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

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

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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.

<u>For Cells</u>

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
1/4th CEP swab	250µL	100µL	350µL
15-50mm^2 S&S 903 paper	150µL	100µL	250µL
3-30mm^2 FTA® paper	150µL	100µL	250µL
Cloth up to 25mm^2	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

<u>Important points before starting</u>

- 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 salive 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 8 Trace	
		Concentration	Yield*	Concentration	Yield	Concentration	Yield	Concentration	Yield	Concentration	Yield	Concentration	Yield
Sample ID	Substrate type	ng/uL	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng
[redacted]	FTA	0.058800	11.877600	0.028700	2.870000	0.006030	0.271350	0.023900	3.585000	0.025700	2.621400	0.018200	1.820000
	Cotton swab	0.007410	1.111500	0.098000	9.800000	0.025800	1.161000	0.096700	14.505000	0.083300	16.826600	0.068900	6.890000
	Cotton cloth	0.001480	0.222000	0.050700	5.070000	0.004880	0.219600	0.014900	2.235000	0.037400	7.554800	0.071900	7.190000
	Denim cloth	0.002360	0.354000	0.028200	2.820000	0.002160	0.097200	0.003250	0.487500	0.041300	8.342600	0.043900	4.390000
	Rayon swab	0.001620	0.243000	0.010000	1.000000	0.000000	0.000000	0.011800	1.770000	0.024000	4.848000	0.031800	3.180000
	Rayon swab	0.001580	0.237000	0.019400	1.940000	0.005050	0.227250	0.018100	2.715000	0.019000	3.838000	0.115000	11.500000
	Rayon swab	0.000000	0.000000	0.015500	1.550000	0.006610	0.297450	0.027400	4.110000	0.011300	2.282600	0.057400	5.740000
	Rayon swab	0.000000	0.000000	0.011200	1.120000	0.007310	0.328950	0.005910	0.886500	0.019700	3.979400	0.029900	2.990000
		0.000800	*0.120000*	*0.014025*	*1.402500*	*0.004743*	*0.213413*	*0.015803*	*2.370375*	*0.018500*	*3.737000*	*0.058525*	*5.852500*
		0.000924	*0.138586*	*0.004291*	*0.429137*	*0.003300*	*0.148490*	*0.009195*	*1.379299*	*0.005285*	*1.067483*	*0.039683*	*3.968336*
	FTA	0.010300	2.080600	0.005790	0.579000	0.005270	0.237150	0.001260	0.189000	0.007510	0.766020	0.005710	0.571000
	Cotton swab	0.000756	0.113400	0.019000	1.900000	0.001480	0.066600	0.031600	4.740000	0.030900	6.241800	0.009500	0.950000
	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
	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
	Rayon swab	0.000558	0.083700	0.005740	0.574000	0.001800	0.081000	0.002860	0.429000	0.002950	0.595900	0.006760	0.676000
	Rayon swab	0.000000	0.000000	0.002560	0.256000	0.001300	0.058500	0.006150	0.922500	0.002020	0.408040	0.001220	0.122000
	Rayon swab	0.000898	0.134700	0.009750	0.975000	0.005570	0.250650	0.006560	0.984000	0.002340	0.472680	0.010200	1.020000
	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
		0.000472	*0.070838*	*0.004513*	*0.451250*	*0.002555*	*0.114975*	*0.004230*	*0.634500*	*0.002835*	*0.572670*	*0.008545*	*0.854500*
		0.000371	*0.055667*	*0.004208*	*0.420765*	*0.002020*	*0.090915*	*0.002536*	*0.380328*	*0.000885*	*0.178801*	*0.006196*	*0.619564*
	FTA	0.008170	1.650340	0.006410	0.641000	0.000000	0.000000	0.000000	0.000000	0.006310	0.643620	0.000000	0.000000
	Cotton swab	0.003710	0.556500	0.012100	1.210000	0.001680	0.075600	0.009130	1.369500	0.003970	0.801940	0.014900	1.490000
	Cotton cloth	0.002600	0.390000	0.010400	1.040000	0.000000	0.000000	0.000355	0.053250	0.005010	1.012020	0.006570	0.657000
	Denim cloth	0.000739	0.110850	0.007630	0.763000	0.015100	0.679500	0.000000	0.000000	0.007770	1.569540	0.000000	0.000000
	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
	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
	Rayon swab	0.000000	0.000000	0.001540	0.154000	0.000000	0.000000	0.003390	0.508500	0.000000	0.000000		
	Rayon swab	0.000739	0.110850	0.003050	0.305000	0.000000	0.000000	0.003360	0.504000	0.000000	0.000000		
		0.000185	*0.027713*	*0.001646*	*0.164550*	*0.000000*	*0.000000*	*0.001862*	*0.279263*	*0.001565*	*0.316130*	*0.010830*	*1.083000*
		0.000370	*0.055425*	*0.000971*	*0.097088*	*0.000000*	*0.000000*	*0.001770*	*0.265562*	*0.001807*	*0.365069*	*0.004200*	*0.420021*
	FTA	0.000000	0.000000	0.000935	0.093500	0.003940	0.177300	0.000000	0.000000	0.001840	0.187680	0.000000	0.000000
	Cotton swab	0.000000	0.000000	0.002900	0.290000	0.000000	0.000000	0.001520	0.228000	0.002280	0.460560	0.000000	0.000000
	Cotton cloth	0.000000	0.000000	0.005010	0.501000	0.001870	0.084150	0.000000	0.000000	0.000741	0.149682	0.000000	0.000000
	Denim cloth	0.000000	0.000000	0.002870	0.287000	0.000227	0.102150	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000
	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
	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
	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
	Rayon swab	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000000	0.000788	0.159176		
		0.000180	*0.027000*	*0.000737*	*0.073675*	*0.000000*	*0.000000*	*0.000910*	*0.136500*	*0.001303*	*0.263105*	*0.002253*	*0.225333*
		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	
[redacted]tion	Yield*	Concentration	Yield	Concentration	Yield	Concentration	Yield	Concentration	Yield	Concentration	Yield	
Sample ID	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng	ng/uL	ng	
[redacted]	355.5	0.482	48.2	2.31	103.95	0.751	112.65	0.00833	1.68266	1.16	116	
	213	0.078	7.8	3.58	161.1	0.754	113.1	0.0066	1.3332	2.61	261	
	76.8	0.356	35.6	3.32	149.4	0.929	139.35	0.0046	0.9292	1.61	161	
	140.1	0.467	46.7	2.46	110.7	0.916	137.4	0.00727	1.46854	2.18	218	
	196.3500	*0.3458*	*34.5750*	*2.9175*	*131.2875*	*0.8375*	*125.6250*	*0.0067*	*1.3534*	*1.8900*	*189.0000*	
	119.8085	*0.1871*	*18.7137*	*0.6270*	*28.2137*	*0.0983*	*14.7451*	*0.0016*	*0.3173*	*0.6361*	*63.6082*	
	32.85	0.238	23.8	0.227	10.215	0.219	32.85	0.00211	0.42622	0.611	61.1	
	12.675	0.198	19.8	1.72	77.4	0.101	15.15	0.000597	0.120594	0.3	30	
	32.4	0.195	19.5	4.59	206.55	0.0673	10.095	0.00128	0.25856	0.251	25.1	
	24.75	0.136	13.6	0.657	29.565	0.0787	11.805	0.00166	0.33532	0.227	22.7	
	25.6688	*0.1918*	*19.1750*	*1.7985*	*80.9325*	*0.1165*	*17.4750*	*0.0014*	*0.2852*	*0.3473*	*34.7250*	
	9.4262	*0.0420*	*4.2019*	*1.9639*	*88.3776*	*0.0698*	*10.4628*	*0.0006*	*0.1294*	*0.1784*	*17.8438*	
	1032	0.0554	5.54	0.0936	4.212	0.094	14.1	0.0126	2.5452	0.154	15.4	
	24.6	0.114	11.4	0.175	7.875	0.0735	11.025	0.00174	0.35148	0.148	14.8	
	42.9	0.145	14.5	0.123	5.535	0.0521	7.815	0.00363	0.73326	0.178	17.8	
	76.95	0.125	12.5	0.0151	0.6795	0.0939	14.085	0.00167	0.33734	0.0819	8.19	
	294.1125	*0.1099*	*10.9850*	*0.1017*	*4.5754*	*0.0784*	*11.7563*	*0.0049*	*0.9918*	*0.1405*	*14.0475*	
	492.4030	*0.0385*	*3.8501*	*0.0668*	*3.0066*	*0.0200*	*2.9991*	*0.0052*	*1.0517*	*0.0411*	*4.1145*	
	6.075	0.0792	7.92	0.0349	1.5705	0.0347	5.205	0.00757	1.52914	0.0766	7.66	
	1.56	0.0566	5.66	0.0454	2.043	0.027	4.05	0.00667	1.34734	0.0923	9.23	
	5.055	0.0847	8.47	0.0386	1.737	0.0197	2.955	0.00544	1.09888	0.0588	5.88	
	4.845	0.109	10.9	0.0276	1.242	0.021	3.15	0.00245	0.4949	0.874	87.4	
	4.3838	*0.0824*	*8.2375*	*0.0366*	*1.6481*	*0.0256*	*3.8400*	*0.0055*	*1.1176*	*0.2754*	*27.5425*	
	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+16	X,X+18	X,X+18
			R15	AI@D13(68%)		
			R16	X,X+18		
			R17	X,X+18		
Dil 1/4	X,X+17	X,X+18	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+18	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+18	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
	(AI@D18)		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(AI@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(AI@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
	AI@FGA		R15	X,X+15		
	AI@D13		R16	X,X+16		
			R17	X,X+8		
Dil 1/4	X,X+9	X,X+18	R10	X,X+NR's	NRNR+2	NSD
		AI@D13	R11	NR/NSD		
			R12	X,NR+2		
			R13	X,NR+NSD		
Dil 1/8	NR/NSD	X,X+14	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. f*orensic*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+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: forensicGEM**

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

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	AI@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(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+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.

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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 f[redacted]ples	2hr	3hr	5hr	3.5hr	1.5hr	5hr
Washing steps in[redacted]remove inhibitors	No	Yes	Yes	Yes	No	Yes
Final extract volu[redacted]	~150	100	45	150	100 for FTA, 200 for other samples	100
% zero quantitati[redacted]or cells	43.750	9.375	37.500	31.250	9.375	24.140
% zero quantitati[redacted]or blood	0.000	0.000	0.000	0.000	0.000	0.000
Cell substrate dis[redacted]ghest quant value for neat cell samples	FTA	Cotton swab	Cotton swab	Cotton swab	Cotton swab	Cotton cloth
Total number of r[redacted]alleles for cells (max 576)	89	282	86	138	209	202
Total number of r[redacted]alleles for blood (max 288)	234	252	284	288	25	264
Total number of a[redacted]loci exhibiting allelic imbalance (max 432)	1	1*	3	5	1	6^
Neat cell samples[redacted] showed inhibition (no profile)	Yes	No	Yes	Yes	No	No
Neat blood sampl[redacted]n swabs showed inhibition (no profile)	No	No	No	No	Yes	No
Amenable to autc[redacted]	No	Yes	Yes	Yes	Yes	Yes
Validated MultiPR[redacted]US automated protocol	No	Yes	No	No	No	No

* Five occurrences [redacted]balance were observed in Amelogenin.
^ One occurrence o[redacted]alance 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.

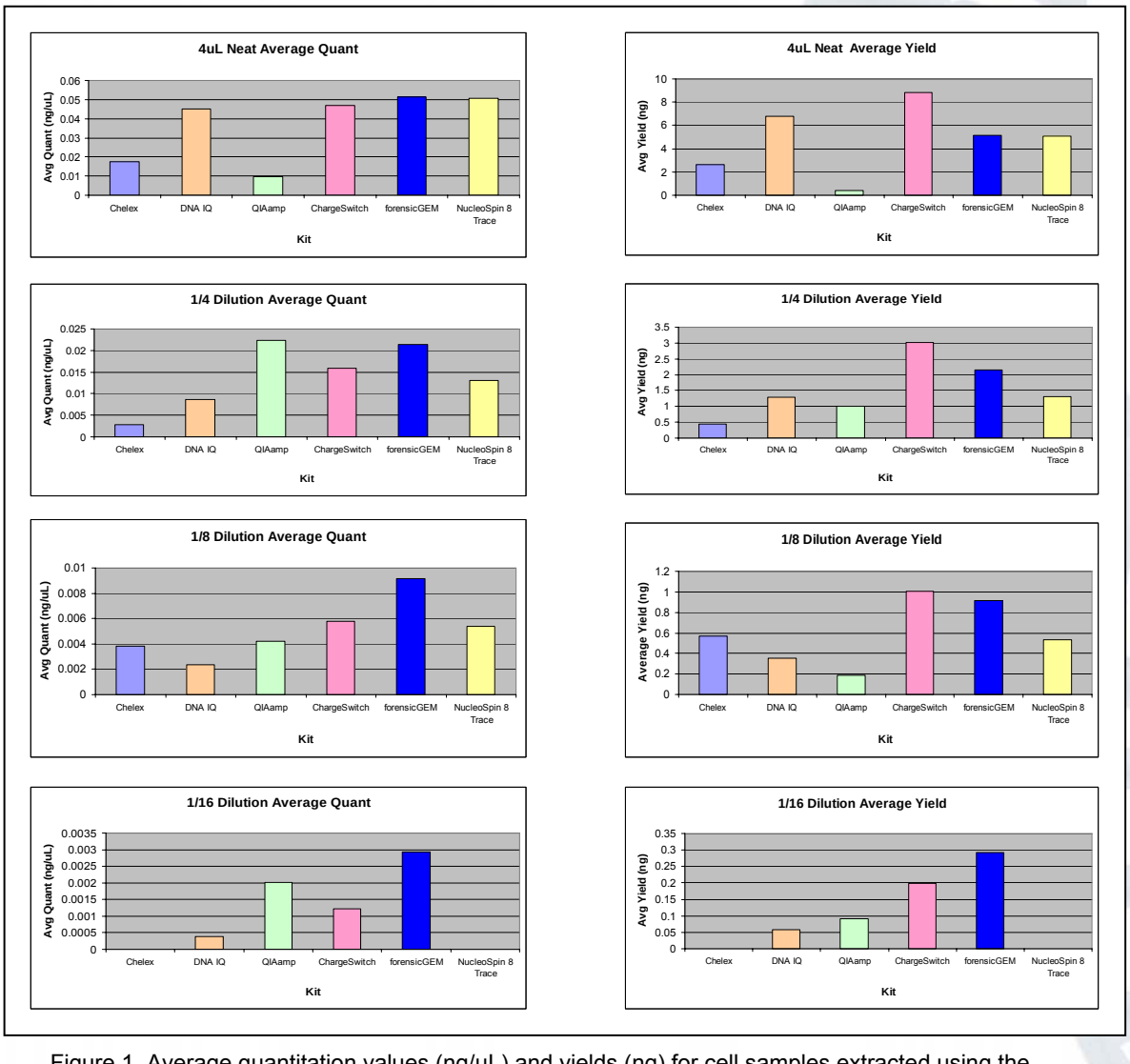

Figure 1. Average quantitation values (ng/µL) and yields (ng) for cell samples extracted using the various extraction chemistries tested, compared to Chelex®.

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 9% 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.

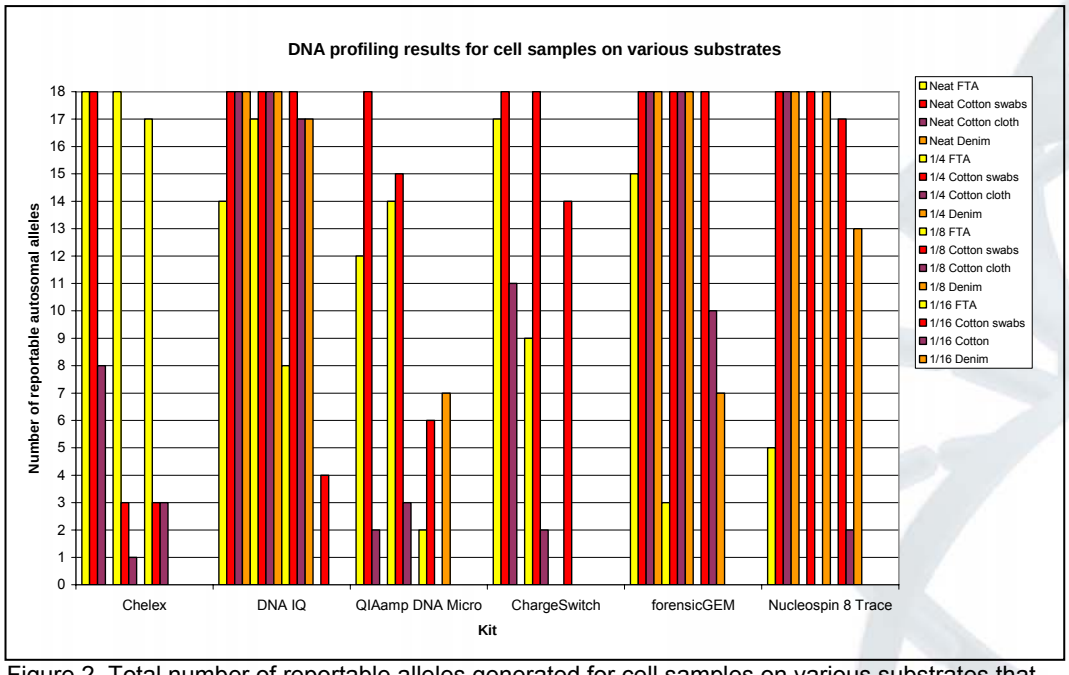

Figure 2. Total number of reportable alleles generated for cell samples on various substrates that were extracted using the various extraction chemistries tested, compared to Chelex®. The kit displaying the most number of full bars (i.e. most full profiles) was found to be DNA IQ™, indicating the superior 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.

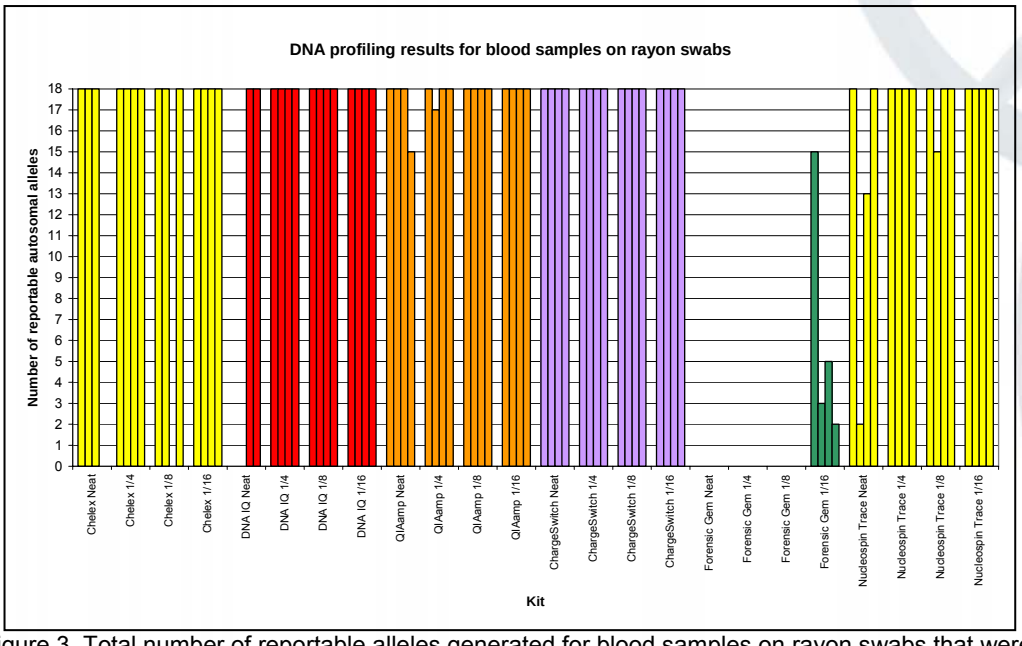

Figure 3. Total number of reportable alleles generated for blood samples on rayon swabs that were extracted using the various extraction chemistries tested, compared to Chelex®. All kits were able to resolve profiles from most dilutions, except *forensic*GEM™ which could only resolve alleles from the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

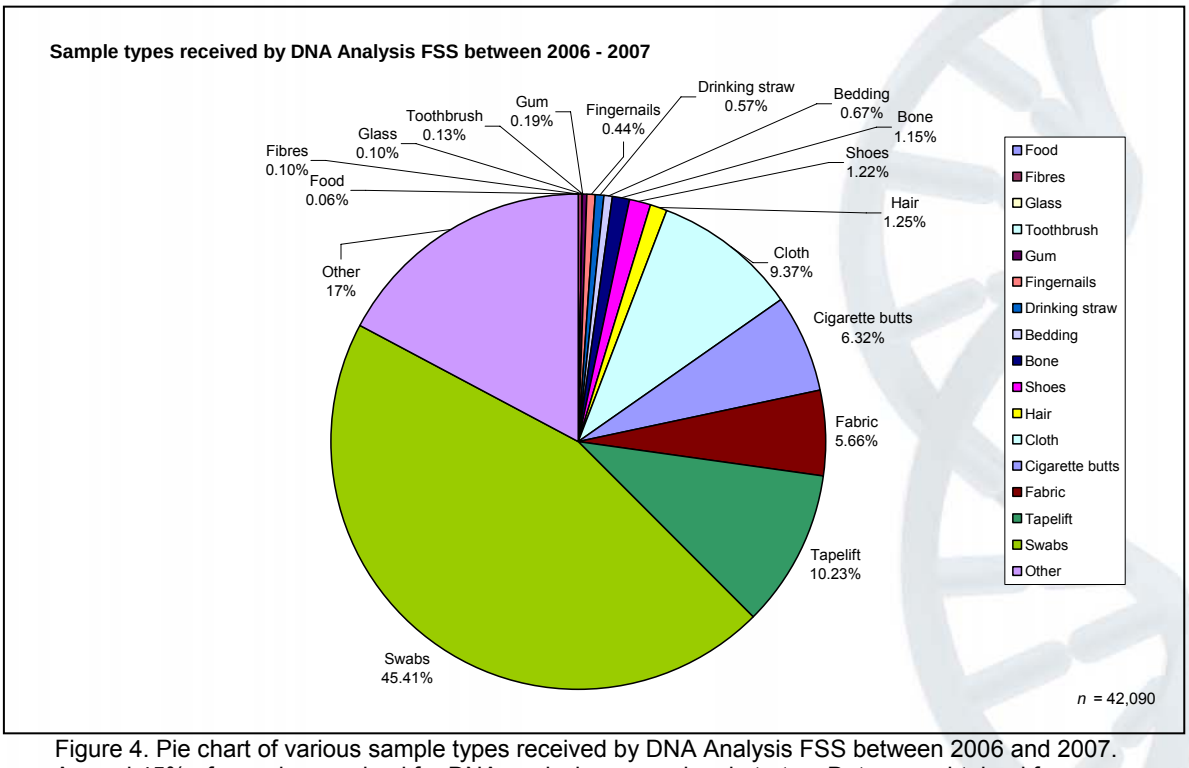

Figure 4. Pie chart of various sample types received by DNA Analysis FSS between 2006 and 2007. Around 45% of samples received for DNA analysis are swab substrates. Data was obtained from AUSLAB on 14 November 2007.

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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