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Guidelines and Considerations for Evaluating or Validating Extraction Chemistry

Introduction

In order to evaluate a DNA extraction method effectively, it is imperative to consider all aspects of chemistry performance, including DNA yield, DNA quality, and performance of the downstream STR reaction. The ideal forensic DNA extraction method maximizes the amount of high-quality DNA recovered while minimizing the presence of extraneous material such as proteins or chemicals in the final eluted DNA. This combination will result in higher amplification success rates and therefore improved laboratory efficiency.

One of the most common causes of poor-quality STR data is the presence of PCR inhibitors that fail to be removed during extraction. Inhibitors may be defined as any chemical or substance that interferes with the PCR process. Common inhibitors found in forensic samples may originate from bodily fluids, substrates, tobacco products, dirt, or reagents used throughout the workflow. Reagent components used in many extraction methods, such as salts, detergents, acids, bases, and alcohols, can also cause poor STR results if not completely removed from the extract at some point prior to completion of the process.

The importance of the choice of extraction method in determining the success of the overall analysis process has been discussed extensively in a previous article ("Maximizing Data Quality Series - Part 1: The Importance of DNA Extraction in the Forensic DNA Workflow," July 2008 issue of *Forensic News*). In this article, we would like to focus on some practical suggestions for evaluating or validating an extraction method, as well as share information on some of the methods used during our own developmental validation of the PrepFiler™ Forensic DNA Extraction Kit and the AutoMate *Express*™ Forensic DNA Extraction System.

Plan for Success

Before beginning any evaluation or validation study involving an extraction method, it is important to outline all aspects of the experimental program. This includes selection of appropriate sample types and identification of sample preparation protocols to ensure the sample types are appropriate to the study. It also includes determination of instrument run schedules and standardization of data analysis parameters and data comparison metrics to prevent downstream analysis processes from introducing additional variation that may cloud the assessment of extraction performance.

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Sample Selection

When defining the sample set to use for evaluation of an extraction method, it is important to consider both the types of samples and the range of substrates to be evaluated.

- *Sample Type*: Certain sample types generate more consistent results than others and are therefore more practical for evaluating and determining the limitations and performance of an extraction method. Some examples of common sample types and their advantages and limitations are as follows:
    - *Blood* – the most homogeneous sample type and therefore best for comparison of performance between methods. Consistency of sample can be maximized by using a single draw from a single donor, as white cell counts can be variable between different blood draws from the same individual.
    - *Semen* – homogeneity of the sample can be maximized by sampling from a single donor and a single ejaculate; however, handling can be difficult, as sample viscosity may lead to inaccurate pipetting.
    - *Saliva* – samples display significant variation both within and between individuals. Liquid samples can be standardized as much as possible by pooling multiple samples from the same contributor to minimize variation between replicates. Buccal scrapes are very difficult to standardize, as cell counts collected can vary greatly between different scrapes.
    - *Hair & Touch Evidence* – samples are very difficult to standardize, and assessment of extraction method performance should be based on success or failure rather than direct yield comparisons.
- *Substrate Type*: The choice and preparation of substrate replicates can have a significant impact on the reproducibility of each replicate and therefore the overall comparison of one extraction method to another.
    - It is important to choose a selection of substrates representative of the sample receipts of the testing laboratory so that the evaluation/validation reflects the expected operational requirements of any new technique.
    - Consistency across substrate replicates can be maximized by cutting or punching (in the case of heavy substrates) equal size sections of each blank substrate, then adding a defined amount of each body fluid, ensuring that the full volume of the fluid remains within the physical boundary of the substrate. Multiple cuttings/punches of uneven sizes, taken from a single larger stain, have the potential for much greater variation.

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Sample Preparation

The content and preparation of a standard sample set is critical to the success of any chemistry evaluation or validation. Frequently, we are asked to provide guidance on how to assemble such a useful resource. The development and validation of the PrepFiler™ Forensic DNA Extraction Kit required the preparation of an extensive, standardized set of source samples and replicate stains for evaluation and comparison purposes. Based on our own investigative experience, we have included suggestions for obtaining, storing, and preparing standard sample sets.

- *Liquid Blood Samples:* To help ensure uniform replicates, mix the source blood thoroughly (e.g., by gentle inversion of the container tube) and prepare multiple small-volume aliquots simultaneously. For the purposes of a forensic evaluation or validation, aliquots of 1 µL can be stored easily in 2 µL screw-cap tubes and stored at -80°C (if available) or -20°C. Aliquots can then be removed, thawed, and mixed thoroughly before use. Always spin the tubes briefly in a microcentrifuge to ensure that all the liquid is collected from the cap before opening the tube. Care should be taken to not centrifuge the blood sample for an extended amount of time, to prevent pelleting of the cellular material.

- *Blood Dilution Series:* To evaluate the sensitivity of a new extraction method, the most uniform sample set can be created using a serial dilution because each concentration originates from a common aliquot. Serial dilutions are easy to make, cover a wide range of sample inputs, and establish a mechanism for evaluating the linearity of an extraction method. When preparing the series, consider spacing dilutions evenly to capture the full analysis range required, and include data points which extend beyond the expected failure point of the assay. Data in the more dilute portions of the series will inherently contain more variability due to sampling variation.

  To prepare a blood dilution scheme on cotton swabs:

  1. Determine the number of swabs to be evaluated.
  2. Thaw a sufficient number of 1 µL aliquots and mix thoroughly.
  3. Pool the thawed aliquots, mix thoroughly, and dilute using the following series: 1:5, 1:10, 1:50, 1:100, 1:250, and 1:500 (example volumes to use for the blood dilution series are shown in Table 1). To minimize premature cell lysis, blood dilutions should be made in 1X PBS containing 1 mM EDTA. Lysis in TE or water can be too harsh on the cell membrane and may lead to a less accurate dilution series.
  4. Place cotton - tipped swabs vertically into a holder with the tip end pointing upwards.
  5. Pipette 5 µL of each dilution onto individual swabs at approximately the same position just proximal to the apex of the cotton tip.
  6. Place the swabs into a laminar flow hood to dry overnight at room temperature.
  7. Once dry, break off each swab tip, as close to the cotton tip as practical. Place the swab tips into individual pre-labeled 1.5 µL snap-cap tubes.

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Dilution Number	Dilution Ratio	Blood Volume / Starting Material	Diluent Volume	Total Volume / Number of Swabs
1	1:5	200 µL / Undiluted liquid blood	800 µL	600 µL / 120 swabs
2	1:10	400 µL / Dilution 1	400 µL	600 µL / 120 swabs
3	1:50	200 µL / Dilution 2	800 µL	600 µL / 120 swabs
4	1:100	400 µL / Dilution 3	400 µL	600 µL / 120 swabs
5	1:250	200 µL / Dilution 4	300 µL	500 µL / 100 swabs
6	1:500	400 µL / Dilution 5	400 µL	800 µL / 140 swabs

Table 1: Example volumes for preparation of a blood dilution series.

- *Blood Stains on Cloth Substrates (Punch):* To standardize replicates of blood stains on cloth substrates, uniform sections of the cloth substrate should be cut prior to adding the bodily fluid. With heavy fabrics, an easy way to generate uniform replicates would be to use a leather punch fitted with a ~4 mm die. Each clean fabric punch should be added to a labeled 1.5 µL microcentrifuge tube and the blood or blood dilutions dispensed directly onto the punch inside the tube. This ensures the full volume of sample applied to the cloth remains within the physical boundary of the fabric. For consistency, the volume of fluid added should be high enough to utilize reliable pipetting techniques and low enough to avoid loss of bodily fluid onto the sides of the tube. For example, with a 4 mm cloth punch, no less than 2 µL and no more than 5 µL of fluid should used. The tubes should be left open and allowed to dry overnight at ambient temperature in a laminar flow hood before experimental use.

- *Blood Dilutions on Cloth Punches (Lower Input Samples):* To minimize variation when preparing lower input blood samples, it is helpful to prepare larger volume dilutions to minimize the pipetting errors associated with low volumes. For example, to prepare a final concentration of 0.04 µL blood (5 µL sample containing 0.2 µL of blood):

    1. Thaw and mix a 1 µL aliquot of EDTA preserved blood.
    2. Dilute 80 µL of blood in 1920 µL 1X PBS + 1mM EDTA to give a final concentration of 0.04 µL blood / µL.
    3. Apply 5 µL of the diluted blood to pre-dispensed cloth punches, prepared as described in the previous section) to yield 0.2 µL "blood equivalents" per sample. This volume is sufficient for approximately 400 samples.

- *Saliva Samples:* Collect multiple saliva samples from an individual contributor in a 5 µL screw capped tube and mix thoroughly with an equal volume of 1XPBS containing 1 mM EDTA. The original undiluted saliva should be used within 4 hours of collection for preparing samples. Quantification, with multiple replicates, of the extraction eluate can be used to approximate the DNA concentration of the source sample and to prepare the dilutions of interest. If available, a haemocytometer may also be used to help determine the concentration of cells in the source sample and to evaluate extraction efficiency.

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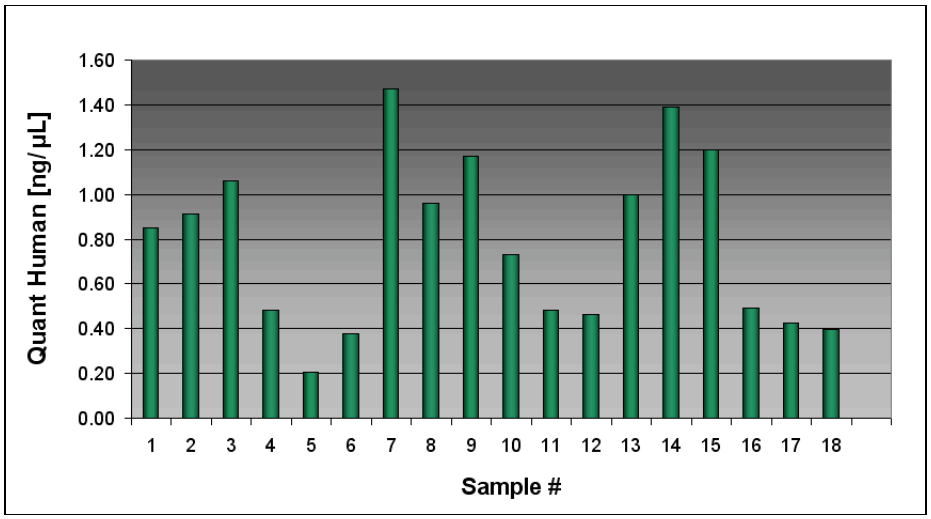

Figure 1: Sample variation shown across a set of identically prepared buccal swab samples from a single individual (source) make it difficult to compare the performance of different extraction methods. In this study, all 18 buccal swabs were collected over time from a single individual, extracted at the same time using the same method, and quantified in one RT-PCR run. Preparing a pooled saliva source sample as described above will help to minimize sample-to-sample variation commonly observed with saliva samples.

- *Semen Samples:* In order to evaluate DNA recovery and prepare appropriately diluted semen samples, the concentration of sperm in the semen sample can be estimated by cell counting. For example, sperm may be diluted to a concentration of ~3,000 sperm per µL in 1X PBS in 1 mM EDTA and the diluted sperm frozen at -80°C (if available) or -20°C until use. Diluted sperm samples which have been thawed at room temperature and stored at 4°C should be used for no more than one month.

- *Inhibitor Spiked Samples:* To assess the effectiveness of an extraction method's ability to remove inhibitors, it can be useful to spike known samples with pre-determined levels of inhibitors, then assess downstream PCR data for any evidence of remaining inhibitors. When creating an inhibition study sample panel, it is important to prepare a control sample set without inhibitors and samples containing inhibitors from the same original source to ensure reproducibility and direct comparability of the inhibited and uninhibited data sets. Samples used during the PrepFiler™ Kit developmental validation study were prepared using 5 µL of whole blood mixed with 1 µL of 1X PBS or inhibitor mix (see note below). Then 3 µL (2.5 µL blood equivalent) of the PBS or inhibitor spiked blood was spotted onto pre-dispensed, 4 mm punches of pre-washed cotton cloth in 1.5 µL tubes and dried overnight at room temperature.

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NOTE: A suggested inhibitor panel could include indigo (12.5 mM, Aldrich Part # 229296), hematin (0.5 mM, Sigma, Part # H3281), humic acid (2.5 mg/µL, Aldrich Part # 53680), and urban dust extract (3%). Extract of urban dust, standard reference material (SRM1649a) from the National Institute for Standards and Technology (Gaithersburg, NC), is prepared by dissolving 300 mg in 10 mM Tris, 0.1 mM EDTA (pH 8.0), shaken 18hr. at room temperature, and the supernatant used to prepare inhibitor mix. Additionally, dilutions of high concentrations of hematin should be first made by dissolving the powder form in 0.1 N NaOH. Water is used for subsequent dilutions from the high concentration stock.

Minimizing Variation in Downstream Analysis Techniques

The content and preparation of a standard sample set is critical to the success of any chemistry evaluation or validation. Frequently, we are asked to provide guidance on how to assemble such a useful resource. The development and validation of the PrepFiler™ Forensic DNA Extraction Kit required the preparation of an extensive, standardized set of source samples and replicate stains for evaluation and comparison purposes. Based on our own investigative experience, we have included suggestions for obtaining, storing, and preparing standard sample sets.

The evaluation or validation of any extraction chemistry is highly dependent upon the downstream processes with which the DNA extracts are analyzed. It is imperative to ensure that those downstream processes impart as little variation as possible onto the result and that extraction methods are evaluated according to both the yield achieved *AND* the quality of the STR profiles.

To minimize variability during quantitation, STR amplification, and electrophoresis and evaluate effectively the capabilities of the extraction technique, it is helpful to consider the following:

- *Pipetting and Chemistry Variation* – Performing multiple replicates will minimize the impact of variation arising from any chemistry and instrumentation used downstream of the extraction. When preparing master mixes and setting up quantitation and STR amplification reactions, consider pipetting larger volumes to minimize pipetting error. And when performing DNA quantitation, consider the detection limitations of the technique. For example, quantification results above 50 ng/µL should be diluted and re-quantified. Results from samples quantifying below 23 pg/µL may be highly variable due to stochastic variation and sampling error, resulting in more difficult direct yield comparisons.

- *Instrument to Instrument and Run to Run Variability* – When possible, utilize the same real-time PCR instrument for all analysis and the same standard curve dilution series if multiple runs are necessary. Place comparative samples on the same run whenever practical to ensure direct comparability of results, and minimize the impact of any run to run variation (Figure 2). For example, to reliably compare extraction efficiency between replicate sets of 5 µL blood spots on cotton extracted with the PrepFiler™ kit and an organic method, all samples should be analyzed in a single real-time PCR run. Perform STR amplification using a single PCR instrument, and dedicate a single CE instrument on which to perform comparative analyses.

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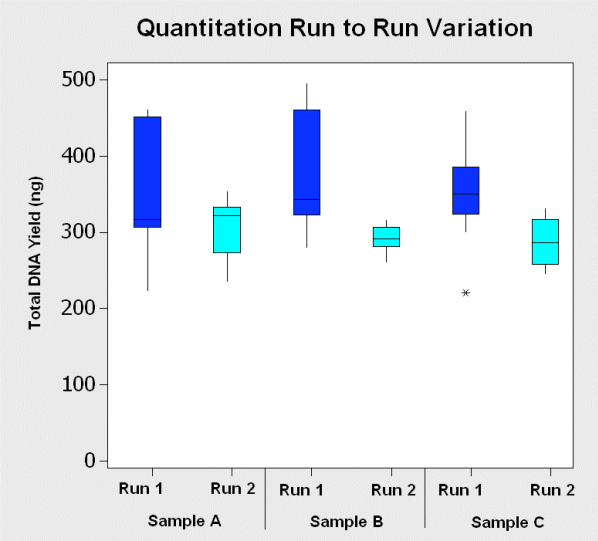

Figure 2: Quantification result variation observed for the same sample set quantified using two different instruments and two different standard curve sample sets. To minimize the impact of this variation, quantitate all comparative samples within a single run whenever possible, to ensure the evaluation focuses on the capability of the extraction method.

Assessing Real-Time PCR Quantitation Results

When evaluating DNA quality and quantity for extracts generated using different extraction techniques, the following considerations may be helpful to ensure accurate interpretation of the real-time PCR quantitation data:

- *Negative Controls* - Run multiple replicate extraction blank samples and No-Template Control (NTC) qPCR negative controls. This may help identify PCR inhibitors originating from the sample, substrate, extraction reagent, or other reagent related issue.

- *Concentration vs. Total Yield* - Data may be expressed in concentration (i.e., ng/µL) or in total yield (i.e., ng). Consider that elution volumes may differ between extracts and extraction methods, and a proper assessment of concentration relative to the volume of each sample is critical.

- *Mean and Standard Deviation* - When evaluating yield between samples, use reliable statistical methods such as calculating the mean of the sample replicates as well as the standard deviations. As an example, samples with identical means but with vastly different standard deviations may indicate poor reproducibility in the set of sample replicates.

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- *Detection of Inhibition* - Compare the IPC C_T values from the quantitation reactions for the samples, extraction blanks, and NTCs. Reviewing real-time quantitation results is one method to help determine the potential presence of PCR inhibitors in the extract. Evaluating the quality of the STR profiles, as described in the next section, is another method.

Assessing STR Profiles Generated by Capillary Electrophoresis

The final step in evaluating an extraction method is to assess STR performance. The impact of PCR contaminants on the STR assay can vary from signal reduction to complete inhibition, resulting in STR profiles that exhibit peak height imbalance (i.e., preferential amplification of the smaller loci generating the 'ski slope' profile morphology), reduced peak heights, locus dropout, minus A or additional peaks, or artifacts. While visual inspection of the electropherogram is a rapid method to evaluate the data quantity and quality, several less subjective methods may be employed to assist the analyst in the evaluation of the STR profile, the identification of PCR inhibitors, and ultimately the overall assessment of each extraction method's performance. Some suggestions are as follows:

- *Peak Height* - Laboratories may establish this criterion on a per locus basis, per dye color, or per profile. Lower than expected peak heights may be a sign that the amplification is less robust and could indicate, among other causes, that the DNA extract is not pure.

- *Peak Height Ratio* - Peak Height Ratio calculations can assist in differentiating between low DNA input amount (poor DNA recovery) and the presence of inhibitors.

- *Split Peak Thresholds* - Split peaks may be caused by the inability of the extraction method to remove a substance which interferes with the terminal transferase activity of the polymerase. It is important to determine whether this effect is reproducible with specific samples or sample types and to rule out non-extract related causes such as capillary electrophoresis or amplification related artifacts.

- *STR Profile Balance* - Profiles can be examined for balance within each dye set (Intracolor Balance) and between different dyes (Intercolor Balance). Inhibitors generally have a greater impact on intracolor balance, resulting in the characteristic 'ski slope' effect associated with inhibition. However, some inhibitors can preferentially affect one dye or one locus over another.

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Summary

When assessing the outcome of an extraction evaluation or validation, it is important to remember that adverse effects on the STR profile can be the result of causes unrelated to the extraction technique as well as the extraction method itself. It is therefore suggested to evaluate any new extraction chemistry as part of a wider, optimized, and fully validated workflow such that any variations in performance can be attributed directly to the extraction technique rather than other processes in the workflow. This will enable more direct comparisons between different methods, and informed decisions can be made on which extraction method is appropriate and/or whether protocol modifications may be required for certain sample types.

Evaluations are designed to compare one method with another, while validations are essential for establishing the performance expectations for any given method. Regardless of the intention of the study, careful attention to detail in experimental planning, sample choice and preparation, and limitation of variables within the wider process will ensure development of a clear understanding of the performance of the extraction system and enable effective troubleshooting of situations encountered during live casework.