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Project 22. A Modified DNA IQ™ Method for Off-Deck Lysis Prior to Performing Automated DNA Extraction

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1. Abstract

Some forensic sample types that were submitted for DNA extraction using the current in-house automated DNA IQ™ protocol could not be efficiently processed due to varying sample sizes, substrate material type and the requirement for retaining supernatant for presumptive screening. An off-deck lysis protocol was investigated as an option to automated lysis on the MultiPROBE® II PLUS HT EX platform, and includes the option to retain supernatant for presumptive testing (see Project 21).

Further testing was performed to investigate the amount of time that samples can be stored in Extraction Buffer after off-deck lysis, prior to presenting the sample lysate to the MultiPROBE® II PLUS HT EX platforms. A comparison of off-deck lysis performed on the Eppendorf Thermomixer Comfort and Ratek hot blocks was also performed.

We found that samples where off-deck lysis was performed could be stored in the fridge for up to 4 days, or frozen, prior to automated DNA IQ™ extraction. Performing off-deck lysis on the hot blocks produced results that were comparable to samples that were incubated on the Thermomixer.

2. Aim

- To test the effect of storage in Extraction Buffer, in the fridge or freezer, for samples where off-deck lysis has been performed.
- To investigate any differences between performing off-deck lysis on the Eppendorf Thermomixer Comfort or Ratek hot blocks.

3. Equipment and Materials

- DNA IQ™ System (Promega Corp., Madison, WI, USA)
- DNA IQ™ Spin Baskets (Promega Corp., Madison, WI, USA)
- TNE buffer (10mM Tris, 100mM NaCl, 1mM EDTA, pH 8.0)
- Proteinase K (20ng/µL)
- 20% w/v SDS
- Thermomixer Comfort (Eppendorf)
- Hot blocks (Ratek)
- Eppendorf 5415C centrifuge
- Rayon swabs (Copan)
- Buccal cell suspension (donor CJA), collected as described previously
- Whole blood (donor VKI), collected as described previously

4. Methods

4.1 Off-deck lysis: storage of lysate prior to automated DNA IQ™ extraction

4.1.1. Sample creation for testing storage time

A total of 25 fresh cell swabs and 25 blood swabs were created. For cell samples, 10µL of donor buccal cell suspension was spotted on to pre-cut rayon swab heads in 1.5mL tubes. For blood samples, 30µL of donor whole blood was spotted onto pre-cut rayon swab heads in 1.5mL tubes. Both sample types were dried on a Thermomixer at 56°C for 2 hours.

In addition to the fresh samples, 25 blood QC swabs were included in the experiments.

4.1.2. Off-deck lysis protocol

Samples that were created for testing were divided equally into 5 sets for testing over 4 days. The off-deck lysis protocol for all samples was identical, except for the storage conditions and storage time for samples after off-deck lysis was performed (Table 1). 500µL of Extraction Buffer was added to each tube. For spiked samples, 30µL of 1M urea was added to each fresh blood sample on rayon swab. Each tube was vortexed, before incubating at 37°C on a Thermomixer Comfort (Eppendorf) at 1000 rpm for 45 minutes. The sample substrate material was transferred to a DNA IQ™ Spin Basket and centrifuged for 2 minutes at room temperature at maximum speed (15800g). The centrifuged lysate was combined with the lysate in the original 1.5mL tube. The samples were incubated at 65°C on a Thermomixer Comfort (Eppendorf) at 1100 rpm for 10 minutes. Samples were then either stored in the fridge or freezer (Table 1) until automated DNA IQ™ extraction was performed up to 4 days later.

Table 1. Storage conditions and storage time for all 5 sets of samples.

Day	Set Number	$T_{storage}$ (°C)	Days of storage prior to extraction
Day 1	Set 1	4	4
	Set 2	-20	4
Day 2	Set 3	4	3
Day 3	Set 4	4	2
Day 4	Set 5	4	1

4.2 Off-deck lysis: assessing differences between hot blocks and Thermomixer

4.2.1. Sample creation for hot block testing

A total of 15 samples each of blood on cotton swab and blood on rayon swab, and 10 of cells on rayon swab were created. Swab heads were cut

off the swab shafts and placed in sterile 1.5mL tubes. For blood samples, 10µL of donor whole blood was spotted on to small cotton swabs and 30µL was spotted on to rayon swabs. For cell samples, 10µL of donor cell suspension was spotted on to rayon swabs. Samples were dried on a hot block (Ratek) at 56°C for 2 hours.

4.2.2. Off-deck lysis protocol

Samples that were created for testing were divided equally into 3 sets, and processed using a similar off-deck lysis protocol, except for minor differences where a hot block (Ratek) or Thermomixer Comfort (Eppendorf) was used, and which vortexing routine was performed (see Table 2). 500µL of Extraction Buffer was added to each tube. Each tube was vortexed, before incubating at 37°C for 45 minutes either on a hot block (Ratek) or Thermomixer Comfort (Eppendorf) set at 1000 rpm. The sample substrate material was transferred to a DNA IQ™ Spin Basket and centrifuged for 2 minutes at room temperature at maximum speed (15800g). The centrifuged lysate was combined with the lysate in the original 1.5mL tube. The samples were then incubated at 65°C for 10 minutes either on a hot block (Ratek) or Thermomixer Comfort (Eppendorf) at 1100 rpm. Samples were stored in the freezer until automated DNA IQ™ extraction was performed.

Table 2. Incubation protocols for the 3 sets of samples.

Sample Set Number	Incubation protocol
Set 1	On hot block; vortex 5 sec before and 5 sec after incubation.
Set 2	On hot block; vortex 5 sec before and after incubation, and 5 sec during incubation.
Set 3	On Thermomixer, shaking at 1100 rpm.

4.3 Automated DNA IQ™ protocol

Sample lysates from the fridge or freezer were allowed to thaw or come to room temperature, then added into a Slicprep™ 96 Device (without basket) using the STORstar instrument. Automated DNA IQ™ was then performed (without the automated addition of Extraction Buffer).

4.4 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.

4.5 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.

4.6 Capillary electrophoresis and fragment analysis

PCR product was prepared for capillary electrophoresis using the manual 9+1 protocol as per QIS 19978. Capillary electrophoresis was performed on an ABI Prism® 3130 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® 3130 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%.

5. Results and Discussion

5.1 Testing the effect of storage condition and storage time of samples that have undergone off-deck lysis prior to automated DNA IQ™

Off-deck lysis was performed on five sets of samples across 5 days, with 2 sets being processed on Day 1, and 1 set processed every day for Day 2, 3 and 4 (see Table 1). One of the sample sets processed on Day 1 was stored in the freezer (-20°C) after off-deck lysis was completed, while all other sets were stored in the fridge (4°C). This resulted in a series of replicate samples that were processed using the same off-deck lysis protocol, but stored in the fridge or freezer for varying lengths of time, up to 4 days. Sample lysates were thawed or allowed to come to room temperature on Day 5, and the automated DNA IQ™ extraction protocol was performed (with the Extraction Buffer addition step removed).

DNA quantitation results for all samples, as determined by the Quantifiler™ system, are outlined in Table 3 below.

Table 3. DNA quantitation results (ng/µL) for samples stored for varying lengths of time in Extraction Buffer, prior to automated DNA IQ™ extraction.

Samples	Number of days in storage				
	1 (Fridge)	2 (Fridge)	3 (Fridge)	4 (Fridge)	4 (Frozen)
NegCtl	*0.0000*	*0.0005*	*0.0006*	*0.0008*	*0.0007*
Blood QC	1.1100	1.7400	1.7200	1.7500	1.2500
Blood QC	0.8140	0.9930	1.4500	1.5700	1.3900
Blood QC	0.2390	0.9240	1.1000	1.5500	1.2200
Blood QC	1.0500	0.9280	0.9490	1.6200	1.0400
Blood QC	1.0400	1.4300	1.4300	1.2600	1.0500
Mean Blood QC	*0.8506*	*1.2030*	*1.3298*	*1.5500*	*1.1900*
Std Dev	*0.36*	*0.37*	*0.31*	*0.18*	*0.15*
Blood Urea	1.0600	1.5900	1.2000	1.0400	1.6000
Blood Urea	1.2700	0.9580	1.5900	0.9770	1.2000
Blood Urea	1.7600	1.7300	1.3400	0.8540	1.0600
Blood Urea	1.1400	1.2900	1.5000	1.4200	1.4300
Blood Urea	1.0900	1.1200	0.8840	1.0700	1.1400
Mean Blood Urea	*1.2640*	*1.3376*	*1.3028*	*1.0722*	*1.2860*
Std Dev	*0.29*	*0.32*	*0.28*	*0.21*	*0.22*
Cells	0.4930	0.1780	0.1060	0.3510	0.1330
Cells	0.3750	0.1140	0.2690	0.3520	0.2070
Cells	0.0999	0.1710	0.1040	0.3380	0.3530
Cells	0.1770	0.2060	0.1960	0.4530	0.2020
Cells	0.1010	0.1930	0.3030	0.3220	0.3360
Mean Cells	*0.2492*	*0.1724*	*0.1956*	*0.3632*	*0.2462*
Std Dev	*0.18*	*0.04*	*0.09*	*0.05*	*0.09*

The combined mean quantitation results for blood QC samples, blood samples spiked with urea and cell samples that were stored in Extraction Buffer across four days of storage (in fridge *or* freezer) were 1.2247, 1.2525 and 0.2453ng/µL respectively. On average, the absolute difference in individual mean results for each set was only 0.0978ng/µL (between 5.7-19.9%) away from the combined mean quantitation result. This indicates little variation in quantitation results between samples that had remained in storage for 1, 2, 3 or 4 days, either in the fridge or freezer.

DNA profiling results (Table 4) also do not indicate any variation between results for samples that had remained in storage over varying lengths of time. All samples generated full profile (18/18) results. Samples spiked with urea to mimic inhibition generated similar results to un-spiked samples, indicating no change in the ability of DNA IQ™ to remove inhibitors, but also no damage to the lysate material while it remained in storage.

Table 4. Number of reportable alleles (out of 18) for samples that were stored for varying lengths of time in Extraction Buffer, prior to automated DNA IQ™ extraction.

Samples	Number of days in storage				
	1 (Fridge)	2 (Fridge)	3 (Fridge)	4 (Fridge)	4 (Frozen)
NegCtl	0	0	0	0	0
Blood QC	18	18	18	18	18†
Blood QC	18	18	18	18	18
Blood QC	18†	18‡	18	18	18
Blood QC	18	18¥	18	18	18
Blood QC	18	18	18	18†	18
Blood Urea	18	18	18	18	18
Blood Urea	18	18	18	18	18
Blood Urea	18	18	18†	18†	18
Blood Urea	18	18	18	18	18
Blood Urea	18†	18	18	18	18
Cells	18	18	18	18	18
Cells	18	18	18¥	18	18
Cells	18	N*	18	18	18
Cells	18	18	18	18†	18
Cells	18	18†	18	18	18

†Allelic imbalance greater than 65% at one locus
‡ Allelic imbalance less than 64.9% but greater than 60% at one locus
¥Allelic imbalance less than 59.9% at one or more loci
*PP and AI with ULP due to bad injection

When compared to results for extraction positive controls (QC blood swabs) that were extracted since January 2008 as part of routine laboratory processes, the positive controls that were included in these series of experiments generated higher quantitation values but similar DNA profile results (Table 5). The off-deck positive controls produced an average DNA concentration of 1.22ng/µL (SD 0.35), compared to 0.27ng/µL (SD 0.12) for routine QC blood swabs, i.e. the concentration of off-deck controls was over 4-fold greater than controls extracted using the current protocol. Positive controls that were extracted using the off-deck method displayed more allelic imbalance compared to routine positive controls, i.e. 20% (5/25) compared to 9% (3/34). Four out of the five occurrences of allelic imbalance in off-deck controls were one locus events with a peak height ratio greater than 60%, and therefore pass the in-house acceptance criteria for extraction positive controls.

Table 5. Comparison of results for extraction positive controls (QC blood swabs) extracted using the off-deck lysis protocol or the current automated method.

	n	Mean $_{[DNA]}$ (ng/µL)	*SD*	% OK Profile	% AI Profile	% Pass QC
Off-Deck Lysis	25	1.22	0.35	80	20	96
Routine Work	34	0.27	0.12	88*	9	97

* One sample was involved in an OQI and excluded from this data pool.

Based on the results in this study, it appears that storage of extracts in extraction buffer for a few days rather than a single day does not affect the quality of the DNA for subsequent use in quantitation and DNA profiling, and the results are comparable to the current method.

5.2 Comparison between hot block and Thermomixer for performing off-deck lysis

For the comparison between hot blocks and Thermomixer, off-deck lysis was performed on a hot block for two sample sets, and a third set was incubated on the Thermomixer. For sets 1 and 2 where the hot block was used, the minor difference between the two sets is a 5 second vortex *during* incubation for Set 2 samples (see Table 2).

Table 6. Quantitation results (ng/µL) for a comparison between off-deck lysis performed on a hot block or a Thermomixer.

Sample type	Set 1 Hot block (vortex before and after)	Set 2 Hot block (vortex before, after, during)	Set 3 Thermomixer (1100 rpm)
10µL blood, cotton swab	0.1060	0.0279	0.1260
	0.0750	0.0871	0.3210
	0.0568	0.0209	0.1240
	0.1020	0.0788	0.3110
	0.0641	0.0267	0.1710
Mean	*0.0808*	*0.0483*	*0.2106*
30µL blood, rayon swab	0.8630	0.6760	0.6850
	0.8440	0.0851	0.7900
	0.7920	0.5060	1.5400
	0.5340	0.4580	0.4530
	0.6430	0.6240	0.3990
Mean	*0.7352*	*0.4698*	*0.7734*
10µL buccal cells, rayon swab	0.0135	0.0102	0.0036
	0.0127	0.0006	0.0016
	0.0023	0.0126	0.0016
	0.0022	0.0027	0.0000
	0.0042	0.0031	0.0007
	0.0083	0.0040	0.0021
	0.0042	0.0022	0.0000
	0.0000	0.0000	0.0000
	0.0079	0.0037	0.0000
	0.0038	0.0007	0.0000
Mean	*0.0059*	*0.0040*	*0.0010*

Quantitation results indicate that all three sets generated similar results, except Set 2 where the mean results were generally lower (Table 6). The vortexing of samples during incubation may have resulted in temperature variations that affected lysis efficiency. Vortexing before and after incubation on a hot block produced similar results to performing lysis incubation on the Thermomixer. For buccal cell samples, however, the Thermomixer samples (Set 3) produced more undetermined results (0ng/µL) compared to the hot block samples (Set 1), but most results were generally low and close to the validated LOD of 0.00426ng/µL and therefore indistinguishable from the background (Hlinka *et al.*, 2006).

For blood samples, either on cotton or rayon swabs, all sample sets produced full (18/18) DNA profiles (data not shown). The results for cell samples were slightly varied and were mostly non-reportable or NSD, as suggested by the low DNA quantitation results (Table 6). Out of 180 possible alleles (excluding Amelogenin) for buccal cell samples, Set 1 samples yielded 12 allele calls and 52 non-reportable alleles, compared to Set 3 that produced 0 allele calls and 14 non-reportable alleles (Table 7). In comparison, Set 2 samples generated only 1 allele call and 39 reportable alleles (Table 7).

Table 7. Number of reportable and non-reportable alleles generated for samples incubated on a hot block and Thermomixer.

	Alleles	NR's
Set 1 (Hot block, vortex before and after)	12	52
Set 2 (Hot block, vortex before, after and during)	1	39
Set 3 (Thermomixer, 1100 rpm)	0	14

Results indicate that performing off-deck lysis on a hot block does not generate results that are worse than samples that were incubated on the Thermomixer. Performing a 5 second vortex before and after incubation appears to be the best method for mixing samples that are incubated on the hot block.

6. Summary and Recommendations

Samples that were lysed off-deck were able to be stored either in the fridge or freezer for up to 4 days without affecting the possibility of obtaining DNA profiles, suggesting stability of the lysate in Extraction Buffer containing inactivated Proteinase K. Furthermore, off-deck lysis was able to be performed using either the Ratek hot blocks or the Eppendorf Thermomixer Comfort without producing significant variation in results.

We recommend:

1. Off-deck lysis using Extraction Buffer can be performed on either a hot block or a Thermomixer, prior to automated DNA IQ™ extraction (without the automated addition of Extraction Buffer). If performed on a hot block, vortexing the sample before and after incubation for 5 seconds each is an adequate substitute for continuous shaking.
2. Samples that have had off-deck lysis performed can be stored either in the fridge or freezer for up to 4 days.

3. Off-deck lysis can be used to processes a greater variety of samples types (e.g. tapelifts) using the DNA IQ™ system.

7. Acknowledgements

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8. References

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