Points to consider document on analytical method development and validation of qPCR methods for nucleic acid biomarkers

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Opinions in this presentation are personal, not represented by MHLW or NIHS

Discussion on biomarker assay validation (BAV)

Making Japanese BAV points to consider document

For harmonized BAV (as a research activity)

Scope: close to Critical-Path Institute document (2019.6)
Molecules： **Endogenous metabolites, peptides, proteins**
Methods: **LC/GC-MS、LBA**
（Excluding IHC, flow cytometry, genomics, MS imaging）
◆Biomarkers as drug developmental tools
(excluding CDx, clinical chemistry)
◆Biomarkers used for regulatory decision
(At first, excluding ones for exploration and decision making in a company)

AMED research group (NIHS, JBF, JPMA, others)

White Paper

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Bioanalysis

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Analytical method validation for biomarkers as a drug development tool: points to consider

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Biomarkers are an important drug developmental tool. Assessment of quantitative analytical methods of biomarkers is not included in any regulatory documents in Japan. Use of biomarkers in clinical evaluations and supporting the post-marketing evaluation of drug efficacy and/or adverse reactions requires assessment and full validation of analytical methods for these biomarkers. The Biomarker Analytical Method Validation Study Group is a research group in Japan comprising industry and regulatory experts. Group members discussed and prepared this 'points to consider document' covering measurements of endogenous metabolites/peptides/proteins by ligand binding assays and chromatographic methods with or without mass spectrometry. We hope this document contributes to the global harmonization of biomarker assay validation.

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Keywords: assay validation • biomarker • Japan • regulatory submission • study sample analysis • white paper

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Table of contents

What's next

Scope: **Quantitative PCR（qPCR）** for gene expression, etc.
Flow cytometry of protein expression

Analytical methods for obtaining data to use the biomarker as a surrogate endpoint or to characterize the drug (e.g., pharmacological effects, occurrence of side effects, etc.) **in drug approval application dossier**.

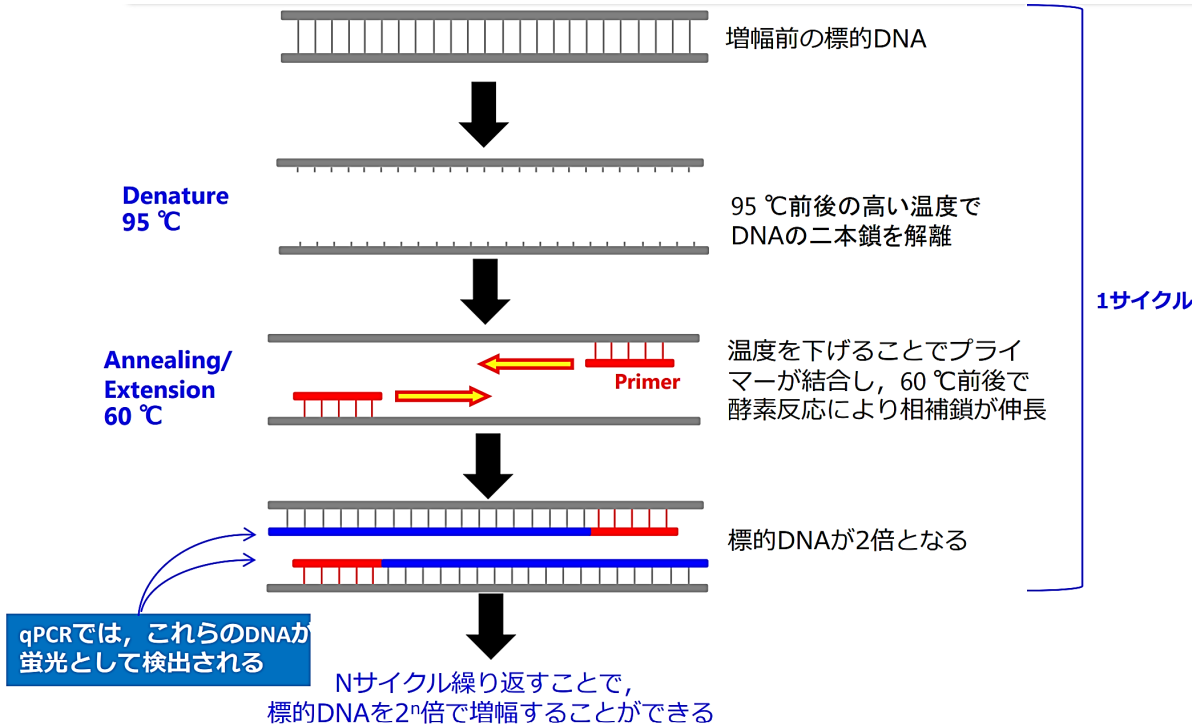
The contents of this document are intended to be used for the description in the drug application dossier (CTD), assuming **analytical method validation and study sample analysis for biomarkers for which reliability of quantitative values of biomarkers is required.**（Discussed assuming the biomarkers described in **CTD module 2**）

qPCR

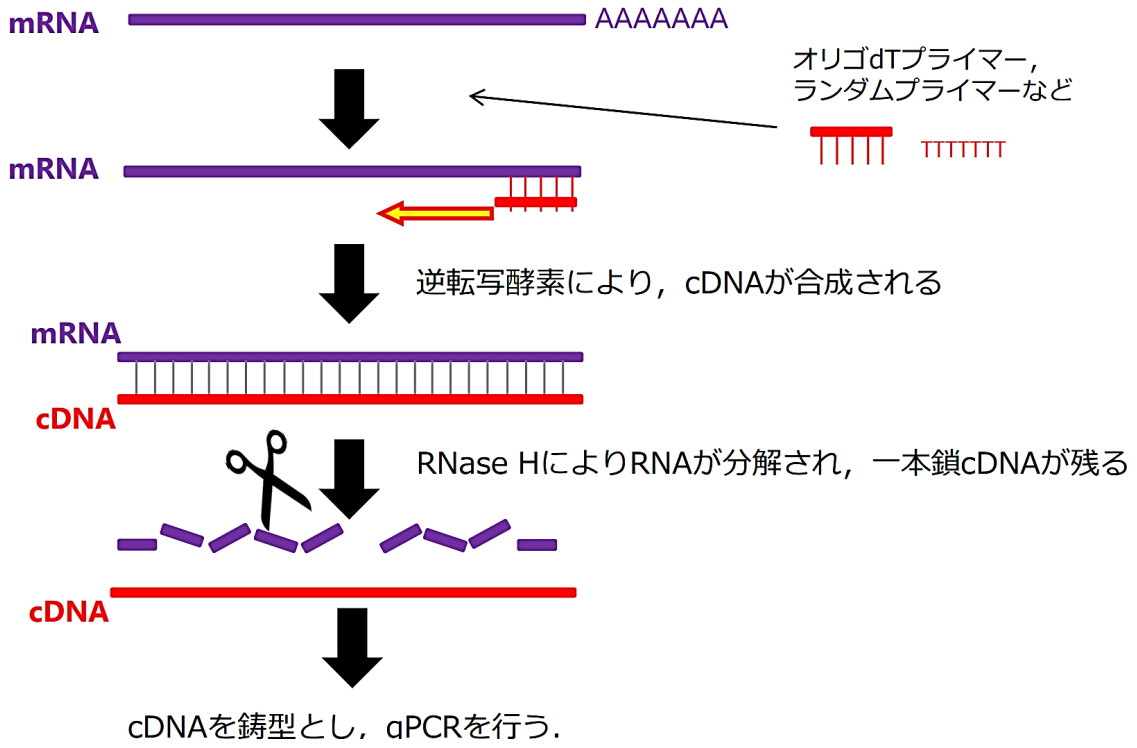
qPCR = quantitative polymerase chain reaction

An analytical method for quantifying the amount of target gene products by serial monitoring PCR reactions using a fluorescent-labeling method.

Detecting DNA（qPCR）



Detecting RNA（RT-qPCR）

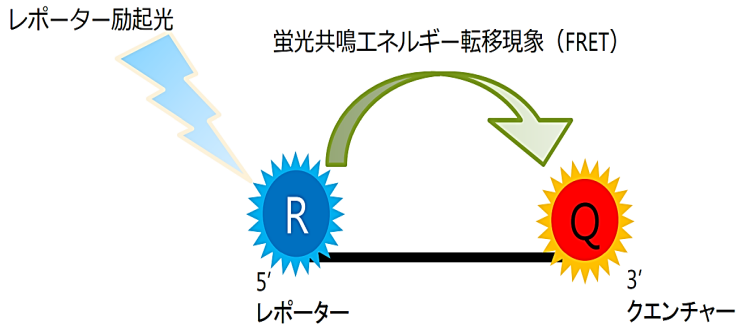


From JBF-DF（http://bioanalysisforum.jp/images/2018_9thJBFS/P6_DG2017-33.pdf）

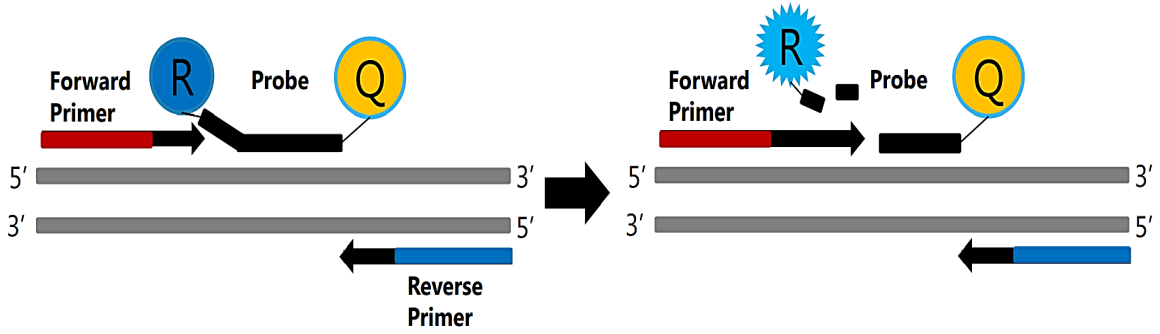
Principles of qPCR

Probe method

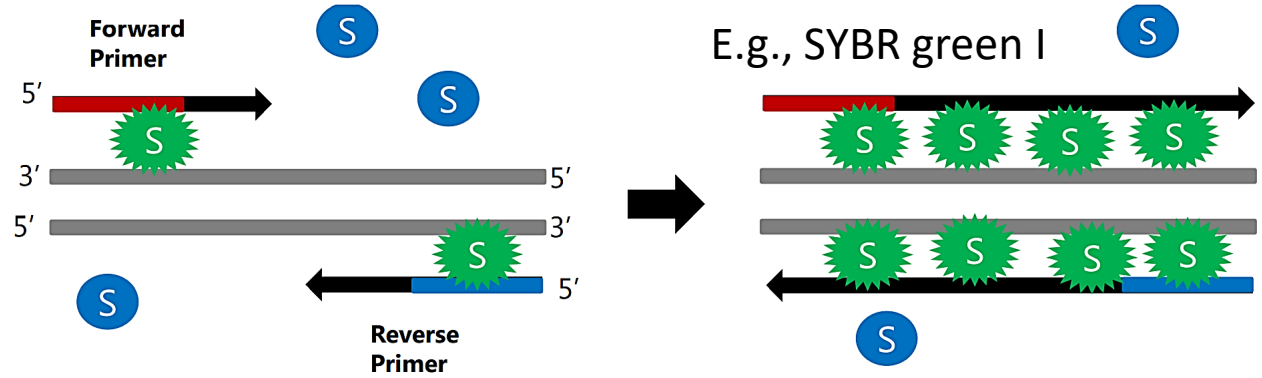
①通常は，FRETによってレポーターの蛍光は吸収されている.

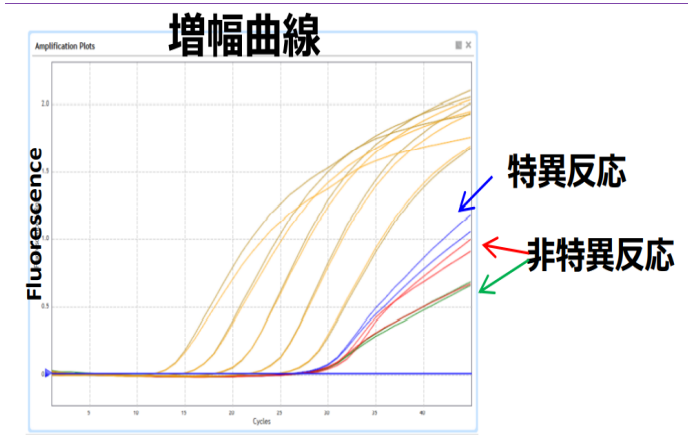


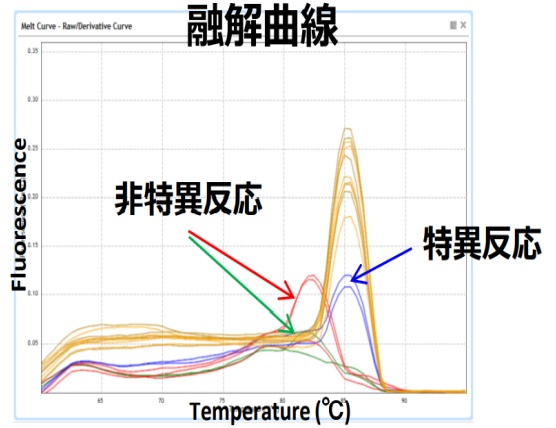
②伸長反応によりプローブが加水分解されることで蛍光を発する.



Intercalator method







From JBF-DG（http://bioanalysisforum.jp/images/2018_9thJBFS/P6_DG2017-33.pdf）

Investigations on the current regulatory situation (qPCR)

FDA List of Qualified Biomarkers

（https://www.fda.gov/drugs/cder-biomarker-qualification-program/list-qualified-biomarkers）

Searched keywords ⇒ ″ RNA″ or “PCR″

Hit ⇒ **1 item**

Hit words ⇒ Plasmodium falciparum 18S rRNA/rDNA blood test

According to the **MIQE guideline (Minimal Information for Publication of Quantitative Real-Time PCR Experiments),** **calibration curve, sensitivity, accuracy/precision, specificity (influence by species differences), matrix effect (influence by plasma samples such as leukocytosis, hemolysis, hyperlipidemia, hyperbilirubinemia), short-term stability, and carryover were validated.**

（Available at Validation documents were https://www.fda.gov/media/136947/download）

Investigations on the current situation (qPCR)

Referred guidelines/white papers

- Guidance/guideline on gene therapy products from FDA/EMA
- White papers from JBF and EBF
- WRIB white papers 2020, 2021
- GCC white paper
- ISO guideline
- qPCR guideline from MIQE

(Clin Chem. 2009;55(4):611-22.)

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines:
Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

D: desirable, E: essential

qPCR validation		
Evidence of optimization (from gradients)		D
Specificity (gel, sequence, melt, or digest)	**Specificity**	E
For SYBR Green I, C_q of the NTC		E
Calibration curves with slope and *y* intercept	**Calibration curve**	E
PCR efficiency calculated from slope	**PCR efficiency**	E
CIs for PCR efficiency or SE		D
r^2 of calibration curve	**R^2 values**	E
Linear dynamic range		E
C_q variation at LOD	**Precision at LOD**	E
CIs throughout range		D
Evidence for LOD	**LOD**	E
If multiplex, efficiency and LOD of each assay		E

Discussion on qPCR

Held 8 meetings

① **Selection of validation parameters for qPCR method on biomarkers**

② **Discussion on selected validation parameters in** ①（Acceptable criteria depends on the Context of Use and are not described in the document）「Standard/measurement control, sensitivity, detection limit, quantification limit, specificity, calibration curve, linearity, PCR efficiency, accuracy, precision (intra- and inter-laboratory precision), matrix effect, stability, recovery rate」＋ISR

③ **Points to consider document will contain development and data analysis of qPCR methods.**
In relation to the above validation parameters, concept and points to consider on the method development are also described in the document.

Table of contents (Final)

Overview + Method development + Validation

Scope & basic principles

Nucleic Acid (NA) biomarkers are used as drug development tools
evaluated in clinical trial end points, or
analytical results are in drug application dossiers as reference information,

the analytical methods to be used must be well validated

- NA biomarkers: mRNAs, miRNAs, cell-free DNA, etc.
- qPCR/RT-qPCR method to quantify the concentration of a single target NA biomarker as an analyte in biological matrices (i.e., a single-plex)

Before validation of an analytical method for each NA biomarker,
➡ define and document the required validation parameters and acceptance criteria,
considering by 'fit-for-purpose' and availability of experimental materials.

Selectivity: not typically required.
(samples containing DNA or RNA other than the analyte are usually analyzed.

NA reference standards & measurement controls

NA reference standards: DNA or RNA containing PCR-amplified regions of the target NA molecule

- ✓ recommended chemically synthesized NAs with known copy numbers
- ✓ advisable to use an advanced purified standard NAs to ensure the reliability of the analytical method (Purity: as high as possible to meet the context of use of the biomarker).
- ✓ RNA: produced RNAs via *in vitro* transcription can be used as standard.

Critical reagents Reagents that directly affect the analytical results are designated in advance

- Primers and probes (for PCR): recommended HPLC or PAGE grade

Method development

- ➢ Cyclic DNA: recommended to linearize as necessary to reduce the influence on PCR efficiency.
- ➢ RNA: recommended to start method development using RNA (not DNA) as a standard to evaluate stability.
- ➢ Recommended that lot-to-lot differences (synthesized or in vitro-transcription) are evaluated.
- ➢ Advisable to evaluate the base lengths, concentrations and degrees of degradation of the reference.

Method validation

- ✓ To verify accuracy, necessary to prepare QC samples (positive controls), extraction blank (Standards+water/buffer to confirm contamination during the extraction) and non-template control (to evaluate non-specific amplification) for each qPCR/RT-qPCR measurement run.
- ✓ QC samples and calibration standards are prepared separately.

Sensitivity (LLOQ & LOD)

Indicated by the lower limit of quantification (LLOQ) and limit of detection (LOD)

LLOQ: minimum conc. that satisfies the predetermined acceptance criteria for accuracy and precision

LOD: minimum conc. at which ≧95% of study samples are detected as positive in the method.

- **LLOQ**: necessary to evaluate in validation
- **LOD**: not included in the quantification range and not used as a validation parameter

Method development

➢ 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.

Method validation

✓ Verify whether the accuracy and precision obtained with QC samples with the same concentration as the LLOQ tentatively determined in the '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

Ability to identify and detect target NA molecules among other NA molecules (DNA, RNA) with similar sequences in the matrix

- Depending on the base sequences of primers and probes used and annealing conditions (temperature, duration) of PCR.
- The development of analytical methods is mainly based on *in silico* analysis results. Advisable to verify the primer/probe sequences with concern for non-specific amplification by validation tests.

Method development

- ➢ Select primers and probes with *in silico* database that have minimal concern for non-specific amplification within the target species. Pay particular attention to the 3'-end sequences.
- ➢ Advisable to obtain various information on the amplified products by DNA sequencing, melting curve analysis, electrophoresis, and restriction enzyme analysis.

Method validation

- ✓ For NA sequences shown in *in silico* analysis to have concern for nonspecific amplification, a confirmation test should be performed to meet the predefined acceptance criteria using water/buffer containing the NA sequences and the analyte at the LLOQ concentration.
- ✓ If non-specific amplification is not estimated for the target sequences in the development stage, the validation test of specificity can be simplified.

Calibration curve

To calculate concentration (copy number per unit volume) of the NA biomarkers, determined from LLOQ to ULOQ

- Recommended that number of calibration standards and number of samples per concentration be determined in advance in the protocol based on the context of use.
- If endogenous concentration is high, surrogate matrix can be used for calibration standard.

Method development

- Considering the concentration range of the endogenous nucleic acid biomarker, establish a quantitative range for the calibration curve that ensures the reliability of the analytical method.
- Described formulae - Linearity (Regression line of the calibration curve)
  - PCR amplification efficiency

Method validation

- ✓ Recommend to perform at least 3 repeated measurements on different days to evaluate PCR amplification efficiency, linearity (r^2 value), and the accuracy and precision of the back-regression concentration.
- ✓ Advisable for the average values - PCR amplification efficiency: 90%-110%
  - linearity (r^2 value): 0.98≤

Accuracy/Precision

Accuracy: Degree of closeness of measured value to nominal or known authentic value
Precision: Closeness of agreement (degree of scatter) among a series of measurements.

- Advisable to evaluate within and between analytical runs using QC samples
- Recommend that either the authentic matrix or an surrogate matrix is selected for preparation of QC samples, depending on the endogenous concentration of the target NA substance.

Method development

➢ Recommend to set several concentrations of QC samples: half of ULOQ for high QC (QC-H), twice the LLOQ for low QC (QC-L), near the middle of calibration curve for medium QC (QC-M) and LLOQ.

Method validation

Repeat measurements ≥3 times on different days in different analytical runs

✓ Evaluate intra- and inter-assay accuracy and precision using 4 conc. QCs.

✓ Advisable to evaluate study samples with 2 different concentrations (low and high) and evaluate precision of them by repeated measurements.

Matrix effect

Effect of contamination of PCR inhibitors, etc. in matrix, on measurement values

- Important to evaluate per matrix
- Inhibitory factors can be collagen, melanin, hemoglobin, urea (endogenous molecules) heparin (anti-coagulant), phenol, surfactants (reagents) and others

Method development

- When preparing calibration curve and QC samples using surrogate matrices, advisable to confirm the matrix effect using the authentic matrix.
- To reduce matrix effect,
  1) Dilution of the study samples
  2) Changing reagents used in the analysis method or extraction method
  3) Using an internal standard

Method validation

Recommend to evaluate precision or matrix factor using 10 different blank matrices

- ✓ Advisable that no matrix effect is observed.
- ✓ When matrix effect is confirmed and if the estimated accuracy corrected by the internal standard is acceptable using internal standard, it can be considered that the matrix effect does not influence the performance of the analytical method.

Parallelism

Confirmation of parallel slope between calibration curve using calibration standard and sample dilution curve

- generally recommended using study samples (Sample dilution leads to a reduction in the effect of PCR inhibitors)
- samples with ≥3 levels of dilution are prepared using authentic or surrogate matrices of high concentrations
  ⇨ confirmed that measured values corresponding to the dilution ratio are obtained.

Stability

Chemical or biological stability of the analyte in a solvent or matrix for a given time and under specified conditions

- Since NA biomarkers are susceptible to degradation, attention should be paid to the analyte stability.

Using **QC samples (low and high conc**. in **authentic matrices)** with **3 repeats**
Essential: '**Benchtop and Short term**', '**Long term**' and '**Freeze-thaw**' stability
Applicable: '**Processed sample stability**'

Numbers of freeze-thaw cycles: expected number of times in the study sample analysis
Short- and long-term: periods longer than expected storage period of the study samples

When suitable study samples are unavailable at validation, 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 incurred sample stability approach using study samples.

Recovery rate

Efficiency of analyte recovery in the pretreatment process of biological samples

Method development

- advisable to confirm that recovery rate 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, advisable to use an internal standard and perform data correction based on the measured value.

Others

Partial validation, **Cross validation** and **Study sample analysis** are largely similar with cases of chromatography and LBAs (Ohtsu et al., 2021)

ISR: recommended to be performed in different matrices on samples from representative clinical trials such as biomarkers is used as end points in the late clinical trial

Points to note

NA adsorption: When endogenous concentration of target analyte is low, the effect of adsorption of the analyte onto laboratory equipment may be significant.

If necessary, use laboratory equipment with low NA adsorption specifications
add carrier NAs to prevent adsorption

White Paper

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Bioanalysis

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

Yuchen Sun[1], Takahiro Nakamura[2], Yoshiaki Ohtsu[3], Masaaki Kakehi[4], Noriyuki Danno[5], Hiroyuki Shimizu[6], Yoichi Tanaka[1], Victoria Serelli-Lee[7,8], Seiji Tanaka[9], Takashige Okayama[10], Yusuke Suda[11], Yuu Moriya[4], Takeshi Hanada[12] & Yoshiro Saito*,[1]

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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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Keywords: method validation • nucleic acid biomarkers • qPCR • regulatory submission • RT-qPCR • study sample analysis • white paper

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