Understanding the issues in qPCR bioanalysis before planning validation

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Our objectives

Why is planning qPCR validation so difficult?

qPCR consists of many steps → Every steps has variabilities and issues → The issues affect the contents of validation & their acceptance criteria

Is it possible to set a standard protocol for qPCR validation?

Maybe?

But before we talk about the details of validation content, we need to understand and discuss the issues that lies in each step so that we can see if they can be standardized.

Application of qPCR in bioanalysis

Topics covered here

Type of products	Type of studies	Target	qPCR or RT-qPCR	Matrix
Gene therapy (virus vector、plasmid etc.)	Test article characterization (concentration)			No
	Biodistribution	vDNA or vRNA or DNA (vector itself)	qPCR or RT-qPCR (depending on vector)	Host tissue derived NA*
	Virus shedding （detection of shedding）			Urine, feces, blood, etc. derived NA
	Virus shedding （infectivity）	vmRNA (virus derived RNA)	RT-qPCR	Cell derived NA
	Gene expression	mRNA (host derived)	RT-qPCR	Host tissue derived NA
Nucleic acids therapeutics	Gene expression	mRNA (host derived)	RT-qPCR	Host tissue derived NA
Cell therapy	Test article characterization (detection of undifferentiated iPS –derived cells)	mRNA (Undifferentiated iPS - derived cells)	RT-qPCR	Cell derived NA
	Biodistribution	DNA (cell derived)	qPCR	Host tissue derived NA
Vaccine	Efficacy (virus titer analysis of challenge study)	vDNA or vRNA (virus itself)	qPCR or RT-qPCR (depending on virus)	Host tissue derived NA

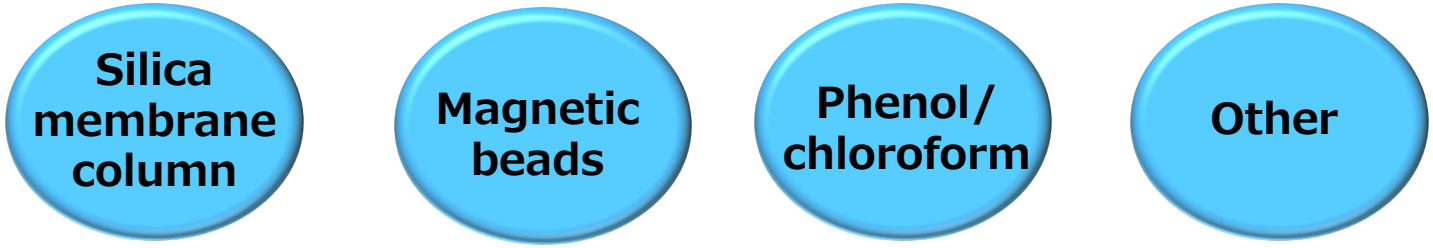
*NA: Nucleic acids

Today's topic

1. Nucleic acids extraction efficiency
2. Matrix
3. Threshold
4. Algorithm in relative quantification
5. Nucleic acids stability
6. View of our discussion group

Nucleic acids extraction efficiency

For quantification of Nucleic Acid (NA) in biological samples, NA extraction is the biggest issue.



Depends on the method, processibility, extraction efficiency, the quality of extracted NA differs.

Problem: There is a lot of Variation in NA extraction efficiency from tissue (5-100%) even if the same method is used.

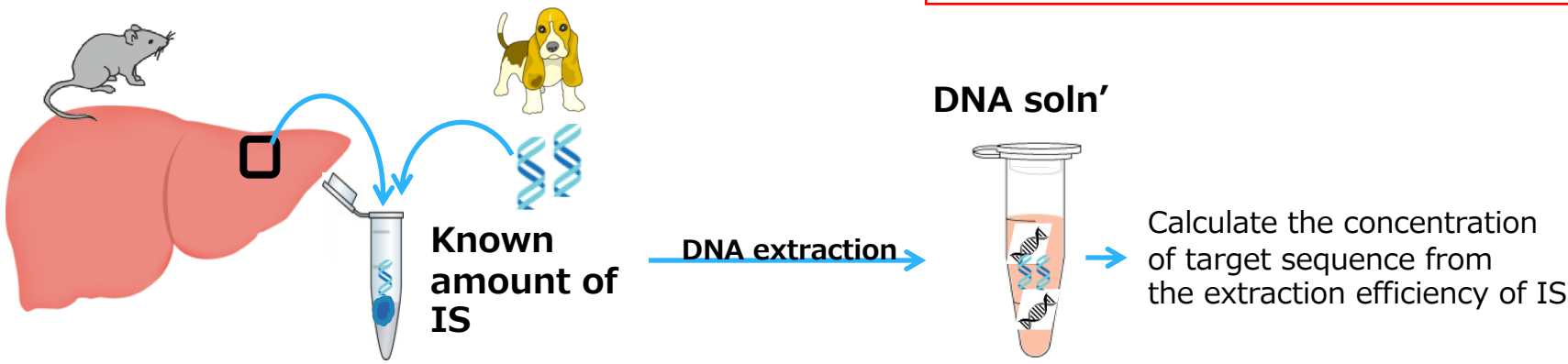
Nucleic acids extraction efficiency

qPCR can quantify the amount of NA with confidence only in extracted DNA/RNA solution, not in tissue due to the varying extraction efficiency.

Can we solve the problem?

Ex : Use of Internal Standard (IS)

*Only for DNA quantification



IS: NA sequence that has low similarity with target/animal seq. (gDNA of organism other than human or animal used)

Concentration of target seq in the tissue can be calculated using the calculated extraction efficiency of IS.

Matrix effect in qPCR

What is the matrix effect in qPCR?

1. PCR inhibitors in a sample retard the PCR reaction
2. Highly concentrated gDNA in the matrix causes depletion of primer/probe/dNTP by secondary binding

PCR inhibition is the main matrix effect

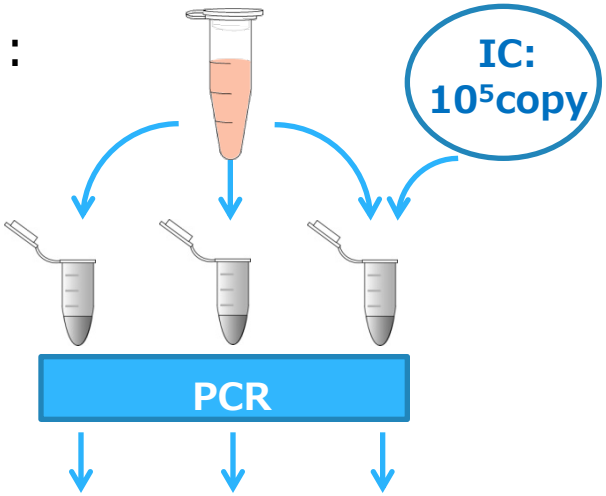
⇒ Concentration of the matrix is not a problem.
Quality of extracted DNA and **dilution factor** are the keys

Validating the matrix effect

Do we need to validate matrix effect?

Use of Internal Control (IC) may prevent the need for validation

Ex :



For each sample to be analyzed, one of the triplicates is spiked with IC sample.

OK →	2.3x10² copy	2.3x10² copy	1.0x10⁵ copy	← spiked sample shows the right copy number.
NG →	2.3x10² copy	2.3x10² copy	1.0x10³ copy	← spiked sample shows a lower number.

Matrix DNA in standard curve

Need to avoid excessive use of animal DNA due to animal welfare.

If we were to use matrix DNA, which DNA is more appropriate?

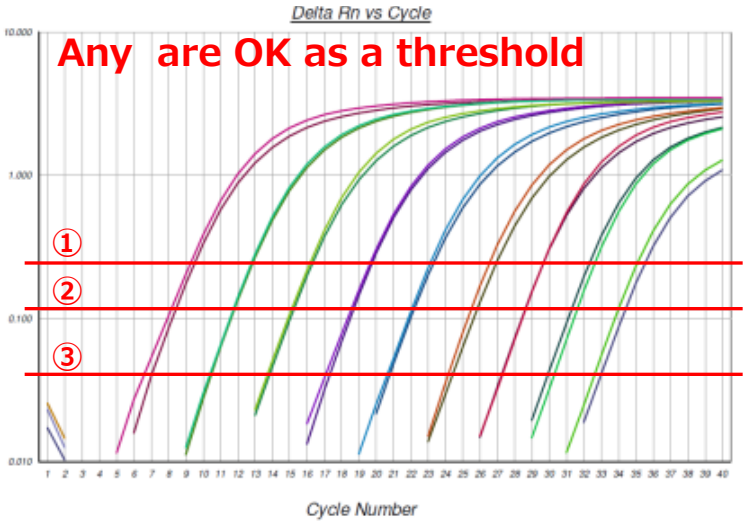
- ✓ tissue type – problem of PCR inhibitor depends on tissue type
- ✓ strain
- ✓ species – may avoid animal welfare issue s (e.g. salmon sperm), but wrong secondary binding

*If the matrix effect can be avoided, we need to consider not using matrix for the standard curve

Threshold

Auto vs. Manual

- ✓ Standard samples are on each plate
- ✓ Compare samples on the same plate



Auto is OK

Compare sample data between plates without standard samples in each plate

Threshold needs to be set

Algorithm in relative quantification

The common method for gene expression studies is comparative quantitation using a reference gene.

PCR machines are equipped with different algorithms for this.

Should we standardize the algorithms?

1. ΔΔCq – commonly used. No standard curve is needed. Assumes amplification efficiency is 100% for both gene of interest & reference genes.

2. Pfaffl – Can be used when amplification efficiency of gene of interest and reference gene are different (only one reference gene)

3. Vandesompele – Can be used for more than two reference gene

Limitation of ΔΔCq

Amplification efficiency (AE): depends on each assay

$$\text{Amount of amplicon after n cycle} = (1+ AE)^n$$

Influence of AE differences in the amount of amplicon;

AE: 100%

$$1+ AE = (1+100\%) = 1+1=2$$

$$\text{Amount of amplicon after n cycles} = 2^n$$

Amount of amplicon after 10 cycles = 1024

AE: 90%

$$1+ AE = (1+90\%) = 1+0.9=1.9$$

$$\text{Amount of amplicon after n cycles} = 1.9^n$$

Amount of amplicon after 10 cycles = 614

Should we standardize the algorithm?

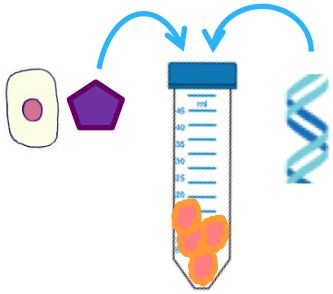
Nucleic Acids stability

1. Stability of extracted DNA solution

→run time-course qPCR on the sample

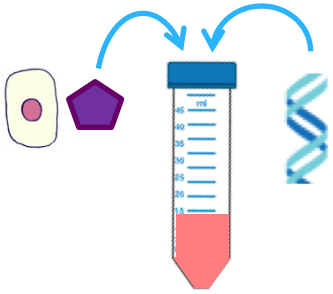
2. Target NA stability in tissue?

It is difficult to create the same environment as the biodistribution sample



Ex. 1 Mix products with solid tissue

Environment for NA does not mimick that of real samples



Ex.2 Mix products with tissue lysate

If tissue samples are lysed immediately after harvesingt and stored as tissue lysate, the environment can be the same

View of our discussion group

- Due to the nature of qPCR, it is difficult to set solid validation guidelines including acceptance criteria.
- Validation protocol should be planned so that it fits the purpose of each assay.
- Setting validation guidelines at this point in time may obstruct the development of new technology.
- Scientists should be familiar with scientific bases of qPCR protocols and analytical methods.

View of our discussion group

Minimum points that can/need to be harmonized:

- How to treat extraction efficiency
  - Ignore the variability or use some methods to normalize the extraction efficiency?
- How to treat matrix effect
  - Do we need to use matrix in a standard curve?
  - Do we need to validate the matrix effect?
  - Is spiking the sample with QC sample enough to assess the matrix effect?
- Threshold set-up
- Standardization of algorithm
- Need for NA stability in tissue

Minimum requirements for validation items

- **Standard curves**
- **Precision & accuracy**
- **Specificity**
- **Limit of quantitation**
- **Limit of detection**
- **Stability of NA**
- **(Matrix effect)**

We can harmonize the minimum requirements for validation items and leave the acceptance criteria to be determined depending on the purpose of the study.