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Best Practice Recommendations 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.

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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 401 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.

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Best Practice Recommendations 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 on a 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 or was not present in sufficient quantity in the aliquot that underwent PCR amplification.

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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
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 profile 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 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 dropout did not occur in mixed DNA sample profiles.

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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.

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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 needs to be conducted and criteria established and documented for each method used.

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.

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 used and protocols being developed.

4.2 Sensitivity

4.2.1 Requirement from ASB Standard 039

NOTE Refer to section 4.1.2 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."

4.2.2 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.

4.2.3 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 dropout is

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152 expected. This is informative for establishing and verifying a stochastic threshold and for the use of
153 probabilistic genotyping software.

154 **4.2.4 Samples Used in Experimental Method**

155 **4.2.4.1** Single-source DNA samples processed in-house with internally validated extraction and
156 quantitation methods should be used for these studies.

157 **4.2.4.2** DNA profiles with heterozygous genotypes having alleles that are multiple repeats apart
158 should be included to account for variation in PCR efficiency due to fragment size.

159 **4.2.4.3** *Pristine DNA:* A minimum of three unique single-source, high-quality, non-degraded DNA
160 samples should be used to perform serial dilutions across a range of DNA template quantities
161 higher and lower than expected to be processed by the laboratory. The serial dilution should
162 include the following:

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

165 b) lower concentrations that produce STR profiles where allele dropout is observed and allow for
166 identification of the lower limits of data analysis and interpretation.

167 **4.2.4.4** *Degraded/Inhibited DNA:* Following initial sensitivity studies using high-quality DNA,
168 additional studies should be performed to gain familiarity with the impact of common
169 environmental insults known to affect forensic DNA samples. The serial dilution should include the
170 following:

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

173 b) DNA degraded using a laboratory defined protocol.

174 **4.2.4.5** *Excess Non-specific DNA*: Following initial sensitivity studies using high-quality DNA for Y
175 Chromosome STR kits, additional studies should be performed to gain familiarity with the impact of
176 the presence of excess DNA from a female individual(s), often co-extracted in forensic DNA samples.
177 The serial dilution should include the following:

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

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

182 **4.2.5 Data Analysis and Results**

183 **4.2.5.1** Variation at different DNA template quantities should be characterized using average
184 peak height (APH), standard deviation, and coefficient of variance for every locus at each DNA
185 template quantity and quality for both homozygotes and heterozygotes. Upper and lower limits of
186 reliable interpretation along with optimal DNA input target/range should be defined as follows:

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

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

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

192 **4.2.5.2** Optimal input for pristine DNA can be a target or a range and will fall within the highest
193 and lowest concentration of DNA template from a single contributor that clearly distinguishes true
194 alleles (homo- and heterozygous) from artifacts, demonstrates intra- and inter-locus and dye color
195 channel balance, and usually results in complete profiles.

196 **4.2.6 Implementation**

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

199 a) amplification conditions as defined by validation studies;

200 b) DNA target input range;

201 c) positive control DNA input target;

202 d) stochastic threshold;

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

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

206 **4.3 Analytical Threshold**

207 **4.3.1 Requirement from ASB Standard 039** *(also available for public comment).*

208 NOTE Refer to section 4.1.3 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
209 *Repeat Profiling on Capillary Electrophoresis Platforms* (also available for public comment).

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

212 **4.3.2 Objective**

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

215 **4.3.3 Considerations**

216 **4.3.3.1** CE instruments should be set on a solid/secure platform that protects against potential
217 vibration or movement that could affect baseline noise.

218 **4.3.3.2** Caution should be taken when utilizing DNA input amounts lower and/or higher than the
219 laboratory's determined optimal DNA input range to set a single AT since this may increase the

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220 potential for loss of interpretable data in low level samples if that single threshold is applied. Based
221 on data generated using known DNA concentrations that straddle the optimum DNA input range,
222 laboratories may determine and apply different AT values (lower or higher than a single AT) to
223 decrease the loss of interpretable data.

224 **4.3.4 Samples Used for Experimental Method**

225 **4.3.4.1** Amplification negatives and samples using a range of DNA template quantities, including
226 the sensitivity study samples, should be used.

227 **4.3.4.2** Non-baseline peaks (e.g., dye-artifacts, other known artifacts, spectral pull-up, and allelic
228 peaks) should be removed from data prior to calculation.

229 **4.3.4.3** Samples displaying off-scale data or excessive artifacts should not be used.

230 **4.3.5 Data Analysis and Results**

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

232 **4.3.6 Implementation**

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

236 **4.4 Peak Height Ratio**

237 **4.4.1 Requirement from ASB Standard 039** *(also available for public comment).*

238 NOTE Refer to section 4.1.4 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
239 *Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

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

242 **4.4.2 Objective**

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

247 **4.4.3 Considerations**

248 **4.4.3.1** Heterozygous loci are required to perform peak height ratio calculations.

249 **4.4.3.2** Measurements of peak area may be substituted for peak height, and if selected, peak area
250 should be used for all studies.

251 **4.4.3.3** Laboratories should evaluate the effects of DNA quantity on PHR.

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

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

4.4.4 Samples Used for Experimental Method

NOTE Refer to Section 4.2.4 for samples to be used.

4.4.5 Data Analysis and Results

4.4.5.1 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.

4.4.5.2 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.

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

4.4.5.4 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).

4.4.5.5 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.

4.4.6 Implementation

4.4.6.1 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.

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

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284 a) a single universal minimal expected PHR threshold;

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

286 **4.4.6.3** The laboratory should document the frequency of events that fall below the minimum
287 threshold(s) in data sets described in 4.4.5.2.

288 **4.5 Stutter**

289 **4.5.1 Requirement from ASB Standard 039** *(also available for public comment).*

290 NOTE Refer to section 4.1.5 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
291 *Repeat Profiling on Capillary Electrophoresis Platforms* *(also available for public comment).*

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

294 **4.5.2 Objective**

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

298 **4.5.3 Considerations**

299 **4.5.3.1** Laboratories utilizing the manufacturer's recommendations should compare internally
300 validated stutter data to manufacturer's developmentally validated data to ensure consistency
301 across the range of loci and alleles represented in the STR kit.

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

304 **4.5.3.2** Laboratories should consider whether to use locus-specific or allele-specific stutter
305 values.

306 **4.5.3.3** Stutter percentages are expected to vary with the type of the repeat unit e.g., tri-
307 nucleotide vs. tetra-nucleotide.

308 **4.5.4 Samples Used for Experimental Methods**

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

311 **4.5.5 Data Analysis and Results**

312 **4.5.5.1** Stutter peaks should be characterized based on size and amplitude relative to an allelic
313 peak. [e.g., = (RFU of stutter artifact)/ (RFU of allele peak)].

314 **4.5.5.2** Off-scale data should not be used to measure stutter since the peak height data is not
315 accurate for the off-scale peak.

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4.5.5.3 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.

4.5.5.4 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.

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

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

4.5.5.5.2 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.

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

4.5.5.7 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.

4.5.5.8 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.

4.5.6 Implementation

4.5.6.1 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.

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4.5.6.2 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).

4.5.6.3 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.

4.6 Stochastic Threshold

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

NOTE Refer to section 4.1.6 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."

4.6.2 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 dropout 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 dropout 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.

4.6.3 Considerations

4.6.3.1 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.

4.6.3.2 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.

4.6.3.3 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.

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

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380 #### 4.6.4 Samples Used for Experimental Method

381 NOTE Refer to Section 4.2.4 for samples to be used.

382 #### 4.6.5 Data Analysis and Results

383 **4.6.5.1** Derive a stochastic threshold using DNA template inputs that exhibit allele drop-out such
384 that the sister allele is not visible and/or drop-out where the sister allele is below the previously
385 established analytical threshold.

386 **4.6.5.2** The stochastic thresholds may be defined using the following:

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

388 b) logistic regression;

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

390 d) plotting PHR vs average RFU.

391 **4.6.5.3** All loci/dye channels should be assessed for differences; however, a single threshold may
392 be implemented where these differences are not determined to be significant.

393 #### 4.6.6 Implementation

394 **4.6.6.1** The derived stochastic threshold should be verified using known case-type samples and
395 the performance evaluated.

396 NOTE If the threshold is set too high, true homozygotes are flagged as possible allelic dropout. If the
397 threshold is set too low, heterozygotes with allelic dropout would not be flagged.

398 **4.6.6.2** If the stochastic threshold does not reflect accurate determination of zygosity, 4.6.6.2.1
399 through 4.6.6.2.3 should be considered to improve the accuracy of the threshold(s)

400 **4.6.6.2.1** Reevaluate the original threshold(s) chosen. If a single threshold has been used,
401 consider multiple thresholds that better separate true homozygotes and heterozygotes where
402 dropout occurs.

403 **4.6.6.2.2** Consider use of another analysis method as described in 4.6.5.2 to define the
404 threshold(s).

405 **4.6.6.2.3** Analyze additional samples.

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406 **4.7 Mixtures**

407 **4.7.1 Requirement from ASB Standard 039** *(also available for public comment).*

408 NOTE Refer to section 4.1.7 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
409 *Repeat Profiling on Capillary Electrophoresis Platforms* *(also available for public comment).*

410 "The laboratory shall conduct studies utilizing mixed DNA samples having a range of DNA
411 ratios, DNA template input quantities, and numbers of contributors with varied degrees of allele
412 sharing expected to be interpreted by the testing laboratory."

413 **4.7.2 Objective**

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

418 **4.7.3 Considerations**

419 **4.7.3.1** This study should be performed after the sensitivity, peak height ratio, analytical
420 threshold, stochastic, and stutter studies have been completed and thresholds have been
421 established.

422 **4.7.3.2** The laboratory should design the study to incorporate one additional contributor beyond
423 the number expected to be interpreted during casework in order to demonstrate the ability to
424 assess the number of contributors and the limitations of the laboratory's mixture interpretation
425 protocol.

426 **4.7.3.3** To support the development of the laboratory's interpretation protocol, a study of the
427 number of contributors paired with several different genomic template quantities (at, above, and
428 below the levels expected to be interpreted by the testing laboratory) and qualities (e.g., degraded,
429 inhibited) should be performed.

430 **4.7.3.4** If a laboratory has validated or is currently planning the validation of an X or Y-STR test
431 kit, then studies should be planned for efficient test design and workflow decisions which can be
432 performed in parallel.

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

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

437 **4.7.3.5** The laboratory mixture interpretation protocol should consider the sample types in which
438 assumed contributors may be present and used to deduce the genotypes of potential contributors.

439 **4.7.4 Experimental Method**

440 **4.7.4.1** Mixture classes, based on the sex, relatedness, and number of contributors, should be
441 selected to include combinations of these factors to be interpreted by the laboratory.

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442 **4.7.4.2** For each mixture class, a mixture series should be created by generating a specified range
443 of ratios including template amounts at, above, and below the optimal input template amount from
444 single-source samples of known genotypes.

445 **4.7.4.2.1** The range of ratios should include minor alleles that fall below stochastic and analytical
446 thresholds.

447 **4.7.4.2.2** The range of ratios should be designed in order to identify the limits of major/minor
448 determination.

449 **4.7.4.2.3** Samples with a range of allele variants should be included to evaluate performance and
450 assess varying degrees of allelic overlap and the effect of alleles in stutter positions.

451 **4.7.4.2.4** Samples with a range of DNA qualities (e.g., degraded, inhibited) should be included.

452 **4.7.4.3** A minimum of two mixture series for each mixture class should be evaluated with each
453 sample tested at least in duplicate, with best practice testing in triplicate.

454 **4.7.5 Data Analysis and Results**

455 **4.7.5.1** Laboratories should apply filters and thresholds developed in the other studies (e.g.,
456 analytical threshold, stochastic threshold (if applicable), peak height ratio, stutter ratios) on data
457 produced for the mixture study.

458 **4.7.5.2** Each mixture series should be evaluated for the following parameters.

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

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

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

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

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

470 **4.7.5.3** Based on the results of the mixture studies, filters and thresholds should be adjusted as
471 needed.

472 **4.7.5.4** The data generated should be used to create interpretation methods and protocols
473 including:

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

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

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

479 **4.7.6 Implementation**

480 **4.7.6.1** Verification of the mixture protocols should be performed on mixed DNA samples of
481 known origin that are different from those in the initial validation studies used to establish the
482 protocol. See ANSI/ASB Standard 020 for more information.

483 **4.7.6.2** To perform replicability studies, a new dataset representative of the number of
484 contributors, DNA ratios and genomic DNA template quantities expected to be interpreted in
485 casework like mixtures should be analyzed using the interpretation methods and protocols.

486 **4.7.6.3** To perform reproducibility studies, at least two analysts should interpret the same data
487 and obtain the same possible contributor genotypes.

488 **4.8 Precision**

489 **4.8.1 Requirement from ASB Standard 039** *(also available for public comment).*

490 NOTE Refer to section 4.1.8 ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
491 *Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

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

495 **4.8.2 Objective**

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

498 **4.8.3 Considerations**

499 **4.8.3.1** Environmental factors may need to be considered prior to placement of CE instrument or
500 adjustments may need to be made to room conditions.

501 **4.8.3.2** DNA sizing variation greater than 0.5 nucleotides may result in incorrect allele calls for
502 alleles that are a single nucleotide apart.

503 **4.8.4 Experimental Method**

504 **4.8.4.1** Depending on the environmental conditions, the stability of ambient room temperature
505 and humidity should be monitored throughout the validation. The periodicity of monitoring should
506 capture the range of conditions under which casework is performed (e.g., running over the
507 weekends when the air conditioning may be turned off).

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

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

4.8.4.4 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.).

4.8.4.5 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.

4.8.5 Data Analysis and Results

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

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

4.8.5.3 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.

4.8.6 Implementation

4.8.6.1 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.

4.8.6.2 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.

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

4.9 Contaminations

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

NOTE Refer to section 4.1.9 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."

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542 **4.9.2 Objective**

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

546 **4.9.3 Considerations**

547 **4.9.3.1** Two categories of exogenous DNA should be evaluated. Contamination where an
548 unexpected source of DNA is detected and allele drop-in where an unexpected allele (possibly two)
549 are detected.

550 **4.9.3.2** Potential sources of contamination may include:

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

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

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

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

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

557 **4.9.4 Experimental Method**

558 **4.9.4.1** No additional studies are necessary for the contamination assessment. Data generated
559 from the other validation studies should be part of the contamination study.

560 **4.9.4.2** Additional controls (e.g., negative controls, or reagent blanks) may be processed to test
561 the system based on the variables described in the considerations above.

562 **4.9.5 Data Analysis and Results**

563 **4.9.5.1** All samples, including controls, should be evaluated for the presence of exogenous DNA
564 (including allele drop-in) which may originate from reagents, consumables, operator and/or
565 laboratory environment.

566 **4.9.5.2** Allele drop-in should be documented and a frequency of occurrence should be
567 determined.

568 **4.9.5.3** Drop-in rate may be estimated by comparing the number of drop-in observances to the
569 total amount of data or samples evaluated during the validation study.

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

572 **4.9.5.4** Contaminating allelic data, if present, should be characterized and attribution of source
573 attempted. The source of the contaminating data (co-processed samples, laboratory operator,
574 consumables etc.) may identify the point in the laboratory process that the contamination event

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

579 **4.9.6 Implementation**

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

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

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

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

585 d) contamination management and necessary corrective measures;

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

587 **4.10 Concordance**

588 **4.10.1 Requirement from ASB Standard 039** *(also available for public comment).*

589 NOTE Refer to section 4.1.1 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
590 *Repeat Profiling on Capillary Electrophoresis Platforms (also available for public comment).*

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

592 **4.10.2 Objective**

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

595 **4.10.3 Considerations**

596 **4.10.3.1** Concordance samples should be evaluated after conditions for casework sample analysis
597 have been established (e.g., target DNA amount, injection time and voltage, PCR cycles/volume).

598 **4.10.3.2** The samples used in the concordance study should reflect the type of STR test kit being
599 validated (e.g., autosomal STR typing test kit should use both male and female DNA, direct
600 amplification typing test kits should use buccal swabs, or stain punches; Y-STR test typing kit
601 should use male DNA).

602 **4.10.4 Experimental Method**

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

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

607 **4.10.5 Data Analysis and Results**

608 **4.10.5.1** A comparison of the observed alleles to the known values (if established) should be
609 performed.

610 **4.10.5.2** The presence of a discordant genotype result at a locus should be documented in the
611 final validation summary and a possible reason provided.

612 **4.10.6 Implementation**

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

618 **4.11 Known References and Casework-like Samples**

619 **4.11.1 Requirement from ASB Standard 039** *(also available for public comment).*

620 NOTE Refer to section 4.1.10.1 of ASB Standard 039, *Standard for Internal Validation of Human Short Tandem*
621 *Repeat Profiling on Capillary Electrophoresis Platforms* *(also available for public comment).*

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

627 **4.11.2 Objective**

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

631 **4.11.3 Considerations**

632 **4.11.3.1** This study may be used to assess relative STR test kit concordance and performance
633 through comparison with the previously validated STR kit data.

634 **4.11.3.2** The new STR test kit and associated standard operating procedures should be robust and
635 generate reproducible results for pristine samples and consistent results for challenged samples.

636 **4.11.3.3** Laboratories should determine if the extraction chemistry procedure(s) used introduce
637 inhibitors that interfere with the amplification of DNA with the STR test kit.

638 **4.11.3.4** DNA samples that have been previously extracted, quantified, and stored should be re-
639 quantified before use in these experiments, as sample quantity and quality may be compromised
640 over time.

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641 **4.11.3.5** Inclusion of previously genotyped, anonymized samples should be included during the
642 evaluation of procedures.

643 **4.11.4 Experimental Method**

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

649 a) Known reference samples should include:

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

651 2) sufficient template to conduct the planned study.

652 b) Casework-like samples should include:

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

654 2) inhibited samples;

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

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

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

659 **4.11.5 Data Analysis and Results**

660 **4.11.5.1** Using the parameters and standard operating procedures established during internal
661 validation of the STR kit, data should be assessed for reproducibility and consistency.

662 **4.11.5.2** STR typing results should be compared to any previous results. Samples should be
663 evaluated for potential contamination or allele drop-out through comparison to expected profile
664 genotypes.

665 **4.11.5.3** To evaluate the standard operating procedures, STR typing results for samples
666 containing more than one contributor should be compared to reference DNA profiles to determine
667 the ability to detect possible contributor genotypes. If detected, the ability to include or exclude
668 contributors should also be determined.

669 **4.11.5.4** Results from the known references and casework-like samples should be evaluated to
670 determine if any adverse effects are observed that may be attributable to extraction chemistry.
671 Some adverse effects include signal reduction, partial or complete inhibition, peak height
672 imbalance, locus imbalance, preferential amplification, incomplete adenylation or other artifacts.

673 **4.11.6 Implementation**

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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 Assessment of Parameters, Protocols and Procedures

4.12.1 An informal assessment 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 assessments (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.

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.

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697 **Annex A**
698 (informative)

699 # Bibliography

700 The following information provides a list of the literature resources:

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705 3] ASB Standard 038, *Standards for Internal Validation of Forensic DNA Analysis Methods*, First
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716 8] Butler, John M. *Advanced Topics in Forensic DNA Typing: Interpretation*. Academic Press, San
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[6] Available from: https://docs.wixstatic.com/ugd/4344b0_6782472e073442ec877085584aaffa36.pdf
[7] Available from: https://www.iso.org/standard/62341.html

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725 threshold and DNA levels on rates of allele and locus drop-out." *Forensic Science*
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727 14] SWGDAM. *SWGDAM Contamination Prevention and Detection Guidelines for Forensic DNA*
728 *Laboratories*[8].

729 15] Wickenheiser, R. and Farrell, L. "Collaborative versus traditional method validation approach:
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