GUIDELINE FOR INTERNAL VALIDATION / VERIFICATION OF VARIOUS ASPECTS OF THE DNA PROFILING PROCESS

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1. AIMS

One of the requirements of the Standard EN ISO/IEC 17025 [1] is that methods used in testing laboratories are validated/verified. As EN ISO/IEC 17025 only determines a general standard, it is the role of the experts in a given field to give more detailed recommendations.

This guideline aims to provide recommendations and advice such as parameters for assessment that may be used by Member laboratories of ENFSI and other forensic DNA laboratories to improve and harmonise their working practices regarding the internal validation/verification of various aspects of the DNA profiling process.

Internal validation studies provide documented evidence of the forensic unit's competence regarding the methods used and are a source of information regarding the parameters for monitoring the validity of results. Further details for monitoring the validity of results are described in the ENFSI Best Practice Manual for Human Forensic Biology and DNA Profiling [2] and the ENFSI Quality Assurance Programme for DNA Laboratories [3].

This Guideline reflects the scientifically accepted principles and applications at the time of creating.

2. SCOPE

This Guideline is aimed at experts in the field and assumes prior knowledge in the discipline. The Guideline is limited to DNA profiling of STR markers and only applies to internal validation/verification of a method, kit, instrument or software that has undergone developmental validation.

The ENFSI DNA Working Group has agreed upon the minimum internal validation/verification criteria as laid down in this document. This document can only serve as a recommendation because each DNA testing laboratory has its own practices and workflows. There might be other approaches to validate/verify a certain protocol, instrument or software [4–9].

In this Guideline, a number of performance characteristics related to method validation are listed. These are based on the definitions in references [4–9]. For each step in the DNA analysis process there are suggestions regarding performance characteristics to be considered in the internal validation/verification (Chapter 5).

3. TERMS AND DEFINITIONS

In this guideline the word "shall" is used where there is a corresponding requirement in the EN/ISO 17025 QA Standard, "should" is used as a recommendation where good practice is expected and "may" where it is permissive / advisory. Can/could is used to signify a possibility.

Bias
Bias refers to the proximity between the measurement value and the true value or alternatively, a reference value. For a qPCR assay, the reference value may be the given concentration of a DNA reference material or of a DNA of known concentration commercially provided and verified (e.g. positive control of PCR).

Carry-over and crosstalk
Carry-over and crosstalk are instrument related artefacts and refer to the fact that one sample being analysed may affect the analysis of other samples.

Concordance Studies
Evaluation of correct allele calling for different kits, chemistries or instrumentation.

Contamination
The undesirable introduction of a substance to an item at any point of the forensic process.

Note 1 to entry: This includes undesirable transfer of a substance within an item or between items (also referred to as cross-contamination).

Developmental validation
The demonstration of the fitness for purpose for the intended use of a method, kit, instrument or software by the manufacturer, technical organization, academic institution, government laboratory, forensic DNA testing laboratory or other party. Developmental validation must precede internal validation of a novel methodology for forensic DNA profiling.

Internal validation
Internal validation is conducted by each forensic DNA testing laboratory and is the in-house demonstration of the reliability and limitations of a method, kit, instrument, or software that has undergone developmental validation. Prior to using a procedure for forensic applications, a laboratory shall conduct and document the outcome of internal validation studies to demonstrate that the method, kit, instrument or software is fit for purpose.

Limit of detection (LOD)
LOD refers to the smallest amount of DNA that with a given probability can be confidently detected by the method.

Note 1 to entry: the LOD of the method can be evaluated by using dilution series of cells/DNA.

Limit of quantification (LOQ)
LOQ refers to the lowest DNA concentration that can be determined with acceptable uncertainty.

Note 1 to entry: the LOQ of the method can be evaluated by using dilution series of cells/DNA.

Matrix effects
Matrix effects refer to the impact such as PCR inhibition from relevant background materials (sometimes called substrates), e.g. denim or cigarette butts. PCR inhibition can also come from the sampling process or DNA extractions chemistry e.g. SDS. Additionally, PCR inhibitors can derive from the biological material itself e.g. hematin in blood.

Mixtures
Mixtures occur when a sample includes DNA from more than one individual. In forensics, mixture studies with cells/DNA from different contributors need to be included in internal validation to establish mixture guidelines, determine minor/major contributions, evaluate separation of different cell types etc.

Precision
Precision refers to the variation between different measurements for a method/instrument. Precision can be evaluated as repeatability or reproducibility.

Recovery
Recovery may also be called yield, and refers to the fraction of the starting material that is made available for analysis. Recovery can be used as a measure of the efficiency of a DNA extraction method.

Repeatability
Repeatability is the variation in results obtained when one person performs the method several times in an identical way.

Reproducibility
Reproducibility is the variation in results under somewhat different conditions e.g. two persons perform the same method or different instruments are used or one person at a different time.

Ruggedness
Ruggedness refers to the effect of consciously made changes on the performance of a method or instrument e.g. changes in incubation time or temperature.

Trueness
Trueness relates to the accuracy of the method. Trueness may be tested by investigating bias or recovery.

Verification
Verification is the provision of objective evidence that a given item, process, measurement procedure, material, compound, or measuring system fulfills specified requirements.

Note 1 to entry: Internal validation should be performed when implementing a new method or installing a new instrument in the laboratory and perform verification, for example, when installing a new instrument of the same model as others already in use at the facility.
Note 2 to entry: Changes to an already validated method have to be evaluated in order to determine if they need internal validation.

Working range
Working range refers to the range of DNA concentrations that can be analysed with the method with acceptable uncertainty.

Note 1 to entry: the working range of the method can be evaluated by using dilution series of cells/DNA.

4. BACKGROUND

This guideline aims to give guidance for internal validation/verification of the methods, instruments and software of the DNA analysis process, including suggestions of which performance characteristics to consider, see Table 1.

5. GUIDELINES

5.1 General Guidelines

The developmental validation report shall be available for the laboratory performing internal validation. If developmental validation has not been carried out, then this shall be carried out for example by the laboratory or another competent body.

The laboratory should formulate an internal validation/verification plan specifying the experimental design, the samples to be tested, equipment to be used, acceptance criteria and data evaluation. Equipment to be used shall be calibrated and competent staff shall be appointed to carry out the validation. Once the internal validation/verification experiments are completed the results and conclusions should be available in an internal validation/verification report. A statement on the validity of the method after completion of the validation shall be issued to the laboratory personnel. Records of all the above shall be kept for reference purposes.

Before implementing any new method, a specific evaluation for risk assessment according to EN ISO/IEC 17025 shall be performed. Monitoring the validity of results may also be planned using validation results as reference.

Whatever the criteria are to validate a method, kit, instrument or software, they shall provide evidence that the procedures and instrument are fit for purpose for the intended use in accordance with EN ISO/IEC 17025.

A new (or change in) method, kit, instrument or software with a potential influence on the results, requires an internal validation/verification.

The validation of an automated or semi-automated method, should also evaluate sample tracking, data storage and evaluation of input/output files.

The validation should include a relevant setup of a number of samples representative of those that are going to be analysed /examined in order to be able to determine if the method is fit for intended purpose. The setup can vary depending on the complexity of the technique and its intended use. Five different samples in triplicate is usually recommended but when analysing a dilution series of cells/DNA having more steps fewer replicates may be as informative.

Proficiency test samples, certified reference standards, reference DNA of known concentration commercially provided and verified and mock samples representative of those that are going to be analysed /examined can be part of the validation experiments.

The performance of a new method, kit, instrument or software may be compared to the one already in use in the facility.

Any modification should be checked in the overall process of DNA analysis.

Each laboratory should implement the new method in accordance with its own standard operating procedures.

Table 1: Performance characteristics to consider investigating in internal validation/verification studies of different steps in the DNA analysis process

Performance characteristics	DNA Extraction and Purification	Quantification Kit	STR Kit	qPCR/PCR instrument	Capillary Electrophoresis	MPS STR kit	MPS instrument	Rapid DNA	Analysis Software
LOD/LOQ/ Working range	-	√	√	√	√	√	√	√	-
Trueness (Bias/Recovery)	√	√	-	-	-	-	-	√	-
Precision (Repeatability/ Reproducibility)	√	√	√	√	√	√	√	√	√
Ruggedness	√	√	√	-	√	√	-	√	√
Concordance	-	-	√	-	√	√	√	√	-
Matrix effects	√	√	√	-	-	√	-	√	-
Mixtures	√	√	√	-	√	√	√	√	√
Contamination/ Carry-over/Crosstalk	√	√	√	-	√	√	√	√	-

5.2 DNA Extraction and Purification

The following performance characteristics should be evaluated /determined:

DNA recovery for a DNA extraction method should be evaluated by applying relevant cell type(s)/biological material(s) of known source/ origin (blood, semen, saliva…). This can be done with a dilution series of cells. When validating a differential lysis method, mixtures of relevant cell types and mixtures ratios should be included.

It is not possible to test all different matrices/substrates that may be found on a crime scene. However, representative matrices/substrates should be included in the validation in order to evaluate relevant matrix effects including PCR inhibition.

Repeatability, one operator performs DNA extraction on replicates from the same sample. For reproducibility, DNA extraction is e.g. performed by another operator or by the same operator on a different instrument or same instrument over a period of time. In these experiments replicates from the same samples as applied in the repeatability test can be used.

Ruggedness can be evaluated if deemed relevant, e.g. if a method makes use of time intervals for incubation or to evaluate stability of the DNA extracts.

Contamination should be evaluated using reagent blanks in the experimental design. Samples with known genotypes can also be used when evaluating contamination.

If possible, the performance of the new extraction method may be compared to the method already in use in the facility. For example, the quality of the STR profile may be evaluated.

5.3 Quantification Kit

The following performance characteristics should be evaluated /determined:

LOD, LOQ and working range should be determined using a range of DNA quantities in replicates.

Matrix effects should be evaluated using DNA extracted from different matrices and biological material. The correlation between DNA concentrations and DNA profiling results should be evaluated.

Trueness can be evaluated comparing the results from the kit to a reference value (e.g. DNA reference material or a DNA of known concentration commercially provided and verified), giving information regarding the method bias.

Repeatability, the same sample (e.g. a DNA extract or reference DNA) should be analysed in replicates within the same run. For reproducibility, replicates from the same sample can e.g. be analysed on a separate run on the same instrument or on another instrument or by another operator.

Ruggedness can be evaluated, e.g. to determine the stability of a prepared master mix or DNA standard.

When validating a quantification kit with a Y target, single source samples (male and female) as well as mixed samples (male/female) with different proportions should be evaluated.

If a degradation index is to be applied in casework, the correlation between this index and corresponding DNA profiling results should be evaluated.

Occurrence of contamination from the PCR setup should be evaluated. For automated PCR setup, this can be done by distributing positive samples and blanks in a check board pattern.

5.4 STR Kit

The following performance characteristics should be evaluated /determined:

LOD, LOQ and the working range, should be determined using a range of DNA quantities in replicates.

Matrix effects should be evaluated using extracted DNA from relevant matrices and biological material. The correlation between given DNA concentrations and STR profiling results should be evaluated.

Repeatability, the same sample should be analysed in replicates in the same run. For reproducibility, replicates from the same sample can e.g. be analysed on a separate run on the same instrument or on another instrument or by another operator.

Ruggedness can be evaluated for example to determine the stability of a prepared master mix or the stability of the amplified material or reduced volume amplification.

Concordance should be evaluated comparing genotypes with those generated using other kits or analysing samples/reference DNA with known STR profiles (e.g. external proficiency test left-over samples and/or certified reference materials). Mixtures in different proportions should be included to investigate minor/major contributions. Evaluation of stutter ratios, artefacts and analysis of intra and inter locus balance should also be performed.

Occurrence of contamination from the PCR and capillary electrophoresis (CE) setup should be evaluated. For automated PCR setup, this can be performed by distributing positive samples and reagent blanks in a check board pattern. Samples with known genotypes can also be used when evaluating contamination.

5.5 qPCR / PCR Instrument

The following performance characteristics should be evaluated /determined:

For a new qPCR/PCR instrument model a number of samples, adapted to the format of the machine, previously quantified/profiled should be repeated using the new instrument model. This should include a dilution series of DNA in order to evaluate LOD, LOQ and working range.

Precision, the samples should be analysed in replicates to evaluate repeatability and on more than one run to evaluate reproducibility.

A homogeneity check between wells in the heating block should be performed either by a temperature control or by analysing replicates.

For a new qPCR/PCR instrument of the same model as others already in use at the facility, a certificate from the manufacturer (technical performance check) should be available and a verification of precision as well as temperature homogeneity check should be performed.

5.6 Capillary Electrophoresis Instrument

The following performance characteristics should be evaluated/determined:

LOD, LOQ and working range should be determined using a range of amplified DNA quantities in replicates.

Concordance should be evaluated comparing genotypes with those generated using other instruments or analysing samples/reference DNA with known STR profiles. Mixtures in different proportions should be included to investigate minor/major contributions.

Repeatability of the instrument should be such that alleles fall within a ± 0.5 bp window around the measured size for the corresponding allele in the allelic ladder. For reproducibility, the precision between runs or between capillaries can be investigated.

Ruggedness can be evaluated for example to determine the effects of different settings, the stability of the polymer or the maximum number of runs with a capillary.

Carry-over (between injections) and crosstalk (within injections) should be evaluated with relevant samples.

For a new capillary electrophoresis instrument that is of the same model as others already in use at the facility, a certificate from the manufacturer (technical performance check) should be available and evaluation of LOD, LOQ and working range should also be performed.

5.7 Massively Parallel Sequencing (MPS) STR Kit

Validation of MPS STR kits implies the same parameters as STR kits for capillary electrophoresis (CE), although working range, LOD and LOQ need not only to take the starting material into account but also the quantity of pooled libraries (number of samples in sequencing or the concentration of the pooled library).

5.8 Massively Parallel Sequencing (MPS) Instrument

The following performance characteristics should be evaluated/determined:

LOD, LOQ and working range should be determined using a range of amplified DNA quantities in replicates.

Concordance should be evaluated comparing genotypes with those generated using other instruments (e.g. CE or MPS) or analysing samples/reference DNA with known STR profiles. Mixtures in different proportions should be included to investigate minor/major contributions.

Repeatability, the same sample should be analysed in replicates in the same run. For reproducibility, replicates from the same sample can for example, be analysed on a separate run on the same instrument or on another instrument.

Contamination should be evaluated using reagent blanks in the experimental design.

For a new MPS instrument that is of the same model as others already in use at the facility, a certificate from the manufacturer (technical performance check) should be available and evaluation of working range should be performed.

5.9 Rapid DNA

Validation of a Rapid DNA system implies the same performance characteristics as for a DNA extraction method, STR kit and CE instrument all together.

5.10 Analysis Software

The following performance characteristics should be evaluated/determined:

Data generated from any software (in house made data transfer tool/spreadsheets, commercial DNA analysis software) may be compared to results generated with a previously used method.

The software's precision (e.g. new functionalities) using the same input data (repeatability) as well as having several operators evaluating the same data set (reproducibility) should be evaluated.

Ruggedness, if relevant, in regards to different settings should be evaluated.

Mixture analysis, if relevant, should be performed in order to determine minor/major contributions.

Updates of already validated and implemented software should be verified e.g. using pre-defined test sets.

Sample tracking, data storage and input/output files should also be evaluated.

5.11 Probabilistic Software

Probabilistic software should be validated according to the ENFSI document for the internal validation of probabilistic software to undertake DNA mixture interpretation [9].

6. REFERENCES

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7. AMENDMENTS TO PREVIOUS VERSION

This document has revised the content and replaces the previous Guideline “Recommended Minimum Criteria for the Validation of Various Aspects of the DNA Profiling Process”, which was referenced as ENFSI DNA WORKING GROUP, issued on November 2010. This new issue is referenced DNA-GDL-002 in order to conform with the correct nomenclature of ENFSI documents. Due to the change of references, this document is at issue 001.

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