Notice number: 9.001

COMMISSION OF INQUIRY INTO DNA PROJECT 13

Section 5(1)(d) of the *Commissions of Inquiry Act 1950*

STATEMENT OF CECILIA IANNUZZI

I, **Cecilia Iannuzzi**, care of Queensland Health Forensic and Scientific Service, retired, do solemnly and sincerely declare that:

1. On 19 October 2023, I was requested to provide a statement responding to Notice 9.001 "Requirement to Give Information in a Written Statement".

Cecilia Iannuzzi

Identification

Question 1(a) - State your full name

2. Cecilia Iannuzzi.

Question 1(b) - State your qualifications, skills or experience relevant to forensic science and DNA

3. I was awarded a Bachelor of Clinical Analysis and Biochemistry, from Buenos Aries, Argentina, in 1979.

4. From 1975 to 1977, I was employed by the Professor R Finochietto District Hospital in Buenos Aries, Argentina as a Laboratory Assistant.

5. From 1979 to 1980, I was employed by the National Institute of Rehabilitation for the Disabled in Buenos Aries, Argentina as a Professional Officer.

6. From 1980 to 1990, I was employed by the Wilde General Hospital as a Professional Officer (from 1980 to 1984) and a Professional Officer in a supervisory capacity from 1984 to 1990.

7. In 1991, I did work experience at the Royal Children's Hospital in Melbourne, Victoria as a Haematology Laboratory Assistant for one month.

8. From 1992 to 1994, I was employed by the University of Queensland as a Research Assistant.

Question 1(c) - State the period(s) of time you have been or were employed by or otherwise engaged with Queensland Health, Queensland Health Forensic and Scientific Services (QHFSS) and/or Forensic Science Queensland, and in what roles and when

[redacted]

Cecilia Iannuzzi

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9. From 1994 to 2003, I was employed by the Royal Brisbane and Women's Hospital as a Professional Officer in Chemical Pathology (Protein section PO304/Technical Officer, endocrinology section).

10. From 2003 to 2019, I was employed by Forensic Science Queensland, in various positions including as a Technical Officer (2003 – 2005), HP2 (2005 – 2007), HP3 (2007 – 2009) and HP4 (2009 – 2019). I was on long service leave from May 2018 to January 2019.

11. I retired from my employment in January 2019.

Manual and Automated DNA Extraction Methods

Question 2 – In relation to the report being the "Project 13. Report on the Verification of an Automated DNA IQ Protocol using the Multiprobe II PLUS HT EX with Gripper Integration Platform", Nurthen, T., Hlinka, V., Muharam, I., Gallagher, B., Lundie, G., Iannuzzi, C., Ientile, V. Automation/LIMS Implementation Project, DNA Analysis FSS (August 2008) (2008 Report)1 and the abstract and introduction therein which state:

1. Abstract

A manual method for extracting DNA from forensic samples using the DNA IQ™ system (Promega Corp., Madison, WI, USA) was validated for routine use in DNA Analysis (FSS). We have verified an automated DNA IQ™ protocol in 96-well format for use on the MultiPROBE® II PLUS HT EX Forensic Workstation platforms (PerkinElmer, Downers Grove, IL, USA). Data indicate that results from the automated procedure are comparable to those from the manual procedure. Contamination checks were performed using samples prepared in checkerboard and zebra-stripe format, and results were as expected. We recommend the use of the MultiPROBE® II PLUS HT EX platforms to perform automated DNA extraction using the DNA IQ™ system.

2. Introduction

The MultiPROBE® II PLUS HT EX FORENSIC WORKSTATION platforms (PerkinElmer, Downers Grove, IL, USA) are equipped to perform automated DNA extractions, as they include a DPC shaker and individual heat controllers to enable on-board lysis and incubation steps. Currently in DNA Analysis, the MultiPROBE® platforms allow walk-away operation of PCR setup protocols for DNA quantitation and amplification.

The DNA IQ™ protocol has been verified or validated by various laboratories for use on the MultiPROBE® II PLUS platform. The laboratories that perform an automated DNA IQ™ protocol include PathWest (Western Australia), Forensic Science South Australia (South Australia) and Centre of Forensic Sciences in Toronto (Ontario). The MultiPROBE® II PLUS instrument comes pre-loaded with an automated DNA IQ™ protocol. Unlike the other laboratories, however, we did not validate the included protocol, but instead validated a manual DNA IQ™ protocol which was based on the CFS automated protocol (PerkinElmer, 2004), followed by verification of an automated protocol based on the validated manual method.

The verified automated DNA IQ™ protocol is identical to the validated manual protocol used in-house: there are no differences in reagents or volumes. The adopted DNA IQ™ protocol differs slightly, however, from the manufacturer's protocol, as it includes a lysis step using Extraction Buffer (10mM Tris, 1mM EDTA, 100mM NaCl, 20% w/v SDS) in the presence of Proteinase K, before incubating in the DNA IQ™ Lysis Buffer. Furthermore, the lysis incubation conditions were lowered from 70°C to 37°C in order to accommodate extraction of DNA from heat labile materials such as nylon and polyester. In addition, the automated protocol utilises the SlicPrep™ 96 Device (Promega Corp., Madison, WI, USA) for simultaneous processing of samples in a 96-wel format.

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Cecilia Iannuzzi Witness

Manual Method

Question 2(a) describe, with precision, the "manual method" for extracting DNA from forensic samples using the DNA IQ™ system referred to in the first line of the Abstract to the 2008 Report (Manual Method), including whether the Manual Method:

(i) was devised within the QFSS Forensic DNA Analysis laboratory (Laboratory); or

(ii) was otherwise a modification of an existing manual method (and if so which method),

12. All information I have set out below has been derived following my review of the:

(a) *Project 9 Report on the Evaluation of Commercial Extraction Chemistries 2007* **(Project 9 Report) annexed and marked Exhibit CI-01**;,

(b) *Project 11 Report on the Validation of a manual method for Extracting DNA using the DNA IQ System* dated August 2008 (**Project 11 Report) annexed and marked Exhibit CI-02** ; and

(c) *Project 13 Report on the Verification of an Automated DNA IQ™ Protocol using the Multiprobe® II PLUS HT EX with Gripper™ Integrated platform* dated 2008 **(Project 13 Report) annexed and marked Exhibit CI-03**.

13. From my review, the Project 13 Report is a draft. As the Project 13 Report, in the form annexed in Exhibit C1-03 appears to be a draft, I have not relied on the accuracy of the contents and do not accept that it contains the final results.

14. To my knowledge, the Manual Method for extracting DNA from forensic samples using the DNA IQ™ system was devised by the biotechnology company Promega.

15. The Manual Method was recorded in protocols issued by Promega, which was provided to the Forensic Science Queensland. I do not have access to a copy of these protocols.

16. From my review of the Project 11 Report, on page 6-7 the manual DNA-IQ™ protocol is set out.

17. From my memory, this protocol accurately sets out how the Automation Team would carry out the manual DNA-IQ™ method.

18. I recall that Chelex extraction system is what we used before DNA IQ™ method. I do not remember much about the Chelex extraction system and how it worked.

19. From memory, the difference between Chelex extraction system and DNA IQ was that DNA IQ was the more advanced system and produced better quality DNA. Chelex is a resin solution with very tiny particles, which is added to the sample solution (which is the sample plus 'Nanopure' water). This combined solution is incubated at 56 degrees celsius, and subsequently into boiling water. The boiling water step can be tough and unsafe for the operator (i.e. risk of burning). Also with the Chelex system there is a risk that tube labels may come off in the boiling process. Further, when trying to remove the Chelex

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Cecilia Iannuz[redacted] Witness [redacted]

particles from the solution, if a particle is left behind in the solution, the quality of the DNA is affected, in that it may affect the quantitation and/or the amplification process, resulting in an impure DNA concentration. We did not consider that this would be an issue for the DNA IQ™ system.

20. I recall that DNA IQ™ system the was already tested by Promega for use in the Multiprobe® II PLUS platform.

21. The rationale for bringing in DNA IQ from memory was that the laboratory intended to implement the Multiprobe® II PLUS, and DNA IQ was a compatible method with the Multiprobe® II PLUS. At the time we had a backlog of samples for testing and we needed a method that was adaptable to the robot. In addition to the other issues referred to above, the Chelex extraction system could not be adapted to the robot.

22. From memory, the Multiprobe® II PLUS was much faster. The DNA IQ method used manually could process 24 or 16 samples manually in 4 hours, whereas the Multiprobe® II PLUS could process 96 samples in the same time.

Question 2(b) describe, with precision, the method by which the Manual Method's "routine use" in DNA Analysis (FSS) was validated

23. I cannot independently recall the formal verification process.

24. From my review of the Project 9 Report annexed at CI-01, the Manual Method's "routine use" in DNA Analysis was evaluated in Project 9. However, I have no independent recollection of this.

25. From my review of the Project 11 Report annexed at CI-02, the Manual Method's "routine use" in DNA Analysis was validated in Project 11. However, I have no independent recollection of this.

26. The difference between evaluation and validation is that, in an evaluation, tests are performed to determine if a process and/or method is appropriate for use in the Laboratory (i.e. to see whether the laboratory requires specific equipment and/or an assessment of whether the process and/or method can be used in the laboratory and suitable for the needs of the laboratory). Validation is the process of ensuring results are comparable to the previous technique used in the laboratory (i.e. to ensure that the results under the new process and/or method is equal to or better than the results obtained using the previous technique.

27. In terms of my specific recollection of how the Manual Method using the DNA IQ system was verified, I recall performing the following tasks within my role in the Automation team:

    (a) The Automation team prepared mock donor samples and ran tests in parallel with Chelex (this was undertaken and recorded in the Project 9 Report).

    (b) Those tests involved the Automation team preparing the work lists, labelling the tubes, sequence checks barcodes of samples performed by another operator and

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Cecilia Ian... Witness

signed. Once this process was complete, the Automation team processed samples with the Manual the DNA IQ™ system extraction.

(c) Once the Automation team were satisfied that the results were comparable, a report reflecting those results was created (potentially by Mr Thomas Nurthen and Mr Iman Muharan, though I cannot recall for certain), being the Project 11 Report.

(d) I assume the report of the Automation team's findings (i.e. the Project 11 Report) was presented by Mr Thomas Nurthen, to the Management team during a meeting (as this is the usual process that occurs when a method/technique is being evaluated or validated). I cannot recall the individuals who were on the Management team at that time.

(e) The Management team review the report and I recall that the Management team have the final say in the finalisation and approval of the report. A report can only be finalised and published with the Management Team's approval.

(f) Once the method received approval, a standard operating procedure (**SOP**) would be created (typically by the Senior Scientists) and once the SOP was finalised the Automation and Analytical team scientists started receiving training in the method.

(g) As soon as the Automation and Analytical team scientists were trained in the method, each team would use the Manual DNA IQ™ system in the laboratory.

(h) The Automation team scientists, after being trained in the Manual DNA IQ™ method, started to calibrate the Multiprobe® II PLUS. Once the performance and calibration was acceptable (e.g. the Multiprobe® II PLUS was pipetting, and dispensing and removing the desired and expected volume of a sample material), the Automation team started testing the Multiprobe® II PLUS with mock samples and performing automatic extractions.

Question 2(c) state whether, and if so how, the Manual Method differed from or otherwise modified the DNA IQ™ protocol that was "verified or validated by various laboratories for use on the Multiprobe® II PLUS platform" (as stated in the second line of the second paragraph of the Introduction to the 2008 Report)

28. I cannot recall any specifics.

29. From memory, the Multiprobe® II PLUS was tested for future use. Therefore, we had to check whether the temperature was acceptable and whether all the calibrations of dispensing volumes with different disposable and fixed tips within the Multiprobe® II PLUS was done correctly, before testing commenced.

30. For example, we tested whether the Multiprobe® II PLUS could dispense and remove the correct volume of liquids from the plate and reagents to ensure the Multiprobe® II PLUS performance is acceptable before deploying to samples.

31. The Automation may have modified the protocol provided by Promega slightly for our purposes, to customise its use in our laboratories. This may have been done by the Automation team in Project 11 (for example, I recall one of the temperature settings was

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Cecilia Iannuzzi Witness

modified because some materials melted such as sticky tape, cigarette butts or gum etc.). I was not involved personally in that modification, though I do recall being informed about it by other members of the Automation team.

Question 2(d) state when the Manual Method was so devised

32. I cannot recall.

33. I assume it was devised before Project 9. On that basis, potentially it was devised in 2006. However, I have no independent recollection of this.

Question 2(e) identify those within the Laboratory responsible for devising the Manual Method

34. I recall the original protocol for the Manual Method was devised by Promega. I cannot recall when the Laboratory received the protocol.

35. I do not know the individual/s within the Laboratory responsible for devising the Manual Method in our Laboratory.

Question 2(f) state the reason(s) why the Laboratory chose to devise and to implement the Manual Method

36. As I am not aware of the individual/s who devised the Manual Method, I do not know, nor can I recall the reasons that the Method was devised and implemented in the Laboratory.

37. Other methods were proposed, these are outlined in Table 1 of the Project 9 Report, at page 2. I do recall that DNA IQ was considered the most appropriate.

CFS Automated Protocol –

Question 2(g) describe, with precision, the "CFS automated protocol (PerkinElmer, 2004)" (CFS Automated Protocol) referred to in the seventh line of the second paragraph of the Introduction to the 2008 Report

38. I cannot recall.

Manual DNA IQ™ Protocol

Question 2(h) describe, with precision, the "manual DNA IQ™ protocol" (Manual DNA IQ™ Protocol)" referred to in the seventh line of the second paragraph of the Introduction to the 2008 Report, including whether it:

(i) was developed or otherwise supplied by the manufacturer of the MultiPROBE® II PLUS HT EX FORENSIC WORKSTATION platform;

(ii) was devised within the Laboratory; or

Cecilia Tannuzzi Witness

(iii) was otherwise a modification of an existing Manual DNA IQ™ protocol (and if so which method)

39. I cannot independently recall the protocol step-by-step.

40. However, after reviewing the Project 9 Report, the Manual DNA IQ™ system is very well documented at section 5.2.2 of the report (pages 4 to 6). From what I can recall, this outline seems accurate.

Question 2(i) describe, with precision, the method by which the Manual DNA IQ™ Protocol was validated

41. I recall that the Automation team ran the same type of mock samples for 'Chelex extraction' and other DNA extraction protocols obtained from different companies, including the Manual DNA IQ™.

42. The Manual DNA IQ™ was shown to have the same or better results when compared to the Chelex extraction protocol (in that the quantitation of DNA in Manual DNA IQ™ was similar or better than the quantitation of Chelex extraction method). Those results are contained within the Report for Project 9.

43. I cannot recall the process we would take if the results between methods were not comparable. I assume we would repeat both experiments, but am not sure.

Question 2(j) state whether, and if so how, the Manual DNA IQ™ Protocol differed from or otherwise modified the DNA IQ™ protocol that was "verified or validated by various laboratories for use on the Multiprobe® II PLUS platform" (as stated in the second line of the second paragraph of the Introduction to the 2008 Report)

44. I cannot recall.

Question 2(k) state when the Manual DNA IQ™ Protocol was so devised

45. I cannot recall.

Question 2(l) identify those within the Laboratory responsible for devising the Manual DNA IQ™ Protocol

46. The original Manual DNA IQ™ Protocol was devised by Promega. I do not know the individual/s within the Laboratory responsible for devising the Manual DNA IQ™ Protocol for use at our laboratory.

47. At the relevant time, the Automation Team comprised of:

(a) Breanna Gallagher;

(b) Generosa Lundie;

(c) Iman Muharam;

(d) Vojteck Hlinka; and

[redacted]

Cecilia Iannuzzi

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Witness

(e) Myself.

48. At that time our supervisor for the Automation Team was Thomas Nurthen.

49. Thomas Nurthen gave the Automation Team different methods to evaluate to adapt to the Multiprobe® II PLUS. Those methods are listed within the Project 9 Report.

50. In Project 9, the Automation team evaluated different methods which could be adaptable to the Multiprobe® II PLUS. The Automation team found the quantitation of DNA in Manual DNA IQ™ method was similar or better than the quantitation of the Chelex extraction method (and the other methods that were tested: which are listed in the Project 9 Report). In addition the Manual DNA IQ™ method was able to be adapted to the Multiprobe® II PLUS.

Question 2(m) state the reason(s) why the Laboratory chose to devise Manual DNA IQ™ Protocol

51. I refer to my answer to Question 2(f) above.

Automated DNA IQ™ Protocol

Question 2(n) state whether the "automated DNA IQ™ protocol" referred to in the first line of the third paragraph of the Introduction to the 2008 Report (Automated DNA IQ™ Protocol) is the same as the automated protocol the subject of the 2008 Report. If it is not, then state the reasons why and describe any differences

52. I do not understand the question.

Question 2(o) state whether, and if so how, the Automated DNA IQ™ Protocol differed from or otherwise modified:

(i) the Manual Method;

(ii) the DNA IQ™ protocol that was "verified or validated by various laboratories for use on the Multiprobe® II PLUS platform" (as stated in the second line of the second paragraph of the Introduction to the 2008 Report);

(iii) the CFS Automated Protocol; and

(iv) the Manual DNA IQ™ Protocol

53. I do not understand the question.

54. As outlined in my response to question 2(c) above, the Automation team may have modified the Manual DNA IQ™ Protocol provided by Promega slightly for our purposes, to customise its use in our laboratories, before the implementation of the Multiprobe® II PLUS platform. I recall that only one step was added to improve performance, which was the addition of an Extraction buffer (referred to at page 6 of the Project 11 Report).

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Cecilia Iannuzzi

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Witness

55. I recall that the Automation team may have also added some steps in the Automated DNA IQ™ Protocol in the Multiprobe® II PLUS platform but, these steps did not affect the method of DNA extraction. The steps added to this protocol that I can recall involved:

    (a) in the step where we used the solution of resin with magnetic beads, (which precipitated in the solution) adding an extra step requiring to mix the magnetic beads within the solution prior to use within the Multiprobe® II PLUS platform (which was found to be very beneficial); and

    (b) to make sure the label of the plate was positioned at the front of the plate.

Question 2(p) state when the Automated DNA IQ™ Protocol was so devised

56. I cannot recall.

Question 2(q) identify those within the Laboratory responsible for devising the Automated DNA IQ™ Protocol

57. I do not know, nor can I recall who was responsible for devising the Automated DNA IQ™ Protocol (i.e. whether it was Promega Corp or Perkin Elmer).

58. The Automation team were provided the Manual DNA IQ™ Protocol from Promega.

59. I otherwise refer to and rely on my answer to question 2(o) above.

Question 2(r) state the reason(s) why the Laboratory chose to devise the Automated DNA IQ™ Protocol rather than use the manufacturer method

60. I do not know, nor can I recall the specific reasons that the Laboratory chose to devise the Automated DNA IQ™ Protocol rather than use the manufacturer method, as I was not involved in that decision.

61. I recall that the modified DNA IQ™ Manual method had been validated for use. I recall that the Automation team had to follow the same validated protocol in the Multiprobe® II PLUS platform.

62. For example, the Multiprobe® II PLUS was running on a program (See Figure 2 at page 5 of the Project 13 Report). The Automation team modified the program and added more steps in line with quality requirements and to ensure better performance (refer to the steps referenced at paragraph 55).

63. From memory, we introduced steps in the process such as "check that plate is in the right position" and "add some reagents". For example, we used to have resin with magnetic beads which precipitated in the solution. In those circumstances we added a step to mix the solution (as mentioned in for the reasons specified in the answer to question 2(o) above).

Multiprobe II PLUS HT EX with Gripper Integration Platform (Multiprobe II Device)

Question 3 - State when the Laboratory received the Multiprobe II Device

.....[redacted]..... .....[redacted].....

Cecilia Iannuzzi Witness

64. I cannot recall.

Question 4 - For each of the Manual DNA IQ™ Protocol and the Automated DNA IQ™ Protocol, describe, with precision and completeness, what, if anything was done to the device to modify it, including whether any of the manufacturer's factory settings were changed, and if so which ones and how (including but not limited to temperature settings, reagents and volumes)

65. I cannot recall.

Question 5 - State when the modifications were made

66. I cannot recall.

Question 6 - Identify those within the Laboratory responsible for the modifications

67. I cannot recall.

Question 7 - State the reasons why the modifications were made

68. I cannot say if or why the modifications were made.

2008 Report

Question 8 - Describe your role in the preparation of the 2008 Report

69. I was not involved in the preparation or the drafting of the 2008 Report. My role was in the calibration of the Multiprobe II Device.

70. From my review of the 2008 Report, it appears that some of the manual tasks which are referenced in the 2008 Report I would have completed. I simply performed the tasks assigned to me, which included from memory:

(a) Preparation of Donor samples, blood and cells with the correspondent dilutions;

(b) Preparation of Reagents for the manual and automated extractions;

(c) Performance of manual DNA IQ extractions of these samples;

(d) Assisting in the verification of Heater tiles temperature;

(e) Cleaning and setting up tiles and labware on the Multiprobe according to the deck layout for each process;

(f) Running the daily and weekly maintenance of the MPII;

(g) Assisting in the Gravimetric evaluation of the pipetting. Setting up the Gravimetric balance on the MPII deck;

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Cecilia Iannazzi Witness

(h) Assisting in the running of the MPII (however cannot recall if I personally ran these plates); and

(i) Entering information into Auslab, (the software we used at that time), where all the sample information, work lists and data obtained from extractions and other process were recorded.

Question 9 - Describe the directions you received in relation to the preparation of the 2008 Report, and identify the person or persons from whom you received those directions.

71. As stated in Question 8, I was not involved in the preparation of the 2008 Report.

72. My supervisor Mr Thomas Nurthen gave me directions in the calibration of the Multiprobe II Device, together with the other staff involved.

73. I assume that the results received from tasks the Automation team were directed to perform, by Mr Nurthen, may have been used in the preparation of this report.

Question 10 - State the substance of the communications (including discussions) that occurred between you, any other authors of the 2008 Report and/or any supervisor or person in a position of management concerning the purpose(s) or intended purpose(s) of the 2008 Report, including by identifying with whom those communications took place and when.

74. I cannot recall.

Question 11 - Identify the persons to whom was the 2008 Report was distributed.

75. I cannot recall.

Question 12 - In relation to the matters stated in the 2008 Report, state

Question 12(a) - how the conclusion on page 1 of the 2008 Report that "Data indicate that results from the automated procedure are comparable to those from the manual procedure" was reached, including:

(i) any discussions or communications between any of the named authors of the 2008 Report and any supervisor or person in a position of management in relation to that conclusion or the referenced data; and

(ii) how that conclusion can be reconciled with the data and figures outlined in part 6.4 of the 2008 Report.

[redacted] [redacted]

Cecilia Iannuzzi Witness

76. I do not know as I was no involved in the drafting of the document.

Question 12(b) - how the recommendations summarised on page 18 of the 2008 Report were decided, including by identifying:

(i) your role in the decision;

(ii) with whom and when you communicated (including by way of discussion) with any other person in connection with the recommendations and the decision to make them

77. I did not contribute to the 2008 Report in any way, including by making recommendations.

78. I did not speak to anyone who made recommendations.

I have not conferred or had any discussion with other witnesses in preparing my statement.

All the facts and circumstances declared in my statement, are within my own knowledge and belief, except for the facts and circumstances declared from information only, and where applicable, my means of knowledge and sources of information are contained in this statement.

I make this solemn declaration conscientiously believing the same to be true and by virtue of the provisions of the *Oaths Act 1867.*

TAKEN AND DECLARED before me at Brisbane, Australia on 24 October 2023

Signature of deponent: .....

Signature of witness: .....

Madeleine Jensen

Australian legal practitioner, MinterEllison

....

Cecilia Iannuzzi Witness

EXHIBITS INDEX

Exhibits Index – Cecilia Iannuzzi Statement

Exhibit	Document Title
CI-01	Project 9: Report on the Evaluation of Commercial DNA Extraction Chemistries 2007
CI-02	Project 11: Report on Validation of a manual method for Extraction DNA using the DNA IQ System, August 2008
CI-03	Project 13. Report on the Verification of an Automated DNA IQ Protocol using the MultiPROBE II Plus HT ES with Gripper Integration Platform.

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Cecilia Iannuzzi

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Witness

Exhibit C1-01

CaSS | Forensic and Scientific Services

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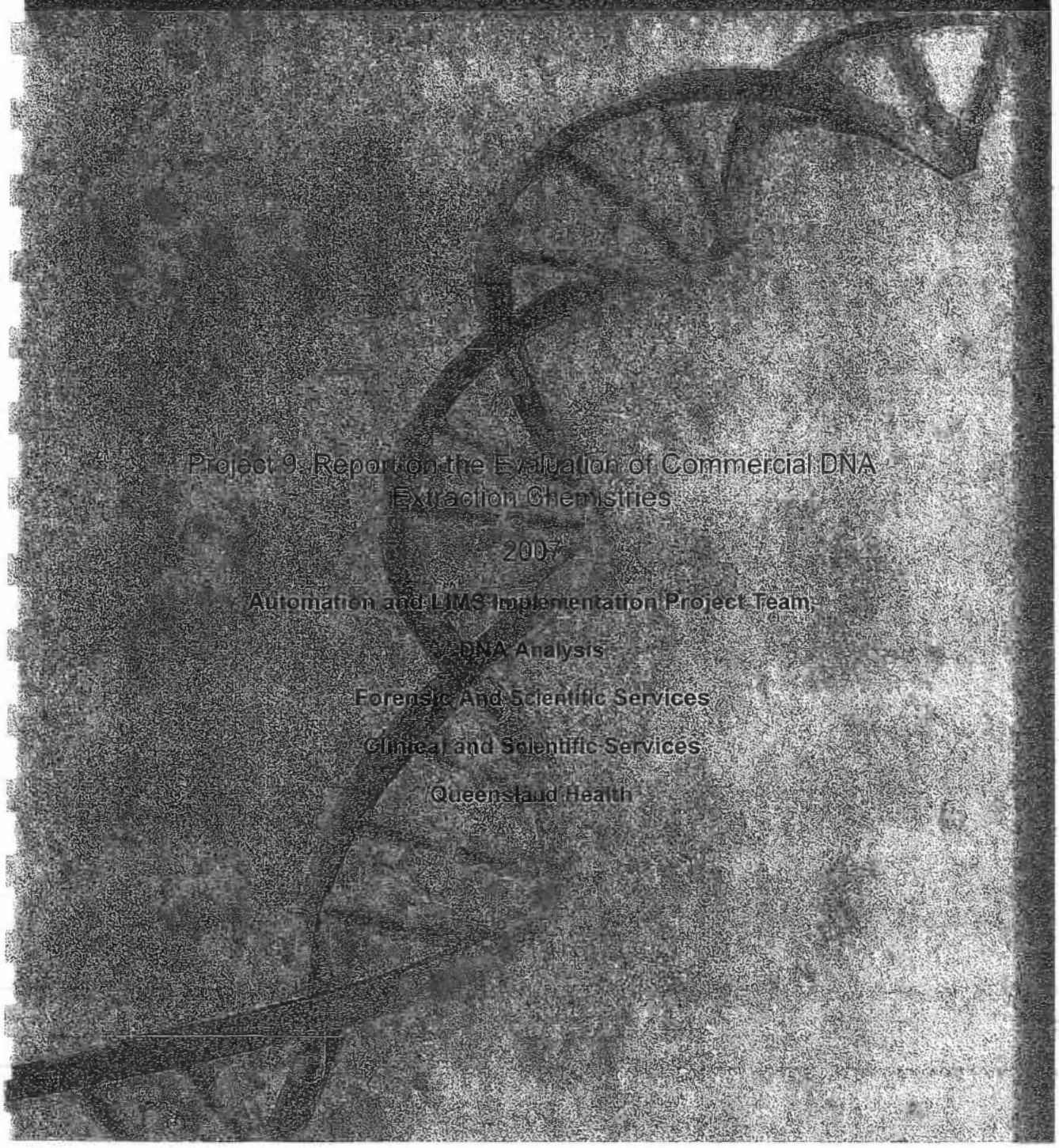

Project 9: Report on the Evaluation of Commercial DNA Extraction Chemistries

2007

Automation and LIMS Implementation Project Team

DNA Analysis

Forensic And Scientific Services

Clinical and Scientific Services

Queensland Health

Exhibit 10-10

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Project 9. Report on the Evaluation of Commercial DNA Extraction Chemistries

Breanna Gallagher^, Vojtech Hlinka^, Cecilia Iannuzzi^, Generosa Lundie^, Iman Muharam^, Thomas Nurthen^, Vanessa Ientile
^ These authors contributed equally.
Automation/LIMS Implementation Project, DNA Analysis FSS (June 2007)

1. Abstract

DNA Analysis FSS performed an evaluation of various commercial DNA extraction chemistries in order to compare their overall performance (quality, yield, user-friendliness and the ability to automate) against the current in-house Chelex® protocol. Out of five commercial kits evaluated, the DNA IQ™ system from Promega Corporation (Madison, WI, USA) was found to be the best out-of-the-box method for DNA extraction of blood and cell samples and will be validated for routine in-house use. This document presents data from the evaluation and provides a discussion of the results observed. For the manual DNA IQ™ validation report, see Project 11. Verification of an automated DNA IQ™ method is reported in Project 13.

2. Introduction

There have been many DNA extraction methods published since DNA was first isolated in 1953 (Butler, 2005). As technology developed and the demand for DNA testing increased, the methods for extracting and purifying DNA have improved. The Chelex® extraction procedure (Walsh *et al.*, 1991) became a quick and easy alternative to the more technically-demanding phenol/chloroform protocol and was more compatible for extracting samples from forensic exhibits, although the resulting DNA extract is still crude and unpurified because inhibitors are not removed from the solution. As the demand for extracting trace DNA samples has increased within the last 10 years to allow interrogation of low copy number forensic samples, coupled with the increase in the need to analyse difficult samples such as touched objects and degraded bone material, new DNA extraction technologies that are designed specifically for forensic samples have increased in availability.

The new DNA extraction chemistries on the market aim to overcome problems encountered in forensic DNA samples as they are designed to:

- Improve removal of inhibitors present in the sample that can affect DNA extraction (e.g. hemoglobin, textile dyes) or prevent successful PCR amplification (e.g. hematin, melanin, polysaccharides, bile salts, humic compounds);
- Maximise recovery of DNA in trace (low copy number) samples by using special buffers that promote cell lysis and integrating a DNA capture system that allows efficient binding and elution of sample DNA, therefore increasing total yields;
- Increase the overall quality and purity of recovered DNA by using special elution or storage buffers, therefore enhancing DNA stability for long-term storage, ensuring reliability and consistency in the sample DNA for reworks and future use.

DNA Analysis FSS obtained various commercial forensic DNA extraction kits (Table 1) in order to evaluate their performance against the in-house Chelex® protocol (see QIS 17171 for detailed information and literature on the Chelex® system).

Table 1. Extraction kits that were evaluated by Forensic Biology FSS.

DNA extraction kit and manufacturer	Technology type
DNA IQ™ (Promega Corp., Madison, WI, USA)	Novel paramagnetic beads
QIAamp® DNA Micro (Qiagen GmbH, Hilden, Germany)	Silica-based membrane
ChargeSwitch® (Invitrogen, Carlsbad, CA, USA)	Magnetic beads
*forensic*GEM™ (ZyGEM, Hamilton, NZ)	Thermophilic proteinase incubation
NucleoSpin® 8 Trace (Macherey-Nagel, Düren, Germany)	Silica-based membrane

Magnetic bead technology is based on the use of magnetic resin that has the capability to bind DNA when subjected to a particular environmental pH or ionic strength. Therefore, by using buffers with different pH values or different ionic components, the binding and elution of DNA can be controlled. Furthermore, whilst the DNA is bound to the resin, the resin-DNA complex can be washed using an alcohol-containing buffer in order to remove inhibitors and residual proteins. A magnetic force is applied during the washing procedure to immobilise the resin-DNA complex and ensure no DNA is lost during washing. Membrane technology is based on a similar principle, except the DNA is immobilised in a thin silica-based membrane within the column.

*forensic*GEM™, the recently-released one-tube proteinase incubation system, uses a thermostable enzyme to digest nucleases in order to yield a crude DNA extract. The enzyme digest method does not incorporate any washing steps, however, and therefore inhibitors are not removed from solution.

3. Aim

To evaluate several commercial DNA extraction kits (as per Table 1) that were specifically designed for forensic DNA samples, using the manufacturer's recommended manual protocols, and compare against the current in-house Chelex® protocol, in order to select a suitable kit for manual validation and automated verification.

4. Equipment and Materials

- Chelex®-100, P/N 143-2832 (Biorad, Hercules, CA, USA)
- DNA IQ™ System, P/N DC6701 (Promega Corp., Madison, WI, USA)
- QIAamp® DNA Micro Kit, P/N 56304 (Qiagen GmbH, Hilden, Germany)
- ChargeSwitch® Forensic DNA Purification Kit, P/N CS11200 (Invitrogen, Carlsbad, CA, USA)
- *forensic*GEM™ (ZyGEM, Hamilton, NZ)
- NucleoSpin® 8 Trace, P/N 740 722.1 (Macherey-Nagel, Düren, Germany)

For preparation of buffers and reagents specific for each kit, see the Methods section that is relevant for that kit.

5. Methods

5.1 Mock sample creation

Refer to document "Mock sample creation for cell and blood samples" (Gallagher *et al.*, 2007) for the detailed protocol.

5.2 DNA extraction kit protocols

The following section provides the principle and protocol for each DNA extraction kit as recommended by the manufacturer. The Chelex® method was as per QIS 17171.

5.2.1. *Chelex®-100 (BioRad)*

Principle

Chelex® is a chelating resin composed of styrene divinylbenzene copolymers, which have a high affinity for polyvalent metal ions. The copolymers contain paired iminodiacetate ions acting as chelating groups which chelate metal ions, including some that degrade DNA while boiling the sample to obtain eluted DNA. Chelex® is the current Forensic Biology FSS standard in-house extraction protocol.

Equipment and Materials

- 20% Chelex® solution (w/v)
- Waterbath
- Magnetic stirrer plate
- 1.5mL sterile tubes
- Spin baskets
- Autoclaved nanopure water
- Vortex
- Centrifuge
- Twirling sticks
- Proteinase K (10mg/mL)
- FTA® Classic Card, P/N WB120205 (Whatman Plc)

Preparation of reagents

- *20% Chelex®-100*
  On balance, to a beaker containing a magnetic stirrer bar, add 2 grams of Chelex®-100 resin. To this, add 10mL of autoclaved nanopure water to make a 20% w/v solution and cover with parafilm. To ensure that the Chelex® is evenly dispersed, place beaker onto a magnetic stirrer plate before pipetting.

Methods (see QIS 17171R9)

1. Label sterile 1.5mL screw-capped tubes which contain sample as well as new elution tubes including extraction controls.
2. Pipette 1mL of autoclaved nanopure water into each tube, vortex gently.
3. Incubate at room temperature for 30 minutes.

The following steps are determined by sample type.

For Cells

4. For buccal FTA® punches, place tubes on multitube vortex for 5min at 12,000rpm.
5. For cell and/or fabric samples, twirl the substrate with a sterile twirling stick for 2min.

Note: Vortex FTA® punches samples then go to "For all sample types."

6. Transfer swab/fabric into spin baskets.
7. Spin tubes with spin basket for 30s at maximum speed (~15,800g or the applicable centrifuge's maximum setting). Discard spin basket with swab.

8. Vortex supernatant, then pour back into original extract tube.

For all sample types

9. Vortex, then spin in centrifuge for 3min at maximum speed (~15,800g or the applicable centrifuge's maximum setting).
10. Carefully remove all but 50µL of supernatant. Leave substrate in tube with pellet.
11. Add 150µL of 20% Chelex® to each tube and vortex.

Note: When pipetting Chelex, the resin beads must be distributed evenly in the solution. Use magnetic stirrer in beaker of Chelex and wide bore pipette tips.

12. Add 4µL of Proteinase K (10mg/mL) to cells and mix gently by vortexing.
13. Incubate in 56°C water bath for 30min for blood and cell samples.
14. Vortex until mixed, then incubate in boiling water bath for 8min.
15. Vortex until mixed, then centrifuge for 3min at maximum speed (~15,800g or the applicable centrifuge's maximum setting).
16. Transfer supernatant to new labelled 1.5mL screw-capped tube, leaving Chelex® beads behind.
17. Samples are stored at -20°C.

5.2.2. *DNA IQ™ System (Promega Corp.)*

Principle

The Promega DNA IQ™ system for small casework samples incorporates two distinct steps. The first step provides an easy, rapid, efficient and almost universal cell lysis method to extract biological materials off stains on solid supports. The second step utilised a specific paramagnetic resin that purifies DNA without extensive washing to remove the lysis reagent. The DNA IQ™ system is designed to purify DNA samples approximately 100ng or less, and is more efficient with samples containing less than 10ng of DNA.

Equipment and Materials

- DNA IQ™ System (100 samples, Cat.# DC6701) containing:
  - 0.9mL Resin
  - 40mL Lysis Buffer
  - 30mL 2X Wash Buffer
  - 15mL Elution Buffer
- MagneSphere® Magnetic Separation Stand, 12-position (Cat.# Z5342)
- DNA IQ™ Spin Baskets (Cat.# V1221)
- Microtube 1.5mL (Cat.# V1231)
- 95-100% ethanol
- Isopropyl alcohol
- 1M DTT
- 65°C heat block
- 70°C heat block
- Vortex mixer

Preparation of Buffers

- *Preparing 1X Wash Buffer*
  i. For DC6701 (100 samples), add 15mL of 95-100% ethanol and 15mL of isopropyl alcohol to 2X Wash Buffer.
  ii. Replace cap and thoroughly mix by inversion.

iii. Mark label to record addition of alcohols.
iv. Label bottle as "1X Wash Buffer".
v. Store bottle at room temperature with lid closed tightly to prevent evaporation.

- *Preparing Lysis Buffer*
  i. Determine the total amount of Lysis Buffer to be used (Table 2) and add 1µL of 1M DTT for every 100µL of Lysis Solution.

Table 2. Total amount of Lysis Buffer required for different sample material types

Material	Lysis Buffer[1]	Lysis Buffer[2]	Total Buffer
Liquid blood	100µL	100µL	200µL
Cotton swab	250µL	100µL	350µL
1/4" CEP swab	250µL	100µL	350µL
15-50mm² S&S 903 paper	150µL	100µL	250µL
3-30mm² FTA® paper	150µL	100µL	250µL
Cloth up to 25mm²	150µL	100µL	250µL

[1] For use in Step 2; [2] For use in Step 9.

ii. Mix by inversion.
iii. Mark and date label to record addition of DTT.
iv. Seal tube and store solution at room temperature for up to one month if required.

Method

DNA isolation from stains on solid material (non-liquid samples)

1. Place sample in a 1.5mL Microtube. The recommended amount of resin can capture a maximum of ~100ng DNA, therefore consider this when determining amount of sample to add.
2. Add 250µL of prepared Lysis Buffer (Table 2). Close lid and place on a 70°C heat block for 30min.
3. Remove tube from heat block and transfer the Lysis Buffer and sample to a DNA IQ™ Spin Basket.
4. Centrifuge at room temperature for 2min at maximum speed. Remove spin basket.
5. Vortex the stock Resin for 10s until it is thoroughly mixed. Add 7µL Resin to the sample. Keep the Resin resuspended while dispensing to obtain uniform results.
6. Vortex sample / Lysis Buffer / Resin mix for 3s. Incubate at room temperature for 5min.
7. Vortex for 2s and place tube in the MagneSphere® Magnetic Separation Stand. Separation will occur instantly.
8. Carefully remove and discard all of the solution without disturbing the Resin on the side of the tube.
9. Add 100µL of prepared Lysis Buffer. Remove the tube from the MagneSphere® Magnetic Separation Stand and vortex for 2 seconds.
10. Return tube to the MagneSphere® Magnetic Separation Stand and discard all Lysis Buffer, without disturbing the resin on the side of the tube.
11. Add 100µL prepared 1X Wash Buffer. Remove tube from the MagneSphere® Magnetic Separation Stand and vortex for 2s.
12. Return tube to the MagneSphere® Magnetic Separation Stand and discard all Wash Buffer, without disturbing the resin on the side of the tube.
13. Repeat steps 11 and 12 once for a total of 2 washes. Make sure that all of the solution has been removed after the last wash.

14. With lid open, air-dry the Resin in the MagneSphere® Magnetic Separation Stand for 5min to 15min.
15. Add 25-100µL Elution Buffer, depending on how much biological material was used. A lower elution volume ensures a higher final concentration of DNA.
16. Close the lid, vortex the tube for 2s and incubate at 65°C for 5min.
17. Remove the tube from the heat block and vortex for 2s. Immediately place on the MagneSphere® Magnetic Separation Stand.
18. Transfer the solution to a fresh tube.
19. Store the DNA extract at 4°C for short-term storage or at -20 or -70°C for long term storage.

5.2.3. *QIAamp® DNA Micro (Qiagen)*

Principle

The QIAamp® DNA Micro kit combines selective binding properties of a silica-based membrane with flexible elution volumes that is suitable for a wide range of sample materials such as small volumes of blood, blood cards, small tissue samples and forensic samples. The basic procedure consists of 4 steps:

- Lysis: the sample is lysed;
- Bind: the DNA in the lysate binds to the membrane of the QIAamp® MinElute column;
- Wash: the membrane is washed;
- Elute: DNA is eluted from the membrane.

Equipment and Materials

- QIAamp® DNA Micro kit containing:
  - QIAamp® MinElute Columns;
  - collection tubes (2mL);
  - Buffer ATL;
  - Buffer AL;
  - Buffer AW1 (concentrate);
  - Buffer AW2 (concentrate);
  - Buffer AE;
  - carrier RNA (red cap);
  - Proteinase K.
- Ethanol (96-100%)
- 1.5mL or 2mL microcentrifuge tubes (for lysis steps)
- 1.5mL microcentrifuge tubes (for elution steps)
- Pipette tips
- Thermomixer
- Microcentrifuge with rotor for 2mL tubes
- Scissors
- Blood collection cards or FTA® card
- Sterile cotton swabs
- DTT

Important points before starting

- Perform all centrifugation steps at room temperature (15-25°C).
- Check whether carrier RNA is required; for purification of DNA from very small amounts of sample, such as low volumes of blood (<10µL) or forensic samples, it is recommended to add

carrier RNA to Buffer AL. For samples containing larger amounts of DNA, addition of carrier RNA is optional.

Steps to perform before starting

- Equilibrate Buffer AE or distilled water for elution to room temperature (15-25°C).
- Set a thermomixer or heated orbital incubator to 56°C for use in step 2, and a second thermomixer or heated orbital incubator to 70°C for use in step 5. If thermomixer or heated orbital incubators are not available, heating blocks or water baths can be used instead.
- If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- If processing semen stains, hair, or nail clippings, prepare an aqueous 1M DTT (dithiothreitol) stock solution. Store aliquots at -20°C. Thaw immediately before use.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions.

Preparation of Buffers

- *Preparing Buffer ATL*
  Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70°C with gentle agitation.
- *Preparing Buffer AL*
  Before starting the procedure, check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70°C with gentle agitation.
- *Preparing Buffer AW1*
  Add 25mL ethanol (96-100%) to the bottle containing 19mL Buffer AW1 concentrate. Tick the check box on the bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15-25°C) for up to 1 year. Note: before starting the procedure, mix the reconstituted Buffer AW1 by shaking.
- *Preparing Buffer AW2*
  Add 30mL ethanol (96-100%) to the bottle containing 13mL Buffer AW2 concentrate. Reconstituted Buffer AW2 can be stored at room temperature (15-25%) for up to 1 year. Note: before starting the procedure, mix the reconstituted Buffer AW2 by shaking.

Method

1. Lysing material stained with blood or saliva: cut out up to 0.5cm^2 of stained material and then cut into smaller pieces. Transfer the pieces to a 2mL microcentrifuge tube. Add 300µL buffer ATL, and 20µL Proteinase K. Close the lid and mix by pulse-vortexing for 10s. Continue this procedure from step 2.
2. Place the tube in a thermomixer or heated orbital incubator, and incubate at 56°C with shaking at 900rpm for at least 1hr. In general, hair is lysed in 1hr. If necessary, increase the incubation time to ensure complete lysis.
3. Briefly centrifuge the tube to remove droplets from the inside of the lid.
4. Add 300µL Buffer AL, close the lid, and mix by pulse vortexing for 10s. To ensure efficient lysis, it is essential that the sample and buffer AL are

thoroughly mixed to yield a homogeneous solution. A white precipitate may form when Buffer AL is added to buffer ATL. The precipitate does not interfere with the QIAamp® procedure and will dissolve during incubation in step 5. Note: if carrier RNA is required, add 1µg dissolved carrier RNA to 300µL buffer AL.

5. Place the tube in the thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900rpm for 10min. If using a heating block or water bath, vortex the tube for 10s every 3min to improve lysis
6. Centrifuge the tube at full speed on a bench top centrifuge (20,000g; 14,000rpm) for 1min.
7. Carefully transfer the supernatant from step 6 to the QIAamp® MinElute column without wetting the rim. Close the lid, and centrifuge at 6,000g (8,000rpm) for 1min. Place the QIAamp® MinElute column in a clean 2mL collection tube, and discard the collection tube containing the flow-through.
8. If lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until QIAamp® MinElute column is empty.
9. Carefully open the QIAamp® MinElute column and add 500µL Buffer WA1 without wetting the rim. Close the lid and centrifuge 6,000g (8,000rpm) for 1min. Place the QIAamp® MinElute column in a clean 2mL collection tube and discard the collection tube containing the flow-through.
10. Carefully open the QIAamp® MinElute column and add 500µL Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6,000g (8,000rpm) for 1min. Place the QIAamp® MinElute column in a clean 2mL collection tube, and discard the collection tube containing the flow-through. Contact between the QIAamp® MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow through, which contains ethanol, coming into contact with the QIAamp® MinElute column. Take care when removing the QIAamp® MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp® MinElute column.
11. Centrifuge at full speed (20,000g; 14,000rpm) for 3min to dry the membrane completely. This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.
12. Place the QIAamp® MinElute column in a clean 1.5mL microcentrifuge tube, and discard the collection tube containing the flow through. Carefully open the lid of the QIAamp® MinElute column and apply 45µL Buffer AE (equilibrated to room temperature) to the centre of the membrane to ensure complete elution of bound DNA. QIAamp® MinElute columns provide flexibility in the choice of elution volume.
13. Close the lid and incubate at room temperature (15-25°C) for 1min. Centrifuge at full speed (20,000g; 14,000rpm) for 1min. Incubating the QIAamp® MinElute columns loaded with Buffer AE or water for 5min at room temperature before centrifugation generally increases DNA yield.

5.2.4. *ChargeSwitch® (Invitrogen)*

Principle

ChargeSwitch® uses a novel magnetic bead-based technology known as ChargeSwitch Technology® (CST®). CST® provides a switchable surface charge, which is switched on and off by changing the pH. With a low pH buffer, the negatively charged DNA backbone binds to the positively charged beads and with a high pH buffer, DNA is eluted by neutralising the charge on the beads.

ChargeSwitch® uses a universal lysis step for all forensic sample types and has been designed to elute DNA from small sample volumes.

ChargeSwitch® uses a basic 4 step principle:

1. Lyse sample;
2. Negatively charged DNA binds to positively charged beads in a buffer with a pH ≤ 6 so charge is switched on;
3. At a pH of 7, charge is still on while beads and bound DNA is washed, removing any contaminants;
4. In a buffer with a pH of 8.5, charge is switched off and DNA is eluted from the beads.

Equipment and Materials

- ChargeSwitch® Forensic DNA Purification kit (stored at room temperature) includes (for 100 preps):
  - ChargeSwitch® Lysis Buffer (L13) – 100mL
  - ChargeSwitch® Magnetic Beads (storage buffer: 10mM MES, pH 5.0, 10mM NaCl, 0.1% Tween 20) – 2 x 1mL
  - Proteinase K (20mg/ml in 50mM Tris-HCl, pH 8.5, 5mM $CaCl^2$ 50% glycerol stored at 4°C) – 1mL
  - ChargeSwitch® Purification Buffer (N5) – 20mL
  - ChargeSwitch® Wash Buffer (W12) – 100mL
  - ChargeSwitch® Elution Buffer (E5; 10mM Tris-HCl, pH 8.5) – 15mL
- MagnaRack™, P/N CS15000 (Invitrogen)
- Sterile, 1.5mL microcentrifuge tubes
- Vortex mixer
- Waterbath set at 55°C

Method

1. Set water bath at 55°C and prepare Lysis master mix in appropriate sized tube using the following formula: *n* x (1mL ChargeSwitch® Lysis buffer + 10µL Proteinase K) where *n* is the number of samples.
2. To tube add 1mL of ChargeSwitch® Lysis Buffer (L13) and immerse forensic sample in mix.
3. Vortex/invert samples for 10-15s to mix then incubate in 55°C water bath for 1hr. Incubation can be shortened to 30min if sample is vortexed or inverted during this step.
4. Remove sample or transfer lysate to clean tube using 1mL pipette tips and pipette.
5. Vortex ChargeSwitch® Magnetic Beads to resuspend evenly in storage buffer.
6. Add 200µL of ChargeSwitch® Purification Buffer (N5) to lysate and mix gently by pipetting up and down.
7. Add 20µL of ChargeSwitch® Magnetic Beads to sample. Pipette-mix to ensure that no bubbles form.
8. Incubate for 1-5min at room temperature to allow the DNA to bind and then place sample tube in MagnaRack™ until a tight pellet has formed. Once this has occurred, aspirate supernatant from tube whilst still in rack and discard, ensuring that the pellet is not disturbed.
9. When supernatant has been completely discarded, remove tube from rack and add 500µL ChargeSwitch® Wash Buffer (W12). Mix gently by pipetting up and down to resuspend the pellet.

10. Allow beads to form a tight pellet by placing tube in MagnaRack™ and remove supernatant completely, without removing from rack or disturbing the pellet and discard.
11. Repeat steps 9 and 10 again.
12. Remove tube from rack, ensuring that supernatant has been completely removed and add 150µL ChargeSwitch® Elution Buffer (E5). Mix by pipetting up and down 10 times.
13. At room temperature, incubate for 1-5min then resuspend pellet and mix like in step 12.
14. Place tube in MagnaRack™ for 1min or until a tight pellet forms. Without removing tube from rack, aspirate DNA supernatant and place in a clean, sterile 1.5mL microcentrifuge tube, ensuring that the pellet is not disturbed. If elution is discoloured repeat steps 12 to 14 again.
15. Discard beads once extraction process is finished and either quantify immediately or store at -20°C.

5.2.5. *forensicGEM™ (ZyGEM)*

Principle

*forensic*GEM™ is a novel thermophilic proteinase developed as a rapid, cheap and effective DNA extraction solution for forensic laboratories that was recently released. It is a simple closed tube forensic DNA extraction method using a thermostable proteinase.

Protocols are available for blood and cell samples.

Equipment and Materials

- *forensic*GEM™ buffer
- *forensic*GEM™
- Heat block or water bath set at 75°C and 95°C
- 20µL sterile Aerosol Resistant Tips
- 0.5-10µL pipettor
- 300µL sterile Aerosol Resistant Tips
- 20-200µL pipettor
- 1mL sterile Aerosol Resistant Tips
- 50µL-1mL pipettor

Method

DNA extraction from buccal swabs using *forensic*GEM™

1. Add buccal swab to tube.
   Note: 1/4 head of swab specified but can utilise up to whole swab.
2. Add 200µL of *forensic*GEM™ buffer.
   Note: if more than 1/4 head of buccal swab is used need to add more *forensic*GEM™ buffer. Moss *et al.* (2003) added 200µL more of the *forensic*GEM™ buffer for trace samples.
3. Add 2µL of *forensic*GEM™.
   Note: *forensic*GEM™ buffer and *forensic*GEM™ can be added as a mastermix.
4. Incubate at 75°C for 15min.
5. Incubate at 95°C for 5min.
6. Remove supernatant to a new tube for storage.

DNA extraction from FTA® containing blood or saliva using *forensic*GEM™

1. UV irradiate plasticware for 5min.

2. Add FTA® punches to each well of a 96-well plate.
   Note: Larger punches can be added but not scalable SOP. PCR tubes can also be used for processing.
3. Add 100µL H_2O and leave at room temperature for 15min.
4. Decant water (remove by pipetting).
5. Add 100µL *forensic*GEM™ buffer and 2µL of *forensic*GEM™.
   Note: The method is not listed as scalable.
6. Incubate at 75°C for 15min.
7. Incubate at 95°C for 5min.
8. Remove supernatant to a new tube for storage.

5.2.6. *NucleoSpin® 8 Trace (Macherey-Nagel)*

Principle

With the NucleoSpin® 8 Trace method, genomic DNA is prepared from forensic samples. Lysis is achieved by incubation of samples in a solution containing chaotropic ions in the presence of proteinase K at room temperature. Appropriate conditions for binding of DNA to the silica membrane in the NucleoSpin® Trace Binding Strips are created by addition of isopropanol to the lysate. The binding process is reversible and specific to nucleic acids. Inhibitors are removed by two washing steps with ethanolic buffer. Pure genomic DNA is finally eluted under low ionic strength conditions in a slightly alkaline elution buffer.

Equipment and Materials

- NucleoSpin® 8 Trace kit, containing:
  - Buffer FLB
  - Buffer B5 (concentrate)
  - Proteinase K (lyophilised)
  - Proteinase Buffer
  - Buffer BE
  - NucleoSpin® Trace Binding Strips
  - MN Wash Plate
  - MN Square-well Blocks
  - MN Tube Strips
  - Cap Strips
  - Self-adhering PE Foil
- NucleoSpin® 8 Trace Starter Set A containing Column Holders A and Dummy Strips
- PVM vacuum manifold (from MultiPROBE® II PLUS HT EX platform)

Preparation of Buffers

- *Proteinase K*
  Add 3mL Proteinase Buffer per vial to dissolve the lyophylised proteinase K and store at -20°C.

- *Buffer B5*
  Add 160mL ethanol to 40mL Buffer B5.
- Store all other components of the kit at room temperature. Storage at lower temperatures may cause precipitation of salts. If a salt precipitate is observed, incubate the bottle at 30-40°C for a few minutes and mix well until all precipitation is redissolved.

Method

1. Premix 25µL Proteinase K and at least 125µL buffer FLB and add to sample. Incubate the sample at room temperature for 3 hours.
2. Insert spacers "MTP/Multi 96 plate" into the vacuum manifold. Place the waste container inside the vacuum manifold and insert a MN Wash Plate into the notches of the spacers. Close the manifold with the lid.
3. Place a NucleoSpin® Trace Binding Strips inserted in Column Holder A into the rubber seal of the vacuum manifold's lid and apply the samples to the wells of the plate.
4. Add 1 volume isopropanol to 2 volumes of lysate, mix three times and transfer to NucleoSpin® Trace Binding Strips.
5. Bind genomic DNA by applying vacuum until all lysates have passed through the columns (-200mbar 2min; -600mbar 10s). Ventilate the vacuum manifold.
6. Wash silica membrane by adding 900µL Buffer B5 to each well of the NucleoSpin® Trace Binding Strips. Apply vacuum (-200mbar 1min) until all buffer has passed through the columns. Ventilate the vacuum manifold.
7. Repeat the wash procedure once.
8. After the final washing step, close the valve, ventilate the vacuum manifold and remove the wash plate and waste container from the vacuum manifold.
9. Remove any residual washing buffer from the NucleoSpin® Trace Binding Strips. If necessary, tap the outlets of the NucleoSpin® Trace Binding Strips onto a clean paper sheet (supplied with the MN Wash Plate) or soft tissue until no drops come out. Insert the column holder with NucleoSpin® Trace Binding Strips into the lid and close the manifold. Apply maximum vacuum (-600mbar) for at least 10min to dry the membrane completely. This step is necessary to eliminate traces of ethanol. Close the valve and ventilate the vacuum manifold.
10. For elution, insert spacers "Microtube Rack" into manifold and rest rack with MN Tube Strips on spacers. Insert Column Holder A with NucleoSpin® Trace Binding Strips into manifold lid. Pipette 100µL Buffer BE directly to the bottom of each well and incubate for 5min at room temperature. Apply vacuum (-400mbar 2min).

5.3 DNA quantitation

All DNA extracts were quantified using the Quantifiler™ Human DNA Quantitation kit (Applied Biosystems, Foster City, CA, USA) as per QIS 19977. Reaction setup was performed on the MultiPROBE® II PLUS HT EX (PerkinElmer) pre-PCR platform.

5.4 PCR amplification and fragment analysis

DNA extracts were amplified using the AmpFℓSTR® Profiler Plus® kit (Applied Biosystems, Foster City, CA, USA) as per QIS 19976. Reaction setup was performed on the MultiPROBE® II PLUS HT EX (PerkinElmer) pre-PCR platform.

5.5 Capillary electrophoresis and fragment analysis

PCR product was prepared for capillary electrophoresis using the manual 9+1 protocol (refer to Project 15 and QIS 19978). Capillary electrophoresis was performed on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3kV injection voltage,10 sec injection time, 15kV run voltage,100µA run current, and 45min run time. Data Collection Software version 1.1 was used to collect

raw data from the ABI Prism® 3100 Genetic Analyzer. Fragment size analysis was performed using GeneScan 3.7. Allele designation was performed using Genotyper 3.7, with thresholds for heterozygous and homozygous peaks at 150 and 300 RFU respectively. The allelic imbalance threshold is 70%.

6. Results and Discussion

6.1 Criteria for acceptance

Various commercial DNA extraction kits (as per Table 1) were evaluated in order to compare their performance against the current in-house Chelex® protocol. These kits were chosen because they were designed specifically for forensic samples and representative of the DNA capture technologies that were out on the market. Furthermore, these kits were manufactured by leaders in the field of DNA extraction technologies with a track record performance in supplying the forensic market with new and reliable products.

We assessed both magnetic bead and silica-based membrane technologies as the automated MultiPROBE® II platforms on which these systems will ultimately be operating on are fully compatible with both systems. The criteria against which the different kits were assessed on include:

1. *Total DNA yield*; the kit must yield sufficient DNA to perform multiple downstream tests such as DNA quantification and PCR amplification.
2. *Quality of the resulting DNA profiles*; the kit should be able to isolate DNA of a suitable quality for PCR amplification of STR loci, in order to generate DNA profiles that are suitable for forensic and human identification purposes.
3. *Ability to remove inhibitors*; the kit must be able to remove common inhibitors present in mock forensic samples (e.g. hemoglobin) using the basic manufacturer's procedure without the use of organic solvents.
4. *Usability*; the kit (and the manufacturer's recommended protocol) must be user-friendly. The necessary steps to prevent cross-contamination should also be described in the protocol. The extraction process should be able to be completed in a reasonable amount of time, comparable to the current procedure.
5. *Availability of validated forensic protocols*; the kit, including the manufacturer's protocol, must be validated for forensic use, either by the manufacturer or by a forensic laboratory, as determined from statements in the manufacturer's protocol or availability of publications in peer-reviewed journals.
6. *Availability of a validated MultiPROBE® II PLUS test file*; the kit should have a validated MPT file for use on the MultiPROBE® II PLUS HT EX platform.

Assessment of points 1, 2 and 3 was performed through experimentation. Point 4 was assessed based on operator feedback. This report provides results for points 1, 2, 3 and 4. A more extensive assessment of Point 3 was performed on the kit that was found to provide the best results for points 1, 2, 3 and 4 and is reported in Project 11. For points 5 and 6, the availability of validated protocols for all kits evaluated is outlined in Table 3.

The acceptance criteria were strictly adhered to in order to objectively evaluate the different systems. Out of all five DNA extraction technologies, there only existed a validated MultiPROBE® II PLUS test file for the DNA IQ™ system (Table 3). Although this was considered an advantage for DNA IQ™, we did not prematurely dismiss any of the other kits prior to evaluation. We decided that if a kit significantly outperformed the rest, and did not have a validated MPT file already created, that we would create a novel program file

with the kit manufacturer's assistance. This, however, would only be decided at the conclusion of the evaluation process.

Table 3. An assessment of available validated protocols for the various kits that were evaluated by Forensic Biology FSS.

Kit	Availability of validated forensic protocol	Availability of validated MPII test file
DNA IQ™	✓	✓
QIAamp® DNA Micro	✓	✓
ChargeSwitch®	✓	✗
*forensic*GEM™	✓	✗
NucleoSpin® 8 Trace	✓	✗

The results and discussion for each of the kits that were evaluated, in comparison to Chelex®, are provided in the following sections. Refer to Tables 4 and 5 for quantitation results for cell and blood samples respectively. Yield calculations for Chelex® samples assume a final elution volume of 150µL.

6.2 Evaluation of DNA IQ™

The DNA IQ™ system uses a novel paramagnetic resin for DNA isolation. It consist of two steps: (1) lysis of the biological material on solid support; (2) using the paramagnetic resin to bind DNA, which allows washing of the resin-DNA complex while the resin is immobilised by a magnetic force, in order to remove the lysis reagent and inhibitors in solution.

The manufacturer's method required the use of the MagneSphere® Magnetic Separation Stand. This magnetic stand is used for the separation of the magnetic pellet in 12 samples at a time. The time to process a batch of 12 samples using the DNA IQ™ system takes about 3 hours, including 30 minutes of incubation time.

Three controls were run with each extraction batch: (1) a negative extraction control (empty tube); (2) a positive extraction control (QC dot saliva or blood depending on the extraction) and (3) a substrate blank (the substrate with only saline).

Samples were extracted using the DNA IQ™ method as described in the Methods section and eluted using 100µL Elution Buffer. Due to volume loss during pipetting, the final elution volume is actually around 95µL. The same set of samples was also extracted using the in-house Chelex® protocol for comparison. Tables 4 and 5 display the DNA concentration (ng/µL) and yield (ng) for all cell and blood samples, compared to the results generated by Chelex®.

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Table 4. Quantitation values for cell samples on different substrates after extraction by Chelex® and the evaluated DNA extraction kits.

Cells samples		Chelex		DNA IQ		QIAamp DNA Micro		ChargeSwitch		forensicGEM		NucleoSpin Tissue	
Sample ID	Substrate type	Concentration ng/uL	Yield* ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng
F5 (4uL, neat)	FTA	0.058800	11.877600	0.028760	2.870000	0.006030	0.271350	0.023900	3.585000	0.025700	2.621400	0.018200	1.820000
S5 (cotton) (4uL, neat)	Cotton swab	0.007410	1.111500	0.098060	9.800000	0.025800	1.161000	0.096700	14.505000	0.083300	16.824600	0.018900	1.890000
C5 (4uL, neat)	Cotton cloth	0.001480	0.222000	0.050700	5.070000	0.004880	0.219600	0.014900	2.235000	0.037400	7.548000	0.019000	1.190000
D5 (4uL, neat)	Denim cloth	0.002350	0.354000	0.028200	2.820000	0.002160	0.097200	0.003250	0.487500	0.041300	8.342600	0.043900	4.390000
R14_Neat 4uL	Rayon swab	0.001620	0.243000	0.010300	1.000000	0.000000	0.000000	0.011800	1.770000	0.024000	4.848000	0.031800	3.180000
R15_Neat 4uL	Rayon swab	0.001580	0.237000	0.019400	1.940000	0.005050	0.227250	0.018100	2.715000	0.019000	3.83800	0.115000	11.500000
R16_Neat 4uL	Rayon swab	0.000000	0.000000	0.015500	1.550000	0.006610	0.297450	0.027400	4.110000	0.018300	3.696600	0.057400	5.740000
R17_Neat 4uL	Rayon swab	0.000000	0.000000	0.011200	1.120000	0.007310	0.328950	0.005910	0.886500	0.013700	2.767400	0.029900	2.990000
Rayon mean (Neat)		*0.000600*	*0.120000*	*0.014025*	*1.402500*	*0.004743*	*0.213413*	*0.015803*	*2.370375*	*0.018500*	*3.737000*	*0.056525*	*5.652500*
Rayon STD (Neat)		*0.000924*	*0.138586*	*0.004291*	*0.429137*	*0.003300*	*0.148490*	*0.009195*	*1.379299*	*0.005285*	*1.067465*	*0.039683*	*3.968332*
F4 (4uL, 1/4 dilution)	FTA	0.010300	2.060600	0.005790	0.579000	0.005270	0.237150	0.001260	0.189000	0.007610	0.766020	0.005710	0.571000
S4 (cotton) (4uL, 1/4 dilution)	Cotton swab	0.000755	0.113400	0.019000	1.900000	0.001480	0.066600	0.031600	4.740000	0.038900	3.841800	0.009500	0.950000
C4 (4uL, 1/4 dilution)	Cotton cloth	0.000541	0.081150	0.015200	1.520000	0.040900	1.840500	0.000000	0.000000	0.011600	2.343200	0.018900	1.890000
D4 (4uL, 1/4 dilution)	Denim cloth	0.000000	0.000000	0.045800	4.580000	0.041800	1.881000	0.001720	0.258000	0.013400	2.706800	0.017800	1.780000
R10_1/4 4uL	Rayon swab	0.000558	0.083700	0.005740	0.574000	0.001800	0.081000	0.002850	0.429000	0.002950	0.595900	0.005760	0.576000
R11_1/4 4uL	Rayon swab	0.000000	0.000000	0.002560	0.256000	0.001300	0.058500	0.000150	0.022500	0.002020	0.408040	0.001220	0.122000
R12_1/4 4uL	Rayon swab	0.000698	0.134700	0.009750	0.975000	0.005570	0.250650	0.005560	0.984000	0.002340	0.472680	0.010200	1.020000
R13_1/4 4uL	Rayon swab	0.000433	0.064950	0.000000	0.000000	0.001550	0.069750	0.001350	0.202500	0.004030	0.814060	0.016000	1.600000
Rayon mean (1/4)		*0.000472*	*0.070838*	*0.004513*	*0.451250*	*0.002555*	*0.114975*	*0.004230*	*0.634500*	*0.009835*	*0.572670*	*0.008545*	*0.854500*
Rayon STD (1/4)		*0.000371*	*0.055657*	*0.004208*	*0.420765*	*0.002020*	*0.090915*	*0.002539*	*0.380328*	*0.000885*	*0.178801*	*0.008196*	*0.819564*
F3 (4uL, 1/8 dilution)	FTA	0.008170	1.650340	0.008410	0.841000	0.000000	0.000000	0.000000	0.000000	0.003170	0.643620	0.000000	0.000000
S3 (cotton) (4uL, 1/8 dilution)	Cotton swab	0.003710	0.556500	0.012100	1.210000	0.001680	0.075600	0.009130	1.368500	0.003970	0.801940	0.014900	1.490000
C3 (4uL, 1/8 dilution)	Cotton cloth	0.002600	0.390000	0.010400	1.040000	0.000000	0.000000	0.000355	0.053250	0.005060	1.012020	0.006570	0.657000
D3 4uL, 1/8 dilution)	Denim cloth	0.000739	0.110850	0.007630	0.763000	0.015100	0.679500	0.000000	0.000000	0.002700	0.539540	0.000000	0.000000
R6_1/8 4uL	Rayon swab	0.000000	0.000000	0.001010	0.101000	0.000000	0.000000	0.000697	0.104550	0.003100	0.626200	0.007860	0.786000
R7_1/8 4uL	Rayon swab	0.000000	0.000000	0.000982	0.098200	0.000000	0.000000	0.000000	0.000000	0.003160	0.638320	0.013800	1.380000
R8_1/8 4uL	Rayon swab	0.000000	0.000000	0.001540	0.154000	0.000000	0.000000	0.003390	0.508500	0.000000	0.000000		
R9_1/8 4uL	Rayon swab	0.000739	0.110850	0.003050	0.305000	0.000000	0.000000	0.003360	0.504000	0.000000	0.000000		
Rayon mean (1/8)		*0.000185*	*0.027713*	*0.001846*	*0.184550*	*0.000000*	*0.000000*	*0.001862*	*0.279263*	*0.001565*	*0.316130*	*0.010830*	*1.083000*
Rayon STD (1/8)		*0.000370*	*0.055425*	*0.000971*	*0.097088*	*0.000000*	*0.000000*	*0.001770*	*0.265582*	*0.001907*	*0.385089*	*0.004200*	*0.420021*
F2 (4uL, 1/16 dilution)	FTA	0.000000	0.000000	0.000935	0.093500	0.003940	0.177300	0.000000	0.000000	0.001840	0.157680	0.000000	0.000000
S2 (cotton) 4uL, 1/16 dilution)	Cotton swab	0.000000	0.000000	0.002900	0.290000	0.000000	0.000000	0.001520	0.228000	0.002250	0.460350	0.000000	0.000000
C2 (4uL, 1/16 dilution)	Cotton cloth	0.000000	0.000000	0.005010	0.501000	0.001870	0.084150	0.000000	0.000000	0.000741	0.149882	0.000000	0.000000
D2 (4uL, 1/16 dilution)	Denim cloth	0.000000	0.000000	0.002870	0.287000	0.000227	0.010215	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
R2_1/16 4uL	Rayon swab	0.000000	0.000000	0.000717	0.071700	0.000000	0.000000	0.000000	0.000000	0.001200	0.242400	0.000000	0.000000
R3_1/16 4uL	Rayon swab	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000632	0.127664	0.002280	0.228000
R4_1/16 4uL	Rayon swab	0.000720	0.108000	0.002230	0.223000	0.000000	0.000000	0.003640	0.546000	0.002590	0.523180	0.004480	0.448000
R5_1/16 4uL	Rayon swab	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000788	0.159176	0.002250	0.225333
Rayon mean (1/16)		*0.000180*	*0.027000*	*0.000737*	*0.073675*	*0.000000*	*0.000000*	*0.000910*	*0.136500*	*0.001303*	*0.263105*	*0.002240*	*0.224012*
Rayon STD (1/16)		*0.000360*	*0.054000*	*0.001051*	*0.105131*	*0.000000*	*0.000000*	*0.001820*	*0.273000*	*0.000891*	*0.180012*	*0.002240*	*0.224012*

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Table 5. Quantitation values for blood samples on rayon swab substrates after extraction by Chelex® and the evaluated DNA extraction kits.

Blood samples	Chelex		DNA IQ		QIAamp DNA Micro		ChargeSwitch		forensicGEM		NucleoSpin 8 Trace	
Sample ID	Concentration ng/uL	Yield* ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng	Concentration ng/uL	Yield ng
R14 (Neat)	2.37	355.5	0.482	48.2	2.31	103.95	0.751	112.65	0.00833	1.66266	1.16	116
R15 (Neat)	1.42	213	0.078	7.8	3.58	161.1	0.754	113.1	0.0066	1.3332	2.61	261
R16 (Neat)	0.512	76.8	0.356	35.6	3.32	149.4	0.929	139.35	0.0046	0.9292	1.61	161
R17 (Neat)	0.934	140.1	0.467	46.7	2.46	110.7	0.916	137.4	0.00727	1.46854	2.18	218
Mean (Neat)	*1.3090*	*196.3500*	*0.3458*	*34.5750*	*2.9175*	*131.2875*	*0.8375*	*125.6250*	*0.0067*	*1.3534*	*1.8900*	*189.0000*
STD (Neat)	*0.7987*	*119.8085*	*0.1871*	*18.7137*	*0.6270*	*28.2137*	*0.0983*	*14.7451*	*0.0016*	*0.3173*	*0.6361*	*63.6082*
R10 (1/4)	0.219	32.85	0.238	23.8	0.227	10.215	0.219	32.85	0.00211	0.42622	0.611	61.1
R11 (1/4)	0.0845	12.675	0.198	19.8	1.72	77.4	0.101	15.15	0.000597	0.120594	0.3	30
R12 (1/4)	0.216	32.4	0.195	19.5	4.59	206.55	0.0673	10.095	0.00128	0.25856	0.251	25.1
R13 (1/4)	0.165	24.75	0.136	13.6	0.657	29.565	0.0787	11.805	0.00166	0.33532	0.227	22.7
Mean (1/4)	*0.1711*	*25.6688*	*0.1918*	*19.1750*	*1.7985*	*80.9325*	*0.1165*	*17.4750*	*0.0014*	*0.2852*	*0.3473*	*34.7250*
STD (1/4)	*0.0628*	*9.4262*	*0.0420*	*4.2019*	*1.9639*	*88.3776*	*0.0698*	*10.4628*	*0.0006*	*0.1294*	*0.1784*	*17.8438*
R6 (1/8)	6.88	1032	0.0554	5.54	0.0936	4.212	0.094	14.1	0.0126	2.5452	0.154	15.4
R7 (1/8)	0.164	24.6	0.114	11.4	0.175	7.875	0.0735	11.025	0.00174	0.35148	0.148	14.8
R8 (1/8)	0.286	42.9	0.145	14.5	0.123	5.535	0.0521	7.815	0.00363	0.73326	0.178	17.8
R9 (1/8)	0.513	76.95	0.125	12.5	0.0151	0.6795	0.0939	14.085	0.00167	0.33734	0.0819	8.19
Mean (1/8)	*1.9608*	*294.1125*	*0.1099*	*10.9850*	*0.1017*	*4.5754*	*0.0784*	*11.7563*	*0.0049*	*0.9918*	*0.1405*	*14.0475*
STD (1/8)	*3.2827*	*492.4030*	*0.0385*	*3.8501*	*0.0668*	*3.0066*	*0.0200*	*2.9991*	*0.0052*	*1.0517*	*0.0411*	*4.1145*
R2 (1/16)	0.0405	6.075	0.0792	7.92	0.0349	1.5705	0.0347	5.205	0.00757	1.52514	0.0766	7.66
R3 (1/16)	0.0104	1.56	0.0566	5.66	0.0454	2.043	0.027	4.05	0.00667	1.34734	0.0923	9.23
R4 (1/16)	0.0337	5.055	0.0847	8.47	0.0386	1.737	0.0197	2.955	0.00544	1.09888	0.0588	5.88
R5 (1/16)	0.0323	4.845	0.109	10.9	0.0276	1.242	0.021	3.15	0.00245	0.4949	0.874	87.4
Mean (1/16)	*0.0292*	*4.3838*	*0.0824*	*8.2375*	*0.0366*	*1.6481*	*0.0256*	*3.8400*	*0.0055*	*1.1176*	*0.2754*	*27.5425*
STD (1/16)	*0.0131*	*1.9577*	*0.0215*	*2.1515*	*0.0074*	*0.3341*	*0.0068*	*1.0274*	*0.0022*	*0.4510*	*0.3993*	*39.9285*

Comparison of quantitation results for cell samples

Refer to Table 4 for observed data. Using DNA IQ™, neat cell samples displayed higher quantitation results for both cotton and rayon swabs, and also for cotton and denim cloth materials. Only for the FTA® card was the result higher for the Chelex® sample. For 1/4 dilutions, DNA IQ™ results were higher than Chelex® results. For 1/8 dilutions, both protocols showed similar results for most sample types. Rayon swabs produced zero quantitation values for Chelex®, but exhibited consistent results for DNA IQ™. For 1/16 dilutions, most Chelex® samples were undetermined, whereas most DNA IQ™ samples yielded quantitation results.

Only three dilution samples extracted by DNA IQ™ gave zero quantitation values. In contrast, fourteen Chelex® samples gave zero quantitation results. This suggests that the DNA IQ™ sample recovery rate is 111% greater than that of the Chelex® protocol for cell samples.

Comparison of quantitation results for blood samples

Refer to Table 5 for observed data. For this experiment, only rayon samples (in quadruplicate) were tested. Neat blood samples showed higher concentration results when extracted using Chelex®. The 1/4 dilutions showed similar results for both methods. The 1/8 dilutions showed better results for Chelex®, but this was primarily due to an outlier result for one of the replicates (highlighted red in Table 4) that resulted in a concentration value 1300% greater than the remaining samples. This occurrence could be the result of inaccurate pipetting during mock sample creation or variability in the Chelex® method, specifically the inconsistent final elution volumes. For the 1/16 dilutions, the DNA IQ™ results were better. All DNA IQ™ results were more consistent and reproducible than Chelex® results.

Overall, samples that were extracted using DNA IQ™ showed quantitation results that were similar to or better than samples that were extracted using Chelex®. For cell substrates, 44% of Chelex® samples gave zero quantitation results, compared to only 9% for DNA IQ™ samples. All blood substrates generated quantitation results that were similar for both methods. Furthermore, DNA IQ™ generated results that were more sensitive, consistent and reproducible across multiple replicates.

Comparison of DNA profiles

Cell samples that were extracted using the DNA IQ™ method gave DNA profiles with more alleles compared to extractions performed using Chelex® (Table 6). Overall, DNA IQ™ resulted in 282 reportable alleles (excluding Amelogenin), compared to 89 alleles resolved by Chelex®, or in other words samples extracted using DNA IQ™ generated 216% more reportable alleles compared to samples extracted using Chelex®. For neat cell substrates, DNA IQ™ samples generated full profiles in all instances except 2: an X,X+14 for the FTA® substrate and an X,X+16 for a rayon swab replicate. All rayon samples extracted by Chelex® did not produce any profiles at all, in contrast to the full profile results using DNA IQ™. DNA IQ™ also gave more reportable alleles for the lower dilutions compared to Chelex®. Additionally, DNA IQ™ was able to yield full profiles from denim substrates, compared to Chelex® which yielded no profiles at all. This observation indicates the superiority of the DNA IQ™ system for removing and overcoming inhibition due to denim dye. Only one occurrence of allelic imbalance (68% at D13S317) was encountered in all 64 samples.

Table 6. Comparison of DNA profiles for cell substrate samples extracted using either Chelex® or DNA IQ™.

CELLS Method: Chelex

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+18	X,X+18	R14	NSD	X,X+8	NR/NSD
			R15	NR/NSD		
			R16	NSD		
			R17	NSD		
Dil 1/4	X,X+18	X,NR+3	R10	NSD	NR+1	NR/NSD
			R11	NSD		
			R12	NSD		
			R13	NSD		
Dil 1/8	X,X+17	X,X+3	R6	NSD	X,NR+3	NR/NSD
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NSD	NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

CELLS Method: DNA IQ

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+14	X,X+18	R14	X,X+18	X,X+18	X,X+18
			R15	A1@D13(6&7)		
			R16	X,X+8		
			R17	X,X+18		
Dil 1/4	X,X+17	X,X+16	R10	X,NR+3	X,X+18	XX+18
			R11	NR/NSD		
			R12	X,X+6		
			R13	NR/NSD		
Dil 1/8	X,X+8	X,X+16	R6	NR/NSD	X,X+17	X,X+17
			R7	NR/NSD		
			R8	NR/NSD		
			R9	NR/NSD		
Dil 1/16	NSD	X,X+4	R2	NR/NSD	NR/NSD	NR/NSD
			R3	NSD		
			R4	NR/NSD		
			R5	NSD		

For blood samples, only rayon substrates were extracted using the DNA IQ™ system as these were deemed sufficient for observing the effects of heme inhibition (without the need to factor variable substrate types). Almost all samples generated full profiles or a sufficient number of reportable alleles for matching purposes (Table 7). For neat samples extracted by Chelex®, no profiles were resulted from the FTA®, cotton swab or denim samples, indicating possible heme inhibition that could not be removed by the Chelex® protocol. For rayon samples, 19% of those extracted by Chelex® did not generate a profile, whereas DNA IQ™ yielded full profiles for all dilutions except two neat samples. Reworks of the two failed samples were performed but yielded the same NSD results. These failed results appear to be outliers, as all other dilutions yielded the expected results. It was observed that results from blood samples on rayon swabs were more likely (32%) to exhibit allelic imbalance at Amelogenin when extracted using the DNA IQ™ system.

Table 7. Comparison of DNA profiles for blood substrate samples extracted using either Chelex® or DNA IQ™.

BLOOD Method: Chelex Method: DNA IQ

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim		Rayon swabs	
	Profile	Profile	Sample#	Profile	Profile	Profile		Sample#	Profile
Neat	NSD	NSD	R14	X,Y+18	X,Y+18	NSD		R14	NSD
			R15	X,Y+18				R15	NSD
			R16	X,Y+18				R16	X,Y+18(AI@AMEL)
			R17	NR/NSD				R17	X,Y+18(AI@AMEL)
Dil 1/4	X,Y+18	X,Y+15	R10	Not Uploaded	X,Y+18	X,Y+18		R10	X,Y+18
			R11	X,Y+18				R11	X,Y+18(AI@AMEL)
			R12	X,Y+18				R12	X,Y+18
			R13	X,Y+18				R13	X,Y+18
Dil 1/8	X,Y+18(AI@D	X,Y+18	R6	X,Y+18	X,Y+18	X,Y+18		R6	X,Y+18(AI@AMEL)
			R7	X,Y+18				R7	X,Y+18
			R8	NR/NSD				R8	X,Y+18
			R9	X,Y+18				R9	X,Y+18
Dil 1/16	X,Y+18	X,Y+18	R2	X,Y+18	X,Y+18	X,Y+18		R2	X,Y+18(AI@AMEL)
			R3	X,Y+18				R3	X,Y+18
			R4	X,Y+18				R4	X,Y+18
			R5	X,Y+18				R5	X,Y+18

We found the DNA IQ™ system yielded results that were either comparable or better than results generated by samples extracted using the in-house Chelex® protocol, both in terms of quantitation values and DNA profile quality and completeness.

6.3 Evaluation of QIAamp® DNA Micro

The QIAamp® DNA Micro kit was designed for the purification of genomic and mitochondrial DNA from small sample volumes or sizes, as often encountered in forensics. The system uses a silica-based membrane to accommodate DNA binding and purification using special buffers, followed by elution in buffer or water, resulting in purified DNA that is free of proteins, nucleases and other impurities.

The QIAamp® DNA Micro system consists of four steps: lysing, binding, washing, followed by elution:

- *Lysis* – Small samples are lysed under highly denaturing conditions at elevated temperatures under the presence of Proteinase K.
- *Binding* – Using Buffer AL and ethanol, DNA is adsorbed into the silica-gel membrane of the column by centrifugation or application of a vacuum. The buffer is formulated so that proteins and other components are not retained in the membrane.
- *Washing* – While DNA is bound to the silica membrane, contaminants are efficiently washed away using a combination of two wash buffers.
- *Elution* – DNA is eluted in a small volume of Buffer AE or sterile water, yielding concentrated DNA.

The QIAamp® protocol involves 5 tube transfers and therefore takes approximately 5 hours to perform a manual extraction of 12 samples. The same set of samples that were used for the DNA IQ™ evaluation was also used to evaluate QIAamp® DNA Micro. Each extraction batch included a positive and negative control, and also a substrate blank. DNA was eluted in 45µL volume.

Comparison of quantitation results for cell samples

Refer to Table 4 for observed data. Twelve samples extracted by QIAamp® gave zero quantitation values, compared to fourteen samples by Chelex®. Despite the low elution volume of 45µL in the QIAamp® protocol that serves to concentrate the purified DNA, quantitation results for all samples were comparable for both DNA extraction methods.

Comparison of quantitation results for blood samples

Refer to Table 5 for observed data. Blood on rayon swab samples displayed wide variation between replicates. For neat samples, the total yield is comparable to Chelex®, however lower dilutions (1/8 – 1/16) suffer from inconsistencies. One of the 1/4 dilution replicates displayed an unexpectedly high quantitation value that was more than 3x greater than the Chelex® average yield, but this can be attributed to inaccurate pipetting, or pipetting of a non-uniform sample mixture, during mock sample creation.

A possible reason as to why the quantitation results for both cell and blood samples were inconsistent is because the QIAamp® DNA Micro protocol uses five sets of collection tubes for supernatant transfer, therefore possibly causing sample lost during multiple sample transfers from one tube to another.

Comparison of DNA profiles

Cell samples that were extracted using the QIAamp® protocol showed profile results that were either comparable or worse than samples that were extracted using the Chelex® protocol (Table 8). Out of 32 samples, only one QIAamp® sample resulted in a full profile (X,X+18). QIAamp® samples failed to produce full profiles for all but one (n = 8) of the neat samples. Overall, QIAamp® resulted in 86 reportable alleles compared to 89 alleles resolved by Chelex®. Some of the QIAamp® allele calls are inconsistent, e.g. the result for 1/4 dilution on cotton cloth was slightly better than the neat sample. This is further exemplified by the denim substrate samples. The QIAamp® method did not appear to effectively overcome inhibition caused by the denim dye as observed from the resulting profiles.

Table 8. Comparison of DNA profiles for cell substrate samples extracted using either Chelex® or QIAamp® DNA Micro.

CELLS Method: Chelex

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+18	X,X+18	R14	NSD	X,X+8	NR/NSD
			R15	NR/NSD		
			R16	NSD		
			R17	NSD		
Dil 1/4	X,X+18	X,NR+3	R10	NSD	NR+1	NR/NSD
			R11	NSD		
			R12	NSD		
			R13	NSD		
Dil 1/8	X,X+17	X,X+3	R6	NSD	X,NR+3	NR/NSD
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NSD	NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

CELLS Method: QIAamp DNA Micro

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+12	X,X+16	R14	NSD	NR,NR+2	NR/NSD
			R15	NR/NSD		
			R16	XNR+6		
			R17	NR/NSD		
Dil 1/4	X,X+14	X,X+15	R10	NSD	NR,NR+3	NR/NSD
			R11	NSD		
			R12	NR,NR+1		
			R13	NR/NSD		
Dil 1/8	NSD+2	X,NR+6	R6	NSD	NR/NSD	XNR+7
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NR/NSD	NR/NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

Table 9. Comparison of DNA profiles for blood substrate samples extracted using either Chelex® or QIAamp® DNA Micro.

BLOOD Method: Chelex Method: QIAamp DNA Micro

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim		Rayon swabs	
	Profile	Profile	Sample#	Profile	Profile	Profile		Sample#	Profile
Neat	NSD	NSD	R14	X,Y+18	X,Y+18	NSD		R14	X,Y+18
			R15	X,Y+18				R15	X,Y+18
			R16	X,Y+18				R16	X,Y+18
			R17	NR/NSD				R17	NR,Y+15
Dil 1/4	X,Y+18	X,Y+15	R10	Not Uploaded	X,Y+18	X,Y+18		R10	X,Y+18
			R11	X,Y+18				R11	X,Y+17
			R12	X,Y+18				R12	X,Y+18
			R13	X,Y+18				R13	X,Y+18
Dil 1/8	X,Y+18(Al@D	X,Y+18	R6	X,Y+18	X,Y+18	X,Y+18		R2	X,Y+18
			R7	X,Y+18				R3	X,Y+18
			R8	NR/NSD				R4	X,Y+18
			R9	X,Y+18				R5	X,Y+18
Dil 1/16	X,Y+18	X,Y+18	R2	X,Y+18	X,Y+18	X,Y+18		R2	X,Y+18(Al@D8,D18)
			R3	X,Y+18				R3	X,Y+18
			R4	X,Y+18				R4	X,Y+18
			R5	X,Y+18				R5	X,Y+18

For blood samples on rayon swabs, 87.5% of QIAamp® samples resulted in full profiles, compared to 81.25% of Chelex® samples (Table 9). Out of all QIAamp® rayon swab samples, only one of the 1/16 replicates displayed allelic imbalance (in D8S1179 and D18S51).

6.4 Evaluation of ChargeSwitch®

The ChargeSwitch® technology (CST) is another magnetic bead-based technology that provides a switchable surface charge dependent on the pH of the surrounding buffer environment to facilitate DNA isolation from small forensic samples. In low pH conditions, the ChargeSwitch® beads have a positive charge that allows negatively-charged DNA to bind. In this environment, proteins and other contaminants are not bound and can be washed away. By using a low salt elution buffer at pH 8.5, the charge on the bead surface is neutralised and DNA can be eluted for immediate use in downstream forensic applications.

The ChargeSwitch® Elution Buffer (E5) that is supplied with the kit is used to provide an environment with a pH of 8.5 that promotes dissociation of bound DNA from the magnetic beads and therefore efficient elution of purified DNA. However, TE buffer with a pH between 8.5 – 9.0 can also be used for elution. TE buffer outside of this pH range should not be used. The use of water for elution is also not recommended.

The manufacturer's method required the use of the MagnaRack™ two-piece magnetic separation rack that consists of two components: a magnetic base station and removable tube rack. The tube rack holds up to 24 microcentrifuge tubes and fits onto the magnetic base station in two different positions associating the row of 12 neodymium magnets with a single row of 12 tubes for simple 'on the magnet' and 'off the magnet' processing. The time to process a batch of 12 samples using the ChargeSwitch® system takes about 3.5 hours, including 30 minutes of incubation time. Each extraction batch included a positive and negative control, and also a substrate blank. Purified DNA samples were eluted in 150µL Elution Buffer (E5).

Comparison of quantitation results for cell samples

Refer to Table 4 for observed data. For cells samples, ChargeSwitch® performed moderately better compared to the current in-house Chelex® method. When comparing the quantitiation values, ChargeSwitch® produced higher quantitation values for cotton and rayon swabs over all dilutions as well as the neat samples of cotton shirt and denim jeans. For other cell samples, ChargeSwitch® performance was comparable to the Chelex® results.

Comparison of quantitation results for blood samples

Refer to Table 5 for observed data. ChargeSwitch® quantitation results for blood samples on rayon swabs were lower but more consistent than Chelex® results.

Comparison of DNA profiles

Cell samples that were extracted using the ChargeSwitch® system showed profile results that were comparable to samples that were extracted using the Chelex® protocol (Table 10). Overall, ChargeSwitch® resulted in 138 reportable alleles compared to 89 alleles resolved by Chelex®. ChargeSwitch® performance for cell samples on FTA® cards was poor for any samples less than the neat dilution. Profiles for both cotton swab and cotton cloth samples were slightly better for ChargeSwitch®, and results for neat samples on rayon

swabs outperformed Chelex®. However, the ChargeSwitch® system was unable to overcome inhibition in denim samples, and did not yield any DNA profiles at all, despite displaying quantitation results for the neat and 1/4 dilution.

Table 10. Comparison of DNA profiles for cell substrate samples extracted using either Chelex® or ChargeSwitch®.

CELLS Method: Chelex

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+18	X,X+18	R14	NSD	X,X+8	NR/NSD
			R15	NR/NSD		
			R16	NSD		
			R17	NSD		
Dil 1/4	X,X+18	X,NR+3	R10	NSD	NR+1	NR/NSD
			R11	NSD		
			R12	NSD		
			R13	NSD		
Dil 1/8	X,X+17	X,X+3	R6	NSD	X,NR+3	NR/NSD
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NSD	NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

CELLS Method: ChargeSwitch

	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+17	X,X+18	R14	X,X+8	X,X+11	NSD
			R15	X,X+13		
			R16	X,X+16		
			R17	X,X+8		
Dil 1/4	X,X+9	X,X+18	R10	X,X+NR+5	NR/NR+2	NSD
			R11	NR/NSD		
			R12	X,NR+2		
			R13	X,NR+NSD		
Dil 1/8	NR/NSD	X,X+12	R6	NSD	NR/NSD	NSD
			R7	NSD		
			R8	NR/NSD		
			R9	NSD		
Dil 1/16	NSD	NR/NSD	R2	NR/NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

For blood samples on rayon swab substrates, all ChargeSwitch® samples consistently yielded full profiles for all dilutions and therefore outperformed Chelex® (Table 11). Two replicates of the lower, 1/16 dilutions displayed allelic imbalance at two different loci: D3S1358 and D7S820, possibly due to stochastic effects that arise from amplifying low concentrations of DNA.

Table 11. Comparison of DNA profiles for blood substrate samples extracted using either Chelex® or ChargeSwitch®.

BLOOD Method: Chelex Method: ChargeSwitch

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim	Rayon swabs	
	Profile	Profile	Sample#	Profile	Profile	Profile	Sample#	Profile
Neat	NSD	NSD	R14	X,Y+18	X,Y+18	NSD	R14	X,Y+18
			R15	X,Y+18			R15	X,Y+18
			R16	X,Y+18			R16	X,Y+18
			R17	NR/NSD			R17	X,Y+18
Dil 1/4	X,Y+18	X,Y+15	R10	Not Uploaded	X,Y+18	X,Y+18	R10	X,Y+18
			R11	X,Y+18			R11	X,Y+18
			R12	X,Y+18			R12	X,Y+18
			R13	X,Y+18			R13	X,Y+18
Dil 1/8	X,Y+18(AI@D	X,Y+18	R6	X,Y+18	X,Y+18	X,Y+18	R6	X,Y+18
			R7	X,Y+18			R7	X,Y+18
			R8	NR/NSD			R8	X,Y+18
			R9	X,Y+18			R9	X,Y+18
Dil 1/16	X,Y+18	X,Y+18	R2	X,Y+18	X,Y+18	X,Y+18	R2	X,Y+18
			R3	X,Y+18			R3	X,Y+18
			R4	X,Y+18			R4	X,Y+18(AI@D3)
			R5	X,Y+18			R5	X,Y+18(AI@D7)

6.5 Evaluation of *forensic*GEM™

*forensic*GEM™ is a novel thermostable proteinase developed as a rapid, cheap and effective single-tube DNA extraction solution for forensic laboratories that was recently released. At the time of testing, the *forensic*GEM™ system was not yet widely used in the field of forensics, however the system has had exposure at various conferences and symposiums, such as the 18th International Symposium on the Forensic Sciences (Fremantle, WA; 2-7 April 2006).

Unlike the other kits that were evaluated, *forensic*GEM™ does not incorporate either magnetic bead or silica membrane technologies, but instead works on the principle action of a thermostable proteinase in an optimised buffer solution. *forensic*GEM™ is based on the work of Moss *et al.* (2003) who developed the use of EA1 proteinase for the DNA extraction of forensic samples. EA1 proteinase comes from the thermophilic *Bacillus* sp. EA1. EA1 proteinase is Ca^{2+} dependent but is unaffected by a concentration of citrate below 5mM and EDTA below 2mM (Moss *et al.* 2003). For EDTA-stabilised blood, the buffer needs to be supplemented to a final concentration of 200µM $CaCl_2$. Heating a sample at 75°C in the presence of *forensic*GEM™ buffer and *forensic*GEM™ lyses the sample and the proteinase hydrolyses nucleases. At 95°C the proteinase is heat inactivated so that an active form will not be carried over into PCR where it would degrade *Taq* DNA polymerase.

The time to process a batch of 12 samples using the *forensic*GEM™ system takes about 1.5 hours. Each extraction batch included a positive and negative control, and also a substrate blank. The final volume was 100µL for FTA® samples and 200µL for all other samples.

Comparison of quantitation results for cell samples

Refer to Table 4 for observed data. For cells samples, *forensic*GEM™ produced higher quantitation results compared to Chelex® across all dilutions. *forensic*GEM™ also generated the highest yield for all samples, including the 1/16 dilutions. *forensic*GEM™ yielded quantitation results for denim samples (neat and 1/4 dilutions).

Comparison of quantitation results for blood samples

Refer to Table 5 for observed data. *forensic*GEM™ performed very poorly for blood samples on rayon swabs, resulting in the lowest observed yield across all kits that were evaluated. The average yield for all four neat replicates processed using *forensic*GEM™ was 0.6% of the average yield for all Chelex® replicates. The best average yield results were observed for 1/16 dilution samples, where the average *forensic*GEM™ yield was around 25% that of Chelex®. This suggests that the *forensic*GEM™ system is prone to heme inhibition if a neat sample is processed, but can slightly overcome the inhibitory effect if the blood sample is diluted prior to extraction.

Comparison of DNA profiles

*forensic*GEM™ resulted in 209 reportable alleles for cell samples compared to 89 alleles resulting from Chelex® extracts (Table 12). *forensic*GEM™ was able to overcome inhibition in denim samples, producing full profiles (X,X+18) for neat and 1/4 dilutions, accurately reflecting the quantitation results. A partial profile (X,NR+7) was obtained for the 1/8 dilution on denim. *forensic*GEM™ results were also superior than Chelex® for cells on cotton swab down to the 1/8 dilution, but FTA® results were considerably poor.

Table 12. Comparison of DNA profiles for cell substrate samples extracted using either Chelex® or *forensic*GEM®.

CELLS Method: Chelex

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+16	X,X+16	R14	NSD	X,X+8	NR/NSD
			R15	NR/NSD		
			R16	NSD		
			R17	NSD		
Dil 1/4	X,X+16	X,NR+3	R10	NSD	NR+1	NR/NSD
			R11	NSD		
			R12	NSD		
			R13	NSD		
Dil 1/8	X,X+17	X,X+3	R6	NSD	X,NR+3	NR/NSD
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NSD	NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

CELLS Method: forensicGEM

	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+15	X,X+16	R14	X,X+17	X,X+16	X,X+16
			R15	X,X+13		
			R16	X,X+3		
			R17	X,X+16		
Dil 1/4	X,NR+3	X,X+16	R10	X,NR+NR/NS	X,X+16	X,X+16
			R11	NR/NSD		
			R12	NR/NSD		
			R13	X,NR+NR/NS		
Dil 1/8	NSD	X,X+16	R7	NR/NSD	X,NR+10	X,NR+7
			R8	NSD		
			R9	NR/NSD		
			R6	NR/NSD		
Dil 1/16	NSD	NR/NSD	R5	NSD	NSD	NR/NSD
			R4	NSD		
			R3	NSD		
			R2	NR/NSD		

For blood samples on rayon swabs, only the 1/16 dilutions generated profile results (Table 13). This is indicatory of potential inhibition for higher blood sample dilutions as predicted by the quantitation data.

Table 13. Comparison of DNA profiles for blood substrate samples extracted using either Chelex® or *forensic*GEM®.

BLOOD Method: Chelex Method: forensicGEM

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim	Rayon swabs	
	Profile	Profile	Sample#	Profile	Profile	Profile	Sample#	Profile
Neat	NSD	NSD	R14	X,Y+18	X,Y+18	NSD	R14	NSD
			R15	X,Y+18			R15	NSD
			R16	X,Y+18			R16	NSD
			R17	NR/NSD			R17	NSD
Dil 1/4	X,Y+18	X,Y+15	R10	Not Uploaded	X,Y+18	X,Y+18	R10	NSD
			R11	X,Y+18			R11	NSD
			R12	X,Y+18			R12	NSD
			R13	X,Y+18			R13	NSD
Dil 1/8	X,Y+18(AI@D	X,Y+18	R6	X,Y+18	X,Y+18	X,Y+18	R6	NSD
			R7	X,Y+18			R7	NSD
			R8	NR/NSD			R8	NSD
			R9	X,Y+18			R9	NSD
Dil 1/16	X,Y+18	X,Y+18	R2	X,Y+18	X,Y+18	X,Y+18	R2	X,Y+15(AI@D13)
			R3	X,Y+18			R3	X,NR+3
			R4	X,Y+18			R4	NR,NR+5
			R5	X,Y+18			R5	NR,NR+2

6.6 NucleoSpin® 8 Trace

The NucleoSpin® 8 Trace kit is designed for extraction of genomic DNA from forensic samples. Cell lysis is achieved by incubating samples in a solution containing chaotropic ions in the presence of proteinase K at room temperature. Adding isopropanol to the lysate creates the appropriate conditions for binding of DNA to the silica membrane, a process that is reversible and specific to nucleic acids. Inhibitors are removed by washing steps using an alcohol-containing buffer. Pure genomic DNA is eluted in a slightly alkaline elution buffer.

The evaluation of this kit was performed with slight alterations in the manual method to incorporate the use of the MultiPROBE® II PLUS PVM vacuum manifold, together with the

NucleoSpin® 8 Trace Starter Set A containing Column Holders A and Dummy Strips to enable use of the vacuum manifold.

The time to process a batch of 12 samples using the NucleoSpin® 8 Trace system takes about 5 hours, including a 3 hour incubation step. Each extraction batch included a positive and negative control, and also a substrate blank. Purified DNA was eluted in a final volume of 100µL.

Comparison of quantitation results for cell samples
Refer to Table 4 for observed data. NucleoSpin® 8 Trace produced greater mean concentration values and mean yields than the Chelex® protocol.

Comparison of quantitation results for blood samples
Refer to Table 5 for observed data. Mean blood quantitation values for samples extracted using NucleoSpin® 8 Trace were comparable to Chelex® results. Yields were variable but comparable to Chelex®.

Comparison of DNA profiles
NucleoSpin® 8 Trace overall yielded higher allele counts compared to Chelex®, resulting in 202 reportable alleles in contrast to the 89 alleles from Chelex®-extracted samples (Table 14). NucleoSpin® 8 Trace was able to yield profiles for cell samples on denim down to 1/8 dilution, but performed poorly with FTA® samples, resulting only in a partial profile (X,X+5) for the neat cell sample. NucleoSpin® 8 Trace performed better for cells on cotton swabs, and performed moderately better for cells on rayon swabs. Profiles from cells on cotton cloth samples were comparable between the two DNA extraction methods.

Table 14. Comparison of DNA profiles for cell substrate samples extracted using either Chelex® or NucleoSpin® 8 Trace.

CELLS Method: Chelex

Dilution	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+18	X,X+18	R14	NSD	X,X+8	NR/NSD
			R15	NR/NSD		
			R16	NSD		
			R17	NSD		
Dil 1/4	X,X+18	X,NR+3	R10	NSD	NR+1	NR/NSD
			R11	NSD		
			R12	NSD		
			R13	NSD		
Dil 1/8	X,X+17	X,X+3	R6	NSD	X,NR+3	NR/NSD
			R7	NSD		
			R8	NSD		
			R9	NSD		
Dil 1/16	NSD	NSD	R2	NSD	NSD	NSD
			R3	NSD		
			R4	NSD		
			R5	NSD		

CELLS Method: NucleoSpin 8 Trace

	FTA	Cotton swabs	Rayon swabs		Cotton	Denim
	Profile	Profile	Sample#	Profile	Profile	Profile
Neat	X,X+5	X,X+18	R14	AI@D3	X,X+18	X,X+18
			R15	X,X+18		
			R16	X,X+15 AI@D3		
			R17	AI@D13		
Dil 1/4	NSD	X,X+18	R10	X,NR+NR/NS	NSD	X,X+18
			R11	X,X+3		
			R12	X,NR+1		
			R13	NR,NR +1		
Dil 1/8	NSD	X,X+17	R6	X,NR+NR/NS	X,NR+2	X,X+13
			R7	NR/NSD		
			R8	X,NR+NR/NS		
			R9	X,NR+NR/NS		
Dil 1/16	NSD	NSD	R2	NSD	NSD/NR	NSD
			R3	NSD		
			R4	NSD		
			R5	X,NR+NR/NS		

For blood samples on rayon swabs, NucleoSpin® 8 Trace profiles were comparable to Chelex®, with several partial profiles being observed in the neat and 1/8 dilutions (Table 15).

Table 15. Comparison of DNA profiles for blood substrate samples extracted using either Chelex® or NucleoSpin® 8 Trace.

BLOOD	Method: Chelex						Method: NucleoSpin 8 Trace	
Dilution	*FTA*	*Cotton swabs*	*Rayon swabs*		*Cotton*	*Denim*	*Rayon swabs*	
	Profile	Profile	Sample#	Profile	Profile	Profile	Sample#	Profile
Neat	NSD	NSD	R14	X,Y+18	X,Y+18	NSD	R14	Al@Amel,&D18+
			R15	X,Y+18			R15	NR/NR+2
			R16	X,Y+18			R16	X,Y+13
			R17	NR/NSD			R17	X,Y+18
Dil 1/4	X,Y+18	X,Y+15	R10	Not Uploaded	X,Y+18	X,Y+18	R10	X,Y+18
			R11	X,Y+18			R11	X,Y+18
			R12	X,Y+18			R12	X,Y+18
			R13	X,Y+18			R13	X,Y+18
Dil 1/8	X,Y+18(Al@D	X,Y+18	R6	X,Y+18	X,Y+18	X,Y+18	R6	X,Y+18
			R7	X,Y+18			R7	X,Y+15
			R8	NR/NSD			R8	X,Y+18
			R9	X,Y+18			R9	X,Y+18
Dil 1/16	X,Y+18	X,Y+18	R2	X,Y+18	X,Y+18	X,Y+18	R2	X,Y+18
			R3	X,Y+18			R3	X,Y+18
			R4	X,Y+18			R4	X,Y+18
			R5	X,Y+18			R5	X,Y+18

6.7 Summary

Findings from the evaluation of various forensic DNA extraction kits, compared to the in-house Chelex® protocol, is summarised in Table 16.

Table 16. Summary of findings from the evaluation of five forensic DNA extraction chemistries.

	Chelex	DNA IQ™	QIAamp® DNA Micro	ChargeSwitch®	*forensic* GEM™	NucleoSpin® 8 Trace
Processing time for 12 samples	2hr	3hr	5hr	1.5hr	1.5hr	5hr
Washing steps included to remove inhibitors	No	Yes	Yes	Yes	No	Yes
Final extract volume (µL)	~150	100	45	50	100 for FTA, 200 for other samples	100
% zero quantitation values for cells	43.750	9.375	37.500	12.500	9.375	24.140
% zero quantitation values for blood	0.000	0.000	0.000	0.000	0.000	0.000
Cell substrate displaying highest quant value for neat cell samples	FTA	Cotton swab	Cotton swab	Cotton swab	Cotton swab	Cotton cloth
Total number of reportable alleles for cells (max 576)	89	282	66	138	209	202
Total number of reportable alleles for blood (max 288)	234	252	251	288	25	264
Total number of autosomal loci exhibiting allelic imbalance (max 432)	1	1*	3	5	1	6^
Neat cell samples on denim showed inhibition (no profile)	Yes	No	Yes	Yes	No	No
Neat blood samples on rayon swabs showed inhibition (no profile)	No	No	No	No	Yes	No
Amenable to automation	No	Yes	Yes	Yes	Yes	Yes
Validated MultiPROBE II PLUS automated protocol	No	Yes	No	No	No	No

* Five occurrences of allelic imbalance were observed in Amelogenin.
^ One occurrence of allelic imbalance was observed in Amelogenin.

Out of all the chemistries tested, only the Chelex® method and *forensic*GEM™ protocols do not incorporate washing steps for the removal of inhibitors and residual proteins. This is because in these protocols, the DNA is free in solution and not immobilised on to a capture device such as magnetic beads, and therefore washing of the sample cannot be performed. Washing steps result in high quality, purified DNA extracts. As such, Chelex® and *forensic*GEM™ extracts are considered to be crude DNA extracts of suboptimal quality that may not yield the best DNA profiles due to the presence of inhibitors that can affect PCR amplification of multiple STR loci. Although the dye in denim material did not appear to result in inhibition for *forensic*GEM™ samples, only 25/288 alleles (8.7%) from blood samples could be resolved by this extraction method.

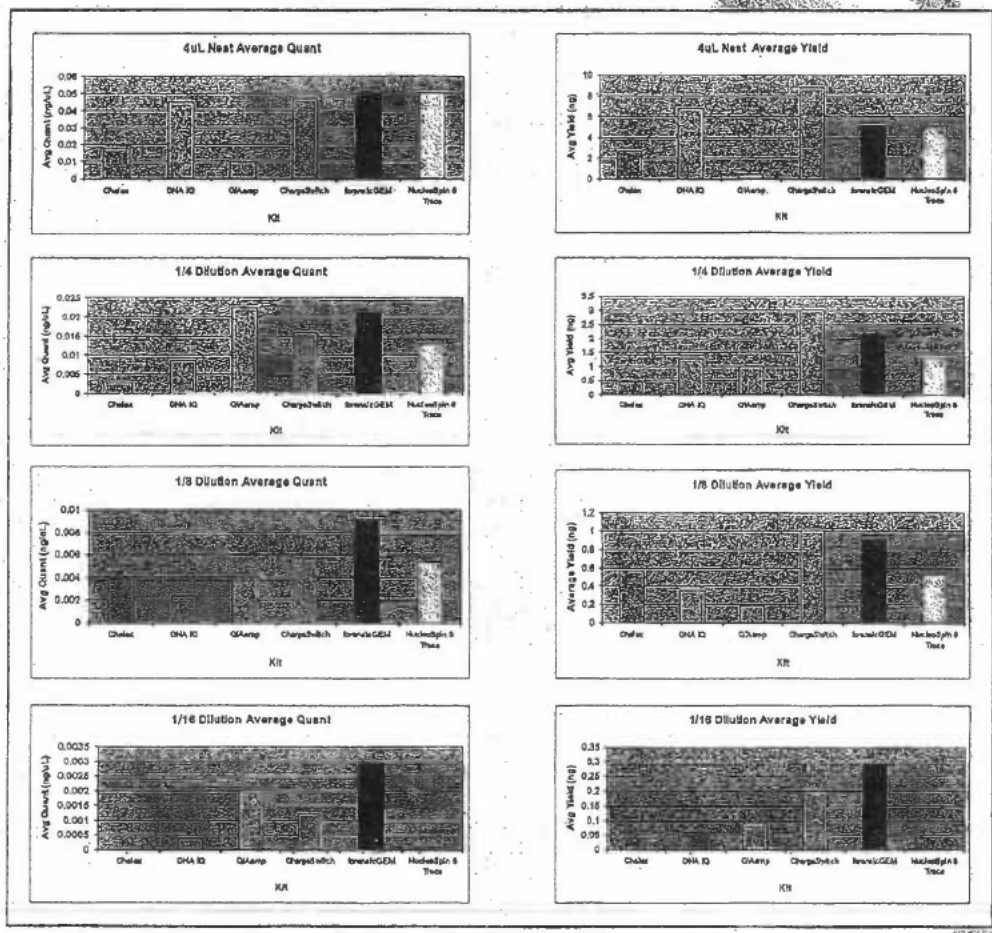

Figure 1. Average quantitation values (ng/µL) and yields (ng) for cell samples extracted using the various extraction chemistries tested, compared to Chelex®.

Although all kits resulted in quantitation results for all blood samples (0% had zero results), the results for cell samples exhibited more variation. Out of the extraction chemistries that incorporate washing steps, the DNA IQ™ system exhibited the best result for zero quantitation values for both cell and blood samples at 0% and 0% respectively. Almost half (44%) of Chelex® cell extracts failed to yield quantitation results. The next worse quantitation results were observed for QIAamp® DNA Micro (37.5% had zero results), followed by ChargeSwitch® (31.25%) and NucleoSpin® 8 Trace (24.14%). For all the different substrate types tested, average quantitation values were comparable for DNA IQ™, ChargeSwitch®, *forensic*GEM™ and NucleoSpin® 8 Trace in neat, 1/4 and 1/8 dilutions (Figure 1). Compared to samples extracted using Chelex®, samples extracted using the evaluated kits displayed higher average quantitation results that were up to 7.7 times higher than Chelex® results. Chelex® and NucleoSpin® 8 Trace were the only two kits that did not result in quantitation values for the 1/16 dilutions. The average yields varied widely due to different elution volumes for the various kits. For neat samples, DNA IQ™ ChargeSwitch®, *forensic*GEM™ and NucleoSpin® 8 Trace resulted in comparable yields for neat samples, which were on average double the yield generated by Chelex® (Figure 1). In all experiments, *forensic*GEM™ resulted in the highest quantitation values, but as discussed in the previous paragraph, this kit produced the least number of reportable alleles for blood samples. It was preferred to have a high quantitation result, coupled with a high yield and high final volume as it allows multiple tests to be performed.

The relationship between quantitation result and the number of resolved reportable alleles is close to proportional. A list of the evaluated chemistries, ranked according to the lowest to highest percentage of zero quantitation results, and also the most to the least number of resolved alleles, is outlined in Table 17.

Table 17. A ranking of the evaluated kits based on quantitation and DNA profile results.

Rank	% zero quantitation values	Total alleles for cells	Total alleles for blood
1	DNA IQ™ & *forensic*GEM™	DNA IQ™	ChargeSwitch®
2	NucleoSpin® 8 Trace	*forensic*GEM™	QIAamp® DNA Micro
3	ChargeSwitch®	NucleoSpin® 8 Trace	NucleoSpin® 8 Trace
4	QIAamp® DNA Micro	ChargeSwitch®	DNA IQ™
5	Chelex®	Chelex®	Chelex®
6		QIAamp® DNA Micro	*forensic*GEM™

The DNA IQ™ system was ranked the highest for most categories and performed the best for both cell and blood samples (see also Figures 2 and 3). For blood samples on rayon swabs, DNA IQ™ received a lower ranking due to 2 outlier results for neat dilutions as discussed above, but overall was considered to produce the best result for all dilutions. In contrast, Chelex® had the lowest rating as it was found to result in the least number of reportable alleles for both cell and blood samples. *forensic*GEM™ also outperformed the other kits for cell samples but performed very poorly for neat blood samples, indicating an inhibitory effect due to dissolved heme, although PCR amplification performance was improved in extracts of diluted blood samples (Figure 3). In contrast, QIAamp® DNA Micro worked well for blood samples, but performed the worst for cell samples. ChargeSwitch®, the alternative magnetic bead system to DNA IQ™, also performed better for blood samples than cell samples. The NucleoSpin® 8 Trace system, another membrane-based technology, performed moderately well and was ranked 3rd for the total number of alleles resolved for both cell and blood samples. Our results did not clearly indicate as to which technology, i.e. magnetic bead or silica membrane, was overall a better DNA extraction technology for forensic samples. However, DNA IQ™ worked the best in our hands as a complete "out-of-the-box" solution for extracting both cell and blood samples on various types of substrates.

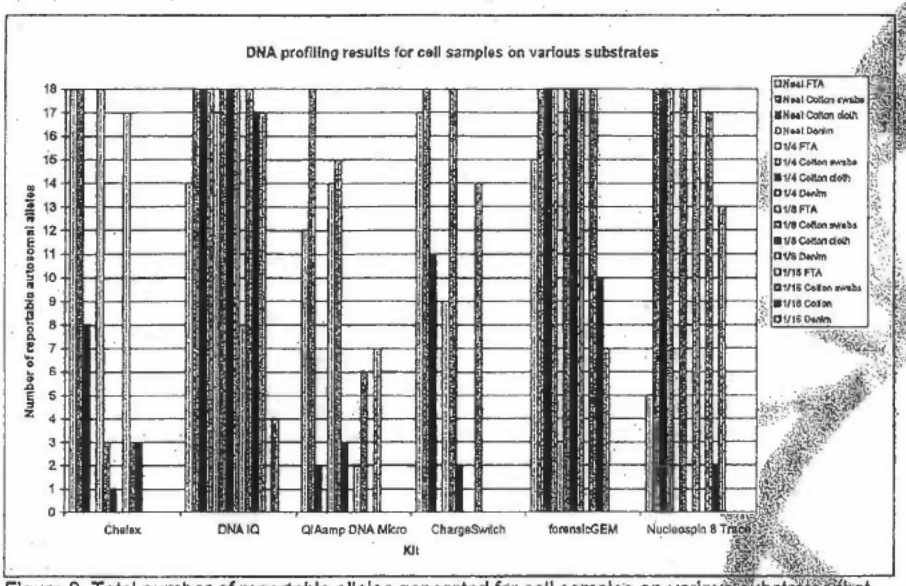

Figure 2. Total number of reportable alleles generated for cell samples on various substrates that were extracted using the various extraction chemistries tested, compared to Chelex®. The kit displaying the most number of full bars (i.e. most full profiles) was found to be DNA IQ™, indicating the superior performance of this kit over the other kits tested. The current in-house Chelex® method did not perform as well as several of the tested kits.

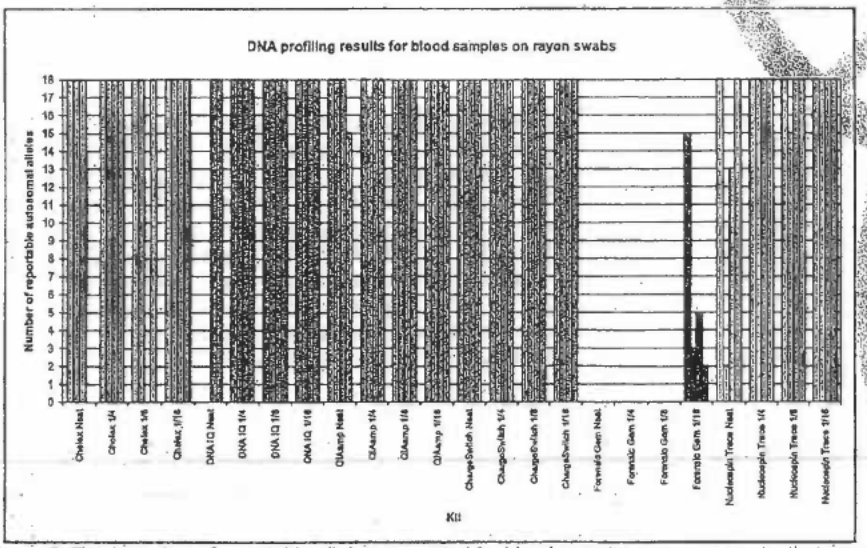

Figure 3. Total number of reportable alleles generated for blood samples on rayon swabs that were extracted using the various extraction chemistries tested, compared to Chelex®. All kits were able to resolve profiles from most dilutions, except *forensic*GEM™ which could only resolve alleles from the 1/16 dilution, indicating an inhibitory effect of heme on the *forensic*GEM™ system.

Out of a total of 432 loci amplified in the assessment of each kit, only one occurrence of allelic imbalance (AI; where peak height ratio is <70%) was detected in each of the Chelex®, DNA IQ™ and *forensic*GEM™ kits (Table 16). QIAamp® DNA Micro and ChargeSwitch® each showed 3 and 5 occurrences of AI respectively, and NucleoSpin® 8 Trace showed the most AI at 6 occurrences observed (Table 16).

Out of 17 occurrences of AI in all samples tested, 9 AI events were observed in cell samples and 8 events were observed in blood samples (Table 18). These results do not suggest any increased likelihood in observing AI in either cell or blood samples. Out of the 9 AmpF*l*STR® Profiler Plus loci interrogated, AI was only encountered in 6 loci: D3S1358, FGA, D13S317, D8S1179, D18S51, and D7S820 (Table 18). Most of the AI (35.29%) occurred in the D13S317 locus, and the least (5.88%) occurred in both D8S1179 and D7S820. The %AI observed was within the range of 52.30% at D13S317 to 69.96% at D3S1358 (data not shown). Most of the AI (58.82%) was ≥60%, and 41.18% of AI was ≥65%. Out of the 7 occurrences of AI that were ≤60%, 4 events (57%) were observed in cell samples extracted using NucleoSpin® 8 Trace. Six additional occurrences of AI were observed in Amelogenin, with all AI events ≥60% (data not shown). The AI data from this evaluation will contribute to further studies on a revised in-house AI threshold.

Table 18. Frequency of all autosomal allelic imbalance observed in the evaluation

Kit	Number of autosomal AI		Profiler Plus loci exhibiting allelic imbalance					
	Cell	Blood	D3S1358	FGA	D13S317	D8S1179	D18S51	D7S820
Chelex	0	1	1					
DNA IQ	1				1			
QIAamp DNA Micro	1	2				1	2	
ChargeSwitch	2	3	1	1	2			1
forensicGEM		1			1			
NucleoSpin 8 Trace	5	1	2	1	2		1	
Total	9	8	4	2	6	1	3	1
	17		23.53%	11.76%	35.29%	5.88%	17.65%	5.88%

Neat cell or blood samples that were extracted using the various kits displayed varying inhibition results for denim dye and heme (Table 16). In several cases, if a kit did not show inhibition for denim dye, it would show inhibition for heme, or vice versa. Only the DNA IQ™ and NucleoSpin® 8 Trace systems did not indicate inhibition for either inhibitor. There did not appear to be a link between the presence or absence of inhibition and the observation of allelic imbalance, although DNA IQ™ and NucleoSpin® 8 Trace generated the most number of total reportable alleles (534 and 466 alleles respectively). These results suggest that the ability to remove inhibitors (such as encountered in the DNA IQ™ and NucleoSpin® 8 Trace protocols) can result in an increase in the number of resolvable alleles, therefore successfully obtaining more DNA profile results more often.

Cotton substrates (e.g. cotton swabs and cotton cloth) make up a large percentage of samples processed in DNA Analysis FSS. For example, cotton swabs make up around 45% of the total number of sample types analysed for DNA analysis (Figure 4). It was therefore considered important that the DNA extraction kits evaluated could process samples and stains on cotton matrices. It was found that the neat cell samples that displayed the highest quantitation values across all extraction kits originated from cotton swab substrates, except for Chelex® results where the best result came from FTA (Table 16).

All of the forensic DNA extraction kits evaluated are amenable to automation, and automated protocols already exist for several kits. However, only the DNA IQ™ kit has been validated for use on the MultiPROBE® II PLUS HT EX platform and a validated protocol was developed by PerkinElmer (PerkinElmer, 2004).

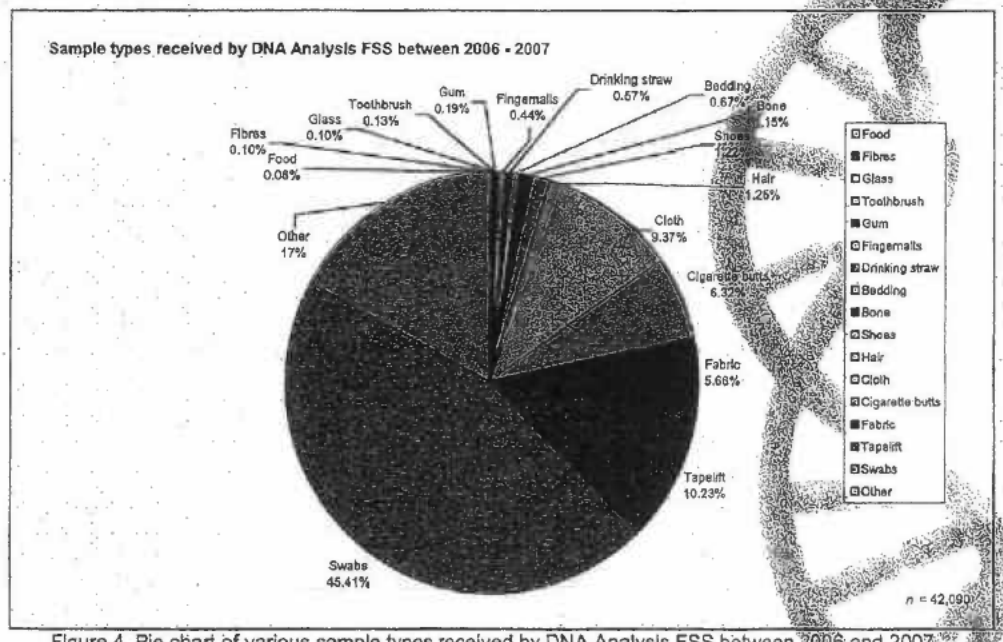

Figure 4. Pie chart of various sample types received by DNA Analysis FSS between 2006 and 2007. Around 45% of samples received for DNA analysis are swab substrates. Data was obtained from AUSLAB on 14 November 2007.

Some of the concerns raised regarding some of the kits tested include:

- QIAamp® DNA Micro involved multiple tube transfers that increased the risk of cross-contamination and also increased processing time to 5 hours for 12 samples.
- An increased risk of contamination was also prevalent in the NucleoSpin® 8 Trace method when coupled with the PVC vacuum manifold, because of the need to fit multiple adapters to ensure seals are maintained for a proper vacuum environment. If the plates and adapters were not assembled correctly, the vacuum environment would fail and possibly cause cross-contamination and, more alarmingly, loss of sample. Furthermore, even when assembled correctly, biohazardous contaminants (e.g. blood) are drawn down the manifold through the vacuum tubing and into the collection containers. Decontamination of the tubing and containers raises serious health and safety concerns.
- The *forensic*GEM™ system was the quickest protocol to perform and yielded crude DNA extracts that produced high allele counts for cell samples. However, the system could not deal with blood samples (and heme inhibition) effectively, therefore causing very low allele counts for blood samples.
- ChargeSwitch® was the alternative magnetic bead system to DNA IQ™. However, ChargeSwitch® did not produce results that were comparable or better than DNA

IQ™. For example, more ChargeSwitch® samples did not yield quantitation results compared to DNA IQ™ and resulted in a lower total allele count. ChargeSwitch® also did not appear to be able to effectively deal with inhibition from the dye in denim material.

Overall, data from the evaluation suggested that DNA IQ™ outperforms all of the forensic DNA extraction kits tested, in addition to the in-house Chelex® protocol. In summary, DNA IQ™:

- Is quick to perform – the amount of time taken to complete the DNA extraction protocol is comparable to the in-house Chelex® method;
- Includes washing steps to remove inhibitors – washing of the immobilised DNA enables purified DNA template to be eluted;
- Produced DNA quantitation values for most (>90%) samples – the percentage of samples that did not yield a quantitation result was one of the lowest for DNA IQ™;
- Generated the highest number of total reportable alleles – samples extracted using DNA IQ™ produced 65% more resolved alleles compared to Chelex®;
- Exhibited minimal allelic imbalance – the occurrence of AI in DNA IQ™ samples was comparable to Chelex®, although increased AI in Amelogenin was observed;
- Was not inhibited by heme in blood samples;
- Was not inhibited by the dye in denim material;
- Has been validated for use on the MultiPROBE® II PLUS HT EX platform.

7. Recommendations

Based on the results from evaluating various commercial DNA extraction kits that were designed specifically for forensic use, and comparing results from each kit to the current in-house Chelex® protocol, we have found DNA IQ™ to be the most suitable kit for extracting cell and blood samples that are analysed in DNA Analysis FSS. We therefore recommend that further studies be performed on the DNA IQ™ system in order to:

1. Validate a manual DNA IQ™ protocol for extracting various DNA Analysis FSS substrate types;
2. Verify an automated DNA IQ™ extraction program on the MultiPROBE® II PLUS HT EX platforms for automated DNA extraction of various DNA Analysis FSS substrate types.

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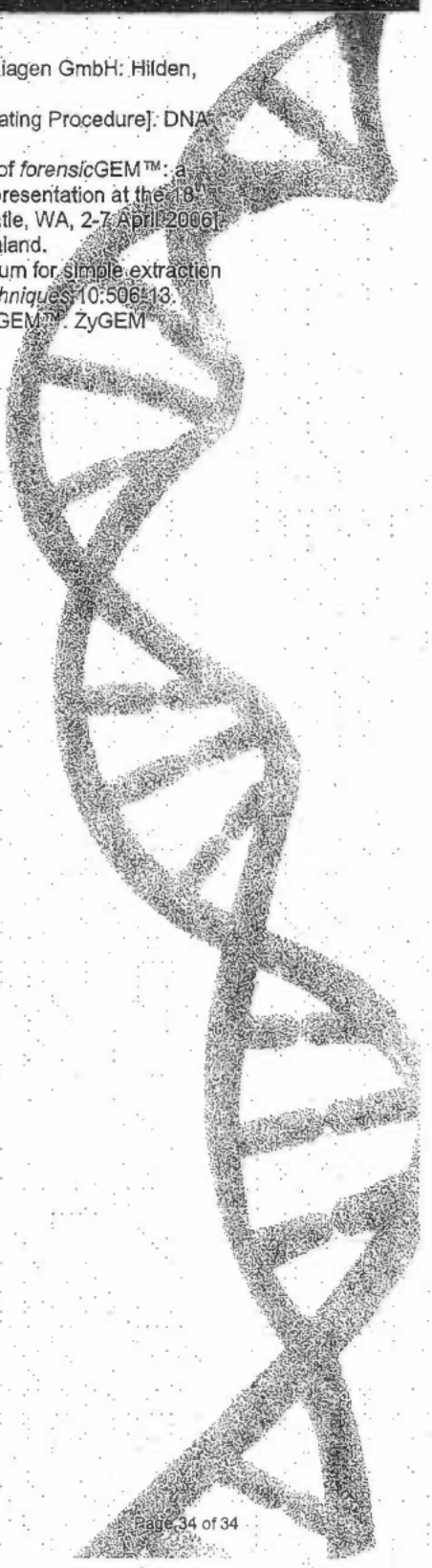

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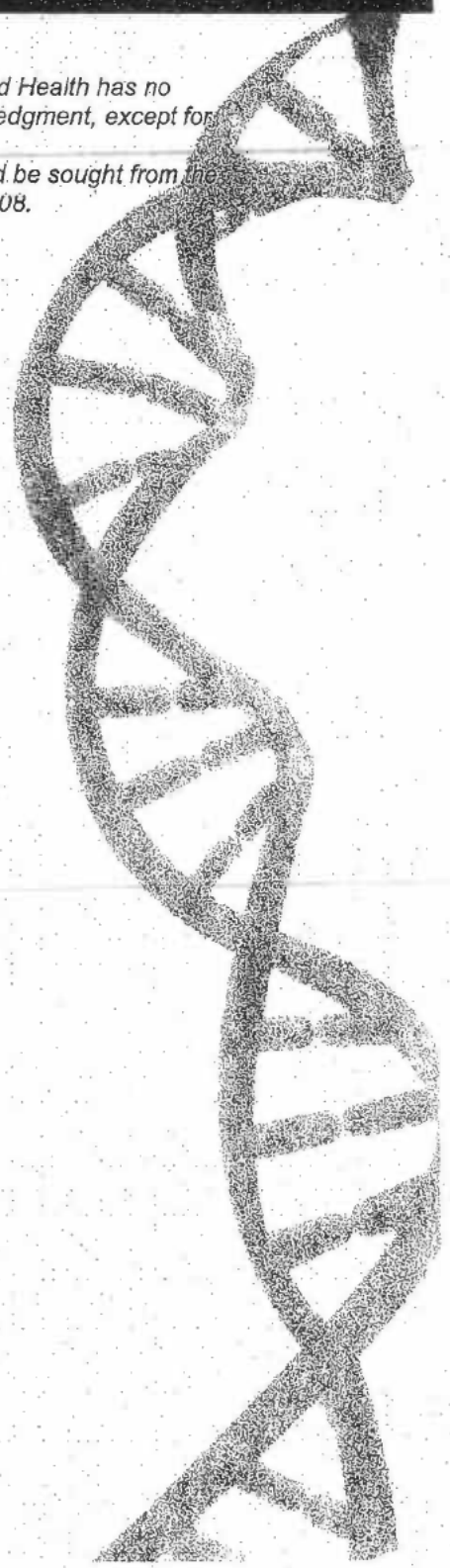

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Project 11. Report on the Validation of a manual method for Extracting DNA using the DNA IQ™ System

August 2008

Automation and LIMS Implementation Project Team,

DNA Analysis

Forensic And Scientific Services

Clinical and Scientific Services

Queensland Health

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Project 11. Report on the Validation of a Manual Method for Extracting DNA using the DNA IQ™ System

Nurthen, T., Hlinka, V., Muharam, I., Gallagher, B., Lundie, G., Iannuzzi, C., Ientile, V.
Automation/LIMS Implementation Project, DNA Analysis FSS (August 2008)

1. Abstract

The DNA IQ™ system was found to be the most suitable kit for extracting cell and blood samples that are analysed in DNA Analysis FSS (refer to Project 9). This DNA extraction system, based on magnetic bead technology, was found to generate results that were comparable or better than the current Chelex®-100 protocol. We have validated a manual DNA IQ™ method for extracting DNA from forensic samples, and incorporated studies on sensitivity and consistency, inhibition, substrate type, substrate size, and mixture studies. This manual DNA IQ™ method is suitable for verification on the automated MultiPROBE® II PLUS HT EX extraction platforms.

2. Introduction

A previous evaluation of various DNA extraction systems that were designed specifically for forensic samples was performed in order to select a suitable extraction technology for extracting various sample types that are processed in DNA Analysis FSS. DNA IQ™ was identified as a suitable kit for extracting forensic samples, and was found to outperform both the current Chelex®-100 protocol and also all the other kits evaluated. The results of the evaluation are reported in Project 9 (Gallagher *et al.*, 2007a).

DNA purification with silica matrices, either in membrane- or bead-form, commonly uses the affinity of DNA for silica without the need for hazardous organic reagents. However, these systems tend to require extensive washing to remove the guanidium-based lysis buffer. The DNA IQ™ system uses a novel paramagnetic resin for DNA isolation (Promega Corp., 2006). The DNA IQ™ System's basic chemistry is similar to other silica-based DNA isolation technologies, except that the specific nature of the paramagnetic resin, coupled with the formulation of the lysis buffer, is unique. In the DNA IQ™ System, negatively-charged DNA molecules have a high affinity for the positively-charged paramagnetic resin under high salt conditions supplied by the lysis buffer. Once DNA is bound to the magnetic resin, and the resin is immobilised by a magnet, the sample can be washed using an alcohol/aqueous buffer mixture. The high alcohol content of the wash buffer aids to maintain the DNA-resin complex in low-salt conditions, while the aqueous component functions to wash away residual lysis buffer and any inhibitors or non-DNA contaminants such as cellular debris and protein residues. DNA is released from the resin by using a low ionic strength elution buffer, and the purified DNA can be used directly in downstream applications such as PCR.

For samples that are in excess (e.g. reference samples), DNA IQ™ resin will only isolate up to a total of approximately 100ng of DNA due to bead saturation (Huston, 2002).

3. Aim

To validate a manual method for DNA extraction of blood and cell stains on forensic samples using the DNA IQ™ system (Promega Corp., Madison, WI, USA).

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4. Equipment and Materials

- DNA IQ™ System (Promega Corp., Madison, WI, USA); 100 samples, Cat.# DC6701), which includes:
    - 0.9mL Resin
    - 40mL Lysis Buffer
    - 30mL 2X Wash Buffer
    - 15mL Elution Buffer
- TNE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0)
- MagneSphere® Magnetic Separation Stand, 12-position (Cat.# Z5342) (Promega Corp., Madison, WI, USA)
- DNA IQ™ Spin Baskets (Cat.# V1221) (Promega Corp., Madison, WI, USA)
- Microtube 1.5mL (Cat.# V1231) (Promega Corp., Madison, WI, USA)
- 95-100% ethanol
- Isopropyl alcohol
- 1M DTT (Sigma-Aldrich, St. Louis, MO, USA)
- Proteinase K (20mg/mL) (Sigma-Aldrich, St. Louis, MO, USA)
- 20% SDS (Biorad, Hercules, CA, USA)
- 0.9% saline solution (Baxter Healthcare, Old Toongabbie, NSW, Australia)
- ThermoMixer Comfort (Eppendorf, Hamburg, Germany)
- Vortex mixer
- Bench top centrifuge
- Cytobrush® Plus Cell Collector (Cooper Surgical, Inc., Trumbull, CT, USA)
- FTA® Classic Cards (Whatman plc, Maidstone, Kent, UK)
- Rayon (155C) and cotton (164C) plain dry swabs (Copan Italia S.p.A., Brescia, Italy)
- Vacuette® K2EDTA blood collection tubes (Greiner Bio-One GmbH, Frickenhausen, Germany)
- Sticky tape (BDF tesa tape Australia Pty Ltd)
- Tannic acid $C_{76}H_{52}O_{46}$ FW1701.25 (Selby's BDH, Lab Reagent >~90%)
- Urea NH_2CONH_2 FW60.06 (BDH, Molecular Biology Grade ~99.5%)
- Indigo carmine $C_{16}H_8N_2Na_2O_8S_2$ FW466.35 PN 131164-100G (Sigma-Aldrich, St. Louis, MO, USA)
- Humic acid sodium salt PN H167520-100G (Sigma-Aldrich, St. Louis, MO, USA)
- Used car motor oil, SW20/SAE50 (Caltex)
- Various clothing materials, including:
    - Best & Less Pacific Cliff, White cotton shirt, XXL
    - Big W Classic Denim, Men's Blue denim jeans, 112
    - Private Encounters, off-white nylon cami, size 14
    - Clan Laird, blue 100% wool kilt
    - Millers Essentials, blue 100% polyester camisole, size 10
    - Unknown, teal green 100% lycra swimwear
    - Leather Belt, brown

5. Methods

5.1 Cell and blood collection

Buccal cells were collected using a modified Cytobrush® protocol (Mulot *et al.*, 2005; Satia-Abouta *et al.*, 2002). Four donors were chosen. Each donor was asked to brush the inside of one cheek for one minute. Then, with another Cytobrush®, the other cheek was also sampled. The cells collected on the brush where then resuspended in 2mL of 0.9% saline solution. Multiple collections were taken on different days.

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Whole blood was collected from three donors by a phlebotomist as per standard collection procedures in EDTA tubes. Blood samples were refrigerated until spotting onto substrate and cell-counting step.

Table 1 lists the donor sample ID's.

Table 1. List of donor samples used for validating a manual DNA IQ™ method.

Donor ID
Cell samples
D1
D2
D3
D4
Blood samples
D1
D2
D3

5.2 Cell counting

Buccal cell suspensions were diluted using 0.9% saline solution to create a 1/10 dilution of the original sample prior to submitting for cell counting. All counts were performed by the Cytology Department, RBWH (QIS 15393).

Blood cell counting was performed on a 1mL aliquot of the original sample also by the Cytology Department, RBWH (QIS 15393).

5.3 Sensitivity, Reproducibility (Linearity) and Yield

Sensitivity and reproducibility of the DNA IQ™ kit was assessed using dilutions of cell and blood samples.

For cell samples, dilutions were made using a sample from donor 4, diluted in 0.9% saline solution. The dilutions used were:

- Neat
- $^1/_{10}$
- $^1/_{100}$
- $^1/_{1000}$

For blood samples, dilutions were made using a sample from donor 2, diluted in 0.9% saline solution. The dilutions used were:

- Neat
- $^1/_{10}$
- $^1/_{100}$
- $^1/_{1000}$

Mock samples were created from rayon and cotton swabs using the above dilutions. The swab heads were removed from the shaft using sterilised scalpel and tweezers. Swab heads were then cut into quarters and each quarter was then added to separate sterile 1.5mL tubes. To each quarter swab, 30µL of each neat sample or dilution was added to

create a total of five replicates. Samples were dried using a ThermoMixer set at 56°C over 2 hours in a Class II biohazard cabinet.

5.4 Inhibition challenge

Quartered cotton swabs in sterile 1.5mL tubes were spotted with 30µL of neat cell suspension and were dried after each addition on a ThermoMixer as described previously. Neat blood samples were also created using the same method.

All the inhibitors except for the motor oil were obtained in powder form. Before making any liquid solution of the powdered inhibitors, research was conducted to determine the likely level of each inhibitor normally encountered in the environment (Hlinka *et al.*, 2007). Each solution was made at concentrations based on the information obtained (Table 2).

Table 2. Concentrations of various inhibitors used in the inhibition study.

Inhibitor	Excess/Neat Solution	Mass	Volume H_2O	Final inhibitor concentration
Tannic acid	Excess	600mg	500µL	0.705M
	Neat	200mg	500µL	0.235M
Humic acid	Excess	1g	5mL	20% (w/v)
	Neat	0.1g	5mL	2% (w/v)
Indigo carmine	Excess	0.47g	10mL	100mM
	Neat	0.047g	10mL	10mM
Urea	Excess	0.06g	1mL	1M
	Neat	0.021g	1mL	0.33M

A total of 30µL of each solution containing specified concentrations of various inhibitors was applied to the buccal cell and blood swabs prepared above. The only exception was motor oil, where only 15µL was added to the cell and blood swabs respectively. Each inhibitor sample was replicated in quadruplicate and left to dry overnight in a Class II biohazard cabinet.

To another set of prepared cell and blood swabs, an excess of each inhibitor was applied in quadruplicate for each inhibitor and allowed to dry overnight. This process was achieved by applying another solution of inhibitor exceeding the normal level (Hlinka *et al.*, 2007).

5.5 Substrates

Swabs

Four cotton and four rayon swab quarters in sterile 1.5mL tubes were loaded with 30µL of neat cell or blood sample and were extracted once the sample had dried on the swab.

Tapelifts

Two donors were sampled using the tape most commonly used within the laboratory (BDF tesa tape). Strips of tape were firmly applied to the inside of the fore arm and lifted off. This process was then repeated until the tape was no longer adhesive. The tape was wrapped around sticky-side-in, forming a cylinder shape, and placed in a sterile 1.5mL tube. These samples were created in quadruplicate. Tape was not used as a substrate in the blood validation.

Fabric

The material types tested included:

- Denim jeans;

- White 100% cotton shirt;
- Blue 100% wool kilt;
- Teal green 100% lycra swimwear;
- White 100% nylon camisole;
- Blue 100% polyester camisole; and
- Brown 100% woven leather belt.

All material types except leather were sampled and ten 2.5cm x 2.5cm pieces were cut from each material and washed in 10% bleach following an in-house washing method to remove any contaminating DNA from outside the laboratory (Gallagher *et al.*, 2007b). As for the leather, one strand of the leather weave was cut from the belt and washed following the same method. Once dry, the material was then cut into 0.5cm x 0.5cm pieces using sterile techniques, placed in 1.5mL tubes and 30µL of both cell sample and blood was applied to separate pieces. Each substrate sample was created in quadruplicate and dried on a ThermoMixer set at 56°C over 2 hours in a Class II biohazard cabinet.

Gum
Two types of chewing gum were chosen: (1) Wriggley's Extra White (peppermint flavour) and (2) Wriggley's Extra Green (spearmint flavour). The donor was asked to chew the gum for 30 minutes and dispose of the gum into a clip-seal plastic bag. The gum was then air dried in a Falcon tube overnight before it was frozen for roughly an hour before cutting into 3mm x 3mm x 3mm pieces and placed into sterile 1.5mL tubes. Gum substrates were not assessed for blood samples.

Cigarette butts
Two brands of cigarettes were smoked all the way through and then the butts collected. The filter paper of the butt was cut into 0.5mm^2 pieces and placed into sterile 1.5mL tubes. Cigarette butts were not assessed for blood samples.

FTA® Classic Card punches
Eight sterile 1.5mL tubes, each containing four 3.2mm FTA® Classic Card punches, were spotted with 30µL of cells or blood before being dried on a ThermoMixer. Four replicates contained sample from one donor, the other remaining four replicate tubes had a different donor sample added.

5.6 Mixture studies

Buccal cells and whole blood were obtained from a male and female donor. Dilutions were made using 0.9% saline solution to ensure that the cell concentration was equal. Dilutions were then performed on the male sample to obtain the correct ratios.

Mock samples were created using the following ratios of female to male:

- 1:1,
- 1:2,
- 1:10,
- 1:25,
- 1:50 and
- 1:100.

A total of 30µL of the female component was spotted first on to a quarter of a cotton swab in a sterile 1.5mL tube and dried on a ThermoMixer before adding another 30µL of the male component. Samples were created in quadruplicate for all ratios, for both cell and blood samples.

5.7 Substrate size

Various sizes of material were cut from a white cotton shirt:

- 0.5cm x 0.5cm,
- 1cm x 1cm,
- 2cm x 2cm.

Each piece of material was stored in individual, sterile 1.5mL tubes and 30µL of cell sample was added to the material and allowed to dry on a ThermoMixer. The same process was followed for blood samples. Five replicates were made for each sample type.

5.8 Extraction using the DNA IQ™ System (Promega Corp.)

The manual DNA IQ™ method used was based on an automated protocol developed by the Centre of Forensic Sciences (CFS) in Toronto, Ontario (PerkinElmer, 2004). A Proteinase K – SDS Extraction Buffer was made as per the recommended protocol. The 1x Extraction Buffer for one sample consisted of:

277.5µL TNE buffer
15µL Proteinase K (20mg/mL)
7.5µL 20% SDS

The TNE buffer consisted of:

1.211g Tris (10mM Tris)
2mL 0.5M EDTA (1mM EDTA)
5.844g NaCl (100mM NaCl)

The adapted manual DNA IQ™ protocol is described below:

1. Set one ThermoMixer at 37°C and another at 65°C.
2. Ensure that appropriately sized samples are contained in a sterile 1.5mL tube. For every sample, prepare three set of labelled tubes: spin baskets (for every tube except the extraction control), 2mL SSI tubes and Nunc™ tubes.
3. Prepare Extraction Buffer and add 300µL to each tube. Close the lid and vortex before incubating the tubes at 37°C on the ThermoMixer at 1000rpm for 45 minutes.
4. Remove the tubes from the ThermoMixer and transfer the substrate to a DNA IQ™ Spin Basket seated in a labelled 1.5mL Microtube using autoclaved twirling sticks. Then transfer the liquid to a labelled 2mL SSI sterile screw cap tube.
5. Centrifuge the spin basket on a benchtop centrifuge at room temperature for 2 minutes at its maximum speed. Once completed, remove the spin basket and collect the remaining solution and pool with the original extract in the 2mL SSI sterile screw cap tube, then vortex.
6. Add 550 µL of Lysis Buffer to each tube.

7. Dispense 50µL of DNA IQ™ Resin – Lysis Buffer solution (7µL Resin in 43µL Lysis Buffer) to each tube. Invert the resin tube regularly to keep the beads suspended while dispensing to obtain uniform results.

8. Vortex each tube for 3 seconds at high speed then place in a multitube shaker set at 1200rpm to incubate at room temperature for 5 minutes.

9. Vortex each tube for 2 seconds at high speed before placing the tubes in the magnetic stand. Separation will occur instantly.

   Note: If resin does not form a distinct pellet on the side of the tube, or if the pellet has accidentally mixed with the solution while in the stand, vortex the tube and quickly place back in the stand.

10. Carefully remove and discard all of the solution without disturbing the resin pellet on the side of the tube. If some resin is drawn up in tip, gently expel resin back into tube to allow re-separation.

11. Remove the tube from the magnetic stand; add 125µL of prepared Lysis Buffer and vortex for 2 seconds at high speed.

12. Return tube to the magnetic stand, allow for separation and then remove and discard the Lysis Buffer.

13. Remove tube from the magnetic stand; add 100µL of prepared 1X Wash Buffer and vortex for 2 seconds at high speed.

14. Return tube to the magnetic stand, allow for separation and then remove and discard all Wash Buffer.

15. Repeat Steps 13 to 14 two more times for a total of three washes. Be sure that all of the solution has been removed after the last wash.

16. In a biohazard cabinet, place the lids of the tubes upside down on a Kimwipe, in their respective order, and the tubes into a plastic rack, and air-dry the resin for 5-15 minutes at room temperature. Do not dry for more than 20 minutes, as this may inhibit removal of DNA. Once dry, screw on the lids.

17. To each samples then add 50µL of Elution Buffer very gently on the top of the magnetic pellet. Do not mix.

18. Close the lid and then incubate the tubes in the ThermoMixer at 65°C for 3 minutes with no shaking and another 3 minutes shaking at 1100 rpm.

19. Remove the tubes and vortex for 2 seconds at high speed. Immediately place the tube in the magnetic stand. Tubes must remain hot until placed in the magnetic stand or yield will decrease.

20. Carefully transfer the supernatant containing the DNA to the respective labelled Nunc™ tubes.

21. Repeat step 17 to 20, transferring the supernatant to the appropriate Nunc™ tube. The final volume after the second elution should be approximately 95µL.

    Note: DNA can be stored at 4°C for short-term storage or at -20 or -70°C for long-term storage.

5.9 DNA quantitation

All DNA extracts were quantified using the Quantifiler™ Human DNA Quantitation kit (Applied Biosystems, Foster City, CA, USA) as per QIS 19977. Reaction setup was performed on the MultiPROBE® II PLUS HT EX (PerkinElmer) pre-PCR platform.

5.10 PCR amplification

DNA extracts were amplified using the AmpFℓSTR® Profiler Plus® kit (Applied Biosystems, Foster City, CA, USA) as per QIS 19976. Reaction setup was performed on the MultiPROBE® II PLUS HT EX (PerkinElmer) pre-PCR platform.

5.11 Capillary electrophoresis and fragment analysis

PCR product was prepared for capillary electrophoresis using the manual 9+1 protocol (refer to Project 15 and QIS 19978). Capillary electrophoresis was performed on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3kV injection voltage, 10 sec injection time, 15kV run voltage, 100µA run current, and 45min run time. Data Collection Software version 1.1 was used to collect raw data from the ABI Prism® 3100 Genetic Analyzer. Fragment size analysis was performed using GeneScan 3.7.1. Allele designation was performed using Genotyper 3.7, with thresholds for heterozygous and homozygous peaks at 150 and 300 RFU respectively. The allelic imbalance threshold is 70%.

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6. Results and Discussion

6.1 Donor sample cell counts

Aliquots of buccal cell samples were counted at Cytology Department (RBWH) to determine the concentration of viable cells, in order to better estimate the number of cells at any particular dilution. A white cell count was not performed on all the blood samples, and therefore an estimate on the number of nucleated cells could not be determined.

6.2 Sensitivity, consistency and yield

To ensure the reliability and integrity of results for samples containing small amounts of DNA, a sensitivity study was conducted to determine the lowest concentration of DNA that provides reliable results. A consistency study was combined into the sensitivity experiment to determine the maximum acceptable difference between the results obtained. All samples were extracted in identical conditions by the same operator at the same time to minimise variability.

The cell sample used for the experiments was from donor sample 4A, which was counted to be around 3,680 nucleated cells (x 10^6/L). The blood sample used was from donor 6A, which was counted to be around 2,540 nucleated cells (x 10^6/L). The estimated amount of DNA present in each dilution is outlined in Table 3.

Table 3. Amount of DNA in each dilution, as calculated from the cell count.

Sample type	Dilution factor	Number of cells (/µL)	gDNA (ng/µL)	Theoretical total DNA on swab (ng)
Cells	Neat	3680	23.552	706.56000
	1/10	368	2.3552	70.65600
	1/100	36.8	0.23552	7.06560
	1/1000	3.68	0.023552	0.07656
Blood	Neat	2540	16.256	487.68000
	1/10	254	1.6256	48.76800
	1/100	25.4	0.16256	4.87680
	1/1000	2.54	0.016256	0.48768

The DNA yields resulted from extracting the above cell dilutions using the DNA IQ™ System is outlined in Table 4. Blood samples produced higher yields compared to cell samples. On average, blood samples on cotton swabs generated the highest yields. Cell samples on rayon and cotton swabs generated similar yields. All blood dilutions down to 1/1000 produced quantitation results, but cell samples only produced reliable quantitation results down to 1/100 dilution, possibly due to the effects of cell clumping.

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Table 4. DNA quantitation data for diluted cell and blood samples on rayon and cotton substrates.

Sample type	Dilution factor	Theoretical Input DNA (ng)	Rayon swab yield (ng)	Alleles	Cotton swab yield (ng)	Alleles	Rayon average yield (ng)	Rayon Std Dev	Recovery Rayon (%)	Cotton average yield (ng)	Cotton Std Dev	Recovery Cotton (%)
Cells	Neat	706.56000	110.0000	18	117.0000	18	134.5400	41.30	19.04	95.2800	32.69	13.48
			130.0000	18	124.0000	18						
			160.0000	18	46.8000	18						
			83.7000	7	76.6000	18						
			189.0000	17	112.0000	18						
	1/10	70.65600	10.1000	18	12.8000	18	10.4520	1.44	14.79	10.4820	2.52	14.84
			12.7000	18	6.3100	18						
			9.5500	18	11.5000	18						
			9.0100	18	10.1000	18						
			10.9000	18	11.7000	18						
	1/100	7.06560	0.6350	0	0.0000	0	0.9254	0.64	13.10	0.1270	0.18	1.80
			0.4930	0	0.0000	0						
			1.4000	5	0.2770	0						
			1.7900	14	0.3580	0						
			0.3090	0	0.0000	0						
	1/1000	0.7656	0.0000	0	0.3630	0	0.0166	0.04	2.17	0.0726	0.16	9.48
			0.0000	0	0.0000	0						
			0.0000	0	0.0000	0						
			0.0831	0	0.0000	0						
			0.0000	0	0.0000	0						
Blood	Neat	487.68000	216.0000	18	718.0000	18	317.0000	102.36	65.00	447.0000	196.46	91.66
			447.0000	18	297.0000	18						
			215.0000	18	595.0000	18						
			383.0000	7	326.0000	18						
			324.0000	18	299.0000	18						
	1/10	48.76800	113.0000	18	126.0000	18	124.7800	28.10	255.86	97.6600	21.66	200.25
			107.0000	18	91.9000	18						
			145.0000	18	75.4000	18						
			95.9000	18	81.0000	18						
			163.0000	18	114.0000	18						
	1/100	4.87680	14.3000	18	15.9000	18	12.4800	1.62	255.91	16.7600	4.69	343.67
			12.5000	13	12.1000	18						
			13.2000	18	20.8000	18						
			9.9000	18	22.4000	18						
			12.5000	18	12.6000	18						
	1/1000	0.48768	0.7300	18	2.3700	18	0.8894	0.20	182.37	3.0200	0.85	619.26
			0.6990	18	3.1300	18						
			1.1800	18	3.6300	18						
			0.8670	18	1.9700	18						
			0.9710	18	4.0000	18						

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The average yield observed within cell and blood samples on either rayon or cotton swabs were comparable (Figure 1). Some inconsistencies were present in cell samples at the lower dilutions of 1/100 and 1/1000 due to unreliable quantitation data at these low dilutions. Blood samples were found to generate higher average yields than cell samples and gave unexpectedly higher recovery values, despite the fact that the input DNA amount was 2-fold higher for cells compared to blood samples (Table 4). This discrepancy may have arisen from inconsistencies in cell suspension uniformity during dilutions of the original cell or blood sample, resulting in inaccurate estimates for average cell concentrations.

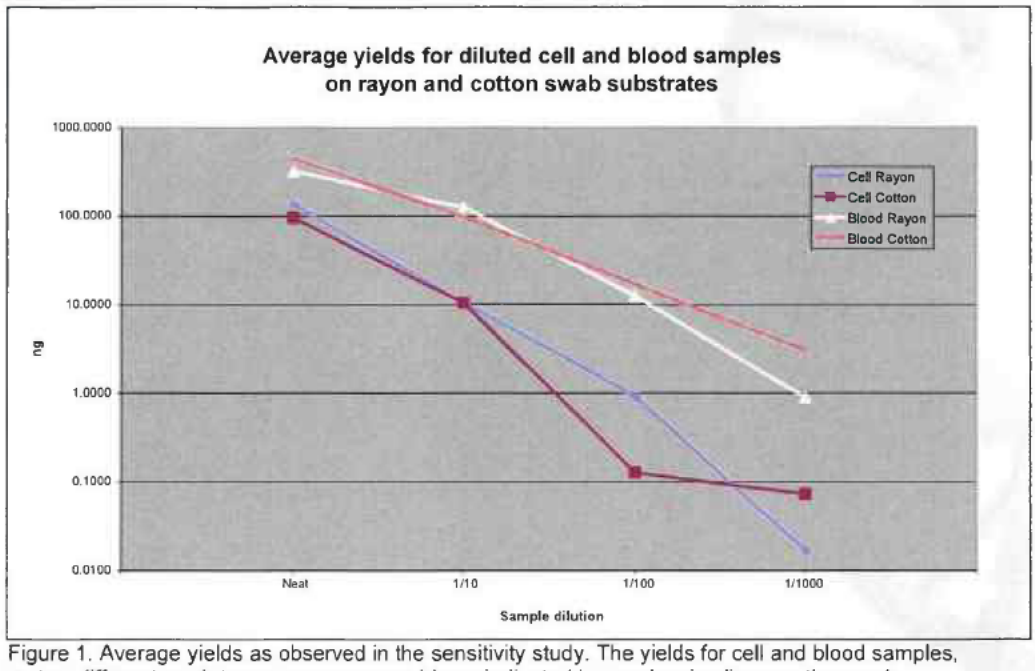

Figure 1. Average yields as observed in the sensitivity study. The yields for cell and blood samples, on two different swab types, were comparable as indicated by overlapping lines on the graph.

The dilution factor was, however, accurately reflected in the average yield for the various dilutions as displayed in Table 4 and Figure 2. An exception to this was the average yields for the neat dilutions (Figure 2). DNA IQ™ isolates a maximum of 100ng DNA as the resin is present in excess, and the system becomes more efficient with samples containing less than 50ng of DNA. Because the amount of DNA was in excess in neat samples, the observed yields varied from sample-to-sample. According to the manufacturer, the DNA IQ™ Database Protocol should be used for samples containing more than 100ng DNA to result in more consistent concentrations between the samples (Huston, 2002).

All five replicates for each neat dilution displayed the highest yields for each dilution series, as expected (Figure 2). For blood samples on rayon and cotton swabs, yields were still around 1ng for samples at the 1/1000 dilution (Figure 3).

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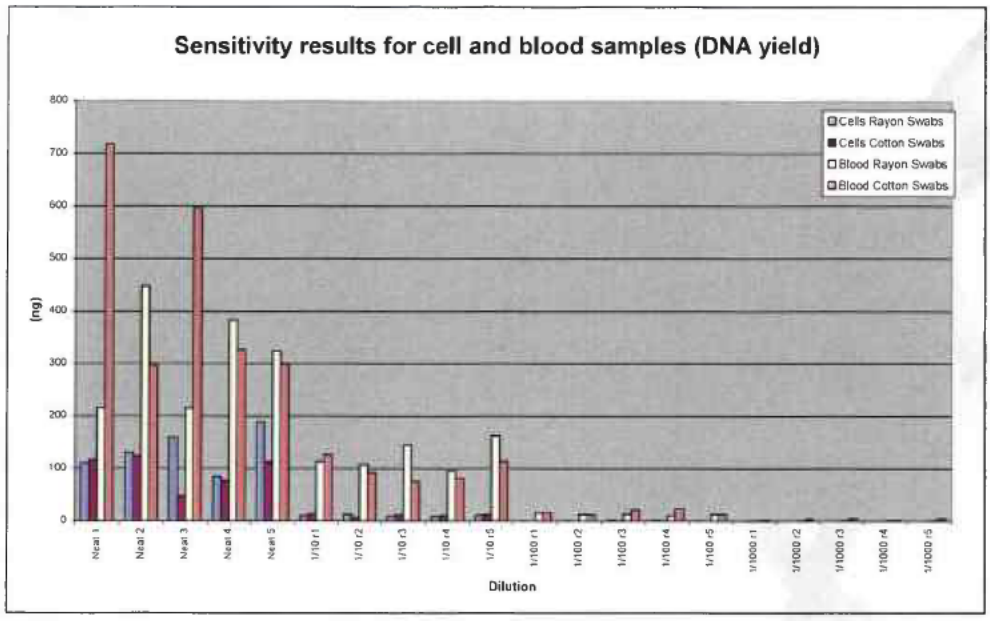

Figure 2. DNA yields (ng) observed for the sensitivity study. As expected, neat samples provided the highest yields. Yields were obtained down to 1/1000 for blood samples and 1/100 for cell samples.

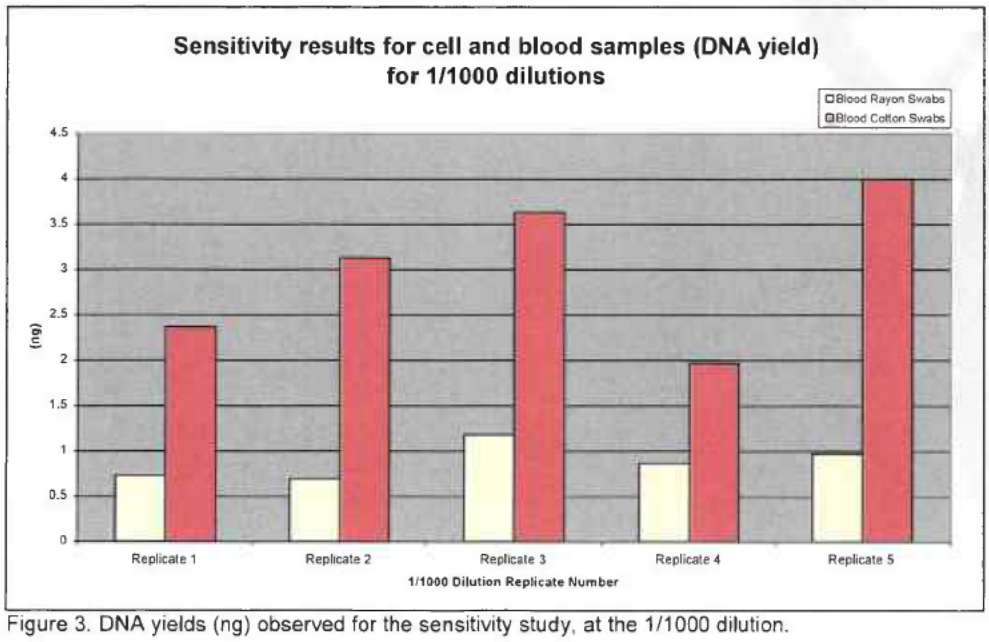

Figure 3. DNA yields (ng) observed for the sensitivity study, at the 1/1000 dilution.

When amplified using a 9-locus STR system, all neat samples produced the expected full DNA profile (18/18 alleles), although one outlier was encountered for a cell rayon sample which produced a 7/18 partial profile (Table 4). For cell samples, full profiles could be obtained for samples that were diluted down to 1/10, with partial profiles generated from samples diluted to 1/100. For blood samples, full profiles were generally obtained from all dilutions down to 1/1000. Although two partial profiles were encountered in blood samples on rayon swabs, all blood cotton swabs produced full profiles at all dilutions.

The apparent discrepancy between the results for cell and blood samples can be attributed to inaccurate cell counts or non-uniform sample suspensions when creating the dilutions, as caused by cell clumping or cellular breakdown and precipitation.

For five replicates of each dilution, consistency was observed to vary depending on the dilution (Figure 4). Consistency, as an indication of reproducibility, was calculated as the percentage of the yield standard deviation over five replicates divided by the mean yield of all five replicates (%[SD_{yield} / $mean_{yield}$]). A value closer to 0% indicates minimal sample-to-sample variation and therefore the results are highly consistent. The mean combined reproducibility for all neat, 1/10, 1/100 and 1/1000 dilutions were 35.31%, 20.63%, 62.14% and 124.32% respectively (Figure 4), indicating that there was high reproducibility between the neat and 1/10 dilutions across the four sample types, and reduced reproducibility at the lower 1/100 and 1/1000 dilutions. Overall, the blood samples on rayon and cotton both exhibited high reproducibility across all dilutions at an average of 30.54% and 22.45% respectively (Figure 5). The cell rayon and cotton samples were more variable across all dilutions, producing lower reproducibility at an average of 84.23% and 105.19% respectively (Figure 5). The poor performance of the cell samples can be attributed to inconsistencies in quantitation data observed at the lower 1/100 and 1/1000 dilutions.

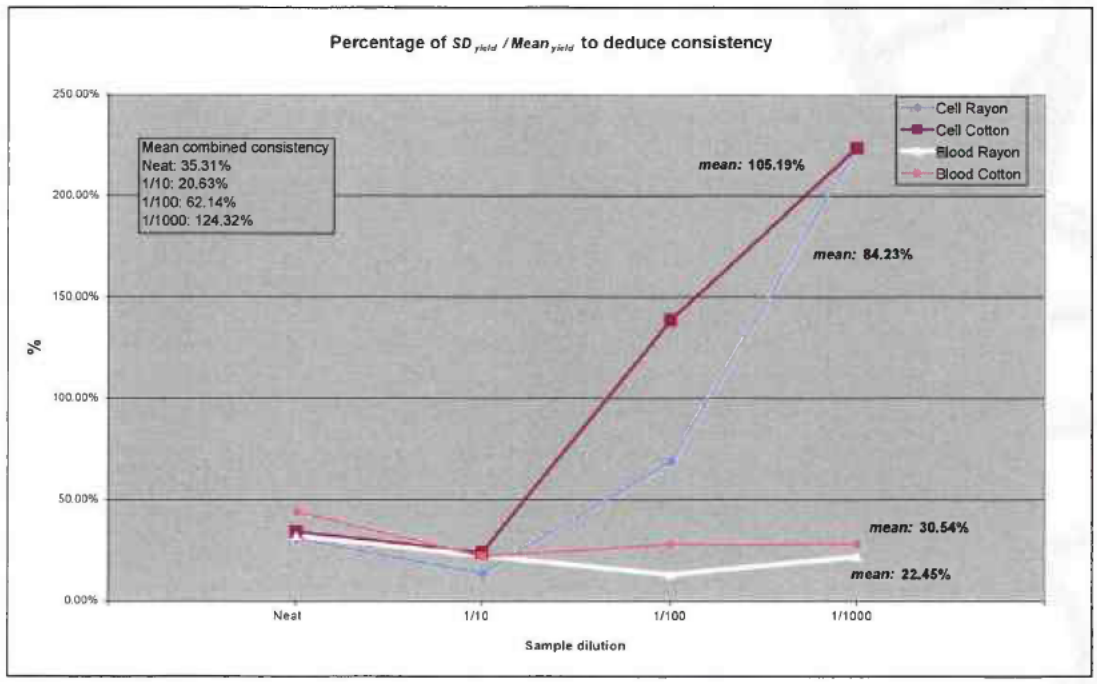

Figure 4. Reproducibility between replicates for cell and blood samples diluted down to 1/1000.

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6.3 Inhibition

Forensic samples that are commonly submitted for DNA analysis often contain inhibitors. These inhibitors may inhibit or significantly reduce the efficiency of a DNA extraction system, either by interfering with cell lysis or interfering by nucleic acid degradation or capture, therefore manifesting as extraction inhibitors (Butler, 2005). Inhibitors can also co-extract with the DNA and inhibit downstream PCR amplification processes, therefore acting as PCR inhibitors (Butler, 2005). For example, inhibitors such as hemoglobin and indigo dye likely bind in the active site of the *Taq* DNA polymerase and prevent its proper functioning during PCR amplification.

For the inhibition study, five substances were chosen for their known ability to inhibit PCR and their likelihood of appearing in routine casework samples:

- Indigo carmine: a component of the blue-dye encountered in denim jeans (Shutler, *et al.*, 1999).
- Tannic acid: a chemical used in the leather tanning process.
- Urea: a component of urine (Mahony *et al.*, 1998).
- Humic acid: a component found in soil and soil products (Tsai and Olson, 1992).
- Motor oil: contains various hydrocarbons and ethanolic compounds that can inhibit PCR.

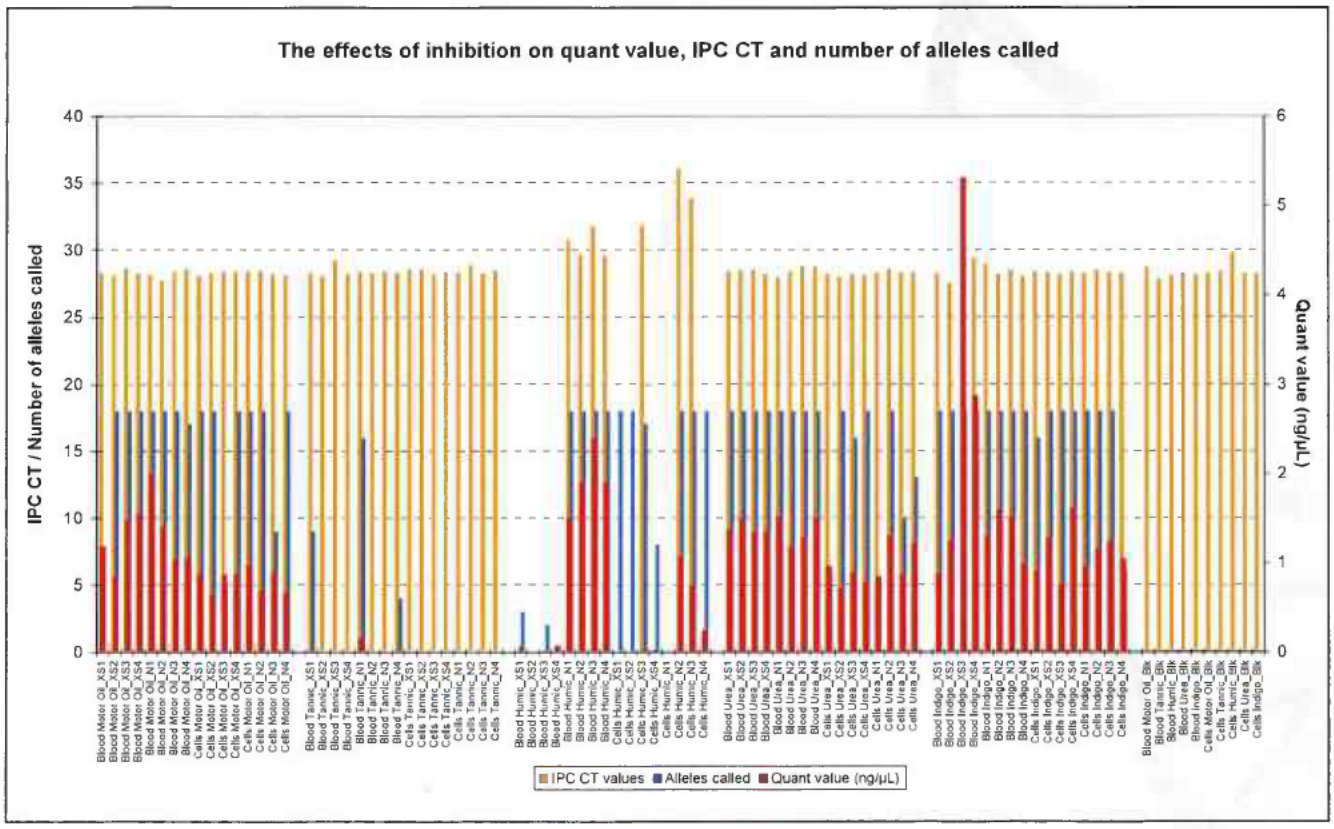

Figure 5. Effects of various inhibitors on quant value, IPC CT and number of resolved alleles.

The observed effects of these inhibitors at neat and excess concentrations on the ability to extract, quantify and amplify various DNA samples are graphed in Figure 5. Samples were quantified using the Quantifiler™ Human DNA Quantitation Kit (Applied Biosystems) as this kit includes a built-in inhibition detector. Reaction efficiency and the presence of inhibitors can be assessed based on the performance of the internal positive control (IPC), which is known to be detected in this laboratory at around 28 cycles.

The observations that were made include:

- Samples that were spiked with motor oil, urea and indigo carmine dye did not show inhibition as determined by the IPC, and resulted in quantifiable DNA templates after extraction using DNA IQ™. The average DNA concentration observed for all samples was around 1ng/µL. The majority of samples yielded full DNA profiles, with the exception of several cell samples that were treated with urea (both at excess and neat concentrations).
- Blood and cell samples that were spiked with tannic acid did not show inhibition in Quantifiler™, as the IPC performed as expected. However, almost no amplifiable template DNA could be quantified and the majority of samples did not produce DNA profiles. This suggests that the original template DNA was degraded by application of tannic acid to the sample. It should be mentioned at this point that the tannic acid used was in the form of a yellow-brown paste substance that was applied directly to the sample swabs. The tannic acid paste, even at the neat concentration, may have been strong enough to severely fragment DNA to result in non-amplifiable templates. It was observed that three blood samples (1 with tannic acid in excess and 2 with tannic acid at neat concentration) yielded partial profiles (between 4-16 reportable alleles), and none of the cell samples produced reportable alleles. This may be caused by: (1) the concentration of viable cells in the buccal cell samples was lower than blood samples; (2) the drying of the blood stain on the substrate may have created a better barrier to protect the blood components from the degradative effects of the tannic acid.
- Blood and cell samples that were treated with humic acid in excess appeared to retain inhibition after extraction using DNA IQ™. However, at neat concentration, the effect of the humic acid inhibitor was overcome and amplifiable DNA template was purified as demonstrated by high DNA concentration yields. Residual inhibition was still present at neat concentration, as evidenced by higher CT values for the IPC (closer to 30), but full profiles were still produced. For some cell samples with humic acid in excess, the Quantifiler™ data suggested full inhibition (undetermined IPC CT and quantitation results), but two samples resulted in full DNA profiles.
- All reagent blanks were undetermined, indicating the absence of contamination in the results.

The results show that the DNA IQ™ system could be used to extract blood or cell samples that were spiked with motor oil, urea and indigo carmine at both excess and neat concentrations. Blood samples that contained humic acid in excess did not yield amplifiable template DNA, but 2 out of 4 cell samples with humic acid in excess appeared to produce full profiles. Samples that were exposed to tannic acid, at both neat and excess concentrations, resulted in non-amplifiable DNA, but the inhibitor was effectively washed out of the extract by DNA IQ™ as evidenced by the amplification of the IPC at the expected CT. Based on these results, we conclude that the DNA IQ™ system effectively removes inhibitors that are present in the original sample, resulting in a DNA extract that is of sufficient quality and is suitable for PCR amplification.

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6.4 Substrates

The substrate types examined included: swabs (cotton and rayon), tapelifts, fabric (denim, cotton, wool, lycra, nylon, polyester, leather), gum, cigarette butts, and FTA® paper. Cell and blood materials were spotted on to the substrates and extracted using DNA IQ™. The results for the two different sample types are presented in Figures 6 and 7 below.

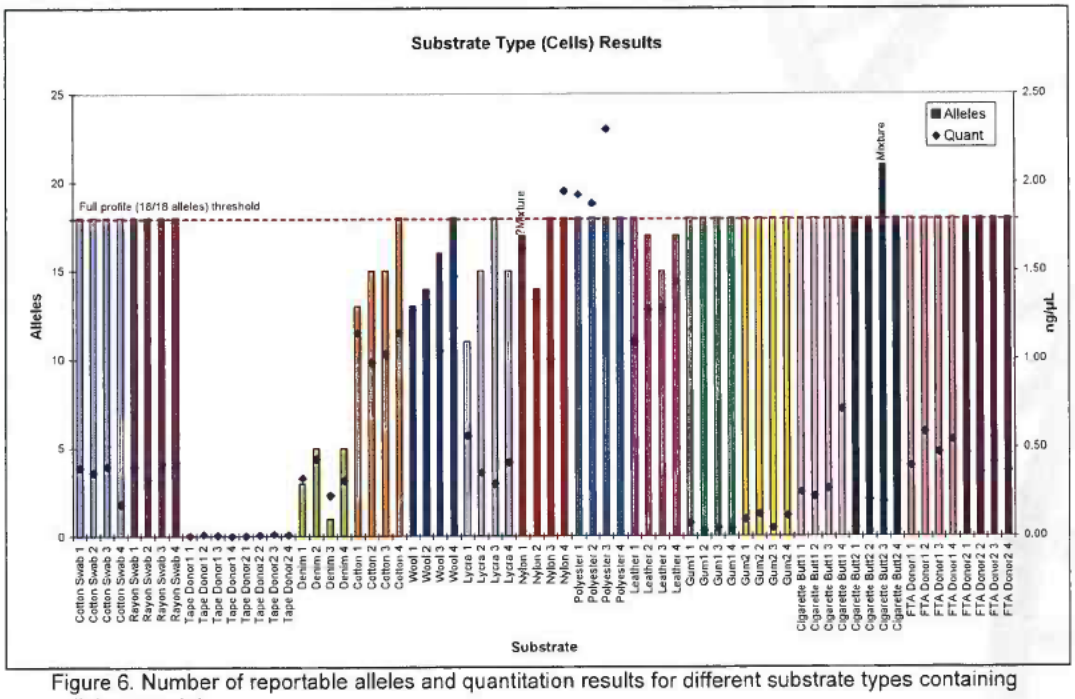

Figure 6. Number of reportable alleles and quantitation results for different substrate types containing cellular material.

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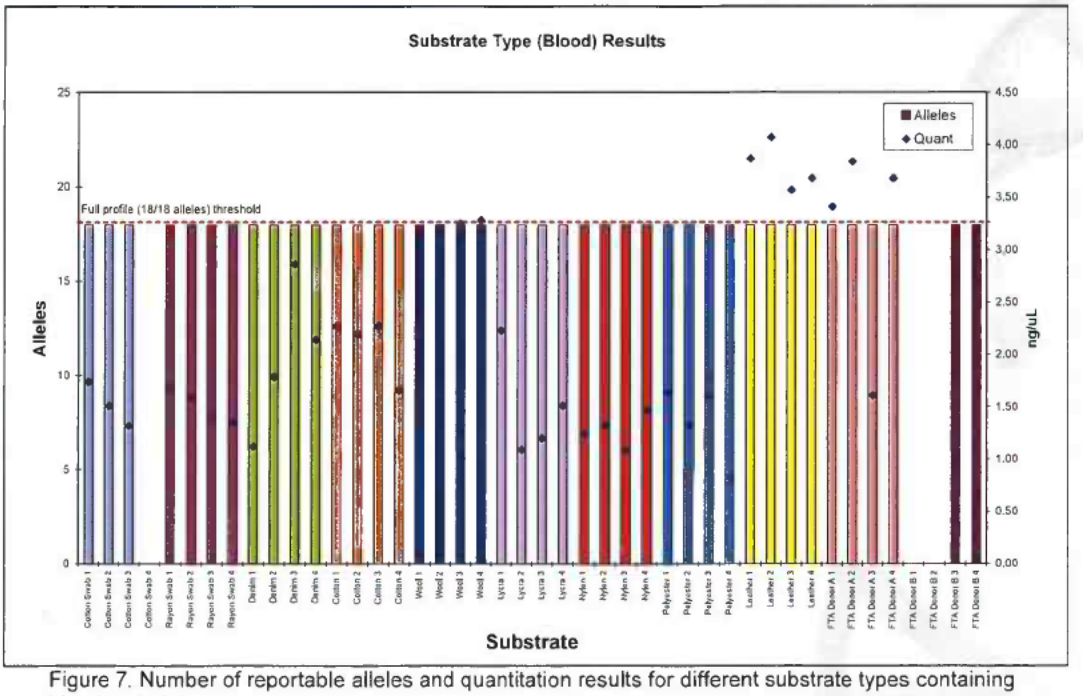

Figure 7. Number of reportable alleles and quantitation results for different substrate types containing blood material.

For cell samples:

- Full DNA profiles (18/18 alleles) were obtained from samples on cotton and rayon swabs, gum, cigarette butts and FTA® paper.
- The quantitation results for most of these samples were less than 0.5ng/µL. For gum samples, the average quantitation result was 0.072ng/µL, and therefore a PCR amplification at maximum volume (20µL) resulted in a total input DNA amount of 1.44ng which is sufficient to result in a full DNA profile.
- Tapelift samples gave an average quantitation result of 0.006ng/µL (just 0.002ng/µL higher than the observed background), and yielded no reportable alleles at all.
- The performance of clothing substrates was variable.
    - Cells on denim yielded quantitation results less than 0.5ng/µL but only partial profiles (maximum 5 reportable alleles), although Quantifiler™ results did not indicate any inhibition of the IPC. The poor performance of these samples may have been a result of sample preparation due to cell clumping.
    - Cells on cotton, wool and nylon resulted in higher quantitation values than lycra, but all substrates generated a similar number of reportable alleles (mean = 14 alleles). Only 25% of samples generated full DNA profiles.
    - Three out of four samples on polyester produced high quantitation results (~2ng/µL) but all samples resulted in a full profile.
    - Cells on leather displayed an average quantitation result of 1.3ng/µL and generated more than 15 reportable alleles.

For blood samples:

- All substrate types generated full DNA profiles.
- On average, the DNA quantitation results for all blood samples was greater than those resulted from cell samples. This is as per expected and was observed previously (see Project 9 report), because the concentration of nucleated cells in the blood samples were hypothesised to be higher than the concentration of buccal cell samples.
- Because of processing error, data was not available for the following samples: Cotton Swab 4, FTA Donor B 1 and FTA Donor B 2.

The results above are initial amplification results that do not take into account any reworking options.

We found that samples on tapelift substrates performed the worst; however this was probably due to the sampling method devised for this experiment, which did not adequately sample a sufficient number of cells.

6.5 Mixture studies

A mixture study was performed as part of the validation, however the results are not presented in this document because the mixture ratio was found to be inaccurate because cell counts were not performed on the saliva samples. Therefore, little information could be deduced from these results.

6.6 Substrate size

Blood on cotton swabs produced full DNA profiles for all sample sizes, ranging from 0.5 x 0.5cm to 2.0 x 2.0cm (Figure 8). Cells on cotton swabs did not perform as well (Figure 8), possibly due to the nature of the cells and difficulties in obtaining full DNA profiles from cell samples as observed in previous experiments.

Although the same starting amount of sample was used, it was observed that the 0.5 x 0.5cm samples generated higher quantitation results (therefore, also higher yields) compared to the 2.0 x 2.0cm samples (Figure 8). It appears that extraction efficiency decreases as the substrate surface area increases. This may be due to insufficient mixing and distribution of the lysis buffer over a larger substrate surface area, causing insufficient lysis of cellular material. This observation is in line with other reports that the DNA IQ™ system works more efficiently with smaller samples (Promega, 2006). The resulting IPC CT fell within the narrow range of 27.91 – 28.43 (mean = 28.10), indicating that both small and larger samples resulted in DNA extracts of similar quality, but the overall yield was lower for larger substrates (Figure 8 & 9).

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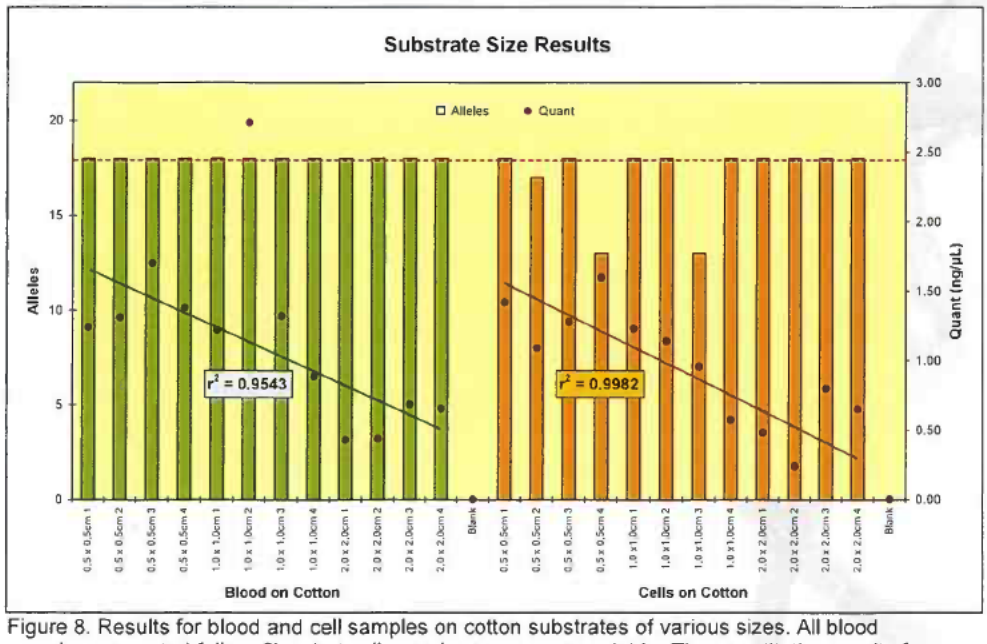

Figure 8. Results for blood and cell samples on cotton substrates of various sizes. All blood samples generated full profiles, but cell samples were more variable. The quantitation results for 0.5 x 0.5cm samples were higher than those for 2.0 x 2.0cm samples (blood r^2 = 0.9543*; cell r^2 = 0.9982; *Note: an outlier was removed from the calculation).

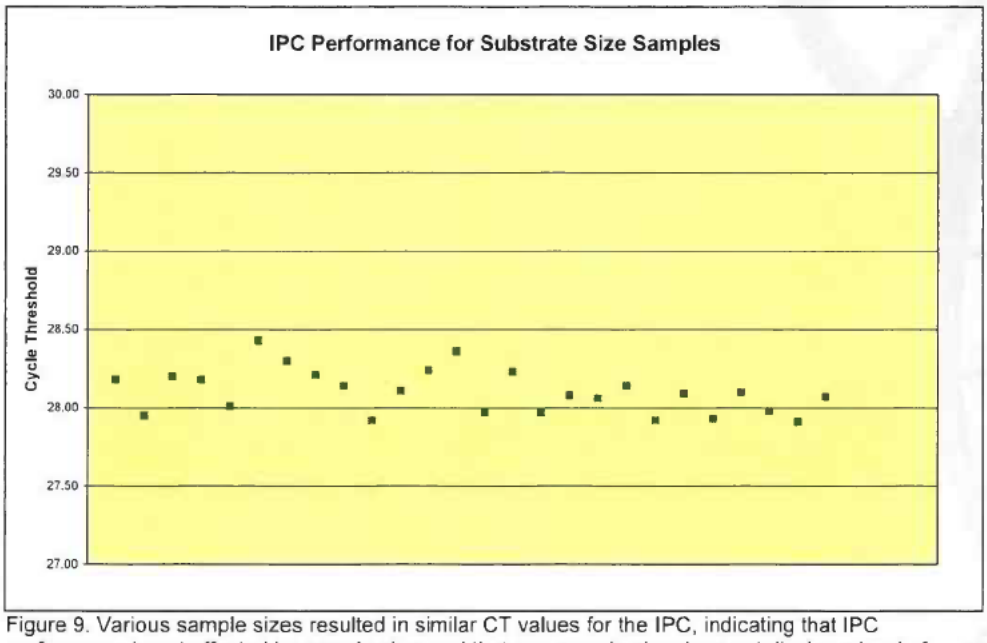

Figure 9. Various sample sizes resulted in similar CT values for the IPC, indicating that IPC performance is not affected by sample size, and that one sample size does not display a level of inhibition that is different to another sample size.

7. Summary and Recommendations

Based on the findings of this validation report, we recommend:

1. To enable processing of cell and blood samples using the validated manual DNA IQ™ protocol, except for samples on tapelift substrates.
2. To design and verify an automated protocol of the validated DNA IQ™ method for use on the MultiPROBE® II PLUS HT EX platforms, for processing blood and cell samples.

8. Acknowledgements

We wish to thank the Cytology Department at the Royal Brisbane and Women's Hospital for assistance with the cell-counting protocols.

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Exhibit CI-03

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Project 13. Report on the Verification of an Automated DNA IQ™ Protocol using the MultiPROBE® II PLUS HT EX with Gripper™ Integration Platform

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Automation/LIMS Implementation Project, DNA Analysis FSS (August 2008)

1. Abstract

A manual method for extracting DNA from forensic samples using the DNA IQ™ system (Promega Corp., Madison, WI, USA) was validated for routine use in DNA Analysis (FSS). We have verified an automated DNA IQ™ protocol in 96-well format for use on the MultiPROBE® II PLUS HT EX Forensic Workstation platforms (PerkinElmer, Downers Grove, IL, USA). Data indicate that results from the automated procedure are comparable to those from the manual procedure. Contamination checks were performed using samples prepared in checkerboard and zebra-stripe format, and results were as expected. We recommend the use of the MultiPROBE® II PLUS HT EX platforms to perform automated DNA extraction using the DNA IQ™ system.

2. Introduction

The MultiPROBE® II PLUS HT EX FORENSIC WORKSTATION platforms (PerkinElmer, Downers Grove, IL, USA) are equipped to perform automated DNA extractions, as they include a DPC shaker and individual heat controllers to enable on-board lysis and incubation steps. Currently in DNA Analysis, the MultiPROBE® platforms allow walk-away operation of PCR setup protocols for DNA quantitation and amplification.

The DNA IQ™ protocol has been verified or validated by various laboratories for use on the MultiPROBE® II PLUS platform. The laboratories that perform an automated DNA IQ™ protocol include PathWest (Western Australia), Forensic Science South Australia (South Australia) and Centre of Forensic Sciences in Toronto (Ontario). The MultiPROBE® II PLUS instrument comes pre-loaded with an automated DNA IQ™ protocol. Unlike the other laboratories, however, we did not validate the included protocol, but instead validated a manual DNA IQ™ protocol which was based on the CFS automated protocol (PerkinElmer, 2004), followed by verification of an automated protocol based on the validated manual method.

The verified automated DNA IQ™ protocol is identical to the validated manual protocol used in-house: there are no differences in reagents or volumes. The adopted DNA IQ™ protocol differs slightly, however, from the manufacturer's protocol, as it includes a lysis step using Extraction Buffer (10mM Tris, 1mM EDTA, 100mM NaCl, 20% w/v SDS) in the presence of Proteinase K, before incubating in the DNA IQ™ Lysis Buffer. Furthermore, the lysis incubation conditions were lowered from 70°C to 37°C in order to accommodate extraction of DNA from heat labile materials such as nylon and polyester. In addition, the automated protocol utilises the SlicPrep™ 96 Device (Promega Corp., Madison, WI, USA) for simultaneous processing of samples in a 96-well format.

Exhibit CL-03

3. Aim

To verify an automated DNA IQ™ protocol for use on the MultiPROBE® II PLUS HT EX platforms to allow extraction of DNA from various sample types.

4. Equipment and Materials

- MultiPROBE® II PLUS HT EX with Gripper™ Integration Platform (PerkinElmer, Downers Grove, IL, USA)
- Gravimetric Performance Evaluation Option with Mettler SAG285/L balance (Mettler-Toledo, Greifensee, Switzerland)
- DNA IQ™ System (Promega Corp., Madison, WI, USA)
- Extraction Buffer (10mM Tris, 1mM EDTA, 100mM NaCl, 20% SDS)
- SlicPrep™ 96 Device (Promega Corp., Madison, WI, USA)
- Nunc™ Bank-It tubes (Nunc A/S, Roskilde, Denmark)
- 175µL non-conductive sterile filter RoboRack tips (PerkinElmer, Downers Grove, IL, USA)
- 1000µL Conductive sterile filter Robotix tips (Molecular BioProducts, San Diego, CA, USA)
- ABI Prism® 7000 SDS (Applied Biosystems, Foster City, CA, USA)
- Quantifiler™ Human DNA Quantification kits (Applied Biosystems, Foster City, CA, USA)
- AmpFℓSTR® Profiler Plus Amplification kits (Applied Biosystems, Foster City, CA, USA)
- GeneAmp® 9700 thermalcycers (Applied Biosystems, Foster City, CA, USA)
- ABI Prism® 96-well optical reaction plates (Applied Biosystems, Foster City, CA, USA)
- ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA)
- GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Foster City, CA, USA)
- Hi-Di™ Formamide (Applied Biosystems, Foster City, CA, USA)
- 3100 POP-4™ Polymer (Applied Biosystems, Foster City, CA, USA)
- Cytobrush® Plus Cell Collector (Cooper Surgical, Inc., Trumbull, CT, USA)
- 0.9% saline solution (Baxter Healthcare, Old Toongabbie, NSW, Australia)
- Stem digital tilting head thermometer
- For mock samples:
  - FTA™® Classic Card (Whatman Inc., Florham Park, NJ, USA)
  - Sterile cotton swabs (Medical Wire & Equipment, Corsham, Wiltshire, England)
  - Sterile rayon swabs (Copan Italia SPA, Brescia, Italy)

5. Methods

5.1 Gravimetric Evaluation of Pipetting Accuracy and Precision

Gravimetric analysis was performed by placing the SAG285/L balance on the platform deck and instructing the MP II to repeatedly pipette certain volumes of system liquid onto the balance pan. Readings were taken automatically by the software and compiled into a results table, which was then used to automatically generate an Excel-based results chart containing mean, %CV and %inaccuracy values. The mean values obtained were used to calculate R^2, slope and Y-intercept (offset) values to calibrate the system's pipetting.

Pipetting performance was assessed for various volumes using three different tips in order to calculate appropriate R^2, slope and Y-intercept (offset) values which were then added to the performance file. Values were calculated for both Blowout (single-liquid transfer) and Waste (multidispense) modes for the 1000µL conductive tips, and Blowout mode only for the 175µL non-conductive tips and fixed tips.

For the addition of resin, a specialised performance file was created based on the performance file for 175µL tips in blowout mode, except the "Blowout Volume" column values were set to 0 to allow pipetting performance that is similar to waste mode. Retesting was performed to confirm accurate and precise pipetting with these settings.

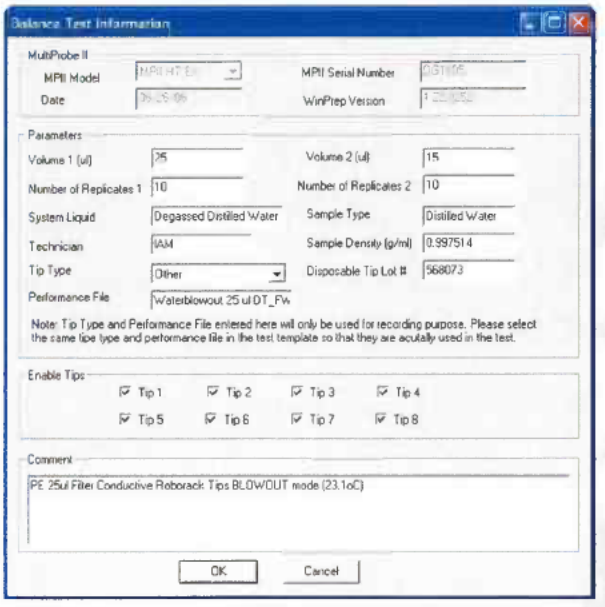

Figure { SEQ Figure * ARABIC }. The Balance Test Information Window as present within the Balance Test DT program. All pipetting parameters are entered here and are subsequently transferred to the result output file.

All gravimetric testing was performed using the Balance Test DT test program within WinPrep®. Parameter values that needed to be entered into the Balance Test Information Window (Figure 1) included those as outlined in Table 1.

Table { SEQ Table * ARABIC }. Input values that are required for the various Balance Test Information parameters.

Parameter(s)	Value
Volume 1 and Volume 2	For 175µL tips: 175, 100, 50, 15µL For 1000µL tips: 1000, 700, 400, 100µL For fixed tips: 1000, 700, 400, 100µL
Number of Replicates	10
System Liquid	Degassed Nanopure Water
Sample Type	Nanopure Water
Technician	Initials of the operator performing the test
Sample Density (g/ml)	The density of water at environmental temperature*
Tip Type	Other
Disposable Tip Lot #	The lot number of the particular tips in use
Performance File	The appropriate Performance File for the tip (175µL, 1000µL or fixed tips) and pipetting mode (Blowout or Waste) in use
Enable Tips (checkboxes)	Select the actual tips (1 to 8) to be tested
Comments	Free text box to add additional information (eg. Tip type, mode, current environmental room temperature, etc).

* Water density values were obtained from http://www.simetric.co.uk/si_water.htm

Pipetting accuracy and precision were examined at four different volumes for each tip size: 175, 100, 50, 15µL for 175µL tips and 1000, 700, 400, 100µL for the 1000µL and fixed tips.

In order to calculate unbiased values for each set of volumes, the slope and offset values in the relevant Performance File were changed to the default 1 and 0 respectively prior to testing. The mean volumes that were pipetted by each tip (10 replicates per tip) at the four designated volumes were used to generate a standard curve. The slope and offset calculated from this curve was used to calibrate the relevant Performance File. The final Performance File settings were then tested at the highest and lowest volumes (as per Table 1) to confirm accurate and precise pipetting.

5.2 Blood Collection

Blood samples were collected from 2 staff donors (DJC/VKI) by a phlebotomist as per normal in three 4mL EDTA vials. Blood samples were stored at 4°C.

5.3 Cell Collection

Buccal cells were collected using a modified Cytobrush® protocol (Mulot *et al.*, 2005; Satia-Abouta *et al.*, 2002). The donor was instructed to brush the inside of one cheek for one minute using a Cytobrush®. Then, with another Cytobrush®, the other cheek was also sampled. Once each cheek was swabbed, the cells on the brush were suspended in 2mL of 0.9% saline solution. Buccal cell samples were stored at 4°C.

5.4 FTA cell Collection

Cells were collected from two staff donors (VKI/CJA) by using a "lolly-pop" swab to sample the inside of the donor's cheek for 15 seconds before pressing the swab onto the FTA™ paper to transfer the DNA. FTA™ cards were stored at room temperature.

5.5 Heater tile temperature verification

Heat tiles supplied with the MultiPROBE® II PLUS HT EX platforms were modified to accept the SlicPrep™ 96 Device. For testing, 1mL of nanopure water (at room temperature) was added to each well. The plate was then placed on a heater tile (controlled by the MP II heater controller) and allowed to reach temperature. The temperatures tested were 37°C and 65°C. Temperature readings for specific outer and inner wells (i.e. A1, A6, A12, D1, D6, D12, H1, H6, H12) were taken at regular intervals up to and including 45 minutes, using calibrated stem digital tilted head thermometer probes. The data were collated and means calculated to determine the distribution of heat over the tile.

5.6 Verification of automated DNA IQ™ Protocol

The automated DNA IQ™ protocol, based on the validated manual method (refer to Project 11), was programmed in WinPrep™ software. The final, optimised protocol was named "DNA IQ Extraction_Ver1.1.mpt". A screenshot of the Test Outline window for this protocol is depicted below in Figure 2. The deck layout is illustrated in Figure 3.

The automated DNA IQ™ protocol was designed to mimic the validated manual method, with minor modifications. Briefly, the changes include:

- Increasing the volume of Extraction Buffer to 500µL;
- A SlicPrep™ 96 Device (Promega Corp., Madison, WI, USA) was used for sample lysis;
- Incubation steps and any shaking steps were performed on the integrated DPC shaker;

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- CRS toroid magnet (P/N 5083175) was used for isolating the DNA IQ™ resin.
- Instead of a single elution of 100µL, a double elution method (2 x 50µL) is used.

Reagents used in the automated protocol were as per the manual method.

Figure 2. The Test Outline window displaying individual nodes within the DNA IQ Extraction_Ver1.1.mpt program test file.

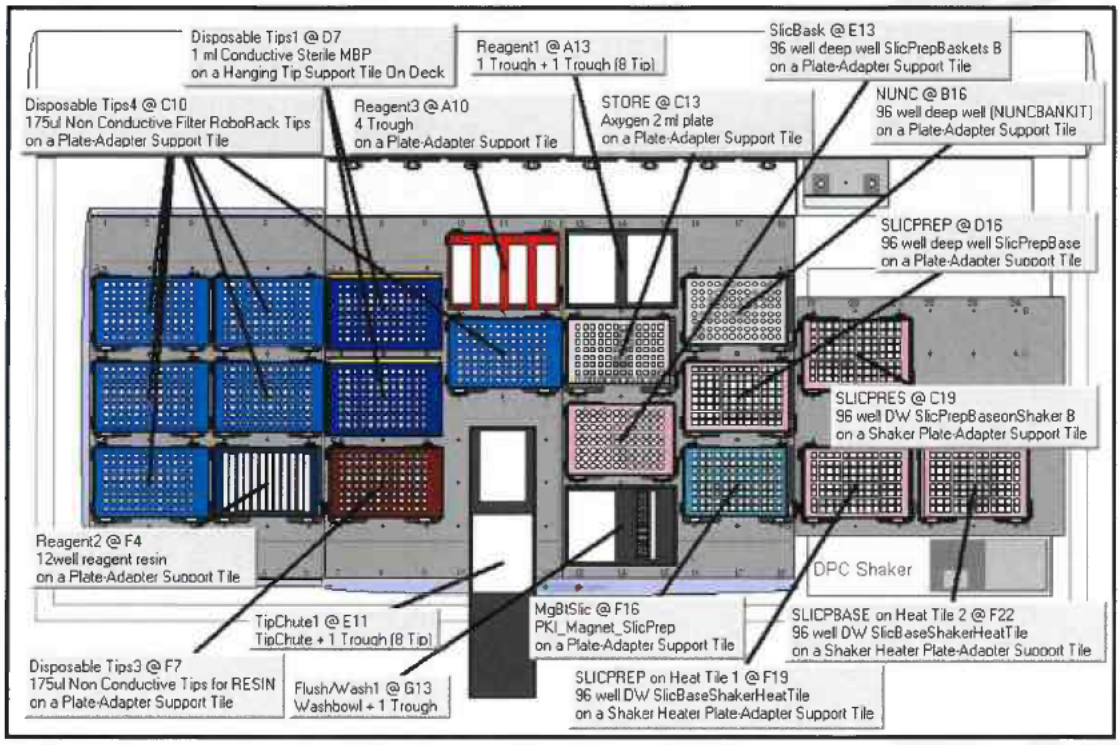

Figure 3. The deck layout for DNA IQ Extraction_Ver1.1.mpt, displaying the required labware on the platform deck.

The automated DNA IQ™ protocol was used to perform the following tests.

5.6.1. Contamination Check via Checkerboard and Zebra-stripe Patterns

Samples consisting of two 3.2mm FTA® discs (containing blood, buccal cells, or blank cards) were arranged in a checkerboard and zebra-stripe pattern (Figure 4) in SlicPrep™ plates using the BSD Duet 600 instrument (BSD Robotics, Brisbane, QLD, Australia) and extracted on the MultiPROBE® II PLUS HT EX platforms using the automated DNA IQ™ protocol. One checkerboard and one zebra-stripe plate was processed on each platform.

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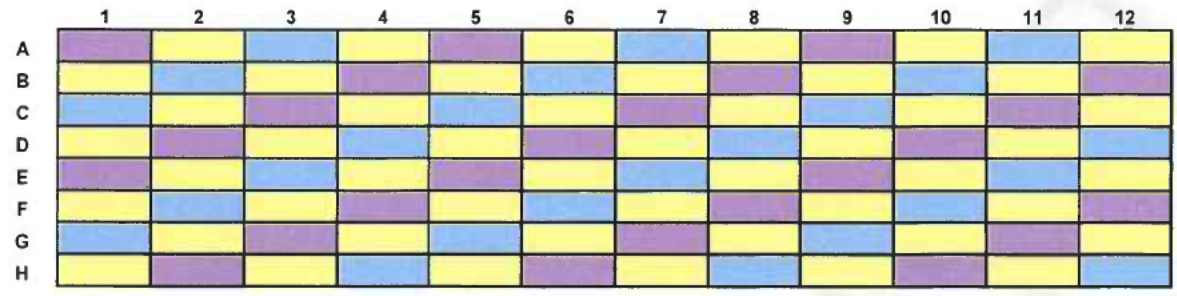
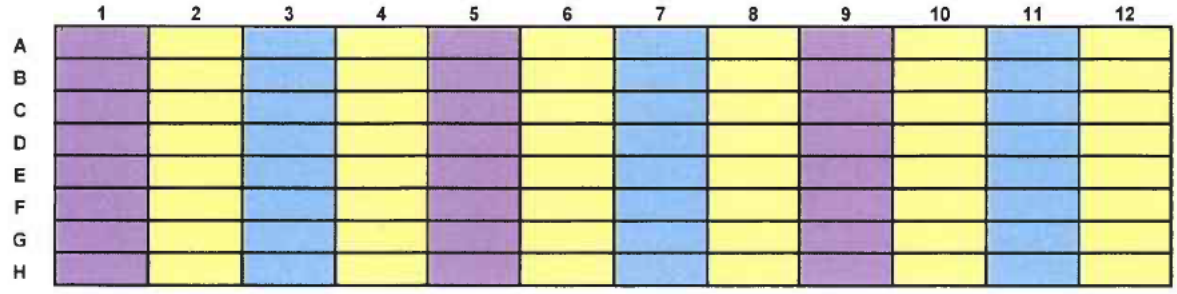


(a) Checkerboard Pattern

b) Zebra Stripe Pattern



Legend:
Blood FTA®
Blank FTA®
Buccal Cell FTA®

Figure 4. Checkerboard and zebra-stripe patterns utilised in the contamination check.

5.6.2. Comparisons with the manual DNA IQ™ method

Comparisons were made between results generated by the automated and manual methods to verify the performance of the automated DNA IQ™ protocol.

Verification samples consisted of different dilutions of blood and cells spotted in 30µL aliquots onto quartered cotton and rayon swabs. Four blood dilutions of neat, 1/10, 1/100 and 1/1000 and four cell dilutions of neat, 1/5.2, 1/52.2 and 1/522 were used to test the sensitivity of both the manual and automated methods. Dilutions were created using 0.9% saline solution for both sample types. Four replicates of each dilution were made up for each substrate and sample type.

The blood was collected using the same method as in 5.2. Four separate extractions were performed for the manual set based on the combination of sample type and swab type: Blood Rayon, Blood Cotton, Cell Rayon and Cell Cotton. For the automated verification, all sample types were extracted together after being transferred to a SlicPrep™ 96 Device to allow automated processing.

5.6.3. Resin volume

The performance of the automated DNA IQ™ protocol was assessed when either 7µL or 14µL of DNA IQ™ resin was used in the protocol to extract blood samples.

5.6.4. Modifying extraction volumes

The performance of the automated DNA IQ™ protocol was assessed for varying volumes of extraction buffer at 300, 350, 400, 450 and 500µL. In each case, the volume of DNA IQ™ Lysis Buffer was kept at 2x the volume of extraction buffer. Samples extracted were blood swabs, prepared as per ???.

5.6.5. Sensitivity of the automated DNA IQ™ protocol

The sensitivity of the automated DNA IQ™ protocol was assessed using dilutions of whole blood at neat, 1:10, 1:50, 1:100 and 1:1000.

6. Results and Discussion

6.1 Gravimetric Evaluation of Pipetting Accuracy and Precision

Pipetting on both automated platforms was assessed gravimetrically as per laboratory practice. Gravimetric results indicate that pipetting performance for five different pipetting behaviours using 500µL syringes on the instruments is accurate and precise to within the established threshold of ±5% (Table 2). The maximum CV at the maximum volume was 0.78%, whereas the maximum CV at the minimum volume was 1.1%. The CV for pipetting at lower volumes is expected to be slightly higher than the CV at higher volumes using 500µL syringes, because accuracy at small volumes is harder to achieve with larger syringe sizes. Nevertheless, pipetting on the extraction platforms is limited to a minimum of 50µL, which exhibited a CV of 0.36%.

Table 2. Gravimetric evaluation results for various performance files used on either MP II EXTN A or MP II EXTN B.

Performance File	Max. Vol. µL	Min. Vol. µL	Max. Vol. µL Mean	Max. Vol. %CV	Max. Vol. %Inac.	Min. Vol. µL Mean	Min. Vol. %CV	Min. Vol. %Inac.
EXTN A								
Water Blowout 175µL DT_FW _13112007RESIN.prf	50µL	N/A	49.98	0.36	0.0	N/A	N/A	N/A
Water Blowout 175µL DT_FW QHSS _13112007.prf	175µL	15µL	172.26	0.21	1.6	[redacted]	[redacted]	[redacted]
WaterWaste 1mL_FW_QHSS 12112007.prf	1000µL	100µL	999.11	0.24	0.1	99.22.	0.71	0.8
Water Blowout 1mL DT_QHSS _09112007.prf	1000µL	100µL	1001.02	0.27	0.1	100.65	0.63	0.7
Water Blowout Fixed Tips_08112007.prf	1000µL	100µL	995.97	0.31	0.4	99.6	0.71	0.4
EXTN B								
Water Blowout 175µL DT_FW_ 25102007RESIN.prf	50µL	N/A	50.12	0.36	0.2	N/A	N/A	N/A
Water Blowout 175µL DT_FW_ 25102007.prf	175µL	15µL	175.58	0.14	0.3	15.23	1.1	1.5
WaterWaste 1mLDT_FW_QHSS 24102007.prf	1000µL	100µL	1002.39	0.78	0.2	99.56	0.89	0.4
Water Blowout 1mL DT_QHSS 23102007.prf	1000µL	100µL	998.2	0.44	0.2	99.44	0.68	0.6

Water Blowout Fixed Tips_FW 26102007.prf	1000µL	100µL	998.87	0.68	0.1	100.37	0.74	0.4

6.2 Heater tile temperature verification

Two heater tiles on each MP II platform was verified to reach either 37°C or 65°C, the optimum incubation temperatures for sample lysis and DNA elution respectively (using the DNA IQ™ kit). Each tile, upon completion of the verification process, could only be used for a specific temperature, and as such was labelled appropriately to ensure use of the correct tile for specific incubation steps (Table 3).

Table 3. Verified heater tiles for use in the automated DNA IQ™ protocol.

Extraction platform	Tile number	Heater Controller Setting	Average °C reached	Verified temperature	Incubation Step
EXTN A	3 (45W)	50°C	37°C	37°C	Sample Lysis
EXTN A	1 (45W)	85°C	[redacted]	65°C	DNA Elution
EXTN B	1 (45W)	50°C		37°C	Sample Lysis
EXTN B	2 (45W)	85°C	65°C	65°C	DNA Elution

A slight variation in the incubation temperature to achieve sample lysis is acceptable, because Proteinase K exhibits stable activity and broad specificity over a wide range of temperatures between 20-60°C, at which the serine protease still retains greater than 80% of its activity (Sweeney & Walker, 1993).

The efficiency of the elution step is dependent on heating the sample to 65°C in the presence of DNA IQ™ Elution Buffer (Huston, 2002). If the sample is not sufficiently heated, the extraction yield may be lower than expected. Two heater tiles were able to be verified for this crucial incubation step, with both tiles exhibiting minimal variation.

6.3 Contamination Check via Checkerboard and Zebra-stripe Patterns

Table 4 below lists the Extraction Batch ID's of the contamination checks.

Table 4. Extraction Batch ID's for the various contamination check plates that were processed on the MP II platforms using the automated DNA IQ™ protocol.

Type of plate	Extraction batch Id	Extraction Platform	Check passed
Checkerboard 1	VALB20070817_02	Extraction A	Invalidated
Checkerboard 2	VALB20070803_02	Extraction B	Yes
Zebra-Stripe 1	VALB20070803_03	Extraction A	Yes
Zebra-Stripe 2	VALB20070817_03	Extraction B	Yes
Checkerboard/Zebra	VALB20071022_01	Extraction A	Yes

Checkerboard 1

Position E3 (Sample Cells 6) was known to have been contaminated prior to the start of the extraction during the STORstar process (???). The result showed a mixed DNA profile, with contributing alleles originating from the expected wells (Table 5). In addition to this contamination event, eight of the designated blank samples (positions D3, A10, F1, H5, C4, E4, B7 and E6), two of the cell samples (A1 and B10) and two of the blood samples (F4

and G7) all exhibited a partial DNA profile that was previously unknown (Table 5). This profile did not match any of the positive control samples present on the batch. The DNA profile was searched against the Staff Database and no matches were found. The source of this contaminating DNA profile could not be identified.

None of the other blank samples yielded any DNA profile. The rest of the cell and blood samples yielded the correct DNA profile. Although there is no evidence of well-to-well contamination, the unknown DNA profile obtained has invalidated this plate. A further checkerboard/Zebra-Stripe combination plate was performed to ensure...

Table 5. The DNA profile of the unknown contaminant that was observed in Checkboard-1.

Sample description	D3	vWA	FGA	Amel	D8	D21	D18	D5	D13	D7
Blk23-E6	14,17	14,17	22,24	X,Y	11,11	29,32.2	14,15	9,11	11,12	11,13
Blk25-B7	14,17	14,17	22,24	X,Y	11,11	29,32.2	14,15	9,11	11,12	
Blk15-E4	14,17	14,17	22,24	X,Y	11,11	29,32.2	14,15	9,11	11,12	11,13
Blk14-C4		14		X	11	32.2		9		
Blk20-H5	14,14	17,17	20,21	X,X	13,16	29,30	14,16	11,13	11,12	11,11
Blk3-F1	14	17		X	13	29,30	14		12	11
Blk10-D3	14,17	14		X,Y	11	29,32.2	14	9,11		11,13
Blk37-A10	14,17	14	22,24	X,Y	11	29	14	9,11		
Cells19-B10	14,17	14,17	20,21,22,24	X,Y	11,13,16	29,30,	14,15,16	11,15	11,12	11,11
Cells13-A1	14,17	14,17	20,21,22,24	X,Y	11,13,16	29,30,32,32.2,33	14,15,16	9,11,13	11,12	11,13
Blood14-G7	NR,17,18	NR,16	20,21	X,Y	NR,13,14	29,30,31,NR	NR,14	NR,12	10,10	10,NR,12
Blood8-F4	NR,17,18	NR,16,17	20,21,NR,24	X,Y	11,13,14	29,30,NR,NR	14,14	9,11,12	10,NR	10,NR,12
Cells 6-E3	14,17,18	16,17	20,21	X,Y	13,14,16	29,30,31	NR,14,16	11,12,13	10,11,12	10,11,12

Checkerboard 2

None of the blank samples yielded DNA profiles; all of the positive cell and positive blood samples yielded the correct DNA profile. Figure 5 illustrates the DNA quantitation results from this plate. DNA was not detected in any of the blank samples.

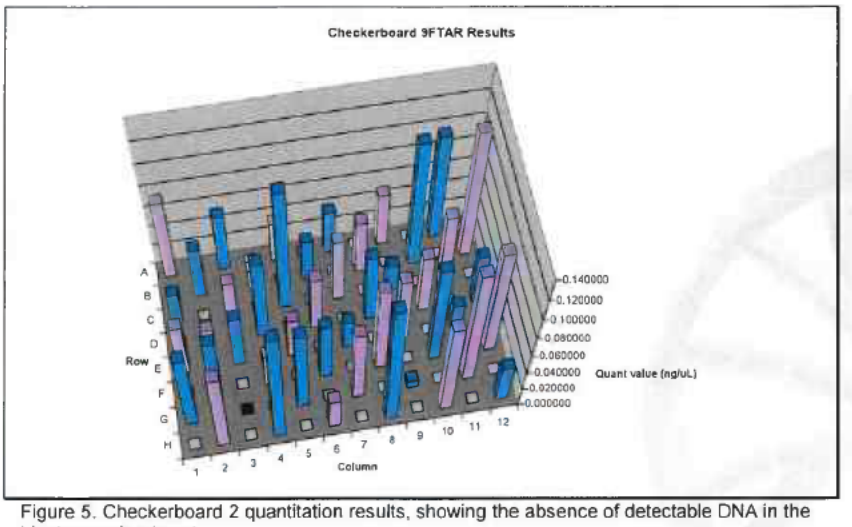


Figure 5. Checkerboard 2 quantitation results, showing the absence of detectable DNA in the blank samples (grey).

Zebra-Stripe 1

None of the blank samples yielded DNA profiles, all of the positive cell and positive blood samples yielded the correct DNA profile. Figure 6 illustrates the absence of detectable DNA in the blank samples.

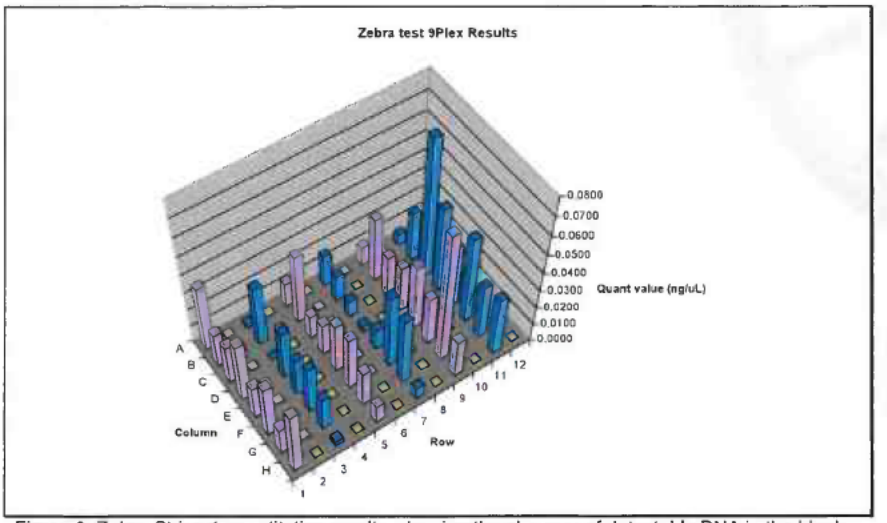


Figure 6. Zebra-Stripe 1 quantitation results, showing the absence of detectable DNA in the blank samples (grey).

Zebra-Stripe 2

None of the blank samples yielded DNA profiles, all of the positive cell and positive blood samples yielded the correct DNA profile. Figure 7 shows the absence of detectable DNA in the blank samples.

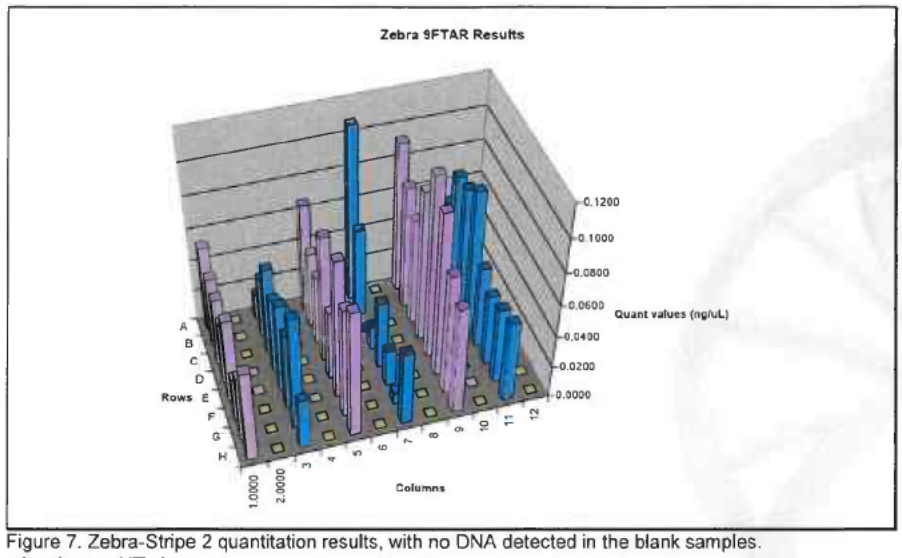


Figure 7. Zebra-Stripe 2 quantitation results, with no DNA detected in the blank samples.

Checkerboard/Zebra

None of the blank samples yielded DNA profiles, all of the positive cell and positive blood samples yielded the correct DNA profile. DNA was undetected in the blank samples (Figure 8).

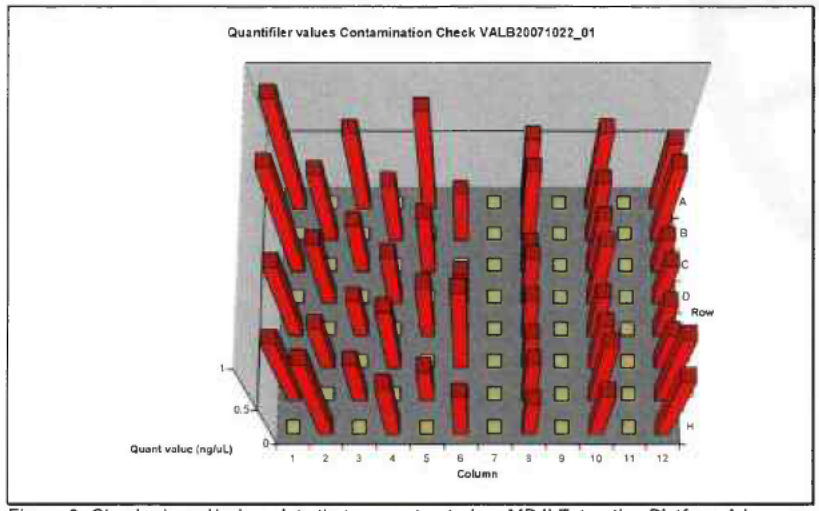


Figure 8. Checkerboard/zebra plate that was extracted on MP II Extraction Platform A because the previous plate was invalidated. DNA was not detected in the blank samples (grey).

6.4 Comparisons with the manual DNA IQ™ method

When dilutions of either blood or cells were applied on to either rayon or cotton swabs, followed by extraction using the DNA IQ™ method, the results of the automated method were always lower in yield compared to the manual method. For blood samples on rayon

swabs, the automated method generated yields that were on average around 8% (SD 8.45%) of the automated method. For blood on cotton swabs, the yield from the automated method was also around 8% (SD 3.62%). The yields for cell samples were higher at around 33% (SD 16.29%) and 25% (10.32%) for cells on rayon and cotton swabs respectively.

The manual method was found to be more sensitive than the automated method. Out of five replicates at the 1/100 and 1/1000 dilutions for blood on rayon swabs that were processed using the manual method, five and three replicates respectively were detected (and none from the automated method) (see Figure 9). The trend is repeated for blood on cotton swabs (Figure 10). For cell samples on either rayon or cotton swabs, the automated method was found to be more sensitive as evidenced by detection of DNA at the 1/522 dilutions (Figure 11 and 12).

Cell clumping may have occurred with the cell dilutions, therefore causing inaccurate dilutions as can be observed in the ratios between each dilution.

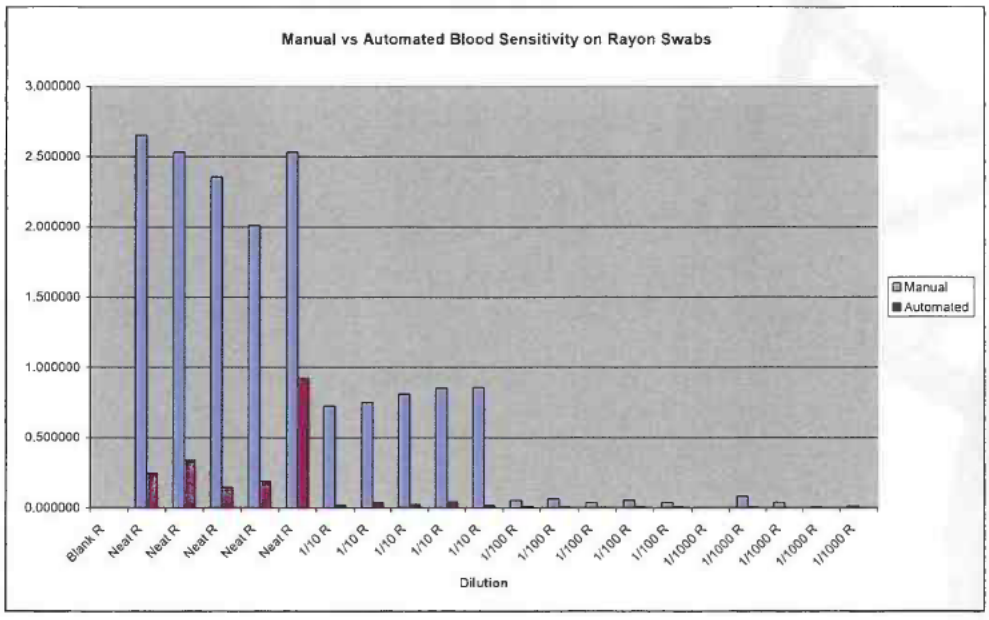


Figure 9. Comparison of sensitivity between the manual and automated DNA IQ™ methods for blood samples on rayon swabs.

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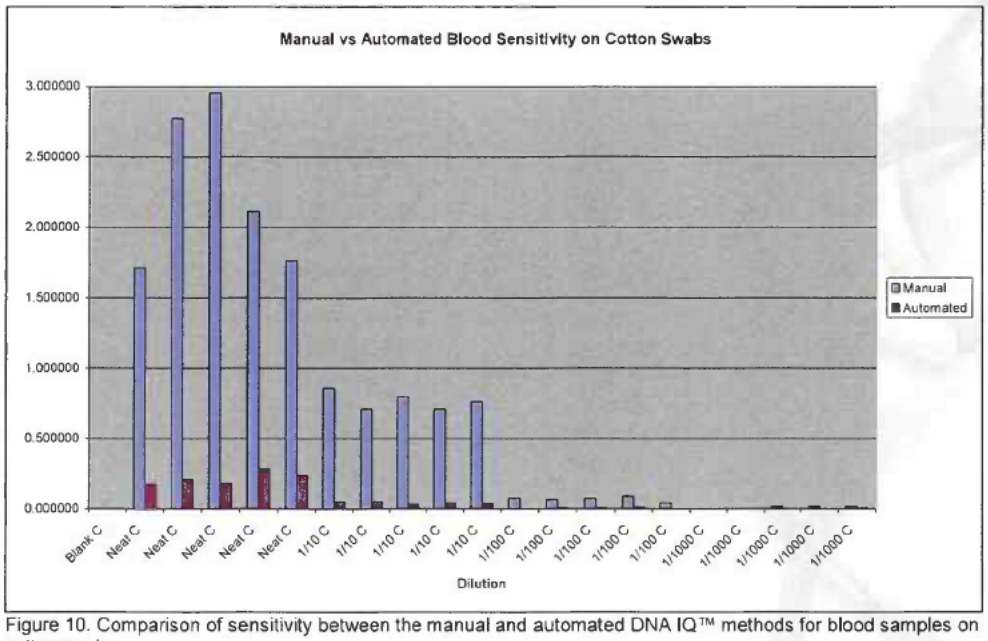

Figure 10. Comparison of sensitivity between the manual and automated DNA IQ™ methods for blood samples on cotton swabs.

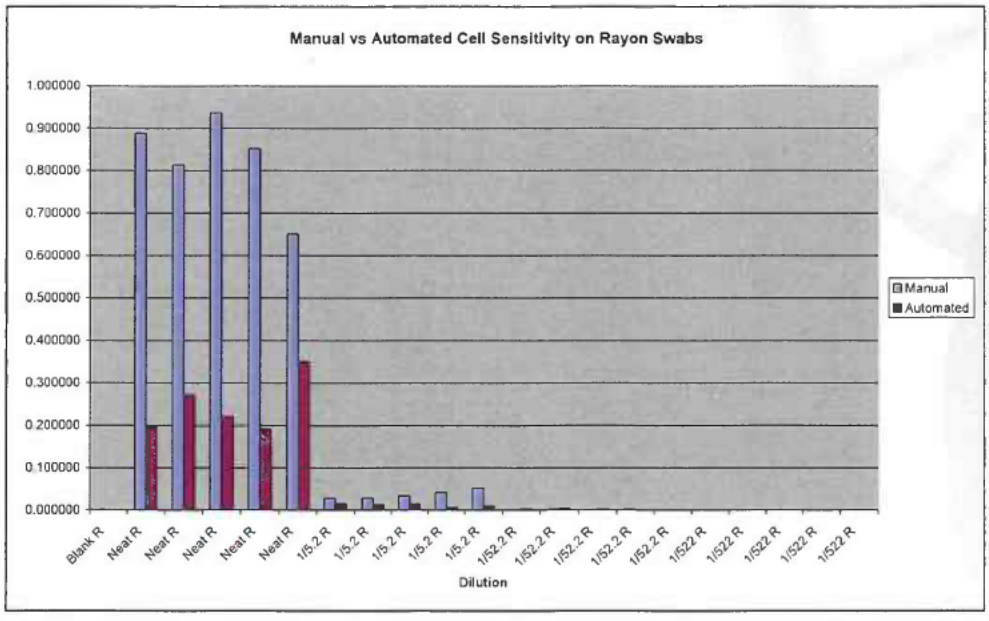

Figure 11. Comparison of sensitivity between the manual and automated DNA IQ™ methods for cells samples on rayon swabs.

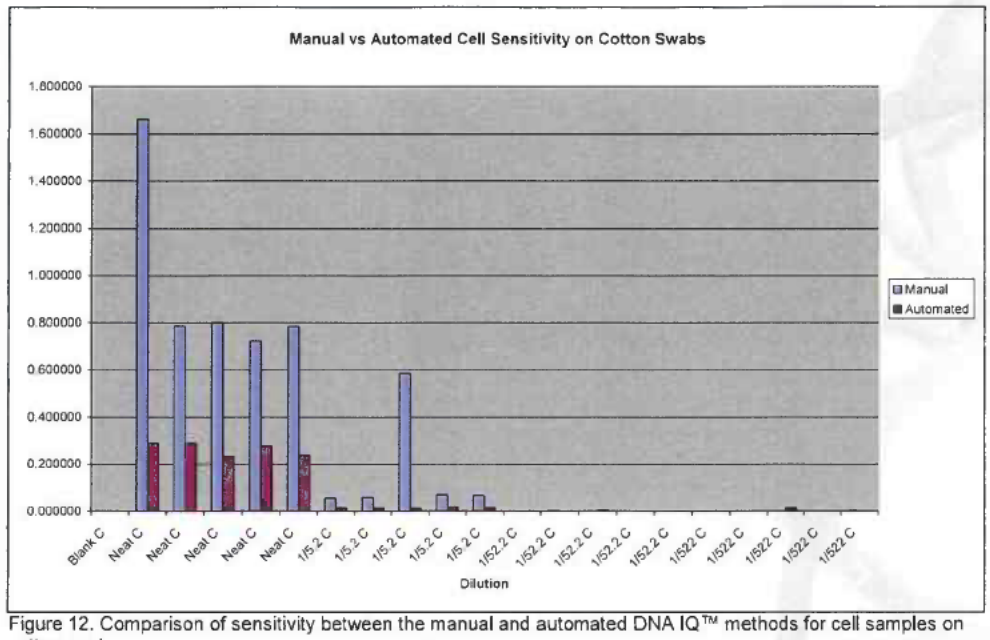

Figure 12. Comparison of sensitivity between the manual and automated DNA IQ™ methods for cell samples on cotton swabs.

6.5 Investigating resin volume

Promega recommends the use of 7µL of DNA IQ™ resin with their protocol. We investigated the performance of the protocol with double the amount of resin (14µL) in order to assess any benefits that may be gained in terms of the resulting yield and quality of the STR profile.

It was observed that doubling the resin resulted in a proportional doubling of the yield. On average, doubling the resin increased the yield by an additional 77.28% (*n*=4). The average yield from an extraction using 7µL of resin was 64.725ng (SD 32.21ng, *n*=4), whereas 14µL resin generated 114.75ng (SD 10.72ng, *n*=4) (Table 6). At the higher resin concentration, the amount of DNA isolated appears to be capped at around 100ng, indicating no change in the ability of the reaction to isolate more DNA due to saturation of resin.

Table 6. Comparison of the effects of doubling the amount of recommended DNA IQ™ resin.

Sample ID	Resin volume	[DNA] ng/µL	Reportable alleles
33383-4216	7µL	0.701	18/18
33383-4225		1.070	18/18
33383-4239		0.319	18/18
33383-4248		0.499	18/18
33383-4252	14µL	1.140	18/18
33383-4261		1.270	18/18
33383-4270		1.010	18/18
33383-4284		1.170	18/18

Samples extracted using either amount of resin generated concordant full DNA profiles (18/18 alleles). Samples processed using the 14µL method produced peaks that were slightly higher. The difference in peak heights between alleles within the same loci ranged from 59-86%, with a mean of 71%, indicating minimal difference between the two methods.

Doubling the amount of resin did not appear to provide any additional benefits compared to the original recommended protocol. More importantly, full DNA profiles were resolved using either method. Therefore, the costs associated with increasing the amount of resin cannot be justified at this stage.

6.6 **Modifying extraction volumes**

An investigation into optimising extraction volumes ranging from 300µL to 500µL was performed in order to ensure that buffer coverage over the samples was sufficient to enable optimal lysis and release of DNA. In addition, the use of an optimum volume of extraction reagents increases efficiency and economy, therefore potentially lowering laboratory costs.

Although the higher extraction volume generated higher yields when processed using the automated DNA IQ™ protocol (Table 7), DNA profile results were comparable across the various extraction volumes tested for eight replicates each (Table 8). Three instances of allelic imbalance were encountered in two samples from the 300µL and 450µL tests. In all instances, allelic imbalance was greater than 69%.

Table 7. DNA profile results for samples extracted using various volumes of Extraction Buffer, for 8 replicates.

Extraction Buffer Volume (µL)	Mean [DNA] (ng/µL)	SD
300	2.04	0.07
350	2.16	0.09
400	1.69	0.10
450	3.14	0.13
500	3.64	0.17

Table 8. DNA profile results for samples extracted using various volumes of Extraction Buffer, for 8 replicates.

Sample	Extraction Buffer Volume (µL)	DNA Profile Result
300-1 swab	300	OK
300-2 swab		OK
300-3 swab		OK
300-4 swab		OK
300-5 swab		OK
300-6 swab		OK
300-7 swab		AI D13
300-8 swab		OK

350-1 swab	350	OK
350-2 swab		OK
350-3 swab		OK
350-4 swab		OK
350-5 swab		OK
350-6 swab		OK
350-7 swab		OK
350-8 swab		OK
400-1 swab	400	OK
400-2 swab		OK
400-3 swab		OK
400-4 swab		OK
400-5 swab		OK
400-6 swab		OK
400-7 swab		OK
400-8 swab		OK
450-1 swab	450	OK
450-2 swab		OK
450-3 swab		OK
450-4 swab		OK
450-5 swab		OK
450-6 swab		OK
450-7 swab		AI vWA, D18
450-8 swab		OK
500-1 swab	500	OK
500-2 swab		OK
500-3 swab		OK
500-4 swab		OK
500-5 swab		OK
500-6 swab		OK
500-7 swab		OK
500-8 swab		OK

6.7 **Sensitivity of the automated DNA IQ™ protocol**

DNA was detected from samples that were diluted down to 1:1000 (Figure 13).

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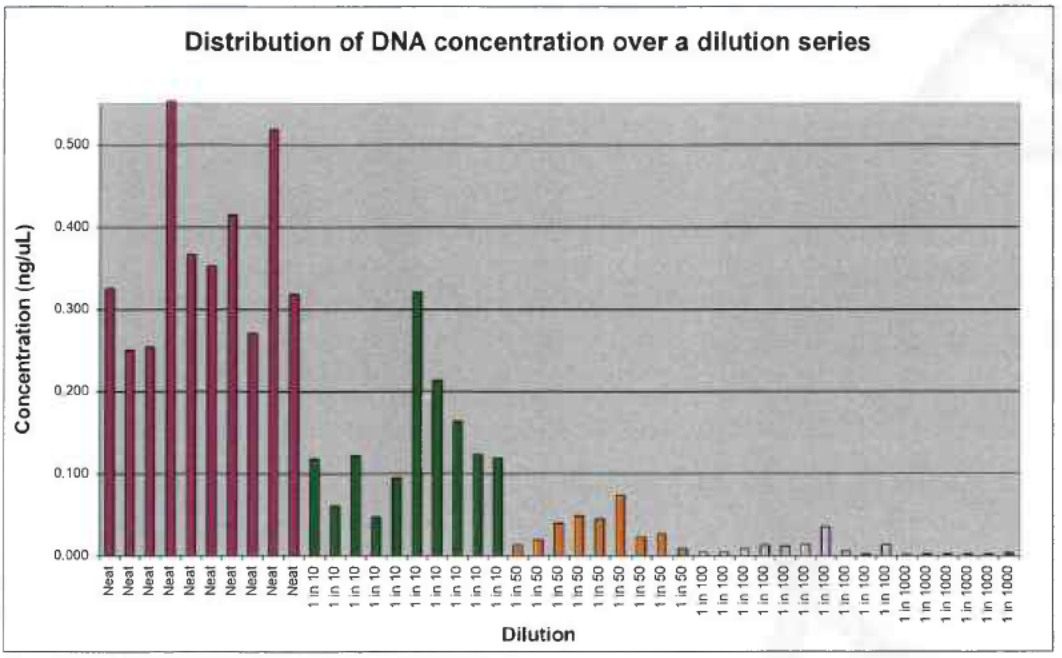

Figure 13. DNA IQ™ sensitivity across various dilutions

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7. Summary and Recommendations

We recommend the following:

- Use of MPII for automated extraction of reference samples
- Use of MPII for automated extraction of casework samples
- Ongoing development of the automated extraction program to increase the efficiency of the extraction

Sweeney, P.J. and Walker, J.M., Burrell, M.M., Enzymes of molecular biology. *Methods Mol. Biol.* Towanam NJ , (1993) **16**, 306