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Determination of multicopy clones containing recombinant papain gene by Real-Time PCR

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ABSTRACT

Multi copy clones also known as "jackpot clones" are transformed cells with a recombinant gene having greater number of recombinant gene copy as compare to normal one. Post Transformational vector amplification (PTVA) is a technique for production of multicopy clones. In PTVA the selection of transformed cell is done on agar medium containing the increasing concentration of antibiotic. A recombinant PUC57 vector containing the artificial synthesized papain gene was used to transform the DH5α strain of *E.coli*. The Transformed cells were selected on LB agar plates with increasing concentration of ampicillin antibiotic (100µg/ml, 250µg/ml, and 500µg/ml), the number of colonies were reduced as ampicillin concentration was increased. Plasmid DNA isolation was carried out from Qiagen-Qiaprep-mini DNA isolation kit. Real time PCR is performed to identify multicopy clone on each plates. The average cycle threshold value i.e. Ct value for 500µg/ml LB-Amp Plate is 19.46, for 250µg/ml LB-Amp Plate is 22.40, and 100µg/ml LB-Amp Plate is 24.68. The Real-time PCR results indicated the higher level of gene expression in cells grown in higher antibiotic concentration.

INTRODUCTION

Recombinant DNA technology is a major DNA-based tool that opens a new age for modern biotechnology. With this technology, a gene or multiple genes can be identified, cut, and inserted into the genome of another organism (Pham, 2018). The recent development of DNA cloning methods has made possible the introduction into *Escherichia coli* of genetic material from a wide variety of sources. Such experiments have involved principally use of cloning vehicles or vectors derived from plasmid (Chang and Cohen, 1978). Foreign genes have been implanted into the DNA of *E.coli* to enable the production of useful protein (Blackstock, 1989). Plasmids are usually maintained in an *E. coli* host by antibiotic selection (Jang and Mangnuson, 2013). There are several essential experimental methods for generating multi-copy clones including in vitro multimerization of the vector before transformation and direct selection of transformants on high concentrations of antibiotics, made possible by the increased use of ampicilln. Due to the low efficiency of generating multi-copy clones via direct selection, in 2008 Sunga *et al*., proposed the method of posttransformational vector (PTVA) (Aw and Polizzi, 2016). PTVA uses a single vector for transformation (containing ampicillin resistance marker). Selection is originally on a low concentration of the corresponding antibiotic, but the cells are increasingly subjected to higher concentrations. Only colonies that have multiple copies of the resistance gene (and therefore multiple copies of the heterologous gene) will be able to survive on the highest concentrations. Jackpot colonies are reported in 6% of all clones tested. Integration into the rDNA locus with PTVA utilizes the repeat sequence of the rDNA, which can prevent tandem head-to-tail integration. Multi-copy clones are generated using PTVA (Aw and Polizzi, 2013). The next step involves the isolation of the multiplied Gene of interest attached with the vector or of the protein encoded by it (Aryal, 2018) single-copy of expression vectors would be sufficient for efficient foreign gene expression (Romanos *et al*., 1998). For the detection and quantification of nucleic acids, Real-time PCR has become the most accurate and sensitive method. Quantitative measurement of specific gene expression using quantitative PCR (qPCR) is necessary for understanding basic cellular mechanisms and detecting of alteration in gene expression levels in response to specific biological stimuli (Yilmaz, *et al.,* 2012). The reaction is going to performed in duplicates for all the samples to rule out-pipetting error and to check the PCR efficiency with one amplification positive control and one amplification negative control.

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ANALYSIS OF RELATIVE GENE EXPRESSION DATA USING REAL-TIME QUANTITATIVE PCR AND $2^{-\Delta CT}$ METHOD

Reverse transcription combines with polymerase chain reaction (RT-PCR) has proven to be a powerful method to quantify gene expression. Real-time PCR has been adapted to perform Quantitative RT-PCR (Livak and Schmittgen, 2001). Real-time PCR (RT-PCR) is also called quantitative PCR or qPCR. The key feature in RT-PCR is that amplification of DNA is detected in real time as PCR is in progress by the use of fluorescent reporter. The fluorescent reporter signal strength is directly proportional to the number of amplified DNA molecules. In 2001–2010, RT-PCR has been used as a powerful tool for genotyping, quantifying viral load, and gene copy number assays. RT-PCR has been the gold standard of gene expression level assay (Jia, 2012).

Real-time PCR uses an increase in the intensity of a fluorescent signal generated by an intercalating dye or from the breakdown of a dye-labeled probe during amplification of a target sequence to detect nucleic acids either for their presence or absence or for their amount. The PCR cycle number where fluorescent signal becomes discernable above background noise is called the Ct value. Between two samples, a decrease in a Ct value (ΔC_T) of one cycle represents a doubling of the amount of target. This relationship can be expressed as.

The fold increase in the amount of amplified product = $2^{-\Delta Ct}$

In the $2^{-\Delta Ct}$ method ΔC_T is equivalent to the change in the target relative to the control (reference):

Ct (target) – Ct (reference) =ΔCt

(Stephenson, 2016). There are two detection method of RT-PCR, the first is based on sequence specific probe such as TaqMan probe, molecular beacon; the second is based on genetic non-specific double stranded DNA binding dye such as SYBR green, RT-PCR is a very sensitive and powerful DNA analysis tool. RT-PCR can be divided into four stages: linear ground phase, early exponential phase, linear exponential phase (log phase) and plateau phase. In the first phase, PCR is just starting, fluorescent signal just rise significantly above background, the cycle at which occurs is called cycle threshold (Ct), in linear exponential phase PCR is in optimal amplification stage with doubling PCR products in every cycle, the last phase is when substrates are exhausted and TaqDNA polymerse is in its end of life, fluorescent signal will no longer increase (Jia, 2012).

Marterials

E. coli Strain DH5α is used as the host systems in the study. The host system were maintained and revived according to TaqGene Training and Research Institude laboratory (TGTRI), Dehradun Culture maintenance guidelines.

The *E. coli* vector, puc57 in which the cloned papain sequence is present is used for Transformation. Selection of transformants was done on Ampicillin antibiotic –LB agar plates (3 different conc. 100ug/ml, 250 ug/ml, and 500 ug/ml) and further Plasmid DNA isolation was carried out from transformed cells for Real-tme PCR analysis.

Qiagen-Qiaprepmini DNA isolation kit (QIAGEN Cat.no-27104) was used to isolate plasmid DNA from transformed cells for efficient purification, high quality and yield. Plasmid DNA isolated by Qiagen kit can be directly used for downstream process like PCR, qPCR, sequencing, and cloning etc.

The qPCR study was carried out on BIO-RAD CFX 96™ Real Time PCR Detection system.

Intercalating dye chemistry is used for real time detection of target sequence. Eva-Green Quantitative-PCR (qPCR) mix from Solis BioDyne is used. The kit comparises all the components necessary to perform qPCR: HOT FIRE Pol DNA Polymerase (DNAPolymerase that is activated by 12 min incubation step at 95°C) dNTPs, MgCl2, and EvaGreen dye.

Transformation of *E. coli* DH5-α cells

DH5 alpha strain of *E. coli* is used in this study to transform with PUC57 plasmid with recombinant Papain gene. The recombinant plasmid was provided by TGTRI.

The selection of *E. coli* is based on different criteria e.g. *E. coli* make useful tools for genetic research because of their relatively small genome size compared to eukaryotes. *E. coli* typically grow much faster than more complex organisms (one generation per 20 minutes under typical growth conditions), easy to transform, and easy to lyse (plasmid DNA isolation) etc.

The transformation protocol laws followed by calcium chloride method. The amount of transformed plasmid DNA was around 4ng used for the transformation of *E. coli* species. After transformation the transformants were selected on increasing concentration of Ampicillin antibiotic containing LB Agar (low salt) plate. Three different concentration of Ampicillin was used to generate the multicopy clone i.e. transformants with more copy number of integrated plasmid.

The LB-Amp agar plates containing three different concentration of antibiotic were prepared as followed:
LB agar plate with ampicillin at conc.100µg/ml: 60ml of LB agar (autoclaved) + 60µl of Ampicillin (100mg/ml).
LB agar plate with ampicillin at conc. 250µg/ml: 60ml of LB agar (autoclaved) + 150µl of Ampicillin (100mg/ml).
LB agar plate with ampicillin at conc.500µg/ml: 60ml of LB agar (autoclaved) + 300µl of Ampicillin (100mg/ml).
Two plates for each concentration were prepared one plate for inoculation of transformed cell and one plate for inoculation of non-transformaed cell (control plate) to see the activity of ampicillin.

The LB agar was autoclaved and ampicillin was added according to the required concentration to the autoclaved medium (after autoclaving, LB agar media was placed in a water bath pre-set at temperature 60°C for 45 min and after 45 min Ampicillin was added to the media inside Laminar air flow, and plates were prepared) After inoculation of transformed cells on LB agar plates, the plates were incubated for overnight. After incubation growth were observed on the plates (AmpR: ampicillin resistant colonies).

Plasmid DNA Isolation from transformed *E. coli* with recombinant gene was carried out by Qiagen-Qiaprepmini DNA isolation kit.

Plasmid DNA isolation

The plasmid DNA isolation was carried out using Qiagen-Qiaprepmini DNA isolation kit. A single colony of AmpR transformed *E. coli* was first inoculated on Low salt LB Medium containing Ampicillin (100µl/ml) and Incubated overnight at 37°C. The plasmid DNA isolation was carried out in triplicates according to the recommended protocols

Figure 1: Fluorescent chart produced in RT-PCR (source of image: Arya M *et al*,(2005)**)**

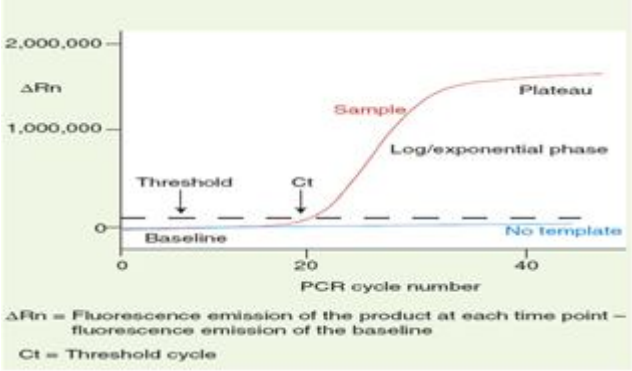
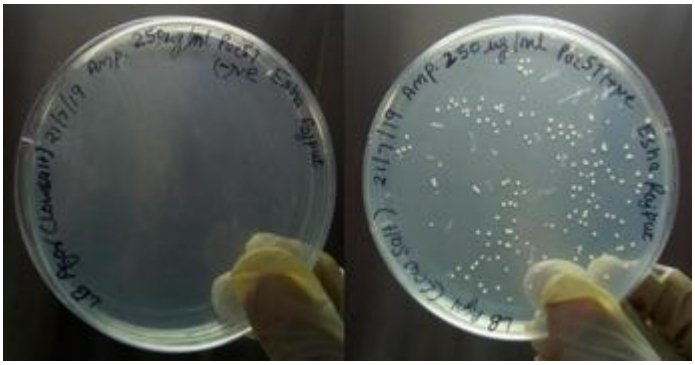
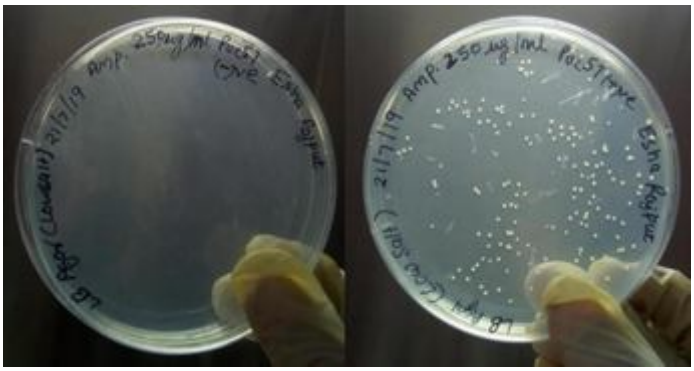


Figure 2 : Plasmid DNA isolation result
Fig-2.1: 100µg/ml LB Amp Plate:



Ampicillin Resistant colonies (transformants) on right side plate vs control plate on left. The concentration of ampicillin in both plates were 100µg/ml.

Fig-2.2:- 250µg/ml LB Amp Plate:



Ampicillin Resistant colonies (transformants) on right side plate vs control plate on left. The concentration of ampicillin in both plates were 250µg/ml.

Fig-2.3:- 500µg/ml LBAmp Plate:

Ampicillin Resistant colonies (transformants) on right side plate vs control plate on left. The concentration of ampicillin in both plates were 500µg/ml.

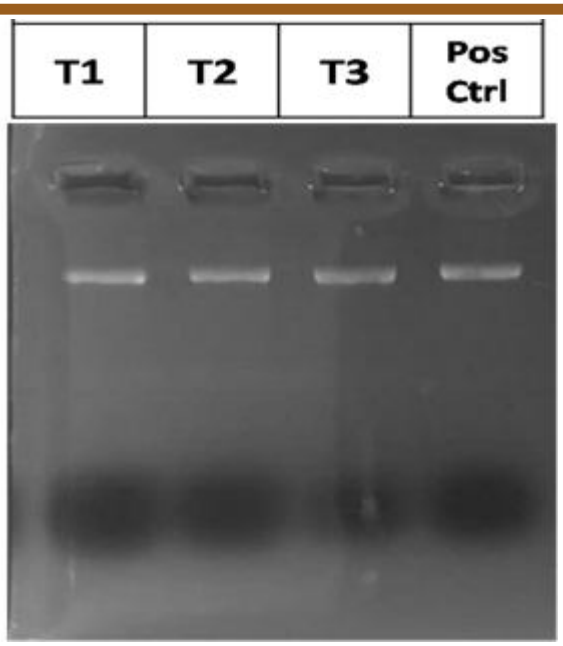


Figure - 3: pUC57 plasmid with r-DNA(papain) isolated by Qiaprepmini kit and visualized on 1.5% agarose gel T1-T3; pUC57plasmid DNA isolated in replicates. T4- Positive control plasmid.

Figure - 4: Amplification curve: 6 standards, 4 known samples, 1 Negative control, 1 Positive control. RP1-A & B; plasmid DNA from 1ooµg/ml Amp Curve, RP2-A & B; cDNA from 250µl/ml curve and RP3-A &om 500µl/ml, positive and negative controls are amplification control

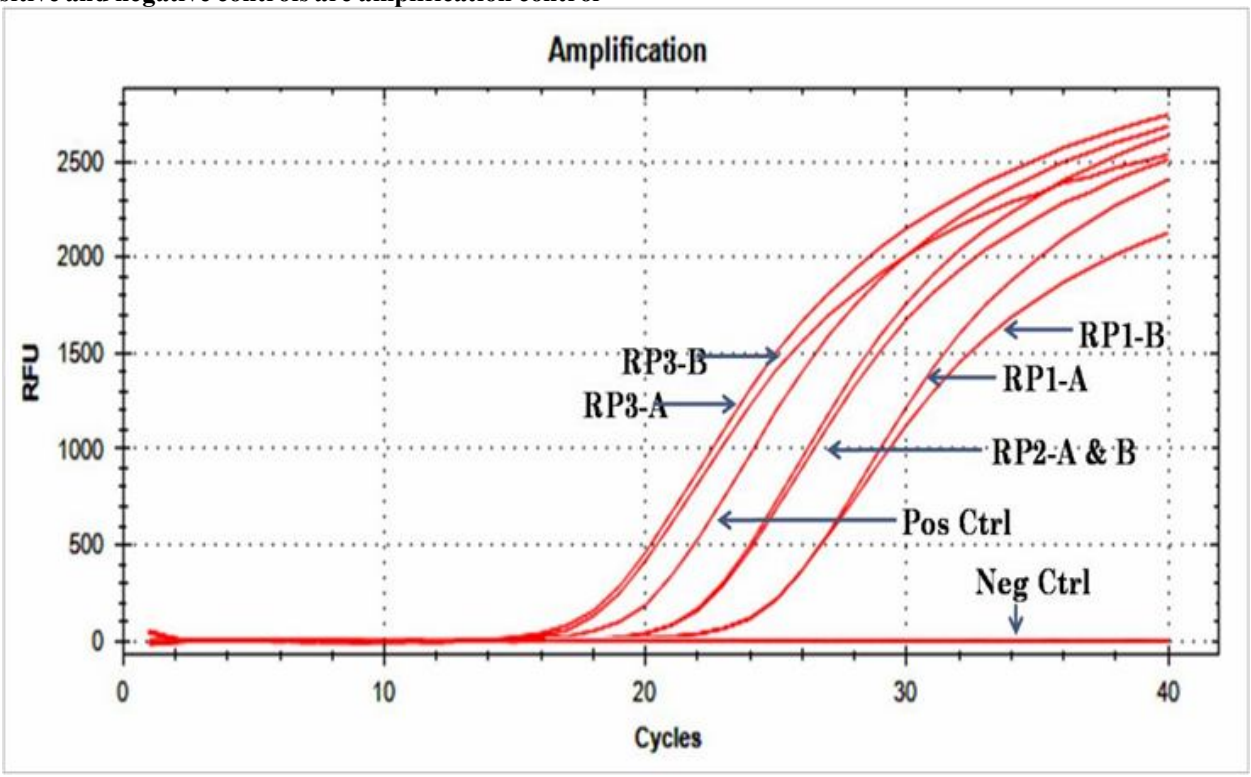


Table -1: qPCR assay results: The value of Ct in different samples

Sample ID	Description	Amplification	Ct Value	Average Ct Value	ΔCT (Ct target-Ct Reference	Fold Change ($2^{-\Delta Ct}$)**
RP-1A	Amp-100 µg	+	24.71	24.68	Reference*	
RP-1B	Amp-100 µg	+	24.65			
RP2-A	Amp-250 µg	+	22.34	22.40	-2.28	4.8
RP2-B	Amp-250 µg	+	22.46			
RP3-A	Amp-500 µg	+	19.78	19.46	-5.22	37.2
RP3-B	Amp-500 µg	+	19.15			
Pos-Ctrl	Positive contol	+	20.65	-	-	-
Neg-Ctrl	Negative control	-	No Ct value	-	-	-

$**2^{\Delta CT}$ = Ct Value of Target – Ct Value of Reference

described in the Qiagen-Qiaprepmini DNA isolation kit. After plasmid DNA isolation the isolated plasmid then visualized on etbr stained 1.5% Agarose gel. The expected length of recombinant plasmid (pUC57+Papain) was 3729bp A positive control plasmid was simultaneously loaded with isolated plasmid for confirmation of specific product. (Fig 3.)

MULTI-GENE COPY (MULTICOPY) CLONES DETECTION BY REAL-TIME PCR

Master mix preparation

1. The reaction volume was 20µl for each reaction. 4ng of DNA samples from amp transformants, 1 negative control (no template control), 1 positive control (Plasmid DNA pUC57with insert), and 6 standards: total 12rxns. The master mix is prepared as follows:

2. In a 1.5 ml sterile water, FIRE POL mix, forward primer, and reverse primer was added and mixed carefully by pipetting.

3. 15µl of master mix is then distributed into 0.1ml QPCR tubes filter tips.

4. 5µl of template DNA/standards /NTC /PC was added by filtered tips carefully.

5. Tubes were placed in CFX96 thermal cycler, and PCR run was proceeded by thermal cycling program.

qPCR Analysis

4µl of DNA was used in the qPCR reaction. The reaction was performed in duplicates for all three samples to rule out-pipetting error. A total of eight reactions were analyzed by qPCR out of them three samples were run in duplicates and one amplification positive control and one is amplification negative control.

RESULTS

Transformation result

The plates were incubated for overnight. After incubation the colony were observed on the plates. No colony growth was observed on the negative control plates that are the plates spread with non-transformed cells. However, the transformation efficiency was highest on 100µg/ml LB-Amp plates. The number of colony reduced as ampicillin concentration was increased. Few colonies appeared on 250µg/ml LB-Amp plates as compared to 100µg/ml Amp plates, and very few colonies appeared on 500µg/ml LB-Amp plates as compared to 100 and 250µg/ml amp plates. No growth was observed on control plates. This results indicated the high concentration effect of ampicillin on transformants as well as indicated that the colonies appeared on high concentration have the tendency to neutralize increased ampicillin concentration suggesting the clones having more copies of antibiotic resistance gene.

Real Time PCR Amplification curve

The Ct values for samples RP3A & B were came early (Fig. 4) as compare to RP2 and RP1 A & B, suggesting high No. of target gene presenting RP3. The early Ct value indicate the high Initial No. of target gene/copy in sample, and the late Ct value indicate low initial No. of target gene/copy in a given sample. As PTVA is a technique to generate the clones of transformed cells with high number of recombinant gene. The above amplification plot indicated the higher level of gene expression in cells grown in high antibiotic concentration.

Relative Quantification Analysis

Relative quantification determines the changes in expression level of Gene of Interest (GOI) in response to different treatments, as in this study the cells were grown on increasing concentration of antibiotic were used to quantify the changes in level of gene expression.

*(Table 1 reference)In this relative quantification study, a comparison is made with the gene expressed in the 2 samples (250µg/ml Amp, and 500µg/ml Amp) considered as target to the 100µg/mlAmp sample considered as reference. The same amount of sample volume is used throughout the all processes starting from Plasmid DNA isolation to qPCR assay. By subtracting the Ct value of target gene from Ct of the reference gene (ΔCT). The fold difference of target gene is calculated by using the resulting differences in cycle number(ΔCT) as the exponent of the base 2 (due to the doubling fiction of PCR). The Real-time PCR results indicated the higher level of gene expression in cells grown in higher antibiotic concentration i.e 500µg/ml Amp.

Discussion

In this study the change in expression levels of a recombinant gene (Papain) was analyzed by qPCR. Real Time PCR is the most commonly used technique for gene expression analysis studies and Quantification of genes/mRNA has been significantly simplified by the improvements and modifications of the technology (Bustin, 2002). It is mostly used for two reasons: either as a primary investigative tool to determine gene expression or as a secondary tool to validate the results of DNA microarrays (Valasek and Repa, 2005).

Post Transformational Vector Amplicfication (PTVA) is a common technique to increase expression levels (Clare *et al.*,1991) of a recombinant gene by an expression system. The Aim of this technique is to enrich strains with multiple copies of an expression vector by selecting transformants that are resistant to high levels of a selective agents for e.g an Antibiotic. Generally, the copy no of plasmid per transformed cells depends on various factors like *ori* sequence, Size of plasmid, Size of insert, and culture conditions etc.,

Here in this study we analyzed the effect of PTVA on gene expression of a recombinant gene by using Real Time PCR. We find out that the increased concentration of antibiotic can be used to generate clone with multiple copies of recombinant gene clones as compared to the routinely used standard antibiotic concentration and which in-turn can express higher amount of recombinant protein.

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