

Development of New Technique for Using *E.coli* DNA Polymerase in PCR

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Abstract

Experiments which can separate polymerase from the reaction mixture before every denaturation step in successive cycle. Later it was verified from comparative analysis of several experiments that the method is working well but is not as efficient as the conventional Taq polymerase amplification protocol. Polymerase chain reaction is one of the most ultimate tools in the field of Genetic Engineering. It employs a thermo stable Taq polymerase for amplifying a target DNA molecule. The ability of Taq polymerase to withstand high temperatures in a PCR cycle makes it most suitable for this special purpose. However, its error rate is high as cycle. A new technique was developed after thoroughly reviewing the literature and conducting several compared to the *E.coli* DNA polymerase. This would therefore produce error after error and will lead to exponential rise in error besides making copies of the target DNA molecule.

This project is all about the development of new technique for using DNA polymerase in PCR for DNA amplification. The objective is to develop a PCR protocol which can protect the *E.coli* DNA polymerase from getting denatured by high temperatures in the initial steps of a normal PCR

Keywords: Polymerase chain reaction, PCR Stages, *E.coli* DNA polymerase, Taq Polymerase, amplification, Error Rate, Denaturation,

Introduction

The polymerase chain reaction (PCR), developed in 1983 by Kary Mullis, is a

scientific technique in molecular biology and bioengineering to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence..

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers, containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as *Taq* polymerase named after the thermophilic bacterium *Thermus aquaticus*, *T. aquaticus* is a bacterium that lives in hot springs and hydrothermal vents, and *Taq* polymerase was identified as an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR. *Taq's* optimum temperature for activity is 75–80°C, with a half-life of greater than 2 hours at 92.5°C, 40 minutes at 95°C and 9 minutes at 97.5°C, and can replicate a 1000 base pair strand of DNA in less than 10 seconds at 72°C. But one of *Taq's* drawbacks is its lack of 3' to 5' exonuclease proofreading activity, which acts as a first line of defense in correcting DNA polymerase errors. A mismatched basepair at the primer terminus is the preferred substrate for the exonuclease activity over a correct basepair and the lack results in relatively low replication fidelity. Originally its error rate was measured at about 1 in 9,000 nucleotides, which is a very huge variation and results so many mutations in replicated DNA.

To overcome this drawback, *E.coli* DNA polymerase was used. DNA polymerase I (or Pol I) is an enzyme that participates in the process of DNA replication and is exclusively found in prokaryotes. It is composed of 928 amino acids, and is an example of a processive enzyme - it can sequentially catalyze multiple polymerizations.). It was initially characterized in *E. coli*, although it is ubiquitous in prokaryotes. In *E. coli* and many other bacteria, the gene that encodes Pol I is known as *polA*. Pol I possesses four enzymatic activities:

1. A 5' → 3' (forward) DNA-Dependent DNA polymerase activity, requiring a 3' primer site and a template strand
2. A 3' → 5' (reverse) exonuclease activity that mediates proofreading
3. A 5' → 3' (forward) exonuclease activity mediating nick translation during DNA repair.
4. A 5' → 3' (forward) RNA-Dependent DNA polymerase activity. Pol I operates on RNA templates with considerably lower efficiency (0.1–0.4%) than it does DNA templates, and this activity is probably of only limited biological significance.

Polymerase I was not the enzyme responsible for most DNA synthesis — DNA replication in *E.coli* proceeds at approximately 1,000 nucleotides/second, while the rate of base pair synthesis by Polymerase I averages only between 10 and 20 nucleotides/second. Moreover, its cellular abundance of approximately 400 molecules per cell did not correlate with the fact that there are typically only two replication

forks in *E.coli*. Moreover, it is insufficiently processive to copy an entire genome, as it falls off after incorporating only 25-50 nucleotides. Its role in replication was proven when, in 1969, John Cairns isolated a viable Polymerase I mutant that lacked the polymerase activity. Cairns' lab assistant Paula De Lucia created thousands of cell free extracts from *E.coli* colonies and assayed them for DNA-polymerase activity.

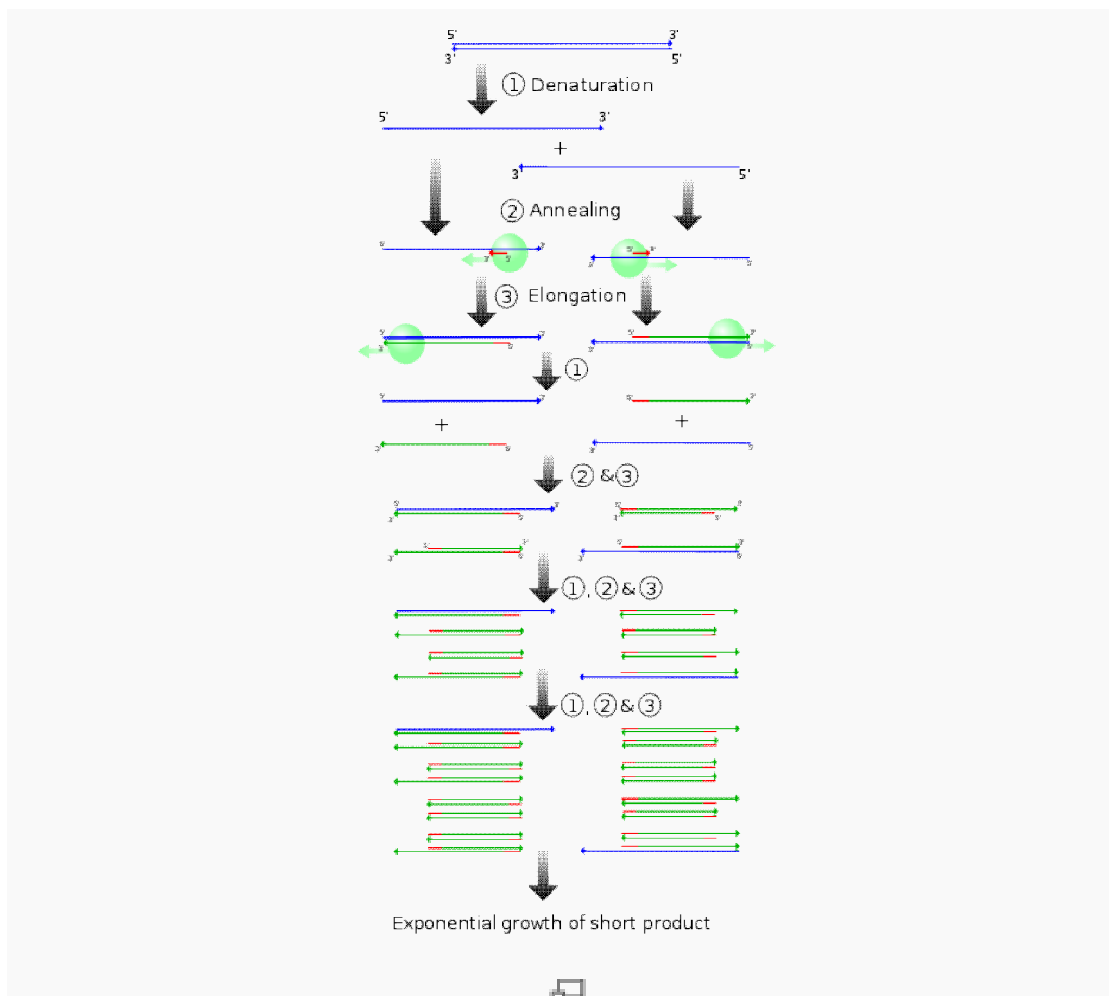


Fig.1: Schematic drawing of the PCR cycle. (1) Denaturing at 94–96 °C. (2) Annealing at ~65 °C (3) Elongation at 72 °C. Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

PCR Principles and Procedure

1. **Initialization step:** This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat

activation by hot-start PCR.

2. **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
3. **Annealing step:** The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
4. **Extension/elongation step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. **Final elongation:** This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. **Final hold:** This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Materials and Methods

A PCR KIT (KT44/44A) GeNei™ is required for the experiment.

Template DNA Source

MIAV1 gene from Mung Bean leaves infected by Yellow mosaic virus

Primers

PCR reaction needs two primers, a forward & a reverse primer. Primers are synthetic oligonucleotides usually ranging from 15 to 30 bases. Primers are designed such that at the 3' ends they do not have more than two bases complimentary to each other and these results in PRIMER-DIMER formation. G+C content is in the range of 40-60 %. The melting temperature (T_m) of both forward & reverse primers is usually same. Low concentrations of primers result in poor yield of the specific product & high concentration of primers may result in the non-specific amplification. Optimal concentration of primers is between 0.1-1 μ M.

Deoxynucleotide-tri-phosphates

The final concentration of each d NTP (d ATP, d GTP, d CTP , d TTP) in a standard amplification reaction is 200 μ M. It is important to keep the d NTP concentrations

above the estimated K_m of each d NTP (10 to 15 μM) for best incorporation.

PCR Buffer

The 10X assay buffer contains 100mM Tris- Cl (pH 9.0), 500 mM potassium chloride, 15mM MgCl_2 & 0.1 % w/v gelatine. Mg^{2+} is an essential cofactor required for the activity of *Taq* DNA polymerase.

Distilled Water: It is the nuclease free water.

Enzymes Involved:

A) *Taq* DNA Polymerase-

It is a 94 k D thermostable DNA polymerase. Optimal temperature for *Taq* DNA polymerase activity is 72° C. It lacks 3' to 5' exonuclease activity but has 5' to 3' exonuclease activity and 5' to 3' polymerase activity. For most amplification reactions 1.5 to 2 units of enzyme is recommended as higher enzyme concentration leads to non-specific amplification.

B) *E.coli* DNA Polymerase-

It is a 109 k D thermostable DNA polymerase. Optimal temperature for *E.Coli* DNA polymerase activity is 37° C. It has 3' to 5' exonuclease activity, 5' to 3' exonuclease activity and 5' to 3' polymerase activity. For most amplification reactions 1.5 to 2 units of enzyme is recommended as higher enzyme concentration leads to non-specific amplification.

Reagents Used

Ethidium bromide (EtBr), Bromo-phenol blue loading dye ($\text{C}_{19}\text{H}_{10}\text{Br}_4\text{O}_5\text{S}$) and Phenol ($\text{C}_6\text{H}_5\text{OH}$) are used as reagents in the experiment.

Method Followed In Experiment Using *Taq* Polymerase in PCR

The master mix was prepared in 1.5 ml eppendorf tubes as per the composition given in the following Table 1-

Table1. Polymerase chain reaction mixture

Reagents	Volume
Sterile water	38 μl
10X Assay Buffer	5 μl
10 mM dNTP Mix	3 μl
Template DNA (100ng/ μl)	1 μl
Forward Primer (100ng/ μl)	1 μl
Reverse Primer (100ng/ μl)	1 μl
<i>Taq</i> DNA Polymerase (3U/ μl)	1 μl
Total reaction volume	50 μl

The master mix of above components was taken in four different microfuge tubes with 50 μ l sample volume and now the microfuge tubes were placed in PCR machine. The thermocycler was programmed to run according to the conditions mentioned Table 2

Table 2: Conditions for polymerase chain reaction cycles

Steps	Temperature and Duration
Initial Denaturation	94 ° C for 5 minutes
Denaturation	94 ° C for 30 seconds
Annealing	54 ° C for 45 seconds
Extension	72° C for 1 minute
Final Extension	72° C for 10 minutes

Analysis on Agarose gel:

Followed PCR amplification, 5 μ l of gel loading buffer to each of the PCR tubes. Mixture was taped thoroughly for a few seconds until the 2 layers were not separated. 15 μ l of reaction mixture was pipette out carefully and loaded onto 1 % Agarose gel. 10 μ l of the ready to use marker provided was loaded. Noted down the order in which the samples has been loaded. The samples were run at 50 volts for first 15 minutes till the tracking dye (bromophenol blue) reached $\frac{1}{4}$ th of the length of the gel and then at 100 volts for about 45 minutes till the tracking dye reached $\frac{3}{4}$ th of the length of the gel. And last gel was visualized under UV transilluminator.

Method Followed In Experiment Using *E.Coli* Polymerase in PCR

The master mix was prepared in 1.5 ml eppendorf tubes as per the composition given in the following Table 3 -

Table3. Polymerase chain reaction mixture

Reagents	Volume
Sterile water	38 μ l
10X Assay Buffer	5 μ l
10 mM dNTP Mix	3 μ l
Template DNA (100ng/ μ l)	1 μ l
Forward Primer (100ng/ μ l)	1 μ l
Reverse Primer (100ng/ μ l)	1 μ l
E.coli DNA Polymerase	1 μ l
Total reaction volume	50 μl

The master mix of above components was taken in four different microfuge tubes with 50 μ l sample volume and now the microfuge tubes were placed in PCR machine. The thermocycler was programmed to run according to the conditions mentioned Table 2

Table 4: Conditions for polymerase chain reaction cycles

Steps	Temperature and Duration
Initial Denaturation	94 ° C for 5 minutes
Denaturation	94 ° C for 30 seconds
Annealing	54 ° C for 45 seconds
Extension	72° C for 1 minute
Final Extension	72° C for 10 minutes

Analysis on Agarose gel

Followed PCR amplification, 5 µl of gel loading buffer to each of the PCR tubes. Mixture was taped thoroughly for a few seconds until the 2 layers were not separated. 15 µl of reaction mixture was pipette out carefully and loaded onto 1 % Agarose gel. 10 µl of the ready to use marker provided was loaded. Noted down the order in which the samples has been loaded. The samples were run at 50 volts for first 15 minutes till the tracking dye (bromophenol blue) reached ¼ th of the length of the gel and then at 100 volts for about 45 minutes till the tracking dye reached ¾ th of the length of the gel. And last gel was visualized under UV transilluminator.

Method Followed In Experiment For The Development Of New Technique For using *E.coli* DNA Polymerase in PCR:

The master mix was prepared in 1.5 ml eppendorf tubes as per the composition given in the following Table 5 –

Table 5: Polymerase chain reaction mixture

Reagents	Volume
Sterile water	38 µl
10X Assay Buffer	5 µl
10 mM dNTP Mix	3 µl
Template DNA (100ng/µl)	1µl
Forward Primer (100ng/µl)	1µl
Reverse Primer (100ng/µl)	1µl
E.Coli DNA Polymerase	1µl
Total reaction volume	50 µl

The master mix of above components was taken in four different microfuge tubes with 50 µl sample volume and now the microfuge tubes were placed in PCR machine. The thermocycler was programmed to run according to the conditions mentioned Table 6.

Table 6: Conditions for polymerase chain reaction cycles

Steps	Temperature and Duration
Initial Denaturation	94 ° C for 5 minutes
Denaturation	94 ° C for 30 seconds
Annealing	54 ° C for 45 seconds
Extension	37° C for 1 minute

These steps included the procedure for the development of new technique for using *E.coli* DNA Polymerase in PCR. The PCR tubes were removed having total reaction volume of master-mix 50 µl each in the four PCR tubes used after completion of 1 cycle from the thermocycler. 50µl phenol was added to each of the four PCR tubes, then contents were mixed gently so that the mixture led to emulsion formation in the PCR tube. The total reaction volume of 100µl was placed in the centrifuge tube & performed centrifugation technique for 10 minutes at 5500g. After centrifugation, the centrifuge tubes were properly removed and observed if there is an interphase layer formed in between the two layers. The formation of interphase between the two layers was led to the formation of upper aqueous layer containing all the reaction mixture from the master mix except the *E.Coli* DNA polymerase that was present in the lower organic solvent phase in soluble form. The lower organic solvent phase was removed properly from the centrifuge tube by using micropipette; remaining upper aqueous layer in the PCR tubes were replaced. The thermocycler was programmed to run for remaining 29 cycles according to the conditions mentioned in Table7 and amplification carried out-

Table 7: Conditions for polymerase chain reaction cycles

Steps	Temperature and Duration
Denaturation	94 ° C for 30 minutes
Annealing	54 ° C for 45 seconds
Extension	37° C for 1 minute

Before elongation step the lower organic solvent phase was added to the master mix and that cycle was completed. Again the process followed the same procedure for next 30th cycles

Analysis on Agarose gel:

Followed PCR amplification, 5 µl of gel loading buffer to each of the PCR tubes. Mixture was taped thoroughly for a few seconds until the 2 layers were not separated. 15 µl of reaction mixture was pipette out carefully and loaded onto 1 % Agarose gel. 10 µl of the ready to use marker provided was loaded. Noted down the order in which the samples has been loaded. The samples were run at 50 volts for first 15 minutes till the tracking dye (bromophenol blue) reached ¼ th of the length of the gel and then at 100 volts for about 45 minutes till the tracking dye reached ¾ th of the length of the gel. And last gel was visualized under UV transilluminator.

Results and Discussion

According to the first experiment using Taq polymerase in PCR, which is also the conventional method, we got to see the brightness of gel. The PCR amplified product with Taq Polymerase run on 1% Agarose gel stained with EtBr, hence by visualizing the gel under UV transilluminator it showed 5 lanes. First lane denoted 100bp DNA Ladder (100bp to 1000bp). Second, Third, Fourth and Fifth lanes included PCR amplified 0.1kb fragment after 15 cycles electrophoresing alongside 0.1kb fragment of 100bp ladder, PCR amplified 0.1kb fragment after 20 cycles electrophoresing alongside 0.1kb fragment of 100bp ladder, PCR amplified 0.1kb fragment after 25 cycles electrophoresing alongside 0.1kb fragment of 100bp ladder and PCR amplified 0.1kb fragment after 30 cycles electrophoresing alongside 0.1kb fragment of 100bp ladder respectively (fig 9). The last four lanes after electrophoresing for various different cycles we found that the illuminescence was very good and prominent. This shows that the experiment was successful as expected with proper amplification of DNA.

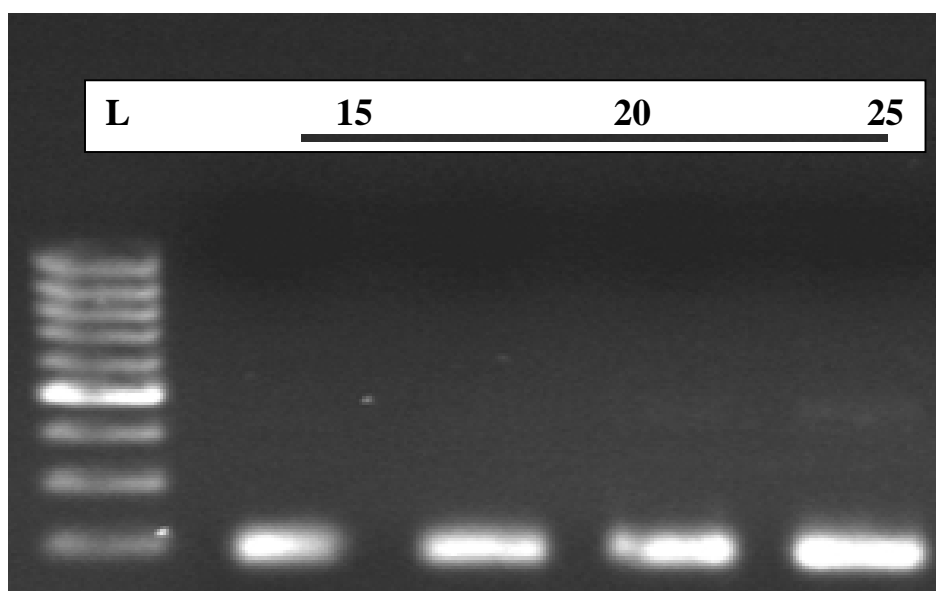


Fig.9: PCR amplified product with Taq Polymerase run on 1% Agarose gel (stained with EtBr)

In the second experiment using *E.coli* polymerase in PCR, the methods and materials used were same as the first experiment except *E.coli* polymerase. The PCR amplified product with *E.coli* Polymerase run on 1% Agarose gel stained with EtBr. The results obtained after visualizing the gel under UV transilluminator were faded and not at all prominent as compared to the earlier one. The experiment was successful and it showed that the amplification of the DNA was not proper as expected.

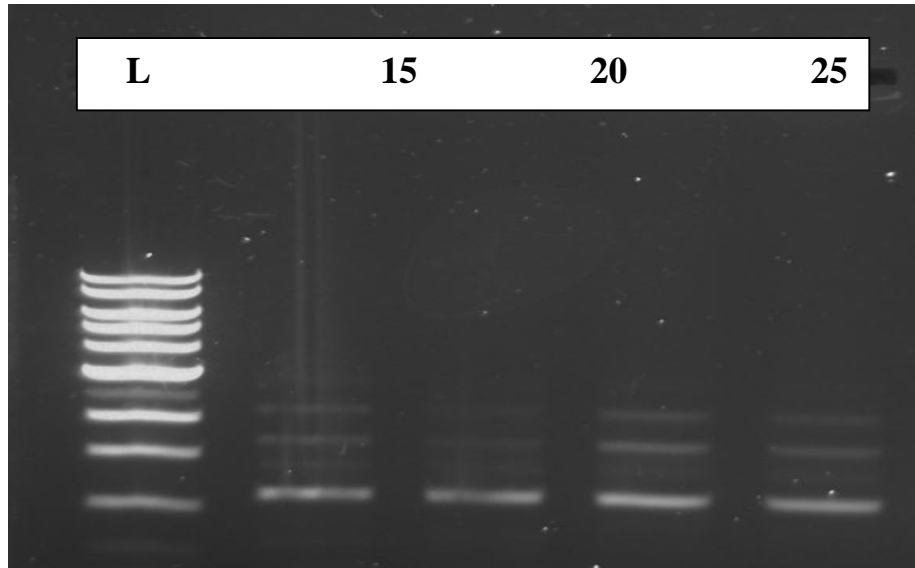


Fig 10. PCR amplified product with *E.coli* Polymerase run on 1% Agarose gel (stained with EtBr)

In the third experiment, PCR amplified product with *E.coli* Polymerase using new developed technique run on 1% Agarose gel stained with EtBr. The result concluded followed that the amplification of the gel was better as compared to the second experiment but not as good as the first experiment where *Taq* Polymerase was used. This was because of the new protocols implied in this experiment which led to the conclusion made.

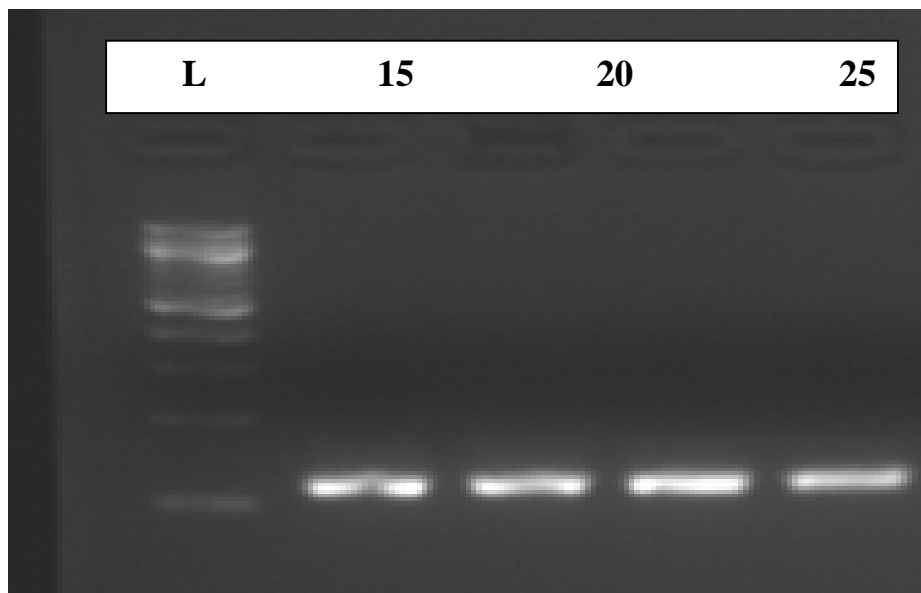


Fig.11. PCR amplified product with *E.coli* Polymerase using new developed technique run on 1% Agarose gel (stained with EtBr)

Hence, it is concluded from the comparative analysis of the three techniques that our new technique is working well but has not amplified DNA to the extent the *Taq* Polymerase did in the first experiment. Moreover the results clearly indicate that this new technique is quite superior to the method in which the *E.coli* DNA polymerase was used under similar conditions of conventional *Taq* Polymerase method.

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