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ISOLATION OF *Taq* POLYMERASE GENE & CLONING INTO *E.coli* USING pGEMT VECTOR

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ABSTRACT

Use of the thermo stable *Taq* polymerase eliminated the need for having to add new enzyme to the Polymerase Chain Reaction (PCR) reaction during the thermo cycling process. A single closed tube in a relatively simple machine can be used to carry out the entire process. Thus, the use of Taq polymerase was the key idea that made PCR applicable to a large variety of molecular biology problems concerning DNA analysis.[1] Considering its huge role in molecular biology research the world market for Taq polymerase is in the hundreds of millions of dollars each year.[2] Gene cloning is the act of making copies of a single gene by introducing it in a host using a suitable vector. Amplified genes are useful in many areas of research and for medical applications such as gene therapy, isolation of genes for further manipulations, DNA fingerprinting, Molecular diagnostics, for cloning purpose leading to cheaper production of gene products, etc. Looking into the importance and ever increasing demands of Taq polymerase, it is important to focus on searching simpler methods for cloning it into easy to maintain host like *Escherichia coli (E.coli).* In the present study, cloning of Taq DNA Polymerase gene in *E.coli* host using pGEMT vector was done successfully.

KEYWORDS: *Taq* DNA Polymerase, Polymerase Chain Reaction (PCR), Cloning, pGEMT vector

INTRODUCTION

Taq DNA Polymerase is a thermostable enzyme obtained from a heat stable bacteria called *Thermus aquaticus.* This enzyme has a molecular weight of about 66,000-94,000 daltons.[3] and is used for the amplification of selective DNA segments using polymerase chain reaction.[4] *Taq* DNA polymerase from Thermus aquaticus was the first characterized

thermostable enzyme.[5] This thermo- stable enzyme enables the amplification reaction to be performed at higher temperatures and makes the automation of PCR possible.[6] The full length 94 kDa *Taq* polymerase has maximal activity and half-life of 9 min at 97.5 °C.[7]

More than 50 DNA polymerase genes have been cloned and sequenced from various organisms including thermophiles and archaea.[8] Several DNA polymerases from the *Thermus* strain have also been studied and some of them like *Tfi, Top, Tfl*, and *Tth* polymerase have been applied to PCR.[9,10,11,12] However, their base-insertion fidelity was found somewhat low since these DNA polymerases do not have 3'→.5' exonuclease activity. The high-fidelity of DNA polymerases, which has 3'→5'exonuclease-dependent proofreading activity, are required for error correction during the polymerization. Many thermostable DNA polymerases like *Vent, deep Vent, Pfu*, and *Pwo* have shown proofreading activity and therefore have also been studied and introduced for high-fidelity PCR amplification.[13,14]

However none of the other thermostable enzymes except *Taq* polymerase could be commercially utilized as many thermostable enzymes were synthesized at very low levels by the thermophilic bacteria, therefore, they were difficult to purify. One advantage with *Taq* DNA polymerase is that it has a terminal transferase activity and it adds an A (adenine) at 3' end[15] which was found to be quite useful and was exploited to produce TA cloning vector (plasmid) which possess a 3'-T (Thymine) overhangs. This allows ligation using DNA ligase to quickly be accomplished with the A overhangs of the PCR product.

MATERIALS AND METHODS

Bacterial strains and plasmids

Culture plate of *Thermus Aquaticus* was obtained from MTCC Chandigarh (acc no.490). Inoculated colonies from this culture plate into LB media and allowed to grow for overnight at 37°C in incubator shaker. The plasmid pGEMT was used as a vector for cloning of desired gene and were obtained from Promega Corporation. Cloning in this vector is easy due to T tailed. *E. coli* strain TOP1O (Invitrogen) pHuLUC3/TOP10 were purchased from Himedia Laboratory. Growth conditions *E. coli* strains were grown at 37°C in Luria Bertani (LB) broth or plated on LB agar containing 80 μg/ml ampicillin as described by Sambrook et al.[16]

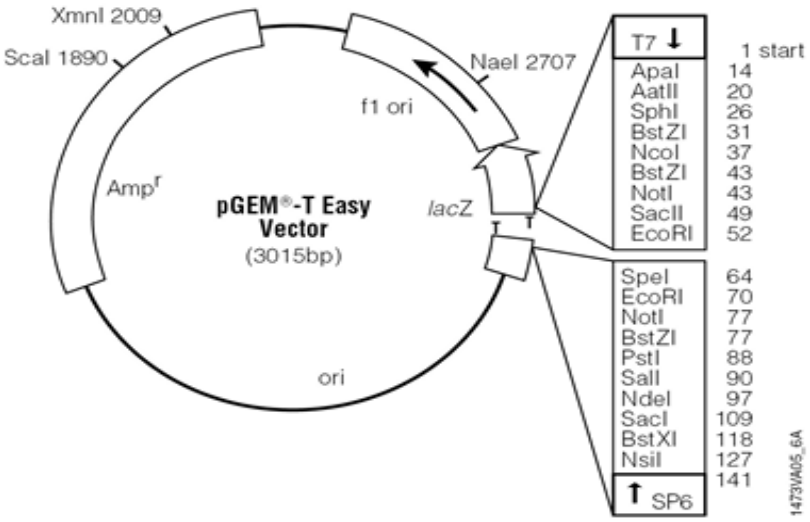

Fig. 1: Plasmid map of pGEMT vector.

Genomic DNA preparation

The Genomic DNA from *Thermus aquaticus* were isolated by simple method which involved the disruption and lysis of cell wall using sodium dodecyl sulphate (SDS) followed by the removal of proteins and other contaminants and finally recovery of the DNA. Removal of proteins is typically achieved by degradation of proteins by proteinase K and separation using phenol chloroform isoamyl alcohol. DNA is usually recovered by precipitation using ethanol or isopropanol. Electrophoresis in 1% agarose gel was used to confirm the size of the isolated DNA.

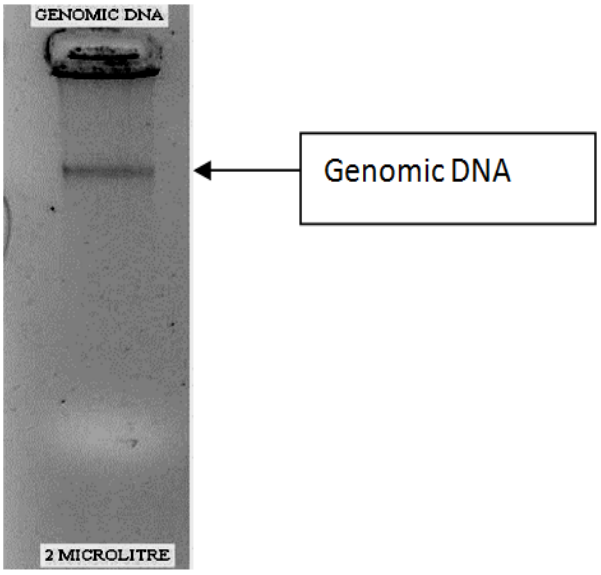

Fig. 2: Genomic DNA from *Thermus aquaticus.*

Amplification protocol

A pair of primers were designed based on the 5' and 3' ends of this gene and were utilized for PCR amplification. The sequences of these primers were as follows:

Forward	5′-CACGAATTCGGGGATGCT GCCCCTCTTTGAGCCCAAG-3′	Reverse	5′-GTGAGATCTATCACTCCT TGGCGGAGAGCCAGTC-3′

The isolated genomic DNA of *Thermus aquatius* was now used as a template to amplify the *Taq* DNA Polymerase gene by Polymerase Chain Reaction (PCR) using gene specific primers. The PCR Reaction was set up using following components in PCR tubes:

Table1: PCR reaction mix composition.

Components	**Volume (µL)**
H_2O	17.4
10X Reaction Buffer	2.5
50mM $MgCl_2$	0.5
10mM dNTPs	1.0
10pmol Forward primer	0.7
10pmol Reverse primer	0.7
Platinum Taq (5U/µl)	0.2
Template DNA	2.0
TOTAL	**25**

These components were mixed properly in PCR tube and incubated in thermocycler (Bio-Rad) using following reaction conditions:

Table 2: Reaction condition for PCR.

No. of cycles	**Temperature (°C)**	**Time**
1	95	5min
35	95	30sec
	60	45sec
	72	2 min
1	72	2 min
1	4	Hold

Confirmation of PCR product

The amplified PCR product was analyzed by electrophoresis in 1.5% agarose gel. The confirmation was done by comparing the size of the band with the 1kb DNA ladder.

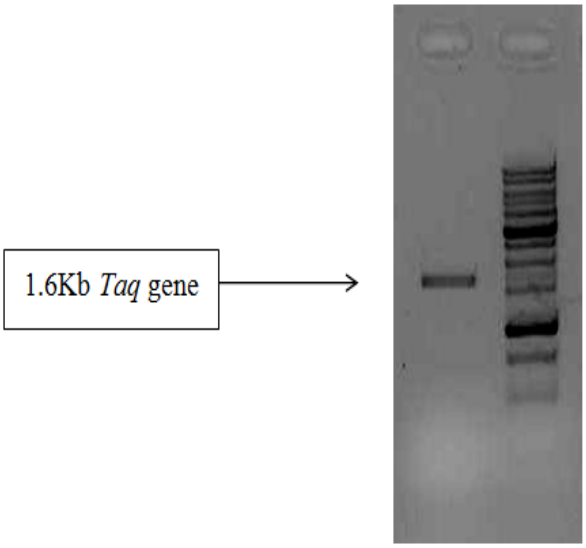

Figure 3: Lane 1-Amplified Taq gene at 1660bp, Lane 2-100bp DNA Ladder.

The amplified PCR product was now excised from the agarose gel using fresh and sterile scalpel. Kept the excised fragment in a micro centrifuge tube and eluted in 30 µl using QIAquick PCR Purification Kit. The eluted fragment was now checked on 1.5% agarose gel. The band was visible at the correct size of the concentration of ~30ng.

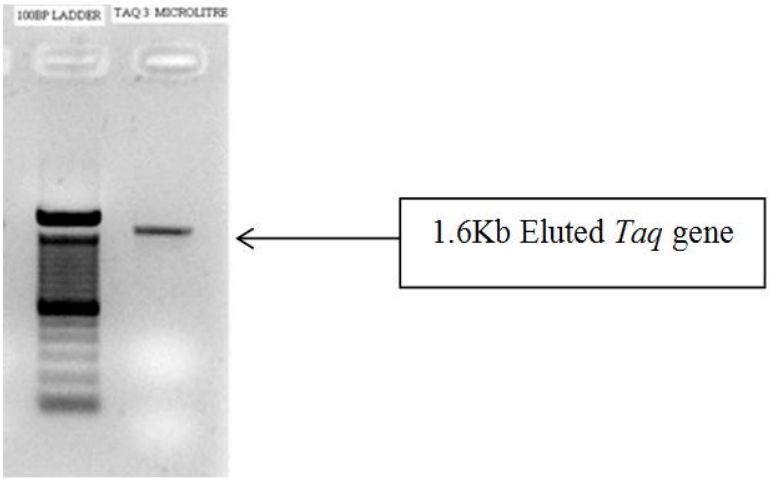

Figure 4: Lane1- 100bp DNA Ladder, Lane2-Eluted Taq gene 2microlitre.

Ligation of *Taq* DNA polymerase gene with pGEMT vector

The eluted *Taq* gene and pGEMT were now ligated using the enzyme T4 DNA ligase. The ligation reaction was carried out at 4°C as this facilitates the higher efficiency for ligation. Following ligation mix was prepared:

Table 3: Reaction mix for ligation reaction.

Contents	Quantity (µl)
H_2O	4.0
Vector (pGEMT)	1.0
Insert (Taq)	3.0
T4 DNA Ligase	1.0
Buffer (10X)	1.0
Total	**10 µl**

Transformation Of *Taq* DNA Polymerase Gene/pGEMT clone in *E. coli*

The ligation mixture of Taq/pGEMT was used for transformation in competent E. coli TOP10 host cell. Whole of the ligated sample was utilized for the transformation procedure. Calcium chloride mediated heat shock method was used for transformation. Transformed cells were plated on LB agar plates containing ampicillin (final concentration 100µg/ml). These plates were kept overnight in incubator at 37°C to grow bacterial cells.

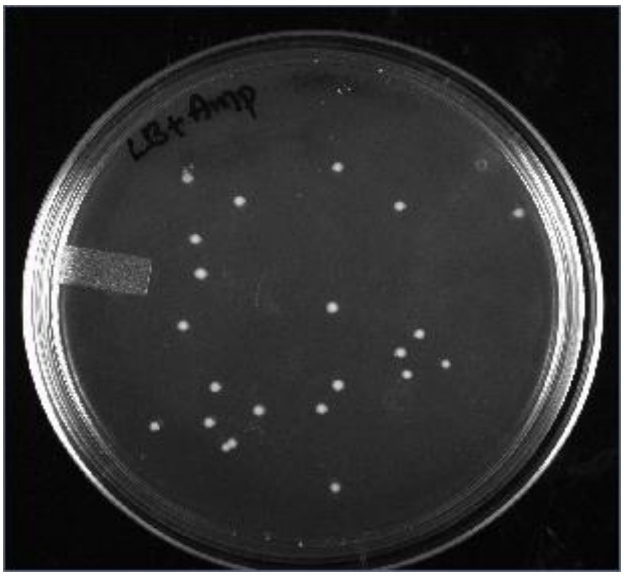

Figure 5: Ampicillin resistant *E.coli* clones after successful transformation.

Plasmid DNA Isolation of *Taq*/pGEMT Clone

One colony from the transformation plate was picked and was inoculated in the LB broth containing ampicillin. The inoculated media was kept at 37°C in incubator shaker for 12-14 hours to grow the bacterial cells. Plasmid DNA isolation was done using this culture. Two microlitre of this isolated plasmid was checked on 1% agarose gel.

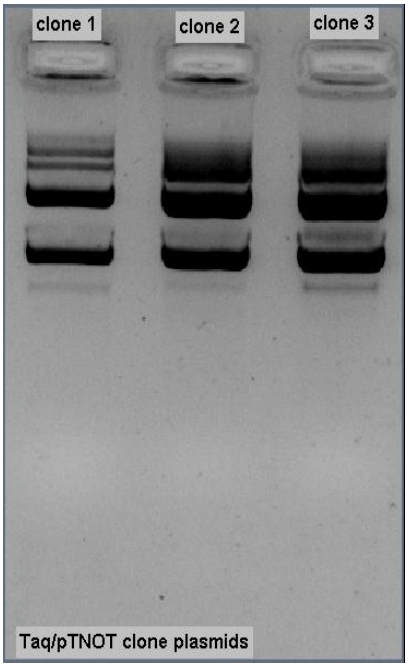

Figure 6: Lane-1 Taq/pGEMT clone plasmid 1, Lane-2 Taq/pGEMT clone plasmid 2, Lane-3 Taq/pGEMT clone plasmid 3.

Restriction Digestion of *Taq*/pGEMT Clone

The restriction digestion of Taq/pGEMT clone was performed using restriction enzymes NOT1 to check the presence of clone according to the following reaction.

Table 4: Reaction mix for Restriction digestion.

Components	Volume Added (µl)
Taq/pGEMT plasmid	1
NEB BUFFER 3	2
NOT 1	1
RNase	1
WATER	5
TOTAL	**10**

The reaction mixture was kept on heating block at 37°C for 3 hours. After three hour, the reaction mixture was mixed with 5X DNA loading dye loaded on 1.5% agarose gel and the product was analyzed by agarose gel electrophoresis.

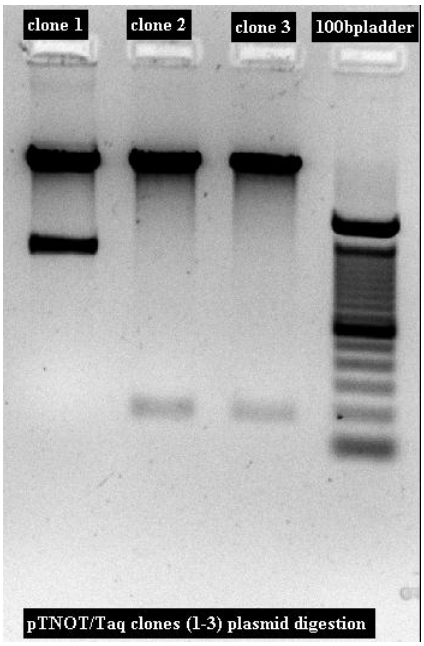

Figure 7: Lane-1 Taq/pGEMTclone 1 plasmid digested with NOT 1, Lane-2 Taq/pGEMT clone 2 plasmid digested with NOT 1, Lane-3 Taq/pGEMT clone 3 plasmid digested with NOT 1, Lane: 4 100bp DNA ladder.

Clone 1 plasmid digestion released approximately 1.7kb band with Not1 enzyme as it is clear from the gel picture. So this is the correct clone of *Taq* gene in pGEMT vector.

RESULTS AND CONCLUSION

Through post PCR analysis it was clear that *Taq* gene was amplified successfully from *Thermus aquaticus* genomic DNA as it shown a band at 1660bp on 1.5% agarose gel. Eluted fragment was also visible on gel, thus according to its concentration it was used for ligation reaction. Colonies on LB/amp plates showed the transformation of plasmid in *E. coli* TOP 10 host. Plasmids showed 3 bands on gel represent linear, circular and super coiled form of plasmid DNA. Finally restriction digestion of these plasmids (pGEMT/Taq) with enzyme NOT1 confirmed the presence of clone as it released approx 1.7kb band on gel. Thus the Taq gene was successfully cloned in pGEMT cloning vector. This vector was used because cloning in this vector is easy, being a T tailed vector.

Cloning is a more convenient and much faster procedure as compared to those used in other studies. Further by sequencing of pGEMT/Taq clone it can be more confirmed that the cloned fragment is only *Taq* gene. Thus this cloning of *Taq* gene would allow us to perform many studies including expression of this gene, mass production of the enzyme and introducing mutations for enhancing its performance.

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