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Research paper

Development of NIST standard reference material 2373: Genomic DNA standards for *HER2* measurements

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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.

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	CTCATCGCTCACAACCAAGT	112 bp	Exon 7 (chr17:37864601–37864620)
HER2-2R	GGTCTCCATTGTCTAGCACG		(chr17:37864693–37864712)
EIF5B-F	GGCCGATAAATTTTTGGAAATG	112 bp	Intron 1 (chr2:99974140–99974161)
EIF5B-R	GGAGTATCCCCAAAGGCATCT		(chr2:99974231–99974251)
RPS27A-F	CGGGTTTGGGTTCAGGTCTT	97 bp	Intron 4 (chr2:55462316–55462335)
RPS27A-R	TGCTACAATGAAAACATTCAGAAGTCT		(chr2:55462386–55462412)
DCK-F	CTCAGAAAAATGGTGGGAATGTT	122 bp	Exon 3 (chr4:71888097–71888119)
DCK-R	GCCATTCAGAGAGGCAAGCT		(chr4:71888199–71888218)
PMM1-F	AGGTCTGGTGGCTTCTCCAAT	78 bp	Intron 7 (chr22:41973739–41973759)
PMM1-R	CCCCTAAGAGGTCTGTTGTGTTG		(chr22:41973682–41973704)

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 CO_2. 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% CO_2, 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% CO_2, 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 Identifiler 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 Cycler (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