Reference materials for GM testing

Food Research Institute, NARO

KAZUMI KITTA

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- Expectation for a Novel Reference Material

Introduction: Reference Materials

Reference Material & Certified Reference Material

ISO Guide 30:2015 Reference materials – selected terms and definitions

reference material
RMmaterials, sufficiently homogeneous and stable with respected to one or more specified properties, which has been established to be fit for its intended use in a measurement process

certified reference material
CRMreference material(RM) characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability

Reference Material & Certified Reference Material

ISO Guide 30:2015 Reference materials – selected terms and definitions

Introduction

Reference materials (RMs) and certified reference materials (CRMs) are widely used for the calibration of measuring apparatus, for the evaluation of measurement procedures and for the internal or external quality control of measurements and laboratories. … RMs and CRMs play an increasingly important role in national and international standardizing activities and in the accreditation of laboratories.

…

Codex Guideline

Guidelines for the assessment of the competence of testing laboratories involved in the import and export control of food
（*CAC/GL 27-1997*）

【SCOPE 】

1. These guidelines provide a framework for the implementation of quality assurance measures to ensure the competence of testing laboratories involved in the import and export control of foods.

2. These guidelines are intended to assist countries in the application of requirements for trade in foodstuffs in order to protect the consumers and to facilitate fair trade.

（cont.）

【REQUIREMENTS 】

3. The following quality criteria should be adopted b laboratories involved in the import and export control of foods:

- Compliance with the general criteria for testing laboratories laid down in ISO/IEC Guide 17025:1999 “General requirements for the competence of calibration and testing laboratories”;

- Participation in appropriate proficiency testing schemes for food analysis which conform to the requirements laid down in “The International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories”. Pure & Appl. Chem. 78(2006) 145-196;

(cont.)

- Whenever available, use methods of analysis which have been validated according to the principles laid down by the Codex Alimentarius Commission; and

- Use internal quality control procedures, such as those described in the "Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories", Pure & Appl. Chem. 67 (1995) 649–666.

4. The bodies assessing the laboratories referred to above should comply with the general criteria for laboratory accreditation, such as those laid down in the ISO/IEC Guide 58;1993: "Calibration and testing laboratory accreditation systems – General requirements for operation and recognition".

Reference Materials for GM Testing

Requirement of GM Reference Materials

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 Sept 2003 on genetically modified food and feed

CHAPTER II **GENETICALLY MODIFIED FOOD**

Article 5 ***Application for authorisation***

3. The application shall be accompanied by the following:

a. the name and the address of the applicant;

…

i. methods for detection, sampling (including references to existing official or standardised sampling methods) and identification of the transformation event and, where applicable, for the detection and identification of the transformation event in the food and /or in foods produced from it;

j. samples of the food and their control samples, and information as to the place where the reference material can be accessed;

…

l. a summary of the dossier in a standardized form.

CHAPTER III **GENETICALLY MODIFIED FEED**

Article 17 ***Application for authorisation***

3. The application shall be accompanied by the following:

…

European Commission, Joint Research Centre, Reference Materials Unit

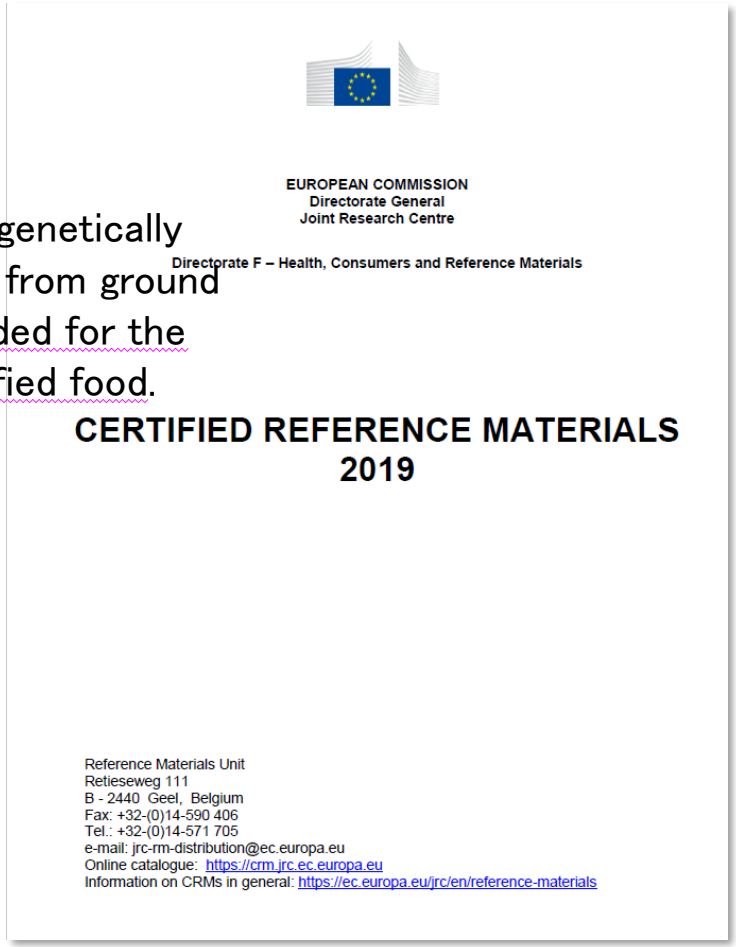
Certified Reference Materials 2019

2.2 Matrix Materials

2.2.1 Certified for GMO content

The materials were prepared by quantitative mixing of non-genetically modified powder and genetically modified powder, produced from ground seed with the help of a dry-mixing technique, and are intended for the calibration of methods for the detection of genetically modified food.

Crop	Number
Soya seed	6
Maize	15
Sugar beet	1
Potato	4
Cotton seed	4
Rapeseed	1
Soya seed (plasmid)	1
Maize (plasmid)	3
Total	35



EUROPEAN COMMISSION
Directorate General
Joint Research Centre

Directorate F – Health, Consumers and Reference Materials

CERTIFIED REFERENCE MATERIALS
2019

Reference Materials Unit
Retieseweg 111
B - 2440 Geel, Belgium
Fax: +32-(0)14-590 406
Tel.: +32-(0)14-571 705
e-mail: jrc-rm-distribution@ec.europa.eu
Online catalogue: https://crm.jrc.ec.europa.eu
Information on CRMs in general: https://ec.europa.eu/jrc/en/reference-materials

https://crm.jrc.ec.europa.eu/graphics/cms_docs/rm_catalogue.pdf

GMO CRM (Reference Materials Unit, EC-JRC)

Name	crop/ plasmid	event/fragment	breakdown
ERM-BF410p	soya seed	GTS 40-3-2	a, b, c, d, e
ERM-BF411	maize	Bt-176	a, b, c, d, e, f
ERM-BF412k	maize	Bt-11	a, b, c, d, e
ERM-BF413k	maize	MON 810	a, c, e*, g
ERM-AD413	plasmid	5'plant-P35S junction *hmg*	plasmid
ERM-BF414	maize	GA21	a, b, c, d, e, f
ERM-BF415	maize	NK603	a, b, c, d, e*, f
ERM-AD415	plasmid	3' insertion-specific *hmg*	pIRMM-0086
ERM-BF416	maize	MON 863	a, b, c, d
ERM-BF417	maize	MON 863 x MON 810	a, b, c, d
ERM-BF418	maize	1507	a, b, c, d
ERM-BF419	sugar beet	H7-1	a, b
ERM-BF420	maize	3272	a, b, c
ERM-BF421	potato	EH92-527-1	a, b
ERM-BF422	cotton seed	281-24-236 x 3006-210-23	a, b, c, d
ERM-BF423	maize	MIR604	a, b, c, d
ERM-BF424	maize	59122	a, b, c, d
ERM-BF425	soya seed	356043	a, b, c*, d

Name	crop/ plasmid	event/fragment	breakdown
ERM-AD425	plasmid	5' insert-to-plant junction *le 1*	pIRMM-0073
ERM-BF426	soya seed	305423	a, b, c, d
ERM-BF427	maize	98140	a, b, c*, d
ERM-AD427	plasmid	5' insert- to-plant junction *hmg*	pIRMM-0090
ERM-BF428	cotton	GHB119	a, b, c
ERM-BF429	cotton	T304-40	a, b, c
ERM-BF430	potato	AM04-1020	a, b
ERM-BF431	potato	AV43-6-G7	a, b
ERM-BF432	soya seed	DAS-68416-4	a, b, c, d
ERM-BF433	maize	DAS-40278-9	a, b, c, d
ERM-BF434	rapeseed	73496	a, b, c, d, e
ERM-BF435	potato	PH05-026-0048	a, b
ERM-BF436	soya seed	DAS-44406-6	a, b, c, d, e
ERM-BF437	soya seed	DAS-81419-2	a, b, c, d, e
ERM-BF438	maize	VCO-01981-5	a, b, c, d, e
ERM-BF439	maize	DP-004114-3	a, b, c, d, e
ERM-BF440	cotton	DAS-81910-7	a, b, c, d, e

* : also certified for the DNA copy number ratio

GMO CRM (AOCS)

Certified Refence Materials (CRMs)

The European Commission (EC) has mandated that as of 2004, a validated published method for detecting a new genetically modified event and a CRM must be available before the EC will authorize a new crop. Several nations outside of Europe require grain and ingredients to be labeled as genetically modified when trait levels exceed mandated thresholds. AOAC CRMs are used by labs that perform those analysis.
These CRMs are intended for use as quality control materials or calibrants in methods for the detection, identification and/or quantification of genetically modified events.

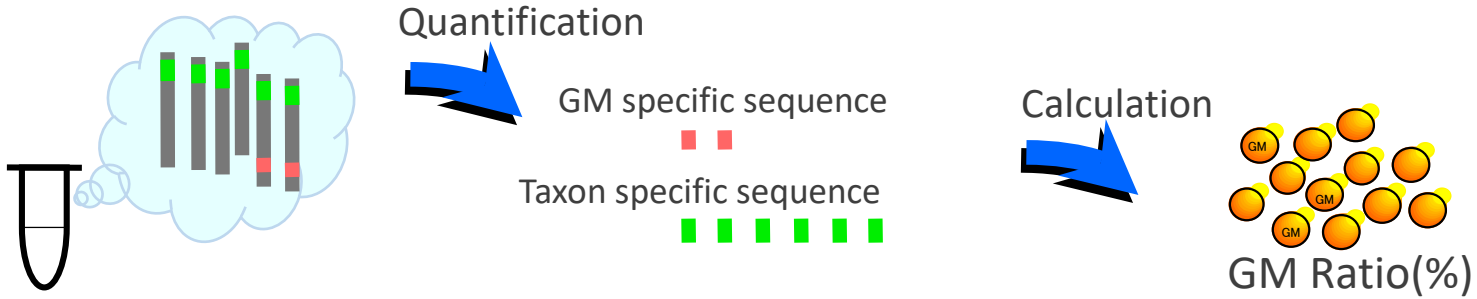
Crop＊1	Number＊2
Canola	11
Cotton	11
Maize	16
Potato	2
Rice	2
Soybean	17
Sugar beet	1
Total	60

＊1：providing form powder/DNA
＊2：including nonGM

https://www.aocs.org/crm

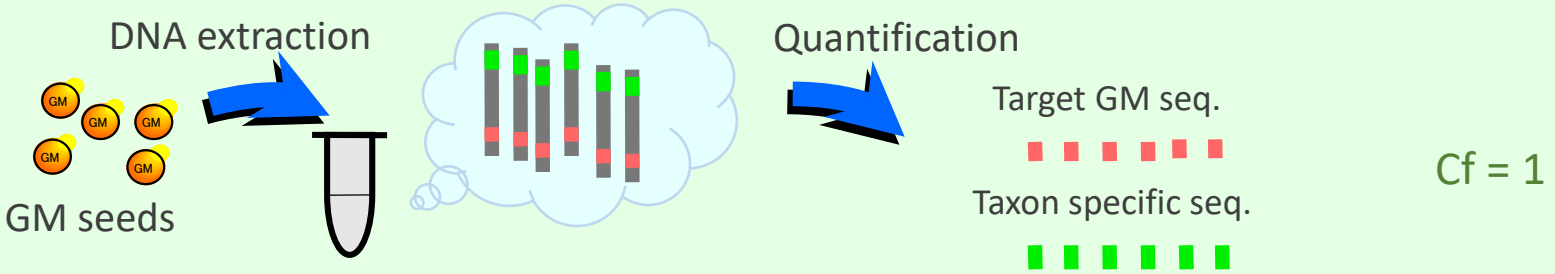
Production and Distribution of GM-CRM by NFRI

How to calculate GM amount?



$$\text{GM Ratio (\%)} = \frac{\text{GM specific seq.}}{\text{Taxon specific seq.}} \times \frac{1}{\text{Cf}} \times 100$$

Cf (conversion factor): ratio of the copy numbers of GM specific to taxon specific DNA sequences

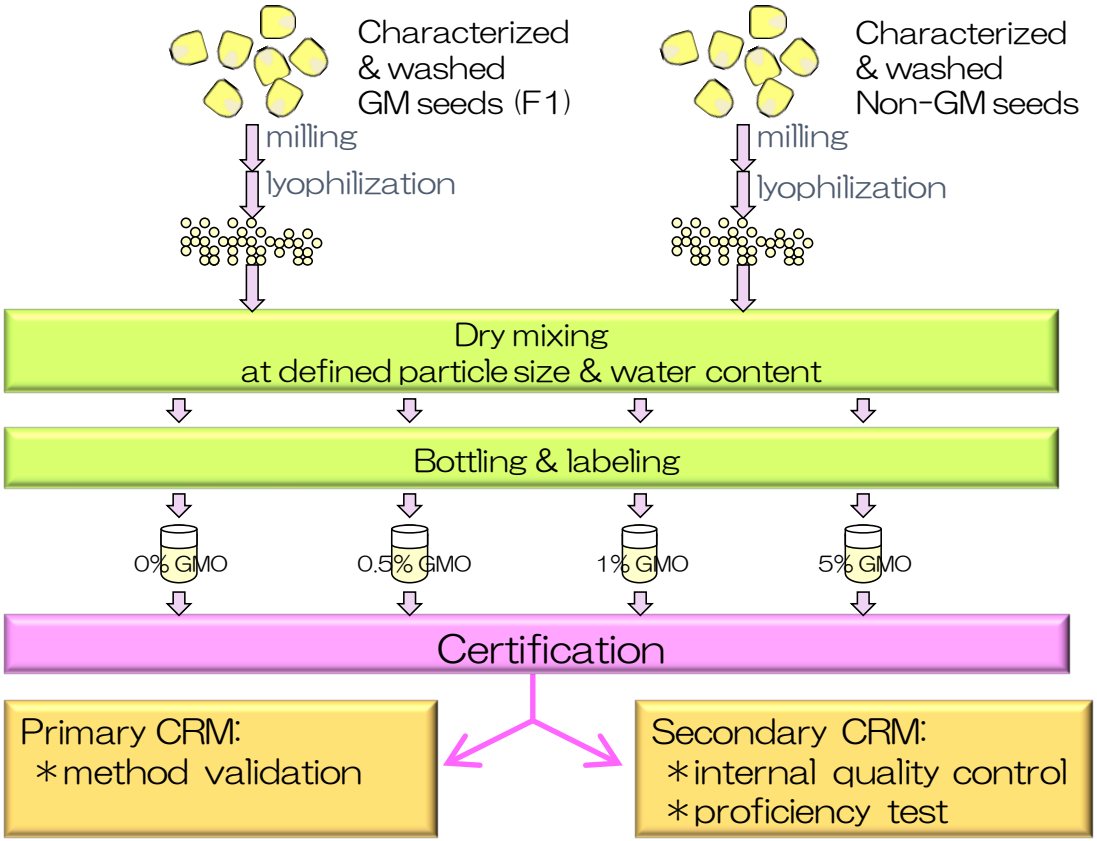


Production and Distribution of GM-CRM by NFRI

Establishment of Quality Control System and accreditation to ISO Guide 34

・ISO Guide 34

General requirements for the competence of reference material producers



Characteristics of GM-CRM by NFRI (～2016)

【Determination of certified values】
based on the measurement values by 5 laboratories using Japanese official method
→certified values with Japanese official method

【Intended purpose】
bias control when using Japanese official method
→internal quality control

【Characteristics】
The materials were prepared by quantitative mixing of non-genetically modified powder and genetically modified powder, produced from ground seed with the help of a dry-mixing technique, and bottled into a brown bottle. The net weight was 1 g
→because CRMs were provided in the form of seed powder, it was possible to perform quality control of measurement procedures including DNA extraction step.

example: 標準物質認証書

【認証標準物質名】
遺伝子組換えダイズ

認証書番号: _____
CRM番号(ボトル番号): NFRI-GM 002b1(　　)†
(製造日:平成21年 5月 21 日)

【認証標準物質の特性】 遺伝子組換えダイズ(RRS)含有率

【使用目的】日本の標準分析法*による測定の真度の確認に用いる。
検量線作成用として使用するものではない。

【製造方法】高純度の遺伝子組換えダイズと非組換えダイズを重量比で混合

【認証値】 (0.177 ±0.076) m/m%

【認証値決定方法】 日本の標準分析法による5試験室の各2点の測定値に基づく。

【認証値不確かさ】 認証値決定のための共同試験で得られた測定値の標準不確かさの2倍(包含係数k=2)で表示

【トレーサビリティ】 日本の標準分析法にトレーサブルである。

【形状等】 本標準物質は、目開き 0.5 mmの篩を通過した粉末であり、褐色のガラス瓶に密封されている。
内容量は1 g である。

【均質性】 調製した300本の小分け容器から無作為に10本を選択し、試料全量から抽出を行い、各抽出液から2点ずつ分析を行い、各小分け容器の均質性を評価した。

【使用方法】 本標準物質は1回の使い切りである。水分量の測定は必要としない。

【保管方法】 未開封状態で－70 ℃程度で保管

【有効期限】 出荷後1年とする。保存試験の結果、有効期限および保管条件に変更がある場合は、ホームペー ジ等を通じて案内する。

【協力者】 製造にあたって、(財)日本食品分析センター及び(財)日本冷凍食品検査協会の協力を得た。また、認証値の決定にあたって、(独)農林水産消費安全技術センター、(財)日本食品分析センター、(財)日本冷凍食品検査協会及び(株)ファスマックの協力を得た。

【備考】 †CRM番号はGM-002ですが、ボトルのラベルはGM-003となっています。
*公定法であるJAS試験ハンドブック遺伝子組換え食品分析・試験マニュアル及び厚生労働省通知記載の組換えDNA技術応用食品の検査方法。

平成21年12月16日
独立行政法人 農業・食品産業技術総合研究機構
食品総合研究所長 林 徹

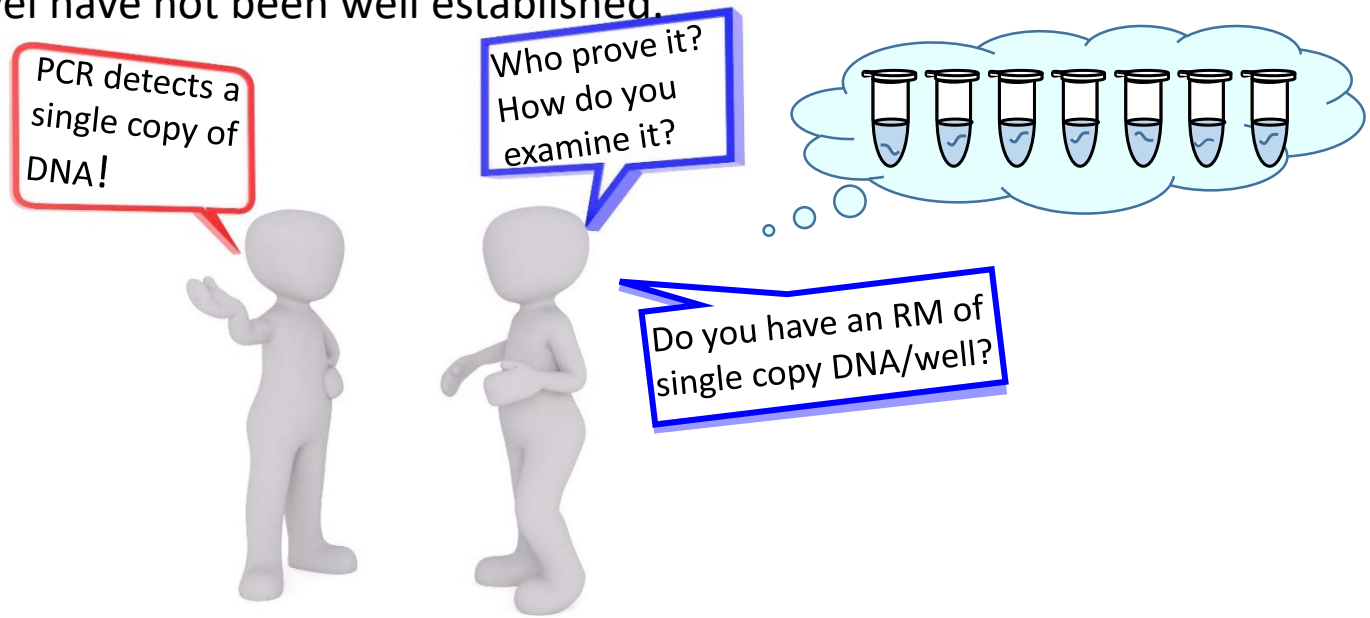
本標準物質に関する質問等は下記にご連絡ください。
独立行政法人　農業・食品産業技術総合研究機構
食品総合研究所　企画管理部　連携共同推進室
〒305-8642 茨城県つくば市観音台2-1-12
Tel　:029-838-7990 Fax　:029-838-7989

複写の制限: 交付された認証書は、複製すると「コピー」(あるいは「複写」等)の文字があらわれます。
RM-18-01.版05

Expectation for a Novel Reference Material

Background

- Along with the sophistication and improvement of analytical instruments, DNA amplification techniques, such as polymerase chain reaction (PCR), have been extensively applied to molecular diagnostics and detections as a gold standard technique. These techniques can detect targets at the level of one to several copies.
- For example, PCR can detect a single copy of DNA. However, there are no measures to assure its performance.
- Compared with the sophistication of analytical instruments, evaluation techniques at low copy number level have not been well established.



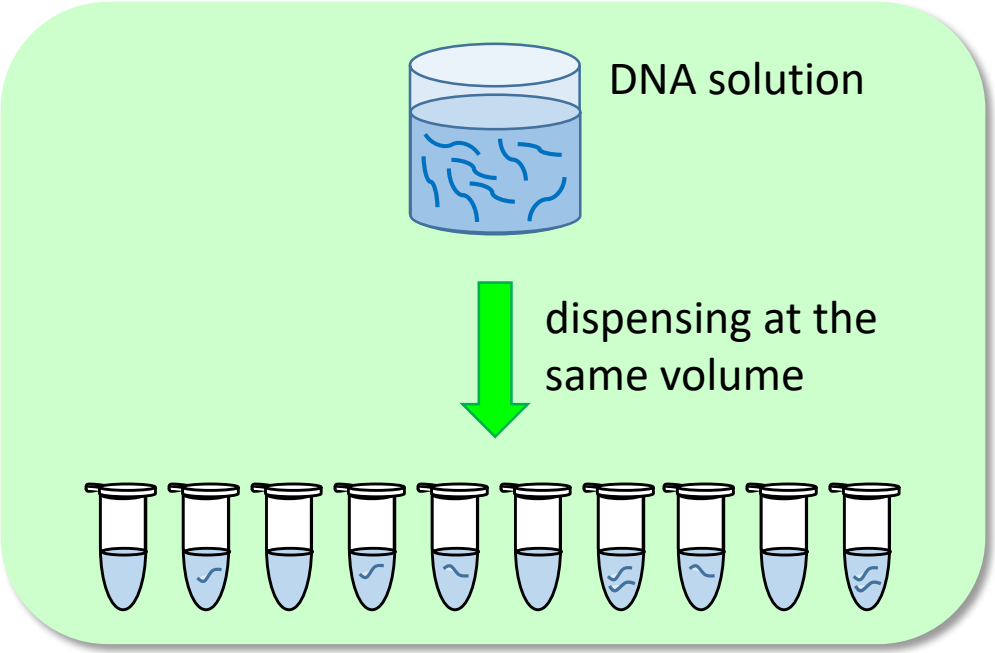
Demand for Quality Control at Low Copy Number Level due to Prevalence of DNA Test

PCR analysis at low copy level

- Qualitative analysis
  Ex. unauthorized GMO, non-GM labeled food, pathogenic microbe

- Negative result…
  - ✓ negative
  - ✓ negative due to sample distribution
  - ✓ negative due to PCR inhibition caused by low quality DNA
  - ✓ negative caused by quality gap of primers, reagents, and so on between lots
  - ✓ negative caused by defects of machine
  - ✓ negative caused by operation mistake

  …



Allotment of DNA at low concentration level

Determination of LOD

- The sensitivity of qualitative real-time PCR methods can be expressed as the limit of detection (LOD). To determine the LOD, a series of standard solutions is prepared by diluting DNA solutions of known concentrations.
- But the copy number of DNA prepared by dilution is not constant because the dilution process is governed by the Poisson distribution. In addition, the relative variation across replicates caused by this distribution tends to become larger at low copy numbers.
- Thus, no matter how accurately the dilution process is performed, it is impossible to control the copy number of target DNA at the single molecule level.

Ex.1: The probability of including the corresponding copy number of DNA in a tube, when DNA solution with a concentration of **1** copy/dispensing volume is allotted.

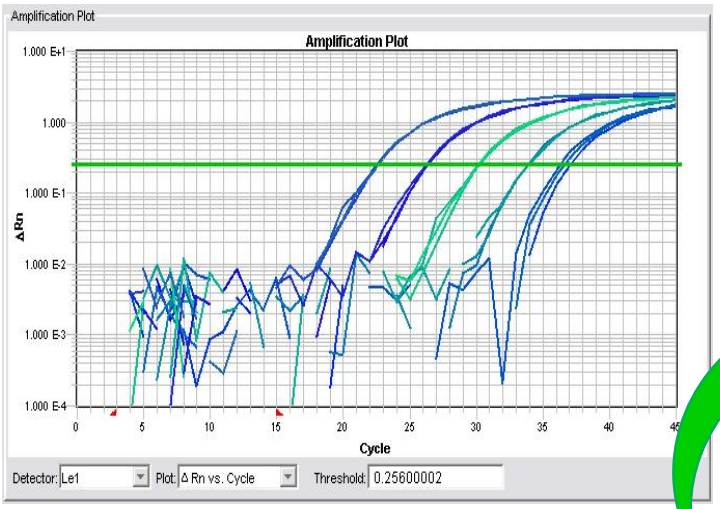
Copy number	0	1	2	3	4	5~
Probability (%)	36.8	36.8	18.4	6.13	1.53	0.34

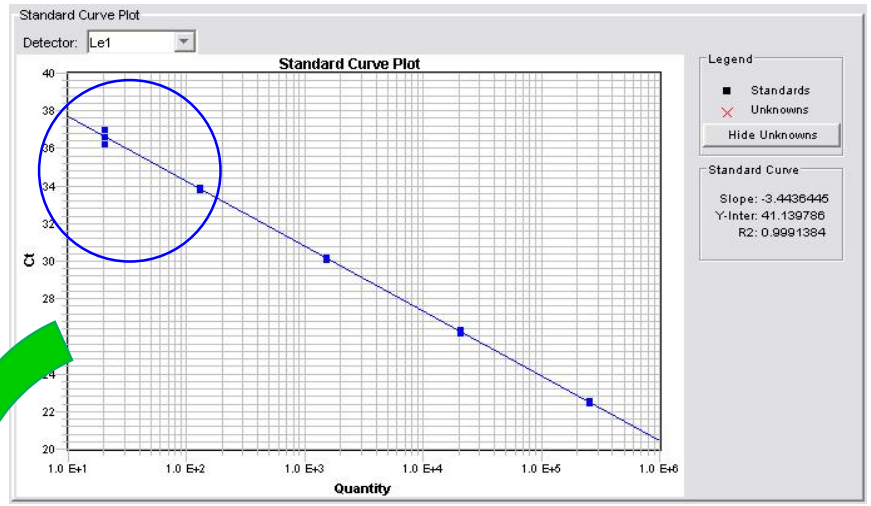
Ex.2: The probability of including the corresponding copy number of DNA in a tube, when DNA solution with a concentration of **2** copy/dispensing volume is allotted.

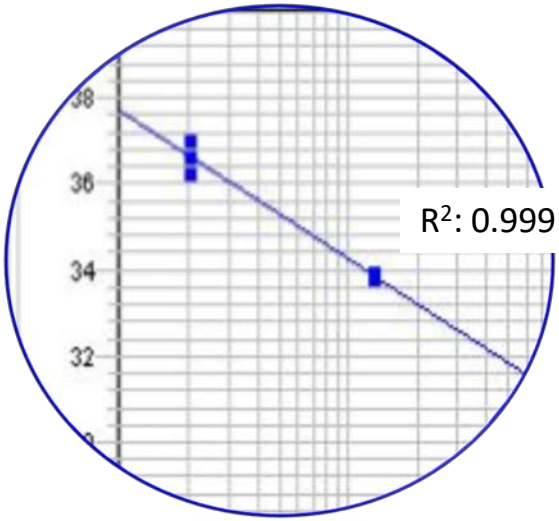
Copy number	0	1	2	3	4	5~
Probability (%)	13.5	27.1	27.1	18.0	9.0	5.3

Amplification Plot and Calibration Curve

--standard plasmid pLLS





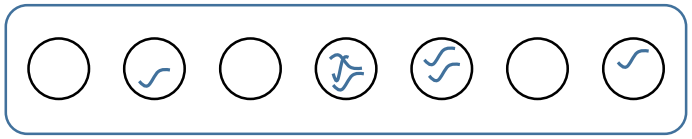


Calibration curve :
20, 125, 1.5k, 20k, 250k copies/reaction

Quality Control of DNA Analysis

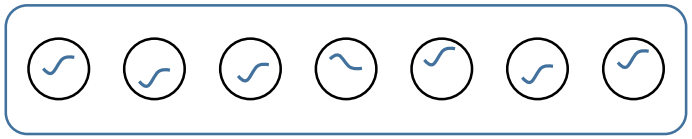
【Current condition】

Example: preparing theoretical xx copy/well DNA solution by dilution, and checking PCR devices, evaluating PCR reagents and methods with the DNA solution



【Copy number defined reference material】

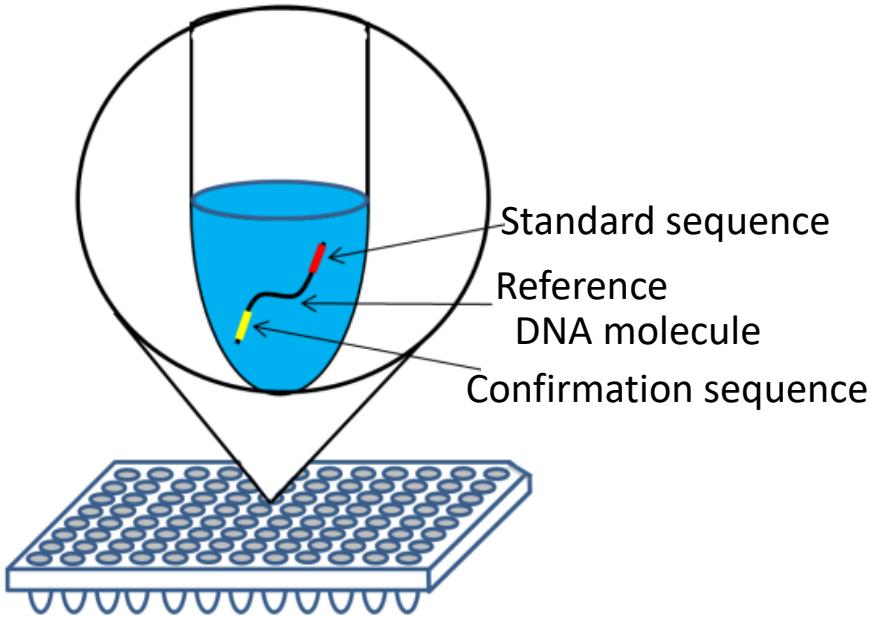
Example: preparing 1 copy/well DNA solution by inkjet system, and checking PCR devices, evaluating PCR reagents and methods with the 1 copy/well DNA solution



Challenge for Development of Single Molecule DNA RM I

【Limiting dilution method】

1. preparing a double-strand linear DNA which has PCR target sequences at the both ends
2. dispensing its highly diluted solution into 96 wells to make the average number of molecules in a well below one
3. checking the presence/absence of the DNA in each well by real-time PCR targeting confirmation sequence
4. after enzymatic treatment, using the positive well as the RM of a single DNA molecule with the standard sequence



【Challenge】

＊productivity

【Outcome】

＊P6093934 (JP)

＊Mano *et al*., *Anal. Chem*., **86**, 8621-8627, 2014

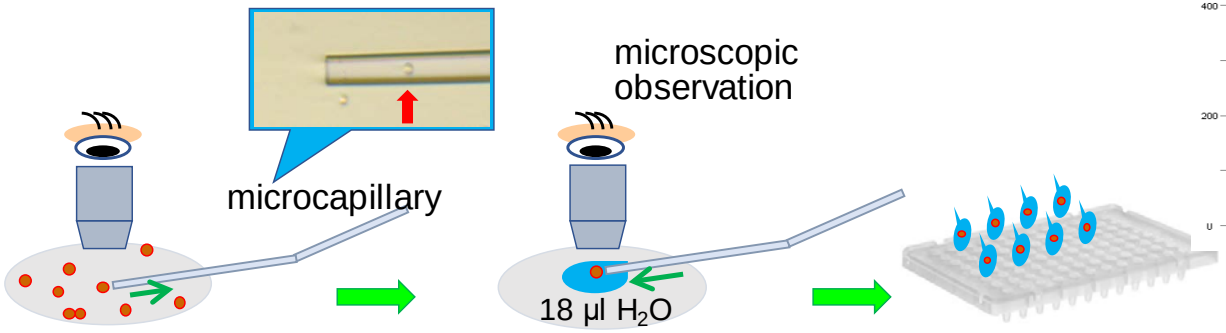
Challenge for development of single DNA molecule RM Ⅱ

【Cell-based RM】

When a eukaryotic cell with a target DNA sequence inserted in the nuclear genome is in G0/G1 phase:

1 cell (G0/G1) = 1 copy of genome DNA = 1 copy of target DNA sequence

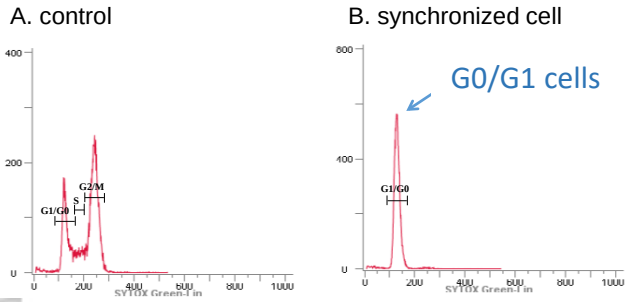
(arresting the target DNA sequence inserted haploid budding yeast cell at G0/G1 phase)



sucking a cell with a micro-manipulator

discharging the sucked cell into H_2O

transferring the cell in H_2O into each well



【Challenge】

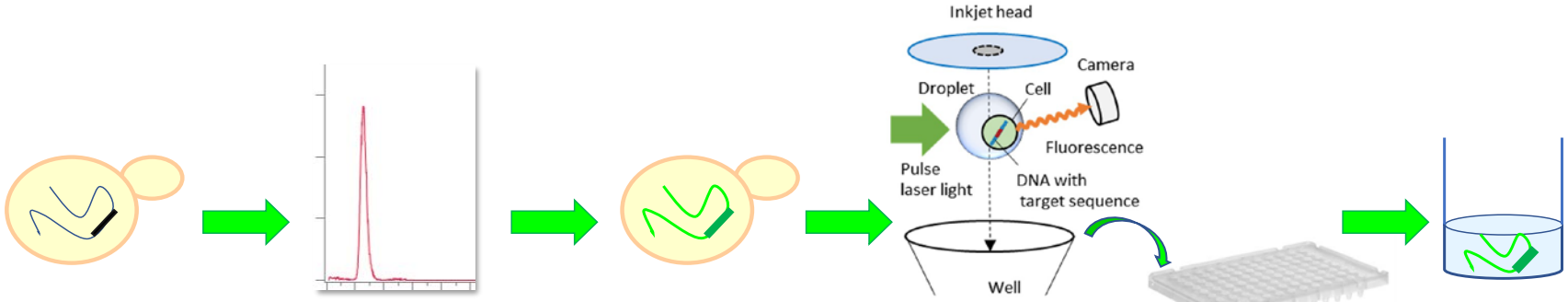
＊productivity

【Outcome】

＊P6366053 (JP)

Development of Novel RM Containing a Defined Copy Number of Target DNA

* inheriting the concept of cell-based RM
* making a breakthrough with inkjet dispensing technique, or dispensing suspension of fine particles



inserting a reference sequence into genomic DNA

arresting cell cycle at G0/G1 phase

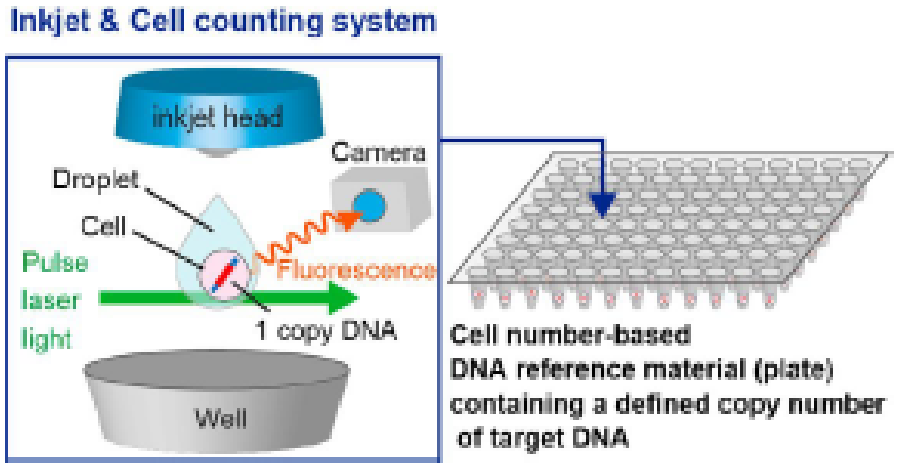
dyeing DNA with fluorescent dye

dispensing one cell each into a well by inkjet technology

treating cells with lytic enzyme

Novel Bioprinting Application for the Production of Reference Material Containing a Defined Copy Number of Target DNA

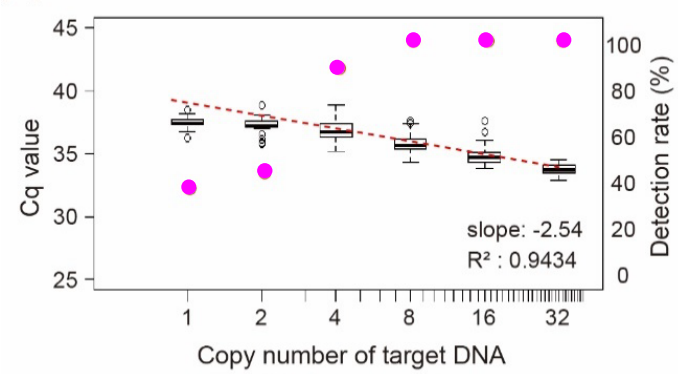
Seo et al., Analytical Chemistry, 91, 12733-12740, 2019



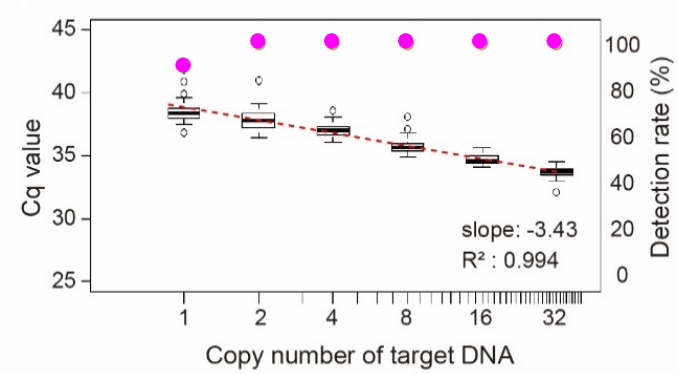
Comparison with a Currently Available Reference Material

Realtime PCR targeting *Le1*

A. dilution process (plasmid)



B. inkjet process (cell)



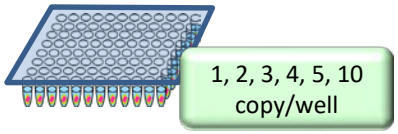
Dilution process						
copy no.	1 copy	2 copies	4 copies	8 copies	16 copies	32 copies
average Cq	37.44	37.24	36.84	35.84	34.82	33.74
max. Cq	38.46	38.87	38.86	37.55	37.61	34.50
min. Cq	36.23	35.80	35.22	34.28	33.79	32.86
CV value	0.0134	0.0186	0.0212	0.0210	0.0216	0.0126
detection rate	42%	48%	90%	100%	100%	100%

Inkjet process						
copy no.	1 copy	2 copies	4 copies	8 copies	16 copies	32 copies
average Cq	38.66	38.04	36.98	35.76	34.72	33.68
max. Cq	46.39	46.97	38.59	38.13	35.65	34.39
min. Cq	36.78	36.48	36.02	34.92	34.12	32.10
CV value	0.0380	0.0406	0.0146	0.0164	0.0112	0.0127
detection rate	92%	100%	100%	100%	100%	100%

Application: performance evaluation of PCR reagents

It is possible to evaluate LOD, amplification efficiency and so on among difference PCR reagents

1. preparing PCR plate with cells including prescribed sequence



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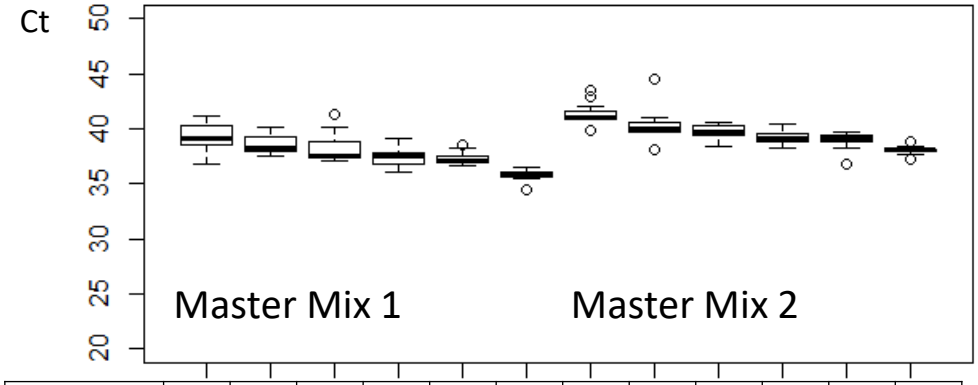
2. adding reagents intended to evaluate

3. analyzing with realtime PCR

4. evaluating performance of PCR reagents



copy no.	1	2	3	4	5	10	1	2	3	4	5	10
Cq ave.	36.7	36.1	35.7	34.9	34.9	33.5	38.7	38.4	37.0	36.5	36.3	35.3
detection rate	88%	100%					75%	94%	100%			
ampl. efficiency	94%						99%					

	Mater Mix 1	Master Mix 2
Cq value (10 copy/well)	33.5	35.3
min. copy no. for 100 % detection rate	2 copy	3 copy
amplification efficiency	94 %	99 %

Application: performance check of PCR device

【Realtime PCR】
Target: artificial sequence (600G), 10 copies/well

【Phenomenon】
A particular well repeatedly produced a high Cq value.

plate 1:

	1	2	3	4	5	6	7	8	9	10	11	12
A	34.5	35.2	34.7	34.7	34.5	34.9	35.0	35.0	35.1	34.5	34.8	34.4
B	35.2	35.0	34.9	34.8	34.9	35.0	35.1	35.1	34.5	34.8	34.7	35.0
C	35.3	35.0	34.9	35.0	35.1	34.9	35.0	34.8	35.0	34.7	34.6	35.0
D	34.8	35.0	35.3	35.2	35.0	35.0	35.3	34.8	35.0	34.9	34.9	35.0
E	35.0	35.2	35.2	35.0	35.1	34.9	34.9	34.8	35.0	35.0	35.0	34.8
F	35.0	34.9	34.6	35.1	34.6	34.7	35.0	35.0	34.8	35.0	34.8	35.1
G	34.9	34.9	34.6	34.6	34.7	34.9	35.3	34.7	35.0	34.8	34.8	35.3
H	35.0	35.3	35.0	35.0	35.2	35.0	35.0	36.7	35.1	34.6	34.6	34.9

Cq Ave	34.94
σ	0.27
CV%	0.77
ΔCq	2.26
Max	36.68
Min	34.42
UD	0
Det. rate	100.0%

plate 2:

	1	2	3	4	5	6	7	8	9	10	11	12
A	35.4	35.3	35.0	35.0	34.9	35.1	34.7	35.0	34.9	35.1	34.9	34.7
B	35.2	35.0	34.8	35.3	34.9	35.0	35.2	35.1	34.9	34.7	34.7	35.0
C	35.4	35.0	35.0	34.2	35.0	34.9	34.9	35.1	35.4	35.4	35.0	35.1
D	35.0	35.0	35.0	35.0	34.8	34.8	35.0	35.2	35.0	35.3	35.1	34.8
E	35.1	35.1	35.1	35.4	35.3	34.8	35.1	35.5	35.1	34.8	35.0	35.6
F	34.6	35.5	35.8	35.3	35.0	35.4	34.9	34.8	35.2	34.7	35.2	34.9
G	34.8	35.2	35.4	34.7	35.0	35.2	35.0	35.0	35.0	35.1	34.9	35.0
H	34.9	35.1	34.9	34.7	35.1	35.3	34.9	36.0	34.9	35.0	35.1	34.9

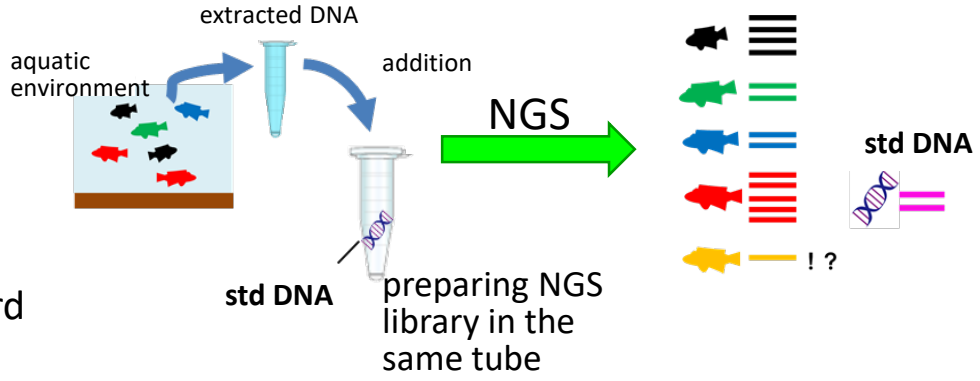
Cq Ave	35.05
σ	0.25
CV%	0.72
ΔCq	1.76
Max	35.98
Min	34.23
UD	0
Det. rate	100.0%

【Solution】
Cleaning the corresponding well resolved the problem.

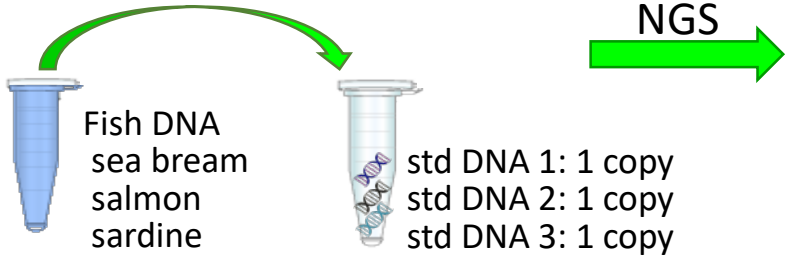
Application: Quality Control on eDNA Analysis

【Challenge】
Does a low-quantity of reads reflect the real existence of corresponding species?

【Proposal】
using a copy number defined DNA as a standard DNA to judge ghost reads



sample: sea bream, salmon, sardine
standard DNA: 3 kinds of DNA (1 copy each)
primer: MiFish-U
analysis pipeline: Usearch



【Results】
sequences with read no. less than that of std DNA was judged as ghost reads

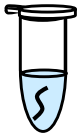
Results of Miseq sequencing

Fish (Japanese name)	Read No.
Pagrus major (madai)	27431
Oncorhynchus keta (sake)	15525
Sardinops melanostictus (maiwashi)	2006
Carassius cuvieri (gengorobuna)	1
Seriola quinqueradiata (buri)	3
Pagrus major (madai): partially mismatch	1
Standard DNA 1	18
Standard DNA 2	10
Standard DNA 3	69

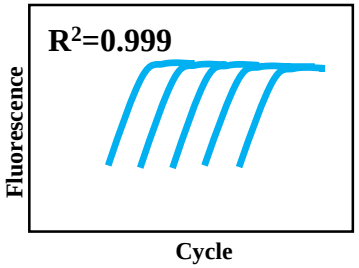
Oosaki et al., 2018

Perspective on Copy Number Defined Reference Material

copy number defined RM

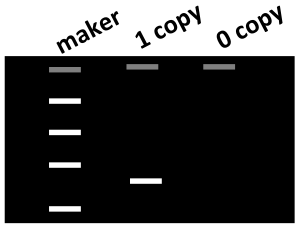


plotting a calibration curve for QN PCR

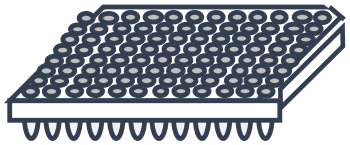


high correlation coefficient covering low to high copy no.

evaluating absolute LOD for QL PCR



checking PCR devices and reagents



and more…

Quality control of PCR and other techniques with copy number defined RM
→International standardization

Thank you for your attention

COI Disclosure Information

KAZUMI KITTA

I have no financial relationships to disclose