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TECHNICAL ADVANCE

Reference Size Matching, Whole-Genome Amplification, and Fluorescent Labeling as a Method for Chromosomal Microarray Analysis of Clinically Actionable Copy Number Alterations in Formalin-Fixed, Paraffin-Embedded Tumor Tissue

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Cancer genome copy number alterations (CNAs) assist clinicians in selecting targeted therapeutics. Solid tumor CNAs are most commonly evaluated in formalin-fixed, paraffin-embedded (FFPE) tissue by fluorescence *in situ* hybridization. Although fluorescence *in situ* hybridization is a sensitive and specific assay for interrogating preselected genomic regions, it provides no information about coexisting clinically significant copy number changes. Chromosomal microarray analysis is an alternative DNA-based method for interrogating genome-wide CNAs in solid tumors. However, DNA extracted from FFPE tumor tissue produces an essential, yet problematic, sample type. The College of American Pathologists/American Society of Clinical Oncology guidelines for optimal tumor tissue handling, published in 2007 for breast cancer and in 2016 for gastroesophageal adenocarcinomas, are lacking for other solid tumors. Thus, cold ischemia times are seldom monitored in non—breast cancer and non—gastroesophageal adenocarcinomas, and all tumor biospecimens are affected by chemical fixation. Although intended to preserve specimens for long-term storage, formalin fixation causes loss of genetic information through DNA damage. Herein, we describe a reference size matching, whole-genome amplification, and fluorescent labeling method for FFPE-derived DNA designed to improve chromosomal microarray results from suboptimal nucleic acids and salvage highly degraded samples. With this technological advance, whole-genome copy number analysis of tumor DNA can be reliably performed in the clinical laboratory for a wide variety of tissue conditions and tumor types. *(J Mol Diagn 2018, 20: 279—288; https://doi.org/10.1016/j.jmoldx.2018.01.004)*

Clinically actionable copy number alterations (CNAs) in the form of amplifications and deletions affect a larger percentage of the tumor genome than other somatic DNA changes. Evaluation of CNAs has thus advanced understanding of tumor biology and the development of targeted cancer therapeutics.[1] Detection of significantly focal amplifications and deletions within an individual patient's tumor genome provides information about activated oncogenes, inactivated tumor suppressor genes, and potential therapeutic targets. Since fluorescence *in situ* hybridization (FISH) was introduced into the clinical laboratory almost two decades ago, it has been the assay of choice for detecting CNAs in solid tumor genomes.[2]

HER2 (*ERBB2*) was the first solid tumor oncogene to be clinically evaluated by FISH as a positive biomarker for predicting response to targeted therapies in breast cancer.[3] Given the importance of breast tumor biospecimens for determining anti-HER2 therapy eligibility, the College of

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American Pathologists/American Society of Clinical Oncology (CAP/ASCO) introduced optimal tissue handling guidelines for HER2 testing, specifying that cold ischemia time should be as short as possible (ideally <60 minutes) until the tissue is fixed in 10% neutral-buffered formalin for 6 to 72 hours.[4] Because approximately 20% of gastric and esophageal adenocarcinomas (GEAs) are *HER2* amplified and eligible for HER2-targeted therapy, similar CAP/ASCO guidelines for GEA tumors were published in 2016.[5–7] Additional *HER2* gene-amplified tumor types include salivary duct (>25%), lung (approximately 15%), ovarian (approximately 15%), bladder (approximately 10%), and pancreatic (approximately 2%).[8–12] However in non-breast, non-GEA tumors, cold ischemia time monitoring and HER2 testing are rarely performed. When *HER2* gene testing is performed and found to be negative, the gene-specific test results provide no information about other potential molecular targets in the tumor genome. Thus, a reliable DNA-based assay for whole-genome copy number analysis, designed for a variety of tissue conditions and tumor types, is an unmet clinical need.

All tumor DNA specimens, regardless of adherence to CAP/ASCO guidelines, are affected by the formalin fixation process, which causes genetic information to be lost through DNA/protein cross linking and generation of apurinic sites. This is particularly true after prolonged cold ischemia times, and further compounding the problem is the long-term storage of blocks before DNA extraction, which contributes to continued nucleic acid degradation.[13] Nucleic acid degradation affects not only the accurate reporting of *HER2* gene status by FISH in breast/GEA tumors, but also the quality of DNA available for genome-wide copy number assessment.[14] Failure of quality control (QC) metrics, because of suboptimal nucleic acid quality, frequently results in tumor samples being considered ineligible for molecular analysis. This analysis may detect clinically actionable therapeutic CNA targets, such as gene amplifications of *RET*, *MET*, *EGFR*, *PDGFRA*, and *KIT* and deletions of *PTEN*, *CDKN2A*, *BRCA1*, and *BRCA2*.

Chromosomal microarray (CMA) technology was developed to allow genome-wide assessment of CNAs from a single germline or tumor sample. First described in 2009 as a DNA-based cytogenetic alternative for evaluating breast cancer with equivocal *HER2*/cep17 ratios by FISH, the test has the added advantage of demonstrating gene copy number within a high-resolution map of a gene's chromosomal locus.[15] Despite the enhanced capabilities of microarrays for scanning the tumor genome, introduction into the clinical arena has been limited by aforementioned preanalytic variables associated with formalin-fixed, paraffin-embedded (FFPE) tissue. Herein, we describe an advanced CMA technical method designed to yield high-quality material for microarray analysis, allowing for consistent DNA labeling and data retrieval from FFPE samples of varying quality. The assay is built on a DNA fragmentation simulation method (FSM) that allows reference size matching, as described by Craig et al.[16] Our study extends the FSM method to a variety of solid tumor types and interrogates a panel of clinically actionable genes, thereby establishing the clinical relevance of the CMA assay. In the extended FSM assay, whole-genome amplification (WGA) is performed with amine modification for efficient incorporation of amine reactive fluorescent dyes into DNA, starting with as little as 75 ng of DNA. The assay reliably detects copy number alterations within a simulated whole chromosome view down to a dilution of 25% tumor DNA with high sensitivity and specificity from FFPE samples. Multiple QC steps (including derivative log ratio spread, signal intensity, background noise, and signal/noise ratio) are evaluated as part of the testing workflow to ensure consistently high-quality microarray results from FFPE tissue. The ability to robustly determine gene copy number across samples of varying DNA quality and quantity makes high-resolution CMA an important addition to the pathologist's toolbox for accurate determination of CNA status in a variety of solid tumors.

Materials and Methods

CNA Specimens

For development of the assay, a total of 45 FFPE tissue and cell line samples were used (Supplemental Table S1). The tissue FFPE samples were composed of a Characterized group ($n = 12$) consisting of FFPE breast and gastric cancer tissue samples previously characterized by Bio-Options (Brea, CA). The Discovery group of FFPE tissue specimens ($n = 23$) represented a variety of solid tumor types of varying block ages, including microsatellite instability positive and negative colorectal, breast, ovarian, thyroid, prostate, salivary gland, esophageal, and bladder. In addition, cell lines ($n = 10$) with previously determined DNA copy number results for cancer-related genes, including *MET*, *MYC*, *ERBB2*, and *PTEN*, were used to validate selected regions of interest across the array.[17–22] Cell lines used were as follows: GM12878 (normal control),[17] SKBR3 (*ERBB2* amp),[18] EBC1 (*MET* amp),[19] MKN-45 (*MET* amp),[20] K562 (*CDKN2A* del),[21] and PC3/CRL-1435 (*PTEN* del).[22] Cell lines were fixed in 10% neutral-buffered formalin for 6 to 72 hours and embedded in paraffin, consistent with CAP/ASCO guidelines for HER2 testing in breast and GEA cancers. A subset of the FFPE cell lines was mixed with normal DNA at concentrations of 100%, 50%, 25%, and 10% to assess array CMA performance in heterogeneous samples. All 12 Characterized samples were purchased from a commercial source. The 23 FFPE Discovery samples, representing other solid tumor types, were deidentified excess tissue obtained from a private pathology laboratory. Institutional review board/ethics review board approval (Integreview IRB, Austin, TX) was obtained for the protocol, including specific waiver of the consent requirement.

Table 1 Cancer-Related Genes Included on the Custom DNA Microarray

Gene	Chromosome location	Gene	Chromosome location	Gene	Chromosome location
MTOR	1p36.22	*BRAF*	7q34	*ERBB3*	12q13.2
NRAS	1p13.2	*SHH*	7q36.3	*CDK4*	12q14.1
NOTCH2	1p12-p11.2	*FGFR1*	8p11.23-p11.22	*MDM2*	12q15
MDM4	1q32.1	*MYC*	8q24.21	*BRCA2*	13q13.1
AKT3	1q43-q44	*JAK2*	9p24.1	*RB1*	13q14.2
MYCN	2p24.3	*CDKN2A*	9p21.3	*NFKBIA*	14q13.2
ALK	2p23.2-p23.1	*CDKN2B*	9p21.3	*AKT1*	14q32.33
ERBB4	2q34	*GNAQ*	9q21.2	*CDH1*	16q22.1
VHL	3p25.3	*NOTCH1*	9q34.3	*TP53*	17p13.1
PIK3CA	3q26.32	*RET*	10q11.21	*AURKB*	17p13.1
FGFR3	4p16.3	*PTEN*	10q23.31	*MAP2K4*	17p12
PDGFRA	4q12	*FGFR2*	10q26.13	*NF1*	17q11.2
KIT	4q12	*MGMT*	10q26.3	*ERBB2*	17q12
RICTOR	5p13.1	*HRAS*	11p15.5	*RARA*	17q21.2
FGF10	5p12	*WT1*	11p13	*TOP2A*	17q21.2
APC	5q22.2	*CD44*	11p13	*BRCA1*	17q21.31
FGF1	5q31.3	*CCND1*	11q13.3	*NOTCH3*	19p13.12
PDGFRB	5q32	*FGF19*	11q13.3	*CCNE1*	19q12
FGFR4	5q35.2	*FGF4*	11q13.3	*AKT2*	19q13.2
CCND3	6p21.1	*FGF3*	11q13.3	*TOP1*	20q12
EGFR	7p11.2	*ATM*	11q22.3	*AURKA*	20q13.2
CDK6	7q21.2	*CCND2*	12p13.32	*NF2*	22q12.2
MET	7q31.2	*KRAS*	12p12.1	*AR*	Xq12
SMO	7q32.1	*CDK2*	12q13.2		

Design of a Custom Oligonucleotide Array

The custom-designed 8× 60K array (Agilent Technologies Inc., Santa Clara, CA) used for the CMA assays includes dense probe coverage across 71 cancer genes in addition to coverage of all chromosome arms, subtelomeric regions, and pericentromeric regions (Table 1).[6–25] The array contains a total of 61,609 probes, of which 3547 represent the 71 cancer-related genes with an average resolution of 6.2 kb in targeted regions. In addition, the array design supports high-resolution *HER2* gene testing with high-density coverage of chromosome 17 and the *HER2* amplicon at 17q12 (Table 2).

Sample Preparation for High-Resolution HER2 Gene Testing

Twelve commercial samples (BioOptions, Brea, CA) were used for development of a high-resolution *HER2* gene testing application for breast and gastric cancer. These previously IHC/FISH-characterized FFPE samples represented the following: eight tumors determined to be highly HER2 positive, two tumors with equivocal HER2 results by FISH (four to six HER2 gene copies), and two HER2-negative tumors. HER2 testing by IHC was performed in our laboratory for the 12 FFPE tissue samples and analyzed by a staff pathologist (S.R.G.). Invasive tumor areas with positive HER2 protein staining (hot spots) were circled, and the percentage IHC positivity was recorded for each sample. Tumors with no detectable IHC staining and those with HER2-positive staining in >70% of the sample were labeled as scrape all, indicating all tissue on the slide should be used for DNA extraction.

DNA Extraction and Quantitation

After macrodissection and deparaffinization, nucleic acid extraction was performed for all FFPE samples, including formalin-fixed cell lines using the QIAmp DNA FFPE Tissue Kit (Qiagen Inc., Valencia, CA), following the manufacturer's instructions, including a heat-treatment step to disrupt protein-DNA cross-links. Extraction was performed manually or on the QIAcube (Qiagen Inc.). Verification of equivalency for the two extraction methods (manual versus automated) was previously performed in our laboratory. Samples were eluted in nuclease-free water. Water was used instead of the manufacturer-supplied buffer, which contains EDTA to avoid chelation of Mg^{2+}, which could compromise enzymes during whole-genome amplification. A minimum of 75 to 150 ng of tumor (test) DNA and 75 to 150 ng of

Table 2 Design of ResearchDx/PacificDx Custom Array

Custom CGH array	Probes, *n*	Average resolution, kb
Overall	61,609	52.7
Target region (71 genes)	3547	6.2
Overall backbone	51,154	56.5
Chromosome 17	4830	16.7

CGH, comparative genomic hybridization.

Table 3 Sonication Conditions for Episonic 2000 System

Criterion	Condition
Sonication duration	2 minutes 15 seconds
Pulse on/pulse off	45 seconds/15 seconds
Amplitude	25%–31% maintained at 110–120 W
DNA amount	150 ng at a concentration of 10 ng/μL
Sample tube type	0.2-mL PCR

sex-matched (Human Genomic DNA Female and Human Genomic DNA Male) reference DNA (Promega, Madison, WI) were required for each assay. The 260/280 ratio was measured on a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA), with acceptable range of 1.6 to 2.0, and the Accublue dsDNA Quantitation Assay (Biotium, Fremont CA) was used to determine the double-stranded DNA concentration of the test and reference DNA samples.

Sonication of DNA and Reference Size Matching

Sample and reference specimens were fragmented in an Episonic 2000 sonicator (Epigentek, Farmingdale, NY) under conditions detailed in Table 3. Fragmentation profiles were generated for each sample and reference pair using the High Sensitivity DNA assay (Agilent Technologies Inc.). Ideal libraries were predicted to have an average bp size of 500 ± 100 bp, with the peak of each sonicated library tapering off at 2000 bp. For undersonicated specimens, additional time and cycles were added for profile optimization. In highly fragmented FFPE DNA samples (post-sonication average bp sizes of <450 bp), fragment matching of an appropriate reference sample was performed. Any specimens <200 bp were rejected because of downstream bead-based cleanup steps that select for short fragment removal. Because size imbalance is known to affect downstream signal processing and analysis, DNA fragmentation simulation and fragment size matching were performed for all test/reference sample pairs, as described by Craig et al.[16]

Whole-Genome Amplification Using the GenomePlex Complete Whole Genome Amplification Kit

Whole-genome amplification was performed using the GenomePlex Complete Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions. A library preparation mastermix was added, consisting of 1 μL of 10× fragmentation buffer and 2 μL of 1× library preparation buffer; 1 μL of library preparation solution was added to the 10 μL sonication products. Samples were incubated at 95°C on a thermocycler, snap chilled on ice, consolidated by centrifugation, and returned to ice. After addition of 1 μL of library preparation enzyme, samples were centrifuged briefly and placed in the thermocycler. After initial library preparation, whole-genome amplification was performed. The mastermix for each sample consisted of 7.5 μL of 10× Amplification Master Mix, 7.5 μL of 20 mmol/L 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate (Thermo Fisher Scientific), 40 μL of nuclease-free water, and 5 μL of WGA DNA polymerase, which were added to the 15 μL product described before placing samples in the thermocycler.

0.8× Ampure Bead Cleanup of Whole-Genome Amplification Product

Ampure XP Beads (Beckman, Pasadena, CA) were equilibrated at room temperature for a minimum of 30 minutes and vortex mixed. WGA product volume was increased to 100 μL with nuclease-free water, and 80 μL of Ampure XP beads was added to each and vortex mixed thoroughly. After incubation at room temperature for 10 minutes, samples were centrifuged to collect residual beads and placed on a magnetic stand for 4 minutes. Supernatant was removed by pipetting. Samples were washed on the magnet two times with 80% ethanol, and residual ethanol was removed by pipette. After air drying at room temperature for 3 to 5 minutes, samples were eluted in 30 μL of nuclease-free water, vortex mixed, and incubated for 4 minutes at room temperature off the magnet. Samples were returned to the magnet, and supernatant was collected. Cleanup product was QC'ed for yield and purity using a microvolume spectrophotometer (Nanodrop). QC acceptance criteria included yields of 2 to 5 μg DNA, with a 260/280 ratio between 1.7 and 1.9. The incorporated d-UTP showed peaks in the Nanodrop trace at 240 and 290 nm. The 2100 dsDNA High Sensitivity assay (Agilent Technologies Inc.) was also performed to confirm size; libraries passing QC showed similar profiles with the original (pre-WGA) libraries, with a peak centered at 400 bp tapering to 2000 bp.

Aminoallylic Dye Incorporation

Dye incorporation was performed with a two-step 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate incorporation and chemical labeling protocol, as described by Cox and Singer.[23] Specimens were concentrated by lyophilization to a final volume of 6 μL. Specimens were briefly denatured on a thermocycler at 95°C for 5 minutes and snap chilled on ice. To each sample, 3 μL of 1 N sodium bicarbonate and 1 μL of either Alexa Fluor (Thermo Fisher Scientific) 555 or 647 NHS ester (alias succinimidyl ester; 40 μg/μL in dimethyl sulfoxide) was added. Specimens were incubated in the dark for a minimum of 1 hour and up to overnight.

Post-Reaction Cleanup

After incubation, samples were vortex mixed and transferred, each to a new 1.5-mL low-bind tube. Cleanup was performed using the Machery Nagel (Düren, Germany) Nucleospin Gel and PCR cleanup kit, following the manufacturer's protocol. Elution was performed by adding 30 μL of warmed (72°C) nuclease-free water to the membrane, with incubation for 5 minutes to increase efficiency and yield.

Post-Cleanup QC

QC was performed using a microvolume spectrophometer (Nanodrop) with degree of labeling (DOL) automatically calculated by the Nanodrop software version 2000 (Thermo Fisher Scientific) using the formula below to derive the number of dyes per 100 bases:

$$A_{base} = A_{260} - \left(A_{dye} * CF\right) \quad (1)$$

$$DOL = \frac{100}{\left(\left(A_{base} * \varepsilon_{dye}\right) / \left(A_{dye} * \varepsilon_{base}\right)\right)} \quad (2)$$

A_{base} is the corrected nucleic acid absorption value. CF is the correction factor adjusted for each dye at 260, which is 0.08 for AlexaFluor 555 and 0.00 (none) for AlexaFluor 647. A_{dye} is the absorbance at 260 nm for each dye.

ε_{dye} is the dye's extinction coefficient, which is 155,000 mol/$L^{-1}cm^{-1}$ for AlexaFluor 555 and 270,000 mol/$L^{-1}cm^{-1}$ for AlexaFluor 647. ε_{base} is the average extinction coefficient for a base in single-stranded DNA (specimens were denatured before measurement), which is 8919 mol/$L^{-1}cm^{-1}$. Typical yields were 2 to 5 μg of DNA, with DOL scores of 1.5 to 3.5 for most samples. A DOL of 2.5 was considered optimal, corresponding to one labeled d-UTP per 40 bases.

Hybridization on Agilent Sure Print G3 Microarrays

Hybridization mastermix was prepared for labeled test and reference DNA libraries in a total volume of 16 μL for the custom 8× 60K array and transferred to the thermocycler. Slides were assembled, per the manufacturer's instructions, and placed in the hybridization chamber for 24 hours at 67°C.

Slide Scanning and Data Analysis

Slides were scanned on a SureScan G4900DA (Agilent Technologies Inc.), and average gene copy number across 71 genes was calculated for each tumor sample from fluorescence intensity values by converting mean $\log_2$ signal intensity ratio value into genomic region copy number with the following formula:

$$\text{Copy Number} = 2\left(2^{\log_2 \text{Ratio}}\right) \quad (3)$$

Whole-genome and chromosome-specific ratio plots were generated using Cytogenomics software version 4.0.2.21 (Agilent Technologies Inc.).

Results

Assay Performance

DNA of sufficient quality/quantity for downstream analysis was obtained for all samples, and gene copy number results for known CNAs were consistent with expected results. Multiple QC steps were incorporated into the CMA workflow and measured throughout the assay to ensure optimal array performance (Figure 1). Across the 30 samples tested, mean DOL was 2.3 (acceptable range, 1.5 to 3.5) and mean labeled DNA yield was 2411 ng (acceptable range, 2000 to 4000 ng).

Pathology Review of FFPE Tissue Samples

Pathology review of the percentage neoplastic content in hematoxylin and eosin–stained tissue sections from the FFPE tissue samples identified 35 of 35 (100%) with adequate (>50%) tumor content. Review of HER2 staining by IHC in eight previously characterized commercially acquired HER2-positive and two HER2 equivocal cases revealed 2 of 10 (20%) of cases with heterogeneous HER2 staining and 8 of 10 (80%) with homogeneous positive staining. Heterogeneously stained HER2-positive areas (hot spots) were circled by the pathologist for macrodissection before DNA extraction. The homogenously stained positive and negative tissue sections were marked as scrape all for DNA extraction.

QC Analysis of DNA Quality and Quantity

Quantity and quality assessment of extracted DNA from FFPE cell lines and tumor tissue showed 47 of 47 (100%) of samples with adequate DNA for downstream CMA analysis. No samples yielded <75 ng DNA. Mean bp size for all samples was 450, with the lowest fragmentation size detected to be 183 bp. Mean bp size for CAP/ASCO processed samples was 555, and it was 359 for non-CAP/ASCO samples.

Fragment Size Matching

Fragment size matching was performed for all samples to produce DNA fragment distributions of paired sizes in the optimal range of ≥400 bp and not <200 bp. It is documented that test and reference specimens of different sizes yield significant differences in the number of dye molecules incorporated per fragment, resulting in a significant difference in signal intensity between the test and reference affecting downstream signal processing and analysis.[16] To generate shorter reference samples, sonication times were increased until an appropriately sized reference was generated to pair with the test sample (Figure 2). One CAP/ASCO-processed sample required sonicated reference DNA as opposed to 13 of the non-CAP/ASCO processed samples requiring sonicated reference DNA.

Whole-Genome Amplification and Dye Incorporation

After whole-genome amplification, mean size was 704.75 bp; WGA products were slightly longer because of primer extension and nonspecific ligation products from the WGA

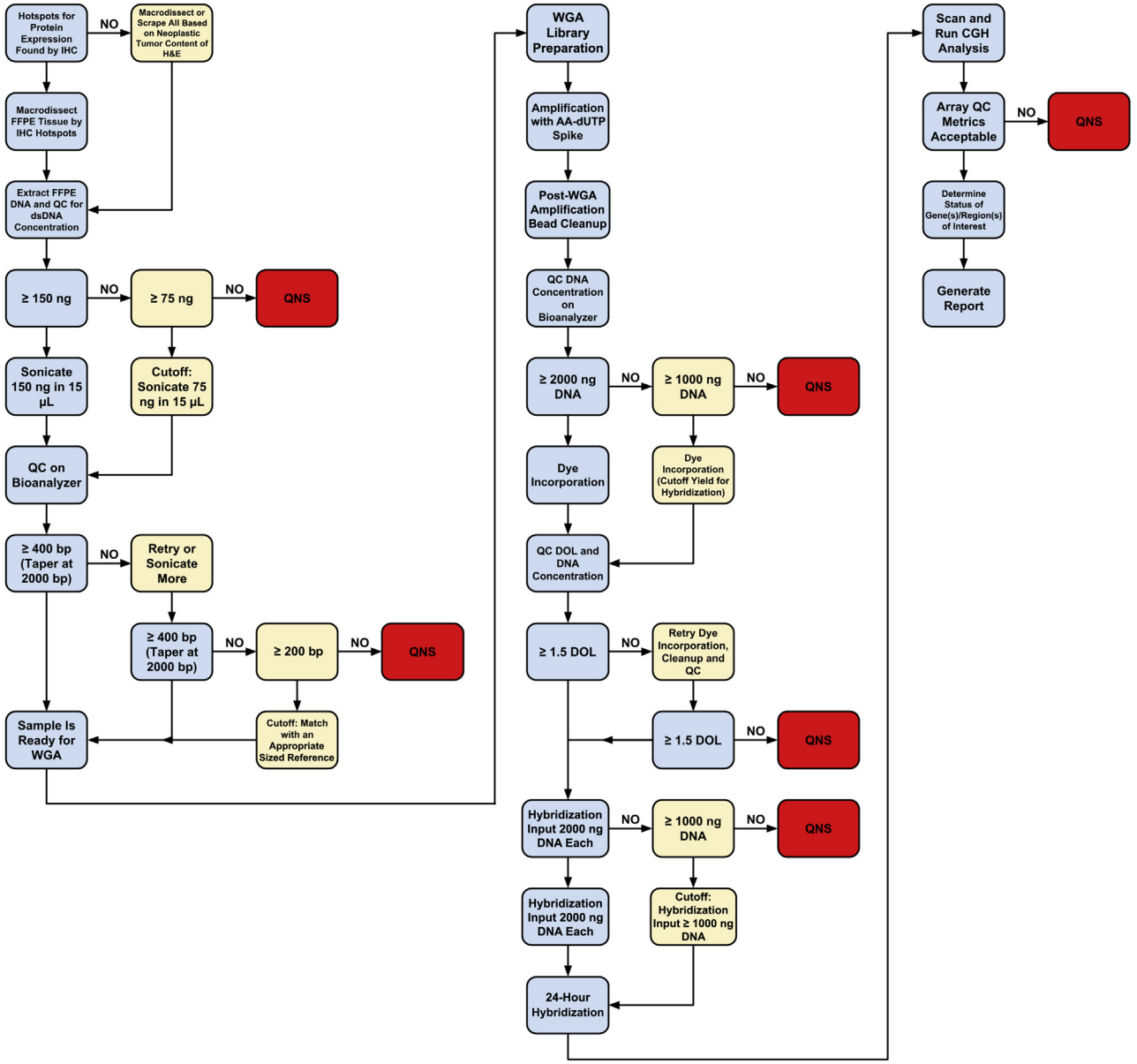

Figure 1 Chromosomal microarray workflow diagram illustrating multiple quality control (QC) steps measured throughout the assay to ensure optimal array performance. AA-dUTP, 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate; CGH, comparative genomic hybridization; DOL, degree of labeling; dsDNA, double-stranded DNA; FFPE, formalin-fixed, paraffin-embedded; H&E, hematoxylin and eosin; IHC, immunohistochemistry; QNS, quantity/quality not sufficient; WGA, whole-genome amplification.

reaction. After dye incorporation, mean DOL and post-cleanup yields were 2.28 and 2.4 µg.

Data QC

QC parameters from the Raw Scanned Data and Cytogenomics software version 4.0.2.21 (Agilent Technologies Inc.) included dLRsd, derivative log ratio spread, red and green signal/noise ratio, signal, background, reproducibility, and IsGoodGrid. QC cutoffs were established from the collective run data, and in conference with technical support (Agilent Technologies Inc.), they were based on sample type (FFPE) and the method described (Supplemental Table S2). Specimens within the upper and lower QC limits and within ±3 SDs of the mean for each metric were passed at this stage for interpretation and reporting.

Detection of Genome-Wide CNAs in Cell Lines and FFPE Samples

Whole-genome landscape views across multiple tumor types accurately identified the expected amplified, deleted, or normal copy number profiles for clinically relevant genes in 24 of 24 (100%) of previously characterized commercially acquired samples.[17–22] The FFPE HAPMAP Somatic Normal cell line (GM12878) used as a copy number control

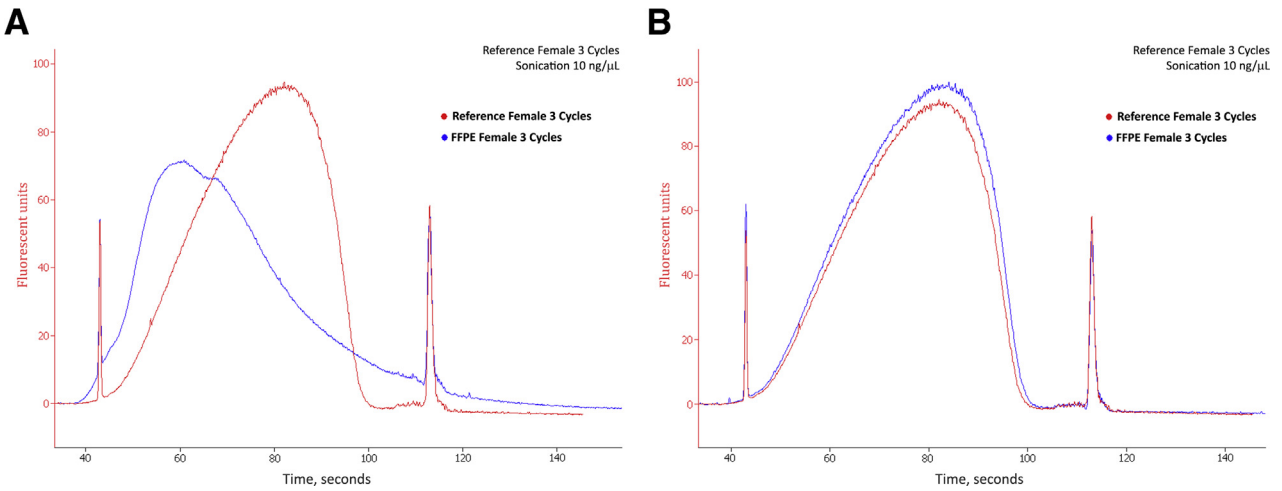

Figure 2 Overlay of 2100 Bioanalyzer (Agilent Technologies Inc.) trace examples. **A:** Inappropriately matched test (blue) and reference (red) DNA specimens. **B:** Appropriately matched test (blue) and reference (red) DNA specimens.

across the 71-gene panel was negative for CNAs. The HER2-negative cancer cell line HCC1143 was negative for *HER2* gene amplification by CMA. The breast cancer cell line, SKBR3, was positive for high-level *HER2* gene amplification on chromosome 17q12; in addition, CMA analysis revealed *MET*, *MYC*, and *AURKA* gene amplifications on chromosomes 7q31, 8q24, and 20q13, respectively. The lung cancer cell line EBC1 was positive for an expected *MET* gene amplification and also revealed *CDK6* and *RICTOR* gene amplifications on chromosomes 7q21 and 5p13, respectively. The myeloid cell line K562 was positive for *CDKN2A* and *CDKN2B* gene deletions on chromosome 9p21. The National Council for International Health 1930 lung cancer cell line was positive for chromosome 4q12 amplification, including the *KIT* gene. The PC3 prostate cancer cell line showed *PTEN* gene deletion on chromosome 10q23. CMA results are summarized in Supplemental Figure S1 and Supplemental Table S3.

HER2 Gene Analysis

High-resolution analysis of the *HER2* gene on chromosome 17 accurately identified positive (amplified), negative (nonamplified), or low copy number in 12 of 12 (100%) breast and gastric cancer FFPE tissue samples (Table 4). Discovery samples [23/23 (100%)] were negative for *HER2* gene amplification.

Titration Studies

Cell lines SKBR3 and PC3 were titrated at 100%, 50%, 25%, and 10% tumor/normal DNA. Copy number of known aberrations was calculated using the following formula:

$$\text{Copy Number} = 2\left(2^{\log_2 \text{Ratio}}\right). \quad (4)$$

Data were plotted in Excel 2016 (Microsoft, Redmond, WA), and R^2 values were calculated using linear regression (Figure 3). R^2 values for all aberrations in both cell lines were $\geq$0.960. Data are summarized in Supplemental Table S4. Although high-level amplifications could be detected in as low as 10% tumor fraction, we determined that specimens should not be <25% tumor content for CMA clinical applications. Deletions were not detectable at <25% tumor fraction (Figure 4).

Discussion

We have developed an advanced CMA technological assay for analysis of CNAs in DNA extracted from FFPE solid tumor tissue. The method expands on the FSM method, described by Craig et al,[16] and is coupled with WGA and incorporation of amine reactive fluorescent dyes into DNA for labeling. We have optimized the minimum DNA concentration from 1 µg, as described in the original FSM method, to a minimum of 75 to 150 ng. This markedly decreased DNA requirement expands

Table 4 HER2 Gene Copy Number Results

Cases, *n*	IHC score	HER2 gene copy no. by FISH	CGH copy no.	Result
8	3+	Positive (>6)	>6	HER2 positive
2	1–2+	4–6	4–6	HER2 low
2	0	Negative (<4)	<6	HER2 negative

CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

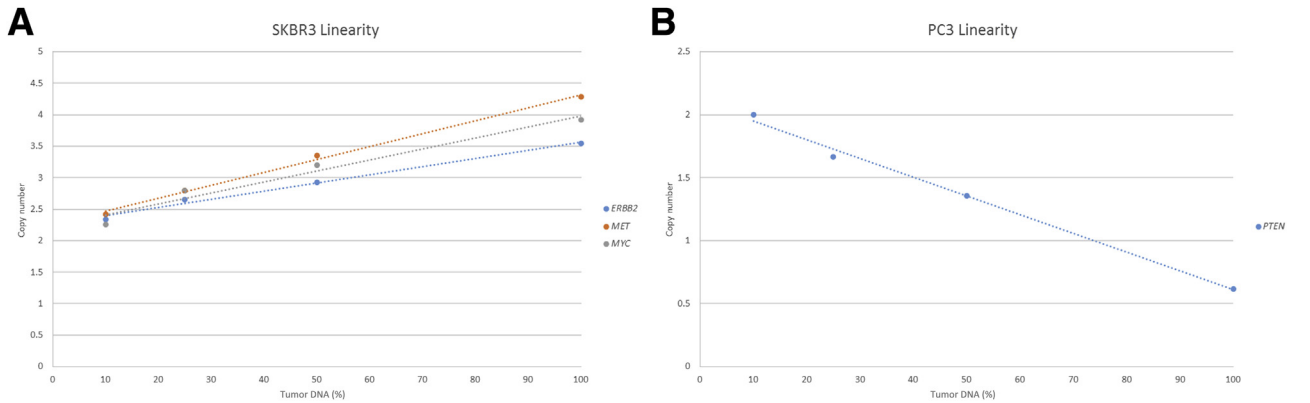

Figure 3 Linearity studies for cell lines SKBR3 and PC3. **A:** Copy number for the SKBR3 cell line amplified genes (*ERBB2*, *MET*, and *MYC*) calculated from LOG2 ratio and plotted against percentage tumor DNA. Calculated R^2 values: *ERBB2* = 0.991, *MET* = 0.996, *MYC* = 0.996. **B:** Copy number PC3 cell line *PTEN* gene deletion calculated from LOG2 ratio and plotted against percentage tumor DNA. R^2 value = 0.995. See also Supplemental Table S4.

the number of samples that would pass QC criteria for CMA eligibility. In addition, we have tested the expanded FSM protocol on a wide variety of tumors, including breast, gastric, pancreatic, colorectal, and thyroid. The original FSM study was performed only on glioblastoma multiforme FFPE tissue, a tumor type known in practice to yield high-quality DNA (S.R.G., unpublished data). To further optimize DNA quality in the expanded FSM assay, thermodegradation of the samples was reduced through Episonic (versus the original heat fragmentation method) and the use of a chemical (aminoallelic dye incorporation) in place of the universal labeling system.[17]

Analysis of 24 previously characterized commercially acquired FFPE cell line and solid tumor samples confirmed accurate identification of genome-wide CNAs in a variety of solid tumor types.[17–22] Copy number status of clinically relevant genes, such as *HER2*, *MET*, and *PTEN*, was reliably determined across relevant genomic regions by both objective and subjective results analysis. In addition, genome-wide analysis of CNAs was satisfactorily performed for 23 samples representing a variety of solid tumor types with unknown CNA status. The CMA assay builds on the FSM method, demonstrating enhancement of array performance when test and reference samples possess similar fragment sizes, and has been further optimized through amine modification probe preparation. The enhanced CMA technology allows salvaging of suboptimal tumor samples for molecular analysis.

The CMA protocol can be incorporated into routine clinical laboratory practice, with many of the procedures replicating existing protocols of the molecular laboratory workflow. These include pathology review, tumor circling and microdissection, DNA extraction from FFPE tissue, DNA library preparation, fluorescent labeling, whole-genome amplification, and data analysis. Thus, the choice of method for detection of CNAs in a tumor sample can be made on the basis of the optimal CNA assay for the clinical scenario rather than the quality and quantity of available tissue. For example, *HER2* gene copy number in a newly diagnosed case of breast cancer is best evaluated per National Comprehensive Cancer Network guidelines using Food and Drug Administration–approved FISH. In contrast, chemoresistant *EGFR*-, *KRAS*-, and *ALK*-negative non–small cell lung cancer would be a candidate for CMA testing to maximize the number of therapeutic targets evaluated from limited tissue. In a third clinical scenario in which only limited tissue is available and clinical questions include the presence or absence of point mutations and insertions/deletions as well as CNAs, a next-generation sequencing assay may be the preferred method for analyzing the tumor genome. An example would be liver core needle biopsy from unresectable colorectal cancer, where extended *RAS* mutation testing and *HER2* gene copy number analysis have been requested by the clinician. In this clinical scenario, next-generation sequencing testing would maximize the biomarker data from a limited tissue sample.

In the current study, whole-genome views were generated for a variety of tumor types representing a spectrum of tissue handling. On the optimal end were breast and GEA tumors, processed with cold ischemia times <60 minutes and 6 to 72 hours' fixation in 10% neutral-buffered formalin, according to CAP/ASCO guidelines. On the other end of the spectrum are tumor types, such as colorectal and pancreatic, traditionally processed according to individual laboratory protocols. Overall, the quality and quantity of DNA extracted from guideline-processed samples was superior to what we extracted from non–guideline-processed tumors. Future studies comparing molecular testing results from guideline-processed samples versus non–guideline-processed tumors will likely contribute to a growing movement toward implementation of tissue handling standards across tumor types to optimize preservation of nucleic acids.

Conclusions

The growing number of solid tumor biomarkers linked to targeted therapies has generated a medical need for clinically available and dedicated methods of whole-genome copy

Figure 4 *PTEN* gene deletions at 100%, 50%, 25%, and 10% dilutions, illustrating 25% tumor content cutoff value for deletion detection. CGH, comparative genomic hybridization.

number analysis. Molecular pathologists can best support precision medicine by having multiple clinical approaches for identification of therapeutic targets in a patient's tumor genome. The molecular tool kit should cover a wide range of clinical scenarios without one single technology being considered necessarily the best. Choice of technology (FISH, PCR, next-generation sequencing, CMA) depends on the tumor type, tissue DNA quality/quantity, types of genomic alterations being tested, and availability of on- and off-label therapeutics for specific aberrations. Obtaining a whole-genome copy number view of an individual patient's tumor using a dedicated copy number assay, such as CMA, can provide information about activated oncogenes, inactivated tumor suppressor genes, and potential therapeutic targets. During the past decade, whole-genome copy number analysis has been frequently termed a landscape view of the tumor because the peaks and valleys of tumor terrain harbor potentially actionable gene amplifications and deletions. The ability to obtain a CMA landscape view from an individual's FFPE tumor tissue DNA contributes an effective precision medicine tool to the pathologist's workbench to help guide therapeutic decisions about a patient's eligibility for receiving National Comprehensive Cancer Network–designated emerging targeted agents.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.01.004*.

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