National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2373

Genomic DNA Standards for *HER2* Measurements

This Standard Reference Material (SRM) is for use to assign ratios of the human epidermal growth factor receptor 2 gene (*HER2*, the official gene symbol *ERBB2*) to unamplified reference genes. Measurements of the amplification (increased copies) of the *HER2* gene in breast cancer samples are used as a biomarker for determining the classification and the best treatment for breast cancers [1]. SRM 2373 consists of genomic DNA extracted from five breast cancer cell lines with different amounts of amplification of the *HER2* gene. The five purified genomic DNAs were solubilized in a buffer consisting of 10 mmol/L tris(hydroxymethyl)aminomethane and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (EDTA) pH 8.0 (TE^{-4}). The five components are genomic DNA materials derived from human cell lines SK-BR-3, MDA-MB-231, MDA-MB-361, MDA-MB-453, and BT-474, labeled A, B, C, D, and E, respectively. A unit of SRM 2373 consists of five vials, one of each component, containing approximately 100 µL of DNA solution. Each of these vials is labeled and is sealed with a color-coded screw cap.

Certified Values: Certified ratios of *HER2* copies per reference genes are shown in Table 1. Certified ratios of *HER2* copies per reference genes with the 95 % uncertainty intervals and prediction intervals at 68 % confidence (one standard deviation) are also shown in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [2].

Information Values: Information values for *HER2* and four Reference Genes Copies are shown in Tables 2 and 3. While measurements were done using the four reference genes, the information values were determined using data from the three reference genes that had the best agreement [3]. A NIST information value is considered to be a value that will be of interest and use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed [2]. Information values cannot be used to establish metrological traceability.

Expiration of Certification: The certification of **SRM 2373** is valid, within the measurement uncertainty specified, until **30 November 2020**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Storage and Use"). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Overall direction and coordination of the technical measurements leading to the certification of SRM 2373 were provided by K.D. Cole and S. Choquette of the NIST Biosystems and Biomaterials Division.

Sample preparation and analytical measurements were done by H.-J. He, K.D. Cole, J. Almeida of the NIST Biosystems and Biomaterials Division. Additional measurements were supplied by S. Maragh and J. Kralj of the NIST Biosystems and Biomaterials Division, and D. Catoe and C.R. Hill both formerly of NIST.

Statistical consultation was provided by S. Lund of the NIST Statistical Engineering Division.

Sheng Lin-Gibson, Chief
Biosystems and Biomaterials Division

Gaithersburg, MD 20899
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Certificate Revision History on Last Page

Steven J. Choquette, Director
Office of Reference Materials

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

NOTICE AND WARNING TO USERS

SRM 2373 IS A HUMAN SOURCE MATERIAL. SINCE THERE IS NO CONSENSUS ON THE INFECTIOUS STATUS OF EXTRACTED DNA, HANDLE THE SRM 2373 COMPONENTS AS BIOSAFETY LEVEL 1 MATERIALS CAPABLE OF TRANSMITTING INFECTIOUS DISEASE [4]. SRM 2373 components and derived solutions should be disposed of in accordance with local, state, and federal regulations.

INSTRUCTIONS FOR STORAGE AND USE

Storage: All vials of SRM 2373 should be stored in the dark between 2 °C to 8 °C. **DO NOT FREEZE.**

Use: Component vials should be mixed briefly and centrifuged (without opening the vial cap) prior to removing sample aliquots for analysis. For the certified and informational values to be applicable, materials should be withdrawn immediately after opening the vials and processed without delay. Dilutions of these materials may be made as appropriate, but the dilutions should be used immediately and not stored. Certified and information values do not apply to any material remaining in recapped vials. There is no minimum sample size requirement, the amount required by any given assay will be dictated by the nature of the assay. **DO NOT EXPOSE ANY DNA SOLUTION TO DIRECT SUNLIGHT**. SRM 2373 is not suitable for immunohistochemical (IHC) or in-situ hybridization techniques (ISH-type tests) that rely upon tissues or cells for the measurements. This standard is suitable only for measurement techniques that utilize purified DNA.

For use with quantitative polymerase chain reaction (qPCR) Assays: Use the provided certified ratio to assign secondary standard DNA solutions for routine use. Users may calibrate their assays to SRM 2373 using one or more dilution series, with each series prepared from one SRM 2373 component. Use the provided information value of *HER2* and reference genes copies per microliter to perform the dilutions.

For use with digital PCR polymerase chain reaction (dPCR) Assays: Use the provided certified ratios to assign secondary standard DNA samples for routine use. Use the information value of *HER2* and reference genes copies per microliter and the approximate concentration values for dilution.

Certified Values: Table 1 contains the certified average ratios of *HER2* copies per copy of the selected reference genes, the approximate 95 % confidence interval about the average ratios, and an approximate one standard deviation prediction interval (68 %). The measurand is the ratio of *HER2* copies per average of the reference gene copies. While measurements were done using the four reference genes, the certified values were determined using data from the three reference genes that had the best agreement [3]. Metrological traceability is to the SI unit for mass.

Table 1. Certified Ratios of *HER2* Copies per Average of the Reference Gene Copies

Component	Cell Line	Color Code	Ratio	95 % Uncertainty Interval	68 % Prediction Interval
A	SK-BR-3	white	9.7	8.7 – 10.7	5.8 – 20.8
B	MDA-MB-231	blue	1.3	1.1 – 1.5	0.6 – 1.9
C	MDA-MB-361	red	6.4	5.7 – 7.1	4.8 – 14.2
D	MDA-MB-453	yellow	2.9	2.6 – 3.2	1.4 – 7.2
E	BT-474	green	17.7	15.9 – 19.5	11.7 – 45.3

The average gene abundance ratio, $Ratio_{sg}$, is based on a collection of gene abundance ratios:

$$Ratio_{sg} = {HER2_s}/{Ref_{sg}}$$

where s denotes one of the five components included in SRM 2373, g denotes one of the reference genes, $HER2_s$ denotes the measured abundance of *HER2* in sample s, and Ref_{sg} denotes the measured abundance of reference gene g in sample s.

The values in Table 1 come from fitting a statistical model to the measurements made on the SRM 2373 materials using both digital and quantitative PCR methods. The Bayesian paradigm with vague priors was used for statistical inference [5]. The expanded uncertainty is an interval calculated in a manner consistent with the ISO/JCGM Guide [6], and it expresses contributions from all recognized sources of uncertainty, including differences between analytical methods and operators, differences among bottles, differences among suitable reference genes, and dispersion of values resulting from sample preparation and replicated measurement. The nominal coverage for the interval is 95 % and can be interpreted in the following manner. The average gene copy ratio between *HER2* and suitable reference genes (examples of suitable reference genes are identified in Table 4) among all bottles prepared for this SRM lies within the provided uncertainty interval with approximately 95 % confidence. The prediction intervals provide the approximate range of values NIST would reasonably expect upon the next single, independent measurement of the *HER2* copy number ratio for each of the five components, based upon the measurement performance of NIST analysts and instruments. The value would be expected to fall within the interval approximately 68 % of the time.

PREPARATION AND ANALYSIS[(1)]

Sample Preparation: NIST SRM 2373 consists of genomic DNA samples prepared from five human breast cancer cell lines. After the initial extraction using the Zymo Quick-gDNA™ midiPrep kit (Zymo Research Corporation, Irvine, CA), the samples were pre-treated with bovine pancreatic ribonuclease A before re-extraction [7]. All purified genomic DNA samples were dissolved or eluted in TE^{-4} buffer (10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0) and stored at 4 ºC. The DNA samples from the cell lines were genotyped using STR analysis and the results agree with the values provided by the cell line repository.

qPCR data analysis: The qPCR assays for *HER2* and reference genes were developed according to The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [8]. The primers and probes for the *HER2* and reference genes for the qPCR assays are listed in Table 4. The efficiency and specificity of the assays were determined [9]. Using a calibration curve based quantitation method, the *HER2* gene copies and the copies of the reference genes were quantified by external standard curves assays using individual PCR assays and SYBER® Green master mix. The qPCR data was evaluated using SRM 2372 component A as a calibrant to produce standard curves. SRM 2372 component A, produced from a single male donor, was shown to contain a single copy of *HER2* gene per haploid genome. The absorbance of SRM 2372 component A is a certified value and the DNA mass concentration derived from the absorbance value is an information value, because the conversion is done using a factor of uncharacterized uncertainty. The following assumptions were used: (1) SRM 2372 component A has a DNA concentration of 57 ng/µL (information value from the Certificate of Analysis) [10]; (2) There are three hundred and thirty-three copies of single-copy genes in 1 ng of human genomic DNA. *HER2* and the reference gene copy number were obtained by qPCR assays by comparing the Cq (Quantitation Cycle from the qPCR instrument) of the samples with their respective standard curves.

dPCR Assays: MIQE guidelines for dPCR were followed and include sample replicates, positive and negative controls, and proper documentation [11]. TaqMan probes are shown in Table 5. BHQ-1™ (Black hole quencher) and FAM labeled probes were obtained from Biosearch Technologies (Novato, CA). MGB® (minor groove binder) and FAM labeled probes were obtained from Life Technologies (Carlsbad, CA). The calculations for DNA copy number were calculated using Poisson sampling statistics assuming that the DNA molecules are partitioning independently from each other into the individual droplets or chambers. We used two restriction enzymes, *Rsa*I and *Mse*I, to test that the DNA with high levels of *HER2* amplification were partitioning as single molecules. Treatment of the genomic DNA with the restriction enzymes did not increase the copy numbers obtained with the dPCR assays. The results were obtained from assays performed by two analysts using a Bio-Rad QX100 droplet digital PCR system; the Bio-Rad software used a droplet size of 0.91 nL for determination of DNA copy number.

Homogeneity and Stability Studies: Each of the components of SRM 2373 was distributed into 350 tubes that were then stored at 4 °C in the dark. Homogeneity studies were accomplished by selecting 10 vials of each of the components that were distributed throughout the order of dispensing. These vials were analyzed by both qPCR and dPCR methods. Analysis of the data did not indicate any detectable changes in the values of any of the five components that varied with dispensing order (given the uncertainty of the measurements). Stability testing was conducted on the samples that were stored at 4 °C in the dark for different periods of time. The *HER2* copy number or the ratio of *HER2* to the reference gene *RPS27A* were measured by dPCR and the data did not show any significant changes in any of the five components for periods of time up to 856 days, the last storage time analyzed (given the uncertainty of the measurements).

[(1)] Certain commercial equipment, instruments or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Information Values: The approximate concentration of components A, B, C, D, and E determined by absorbance measurements at 260 nm are 23.4 µg/mL, 22.2 µg/mL, 22.3 µg/mL, 22.3 µg/mL, and 22.4 µg/mL, respectively. The absorbances at 260 nm of the components (six replicates) were measured and a conversion factor (one optical density at 260 nm with a pathlength of 1 cm is 50 ng DNA/µL). The approximate values are used to determine the appropriate dilution of the samples for use with the desired measurement technique. It is important to consider the level of *HER2* amplification in the samples and appropriate dilutions needed to ensure that the *HER2* copy number is within the working range of the assay.

Tables 2 and 3 contain the information values for the concentrations (copies per microliters) of the *HER2* gene and the reference genes in the components of SRM 2373, determined by qPCR and dPCR, respectively. This data is a subset of the data used to calculate the certified ratios in Table 1. The samples were measured using qPCR and dPCR using the reference genes indicated in the respective tables.

Table 2. Information Values for *HER2* and Reference Genes Copies per Microliter by qPCR[(a)]

Component	*HER2*	Mean of 3 Reference Genes
A	64358	6912
B	8839	6525
C	47128	7014
D	17493	6613
E	99440	6030

[(a)] Mean values of ten samples, the average coefficient of variation for the measurements was 8.1 %.

Table 3. Information Values for *HER2* and Reference Genes Copies per microliter by dPCR[(a)]

Component	*HER2*	Mean of 3 Reference Genes
A	66964	6547
B	8707	6501
C	46277	6942
D	19218	6196
E	104655	5634

[(a)] Mean values of ten samples, the average coefficient of variation for the measurements was 7.3 %.

Table 4. PCR Primer Information for Reference Genes and *HER2* Assays

Primer Name[(a)]	Sequence	PCR Amplicon	Gene Name	Location (GRCh37/hq19 nucleotide number)
HER2-2F	CTCATCGCTCACAACCAAGT	112 bp	*HER2* (*ERBB2*)	Exon 7 (chr17:37864601-37864620)
HER2-2R	GGTCTCCATTGTCTAGCACG			(chr17:37864693-37864712)
EIF5-F	GGCCGATAAATTTTTGGAAATG	112 bp	*EIF5B*	Intron 1 (chr2:99974140-99974161)
EIF5-R	GGAGTATCCCCAAAGGCATCT			(chr2:99974231-99974251)
2PR4-F	CGGGTTTGGGTTCAGGTCTT	97 bp	*RPS27A*	Intron 4 (chr2:55462316-55462335)
2PR4-R	TGCTACAATGAAAACATTCAGAAGTCT			(chr2:55462386-55462412)
R4Q5-F	CTCAGAAAAATGGTGGGAATGTT	122 bp	*DCK*	Exon 3 (chr4:71888097-71888119)
R4Q5-R	GCCATTCAGAGAGGCAAGCT			(chr4:71888199-71888218)
22C3-F	AGGTCTGGTGGCTTCTCCAAT	78 bp	*PMM1*	Intron 7 (chr22:41973739-41973759)
22C3-R	CCCCTAAGAGGTCTGTTGTGTTG			(chr22:41973682-41973704)

[(a)] F: Forward primer.
R: Reverse primer.

Table 5. TaqMan® Fluorescent Probe Sequences

Probe Name	Sequence	5' Label	3' Quencher
HER2-2 (BHQ)	ACCCAGCTCTTTGAGGACAACTATGC	FAM	BHQ-1
HER2-2 (MGB)	AGCTCTTTGAGGACAACTA	FAM	MGB
EIF5-P	TTCAGCCTTCTCTTCTCATGCAGTTGTCAG	FAM	BHQ-1
2PR4-P	TTTGTCTACCACTTGCAAAGCTGGCCTTT	FAM	BHQ-1
R4Q5-P	CCTTCCAAACATATGCCTGTCTCAGTCGA	FAM	BHQ-1
22C3-P	CAAATCACCTGAGGTCAAGGCCAGAACA	FAM	BHQ-1

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Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at https://www.nist.gov/srm.