Afyon Kocatepe Üniversitesi Fen ve Mühendislik Bilimleri Dergisi

Afyon Kocatepe University Journal of Science and Engineering

AKÜ FEMÜBİD **23** (2023) 061003 (1363-1372) AKU J. Sci. Eng. **23** (2023) 061003 (1363-1372)

DOI: 10.35414/akufemubid.1322267

Araştırma Makalesi / Research Article

Optimization Studies and Results of Recombinase Polymerase Amplification Technique for Gene Mutation Detection

Beste ÇAĞDAŞ[1*], Sebastian KERSTING[2]

[1] *Kırıkkale University, Faculty of Engineering and Natural Sciences, Bioengineering Department, Kırıkkale, Türkiye.*

[2] *Fraunhofer Institute for Cell Therapy and Immunology, Branch Bioanalytics and Bioprocesses IZI-BB, Potsdam, Germany*

**Corresponding author* e-mail: beste7540@yahoo.com* *ORCID ID: https://orcid.org/0000-0003-2534-382X*

sebastian.kersting@izi-bb.fraunhofer.de *ORCID ID: https://orcid.org/0000-0003-0474-7825*

Geliş Tarihi: 4 Temmuz 2023 ; Kabul Tarihi: 30 Kasım 2023

Keywords

Single Nucleotide Polymorphism; Isothermal Amplification; Polymerase Chain Reaction; Recombinase Polymerase Amplification; Gene Mutation; PIK3CA

Abstract

Single nucleotide polymorphisms (SNPs) in human genes are very significant genetic changes and PCR (polymerase chain reaction) or NGS (next-generation sequencing) are extensively employed in SNP analysis. Thanks to the studies on the progress of new technologies, interest in the isothermal nucleic acid amplification approach has increased. As one of these methods, recombinase polymerase amplification (RPA) represents an attractive option for point-of-care nucleic acid quantification. The target SNPs selected within the scope of the study are mutations identified in the PIK3CA gene region (E542K, E545K), and DNA samples which were evaluated about PIK3CA mutations were isolated from the cancer cells MCF7, BT474, and also SKBr3. The optimization studies for the RPA reaction conditions were carried out for parameters such as assay time, temperature, primer, and also magnesium acetate concentration. According to the results of the reaction optimization studies, in which the RPA products can be obtained in the most efficient way, the assay time was determined as 20 min; the temperature as 40°C; the primer concentration as 10 µM and the MgOAc concentration as 140 mM.

Gen Mutasyonunun Belirlenmesinde Rekombinaz Polimeraz Çoğaltım Tekniği Optimizasyonu Çalışmaları ve Sonuçları

Anahtar kelimeler

Tek Nükleotid Polimorfizmi; İzotermal Çoğaltım; Polimeraz Zincir Reaksiyonu; Rekombinaz Polimeraz Çoğaltım; Gen Mutasyonu; PIK3CA

Öz

İnsan genlerindeki tek nükleotid polimorfizmleri (SNP'ler) çok önemli genetik değişikliklerdir ve PCR (polimeraz zincir reaksiyonu) veya NGS (yeni nesil dizileme), SNP analizinde yaygın olarak kullanılır. Yeni teknolojilerin ilerlemesi üzerine yapılan çalışmalar sayesinde izotermal nükleik asit amplifikasyon yaklaşımına ilgi artmıştır. Bu yöntemlerden biri olarak, rekombinaz polimeraz amplifikasyonu (RPA), hasta başı nükleik asit ölçümünde çekici bir alternatif oluşturmaktadır. Çalışma kapsamında seçilen hedef SNP'ler, PIK3CA gen bölgesinde (E542K, E545K) tanımlanan mutasyonlardır ve PIK3CA mutasyonlarının değerlendirildiği DNA örnekleri, MCF7, BT474 ve ayrıca SKBr3 kanser hücre hatlarından izole edilmiştir. RPA reaksiyon koşulları için optimizasyon çalışmalarında analiz süresi, sıcaklık, primer ve ayrıca magnezyum asetat konsantrasyonu parametreleri değerlendirilmiştir. RPA ürünlerinin en verimli şekilde elde edilebildiği reaksiyon optimizasyon çalışmaları sonuçlarına göre reaksiyon süresi 20 dk, sıcaklık 40°C, primer konsantrasyonu 10 µM ve MgOAc konsantrasyonu ise 140 mM olarak değerlendirilmiştir.

1. Introduction

Cancer is a leading cause of death globally and was reported to be responsible for nearly 10 million deaths in 2020, or in other words approximately 1 in 6 deaths. Mortality in cancer is quite high due to delayed diagnosis and inadequate treatment. According to 2020 data, the highest death rate (1.8 million deaths) was seen in lung cancer, followed by colon and rectum (916 thousand deaths), stomach (769 thousand deaths), liver (830 thousand deaths), and breast (685 thousand deaths) cancers (WHO 2020). Therefore, early diagnosis, which decreases the treatment costs and

increases the patient's life expectancy, although it varies according to the cancer type, has become a very important issue (Coley et al. 1997). Although physical examinations are used today for early diagnosis, the biopsy is accepted as the gold standard for definitive diagnosis. However, recently, it has come to the fore that single nucleotide polymorphisms (SNP) can also be used in the diagnosis of cancer. Single nucleotide polymorphisms (SNPs) are seen in several genes linked to different cancer types and clinically, SNPs have the potential to be both therapeutic and diagnostic biomarkers for a variety of cancer types (Deng et al. 2017). The target SNPs selected within the scope of the project are mutations identified in the PIK3CA gene region (E542K, E545K) and have been chosen as the target region (180 bp) because they are widely detected in a wide variety of cancer types such as brain, breast, stomach, colon, lung, and liver, (Bader et al. 2006, Banerji et al. 2012, Dawson et al. 2013, Wang et al. 2013). For the purpose of amplifying the nucleic acid target sequence, PCR requires many cycles of three or two temperature-dependent steps and is used by the great majority of systems created for nucleic acid analysis (Zanoli and Spoto 2013). However, due to inhibition or degradation of priming sites, which causes allelic dropout, trace DNA such as short tandem repeats (STRs) markers and SNPs frequently fails to amplify in PCR-based approaches. Although several PCR optimization procedures have been created, it is still difficult to analyze DNA samples. The issues with PCR amplification have been solved by universal adapter ligation-based massively parallel sequencing (MPS) techniques. Cost-effective MPS analysis necessitates the enrichment of target DNA regions. Although hybridization capture techniques have been created to enrich SNPs, their widespread use is hampered by the high cost of the baits. Therefore, it is suggested that create techniques to provide affordable non-PCR-based target enrichment baits for MPS analysis (Gorden et al. 2021). Because of these mentioned drawbacks and also some challenges to implement for on-site testing or in environments with limited resources, it is an important requirement to try to develop alternative techniques to PCR and DNA sequencing. On the other hand, thermal cycling is not necessary for low-temperature isothermal amplification techniques, so implementing these methods is cheaper, faster, and more practical than traditional PCR-based methods (Zanoli and Spoto 2013). Over the past 20 years, numerous isothermal amplification techniques have been created. The most common ones are recombinase polymerase amplification (RPA), rolling circle amplification (RCA), helicase-dependent amplification (HDA), loop-mediated amplification (LAMP), and multiple displacement amplification (MDA) (Asiello and Baeumner 2011) and all of these techniques differ from PCR in that they don't need the same quick heating and cooling mechanisms or temperature cycling that PCR systems do. Nucleic acid sequence-based isothermal amplification methods such as rolling circle amplification (RCA), helicase-dependent amplification (HDA), and loop-mediated isothermal amplification (LAMP) are frequently used today although such problems like complex primer design (Kersting et al. 2018), pretreatment of the target DNA (Zanoli and Spoto 2013), the success of the process due to the synchronization between the amplification components (Zanoli and Spoto 2013), or the increased cost due to the enzymes used (Zaghloul and El-shahat 2014) are encountered (Liu et al. 2016). In recombinase polymerase amplification (RPA), which is an alternative isothermal amplification method, high yield product can be obtained with a low temperature (37°C), easy primer design, and high amplification rate. Therefore, although they are still new, their use in the determination of human gene mutations has come to the fore (Liu et al. 2016). Considering the advantages and disadvantages of all these methods, it was decided to use the RPA method for the enrichment of target mutant sequences selected within the scope of the study. RPA is a low-temperature (approximately 37°C) isothermal amplification method that combines isothermal recombinase-driven primer targeting of the template sequence with strand displacement DNA synthesis. In the method developed by Piepenburg et al. (Piepenburg et al. 2006), DNA sequences are

amplified using recombinase, DNA polymerase, and DNA binding proteins with a new probe-based detection approach. In the RPA method, primer binding to the template DNA is facilitated by nucleoprotein complexes formed by oligonucleotide primers and recombinase proteins. Recombinase primer complexes screen double-stranded DNA by promoting primer binding to the target dsDNA sequence and displacement of the non-pattern strand.

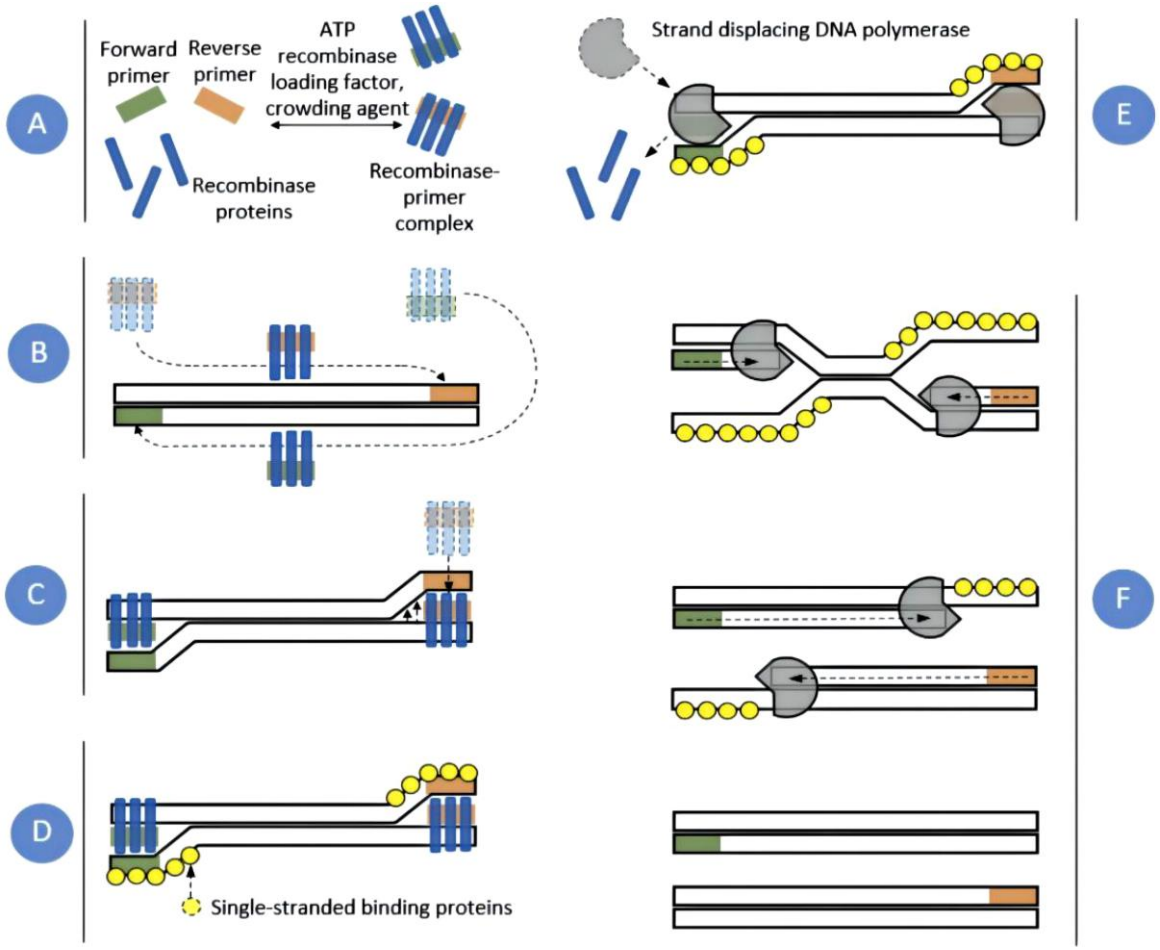

Figure 1. RPA amplification scheme. Recombinase proteins formed complexes with each primer (A), which scanned DNA for homologous sequences (B). The primers were then inserted at the cognate site by the strand-displacement activity of the recombinase (C) and single-stranded binding proteins stabilized the displaced DNA chain (D). The recombinase then disassembled leaving the 3` end of the primers accessible to a strand displacing DNA polymerase (E), which elongated the primer (F). Exponential amplification was achieved by cyclic repetition of this process (Lobato and O'Sullivan 2018).

The displaced strand is stabilized by ssDNA binding proteins, while deconstruction of the recombinase makes the 3'-end of the primer accessible to the DNA polymerase (Figure 1). As a result, the target sequence is exponentially amplified by the cyclic replication of the process (Zanoli and Spoto 2013). Here in this study, the optimization studies of the RPA reaction were carried out for parameters such as assay time, temperature, primer, and also medium (magnesium acetate) concentrations, then the RPA products obtained were purified and confirmed by the electrophoretic method.

2. Material and Methods

2.1. Designing of the Primers

The oligonucleotide sets were designed by an online software of Basic Local Alignment Search Tool (Int.Res-1) and supplied (©Biomers.net GmbH) for the analysis of two important hotspots in the PIK3CA gene (Table 1). The two codons with the highest mutation rates in exon 9's helicase

domain are codon 542 (E542K) and codon 545 (E545K).

Table 1. List of tested DNA sequences of primers for the PCR and RPA Assay

Strands	Sequence (5´- 3´)
FP-1	TCA AAG CAA TTT CTA CAC GAG ATC CT
RP-1	CTG TGA CTC CAT AGA AAA TCT TTC TC
FP-2	ACA ATG AAT TAA GGG AAA ATG ACA AAG AAC
RP-2	TTA CCT GTG ACT CCA TAG AAA ATC TTT CTC

FP: upstream or forward primer; RP: downstream or reverse primer

2.2. DNA Extraction

PIK3CA E542K and E545K mutations including human breast cancer cell lines SKBr3, BT474, and MCF7 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM medium supplemented with 4.5 g/L glucose, %10 fetal bovine serum, 1.5 mM L-glutamine, 100 U/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified incubator containing 5% CO_2 and 95% air. Genomic DNA (gDNA) was extracted from BT474, MCF7, and SKBr3 cells using a DNA purification kit (Thermoscientific GeneJet, Lot no: 01001401) according to the manufacturer's instructions. The DNA concentrations (ng/µL) were measured using the 260/280 ratio obtained with a NanoDrop. The samples were stored at -20°C until use.

2.3. Amplification of target DNA using PCR

Detection of target DNA in three breast cancer cell lines was performed by PCR amplification with a PCR amplification kit (Jena Biosciences, Taq Core Kit; 214S.1). The primers and tested PCR programs for amplification of PIK3CA mutations were as described in Table 2. The general PCR mixture contains 1 µL of gDNA A, 1 µL of each primer (10 µM), 1 µL of dNTPs, 0.25 µL of 2x EasyTaq®PCR Supermix, 5 µL of hybridization buffer, 41.75 µL of ddH2O; the thermal cycling conditions were given in Table 2 (PCR Programme takes approximately 2 hours). After rapid purification of DNA from PCR (GeneJet PCR purification kit (ThermoScientific, Lot no: 00463783) the purified DNA was then measured by NanoDrop-Spectrophotometer (NanoDrop ND-1000 Spectrophotometer).

2.4. Amplification of target DNA using RPA

Detection of PIK3CA mutations in three breast cancer cell lines was also performed by RPA amplification with a TWISTAmp/Liquid Basic Kit (TWISTDX, Lot no: 111112, England) and after rapid purification of DNA from RPA (GeneJet PCR purification kit (ThermoScientific, Lot no: 00463783)), the bands were seen on the agarose gel. The primers for the amplification of PIK3CA mutations were as described in Table 1. The general RPA mixture for samples contains 25 µL of 2x reaction buffer, 2.4 µL of each primer (10 µM), 9 µL of dNTPs (10 mM), 5 µL of 10x Basic E-Mix, 2.5 µL of 20x Core reaction mixture, 0.2 µL of ddH2O, 1 µL of gDNA and 2.5 µL of Magnesium Acetate (MgOAc, 280 mM); the conditions of RPA was generally set to 39°C for 30 min, and 4°C for forever (To terminate the RPA reaction by rapidly lowering the temperature, the samples were taken to the freezer and kept in the freezer until the time of purification.).

Table 2. PCR Protocol for Optimization Studies

PCR Code	*Primer Set*	*Template Amount*	*PCR-Program*
			Initial denaturation (95°C, 5 min)
A	FP1-RP1	1 µL	Denaturation (95°C (30 sec)) -Annealing (53°C (40 sec)) -Extension (72°C (40 sec))/Cycle Number 35
			Final extension (72°C, 5 min)
			Initial denaturation (95°C, 2 min)
B	FP2-RP2	5 µL	Denaturation (95°C (15 sec)) -Annealing (55°C (15 sec)) -Extension (72°C (30 sec))/Cycle Number 45
			Final extension (72°C, 5 min)

2.5. Optimization of Recombinase Amplification Reaction Conditions

2.5.1. Optimization of RPA Reaction Assay Time

RPA was carried out in accordance with the manufacturer's recommendations (TwistAmp Liquid Basic kit, TwistDx, Cambridge, UK). Briefly, 1

µL of dsDNA (RPA product (which is firstly amplified by PCR) of SKBR3) was added to 50 µL of RPA reagents (2x Rehydration buffer (25 µL), 10x Basic E-mix (5 µL), 20x Core Reaction mix (2.5 µL), 10 µM of primers (16F and 125R; 2.4 µL), 10 mM dNTPs (9 µL), H2O (0.2 µL) 280 mM MgAcetate (2.5 µL) and were incubated for 10, 20, 30, 40, 50 and 60 min at 39°C in the shaker incubator. The samples were put at -20°C for 5 min to stop the RPA reaction.

8 µL of RPA amplified products were mixed with 2 µL of 5x Loading dye and run in 3% (w/v) AGE prepared in 1x TAE buffer (Tris-Acetate-EDTA, pH 8) at 100 mV for 40 min. The gel was pre-stained with 5 µL of RotiSafe nucleic acid stain and HyperLadder V (500 bp) was loaded to the gel as a nucleic acid standard then the gel was imaged with a UV lamp (λ = 254 nm).

2.5.2. Optimisation of RPA Reaction Temperature

RPA was carried out following the manufacturer's recommendations (TwistAmp Liquid Basic kit, TwistDx, Cambridge, UK). Briefly, 1 µL of dsDNA (RPA product (which is firstly amplified by PCR) of SKBR3) was added to 50 µL of RPA reagents (2x Rehydration buffer (25 µL), 10x Basic E-mix (5 µL), 20x Core Reaction mix (2.5 µL), 10 µM of primers (16F and 125R; 2.4 µL), 10 mM dNTPs (9 µL), H2O (0.2 µL) 280 mM MgAcetate (2.5 µL) and were incubated for 20 min at 25°C-30°C-35°C-40°C-45°C in the shaker incubator. The samples were put at -20°C for 5 min to stop the RPA reaction. 8 µL of RPA amplified products were mixed with 2 µL of 5x Loading dye and run in 3% (w/v) AGE prepared in 1x TAE buffer (Tris-Acetate-EDTA, pH 8) at 100 mV for 40 min. The gel was pre-stained with 5 µL of RotiSafe nucleic acid stain and HyperLadder V (500 bp) was loaded to the gel as a nucleic acid standard then the gel was imaged with a UV lamp (λ = 254 nm).

2.5.3. Effect of primer concentration on RPA reaction yield

Primers that interact with the target sequence (dsDNA) during the RPA reaction (in the same molar ratio) should be in a concentration in the range of 0.1-10 µM in the reaction final volume of 50 µL was added to the reaction medium (in the range of 5 nM-0.5 µM as final concentration) to determine the optimum primer concentration forthe RPA reaction (Liu et al. 2016, Martorell et al. 2018).

2.5.4. Effect of medium composition on RPA reaction yield

After the RPA reaction mixture is prepared, the magnesium acetate buffer required to start the reaction was added to the reaction medium at concentrations ranging from 17 mM to 280 mM in molarity (in the range of 0.85 mM - 14 mM as final concentration in reaction medium), and the optimum concentration of the buffer was determined.

3. Results and Discussion

3.1. Genomic DNA extraction

To evaluate the amount of extracted genomic DNA from MCF7, BT474, and SKBr3 cell cultures, the DNA concentrations were measured using the 260/280 ratio with a NanoDrop spectrophotometer (Table 3, Figure S1). According to the ratio of absorbance (~1.8) at 260 nm and 280 nm, DNA samples accepted as “pure”. And as a secondary measure of nucleic acid purity, the 260/230 values were also higher than the respective 260/280 values that were common in the range of 2.0-2.2 indicating the purity of nucleic acids in the samples.

Table 3. Concentrations of gDNA from cancer cells

Cell Line	A260/280	Concentration (ng/µL) Mean (± std. dev.)
BT474	1.89	57.9 ± 1.4
MCF7	1.82	54.6 ± 4.1
SKBR3	1.82	44.0 ± 6.3

3.2. Amplification of target DNA using PCR

Target DNA of BT474, MCF7, and SKBR3 cell lines were amplified with the specified primer sets (FP1-RP1/ FP2-RP2) under various PCR program settings (Table 2), followed by agarose gel electrophoresis detection. Primer set with the best amplification efficiency was chosen for PCR assay according to

the detected products by agarose gel electrophoresis. Then purified amplicons' (B coded PCR run) DNA concentrations were measured (Table 4). Among the two candidate pairs of primers, the amplification effect of the FP2-RP2 primer set was significantly higher than that of the FP1-RP1 set because they had brighter bands. The reaction conditions of PCR were also optimized.

Table 4. Concentrations of cleaned-up gDNA from PCR

Sample Name	Concentration of DNA (ng/µL)
BT474	51.1 ± 4.3
MCF7	37.3 ± 2.6
SKBR3	39.6 ± 2.4
Positive control (+) (Human DNA)	32.7 ± 3.1
Negative Control (-)	26.6 ± 2.2

The results revealed that optimal PCR program was as Initial Denaturation (95°C, 2 min), Denaturation-Annealing-Extension ((95°C (15 sec)-55°C (15 sec)-72°C (30 sec)/Cycle Number 45) and Final Extension (72°C, 5 min).

Gel electrophoresis results showed that the assay produced clearly visible bands at approximately 150 base pairs (bp) which are close to the expected size (Figure 2). In the PCR reactions, the genomic DNA template was used in two different amounts as 1 and 5 µL in the reaction mixture, and according to the gel electrophoresis analysis, the expected bands could obviously be detected when the template was used as 5 µL. In addition, according to the analysis of the products amplified by the PCR method by gel electrophoresis, no unwanted products were detected in the other samples, except for the PCR amplicon obtained from the DNA sample purified from the MCF7 cell line (Figure 2B, Lane 3). Although it was imperceptible, the presence of a band in the expected region in the negative control group indicated that a small amount of contamination occurred between the samples during the sample preparation or DNA purification stages, but this contamination could not be prevented in repeated trials (Figure 2B, Lane 6).

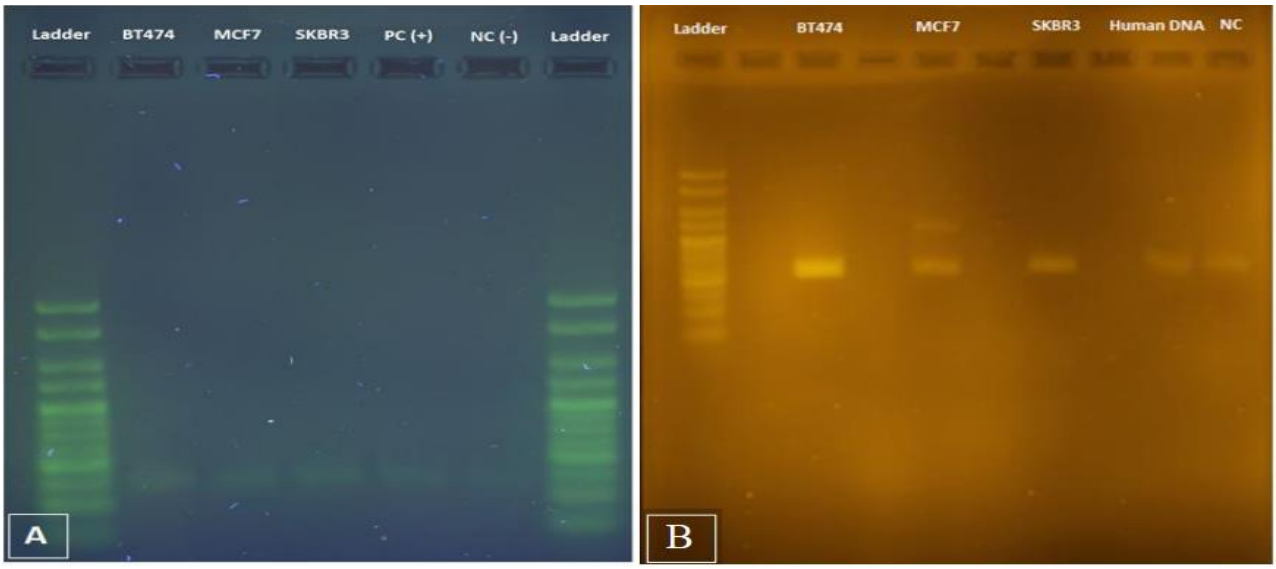

Figure 2. Optimization of PCR for amplification of gDNA from cancer cell lines. A) A coded PCR run-Amount of the template is 1 µL and PCR cycle number is 35 - non cleaned-up; B) B coded PCR run- Amount of template is 5 µL, PCR cycle number is 45 – cleaned-up. (Data of products obtained from PCR reactions using the FP2-RP2 primer set)

3.3. Amplification of target DNA using RPA

The RPA reactions were performed using 1 µL of the genomic DNA of three breast cancer cell lines as a template and cleaned-up amplicons' DNA concentrations were measured (Table 5). When employing the same primer set as PCR, the size of the amplified product acquired by RPA was comparable to the amplicon generated by PCR. (FP2-RP2) (Figure 3). Since RPA operates at low temperatures, it is known that it does not force the system to regenerate base pairs in accordance with

Watson-Crick guidelines. As a result, significant amounts of undesirable products known as primer-dimers are formed, which impedes the amplification of the intended product and is a common issue in isothermal amplification techniques (Piepenburg et al. 2006).

Table 5. Concentrations of cleaned-up gDNA from RPA

Sample Name	Concentration of DNA (ng/µL)
BT474	99.1 ± 2.8
MCF7	120.2 ± 10.7
SKBR3	104.8 ± 8.3
Positive control (+) (PC 1/ Human DNA)	175.5 ± 0.0
Positive control (+) (PC 2/ Human DNA)	72.6 ± 3.1
Negative Control (-)	84.5 ± 10.0

Therefore, we think that this band formation in the negative control group (NC) may be caused by this characteristic of RPA. We also noticed that band formation was not good for the positive control group (PC2) provided by the company, and unfortunately, we saw that this result did not change in our repeated trials.

Figure 3. Target DNA amplification by RPA (PC1 refers to the Human DNA as the positive control group; PC2 refers to the Positive control DNA as the other positive control group which was supplied by the company; NC refers to the negative control group)

3.4. Optimization of RPA reaction

The optimal incubation time, temperature, primer concentration, and medium composition of the RPA reaction for the detection of the target gene were determined. According to the findings, the RPA amplicon was visible after only 10 minutes and reached saturation after 20 minutes of incubation (Figure 4A) and the highest amount of amplified product was observed at 40°C (Figure 4B). These RPA-based assays achieve sensitivities that are comparable to those obtained with PCR, but with a markedly shorter reaction time between 10 and 20 min for the complete reaction (Kunze et al. 2016). Similar product formation was obtained when the RPA reaction was produced within the 30–40°C range, showing high tolerance to temperature fluctuations. It has also been shown by other studies that RPA exhibits similar amplification ability over a wide temperature range (Martorell et al. 2018, Guo et al. 2021). Subsequently, a range of concentrations of the primers for the target fragments (0.1-10 µM) were tested. As shown in Figure 4C, there were no obvious differences between the primer concentrations from 0.1 to 1 µM, for 5 µM the band density was slightly increased and for 10 µM the band density got saturation. Thus, 10 µM primer concentration was found to provide optimal performance for the RPA. In this study, when the primer was used at 0.25 µM in the final reaction medium (5 µM as stock concentration), a band could be seen in the gel, and the amount of primer used was similar to the amount (0.24 µM) used in the study of Rathore et al. (Rathore et al. 2019). Finally, five magnesium ion concentration gradients of 17.5, 35, 70, 140 and 280 mM were tested and the magnesium ion concentration that resulted in a clearly visible band was selected as 140 mM (Figure 4D). The amount employed in this study, 140 mM stock MgOAc concentration, was less than the amount often used in other experiments (280 mm) (Zhang et al. 2020, Daddy Gaoh et al. 2023), yet it was shown to be adequate for the production of visible bands. At a low temperature of 40°C, the assay could successfully amplify the target sequence in just 20 minutes. However, we encountered a problem that is frequently mentioned in the literature regarding this assay. RPA never compels the system to recreate base pairs in accordance with Watson-Crick guidelines because it operates at low

temperatures and as a result, it inevitably creates significant amounts of undesirable products, known as primer-dimers, which prevent the intended product from being amplified (Piepenburg et al. 2006). Additionally, uncharacterized artifacts are created to add to the background signal (TwistDx 2013). Because the system is not required (at any time in the cycle) to rebuild the Watson-Crick base pairs by gently annealing from high to low temperatures, this is a common issue with isothermal amplification techniques (Sharma et al. 2014).

According to the literature research, it has been seen that a number of solutions have been developed to overcome this problem. In one of these studies, it has been shown that if the primers incorporate elements of a self-avoiding molecular recognition system (SAMRS), the majority of these undesirable side products can be avoided (Sharma et al. 2014). Another suggestion on the subject is the application of the ‘‘RPA-PCR couple’’ approach. DNA extraction procedures that take a lot of time and provide difficulties can be avoided by combining PCR and slower reaction kinetics RPA.

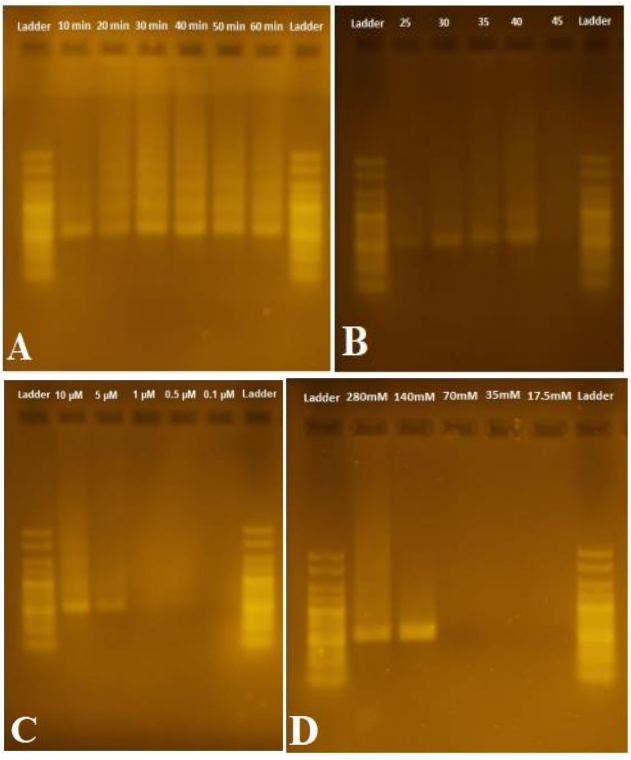

Figure 4. Optimization of RPA assay components. (A) Optimization of the RPA reaction time. (B) Temperature gradient for the RPA assay. (C) Optimization of the primer concentration. (D) Optimization of the magnesium ion concentration.

The technique is also anticipated to address RPA's disadvantages of nonspecificity (background amplification), small amplicon size (optimal range: 100–200 bp), and subpar quantification resolution. In the linked amplification, RPA amplifies the source directly in the first round while also gradually amplifying the target and some flanking regions. The target region is then immediately amplified by PCR in the second round of amplification from the RPA reaction. Longer amplicons are generated more frequently when RPA reaction kinetics are slower. Similar to this, the slower pace of RPA amplification produces less background noise and target amplicons that can be used straight for PCR (without dilution). While background amplification issues plague RPA and other isothermal nucleic acid amplification techniques frequently, its use for initial and limited target nucleic acid amplification to replace the DNA extraction phase offers new insight (Munawar et al. 2020) In practical applications, the quick testing period and low incubation temperature are advantageous for the early identification of the target gene. The fact that a specific thermocycler is not necessary for this assay makes it favorable as well. The tests could be carried out in a heating block or water bath. According to numerous studies, the RPA reaction can be carried out at any temperature, including body temperature (Lillis et al. 2014, Wang et al. 2017, Ma et al. 2020). With these benefits, the RPA assay is time and money efficient in remote locations with limited resources.

4. Conclusion

Here, we reported the optimization studies and results of RPA reaction as a quick and simple way for the amplification of the target gene from the breast cancer cell line. In conclusion, it was clear that the RPA used in this investigation could quickly and accurately detect the target gene. The test also permits independence from the exacting laboratory environmental standards and equipment.

Acknowledgments

We acknowledge the Scientific and Technological Research Council of Turkey (Tubitak 2219 Award # 1059B192001062) for Dr. Beste ÇAĞDAŞ. We are also grateful to the Fraunhofer Institute For Cell Therapy and Immunology for supporting this study at Dr. Markus von Nickisch-Rosenegk's laboratory.

5. References

Asiello, P.J. and Baeumner, A.J., 2011. Miniaturized isothermal nucleic acid amplification, a review. *Lab on a Chip*, **11**(8), 1420-1430. https://doi.org/10.1039/C0LC00666A

Bader, A.G., Kang, S. and Vogt, P.K., 2006. Cancer-specific mutations in PIK3CA are oncogenic in vivo. *Proceedings of the National Academy of Sciences*, **103**(5), 1475-1479. https://doi.org/10.1073/pnas.0510857103.

Banerji, S., Cibulskis, K., Rangel-Escareno, C., Brown, K.K., Carter, S.L., Frederick, A.M. and Meyerson, M., 2012. Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature*, **486**(7403), 405-409. https://doi.org/10.1038/nature11154

Coley, C.M., Barry, M.J., Fleming, C., Fahs, M.C. and Mulley, A.G., 1997. Early detection of prostate cancer. Part II: Estimating the risks, benefits, and costs. American College of Physicians. *Annals of internal medicine*, **126**(6), 468-479. https://doi.org/10.7326/0003-4819-126-6-199703150-00010

Daddy Gaoh, S., Kweon, O., & Ahn, Y. 2023. Propidium Monoazide (PMAxx)-Recombinase Polymerase Amplification Exo (RPA Exo) Assay for Rapid Detection of Burkholderia cepacia Complex in Chlorhexidine Gluconate (CHX) and Benzalkonium Chloride (BZK) Solutions. *Microorganisms*, **11**(6), 1401. https://doi.org/10.3390/microorganisms11061401

Dawson, S.J., Tsui, D.W.Y., Murtaza, M., Biggs, H., Rueda, O. M., Chin, S.-F., Dunning M. J., Gale D., Forshew T., Mahler-Araujo B., Rajan S., Humphray S., Becq J., Hallsal D., Wallis M., Bentley D., Caldas C., Rosenfeld, N., 2013. Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. *New England Journal of Medicine*, **368**(13), 1199-1209. https://doi.org/10.1056/NEJMoa1213261

Deng, N., Zhou, H., Fan, H. and Yuan, Y., 2017. Single nucleotide polymorphisms and cancer susceptibility. *Oncotarget,* **8**(66), 110635–110649.https://doi.org/10.18632/oncotarget.22372.

Gorden, E. M., Sturk-Andreaggi, K. and Marshall, C. 2021. Capture enrichment and massively parallel sequencing for human identification. Forensic Science International: *Genetics*, **53**, 102496. https://doi.org/10.1016/j.fsigen.2021.102496

Guo, Q., Zhou, K., Chen, C., Yue, Y., Shang, Z., Zhou, K., Fu Z., Liu, J., Lin, J., Xia, C., Tang, W., Cong, X., Sun X., and Hong, Y. 2021. Development of a recombinase polymerase amplification assay for Schistosomiasis Japonica diagnosis in the experimental mice and domestic goats. *Frontiers in Cellular and Infection Microbiology*, 1136. https://doi.org/10.3389/fcimb.2021.791997

Kersting, S., Rausch, V., Bier, F.F. and von Nickisch-Rosenegk, M., 2018. A recombinase polymerase amplification assay for the diagnosis of atypical pneumonia. *Analytical biochemistry*, **550**, 54-60. https://doi.org/10.1016/j.ab.2018.04.014

Kunze, A., Dilcher, M., Abd El Wahed, A., Hufert, F., Niessner, R., and Seidel, M., 2016. On-Chip Isothermal Nucleic Acid Amplification on Flow-Based Chemiluminescence Microarray Analysis Platform for the Detection of Viruses and Bacteria. *Analytical Chemistry* **88** (1), 898–905. https://doi.org/10.1021/acs.analchem.5b03540

Lillis, L., Lehman, D., Singhal, M.C., Cantera, J., Singleton, J., Labarre, P., Toyama, A., Piepenburg, O., Parker, M., Wood, R., Overbaugh, J. and Boyle, D.S., 2014. Non-instrumented incubation of a recombinase polymerase amplification assay for the rapid and sensitive detection of proviral HIV-1 DNA. *PloS one*, **9**(9), e108189. https://doi.org/10.1371/journal.pone.0108189

Liu, Y., Lei, T., Liu, Z., Kuang, Y., Lyu, J. and Wang, Q., 2016. A novel technique to detect EGFR

mutations in lung cancer. *International journal of molecular sciences*, **17**(5), 792. https://doi.org/10.3390/ijms17050792

Lobato, I.M., O'Sullivan, C.K., 2018. Recombinase polymerase amplification: Basics, applications and recent advances. *Trends Analytical Chemistry* **98**, 19–35. https://doi.org/10.1016/j.trac.2017.10.015

Ma, B., Li, J., Chen, K., Yu, X., Sun, C. and Zhang, M., 2020. Multiplex recombinase polymerase amplification assay for the simultaneous detection of three foodborne pathogens in seafood. *Foods*, **9**(3), 278. https://doi.org/10.3390/foods9030278

Martorell, S., Palanca, S., Maquieira, Á., and Tortajada-Genaro, L.A., 2018. Blocked recombinase polymerase amplification for mutation analysis of PIK3CA gene. *Analytical biochemistry,* **544**, 49-56. https://doi.org/10.1016/j.ab.2017.12.013

Munawar M., Martin F., Toljamo A., Kokko H. and Oksanen E., 2020. RPA-PCR couple: an approach to expedite plant diagnostics and overcome PCR inhibitors, *BioTechniques*, doi: 10.2144/btn-2020-0065. https://doi.org/10.2144/btn-2020-0065

Piepenburg, O., Williams, C.H., Stemple, D.L. and Armes, N.A., 2006. DNA detection using recombination proteins. *PLoS biology*, **4**(7), e204. https://doi.org/10.1371/journal.pbio.0040204

Rathore, H., Biyani, R., Kato, H., Takamura, Y., & Biyani, M. 2019. Palm-size and one-inch gel electrophoretic device for reliable and field-applicable analysis of recombinase polymerase amplification. *Analytical Methods*, **11**(39), 4969-4976. https://doi.org/10.1039/C9AY01476D

Sharma, N., Hoshika, S., Hutter, D., Bradley, K.M. and Benner, S.A., 2014. Recombinase-Based Isothermal Amplification of Nucleic Acids with Self-Avoiding Molecular Recognition Systems (SAMRS). *ChemBioChem*, **15**, 2268 – 2274. https://doi.org/10.1002/cbic.201402250

Wang, R., Zhang, F., Wang, L., Qian, W., Qian, C., Wu, J. and Ying, Y., 2017. Instant, visual, and instrument-free method for on-site screening of GTS 40-3-2 soybean based on body-heat triggered recombinase polymerase amplification. *Analytical chemistry*, **89**(8), 4413-4418. https://doi.org/10.1021/acs.analchem.7b00964

Wang, W., Xie, Y., Zhou, Z., Qin, Z., Wu, J. and He, J., 2013. PIK3CA hypomethylation plays a key role in activation of the PI3K/AKT pathway in esophageal cancer in Chinese patients. *Acta Pharmacologica Sinica*, **34**(12), 1560-1567. https://doi.org/10.1038/aps.2013.163

Zhang, S., Sun, A., Wan, B., Du, Y., Wu, Y., Zhang, A., Jiang, D., Ji, P., Wei Z., Zhuang G. and Zhang, G., 2020. Development of a directly visualized recombinase polymerase amplification–sybr green i method for the rapid detection of African swine fever virus. *Frontiers in microbiology*, **11**, 602709. https://doi.org/10.3389/fmicb.2020.602709

Zaghloul, H. and El-Shahat, M., 2014. Recombinase polymerase amplification as a promising tool in hepatitis C virus diagnosis. *World Journal of Hepatology*, **6**(12), 916. https://doi.org/10.4254/wjh.v6.i12.916

Zanoli, L.M. and Spoto, G., 2013. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. *Biosensors*, **3**(1), 18-43. https://doi.org/10.3390/bios3010018

Internet References

1-https://blast.ncbi.nlm.nih.gov (Basic Local Alignment Search Tool) (01.03.2022)

2-https://www.who.int/news-room/fact-sheets/detail/cancer (World Health Organisation, 2020. (10.06.2023)

3-http://www.twistdx.co.uk/images/uploads/docs/Appendix.pdf, TwistDx, Babraham, Cambridge. (30.03.2023).