Project #148 – to optimise the cleaning protocol for bone crusher vials

Timothy GARDAM, Sharon JOHNSTONE, Cathie ALLEN

May 2015

Project #148 – to optimise the cleaning protocol for bone crusher vials

Published by the State of Queensland (Queensland Health), May 2015

For more information contact:
Forensic DNA Analysis, Department of Health, P.O. Box 594, Archerfield QLD 4108, email forensics@health.qld.gov.au, phone (07) 3274 9111.

Document details

Contact for enquiries & proposed changes

If you have any questions regarding this document or if you have a suggestion for improvements, please contact:

Contact officer: Timothy GARDAM

Title: Scientist, Intelligence Team

Phone: [redacted]

Email: [redacted]

Version history

Version	Date	Changed by	Description
1	08-May-2015	Timothy GARDAM	1st Version
1.1	23-Jun-2015	Timothy GARDAM	Incorporated Management Team feedback

Document sign off

This document has been approved by:

Name	Position	Signature	Date
Cathie Allen	Managing Scientist		

This document has been endorsed by:

Name	Position	Signature	Date
Justin Howes	Team Leader FRIT		
Kirsten Scott	A/Team Leader ER&Q		
Emma Caunt	A/Senior Scientist Q&P		
Sharon Johnstone	Senior Scientist Intel		
Amanda Reeves	Senior Scientist Reporting 1		
Kylie Rika	Senior Scientist Reporting 2		
Allan McNevin	Senior Scientist ER		
Luke Ryan	Senior Scientist Analytical		

Contents

1. Abstract 5
2. Materials 6
2.1 Reagents 6
2.2 Materials 7
2.3 Equipment 7
3. Methods 8
3.1 Sample Selection 8
3.2 Reagent Preparation 9
3.3 Miele Dishwasher “SPECIAL” cycle 9
3.4 DNA Extraction 9
3.5 DNA Quantification 9
3.6 DNA Amplification 9
3.7 DNA Fragment Analysis 10
3.8 Profile Interpretation 10
3.9 Experimental Design 11
3.9.1 Experiment 1 – Detergent 11
3.9.2 Experiment 2 – Inhibition Test 12
3.9.3 Acceptance Criteria 12
4. Results 13
4.1 Experiment 1 – Detergent 13
4.2 Experiment 2 – Inhibition Test 15
5. Summary & discussion 16
6. Recommendations 18
7. Abbreviations 18
8. References 18

Tables

Table 1 PCR cycling conditions for PowerPlex®21 System. 10
Table 2 CE protocol conditions..... 10
Table 3 Quantitation Values (pg/µl)..... 13
Table 4 Alleles (≥16 RFU) 14
Table 5 Summary of Peak Data from Experiment 2 15

Figures

Figure 1 Quantitation Values (pg/µl) with standard deviation 13
Figure 2 Alleles (≥16 RFU) with standard deviation..... 14
Figure 3 Peak Heights with Standard Deviation 15

1. Abstract

The Forensic DNA Analysis laboratory prepares bone samples by crushing in a SPEX 6750 Freezer Mill. The vials where the crushing takes place are a re-usable component. The manufacturer lists basic cleaning protocols for the crushing vials but no specific protocols suitable for Forensic DNA identification purposes are listed, nor are they present in the literature.

Before crushing a bone sample in the Forensic DNA Analysis laboratory the crushing vial components are swabbed and this swab is submitted for DNA profiling along with the bone sample. The purpose of this 'Equipment Control' is to show that the crushing vial is free from contaminating DNA. The laboratory has recently changed from the Profiler Plus multiplex to the PowerPlex 21 (PP21) multiplex. PP21 appears to have much greater sensitivity for low levels of DNA than Profiler Plus and we are now frequently seeing low-level DNA profiles in Equipment Controls – approximately 70% of Equipment Controls amplified with PP21 have one or more peaks above our limit of detection as compared to less than 10% for those amplified with Profiler Plus.

To have confidence in our results for crushed bones we investigated alternative cleaning protocols to try to ensure that the amount of contaminating DNA in the crushing vials was sufficiently reduced. This experiment compared several alternative cleaning protocols to the current cleaning protocol to see whether they reduced the amount of contaminating DNA.

The use of the autoclave as part of bone vial cleaning was dropped from the project plan early on as initial testing showed that it had only minimal impact on the cleaning process.

Any suitable cleaning protocol must not damage the stainless steel components of the crushing vials by causing rusting or pitting. Such damage weakens the vials and increases the risk that they will break during crushing. It also makes them far more difficult to clean properly, increasing retention of contaminating DNA.

This project found that Tergazyme (the current detergent) was the most effective cleaning agent but the Miele dishwasher 'SPECIAL' cycle offered equivalent performance with the added benefit of being automated.

2. Materials

The following resources are required for this project and are currently in use within the Forensic DNA Analysis Laboratory:

2.1 Reagents

- Terg-a-zyme® enzyme detergent (Alconox Inc.)
- Decon 90 Cleaning solution (Decon Laboratories Ltd.)
- Trigene Advance (CEVA DEIVET Pty. Ltd., Seven Hills, NSW, AU)
- Miele Dishwasher Detergent: Asepti Advantage and Asepti Neutraliser (Miele Australia Pty. Ltd., AU)
- Promega 2800M Positive Control DNA (Promega Corporation, Sydney, AU)
- 5% v/v Hypo 10 bleach (elite Chemicals Pty. Ltd.,Lytton, QLD, AU)
- Proteinase K (20 mg/mL) (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Dithiothreitol (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Ethanol (Recochem Incorporated, Wynnum, QLD,AU)
- Amphyl (Reckitt Benckiser Inc. Parsippany, NJ, US)
- Sarcosyl (Sigma-Aldrich® Corporation, St Louis, MO, US)
- Nanopure water: from Millipore Milli-Q Advantage A10
- Positive controls (Forensic DNA Analysis Unit, Brisbane, QLD, AU)
- TNE (Forensic DNA Analysis Unit, Brisbane, QLD, AU)
- Hi-Di™ Formamide (Life Technologies Applied Biosystems, Foster City, CA, US)
- 3130 POP-4™ Polymer (Life Technologies Applied Biosystems, Foster City, CA, US)
- Running Buffer (Life Technologies Applied Biosystems, Foster City, CA, US)
- Promega PowerPlex® 21 system (Promega Corp., Madison, WI, US)
- Promega CC5 Internal Lane Standard 500 (Promega Corp., Madison, WI, US)
- Promega PowerPlex 5 Dye Matrix Standard (Promega Corp., Madison, WI, US)
- Promega PowerPlex® 21 Allelic Ladder Mix (Promega Corp., Madison, WI, US)
- Water amplification grade (Promega Corp., Madison, WI, US)
- DNA IQ™ Casework Pro Kit for Maxwell® 16 (Promega Corp., Madison, WI, US)

2.2 Materials

- Tubed Sterile DrySwab™ MW1048, wood shaft, cotton bud (Medical Wire & Equiment, Wiltshire, UK)
- Small Stainless Steel End Plugs 6751E (SPEX SamplePrep, Metuchen, NJ, US)
- 96-well PCR micro-plates (Axygen Scientific Inc., Union City, CA, US)
- Tape pads (Qiagen Pty. Ltd., Doncaster, VIC, AU)
- 96-well plate Septa mats (Life Technologies Applied Biosystems, Foster City, CA, USA)
- Sterile 2 mL screw-cap tubes (Axygen Scientific Inc., Union City, CA, US)
- ART Filtered 1000, 300 & 20p pipette tips (Molecular BioProducts Inc., San Diego, CA, US)
- One Touch filtered 10 µL and 200 µL pipette tips (Quantum Scientific Lab Advantage, Murrarie, QLD, AU)
- Rediwipes (Cello Paper Pty. Ltd., Fairfield, NSW, AU)
- Adhesive film (QIAGEN, Hilden, DE)
- Sterile conductive filtered Roborack 25 µL disposable tips (PerkinElmer, Downers Grove, IL, USA)

2.3 Equipment

- Sonicator: Elma Transsonic T310 (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany)
- Dishwasher: Miele Professional G7883 CD (Miele Professional USA, Princeton, USA)
- LaboGene Scanspeed 1248 Centrifuge (Labgear, Lynge, Denmark)
- Hot-block (Ratek Instruments Pty. Ltd., Boronia, VIC, AU)
- Biological safety cabinets class II (Westinghouse Pty. Ltd., Newport, AU)
- Refrigerators and freezers (Westinghouse Pty. Ltd., AU)
- GeneMapper-IDX ver.1.4 (Life Technologies Applied Biosystems, Foster City, CA, USA)
- AB 7500 Real Time PCR System (Life Technologies Applied Biosystems, Foster City, CA, USA)
- GeneAmp PCR system 9700 (Life Technologies Applied Biosystems, Foster City, CA, USA)
- ABI 3130*xl* Genetic Analyzer (Life Technologies Applied Biosystems, Foster City, CA, USA)
- STORstar instrument (Process Analysis & Automation, Hampshire, GB)

- MultiPROBE II PLUS HT EX with Gripper Integration Platform (PerkinElmer, Downers Grove, IL, US)
- Thermomixer (Eppendorf AG, Hamburg, DE)
- MixMate (Eppendorf AG, Hamburg, DE)
- Vortex (Ratek Instruments Pty Ltd, Melbourne, VIC, AU)
- Micro centrifuge (Tomy, Tokyo, JP)
- Pipettes (Eppendorf, Hamburg, DE and Thermo Fisher Scientific(Finnpipette), Waltham, MA, US)

3. Methods

3.1 Sample Selection

For Experiment 1 buccal swabs and saliva from donor "FBSTF00049" were used. Each end plug was coated with approximately 250 µL of saliva and smeared with buccal cells from a fresh buccal swab (one swab per end plug). The end plugs were air-dried for 24 hours to ensure the saliva-buccal cell mix was adhered to them.

For experiment 2 a dilution of the Promega 2800M Positive Control was used as template DNA. This ensured improved consistency for the inhibition test. A Positive control DNA concentration of 0.3 ng/µL using a total of 0.3 ng per reaction was used to maximise the amount of sample from the end plug that could be added and therefore maximise the sensitivity of inhibition detection.

For each of the experiments crushing vial end plugs were tested by swabbing with a swab moistened with Nanopure water as per SOP 22904 "Procedure for Crushing Bone and Teeth using the SPEX 6750 Freezer Mill". These swabs were submitted for DNA profiling as outlined below (Methods 3.4 to 3.8).

3.2 Reagent Preparation

- Tergazyme was prepared as a saturated solution.
- 5% v/v Decon 90 was prepared as per SOP 17165 “Receipt, Storage & Preparation of Chemicals, Reagents & Kits”
- 5% v/v Trigene Advance was prepared as per the procedure for Trigene II in SOP 17165 “Receipt, Storage & Preparation of Chemicals, Reagents & Kits” but with Trigene Advance rather than Trigene II.
- 0.3 ng/µL Promega 2800M Positive Control was prepared as per the procedure in SOP 19994 “Procedure for testing DNA Quantification Standards, DNA Quantification and Amplification kits & Reagents, and Quality Control Samples” except that 3 µL of 10 ng/µL control was added to 97 µL water.

3.3 Miele Dishwasher “SPECIAL” cycle

Main Wash: Cold Water, Detergent, 93 ºC 10 min

Rinse: Hot Water, Detergent Neutraliser

Rinse: Hot Water

Final Rinse: Distilled Water, 75 ºC 3 min

Drying: 99 ºC 35 min

3.4 DNA Extraction

Swabs were submitted for bone extraction as per “Extracting DNA from Bone and Teeth” (QIS 17182). This is the same method used for Equipment Controls.

3.5 DNA Quantification

All reactions were prepared by manual methods or using the MultiPROBE II plus HT EX platform according to QIS 19977 “Quantification of Extracted DNA using the Quantifiler™ Human DNA Quantitation Kit”.

3.6 DNA Amplification

All amplification set ups were performed using the MultiPROBE II plus HT EX platform and amplification using the GeneAmp PCR system 9700 according to QIS 31511 “Amplification of Extracted DNA using the PowerPlex®21 System”.

Table 1 lists the PCR cycling conditions used for this project.

Table 1 **PCR cycling conditions for PowerPlex®21 System.**

PowerPlex® 21 Kit	Standard
GeneAmp 9700 mode	Max
	30 cycles
Activation	96 °C for 1 minute
Cycling	94 °C for 10 seconds
	59 °C for 1 minute
	72 °C for 30 seconds
Extension	60 °C for 10 minutes
	4 °C Soak

3.7 DNA Fragment Analysis

The plates for DNA fragment analysis were prepared and the PCR fragments separated by capillary electrophoresis (CE) using a 3130*xl* Genetic Analyser according to QIS 15998 "Procedure for the Use and Maintenance of the AB 3130*xl* Genetic Analysers". Table 2 outlines the 3130*xl* Genetic Analyser running conditions used.

Table 2 **CE protocol conditions**

Injection time	Injection voltage	Run time
5 s	3 kV	1500 s

3.8 Profile Interpretation

All samples were analysed with GeneMapper ID-X v1.4 under current casework conditions by an independent scientist with no knowledge of the profiles being analysed. The "16RFU Negative Controls" analysis method was used as this is the analysis method used for Equipment Controls.

3.9 Experimental Design

For each experiment 25x bone crusher end vials were cleaned using the current cleaning procedure then labelled '1' to '25' with black marker pen. All of the end vials were cleaned at the same time in individual 70 ml plastic screw-cap vials, except for the dishwasher samples which were instead washed together in a wire basket in the dishwasher.

3.9.1 Experiment 1 – Detergent

Bone crusher vial end plugs were subjected to a 15 minute soak in the following cleaning reagents:

a) Nanopure Water
b) Tergazyme (current procedure)
c) 5% v/v Decon 90
d) 5% v/v Trigene Advance
e) Miele Dishwasher "SPECIAL" program*

* These end plugs were not soaked and scrubbed but were instead washed in the dishwasher using the "SPECIAL" program

With the exception of e) – washed in the dishwasher – the end plugs were subjected to the following physical cleaning measures after the 15 minute detergent soak:

- Sonication (15 min) in Nanopure water
- Scrub with clean nail brush under hot tap water

25x bone crusher vial end plugs were coated with a mixture of buccal cells/saliva and air-dried for a period of 24 hours. One of the above cleaning reagents (a. to e.) was applied to each end plug (5x end plugs for each cleaning reagent). End plugs were swabbed and submitted for DNA Profiling.

NOTE: A 15 minute soak was chosen as that is the period that is used currently for crushing vial components. Longer periods (e.g. overnight) significantly increase the risk of corrosion and rusting.

3.9.2 Experiment 2 – Inhibition Test

Bone crusher vial end plugs were subjected to a 15 minute soak in the following cleaning reagents:

a) Nanopure Water
b) Tergazyme (current procedure)
c) 5% v/v Decon 90
d) 5% v/v Trigene Advance
e) Miele Dishwasher “SPECIAL” program*

* These end plugs were not soaked and scrubbed but were instead washed in the dishwasher using the “SPECIAL” program

With the exception of e) – washed in the dishwasher – the end plugs were subjected to the following physical cleaning measures after the 15 minute detergent soak:

- Sonication (15 min) in Nanopure water
- Scrub with clean nail brush under hot tap water

25x bone crusher vial end plugs were cleaned by one of the cleaning reagents listed above. One of the above cleaning reagents (a. to e.) was applied to each end plug (5x end plugs for each cleaning reagent). End plugs were swabbed and submitted for DNA Profiling. After extraction and quantitation and prior to the amplification step each sample was spiked with 1 µL of 0.3 ng/µL Promega positive control DNA to test for inhibition from detergent residues.

3.9.3 Acceptance Criteria

The optimum cleaning reagent was selected based on the combination of:

- No rusting or damage to the end plugs during the 15 minute soak
- No indication of inhibition (Experiment 2), and
- The lowest amount of DNA detected by quantitation and the fewest amplified peaks (Experiment 1).

4. Results

4.1 Experiment 1 – Detergent

The quantitation values are listed in Table 3 and plotted with standard deviation in Figure 1. For clarity the quant values are shown in pg/µL rather than the usual ng/µL.

Table 3 Quantitation Values (pg/µL)

	a. Nanopure Water	b. Tergazyme	c. Decon 90 (5% v/v)	d. Trigene Advance (5% v/v)	e. Dishwasher "SPECIAL"
	28.100	0.000	1.890	41.600	0.000
	4.780	0.000	1.680	65.300	0.490
	0.110	0.000	1.100	138.000	0.000
	14.900	0.000	0.000	68.300	0.000
	18.700	0.000	0.000	17.500	0.000
Average:	**13.318**	**0.000**	**0.934**	**66.140**	**0.098**

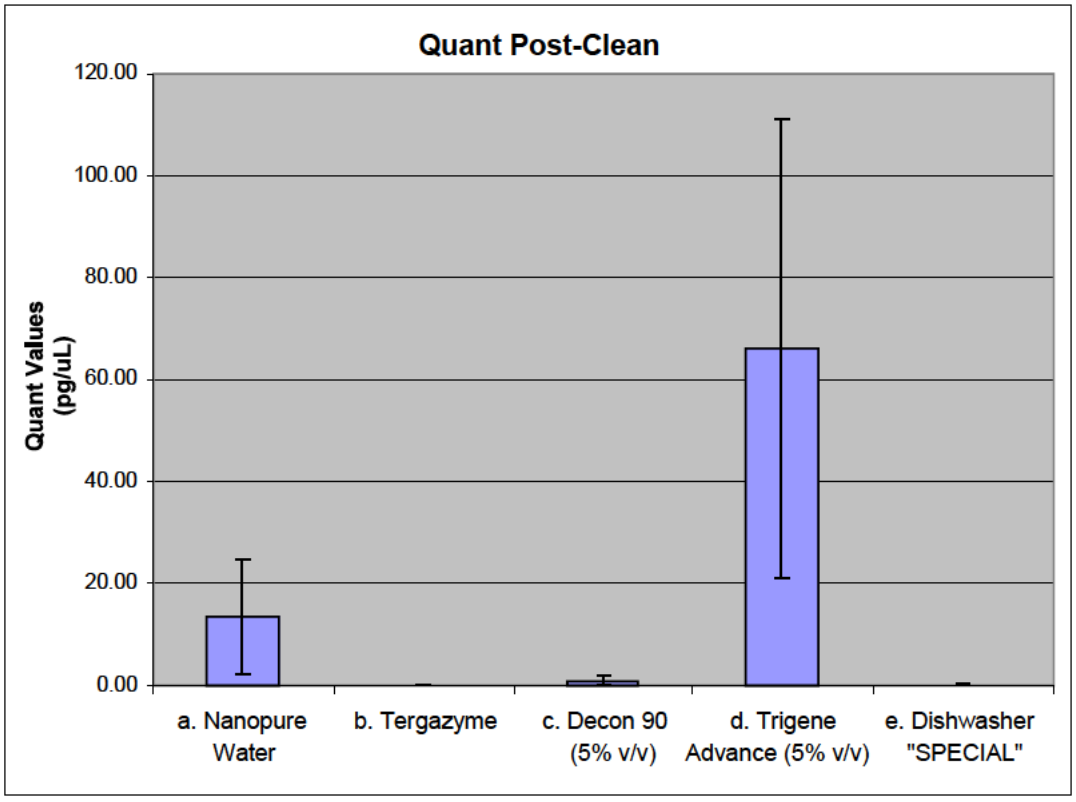

Figure 1 Quantitation Values (pg/µL) with standard deviation

The extracts above were DNA profiled. The DNA profiles were read at a threshold of 16 rfu – the more sensitive level used for checking negative controls such as bone vial equipment controls. The number of peaks seen (maximum of 40) is listed in Table 4 and plotted in Figure 2 with standard deviation.

Table 4 **Alleles (≥16 RFU)**

	a. Nanopure Water	b. Tergazyme	c. Decon 90 (5% v/v)	d. Trigene Advance (5% v/v)	e. Dishwasher "SPECIAL"
	40	0	7	40	0
	40	0	13	40	2
	18	1	6	40	0
	40	2	1	40	0
	38	0	1	39	0
Average:	**35.2**	**0.6**	**5.6**	**39.8**	**0.4**

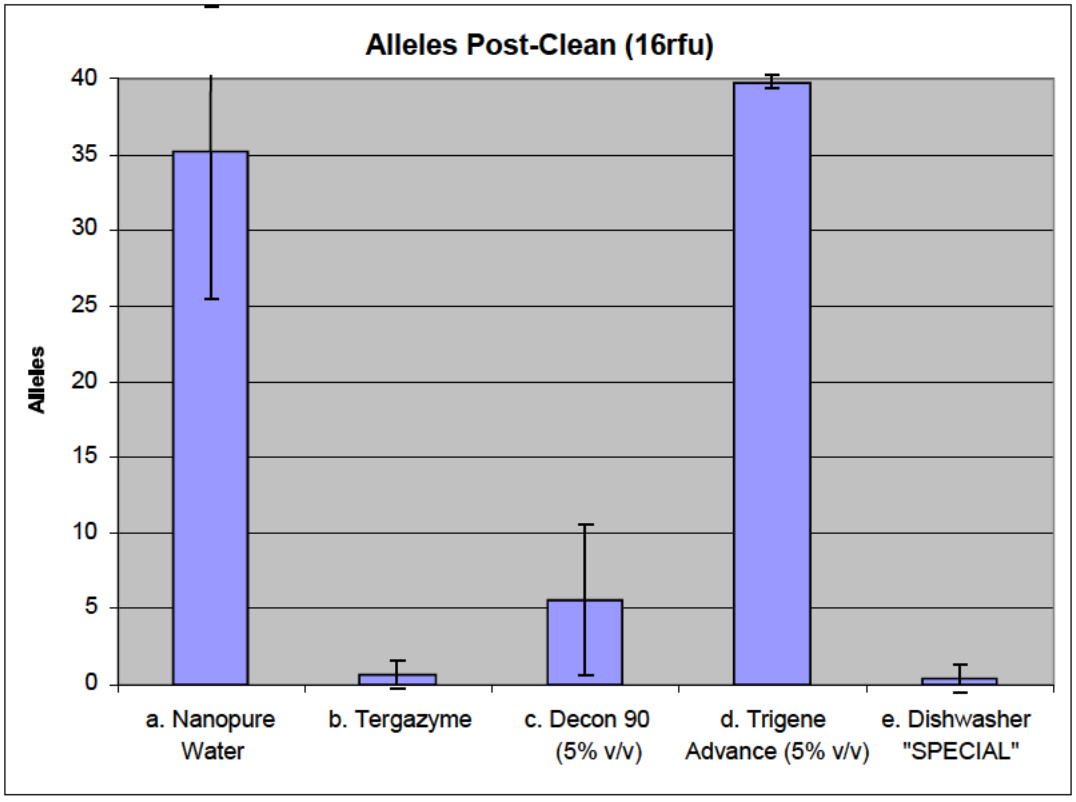

Figure 2 **Alleles (≥16 RFU) with standard deviation**

5% Trigene Advance (average 66.1 pg/µL, 39.8 peaks) gave a greater DNA yield and a greater number of peaks than water (average 13.3 pg/µL, 35.2 peaks). Because of this result Trigene Advance was considered not suitable for cleaning bone vials and so was not tested in Experiment 2.

5% Decon 90 (average 0. 9 pg/µL, 5.6 peaks) gave a much lower DNA yield and fewer peaks than water (average 13.3 pg/µL, 35.2 peaks). Tergazyme (average 0 pg/µL, 0.6 peaks) and the Miele dishwasher "SPECIAL" cycle (average 0.1 pg/µL, 0.4 peaks) gave a much lower yield and fewer peaks again than 5% Decon 90.

4.2 Experiment 2 – Inhibition Test

After soaking, sonicating and scrubbing then DNA profiling, no inhibition was noted – all samples gave full 40-allele DNA profiles from the positive control (all peaks ≥ 40 RFU).

To assess more subtle effects of inhibition the average Peak Height, Heterozygote Balance and Stutter Percentage for each reagent were calculated from the peak height data (Table 5). Stutter percentage could only be calculated for stutter peaks ≥16 RFU but these peaks were considered representative. The Peak Height data is shown in Figure 3 with standard deviation.

Table 5 Summary of Peak Data from Experiment 2

	a. Nanopure Water	b. Tergazyme	c. Decon 90 (5% v/v)	e. Dishwasher "SPECIAL"
Average Peak Heights (RFU)	658	544	594	510
Average Heterozygote Balance (%)	82.8	82.7	81.4	80.6
Average Stutter (%)	7.83	7.81	7.59	7.67

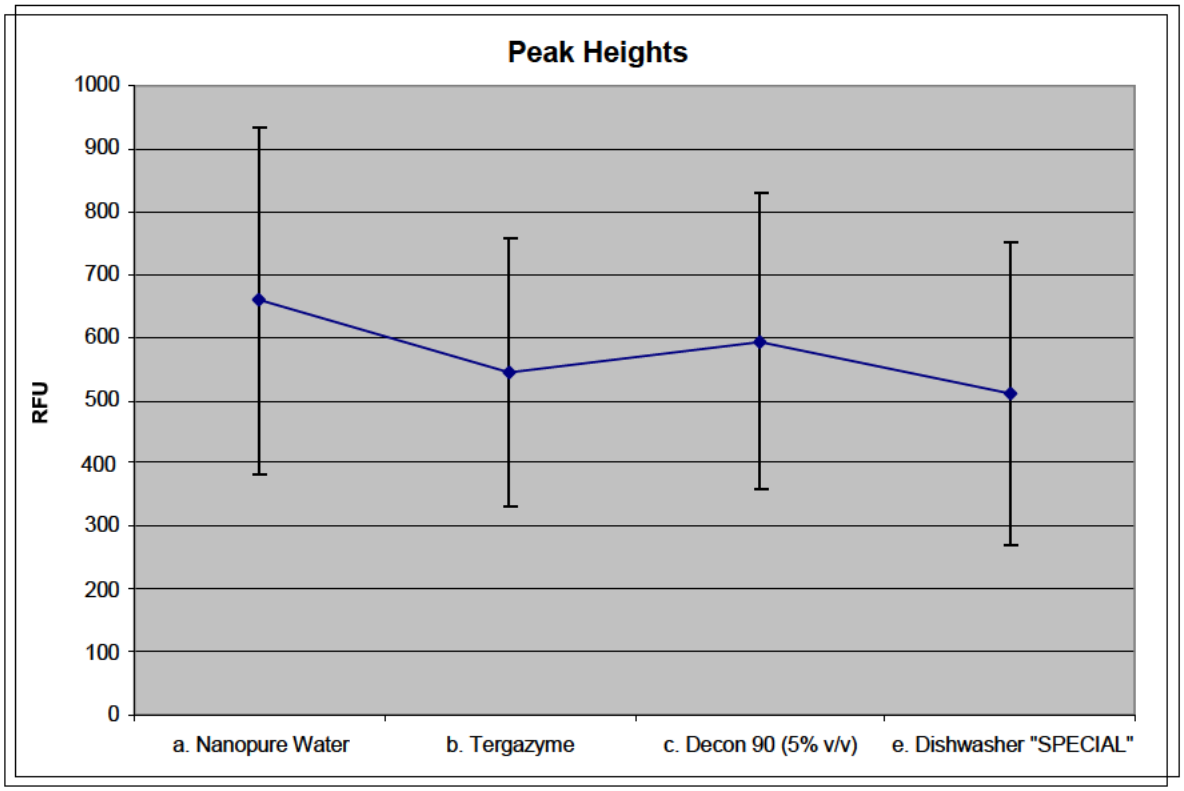

Figure 3 Peak Heights with Standard Deviation

Although there appeared to be some difference in average peak heights between the different reagents the variance within each group was very high (CV~40%) making it difficult to assess whether this difference was significant or not.

A two-tailed student's t-test was used to compare the results for the Miele "SPECIAL" cycle and Tergazyme to assess whether there was likely to be a significant difference between the data sets. A p-value of < 0.05 was considered to be significantly different. For Heterozygote Balance ($p=0.27$), Stutter percentage ($p=0.65$) and Peak Heights ($p=0.13$) there was no significant difference between the Miele "SPECIAL" cycle and Tergazyme.

Another possible indicator of inhibition is the pattern of 'degradation' seen in the DNA profiles. All of the DNA profiles from Experiment 2 were run through the STRmix software[3] to assess the degree of degradation. For all samples STRmix modelled degradation of 0.0 RFU/bp – meaning no degradation.

No significant difference was noted between the Miele "SPECIAL" cycle and Tergazyme (the current cleaning method) in Experiment 2 with regard to peak heights, heterozygote balance, stutter or degradation.

5. Summary & discussion

The results of Experiment 1 showed a large variation between the different cleaning reagents in their ability to remove DNA from dried-on saliva. Although there was significant variation between the quantitation values for some of the replicates – especially those with higher quant values – there was certainly a clear trend for each of the reagents. The number of alleles seen after amplification (Table 4, Figure 2) was consistent with the quant value results (Table 3, Figure 1) and was a more sensitive measure for differentiating between the reagents with lower quant values.

The most striking results are those of 5% Trigene Advance which gave significantly higher DNA yields and peak counts than water. This surprising result is similar to what was found by Ballantyne *et al.* (2015) when testing Virkon with wet and dry saliva samples[1]. They speculated that cellular and extracellular components of the saliva may inhibit the active ingredients in Virkon. In this case it appears that the Trigene Advance is actually increasing the yield from dried saliva stains, relative to water. This may be because Trigene Advance is not damaging the DNA but is damaging some of the 'stickier' proteins so more nuclei and nuclear fragments are released to the swab after treatment with Trigene Advance versus washing with water. This result also conflicts with other testing at this laboratory using whole blood dried onto petri dishes, where it was found that Trigene Advance and Virkon are the most effective cleaning agents[2]. It is possible that depending on the surface to be cleaned (metal, plastic) and the contaminant (dried saliva, dried whole blood, extracted DNA, amplified DNA) all of the different cleaning agents will perform quite differently. It may be that no one cleaning agent will be suitable for all cleaning tasks in a forensic DNA laboratory. Because of the results in Experiment 1, Trigene Advance was not considered suitable for cleaning the bone vials.

Although the Decon 90 was considerably more effective than Nanopure water for removing dried saliva stains the Tergazyme and the Miele "SPECIAL" cycle were much more effective again.

From Experiment 2 none of the reagents tested appears to show any significant inhibition at the concentrations tested. Additionally after analysing peak heights, heterozygote balance, stutter percentage and degradation there was no significant difference seen between the Miele "SPECIAL" cycle and Tergazyme (the current cleaning method).

All of these detergents are classified as 'Corrosive' and 'Irritant' and there is no significant safety benefit to using any one over any other, with the exception of the dishwasher where operator exposure to the detergent is minimal. None of these cleaning agents caused rusting or damage to the bone crusher end plugs during the 15-minute soak steps.

The suitability of these reagents for cleaning bone vials then comes down to their performance in Experiment 1. Because of their lower quantitation results and lower peak counts, Tergazyme and the Miele "SPECIAL" cycle are the preferred options. The Miele "SPECIAL" cycle is an automated process whereas cleaning with Tergazyme is a manual process. The automated process requires less operator hands-on time, less risk of operator exposure to detergents, and is likely to be subject to less operator-to-operator variability than the manual cleaning process. The Miele "SPECIAL" cycle is therefore the preferred option.

6. Recommendations

From the outcome of this report, the authors suggest the following recommendations for implementation within the Forensic DNA Analysis laboratory:

1. The Miele "SPECIAL" cycle is recommended as the primary cleaning method as it requires less operator hands-on time, less operator exposure to detergents, and is likely to be less susceptible to operator variation.
2. Cleaning with Tergazyme should remain a viable backup method if the dishwasher is unavailable for any reason.

7. Abbreviations / Glossary

DNA	Deoxyribonucleic Acid.
PCR	Polymerase chain reaction.
Quant	DNA Quantification/Quantitation – determining the amount of amplifiable DNA present in the sample.
RFU	Relative fluorescence units – a unit-less measure of peak intensity for DNA profiles.
CV	Coefficient of Variation – the ratio of the standard deviation to the mean.
bp	Base pairs (of a DNA strand).
Degradation	When referring to a DNA profile, a pattern of sharply decreasing peak heights as peak molecular weight increases. Seen when amplifying poor quality degraded DNA where shorter fragments predominate, but also if the PCR is impaired for other reasons (e.g. PCR inhibitors are present in the sample).

8. References

1. Ballantyne K, Salemi R, Guarino F, Pearson J, Garlepp D, Fowler S, van Oorschot R. “DNA contamination minimisation – finding an effective cleaning method”. *Australian Journal of Forensic Sciences*, DOI: 10.1080/00450618.2015.1004195
2. Thompson S, Kaity A, Mathieson M, Ryan L, McNevin A, Allen C. *Project#153 - Verification of Cleaning Reagents (Trigene Advance, Viraclean, Virkon, Pyroneg, Decon, Cavicide, F10SC) for use in Forensic DNA Analysis*. 2015
3. STRmix™ software v2.06, http://strmix.esr.cri.nz