Scientific Working Group on DNA Analysis Methods

Module for Modified Rapid DNA Internal Validation Guidance for Database, Known or Casework Reference Samples

The following are minimum requirements and best practice guidelines to help laboratories design internal validation experiments as required by Forensic Standard 8.6 and Database Standard 8.7 for the use of Rapid DNA systems for modified Rapid DNA Analysis on database, known or casework reference samples. Additional tests and samples may be included as determined by the laboratory technical leader. It is recognized that laboratories may have validation experimental approaches that differ from those listed in this document. Such approaches can be utilized as long as they generate enough empirical data to substantiate the findings and support the laboratory's procedures.

This Modified Rapid DNA Internal Validation Guidance Module should be used in conjunction with the SWGDAM Validation Guidelines for Forensic DNA Analysis Methods: Overview Document available at https://www.swgdam.org/publications.

1. General Considerations

1.1 The studies within this module are not synchronized to the FBI QAS; instead, they are presented in a suggested order to conserve resources such as time, reagents, samples and consumables.

1.2 For the purposes of this validation guidance module, when a laboratory chooses to transfer and/or analyze the generated raw data outside the set parameters of the manufacturer provided Expert System, all data generated should be imported into such software and evaluated therein to ensure that the appropriate interpretation parameters are established. It

is possible to observe some data variations between software packages due to differences between applied thresholds, baseline smoothing, bins, panels and/or sizing algorithms.

1.3 In accordance with NDIS Operational Procedures Manual Section 4.6.1, the modified analysis of Rapid DNA System generated data should only be performed if the Rapid DNA System utilizes **an NDIS approved** PCR chemistry and documentation of such NDIS approved chemistry is available. See NDIS Operational Procedures Manual Section 4.6.1.

2. Contamination Study

2.1 *Study Purpose:*

To establish the detection of exogenous DNA in Rapid DNA reagents and consumables.

2.2 *Study Design and Considerations:*

Rapid DNA reagent cartridges are single use/'self-contained' items, therefore, instances of environmental and/or user introduced reagent contamination are greatly reduced. Nevertheless, reagent contamination during the manufacturing process and sample-to-sample contamination due to faulty cartridge lots or capillary cross-talk (depending on the instrument used) remain a possibility. To assess contamination of Rapid DNA components, the following is recommended:

2.2.1 Single sample cartridges: In order to assess contamination, at least ten consecutive runs alternating sample and empty cartridge/clean swab, corresponding to five blanks, should be performed. The initial run should be conducted using a sample rather than a blank.

2.2.2 Multi-sample cartridges: A minimum of three consecutive runs alternating blanks (empty wells or clean swabs) is recommended. This approach will generate anywhere between 6 and 11 blanks (depending on the instrument/cartridge being used) and demonstrate if cross-contamination between runs and/or samples exists.

2.2.3 If using swabs in place of an empty sample well, it will be unclear whether any observed contamination originates from the swab itself, the reagents, or as a consequence of the data generation process. If this is the case, the study should be repeated with empty wells or different swabs.

2.2.4 It is recommended that a laboratory performs a contamination assessment for each new lot of cartridges received throughout the validation to ensure that the chemistry and cartridges are free of contaminants.

2.2.5 Contamination studies can be performed in combination with other studies.

2.3 *Study Outcome:*

At the completion of the contamination study, the laboratory should be confident that the Rapid DNA process and the cartridges within the tested lot(s) are free of contaminants (e.g., no peaks detected above the analytical threshold, no peaks present in a marker bin).

3. Sensitivity and Stochastic Studies

3.1 *Study Purpose:*

Conventionally, sensitivity studies are conducted to define the dynamic range, limit of detection, peak height ratios, and signal-to-noise thresholds associated with an instrument or assay. Assessing the dynamic range of a Rapid DNA instrument for reference sample applications is not necessary (or viable) as the process currently does not allow for the laboratory to pre-determine the sample input. A laboratory could, however, perform similar sensitivity assessments to establish general expectations with regards to the results from a Rapid DNA instrument.

3.2 *Study Design and Considerations:*

Based on the intended use of the Rapid DNA system, one or more of the following options can be performed to assess the limit of detection of the Rapid DNA instrument. **NOTE:** The options provided are not equivalent. A laboratory can choose to perform one or more of the listed approaches. If choosing to perform just one, Option 1 is the recommended choice as it will provide the best sensitivity range for Rapid DNA instruments.

3.2.1 <u>Option 1:</u>

A modified sensitivity study can be performed by applying decreasing volumes of a given biological fluid (e.g., saliva) to a swab to establish the extraction

efficiency of the instrument and determine if the expected decreasing linear response is obtained. The results obtained will be dependent on the donor.

3.2.2 Option 2:

Buccal reference swabs are typically obtained by rubbing the inside of the cheek with a sterile swab. A study aimed at determining the number of collection 'swipes' required to generate a successful profile could be performed to establish the limitations of the instrument.

NOTE: Varying results are expected based on the donor, type of swab used for collection, and the fact that the laboratory ultimately has no control over how many swipes were performed during the collection.

3.2.3 Option 3:

Though Rapid DNA systems are designed to work optimally with non-extracted DNA, a laboratory could perform a conventional sensitivity assessment by adding pre-extracted, pre-quantified volumes of DNA on individual swabs and processing these using the Rapid DNA instrument being validated to determine if the expected linear response to decreasing amounts of input material is obtained. This study could also provide an assessment of instrument saturation, if applicable.

3.2.4 Option 4:

Alternatively, a cell counting instrument (hemocytometer) can be used to estimate the number of cells deposited in a swab or into a particular biological fluid volume. A decreasing number of cells can then be loaded on a swab and into the instrument to establish the limit of detection.

3.3 *Study Outcome:*

At the completion of these experiments, the laboratory should be able to utilize the data generated to gauge the instrument's limitations and establish general expectations for Rapid DNA analysis in comparison to other technologies. Data generated could be used to help establish analytical and stochastic thresholds to be used if a laboratory chooses to transfer and/or analyze the generated raw data outside the set parameters of the manufacturer provided Expert System. Data can also guide best practice for directed swab collections (e.g., optimal number of swipes).

3.3.3 If a laboratory chooses to transfer and/or analyze the generated raw data outside the set parameters of the manufacturer provided Expert System, the data generated for these studies can be combined with that obtained from other studies to evaluate stutter, baseline noise, peak resolution, and other interpretation thresholds.

4. Known Sample Concordance Studies

4.1 *Study Purpose:*

To demonstrate that the Rapid DNA instrument is capable of precise allele sizing and allele calling.

4.2 *Study Design and Considerations:*

4.2.1 A minimum of ten unique known samples should be evaluated for concordance using already validated system-generated data as a point of comparison.

4.2.2 When possible, the selection of samples should include heterozygous genotypes which include alleles differing by one base and other rare allelic occurrences (e.g., tri-alleles).

4.2.3 Some Rapid DNA cartridges might contain chemistries that will generate data for markers that are not contained in commonly used multiplexes. It is therefore recommended that some or all of the exemplars used in this study are also used in the repeatability studies to enable data comparison and concordance determination between samples.

4.3 *Study Outcome:*

The percent success of the Rapid DNA system should be determined and measured against the results obtained from methods already validated for use in the laboratory. If a laboratory chooses to transfer and/or analyze the generated raw data outside the set parameters of the manufacturer provided Expert System, the data generated as part of this study should be merged with data from other studies to determine stutter and other interpretation thresholds.

5. Repeatability and Reproducibility

5.1 *Study Purpose:*

To establish if the Rapid DNA system can consistently generate accurate genotype results.

5.2 *Study Design and Considerations:*

When possible, the selection of samples for this study should include heterozygous genotypes which include alleles differing by one base, and other rare allelic occurrences (e.g., tri-alleles).

5.2.1 Repeatability: This metric will establish if the instrument can consistently generate the same genotype from replicates of the same sample. To demonstrate repeatability a minimum of five unique samples should be processed at least three times by the same analyst.

5.2.2 Reproducibility: To establish reproducibility, replicates of at least three unique samples should be processed by at least two operators/analysts in at least two separate rapid runs.

NOTE: Because the amount of input DNA is not determined, differences in peak heights (and even occasional drop-outs) are to be expected.

5.3 *Study Outcome:*

5.3.1 Repeatability and reproducibility data will establish the accuracy and robustness of the Rapid DNA system.

5.3.2 If a laboratory chooses to transfer and/or analyze the generated raw data outside the set parameters of the manufacturer provided Expert System, suitable data generated as part of these studies can be combined with data from other studies to determine stutter and other interpretation thresholds.

6. Mixture Studies

6.1 *Study Purpose:*

To ensure that the Rapid DNA software, when challenged with mixed reference samples, can consistently and accurately ‘flag’ loci and/or profiles suspected of having originated from a mixture of genetic material (e.g., from a contamination event).

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178 6.2 *Study Design and Considerations:*

179 6.2.1 Mixture samples should be run simultaneously using conventional methods to
180 establish a limit of detection comparison between the Rapid DNA instrument and
181 conventional methods.

182 6.2.2 At a minimum, two sets of two-person mixtures should be evaluated. Within the
183 scope of the assay's sensitivity, the following approximate ratios should be used:
184 20:1, 15:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:15 and 1:20.

185 6.2.3 It is suggested that one or more of the following approaches are used for mixture
186 preparation:

187 6.2.3.1 Pre-quantifying the genetic material and spiking swabs with amounts
188 corresponding to the ratios listed above.

189 6.2.3.2 Volume-based ratios using the same fluid type (e.g., saliva – saliva) and/or
190 different fluid types (e.g., saliva – blood).

191 6.2.3.3 Although less commonly used, alternative mixture preparation approaches
192 that are more 'realistic' could be evaluated if deemed appropriate. For
193 example:

194 6.2.3.3.1 Swab and spike: Collect a reference swab by swabbing the inside of a
195 donor's cheek, then spike the collected swab with a set volume of a
196 biological fluid from a second donor.

197 6.2.3.3.2 Swab and swab: Donors 1 and 2 swab the inside of their respective
198 cheeks using the same swab.

199 6.2.3.3.3 Naturally occurring mixtures: Swab inside cheek of Donor 1 after
200 he/she has had intimate contact with Donor 2.

201 6.3 *Study Outcome:*

202 6.3.1 Upon completion, the laboratory should be able to establish if, and to what extent
203 (mixture ratio), the Expert System software can accurately and reliably identify
204 and 'flag' mixed DNA profiles.

205 6.3.2 Based on the results of the mixture studies, the laboratory may determine suitable
206 analysis parameters that can be utilized by a qualified analyst to interpret

presumed single source/reference samples whose profiles resulted to be a mixture and assign a major/minor contributor if possible and when appropriate.

7. Determination of analysis parameters for modified Rapid DNA purposes of database, known or casework reference samples

7.1 When a laboratory is validating modified Rapid DNA analysis, a general analysis method with the appropriate bins and panels should be used to analyze the data initially. Once the appropriate thresholds are established, the data should be reanalyzed with the empirically established parameters to ensure proper results.

7.2 Precision

7.2.1 Accurate allele calling is in part dependent on precise amplification kit ladder sizing. Demonstrating the precision of ladder sizing for any particular amplification chemistry instills confidence regarding the reliability of allele calls when using the tested kit for sample processing. The precision parameter is determined by evaluating the standard deviations of the allele sizes generated by multiple ladder runs/injections. Ideally, the maximum standard deviation of any allele at a locus would not be greater than ~0.167 bp, to ensure that 3 standard deviations are less than 0.5 bp from an adjacent allele in either direction (0.167*3 = 0.5).

7.2.1.1 To establish precision, export run generated ladder files, and any virtual ladder files utilized throughout the validation of the instrument and import such files into the data analysis software of choice. After analysis, the size data should be exported into Microsoft Excel (or similar software) to determine the standard deviation of the sizes obtained for each allele.

7.3 Analytical Threshold (Noise)

7.3.1 The analytical threshold is the lowest relative fluorescent unit (RFU) value at which one would reliably expect a true allele peak to be distinguished from background noise. To establish this threshold, a laboratory needs to measure the background noise, associated with both the chemistry and instrumentation.

7.3.1.1 To establish the peak amplitude threshold, the laboratory should import all the data generated throughout the studies (except for those associated with sensitivity and mixture studies). The laboratory should then set the peak amplitude threshold within the software of choice to the lowest setting allowed to enable the labeling of all peaks within a particular sample. The peaks corresponding to true alleles, stutter, or known artifacts (spectral bleed-through, incomplete adenylation) should be manually deleted, leaving only data associated with noise, prior to exporting the data (allele calls, size, height) to Microsoft Excel (or similar software) for analysis.

NOTE: Blank samples should not be used for analytical threshold determination as they are known to have a lower baseline than that observed for electropherograms with actual data.

7.3.1.2 Once all known peaks are removed from the data, the laboratory should calculate the minimum, maximum, average noise peak heights and associated standard deviations for each marker and dye channel (as applicable to the limitations of the analysis software), or as a global setting.

7.3.1.3 A laboratory should then apply the newly determined analytical threshold(s) (Bregu, 2013) to the analysis method within the chosen data analysis software to analyze all data generated via the instrument being validated and calculate the remainder of the metrics.

7.4 Peak Height and Peak Height Ratio

7.4.1 To determine the average peak height, the laboratory should export the analyzed data to Microsoft Excel (or similar software) and average all peak heights observed within each locus. The average peak heights between loci should be used to establish inter-locus variability or the overall balance of the chemistry being utilized.

7.4.1.1 To establish the expected peak height ratios, data corresponding to heterozygous loci should be used. Calculations are performed by dividing the lowest peak of a heterozygous pair by the highest peak and expressing it as a percentage (Kelly, 2012).

7.4.2 A laboratory should extrapolate variations in peak height ratios based on average peak heights using the data calculated above. If desired, these variation trends can be incorporated into the data interpretation process.

7.5 Stutter

7.5.1 Stutter calculations are used to assist analysts with the determination of true alleles versus stutter artifact peaks should the data be analyzed using a software package other than the manufacturer provided Expert System.

7.5.1.1 To determine stutter percentages, peaks located at *n-0.5, n-1, n-2, n+1,* and *n+2* repeat units from the true allele call are identified as stutter and used for the analysis. The percent stutter for each position and locus is calculated by dividing the height of the stutter peak by the corresponding true allele (Brookes, 2012).

7.5.2 Based on the results obtained, a laboratory can apply a global stutter filter to the analysis method, assign locus specific or allele specific stutter filters to aid with data interpretation.

7.6 Stochastic Threshold

7.6.1 Stochastic thresholds are used to determine the RFU value below which it is reasonable to assume the sister allele of a heterozygous pair may have dropped out.

7.6.1.1 For this threshold, sensitivity data and/or all data in which one of two peaks in a heterozygous locus has dropped out can be used. To calculate the stochastic threshold, for example, determine the highest peak height of the heterozygous peak observed when the partner drops-out (Gill, 2009). This threshold can be established per locus, dye channel, or as a more general overall profile determination.

8. Additional Comments

8.1 Rapid DNA system manufacturers provide the users with different sample cartridge options. There are cartridges designed to process high quality/quantity samples and cartridges intended to work best with samples of lesser quality/quantity. Though the

297 experimental approach for validating both types of cartridges is the same, the
298 determination of software analysis metrics to allow modified Rapid DNA analysis should
299 be empirically established separately. The number of cycles used and sample
300 concentration modules, among other factors, can have a significant impact on the noise
301 levels, the peak height detected, peak height ratio, and stutter percentage determinations.
302 8.2 A laboratory can apply data generated from one experiment to another. For example, a
303 laboratory may choose to perform their repeatability/reproducibility studies using the
304 same samples utilized in the 'Known Sample Concordance Studies' to obtain data that
305 would suffice the requirements for both studies.
306 8.3 A laboratory with multiple Rapid DNA instruments of the same make and model must
307 performance check each individual instrument prior to its implementation. At a
308 minimum, a positive control run must be performed to ensure the proper operation of the
309 instrument. Data pertaining to all reference or control samples used for such runs should
310 be verified for genotype accuracy.
311 8.4 Rapid DNA cartridges are considered critical reagents and an evaluation is required for
312 each new lot or shipment of reagents.
313 8.5 A laboratory may choose to perform additional studies or use additional samples based
314 on the results of the individual studies listed above. For example, if a sample fails or if
315 non-expected results are obtained, an increased number of samples for the affected
316 studies are appropriate.

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References:

Bregu, J., et al. (2013) *Analytical Thresholds and Sensitivity: Establishing RFU Thresholds for Forensic DNA Analysis*. J. Forensic Sci. 58:120-129.

Kelly, H., et al. (2012) *Modelling heterozygote balance in forensic DNA profiles*. FSI: Genetics 6:729-734.

Brookes, C., et al. (2012) *Characterising stutter in forensic STR multiplexes*. FSI: Genetics 6:58-63.

Gill, P., et al. (2009) *The low-template (stochastic) threshold - Its determination relative to risk analysis for national DNA databases*. FSI: Genetics, 3:104-111.