

# PIK3CA Hotspot Mutation Scanning by a Novel and Highly Sensitive High-Resolution Small Amplicon Melting Analysis Method

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**Somatic mutations in the *PIK3CA* gene have been discovered in many human cancers, and their presence correlates to therapy response. Three “hotspot” mutations within the *PIK3CA* gene are localized in exons 9 and 20. High-resolution melting analysis (HRMA) is a highly sensitive, robust, rapid, and cost-effective mutation analysis technique. We developed a novel methodology for the detection of hotspot mutations in exons 9 and 20 of the *PIK3CA* gene that is based on a combination of PCR and HRMA. The *PIK3CA* HRMA assay was evaluated by performing repeatability, sensitivity, and comparison with DNA sequencing studies and was further validated in 129 formalin-fixed paraffin-embedded breast tissue samples: 99 tumors, 20 noncancerous, and 10 fibroadenomas. The developed methodology was further applied in a selected group of 75 breast cancer patients who underwent Trastuzumab treatment. In sensitivity studies, the assay presented a capability to detect as low as 1% of mutated dsDNA in the presence of wtDNA for both exons. In the 99 tumor samples (validation group), 12/99 (12.1%) exon 9 mutations and 20/99 (20.2%) exon 20 mutations were found. No mutations were found in noncancerous tissues. In fibroadenomas, we report one *PIK3CA* mutation for the first time. In the selected group, 30/75 (40%) samples were detected as mutants. The *PIK3CA* HRMA assay is highly sensitive, reliable, cost-effective, and easy-to-perform, and therefore can be used as a screening test in a high-throughput pharmacodiagnostic setting. (*J Mol Diagn* 2010, 12:697–704; DOI: 10.2353/jmoldx.2010.100008)**

Somatic mutations and gene amplification for the gene encoding for phosphatidylinositol 3-kinase (PI3K) p110 $\alpha$  catalytic subunit, *PIK3CA*, located in chromosome 3, have been discovered in many different human can-

cers.<sup>1–9</sup> *PIK3CA* mutations in exons 9 and 20 account for more than 90% of mutations reported for this gene, according to COSMIC Database (Catalogue Of Somatic Mutations In Cancer Database, v41 release; Wellcome Trust Genome Campus, Hinxton, Cambridge; Accessed May 2009; <http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=PIK3CA>). There are three recurrent or “hotspot” mutations within these exons: c.1624G>A(E542K), c.1633G>A(E545K) in exon 9 (helical domain), and c.3140A>G(H1047R) in exon 20 (kinase domain). These hotspot mutations have been shown to have oncogenic effects.<sup>1,10,11</sup>

*PIK3CA* mutations appear to have a clinical significance.<sup>12</sup> Recent studies have shown that *PIK3CA* mutations can independently hamper the therapeutic response to anti-EGFR biological therapies (panitumumab or cetuximab) in metastatic colorectal cancer<sup>13</sup> and demonstrate resistance to dietary restriction therapies.<sup>14</sup> Moreover, several efforts are underway nowadays to target the PI3K pathway with therapeutic inhibitors.

Additionally, keeping in mind that not all patients with HER2-overexpressing metastatic breast cancer respond to Trastuzumab (Herceptin), activated PI3K signaling has been proposed to predict Trastuzumab resistance.<sup>15,16</sup> Loss of the PTEN (Phosphatase and Tensin Homolog) protein has been suggested as a key factor for the development of resistance to this drug.<sup>15,16</sup> However, PTEN loss alone,<sup>16,17</sup> and in combination with phosphorylated AKT expression,<sup>17</sup> proved inadequate to predict response to therapy. Conversely, combining PTEN loss and gain-of-function mutations of the *PIK3CA* gene, resistance to Trastuzumab was successfully predicted.<sup>16</sup>

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The introduction of High-Resolution Melting Analysis (HRMA) technique in 2003<sup>18</sup> came with several advantages. HRMA, a closed-tube probe-free technique, with genotyping and mutation scanning capabilities, is rapid, simple, cost-effective, and nondestructive. Especially, HRMA of small amplicons (<100bp) overcomes the difficulty of classic HRMA analysis to detect mutant homozygotes, and demonstrates a higher diagnostic sensitivity in detecting homozygotes and heterozygotes,<sup>19–22</sup> with an increase in rapidity.<sup>20</sup> Moreover, small amplicons display a higher analytical sensitivity compared with larger amplicons.<sup>23,24</sup> This is a major advantage when scanning for somatic mutations, where the tumor tissue is contaminated with adjacent normal tissue.

Archival tissue specimens, such as formalin-fixed paraffin-embedded (FFPE) tissues, represent a vast source of tissue genomic DNA (gDNA), easily obtained from clinical archives. However, they present difficulties in amplification due to DNA degradation, therefore demanding a reduction of PCR amplicons' length. In addition, use of FFPE tissue decreases diagnostic specificity when used as a template for HRMA.<sup>25</sup>

Until recently, several techniques have been used for scanning and/or genotyping somatic mutations of the *PIK3CA* gene. These include DNA sequencing,<sup>1–3,5,6,8</sup> denaturing High Performance Liquid Chromatography (dHPLC), Single Strand Conformation Polymorphism (SSCP),<sup>5</sup> multiplex PCR amplification and primer extension,<sup>26</sup> and Amplification Refractory Mutation System (ARMS) PCR.<sup>27</sup> ARMS PCR has the highest analytical sensitivity reported; however, it does not allow mutation scanning capabilities and the use of Scorpion probes carries a high cost. A HRMA method for hotspot regions of the *PIK3CA* gene has been reported, using amplicons larger than 100bp and not applied in archival tissue specimens.<sup>28</sup>

Herein, we report a novel methodology for the detection of *PIK3CA* hotspot mutations using HRMA of small amplicons. By performing novel *in silico* primer design and extensive optimization studies we maximized analytical sensitivity and obtained very accurate results for FFPE samples, as verified by DNA sequencing. After validation using a group of 129 breast tissue samples, the method was applied on a selected group of 75 patients who underwent Trastuzumab treatment.

## Materials and Methods

### DNA Samples

Five gDNA samples isolated from peripheral blood (PB) of healthy volunteers served as wild-type controls. Two gDNA samples isolated from MCF-7 (c.1633G>A:E545K; heterozygous), and T47D (c.3140A>G:H1047R; heterozygous)<sup>29</sup> breast cancer cell lines served as mutant controls. Genomic DNA was also isolated from 129 FFPE breast tissues (validation group): 99 carcinomas, 10 histologically normal samples adjacent to tumors, 10 fibroadenomas, and 10 reduction mammoplasty samples. All carcinoma patients (validation group) were diagnosed with early-stage breast cancer, and their clinical characteristics were previously described.<sup>30</sup> Additionally, gDNA was isolated from 75 FFPE breast tissues (selected group) from patients with HER2-positive metastatic breast cancer. These patients received Trastuzumab in combination with first-line chemotherapy. All patients gave their informed consent, and the study has been approved by the Ethical and Scientific Committees of our Institutions.

From PB, gDNA was extracted using a salting-out DNA extraction protocol.<sup>31</sup> From cell lines and FFPE samples, gDNA was extracted using the High Pure PCR Template Preparation Kit (Roche, Germany). DNA purity and quantity was determined using the Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

### Primer Design

Two sets of primers were *in silico* designed for each of *PIK3CA* exons 9 and 20, by using the PrimerPremier 5 software (Premier Biosoft International, Palo Alto, CA), and synthesized by FORTH (Greece). For each exon, one primer set was designed to span the hotspot region, with a reduced length (<100 bp), and another set to span the exons' boundaries. Primer oligonucleotide sequences are presented in Table 1.

For exon 9, set S9 was designed to amplify the region (92 bp) that includes the hotspot mutations of exon 9 (Figure 1A), while set L9 embrace the whole exon (261 bp). Both our primer sets were designed with either 1 (L9)

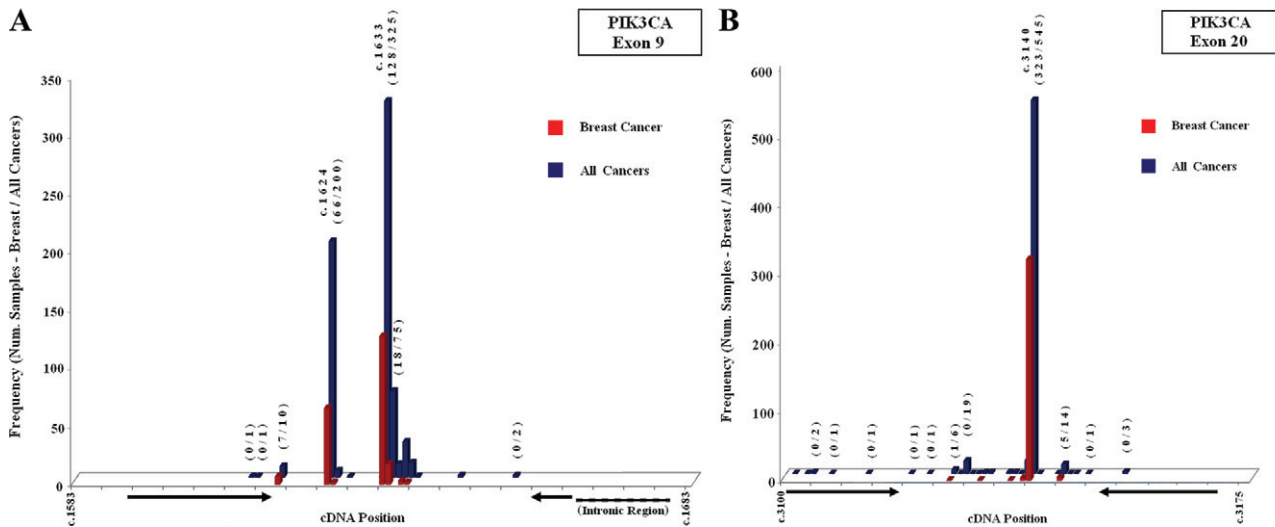
**Table 1.** *PIK3CA* Oligonucleotide Sequences and Locations Used in this Study

Exon	Primers	Primer set	Amplicon position*	Amplicon size	Amplicon Tm (°C) <sup>†</sup>	Recurrent mutations <sup>‡</sup>	Mutation position within amplicon
9	F: 5'-GCTCAAAGCAATTTCTACACGAGA-3'	S9	180, 418, 742–180, 418, 833	92 bp	81.0	c.1624G>A	35
	R: 5'-TCCATTTTAGCACTTACCTGTGAC-3'						44
	F: 5'-ATCCAGAGGGGAAAAATATG-3'	L9	180, 418, 634–180, 418, 894	261 bp	82.5	c.1624G>A	143
	R: 5'-ATGCTGAGATCAGCCAAAT-3'						152
20	F: 5'-GAGGCTTTGGAGTATTTTCAT-3'	S20	180, 434, 739–180, 434, 808	70 bp	79.5	c.3140A>G	41
	R: 5'-AATCCATTTTTGTGTCCAG-3'						
	F: 5'-TCATTTGCTCCAACTGACCAA-3'	L20	180, 434, 534–180, 434, 885	352 bp	83.5	c.3140A>G	246
	R: 5'-TGGAATCCAGAGTGAGCTTTCA-3'						

\*According to GenBank accession no. NC\_000003.10.

<sup>†</sup>The stated Tms are according to the wild-type gDNA samples.

<sup>‡</sup>According to GenBank accession no. NM\_006218.2.



**Figure 1.** Diagrams of mutation frequencies of hotspot regions of exon 9 (A) and exon 20 (B) of the *PIK3CA* gene. Frequencies are reported for breast cancer (red color) and for all cancer (blue color) types. **Arrows** indicate primer sites of small amplicons (Sets S9 and S20). Mutation frequency data are according to COSMIC database v41 release.

or 2 (S9) mismatches at the 3'-end of the reverse primer, to a highly homologous (98%) genomic region (chromosome 22), to avoid false positives. For exon 20, set S20 was designed to amplify the region (70 bp) including the hotspot mutation (Figure 1B), and set L20 to amplify the whole exon 20 into the 5'-UTR (352 bp).

Our primer sets S9 and S20 for the small amplicons were designed so that all reported somatic mutations in breast cancer, in the hotspot regions, according to the data registry at the COSMIC database, were included within the amplified region while excluded from primer sites (Figure 1). Hotspot mutations were placed as close to the center of the amplicons as possible (Figure 1 and Table 1). Amplicon melting domains were *in silico* tested by using the POLAND program<sup>32</sup> (POLAND program; Institut für Physikalische Biologie, Heinrich-Heine-Universität, Düsseldorf, Germany; Accessed May 2008; [http://www.biophys.uni-duesseldorf.de/local/POLAND/poland\\_help.html#Poland](http://www.biophys.uni-duesseldorf.de/local/POLAND/poland_help.html#Poland)), and presented a single melting transition with the exception of the 261bp amplicon of exon 9 (Set L9), which presented a double melting transition.

### PCR

Real-time PCR was performed in the LightCycler instrument (Roche Applied Science, Mannheim, Germany) using glass capillary tubes (Roche Applied Science). Extensive optimization experiments were performed to maximize PCR amplification efficiency, including PCR program parameters, Mg<sup>2+</sup>, primer, and template concentrations. The PCR reaction mix consisted of 1× PCR Buffer (Invitrogen, Carlsbad, CA), 0.4 mmol/L for each dNTP (Invitrogen), 0.05 U/μl Platinum TaqDNA Polymerase (Invitrogen), 0.15 μg/μl BSA (Sigma, Munich, Germany), 1× LCGreen Plus Dye (Idaho Technology, Salt Lake City, UT), 0.3 μmol/l primers, 25 ng gDNA and Mg<sup>2+</sup> (2.5 mmol/L for S9 and 3.0 mmol/L for all other sets). Sterile water was used to supplement up to 10 μl.

The real-time PCR protocols began with one cycle at 94°C for 2 minutes followed by 60 cycles of the following: Set S9: 94°C for 10 s, 67°C for 20 seconds and 72°C for 20 s; Set L9: 94°C for 20 s, 61°C for 20 seconds and 72°C for 30 s; Set S20: 94°C for 10 s, 59°C for 15 seconds and 72°C for 20 s; Set L20: 94°C for 20 s, 58°C for 20 seconds and 72°C for 40 s.

After amplification, all protocols were followed by one cycle at 95°C for 1 minute and a rapid cooling to 40°C for 1 minute. Real-time fluorescence acquisition was set at 72°C (F1 channel). All PCR reactions were performed in triplicate for each sample.

### Melting Analysis

After real-time PCR, melting curves were obtained using the HR-1 High-Resolution Melter (Idaho Technology). Melting with data acquisition began 10°C below and ended 6°C above the T<sub>m</sub> of each amplicon (Table 1), rising at 0.1°C/s. Data processing included normalization, temperature shifting (2 to 5%), and fluorescence difference plots, using the accompanied software. The accompanied software did not allow processing of more than 32 samples simultaneously. In cases when more samples were needed for comparison, data were exported and curves were generated using Microsoft Excel 2003 software.

### Sensitivity Study

We evaluated the sensitivity by mixing mutated gDNA from cell lines, with wild-type from PB at ratios of 50%, 30%, 20%, 10%, 5%, 2%, 1%, and 0.2%. Samples that were used for dilutions were selected to match gDNA quantity, quality and quantification cycle (C<sub>q</sub>), to minimize PCR bias. Evaluation of sensitivity was performed for both small and large amplicons for both exons.

## DNA Sequencing

All samples from both validation and selected groups that presented mutant melting transitions and ten samples presenting wild-type transitions were subjected to bidirectional DNA sequencing using the small amplicons. After HRMA, 7.5  $\mu$ l of the PCR reaction mix was treated with 3  $\mu$ l of ExoSAP-IT (USB). Five microliters of the treated PCR mix was used as a template for cycle sequencing, using the Big Dye Terminator v1.1 kit (Applied Biosystems, Carlsbad, CA). The reaction mix consisted of 1  $\mu$ l ready reaction terminator premix, 7  $\mu$ l buffer, 1  $\mu$ l of 0.5  $\mu$ mol/L primer (same used for PCR), and 6  $\mu$ l of sterile water. The reactions were performed according to manufacturer's instructions, purified with NucleoSeq Columns (Macherey-Nagel, Düren, Germany), ran on an ABI 310 Genetic Analyzer (Applied Biosystems), and manually called.

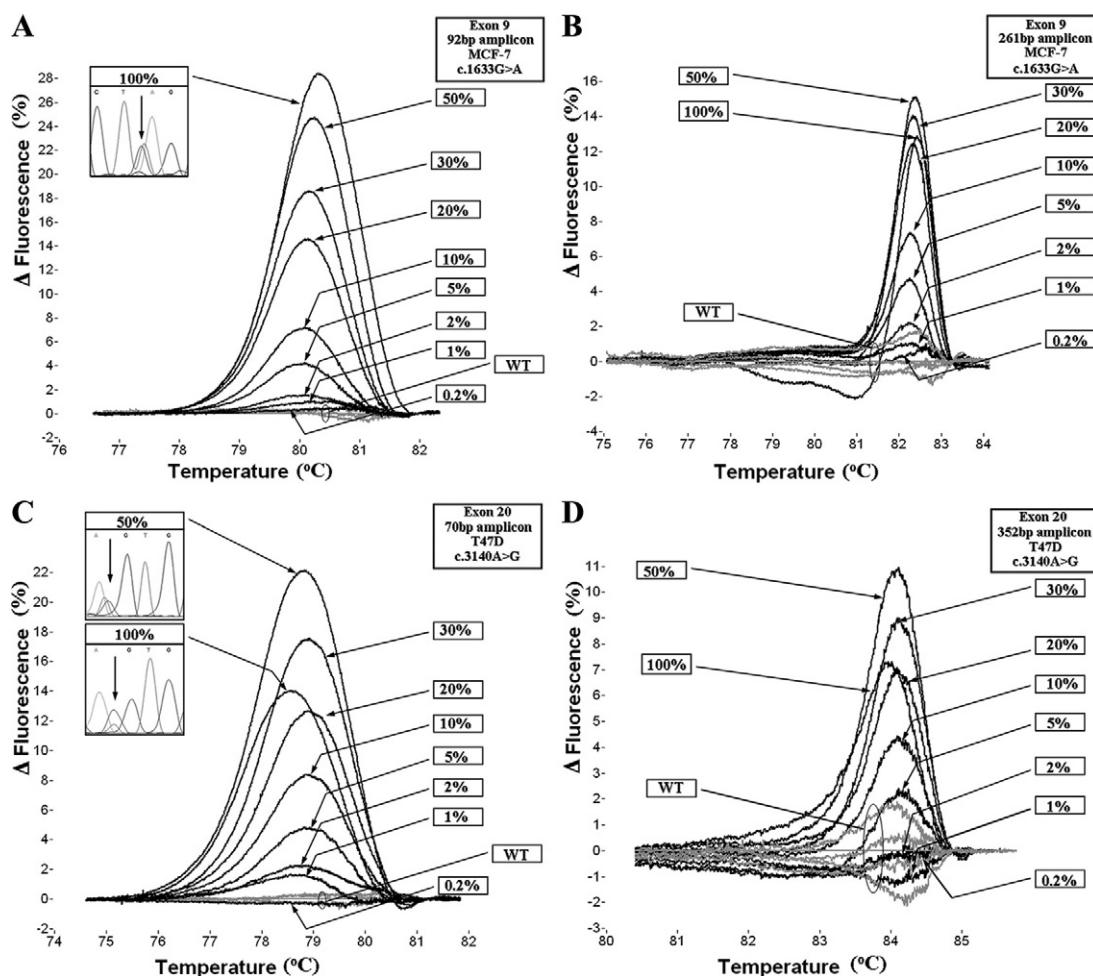
## Results

Genomic DNA from the cancer cell lines MCF-7 and T47D as well as wild-type PB samples was first used to evaluate the developed methodology. HRMA was able to

readily discriminate between different wild-type controls and cell line samples in both small and large amplicons (see Supplemental Figure S1 at <http://jmd.amjpathol.org>). No dimers or "nonspecific" products were observed.

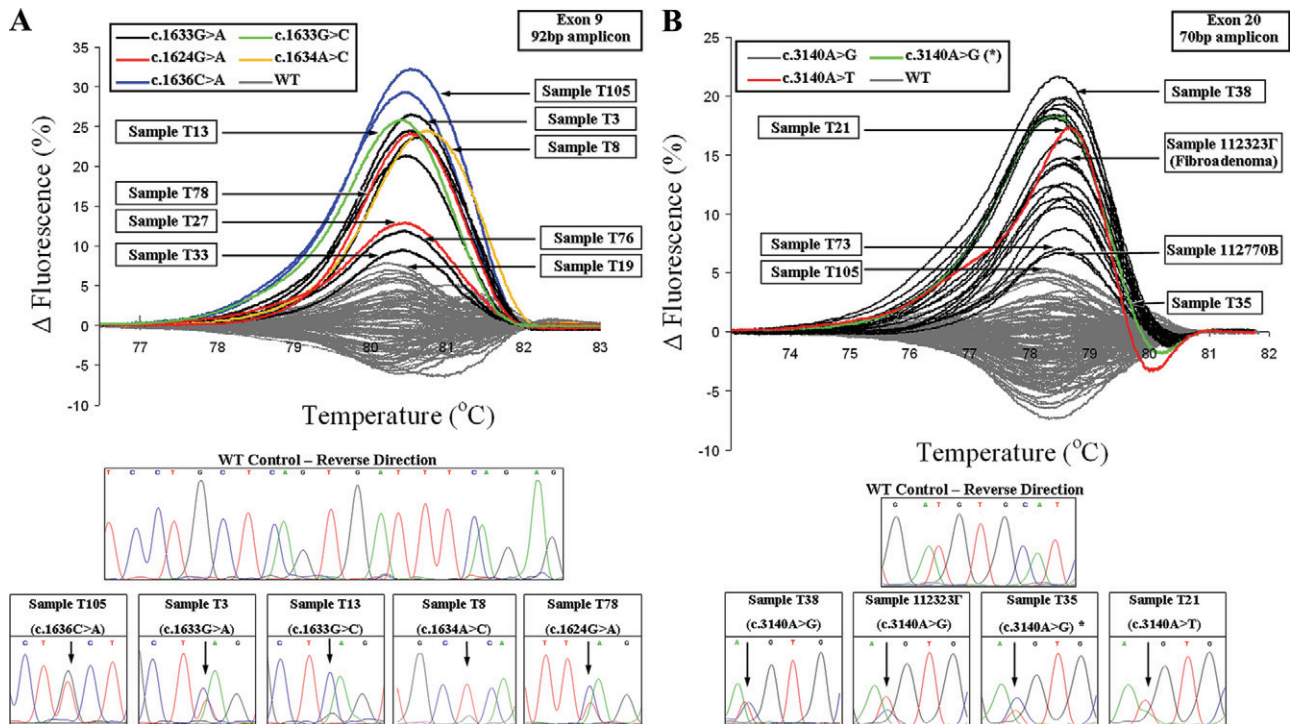
During optimization studies we observed a high baseline variation for FFPE samples. In an effort to reduce the wild-type baseline variation, we increased the number of PCR cycles to have FFPE samples fully amplified (see Supplemental Figure S2, A and B at <http://jmd.amjpathol.org>). It has been previously shown that this could be an important issue in HRMA.<sup>24,25</sup> We also tested the amount of template used (100 ng, 50 ng, and 25 ng) in PCR reactions, using two wild-type FFPE samples presenting high and one presenting low variation. The samples that presented high variation showed a reduction of variation with template amount of 25 ng (see Supplemental Figure S2, C and D at <http://jmd.amjpathol.org>). By adjusting these two parameters (high number of cycles and low DNA input) a reduction of FFPE wild-type variation was apparent in a global level.

To evaluate the analytical sensitivity, dilutions of each cell line to wild-type gDNA (50–0.2%) were assessed. Fluorescence difference plots were generated, and the ability to discriminate melting transitions of the cell line



**Figure 2.** Fluorescence difference plots of sensitivity studies. The 92-bp amplicon (A) and 261-bp amplicon (B) of exon 9, and 70-bp amplicon (C) and 352-bp amplicon (D) of exon 20 of the *PIK3CA* gene. Figure windows present sequencing results of indicated ratios of the cell lines in the reverse direction.





**Figure 3.** Fluorescence difference plots of HRMA, and sequencing results of the 129 formalin-fixed paraffin-embedded tissues, 92-bp amplicon for exon 9 (A) and 70-bp amplicon for exon 20 (B). Sequencing results of samples indicated by **arrows** can be found in the lower section of each of the figures or Supplemental Figure S3 at <http://jmd.amjpathol.org>. The sample indicated with an **asterisk**, in HRMA and sequencing, is the sample found to bear a high proportion for the c.3140A>G mutation, not compatible with a heterozygote.

dilutions from that of five wild-type samples was assessed. For exon 9, with the 92-bp amplicon we could discriminate a dilution corresponding to 2% of MCF-7 cell line, while with the 261-bp amplicon 5% could be discriminated (Figure 2, A and B). For exon 20, with the 70-bp amplicon we could discriminate a ratio of 1% of T47D cell line dilution, whereas with the 352-bp amplicon the detectable ratio was 10% (Figure 2, C and D). Melting curves were highly reproducible.

In the case of exon 20, where T47D cell line was used, we observed that the 50% dilution presented a melting transition with higher differentiation than the undiluted cell line. This phenomenon was observed for both small and large amplicons (Figure 2, C and D). We considered this observation to be inconsistent with previous findings demonstrating maximum heteroduplex yield with fully heterozygous samples.<sup>33</sup> Indeed, as can be seen in Figure 2C, sequencing results demonstrated that the 50% dilution of T47D simulated more authentic heterozygous sequence. We hypothesize that this may be due to preferential amplification of the mutant allele, because there are reports demonstrating gene amplification of this cell line.<sup>34</sup>

Our purpose of developing HRMA methodology for the larger amplicons was not limited to test and compare the analytical sensitivity with respect to the smaller amplicons. The objective was to detect mutations missed within these exons, not included in the small amplicon region. However, using larger amplicons, approximately 50% of the FFPE samples presented low or no PCR

amplification. Therefore, only small amplicons were used for mutation scanning.

In the validation group, scanning the 20 noncancerous FFPE tissue samples no mutant melting transitions were observed. Scanning the 99 FFPE breast tumor samples, 12 samples in exon 9 and 20 in exon 20 were found to be mutated (in total: 32/99, 32%) (Figure 3). Interestingly, one FFPE fibroadenoma sample (1/10, 10%) presented a mutant transition, which was further defined by DNA sequencing as a c.3140A>G substitution (Figure 3B). All mutations detected were further defined by DNA sequencing (Figure 3; Supplemental Figure S3, A and B at <http://jmd.amjpathol.org>) and demonstrated in Table 2. All mutations were mutually exclusive. All samples were detected as heterozygous.

However, one sample was found to bear a high proportion for the c.3140A>G mutation (higher than 50%). This sample presented a transition with minor differences from the rest of the samples bearing this mutation (Figure 3B, green color & marked with asterisk).

Additionally, only one wild-type FFPE sample and in only one out of the three PCR amplification experiments for exon 9 presented a transition wrongly assigned as mutant. Although this occurred in only one of the three runs, it was forwarded for DNA sequencing to be verified as wild-type. This false positive call corresponds to 1/675 PCR runs (0.15%) performed for wild-type FFPE samples.

The high baseline variation of the wild-type FFPE samples that was observed (Figure 3) has also been reported in our previous work on *BRCA1* exon 20 HRM scanning.<sup>24</sup>

**Table 2.** *PIK3CA* Gene Variants in Breast Cancer (FFPE) Samples, as Detected by HRMA, and Defined by DNA Sequencing

Nucleotide change	Protein change	Number of samples	Relative (%)	Total (%)
Validation group				
Exon 9				
c.1633G>A	E545K	6	18.8	6.1
c.1624G>A	E542K	2	6.2	2.0
c.1636C>A	Q546K	2	6.2	2.0
c.1633G>C	E545Q	1	3.1	1.0
c.1634A>C	E545A	1	3.1	1.0
Exon 20				
c.3140A>G*	H1047R	19	59.5	19.2
c.3140A>T	H1047L	1	3.1	1.0
Total		32	100	32.3 <sup>†</sup>
Selected group				
Exon 9				
c.1633G>A	E545K	3	9.7	4.0
c.1624G>A	E542K	2	6.5	2.7
c.1636C>G	Q546E	1	3.2	1.3 <sup>‡</sup>
Exon 20				
c.3140A>G	H1047R	23	74.2	30.7
c.3140T>G	H1047L	1	3.2	1.3
c.3136G>A	A1046T	1	3.2	1.3 <sup>‡</sup>
Total		31	100	40.0 <sup>‡§</sup>

\*One fibroadenoma sample, found to bear this mutation, is not included.

<sup>†</sup>Of the 99 tumor samples.

<sup>‡</sup>The two mutations c.1636C>G and c.3136G>A were detected in the same sample, and therefore counted as a single mutated sample.

<sup>§</sup>Of the 75 tumor samples.

Some MUT samples presented a transition close to the wild-type baseline spread. The threshold for calling these transitions as MUT was attributed to the shape of the curve. In the case of exon 9 (92-bp amplicon), the broader range of the transitions with respect to the wild-type baseline assisted in calling (Figure 3A). For exon 20 (70-bp amplicon), a shift to the right of the curve peak assisted in calling these samples as MUT (Figure 3B). Because the MUT proportion varies, melting transitions from the same genotype do not present cluster together. Nevertheless, samples with the same genotype were similar or identical in shape. HRMA results were highly reproducible (see Supplemental Figure S4A at <http://jmd.amjpathol.org>).

When we applied the developed methodology in a high-throughput format, using a 96-well plate format LightCycler 480 (II) instrument (Roche, Germany) (data not shown), melting transitions presented almost identical profiles to those obtained by the HR-1 instrument (See Supplemental Figure S4, B and C at <http://jmd.amjpathol.org>).

Finally, the developed methodology was applied in a selected group of 75 breast cancer patients treated with Herceptin. When scanning these 75 FFPE tumor samples, six samples in exon 9 and 25 in exon 20 were found to be mutated. All mutations were further defined by DNA sequencing (Table 2). Interestingly, one sample presented mutant transitions in both exons. These transitions were defined by DNA sequencing (See Supplemental Figure S3C at <http://jmd.amjpathol.org>) as c.1636C>G and c.3136G>A substitutions and were the only muta-

tions not encountered in the validation group. In total, 30/75 samples (40%) were detected as mutant. Only one wild-type FFPE sample in only one of the three PCR amplification experiments (exon 9) presented a transition wrongly assigned as mutant, to be verified as wild-type by sequencing.

## Discussion

HRMA was introduced in 2003 as a cost-effective, probe-free, rapid, closed-tube, and simple approach for genotyping and/or mutation scanning.<sup>18</sup> The ability of this technique to scan for mutations in samples from archival tissue specimens such as FFPE tissues is of high importance due to their ease of recovery and frequent use. However, FFPE samples present difficulties in PCR amplification and a reduction of diagnostic specificity when analyzed by HRMA.<sup>24,25</sup>

Herein, we report the development and validation of a highly sensitive and reliable HRMA mutation scanning methodology, using small amplicons, for hotspot regions of the *PIK3CA* gene. These mutations present a high prevalence in breast, as well as in other cancer types, and have an important clinical significance.<sup>35</sup> We validated this methodology on 129 FFPE tissue samples and managed to minimize their disadvantages during PCR and HRMA, as shown by comparison studies with DNA sequencing. We further applied this methodology on FFPE tissue samples from 75 breast cancer patients who underwent treatment with Herceptin. All mutations in this group were also verified by DNA sequencing. However, we avoid reporting any clinical significance of these findings, because this is beyond the scope of this article.

The two small amplicons were used to scan within hotspot regions in exons 9 and 20 of the *PIK3CA* gene that bear the three recurrent mutations. According to COSMIC database, these three recurrent mutations represent the majority (80%, 488/609) of the reported mutated breast cancer samples of the *PIK3CA* gene. However, within these regions the presence of several additional mutations has been reported. Our primer sequences are *in silico* designed to amplify a region spanning 92% (561/609) of the reported mutated breast cancer samples (Figure 1). Moreover, in respect to mutated samples reported for all cancer types, the regions cover 84% of mutations (1305/1558).

In addition to our effort to include all frequent mutations within the small amplicons, we also tried to avoid mutations on primer sites, because this could compromise PCR amplification. Thus, regions bearing reported mutations in breast cancer were completely avoided, while regions bearing mutations in all other cancers that we were unable to exclude from primer sites presented a negligible frequency (0.008–0.024%) (Figure 1).

The high wild-type baseline variation detected in the FFPE samples tested is generally present in such samples.<sup>24,25,36</sup> Additionally, reduction of diagnostic specificity has been reported when using HRMA of FFPE samples and is compounded by longer amplicons.<sup>25</sup> In our validation study, only one false positive of the 129 sam-

ples occurred, and this in only one of the three runs performed for this sample. We believe that minimization of false positive calls and wild-type baseline variation may be credited to the reduction of amplicon length, and the optimization performed in terms of maximizing PCR amplification efficiency, increasing the number of PCR cycles and reducing DNA input.

Of the 2411 breast cancer samples reported in the COSMIC database that have been tested for *PIK3CA* mutations, 609 (25%) bear mutations. The regions covered by the two small amplicons include 23% (561/2411). However, in our study, we report a mutant frequency of 32% (32/99) of tumor samples in the validation (unselected) group. This difference was statistically significant from the expected ( $P < 0.05$ , using binomial test). This could be attributed to the higher analytical sensitivity of our methodology. Specifically, the best ever reported sensitivity for DNA sequencing has been demonstrated as being 5%,<sup>37</sup> and as can be seen from our sequencing results (Figure 3; Supplementary Figure S3 at <http://jmd.amjpathol.org>), several mutant samples could easily be missed as wild-type. Our HRMA sensitivity study from the two small amplicons demonstrates an ability to detect as low as 1% of MUT alleles to total DNA, in a background of five different PB wild-type samples (Figure 2, A and C). On the contrary, larger amplicons have a lower analytical sensitivity (2.5% for 261-bp and 10% for 352-bp amplicon) (Figure 2, B and D), higher baseline variation, and increased difficulties in FFPE amplification. However, HRMA of FFPE tissue increases wild-type baseline variation, which may cause a reduction of analytical sensitivity. We estimate an analytical sensitivity of ~2.5 to 5% when scanning FFPE samples, for the two mutations for which a sensitivity study was performed. As the estimated analytical sensitivity of HRMA is higher than this of DNA sequencing, in the case of a low MUT/wild-type proportion that cannot be verified by sequencing, digital PCR techniques can be used to confirm the results. Although digital PCR could be a labor-intensive method, recent advances in microfluidics and real-time PCR allow a less laborious setting.<sup>38</sup>

In this study, we report for the first time a fibroadenoma sample mutated in the exon 20 of the *PIK3CA* gene (c.3140A>G), and it could be very interesting and instructive to monitor the disease outcome of this patient. Moreover, this result, along with recent findings demonstrating that *PIK3CA* mutations occur in preinvasive breast lesions<sup>39</sup> and present high prevalence in papillary breast lesions,<sup>40</sup> could have important implications with regard to therapeutic targeting of the PI3K pathway.

We also report for the first time c.1636C>A (validation group) and 3136G>A (selected group) substitutions in breast cancer. One of the tumor samples bearing a c.3140A>G mutation had a high percentage of the mutant allele (Figure 3B). This could be explained by a homozygous tumor sample contaminated with normal adjacent tissue or preferential gene amplification of the mutant allele.

In the selected group of 75 breast cancer patients who underwent Trastuzumab therapy, 40% (30/75) of the samples were detected by HRMA as mutants and were

all verified by DNA sequencing. The clinical importance of these findings with respect to the connection of *PIK3CA* mutations resistance to Trastuzumab is to be reported in the near future by an extensive statistical analysis and in combination with results from PTEN immunohistochemistry staining.

The developed HRMA methodology has many advantages because it is rapid, demanding less than one hour for PCR, and approximately two hours for high-resolution melting per 30 samples, when using the HR-1 instrument and a heating rate of 0.1°C/s. Moreover, it can be performed in a high-throughput HRMA instrument and apart from the (96- and 384-well) plate-format HRMA instruments available (LightScanner, LightCycler 480), the recent introduction of the LightScanner32 instrument could minimize the turnaround time of this methodology, using the same (glass capillary) format.

The developed HRMA methodology represents an inexpensive approach for the detection of *PIK3CA* mutations because reaction volume is limited to 10  $\mu$ l and does not require gene-specific probes. Samples are loaded for HRMA without postamplification processing, and because there is no destruction of the sample, we were able to forward PCR samples to sequencing. Although we were unable to undoubtedly define mutations by their melting transitions, we were able to reduce the amount of samples for sequencing down to ~13% (33/258) for the validation and ~21% (31/150) for the selected group.

The presence of *PIK3CA* mutations in many cancer types is connected with response to therapy. Furthermore, recent studies provide strong rationale for the clinical testing of combination therapies that target PI3K,<sup>41</sup> while additional inhibitors of PI3K are expected to become available. Therefore, rapid and reliable screening for *PIK3CA* mutations will be essential as a marker of response to therapy. HRMA methodology based on the amplification of small amplicons is highly sensitive, reliable, cost-effective, easy-to-perform, and can be used as a screening test before DNA sequencing in a high-throughput pharmacodiagnostic setting.

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