Improvement of HER2^{I655V} TARMS-PCR Performance by DNA Quality Analysis

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Abstract

Reliable TARMS-PCR is a prerequisite in constructing a solid conclusion in genetic diagnostics. The validity of data generated by this molecular technique in most cases is hampered by a false positive result. In attempt to develop a TARMS-PCR for HER2^{I655V} genotyping with no interfering of bias we used DNase I to eliminate DNA contaminant resided in PCR reagent. TARMS-PCR with no enzymatic pretreatment on PCR master mix kit produced a false positive result on HER2^{I655V} TARMS-PCR using as recombinant plasmids system model proven by the presence of multiple PCR products in Non-Template Control (NTC). A dose of 0.1 U of the enzyme could eliminate this DNA contaminant effectively, although this pretreatment altered the specificity of HER2^{I655V} TARMS-PCR genotyping on certain genotype. Combination of touchdown TARMS-PCR with another allele-specific primer recovered specificity of detection on this model system. Interestingly, this optimized HER2^{I655V} TARMS-PCR can only be used for genotyping the clinical samples if only further optimization was done using genomic DNA as template.

Keywords: TARMS-PCR, HER2^{I655V}, DNase I, Polymorphism

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Introduction

TARMS-PCR (Tetra Amplification Refractory Mutation System-Polymerase Chain Reaction) is one of DNA amplifying-based molecular method used for detecting any type of a base change in the genome. This technique uses four primers in which two of them, named inner primers are engineered for a base at 3 end position of primer so that mismatch with a corresponding base which represents polymorphism or mutation within genome. Under appropriate condition this mismatched 3'-residue in inner primers will not function as primers in the PCR (Newton *et al.*, 1989). Additional mismatched bases are also introduced at penultimate or at bases number 3 from 3 end position of primer in order to increase detection accuracy (Tanha *et al.*, 2015). TARMS-PCR has been used widely in medical, agricultural and food science for genotyping of the genes that play an important role in certain pathological condition, quality improvement of the traits, or foodborne pathogen (Etlik *et al*., 2011; Dubey *et al*., 2012).

Recently, the development of TARMS-PCR as molecular tools for genotyping is very popular among researchers, not only claimed giving high accuracy but also technically this method offering rapidness, simplicity, and cost-effective detection. Randawa *et al.* (2017) have developed TARMS-PCR for genotyping SNP rs12303764(G/T) of human Unc-51 like kinase 1t gene and showed 100% concordance with DNA sequencing result which is a gold standard method for genotyping. Another study has evaluated the performance of TARMS-PCR versus TaqMan Real-Time PCR in diagnosing

Rs 8066560 polymorphism giving complete concordance of both methods compared (Miranzadeh-Mahabadi *et al.*, 2015). TARMS-PCR also produces a consistent result when it was compared to RFLP-PCR to detect polymorphisms of the CYP2E1 Gene (Suhda *et al.* 2016). These evidence highlight that TARMS-PCR is reliable, accurate and simple technique and it can be used as a complementary method for diagnostics cases or in performing large-scale epidemiological studies.

Although research on this technique is very rapidly growing and giving a promising result as supported by massively published articles, yet only a few studies have concerned on the importance of good quality of method in TARMS-PCR development (Baris *et al*., 2010; Aloui *et al*., 2015; Furtado and Rabelo, 2015). In fact, methodological errors during method development approximately contribute to incorrect detection as much as 14 up to 58% (Hosking *et al*., 2004). This bias in genotyping analysis mostly happens due to false PCR product as result of lacking the laboratory quality assurance programs (Lievano *et al.*, 2002). Adapting a protocol to obtain high purity of nucleic acid during extraction step is an effective strategy to eliminate false-negative PCR reaction caused by inhibitors, while more complex approach is applied to eliminate false-positive PCR reaction such as physical, chemical and biochemical treatments into the apparatus or PCR reagents which were used in PCR activity (Borst *et al*., 2004; Schrader *et al*., 2012).

Contaminating DNA molecules is the most common problem not only in the development of a reliable nucleic acid amplification-based molecular detection (Muhl *et al*., 2010), but also in detection of genetics-related particular conditions using established molecular methods (Salter *et al*., 2014). In particular, genetics analysis using PCR methodology has been hampered by the presence of microbial DNA contained in PCR reagents that significantly give an impact on data validity generated (Silkie *et al*., 2008; Czurda *et al*., 2015; Glassing *et al*., 2016). In fact, several population studies, with different background of races, have highlighted the important role of HER2^{I655V} gene (HGVS:NC_000017.10:g.37879588A > G, dbSNP: rs1136201) in pathogenesis of breast cancer (Xie *et al*., 2000; Lu *et al*., 2010). On the contrary, these findings were argued due to laboratory artifacts, bad experiment procedure, or publication bias (Dahabreh and Murray, 2011). Hence, an effort to eliminate this type of contamination is an essential step in developing TARMS-PCR for HER2^{I655V} SNP detection in our study. The purpose of this study are (1) to identify the source of PCR contamination, (2) to decontaminate PCR reagent using DNase I treatment, and (3) to optimize TARMS-PCR.

Materials and Methods

Materials. DreamTaq Green PCR Master Mix (2X) and O'gene ruler ultra low ranger DNA ladder were purchased from Thermo Fisher Scientific Inc, USA. RNase-free DNase I (2U/μL) was purchased from geneaid Biotech Ltd, Taiwan. All primers used in this study were purchased from Integrated DNA Technology Inc, USA. Betaine was purchased from Sigma-aldrich Inc, USA and was stored as 10 M stock in ddH_2O at -20^{o}C. Primers for TARMS-PCR were constructed using batchprimer3 software (You *et al*., 2008) as listed in figure 1. An additional primer from previous work (Budiarto & Desriani, 2016) was also included in this study for further TARMS-PCR optimization. pGEM-Ts containing HER2 gene fragment that each harbor A or G allele of HER2^{I655V} have been constructed in our laboratory. HER2 gene fragment with G allele was inserted into pGEM-T generating recombinant plasmid in a size of ~3.5 kbp, while the other allele was inserted into the same vector generating recombinant plasmid in a size of~5.3 kbp.

Figure 1. Primers used in this study

Name	Nucleotide sequence	Ref.	Purpose
Outer_HER2_F	5'-ACCCTTCCGACTTCCCTTTC-3'	In this study	for TARM-PCR optimization using pGEM recombinant as DNA template. These primers were constructed using batchprimer3 software
Outer_HER2_R	5'-TGTACTTCCGGATCTTCTGCTG-3'		
InWTHER2_1_F	5'-CAGCCCTCTGACGTCCATAA-3'		
InMTHER2_1_R	5'-GCCAACCACCGCAGAGGC-3'		
InWTHER2_2_F	5'-CCAGCCCTCTGACGTCCAGCT-3'	In this study	together with Outer_HER2_F/R and InMTHER2_1_R For TARMS-PCR evaluation using genomic DNA as template
HER2AA_F	5'-CCAGCCCTCTGACGTCCAGCA-3'	(Budiarto & Desriani, 2016)	

DNA extraction from patients breast cancer tissue. Genomic DNA was obtained from frozen sections of breast tissue samples collected from M. Djamil Hospital Padang, West Sumatera Province. Genomic DNA was then extracted following manual tissue DNA extraction protocol (Pure Link Genomic DNA Mini Kit; Invitrogen, Thermo Fisher Scientific Inc, USA). The types of HER2^{I655V} alleles of this DNA have been confirmed using Sanger DNA sequencing (unpublished data). Two genomic DNA that represent for A allele or G allele of HER2^{I655V} then were chosen for the optimized TARMS-PCR evaluation.

TARMS-PCR for HER2^{I655V} to detect reagent contamination. DNA contamination was evaluated by doing Tetra ARMS-PCR on each gene in which different ratio of primers (inner primer to outer primer) were used. The PCR reagent contained 6.25 µL 2x master mix PCR, primers with a different ratio (1:4 and 1:6), 0.16 ng of plasmid DNA template and ddH_2O up to 12.5 µL. PCR profile for this purpose is as follow: Pre- denaturation at 95 ºC for 5 min followed by 35 cycles of PCR with denaturation at 95 ºC for 20 sec, annealing temperature -it was as temperature gradient ranging from 54.4ºC up to 59.3ºC- for 30 sec and, and extension at 72 ºC for 30 sec (Kyratec Super Cycler Thermal Cycler, Australia). All PCR tubes, distilled water, pipette tips, and pipettes were pretreated by exposing them on UV-light for 15-20 minutes prior to use. All PCR reagent mixing was done under laminar air flow. DNA electrophoresis was done on PCR product using 3% of agarose for 30 minutes and, stained on EtBr solution then visualized on UV-transilluminators.

PCR reagent decontamination by DNase I treatment. As much as 62.5 µL of DreamTaq Green PCR Master Mix (2X) was added with enzyme to obtain final concentration of 0.1 and 0.2 U enzyme/reaction with or without the addition of DNase I reaction buffer. DNA digestion was done by incubating the solution at 37ºC for 1 hour then the reaction was stopped by increasing the temperature up to 95ºC for 1 hour.

TARMS-PCR optimization. DNase I–treated PCR master mix reagent was included in PCR solution using PCR formulation as mentioned above with ratio of primer was fixed at 1:4. Betaine was added during the optimization step and was applied as much as 0.5 M and 1 M. Touchdown PCR approach was also used in attempt to obtain TARMS-PCR with more specific result (Aloui *et al*., 2015) with profile as followed: Pre-denaturation at 96 ºC for 5 min followed by first 10 cycles with denaturation at 95 ºC for 20 sec, annealing temperature from 65 ºC down to 56.8 ºC for 30 sec and extension at 72 ºC for 30 sec. The remaining 25 cycle is

consisting of denaturation at 95 °C for 20 sec, annealing temperature at 56.8°C for 30 sec, and extension at 72 °C for 30 sec. Optimized TARMS-PCR was tested on genomic DNA templates that contain each type of allele variant of HER2^{I655V} gene where pre-denaturation and denaturation temperature were set at 96°C.

Results

HER2^{I655V} TARM-PCR strategy. TARMS-PCR to detect polymorphism of HER2^{I655V} as illustrated in figure 1 have developed. Initially, primers were constructed based on original primer settings provided in the software. As suggested, A/G as a target SNP at codon 655 of HER2 gene must first be converted to R to be computed by the software. The data obtained showed no any primers could be generated by this parameters. Another strategy in which R was changed with a code of SNP other than Y (T/C) to overcome this obstacle was applied. The data generated has succeeded to produce primers as listed in table 1. To increase the specificity of detection, destabilization strength approach as suggested by Bui and Liu, (2009) was used to replace penultimate base of both inner primers with G/A due to a weak destabilizing effect produced by SNP mismatch at 3'end terminal base of primers (AC/GT).

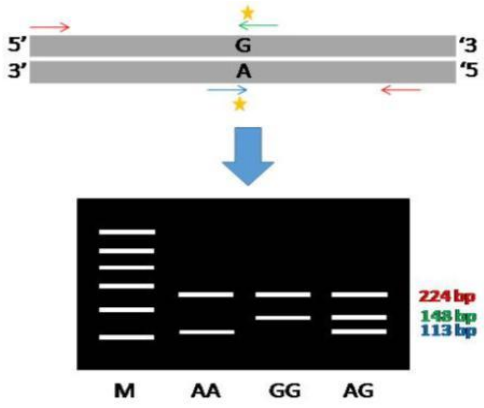
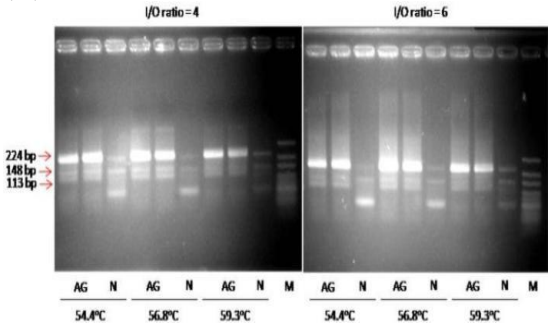


Figure 1. Illustration of HER2^{I655V} TARMS-PCR. Stars represent SNP position for [A/G] in HER2 gene, Red arrows represent a pair of outer primers that amplify HER2 gene in the size of 224 bp, green arrow represents allele-specific primer for G allele paired with forward primer of outer primer producing amplicon in the size of 148 bp, blue arrow represents allele-specific primer for A allele paired with reverse primer of outer primer producing amplicon in the size of 113 bp.

HER2^{I655V} TARMS-PCR contamination. PCR reagent contaminated by foreign DNA molecules could be detected in a HER2^{I655V} TARMS-PCR product without adding the DNA template (Fig. 2) as multiple PCR products due to unspecific binding of primers on contaminating DNA molecules. Even, this unspecific PCR appeared on NTC can not be eliminated by either reformulating the primer ratio (Fig. 2A) or increasing the temperature melting of PCR Fig. 2B). This result shows that PCR master mix reagent used in HER2^{I655V} TARMS-PCR contained contaminating DNA molecules.

(A)



(B)

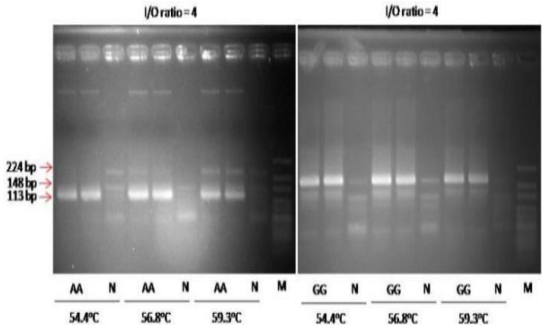


Figure 2. PCR contamination occurred during HER2^{I655V} TARMS-PCR optimization. False positive amplification in all sample N (Non-Template Control) was produced either in (A)

optimization step using mixed plasmid templates that each contained variant allele of HER2^{I655V} or (B) using each plasmid template at defined I/O primer ratio.

Elimination of contaminating DNA molecules from PCR reagent using DNase I. As much as 0.1 U enzyme/ PCR reaction can eliminate DNA contamination from reagent as shown by no any unspecific PCR product developed in NTC after HER2^{I655V} TARMS-PCR genotyping (Fig. 3A). To evaluate the impact of DNase I treatment on HER2^{I655V} TARMS-PCR accuracy, the recombinant plasmid for both variant allele was tested separately and in a mixed template. As shown in figure 3B, DNase I not only altered the specificity of detection, especially in TARMS-PCR with recombinant pGEM-T that contains GG genotype of HER2^{I655V}, but also eliminated the internal control PCR product in case of TARMS-PCR with recombinant pGEM-T that contains AA genotype of HER2^{I655V} gene.

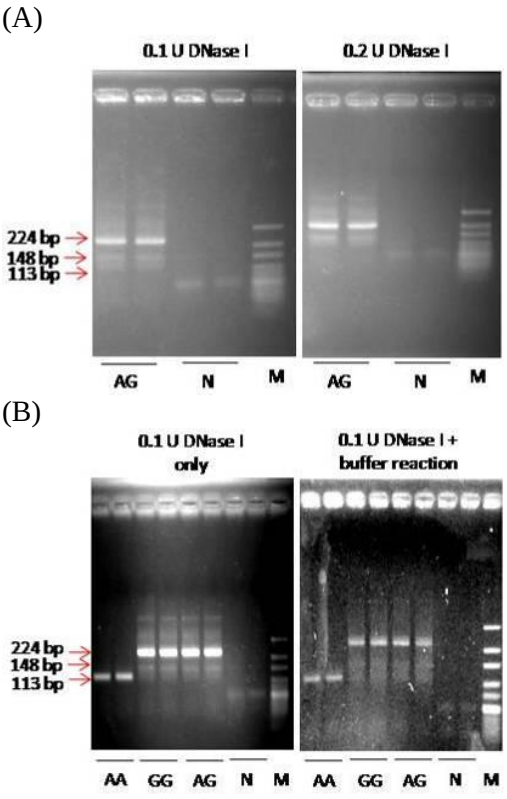


Figure 3. Elimination of PCR reagent master mix contamination by DNase I treatment. (A) TARMS-PCR of the mixed template after reagent DNase treatment using 0.1 U or 0.2 U enzyme for 1 hour incubation followed by 95^{o}C for 1 hour for enzyme inactivation. (B) TARMS-PCR using the template with represented HER2^{I655V} genotype in reagent contained 0.1 U inactivated DNase I.

Strategies to recover the specificity of HER2^{I655V} TARMS-PCR. Modification in PCR formulation and PCR profile were applied for this purpose. Betaine at 1 M recovered only the specificity of HER2^{I655V} TARMS-PCR for GG genotype but eliminate PCR product of 113 bp in TARMS-PCR product with a mixed pGEM-T template. No significant effect of betaine in recovering the internal control in TARMS-PCR product of pGEM-T template with AA genotype (Fig. 4A). Touchdown-thermal profile approach only recovered the specificity of the HER2^{I655V} TARMS-PCR result when the inner primer with forward direction has been changed as showed in figure 4B.

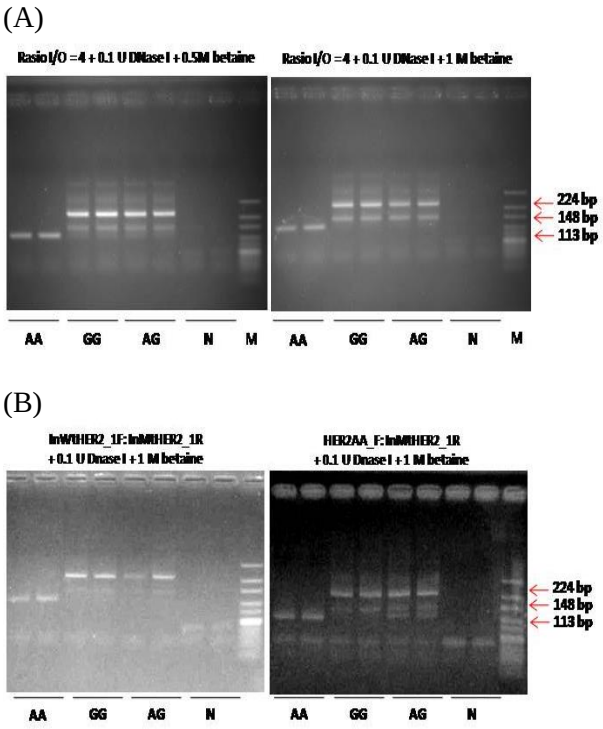


Figure 4. Effect of modified TARMS-PCR conditions on the accuracy of HER2^{I655V} SNP-containing plasmids genotyping. (A) HER2^{I655V} TARMS-PCR was done using standard TARMS-PCR profile. (B) HER2^{I655V} TARMS-

PCR was done using touchdown TARMS-PCR profile.

Evaluation of the optimized HER2^{I655V} TARMS-PCR using genomic DNA from clinical samples. The optimized condition for genotyping the polymorphism of HER2^{I655V} as obtained from recombinant plasmids genotyping using TARMS-PCR as mentioned in the previous result was applied to test the variant alleles of HER2^{I655V} in clinical samples. With this condition, only two type of genotypes, AA and GG genotypes, can be detected with limit detection ranging from 10 to 20 ng of template (Fig. 5). This result indicated that the plasmid-based TARMS-PCR optimization can not be directly used for genotyping the HER2^{I655V} gene from the more complex DNA forming such as genomic DNA.

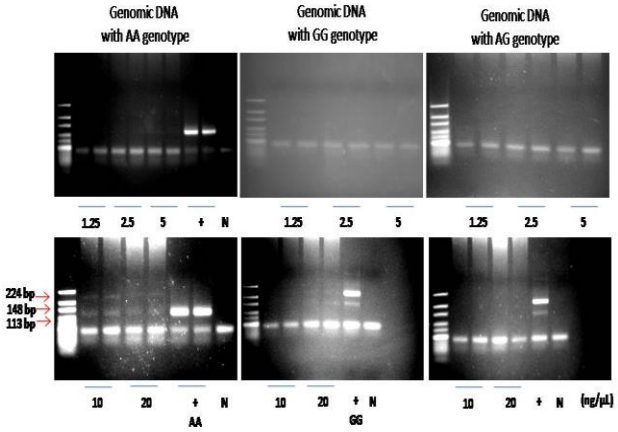


Figure 5. HER2^{I655V} TARMS-PCR on clinical genomic DNA samples using optimized protocol obtained from genotyping the recombinant pGEM-T HER2^{I655V}gene.

Further optimization of HER2^{I655V} TARMS-PCR using genomic DNA template. Increasing the denaturation temperature only give specificity of detection for PCR product with AA or GG genotype, while PCR product with a size of 148 bp was failed to be amplified in mixed DNA template (Fig. 6A). Changing the inner primer for forward direction eliminate PCR product with a size of 113 bp both in genomic DNA with AA genotype and in mixed genomic DNA as template (Fig. 6B). The specificity of HER2^{I655V} TARMS-PCR was fully recovered only when two types of inner primer with forwards direction were mixed in equal molar with keeping the I/O primer ratio of 1:4 (Fig. 6C).

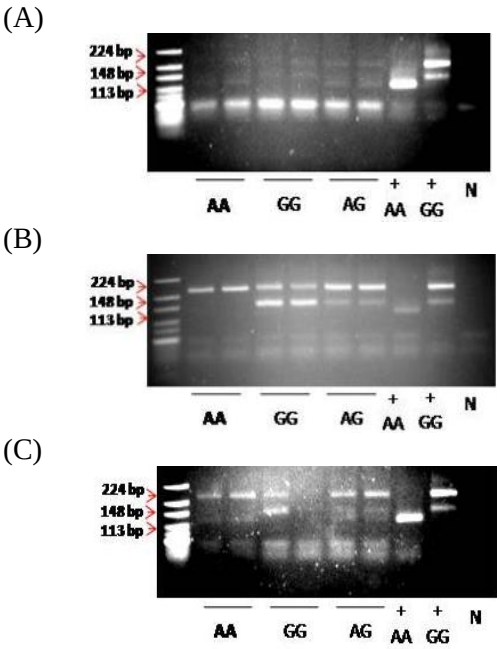


Figure 6. HER2^{I655V} polymorphism detection by touchdown TARMS-PCR on genomic DNA samples. 10 ng of genomic DNA template was used per PCR reaction. Mixed genomic DNA to obtained AG genotype was done by mixing 5 ng of genomic DNA with AA genotype with 5 ng of genomic DNA with GG genotype. (A) inner primer in forward direction used primer HER2AA_F, (B) inner primer in forward direction used primer InWTHER2_2_F, (C) inner primer in forward direction used mixed of HER2AA_F and InWTHER2_2_F with total concentration was 0.4 µM.

Discussion

Constructing the allele-discriminating primers is a critical step in the success of TARMS-PCR development. Currently, Batchprimer3 is one of a popular web-base program accessible to construct primers for this purpose (Islam *et al.*, 2014; Soler *et al*., 2011) besides Primer1 software developed by Ye *et al*. (2001). Islam *et al.* (2014) have successful constructed allele specific ARMS primers using

Batchprimer3 to screen PPARG (Pro12Ala, CCA[GCA) SNP as a risk factor for Type 2 Diabetes. Another study came from Soler *et al.* (2011) who has successful applied allele-specific TARMS primers to be used in TARMS-PCR genotyping of LTA+250 C>T SNP. We noticed from both experiments that DNA sequence input in step of input sequences no need further modification especially in term of their SNP code. In contrast, it is unlikely that primer designing for HER2^{I655V} TARMS-PCR purpose using Batchprimer3 no need modification. Based on our experiment, changing the code of the original SNP in DNA reference with the other code is the simplest way in order that our DNA reference could be readable by the software. You *et al*. (2008) stated that the success rate of picking primers using Batchprimer3 is affected by sequence quality, target polymorphism location (for SNP genotyping primers), and Parameter settings. Modification in several primer parameters and the input sequence of HER2 gene in Batchprimer3 application in our study has succeeded to generate a pair of TARMS-PCR primers which capable of genotyping the HER2^{I655V} SNP inserted in recombinant pGEM-T (Fig. 1).

Foreign DNA contamination in nucleic acid amplification-based diagnostics tools development contributes significantly to bias result because of false detection. In fact, bias amplification mostly produced artifact PCR product with size was similar to that of the target PCR amplicon as proven in our experiment (Fig. 1A and B), indicating that the primers used in HER2^{I655V} TARMS-PCR genotyping are also able to anneal to the bases sequence of foreign DNA in a random event. This situation also oftenly observed in bacterial genotyping where 16s rRNA gene was used as target amplification **(**Rand and Houck, 1990; Carroll *et al*., 1999). Some reports have suggested to increase the annealing temperature of PCR or to reformulate the composition of primers to eliminate bias amplification due to DNA contamination (Guan et al., 2014; Suhda *et al*., 2016), yet those approaches still can not eliminate this event in our experiment.

The sources of contamination during PCR processing can be from lab environment, PCR tools and equipment or brought in PCR reagents due to the sub-optimum method in protein purification steps. In our study, we have applied very strick treatment in the pre-PCR processing step in which the PCR solution mixing was done in pre-treated UV-laminar air flow, thus excluding the environmental-derived contaminating DNA molecules to be accidentally included in PCR reaction. Therefore, HER2^{I655V} TARMS-PCR developed in our experiment has been contaminated by the foreign DNA molecules contained in PCR reagent master mix (Fig. 2A). Spangler *et al*. (2009) has identified and quantified the bacterial DNA molecules that contaminated several types of commercial Taq polymerase such as Amplitaq Gold (ABI, CA; Roche lot # J02913), Platinum Taq (Invitrogen, CA; cat # 10966–026; lot 1169610), Platinum HiFi Taq (Invitrogen, CA; cat# 11304–011; lot# 1267490), HotStar Taq (Qiagen, CA; Mat # 1007837; lot # 124125007), JumpStart Taq (Sigma, MO; cat # D-6558; lot # 71K9029) and the bacterial copy number that contaminated those of commercial Taq polymerases ranging from 1 up to 45, respectively.

Several strategies have been deeply investigated to eliminate inherent contaminating DNA molecules from PCR reagent either using physical or biochemical approach (Champlot *et al*., 2010). The first approach is mostly using ultra-violet (UV) light**.** UV irradiation of DNA results in the formation of pyrimidine dimers and thus prevents them from being effective templates in subsequent PCR (Ou, *et al*., 1991). However, the application of UV irradiation on Taq polymerase reagent can significantly reduce the activity of enzyme (Pao *et al*., 1993). The second approach is PCR reagents treatment using DNase I. Endonuclease will breakdown DNA double strand, while leaving single strand DNA or oligonucleotide such as primer from degradation. It seems that the second approach is more effective and practical to eliminate contaminating DNA molecules in PCR reagent and also, more importantly, no adverse effect was shown by DNase I on Taq polymerase's activity (Heininger *et al*., 2003). DNase I has been proven as a strong biochemical agent to eliminate contaminating DNA molecules contained in PCR reagent in HER2^{I655V} TARMS-PCR development (Fig. 3A). The DNase I

concentration used in our study, 0.1 U/PCR reaction was similar with those of DNase I concentration used in treating the contaminating DNA molecules from TaqMan Universal PCR 2× master mix (UMM), (Applied Biosystems, Foster City, CA) (Silkie *et al*., 2008) and AmpliTaq Gold and AmpliTaq 360 DNA Polymerase (Applied Biosystems, CA, USA), FastStart Taq DNA polymerase (Roche Applied Science, Mannheim, Germany), HotStarTaqH DNA polymerase (Qiagen, Du¨ sseldorf, Germany), and GoTaqH Hot Start Polymerase (Promega Corporation, Madison, WI, USA) (Champlot *et al*., 2010).

Residual DNase I, in decontaminated PCR reagent, has affected the specificity of HER2^{I655V} TARMS-PCR genotyping as proven in recombinant plasmid model system (Fig. 2B). Silke *et al*., (2008) have shown that residual DNase I activity in treated PCR reagent significantly influences Taq polymerase performance. It was noted that the heat inactivation of DNase I in their experiment only for 15 minutes at 95ºC. Hanaki *et al*. (2000) have documented residual DNase activity after 50 min of heating at 95 °C. It was noted that active-stated DNase I after heat treatment that plays important role in reducing the sensitivity of Taq polymerase as observed by Silke *et al*., (2008). In contrast, we did not observe any effect of residual DNase I activity on the sensitivity of HER2^{I655V} TARMS-PCR genotyping even though heat inactivation of this enzyme has been prolonged up to 60 min at 95 °C. Surprisingly, we found that DNase I-treated PCR reagent altered the specificity of HER2^{I655V} TARMS-PCR genotyping especially for those of recombinant plasmid containing HER2 gene with GG genotype or AG genotype (Fig. 3). We predict that active-stated DNase I interacts direct or indirectly with Taq polymerase in such ways that alters the preferential binding of Taq polymerase on allele-specific primers.

The effective binding of Taq polymerases on a template:primer duplex in starting the amplification process largely depends on base composition at 3 ends the duplex (Ayyadevara *et al*., 2000). Simsek and Adnan, (2000) have proven that the primer with G/C mismatch was as efficient as the normal primer G/C match, while refractory to amplification occurred when the primers contained A/G or G/G mismatch at the 3 end with template DNA. This fact is in agreement with our result where allele-specific primers used in our study also followed this role only when HER2^{I655V} TARMS-PCR genotyping used PCR reagent without pre-treated with Dase I. Interestingly, DNase I reduces refractory to genotyping of recombinant plasmid containing HER2 gene G variant allele with primer A/G mismatch (Fig. 3B) and this phenomenon can not be simply eliminated by reformulating the PCR condition with the addition of betaine, because primer with A/G mismatch lost its binding capacity when two recombinant plasmids were mixed together as template (Fig. 4A). To recover the specificity of HER2^{I655V} TARMS-PCR, Touchdown PCR condition was applied. This strategy has been reportedincreasing the specificity of TARMS-PCR in CD40 ligand gene (Aloui *et al*., 2015), GJB2L gene (Han *et al*., 2013), PNPLA3 gene (Wang *et al*., 2015), and bovine ACADVL gene polymorphism studies (Zhang *et al*., 2015). Touchdown condition recovers specificity of HER2^{I655V} TARMS-PCR (Fig 4B) only when an allele specific primer with forward direction was replaced with an allele-specific primer in the size of 21 bp in which base composition at the third position from 3'end of primer was G/A mismatch. As stated by Liu *et al*., (2012) that A/A, C/A or G/A base mismatch at 3 third position of primer with A/G SNP at its 3'end will produce 28.3 up to 46.8 of polymorphism percent of primers.

We observed that the optimized condition of TARM-PCR for HER2^{I655V} genotyping obtained from recombinant plasmid genotyping model system can not be directly applied on genomic DNA samples as proven by the result as pointed in figure 5. We noticed that only homozygote type of HER2^{I655V} gene can be detected by TARMS-PCR (Figure 5). We assumed that the difference in the complexity of DNA structure between recombinant plasmid and genomic DNA contributes to this PCR failure. Two combined approaches has been applied to recover the specificity of HER2^{I655V} TARMS-PCR genotyping on genomic DNA through mixing the allele-specific primer of forward direction that mimics degenerate primer and then using 96ºC of denturation temperature in

TARMS-PCR as pointed in figure 6C. Increasing the denaturation temperature up to 96°C along PCR reaction will turn genomic DNA into a complete denaturation, thus giving more access for primers to bind its target DNA sequence (Ray and Handyside, 1996). Moreover, mixed allele-specific primers for forward direction give equal amplification of HER2^{I655V} gene with heterozygote, preventing the preferential amplification for either type of HER2^{I665V} homozygote as shown in figure 6A and B. We predicted that this recovery effect may be result of the difference in mismatch bases between base at penultimate and 3rd position of primers, or destabilization strength of terminal mismatch bases in primers used (Bui and Liu, 2009).

Our experiment has proven the significance of recombinant plasmids usage in developing the TARMS-PCR for HER2^{I655V} genotyping using genomic DNA-derived clinical samples. This approach is suitable for genotyping some clinical importance of the gene where tissues as the source of material genetics are very limited such as in cancer diagnosis or preimplantation genetics diagnosis. For TARMS-PCR used in SNP study in cattle where the tissues can be obtained abundantly (blood), genomic DNA for optimizing PCR condition is more preferable.

In conclusion, the reliable TARMS-PCR for genotyping the HER2^{I655V} polymorphisms has been developed. Reliability of this method can be obtained by eliminating the contaminating DNA molecules from PCR reagent by DNase I followed by some modifications in TARMS-PCR condition to recover its specificity.

Acknowledgements

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