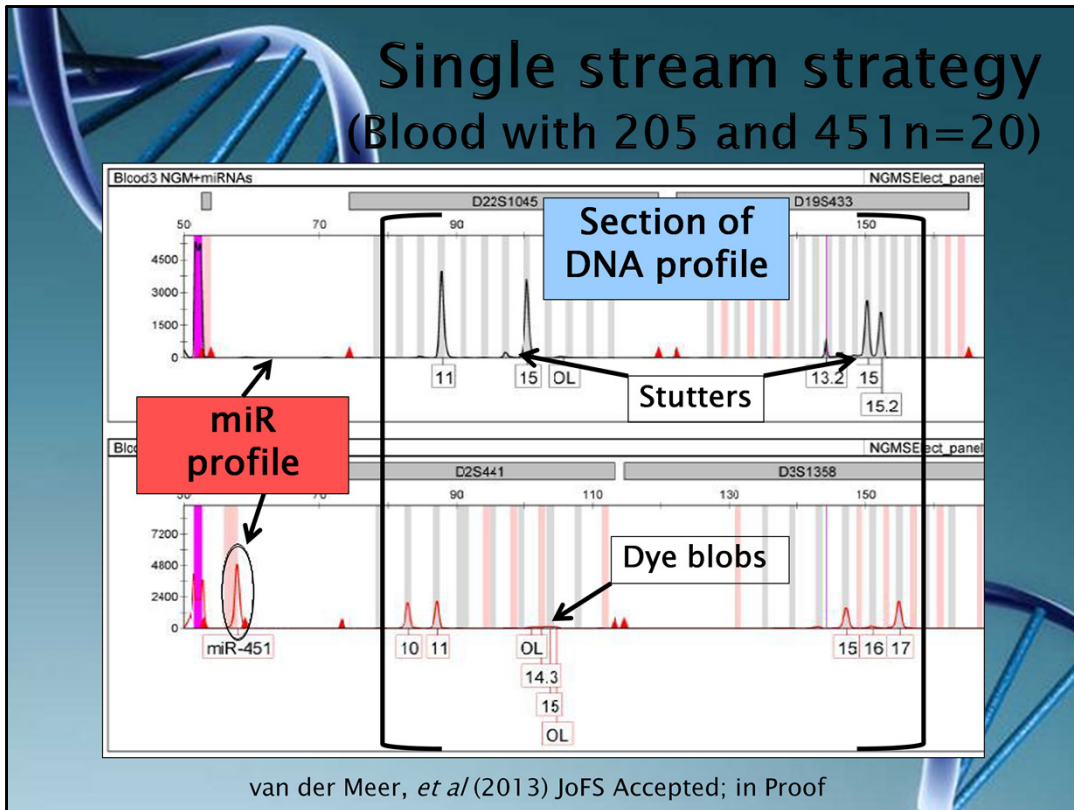
Sample A: Blood sample with 205 marker no peak present

Sample B: Blood sample with 451 marker and a peak is present about 2,300 rfu

Sample C: Saliva sample with 205 marker and a peak of 1800 is also present

Sample D: Saliva sample with 451 marker and a peak is absent.

This then brought us to our single stream strategy

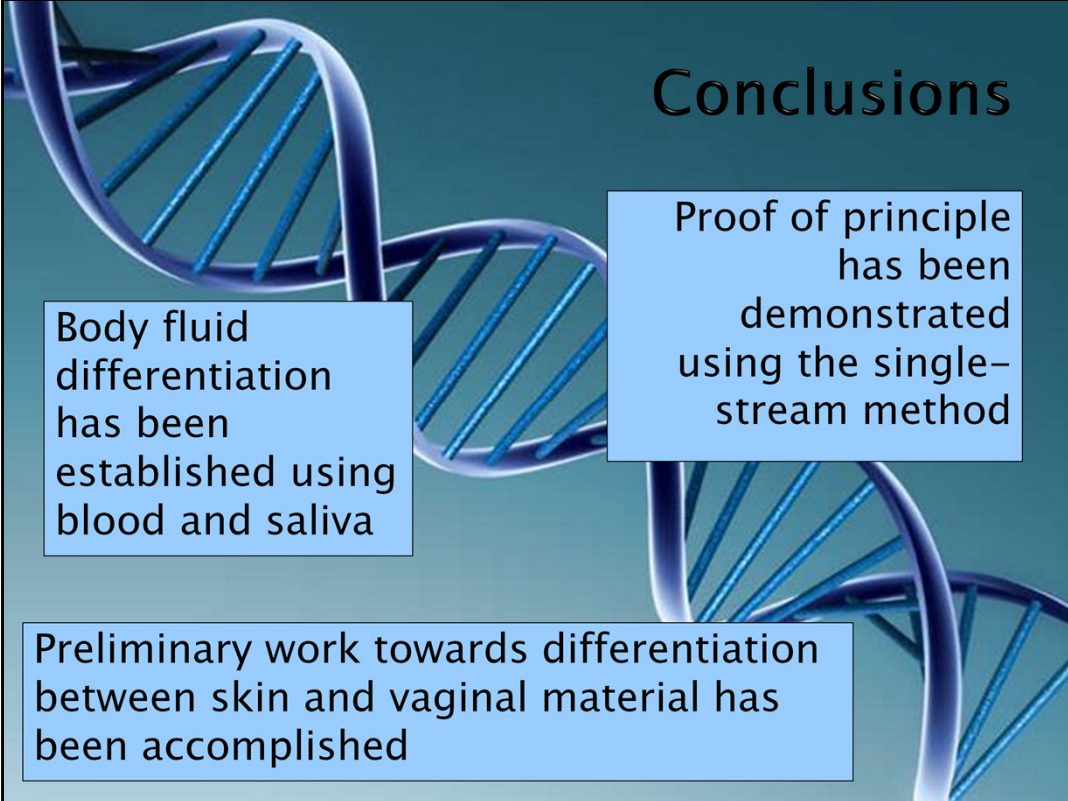


Single-stream strategy

Multiplexed custom miR markers 205 and 451.

Added blood sample.

Here you can see section DNA profile
Stutters



Conclusions

Body fluid differentiation has been established using blood and saliva

Proof of principle has been demonstrated using the single-stream method

Preliminary work towards differentiation between skin and vaginal material has been accomplished



Current goals

Extend our body fluid marker panel to include menstrual blood and seminal fluid

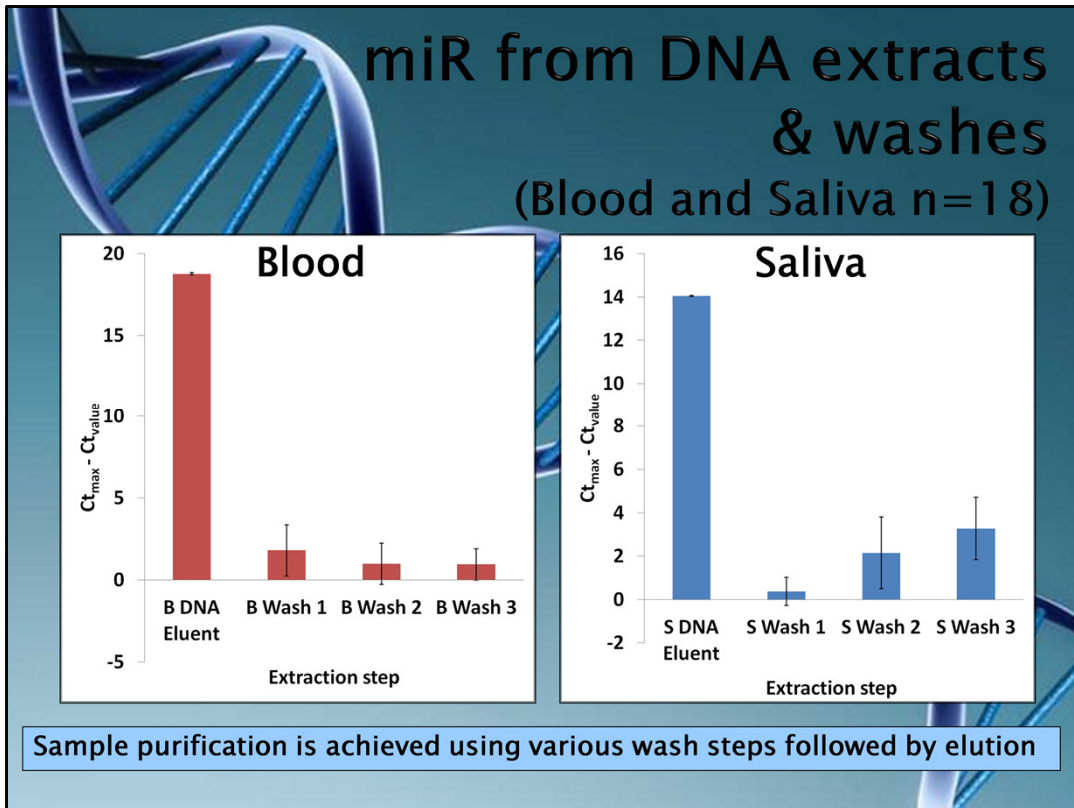
Optimize the single-stream method for DNA and miR analysis



Acknowledgements

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 - Damian World
 - Emma Beasley
 - Donny van der Meer
- Thanks to the volunteers for donating samples





We then wanted to see if we could get miR from a DNA extract and if not from the wash steps.

In theory we should only get DNA in a DNA extraction.

Anything else ie. miR should be in the washes.

We performed DNA extraction on blood and saliva and retained the wash steps.

We found that the eluent gave the most amt miR.