Optimization and Validation of the forensicGEM® Rapid Extraction Method for High-Throughput Processing of Cotton Buccal Swabs

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AFDIL
Armed Forces DNA Identification Laboratory

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ABSTRACT

Most extraction methods are time-consuming and have a high risk for contamination due to numerous steps involved in the purification of the DNA. ForensicGEM® (ZyGEM NZ Ltd., New Zealand) chemistry is rapid, compatible with downstream processing methods, adaptable to tubes or plates, amenable to automation, and inexpensive. The ability to do a single step extraction without purification will facilitate the rapid, low-cost HTP processing of cotton buccal swabs.

For use at the Armed Forces DNA Identification Laboratory (AFDIL), the forensicGEM® Storage Card (Saliva) kit was optimized and validated for high-throughput (HTP) processing of cotton buccal swabs. The optimization experiments examined cell elution, eluate input volume, and extraction buffer volume. To further improve DNA yield, the eluate input volume and extraction buffer volume were also investigated.

INTRODUCTION

Currently at the AFDIL, DNA IQ™ is used for the HTP processing of cotton buccal swabs. This processing method is dependable but also expensive and time-consuming. Bode buccal collectors have recently been used for family reference sample collection but a high reprocessing rate requires a switch to cotton buccal swabs. A more efficient extraction method is needed to fulfill the needs of the AFDIL.

Recently, single-tube rapid extraction methods have been developed that lyse cells and tolerate inhibitors. Three of these methods were evaluated and forensicGEM® was chosen because it was the most compatible with the needs of the laboratory, as well as inexpensive and comparable to current extraction methods. The forensicGEM® method uses a proteinase from the thermophilic *Bacillus* sp. EA1 to extract DNA without the need for purification [1, 2]. This optimal method uses a short two-step incubation during which the proteinase lyses the cells and degrades the proteins and nucleases in a buffer compatible with PCR. The single stranded extract is transferred directly into the amplification reaction. Along with being compatible with multiple STR kits and mitochondrial DNA sequencing, forensicGEM® allows for stable, long-term storage and has enough volume for many amplification reactions. This method can also be used with automation, which makes it an optimal method to reduce the time needed to process family reference samples.

MATERIALS AND METHODS

forensicGEM® Optimization

The forensicGEM® optimization consisted of five separate experiments to develop a protocol most suitable for the processing of family reference samples and purposes of the AFDIL.

Experiment A

Experiment A evaluated rapid extraction buffers. Prep-n-Go Buffer (Applied Biosystems, Carlsbad, CA), SwabSolution Kit (Promega, Madison, WI), and forensicGEM® Saliva Kit were evaluated because they exhibited the most potential for the AFDIL's needs. Each extraction method includes the addition of buffer to a cotton buccal swab followed by a short incubation.

Experiment B

Experiment B evaluated the impact of cellular input by observing the influence of agitation time and eluate input volume on the amount of DNA yielded from the extraction. Previously, agitation was 5 seconds, thus longer agitation times and increased eluate inputs were tested.

Extraction Condition	A	B	C	D	E	F
Agitation Time	5 sec	5 sec	60 sec	60 sec	120 sec	120 sec
Eluate Volume	20 µL	40 µL	20 µL	40 µL	20 µL	40 µL

Table 1. Experiment B extraction conditions - the same eluate was used with all conditions.

Experiment C

Experiment C evaluated the five forensicGEM® buffers that are optimized for different substrates for their functionality with cotton buccal swabs. These buffers were evaluated to determine the most optimal buffer and if a single buffer would be suitable for the extraction of all substrate types.

Extraction Condition	A	B	C	D	E
Buffer (Kit)	Blue (Saliva)	Sky (Saliva Storage Card)	Magenta (Blood Storage Card)	Red (Blood)	Red Plus (Blood - Enhanced)

Table 2. Experiment C extraction conditions – the same eluate was used with all conditions.

Experiment D

Experiment D evaluated elution with plate agitation for the purpose of manual HTP processing. Two agitation speeds (900 RPM and 2000 RPM) were tested on the ThermoMixer C to determine the optimal DNA yield.

Experiment E

Experiment E evaluated the eluate input and buffer volumes when performing elution on a plate for the purpose of manual HTP processing. Various buffer volumes were evaluated with different amounts of eluate input to maximize DNA yield.

	Condition A	Condition B	Condition C	Condition D	Condition E
SKY buffer (µL)	11	25	10	17.5	10
ForensicGEM Enzyme (µL)	1	1	1	1	1
dH_2O (µL)	-	-	19	11.5	49
Eluate input (µL)	100	100	70	70	40
Total Rxn Volume (µL)	112	126	100	100	100

Table 3. Experiment E extraction conditions. Conditions B and D evaluated increased buffer volumes.

Validation

forensicGEM® Validation

Validation studies were performed for the optimized manual and manual HTP protocols using the recommended SWGDAM Internal Validation Guidelines. Sensitivity was evaluated with the manual protocol only. Concordance, precision, accuracy, and contamination were evaluated with both protocols. Manual protocol processed 24 cotton buccal swabs, whereas manual HTP protocol processed 90 cotton buccal swabs.

Manual Protocol

1. Prepare master mix – 49 µL dH_2O, 10 µL SKY Blue Buffer, 1 µL forensicGEM® enzyme
2. Add 60 µl master mix to a 96 well plate (extraction plate).
3. Cut whole swab head into 1.7 mL tube.
4. Add 400 µL dH_2O to swab head and vortex at high speed for 60 seconds.
5. Add 40 µL of eluate to the extraction plate.
6. Perform thermal cycling: 75° C for 5 minutes, 95° C for 5 minutes, 4° C hold

HTP Protocol

1. Prepare master mix – 19 µL dH_2O, 10 µL SKY Blue Buffer, 1 µL forensicGEM® enzyme
2. Add 30 µl master mix to a 96 well plate (extraction plate).
3. Cut whole swab head into deep square well plate
4. Add 400 µL dH_2O to swab head and agitate on Thermomixer C at 2000 RPM for 5 minutes. Centrifuge plate.
5. Add 70 µL of eluate to the extraction plate.
6. Perform thermal cycling: 75° C for 5 minutes, 95° C for 5 minutes, 4° C hold

RESULTS

Experiment A: Evaluation of rapid extraction buffers

	Prep-n-Go (N=10)	SwabSolution (N=10)	forensicGEM (N=10)	DNA IQ (N=10)
Incubation Time	20 minutes	30 minutes	10-20 minutes	1 - 1.5 hours
QuantDuo Results				
Average Human Quant	Undetermined (1:5 dilution = 3.15 ng/µL)	0.37 ng/µL	0.31 ng/µL	2.76 ng/µL
IPC Values	Undetermined	Slightly elevated	Normal	Normal
STR Results (full profiles)				
Yfiler Results	1/6	6/6	6/6	6/6
PP16HS Results	4/10	9/10	7/10	10/10
ID+ Results	10/10	10/10	8/10	10/10
mtDNA Results				
CR Amplicon Concentration	1.21 ng/µL	6.14 ng/µL	6.23 ng/µL	9.40 ng/µL
Full Sequence Data	8/10	10/10	8/10	10/10
Average RFUs	731	1824	1505	2151
Comments	Severe inhibition	Imbalanced profiles	The same donor (processed in duplicate) failed across all amps	
Cost/sample (estimate)	$5	$8-10	$1.50	$1.75

Table 4. Comparison of time, success, and cost of rapid extraction methods and the DNA IQ extraction method.

Experiment B: Evaluation of cellular input

	20 µL	40 µL
5 seconds vs. 60 seconds	722%	906%
5 seconds vs. 120 seconds	773%	2395%

Table 5. Agitation time comparison - average percent change of the extract concentration within samples. Green is significantly different ($p<0.05$).

Experiment C: Evaluation of forensicGEM® buffer

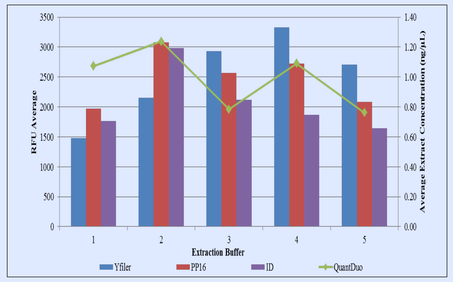


Figure 1. Yfiler, PP16, and ID RFU averages along with the average extract concentration values for comparison.

Experiment D: Evaluation of plate elution

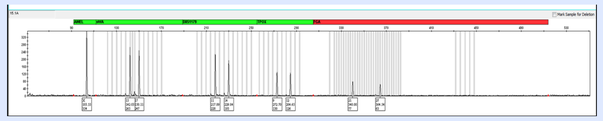
	900 RPM	2000 RPM
Yfiler (1/2 rxn)	46% (41/90)	79% (71/90)
PP16 (1/2 rxn)	86% (77/90)	94% (85/90)

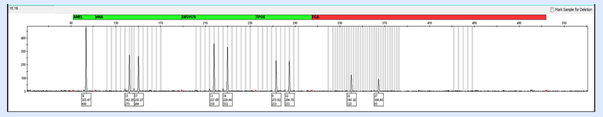
Table 6. Yfiler and PP16 success rate at the two agitation conditions

Experiment E: Evaluation of eluate input and buffer volumes

	Condition A	Condition B	Condition C	Condition D	Condition E
CR	97% (29/30)	100% (30/30)	97% (29/30)	90% (27/30)	93% (28/30)
PP16 (1/2 rxn)	73% (22/30)	87% (26/30)	90% (27/30)	77% (23/30)	13% (4/30)
Yfiler (1/2 rxn) - 3130		90% (18/20)	90% (18/20)	75% (15/20)	
Yfiler (1/2 rxn) - 3500		90% (18/20)	90% (18/20)	90% (18/20)	
Fusion		93% (28/30)	97% (29/30)	80% (24/30)	

Table 7. CR and STR amplification success. CR success was based on the detection of the expected amplicon peak with the QIAxcel. STR success was calculated based on the generation of a full profile.





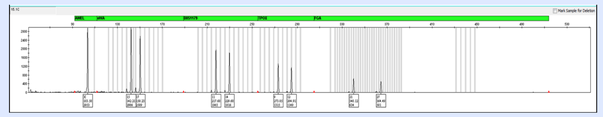


Figure 2. Conditions A (top), B (middle), and C (bottom) typed with PP16 – showing the yellow color channel.

Validation

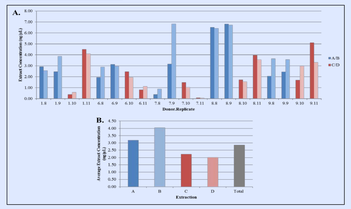


Figure 3. Individual (A) and average (B) extract concentrations from the manual validation testing.

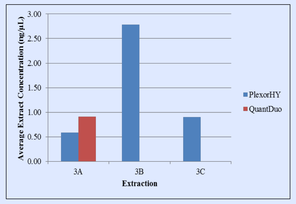


Figure 4. Average extract concentration across all HTP forensicGEM® extractions. QuantDuo data only available for Extraction 3A.

	3A	3B	3C	3D
Yfiler (1/2 rxn)	67%	90%	93%	100%
Yfiler	93%	97%	100%	100%
PP16 (1/2 rxn)	67%	93%	84%	100%
Fusion	82% (89%*)			

Table 8. Yfiler, PP16, and Fusion STR amplification success of all HTP extracts, as calculated based on the generation of a full profile. The * indicates locus DYS391 was excluded from the Fusion profile.

	3A	3C
Original	67%	93%
Retype - 2 sec	70%	97%
Retype - 5 sec	97%	100%
Retype - 3500xL	97%	

Table 9. Yfiler ½ rxn success with additional typing conditions.

Extraction	A	B	C	D	E
Yfiler (1/2 rxn)	8/8	8/8	7/8	7/8	4/4
PP16 (1/2 rxn)	10/10	10/10	9/10	9/10	5/5
ID	10/10	10/10	9/10	9/10	5/5
ID+	10/10	10/10	9/10	9/10	5/5
CR	10/10	10/10	10/10	10/10	5/5

Table 10. Amplification success of all manual validation extracts. STR success was calculated based on the generation of a full profile. CR success was based on the detection of the expected amplicon peak.

DISCUSSION & CONCLUSIONS

Experiment A concluded that SwabSolution and forensicGEM® performed similarly except for the fact that SwabSolution extracts were inhibited. Both methods outperformed Prep-n-Go, but none of the methods matched the fully optimized DNA IQ™ method. ForensicGEM® was selected to continue with optimization because it showed no signs of inhibition, a short incubation time, and lower cost (Table 4). Further, optimization would likely increase DNA yield.

Experiment B concluded that increasing elution agitation time and eluate input volume resulted in higher yields of DNA without increased inhibition. The 60 second agitation time and 40 µL eluate input yielded a sufficient amount of DNA in a short period of time (Table 5).

Experiment C concluded that all 5 extraction buffers performed similarly, although the sky buffer from the saliva card kit performed slightly better. All of the buffers were also tested with saliva and blood cards. The buffer performance was similar with the sky buffer being slightly better. The sky buffer was selected for use with cotton buccal swabs, with the potential to use this same buffer with other substrates (Figure 1).

Experiment D concluded that the 2000 RPM agitation condition yielded notably more DNA than the 900 RPM agitation condition. The faster agitation speed is likely releasing more buccal cells from the buccal swabs allowing for a greater DNA yield (Table 6).

Experiment E concluded that when eluting on a plate, an eluate input larger than 40 µL was needed, but 100 µL produced too much inhibition. When an intermediate volume (70 µL) was used, the success rate improved. The success rate also improved when a larger buffer volume was used with the 100 µL eluate input. Yet, the intermediate eluate input of 70 µL produced the best results. Overall, forensicGEM® was found to be a suitable rapid extraction method for the needs of the AFDIL (Table 7, Figure 2).

Validation of the forensicGEM® extraction method for both manual (tube elution) and manual HTP (plate elution) was performed according to SWGDAM guidelines. The sensitivity study showed that as the elute dilution increased, the DNA concentration consistently decreased. Based on the small sample set, the sensitivity of the manual extraction can be established at 1:5 dilution of neat DNA. The manual forensicGEM® validation concluded that the forensicGEM® manual extraction protocol is a repeatable, reproducible, and accurate extraction method that results in minimal to no contamination. While buccal swab collection variability resulted in a range of extract concentrations (Figure 3), the results show concordance between STR and CR profiles. Sufficient DNA was generated to produced in full STR profiles more than 93% of the time and 100% of the time with CR profiles (Table 10). One sample in particular resulted in less than 100 pg for both extractions (A and B) and consistently produced partial STR profiles. The partial profiles for this sample resulted from insufficient amounts of DNA, not due to inhibition. While the manual protocol will produce higher DNA yields than the HTP method, the manual method will not be used as much in practice as the HTP method because it was developed specifically for processing large amounts of samples. As was observed with the manual extraction, the forensicGEM® HTP protocol is repeatable, reproducible, and an accurate extraction method that results in minimal to no contamination. While buccal swabs have a large range of variability, this study showed concordant results between STR and CR profiles and was confirmed by the validated DNA IQ™ method (Figure 4 and Table 8). STR success rates were high but could still be improved, and so adjustments were made to processing techniques (Table 9). The HTP forensicGEM® validation consistently yielded more than 100 pg/µL of DNA and resulted in concordant STR and CR results.

REFERENCES

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