

Molecular Cloning Techniques for Applications in Biotechnology



INTRODUCTION

Molecular cloning

In order to make plentiful identical specimen of a particular DNA fragment, molecular cloning is required. This is a collection of experimental approaches. DNA cloning is a powerful technique that may be used to create complicated DNA sequences that can be used in a diversity of applications, as well as study of one specific DNA sequence. All modern biomedical research studies and translational applications are based on this process (Bertero, Brown, & Vallier, 2017).

Molecular cloning inserts a gene from any species into a propagation vector without changing the DNA sequence. Molecular clones can be used to analyse gene sequences or express proteins for study or practical usage. In vitro clone changes and mutations are possible. The fundamental cloning workflow involves creating recombinant molecules (plasmids), ligating target DNA pieces into a cloning vector, and separating the target DNA fragments (commonly referred to as inserts). Once transformed into bacteria or another host for proliferation, recombinant plasmid can be used to identify or test potential hosts. In the late 1960s and early 1970s, numerous laboratories carried out four groundbreaking processes (Davis, 2012).

Cloning involves isolating and amplifying a DNA segment. Restrictive enzyme digestion or PCR targeting produce these fragments. Complementary oligos can be used to make tiny inserts of less than 100 base pairs that can be annealed to create double-stranded fragments. Target DNA is ligated to a plasmid vector, a circular piece of double-stranded DNA that can be generated in *E. coli*. Laboratory-created vectors are scaled-down analogues of naturally occurring plasmids. They have a replication origin, drug-resistance gene, and specific restriction sites to insert DNA fragments. Polylinker regions or "multiple cloning sites" make it easier to identify feasible restriction enzyme combinations for a wide range of inserts. Cloning strategies must carefully address restriction enzymes. After cutting double-stranded DNA in one spot, a few bases remain, generating "blunt" ends. Successful cloning is more likely if the "sticky" ends can find each other during ligation. Restriction enzymes can also be utilised to modulate insert directionality (Sharma, Mishra, Mehraj, & Duraisamy, 2014).

Glycoside hydrolases family gene – cel48

Certain cellulases depend on family 48 glycoside hydrolases. Anaerobic fungi like *Piromyces equi*, free enzyme systems like *Thermobifida fusca*, multifunctional enzymatic systems like *Caldicellulosiruptor saccharolyticus*, and every cellulosome system reported so far contain them. Most cellulosome-producing bacteria include GH48 cellulase. Some bacteria have two or more copies of the GH48 gene, although most only have one. Cel48Y is a free, non-cellulosomal enzyme that binds to cellulose through CBM3. Cel48S is a cellulosomal enzyme that contains dockerin (Smith et al., 2012).

PCR Amplification of the glycoside hydrolases family gene – cel48

Sustainable development necessitates using lignocellulosic biomass to replace petroleum-based manufacturing processes. Despite lignin's potential as a bioresource, its resistance hinders its use in lignocellulosic biomass. Before using cell wall components, lignin must be removed. Degradation processes in nature include depolymerization of lignin and mineralization of the resulting heterogeneous low-molecular-weight aromatic compounds. Using bacterial catabolic systems for lignin-derived aromatics may help produce taste compounds, polymer building blocks, and energy storage molecules. This may be because these two processes synergize (Kamimura, Sakamoto, Mitsuda, Masai, & Kajita, 2019).

Without GHs, biomaterial degradation is impossible (glycoside hydrolases). GHs hydrolyze glycosidic bonds between two or more carbohydrates or a carbohydrate and a non-carbohydrate substance. This relationship might also be between two carbohydrate-free substances. This work involves amplifying glycoside hydrolase cel48 from genomic DNA and cloning it into the T-Easy vector. The circular vector with an insert will be utilized to produce competent cells that carry lignocellulosic enzyme.

MATERIALS AND METHODS

1. DNA Quantification

Before moving on to subsequent investigations, DNA quantification, also known as nucleic acid quantification, is frequently carried out to ascertain the average concentration of DNA in a sample. To accurately determine the concentration of DNA or RNA in a sample, sample purity is a crucial factor.

The amount of DNA in a 2-L drop on a pedestal can be measured using a NanoDrop spectrophotometer, a typical lab tool.



Figure 1. Nanodrop
www.thermoscientific.com/nanodrop

- Launch ND-1000 V3.8.1 on the Nanodrop's PC.
- After opening the software, the appropriate module was chosen. Open the sampling arm and input DNase-free H₂O.
- Machine's arm opened to cover blank sample. "OK" read the blank.
- Used a Kimwipe to clean the upper and lower pedestal blanks.
- Closed the RNA arm. Sample type was selected in the software's upper right. Clicked "Measure" in software's upper left corner.
- After reading the sample, the equipment read 260/280, 260/230, and concentration (ng/L).

2. Polymerase Chain Reaction

A single DNA fragment can be amplified into millions or billions of copies using PCR. This allows deeper study. Primers are synthetic DNA fragments used in PCR to select the amplified region of the genome. This region is then amplified by DNA synthesis.

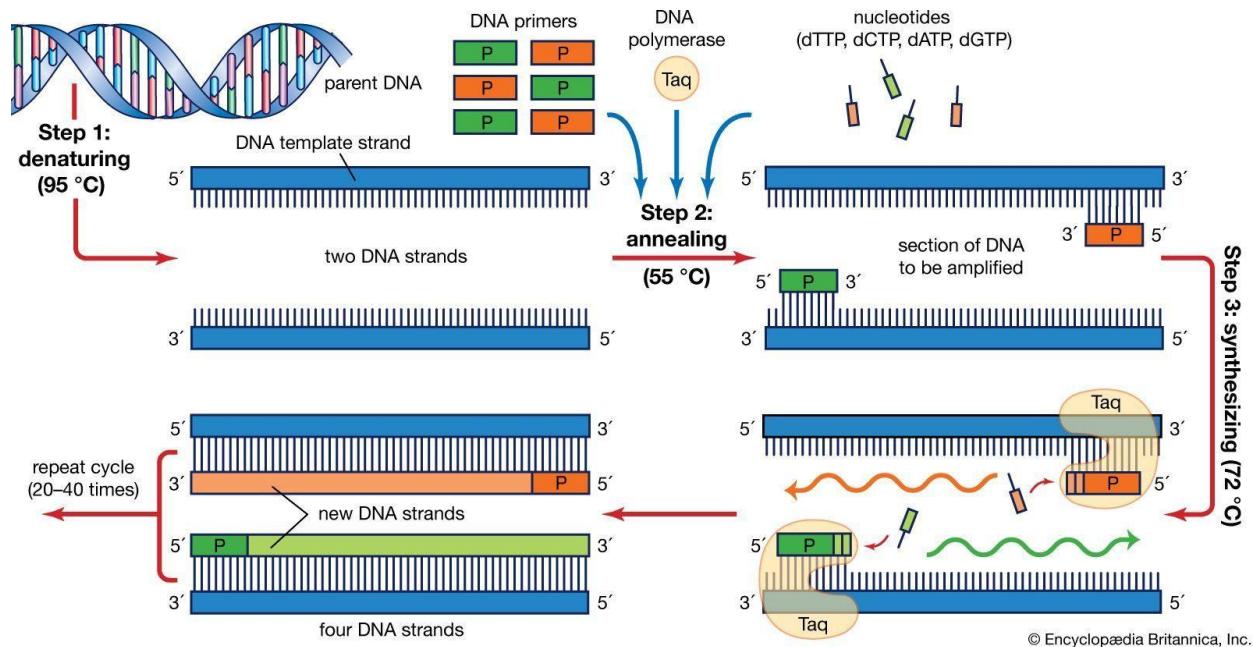


Figure 2. Polymerase Chain Reaction
<https://www.britannica.com/science/polymerase-chain-reaction>

Procedure

Genomic DNA, Master Mix (Table No.1) and sterile water were mixed in an Eppendorf tube.

Master mix Recipe

Table No. 1 Master Mix

Reagents	Concentration
PCR buffer,	10mM KCl, 10mM $(\text{NH}_4)_2\text{SO}_4$
Tris HCl	20mM pH 8.8
MgSO ₄	2mM
Triton-X-100	0.1%
Primer cel48_490F	0.2 μ M
Primer cel48_920R	0.2 μ M
dNTPs	200 μ M
Taq polymerase	1 unit/30 μ l

Primer sequences

Table No. 2 Primers

cel48_490F	TAATGGTTGAAGCTCCAGATTATGG
cel48_920R	CCAAAGCCGTACCAGTTATCAACGTC

Primers were optimized for maximizing the yield of PCR product. The PCR conditions and recipe was also optimized and are listed in the given tables.

Table No. 3 Recipe for PCR

Serial No.	Working Reagents	Quantity (μL)	Conc.
i.	DNA	1.0	50 ng/ μ L
iv.	PCR Master Mixture	10	
v.	(Distilled) Water	9.0	
Total vol.		20	

Table No. 4 PCR Steps

Sr. no.	Steps of PCR	Temp (°C)	Time
1.	Initial denaturation	95	3 minutes
2.	Denaturation	95	40 seconds
3.	Annealing	56	30 seconds
4.	Extension	72	30 seconds
Repeat 2-3-4 (45 cycles)			
3.	Final extension	72	7 minutes

- The amplified samples were kept at -20°C for later your use.

3. Agarose gel electrophoresis

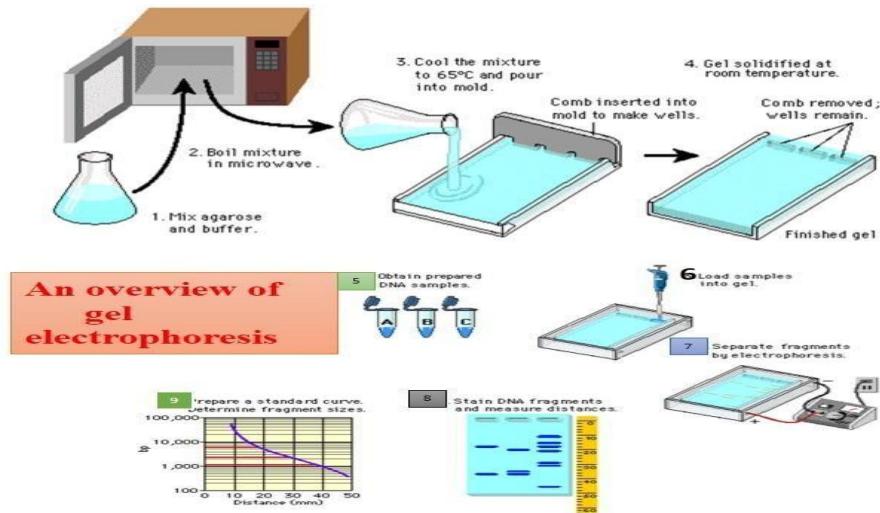


Figure 3. Overview of Agarose Gel Electrophoresis
<https://universe84a.com/collection/gel-electrophoresis/>

- The amplified PCR product was examined by 1 % agarose gel electrophoresis. Gel was prepared by using following method.
- One gram of agarose gel powder was weighed.
- 100ml of 1X TBE buffer was measured in a flask.
- The weighed agarose gel powder was added into the flask.
- Flask was held in microwave oven and heated until the mixture boiled and became clear.
- The mixture was allowed to cool down and then 10 μ L ethidium bromide dispensed into the mixture.
- The gel caster and comb was set and gel was poured into it.
- The gel was left to cool down and solidify. The comb was removed carefully after solidification.
- The gel was then settled into tank containing the same buffer (1X TBE buffer).

Sample Preparation and Loading

- PCR product (10 μ L) was mixed with 5 μ L of bromo-phenol blue.
- The mixture was loaded into wells.
- Gel was run for 30 minutes at 110 volts.

Visualization

- Amplicons were visualized under UV light using UV-Transilluminator.

4. DNA extraction from the agarose gel

This study employed the 28706X4 Gel Extraction Kit (1000) to extract DNA amplicon from gels. The QIAquick Gel Extraction Kit removes salts, agarose, ethidium bromide, and other contaminants from samples, allowing for 80% DNA recovery.

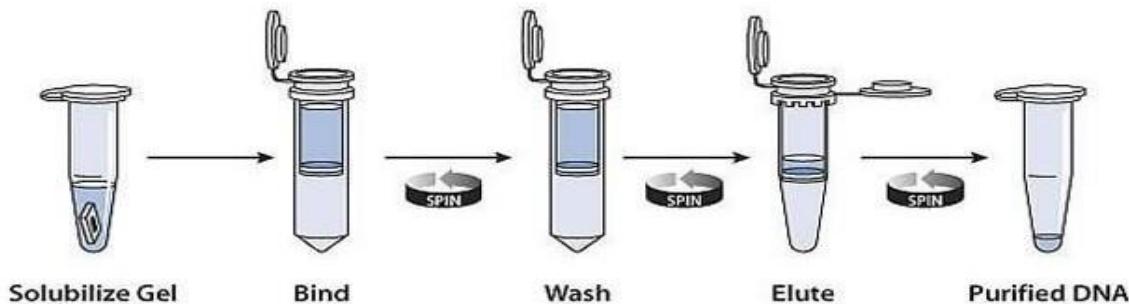


Figure 4. DNA extraction from the agarose gel

https://www.researchgate.net/figure/Flow-chart-of-the-Protocol-for-gel-purification-by-using-Isolate-II-PCR-and-Gel-kit_fig3_316828918

Procedure

1. After weighing an empty Eppendorf tube, weighed it again with agarose gel slice.
2. Noted the gel slice's mass.
3. Added 300 μ l of Buffer QG for every 100 mg of gel, or 3 volumes of Buffer QG to 1 volume of gel.
4. Incubated for 10 minutes at 50 °C (or until the gel slice has dissolved entirely).
5. Mixed by vortexing the tube every 2-3 minutes during the incubation to aid in the gel's dissolution.
6. Verified that the fluid is yellow after the gel slice has completely dissolved (similar to Buffer QG without dissolved agarose)
7. Mixed the sample with 1 gel volume of isopropanol.
8. Inserted a QIAquick spin column into the 2 ml collection tube that is provided.
9. Applied the material to the QIAquick column and centrifuge for 1 minute to bind DNA.
10. Throwed away the flow-through and re-insert the QIAquick column into the original collecting tube.
11. Discarded the flow through and centrifuge the column for 1 minute while washing with 0.5 ml of buffer QG.
12. Washed the column with 0.75 ml of buffer PE.
13. Removed any remaining PE buffer by centrifuging an empty QIAquick column for an additional minute after discarding the flow-through.
14. Inserted a clean 1.5 ml microcentrifuge tube with QIAquick column inside.
15. Added 50 μ l of warm, sterile water to the QIAquick membrane to elute the DNA. Centrifuge for 1 minute, then let the column stand for 1 minute. Saved the purified PCR result for the following step.

5. Ligation

The ligation of linear DNA into a cloning vector is one of the crucial steps in the cloning procedure. Many fundamental biotechnology investigations depend on the ability to connect DNA fragments using recombinant technology.

T Easy Vector

The pGEM-T Easy Vector MCS has restriction enzyme-recognizable sequences on both sides of the insert. Each insert side has sequences. Because of this, insert DNA can be deleted with just one restriction enzyme round.

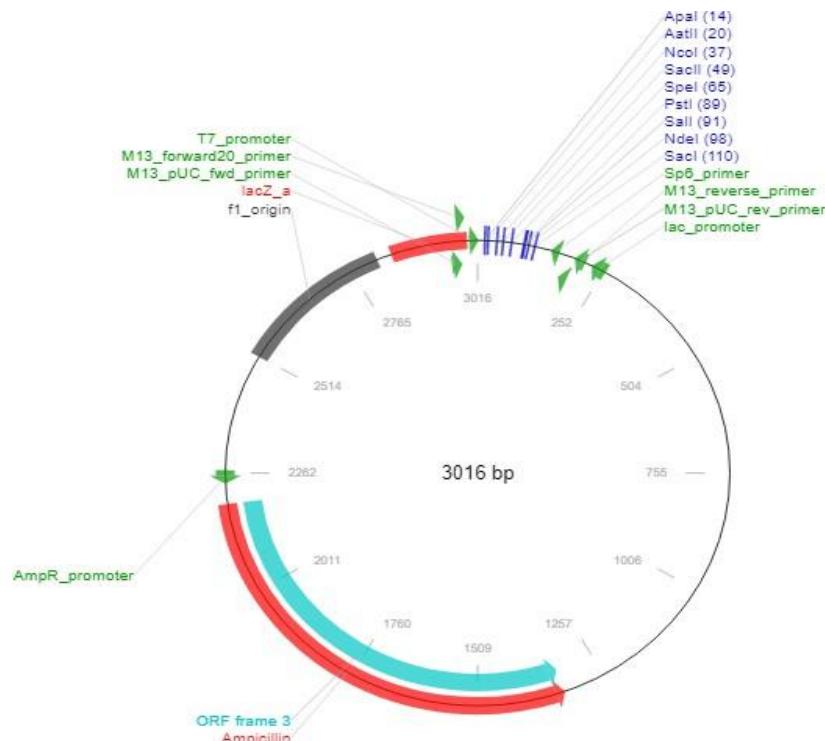


Figure 5. pGEM-T Easy Vector
<https://www.addgene.org/vector-database/2853/>

Procedure

The ligation mixture was prepared in the 0.5 ml tube and was mixed by centrifugation. Incubation was done for 24 hours and then stored at -20°C for later use.

Table 5. Ligation Mixture

Reagents	Concentration
Purified PCR product	3.5 µl
T-easy vector,	0.5 µl
2x buffer	5 µl
T4 ligase	1 µl

6. Transformation of the ligation mixture in TOP10 competent cells

The ability of a cell to absorb foreign (extracellular) DNA from its environment is referred to as cell competence. Transformation describes the process of genetic absorption.

Procedure

1. Put ice on top of the ligation mixture.
2. Two 50 L vials of One Shot® cells were thawed on ice for each ligation or transformation.
3. Pipetted 5 L of the ligation reaction into the competent cell container and blend by gently tapping.
4. Pipetted 5 l of pUC19 that has not been digested (this is a positive control; this supplied straight into the container at a concentration of 0.2 ng/l) competent cells, then gently tapped. Added a positive control label on the tube.
5. The leftover ligation mixture was kept at 20°C.
6. The vials were kept on ice for 5 minutes.
7. Spreaded your cells on two LB agar plates that have been preheated and labelled having ampicillin 100 g/mL.
8. Overnight incubation of the plates at 37 °C was done.

RESULTS

Nanodrop

The results observed were according to the standard range. A260/280= 1.80, A260/230=1.76 and concentration= 247.9.ng/ μ L

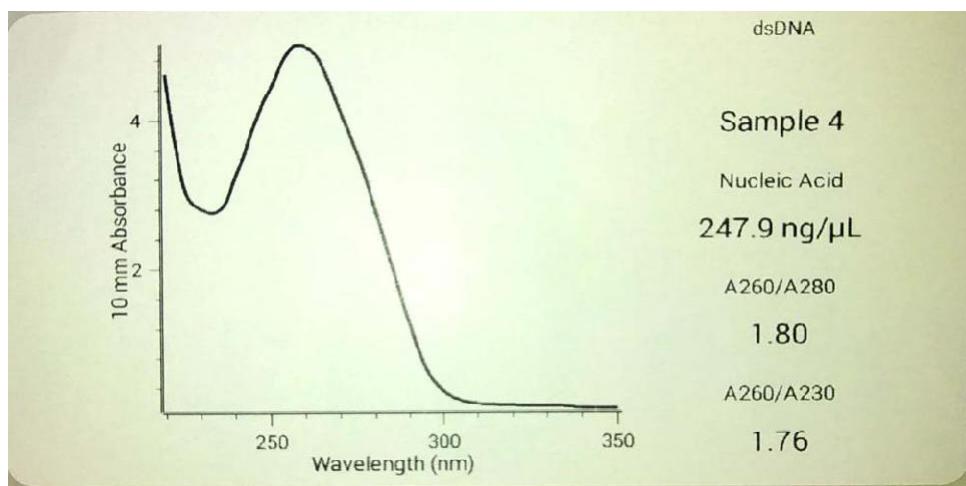


Figure 6. Nanodrop Readings

Polymerase Chain Reaction

The first gel picture (figure 7. (a)) shows the reference band size of cel48 amplicon that can not be observed hence from the results (Figure 7. (b)) it is interpreted that the amplification of the desired gene i.e. cel48, has not been accomplished.

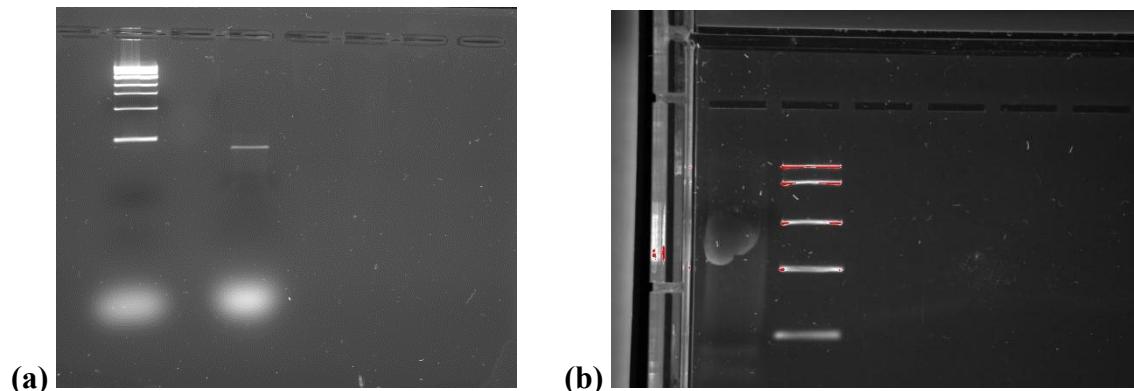


Figure 7. (a) Reference cel48 amplified on Gel. (b) Amplification of cel48 gene in sample DNA

Transformation

Table 6. Transformation results

Sample	White colonies	Blue colonies	Recombination efficiency
Ligation control	0	103	7×10^{-6} cfu / μg of DNA
Ligation containing insert	75	47	8×10^{-6} cfu / μg of DNA

Analysis of sequence

Table 7. Sequence for analysis

Bilal Shafiq	caagtgaagc tatgagttac tatatgaggc ttgaagcaat gaatggaaaa ttcagtggag atccctctgg ctttgaagaa gcatggatg ttacagagaa atatttgatc ccatctgaca aggatcaacc aaacagtagt atgagtagat ataatcctag tgacggtgca atggatgctc cagaatggaa aacacacctgaa aaatatccat cacagttaga ttttgatgct aagcttggtc aggaccat aatttagagag ttagtgtctt cctacggcat
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Uncultured bacterium clone C48C02TR328CT08 glycosyl hydrolase family 48 protein gene, partial cds

Sequence ID: [KC789656.1](#) Length: 321 Number of Matches: 1

Range 1: 11 to 287 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
		263/277(95%)	0/277(0%)	Plus/Plus
435 bits(235)	2e-117			
Query 1	CAAGTGAAGCTATGAGTTACTATGAGGCTTGAAGCAATGAATGGAAAATTCACTGGAG	60		
Sbjct 11 T	70		
Query 61	ATCCCTCTGGCTTGAAGAAGCATGGATGTTACAGAGAAATATTGATCCCCTGACA	120		
Sbjct 71	.. TTT	130		
Query 121	AGGATCAACCAAACAGTAGTATGAGTAGATATAATCCTAGTGACGGTGCAATGGATGCTC	180		
Sbjct 131 CC CTT	190		
Query 181	CAGAATGGAAACACCTGAAAAATATCCATCACAGTTAGATTTGATGCTAACGCTTGGTC	240		
Sbjct 191 CCTG	250		
Query 241	AGGACCCCATAATTAGAGAGTTAGTGTCTTCTACGG	277		
Sbjct 251 A	287		

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Uncultured bacterium clone CEL48OTU10TRF329WWTP04C06 glycosyl hydrolase family 48 protein gene, partial cds

Sequence ID: [KT336183.1](#) Length: 321 Number of Matches: 1

Range 1: 11 to 287 [GenBank](#) [Graphics](#)

[▼ Next Match](#) [▲ Previous Match](#)

Figure 8. Sequence results on BLAST

Table 8. Interpretation of sequence

Position	Mutation	Change
27	Subsitition	T -> A
63-65	Subsitition	TTT -> CCC
165-166	Subsitition	CC -> GG
172-174	Subsitition	CTT -> TGG
231-234	Subsitition	CCTG -> AAGC
254	Subsitition	A -> T

DISCUSSIONS

Nucleic acid concentration can be estimated by measuring the absorbance of a sample at 260 nm (Gallagher, 1994). There are three absorbance ratios that are used to measure the purity of DNA extracted from biological materials during the extraction process: 260/280, 260/230, and 260/325. For DNA, pure nucleic acids typically produce a 260/280 ratio of 1.8 to 2.0 (Sukumaran, 2011). The 260/230 purity ratio is a second metric for DNA purity, with values typically falling between 1.8 and 2.2 for "pure" nucleic acids. At least 50 ng/ μ L is the minimum acceptable concentration of double stranded DNA. The results observed were according to the standard range. A_{260/280}= 1.80, A_{260/230}=1.76 and concentration= 247.9.ng/ μ L (Figure 6). In a study A total of 91.55 ng/L of human DNA was extracted from peripheral blood; the initial concentrations for Sprague Dawley rats and NIST SRM 2372 were 56.7 and 57.9 ng/L, respectively, with a purity of 1.95 and 2 (Ponti et al., 2018).

From the (Figure 7. (b)) it is interpreted that the amplification of the desired gene i.e. cel48, has not been accomplished. The reason for the negative results was figured out to be the fault in the PCR machine locking. The device was not properly closed which caused the temperature fluctuations and the desired temperature was not attained that is crucial for thermocycler. The use of heated lids reduces the development of nonspecific annealing and primer dimers by allowing samples to be heated more efficiently. In addition, this feature helps to keep water from condensing on the lid and contaminating your samples (Tahamtan & Ardebili, 2020).

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