White Paper

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Bioanalysis

Development and validation of qPCR methods for nucleic acid biomarkers as a drug development tool: points to consider

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Nucleic acid (NA) biomarkers play critical roles in drug development. However, the global regulatory guidelines for assessing quantification methods specific to NA biomarkers are limited. The validation of analytical methods is crucial for the use of biomarkers in clinical and post-marketing evaluations of drug efficacy and adverse reactions. Given that quantitative polymerase chain reaction (qPCR) and reverse transcription qPCR (RT-qPCR) methods are the gold standards for the quantification of NA biomarkers, the Biomarker Analytical Method Validation Study Group in Japan has discussed considerations and made recommendations for the development and validation of qPCR- and RT-qPCR-based analytical methods for endogenous NA biomarkers as drug development tools. This white paper aims to contribute to the global harmonization of NA biomarker assay validation.

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A biomarker is defined as a measurable characteristic that indicates the biological response to a normal biological process, pathogenic process, exposure or intervention (including therapeutic interventions). Biomarkers have molecular, histological, radiological and physiological properties [1]. The use of biomarkers in drug development is crucial for improving the success rate and safety of drug development, and has been increasing annually. Before using biomarkers as drug development tools, such as end points for clinical evaluation, or including them in drug application dossiers as reference information, their analytical methods must be adequately validated. In addition, the validation of analytical methods for assaying biomarkers based on their context of use is important. At present, it is difficult to define a uniform acceptance criterion for biomarker validation (as in the case of bioanalytical method validation used in drug development). Therefore, applying the 'fit-for-purpose' concept is useful for evaluating biomarker analysis methods [2–5]. Unlike in drug bioanalytical methods, careful attention must be paid to the limitations of the characteristics and availability of reference substances for biomarkers during analytical method development and validation. Moreover, the possible presence of analytes in sample matrices and problems unique to

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biomarker analysis methods (such as interindividual differences and diurnal variations in biomarker concentrations) also need to be considered.

In addition to biomolecules, such as endogenous metabolites and proteins, nucleic acids, such as DNA and RNA, can be used as biomarkers. For example, the US FDA has proposed virus-derived DNA and RNA as alternative end point biomarkers for the development and approval of antiviral drugs [6]. Circulating microRNAs (miRNAs) have also been used as biomarkers to detect adverse drug reactions [7]. Furthermore, the use of nucleic acid (NA) biomarkers as predictive biomarkers of the therapeutic effects of anticancer drugs and their clinical applications has been reported [8]. Quantitative polymerase chain reaction (qPCR)/reverse transcription qPCR (RT-qPCR)-based analytical methods are commonly used for the detection and quantification of NA biomarkers.

qPCR and RT-qPCR are highly specific, sensitive and reproducible NA analysis methods that incorporate PCR-based amplification of target NA sequences with detection using fluorescent substances. With the recent increase in the number of gene and cellular therapy products and vaccines, the importance of qPCR and RT-qPCR for assessing the quality, safety and efficacy of these drugs is increasing. For example, the Nonclinical Biodistribution Considerations for Gene Therapy Products (ICH S12 guideline) [9], published by the International Conference on Harmonization of Medicines Regulations (hereafter referred to as ICH) in March 2023, proposed the use of qPCR in the biodistribution studies of gene therapy products as an assay method. In addition, the 'General Principles to Address Virus and Vector Shedding' [10], published in June 2009 as the opinion of the ICH expert meeting, recommends the use of qPCR for the detection of emitted viruses/vectors. However, the acceptance criteria for the analytical method validation of qPCR and RT-qPCR are not described in the aforementioned guidelines or points-to-consider documents.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were first published in 2009 to ensure the accuracy and reproducibility of analytical methods using qPCR and RT-qPCR that were submitted to scientific journals [11]. In addition, the American Association of Pharmaceutical Scientists, Workshop on Recent Issues in Bioanalysis (WRIB), Discussion Groups of the Global CRO Council and Japan Bioanalysis Forum have discussed the acceptance criteria for qPCR/RT-qPCR-based bioanalytical method validation of biological samples for gene and cellular therapy products. The content of the agreement on the validation parameters and their acceptance criteria in the validation tests has been published [12–16]. Although biomarkers are not yet covered, regulatory bodies in Europe and the USA have issued guidelines for the validation of qPCR/RT-qPCR-based analytical methods for the detection and quantification of specific NA sequences derived from genetically modified organisms present in food and feed [17,18]. In addition, the International Organization for Standardization (ISO) has established guidelines for the development and validation of qPCR/RT-qPCR-based quantitative methods [19].

In cases where NA biomarkers are to be used as drug development tools evaluated in clinical trial end points or where their analytical results are to be included in drug application dossiers as reference information, the analytical methods to be used must be well validated, as is done for protein and small-molecule biomarkers. Regarding assay validation for biomarkers, points-to-consider documents were jointly published by the Critical Path Institute and the FDA in June 2019 [4], and by the Japan Agency for Medical Research and Development (AMED) study group in Japan [5]. Although qPCR/RT-qPCR-based analytical methods are not covered, many critical points described in these documents can be applied to NA biomarker analytical method validation when determining validation parameters as well as their acceptance criteria.

As described above, guidelines and white papers for qPCR/RT-qPCR method validation are being actively prepared and examined globally, mainly in the pharmaceutical and food industries. However, documents specific to the evaluation of NA biomarkers as drug development tools have not yet been published. Therefore, it is necessary to discuss method validation and study sample analysis using qPCR/RT-qPCR-based methods for NA biomarkers as well as to prepare a white paper and other regulatory documents for consensus to promote the use of NA biomarkers in drug development.

For this reason, initial discussions were held by researchers in the National Institute of Health Sciences belonging to the AMED research group on the 'Studies for qualification of biomarker candidates in drug-induced interstitial lung diseases and severe cutaneous adverse reactions, and for drafting the related guidance'. Based on these details, the expert members from the AMED research group (hereafter referred to as the Study Group) on 'Studies of the Acceleration of Global Harmonization for Regulating Safety and Quality Assurance of Pharmaceuticals' had further discussions. The current points-to-consider document was prepared as a result of the discussion of the Study Group

and is expected to contribute to the international harmonization of qPCR/RT-qPCR-based analytical method validation and study sample analysis for NA biomarkers in drug development.

Scope & basic principles

This document includes the opinions of the Study Group regarding the points to consider during the development, validation and study sample analysis of qPCR/RT-qPCR-based analytical methods for NA biomarkers used in drug development. The descriptions in this document are assumed to apply to qPCR/RT-qPCR-based analytical methods for quantifying NA biomarkers, the results of which are described in the Common Technical Document summaries [Module 2] (part of the application dossiers for drug approval). Therefore, *in vitro* diagnostic agents, such as companion diagnostics and clinical tests, are excluded from the scope of this document.

Prior to the validation of an analytical method for each NA biomarker used during drug development, it is necessary to define and document the required validation parameters and acceptance criteria in advance, considering the principle of 'fit-for-purpose' and availability of experimental materials.

This document applies to DNA and RNA-based biomarkers as analytes within qPCR and RT-qPCR analyses. Examples of DNA-based biomarkers include genomic DNA, mitochondrial DNA and cell-free DNA, while examples of RNA-based biomarkers include mRNAs and miRNAs. Digital PCR methods, wherein the measurement principle and data analysis method differ from the qPCR method, are out of scope in this document. This document describes a qPCR/RT-qPCR method used to quantify a single target NA biomarker as an analyte in biological matrices (a single-plex).

This document describes the development and validation of analytical methods that can be used to quantify the concentration (e.g., copy number per unit volume) of NA biomarkers in biological samples using qPCR/RT-qPCR. Genotyping analysis of gene variants using qPCR was beyond the scope of this document.

The number 'n' described in each validation parameter of this document indicates the number of samples for which preanalytical steps (such as NA extraction) was performed, as is done for study samples. In addition, during the quantitative analyses of NA biomarkers or validation runs prior to study sample analyses, obtaining duplicate (or more) measurements for each sample can improve the accuracy of the measurement results and support the evaluation of variation in the measured values. Finally, the validation of qPCR/RT-qPCR methods does not typically require the evaluation of selectivity because samples containing DNA or RNA other than the analyte are usually analyzed.

NA reference standards & measurement controls

For the quantitative analysis of NA biomarkers using qPCR/RT-qPCR, it is advisable to use DNA (single- or double-stranded) or RNA containing PCR-amplified regions of the target NA molecule as the NA reference standard. For NA reference standards, chemically synthesized NAs with known copy numbers are generally recommended. Regarding the purification method for chemically synthesized NA reference standards, it is advisable to use an advanced purification method to ensure the reliability of the analytical method, considering the characteristics and intended use (context of use) of the biomarker. The desired purity of reference standards should be as high as possible to meet the context of use of the biomarker. When the analyte is RNA, RNA produced via *in vitro* transcription can be used as a NA reference standard. For standard NA substances, their stability in standard solutions should be considered.

Critical reagents

Reagents that directly affect the analytical results are designated in advance as critical reagents which are recommended to be PCR grade and nuclease free. The primers and probes used in PCR are defined as critical reagents, and purification using high-performance liquid chromatography or polyacrylamide gel electrophoresis is recommended. The desired purity of primers and probes should be as high as possible, and needs to be determined based on the context of use of the biomarker. The stability of these primers and probes should also be considered. It is not necessary to specify water as a critical reagent, but attention should be paid to the contamination of water with substances such as nucleases.

Points to note in analytical method development

If an analyte is DNA based, it is desirable to use single- or double-stranded DNA containing the target amplification region as the NA reference standard. However, when using single-stranded DNA as a NA reference standard in

quantification of double-stranded DNA, it should be noted that the amplification reaction of single-stranded DNA does not occur in the first PCR cycle; therefore, to reflect the quantification cycle (Cq) difference of 1 unit, the data need to be corrected by subtracting 1 from the detected Cq values of each calibration standard in the construction of calibration curve. In addition, when using cyclic DNA as a NA reference standard, it is recommended to implement a linearization treatment as necessary to reduce the influence of the 3D structure of the NA molecule on PCR efficiency. Furthermore, it is necessary to consider the residual effect of the restriction enzymes used for linearization treatment and sample loss during purification.

When an analyte is RNA based, such as mRNA or miRNA, it is recommended to start analytical method development using RNA as a NA reference standard to reflect the influence of reverse transcription efficiency on the analysis. Because RNA is more easily degradable than DNA, it is advisable to analyze RNA stability and add a degradation inhibitor (e.g., an RNA-degrading enzyme inhibitor) as necessary when RNA is used as a NA reference standard. When quantifying RNA using DNA as a NA reference standard, it is important to demonstrate validity by evaluating the parallelism of the calibration curve using NA reference standards derived from multiple reverse-transcribed products in addition to evaluating the constant reverse transcription efficiency during analytical method development.

It is recommended that lot-to-lot differences in chemically synthesized or *in vitro*-transcribed NA reference standards be evaluated during the method development phase. In addition, it is advisable to evaluate the base lengths, concentrations and degrees of degradation of the reference standards in advance using methods such as electrophoresis or spectrophotometry.

Points to note in analytical method validation

To verify qPCR/RT-qPCR accuracy, it is necessary to prepare suitable and multiple concentrations of quality control (QC) samples (positive controls), an extraction blank (water or buffer to confirm contamination during the extraction procedure) and a nontemplate control (NTC) for each qPCR/RT-qPCR measurement run. A QC sample is a control sample used to confirm correct gene amplification during PCR; a solution containing a certain amount of a NA reference standard is used as the QC sample. In principle, QC samples and calibration standards are prepared separately and used during the period in which stability is confirmed. NTC is a sample used to evaluate nonspecific gene amplification and contamination of reaction solutions and samples. Ultrapure water and buffer solutions used for diluting NAs are generally used as the NTC.

Sensitivity (lower limit of quantification & limit of detection)

The sensitivity of qPCR/RT-qPCR is indicated by the lower limit of quantification (LLOQ) and limit of detection (LOD). The LLOQ is defined as the minimum concentration that satisfies the predetermined acceptance criteria for accuracy and precision. The LOD is generally defined as the minimum concentration at which 95% or more of the study samples are detected as positive in a given analytical method. Evaluation of the LLOQ is necessary when validating analytical methods for NA biomarkers used in drug application dossiers. LOD is not included in the quantification range and not used as a validation parameter in the quantification of NA biomarkers. Therefore, LOD is not a subject of validation in this document.

Points to note in analytical method development

The LLOQ can be provisionally determined by measuring various low-concentration samples with a Cq value of less than 40. The analytical results of the provisionally determined LLOQ should satisfy the predefined acceptance criteria of precision for the Cq value.

Assessments in analytical method validation

For analytical method validation, it is important to verify whether the accuracy and precision obtained from the measured values of QC samples with the same concentration as the LLOQ tentatively determined in the 'Points to note inanalytical method development' section (QC-LLOQ, n = 3 or more, evaluation via repeated analysis at least three times on different days is recommended) satisfy the predefined acceptance criteria.

Specificity

Specificity is defined as the ability to identify and detect target NA molecules among NA (DNA and RNA) molecules with similar sequences in the matrix, which can potentially affect the measurements. The specificity

of qPCR/RT-qPCR depends on the base sequence of the primers and probes used, and the annealing conditions (temperature and duration).

To evaluate specificity, it is advisable to conduct an evaluation centered on *in silico* analyses during the method development stage. Validation tests should be conducted using synthetic NAs with sequences where there is a concern for nonspecific amplification. Nonspecific amplification can be confirmed using DNA sequencing, melting curve analysis, electrophoresis and restriction digestion. If nonspecific amplification exceeds the predetermined criteria, the primer and probe need to be redesigned, and PCR annealing conditions should be further optimized.

Points to note in analytical method development

Using *in silico* databases (e.g., BLAST [20] and Primer-BLAST [21]), primers and probes with a low possibility of gene amplification for sequences other than the analyte should be selected in animal species where NA biomarkers are present (human genome or transcriptome if the analytical target exists in humans). Particular attention should be paid to the similarity of the 3′-end sequences. In addition, it is advisable to confirm the status of nonspecific amplification of sequences similar to the analyte using *in silico* analysis for the NA sequence of synthetic impurities that are approximately 1 base short of the 3′-end of the selected primers. Furthermore, it is advisable to obtain multifaceted information with regard to gene amplification products from the designed primers and probes using methods such as DNA sequencing, melting curve analysis, electrophoresis and restriction enzyme treatments.

Assessments in analytical method validation

For NA sequences shown in the *in silico* analysis to have the possibility of nonspecific gene amplification, a confirmation test of the status of nonspecific gene amplification should be performed using ultrapure water or a NA dilution buffer containing the NA sequences along with the analyte at the LLOQ concentration. If nonspecific gene amplification is observed, it should be confirmed to be within the predefined acceptance criteria. In addition, if a similar sequence with the possibility of nonspecific amplification is not identified at the analytical method development stage, the validation of specificity may be simplified.

Calibration curve

A calibration curve is used to calculate the concentration (copy number per unit volume) of NA biomarkers in the study samples. Calibration range is defined as the range from the LLOQ to the upper limit of quantification (ULOQ); LOD is not within the calibration range. Calibration curves are constructed for each qPCR/RT-qPCR run to quantify NA biomarkers. It is recommended that the number of calibration standards and the number of samples per concentration be determined in advance in the protocol for each analytical method based on the context of use.

Calibration standards are prepared by adding a known concentration of a NA reference standard to the authentic biological matrix. However, when the endogenous concentration of the target NA molecule is high or when a rare matrix is used, a surrogate matrix can be used to construct a calibration curve.

For the matrix of QC samples, see the 'Accuracy & precision' section below.

Points to note in analytical method development

During the analytical method development stage, it is important to consider the fluctuation range of the target analyte and set the quantitative range of the calibration curve to ensure reliability of the analytical method. The quantitative range of the calibration curve can be tentatively determined as the concentration range where the accuracy of the back-calculated concentration of the calibration standards satisfies the predefined acceptance criteria.

The regression line of the calibration curve in qPCR/RT-qPCR is generally obtained using the least-squares method, with the logarithmic concentrations of the calibration standards on the x-axis and the Cq values obtained from the measurement on the y-axis; it is often represented by a linear function (Equation 1). PCR amplification efficiency is calculated using the slope of the regression line of the calibration curve (Equation 2).

$$y = ax + b \qquad \text{(Equation 1)}$$

where y is the Cq value obtained from the measurement, x is the logarithmic concentration of the calibration standard, a is the slope of the regression line, and b is the intercept of the regression line.

$$\text{PCR amplification efficiency } (\%) = \left[10^{\left(\frac{-1}{a}\right)} - 1\right] \times 100 \quad \text{(Equation 2)}$$

(when the common logarithm is used for calibration standards for the x − axis of the calibration curves)

where a is the slope of the regression line determined in Equation 1.

Assessments in analytical method validation

It is recommended that at least three or more repeated measurements be performed on different days to evaluate the PCR amplification efficiency, linearity (R^2 value of the regression line of the calibration curve), accuracy and precision of the back-calculated concentration of each calibration standard.

It is advisable that the average value of PCR amplification efficiency (Equation 2) obtained from the slope of the regression line of the calibration curve be 90–110% (slope between -3.1 and -3.6). It is advisable that the average R^2 value of the calibration curve regression line not be less than 0.98. Furthermore, the average values of the accuracy and precision of the back-calculated concentration of each calibration standard must satisfy the predefined acceptance criteria. When calibration standards are prepared using a surrogate matrix, it is advisable to confirm that the PCR amplification efficiency of the calibration standards prepared using an authentic matrix is within the range of the aforementioned acceptance criteria.

Accuracy & precision

It is advisable to evaluate the accuracy of qPCR/RT-qPCR within (intra-assay) and between (inter-assay) analytical runs using QC samples of known concentrations. For the matrix used in preparing QC samples, it is recommended that either an authentic matrix or a surrogate matrix be selected according to the endogenous concentration of the NA biomarker (analyte). When preparing QC samples using authentic matrices, the endogenous concentrations of the analytes in the blank matrices should be evaluated.

Accuracy is calculated using Equations 3 or 4. The consistent use of the selected equation, considering the characteristics of the biomarker and the purpose of the evaluation, is advisable.

$$\text{Accuracy } (\%) = \frac{\left(\text{concentration of the analyte in the sample} - \text{concentration of endogenous substance}\right)}{\text{concentration of spiked NA reference standard}} \times 100 \quad \text{(Equation 3)}$$

$$\text{Accuracy } (\%) = \frac{\text{concentration of analyte in the sample}}{\left(\text{concentration of endogenous substance} + \text{concentration of spiked NA reference standard}\right)} \times 100 \quad \text{(Equation 4)}$$

Points to note in analytical method development

At the method development stage, it is recommended that the reproducibility of the method in the quantitative target range be confirmed using multiple concentrations of QC samples. For validation purposes, it is recommended that multiple points of the concentration of QC samples be set with the high-concentration QC (QC-H) at half the ULOQ, the medium-concentration QC (QC-M) near the middle of the calibration curve, and the low-concentration QC (QC-L) at twice the LLOQ.

Assessments in analytical method validation

Accuracy and precision can be evaluated using QC samples of known concentrations prepared from surrogate or authentic matrices. When evaluating precision, in addition to using QC samples of known concentrations, it is advisable to prepare two study samples (low- and high-concentration samples) with different concentrations and to perform an evaluation using repeated measurements of the samples. During validation, four different known concentrations of QC samples (QC-LLOQ, QC-L, QC-M and QC-H; n = 3 or more for each concentration) should be measured at least three times on different days in different analytical runs to evaluate intra- and inter-assay accuracy and precision. It is recommended that the intra- and inter-assay accuracy and precision meet predefined

acceptance criteria. The number of repeated measurements (three or more) in different analytical runs should be predetermined based on the context of use of the target NA biomarker.

Matrix effect

When quantifying NA biomarkers (analytes) in biological samples using qPCR/RT-qPCR, it is important to consider the matrix effect caused by, for example, the presence of contaminating PCR inhibitors. Considering that the types and amounts of PCR inhibitors that can be the main cause of the matrix effect are different depending on the type of biological matrices being measured (e.g., tissues, blood or urine), evaluation of the matrix effect for each matrix being measured is important. The use of internal standard molecules may facilitate the evaluation of matrix effects.

Collagen, melanin and myoglobin are known as typical tissue-derived PCR inhibitors, while hemoglobin, hematin and immunoglobulin G have been reported to be blood-derived inhibitors [22,23]. Urea has been reported to inhibit PCR in urine samples [24]. In addition to components derived from biological samples, residues of anticoagulants used during blood sampling (such as ethylenediaminetetraacetic acid and heparin) and reagents added during NA extraction (including alcohols, phenols and surfactants) have also been reported to cause matrix effects [25]. Furthermore, there have been reports of PCR inhibitory effects due to contamination with antiviral drugs (e.g., acyclovir) that have NA-like structures [23,26]. At any stage during the development of the analytical method or validation, it is advisable to evaluate the residues and contamination of PCR inhibitors in the study samples.

Points to note in analytical method development

To establish a reliable analytical method, it is recommended that the matrix effect in the same matrix as the study samples be confirmed during the analytical method development stage. When preparing calibration standards and QC samples using surrogate matrices, it is advisable to confirm the presence of matrix effects using an authentic matrix. For example, the matrix effects in qPCR/RT-qPCR can be evaluated by comparing the measured NA concentration of:

Sample 1) a preprocessed authentic matrix spiked with a known amount of NA reference standard;
Sample 2) a preprocessed blank authentic matrix; and
Sample 3) NA dilution buffer spiked with the same amount of NA reference standard as sample 1.

In this evaluation, it is advisable to measure each sample at a frequency of n = 3 or more. In general, if there is no difference between the measured concentration of sample 1 and the sum of that of samples 2 and 3, then the matrix effect can be deemed negligible. However, if the measured concentration of sample 1 is less than or greater than the sum of samples 2 and 3, the matrix effect can be considered present. The slope of a calibration curve can also be used as an indicator of the matrix effect.

To counteract the matrix effect, diluting the study samples to mitigate the influence of PCR inhibitors, changing reagents used in the analysis method, using an internal standard (different from the target analytes) and modifying the NA extraction method may be considered.

Assessments in analytical method validation

It is recommended that the matrix effect be evaluated using 10 different blank matrices in which NA extraction has been performed, and the blank matrix extract and NA dilution buffer spiked with known amounts of NA reference standard. In such an evaluation, it is advisable that the matrix effect is not observed. Even if the matrix effect is confirmed for the study samples, if the accuracy calculated from the measured concentrations after data correction using an internal standard of NAs is within the acceptance criteria, it can be considered that the matrix effect does not influence the performance of the analytical method.

Parallelism

In the analysis of NA biomarkers using qPCR/RT-qPCR, it is generally recommended that parallelism be evaluated using study samples because sample dilution leads to a reduction in the effect of PCR inhibitors (see 'Matrix effects' section) present in biological samples. Although either surrogate matrices or authentic matrices can be used to dilute study samples, it should be noted that the evaluation content varies depending on the type of diluent used.

When a surrogate matrix is used as a diluent, it is possible to evaluate the effect of reduction in PCR inhibitors by sample dilution, as described in the evaluation of the matrix effect. When authentic matrices are used for sample dilution, the amount of matrix-derived PCR inhibitors in the study samples remains the same. However, the effect of coexisting PCR inhibitors on the measured values of the analyte at different concentrations can be evaluated. In addition, the differences between NA reference standards and endogenous NAs can also be evaluated.

In the evaluation of parallelism, samples with at least three levels of dilution are prepared using authentic or surrogate matrices of high concentrations; it should be confirmed that measured values corresponding to the dilution ratio are obtained.

Stability

Since NA biomarkers are susceptible to degradation by nucleases present in biological matrices, careful attention should be paid to the possibility of analyte degradation by degrading enzymes at the analytical method development stage. If analyte degradation is observed, the use of commercially available NA-stabilization reagents is recommended.

In the evaluation of stability in validation tests, it is essential to evaluate 'Benchtop and Short term', 'Long term' and 'Freeze-thaw' stability using QC samples prepared with authentic matrices. As the storage and dilution of preprocessed samples are assumed during the sample analysis, it is advisable to evaluate the processed sample stability of QC samples under the same conditions as the preprocessed study samples (including cases such as storing cDNA reverse transcribed from mRNA). In these evaluations, it is recommended that stability be confirmed using low- and high-concentration QC samples within the assumed concentration range of the study samples. The frequency of evaluation repetitions should be at least three. It is recommended that the number of freeze–thaw cycles be evaluated for the expected number of times in the study sample analysis. For short- and long-term stabilities, evaluation for periods longer than the expected storage period of the study samples is advisable.

For biomarker analysis, there may be limitations in sample availability at the time of validation tests. Therefore, stability should be evaluated to the extent possible using available samples. After the start of the study sample analysis, it is also possible to confirm stability based on the incurred sample stability approach using study samples. A sample spiked with a surrogate NA reference standard can be used to evaluate the stability in the validation of an analytical method before the study samples are available. However, in that case, it is important to confirm the stability at low and high concentrations within the target concentration range using incurred sample stability after the start of the study sample analysis.

Recovery rate

At the method development stage, it is advisable to confirm that the recovery rate of the NA extraction method for NA biomarker analysis is within a certain range based on the context of biomarker use. When the recovery rate varies due to the characteristics of the biological matrix used for analysis, it is advisable to use an internal standard and perform data correction based on the measured value.

Partial validation

The points to consider for partial validation in the previous white paper for biomarker assays using ligand-binding assays (LBAs) and chromatographic methods [5] are largely applicable to qPCR/RT-qPCR-based analytical methods.

If minor changes are made to the analytical method for which full validation has been performed, partial validation should be performed. The parameters evaluated via partial validation are set according to the degree of change in the analytical method and its nature.

Typical examples of partial validation include transferring analytical methods to other facilities, changing analytical instruments, changing quantitation ranges, changing the amount of sample matrices used for analysis, changing NA extraction methods and analytical conditions, changing sample storage conditions, using rare matrices additionally, and changing the number of critical reagents.

In principle, the predefined acceptance criteria for full validation are also used for partial validation.

Cross-validation

The points to consider for cross-validation in the previous white paper for biomarker assays using LBAs and chromatographic methods [5] are largely applicable to qPCR/RT-qPCR-based analytical methods.

For example, cross-validation is performed when samples from a single clinical trial are analyzed at multiple sites, when measured values are compared between analytical methods on different platforms [5], or when laboratories are moved between studies (not within a study). Cross-validation comparisons are performed after the full or partial validation of each analytical method. Cross-validation can be evaluated by comparing the mean accuracy of each concentration of the QC samples and by comparing differences in the measured concentrations of the study samples.

As a specific method of cross-validation, it is possible to evaluate the mean accuracy of the QC sample (low, middle and high concentrations) by repeating the analysis three or more times, considering intra- and interlaboratory reproducibility. Although the evaluation using study samples depends on the characteristics of the analyte and sample matrices, the number of samples can be at least 30 (if available) to span the study sample concentration. The evaluation using study samples are recommended, especially when the composition of sample matrices significantly vary due to the severity or condition of diseases, since performance of the analytical method cannot be fully evaluated only using QC samples in such cases. When selecting samples, it is advisable to consider the concentration distribution to the largest extent possible and include samples from many individuals. A single analytical run is considered acceptable when evaluating study samples.

Study sample analysis

The points considered in the study sample analysis in the previous white paper for biomarker assays using LBAs and chromatographic methods [5] are largely applicable to qPCR/RT-qPCR-based analytical methods.

The study samples are specimens subjected to bioanalysis in which a validated analytical method should be used. During study sample analysis, the study samples should be handled under conditions where stability is validated. These samples should be analyzed with calibration standards and QC samples within the validated stability period.

Unless otherwise specified, the following parameters (see 'Calibration curve', 'QC samples' and 'Incurred sample reanalysis' sections) and their acceptance criteria are determined based on the intended use and characteristics of the biomarker before the start of the analysis and are described in the study plan. Depending on the results of the validation, it may be necessary to perform confirmatory runs for the analytical method using subject samples before the study sample analysis.

If a target analyte is RNA based, it is recommended that the quality of the RNA in the study samples be evaluated using methods, such as electrophoresis or spectrophotometry.

Calibration curve

A calibration curve is used to calculate analyte concentrations in study samples. It is recommended that calibration curves used for study sample analysis be constructed for each analytical run using a validated method.

The validity of the analytical method for sample analysis is evaluated using the accuracy of each concentration of the calibration standard obtained from the regression formula. In the analysis of the study samples, if the LLOQ or ULOQ of the calibration standards does not meet the predefined acceptance criteria, the higher calibration standards just above the LLOQ or the lower calibration standards just below the ULOQ are used as the LLOQ or ULOQ.

QC samples

QC samples are analyzed to evaluate the validity of the analytical methods used in the analysis of the calibration curves and study samples. It is recommended that QC samples be evaluated in each analytical run. In principle, it is recommended that three concentrations (low, medium and high) of QC samples be prepared using authentic matrices, authentic matrices diluted with surrogate matrices or surrogate matrices spiked with NA reference standards, as with the validation tests, and analyzed in each analytical run. It is also advisable to simultaneously analyze an NTC sample and extraction blank. Use of QC samples consisting of authentic matrices is considered wherever possible.

When only QC samples prepared with surrogate matrices spiked with NA reference standards are used, it is advisable to perform additional analysis of QC samples using authentic matrices (not included in the acceptance criteria) because useful information specific to the true analyte/matrix may be obtained when comparing the results of analytical runs and studies. The validity of the sample analysis method is assessed by evaluating the accuracy of the QC samples using the same predetermined acceptance criteria used for the evaluation of accuracy in the assay validation.

Incurred sample reanalysis

Based on the context of use and positioning as a clinical end point, it is recommended that incurred sample reanalysis (ISR) be performed in different matrices on samples from representative clinical trials, such as when the biomarker is used for important evaluations to characterize a drug (e.g., as end points in the later stages of a clinical trial).

ISR should be conducted within a period of guaranteed stability. It is recommended that 10% of study samples (when $n < 1000$) be reanalyzed during ISR. When sample counts exceed 1000, it is recommended to additionally perform ISR on approximately 5% of the sample count after the first 1000. The sample concentration range is selected based on the concentration range of the biomarkers in the study samples. The validity of the analytical method is evaluated by assessing the accuracy of the study samples.

Points to note

NA adsorption

When the endogenous concentration of the target analyte is low, the effect of adsorption of the analyte onto the laboratory equipment may be significant. If necessary, laboratory equipment with low NA adsorption specifications should be used. Alternatively, it is possible to utilize carrier NAs to prevent adsorption while maintaining the desired analytical performance.

Commercial kits

The points to consider for commercial kits in the previous white paper for biomarker assays using LBAs and chromatographic methods [5] are largely applicable to qPCR/RT-qPCR-based analytical methods.

Commercial kits can be used to analyze NA biomarkers. These kits include those approved for manufacturing and sale as *in vitro* diagnostic reagents, medical devices for clinical testing and research kits. When using commercially available kits, validation in each testing facility needs to be conducted for each target biomarker.

The validation parameters and contents of clinical test kits can be determined on a case-by-case basis according to the intended use and characteristics of the biomarker.

For research-use-only kits, full validation is performed without relying on the validation information included in the kit. Before deciding to use the kits, it is advisable to sufficiently evaluate whether the kit is suitable for the intended use and the characteristics of the biomarker (e.g., including the calibration curve range and specificity). It is possible to refer to information about the expiration date of the kit as confirmed by the manufacturer. In addition, the suitability of the NA reference standards included in the research kits should be considered. When switching to a different lot (or batch) of the kit, it is recommended to verify whether the difference in the measured concentration of the target analyte in the same sample between the lots is acceptable. Additionally, it is important to have contingency plans in place as kits for research use may become unavailable.

Reanalysis

The points to consider for reanalysis in the previous white paper on biomarker assays using LBAs and chromatographic methods [5] can be largely applied to qPCR/RT-qPCR-based analytical methods.

Before commencing the analysis of study samples, it is important to specify the reasons for performing the reanalysis of study samples, the number of reanalyses to be performed and the selection criteria for the measured values to be reported.

The reasons for reanalyses of study samples include, but are not limited to, the following:

- Calibration standards or QC samples do not meet the predefined acceptance criteria;
- The measured concentration exceeds ULOQ;
- The measured concentration was lower than the changed LLOQ in the analytical run, whereas the LLOQ after the change was higher than that in the other analytical runs because of the rejection of the lowest calibration standard for the calibration curve;
- Malfunction of the analytical instrument;
- The concentration of the study sample measured by dilution is lower than the LLOQ;
- Amplification cannot be conducted for study samples due to the matrix effect.

It is important to keep records of the following information for reanalyzed samples: the name of the sample, the reason for the reanalysis, the finally adopted measured value and the basis for its adoption, including any relevant information used in the decision-making process.

If the initial analysis does not produce reportable results (e.g., concentrations above the ULOQ or instrument failure), one reanalysis is sufficient. If measured values must be confirmed, multiple iterations of analysis are recommended if a sufficient sample volume is available.

It is important to note that in clinical trials, the safety of the participants takes precedence. Therefore, a reanalysis of a particular study sample may be necessary for investigative purposes to ensure the safety of the subject.

Conclusion

In this paper, the authors have discussed and summarized the points to consider for the method development and validation of qPCR- and RT-qPCR-based analytical methods for NA biomarkers as drug development tools. This paper is expected to be a valuable resource for promoting the development and ensuring the reliability of NA biomarker assays included in the application dossier for drug approval. The authors also hope that this white paper will stimulate further dialogue among industry professionals and regulatory authorities, leading to the establishment of guidelines and the achievement of international harmonization in this field.

Summary points

- Nucleic acid (NA) biomarkers, such as microRNAs, have come to play critical roles in drug development.
- The appropriate validation of analytical methods for NA biomarkers is crucial to ensure their clinical and post-marketing usage in drug efficacy and safety evaluations.
- However, the global regulatory guidelines for assessing quantitative analytical methods specific to NA biomarkers have not yet been issued.
- Quantitative polymerase chain reaction (qPCR) and reverse-transcription qPCR (RT-qPCR) methods are considered as the gold standards for the quantification of NA biomarkers, such as DNA and RNA, respectively.
- The Biomarker Analytical Method Validation Study Group in Japan has discussed points to consider and made recommendations for the development and validation of qPCR/RT-qPCR-based analytical methods for endogenous NA biomarkers.
- The discussed validation parameters include sensitivity, specificity, calibration curve, accuracy, precision, matrix effect, parallelism, stability and recovery rate.
- In addition to these parameters, the recommendations for study sample analysis using qPCR or RT-qPCR were also summarized.
- This white paper aims to contribute to the global harmonization of NA biomarker assay validation.

Author contributions

Y Sun: conceptualization, discussion, writing-original and revising the draft. T Nakamura, Y Ohtsu, M Kakehi, N Danno, H Shimizu, Y Tanaka, V Serelli-Lee, S Tanaka, T Okayama, Y Suda, Y Moriya and T Hanada: discussion, revision of manuscript. Y Saito: project administration, fund acquisition, supervision, revision of manuscript.

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References

Papers of special note have been highlighted as: ● of interest; ●● of considerable interest

1. FDA-NIH Biomarker Working Group. *BEST (Biomarkers, Endpoints, and Other Tools) Resource* US FDA, NIH, both MD, USA (2016).
2. Lee JW, Devanarayan V, Barrett YC *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm. Res.* 23(2), 312–328 (2006).
3. Piccoli SP, Garofolo F. Biomarker assay validation. *Bioanalysis.* 10(12), 889–891 (2018).
4. Biomarker Assay Collaborative Evidentiary Considerations Writing Group, Critical Path Institute. Points to consider document. Scientific and regulatory considerations for the analytical validation of assays used in the qualification of biomarkers in biological matrices (2019). https://c-path.org/wp-content/uploads/2019/06/evidconsid-whitepaper-analyticalsectionv2019.pdf
5. Ohtsu Y, Tanaka S, Igarashi H *et al.* Analytical method validation for biomarkers as a drug development tool: points to consider. *Bioanalysis.* 13(18), 1379–1389 (2021).
6. US FDA. Endpoints that were the basis of drug approval or licensure. https://www.fda.gov/drugs/development-resources/table-surrogate-endpoints-were-basis-drug-approval-or-licensure
7. Schofield AL, Brown JP, Brown J *et al.* Systems analysis of miRNA biomarkers to inform drug safety. *Arch. Toxicol.* 95(11), 3475–3495 (2021).
8. Goossens N, Nakagawa S, Sun X, Hoshida Y. Cancer biomarker discovery and validation. *Transl. Cancer Res.* 4(3), 256–269 (2015).
9. ICH. Nonclinical biodistribution considerations for gene therapy products S12 (2023). https://database.ich.org/sites/default/files/ICH_S12_Step4_Guideline_2023_0314.pdf
10. ICH. General principles to address virus and vector shedding (2009). https://admin.ich.org/sites/default/files/2019-04/ICH_Considerations_Viral-Vector_Shedding_.pdf
11. Bustin SA, Benes V, Garson JA *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55(4), 611–622 (2009).

●● **First published guideline for qPCR-based assays and the basis for current guidelines and white papers.**

12. Hays A, Islam R, Matys K, Williams D. Best practices in qPCR and dPCR validation in regulated bioanalytical laboratories. *AAPS J.* 24(2), 36 (2022).
13. Corsaro B, Yang TY, Murphy R *et al.* 2020 white paper on recent issues in bioanalysis: vaccine assay validation, qPCR assay validation, QC for CAR-T flow cytometry, NAb assay harmonization and ELISPOT validation (Part 3 – Recommendations on immunogenicity assay strategies, NAb assays, biosimilars and FDA/EMA immunogenicity guidance/guideline, gene & cell therapy and vaccine assays). *Bioanalysis* 13(6), 415–463 (2021).
14. Wissel M, Poirier M, Satterwhite C *et al.* Recommendations on qPCR/ddPCR assay validation by GCC. *Bioanalysis* 14(12), 853–863 (2022).

●● **Comprehensive summary for the current status of assay validation for qPCR and digital PCR in contract research organizations. Also offers consensus recommendations on validation parameters and acceptance criteria for qPCR assays for gene and cell therapy products, and vaccines.**

15. Ma H, Bell KN, Loker RN. qPCR and qRT-PCR analysis: regulatory points to consider when conducting biodistribution and vector shedding studies. *Mol. Ther. Methods Clin. Dev.* 20, 152–168 (2021).

● **Provides points to consider for method development and validation of qPCR methods for biodistribution and vector shedding studies.**

16. Uchiyama A, Naritomi Y, Hashimoto Y *et al.* Understanding quantitative polymerase chain reaction bioanalysis issues before validation planning: Japan Bioanalysis Forum discussion group. *Bioanalysis* 14(21), 1391–1405 (2022).

●● **Describes points to consider for qPCR-based bioanalytical methods and provides validation parameters along with their acceptance criteria for cell and gene therapy products.**

17. US FDA. Guidelines for the validation of analytical methods for nucleic acid sequence-based analysis of food, feed, cosmetics and veterinary products (2020). https://www.fda.gov/media/121751/download

18. European Network of GMO Laboratories. Definition of minimum performance requirements for analytical methods of GMO testing (2015). https://gmo-crl.jrc.ec.europa.eu/doc/MPR%20Report%20Application%2020_10_2015.pdf
19. ISO. Biotechnology – Requirements for evaluating the performance of quantification methods for nucleic acid target sequences – qPCR and dPCR. ISO 20395 (2019). https://www.iso.org/obp/ui#iso:std:iso:20395:ed-1:v1:en
20. NIH. BLAST database. https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
21. NIH. Primer-BLAST. https://www.ncbi.nlm.nih.gov/tools/primer-blast/
22. Sidstedt M, Rådström P, Hedman J. PCR inhibition in qPCR, dPCR and MPS-mechanisms and solutions. *Anal. Bioanal. Chem.* 412(9), 2009–2023 (2020).
23. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors – occurrence, properties and removal. *J. Appl. Microbiol.* 113(5), 1014–1026 (2012).
24. Khan G, Kangro HO, Coates PJ, Heath RB. Inhibitory effects of urine on the polymerase chain reaction for cytomegalovirus DNA. *J. Clin. Pathol.* 44(5), 360–365 (1991).
25. Demeke T, Jenkins GR. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal. Bioanal. Chem.* 396(6), 1977–1990 (2010).
26. Yedidag EN, Koffron AJ, Mueller KH *et al.* Acyclovir triphosphate inhibits the diagnostic polymerase chain reaction for cytomegalovirus. *Transplantation* 62(2), 238–242 (1996).