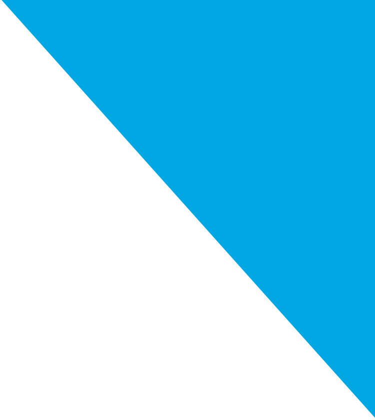

15th JBF Symposium, D1-A3-03

Validation of PCR assays for preclinical studies with real-world data

Shin Nippon Biomedical Laboratories, Ltd.
Drug Safety Research Laboratories

Haruka Inoue, Akiko Suzuki, Kuniyoshi Abe, Naoki Kaname, Chihiro Masuda,
Mirano Yoshikawa, Yurie Okawa, Yukiko Arimura, Asako Uchiyama, Tetsuya Yoshikawa

Outline

01 Introduction

02 Standard Design for qPCR Method Validation

03 Current Issues in Validation

04 Real-world Examples of Validation Studies

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Introduction: Our Capabilities

Scientific Personnel	Study Directors	5
	Analysts	10
Number of Studies (2019 to 2023)	Validation studies	40<
	Analysis studies	100<
Nucleic Acid Extraction Method	Column : manual/automated (2)	
	Magnetic beads: automated (1)	
PCR equipment	qPCR	5
	ddPCR	1

KingFisher Apex

QIAcube Connect

Fast 7500

CFX 96

Touch

Opus

QX200
Auto DG

Image source：https://www.thermofisher.com, https://www.qiagen.com, https://www.bio-rad.com

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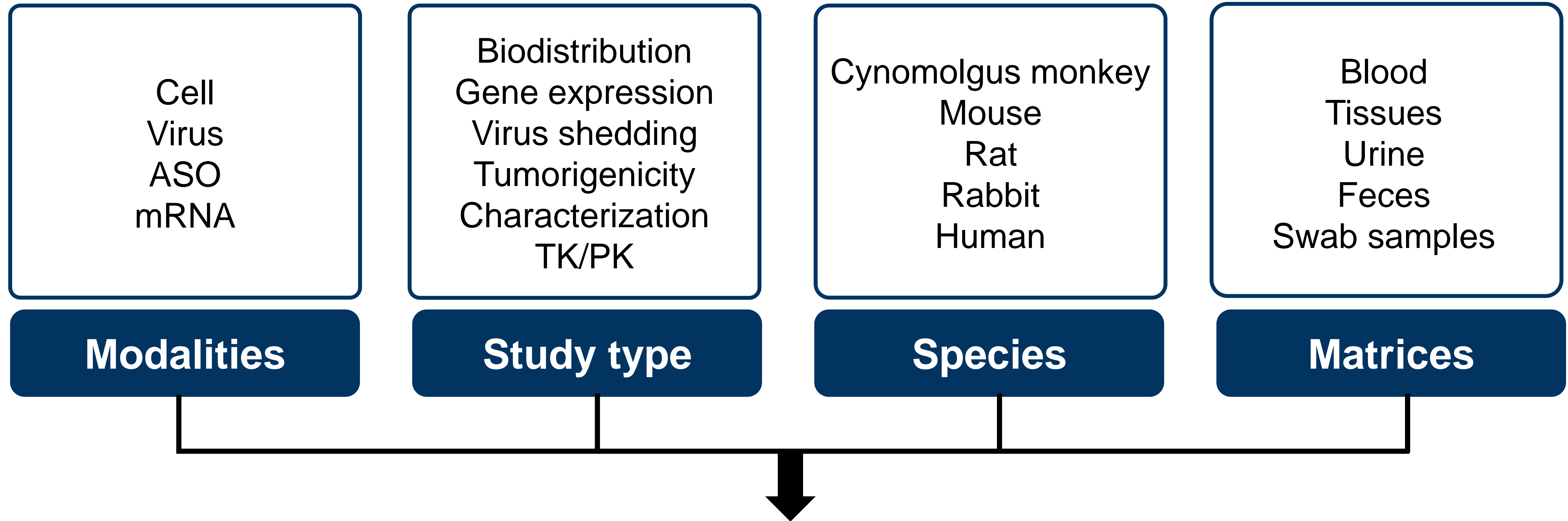
Introduction: Various Validation Studies at SNBL



Need to customize the design for qPCR method validation

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Outline

01 Introduction

02 **Standard Design for qPCR Method Validation**

03 Current Issues in Validation

04 Real-world Examples of Validation Studies

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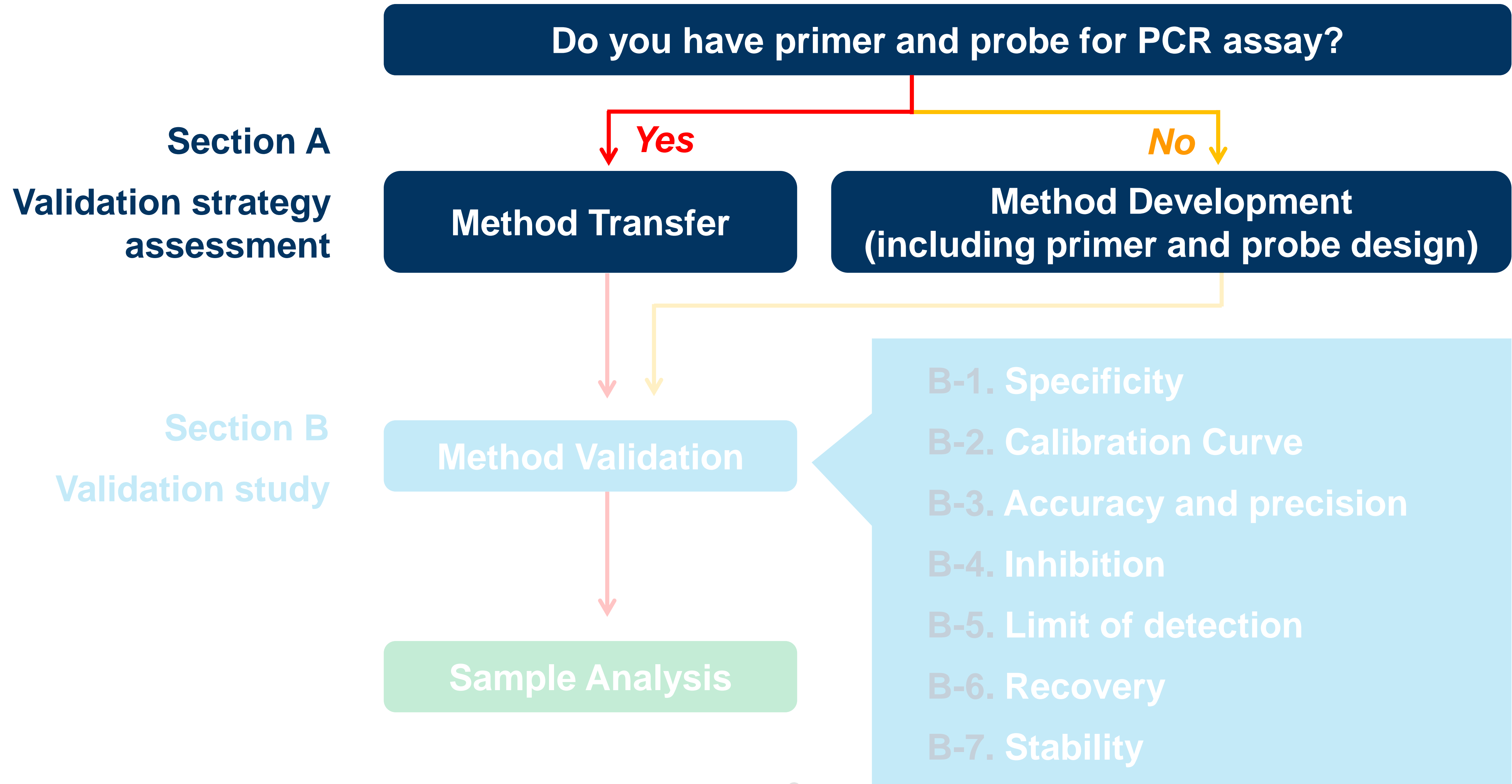
Study flow



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Section A: validation strategy assessment

Target: DNA
Standard substance: plasmid DNA or synthetic DNA
Replicate: triplicate or duplicate

Strategy	Sample	n
Confirm specificity	No template control (DNase/RNase-free water)	1
	gDNA of blank biological samples	
Confirm inhibition Determine DNA template	gDNA of blank biological samples spiked with Standard substance	
Confirm dynamic range	Dilution series of standard substance with matrix DNA	2

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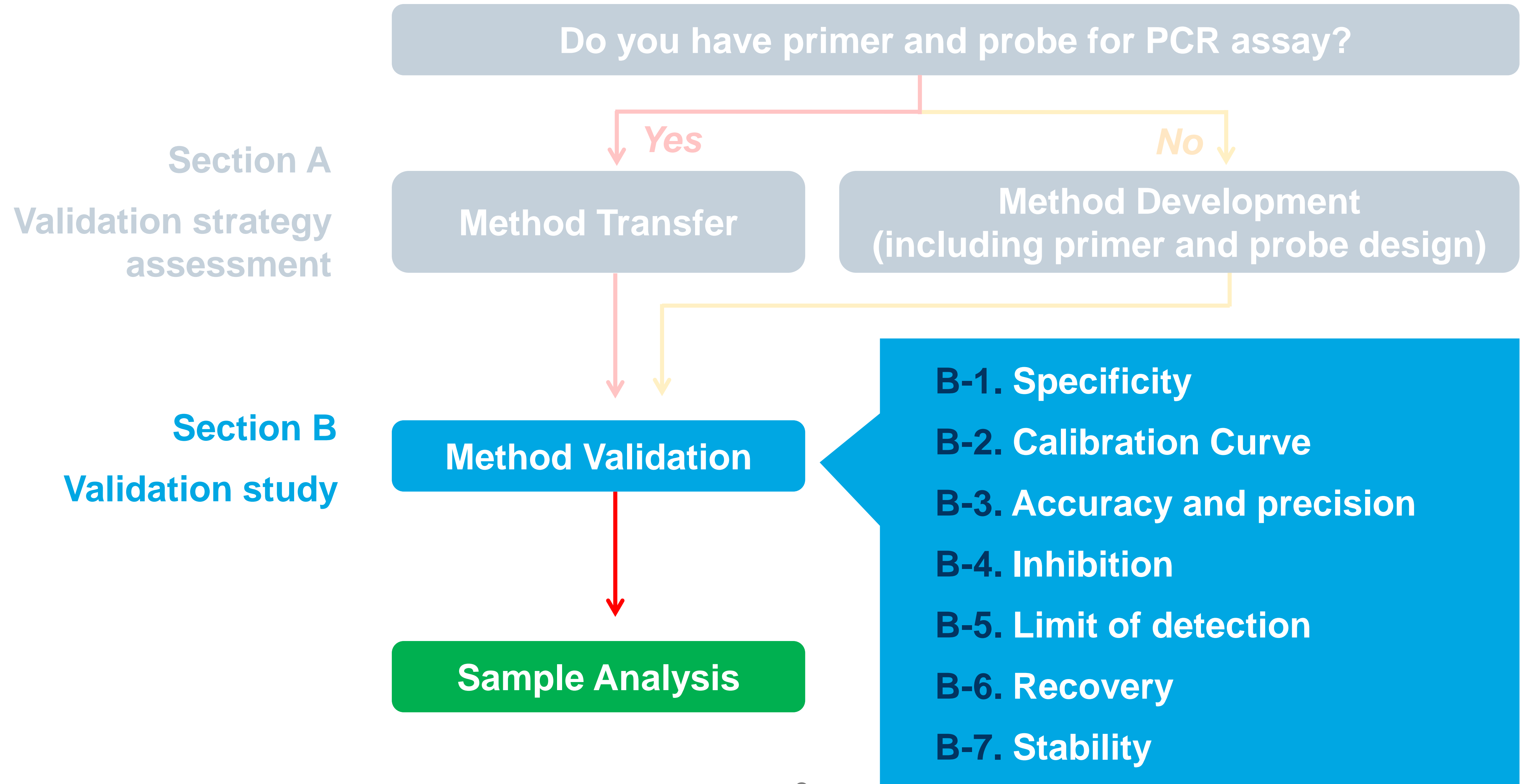
Study flow



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Section B: Validation Design

B-1. Specificity

Sample	• NTC (no template control): DNase/RNase-Free Water, n=1 • NC (negative control): pooled gDNA of liver, lung, and spleen, n=1
Acceptance criterion	• Cq values are undetermined or are equal to or higher than 38.5. (terminal cycle: 40)

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Section B: Validation Design

B-2. Calibration curve

Sample

- Standard solution for calibration curve (STD) with matrix, n=3

Acceptance criteria

- The difference of the Cq values is equal to or lower than **1.5**.
- The amplification efficiency (E) is **between 85% and 110%.**
- Linearity of the calibration curve (R^2) is equal to or higher than **0.980**.

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Section B: Validation Design

B-3. Accuracy and precision

Sample

- QC sample (LQC, MQC, HQC): 3 preparations/run, 3 runs

  Within-run: evaluation is conducted on each run

  Between-run: evaluation is conducted on each concentration

Acceptance criteria

- Accuracy within **100% ± 35%**
- CV is within **35%**

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Section B: Validation Design

B-4. Inhibition

Sample

- gDNA of blank samples (**5 biological samples**)
 + **spike standard substance** , n=1

Injection site (e.g., cephalic vein and adjacent tissue), target organ (e.g., muscle), blood, liver, and gonads (testis/ovary)

Acceptance criterion

- The difference between the mean of the Cq values in sample solutions for inhibition and the mean of the Cq values of standard substance (e.g., LLOQ) is equal to or lower than **1.5**.

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Section B: Validation Design

B-5. Limit of detection

Sample	• Sample solution for limit of detection: LLOQ or its diluted solution analyze in 20 wells
Acceptance criterion	• Cq values are determined in **19** wells or more.

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Section B: Validation Design

B-6. Recovery

Sample	• DNA extracted from 5 biological samples containing test article: 2 concentrations, n=3 per biological sample and concentration
Acceptance criterion	• None

The purpose of the recovery experiment is to detect the target sequence in biological samples by qPCR.

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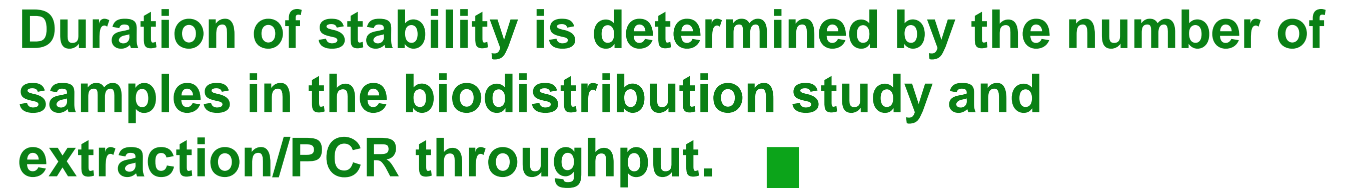
Section B: Validation Design

B-7. Stability



Sample

- 2 concentrations, n=1 to 3 per concentration

Frozen stability of DNA in biological sample

- Blood samples containing test article, storage in a deep freezer for **28 and 56 days**

Frozen stability of extracted DNA

- DNA extracted from blood samples containing test article, storage in deep freezer for **28 and 56 days**

Freeze-thaw stability of extracted DNA

- DNA extracted from blood samples containing test article, **3 or 5** freeze-thaw cycles are performed

Acceptance criterion

- The difference between the mean of the Cq values in triplicate after storage and the mean of the Cq values immediately after preparation is equal to or lower than 1.5.

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Outline

01 Introduction

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04 Real-world Examples of Validation Studies

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Current issues in qPCR validation

SNBL

1 Evaluation of replicate variation

The difference of Cq values or CV% of Cq values?

SNBL evaluate the difference of the Cq values as the replication variation.
(acceptance criteria: difference of the Cq is lower than 1.5)

Actual values	Copy number of standard solutions for the calibration curve (copies/reaction)					
	1.00E+03	1.00E+04	1.00E+05	1.00E+06	1.00E+07	1.00E+08
Cq value	29.86	26.57	23.38	19.84	16.71	13.21
	29.76	26.63	23.24	19.89	16.59	13.21
	29.74	26.61	23.38	19.93	16.62	13.33
Mean	29.79	26.60	23.33	19.89	16.64	13.25
Difference of the Cq values	**0.12**	**0.06**	**0.14**	**0.09**	**0.12**	**0.12**
CV% of Cq values	**0.2**	**0.1**	**0.3**	**0.2**	**0.4**	**0.5**

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Current issues in qPCR validation

SNBL

1 Evaluation of replicate variation

values	Copy number of standard solutions for the calibration curve (copies/reaction)					
	1.00E+03	1.00E+04	1.00E+05	1.00E+06	1.00E+07	1.00E+08
Cq value	30.00	26.67	23.34	20.01	16.68	13.35
	28.50	25.17	21.84	18.51	15.18	11.85
	28.50	25.17	21.84	18.51	15.18	11.85
Mean	29.00	25.67	22.34	19.01	15.68	12.35
Difference of the Cq values	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**
CV% of Cq values	**3.0**	**3.4**	**3.9**	**4.6**	**5.5**	**7.0**

If the difference of the Cq values is 1.5, the CV of Cq values would be below 10%.

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Current issues in qPCR validation

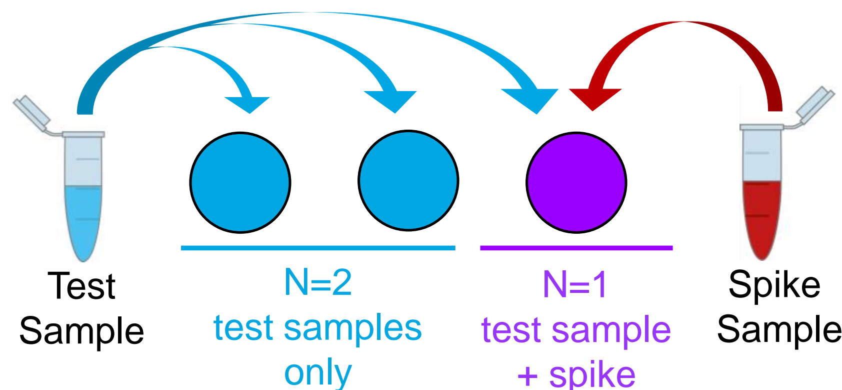
SNBL

2 **Evaluation of PCR inhibition for all matrices**

Is it necessary to validate for all organs?

- Representative organ (liver) and samples with a **high risk of inhibition** (e.g., blood, skin and feces) should be evaluated within the validation.
- When measuring test samples, **all samples are spiked with standards to confirm PCR inhibition**.

Testing Biodistribution samples



1 well containing test sample is spiked with a known amount of the target gene (spike sample) to evaluate potential qPCR inhibition

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Current issues in qPCR validation

2 Evaluation of PCR inhibition for all matrices

- The degree of inhibition **varies with individuals**.

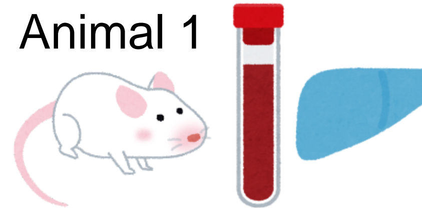
Validation study

From one source

No matrix effect

Biodistribution study

Animal 1



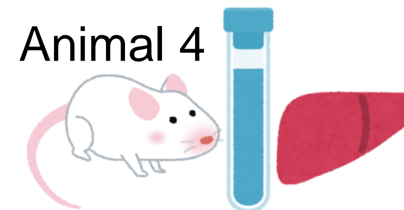
Low matrix effect

Animal 2

High matrix effect

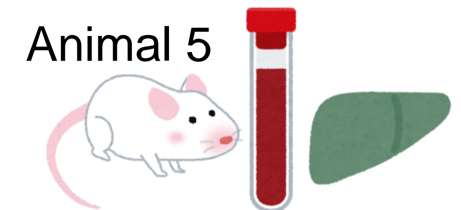
Animal 3

Animal 4



Low matrix effect

Animal 5



High matrix effect

We **do not** believe that it is necessary to confirm inhibition for all biological samples in a validation study.

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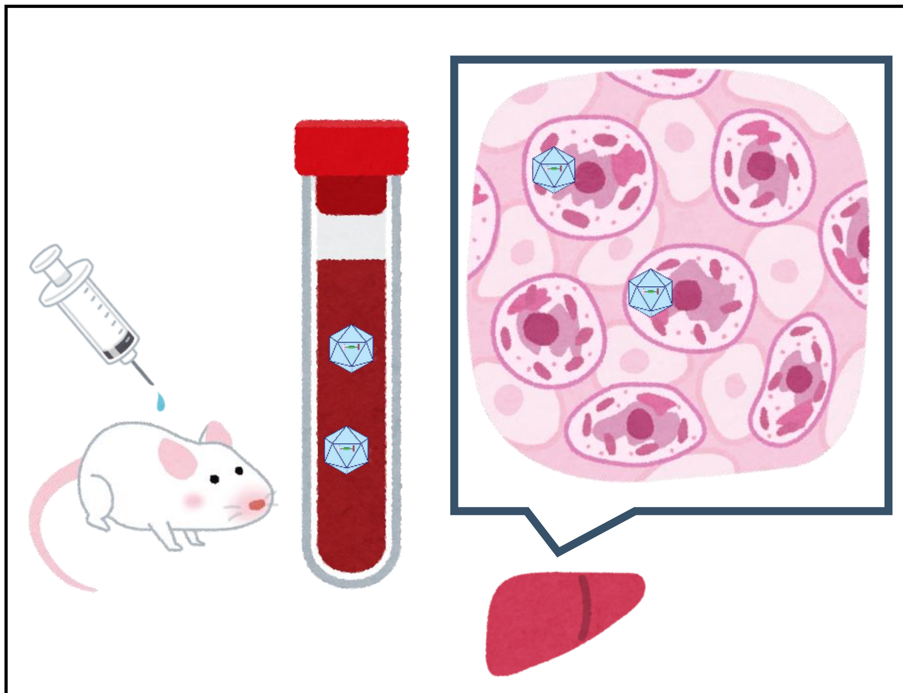
Current issues in qPCR validation

SNBL

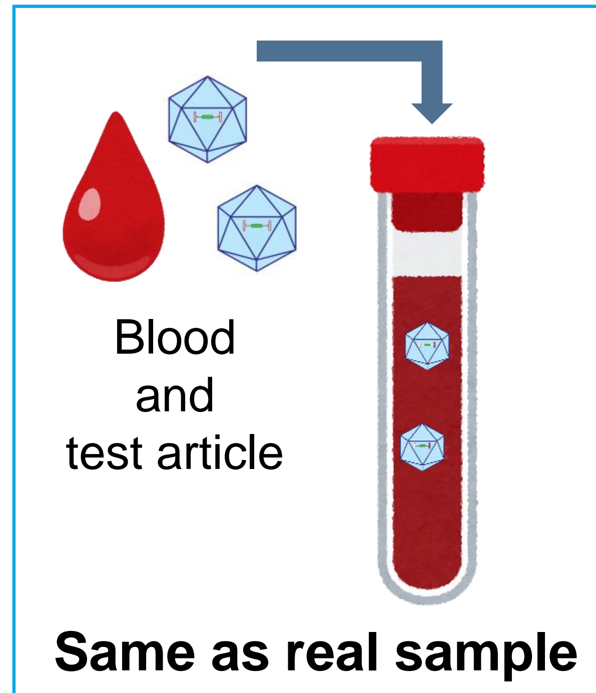
3 **Stability in tissue**

Should it be performed on both blood and tissue?

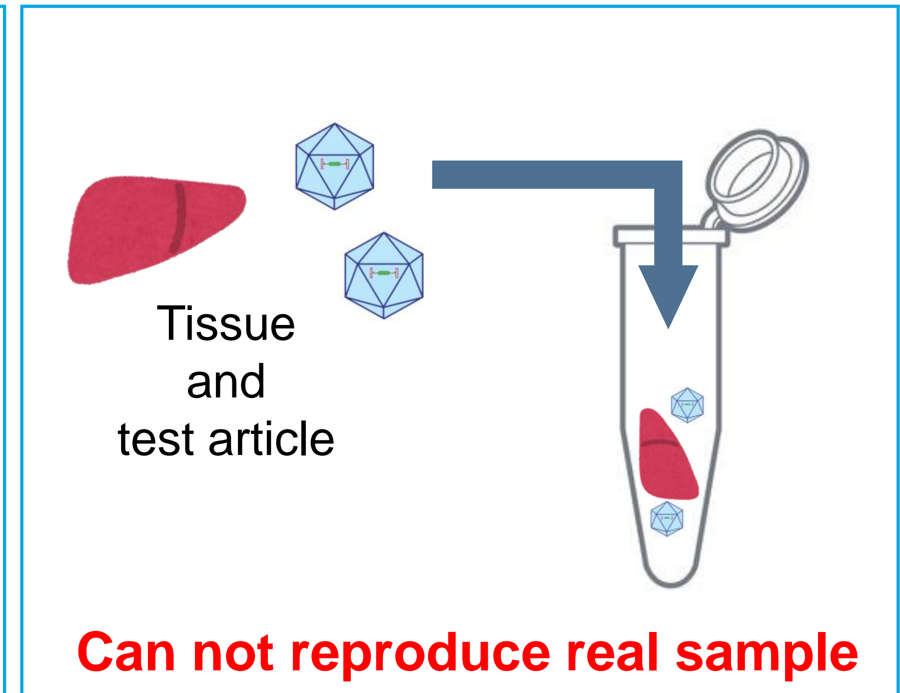
- It is difficult to reproduce the biological condition in which the test article is distributed and to prepare uniform samples. We typically use blood to confirm the stability of the test article in biological sample.



In real sample



Same as real sample



Can not reproduce real sample

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Outline

01 Introduction

02 Standard Design for qPCR Method Validation

03 Current Issues in Validation

04 Real-world Examples of Validation Studies

Please visit our poster presentation!
Abstract No. P3-11

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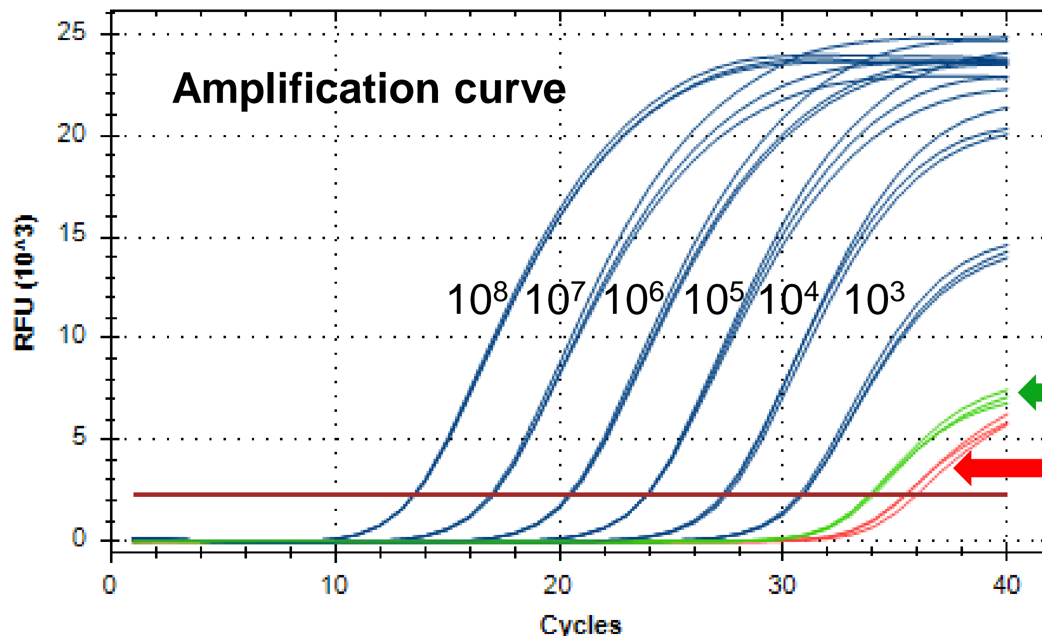
Real-world example 1: Alu sequence

Equipment/software	CFX96 Touch/CFX Maestro v1.0, Bio-Rad Laboratories, Inc.
Standard substance	125 bp of synthesized DNA
Matrix DNA	Pooled gDNA from liver and spleen of naïve NOG mouse or nude rat (10 ng/reaction)
Specificity	Difference of the Cq values between the Cq value of NTC or NC and the mean Cq value of LLOQ: 6.85 to 7.57 and 4.13 to 4.32
Calibration curve	**1.00E+03 to 1.00E+08 copies/reaction** Amplification efficiency: 92.3% to 100.3% Linearity (R^2): 0.999 or 1.000



Due to the reagent-derived contamination, The amplification curve shows up. Since Alu-1 targeting PCR is highly sensitive, the assay is prone to the contamination.

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Real-world example 1: Alu sequence

<table>
<tr><td>Accuracy (within-run)</td><td colspan="3">101.5% to 121.4%</td></tr>
<tr><td>Accuracy (between-run)</td><td colspan="3">96.4% to 134.3%</td></tr>
<tr><td>Precision (within-run)</td><td colspan="3">CV%: 1.7% to 28.1%</td></tr>
<tr><td>Precision (between-run)</td><td colspan="3">CV%: 2.3% to 17.5%</td></tr>
<tr><td>Inhibition</td><td colspan="3">The gDNAs (10 ng/reaction) extracted from 5 different biological organs (blood, liver, lung, spleen, and testis) were confirmed to have no effect on this qPCR analysis.
Spike sample: LLOQ of standard substance (1.00E+03 copies/reaction)</td></tr>
<tr><td>Limit of detection</td><td colspan="3">500 copies/reaction</td></tr>
<tr><td rowspan="5">Recovery of Alu-1 sequence derive from A549 cell</td><td rowspan="2">Added cell number</td><td colspan="2">Copy number (copies/reaction)</td></tr>
<tr><td>Blood</td><td>Liver</td></tr>
<tr><td>100</td><td>3.51E+04 to 5.49E+04</td><td>BLOQ (8.36E+02 to 9.18E+02)</td></tr>
<tr><td>1000</td><td>2.85E+05 to 4.00E+05</td><td>6.80E+03 to 1.06E+04</td></tr>
<tr><td>10000</td><td>6.94E+06 to 7.46E+06</td><td>7.61E+04 to 1.03E+05</td></tr>
</table>

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Real-world example 1: Alu sequence

SNBL

Conclusion

- The analysis method targeting Alu-1 is has been successfully validated, and the analytical method was judged suitable to quantify Alu-1 sequences in biological samples from NOG mouse and nude rat.

Alu sequences are present in the genome of experimental staff, need to prevent human-derived Alu contamination.

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Preventing contamination of human-derived Alu

SNBL

Sample collection (necropsy)

- Minimize access to the labs as much as possible (except for necropsy staff)
- Use PCR-grade collection containers (**guaranteed human DNA free**)
- Use apparatus wiped with a paper towel soaked in nucleic acid removal reagent and then rinsed with a PBS
- Collecting tissues for PCR first
- Wash the tissues with PBS
- Use UV irradiated materials

Nucleic acid removal reagent
DNAAWAY
Thermo Fisher Scientific

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Preventing contamination of human-derived Alu

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Extraction and qPCR measurement

PCR workstation

- Minimize air flow
- Wear a mask, a cap, gloves, and a disposable gown
- Use PCR-grade, DNA low-bind tubes and filtered tips
- Wipe apparatus with a paper towel soaked in nucleic acid removal reagent
- Set up reaction in a PCR workstation
- Master Mix, test samples, and standards are handled in separate workstations

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Real-world example 2: miRNA qPCR/ddPCR

Equipment/ software	qPCR	CFX96 Touch/CFX Maestro Security Edition v1.0, Bio-Rad Laboratories, Inc.
	ddPCR	Auto DG + QX200/Quanta soft Security Edition v1.0, Bio-Rad Laboratories, Inc.
Analyte	miRNA in serum of naïve cynomolgus monkeys	

Validation Item		ddPCR		qPCR	
Target		miR-223	miR-16	miR-223	miR-16
Specificity		Positive droplet was not detected.		Cq values were not determined.	
Dilution linearity	Range (copies/reaction)	737 to 1.12×10^5	686 to 1.06×10^5	73.7 to 7.37×10^5	59.8 to 5.98×10^5
	Amplification efficiency (%)	-	-	99.9 to 104.0	95.5 to 99.4
	R^2	0.998 or 0.999	0.994 to 0.999	0.999 or 1.000	0.999
Reproducibility (%)		4.9	-	3.6	-
Limit of detection (copies/μL)		4.18	3.40	7.37	5.98

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Real-world example 2: miRNA qPCR/ddPCR

SNBL

Accuracy and precision (**miR-223**, n=3)

Assay	Theoretical concentration (copies/reaction)	Within-run Concentration (copies/μL)		Between-run Concentration(copies/μL)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
ddPCR	7.37E+02	4.4 to 5.5	99.3 to 130.0	12.6	113.8
	7.37E+03	0.5 to 4.8	93.3 to 125.3	13.4	107.3
	7.37E+05	3.8 to 15.4	87.4 to 89.1	9.0	88.4
qPCR	7.37E+01	1.0 to 11.0	89.0 to 108.2	11.9	101.5
	7.37E+03	1.7 to 3.9	109.2 to 118.6	4.6	114.8
	7.37E+05	3.3 to 7.0	**87.1 to 99.6**	7.7	92.3

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Real-world example 2: miRNA qPCR/ddPCR

Accuracy and precision (**miR-16**, n=3)

Assay	Theoretical concentration (copies/reaction)	Within-run Concentration (copies/µL)		Between-run Concentration(copies/µL)	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
ddPCR	5.98E+02	**0.7 to 5.1**	101.1 to 128.1	10.8	113.5
	5.98E+03	**0.7 to 2.2**	97.1 to 121.0	11.0	105.7
	1.20E+05	8.0 or 10.0	89.1 to 92.0	7.6	90.5
qPCR	5.98E+01	4.3 to 19.3	93.0 to 98.4	13.2	94.9
	5.98E+03	4.2 to 8.5	94.5 to 105.3	7.4	99.4
	5.98E+05	5.5 to 9.4	**92.0 to 101.5**	7.6	96.7

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Real-world example 2: miRNA qPCR/ddPCR

SNBL

Conclusion

- Both the qPCR and ddPCR methods were successfully validated to quantify miR-223 and miR-16 from serum obtained from non-treated cynomolgus monkeys.
- The qPCR method had a wider quantification range and higher accuracy at high concentration when compared with the ddPCR method.
- Depending on the target, the ddPCR method had higher precision than qPCR in the low concentration range.

The ddPCR and qPCR methods have different quantitative ranges and different concentration range of accurately measured, so it is necessary to select the appropriate method depending on the purpose of study.

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SNBL

Thank you!

Please visit our poster presentation!

Abstract No. P3-11

Core time: 10:10 to 11:10 on February 7th

Validation design

Validation sample (except for limit of detection)

Acceptance criterion	• The difference between the mean of the Cq values in triplicate is equal to or lower than 1.5.

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System suitability

System suitability (DNA)

- NTC (no template control): DNase/RNase-Free Water
- NC (negative control): pooled gDNA of liver, lung, and spleen
- Calibration curve

Acceptance criteria

NC, NTC

- Cq values are undetermined or are equal to or higher than 38.5 (terminal cycle: 40).

Standard curve

- The difference of the Cq values (maximum value − minimum value) is equal to or lower than **1.5**.
- The amplification efficiency (E) is **between 85% and 110%.**
- Linearity of the calibration curve (R^2) is equal to or higher than **0.980**.

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System suitability

System suitability (mRNA)

GOI and reference gene

- NTC (no template control): DNase/RNase-Free Water

GOI

- Calibration curve

Acceptance criteria

NTC

- Cq values are undetermined or are equal to or higher than 38.5. (terminal cycle: 40)

Calibration curve

- The difference of the Cq values (maximum value − minimum value) is equal to or lower than **1.5**.
- The amplification efficiency (E) is **between 85% and 110%.**
- Linearity of the calibration curve (R^2) is equal to or higher than **0.980**.

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PCR measurement in biodistribution study

SNBL

Test sample (DNA)

Unspiked test sample

- Measure in duplicate

Spiked test sample

- A known amount of the target gene (spike sample) is spiked to 1 well containing test sample to evaluate potential qPCR inhibition

Acceptance criteria

Unspiked test sample

- The difference of the Cq values in duplicate is equal to or lower than 1.5 (when the Cq values of the test sample is higher than that of LLOQ, the values are adopted and expressed as BLOQ).

Spiked test sample

- The difference between the Cq value and the mean of the Cq values of spike sample is equal to or lower than 1.5, or the Cq value is lower than the mean of the Cq values of spike sample.

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PCR measurement in biodistribution study

SNBL

Test sample (mRNA)	• Measure in triplicate targeting GOI/reference gene, reference gene is used for normalization
Acceptance criterion	• The difference of the Cq values in triplicate is equal to or lower than 1.5 (when the Cq values of the test sample is higher than that of LLOQ, the values are adopted and expressed as BLOQ).

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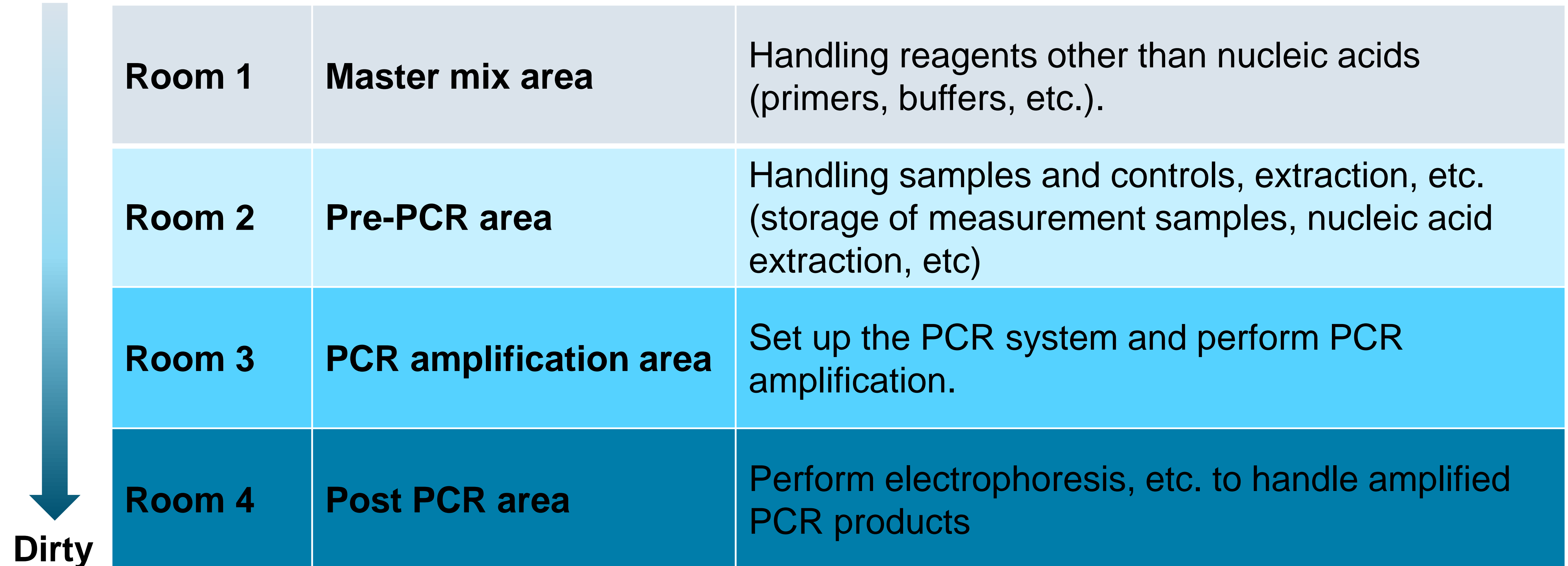
Preventing contamination of Alu sequence

Facility

Applying European Pharmacopoeia 2.6.21 to prevent contamination

Clean



Room 1	**Master mix area**	Handling reagents other than nucleic acids (primers, buffers, etc.).
Room 2	**Pre-PCR area**	Handling samples and controls, extraction, etc. (storage of measurement samples, nucleic acid extraction, etc)
Room 3	**PCR amplification area**	Set up the PCR system and perform PCR amplification.
Room 4	**Post PCR area**	Perform electrophoresis, etc. to handle amplified PCR products

Dirty

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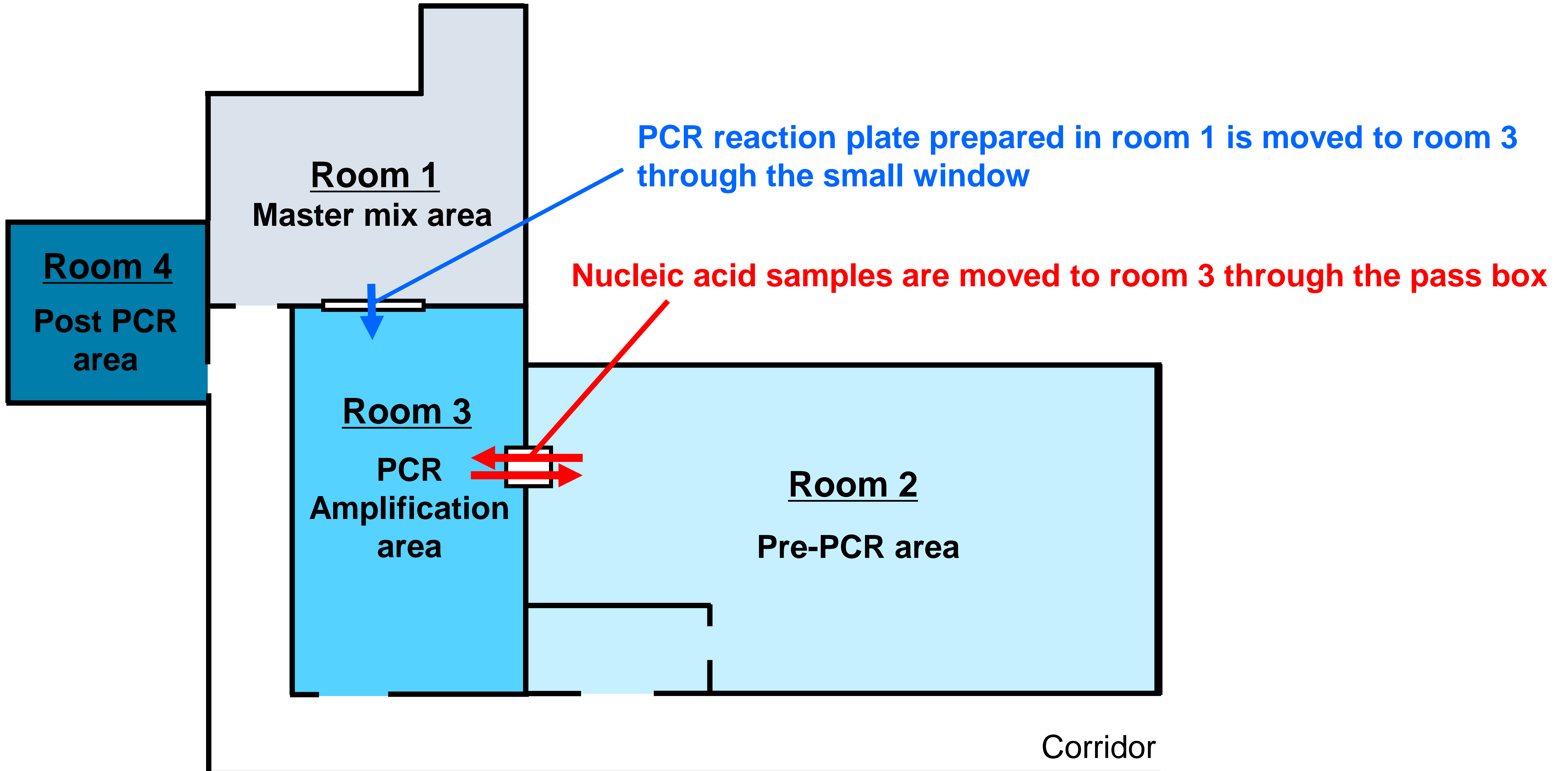
Preventing contamination of Alu sequence



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