Characterisation of promoter for anti-sense RNA expression in *Toxoplasma gondii*

by

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Certificate

This is to certify that the work incorporated in this Major Project Report entitled, "**Characterisation of promoter for anti-sense RNA expression in *Toxoplasma gondii***" submitted by **Manali Bajpai** to the Academy of Scientific and Innovative Research (AcSIR), in partial fulfilment of the requirements for the award of the Degree of **Master of Technology**, embodies original research work carried-out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material(s) obtained from other source(s) and used in this research work has/have been duly acknowledged in the thesis. Image(s), illustration(s), figure(s), table(s), etc., used in the thesis from other source(s), have also been duly cited and acknowledged.

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Abstract

Toxoplasma gondii is apicomplexan parasite that is responsible for toxoplasmosis in felines and almost all warm-blooded animals. This parasite has three stages tachyzoite, bradyzoite and oocyst in its lifecycle, hence differential gene expression is observed in different stages which is mediated by promoter or anti-sense mediated regulatory mechanism. Researchers have unveiled the fact that in a typical eukaryotic species, transcription occurs in over 80% of genomic sequence, even though proteins are encoded by 1-2% of the genome only. Much of the transcribed RNA is the so-called non- coding RNA (ncRNA) molecules which may have a wide variety of functions. These ncRNAs are thought to have major role in regulation of cellular processes like growth, differentiation, cell cycle, gene silencing and much more. Some genes in *Toxoplasma* are observed to be mediated by anti-sense RNA, and one such example is the FAS-I gene. Evidence from RNA sequencing data of *T. gondii* proves the existence of anti-sense activity in FAS-I gene expression regulation. The goal of this project is to carry out reporter-based characterization of the promoter responsible for antisense RNA expression in *T. gondii*. This will help in understanding how stage specific expression of the FASI gene is achieved using anti-sense regulatory mechanism by the parasite.

Keywords – Anti-sense RNA, differential gene expression, FAS-I gene, non-coding RNA, transcriptomics, *Toxoplasma*

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List of Abbreviations

S. No.	Abbreviation	Full Form
1	ASR	Anti-sense RNA
2	DHFR	Dihydrofolate reductase
3	FAS-I	Fatty Acid Synthase-I
4	FBS	Fetal Bovine Serum
5	HCM	Host cell medium
6	HFF	Human foreskin fibroblast
7	IHC	Immunohistochemistry
8	LA	Luria Bertini Agar
9	LB	Luria Bertini Broth
10	lncRNA	Long non-coding RNA
11	miRNAs	Micro RNA
12	NATs	Natural anti-sense transcripts
13	ncRNA	Non-coding RNA
14	nts	nucleotides
15	PBS	Phosphate buffered saline
16	siRNA	Small interfering RNA
17	TgFASI	*Toxoplasma gondii* type I fatty acid synthase
18	ToxoDB	Toxoplasma genome database
19	wt	Wild type
20	YFP	Yellow fluorescent protein

1. Introduction

Toxoplasma gondii is an apicomplexan parasite with a crescent-shaped cell morphology, thus it is named as *Toxoplasma* meaning 'arc' in Greek (Innes, 2010). It is the most successful single-celled parasite worldwide as it is responsible for causing toxoplasmosis in approximately one-third of the world's population and can infect a wide range of warm-blooded hosts (Hill, Chirukandoth and Dubey, 2005). Cat is the definite host of this parasite while, it has many intermediate hosts including humans, sheep, etc (Freppel *et al.*, 2019). The disease caused by *Toxoplasma* is often asymptomatic but, might cause serious complications that include mental retardation, blindness, disorientation, convulsions (Lehmann *et al.*, 2006). In humans, enlarged lymph nodes associated with fatigue, headache, fever, etc. is common manifestation along with lethargy, necrosis, ataxia in immunocompromised individuals. Though tissue cysts are not the major cause of the disease but in AIDS patient or patient who has undergone organ transplant and is under immunosuppressant drugs, rupture of tissue cyst will lead to conversion of bradyzoites to tachyzoites hence establishing recurring infection (Lyons, McLeod and Roberts, 2002).

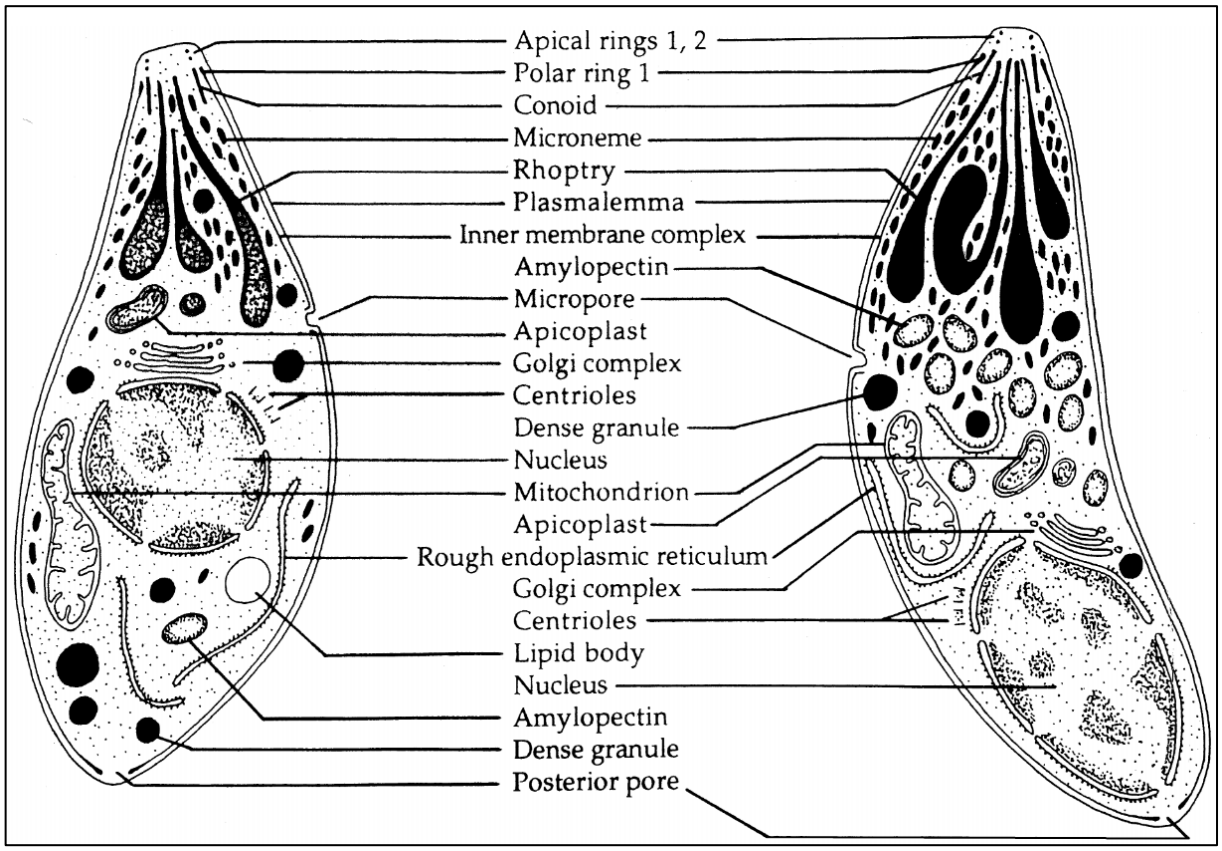


Figure 01 – Schematic diagram of asexual stages of *T. gondii* (Dubey, Lindsay and Speer, 1998)

T. gondii exhibits three prominent life cycle stages i.e., tachyzoites, bradyzoites and oocysts. Tachyzoites are 2 by 6 µm in size, capable of actively multiplying in any intermediate host cell and non-intestinal epithelial cells of the cats. Bradyzoites (cystozoites) of approximate 7 by 1.5 µm size can grow and multiply in the tissue cysts. While, oocysts are spherical with 10 by 12 µm of diameter (Dubey, Lindsay and Speer, 1998). At different stages of lifecycle, differential gene expression is observed which is mediated by promoter or anti-sense expression. Prior studies on parasite transcriptome analysis have demonstrated the anti-sense RNA mediated regulation of gene expression (Crater *et al.*, 2017). One such example is FAS-I gene (Fatty Acid Synthase-I gene) with encodes for multiple functional domains. Type I fatty acid synthesis (FAS-I) pathway encodes for single polypeptide chain components. FAS-I pathway have important role to play in unique extracellular walls synthesis for one stage of lifecycle that is oocysts (Ramakrishnan *et al.*, 2015). The ongoing studies depicts its significance in cell biology and growth of the organism. This study focuses on the reporter-based promoter characterization in ASR region of FAS-I gene in ME49 strain of *T. gondii*.

1.1 Life cycle of *T. gondii*

Toxoplasma is highly prevalent parasite worldwide due to its environmental resistance in oocyst stage and has complex lifecycle including sexual stage as well as asexual stage (Hutchison *et al.*, 1971). This coccidian parasite can infect almost all warm-blooded animals including humans and birds as their intermediate hosts and cats as their definite hosts (Dubey, 2009). Lifecycle of this apicomplexan parasite alternates between felines and warm-blooded animals. Sexual phase occurs in cats and asexual phase may occur either in felids or intermediate hosts (Freppel *et al.*, 2019). Lifecycle of *T. gondii* in cats differ from its intermediate hosts. When fed with bradyzoites cats excrete oocyst in faeces after 3 to 10 days, when ingested with tachyzoites they excrete oocysts in ≥13 days whereas, it takes ≥18 days to excrete oocysts after ingestion of sporulated oocysts (Dubey, 1998). Intestinal infection by the parasite in felines is because of carnivorism or consumption of sporulated oocysts. Only in the intestinal epithelium of cats, parasites undergo sexual differentiation to produce oocyst that is released into the intestinal lumen and excreted in the faeces.

Upon exposure to ambient environmental conditions, these diploid oocysts undergo rapid meiotic reduction to produce haploid sporozoites. On the other hand, wide range of intermediate hosts of this apicomplexan parasite can be infected mainly due to ingestion of water and other food items contaminated with these sporulated oocysts after egestion in the

faeces of cats; intake of viable tissue cysts in uncooked or undercooked meat; congenital transmission of infection from the mother through the placenta (Hill and Dubey, 2002); blood transfusion; organ transplantation, where the organs may contain parasite contamination (Attias *et al.*, 2020). In stomach, due to acidic pH excystation occurs leading to release of sporozoites or bradyzoites within the small intestine which then differentiate into tachyzoites further lysing the infected cell and invading other cells throughout the body (Diaz-Miranda, 2017).

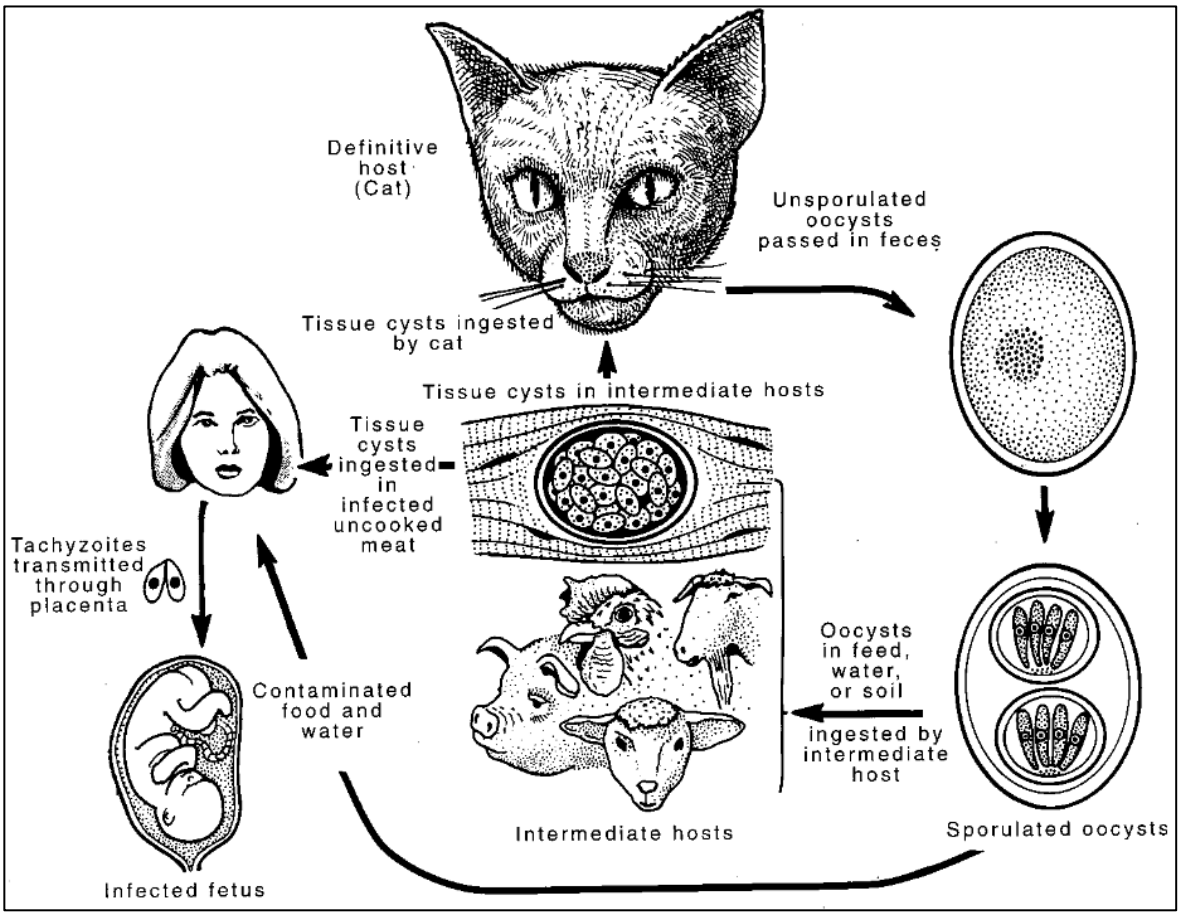


Figure 02 – Life cycle of *T. gondii* (Dubey, Lindsay and Speer, 1998)

1.2 Anti-sense RNA expression in *T. gondii*

Transcriptomics studies have revealed that RNA is transcribed from ~90% of a typical eukaryotic genome. Out of which, protein coding transcripts are nearly 1-2% only. Much of the non-protein-coding RNAs (ncRNA) have important regulatory functions. These ncRNAs are thought to have major role in regulation of cellular processes like growth, differentiation, cell cycle, gene silencing and much more (Fahim, 2019). Based on their length ncRNA can be divided into siRNA (small-interfering RNAs), miRNA (micro RNAs) and lncRNAs (long non-

coding RNAs) (Xu, Zhang and Zhang, 2018). Our study focuses on lncRNAs that ranges from 200 to 100000 nts in length. The lncRNAs are classified into intergenic, intronic, sense lncRNA and anti-sense lncRNA based on their origin and location within the genome. Anti-sense lncRNAs also known as natural anti-sense transcripts (NATs) have some part overlapping with the sense RNA and are transcribed from the opposite strand of the protein-coding gene (Faghihi and Wahlestedt, 2009). From transcriptome data of *Toxoplasma* (available in ToxoDB database) the anti-sense RNA can be identified.

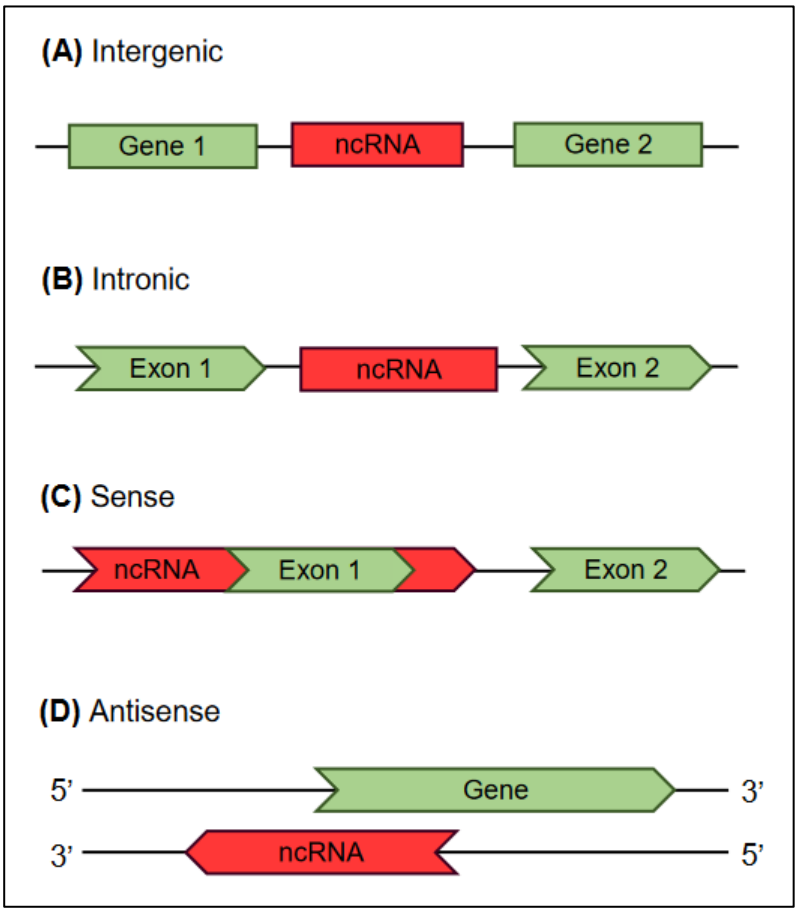


Figure 03 – Classification of non- coding RNAs by their origin (Fahim, 2019)

In our laboratory the regulation of FAS-I gene expression is studies in two different strains of the parasite; the ME49 strain which is avirulent and can convert into bradyzoite tissue cyst in mammalian hosts and the RH strain which is virulent and is not capable of developing into bradyzoites. Moreover, the RH strain also lacks the potential to undergo sexual reproduction in felines while the ME49 strain has this capability (Pricop, 2009). Based on available expression datasets in the public domain, it appears that the expression of the FAS-I gene in both these strains may be under the control of antisense RNA. As figure 04 suggests, this anti-sense is not expressed in the oocyst stage of the parasite where the *TgFASI* gene is

expressed maximally and completely. On the other hand, FAS-I gene anti-sense regulation is observed in tachyzoite stage of both Me49 and RH wt strains resulting in detection of degraded RNA and no full-length expression profiles.

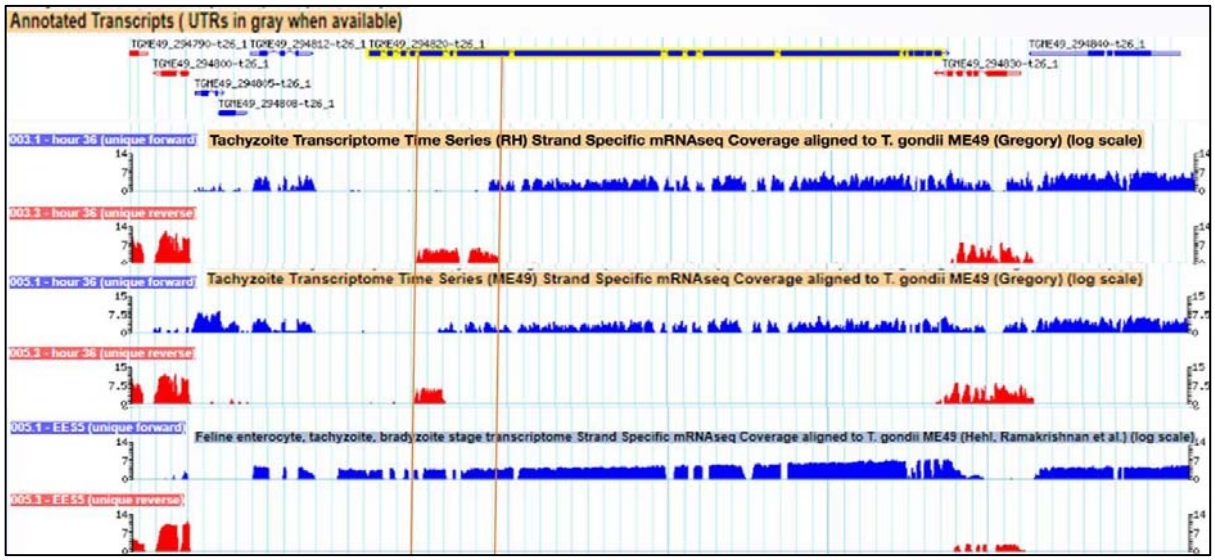


Figure 04 – Representation of anti-sense transcript expression in *T. gondii* FAS-I gene (Gene ID - ME49_294820) in tachyzoite and oocyst stages of parasite. The top panel depicts RNA sequencing data from RH strain tachyzoites, while the middle and bottom panels show expression data from ME49 strain tachyzoites and oocysts. The region highlighted by vertical orange lines highlights the antisense expression (in red) and sense expression (in blue) of the FAS-I gene (Source - toxodb.org/toxo/app)

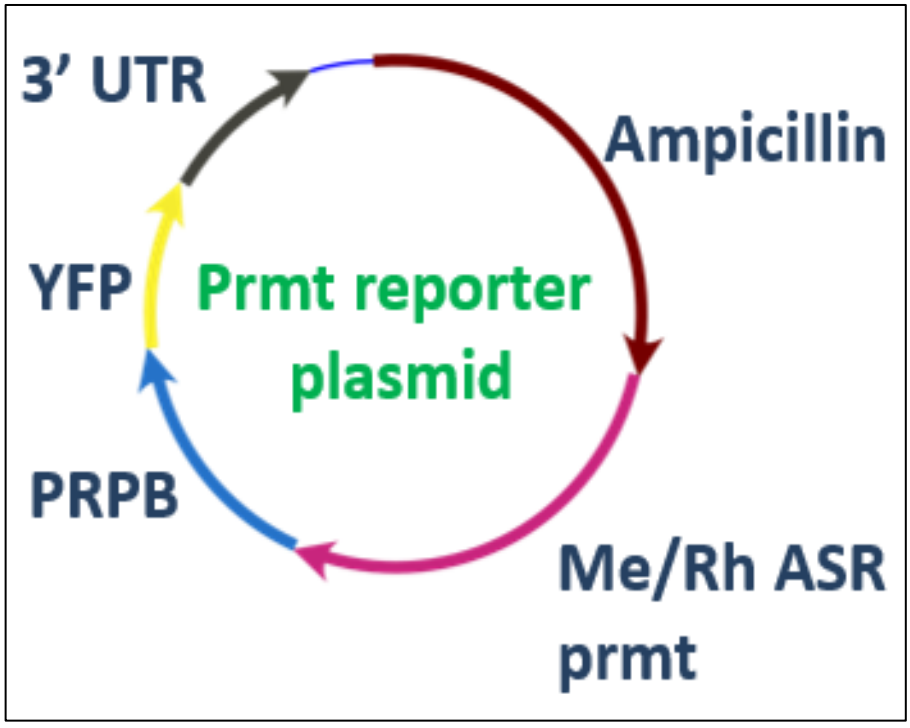


Figure 05 – Diagrammatic representation of the plasmid construct with reporter for promoter characterisation studies

This project is planned focusing on the reporter-based promoter characterization of the antisense RNA in ME49 strain of *T. gondii* to further evaluate the mechanism by which the expression of *TgFASI* gene is under the control of antisense RNA. Promoter characterization is the first step towards research in getting a better idea about gene expression regulation in apicomplexans. Furthermore, it will pave way towards several other studies related to pathway characterization and gene silencing studies. Also, there is little known about how promoters responsible for sense strand and anti-sense strand expression work in the parasite. Likewise, whether the promoter characterized is locus specific for anti-sense transcripts or it can work for other anti-sense transcripts also is not known.

2. Materials and Methods

2.1 Primer Design – Referring to the datasets mentioned in the public domain, anti-sense RNA (ASR) expression region of FAS-I gene (Gene ID - ME49_294820) from ME49 strain of *T. gondii* was of interest. Four forward primers (Table 02) were designed targeting different length of the ASR region with HindIII restriction digestion site at 5' end of PCR fragment and one common reverse primer with NheI restriction digestion site at 3' end of PCR fragment, including start codon for downstream YFP expression. The length of the promoter region of interest between P1, P2, P3, P4 and reverse primer is 2094 bp, 1589 bp, 1074 bp and 579 bp respectively.

Table 01 – ME49 strain *TgFASI* gene specific primers for ASR promoter characterisation

Sequence 5' to 3'	Description	Melting Temperature	GC content
agctagctAAGCTTCAGCCCCGAGAATCAGCG	P1	69.4 ºC	56 %
agctagctAAGCTTGGAGCAGGCAGTGTCGATTG	P2	69.3 ºC	53 %
agctagctAAGCTTCGAGTAACTTTGGCTGTGATGTC	P3	67.1 ºC	46 %
agctagctAAGCTTCGTGTACGAATGGTGTATGTGC	P4	67.3 ºC	47 %
agctagctGCTAGCCATCTCCTCTACATGAACTTGGTGTTGAC	RP	70.4 ºC	49 %

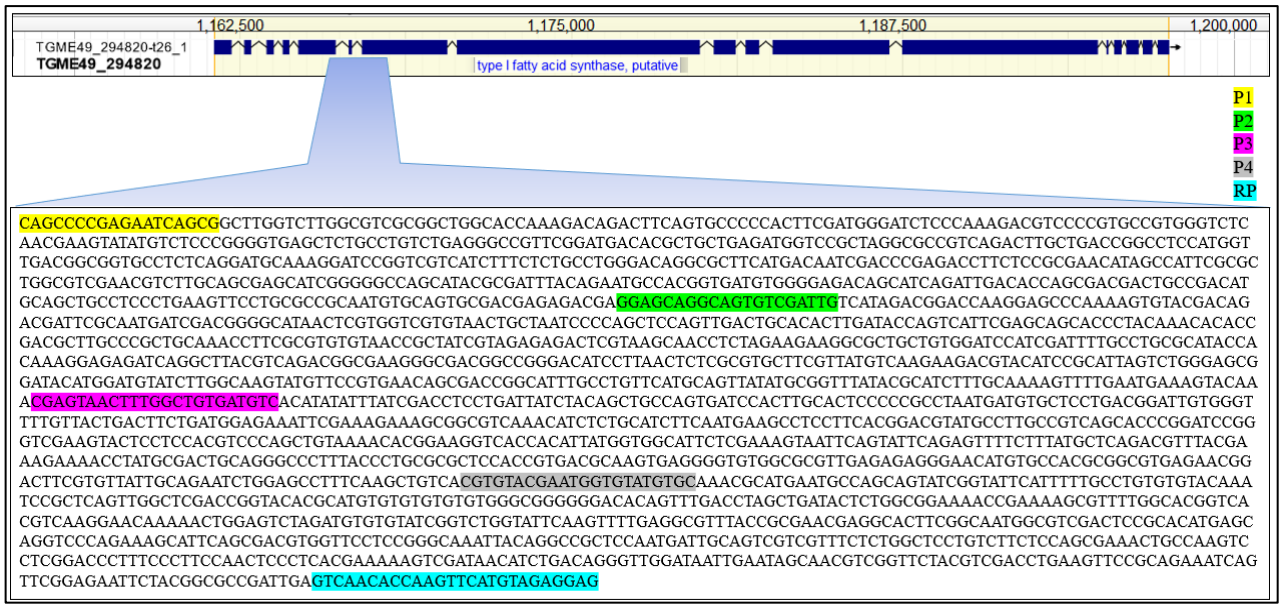


Figure 06 – Representation of primers on the targeted region of *TgFASI* gene for ASR study

2.2 PCR amplification – PCR was setup for four ME49 constructs using LongAmp® Taq 2X Master Mix | NEB kit. Primers used were P1, P2, P3 and P4 as forward primers and RP as reverse primer for all four reactions. For each construct, 25 µl amplification reaction was setup using 1 µl RH wt DNA template, 5 µl LongAmp buffer, 0.75 µl dNTPs, 1 µl forward primer, 1 µl reverse primer and volume makeup to 25 µl by nuclease free water. PCR conditions to get required results was optimised accordingly where initial denaturation was carried out at 94ºC (4 min), denaturation at 94ºC (30 sec), annealing at 56ºC (1 min), extension at 65ºC (2.5 min), final extension at 65ºC (5 min) and finally hold at 4ºC. Results were checked on 1% agarose gel.

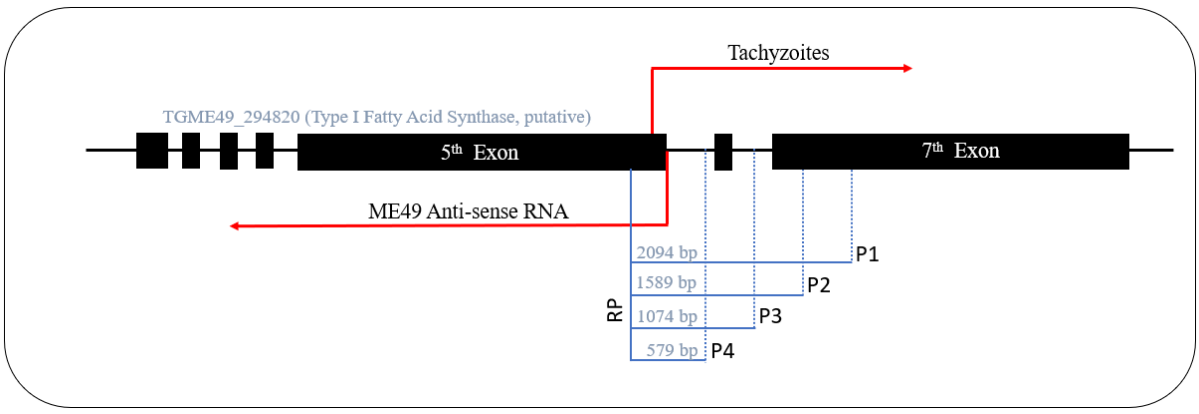


Figure 07 -Schematic representation of primers of ME49 constructs in FAS-I gene

2.3 TA Ligation – All the four PCR fragments (P1, P2, P3, P4) created using LongAmp kit has high chances of having 3'-Adenine overhangs in all products as 3' to 5' proofreading

activity is absent in *Taq* polymerase. The vector used here is pGEM®-T Easy vector that serves a good platform for TA cloning as it is linearised with 3'-Thymidine overhangs at both the ends. Hence, it provides compatible overhang for PCR amplified products. LigaFast™ Rapid DNA Ligation System - Promega kit was used for ligation of all four constructs with pGEM®-T Easy vector. 5 µl 2X rapid buffer, 1 µl insert, 1 µl vector, 1 µl ligase enzyme and 2 µl nuclease free water were added for setting up 10 µl ligation reaction for each construct. Reaction was kept for overnight incubation at 4ºC before transformation in *E. coli*.

2.4 Transformation of ligated products – DH5-α *E. coli* competent cells prepared in laboratory were used for transforming TA ligated products. Whole 10 µl ligation mixture for each construct was added in competent cells and kept on ice for 30 minutes incubation. Then heat shock was given at 42ºC for 60 seconds following which 10 minutes ice incubation was done. After adding 200 µl of LB media the transformed mixture was kept at 37ºC for one hour. Lastly, about 100 µl of the resulting culture was spread on ampicillin agar plates and was allowed to grow overnight at 37ºC.

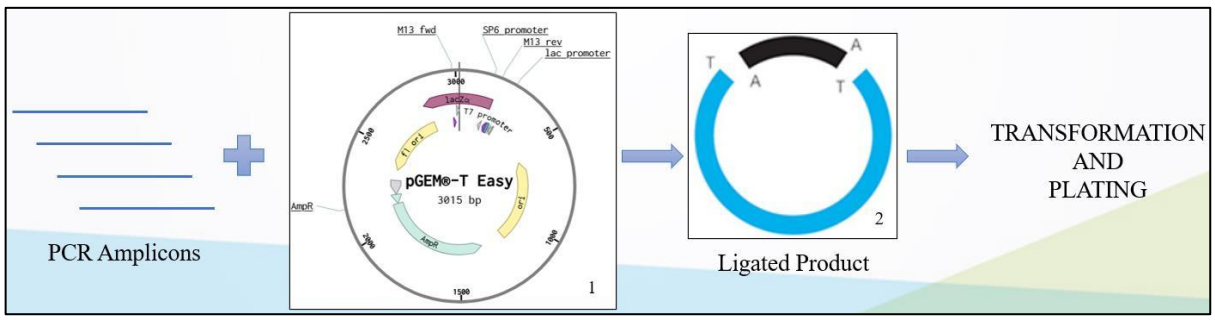


Figure 08 – Schematic diagram for basic principle of TA cloning

(Source – 1. https://benchling.com/1/api/sequences/seq_qRNWvavM.svg, 2. www.promegaconnections.com)

2.5 Colony PCR – 16 µl reaction was setup using GoTaq® DNA Polymerase | Taq Polymerase | PCR - Promega kit to confirm positive colonies after ligation for P1, P2, P3 and P4 constructs. 4 µl of 4X GoTaq master mix, 1.25 µl forward primer, 1.25 µl reverse primer, 9.5 µl nuclease free water and then finally colony was added to the PCR reaction mix. PCR conditions to get required results was optimised accordingly where initial denaturation was carried out at 95ºC (5 min), denaturation at 95ºC (30 sec), annealing at 58ºC (30 sec), extension at 72ºC (3 min), final extension at 72ºC (5 min) and finally hold at 4ºC. Results were checked on 1% agarose gel. Master plate was also prepared for all the colonies picked from P1, P2, P3 and P4 transformations.

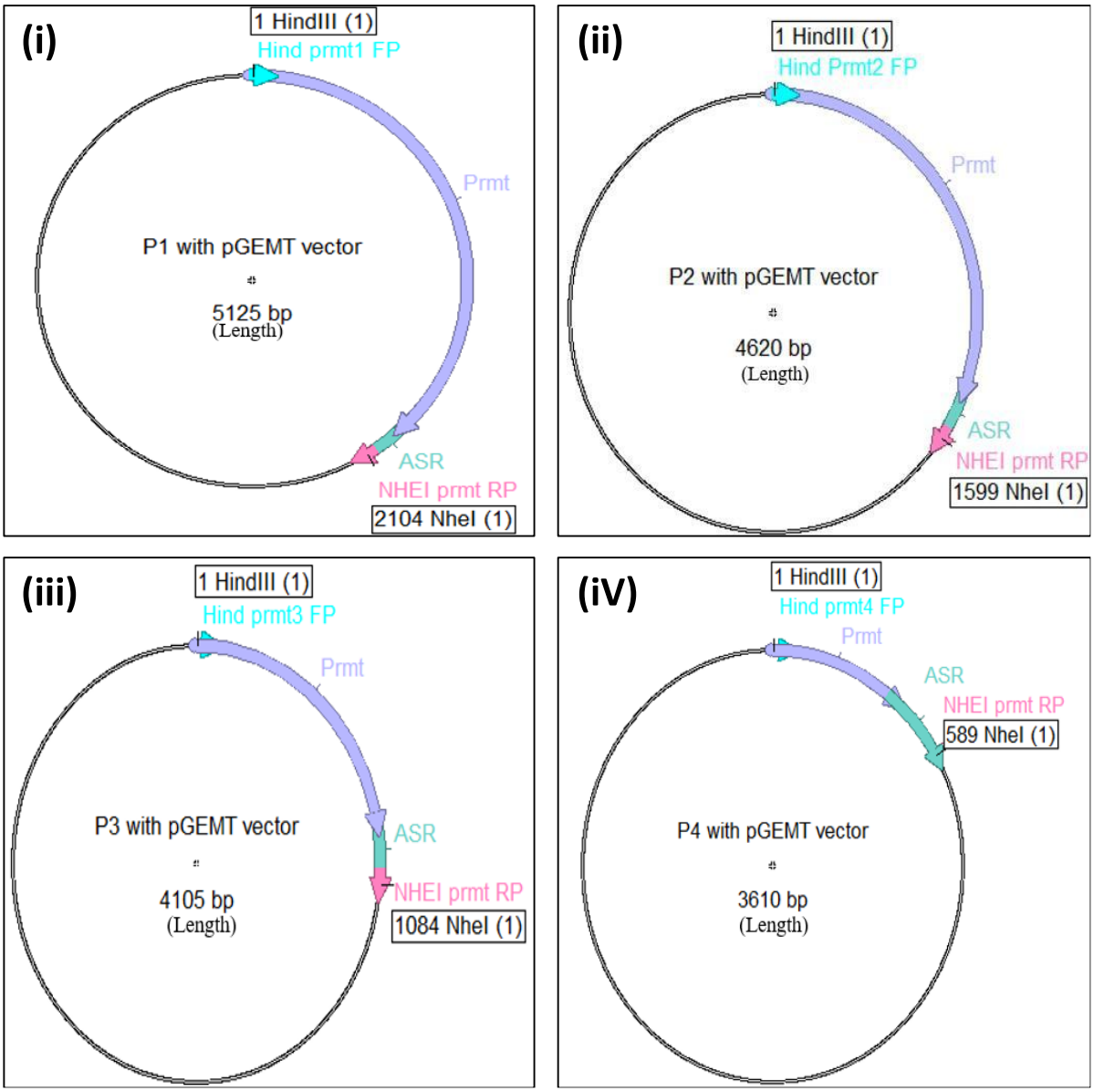


Figure 09 – Representation of TA ligated plasmids showing HindIII and NheI restriction digestion sites (i) P1 TA clone (ii) P2 TA clone (iii) P3 TA clone (iv) P4 TA clone

2.6 Primary and secondary culturing – Positive colonies were picked from master plate and inoculated in 5 ml LB media containing ampicillin. This primary culture was kept for incubation and growth at 37ºC on shaker incubator for 4 to 6 hrs. Later, the secondary culture was done by adding 5 ml primary culture to 45 ml fresh LB media containing ampicillin. Secondary culture was kept in shaker incubator for 16 hrs or overnight incubation at 37ºC.

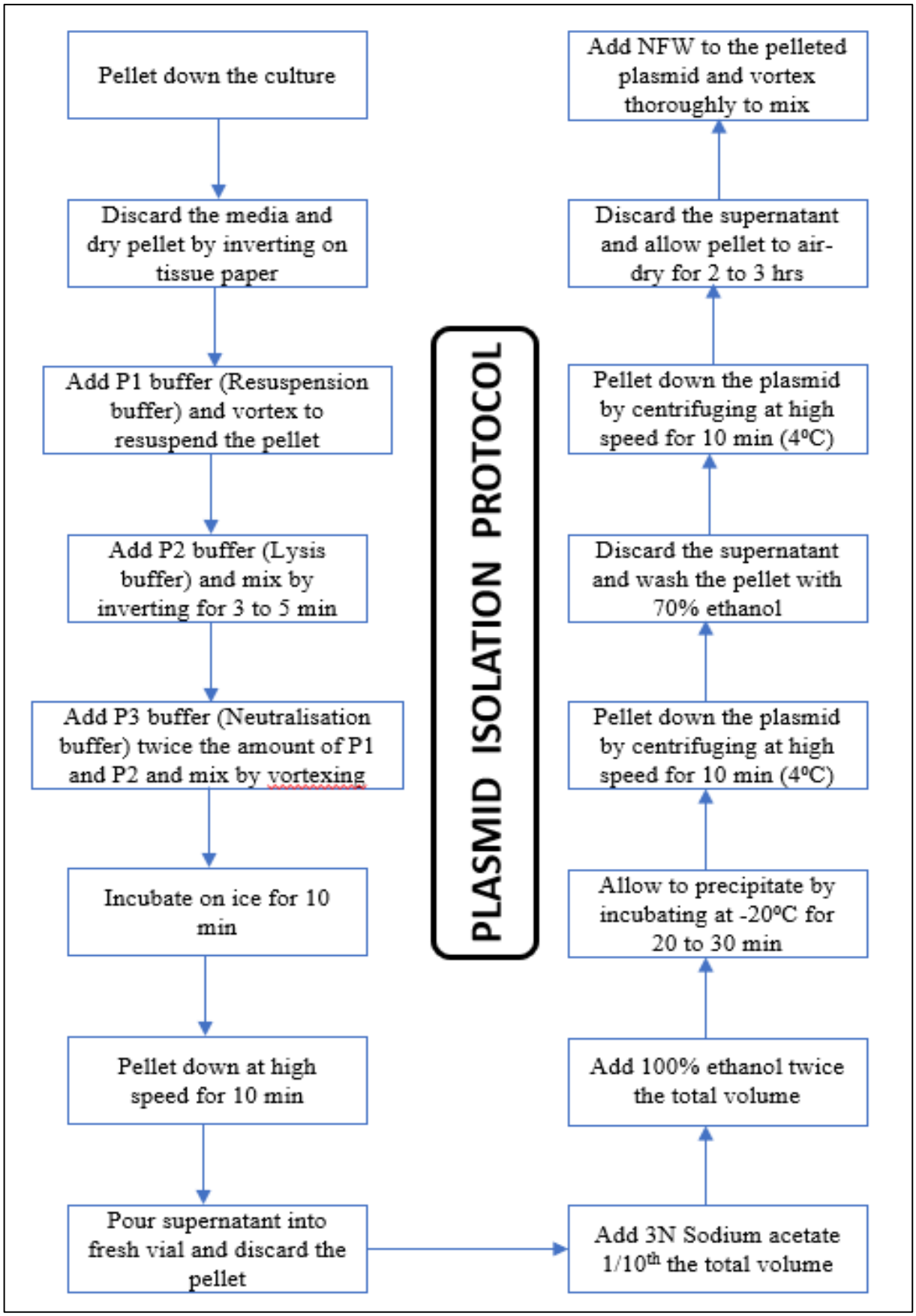


Figure 10 – Schematic diagram representing plasmid isolation by alkaline lysis method

2.7 Plasmid isolation and restriction digestion for confirmation – Plasmid isolation of all positive secondary cultures was done by alkaline lysis method as given in the figure below. Plasmids isolated were confirmed by restriction digestion using EcoRI enzyme. Reaction was setup by adding 1 µg template, 0.2 µl EcoRI enzyme, 2 µl buffer and 12.8 µl NFW. Reaction mixture was then allowed to incubate at 37ºC overnight and bands were checked on 1% agarose gel.

2.8 Restriction digestion for cloning – Digestion was setup for all four TA clones of ME49 promoter region P1, P2, P3 and P4 using HindIII and NheI enzyme to obtain 2103 bp, 1598 bp, 1083 bp and 588 bp bands respectively. 50 µl reaction was setup for 20 µg of template for each construct using both enzymes and compatible buffer. On the other hand, PRPB plasmid with HindIII and AvrII restriction site was used as backbone to provide 4424 bp size band with YFP gene and ampicillin resistance gene. 20 µg of pTub plasmid (used for expression studies in *T. gondii*) was also digested using HindIII and AvrII enzymes by setting 50 µl reaction to obtain 4424 bp band on agarose gel.

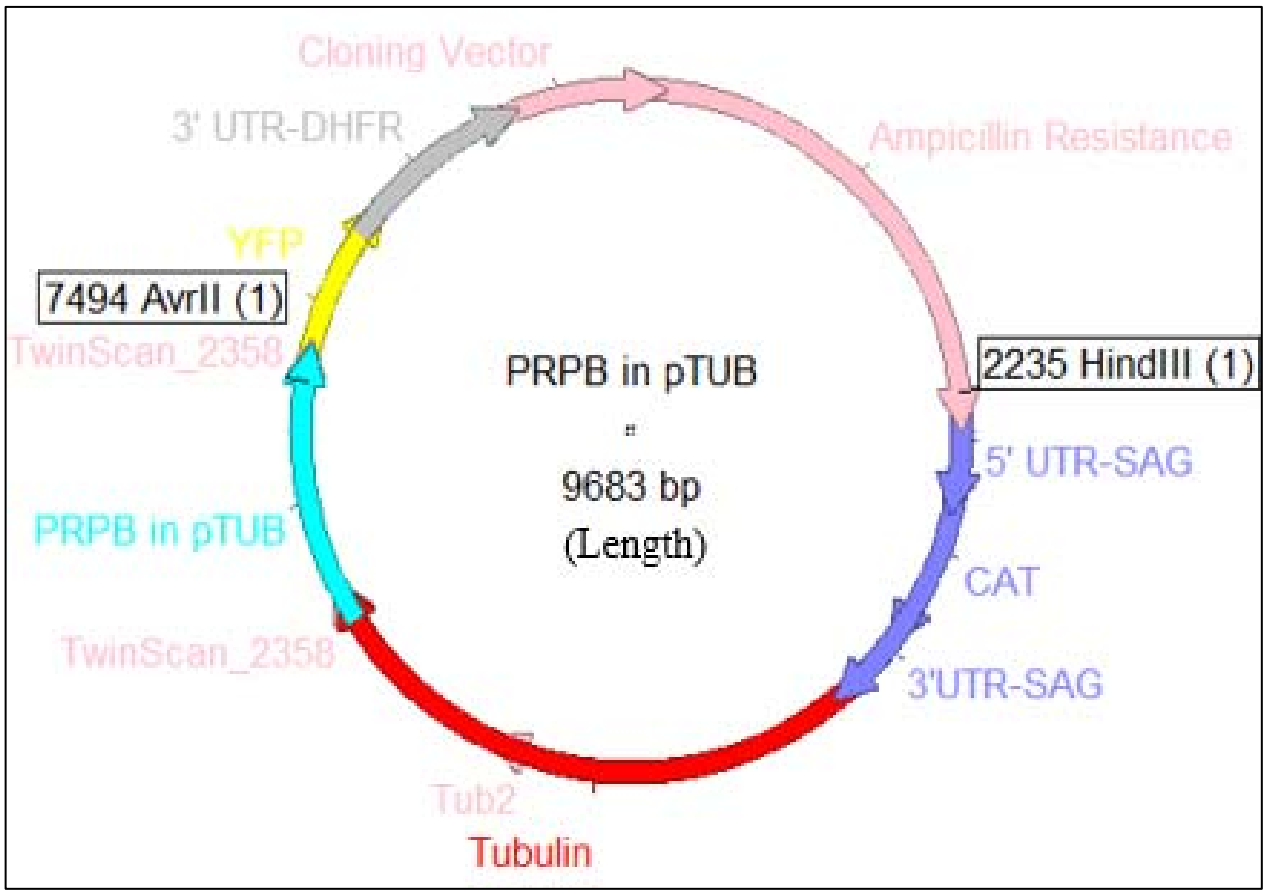


Figure 11 – Representation of pTub plasmid with AvrII and NheI restriction digestion sites

2.9 Gel isolation – DNA band of desired size was cut on UV-transilluminator after agarose gel electrophoresis. Gel isolation of P1, P2, P3, P4 and PRPB bands of size 2103 bp, 1598 bp, 1083 bp, 588 bp and 4424 bp was done respectively. Promega Wizard™ SV Gel and PCR Cleanup System - Fisher Sci kit was used for gel isolation of desired band sizes and concentrations were noted down using nanodrop.

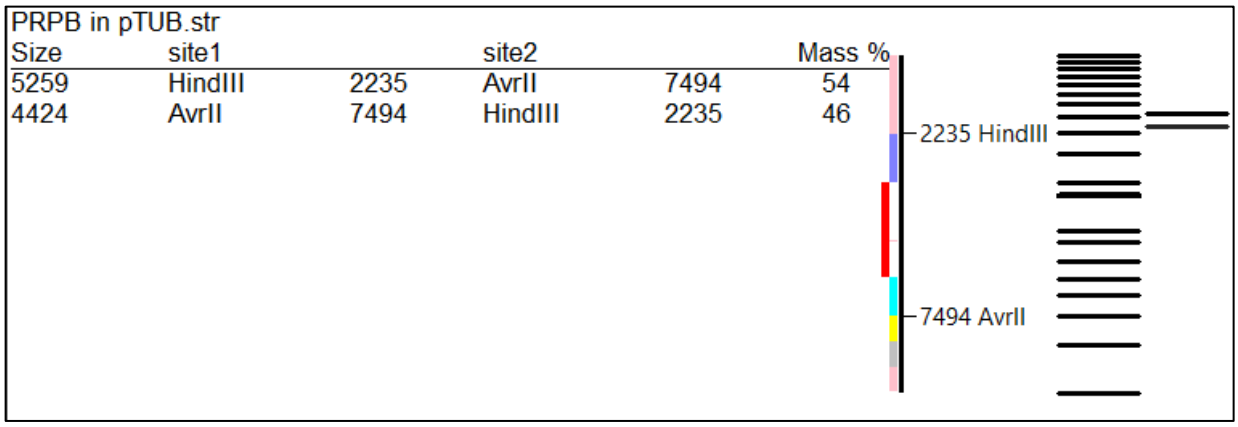
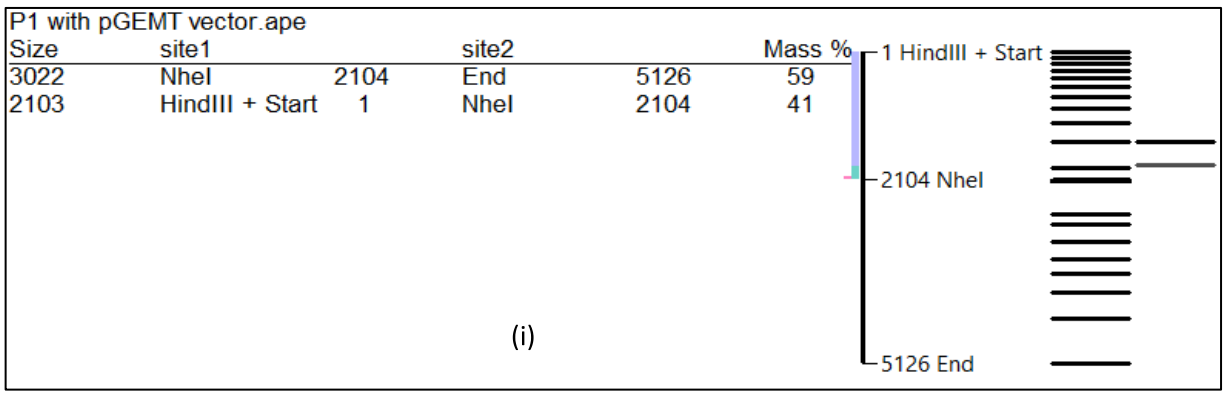
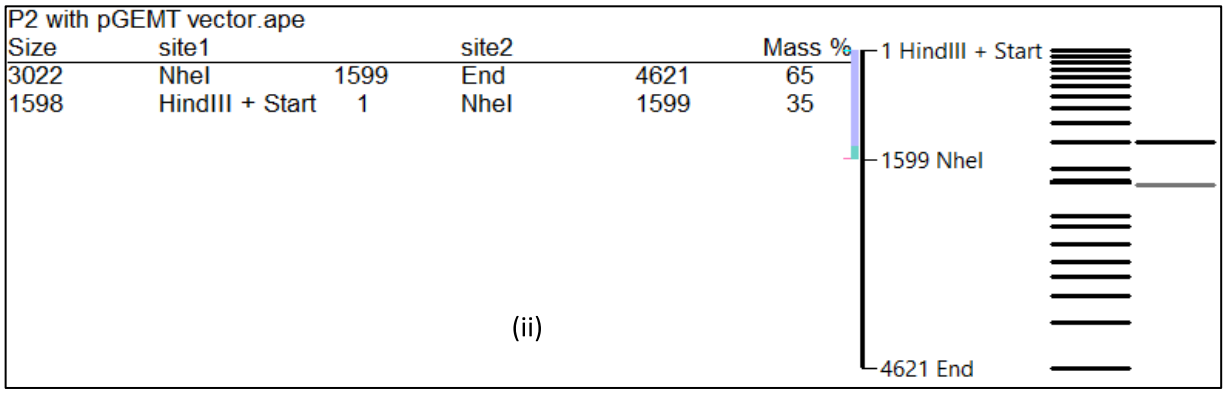


Figure 12 – Band size obtained on agarose gel after restriction digestion of pTub plasmid by AvrII and NheI





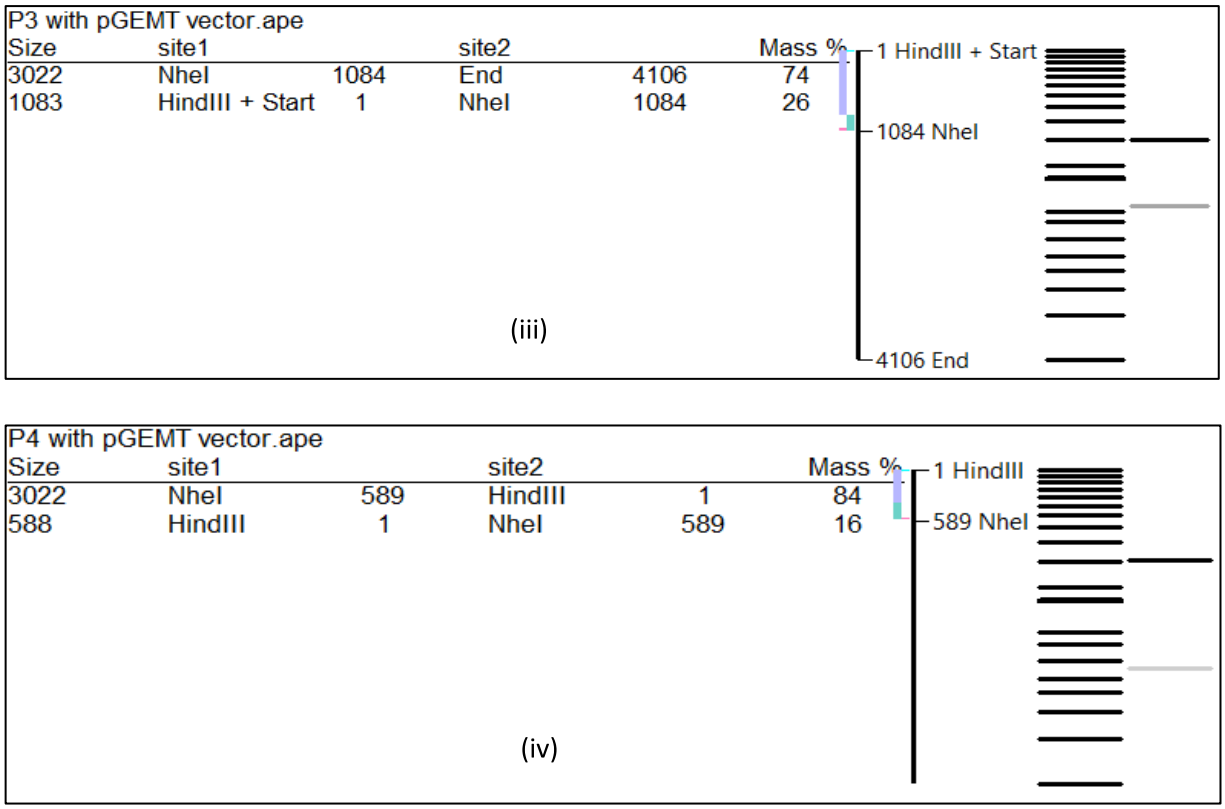


Figure 13 – Band size obtained on agarose gel after restriction digestion by HindIII and NheI (i) P1 TA clone (ii) P2 TA clone (iii) P3 TA clone (iv) P4 TA clone

2.10 T4 DNA ligation and transformation – P1, P2, P3, P4 bands isolated from gel were used as insert to setup ligation reaction with PRPB 4424 bp band as vector. Ligation reaction was done for 100 µg of vector and insert was calculated for 1:3 molar ratio of vector:insert using Ligation Calculator - NEB calculator. T4 DNA Ligase | Ligase | DNA Ligation - Promega kit was used for ligation reaction. 10 µl ligation reaction mixture was prepared containing the vector, insert, enzyme, buffer, NFW and the reaction was incubated overnight at 4ºC. Ligation mixture was then transformed into DH5-α competent cells and plated on ampicillin containing agar plates.

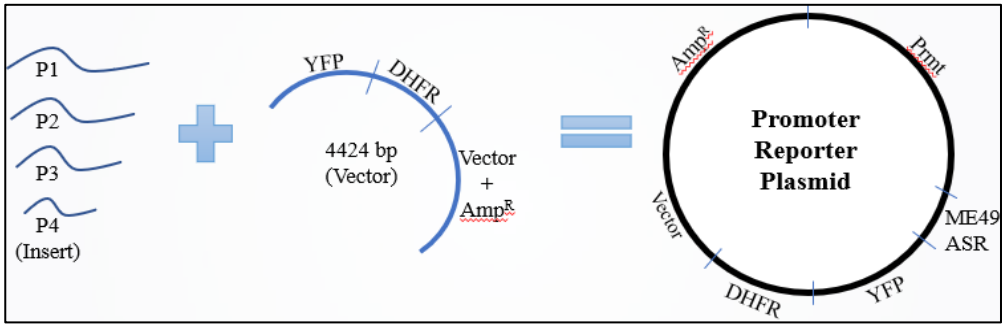


Figure 14 – Schematic diagram of the resulting plasmid after T4 DNA ligation of PCR amplified promoter constructs and reporter

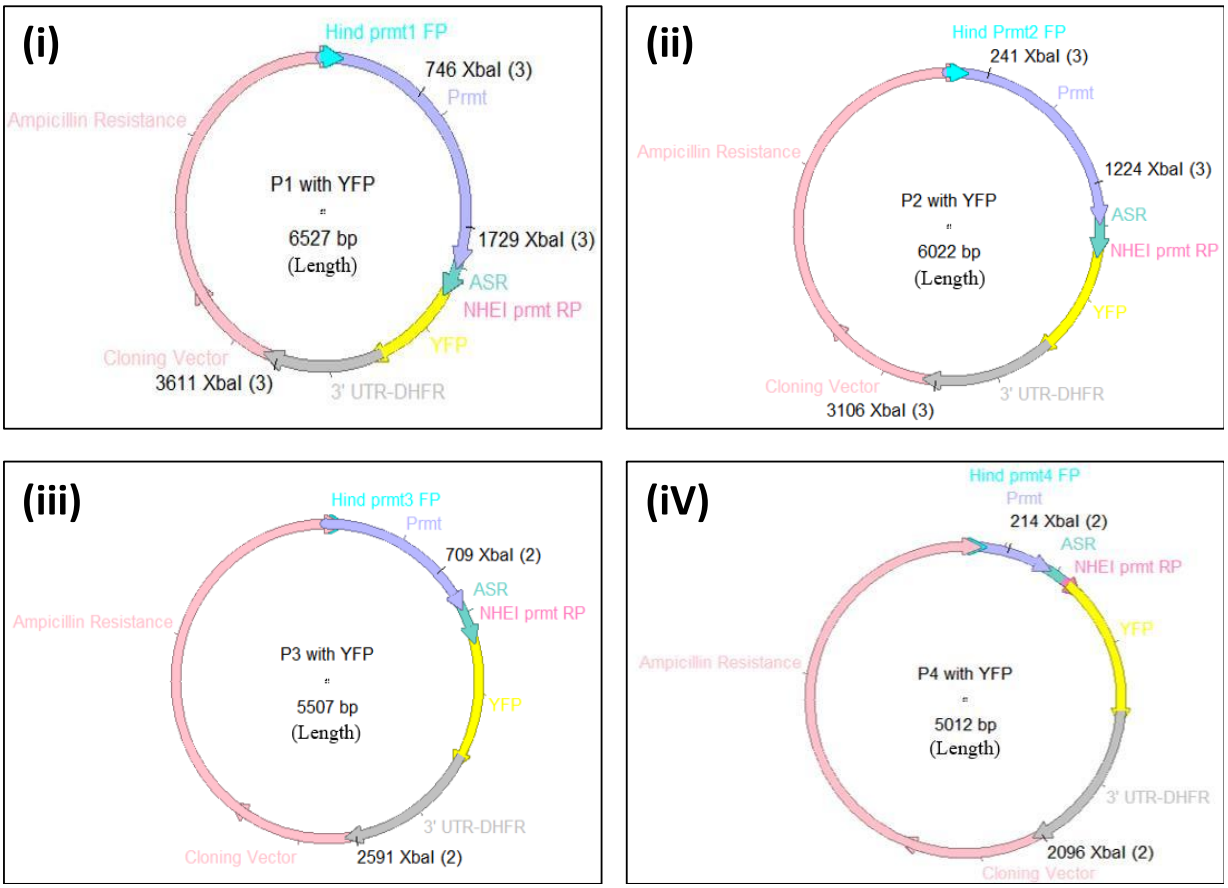


Figure 15 – T4 DNA ligated final clones in pTub backbone showing XbaI restriction digestion sites in (i) P1 final clone (ii) P2 final clone (iii) P3 final clone (iv) P4 final clone.

2.11 Primary culture and plasmid isolation – Colonies on the plates were picked under sterile conditions and inoculated in 5 ml LB media with ampicillin for further plasmid isolation by alkaline lysis method.

2.12 Restriction digestion to confirm final clones – Plasmids isolated were checked by XbaI enzyme for confirming the final clones of P1, P2, P3 and P4 plasmid constructs. 20 µl restriction digestion reaction was setup containing 1 µg of template, 0.2 µl XbaI enzyme, buffer and NFW. The reaction was incubated at 37ºC overnight and results were observed on agarose gel electrophoresis. Expected band size to be observed on agarose gel for final clones P1 is 3662 bp, 1882 bp, 983 bp; P2 is 3157 bp, 1882 bp, 983 bp; P3 is 3625 bp, 1882 bp; and P4 is 3130 bp, 1882 bp.

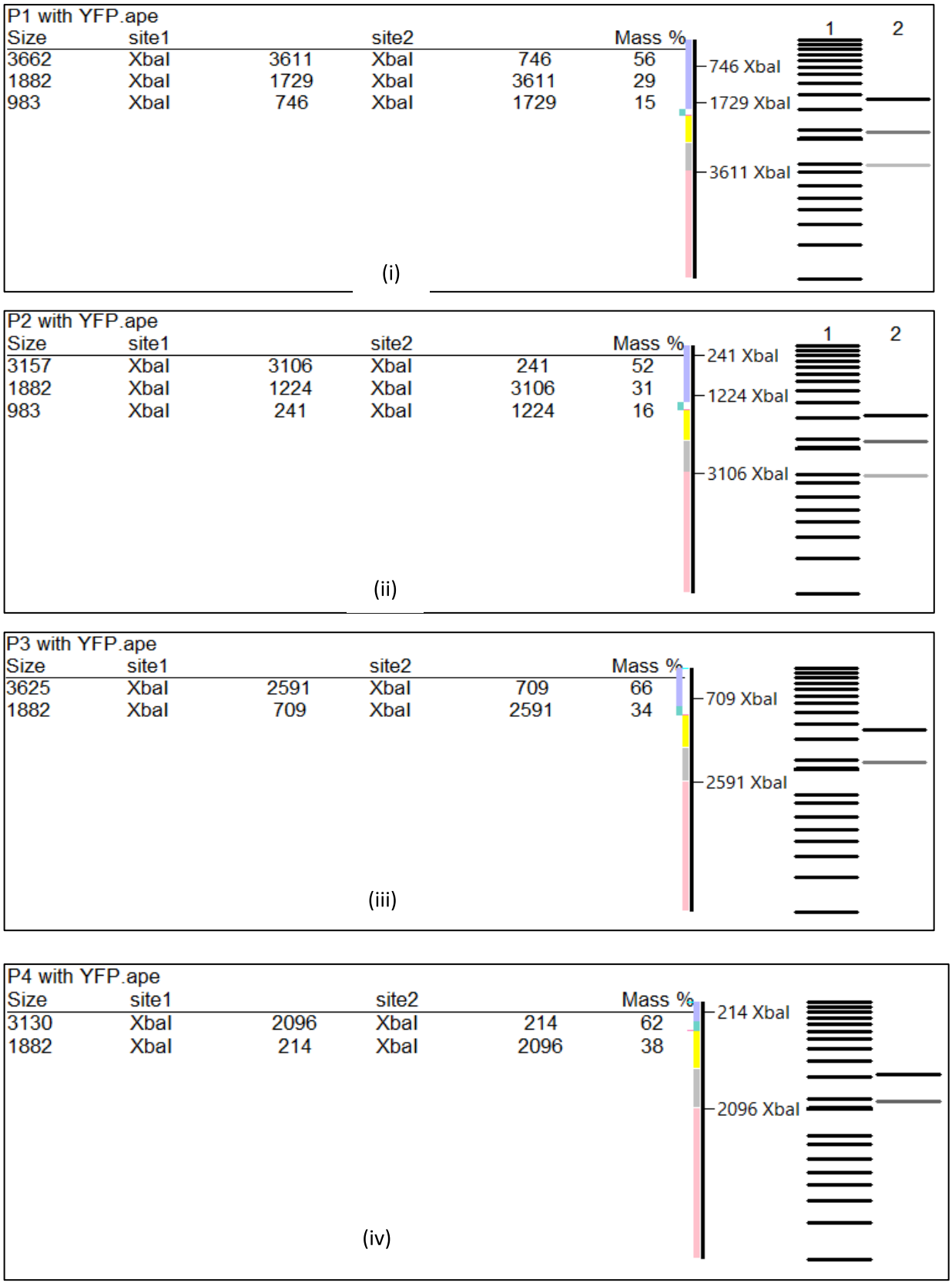


Figure 16 – Band size expected to be obtained on agarose gel after restriction digestion with XbaI for confirming final plasmid constructs of (i) P1 with YFP (ii) P2 with YFP (iii) P3 with YFP (iv) P4 with YFP

2.13 HFF 6 well plate preparation – Sterile 6 well plate is prepared by putting coverslips and HCM (host cell medium) in each well to prepare for host cells growth on them. Trypsinization is done to dissociate adherent cultured HFF host cells in T25 flask. Then, after neutralization with HCM some amount is inoculated on the coverslips. The plate is kept for incubation at 37ºC in 5% CO_2 and host cells are allowed to grow. All the steps are to be performed in sterile conditions and proper safety measures to avoid contamination.

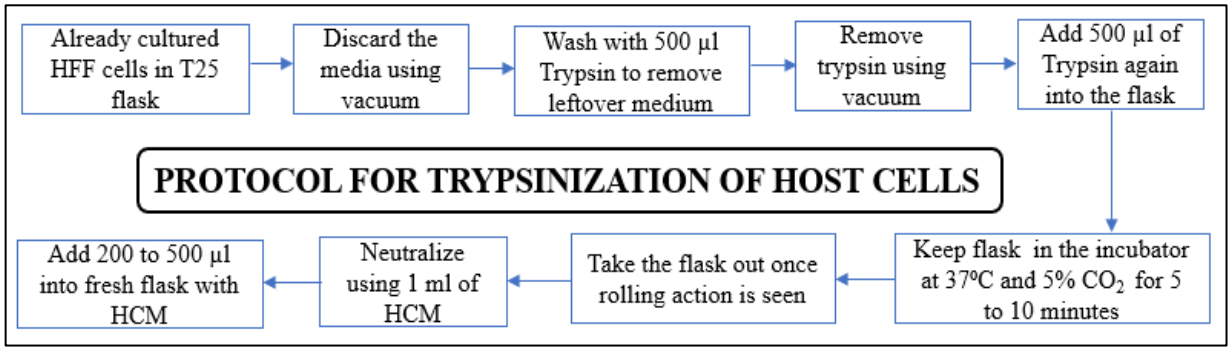


Figure 17 – Protocol for trypsinization of host cells

2.14 Transient transfection in RH strain – Transfections were done for three ME49 clones P2, P3 and P4 in duplicates. RH wild-type strain was chosen for insertion of the desired plasmid into the *Toxoplasma gondii* cells. Human foreskin fibroblast (HFF) was used as host cells to infect the transfected parasites and replicate.

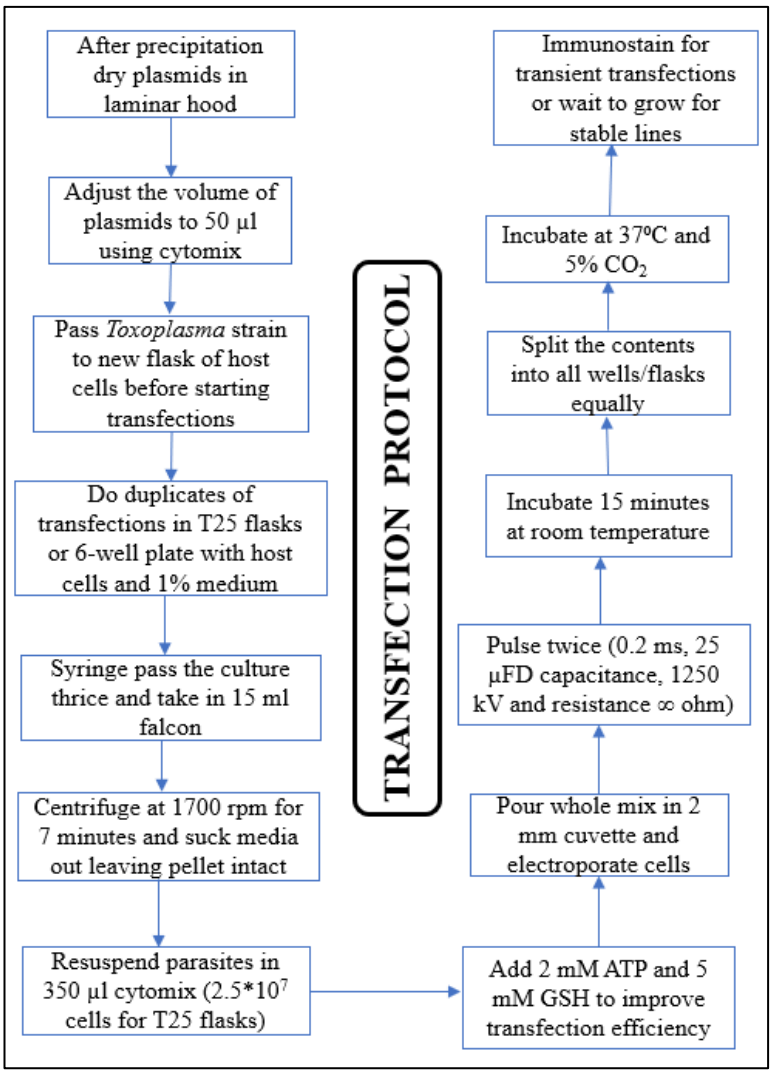


Figure 18 – Diagrammatic representation of steps for transfection in *T. gondii*.

2.15 Immunostaining and slide visualization under microscope – Immunofluorescence staining is used for the visualization of specific protein in tissue or in individual cells. It involves four main steps that include fixation, permeabilization, blocking and antibody binding. All buffers must be freshly prepared. Fixative is prepared with 1% paraformaldehyde, 1% sucrose and volume makeup by 1x PBS. Permeabilization solution or washing buffer is 0.25% Triton X in 1x PBS. Whereas, 2% FBS in 1x PBS is used as blocking buffer. After immunostaining coverslips can be mounted on slides and be visualized under inverted microscope at 40x or 63x magnification.

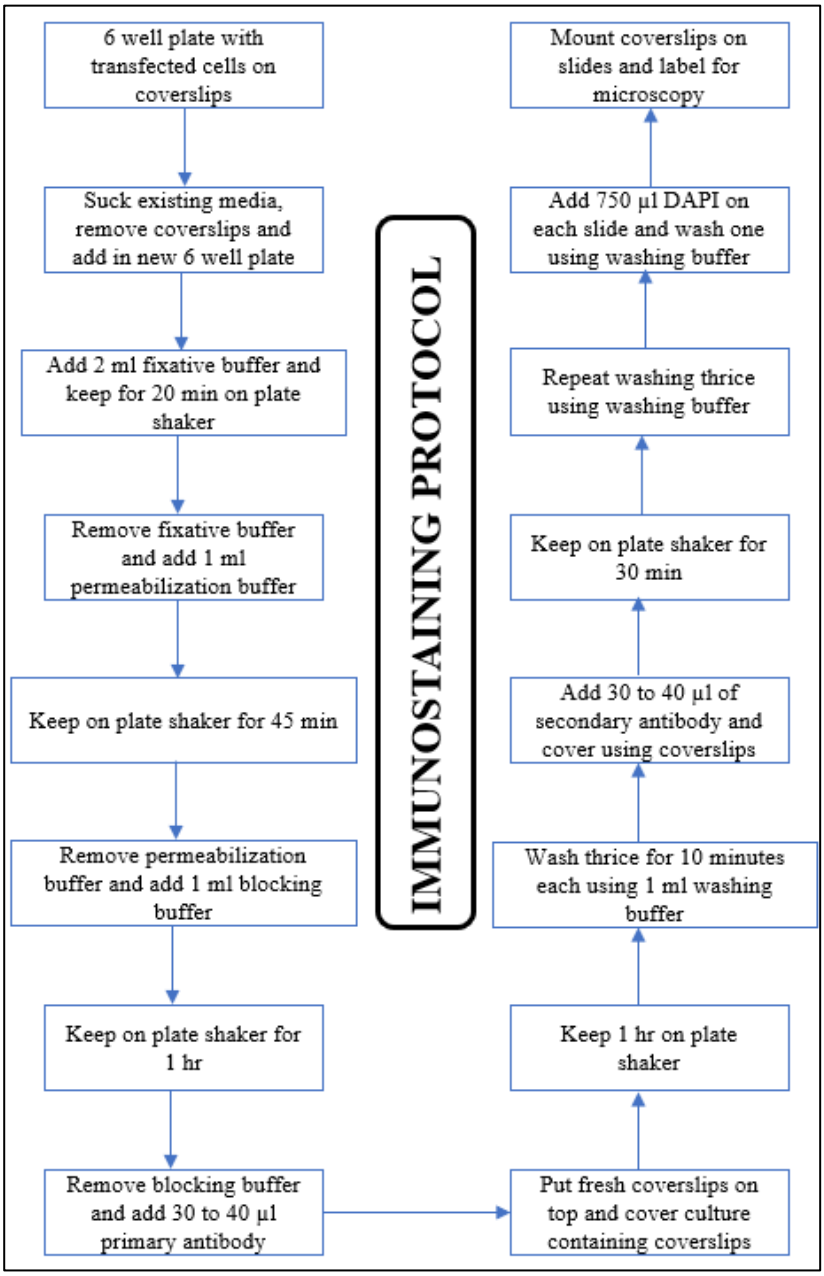


Figure 19 – Diagrammatic representation of steps for immunofluorescence staining of *T. gondii*

3. Results

In this study the four different promoter regions were cloned based on the FAS-I gene anti-sense RNA expression as seen in ME49 strain of the parasite. The experiments were performed as discussed earlier and results are given below.

3.1 Amplification by PCR – The length of the promoter region of interest between the forward primers P1, P2, P3, P4 and reverse primer (RP) is 2094 bp, 1589 bp, 1074 bp and 579 bp respectively. The gel image for the PCR amplified products is shown in figure 20.

3.2 Confirmation of TA clones by colony PCR – TA clones were confirmed by setting up colony PCR reaction for all four constructs. Positive constructs with 2094 bp, 1589 bp, 1074 bp and 579 bp band for P1, P2, P3 and P4 respectively were chosen for plasmid isolation and further cloning. The results of colony PCR are shown in figure 21.

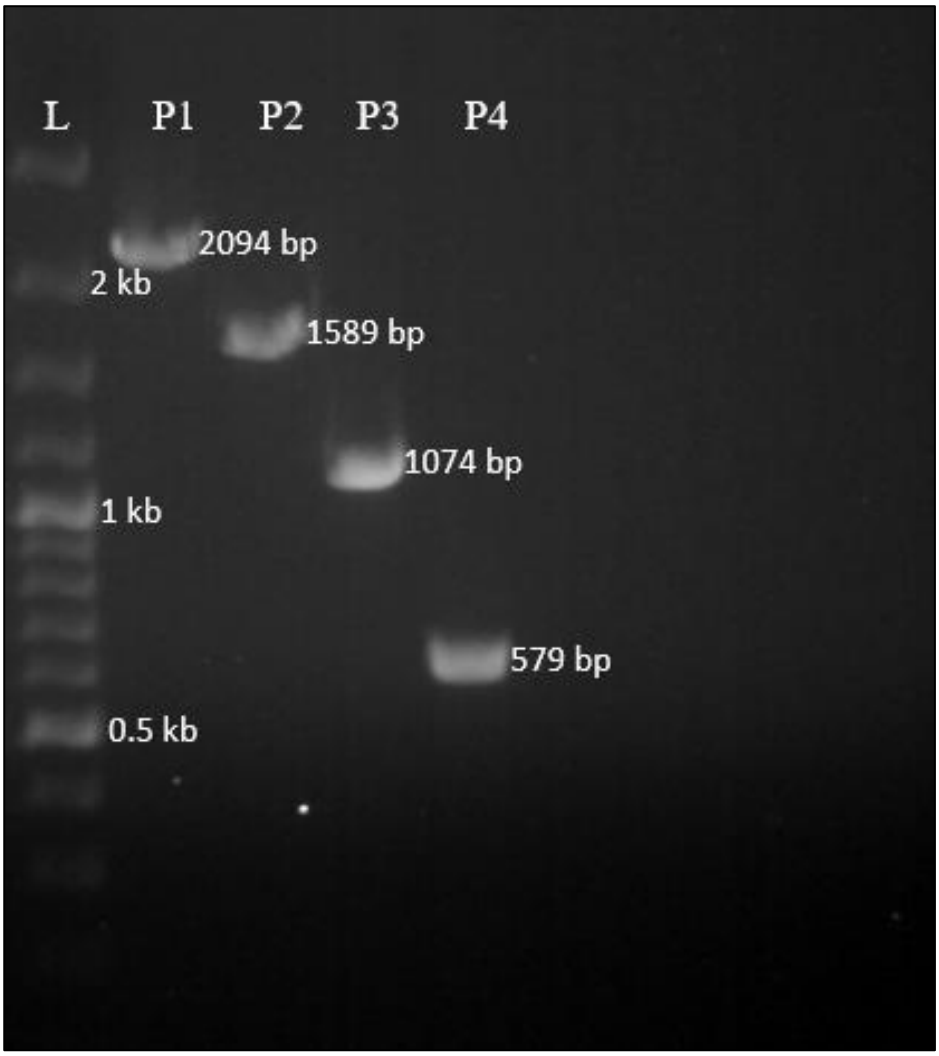


Figure 20 – Amplified promoter fragments for all four targeted regions

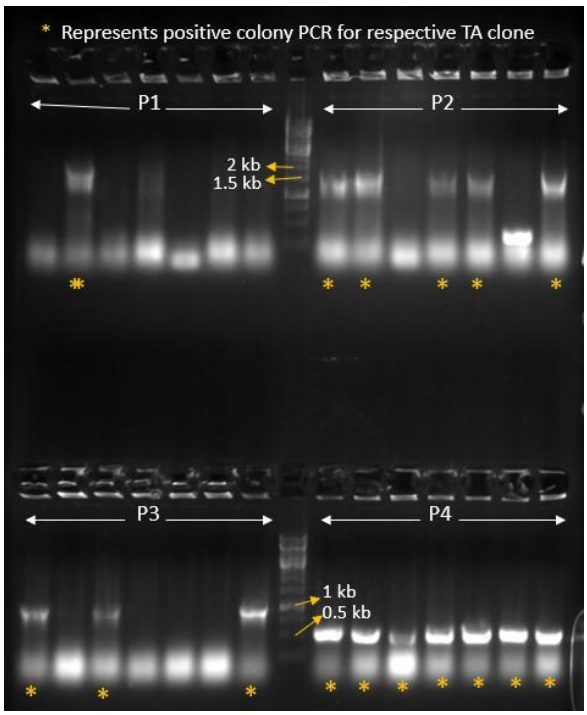


Figure 21 – Gel image of colony PCR to confirm TA clones

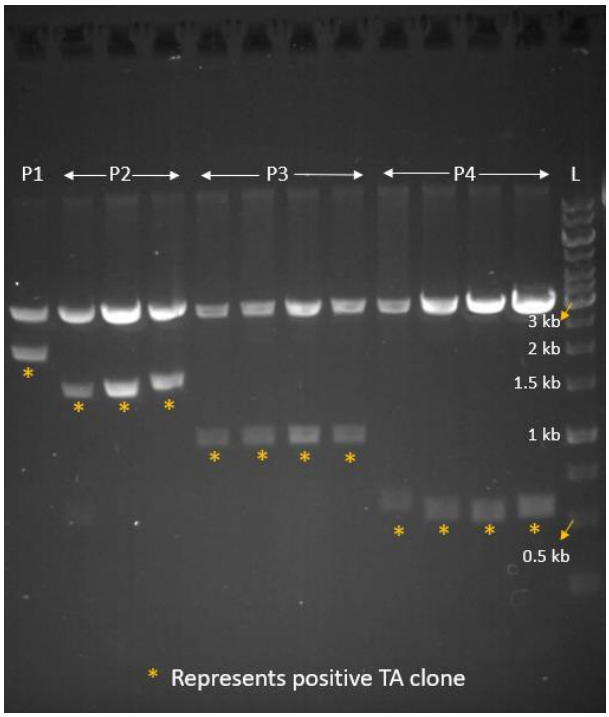


Figure 22 – Confirmed P1, P2, P3 and P4 TA clones by EcoRI enzyme digestion

3.3 Confirmation of TA clones by restriction digestion – pGEM-T easy vector has multiple cloning site with EcoRI digestion site. Thus, EcoRI was used to confirm TA ligated products as shown in the gel image in figure 22. Vector backbone is separated from ME49 ASR constructs P1, P2, P3 and P4 after digestion with EcoRI hence, confirming the TA ligation.

3.4 Restriction digestion for cloning – After confirming TA clones, restriction digestion was setup by HindIII and NheI for ligating the resulting products with pTub plasmid backbone having ampicillin and YFP gene including HindIII and AvrII digested sites. Expected band size with HindIII and NheI digestion for P1 was 2103 bp, P2 was 1598 bp, P3 was 1083 bp and P4 was 588 bp including 3022 bp band in each. Whereas, pTub plasmid digestion with HindIII and AvrII yields 5259 and 4424 bp bands. Bands were cut from gel and isolated for further ligation. The agarose gel image is shown in figure 23.

3.5 Restriction digestion for confirmation of final clones – After successful ligation, transformation and plasmid isolation, final ME49 clones were confirmed with restriction enzyme digestion using XbaI enzyme. Band size observed on agarose gel for final clones P1 is 3662 bp, 1882 bp, 983 bp; P2 is 3157 bp, 1882 bp, 983 bp; P3 is 3625 bp, 1882 bp; and P4 is 3130 bp, 1882 bp. The results are depicted in figure 24.

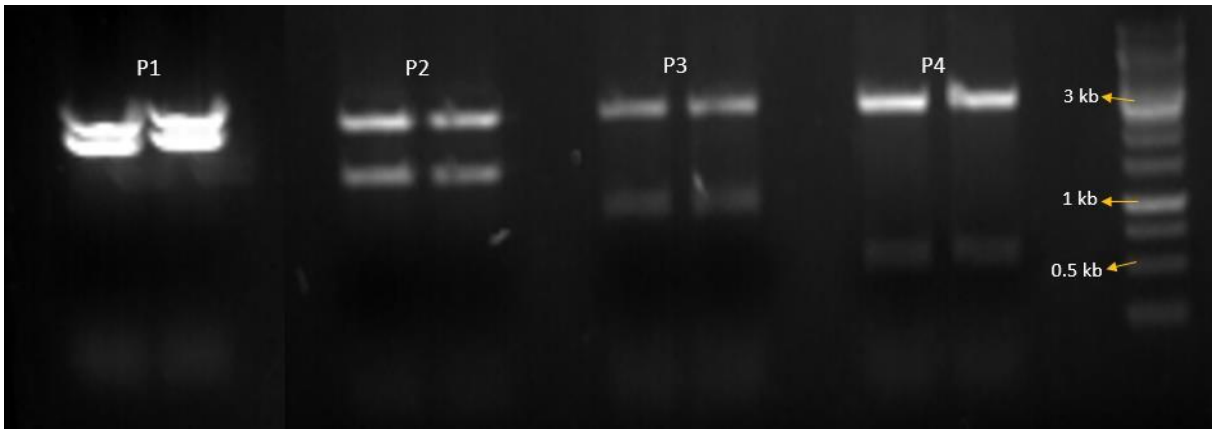


Figure 23 – Restriction digestion of P1, P2, P3, P4 with HindIII and NheI for gel isolation

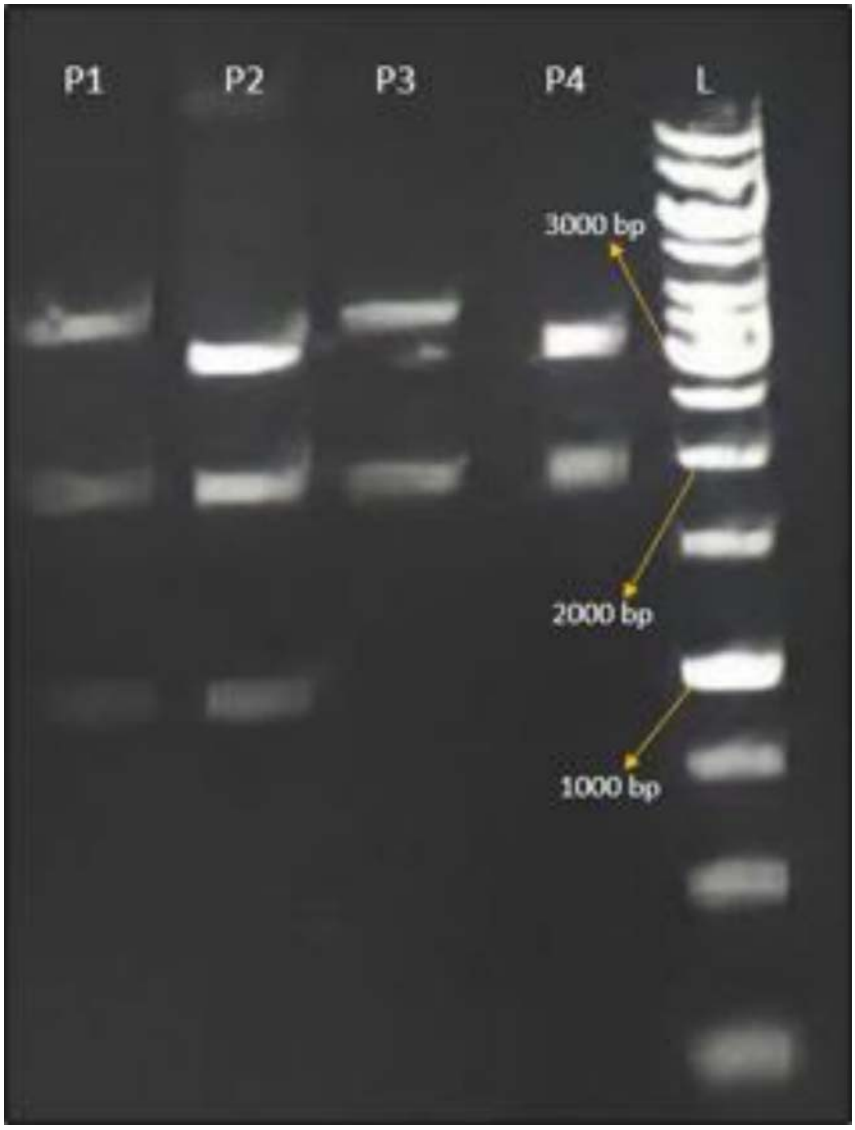


Figure 24 – XbaI restriction digestion to confirm final clones P1, P2, P3 and P4 of ME49 strain of *Toxoplasma gondii*

3.6 Transfection and immunostaining – Transfection was done using electroporation method for inserting ME49 clones into RH strain and allowed to invade in HFF cells. Transfected parasites were allowed to grow in host cells for one day at 37ºC and 5% CO_2. Cells were later immuno-stained and seen under inverted microscope at required magnification. No fluorescence was observed in the vacuoles after parasites infected host cells. Hence, expression of YFP in transfected RH wt parasite is not visualized for all the clones in both the duplicates.

4. Discussion

With emerging advancements in research, study on anti-sense RNA and their functions is gaining lot of interest. Natural anti-sense transcripts are a common feature in apicomplexan transcriptome and is expected to play important role in differential gene regulation. Our work mainly focuses on anti-sense long non-coding RNA (lncRNAs) that is a unique transcript of DNA that can range from 200 bp to 100 kbp approximately and is complementary to sense strand of protein coding gene. It plays important role in regulating expression of gene at replication, transcription and translation levels (Xu, Zhang and Zhang, 2018). High-throughput RNA sequencing, strand-specific PCR, northern blotting, RNase protection assay, etc. are some famous techniques that can be used to study anti-sense transcript and gene transcriptional orientation (Zhang *et al.*, 2016).

In this study, expression of anti-sense RNA is studied on the basis of reporter-based promoter characterisation. Taking the reference of the RNA sequencing data anti-sense RNA regions on 5th exon of FAS-I gene are identified and four constructs are cloned for identification of anti-sense promoter in ME49 strain of *T. gondii*. Expression studies are carried out by transfecting the final constructs with expected promoter region into RH wt strain and infecting the transfected parasite in HFF host cells for growth. YFP protein was expected to be expressed after transfection which was not observed on microscopy.

5. Conclusions

The impact of anti-sense RNAs at different levels of translation, transcription and replication as well as their role in regulating gene expression is still not completely known. But as ASR is considered to be a powerful tool with potential applications in regulating gene expression it becomes crucial to study more about its mechanism and its effects on the organisms. In this study, the goal was to characterize the promoter activity for the anti-sense

RNA corresponding to the *T. gondii* FAS-I gene. The results after transfections were not conclusive as no expression of reporter gene is observed. The reasons for the failed result may be due to technical reasons, including because of lack of proper genomic context for obtaining constitutive promoter activity. It is also possible that the promoter activity needs to be tested in the ME49 strain to yield expected results. In addition, other reason might be that the promoter region has been partially cloned or is far away from the expected location upstream of the anti-sense expression.

6. Future Prospects

Taking all the conclusions under consideration, experiment must be carried out again with the same clones of ME49 strain and transfection can be done in both ME49 and RH strain of *T. gondii*. Also, the promoter construct can be modified by the introduction of 5' UTR region to facilitated YFP reporter expression. More sensitive reporter like luciferase gene can be cloned instead of YFP for easier detection of expression. Once, the promoter is identified further research on effect of anti-sense RNA on the mRNA expression and their gene regulation mechanism can be studied in detail.

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