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D3.4 SOP for Biomarker Validation

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Document History

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Standard operating procedure (SOP) for technical method validation

Introduction

A technical validation of an assay should be as extensive as the customer/sponsor requests, which depends on the intended use. For example, if the assay is going to be used in an exploratory fashion then a limited validation, where only precision and measurement range are evaluated, may suffice. The demands on the different parameters should be set and documented in a validation plan, that should be approved by the customer/sponsor, before any data collection begins. Results and conclusions, in relation to what is stated in the validation plan, should be summarized in a validation report. Finally, the customer/sponsor has to approve the report before the assay is put into service.

The current SOP is based on an article [1] that was an outcome of the BIOMARKAPD project in the EU Joint Programme – Neurodegenerative Disease Research initiative. An overview of different validation parameters is shown in Table 1. In addition to these there are others that have been excluded based on different assumptions. First, robustness is excluded since this should be done by the manufacturer during method development. Second, trueness and uncertainty demand the existence of a reference material, which in most cases does not exist.

Table 1. Validation parameters

Parameter	Description
Precision	The closeness of agreement between independent test results obtained under stipulated conditions.
Limits of quantification	Highest and lowest concentrations of analyte that have been demonstrated to be measurable with acceptable levels of precision and accuracy.
Dilutional linearity	Dilutional linearity is performed to demonstrate that a sample with a spiked concentration above the upper limit of quantification can be diluted to a concentration within the working range and still give a reliable result.
Parallelism	Relative accuracy from recovery tests on the biological matrix or diluted matrix against the calibrators in a substitute matrix.
Recovery	The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in the solvent.
Selectivity	The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present.
Sample stability	The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Precision

Assuming that a validation is performed in one laboratory there are two types of imprecisions that need to be reported: repeatability (aka within day variability) and intermediate precision (aka between day variability). Repeatability is the variability observed when as many factors as possible, *e.g.*, technician, days, instrument, reagent lot, are held constant and the time between the measurements is kept to a minimum as opposed to intermediate precision conditions where all factors that can be changed naturally, from day to day when the assay has been put into service, are varied.

Procedure

1. Collect samples with known high and low concentrations of the measurand. Pool samples if necessary.

2. Make 25 aliquots of each sample and store at -80°C pending analysis.

NOTE 1: If large volumes of the samples are available, more aliquots than the ones needed for the precision measurements can be prepared for use as internal quality control samples when the method has been put into service.

3. At day 1-5 measure 5 replicates on each sample.

NOTE 2: The days do not have to be consecutive, only different. NOTE 3: Vary as many factors as possible between the days but keep as many as possible constant within a day.

4. Insert data, separate days on different rows, in the excel file “Data Sheet 3.xlsx” (supplementary material in ref. [1]) that calculates the mean value, SD, %CV for both the repeatability and intermediate precision.

Limits of quantification

The working range for a method is defined by the lower and upper limits of quantification (LLoQ and ULoQ, respectively). At least for the LLoQ, there is more than one definition, and these can be classified as either determined based on the signals from the instrument or the calculated concentrations from samples. There are *pros* and *cons* with each procedure but procedure 2 is the one that may be most relevant to the measurement of real samples.

Procedure 1

1. Run 16 blank samples (immunodepleted matrix or sample diluent)

2. Calculate the mean and standard deviation (SD) of the signal.

3. Determine the concentration based on a signal of 10 standard deviations above the mean of the blank. NOTE 4: This procedure gives only the LLoQ but not the ULoQ.

To determine the concentration based on a signal, the inverse of the calibration function must be used. Two common models used are the four and five parametric logistic models. The four parametric function and its inverse are:

$$Signal = \frac{A - D}{1 + \left(\frac{Concentration}{C}\right)^{B}} + D \quad \Leftrightarrow \quad Concentration = C\left(\frac{A - D}{Signal - D} - 1\right)^{\frac{1}{B}}$$

For the 5-parameter logistic model the corresponding functions are:

$$Signal = \frac{A - D}{\left(1 + \left(\frac{Concentration}{C}\right)^{B}\right)^{E}} + D \Leftrightarrow Conncentration = C\left(\left(\frac{A - D}{Signal - D}\right)^{\frac{1}{E}} - 1\right)^{\frac{1}{B}}$$

The parameters A-E should be available from the software used for data acquisition and analysis.

Based on the concentrations of samples the LLoQ and ULoQ can also be defined as the endpoints of an interval in which the %CV is below the accepted intermediate precision, as stated in the validation plan, with a high probability, *e.g.*, 95%.

Procedure 2

1. Analyse, in duplicates, samples with very low and very high concentrations of the measurand.

2. Calculate the average concentration and %CVs for the samples.

3. Make a scatter plot of the %CV as a function of concentration for all samples.

4. Determine the LLoQ by identifying the lowest mean level above which the %CV is below the accepted intermediate precision for the prespecified majority of the samples.

5. Determine the ULoQ by identifying the highest mean level below which the %CV is below the accepted intermediate precision for the prespecified majority of the samples.

A third way to determine the LLoQ and ULoQ is to use the data from calibration curves (procedure 3).

Procedure 3

1. Plot the error of the back-calculated concentrations relative to the assigned concentrations (%RE) as a function of the assigned concentration using data from the five calibration curves produced in the precision measurements. To back-calculate the concentration for the calibrator points the formulas for the 4- or 5-parameter functions above can be used. The %RE is then calculated as:

$$\%RE = \frac{Measured\ calibrator\ concentration - Assigned\ calibrator\ concentration}{Assigned\ calibrator\ concentration} \cdot 100$$

2. Determine the working range as the interval where the %RE is below the accepted intermediate precision for the prespecified majority of the samples.

Dilution linearity

Dilution linearity is performed to demonstrate that a sample with a spiked concentration above the ULoQ can be diluted to a concentration within the working range and still give a reliable result. At the same time the presence of a hook effect, *i.e.*, suppression of signal at concentrations above the ULoQ, is investigated.

Procedure

1. Spike three neat samples with calibrator stock solution. NOTE 5: If possible, spike samples with 100- to 1000-fold the concentration at ULoQ using the calibrator stock solution.

2. Make serial dilutions of the spiked samples, using sample diluent in small vials until the theoretical concentration is below LLoQ.

3. Analyse the serial dilutions in duplicates and compensate the measured concentrations for the dilution factor.

4. Calculate for each sample the mean concentration for the dilutions that fall into the working range of the calibrator curve. After compensating for the dilution factor these should not deviate more from the spiked concentration than the intermediate precision stated in the validation plan. Plot the signal against the dilution factor to investigate if the signal is suppressed at high concentrations ("hook effect").

Parallelism

For parallelism, only samples with high endogenous concentrations, but below ULoQ, of the analyte must be used.

Procedure

1. Identify four samples with high, but below ULoQ, endogenous concentration of the measurand.

2. Make at least three, two-fold serial dilutions using sample diluent in vials until the calculated concentration is below LLoQ.

3. Analyse the neat samples and the serial dilutions in duplicates, in the same run, and compensate for the dilution factor.

4. For each sample, calculate the %CV using results from neat sample and the dilutions. The %CV should be lower than the accepted intermediate precision stated in the validation plan.

Recovery

A spike recovery test is conducted to investigate if the concentration-response relationship is similar in the calibration curve and the samples. A bad outcome of the test suggests that there are differences between the sample matrix and calibrator diluent that affect the response in signal.

Procedure

1. Collect five samples where the concentrations of the measurand have previously been determined and divide each sample into 4 aliquots.

2. Spike three of the aliquots, using calibrator stock solution, to expected concentrations that are evenly distributed over the linear range of the standard curve (low, medium, high). NOTE 6: All additions should be in the same volume, preferable <10% of the sample volume. The same volume of measurand-free calibrator diluent must also be added to the neat sample (4th aliquot) to compensate for the dilution. NOTE 7: The theoretical concentration in the spiked samples should be lower than the ULoQ. Different spiking concentrations should be used to investigate possible dependency on the

amount of added substance. The low spike should be slightly higher than the lowest reliable detectable concentration. NOTE 8: Alternatively, samples can be spiked after dilution, if there is limited availability of the calibrator and high working dilutions.

3. Analyse both the neat and spiked samples in the same run. Dilute each sample as advised for each assay to be used,

4. Calculate the recovery using the formula below. NOTE 9: Acceptance range for the recovery is usually 80-120%.

$$\%Recovery = \frac{Measured\ concentration_{\ spiked\ sample} - Measured\ concentration_{\ neat\ sample}}{Theoretical\ concentration_{\ spiked}} \cdot 100$$

Selectivity

Of the different validation parameters, selectivity is in principle the only one for which a certain amount of knowledge about the analyte and related substances is demanded. For example, if the analyte is a peptide of a specific length, do slightly longer or shorter peptides also give rise to a signal in the assay? Do metabolites of the analyte or post-translational modifications of a protein analyte interfere with the assay?

Procedure

1. Identify substances that are physiochemically similar to the one that the assay is developed for.

2. Investigate to what degree the measurements are interfered by spiking samples with substances identified in step 1. NOTE 10: If information is available regarding the endogenous concentration of an investigated substance the spiking concentration should be at least two times the reference limit. Otherwise a titration is recommended.

3. For antibody-based methods, an epitope mapping should be performed.

Sample stability

Sample handling prior to analysis has the potential to dramatically influence the results of a measurement. Examples of factors that potentially affect the results of an analysis, but are not included in the following procedure includes, sample tube, type of plasma anticoagulant, gradient effects (concerns cerebrospinal fluid samples), centrifugation conditions, extended mixing, and diurnal variations. If data is not available on how these factors influence the measurement, the sample handling instructions should be written in a way to prevent variations potentially induced by these.

Procedure

1. Repeat the following steps for three independent samples, preferably with different concentrations of the measurand (low, medium, high).

2. Divide the sample into nineteen aliquots with equal sample volume.

NOTE 11: It is important that every aliquot contains the same sample volume and to use the same kind of storage tubes, since unequal sample volumes may affect the concentration of the measurand due to adsorption.

3. Place aliquots #1-6 at -80°C.

4. Thaw aliquots #2-6 and store again at -80°C.

NOTE 12: Thaw for 2 hours at room temperature and next store the sample at least 12 h at -80°C for each freeze/thaw cycle.

5. Thaw aliquots #3-6 and store again at -80°C.

6. Thaw aliquots #4-6 and store again at -80°C.

7. Thaw aliquot #5-6 and store again at -80°C.

8. Thaw aliquot #5-6 and store again at -80°C.

9. Thaw aliquot #6 and store again at -80°C.

10. Thaw aliquot #6 and store again at -80°C.

11. At time point 0, store aliquots #7-12 at room temperature and another six aliquots #13-18 at 4^0C.

12. At time points t=1 h, t=2 h, t=4 h, t=24 h, t=72 h, t=168 h, transfer one sample stored at each temperature, RT and 4^0C, to -80^0C.

13. Store aliquot #19 at -20^0C during one month before transfer to -80^0C.

14. Thaw all aliquots for a given sample simultaneously and analyse them in duplicates in the same run.

15. Insert raw data of aliquots #1-19 (replicates of observed concentrations) in the Excel file “Data Sheet 4.xlsx” (supplementary material in ref [1]). The file calculates the mean value, standard deviation (SD), and coefficient of variation (%CV) for both the observed concentration and normalized concentration. NOTE 13: The standard deviation for the storage conditions and the freeze/thaw aliquots should be within the acceptance criteria for the intermediate precision defined validation plan. NOTE 14: The above conditions tested should only serve as an example and the can be modified to better suit the environment and different routine handling of samples at the individual laboratories.

Reference

1. Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn L, Blennow K, Chiasserini D, Engelborghs S, Fladby T, Genc S, Kruse N, Kuiperij B *et al*: **A practical guide to immunoassay method validation**. *Frontiers in Neurology* 2015, **6**.