Understanding qPCR* bioanalysis issues before validation planning (DG2017-33) –Update-

Daiichi Sankyo Co., Ltd.
Takeshi Hanada

* Quantitative Polymerase Chain Reaction

DG2017-33「qPCR for Bioanalysis」

Members

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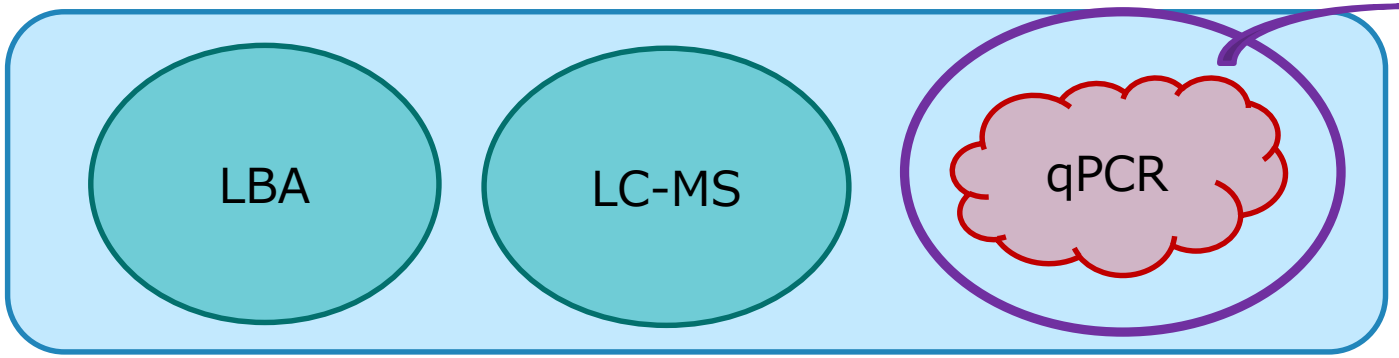
DG2017-33「qPCR for Bioanalysis」



There are no guideline regarding qPCR as bioanalysis tool

Current Status

To investigate the validation contents, there is a lack of “common awareness” on experimental methods, handling of values, etc.

we summarized the characteristics of qPCR as a tool and the points to consider when using it for Bioanalysis.

Review

For reprint orders, please contact: reprints@future-science.com

Bioanalysis

Understanding quantitative polymerase chain reaction bioanalysis issues before validation planning: Japan Bioanalysis Forum discussion group

Asako Uchiyama[1], Yoichi Naritomi[2], Yoshitaka Hashimoto[3], Takeshi Hanada*[,4], Kyoko Watanabe[4], Kumiko Kitta[5], Genki Suzuki[6], Takao Komatsuno[7] & Takahiro Nakamura[1]

Bioanalysis **14(21), 1391–1405 (2022)**

Referred qPCR guideline and white papers

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]

Guidance for Industry

Gene Therapy Clinical Trials – Observing Subjects for Delayed Adverse Events

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research
November 2006

ICH harmonisat

ICH S12 Guideline on nonclinical biodistribution considerations for gene therapy products

Step 5

19 April 2023

IPRP International Pharmaceutical Regulators Programme

Expectations for Biodistribution (BD) Assessments for Gene Therapy (GT) Products

WRIB

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)

GCC GLOBAL CRO COUNCIL

Recommendations on qPCR/ddPCR assay validation by GCC

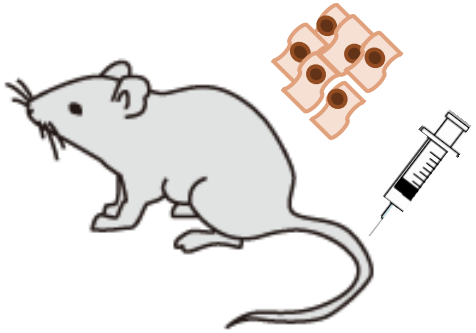
Contents

1. Consideration common by qPCR, RT-qPCR
2. RT-qPCR considerations
3. Specific issues for cell quantification
4. Assay assurance

Contents

General Experimental Flow for qPCR, RT-qPCR

①Administration



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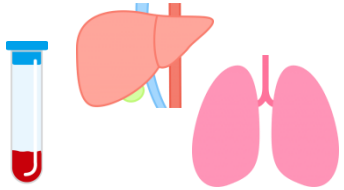

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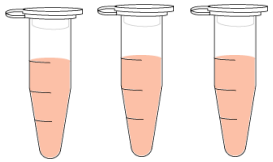

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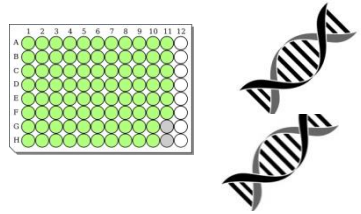
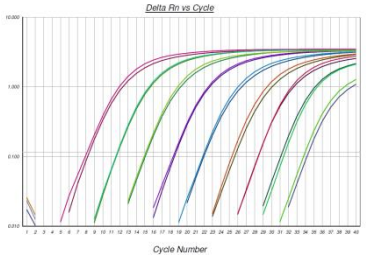


qPCR for DNA

Add equivalent amounts of NA in 1 reaction



⑤qPCR Calculation of copies/rxn

qPCR step

RT-qPCR for RNA

cDNA synthesis (RT step)

☆Primary issues for qPCR is to prevent comtamination.

PCR facilities

To prevent contamination, the area should be segmented for each procedure, and one-way movement is recommended. Use of PCR cabinet is also effective to prevent contamination.

Clean

master-mix area — Handle reagents other than NAs (primers, buffers, etc.)

pre-PCR area — Handle samples and control NAs

PCR amplification — PCR devices are placed and PCR amplified

PCR cabinet

https://www.medicalexpo.com/ja/seizomoto-iryo/kiwado-40390.html

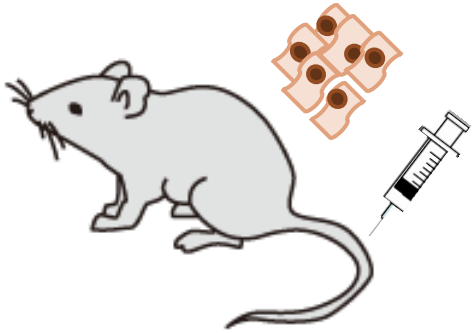
Post-PCR area — Handle the amplified PCR products

Dirty

From European Pharmacopoeia 10th edition (2019)

General Experimental Flow for qPCR, RT-qPCR

①Administration



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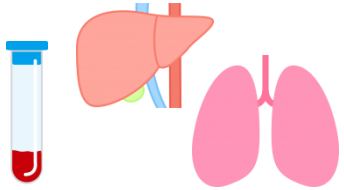

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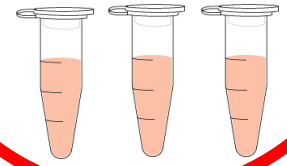

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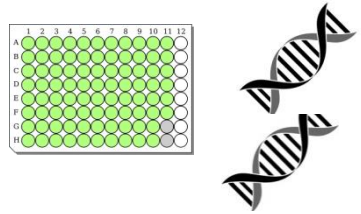

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RT-qPCR for RNA

cDNA synthesis (RT step)

qPCR step

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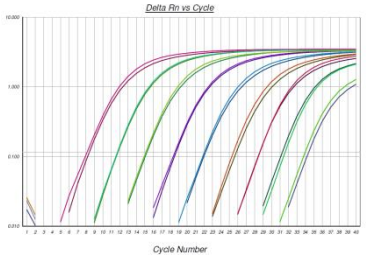


⑤qPCR Calculation of copies/rxn

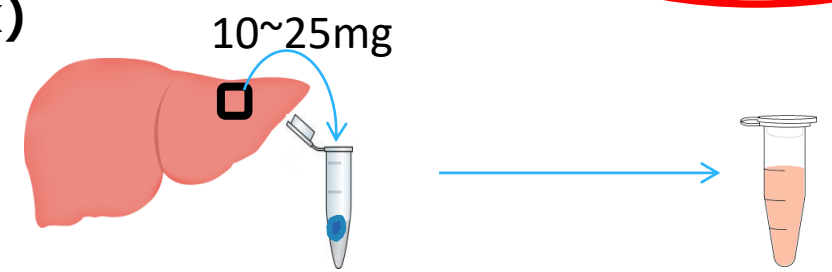
http://bioanalysisforum.jp/

NA extraction（Step③）

Tissue Excision → Milling homogenize → NA extraction → DNA/RNA

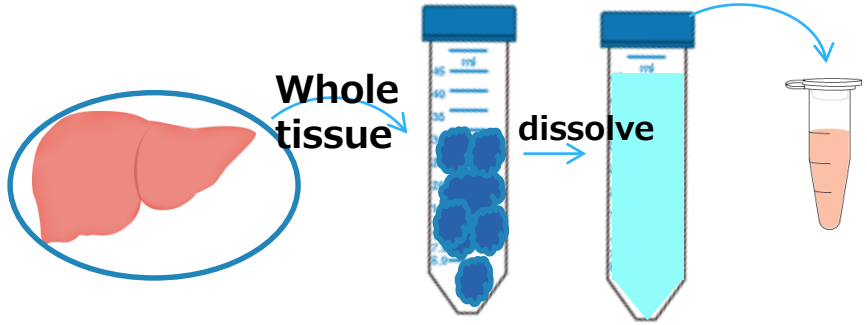
Ex)

A.



- A portion of tissue are sampled and extracted
  Confirmation of no difference in distribution is required.

B.



- Lyse whole tissue and extraction from part of it
  Pro: Available even if if there is a bias in the tissue distribution
  Con: A lot of solution are needed.

C.

- Grind whole tissue, then lyse and extraction from part of it
  Pro: Available even if if there is a bias in the tissue distribution
  Con: High contamination risk

NA extraction（Step③）

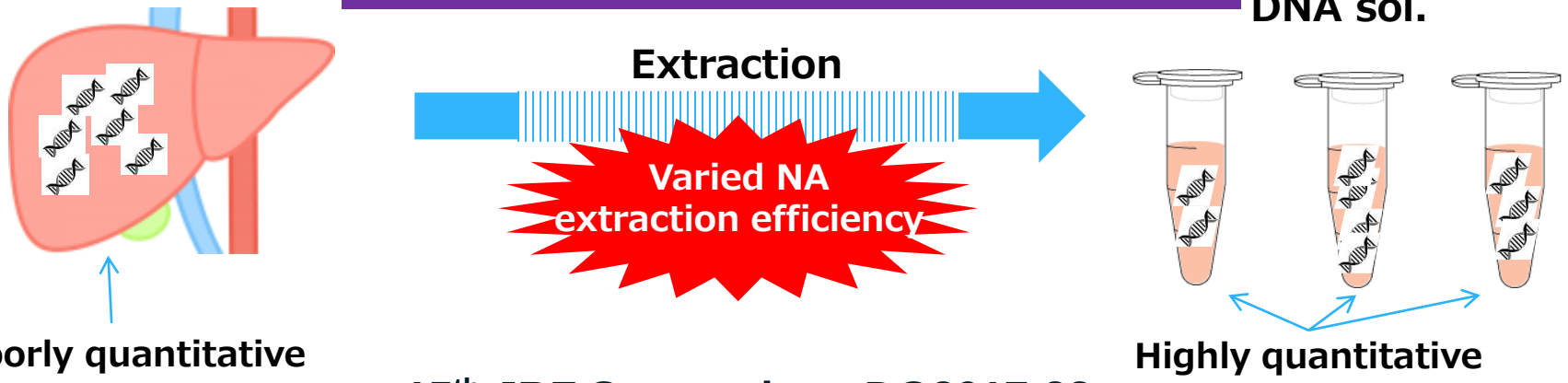
Tissue Excision → Milling homogenize → NA extraction → DNA/RNA

There are many methods such as column method using silica, magnetic bead method, phenol/chloroform method.

The extraction efficiency from tissue is varied from 5 to 100%
（Different from sample, not constant ）

⇒Conventional methods cannot accurately calculate the concentration of a target NA in a tissue.

Usually, PCR can quantitate only the amount of NA contained in the NA solution after extraction



NA extraction（Step③）

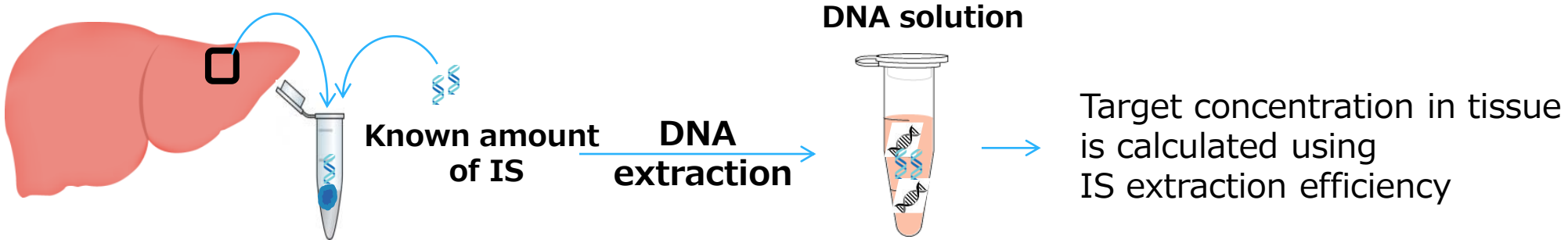
There are many methods such as column method using silica, magnetic bead method, phenol/chloroform method.

	Principle	Pros	Cons
Phenol/ chloroform method	Protein is denatured by organic solvent.	High-purity NAs can be recovered at high concentrations.	However, the treatment process is complicate and low-throughput
Membrane method	NA is purified by column	Simple, convenient and widely used High-throughput	Extraction efficiency of is lower than that of other methods.
Magnetic bead method	NA is isolated by magnetic beads	Most suited for automation and high throughput. High extraction efficiency	May not function in highly viscous samples Residual beads may contaminate samples

Possible solution for extraction efficiency

Ex) Using Internal Standard (IS)

Only for DNA detection

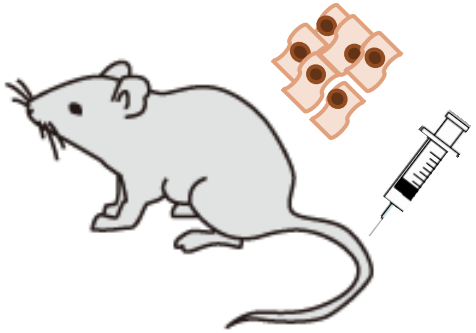


IS: NA that do not cross the sequences of the host and analyte
(NA other than human and animal used)

Target concentration in tissue can be calculated by enabling calculation of extraction efficiency.

General Experimental Flow for qPCR, RT-qPCR

①Administration



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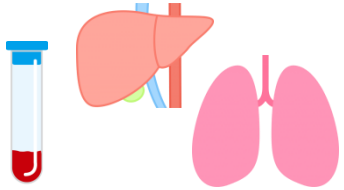

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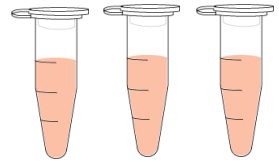

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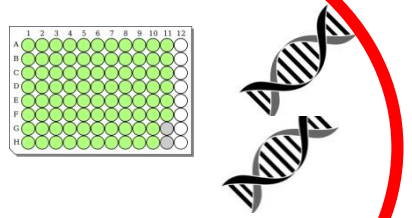


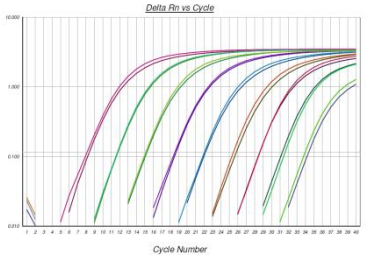
qPCR for DNA

RT-qPCR for RNA

cDNA synthesis (RT step)

qPCR step

Add equivalent amounts of NA in 1 reaction



⑤qPCR Calculation of copies/rxn

Measurement of NA concentration（Step④）-Methods-

Note : Accurate measurement is important minimize matrix effect by setting the amount of NA per reaction to be constant.

Absorbance method

- Calculated from OD_{260} absorbance

Pro: Simple

Con: Unidentifiable DNA and RNA
Effect of protein contamination

Ex) Nanodrop
Nanovue
etc.

Fluorescence method

- Specifically detect DNA or RNA by fluorescence

Pro: Not affected by materials other than analyte present in the sample

Con: Not yet generalized

Ex) PicoGreen
RiboGreen
Qubit

Measurement of NA concentration (Step④) - Evaluation of NA quality -

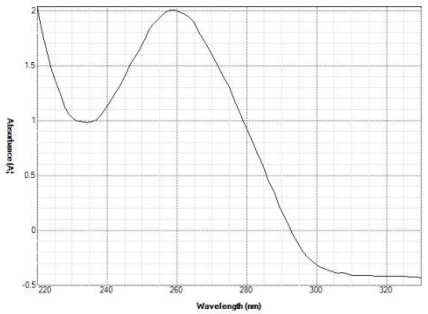
Poor quality may inhibit the reaction and reduce the number of template DNA sequences due to NA fragmentation.

Absorbance

OD260/280 : 1.8~2.0
OD260/230 : 2.0~2.2

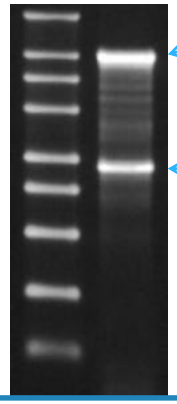
Showing the proportion of impurities absorbed by 230,280 nm

Absorbance chromatogram



Visually impurity contamination

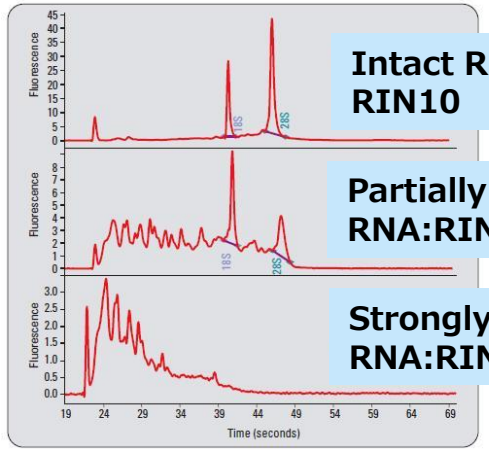
Agarose gel Electrophoresis (RNA)



RIN (RNA Integrity Number)
DIN (DNA Integrity Number)

Degradation of NA from the electrophoresis image is automatically displayed (full score of 10)

RIN

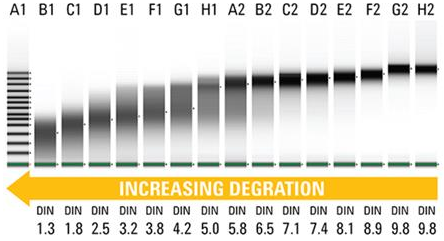


Intact RNA: RIN10

Partially degraded RNA:RIN5

Strongly degraded RNA:RIN3

DIN



https://www.agilent.com/cs/library/applications/5991-5258EN.pdf

General Experimental Flow for qPCR, RT-qPCR

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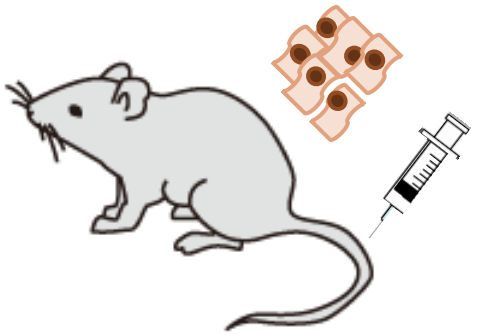
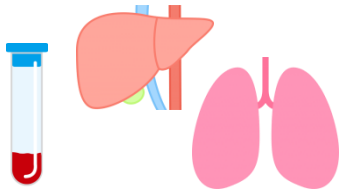
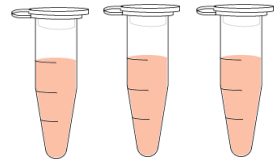
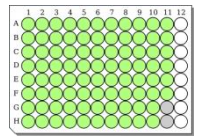
②Tissue sampling

③Dissolution, Nucleic acid (NA) extraction









④Analysis of NA

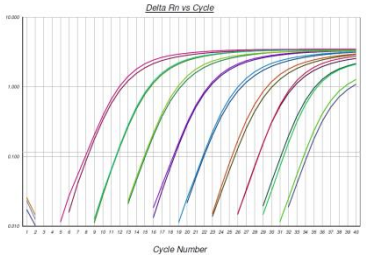
RT-qPCR for RNA

cDNA synthesis (RT step)

qPCR step

qPCR for DNA

Add equivalent amounts of NA in 1 reaction



⑤qPCR Calculation of copies/rxn

http://bioanalysisforum.jp/

qPCR measurement（Step⑤）
- Reference Standard -

For absolute quantification, reference standards with known copy numbers are required.

Types of reference standard

	Pros	Cons
Plasmid	・Highly stable ・Easy to produce large amounts (initial cloning is necessary)	・Equivalence to Analyte
Synthetic NA	・Flexibility of sequencing ・No cloning required	・Equivalence to Analyte
Genomic NA	・Equivalent to analyte	・Low stability ・Difficult to produce large amounts, and take long time ・Lot-to-lot variation

In general, plasmids are often used.

qPCR measurement（Step⑤）
- Matrix Effect -

Matrix effect in qPCR : Various type of inhibitors

Table 2. List of inhibitors derived from matrices.

Matrix	Inhibitor	Mode of action
Blood	Heme/hemoglobin	Release of iron ions/competition with template
	Leukocyte DNA	Competition with template
	IgG	Binding to ssDNA
	Lactoferrin	Release of iron ions
	Antiviral drugs (e.g., acyclovir)	
Blood (anticoagulant)	EDTA	Chelation of Mg^{2+}
	Heparin	Binding to DNA, competition with template and direct interaction with polymerase
Muscle, eye fluid	Myoglobin	Release of iron ions
Hair, skin	Melanin	Binding to polymerase
Stomach	Bile acids	
Bone	Collagen	Alteration of ion composition by binding cations
Feces	Bile salts	Direct effect on polymerase
	Phytic acid	Chelation of Mg^{2+} or change in ion content if present as salt
	Polysaccharides	Binding to polymerase
	Bilirubin	Competition with template
Urine	Urea	Prevention of noncovalent bonding, acting directly on polymerase or hindering primer annealing

Bioanalysis 14(21), 1391–1405 (2022)

http://bioanalysisforum.jp/

Effect of PCR inhibitors in the extracted DNA is important for reliability of data

qPCR measurement（Step⑤）
- Matrix Effect -

Need for addition of matrix to the reference standard sample

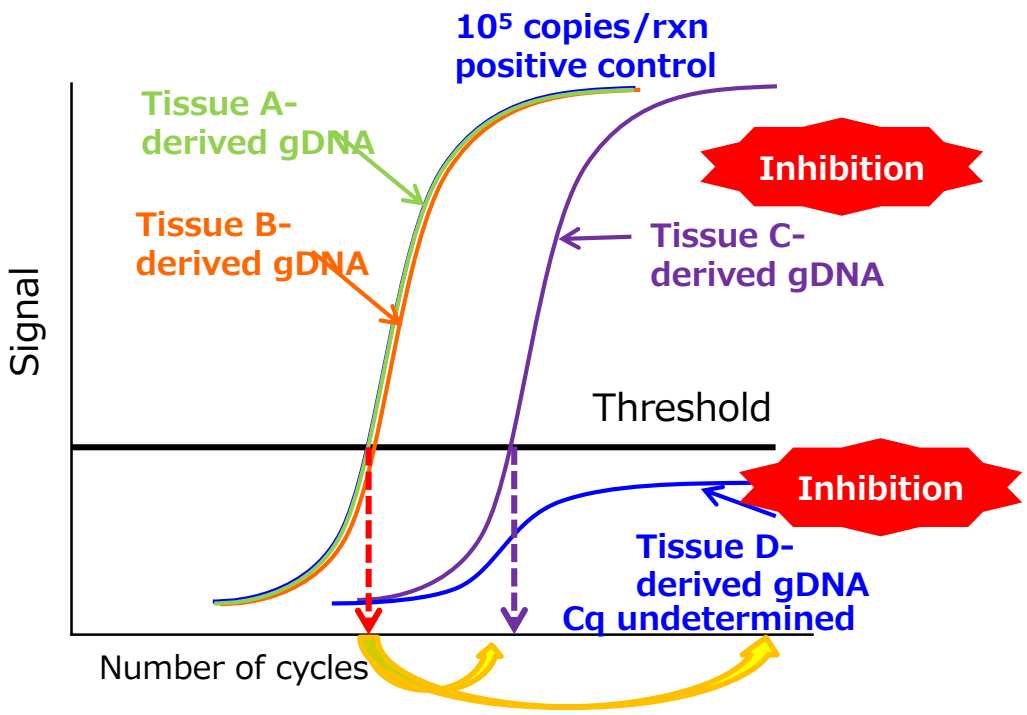
- ✓ In general, the matrix of the standard sample should be the same as that of the measurement sample.
- ✓ If it is shown that there is no effect on the calibration curve data due to the presence or absence of matrix DNA, there may be no need to add matrix DNA.
- ✓ When measuring multiple organs, representative organ-derived DNA (ex. liver) may be used for matrix of standard sample, if the matrix effects are same between organs.

Note: The extracted nucleic acids may differ in quality depending on the tissue of origin.

Ex. Skin – High levels of melanin, a PCR inhibitor
Feces – Abundant PCR inhibitors

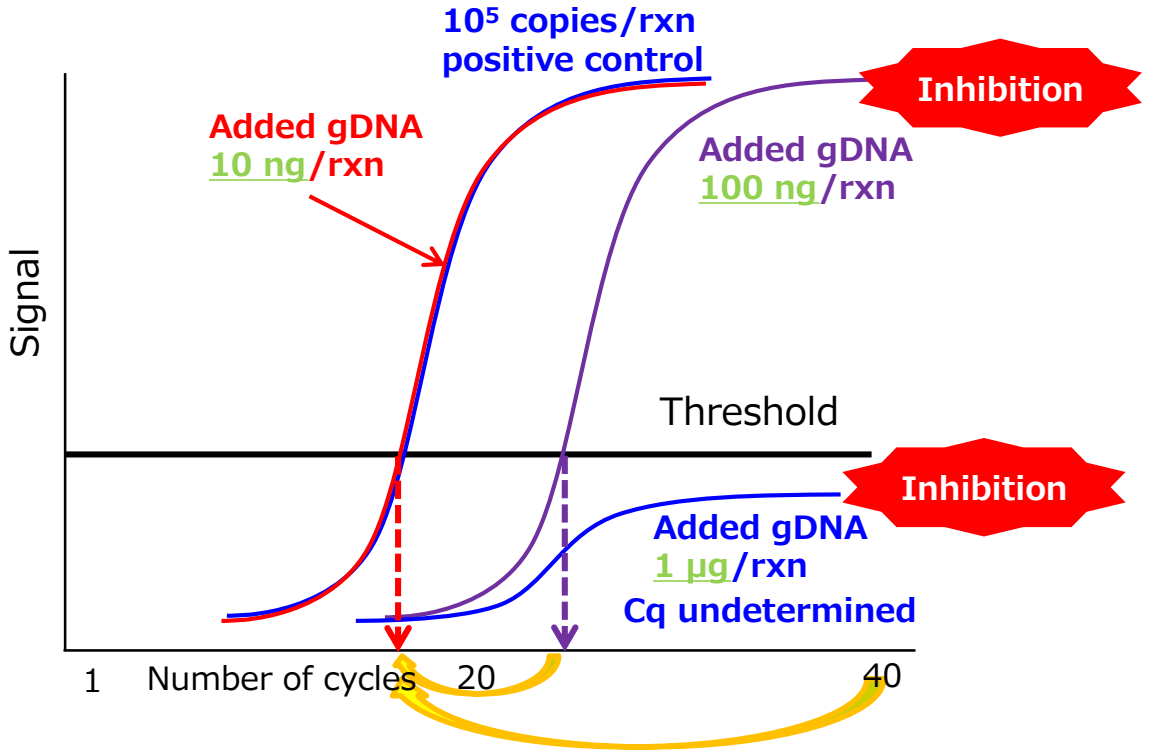
qPCR measurement（Step⑤）
- Example of Matrix Effect -

Ex1) 10^5 copies/rxn of positive control is added to gDNA from various tissues



Cq levels are usually observed in the same way as positive controls, but Cq are higher or not observed if there is an inhibitor in gDNA.

Ex2) Inhibitory effect is improved by dilution of gDNA



When the added gDNA is 1 µg/rxn, Cq level is ND.
When diluted to 100 or 10 ng/rxn, Cq levels recovered in a dose-dependent manner.
In 10 ng/rxn, Cq level is same as positive control.

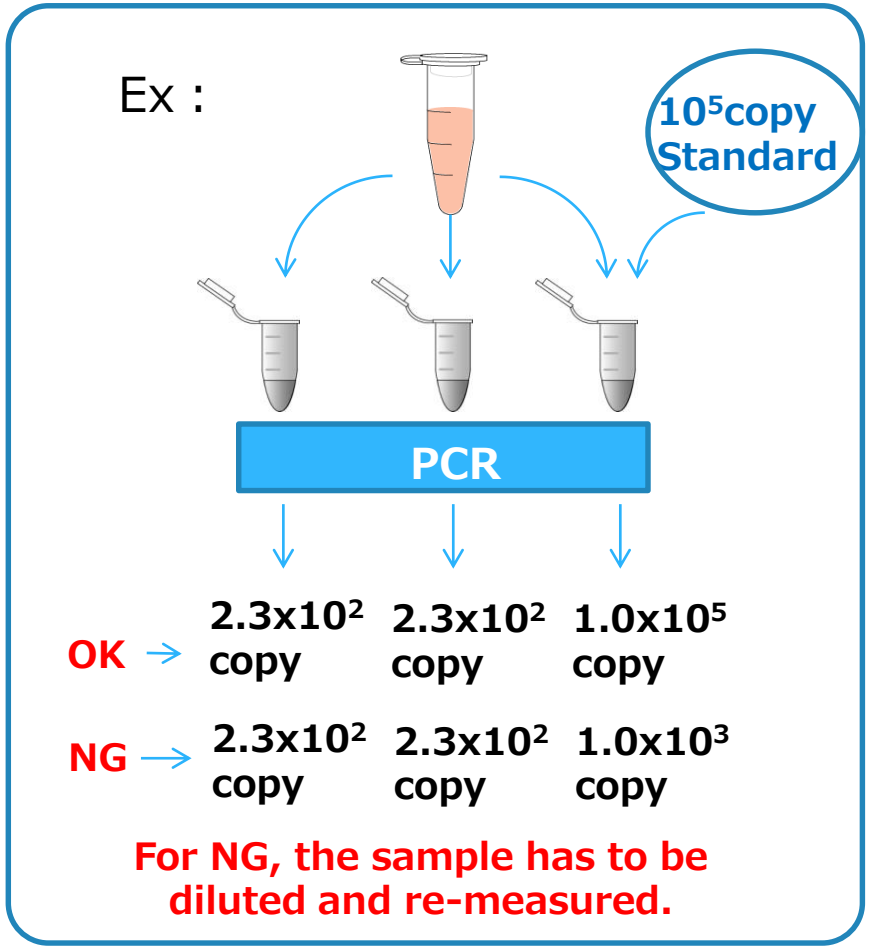
qPCR measurement（Step⑤）
- Matrix Effect -

Measures against inhibition

1. Sample dilution (See previous page)
   - The simplest and most effective response
   - Result in reduction of assay sensitivity

2. Selection of appropriate reagents
   - Selection of appropriate DNA polymerase
   - Optimize buffer system

3. Use of appropriate controls



FDA Guidance for industry: gene therapy clinical trials (2006)

Samples should be assured individually, as the amounts of PCR inhibitors may be vary from sample to sample.

Contents

1. Consideration common by qPCR, RT-qPCR
2. **RT-qPCR considerations**
3. Specific issues for cell quantification
4. Assay assurance

Assay method

Category	Method	Std curve	Description	Application
absolute quantification (mainly DNA)		○	Quantification using calibration curves generated with standard material of known copy number	Quantification of GMO* and microorganisms
Relative quantification (mainly RNA)	Calibration curve method	○	Calibration curves are generated with relative values such as dilution rate. With this calibration curve, comparative quantification can be performed.	Gene expression analysis
	ΔΔCq method	X	The difference in Cq values between the target and reference genes in the sample is compared to the control sample and calculate a relative amount.	Gene expression analysis

*Genetically Modified Organism

Reference gene (endogenous control gene)

Objective: Correct the expression level of target genes

- The expression level of endogenous control genes reflects the **quality of the RNA** and the **efficiency of the RT response**.
- Traditionally, **GAPDH and β-actin** have been used, but there are many reports that their expression levels are fluctuating.

Incorrect setting of endogenous control genes may lead to an overestimate/underestimate of the expression level of target genes.

Caution :
1. Is the level of expression in the target cell or tissue appropriate?
2. To investigate the expression in response to stimuli

Expression level are corrected using the average of two to three reference genes

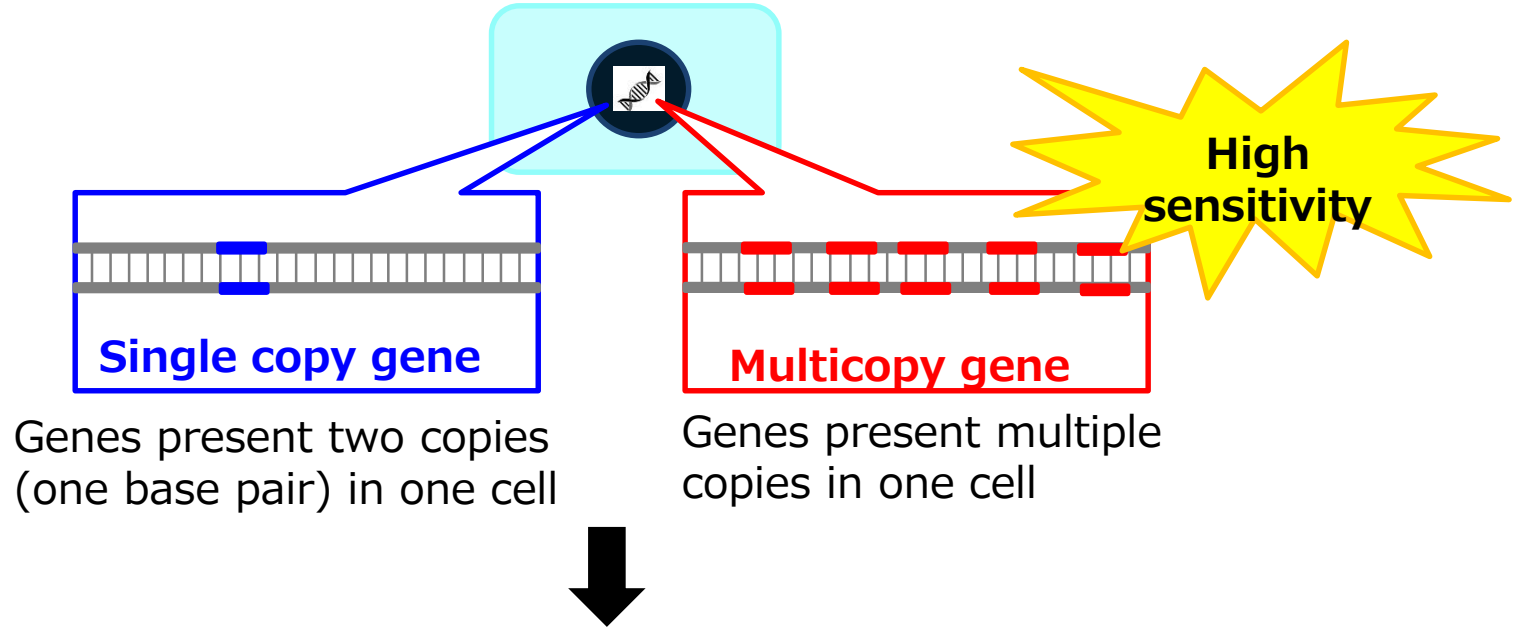
Reference : *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes:* Vandesompele et al., 2002. 3(7) Genome Biology 2002.

Quantification of mRNA using real-time reverse transcription of PCR (RT-PCR): trends and problems: Bustin, 2002.29, 23-39. J Mol Endo

Contents

Target genes for detecting cell therapy products (CTPs)

Target genes in CTPs



Genes present two copies (one base pair) in one cell

Genes present multiple copies in one cell

In the case for biodistribution of CTPs, **multicopy gene** should be used.

The most studied target sequence

Alu sequence

The most studied target for qPCR detection of CTPs

- ✓ Primate-specific repeat
- ✓ Length : about 300 bases
- ✓ Among the SINE (short interspersed element) in human gene, Alu has the most copies (about 10^6 copies)
- ✓ Alu sequence insersions into genes differ in individuals.

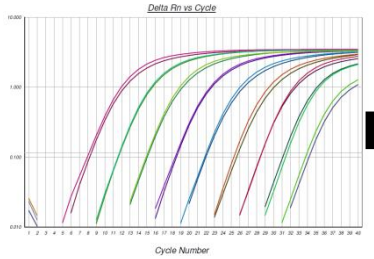
Although the sensitivity is high…

Because PCR target is abundant in human cells, it is **difficult to remove contamination.**

Human DNA contamination from reagents and consumables cannot be completely removed

Considerations when targeting multicopy genes

From PCR data…



①Synthetic NA as Std… Copy number/reaction

②gDNA derived from cell as Std…DNA amount/reaction

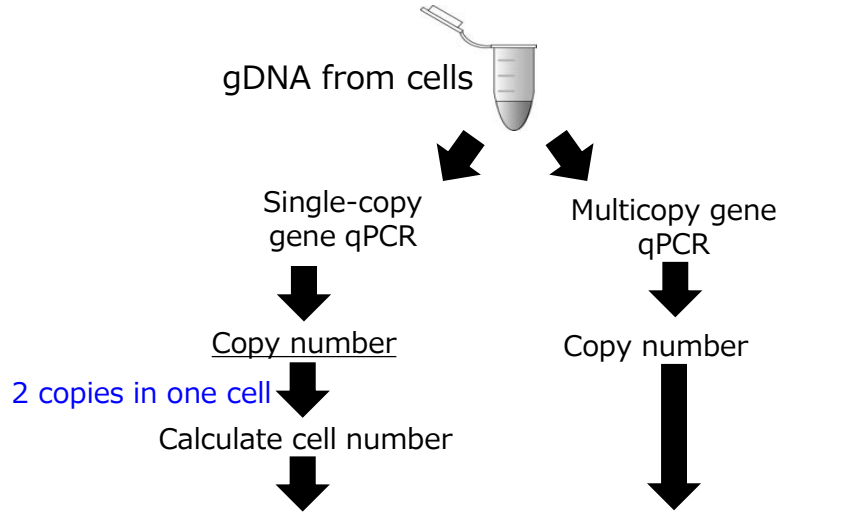
What we want to evaluate…

Cell number

Need to be converted to cell number

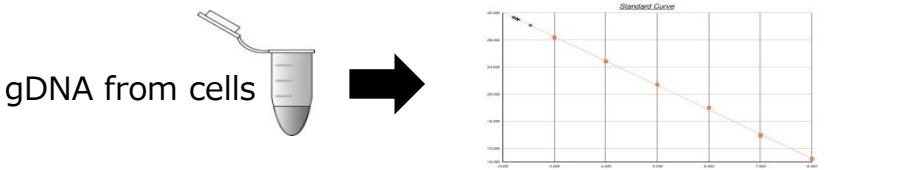
Conversion of cell number

① Calculate the number of copies per cell from single-copy gene measurement data



The number of cells is converted from the number of copies obtained in the actual measurement

② Converted from theoretical genomic DNA per cell



The theoretical genome mass per cell is 6.67 pg*

Converted to the cell number

Cytotherapy 19, 384–394 (2017)

Contents

Lower limit of quantification (LLOQ)

The FDA guidance on gene therapy[1] recommends that

LLOQ be 50 copies of vector per µg of gDNA, with 95% CI*

*Confidence interval

Q: Actually 1µg gDNA is added in one PCR reaction?

- **Typically, Total DNA amount is up to 200 ng in one PCR reaction**
- **Only small amount of DNA may be extracted in small tissue**
- **Large amounts of gDNA may increase the amount of PCR inhibitors**

→ **1 µg gDNA per reaction is impractical**

However,…

50 vector -------1µg gDNA
5 vector ---------100ng gDNA
0.5 vector -------10ng gDNA

→ Detection with 95% CI is almost impossible, because theoretical limits detectable with 95% CI are 3 Copies[2].

To set an adequate LLOQ, the greatest amount of attainable gDNA having no effect on the measurement should be used for qPCR.

1) FDA Guidance for industry: gene therapy clinical trials (2006)
2) The MIQE Guidelines. Bustin et al., 2009. Clinical Chemistry 55:4, 611-622

Accuracy and precision

- ✓ Based on our experiences, the acceptable variation in qPCR Cq values is ± 0.25 or less with technical issues and ± 0.5 or less with biological variations, which makes a total acceptable variation of ± 0.75 or less.
- ✓ This corresponds to a variation of -41 to +68% when Cq values are converted to copy numbers.

Considering the above, realistic criteria of accuracy and precision is around ±50%.

Validation items and criteria for qPCR（TBD）

Item	Criteria
Accuracy & Precision	Around 50%
Calibration curve	$r^2 \geqq 0.98$, Amplification efficiency: 85～110%
Specificity	No amplification in NTC and NC are desired. However, if amplification is observed, itmight be considered appropriate if the Cq values of these controls are higher than LLOQ by 1.5.
Matrix effect	QC samples are spiked into the control gDNA stock and H2O, with Cq differences of <1.5 and calibration curve slope of-3.6 to-3.1. If inhibitions are observed, reexamination of DNA extraction method and/or sample dilutions should be considered.
LLOQ	Minimum concentration that meets the criteria for accuracy & precision
LOD	Concentration detectable with 95% reliability.
Stability	Cq change <1.5
Recovery	Recovery rate is more consistent and reproducible than the value itself.

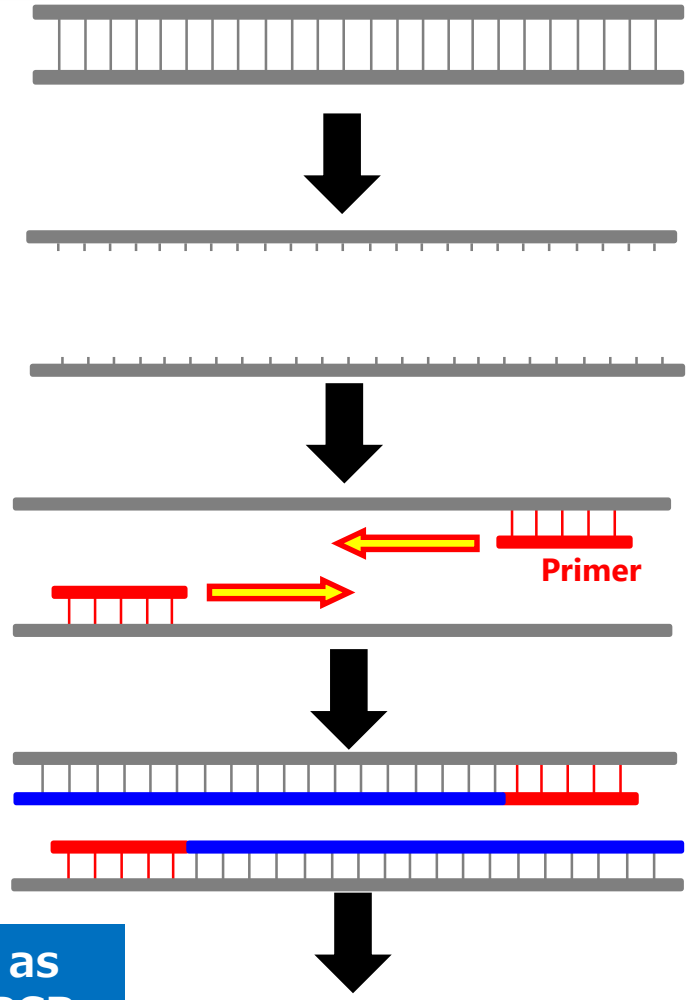
The 'fit-for-purpose' approach is considered as important in the qPCR assay.

Summary

- ✓ Development of cell and gene therapy products is also expected to continue at a rapid pace, leading to the increased use of qPCR under regulation.
- ✓ However, numerous challenges exist in the development of a qPCR method with excellent accuracy and precision.
- ✓ For bioanalysts attempting qPCR, it is vital that its advantages and disadvantages be understood and validation criteria be established according to the study purposes.
- ✓ Therefore, continued discussion and dissemination of information are critical for its future use.
- ✓ Although digital PCR was not discussed herein, the latest technologies should be considered, as the increased demand for qPCR may catalyze the development of next-generation quantitative techniques.

Back up

Basic mechanism of PCR –Quantification of DNA-



Target DNA (before amplification)

Denature
95 ℃

Dissect DNA duplexes at 95 ℃

Annealing/
Extension
60 ℃

Primers bind by lowering temperature, and the complementary strand is extended by enzymatic reaction at 60 ℃.

The target DNA is doubled.

1 Cycle

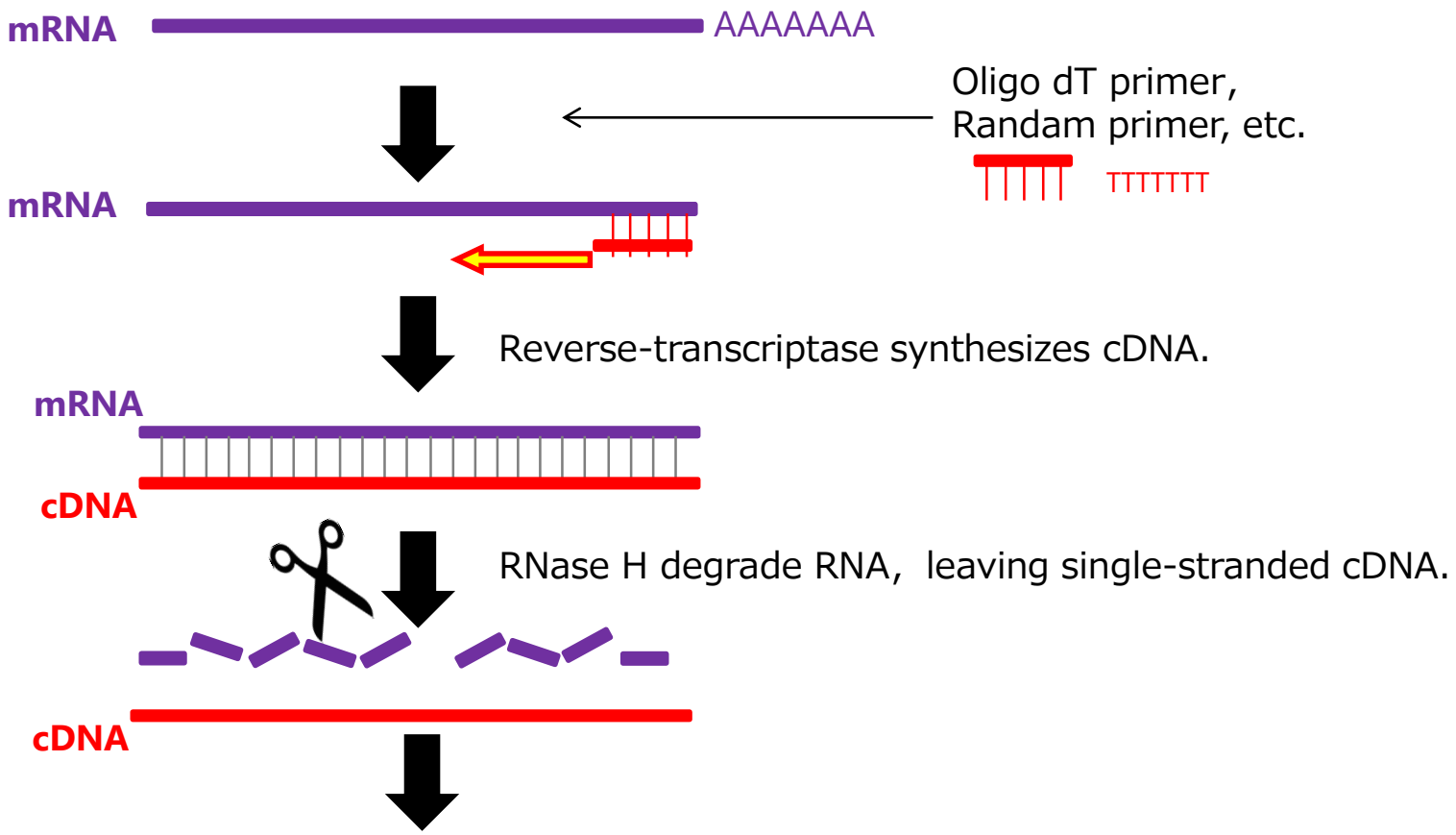
DNA is detected as fluorescent in qPCR.

By repeating the N cycle,
The target DNA can be amplified by 2^n fold.

Basic mechanism of PCR –Quantification of RNA-

In the case of expression analysis and RNA virus quantification, analytical target is RNA.

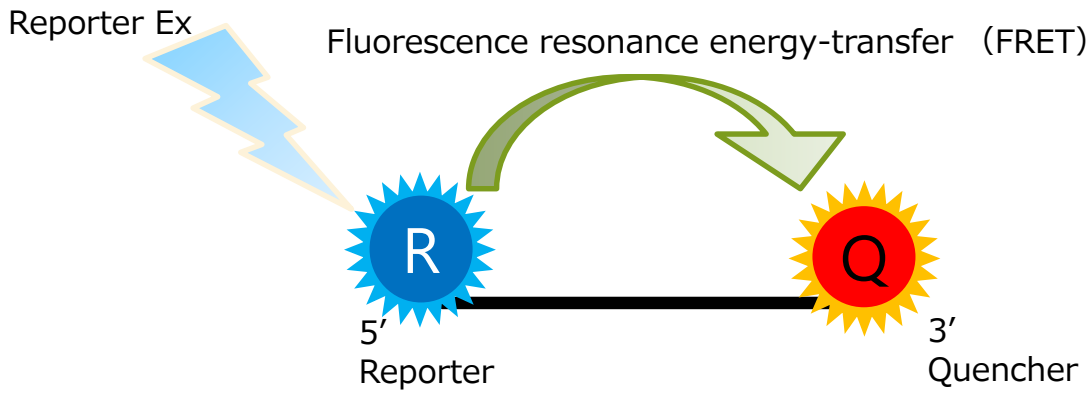
Reverse transcription (RT) step is required.



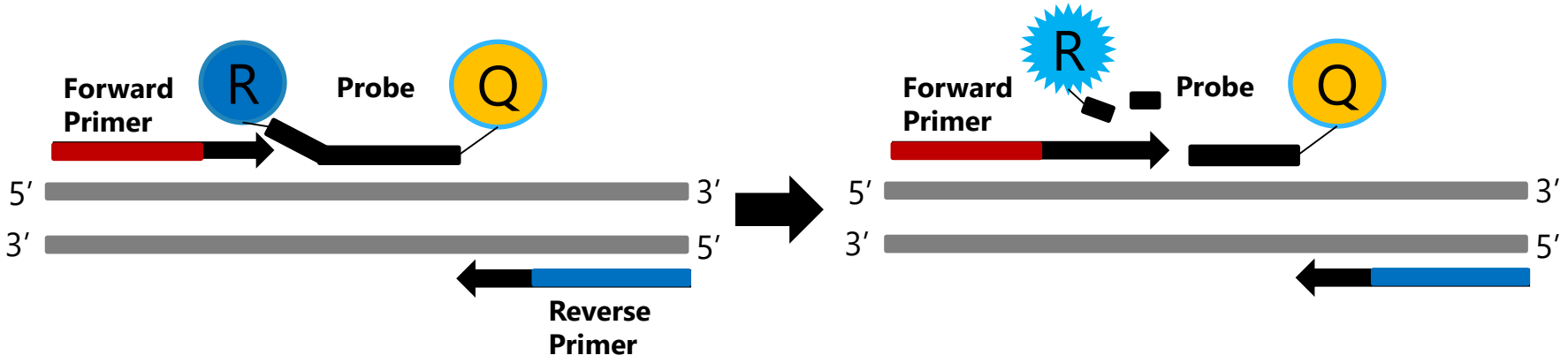
qPCR is performed using cDNA as a template.

Basic mechanism of PCR
–Hydrolysis Probe labeled with fluorescent-

①Fluorescence of the reporter is absorbed by quencher (FRET).



② Fluorescence is generated by the hydrolysis of the probe by the extension reaction.

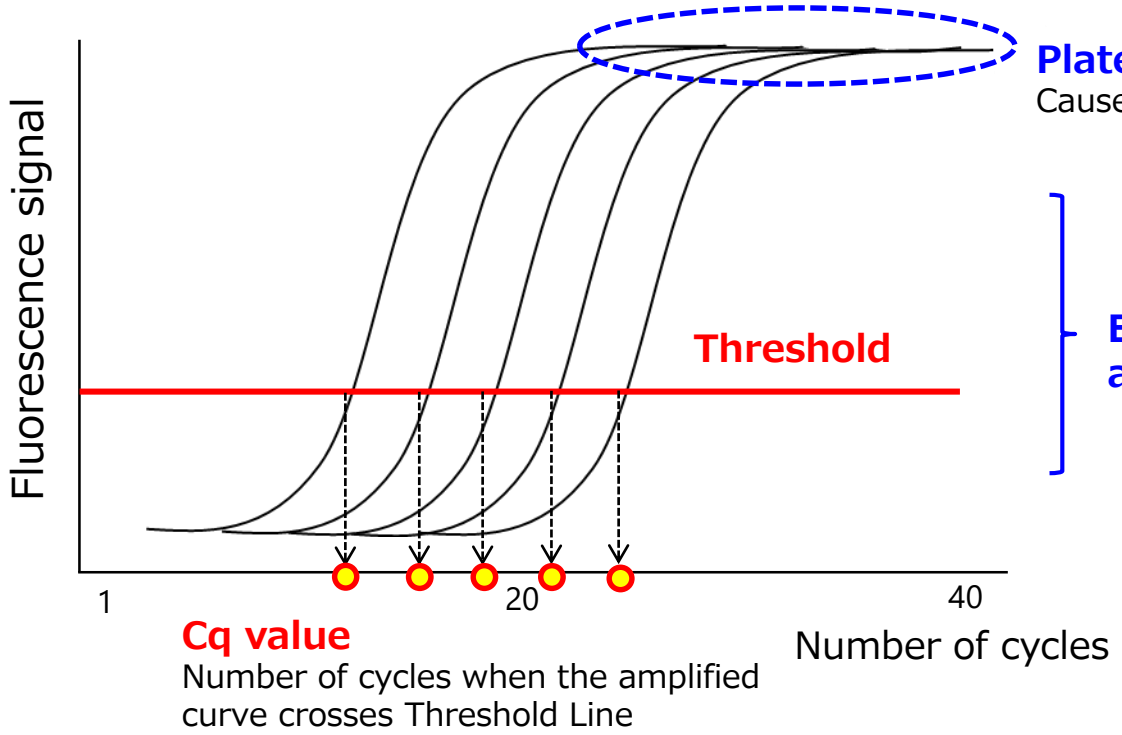


Probes have highly specificity for the target due to specific sequences.

Basic mechanism of PCR –Data analysis-

Amplification Plot

Plotted fluorescence signal for each cycle



Plateau state

Caused by depletion of dNTP and/or PCR Primer

Exponential amplified region

Cq value

Number of cycles when the amplified curve crosses Threshold Line

If there is a 2-fold difference in the theoretical amount of the original sample, the amplification curve is obtained in one cycle difference.

Term

Cq: quantification cycle
Ct: threshold cycle

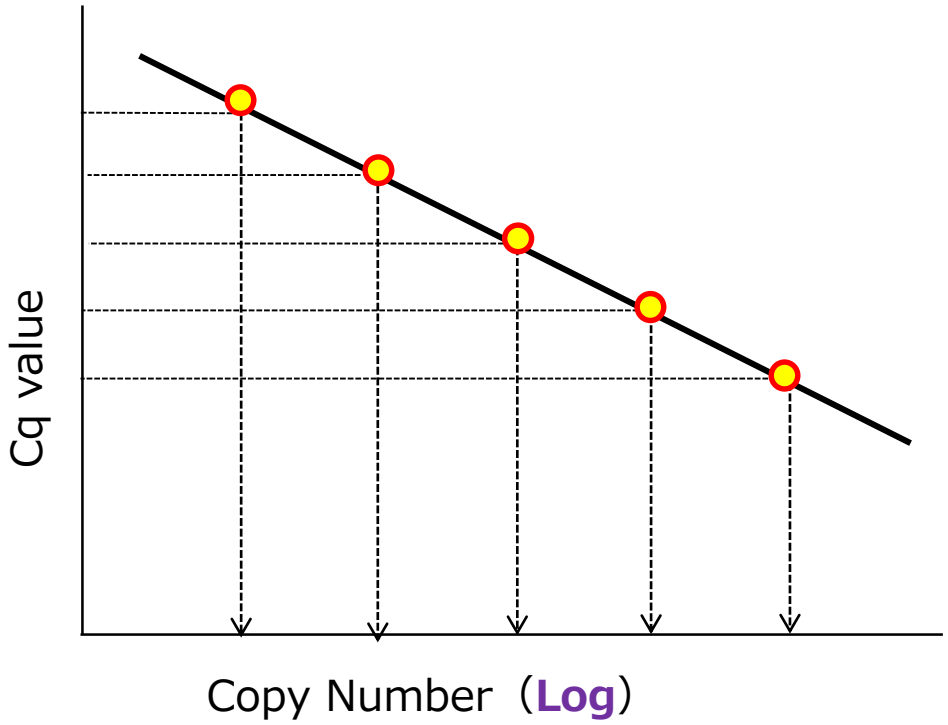
Here unified Cq

http://bioanalysisforum.jp/

Basic mechanism of PCR –Data analysis-

Standard Curve

By plotting Cq of the calibration curve samples against the copy-number



If there is a 2-fold difference in the theoretical amount of the original sample, the amplification curve is obtained in one cycle difference.

PCR efficiency can be calculated from the slope of the calibration curve.

PCR Efficiency

$$E\ (\text{PCR Efficiency}) = 10^{(-1/\text{slope})} - 1$$

※In the case of theoretical amplification,
PCR efficiency =**100 %**
Slope of the calibration curve ≒ **-3.32**

Application of qPCR

Discussed in DG2017-33

Table 1. Application of quantitative polymerase chain reaction for bioanalysis.

Type of product	Type of test	Target to be detected	qPCR or RT-qPCR	Matrix
Gene therapy (viral vector, plasmid, etc.)	Property analysis (determining concentration)	vDNA, vRNA, vector DNA	Both (depends on vector)	None
	Biodistribution	vDNA, vRNA, vector DNA	Both (depends on vector)	Host tissue
	Viral shedding (detection of shedding)			Urine, feces, blood, etc.
	Viral shedding (confirmation of infectivity)	vmRNA	RT-qPCR	Host tissue
	Gene expression analysis	mRNA	RT-qPCR	Host tissue
Oligonucleotide therapeutics	Gene expression analysis	mRNA	RT-qPCR	Host tissue
Cell therapy	Property analysis (detection of undifferentiated iPSCs)	mRNA (derived from undifferentiated iPSCs)	RT-qPCR	Cell product
	Biodistribution	gDNA (derived from cell product)	qPCR	Host tissue
Vaccine	Challenge test (analysis of viral titer)	vDNA or vRNA	Both (depends on virus)	Host tissue

iPSCs: Induced pluripotent stem cells; qPCR: Quantitative polymerase chain reaction; RT-qPCR: Reverse transcription quantitative polymerase chain reaction.

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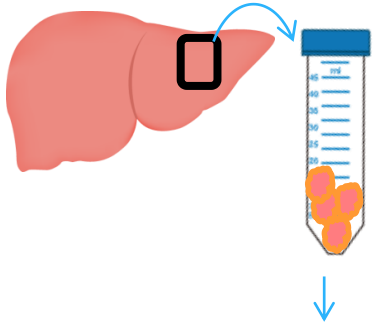
NA stability（After Step④）

There are several issues regarding evaluation of NA stability

1． Stability assessment of the extracted DNA is feasible.

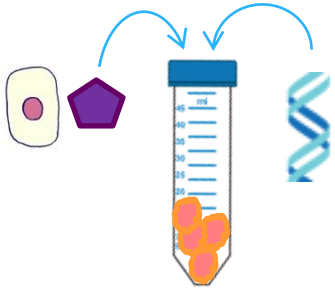
→Stability can be assured by measuring qPCR over time.

2． Stability in tissues？



Issue ①

It is difficult to create a state in which target nucleic acids are present in tissues.

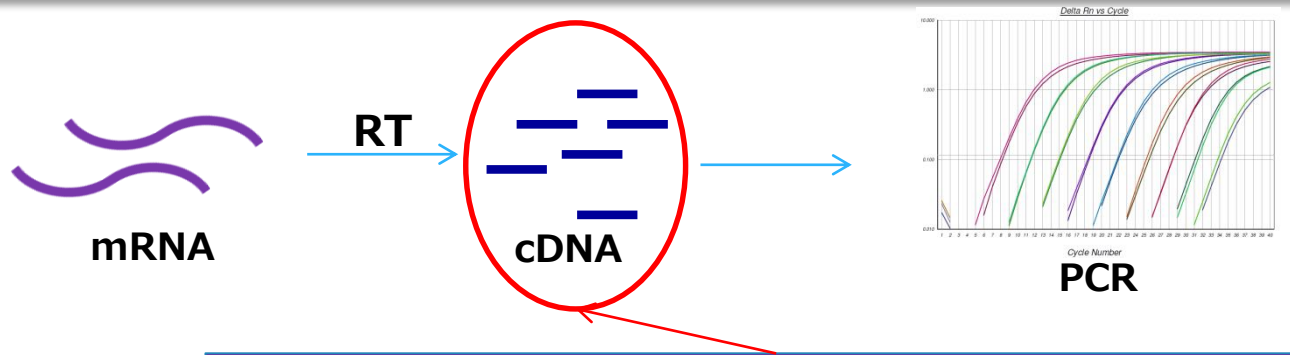


adding NA or virus/cell to tissue sections or homogenates, and cryopreservation

Issue ②

Taking into account the variation of extraction efficiency, stability evaluation may be challenging.

Reference Standard for RNA assay



Efficiency of RT is not constant.
The reference material is not RNA but DNA

	Pros	Cons	Application
Plasmid	・Highly stable ・Easy to produce large amounts (initial cloning is necessary) ・Known copy number	・Equivalence to Analyte	・Absolute quantification
Synthetic NA	・Flexibility of sequencing ・No cloning required ・Known copy number	・Equivalence to Analyte	・Absolute quantification
cDNA	・Equivalent to analyte	・Copy number unknown	・Relative quantification