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Sensitive-Detection of PIK3CA Exon 20 H1047R Breast Cancer Based on Low-Cost Intercalary Dye SYBR Green I Real-Time qPCR Assay

(Pengesanan Sensitif PIK3CA Exon 20 H1047R Kanser Payudara Berdasarkan Pewarna Interkalari Kos Rendah SYBR Green I Ujian Asai qPCR Masa Nyata)

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ABSTRACT

Breast cancer is associated with an excessive function of somatic mutation in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA), specifically in exon 9 and 20 hotspots. Exon 20, which contains H1047R, has more potential for an oncogenic mutation than exon 9. Detection of PIK3CA/H1047R mutation has also been reported for molecular diagnosis and therapeutic application. Sensitive methods are required because mutants are mostly present at low levels in a mixture with the wild type. Therefore, this study aimed to explore the development of a low-cost and sensitive PIK3CA exon 20 H1047R detection method using intercalary dye real-time qPCR (quantitative polymerase chain reaction), SYBR Green I. The method was primer design, formulation, specificity, limit of detection, reproducibility, repeatability, and genotyping of 15 DNA (deoxyribonucleic acid)-frozen tissue patient samples, which were further confirmed by PCR sequencing for validation. DNA primer proportion formulations were 0.25 µM PIK3CA exon 20 (WT), 0.65 µM PIK3CA exon 20 H1047 (MT), and 0.2 µM reverse primer in 10 µl total volume. The results showed that compared to the gold standard genotyping PCR sequencing (6.6-20%), the developed method had a higher sensitivity of 5%. The coefficients of intra- and inter-variability were between 0.01-0.19%, suggesting the developed method was repeatable and reproducible with a low mean pipetting error. Genotyping of 15 DNA breast cancer patient samples showed a wild-type genotype, which was in 100% agreement with the PCR sequencing result. The developed method showed sensitive, reproducible, and repeatable results, potentially applicable in prognosis and therapeutic predictions.

Keywords: Breast cancer; Exon 20; H1047R; mutation; PIK3CA

ABSTRAK

Kanser payudara dikaitkan dengan fungsi berlebihan mutasi somatik dalam subunit pemangkin fosfatidilinositol-4,5-bisfosfat 3-kinase α (PIK3CA), khususnya di exon 9 dan 20 titik panas. Exon 20 yang mengandungi H1047R lebih berpotensi untuk mutasi onkogenik berbanding exon 9. Pengesanan mutasi PIK3CA/H1047R juga telah dilaporkan untuk diagnosis molekul dan aplikasi terapeutik. Kaedah sensitif diperlukan kerana mutan kebanyakannya terdapat pada tahap rendah dalam campuran dengan jenis liar. Oleh itu, kajian ini bertujuan untuk meneroka pembangunan kaedah pengesanan PIK3CA exon 20 H1047R yang kos rendah dan sensitif menggunakan qPCR masa nyata pewarna interkalari (tindak balas rantai polimerase kuantitatif), SYBR Green I. Kaedahnya ialah reka bentuk primer, formulasi, kekhususan, had pengesanan, kebolehasilan, kebolehulangan dan genotaip 15 sampel pesakit tisu DNA (asid deoksiribonukleik)-beku, yang

selanjutnya disahkan oleh penjujukan PCR untuk pengesahan. Formulasi perkadaran primer DNA ialah 0.25 µM PIK3CA exon 20 (WT), 0.65 µM PIK3CA exon 20 H1047 (MT) dan 0.2 µM *primer* songsang dalam 10 µL jumlah isi padu. Keputusan menunjukkan bahawa berbanding dengan penjujukan PCR genotaip piawaian emas (6.6-20%), kaedah yang dibangunkan mempunyai kepekaan yang lebih tinggi sebanyak 5%. Pekali intra-dan inter-kevariabelan adalah antara 0.01-0.19% menunjukkan kaedah yang dibangunkan boleh diulang dan boleh dihasilkan semula dengan ralat pipet min yang rendah. Genotaip 15 sampel pesakit kanser payudara DNA menunjukkan genotaip jenis liar yang 100% bersetuju dengan hasil penjujukan PCR. Kaedah yang dibangunkan menunjukkan keputusan yang sensitif, boleh dihasilkan semula dan boleh diulang, berpotensi digunakan dalam ramalan prognosis dan terapeutik.

Kata kunci: Exon 20; H1047R; kanser payudara; mutasi; PIK3CA

INTRODUCTION

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) is part of lipid kinase PI3K, which plays an important role in cell division and growth, apoptosis, and migration in breast cancer cells. Mutation of PIK3CA gene will enhance PI3K pathway in relation to oncogenic cellular transformation and cancer. Catalog of Somatic Mutations in Cancer (COSMIC) database shows that PIK3CA H1047R is the most common mutation found in human tumors, accounting for about 55%. Furthermore, approximately 25% of PIK3CA mutation was found in breast carcinoma and activated PI3K/AKT (protein kinase B)/mTOR (mammalian target of rapamycin) pathway. Two hotspots of PIK3CA were located in the kinase at exon 20 site (H1047R) and the helical domain at exon 9 sites of p110α (E542K & E545K). The hotspot mutations work synergistically but also independently (Arsenic et al. 2014; Hanker et al. 2013; Samuels et al. 2004; Zhao & Vogt 2008). Based on the molecular dynamic simulation, E454K mutant showed communication loss between regulatory and catalytic subunits caused by spontaneous releasing among both subunits. Meanwhile, H1047R showed a loss of crucial intermolecular interaction that affected mutant protein accumulating positive charge (Gkeka et al. 2014; Leontiadou et al. 2018).

PIK3CA exon 20 H1047R was shown as the most common mutation that increased lipid kinase activity (Campbell et al. 2004; Samuels et al. 2004; Yuan et al. 2013). The mutation has the potential to be more oncogenic since the apoptosis suppressor ability increased, as shown in the *in vivo* study compared to PIK3CA E545K (Meyer et al. 2013). Mutation in this area affected constitutively active and increased tumorigenicity in the xenograft model. Further xenograft mouse models could be used to understand tumors as well as the initiation and progression. The mouse could also be a model for the development of therapeutic strategies (Yuan et al. 2013). PIK3CA H1047R was reported to affect the drug resistance problem of anti-HER-2 therapies such as single trastuzumab or in combination with lapatinib or pertuzumab (Hanker et al. 2013). In triple-negative breast cancer, patients with mutations at this site are less likely to have a pathological complete response (Guo et al. 2020). Mutation at PIK3CA/H1047R also showed a potential biomarker for PIK3CA inhibitor, everolimus. Patients with this mutation, under everolimus treatment, demonstrated longer progression-free survival in hormone receptor-positive breast cancer patients (Yi et al. 2019). Therefore, detecting the mutation could provide both molecular diagnosis and therapeutic accuracy.

The divergence of mutations in the somatic gene PIK3CA H1047R in several countries in Asia has been identified, including Japan 6 out of 30 (20%) (Shimoi et al. 2018), Malaysia 2 out of 20 (10%) (Leong et al. 2008), Taiwan 15 out of 158 (9.5%) (Lai et al. 2008), India 31 out of 185(16.7%) (Ahmad et al. 2016) and China 30 out of 149 (20.1%) (Chen et al. 2018). Several methods have been developed for PK3CA H1047R detection, including high resolution melting (Daneshmand et al. 2012; Dirican et al. 2014), PCR RFLP (Polymerase Chain Reaction Restricted Fragment Length Polymorphism) (Li et al. 2016), Droplet PCR (Shimoi et al. 2018), allele-specific real-time qPCR (quantitative PCR) (Alvarez-Garcia et al. 2018), full-COLD PCR/HRM (full Coamplification at Lower Denaturation Temperature PCR/High-Resolution Melting) (Ghalamkari et al. 2019), LAMP (Loop-Mediated Isothermal Amplification) (Kalofonou et al. 2020), RCA (rolling circle amplification)-CRISPR/Cas12a (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 12a) (Cao et al. 2021), and SDA (Strand Displacement Amplification)-CRISPR/Cas12a (Deng et al. 2021). PCR sequencing is considered the gold standard for gene mutation screening, but the sensitivity is limited to 6-20%. Although next-generation sequencing has shown a better sensitivity of 5%, it is not recommended for routine use (Ihle et al. 2014). Considering mutant sequences are usually found at low levels in a mixture of wild-type DNA (deoxyribonucleic acid), finding a sensitive detection method could be challenging. This study performed allelic discrimination using low-cost intercalary dye SYBR Green I method. SYBR Green I is the most widely used economical reagent compared to other DNA-binding dyes in real-time qPCR reactions (Mote et al. 2021).

This study used two forward primers specific for the target allele and a common reverse primer developed in a single closed tube. Melting patterns and temperature were investigated to discriminate between the alleles. The method was validated by PCR sequencing and confirmation on DNA isolated from frozen breast cancer tissue. The specificity, sensitivity, repeatability, and reproducibility were also examined. The development of real-time qPCR with SYBR Green I can be used to reduce the cost of detection compared to real-time probe labeling (Wang et al. 2017). It showed high-sensitivity results and efficient time, without the need for post-PCR processing (Paudel et al. 2011).

MATERIALS AND METHODS

ETHICAL APPROVAL AND MATERIALS SAMPLES

The ethical approval number 053/PEP/01/2012 was approved by the Indonesian Ministry of Health, and informed consent was obtained from all individual participants included in this study. In addition, 15 DNA extraction samples from a tissue sample of breast cancer patients in West Sumatera Province, Indonesia, were isolated using the Purelink Invitrogen DNA Kit according to the manufacturer's protocol. The purified DNA was confirmed with A260 and A280 values between 1.8 and 2.0.

PRIMER DESIGN

The primer sequence for specific PIK3CA exon 20 H1047 (MT) forward orientation contains 14 base GC-rich tails in the 5' area (bold nucleotide): 5' GCGGGCAGGGCGGC GAAACAAATGAATGATGCGCG 3'. At the 3 bases from 3' orientation, one mismatch was introduced where A was changed to G, and forward primer for PIK3CA exon 20 (WT) also introduced 6 GC rich tails (bold nucleotide): 5' GCGGGC GAAACAAATGAATGATGCACA 3'. Reverse primer that amplified both alleles was 5' TTT AAT TGT GTG GAA GAT CC 3'. Introducing GC tails for both forward primers was based on Wang et al. (2005) to increase the ability of the method to discriminate PCR products by temperature melting.

PIK3CA EXON 20 H1047 (MT) AND PIK3CA EXON 20 (WT) DETECTION DEVELOPMENT BY QUANTITATIVE REAL-TIME qPCR

PCR sequencing confirmed that each plasmid contained MT and WT. Real-time qPCR was optimized by comparing up to three different primer ratio formulations. The formulation ratios of WT:MT: reverse primer were 1 (0,25: 0,65: 0,2), 2 (0,26: 0,65: 0,2), and 3 (0,275: 0,65: 0,2). Real-time qPCR for each primer ratio formulation was carried out using two PCR tube sets, one containing WT plasmid and the other MT plasmid as DNA template. Each PCR tube set consists of three repetitions and as a negative control, DNA template was replaced by MQ, also with three repetitions. All tubes contain the same PCR mix and DNA primer ratio for each formulation. A total of 10 µL in one PCR tube was used with 5 µL KAPA SYBR Fast qPCR master mix # KK4600 KAPA (Biosystems), DNA template of 1 µL (MT or WT), Forward primer for H1047R, and PIK3CA exon 20, as well as reverse primer. TE (Tris-EDTA) Buffer pH 7.8 was adjusted until 10 µL, while the thermal cycling program consisted of initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C denaturation at 30 s, and 62 °C annealing at 30 s. Melt curve and peak analysis were performed immediately with a melting rate value of 0.2 °C/5 s, starting from 65 °C up to 95 °C. Melting peaks were plotted by the -dF/dT against the T formula (F is fluorescence, T is temperature). Real-time qPCR result was verified in 2% agarose at the start of the detection development, to ascertain that the correct size of each target was obtained. The best formulation DNA-primer ratio was further validated by PCR sequencing. Fifteen DNA samples isolated from breast cancer patients were genotyped using the developed method and validated against PCR sequencing.

SPECIFICITY AND SENSITIVITY ASSAY

The specificity and sensitivity assays were performed by varying the mix of MT and WT plasmid with a combination at 100%, 50%, 25%, 10%, 5%, 1%, and 0% for MT. DNA concentration of the sample was 1 ng/µL. As a negative control, DNA template was replaced by Milli-Q Water (MQ). The activities were performed in three repetitions for intra-assay repeatability of real-time qPCR and similar activities were carried out the following day for reproducibility.

LIMIT DETECTION

Limit detection was performed starting from 1 ng at 10x dilution to 0.0001 ng of DNA sample concentration. PCR detection limit study was determined from Cycle Threshold (CT) values of the serial plasmid dilutions. As a negative control, DNA template was replaced by MQ. The activities were also performed in three repetitions.

DATA ANALYSIS INTER AND INTRA-ASSAY VARIABILITY

The specificity of the developed method was analyzed descriptively based on melting curve and pattern analysis confirmed by 1% agarose electrophoresis. The repeatability and reproducibility were assessed using inter- and intra-assay variability in Microsoft Excel. Intra- and inter-assay CT values were used to calculate the mean, SD (Standard Deviation), and CV (Coefficient of Variation).

RESULTS AND DISCUSSION

PIK3CA protein was found as a common mutation in the breast, prostate, colon, and endometrium. The most commonly observed PIK3 mutations are E542K, E545K, and H1047R. These mutations reportedly induce high efficiency of oncogenic transformation by increasing lipid kinase activity of p110α and have a poor prognosis in breast cancer with anti-HER2 agents. H1047R was most frequently detected and also predicted as a stronger driver mutation compared to others. Furthermore, mutation in H1047R has significantly the worst overall survival (Arsenic et al. 2014). It increases the amplification of downstream signaling affected to cellular transformation (Kang, Bader & Vogt 2005). Mutations in H1047R were also reported to affect the drug resistance problem of anti-HER-2 therapies. In triple-negative breast cancer, patients with mutations at this site are less likely to have a complete pathological response. Moreover, mutation at H1047R is a potential biomarker for everolimus treatment due to the characteristic longer progression-free survival in hormone receptor-positive breast cancer patients (Yi et al. 2019).

SYBR GREEN I QUANTITATIVE REAL-TIME qPCR DETECTION AND ANALYSIS OF PIK3CA H1047R MUTATION

Target amplification of PIK3CA H1047R or WT was conducted using KAPA SYBR Fast qPCR Master Mix # KK4600 (KAPA Biosystems). To increase specificity of the primers for WT and MT discrimination, an additional GC-rich tail was added, following Wang et al. (2005). For MT primers, mismatches were added at the third base from the 3' end. Due to the limited sample, recombinant plasmids were used for method development, namely pGEMT-easy_PIK3CA_ex20_H1047R (MT) and pGEMT-easy_PIK3CA_ex20 (WT), which were confirmed by PCR sequencing and available in the laboratory. All SYBR Green I quantitative real-time qPCR formulations successfully discriminated between WT and MT, with no significant melting curve pattern and temperature differences. CT value of MT showed only in PCR tube with MT plasmid as DNA template at melting temperature of around 81 °C. Meanwhile, CT value of WT showed only in PCR tube with WT plasmid as DNA template at 77 °C. The primer design allowed specific amplification for each target with a sharp melting pattern. Based on the result, the first primer formulation containing 0.25 μM PIK3CA exon 20 forward primer, 0.65 μM PIK3CA exon 20 H1047R forward primer, and 0.2 μM universal reverse primer were selected. NTC showed no CT value and no melting curve. This implies that there was no PCR contamination, no self-amplification, and no primer dimer formation. Furthermore, the designed primer had high target specificity and no cross-template binding. In addition, sensitivity, specificity, repeatability, and reproducibility were tested. The discrimination of WT and H1047R with this system was based on melting temperature and pattern that perfectly discriminated between mutant and wild type, confirmed with 2% agarose. The 2% agarose confirmation was only performed at the start of the detection development, not for routine activity. Considering mutant has a longer, additional GC-rich tail, the band showed an increased size of about 20 bases when confirmed with 2% agarose (Figure 1). According to Bustin et al. (2009), qPCR specificity must be validated empirically using electrophoresis gel, melting profile, DNA sequencing, amplicon size, and restriction enzyme digestion.

For MT primer, one mismatch was introduced at the third from the 3' end and also at the last base at the 3' end base, which recognizes the mutated base for MT recognition only. An additional mismatch at the third base from 3' end will significantly affect the amplification target compared to the fifth base from the 3' end, where at least two mismatches are required to prevent non-target amplification (Appelt et al. 2019). In addition, different mismatch sites have varying effects on allelic discrimination. Based on statistical data, a mismatch (CA) in the third nucleotide from the 3' showed 81.9% specificity (Liu et al. 2012). A previous study proved that these positions are the best for allelic discrimination (Desriani & Al-Ahwani 2018; Desriani et al. 2021). Aside from introducing the mismatch, for each F_w primer that recognized PIK3CA exon 20 and H1047R, GC-rich tails of different sizes were added following Wang et al. (2005). Considering both PCR products have different T_m depending on length and GC content, this shifts melting temperature, which could be used to recognize and identify each product. This explains why the developed method is effective for discriminating between PIK3CA exon 20 and H1047R.

The developed method was tested on DNA genome of breast cancer patients extracted from frozen tissue samples and validated with PCR sequencing. In practical terms, the application of the method needed only a closed single tube to discriminate between MT and WT. A total of 15 frozen patient samples were randomly selected, and PCR sequencing was performed for the validation study. Based on screening, all patients were found to have WT genotype, which was validated with gold-standard genotyping (PCR sequencing) and showed 100% agreement (Table 1). PIK3CA exon 20 has no pseudogene, in contrast to exon 9, which reportedly contains a pseudogene on chromosome 22q11.2 in the cat eye syndrome region (GI 5931525) and on chromosome 16 (GI 28913054). The absence of the pseudogene provides an advantage for PIK3CA exon 20 H1047R detection, avoiding false positive or negative results.

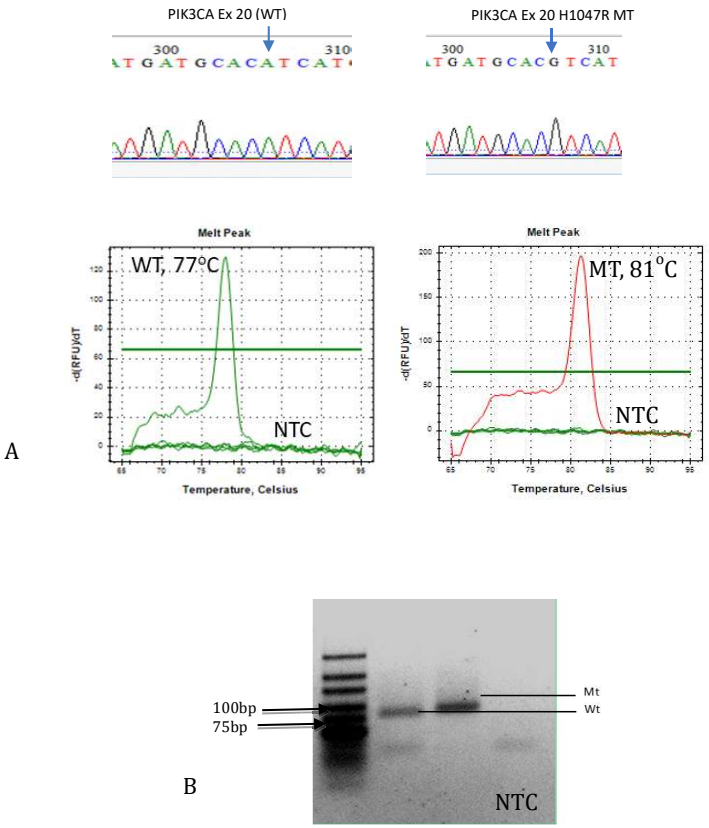


FIGURE 1. Development of PIK3CA H1047R detection by real-time qPCR with the intercalary dye SYBR Green I using the recombinant plasmids MT and WT as DNA templates. (A) DNA sequence and melting patterns of PIK3CA WT (left) and MT (right); (B) 2% agarose to confirm each real-time qPCR result

TABLE 1. Comparison of PIK3CA H1047R real-time qPCR and PCR sequencing methods

Sample Code	Detection with PCR seq	Detection with real-time qPCR
1	WT	WT
2	WT	WT
3	WT	WT
4	WT	WT
5	WT	WT
6	WT	WT
7	WT	WT
8	WT	WT
9	WT	WT
10	WT	WT
11	WT	WT
12	WT	WT
13	WT	WT
14	WT	WT
15	WT	WT

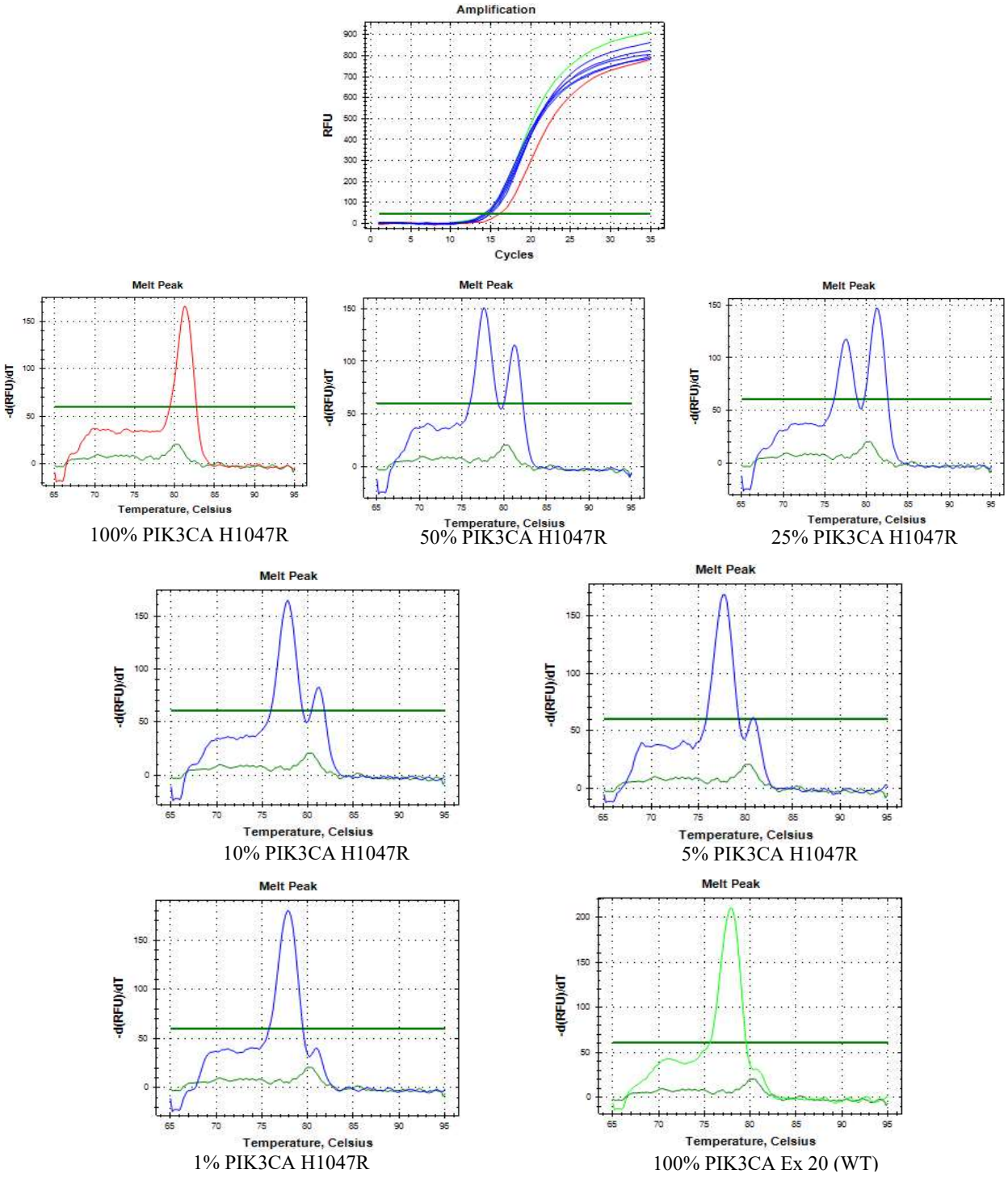
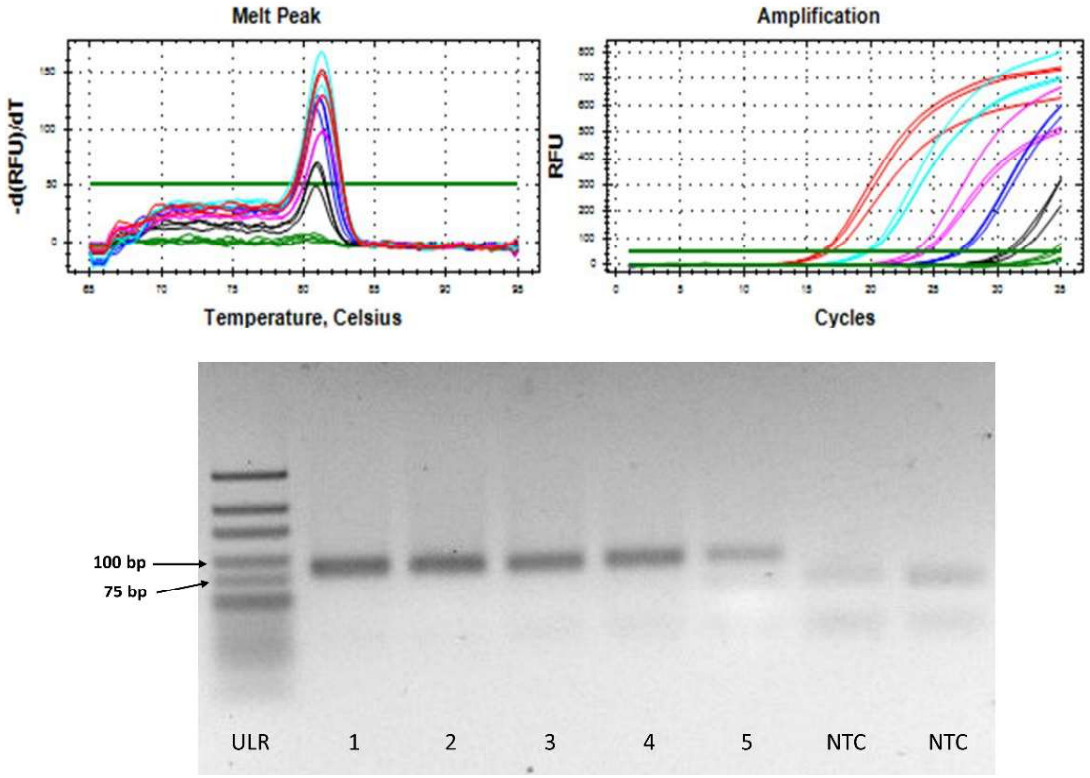


FIGURE 2. Specificity and sensitivity of the detection of PIK3CA H1047R when MT DNA plasmid is mixed with WT DNA plasmid at a ratio of 100%, 50%, 25%, 10%, 5%, 1%, and 0% for MT, respectively



1. 1; 2. 0.1; 3. 0.01; 4. 0.001; 5. 0.0001 ng/µL)

FIGURE 3. The limit detection study of PIK3CA H1047R starts at 1 ng/µL with 10x dilutions from left to right (top) and its 1% agarose confirmation (ULR: ultra-low range DNA ladder, 1. 1; 2. 0.1; 3. 0.01; 4. 0.001; 5. 0.0001 ng/µL)

TABLE 2. CV intra- and inter-assay variability study

Category	Mix MT in WT DNA (%)	Mean (Ct)	SD	CV (%)
Intra-assay	100	16.62	0.61	0.03
	50	15.81	0.17	0.01
	25	15.57	0.44	0.02
	10	15.04	0.18	0.01
	5	15.84	0.34	0.02
	1	15.48	0.14	0.01
	0	15.57	0.38	0.02
Inter-assay	100	16.48	0.188	0.01
	50	15.39	0.59	0.03
	25	15.28	0.4	0.02
	10	14.75	0.41	0.02
	5	15.24	0.84	0.05
	1	14.75	1.03	0.07
	0	15.79	0.31	0.01

SYBR GREEN I QUANTITATIVE REAL-TIME qPCR SPECIFICITY AND SENSITIVITY

Figure 2 shows that mixed DNA plasmid samples could be differentiated between MT and WT. In PCR tubes with 100% WT plasmid, a sharp melting curve at a temperature of 77 °C was observed, while in PCR tubes with 100% MT, a sharp melting curve at a temperature of 81 °C was observed. Tubes containing mixed MT and WT recombinant plasmid with different proportion percentages showed two peaks of melting curve around 77 °C and 81 °C. Furthermore, proportional percentages of 100%, 50%, 25%, 10%, 5%, 1%, and 0% for MT could differentiate WT and MT mixed up to 5% mutant. This implies that the developed method can detect a 5% mutant among 95% wt. It showed high specificity and sensitivity for discriminating between WT and MT compared to the gold standard method of PCR sequencing. Sensitivity of PCR sequencing was 6.6-20%, while that of the developed method was 5%.

SYBR GREEN I QUANTITATIVE REAL-TIME qPCR LIMIT DETECTION, REPEATABILITY, AND REPRODUCIBILITY

Using the prepared plasmid, PCR detection limit can be observed down to 0.0001 µg/µL (Figure 3). The inter- and intra-variability for the developed method was between 0.01 - 0.19 and 0.01 - 0.1, respectively (Table 2). CV value ≤ 5% indicates that the data is qualified and acceptable (Zheng et al. 2019). All these values showed that the developed method has precision and robustness repeatedly on different days, which means accurate pipetting, high repeatability, and significant reproducibility.

A previous study proved the success of the developed detection method using DNA plasmids, which also showed consistent results for DNA genome patient detection (Desriani & Al-Ahwani 2018; Desriani et al. 2021). In general, DNA plasmids are comparable to the genome for target genotyping and have produced similar results. Han et al. (2023) reported that synthetic fragments, plasmids, or genomic DNA extracted from soil were comparable to quantitative PCR standards for the quantification of microbial genes. The synthetic DNA fragment was more time- and cost-efficient. In this study, the detection method was also developed using DNA plasmids due to limited samples. Random screening showed that all samples had WT genotype with a consistent 100% PCR sequencing result. This method proved to have high specificity, sensitivity, repeatability, and reproducibility. DNA genome of MT (PIK3CA exon 20 H1047R) genotype has a high probability of being detected as mutant, with CT value, and melting temperature of around 81 °C. Further studies should collect a larger number of samples to improve data quality.

The developed method showed simple, specific, sensitive, and repeatable results compared to the previous methods, such as PCR RFLP for PIK3CA H1047R detection, which requires post-PCR modification and has a high risk of contamination (Li et al. 2016) or detection with digital droplet PCR, which has expensive chemical reagents and equipment (Borkowska et al. 2021). Considering the method was developed in a single closed tube, it is possible to screen a large number of samples, which saves time and is cost-effective. Furthermore, the method can potentially be applied to prognosis and predictive therapy in different breast cancer subtypes. Oncologists may use the method to improve diagnosis and the quality of breast cancer treatment.

CONCLUSION

In conclusion, this study successfully developed a specific and sensitive detection method for PIK3CA exon 20 H1047R, considered the most oncogenic mutation and also a predictive factor for breast cancer therapy. The developed real-time qPCR SYBR Green I method proved to be fast, simple, highly sensitive, repeatable, and reproducible, as well as cost-effective for PIK3CA H1047R genotyping. DNA primer proportion formulations were 0.25 µM PIK3CA exon 20 µM (WT), 0.65 PIK3CA exon 20 H1047 (MT), and 0.2 µM reverse primer in 10 µL total volume. Compared to the gold standard genotyping PCR sequencing (6.6-20%), the developed method had a higher sensitivity of 5%. The coefficients of intra- and inter-variability were between 0.01-0.19%, suggesting the developed method is repeatable, reproducible, and has low intra- and inter-variability with a low mean pipetting error. Genotyping of 15 DNA breast cancer patient samples showed a wild-type genotype, which was in 100% agreement with PCR sequencing result. In addition, the developed method can potentially be applied to prognosis and therapeutic predictions.

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