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BIOLOGY VALIDATION PROJECT WORKING GROUP

Requirements of Validation Parameters: Quantification Systems

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ANZPAA NIFS Document Position Statement

Australia New Zealand Forensic Executive Committee

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Review Date: 3 years from release date. The recommendations in this document are applicable until the review date. However, forensic service providers may continue using the document as guidance if deemed appropriate.

Purpose

Developed collaboratively by a cross-agency working group, this document outlines a shared view of accepted practices in the relevant forensic science field.

This document has been prepared with the aim of enhancing practice via continuous quality improvement and supporting greater consistency and standardisation within disciplines.

Recommendation

ANZFEC members recommend adopting the outlined practices wherever possible, as they enhance shared knowledge and experience, foster collaborative improvement, and support cross-organisational technical reviews, including laboratory accreditation assessments.

Application

The document serves as recommendations only and does not compel forensic service providers to adhere strictly to its contents. Where appropriately justified, applying alternative practices may also be considered effective and acceptable.

It is acknowledged that variations in legislation, organisational policies, infrastructure, equipment, resources and customer requirements may necessitate deviations in the adoption and application of the document's content.

The document's content is intended to guide the development and implementation of organisational practices. The contents are not intended to apply retroactively to organisational practices that were implemented and validated before the document's release date.

Requirements of Validation Parameters: Quantification Systems

1.1. Scope

The scope of this document outlines the internal validation requirements for the evaluation of the reliability, reproducibility and limitations of a quantification (also referred to as quantitation) system as part of manual and/or automated workflows within forensic casework. The data derived from this study will assist in the determination of laboratory specific DNA quantification, Quality Assurance parameters and interpretation guidelines.

1.2. Introduction

Following DNA extraction, quantification provides information around sample DNA quality and quantity, which is critical to ensuring the optimal DNA input is added for downstream processing. Quantification also facilitates the assessment of the concentration, degradation and inhibition of human DNA extracted from forensic samples. This step is important for guiding workflow decisions and ensuring optimal performance of downstream assays (1). Consequently, a robust validation is required to establish that the quantification processes are accurate and reliable.

Crucial parts of a quantification validation are outlined in the experimental design. The validation aims to demonstrate the quantification system as reliable for estimating (human) DNA concentrations within a sample, and to identify any significant discrepancies or inherent biases between additional instruments.

1.2.1. Relevant Guiding and Critical Documents

Reference should be made to documentation which has informed the experimental design including but not limited to:

- manufacturer user guide(s)
- published developmental validation data
- accreditation standards, and
- *ANZPAA NIFS Guideline for the Validation of Forensic Science Methods.*

1.3. Experimental Design

1.3.1. Sample Collection

The laboratory should evaluate the appropriate sample number and type, based on the methodology and/or application necessary to demonstrate the potential limitations and reliability. Methods intended for casework samples should be evaluated and tested using known samples and mock casework samples (or non-probative evidence samples), reflective of the range of samples commonly encountered within the laboratory.

1.3.2. Testing

The attached document does not explicitly use all terms from the *ANZPAA NIFS Guideline for the Validation of Forensic Science Methods*; however, the work outlined in this document addresses the relevant requirements.

Accuracy is demonstrated through replicate analysis using samples of known composition, sometimes referred to as concordance studies. Linearity is demonstrated through sensitivity studies using dilutions across the method's working range. Ruggedness is addressed through reproducibility studies were applicable.

Note: When validating quantification kits, the inhibition and Degradation Index (DI) studies outlined in this document are recommended. However, when validating quantification instruments, this series of work is not required, as these studies relate to the chemistry used and not the detection system.

The sample requirements for each aspect of testing are listed in the sub-headings below.

1.3.2.1. Sensitivity Studies

The sensitivity of a method may be determined via end-point dilution analysis of samples from known positive samples, to define the dilution in which the analyte is no longer detectable. The requirements for sensitivity testing include:

- Quantification of sensitivity series completed in triplicate (2).
- Sensitivity series created using commercially available or robust internal positive controls. Male DNA (XY) should be used to ensure the results of both total DNA and male DNA target can be assessed using the same sensitivity data.
- Each series diluted to span DNA concentrations both within and beyond the limits of the standard curve, for example, 100 ng/μL to 0.0001 ng/μL, to evaluate the full dynamic range of the assay.
- The range of values tested should reflect what is encountered in casework, based on the kit in use, including outliers. The series should incorporate 10 concentrations that represent 10-fold increases in DNA quantities, for example, 0.0001 ng/μL, 0.001 ng/μL, 0.01 ng/μL, 0.1 ng/μL 1 ng/μL, 10 ng/μL and 100 ng/μL.
- Calculate the average and standard deviation (SD) (assuming >2 replicates) of all quantification markers present, for example, small autosomal (SA), large autosomal (LA) and Y-Chromosomal marker values (Y). This allows comparisons to be made via determining the coefficient of variation (CV) for each DNA concentration by dividing the SD by the average, which can be presented as a percentage.
- If quantification will be undertaken on a robotic platform and manually, a sensitivity series must be produced for each method followed by comparative analysis of the results obtained by both methods. The average autosomal/Y ratio for each set of triplicate results should be assessed to determine the relationship of the two targets across a range of template amounts.

1.3.2.2. Limit of Detection (LOD) Studies

The detection limit is determined by the analysis of samples with known and decreasing concentrations of analyte to establish the minimum level at which the analyte can be reliably detected. The requirements for limit of detection (LOD) testing include:

- Twenty quantification replicates performed across ten DNA concentrations, from a male donor, spanning the lower end of the sensitivity series, including values below the limits of the standard curve. Run replicates on each available machine, as detailed in **Table 1**.
- The LOD for each quantification marker is determined as the lowest concentration at which the system(s) correctly reports concentration values with 95% confidence., Specifically, the concentration at which a maximum of one sample out of 20 does not produce a quantification value (3).
- This LOD should represent the quantification value at which background noise cannot be differentiated from human DNA.

- Where results were obtained from only some of the replicates, consideration should be given to documenting the number of replicates for which results are obtained.
- Samples should be amplified to understand the correlation between these quantification values and obtaining profiling results from each of the amplification kits used in the laboratory.

Table 1 – Empirical set up to investigate the lower end of the sensitivity series.

Sample Number	Concentration	Replicates
1	0.0001 ng/μL	20
2	0.0002 ng/μL	20
3	0.0003 ng/μL	20
4	0.0004 ng/μL	20
5	0.0005 ng/μL	20
6	0.0006 ng/μL	20
7	0.0007 ng/μL	20
8	0.0008 ng/μL	20
9	0.0009 ng/μL	20
10	0.0010 ng/μL	20

1.3.2.3. Negative Control Studies

Quantification of 100 known negative control samples across multiple batches should be used to assess the occurrence and extent of false positive quantification results. The requirements to determine the extent of false positive results include quantifying extraction negative controls that:

- Previously returned a quantification value of undetermined (Undet) at all quantification markers, for example SA, LA and Y, and produced no allelic information in amplification.
- Were recently extracted including negative controls representing all routine extraction types used in the laboratory, allowing for the assessment of reagent impact at elevated concentrations, such as dithiothreitol (DTT).
- Incorporate variables relevant to this work, such as timeframe between extraction and quantification.
- Reflect the standard process for testing controls applied to case samples.

Any negative controls which generate quantification values should be amplified to understand the correlation between these values and the presence of profiling information.

The extent of false positive quantification values for all markers within quantification kit should also be determined to ensure this aligns with the LOD. Consideration should be given to interpretation guidelines or re-work action for controls based on the validation data to inform scenarios of observing negative controls in casework.

1.3.2.4. Mixture Studies

Mixture studies pertaining to male and female donors at various ratios may inform the accuracy in determining male versus female inputs via the assessment of quantification output ratios of SA to Y. The requirements for mixture studies include human male and female DNA mixtures created in triplicate (**Table 2**). The ratios can be

undertaken on different starting concentrations subject to ratios routinely encountered in casework samples, such as 0.005-0.1 ng/μL. While evaluating the impact of male DNA with increased quantities of female DNA is an important focus for testing to reflect samples encountered in sexual assault casework, consideration should also be given to testing a subset of mixtures where the major DNA contribution is male and the minor DNA contribution is female.

Table 2 – Example of mixture studies within quantification validation.

Sample Number	Ratio	Replicates
1	1:0	3
2	1:1	3
3	1:5	3
4	1:10	3
5	1:25	3
6	1:50	3
7	1:100	3
8	1:1000	3
9	1:2000	3
10	0:1	3

The accuracy can be assessed by determining male and female input according to the ratios of expected versus observed SA:Y markers. Subsequent conclusions can be drawn about the limitations in interpreting quantification values at ratios/DNA concentrations below a specific value.

1.3.2.5. Repeatability and Reproducibility Studies

Repeatability is the precision estimate that may be produced where tests are performed on identical test items during a short interval of time by one operator using the same equipment under conditions that are as constant as possible. Reproducibility is the precision estimate obtained when a series of measurements are made under more variable conditions, such as the same method on identical systems and different operators on different instruments.

The repeatability and reproducibility requirements include testing a DNA concentration range comprising of five to ten points within the confines of the standard curve quantified using five replicates (4). Additionally:

- Operators perform testing using different instruments to evaluate reproducibility.
- Position each DNA concentration across 96-well plates to assess well-to-well variability.
- Perform replicate tests manually and within automated workflows, as appropriate.
- Run on all available quantification instruments to determine machine-to-machine variability.

1.3.2.6. Inhibition Studies

The tolerance limit of inhibitor presence of a quantification system may be assessed by any reported absence of DNA at the SA or LA markers and/or the presence of an Internal PCR Control (IPC) flag. The requirements for inhibition studies include:

- Inhibition study samples quantified in triplicate (2).
- Three concentrations of common PCR inhibitors, such as hematin, humic acid, added to DNA samples of low (0.01 ng/μL), medium (0.1 ng/μL) and high concentrations (1 ng/μL).

- An evaluation of tolerance limits to inhibitors based on comparing SA, LA and IPC flags, along with the passive reference dye, for kits that include a measure of degradation, to results obtained from the same concentration without inhibitor presence in the sensitivity series.
- Experimental work undertaken on samples with a template less than 5 ng/μL as the IPC can be an indicator of high DNA concentration in a sample.
- A comparison of results with the findings of the developmental validation/findings from the manufacturer, if available.

The results of the inhibition study should be used to inform analysts about any rework action post quantification or DNA profiling, or simply to understand the limitations/expected results for a sample impacted by inhibition.

1.3.2.7. Degradation Index (DI) Studies

Subject to the chemistry used, the DI may be an indicator of inhibition as well as degradation. The effects of DNA degradation on quantification results may be assessed by any reported absence of DNA at the SA or LA markers, and/or the system calculated DI. The requirements for degradation testing include:

- Degradation study samples quantified in triplicate (2).
- DNA inputs, consistent with DNA concentrations tested within the sensitivity series, be subjected to various levels of artificial degradation, such as the addition of at least five inputs of DNase enzyme into the DNA extract or mechanical degradation through ultra-violet treatment.
- An assessment of degradation impact on quantification results should be based on a comparison of the SA and LA markers, along with the reported DI (for the Quantifiler™ Trio DNA Quantification Kit only), against the results obtained from the same concentration in the sensitivity series that were not exposed to degradation.
- A comparison of results with the findings of the developmental validation or findings from the manufacturer, if available.

The results of the degradation study should be used to inform analysts about any rework action post quantification or DNA profiling, or simply to understand the limitations or expected results for a sample impacted by degradation.

1.3.2.8. Limit of Profiling / Profiling Outcomes Studies

The limit of profiling study serves as an assessment of how a range of quantification values overlap with DNA profiling outcomes of each short tandem repeat (STR) chemistry used within the laboratory. This can be achieved by amplification of the sensitivity series and should be reviewed in conjunction with the results of the LOD to understand at what point quantification results correlate with profiling results. If quantification values are different from previous systems, the optimal template amount for amplification may need to be reassessed to determine the optimal template at which point peak heights within an acceptable or optimal range are obtained.

1.3.2.9. Mock Forensic Sample Studies

Samples which mimic casework samples, such as clothing items, drinking vessels, touched surfaces, blood, semen and saliva samples, should be tested and compared with results from existing laboratory quantification kits. Samples should be extracted using routine laboratory processing, with five to ten replicates of each sample type considered for testing.

1.3.3. Data Analysis

Data analysis will include graphical plots of the DNA concentrations to compare:

- inter-plate (also acts inter-operator variability) variability (e.g. box plot)
- inter-instrument variability per dilution level (e.g. box plot)
- sample spread per dilution level (e.g. box plot)
- expected vs known DNA concentrations for each instrument (e.g. scatter plot), and
- bias (e.g. Bland-Altman plot).

If further analysis is required due to a lack of alignment in the boxplots, a one-way ANOVA analysis could be performed. This will identify if there are any significantly different groups. If significantly different groups are identified, then further investigation is required before acceptance can be passed.

DNA concentrations in the blank wells should be assessed to identify any that exceed the LOD.

A linear model could be used to further examine any major sources of variance. This model would consist of the following factors:

- known quantification value (continuous variable)
- instrument (factor with two levels - one per instrument), or
- plate (factor with five levels - one per plate).

Note: When applying statistical tests to the data, remember there are assumptions that must be tested and met for these tests to be considered valid. Seek statistical advice if unsure what the assumptions are and how to test them.

1.3.4. Assessment Against Acceptance Criteria

Acceptance criteria need to consider the level of variation between replicates in conjunction with average values to reflect that differing amounts of variation will be seen at different concentrations. Therefore, to measure CV, the standard deviation should be divided by the average values. Moreover, increased variation may be seen at low concentrations due to the impact of stochastic effects but also if concentrations exceed the upper and lower limits of the standard curves. For example, acceptance criteria may be set based on CV or other downstream implications such as modifications to the dilution/input.

Further to this, when drawing conclusions from results from different instruments, the following should be considered:

- If different instruments are being compared that have a different detection technology, some variability in results may be expected.
- If same model instrument is used as a point of comparison, CV would be expected to be low relative to the average value.

1.4. Limitations

The following limitations are noted with quantification system validations:

- Where ground truth samples are used, changes to the production of a DNA standard by the manufacturer may require reverification of the data.
- Studies are performed using mock casework samples, as it is not appropriate to use real casework samples where the ground truth is unknown. Samples were created in the laboratory and are designed

to mimic typical and real casework samples as closely as possible across the range of quantification values expected. However, it is acknowledged that mock samples can never fully represent all conditions and variables that exist in real casework samples, such as the number of contributors, DNA degradation, and the presence of non-human DNA and inhibitory substances.

1.5. Verification

All additional quantification machines of the same make and model, operating under identical conditions, should be verified against the validated quantification instrument to confirm reliability of results. At a minimum, a sensitivity series which covers the expected range of templates for the process being verified, and repeatability testing should be conducted.

1.6. References

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ANZPAA NIFS is responsible for the management and co-ordination of the Specialist Advisory Groups and has reporting accountability to the Australia New Zealand Forensic Executive Committee (ANZFEC).

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