



Research paper

Development of NIST standard reference material 2373: Genomic DNA standards for *HER2* measurementsHua-Jun He^a, Jamie L. Almeida^a, Steve P. Lund^b, Carolyn R. Steffen^c, Steve Choquette^a, Kenneth D. Cole^{a,*}^a Bioassay Methods Group, Biosystems and Biomaterials Division, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD 20899, USA^b Statistical Engineering Division, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD 20899, USA^c Applied Genetics Group, Biomolecular Measurements Division, National Institute of Standards and Technology, 100 Bureau Drive, Gaithersburg, MD 20899, USA

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ABSTRACT

NIST standard reference material (SRM) 2373 was developed to improve the measurements of the *HER2* gene amplification in DNA samples. SRM 2373 consists of genomic DNA extracted from five breast cancer cell lines with different amounts of amplification of the *HER2* gene. The five components are derived from the human cell lines SK-BR-3, MDA-MB-231, MDA-MB-361, MDA-MB-453, and BT-474. The certified values are the ratios of the *HER2* gene copy numbers to the copy numbers of selected reference genes *DCK*, *EIF5B*, *RPS27A*, and *PMM1*. The ratios were measured using quantitative polymerase chain reaction and digital PCR, methods that gave similar ratios. The five components of SRM 2373 have certified *HER2* amplification ratios that range from 1.3 to 17.7. The stability and homogeneity of the reference materials were shown by repeated measurements over a period of several years. SRM 2373 is a well characterized genomic DNA reference material that can be used to improve the confidence of the measurements of *HER2* gene copy number.

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1. Introduction

The human epidermal growth factor receptor 2 (*HER2*, official symbol *ERBB2*) is a proto-oncogene protein, a 185-kDa transmembrane glycoprotein with tyrosine kinase activity [1]. Amplifications of the *HER2* gene, resulting in protein overexpression, are present in approximately 20% of breast cancers, and have been associated with poor patient prognosis [2,3]. The most common methods for *HER2* measurements in clinical laboratories are the detection of protein overexpression by immunohistochemistry (IHC) and the evaluation of gene amplification by *in situ* hybridization (ISH) methods. A number of studies have reported problems with the accuracy and the concordance of the results obtained from different laboratories using IHC and fluorescence ISH (FISH) methods [4,5]. The American Society of Clinical Oncology and the College of American Pathologists published guidelines to improve the performance of *HER2* testing by IHC and ISH methods in 2007, and an update in 2013 [6,7]. However, standardization of both IHC and ISH methods

across laboratories remains a major challenge. Approximately 20% of *HER2* testing performed may be inaccurate [8].

Recently, genomic analytical methods have been developed that enable DNA copy number variations (CNV) to be measured with high sensitivity and specificity. As few as 50 cells extracted from archival formalin-fixed paraffin-embedded (FFPE) tissues may be quantified using quantitative PCR (qPCR) [9,10]. The results from qPCR of *HER2* measurements have been positively correlated with the results from IHC and FISH analysis [10–13]. Koudelakova et al. compared qPCR to IHC and FISH data in breast cancer samples, and found that high sensitivity and specificity of the new method was achieved and the results obtained with the qPCR method and FISH/IHC agreed [14]. Digital PCR (dPCR) was used to measure *HER2* copy number in FFPE breast cancer tissue and these results agreed with the results from FISH and IHC analysis [15]. Garcia-Murillas used dPCR to measure the gene copy ratio of *HER2* to reference genes in the microdissected DNA from *HER2* amplified and *HER2* non-amplified cancers. They too, obtained high sensitivity and specificity and good agreement with the traditional detection methods [16].

Many cancer cells, including these breast cancer cell lines, have highly abnormal karyotypes, with multiple chromosome copies

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Table 1
PCR assays gene locations and primers used for certification values.

Primer name	Sequence	PCR amplicon	Location (GRCh37/hq19 nucleotide number)
HER2-2F	CTCATCGCTCACAAACCAAGT	112 bp	Exon 7 (chr17:37864601–37864620) (chr17:37864693–37864712)
HER2-2R	GGTCTCCATTGCTAGCACG		
EIF5B-F	GGCCGATAAATTTTGGAAATG	112 bp	Intron 1 (chr2:99974140–99974161) (chr2:99974231–99974251)
EIF5B-R	GGAGTATCCCAAAGGCATCT		
RPS27A-F	CGGGTTTGGGTTCAAGTCTT	97 bp	Intron 4 (chr2:55462316–55462335) (chr2:55462386–55462412)
RPS27A-R	TGCTACAATGAAAACATTGAAAGTCT		
DCK-F	CTCAGAAAATGGTGGGAATGTT	122 bp	Exon 3 (chr4:71888097–71888119) Intron 4 (chr2:55462316–55462335)
DCK-R	GCCATTTCAGAGAGGCAAGCT		
PMM1-F	AGGTCTGGTGGCTTCTCCAAT	78 bp	Intron 7 (chr22:41973739–41973759) (chr22:41973682–41973704)
PMM1-R	CCCTAAGAGGTCTGTTGTGTTG		

F: forward primer; R: reverse primer.

and major structural changes [17]. The selection of the reference genes is important in cancer cells because of the frequent gene mutations and gains or losses of DNA that have occurred. Chromosomal alterations in 15 breast cancer cell lines detected frequent gains at 1q, 8q, 20q, 7, 11q, 13, 17q, 9q and 16p and frequent losses at 8p, 11q14-qter, 18q and Xq [18]. Spectral karyotyping (SKY) using fluorescent staining for each chromosome showed a large number of complex alterations in the chromosome complement of breast cancer cell lines [18] (<http://old-www.path.cam.ac.uk/~pawefish/index.html>). A study of *HER2* amplified tumors showed increased gains at 1q, 8q, 20q and losses at 18q, 13q, and 3p [19]. Comparative Genomic Hybridization (CGH) of 89 breast cancer tumors detected frequent gains at 1q, 8q, 11q, and 16p and losses at 4q, 5q, 6q, 8p, and 14q [20]. CGH was used to examine the chromosome complement of 51 breast cancer cell lines and 145 primary breast cancer tumors showed similar genetic changes in the cell lines and tumor samples with some differences: losses in 5q and losses in chromosome 18 [21]. These studies showed that the chromosomal locations of the reference genes need to be carefully considered and the assays have to be tested to ensure that the reference genes have not been specifically amplified or deleted.

Suitable reference materials are needed for the new generation of nucleic acid measurement methods for cancer that are now being implemented in clinical laboratories [22]. This report describes the development of NIST SRM 2373 from five human breast cancer cell lines with different degrees of amplification of the *HER2* gene. Assays were developed for *HER2* and reference genes that are located at different chromosomal regions that are not frequently mutated in cancer. The use of reference genes that are not located on chromosome 17 (where the *HER2* gene is located) allows the detection of *HER2* amplification, due to the occurrence of chromosome 17 polysomy [23]. The copy numbers of the *HER2* gene and selected reference genes were measured using both qPCR and dPCR and used to calculate the ratio of *HER2* amplification.

2. Methods

2.1. Breast cancer cell lines

DNA samples from five human breast cancer cell lines, SK-BR-3, MDA-MB-231, MDA-MB-361, MDA-MB-453, and BT-474, which were used to prepare components A, B, C, D, and E, respectively. The cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) as frozen stocks and cultured in the NIST laboratory using standard cell culture methods. MDA-MB-231, MDA-MB-361, and MDA-MB-453 cells were cultured in Leibovitz's L-15 Medium (ATCC # 30-2008) supplemented with 10% fetal bovine serum (FBS, Gibco # 10437-028, except MDA-MB-361 where 20% FBS was used) at 37 °C in an air atmosphere without added CO₂. SK-BR-3 cells were grown in the McCoy's 5A modified medium (ATCC # 30-2007) supplemented with 10% FBS at 37 °C in

a humidified (5% CO₂, 95% air) atmosphere. BT-474 cells were cultured in the Hybri-Care Medium (ATCC # 46-X) supplemented with 1.5 g/L sodium bicarbonate and 10% FBS at 37 °C in a humidified (5% CO₂, 95% air) atmosphere.

2.2. Scale-up of DNA extraction and purification

Large batches of cells were prepared from each cell line for DNA extraction. The cells were sub-cultured for 4 to 5 passages, and harvested when they reached ~90% confluence in ten T-175 cell culture flasks. The culture medium was removed, the cells were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS), and the cells were treated with 0.25% (w/v) trypsin in 0.53 mM EDTA solution (Life Technologies, Grand Island, NY). Large scale DNA extraction was accomplished using Zymo Quick-gDNA™ midiPrep kits (Zymo, Irvine, CA). After the initial extraction, the samples were pre-treated with bovine pancreatic ribonuclease A before re-extraction. All purified genomic DNA samples were dissolved or eluted in 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0 buffer (TE⁻⁴) and stored at 4 °C.

2.3. Cell line genotyping

The five breast cancer cell lines were genotyped upon receipt from ATCC and after expansion using the AmpFLSTR Identifier Plus PCR Amplification Kit (Life Technologies Cat# 4427368) on a 3500xl Genetic Analyzer with a 36-cm capillary array and POP-4 polymer (Life Technologies). PCR amplification was carried out in a total 12.5 μL reaction volume (1/2 reactions) with 1 μL of purified genomic DNA (1.0 ng/μL) on GeneAmp PCR System 9700 Cyclor (Life Technologies) for 28 cycles according to the conditions specified by the manufacturer. Fifteen short tandem repeat (STR) loci with Amelogenin (sex-typing marker) were co-amplified in a single tube. After the reaction, 0.5 μL of GeneScan 600 LIZ Size Standard v2.0 and 8.5 μL Hi-Di Formamide (Life Technologies) were added to 1 μL of the PCR product or allelic ladder for a total volume of 10 μL. The samples were analyzed on the 24-capillary 3500xl Genetic Analyzer without prior denaturation of samples. Samples were injected electrokinetically for 15 s at 1.2 kV. The STR alleles were then separated at 15 kV at a run temperature of 60 °C. Data from the 3500xl was analyzed using GeneMapper ID-X software (version 1.3; Life Technologies).

2.4. Preparation of DNA samples

The concentrated DNA stock solutions prepared from the cell lines were diluted to an approximate concentration of 20–25 ng/μL (based on absorbance at 260 nm) in TE⁻⁴. The individual DNA solutions (approximately 45 mL) were placed in 100 mL beakers (polytetrafluoroethylene, VWR#89026-012) containing a polytetrafluoroethylene magnetic stir bar. The beaker and stir bar had

been pre-treated with 10% bleach for 1 h, and then rinsed several times with sterile water and finally with ethanol. The beakers and stir bars were then autoclaved (dry cycle) at 121 °C for 1 h. The DNA solutions were gently stirred for 3–4 h in a sterile laminar flow hood before dispensing. Samples (110 µL) were pipetted into sterile 0.5 mL Sarstedt polypropylene tubes (#72.730.105, VWR #10193-760). The samples were stored in the dark at 4 °C.

The molecular mass and integrity of the DNA were assessed using gel electrophoresis (Cambrex Bio Science Rockland, Inc., Rockland, ME). The gel was loaded with 2 µL of each component with loading buffer; and the FlashGel DNA Marker was used as a molecular weight indicator. The gel was electrophoresed for about 7 min at 250 V.

2.5. HER2 assay design and PCR conditions

The minimum information for publication of quantitative real-time PCR experiments (MIQE) [24] and for digital PCR experiments [25] guidelines were followed during the assay development and for the measurements of the samples. The sequences of the oligonucleotide primers for *HER2* and reference genes are shown in Table 1. Primers were obtained from Life Technologies (Carlsbad, CA) or IDT (Coralville, Iowa) and were gel purified. All oligonucleotide sequences were designed with Primer3 (<http://frodo.wi.mit.edu/>) or Primer Express (Version 3.0.1, Life Technologies) and checked for specificity using BLAST searches of NCBI Nucleotide database. PCR was performed on a Veriti Thermal cycler (Applied Biosystems, Waltham, MA) in a total reaction volume of 20 µL, containing 0.1 ng linearized *HER2* plasmid (for *HER2* primers only) or 1 ng of genomic DNA, 0.4 µmol/L each primer, 0.2 mmol/L each deoxynucleotide triphosphate, and 0.5 U of Taq DNA polymerase in 1× PCR buffer. All reagents were obtained from Roche Diagnostics (Pleasanton, CA). Non-template control samples were also run consisting of TE⁻⁴ buffer instead of the DNA samples. The following PCR cycling conditions were used: an initial denaturation step of 1 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 20 s at 60 °C, and 15 s at 72 °C, with a final extension step for 10 min at 72 °C. The product of PCR amplification was monitored by electrophoretic separation on a FlashGel system.

2.6. HER2 calibration

The plasmid pORF9-herBB2 containing the full length of *HER2* cDNA was purchased from Invivogen (San Diego, CA). The construct frame and the cDNA sequence were verified by restriction enzyme analysis and DNA sequencing. One microgram of *HER2* plasmid was linearized by 20 units of the restriction enzyme NotI (New England Biolabs, Ipswich, MA) treatment in a 50 µL reaction at 37 °C for 4 h. The linearized *HER2* plasmid was purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and was eluted in TE⁻⁴, then the DNA concentrations were quantitated by OD₂₆₀ measurements. A more sensitive Quant-iT PicoGreen dsDNA assay (Life Technologies, Grand Island, NY) was also used to quantitate the DNA samples, according to the supplier's protocol (BioTek Synergy MX, Winooski, VT). Component A of SRM 2372 (2372-A), used for calibration of these assays, was derived from a single male donor [26].

The copy number of *HER2* plasmid was calculated through the following formula:

$$\text{Copy number} = 6.022 \times 10^{23} \times \frac{[\text{Quantity}]}{\text{molecular weight}}$$

The *HER2* plasmid has 6780 bp (*HER2* cDNA size is 3768 bp) resulting a molecular weight (MW) of approximately 4,190,000 Da. One copy of the *HER2* cDNA equals 6.96×10^{-18} g of *HER2* plasmid. For genomic DNA, the assumption used was that one genome copy is approximately 3×10^9 bp, with a resultant molecular mass of

approximately 3 pg, and therefore, 1 ng of human genomic DNA would have approximately 333 copies of a single copy gene [27].

The *HER2* copy number of 2372-A was confirmed using a linearized *HER2* plasmid with the calibration curve method. A dilution curve was constructed using five serial dilutions, in triplicate, of 2372-A ranging from 0.3 ng to 30 ng (100 to 10,000 haploid genome copies) per reaction. A calibration curve using the *HER2* plasmid was constructed using five serial dilutions ranging from 0.696 fg to 6.96 pg (100 to 1,000,000 *HER2* copies) per reaction. Each dilution sample was run 4 times. Analysis was performed with SDS software v2.4 (ABI) using a manual threshold of 0.2 and an automatic baseline setting. The genomic *HER2* gene copy number of 2 per diploid genome in 2372-A was confirmed using this method.

2.7. Quantification of gene copy number using SYBR Green qPCR

Each PCR reaction has a final volume of 20 µL: 10 µL 2× SYBR Green qPCR Supermix (Life Technologies), 0.2 µL 5 µmol/L ROX, 0.4 µL 10 µmol/L primer pair for *HER2* or the reference genes, 5.4 µL water, and 4 µL DNA template or non-template control. In each 96 well plate, half of the plate contained a calibration curve using 2372-A for *HER2* (and three of the reference genes) with serial dilutions (5-fold) resulting in approximately (4, 0.8, 0.16, and 0.032) ng/µL. The other half of the plate had one of the five breast cancer cell line components. Assays for the reference genes in the components were done at approximately (4, 0.8, 0.16, and 0.032) ng/µL. Assays for the *HER2* copy number measurements of components B and D were done at concentrations of approximately (4, 0.8, 0.16, and 0.032) ng/µL. Assays for the *HER2* copy numbers of components A, C, and E, were done at (0.8, 0.16, 0.032, and 0.0064) ng/µL (due to the high *HER2* amplification). All of the samples were measured in triplicate. Fresh dilutions were prepared for the 2372-A calibrant and the *HER2* standard components each day.

Thermal cycling conditions for the ABI 7500 Real Time PCR systems are as follows: 50 °C for 2 min (UDG incubation), 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Melting curve analysis is performed from 60 °C to 95 °C. The dissociation curve of PCR products appears as a single peak, indicating the amplified genes have similar sequences (G+C content) and there was no evidence of nonspecific binding of primers. For each plate, a calibration curve was constructed for each target gene (*HER2* and three reference genes) using the logarithm of the initial copy number of the 2372-A calibrant plotted along the X-axis and their respective C_q values plotted along the Y-axis. The copy number of *HER2* and reference genes in 2372-A was calculated based on the assumption that 2372-A has a DNA concentration of 57 ng/µL (information value from the SRM 2372 certificate of analysis, <http://www.nist.gov/srm/>) and that 1 ng of 2372-A contains 333 copies of *HER2* and the reference genes. The equation and linear regression line for each target was calculated using the following formula $Cq = m \times \log(\text{copy number}) + b$.

A dilution curve similar to the calibration curve was constructed for each target of each SRM 2373 component. The efficiency of the qPCR amplification was assessed using the equation:

$$\text{efficiency} = (10^{(-1/m)} - 1) \times 100\%$$

Inhibitors in the template DNA will reduce the efficiency and increase the slope of the standard curve.

2.8. Quantification of gene copy numbers using chamber dPCR

The chamber dPCR reactions were run on a BioMark platform (Fluidigm, San Francisco, CA). The PCR reactions consisted of 1× TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies), 1× GE sample loading reagent (Fluidigm), with final

Table 2
TaqMan fluorescent probe sequences.

Probe name	Sequence	5' Label	3' Quencher
HER2-2 (BHQ)	ACCCAGCTCTTGAGGACAACTATGC	FAM	BHQ-1
EIF5B-P	TTCAGCCTTCTTCTCATGCAGTTGTACG	FAM	BHQ-1
RPS27A-P	TTTGCTACCACTTGCAAAGCTGGCCTTT	FAM	BHQ-1
DCK-P	CTTCCAAACATATGCCTGTCTCAGTCGA	FAM	BHQ-1

BHQ-1TM (Black hole quencher) and FAM labeled probes were obtained from Biosearch Technologies (Novato, CA).

concentrations of 900 nmol/L primer and 250 nmol/L probe. The concentration of DNA molecules in the amplification reaction mix was adjusted so that there were 200–500 positive chambers in a 770 chamber/panel. The sequence information of primers and probes are shown in Tables 1 and 2. Approximately 20 ng of genomic DNA was applied in each 20 μ L reaction except for *HER2* target in SRM 2373 component A, C and E, where 4 ng of genomic DNA was applied (due to high *HER2* amplification). Then 4 μ L reaction mixtures were loaded to each well on the array.

The PCR reactions were loaded into a 48.770 Digital Array (36,960 reactions per run), a 37K quantitative dPCR integrated fluidic circuits (IFCs) chip which divides each sample well into 770 chambers. The fluorescent signal for each reaction chamber was read after every cycle of amplification. Thermal cycling was as follows: 95 °C for 10 min, and 60 cycles of 94 °C for 30 s and 60 °C for 1 min, with fluorescence measured at the end of the 60 °C step.

The number of positive chambers containing >1 DNA target molecule were used to estimate the total number of targets per reaction using Poisson statistics. The Fluidigm Digital PCR Analysis Tool was used to determine the count of positive chambers in each panel of each 37K chip. The *HER2* and three reference genes nominal concentrations were calculated for each component using the equation (Copies/ μ L):

$$[\text{DNA}] = \frac{-\ln(1 - (\text{Number positive chambers}/770))}{0.85 \times 10^{-3} \mu\text{L}},$$

where 0.85 nL is the nominal chamber volume stated by the manufacturer. Two samples were measured using 3–6 replicates of each target gene for each component. Four 48.770 arrays were used over four days. The copy number ratios (*HER2* divided by the selected reference gene) were then calculated.

2.9. Quantification of gene copy number using droplet dPCR

The Bio-Rad QX100 droplet digital PCR system which consists of two components: the QX100 droplet generator and the QX100 droplet reader were used. The droplet generator utilizes microfluidics to partition samples randomly into approximately 20,000 nanoliter-sized droplets. The TaqMan PCR reaction mixture consists of 1 \times Droplet dPCR Supermix for probes (Bio-Rad, Hercules, CA), 900 nmol/L primers, and 250 nmol/L probe (final concentrations) and genomic DNA template or non-template control in a total volume of 25 μ L. The copy number of *HER2*, *EIF5B*, *DCK*, and *RPS27A* was determined in the five components (primers and probes in Tables 1 and 2). Approximately 20 ng of genomic DNA was applied in each reaction except for *HER2* target in SRM 2373 component A, C and E, where 4 ng of genomic DNA was applied.

Twenty microliters out of the 25 μ L droplet dPCR reaction mixture was transferred to the droplet generator DG8 cartridge (Bio-Rad). Droplet generation oil (70 μ L, Bio-Rad) was added into the oil well for each channel. The wells were covered with a droplet generator DG8 gasket, and the cartridge was placed into the droplet generator (Bio-Rad). After the droplet generation was complete, the droplets were then carefully transferred to a 96-well PCR plate using an 8-channel pipette. The plate was covered with foil using a heat sealer (Eppendorf, Hamburg, Germany) and then was placed

on an Applied Biosystems Veriti 96-well thermal cycler. The following thermal cycling conditions were used: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 1 min, then 98 °C for 10 min, temperature ramp rate at 50% (3 °C/s). After PCR, the 96-well PCR plate was loaded onto the QX100 droplet digital reader (Bio-Rad), which automatically reads the droplets from each well of the plate. PCR-positive and PCR-negative droplets are counted to provide nominal quantification of target DNA using the equation (Copies/ μ L):

$$[\text{DNA}] = \frac{-\ln(1 - (\text{Number positive droplets}/\text{Number of counted droplets}))}{(0.91 \times 10^{-3} \mu\text{L})}$$

where 0.91 nL is the nominal droplet volume specified by the manufacturer. As described below 10 samples were measured in triplicate for the *HER2* and reference genes. The stock concentration was obtained by multiplying the dilution factor by the measured concentration.

2.10. Homogeneity and stability measurements

To determine homogeneity, ten sets of the five components (total of 50 vials) were selected for qPCR and dPCR measurements (done as described above using two different ABI 7500 qPCR instruments and a Bio-Rad QX100 dPCR instrument by two operators). The vial numbers (order of filling) selected were: 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300.

To further evaluate the effects of different operators and qPCR measurement platforms, two sets of the five components (vial numbers 213 and 245) were randomly selected and evaluated using ABI 7900 and ABI ViiA7 instruments (both in a 96-well format) according to the above protocol except for using SYBRGreen PCR Master Mix (Catalog# 4309155, Life Technologies). These samples were analyzed by a total of five different operators using four qPCR instruments at the NIST laboratories.

Another single independent component set was analyzed using a ViiA7 (384-well format) instrument. For this instrument the copy number was determined using a TaqMan *HER2* copy number assay (Assay ID: Hs00783404_cn, Life Technologies) for *HER2* gene, and using TaqMan Copy Number Reference Assay of *RNase P* (Catalog#: 4403326, Life Technologies) and *RPS27A* (Assay ID: Hs03422139_cn, Life Technologies) as reference genes. Data from these experiments were utilized in the combined *HER2* ratio and uncertainty analysis, described below.

Stability measurements were done using the master stock sample used to prepare the standards stored at 4 °C in the dark for different periods of time (up to 856 days) using the droplet dPCR method. Digital PCR measurements of *HER2* and the reference gene, *RPS27A*, were done in triplicate on the master stock sample to determine any trends in the values with time within the uncertainty of the measurements.

2.11. Analysis of qPCR and dPCR data for a combined *HER2* ratio and uncertainty analysis

This section provides an overview of the modeling methods that were used to produce certified values for the *HER2* copy number

ratios and to determine the uncertainties in SRM 2373 ratio values. A more detailed description of the analysis and model can be found in the Supplemental Materials section. SRM 2373 contains certified values for the geometric average copy number ratio between *HER2* and suitable reference genes. Let $\text{Ratio}_{gs} = \text{HER2}_s / \text{Ref}_{gs}$ where HER2_s is the copy number of *HER2* in component s and Ref_{gs} is the copy number of reference gene g in component s . The ratios, rather than the *HER2* copy number itself, were certified due to uncertainty in the true DNA copy number of the calibrator used for the qPCR measurements and the true chamber and droplet volumes. These issues cancel out when taking the ratio between copy numbers of two genes from the same sample.

The qPCR instrument measures the fluorescence intensity of the reaction to detect the cycle number when the intensity reaches the threshold value. This value will depend upon not only the number of target DNA copies initially added to the reaction but also other factors specific to the particular PCR reaction, including the sequence of the target amplicon, the primer sequences, binding efficiencies, the polymerase used, and the SYBR Green dye binding. In order to compensate for these differences in the PCR reactions of *HER2* and the reference genes we chose to use 2372-A, purified from the white blood cells of from a single male donor, as the human genomic DNA calibrator. Other genomic DNA samples that have normal copy numbers of *HER2* and the selected reference genes could also be used as a calibrator. To normalize for the differences in the fluorescence signal development of the *HER2* and the reference genes PCR reactions, a calibration curve for 2372-A was always run on the same plate as the SRM 2373 samples. The copy number ratio between *HER2* and reference gene g in sample s from plate p and initial gene copy number, is given by:

$$\text{Ratio}_{gsp} = \frac{R_{\text{HER2},sp} / R_{gsp}}{R_{\text{HER2},2372,p} / R_{g,2372,p}},$$

where $R_{\text{HER2},2372,p}$ and $R_{g,2372,p}$ correspond to the initial abundance of *HER2* and reference gene g respectively, in a sample of 2372-A on plate p . This step corrects for the differences in the development of fluorescence signal between the different PCR reactions for *HER2* and the reference genes.

The qPCR data as reported as pairs of dilution factor and corresponding Cq values resulting from a single dilution experiment, applied to a single targeted gene in a single biological sample between *HER2* and a given reference gene in a single replicate of a given component. A linear regression model is fit to these data and used to estimate the abundance of gene g in sample s from plate p , R_{gsp} . Uncertainty and bias in slope estimates derived from qPCR data for a single gene in a single sample frequently cause the ordinary least squares analysis to perform poorly [28]. This may be due to variability in the pipetting process to create the dilution series. To estimate and account for the variability around the desired dilutions, all qPCR data (comprised of 6423 pairs of dilution fractions and corresponding Cq values from 556 unique combinations of target gene and dilution series) were combined into a single Bayesian analysis, which was evaluated via Markov Chain Monte Carlo (MCMC) (Supplementary Materials Section).

For dPCR experiments, the abundance of gene g in sample s from plate p is estimated from the proportion of chambers or droplets in which no copies were detected. Suppose that out of N_{gsp} total droplets/chambers, there were Y_{gsp} droplets/chambers within which no copies of gene g were detected. The abundance of gene g in sample s from plate p is estimated by modeling Y_{gsp} as following a binomial distribution with N_{gsp} observations and probability $e^{-\lambda_{gsp}}$ where λ_{gsp} is the average number of copies of gene g per droplet/chamber in sample s on plate p . The relationship between the average number of copies per droplet/chamber and the probability of not observing any copies in a given droplet comes from

assuming that gene copies within droplets are distributed according to the Poisson distribution, for which

$$\text{Pr}(\text{no copies in droplets}) = e^{-\text{average \# of copies per droplet}}$$

The copy number ratio between *HER2* and reference gene g in sample s from plate p when evaluated using digital PCR, is then given by $\text{Ratio}_{gsp} = \lambda_{\text{HER2},sp} / \lambda_{gsp}$.

The geometric average copy number ratio for each reference gene in each component was evaluated from the collection of copy number ratios for individual replicates using a mixed effects model that included random effects for combination of operator and instrument, date, bottle and plate (*i.e.* a catch-all for random errors other than those previously stated). To generalize the results across a broader population of reference genes from which the chosen reference genes were considered a representative sample (*i.e.* those with similar copy number stability across biological samples), the log copy number ratio for each reference gene in a given sample was modeled as a random draw from a normal distribution with unknown mean $\mu = \log(\text{Ratio}_s)$ and unknown standard deviation. The posterior credible interval for Ratio_s is used to establish the reported uncertainty for the certified copy number ratio in sample s on the certificate of SRM 2373. The relative effect of each of these variables is shown as the percentage of the largest source of variability that was the plate (catch all for the experimental setup of a microplate) (Fig. 4).

3. Results and discussion

3.1. Breast cancer cell line and preparation of the standard

Cell lines are a renewable source of materials for developing reference materials, but the products need to be extensively characterized for each lot of materials. We selected five breast cancer cell lines with different *HER2* copy number to make the reference materials. The breast cancer cell lines used for these samples are frequently used in research studies. These cancer cell lines and many others, because of their importance to basic research and therapeutic screening, are now the subject of sequencing projects, such as the Cancer Cell line Encyclopedia (<http://www.broadinstitute.org/ccle/home>) and the Catalogue of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cell_lines).

The identities of the cell lines were confirmed using STR genotyping before and after scale-up of the production of the standard. The STR profiles from the genotyping were the same for the DNA samples prepared before and after scale-up (Supplemental Table 1). The STR profiles matched the nine loci STR profile provided by ATCC.

The cell lines were harvested for DNA extraction after no more than eight passages of the initial cell stocks. The ratio between absorbance at 260 nm and 280 nm ($\text{OD}_{260}:\text{OD}_{280}$) provides an estimate of the purity of the nucleic acid. A ratio between absorbance at 260 nm and 230 nm ($\text{OD}_{260}:\text{OD}_{230}$) that is lower than 2 can be due to contamination by phenol, thiocyanates, and other organic compounds, whereas absorbance at 330 nm (OD_{330}) is usually caused by light scattering and indicates the presence of particulate matter. The results shown in Supplemental Table 2 indicate the high quality of the DNA samples. Gel electrophoresis did not show any indication of low molecular weight contaminants (Supplemental Fig. 1).

The packaging, storage conditions, and characterization of SRM 2373 were greatly aided by the experience gained from development of SRM 2372 [26]. The sample volume of 110 μL (approximately 2 μg of DNA) per tube provides enough material for *HER2* copy number assays, including next-generation sequencing. The samples are stored at 4 °C, protected from light.

3.2. qPCR assays and copy number measurements

The MIQE guidelines were followed to ensure the reliable reporting of the assay conditions [24]. In addition to reporting the relevant information about the assay design and conditions, the qPCR assays must be checked for specificity and efficiency [29]. Specificity was determined by a single PCR amplicon being produced. BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were done to ensure that the primers and probes for the dPCR assays only bind to the target sequences in the human genome. Running the PCR products on a gel to ensure a single band and melting curve analysis of the PCR products after SYBR Green PCR amplification were also done to confirm the assay specificity.

2372-A, genomic DNA from a single male donor, was used as the calibrant for the qPCR measurements and to confirm the dPCR assays. For each qPCR measurement on a 96 well plate a calibration curve was done using SRM 2372 and a dilution curve was made using the SRM 2373 components.

SYBR Green single-analyte assays were used for the qPCR reactions because of the higher precision and lower coefficient of variation compared to TaqMan and probe hybridization assays [30]. The *HER2* gene has a total length of 40,523 bp. As shown in Supplemental Table 3, five primer pairs were designed to span the *HER2* gene. The amplicon for HER2-1 primer set is located in intron 4, and amplicons of HER2-2, HER2-3, and HER2-5 primer sets are located in exons 7, 13, and 21, respectively, while the amplicon for HER2-4 primer set spans the regions of exon 14 and exon 15. PCR was performed using either 2372-A or *HER2* cDNA plasmid as templates. The results of the gel electrophoresis of the PCR products are shown Supplemental Fig. 2. The PCR product bands using genomic DNA gave the expected amplicon sizes. When using the *HER2* cDNA plasmid as a template (panel B of Supplemental Fig. 2), no amplicon was detected from HER2-1 primers, because there are no introns present in the *HER2* cDNA plasmid and the amplicon from primer set HER2-4 produced the expected shorter PCR fragment due to the elimination of the intron region between exon 14 and exon 15 in the *HER2* cDNA plasmid.

The SYBR Green based qPCR assays were done using the five *HER2* primer pairs. The amplification efficiency of the qPCR reactions were calculated based on the slopes of the calibration curves and the primer specificities were determined by the dissociation curves and gel electrophoresis. All five *HER2* primer pairs had satisfactory amplification efficiencies (within the range of 90–110%) and primer specificity indicated a single product using gel electrophoresis and melting curve analysis. All of the assays had high efficiencies and specificities, however it was decided to use HER2-2 assay for the extensive measurements of the *HER2* copy number in the components of SRM2373.

A linearized *HER2* plasmid was used to confirm the copy number of *HER2* in 2372-A. The linearized plasmid concentration was determined by spectrophotometric analysis and PicoGreen assays. The results from absorbance and PicoGreen assays differed by approximately 2.5% (results not shown). The *HER2* plasmid was used to construct the calibration curve for the qPCR assays. The relationship of the 2372-A copy number and *HER2* plasmid copy number had a correlation coefficient of 0.9924. This data confirmed that 2372-A has a single *HER2* copy number per haploid genome and is a suitable calibrant for the *HER2* gene copy measurements. The *HER2* and reference gene assays were run using the same sample dilutions and on the same plate.

3.3. Digital PCR measurements

Three TaqMan assays for the reference genes, *EIF5B*, *RPS27A*, and *DCK* were developed. The gene locations and primer sequences for

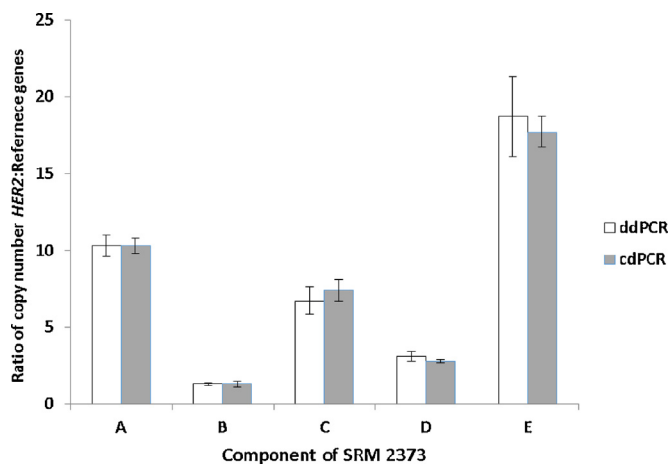


Fig. 1. A comparison of the average ratios of *HER2* copies with respect to three reference genes for droplet dPCR and chamber dPCR for each component of SRM 2373. Error bars are 1 standard deviation, where $N = 10$ samples for the droplet dPCR (ddPCR) and $N = 3$ samples for the chamber dPCR (cdPCR).

the reference genes are shown in Table 1 and the probes developed are shown in Table 2 (used for the dPCR measurements).

Measurements using qPCR have been shown to be able to detect gene copy number differences of 1.5 fold [31]. However, qPCR has limitations such as PCR efficiency, which will change the Cq values and the calculated fold differences [32]. dPCR can be considered an end-point assay where each reaction is either positive or negative and therefore differences in amplification efficiencies are not as critical as in qPCR measurements.

The calculations for DNA copies were evaluated using Poisson sampling statistics with the assumption that the DNA molecules were partitioning independently from each other into the individual droplets or chambers. To calculate the concentration of DNA, the sizes or the numbers of droplets or chambers must be accurately known. The uncertainty of the volume measurements of the droplets generated in the Bio-Rad instrument has not yet been fully investigated. The National Measurement Institute (Australia) has measured the volume of droplets [33] and NIST is currently characterizing the volumes formed under different conditions. A comparison of ratios of *HER2* to the reference genes for the droplet dPCR and the chamber dPCR instruments is shown in Fig. 1. The two instruments yielded very similar *HER2* gene ratio values for the five components.

3.4. Agreement of qPCR and dPCR measurements

The results of the different reference genes for the copy number (copies/ μL) for the different components determined using qPCR and dPCR are shown in Fig. 2 (data in Supplemental Tables 3 and 4). The results show that the reference genes are consistent for each of the components and also consistent between the two measurement methods. The assays for *HER2* and the reference genes were validated for each component. Different reference genes were used for the qPCR assays and dPCR measurements, and they gave similar results (Fig. 2). The agreement between results of the two methods is encouraging, given that the two methods use different assumptions for the final calculation of the copy number.

The digital PCR calculation requires the assumption of the independent partitioning of DNA fragments and also the accurate determination of the volume of the droplets (or chambers). The five components of SRM 2373 did not show an increase in *HER2* copy number after digestion of the DNA with the restriction enzymes *MseI* or *RsaI* (results not shown), indicating that the *HER2* gene

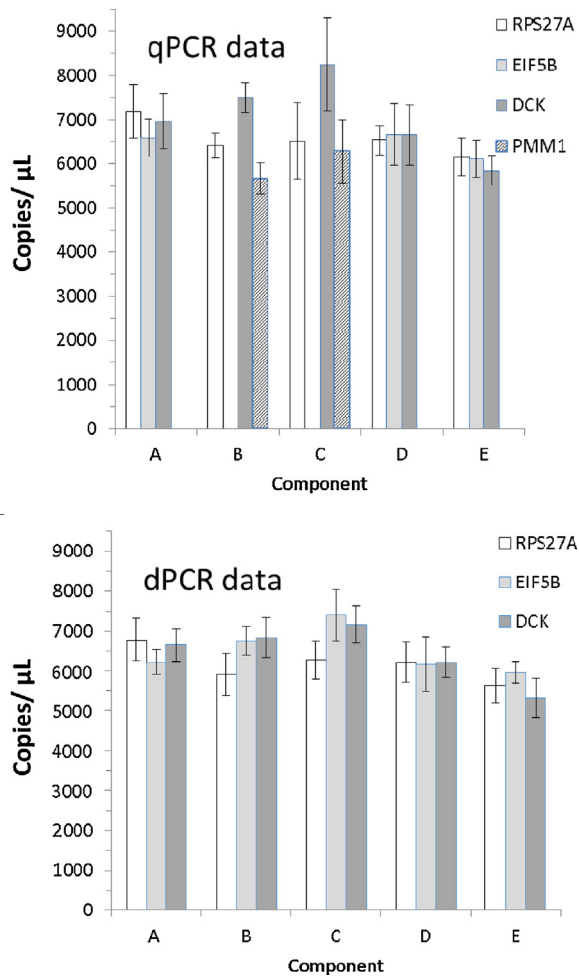


Fig. 2. Comparison of copy number of the reference genes using qPCR (top) and ddPCR (bottom) and the indicated reference genes (gene copy concentration in SRM 2373 components). Error bars are 1 standard deviation, where $N=10$ independent samples for the qPCR and ddPCR measurements.

fragments were partitioning independently of each other into the droplets.

3.5. Calculation of the HER2 to reference gene copy number ratios

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 accounted for by NIST [34]. The certified ratios for *HER2* to the reference genes based on all of the data along with the calculated 95% confidence interval are listed in Table 3. The prediction intervals are the approximate range of values that NIST would reasonably expect upon the next single, independent measurement of the *HER2* copy number ratio approximately 68% of the time, based on our measurements.

3.6. Homogeneity and stability studies

The analysis of ten sets of samples (out of approximately 350) distributed throughout the dispensing order were analyzed. There were no significant trends in the *HER2* copy number values that were detectable within the detection limits of the assays. The *HER2* copy number or the ratio of *HER2* to the reference gene *RPS27A* 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) (Fig. 3).

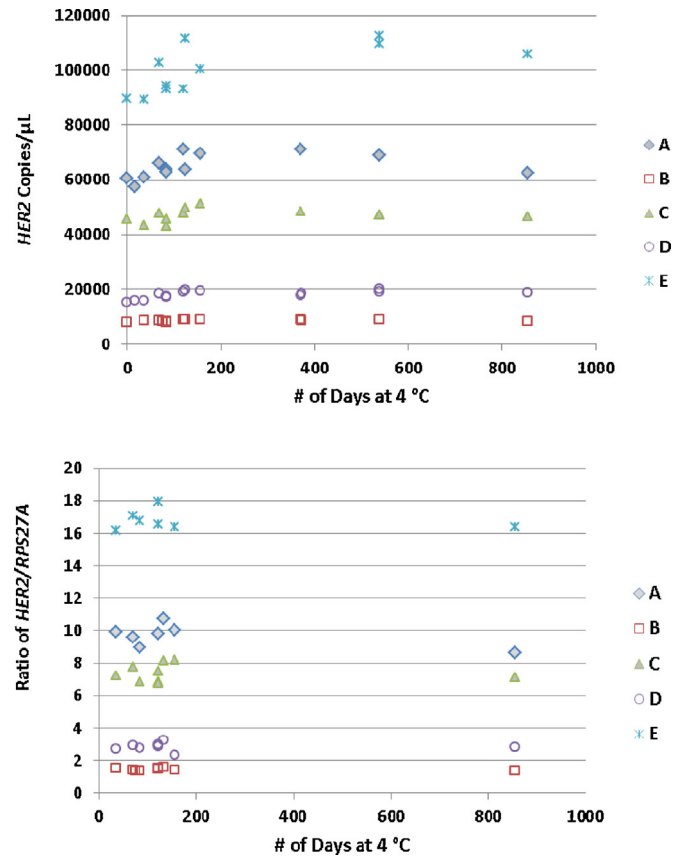


Fig. 3. Stability studies of SRM 2373. Copy number of the *HER2* copy number (top, *HER2* copy concentration in SRM 2373 components) and ratios of the *HER2* copy number to *RPS27A* reference gene (bottom). Individual samples from the master stock sample used to prepare the standards stored using the same conditions as the samples, measurements in triplicate.

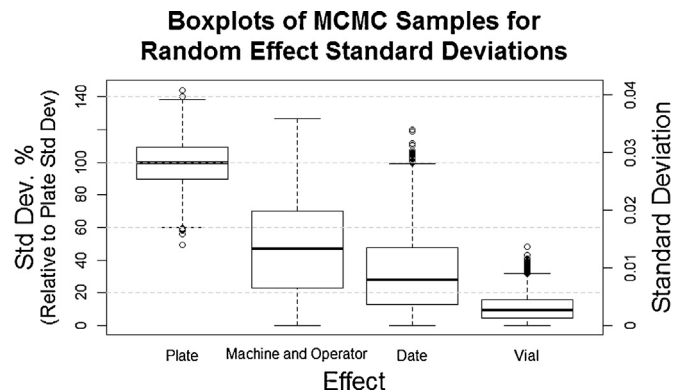


Fig. 4. The results from the random effects model were broken down into the plate (microplate experimental setup), the machine (instruments) and operator, the date (of analysis), and the vial (sample). In the boxplot for each effect, the bold horizontal line depicts the median of the MCMC samples, the horizontal lines at the top and bottom of the box depict the 75% and 25% quantiles, respectively; and the whiskers and individual circles characterize the tails of the MCMC sampling distribution. The combined data from the effects data from qPCR (using five instruments and five operators) and ddPCR (using two instruments and two operators) were analyzed using the model detailed in Supplemental materials.

3.7. Effect of variables on the measurement uncertainty

The results from the random effects model were broken down into the plate (catch all microplate experimental setup), the machine (instruments) and operator, the date (of analysis), and the vial (sample) (Fig. 4). This analysis suggests that the largest compo-

Table 3The certified values, ratios of *HER2* copies to the reference genes, 95% uncertainty interval and 68% prediction interval.

Component	Cell line	Ratio	95% uncertainty interval	68% prediction interval
A	SK-BR-3	9.7	8.7–10.7	5.8–20.8
B	MDA-MB-231	1.3	1.1–1.5	0.6–1.9
C	MDA-MB-361	6.4	5.7–7.1	4.8–14.2
D	MDA-MB-453	2.9	2.6–3.2	1.4–7.2
E	BT-474	17.7	15.9–19.5	11.7–45.3

The uncertainty intervals characterize the uncertainty in the long-run averages of many repeated measurements. The 68% prediction interval is used for single measurements. The distribution of individual observations is expected to exhibit substantially greater variability, as evidenced by the much broader and lower coverage (68% vs 95%) prediction intervals.

ment of variability was in the plate category, which is the catch-all random error associated with setting up a microplate between different measurements. The variability attributed to the combination of instrument and operator, the date or the vial were moderate. The vertical and horizontal lines in Fig. 4 display the 95% posterior credible intervals and posterior expectations, respectively, for the standard deviations of these random effects from the combined analysis of the data from qPCR (using 5 instruments) and dPCR (using two instruments).

Disclaimer

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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bdq.2016.02.001>.

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