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Best Practice Recommendation for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms

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Foreword

This document outlines best practice recommendations for the internal validation of human short tandem repeat DNA profiling on capillary electrophoresis platforms utilized in forensic laboratories.

The validation of STR profiling kits is one part of the process of generating a DNA result. There are steps prior to and after this amplification step and their impact on the STR profiling kit validation studies need to be considered.

The American Academy of Forensic Sciences established the Academy Standards Board (ASB) in 2015 with a vision of safeguarding Justice, Integrity, and Fairness through Consensus Based American National Standards. To that end, the ASB develops consensus based forensic standards within a framework accredited by the American National Standards Institute (ANSI), and provides training to support those standards. ASB values integrity, scientific rigor, openness, due process, collaboration, excellence, diversity and inclusion. ASB is dedicated to developing and making freely accessible the highest quality documentary forensic science consensus Standards, Guidelines, Best Practices, and Technical Reports in a wide range of forensic science disciplines as a service to forensic practitioners and the legal system.

This document was revised, prepared, and finalized as a standard by the DNA Consensus Body of the AAFS Standards Board. The draft of this standard was developed by the Human Forensic Biology Subcommittee of the Organization of Scientific Area Committees (OSAC) for Forensic Science.

Questions, comments, and suggestions for the improvement of this document can be sent to AAFS-ASB Secretariat, asb@aafs.org or 410 N 21st Street, Colorado Springs, CO 80904.

All hyperlinks and web addresses shown in this document are current as of the publication date of this standard.

ASB procedures are publicly available, free of cost, at www.aafs.org/academy-standards-board.

Keywords: *internal validation, DNA, short tandem repeat profiling, capillary electrophoresis.*

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Best Practice Recommendation for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms

1 Scope

This document provides best practice recommendations for performing an internal validation of a human short tandem repeat (STR) multiplex kit using capillary electrophoresis (CE). This document is to be used as a companion document to the ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

2 Normative References

There are no normative reference documents. Annex A, Bibliography, contains informative references.

3 Terms and Definitions

For purposes of this document, the following definitions apply.

3.1
analytical threshold
The minimum height requirement at and above which detected peaks in an STR DNA profile electropherogram can be reliably distinguished from instrument background noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles.

3.2
artifact
A non-allelic product of the amplification process (e.g., stutter, non-templated nucleotide addition, or other non-specific product), an anomaly of the detection process (e.g., single or multi-channel voltage spikes or "pull-up"), or a by-product of primer synthesis (e.g., "dye blob") that may be observed on an electropherogram.

3.3
contamination
Exogenous DNA or other biological material in a DNA sample, PCR reaction, or item of evidence; the exogenous DNA or biological material could be present before the sample is collected or introduced during collection or testing of the sample.

3.4
drop-in
Allelic peak(s) in an electropherogram that are not reproducible across multiple independent amplification events.

3.5
drop-out
Failure of an otherwise amplifiable allele to produce a signal above analytical threshold because the allele was not present in sufficient quantity in the aliquot that underwent PCR amplification.

3.6
interpretation
The process of evaluating DNA data for purposes including, but not limited to, defining assumptions related to mixtures and single-source profiles, distinguishing between alleles and artifacts, assessing the possibility of degradation, inhibition, and stochastic effects, and determining whether the data are suitable for comparison.

3.7
peak height ratio
PHR
The relative ratio of two peaks at a given locus in a diploid heterozygous single-source sample.

3.8
precision
The degree of mutual agreement among a series of individual measurements, values and/or results.

3.9
probabilistic genotyping
The use of biological modeling (i.e., statistical modeling informed by biological data), statistical theory, computer algorithms, and/or probability distributions to infer genotypes and/or calculate likelihood ratios.

3.10
repeatability studies
Studies to evaluate the degree of variability in multiple measurements (e.g., replicate samples) under constant conditions, such as studies by the same operator using the same equipment in the same laboratory within short intervals of time.

3.11
reproducibility studies
Studies to evaluate the degree of variability in multiple measurements (e.g., replicate samples) under varying conditions, such as studies using the same methods with different operators or different equipment.

3.12
sensitivity studies
Studies performed during developmental and/or internal validation of DNA or other test methods designed to define the lower and upper limits/bounds of an assay to accurately detect an analyte.

3.13
stochastic threshold
The peak height value in a DNA electrophoretic profile above which it is reasonable to assume, based on validation studies, that at a given locus, allelic drop-out of a sister allele in a heterozygous pair has not occurred in a single-source DNA sample; due to the possibility of shared alleles in mixed samples, the presence of allele peaks above the stochastic threshold is no guarantee that allele drop-out did not occur in mixed DNA sample profiles.

3.14
stutter
An artifact of polymerase chain reaction (PCR) amplification typically observed one or more repeat units smaller or larger than a short tandem repeat (STR) allele in a DNA profile, may result from strand slippage during PCR amplification. A stutter peak is generally of lower relative fluorescence units (RFU) than the allele peak.

3.15
validation
The process of performing and evaluating a set of experiments that establishes the efficacy, reliability, and limitations of a method, procedure or modification thereof; establishing recorded documentation that provides a high degree of assurance that a specific process will consistently produce an outcome meeting its predetermined specifications and quality attributes. May include developmental and/or internal validation.

4 Recommendations

4.1 Considerations for Validation Studies

4.1.1 A validation plan should be developed defining questions to be addressed and the studies designed to answer these questions.

4.1.2 Laboratories validating X and Y chromosome STR kits should perform the same studies, where applicable, as those validating autosomal STR kits, taking care to consider appropriate sample selection and data interpretation.

4.1.3 Human DNA samples (whether collected or generated internally or externally to the laboratory) should be selected to meet agency policy regarding human subjects and privacy concerns as the DNA profiles from these samples may need to be shared for independent review of validation data.

NOTE Use of DNA samples from staff members to perform validation experiments can cause complications regarding privacy.

4.1.4 In order to characterize variability that may exist in the system, single-source DNA samples from multiple donors with a variety of alleles and with a high degree of heterozygous genotypes, should be used.

4.1.5 Samples tested should include extracts prepared using all extraction chemistries in use by the laboratory.

4.1.6 The DNA concentration of each extract used in the studies should be established using the current laboratory quantitation method.

4.1.7 Applicable controls (e.g., reagent blanks, negative and positive amplification controls) should be run with all sample sets throughout the validation process.

4.1.8 Variation between lots of critical reagents and supplies are known to exist and should be assessed, monitored, and understood. At least two lots of each critical PCR amplification reagent should be tested during validation studies conducted.

4.1.9 Instrumentation should meet the laboratory's criteria for use in casework including being current on calibrations and maintenance.

4.1.10 Variation in peak position and peak height should be assessed, monitored, and understood within and between instruments (e.g., using replicate samples, positive controls, and/or allelic ladders). Variation may result from intrinsic differences between platforms or instruments and extrinsic factors such as room temperature. Significant differences should be addressed in protocols and procedures.

4.1.11 If a laboratory plans to use methods to enhance detection sensitivity (e.g., increased amplification cycle number, increased injection time, and/or post-amplification purification), prior to implementation, additional validation studies to determine the effect of these methods on interpretation should be performed. After studies are performed, an evaluation of the benefits and risks should be conducted and criteria established and documented for each method used.

As stated in section 4.1.2 of ASB Standard 039, "The laboratory shall perform sensitivity studies that will include replicates for each set of assay parameters (e.g., PCR cycle number, injection time, injection voltage, and PCR reaction volume) that the laboratory utilizes with the new STR kit."

4.1.12 If alterations are made to amplification parameters (e.g., reaction volume, reaction components and concentrations, amplification cycle number and thermal cycling conditions), prior to implementation, additional validation studies to determine the effect of these methods should be performed.

As stated in section 4.2.2 of ASB Standard 039, "The laboratory shall perform sensitivity studies that will include replicates for each set of assay parameters (e.g., PCR cycle number, injection time, injection voltage, and PCR reaction volume) that the laboratory utilizes with the new STR kit."

4.1.13 If alterations are made to the data analysis parameters that impact sizing, peak height, or peak detection (e.g., smoothing, peak half-widths, sizing algorithm selection), the validation data should be re-analyzed with any software that is used for data analysis.

4.1.14 Outliers or discrepancies identified during data analysis should be further evaluated. Potential explanations for the discrepancies should be provided and where possible, additional data and research studies supporting the explanations documented. These results may dictate the need for additional testing and/or modifications to the procedures and protocols under development.

4.2 Concordance

4.2.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.1 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

"The laboratory shall conduct an STR genotyping concordance study."

4.2.2 Objective

The purpose of concordance testing is to demonstrate agreement between STR typing results obtained compared to those using previous methods or published data.

4.2.3 Considerations

4.2.3.1 Concordance samples should be evaluated after conditions for casework sample analysis have been established (e.g., target DNA amount, injection time and voltage, PCR cycles/volume).

4.2.3.2 The samples used in the concordance study should reflect the type of STR test kit being validated (e.g., autosomal STR typing test kit should use both male and female DNA, direct amplification typing test kits should use buccal swabs, or stain punches; Y-STR test typing kit should use male DNA).

4.2.4 Experimental Method

The sample types used in the concordance study should include an appropriate certified reference material and may include proficiency test samples, amplification positive controls, purchased blood samples, or genomic DNA derived from stable cell lines.

NOTE Refer to Section 4.1.2. for information on use of DNA samples from staff members.

4.2.5 Data Analysis and Results

4.2.5.1 A comparison of the observed alleles to the known values (if established) should be performed.

4.2.5.2 The presence of a discordant genotype result at a locus should be documented in the final validation summary and a possible reason provided.

4.2.6 Implementation

Observed discordant results may not invalidate the concordance study. Common reasons for discordance may include a null allele resulting from a primer binding site mutation, a difference in allele call due to different PCR primer sets, or a different method of DNA separation affecting resolution or migration. Stochastic effects from amplifying low levels of DNA can also produce discordant results due to elevated stutter or allele drop-out.

4.3 Sensitivity

4.3.1 Requirement from ASB Standard 039

NOTE Refer to section 4.2.2 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall perform sensitivity studies that will include replicates for each set of assay parameters (e.g., PCR cycle number, injection time, injection voltage, and PCR reaction volume) that the laboratory utilizes with the new STR kit."

4.3.2 Objective

The purposes of these studies are to define the upper and lower limits for the STR test kit and capillary electrophoresis platform used, and to determine the sensitivity and optimal DNA template target or target range for developing interpretable DNA profiles.

4.3.3 Considerations

Performing replicates of the samples selected with an emphasis on testing DNA below the optimal template range is recommended for collecting and examining STR data where allele drop-out is expected. This is informative for establishing and verifying a stochastic threshold and for the use of probabilistic genotyping software.

4.3.4 Samples Used in Experimental Method

4.3.4.1 Single-source DNA samples processed in-house with internally validated extraction and quantitation methods should be used for these studies.

4.3.4.2 DNA profiles with heterozygous genotypes having alleles that are multiple repeats apart should be included to account for variation in PCR efficiency due to fragment size.

4.3.4.3 *Pristine DNA:* A minimum of three unique single-source, high-quality, non-degraded DNA samples should be used to perform serial dilutions across a range of DNA template quantities higher and lower than expected to be processed by the laboratory. The serial dilution should include the following:

a) a minimum of five different template quantities performed in triplicate (e.g., three unique DNA samples × five dilutions × three replicates = 45 total amplifications);

b) lower concentrations that produce STR profiles where allele drop-out is observed and allow for identification of the lower limits of data analysis and interpretation.

4.3.4.4 *Degraded/Inhibited DNA:* Following initial sensitivity studies using high-quality DNA, additional studies should be performed to gain familiarity with the impact of common environmental insults known to affect forensic DNA samples. The serial dilution should include the following:

a) a biologically relevant serial dilution of inhibitor added to a serial dilution of DNA and multiple inhibitors may be tested (e.g., hematin, humic acid);

b) DNA degraded using a laboratory defined protocol.

4.3.4.5 *Excess Non-specific DNA*: Following initial sensitivity studies using high-quality DNA for Y chromosome STR kits, additional studies should be performed to gain familiarity with the impact of the presence of excess DNA from a female individual(s), often co-extracted in forensic DNA samples. The serial dilution should include the following:

a) a minimum of three unique single-source DNA samples across a range of DNA template quantities higher and lower than expected to be processed by the laboratory;

b) a biologically relevant serial dilution of DNA from an individual providing an excess of the non-target sex chromosome.

4.3.5 Data Analysis and Results

4.3.5.1 Variation at different DNA template quantities should be characterized using average peak height (APH), standard deviation, and coefficient of variance for every locus at each DNA

template quantity and quality for both homozygotes and heterozygotes. Upper and lower limits of reliable interpretation along with optimal DNA input target/range should be defined as follows:

a) Limits should be evaluated using RFU and/or DNA template quantity and quality.

b) Upper limits should be informed by evaluation of spectral pull-up, excessive stutter, off scale signal, increased artifact detection, and decreased locus and allelic balance.

c) Lower limits should be informed by peak height balance, allelic and locus drop-out, allelic drop-in, and elevated stutter.

4.3.5.2 Optimal input for pristine DNA can be a target or a range and will fall within the highest and lowest concentration of DNA template from a single contributor that clearly distinguishes true alleles (homo- and heterozygous) from artifacts, demonstrates intra- and inter-locus and dye color channel balance, and usually results in complete profiles.

4.3.6 Implementation

Data obtained from the sensitivity studies should inform the development of the laboratory's protocols on the following:

a) amplification conditions as defined by validation studies;

b) DNA target input range;

c) positive control DNA input target;

d) stochastic threshold;

e) upper limit of data analysis, that prevents an increased observation of off-scale/artifact peaks;

f) lower limit of data analysis, that informs processing and interpretation strategies used for low template samples that results in the highest number of complete and accurate allele calls.

4.4 Analytical Threshold

4.4.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.3 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

"The laboratory shall determine the analytical threshold for each dye channel of the new STR test kit using a range of sample types and DNA input quantities across multiple analyses."

4.4.2 Objective

The purpose of setting an analytical threshold (AT) is to define the RFU value above which artifacts and true allelic signal are differentiated from baseline noise.

4.4.3 Considerations

4.4.3.1 CE instruments should be set on a solid/secure platform that protects against potential vibration or movement that could affect baseline noise.

4.4.3.2 Caution should be taken when utilizing DNA input amounts lower and/or higher than the laboratory's determined optimal DNA input range to set a single AT since this may increase the potential for loss of interpretable data in low level samples if that single threshold is applied. Based on data generated using known DNA concentrations that straddle the optimum DNA input range, laboratories may determine and apply different AT values to decrease the loss of interpretable data.

4.4.4 Samples Used for Experimental Method

4.4.4.1 Amplification negatives and samples using a range of DNA template quantities, including the sensitivity study samples, should be used.

4.4.4.2 Non-baseline peaks (e.g., dye-artifacts, other known artifacts, spectral pull-up, and allelic peaks) should be removed from data prior to calculation.

4.4.4.3 Samples displaying off-scale data or excessive artifacts should not be used.

4.4.5 Data Analysis and Results

Multiple methods for calculating an AT are acceptable, and examples can be found in the literature.

4.4.6 Implementation

Analytical thresholds should be established early in the validation process and applied to all other validation studies. The thresholds should be modified if necessary, based on other studies conducted at a later time.

4.5 Peak Height Ratio

4.5.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.4 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall characterize peak height ratio variation observed for each locus of the STR test kit utilizing single-source samples amplified over a range of DNA input amounts."

4.5.2 Objective

Characterizing peak height ratios during internal validation provides the laboratory with data to assist in understanding allelic imbalance, identifying the possibility of allelic drop-out, and the development of interpretation guidelines. This study is not applicable to single-copy Y-STR loci as they do not have heterozygous loci.

4.5.3 Considerations

4.5.3.1 Heterozygous loci are required to perform peak height ratio calculations.

4.5.3.2 Measurements of peak area may be substituted for peak height, and if selected, peak area should be used for all studies.

4.5.3.3 Laboratories should evaluate the effects of DNA quantity on PHR.

4.5.3.4 Off-scale data should not be used to measure PHR since the peak height data is not accurate for off-scale peaks.

4.5.3.5 Laboratories should calculate PHRs using alleles from heterozygous pairs separated by two or more repeats to avoid contribution from stutter artifacts.

4.5.4 Samples Used for Experimental Method

NOTE Refer to Section 4.2.4 for samples to be used.

4.5.5 Data Analysis and Results

4.5.5.1 PHR for each heterozygous pair of alleles should be calculated (e.g., low RFU peak/high RFU peak) as follows:

a) average PHR;

b) standard deviation;

c) minimum and maximum PHR; and

d) across the full range of data generated from various DNA template amounts.

4.5.5.2 PHR trends should be assessed for the following data sets:

a) differences based upon peak height RFU (e.g., low RFU vs. high RFU);

b) differences based on locus;

c) PHR balance within loci and PHR balance among loci.

4.5.5.3 The laboratory should create a plot of peak height and/or input value vs. PHR to determine data linearity.

4.5.5.4 PHR variation should be characterized to determine the expected value(s) for loci under defined conditions (e.g., template quantity seen across the range of samples tested in case work, peak heights) to be used in profile interpretation. This can be accomplished using various methods (e.g., using the average PHR minus three standard deviations).

4.5.5.5 Laboratories employing probabilistic genotyping software for mixture deconvolution should evaluate PHRs at a range of DNA input amounts to assist with preliminary profile interpretation (e.g., assessing the potential number of contributors) prior to software deconvolution.

4.5.6 Implementation

4.5.6.1 The results from this study can form the basis for the laboratory's minimum PHR expectations for assistance in data interpretation, including evaluation of mixed DNA profiles.

4.5.6.2 The following PHR threshold approaches may be implemented based on laboratory requirements:

a) a single universal minimal expected PHR threshold;

b) multiple locus-specific or RFU-specific minimal expected PHR thresholds.

4.5.6.3 The laboratory should document the frequency of events that fall below the minimum threshold(s) in data sets described in 4.4.5.2.

4.6 Stutter

4.6.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.5 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall characterize PCR stutter artifacts observed for each STR locus of the new STR test kit."

4.6.2 Objective

Characterizing stutter ratios during internal validation provides the laboratory with data to ensure the STR kit displays stutter frequencies comparable to those reported during developmental validation and can assist with the development of interpretation protocols.

4.6.3 Considerations

4.6.3.1 Laboratories utilizing the manufacturer's recommendations should compare internally validated stutter data to manufacturer's developmentally validated data to ensure consistency across the range of loci and alleles represented in the STR kit.

NOTE When creating a validation plan, the laboratory defines the level of acceptable variation from developmentally validated values.

4.6.3.2 Laboratories should consider whether to use locus-specific or allele-specific stutter values.

4.6.3.3 Stutter percentages are expected to vary with the type of the repeat unit e.g., tri-nucleotide vs. tetra-nucleotide.

4.6.4 Samples Used for Experimental Methods

NOTE Refer to Section 4.2.4. for samples to be used. Additional samples from population data representing a wide allele range can assist in determining allele-specific stutter values.

4.6.5 Data Analysis and Results

4.6.5.1 Stutter peaks should be characterized based on size and amplitude relative to an allelic peak. [e.g., = (RFU of stutter artifact)/ (RFU of allele peak)].

4.6.5.2 Off-scale data should not be used to measure stutter since the peak height data is not accurate for the off-scale peak.

4.6.5.3 Stutter characterization should be classified into categories based on relation to the true allele peak:

a) one repeat unit smaller;

b) one repeat unit larger;

c) two repeat units smaller; and

d) partial or other repeat unit differences.

4.6.5.4 The data analysis software employed should have all stutter filters removed and the allele-calling threshold set to a value low enough to capture all non-template peaks (e.g., 20 RFU). Non-stutter artifacts should be edited out prior to data export, such as spectral pull-up, incomplete adenylation, CE spikes or dye artifacts.

4.6.5.5 Stutter should not be calculated for the conditions in 4.5.5.5.1 and 4.5.5.5.2.

4.6.5.5.1 When two alleles at an individual locus are one repeat unit different in size, as stutter contribution cannot be decoupled from the allele height.

4.6.5.5.2 When two alleles are two repeat units apart, as the n+1 stutter from the first allele is additive with the n–1 stutter of the second allele.

4.6.5.6 Allele designation, base pair size and peak height data should be exported for accurate stutter analysis.

4.6.5.7 At a minimum, the following characteristics for stutter should be calculated using the above data:

a) average stutter per locus;

b) standard deviation; and

c) minimum and maximum stutter observed at each locus.

4.6.5.8 A test of the success of the stutter thresholds should be performed using single-source known references and casework-like samples until the laboratory determines an acceptable level of stutter peaks are filtered across several different single-source samples.

4.6.6 Implementation

4.6.6.1 The laboratory may determine stutter thresholds using one of the following methods.

a) Maximum stutter observed per locus, or per allele.

b) Average stutter plus a determined number of standard deviations per locus, or per allele.

c) If fewer than five observations per allele occurred at a particular locus the largest observed stutter value for that locus may be used.

4.6.6.2 Each laboratory should decide if they wish to implement stutter thresholds only for the most commonly observed (e.g., n–1 repeat unit) stutter in the data analysis software used by the laboratory or to implement additional stutter thresholds (e.g., n+1, n-2 repeat unit).

4.6.6.3 At a minimum, the less common stutter values (e.g., n+1, n–2 repeat unit) and their ranges should be documented within the validation summary. This information is valuable for staff to consider during interpretation of casework results.

4.7 Stochastic Threshold

4.7.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.6 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall determine stochastic threshold(s) for any manual binary method used to interpret the new STR test kit data."

4.7.2 Objective

Identification of a stochastic threshold allows the laboratory to determine the peak height value above which it is reasonable to assume that, at a given locus, allelic drop-out of a sister allele in a heterozygous pair has not occurred; due to the possibility of shared alleles in mixed samples, the presence of allele peaks above the stochastic threshold is no guarantee that allele drop-out did not occur in mixed DNA sample profiles. This study is not applicable to single-copy Y-STR loci as they do not have heterozygous loci.

4.7.3 Considerations

4.7.3.1 Data to assess stochastic threshold may be generated using the sensitivity samples.

If additional samples are needed beyond the sensitivity study, these should be selected from below the optimal DNA template range.

4.7.3.2 Due to amplification efficiency variation across loci, stochastic effects within an amplification reaction may affect one or more loci irrespective of locus or allele size. As such, the stochastic threshold may be designated per locus. Laboratories may determine separate stochastic thresholds based on validation data.

4.7.3.3 The following are contributing factors when establishing a stochastic threshold:

a) amplification conditions (e.g., DNA template input, reaction volume, or cycle number);

b) CE parameters (e.g., injection time or voltage);

c) post-amplification sample cleanup.

4.7.3.4 Stochastic threshold values should be calculated for all amplification conditions and capillary electrophoresis parameters the laboratory plans to use.

4.7.4 Samples Used for Experimental Method

NOTE Refer to Section 4.2.4 for samples to be used.

4.7.5 Data Analysis and Results

4.7.5.1 Derive a stochastic threshold using DNA template inputs that exhibit allele drop-out such that the sister allele is not visible and/or drop-out where the sister allele is below the previously established analytical threshold.

4.7.5.2 The stochastic thresholds may be defined using the following:

a) average peak height plus a determined number of standard deviations;

b) logistic regression;

c) value above highest peak height where most severe imbalance is observed;

d) plotting PHR vs average RFU.

4.7.5.3 All loci/dye channels should be assessed for differences; however, a single threshold may be implemented where these differences are not determined to be significant.

4.7.6 Implementation

4.7.6.1 The derived stochastic threshold should be verified using known case-type samples and the performance evaluated.

NOTE If the threshold is set too high, true homozygotes are flagged as possible allelic drop-out. If the threshold is set too low, heterozygotes with allelic drop-out would not be flagged.

4.7.6.2 If the stochastic threshold does not reflect accurate determination of zygosity, 4.6.6.2.1 through 4.6.6.2.3 should be considered to improve the accuracy of the threshold(s)

4.7.6.2.1 Reevaluate the original threshold(s) chosen. If a single threshold has been used, consider multiple thresholds that better separate true homozygotes and heterozygotes where drop-out occurs.

4.7.6.2.2 Consider use of another analysis method as described in 4.7.5.2 to define the threshold(s).

4.7.6.2.3 Analyze additional samples.

4.8 Mixtures

4.8.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.7 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall conduct studies utilizing mixed DNA samples having a range of DNA ratios, DNA template input quantities, numbers of contributors, and degrees of allele sharing (including mixtures of first-degree biological relationships i.e., parent and child; siblings), expected to be interpreted by the testing laboratory."

4.8.2 Objective

These studies should assist the laboratory in establishing a mixture interpretation protocol; this includes an estimate of the number of contributors to the mixture, determination of the major and minor contributor profiles, and contributor ratios. See ANSI/ASB Standard 020 and ANSI/ASB Standard 040 for further requirements.

4.8.3 Considerations

4.8.3.1 This study should be performed after the sensitivity, peak height ratio, analytical threshold, stochastic, and stutter studies have been completed and thresholds have been established.

4.8.3.2 The laboratory should design the study to incorporate at least one additional contributor beyond the number expected to be interpreted during casework in order to demonstrate the ability to assess the number of contributors and the limitations of the laboratory's mixture interpretation protocol.

4.8.3.3 To support the development of the laboratory's interpretation protocol, a study of the number of contributors paired with several different genomic template quantities (at, above, and below the levels expected to be interpreted by the testing laboratory) and qualities (e.g., degraded, inhibited) should be performed.

4.8.3.4 If a laboratory has validated or is currently planning the validation of an X or Y-STR test kit, then studies should be planned for efficient test design and workflow decisions which can be performed in parallel.

NOTE 1 Decisions about downstream STR typing may be impacted by the autosomal/Y-STR mixture ratios and upstream quantification values.

NOTE 2 An important consideration is that Y-STR loci are linked and therefore the full haplotype for each contributor needs to be considered when mixtures are deconvoluted or are interpreted.

4.8.3.5 The laboratory mixture interpretation protocol should consider the sample types in which assumed contributors may be present and used to deduce the genotypes of potential contributors.

4.8.4 Experimental Method

4.8.4.1 Mixture classes, based on the sex, relatedness, and number of contributors, should be selected to include combinations of these factors to be interpreted by the laboratory.

4.8.4.2 For each mixture class, a mixture series should be created by generating a specified range of ratios including template amounts at, above, and below the optimal input template amount from single-source samples of known genotypes.

4.8.4.2.1 The range of ratios should include minor alleles that fall below stochastic and analytical thresholds.

4.8.4.2.2 The range of ratios should be designed in order to identify the limits of major/minor determination.

4.8.4.2.3 Samples with a range of allele variants should be included to evaluate performance and assess varying degrees of allelic overlap and the effect of alleles in stutter positions.

4.8.4.2.4 Samples with a range of DNA qualities (e.g., degraded, inhibited) should be included.

4.8.4.3 A minimum of two mixture series for each mixture class should be evaluated in triplicate.

4.8.5 Data Analysis and Results

4.8.5.1 Laboratories should apply filters and thresholds developed in the other studies (e.g., analytical threshold, stochastic threshold (if applicable), peak height ratio, stutter ratios) on data produced for the mixture study.

4.8.5.2 Each mixture series should be evaluated for the following parameters.

a) Decipher the possible genotypes of major and minor donors.

b) Determine the ratio at which alleles from the minor contributor fall below established thresholds.

c) The observed mixture ratio should be assessed for all samples and compared to the expected mixture ratio. Variation from the expected mixture ratio should be explored and additional testing performed if warranted.

NOTE Alleles that exhibit masking are not appropriate for use in manual mixture ratio estimation. Masking is defined as alleles that are shared between contributors, or when alleles of one donor fall into the stutter position of another donor.

d) Instances in which the major and minor contributors become indistinguishable should be identified.

4.8.5.3 Based on the results of the mixture studies, filters and thresholds should be adjusted as needed.

4.8.5.4 The data generated should be used to create interpretation methods and protocols including:

a) the criteria for establishing minimum and assumed numbers of contributors;

b) the mixture ratios when deconvolution can be used to assign possible genotypes to contributors;

c) the limitations of the method i.e., degradation, inhibition, number of contributors, and stochastic effects.

4.8.6 Implementation

4.8.6.1 Verification of the mixture protocols should be performed on mixed DNA samples of known origin that are different from those in the initial validation studies used to establish the protocol. See ANSI/ASB Standard 020 for more information.

4.8.6.2 To perform replicability studies, a new dataset representative of the number of contributors, DNA ratios and genomic DNA template quantities expected to be interpreted in casework-like mixtures should be analyzed using the interpretation methods and protocols.

4.8.6.3 To perform reproducibility studies, at least two analysts should interpret the same data and obtain the same possible contributor genotypes.

4.9 Precision

4.9.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.8 ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The laboratory shall demonstrate allelic sizing precision and calling accuracy of the new STR test kit and capillary electrophoresis instrument through repeatability and reproducibility studies."

4.9.2 Objective

The purpose of evaluating precision for base pair sizing is to assess the analytical performance of the separation mechanism and software sizing of DNA fragments included in the STR test kit.

4.9.3 Considerations

4.9.3.1 Environmental factors may need to be considered prior to placement of CE instrument or adjustments may need to be made to room conditions.

4.9.3.2 DNA sizing variation greater than 0.5 nucleotides may result in incorrect allele calls for alleles that are a single nucleotide apart.

4.9.4 Experimental Method

4.9.4.1 Depending on the environmental conditions, the stability of ambient room temperature and humidity should be monitored throughout the validation. The periodicity of monitoring should capture the range of conditions under which casework is performed (e.g., running over the weekends when the air conditioning may be turned off).

4.9.4.2 DNA sizing precision measurements should be performed using injections of allelic ladders in different capillaries throughout the course of the validation studies.

4.9.4.3 A DNA sizing precision study should be performed for each CE instrument in use in the laboratory/laboratories.

4.9.4.4 A DNA sizing precision study should be performed for each instrument parameter/run condition that may affect migration (e.g., run voltage, polymer type, etc.).

4.9.4.5 Allelic ladders should be evaluated to allow for the characterization of variation across capillaries, injections, and time. The frequency of injection should be performed to capture the variation on DNA sizing precision due to the environmental condition encountered.

4.9.5 Data Analysis and Results

4.9.5.1 Calculate the range of maximum and minimum DNA sizes across all allelic ladders to assess run-to-run variation.

4.9.5.2 The precision for DNA sizing is calculated by using the DNA size for each allele in the allelic ladder generated from the analysis software.

4.9.5.3 The average nucleotide size of each allele is calculated using these data and the standard deviation calculated. Three times standard deviation (a confidence interval of 99.7%) provides precision estimates for each allele of a locus and should be less than 0.5 nucleotides.

4.9.6 Implementation

4.9.6.1 If precision is determined to be greater than 0.5 nucleotides for any allele within a locus, sources of the cause of this deviation should be examined (environmental conditions, run parameters, etc.). Once the source of the deviation is remedied, precision should be reassessed.

4.9.6.2 Results should allow the laboratory to determine the frequency of ladder injections and to identify if re-injections related to off-ladder allele designations are necessary.

4.9.6.3 Environmental factors may need to be considered for placement of CE instrument or adjustments to room conditions.

4.10 Contamination

4.10.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to section 4.2.9 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

"The laboratory shall determine the susceptibility of the genotyping process to the introduction and detection of exogenous DNA by documenting allelic drop-in and contamination through the evaluation of controls (i.e., reagent blanks, negative and positive amplification controls) and samples with known genotypes.

The laboratory shall document contamination events and calculate drop-in rates in accordance with its quality system."

4.10.2 Objective

The purpose of the contamination study is to inform the laboratory as to the susceptibility of the STR analysis system to the detection of exogenous DNA originating from reagents, consumables, laboratory operator, instrument, and environment.

4.10.3 Considerations

4.10.3.1 Two categories of exogenous DNA should be evaluated. Contamination where an unexpected source of DNA is detected and allele drop-in where an unexpected allele (possibly two) are detected.

4.10.3.2 Potential sources of contamination may include:

a) laboratory environment (e.g., possible new cleaning schedule/procedure, HVAC systems, workflow/dedicated lab space);

b) consumables (e.g., tubes, reagents, pipette tips);

c) operator (e.g., appropriate training, personal protective equipment);

d) capillary electrophoresis instrument (e.g., capillary injection carry-over, capillary cross-talk);

e) automation equipment (e.g., normalization, amplification setup, post-amplification loading).

4.10.4 Experimental Method

4.10.4.1 No additional studies are necessary for the evaluation of potential contamination events. Data generated from the other validation studies should be part of the contamination study.

4.10.4.2 Additional controls (e.g., negative controls, or reagent blanks) may be processed to test the system based on the variables described in the considerations above.

4.10.5 Data Analysis and Results

4.10.5.1 All samples, including controls, should be evaluated for the presence of exogenous DNA (including allele drop-in) which may originate from reagents, consumables, operator and/or laboratory environment.

4.10.5.2 Allele drop-in should be documented and a frequency of occurrence should be determined.

4.10.5.3 Drop-in rate may be estimated by comparing the number of drop-in observances to the total amount of data or samples evaluated during the validation study.

NOTE If no drop-in events are identified, the drop-in rate can be described as less than one event in the number of samples tested.

4.10.5.4 Contaminating allelic data, if present, should be characterized and attribution of source attempted. The source of the contaminating data (co-processed samples, laboratory operator, consumables, etc.) may identify the point in the laboratory process that the contamination event

occurred and inform the laboratory on how to adjust procedures to prevent recurrence, and identify potential systemic problems that may require further improvements. The creation of an elimination database containing DNA profiles from laboratory personnel and crime scene investigators may provide a method for identifying the source of contamination.

4.10.6 Implementation

The results from the contamination study should form the basis for the laboratory's policies on the following:

a) appropriate procedure setup including placement and number of controls;

b) laboratory environment (design, workflow, cleanup/maintenance);

c) level of tolerance (drop in frequency/expectations);

d) contamination management and necessary corrective measures;

e) control measures (e.g., personal protective equipment).

4.11 Known References and Casework-like Samples

4.11.1 Requirement from ASB Standard 039 *(also available for public comment).*

NOTE Refer to sections 4.2.10.2 and 4.2.10.3 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

> "The evaluation shall include studies utilizing known and casework-like samples with a range of sample types representative of those expected to be encountered by the testing laboratory.
>
> These studies shall minimally include processing a set of samples using all DNA extraction and quantification chemistries utilized by the laboratory to verify compatibility with the new STR test kit."

4.11.2 Objective

The purpose of the known references and casework-like samples study is to assess the performance of validated parameters of the STR test kit using case-type samples processed using upstream laboratory procedures.

4.11.3 Considerations

4.11.3.1 This study may be used to assess relative STR test kit concordance and performance through comparison with the previously validated STR kit data.

4.11.3.2 The new STR test kit and associated standard operating procedures should be robust and generate reproducible results for pristine samples and consistent results for challenged samples.

4.11.3.3 Laboratories should determine if the extraction chemistry procedure(s) used introduce inhibitors that interfere with the amplification of DNA with the STR test kit.

4.11.3.4 DNA samples that have been previously extracted, quantified, and stored should be re-quantified before use in these experiments, as sample quantity and quality may be compromised over time.

4.11.3.5 Inclusion of previously genotyped, anonymized samples should be included during the evaluation of procedures.

4.11.4 Experimental Method

The testing laboratory should analyze known references and casework-like samples representative of those expected to be encountered by the laboratory for casework using the new STR test kit and different from those previously used for other portions of this validation. All extraction methods currently in use by the testing laboratory should be represented in the tested samples. Sample type considerations include the following.

a) Known reference samples should include:

    1) single-source DNA samples of good quality, including multiple male and female individuals;

    2) sufficient template to conduct the planned study.

b) Casework-like samples should include:

    1) different genomic template quantities including limited DNA template amounts;

    2) inhibited samples;

    3) adulterated samples (latent print processing reagents, gun oil, condom lubricants, etc.);

    4) samples containing more than one contributor in varying template amounts and similar levels of complexity to the samples tested in the mixture studies;

    5) degraded samples, including differential degradation in mixed samples.

4.11.5 Data Analysis and Results

4.11.5.1 Using the parameters and standard operating procedures established during internal validation of the STR kit, data should be assessed for reproducibility and consistency.

4.11.5.2 STR typing results should be compared to any previous results. Samples should be evaluated for potential contamination or allele drop-out through comparison to expected profile genotypes.

4.11.5.3 To evaluate the standard operating procedures, STR typing results for samples containing more than one contributor should be compared to reference DNA profiles to determine the ability to detect possible contributor genotypes. If detected, the ability to include or exclude contributors should also be determined.

4.11.5.4 Results from the known references and casework-like samples should be evaluated to determine if any adverse effects are observed that may be attributable to extraction chemistry.

Some adverse effects include signal reduction, partial or complete inhibition, peak height imbalance, locus imbalance, preferential amplification, incomplete adenylation or other artifacts.

4.11.6 Implementation

When evaluating known reference and casework-like samples, if parameters developed during internal validation of the STR test kit fail to produce the expected outcome, the result(s) should be documented and a possible reason should be provided. If there are deviations from the expected outcomes, they should be documented and a possible reason provided. These results may dictate the need for additional testing and subsequent review of standard operating procedures and interpretation protocols.

4.12 Periodic Evaluation of Parameters, Protocols and Procedures

4.12.1 An informal evaluation for expected values (e.g., peak height ratio threshold, stutter percentages, average peak height) during data analysis can increase the laboratory's confidence in conclusions drawn from the data set collected during validation and ongoing testing (see 4.1.14). Possible variables to assess could include variation in reagent lots, in results from individual instruments, in negative controls and in changes in background noise levels.

4.12.2 The technical leader should perform periodic evaluations (e.g., at least every six months) after casework implementation for concordance of the results to the validation data and if necessary, create additional data that may guide adjustments to the workflow, thresholds, and interpretations.

NOTE Additional information on routine evaluation of laboratory protocols can be found in ANSI/ASB Standard 123.

4.12.3 Changes to critical reagents or critical equipment used for extraction, quantitation, amplification, or separation and detection may require additional testing to identify impacts on the STR kit performance and demonstrate that the results are concordant and reproducible. If not, additional validations studies should be performed and appropriate changes made to the standard operating procedures and interpretation guidelines.

Annex A
(informative)

Bibliography

The following bibliography is not intended to be an all-inclusive list, review, or endorsement of literature on this topic. The goal of the bibliography is to provide publications cited informationally, and publications relevant to the standard

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