# Development of a Direct Amplification Method for Exemplar and Pseudo-exemplar Reference Samples Using Identifiler® Plus

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## Abstract

The New York City Office of Chief Medical Examiner's Department of Forensic Biology compared two direct amplification methods. The first method used Identifiler® Direct, which allows samples to be directly amplified from FTA®-treated collection cards. The second method used Identifiler® Plus with an in-house extraction buffer. It was determined that using Identifiler® Plus with the in-house extraction buffer proved to be more cost-effective, in addition to allowing a wider array of sample types to be analyzed. A direct amplification method was developed for buccal swab and blood card reference samples as well as various pseudo-exemplar samples (bottles, cans, cups, straws, chewing gum and cigarette butts).

## Introduction

A common problem in crime laboratories is the backlog of DNA evidence waiting to be processed. Typical DNA analysis takes more than one day and requires a series of steps consisting of extraction, purification, quantitation, PCR amplification, capillary electrophoresis and data analysis. Recently, direct amplification kits have been developed for exemplar reference samples that eliminate the need for purification and quantitation. This reduces the time and costs for processing reference samples, allowing laboratories to invest more resources into analyzing forensic samples.

Reference samples can consist of:

- **Exemplars:** reference samples professionally collected from an individual (ex. buccal swab or blood)
- **Pseudo-exemplars:** reference samples collected from items used by an individual (ex. bottles, cans, cups, straws, chewing gum and cigarette butts)

Identifiler® Direct is an amplification kit optimized for directly amplifying samples spotted on FTA®-treated collection cards. Use of non-FTA® substrates requires pretreatment with Prep-n-Go™ Buffer, at an additional cost. Currently, the NYC OCME does not use FTA®-treated collection cards. The purpose of this project was to develop a direct amplification method that would be cost-effective and adhere to collection techniques employed by the NYC OCME. Identifiler® Direct and Identifiler® Plus, an amplification kit optimized to overcome inhibition, were tested for reference samples. The Identifiler® Plus method involved use of an in-house extraction buffer prepared from reagents readily available in the laboratory.

## Materials and Methods

All samples were analyzed using the following:

**Amplification:** Identifiler® Direct or Identifiler® Plus on GenaAmp® PCR System 9700

**Capillary Electrophoresis:** 3130*x*/ Genetic Analyzer (first injection 1kV 22sec, re-injection 5kV 20sec if drop-out occurred)

**Data Analysis:** GeneMapper® ID v.3.2.1 (20% filter with 75RFU threshold)

### Identifiler® Direct

Samples were amplified with full reactions (12.5µL primers + 12.5µL reaction mix) for 27 or 28 cycles.

- Buccal swabs incubated in $H_2O$ or PBS and an aliquot of lysate directly added to IDD
- Blood cards directly added to IDD

## Materials and Methods Continued

### Identifiler® Plus

Samples were treated with in-house extraction buffer (0.2% Tween® 20, 0.1mg/mL Proteinase K and 2.4% Trehalose in in $TE^{-4}$ buffer) for 30 minutes at 56°C followed by inactivation at 99°C for 5 minutes. An aliquot of extract was directly added to a half reaction of ID+ (2.5µL primers + 5.0µL reaction mix) and amplified for 28 or 29 cycles.

- Buccal swabs and blood cards tested to optimize cutting size, extraction buffer volume, PCR input volume and cycle number
- Pseudo-exemplar samples (bottles, cans, cups, straws, chewing gum, cigarette butts) tested to optimize PCR input volume with 100µL extraction buffer and 29 cycles

**Figure 1.** Sampling methods for pseudo-exemplar reference samples. Cigarette butt paper (3mmx3mm) cut off filter. Gum frozen at -20°C and cut into small pieces and filled to 1mL mark of tube. Bottles, cans, cups and straws were swabbed and outer layer of swab peeled.

## Results

### Identifiler® Direct Buccal Swabs

- 1/3 cutting in 200µL PBS; 2µL PCR input amplified for 27 cycles and injected at 1kV 22sec yielded maximum of 2 alleles for a sample
- Re-injection at 5kV 20sec increased allele calls but no full profiles obtained
- 1/3 cutting in 100µL PBS; 2µL PCR input amplified for 28 cycles and injected at 1kV 22sec yielded 40% full profiles

### Identifiler® Direct Blood Cards

- 40% profiles full profiles and 40% partial profiles
- Majority of peaks in all samples were split peaks
- Off-ladder peak taller than allele peak ~50% of the time

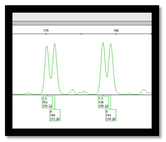

**Figure 2.** Split peaks observed for blood cards. Example of OL peak that is taller than allele peak.

### Identifiler® Plus Buccal Swabs

- ID+ already undergoing validation for 29 cycles so 29 cycles chosen for subsequent studies
- No samples required re-amplification

| | % Reportable Profiles | % Re-injected at 5kV 20sec | % Diluted |
|---|---|---|---|
| **2µL** | **80** | **-** | **20** |
| 1µL | 80 | 10 | 10 |
| 5µL (1/10) | 60 | 40 | - |

**Table 1.** 1/3 swab cutting in 200µL extraction buffer and amplified for 28 cycles. Various PCR input volumes tested.

## Results Continued

### Identifiler® Plus Buccal Swabs Continued

| | % Reportable Profiles | % Re-injected at 5kV 20sec | % Diluted |
|---|---|---|---|
| 3µL | 20 | - | 80 |
| **2µL** | **60** | **-** | **40** |
| 1µL | 70 | 10 | 20 |

**Table 2.** 1/6 swab cutting in 200µL extraction buffer and amplified for 29 cycles. Various PCR input volumes tested.

### Identifiler® Plus Blood Cards

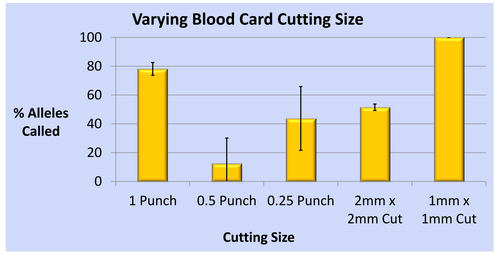


**Figure 3.** Various cutting sizes were tested in 200µL extraction buffer with 2µL PCR input volume.

| | % Reportable Profiles | % Re-injected at 5Kv 20sec | % Diluted |
|---|---|---|---|
| 200µL, 2µL | 30 | 50 | 20 |
| 200µL, 3µL | 50 | 30 | 20 |
| **200µL, 5µL** | **80** | **-** | **20** |
| 300µL, 2µL | 50 | 30 | 20 |

**Table 3.** 1mm x 1mm cuttings in various extration buffer volumes with differing PCR input volumes.

- 1mm x 1mm cut was only size to give full profile – attributed to decreased concentration of heme added to PCR reaction
- 1mm x 1mm determined to be too small – increased to 2mm x 2mm cut in 800µL extraction buffer with 5µL PCR input volume
- No samples required amplification

### Identifiler® Plus Pseudo-exemplar Samples

- Overall 3µL PCR input volume gave best results
- If substrate specific protocols implemented can increase initial run success rate
- Only 1 out of 59 samples required re-amplification (cigarette butt)
- Recommended cigarette butt size be increased to 5mm x 5mm

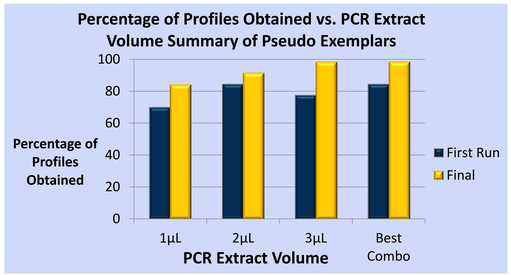


**Figure 4.** Various PCR input volumes tested for pseudo-exemplars. First run at 1kV 22sec. Second run at 5kV 20sec if drop-out occurred. Final gives total profiles obtained without re-amplification.

## Conclusion

Identifiler® Direct would require an initial extraction step in order to obtain reportable results and would not serve as a viable option for the NYC OCME. Identifiler® Plus proved to be more cost-effective using an in-house buffer prepared from reagents readily available. Methods were developed for all substrates and sample types tested with 100% exemplar and 98% pseudo-exemplar reference samples having reportable profiles without need for re-amplification. The following protocols were developed for half reactions of Identifiler® Plus amplified for 29 cycles:

- **Buccal Swabs:** 1/6 cutting in 200µL extraction buffer, 2µL PCR input volume
- **Blood Cards:** 2mm x 2mm cutting in 800µL extraction buffer, 5µL PCR input volume
- **Bottles, Cans, Cups and Straws:** 1/2 outer layer swab peeled in 100µL extraction buffer, 3µL PCR input volume
- **Chewing Gum:** frozen at -20°C and cut into small pieces, fill tube to 0.1mL mark, 100µL extraction buffer, 3µL PCR input volume
- **Cigarette Butts:** 5mm x 5mm cutting in 100µL extraction buffer, 3µL PCR input volume

## Discussion

The direct amplification method developed can reduce the time and costs associated with processing reference samples in several ways. First, using an in-house extraction buffer is cheaper than the alternative extraction methods for Identifiler® Direct (FTA®-treated cards or Prep-n-Go™ Buffer). Second, eliminating purification and quantitation steps reduces materials required to perform them and cuts hours off sample processing time. Finally, using half reactions of Identifiler® Plus allows for the amplification of twice as many samples per kit.

Implementing this method reduces reference sample processing time to less than a day. Starting in the morning, a sample can be cut and extracted and the final profile can be reported by the end of the work day. This cuts the time from the 2-3 days it currently takes.

Further studies were completed to test the reproducibility, sensitivity, concordance, reliability and stability of the method.

## References

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