CHAPTER IV

EXPRESSION OF GREEN FLUORESCENT PROTEIN IN *DICTYOSTELIUM DISCOIDEUM*

4.1 Introduction: Green fluorescent protein (GFP)

Light is produced by the bioluminescent jellyfish *Aequorea victoria* when calcium binds to the photoprotein aequorin (Shimomura *et al.,* 1962). Although activation of aequorin *in vitro* or in hetreologous cell produces blue light, the jellyfish produces green light. This light is the result of a second protein in *Aequorea victoria* that derives its excitation energy from aquorin (Moorin and Hastings, 1971), appropriately named the green fluorescent protein (GFP), responsible for the green lining along the margins of the jellyfish bell. In 1991 Prasher et.al. cloned the gene for GFP from *Aequorea victoria* as part of their effort to understand the mechanisms of light generation in the bioluminescent jellyfish organ (Prasher *et al.,* 1992). By cloning the gene they triggered an avalanche of applications for GFP covering most major areas of biological research.

4.1.1 Characteristics

GFP is unique among light-emitting proteins in that it does not require the presence of any cofactors or substrates for the generation of its green light. In the jellyfish, GFP is activated in Ca-dependent manner when Ca^{2+} binds aquorin, which transfers energy indirectly to GFP to trigger the release of green light (Prasher *et al.,* 1995). This energy transfer can be mimicked experimentally by simple exposure of GFP to standard long wave ultraviolet (UV) light (e.g. from a conventional fluorescence microscope or a handheld UV source); making it a versatile marker for protein tagging and gene expression in living cells.

4.1.2 Structure

Purified GFP, a protein of 238 aa , absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission 509 nm with a shoulder at 540 nm). This fluorescence is very stable, and virtually no photo-bleaching is observed. The crystal structure of GFP has been resolved and shown to be a remarkable barrel like arrangement: Eleven ß sheets surround a central alpha- helix, containing the fluorescent center, which is formed by a hexapeptide (Ormo *et al.,* 1996; Yang *et al.*, 1996; Wachter *et al.*, 1997). The chromophore itself is formed by a Ser65-Tyr66-Gly67 tripeptide, which forms in as an yet uncharacterized cyclization process in the presence of oxygen (Heim *et al.,* 1994). Although the intact protein is needed for fluoresence, the same absorption spectral

properties are found in the denatured protein at amino acid 64. Mutational analysis of the protein have produced " improved" GFP isoforms, one of which emits blue instead of green light (Tyr66/His (Heim and Tsien, 1996), Tyr145/Phe), a red shift varient (Heim and Tsien, 1996); and another isoform significantly brighter than the wild type (Ser65/Thr, Phe64/Leu) (Cormack *et al.,* 1996). Using specific filters, three different isoforms have been detected simultaneously in samples of living bacteria (Heim and Tsien, 1996). An important step towards the widespread application of GFP in various organisms has been the generation of GFP forms that are tailored for efficient expression in a particular species by modifications such as introduction of introns or codon optimization e.g. bacteria (Siemering *et al.,* 1996), mammal (Siemering *et al.,* 1996; Zolotukhin *et al.,* 1996; Zernicka-Goetz *et al.,* 1996), *Xenopus Laevis* (Zernicka-Goetz *et al.,* 1996), yeast (Siemering *et al.,* 1996; Kahana and Silver, 1996), plants (Chiu *et al.,* 1996; Haseloff *et al.,* 1997) as well as *Drosophila melanogaster, C. elegans,* and Zebrafish.

4.1.3 Gene sequence

Accession # M62653

tacacacgaataaaagataacaaagATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGT
CCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAG
TGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTT
GCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTT
ATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAACAGCATGACTTTT
TCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAA
GATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCT
TGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTT
GGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGAC
AAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGAT
GGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGG
CCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAA
AGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTG
CTGGGATTACACATGGCATGGATGAACTATACAAATAAatgtccagacttccaattgacac
taaagtgtccgaacaattactaaaatctcagggttcctggttaaattcaggctgagatattatttatatatttatagattcat
taaaattgtatgaataatttattgatgttattgatagaggttattttcttattaaacaggctacttggagtgtattcttaattctat
attaattacaatttgatttgacttgcaaa

4.1.4 Application

Apart from its use as a protein tag, GFP is also used to moniter gene expression in single, living cells noninvasively. The GFP gene under the control of any promoter of interest directly indicates the gene expression level in living cells or tissues. This approach has major advantages over commonly used expression reporters such as *chlorempheniçol-acetyltransferase* or *ß-galactosidase* requiring exogenous addition of substrates or cofactors and thus rendering them with limited use to living tissues. Because the detection of intracellular GFP requires only irradiation by near UV or blue light, it is not limited by the availability of substrates. Thus, it provides an excellent means for monitoring gene expression in living cells, and repeated readings, under various conditions, can be taken on the same cell. Also fixation and sample prepration techniques are not necessary (Subramanian and Srienc, 1996). Since it does not appear to interfere with cell growth and function, GFP should also be a convenient indicator of transformation and one that could allow cells to be separated with fluorescence-activated cell sorting.

Several restrictions must be considered when using GFP as a gene reporter: the GFP signal cannot be amplified in a controlled manner, possibly preventing detection of low expression levels. In addition, folding of GFP into its active, fluorescent form is fairly slow and occurs in the order of hours. Although, a "faster" folding form of GFP has been generated. This makes the study of fast transcriptional activation processes difficult. Possibly the most serious concern regarding quantitative use of GFP must be the non-linearity of fluorescent signals. The linear range of fluorescence signals has to be determined for each application, and calibration curves has to be established. For these reasons GFP is per se not superior to other gene expression reporters such as *luciferase* in applications that require quantitative measurement of gene expression. It does represent, however, a simple alternative for detection of highly transcribed messages or for use in all-or-none type of expression assays.

4.2 Construction of pA15S(2)GFP

In order to test the secretory property of the newly constructed vectors, GFP was used as a reporter molecule. First step in the expression of GFP in *D. discoideum* was to clone it in-frame with the secretory sequence in one of the secretory vectors

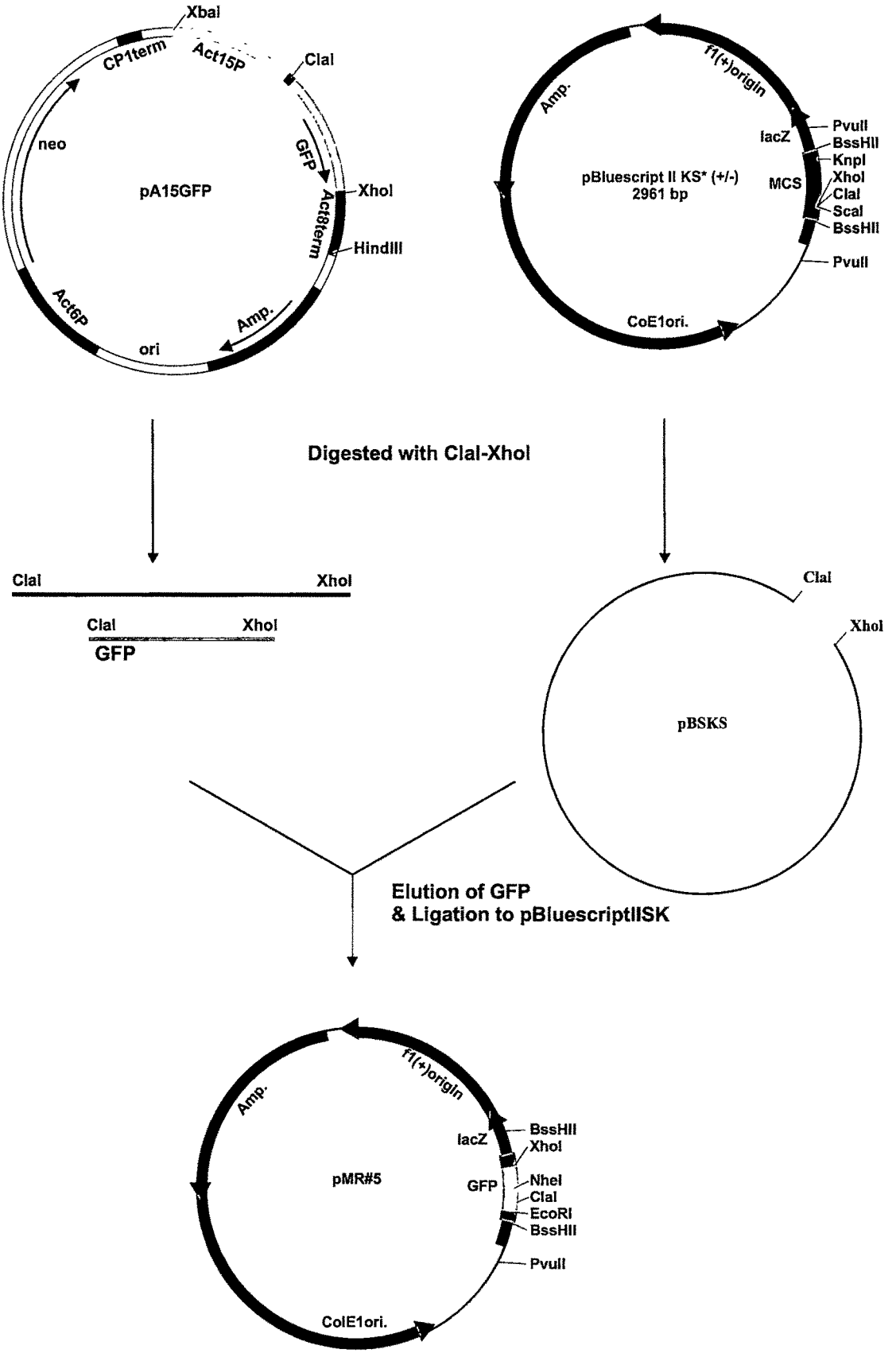
[pA15S(1), pA15S(2), pA15S(3)]. GFP gene was in-frame with the secretory sequence in pA15S(2). Therefore it was decided to clone GFP from pA15GFP in the MCS of pA15S(2).

In pA15GFP, GFP is flanked by *ClaI* at 5' end and *XhoI* at 3' end. Since these two sites are present in the backbone of pA15S(2), there was a need to flank GFP with another RE sites which are also present in the MCS of the pA15S(2). MCS present in pBluescript II SK phagmid (BSSK) contains many RE sites **(section 2.1.5.3)**. Therefore GFP was subcloned in the MCS of pBluescript II SK phagmid. pA15GFP was double digested with *ClaI-XhoI* to release GFP as a 750 bp band **[Fig. IV(b), lane 2]**. It was eluted from gel and purified **(section 2.3.8)**. Purified DNA was ligated to pBSSK **(section 2.3.10)** linearized with *ClaI-XhoI* **[Fig. IV(b), lane 3]**. The resulting vector was henceforth named as pMR#5 (3.71 kb) **[Fig. IV(a)]**. In pMR#5, GFP is flanked on either side by several RE sites. *E. coli* cells were transformed with pMR#5 **(section 2.3.1.1)** and amp^+ clones were screened for the insert. 160 amp^+ clones were obtained out of which 12 were screened for the insert.

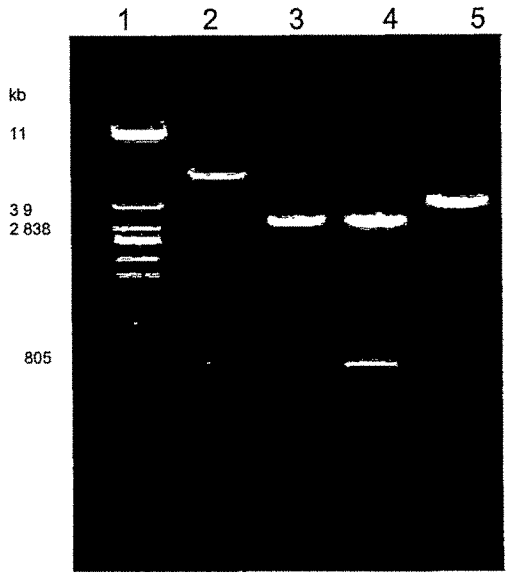
Confirmation of clone of pMR#5 by RE digestion **(section 2.3.5) [Fig. IV(b)]**:

i) *ClaI-XhoI*: *ClaI-XhoI* sites were used for cloning of GFP in pMR#5. Release of 750 bp band on digestion of pMR#5 with *ClaI-XhoI* confirmed the presence of GFP in pMR#5 **[Fig. IV(b), lane 3]**.
ii) *NheI*: *NheI* site is present in GFP gene and absent in pBSSK. Linearization of pMR#5 with *NheI* confirmed the presence of GFP in pMR#5 **[Fig. IV(b), lane 4]**.

After confirmation of presence of GFP in pMR#5, pMR#5 was digested with *EcoRI* and *XhoI* to release GFP fragment of 750 bp **[IV(d), lane 2]**. GFP was eluted from gel and purified **(section 2.3.8)**. Purified DNA was ligated **(section 2.3.10)** to pA15S(2) linearized with *EcoRI-XhoI* **[IV(d), lane 3]**. The resulting vector was henceforth named as pA15S(2)GFP **[Fig. IV(c)]**. *E. coli* cells were transformed with pA15S(2)GFP **(section 2.3.1.1)** and amp^+ clones were screened for the insert. 52 amp^+ clones were obtained out of which 12 were screened for the insert.

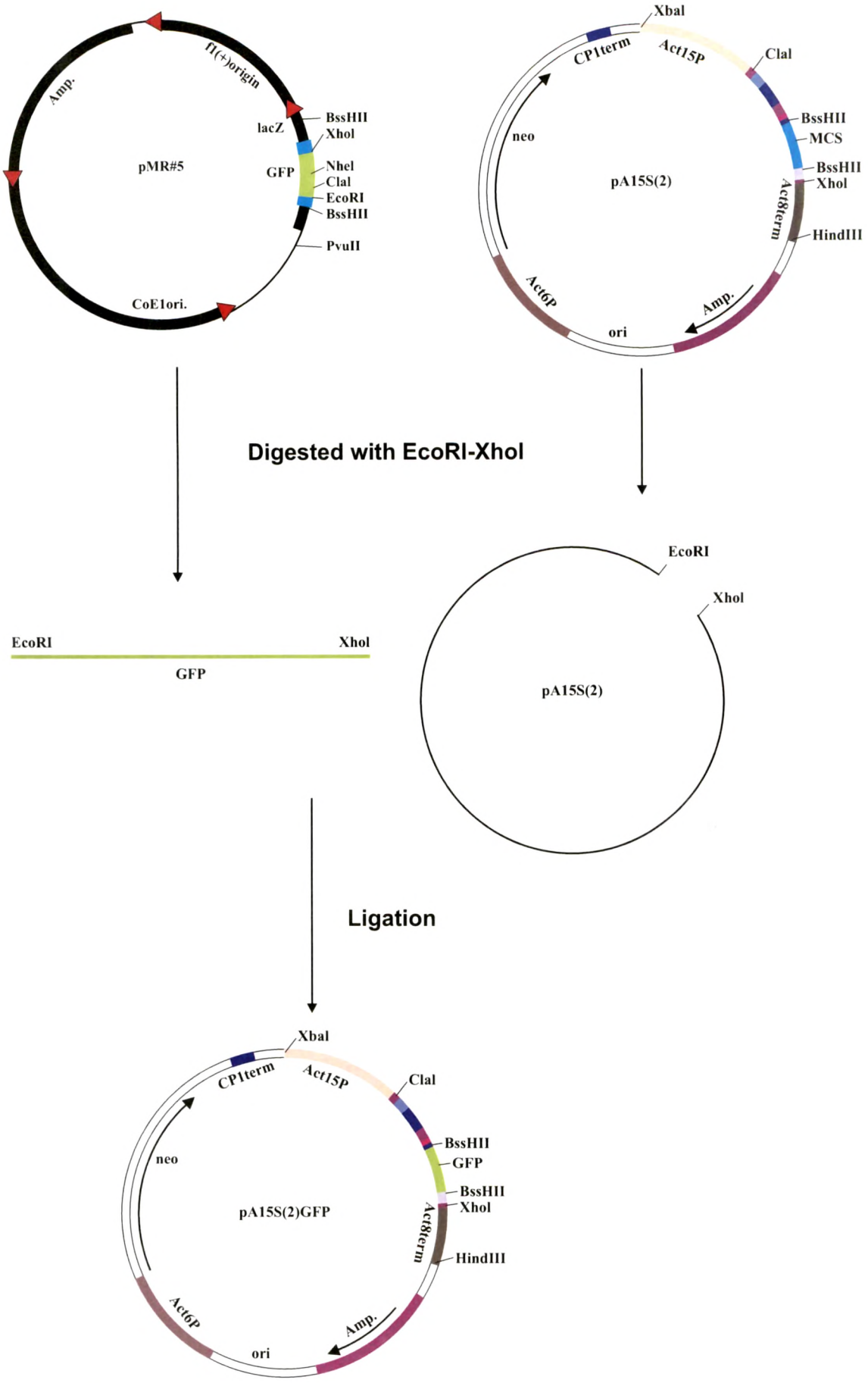


4.2 Fig. IV(a) - Schematic summary of construction of pMR#5



4.2 Fig. IV(b) - Construction and RE digestion pattern of pMR#5 on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pA15GFP digested with *ClaI-XhoI*
Lane 3: pBSSK digested with *ClaI-XhoI*
Lane 4: pMR#5 digested with *ClaI-XhoI*
Lane 5: pA15GFP digested with *NheI*



4.2 Fig. IV(c) - Schematic summary of construction of pA15S(2)GFP

Confirmation of clone of pA15S(2)GFP by RE digestion **(section 2.3.5)** **[Fig. IV(d)]** :

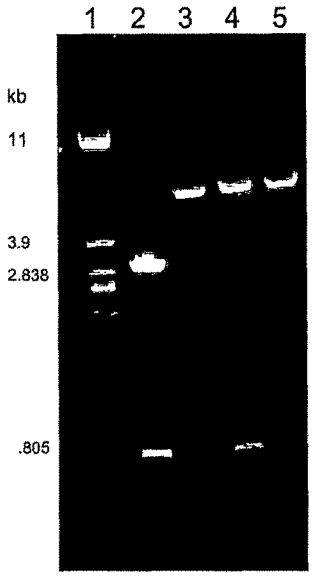
i) *EcoRI-XhoI*: *EcoRI-XhoI* sites were used for cloning of GFP in pA15S(2)GFP. Release of 750 bp band on digestion of pA15S(2)GFP with *EcoRI-XhoI* confirmed the presence of GFP in pA15S(2)GFP **[IV(d), lane 3]**.

ii) *NheI*: *NheI* site is present in GFP gene and absent in pA15S(2). Linearization of pA15S(2)GFP with *NheI* confirmed the presence of GFP in pA15S(2)GFP **[IV(d), lane 4]**.

To further confirm the GFP sequence and its reading frame in pA15S(2)GFP, GFP along with HP1:HP2 was sequenced **(section 2.3.14)** from pA15S(2)GFP using primer MR01 **(section 2.1.2)**. The GFP sequence showed no mutation and was in proper reading frame **[Fig. IV(e)]**.

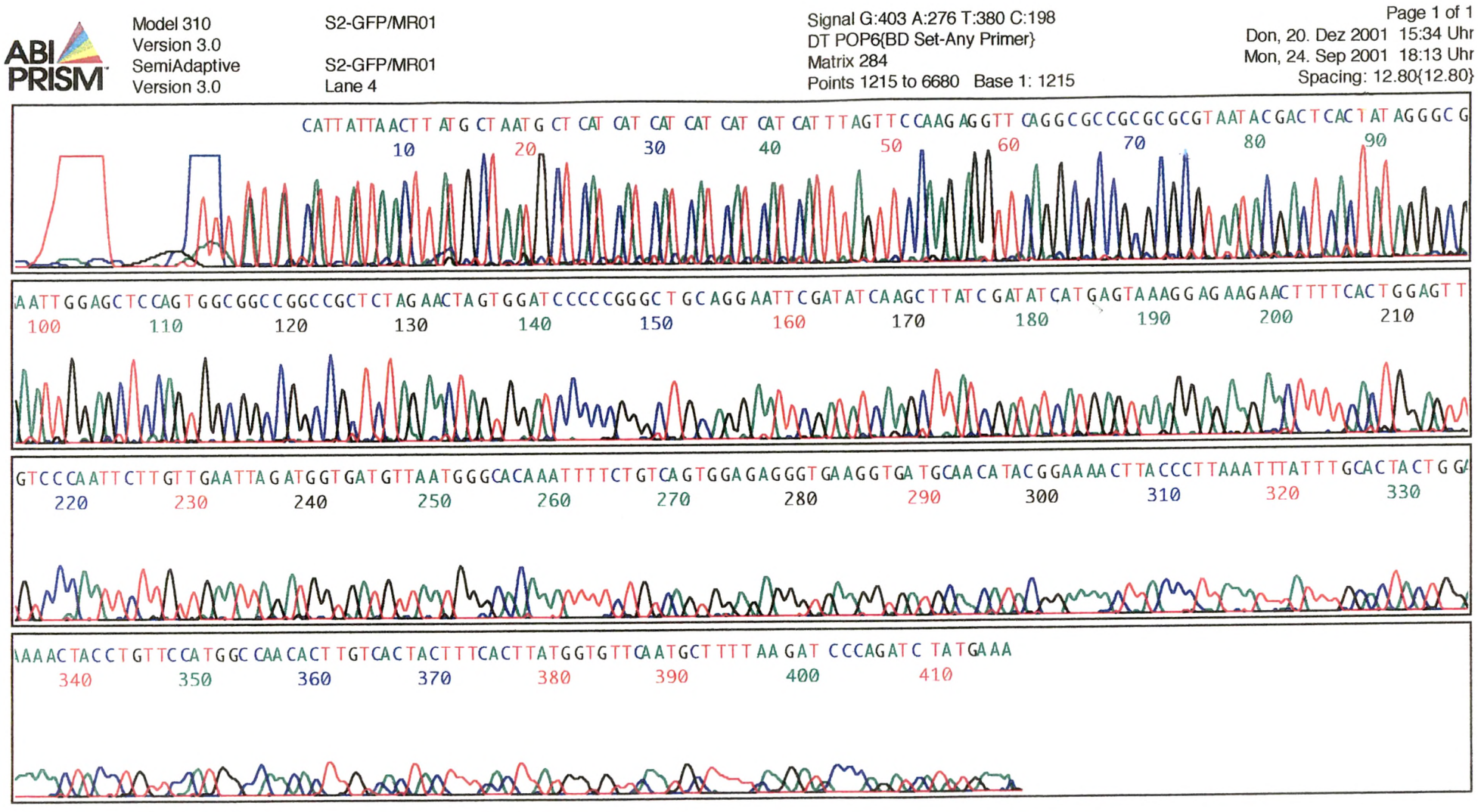
4.3 Transformation of *Dictyostelium discoideum* (Ax-2) cells with pA15S(2)GFP

Cells of Ax-2 strain of *Dictyostelium discoideum* in log phase (2 X 10^6 cells/ml, 10 ml per plate) were transformed with pA15S(2)GFP (7-8 μg) by $CaPO_4$ method **(Section 2.4)** along with proper controls. pA15GFP transformed cells were used as positive control and untransformed cells as negative control for fluorescence and antibiotic resistance. 10-50 clones/plate were observed after 10-15 days. Clones growing on plates containing G418 were transferred to 24 well plates for further growth and observation. Transformants grew at rates comparable to the Ax-2 cells (doubling time of ~12 hours) and went through the developmental cycle normally upon starvation. No difference in the transformation efficiency as well as in growth and development pattern was observed with different GFP constructs. Spores as well as glycerol stock of the transformants retained the G418 resistance on revival in the absence of the antibiotic indicating the stability of the integrating plasmid over the generations. Transformants were highly resistance to G418 - growing upto 75 μg/ml concentration at 21°C.



4.2 Fig. IV (d) - Construction and RE digestion pattern of pA15S(2)GFP on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#5 digested with *EcoRI-XhoI*
Lane 3: pA15S(2) digested with *EcoRI-XhoI*
Lane 4: pA15S(2)GFP digested with *XhoI*
Lane 5: pA15S(2)GFP digested with Nhe*I*



4.2 Fig. IV(e) - Sequence of pA15S(2)GFP : In pA15S(2)GFP, GFP gene from pMR#5 has been cloned in the *EcoRI - XhoI* site of pA15S(2) and was found to be in frame with the secretory sequence (182 onwards).

4.3.1 GFP fluorescence images of transformed cells

Since the growth medium (HL-5) of *D. discoideum* itself appears to be green under fluorescence microscope using blue filters, cells from log phase cultures were washed and suspended in KK2 buffer for observation under the fluorescence microscope. No significant fluorescence was observed in the transformants as compared to the positive control which appeared bright green under fluorescence microscope **[Fig. IV(k)]**.

4.3.2 GFP fluorescence in transformed cells

To further verify the GFP fluorescence from the transformants, 1 X 10^6 log phase cells were washed, suspended in 1 ml of KK2 buffer and fluorescence was recorded at Ex: 488 nm and Em: 507 nm. No significant fluorescence was observed **[Fig.IV(l)]**.

Since in pA15S(2)GFP, GFP sequence is preceded by the secretory sequence, GFP is expected to be secreted out in the growth medium. The growth medium itself shows fluorescence at Ex: 488 nm and Em: 507nm. Therefore medium was TCA precipitated (10%) for the removal of small fluorescent peptides from it (section 2.5.1). TCA precipitate of the growth medium was tested for fluorescence at Ex: 488 nm and Em: 507nm but no significant fluorescence was observed indicating low expression of GFP in the transformants [Fig.IV(l)]. As discussed in section 4.1.3, GFP signals are detectable for highly transcribed/translated messages and are uses in all-or-none type of expression assays. The non-linearity of fluorescent signals of GFP might possibly be responsible for the insignificant fluorescent observed.

In pA15S(2)GFP, GFP is preceded by part of the MCS containing stretch of GC sequences recognized by restriction enzymes. *D. discoideum* genome has an AT content of > 75% and codon usage is highly biased towards AT-rich codons. GC rich sequences are known to interfere with *D. discoideum*'s translational machinary by causing ribosomal instability. Since GFP signals are detectable for highly transcribed/ translated messages, presence of GC sequences might be responsible for the unstability of transcripts and slow rate of translation of GFP resulting into

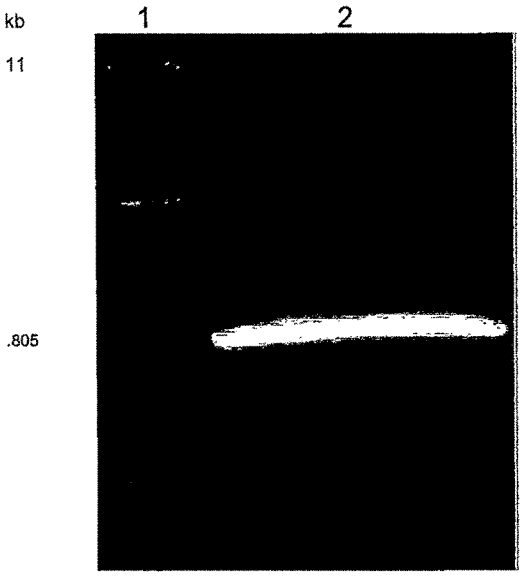
lack of detectable fluorescence. Therefore it was decided to remove the extra GC sequences present at 5' to GFP gene by cloning it into the first site (*SacI*) of the MCS of the secretory vector.

4.4 Construction of pA15S(1)GFP

In order clone GFP into the *SacI* site of MCS of pA15S(1), GFP from pA15GFP was PCR amplified **(section 2.3.11)**. Twentymer primers MR09 and MR10 **(section 2.1.2)** were designed for *SacI* site at both ends of GFP cDNA. Extra nucleotides were added to the 5' of the primers to make GFP in-frame.

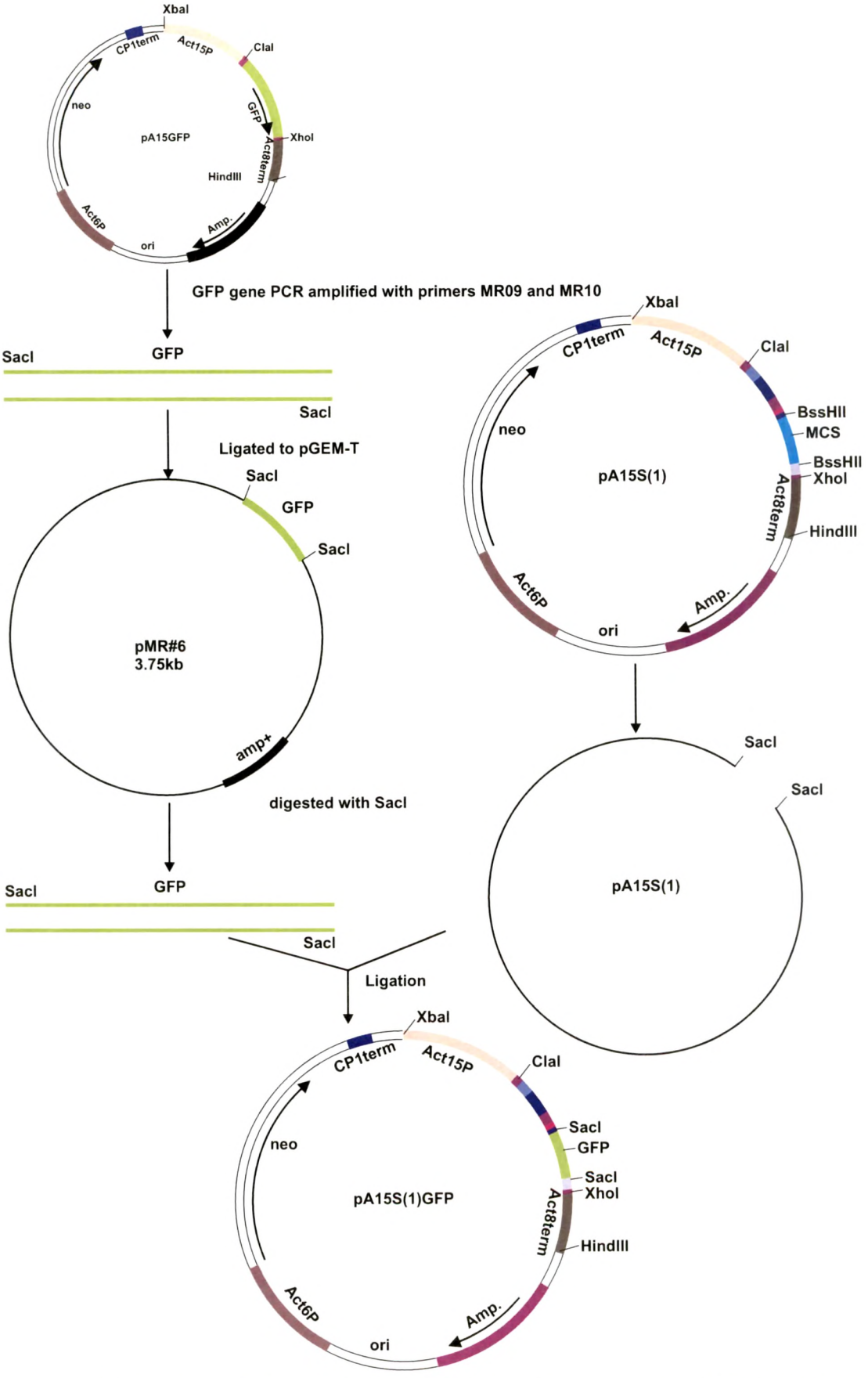
Amplification of GFP was confirmed by the presence of 750 bp band on the gel **[Fig. IV(f), lane 2]**. Amplified DNA was eluted from gel, purified and ligated using poly[A] tail of the recovered band and poly[T] region of pGEM-T (Promega) vector. The new vector formed was henceforth named as pMR#6 (3.750 kb). *E. coli* cells were transformed with pMR#6 by electroporation method **(section 2.3.1.2)** and blue/white selection of transformants was done on amp-Xgal-IPTG plates **(section 2.3.12)**. Many white colonies were obtained, out of which 12 were screened for the insert by digestion with *SacI*. To check for any point mutation in GFP during PCR, GFP along with HP1:HP2 was sequenced from pMR#6 **(section 2.3.14)** and showed no mutation (data not shown).

After confirming the sequence of GFP cDNA in pMR#6, pMR#6 was digested with *SacI* to release the 750 bp GFP cDNA **[Fig. IV(i), lane 2]**. GFP gene was gel eluted and purified using Quagene DNA elution kit. Purified DNA was ligated **(section 2.3.10)** to *SacI* digested and dephosphorylated pA15S(1) **[Fig. IV(i), lane 3]**. The new vector formed was henceforth named as pA15S(1)GFP (6.759 kb) **[Fig. IV(g)]**). *E. coli* cells were transformed with pA15S(1)GFP by electroporation method **(section 2.3.1.2)**. Since there was no antibiotic selection for the recombinants, colonies obtained on ampicillin plates were replica plated for colony blot assay **(section 2.3.13)**. GFP cDNA radiolabelled with ^{32}P by nick translation was used as a probe for detecting recombinants **[Fig. IV(h)]**.

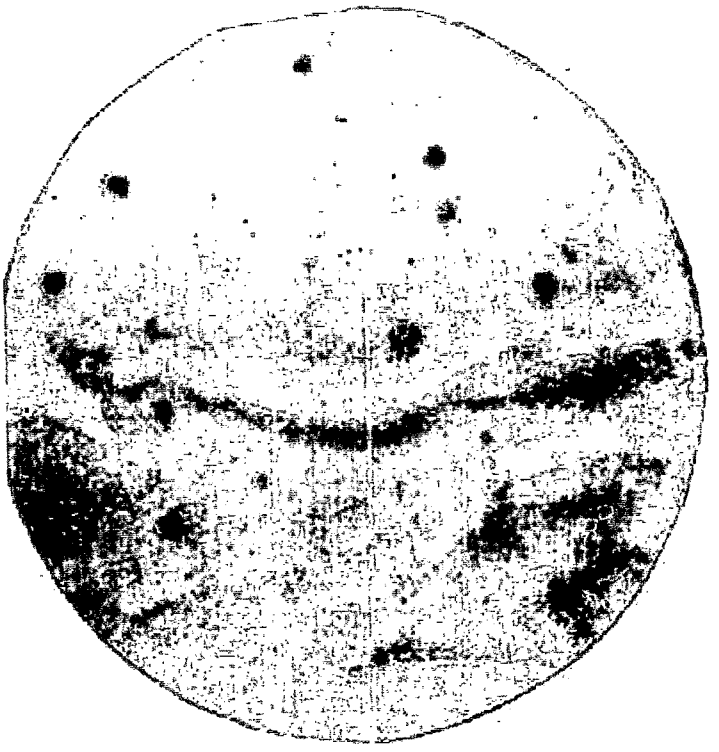


4.4 Fig. IV(f) - PCR amplified GFP gene from pA15GFP on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: PCR amplified GFP from pA15GFP



4.4 Fig. IV(g) - Schematic summary of construction of pA15S(1)GFP



4.4 Fig. IV(h) - Colony blot of *E.coli* cells transformed with pA15S(1)GFP. GFP gene radiolabelled with ^{32}P was used as a probe for detecting recombinant colonies

Positive clones containing GFP insert in pA15S(1)GFP were further confirmed by RE digestion **(section 2.3.5) [Fig. IV(i)]**:

i) *SacI: SacI* site was used for cloning of GFP in pA15S(1)GFP.Release of two fragments of 6 kb and 750 bp from pA15S(1)GFP on digestion with *SacI* confirmed the presence of GFP in pA15S(1)GFP **[Fig. IV(i), lane 4]**.

ii) *XbaI-NdeI*: *XbaI* site is present in the GFP sequence and *NdeI* site in the pA15S(1) backbone. Release of lower fragment of 690 bp from pA15S(1)GFP on digestion with *XbaI-NdeI* confirmed the correct orientation of GFP in pA15S(1)GFP **[Fig. IV(i), lane 5]**.

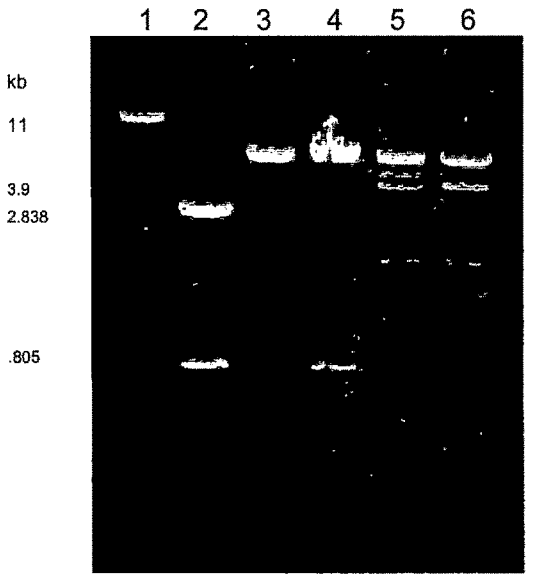
To further confirm the GFP sequence and its reading frame in pA15S(1)GFP, GFP along with HP1:HP2 was sequenced **(section 2.3.14)** from pA15S(1)GFP using primer MR01 **(section 2.1.2)**. The GFP sequence showed no mutation and was in proper reading frame **[Fig. IV(j)]**.

4.5 Transformation of *Dictyostelium discoideum* (Ax-2) cells with pA15S(1)GFP

Cells of Ax-2 strain of *Dictyostelium discoideum* in log phase (2 X 10^6/ml, 10 ml per plate) were transformed with pA15S(1)GFP (7-8 μg) by $CaPO_4$ method **(Section 2.4)** along with proper controls. pA15GFP transformed cells were used as positive control and untransformed cells as negative control for fluorescence and antibiotic resistance. Transformants growing in medium containing G418 at 10μg/ml concentration were selected and subsequently maintained at 5μg/ml.

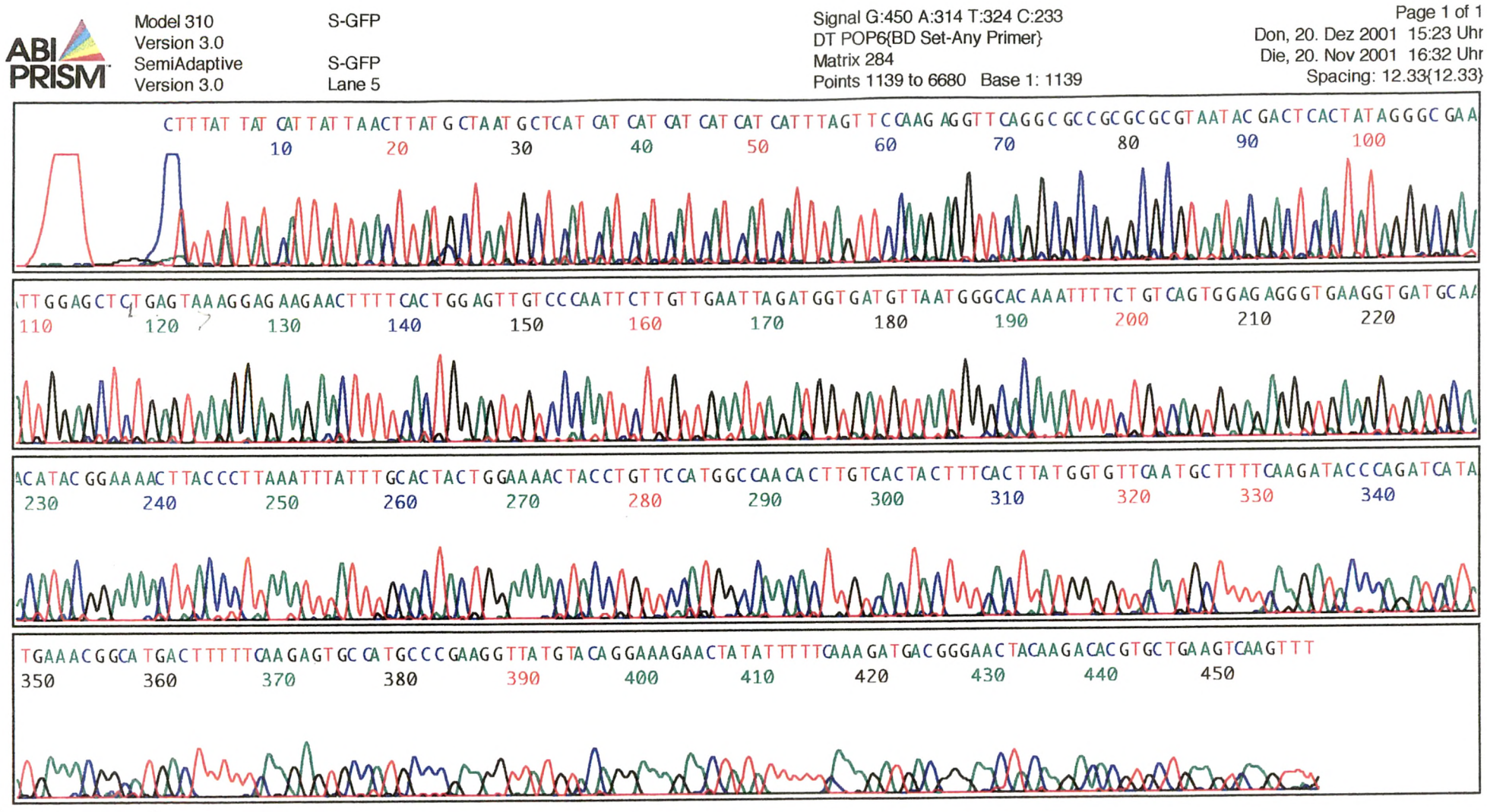
4.5.1 GFP fluorescence images and fluorescence in transformed cells

Clones were observed after 10-15 days and positive clones growing on plates containing G418 were transferred to 24 well plates for further growth and observation. Cells were washed and suspended in KK2 buffer for observation. No significant fluorescence was observed in the transformants either under the flurescence microscope **[Fig. IV(k)]** or with the spectrofluorimeter at Ex: 488 nm and Em: 507 nm **[Fig. IV(l)]**.

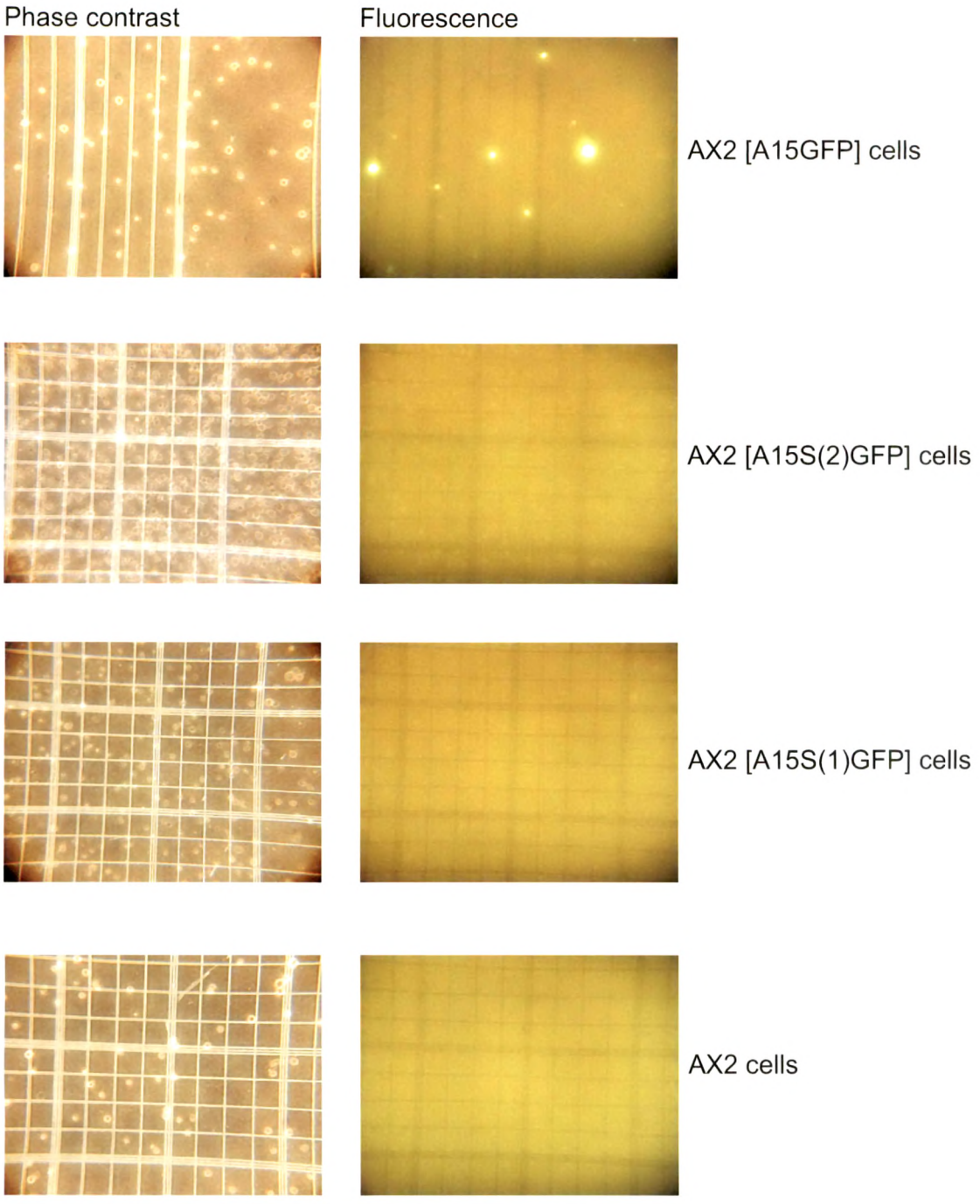


4.4 Fig. IV(i) - Construction and RE digestion pattern of pA15S(1)GFP on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#6 digested with *SacI*
Lane 3: pA15S(1) digested with *SacI*
Lane 4: pA15S(1)GFP digested with *SacI*
Lane 5: pA15S(1)GFP digested with *XbaI-NdeI*
Lane 6: pA15S(1) digested with *XbaI-NdeI*



4.4 Fig. IV(j) - Sequence of pA15S(1)GFP : In pA15S(1)GFP, GFP gene has been cloned in the *SacI* site of pA15S(1) and was found to be in frame with the secretory sequence (118 onwards).



4.5.1 Fig. IV(K) - Phase contrast and fluorescence images of Ax2 cells transformed with various GFP constructs (40X)

TCA precipitate (10%) of medium **(section 2.5.1)** of pA15S(1)GFP transformants showed marginal increase in fluorescence (at Ex: 488 nm & Em: 507 nm) as compared to the negative control (untransformed cells) and pA15GFP transformants where GFP is cytoplasmic, indicating the secretion of GFP in the medium **[Fig. IV(l)]**. As discussed in section 4.1.4 GFP is a poor quantitative marker and this might be the reason for the marginal increase in fluorescence observed in the medium of pA15S(1)GFP transformants.

4.5.2 PCR amplification of GFP from genomic DNA of transformants

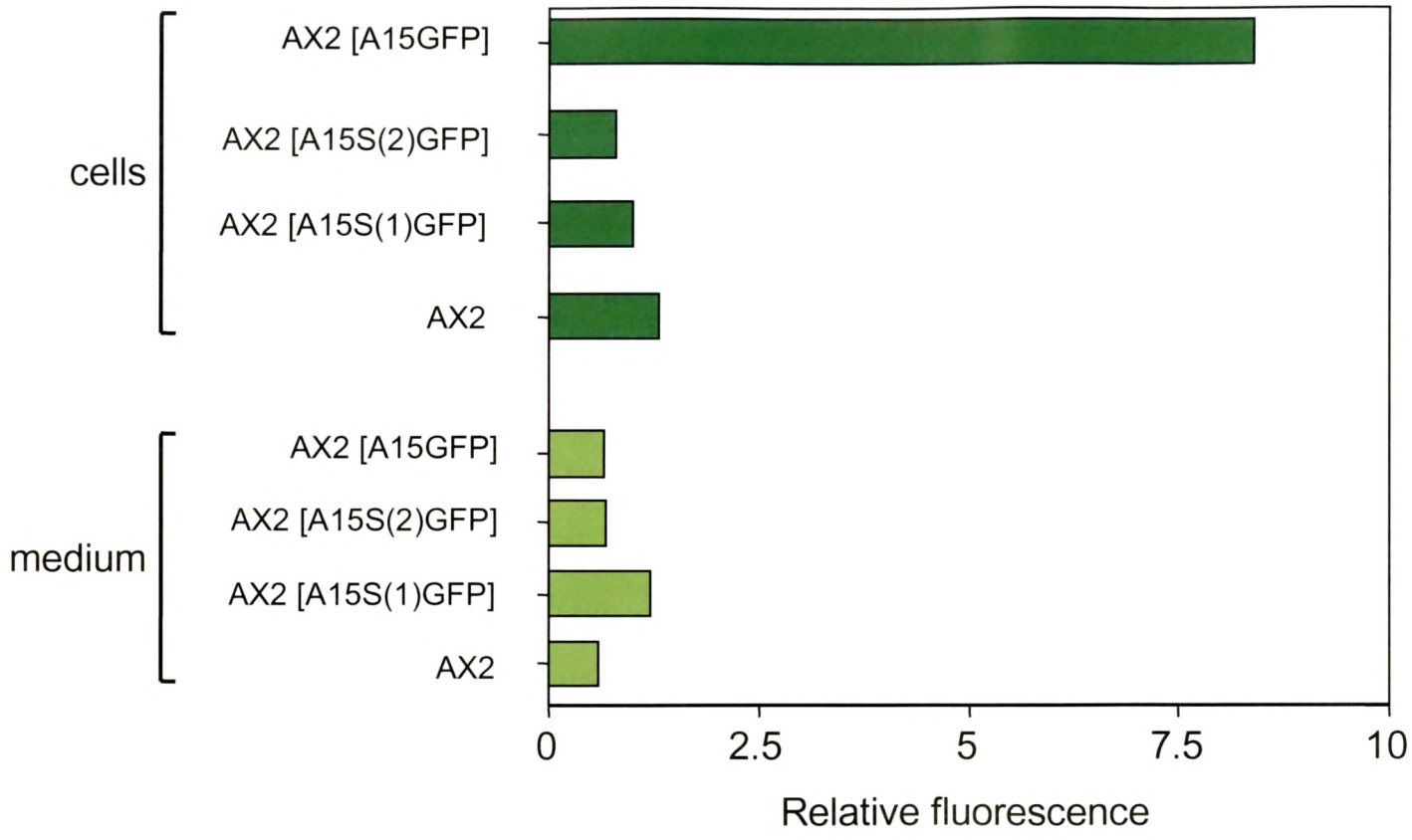
Since no fluorescence was detectable in the transformed cells and only marginal fluorescence was detected in the medium of pA15S(1)GFP transformants, genomic DNA was extracted from transformants **(Section 2.5.2)** and PCR amplified to check for the presence GFP gene sequence. MR09 and MR10 primers **(Section 2.1.2)** were used for amplification of genomic DNA **(Section 2.5.3)**.

Presence of GFP sequence in the genome of the transformants was reaffirmed by the amplification of 750 bp band **[Fig. IV(m), lane 4,5]** as compared to the untransformed cells **[Fig. IV(n), lane 6]**.

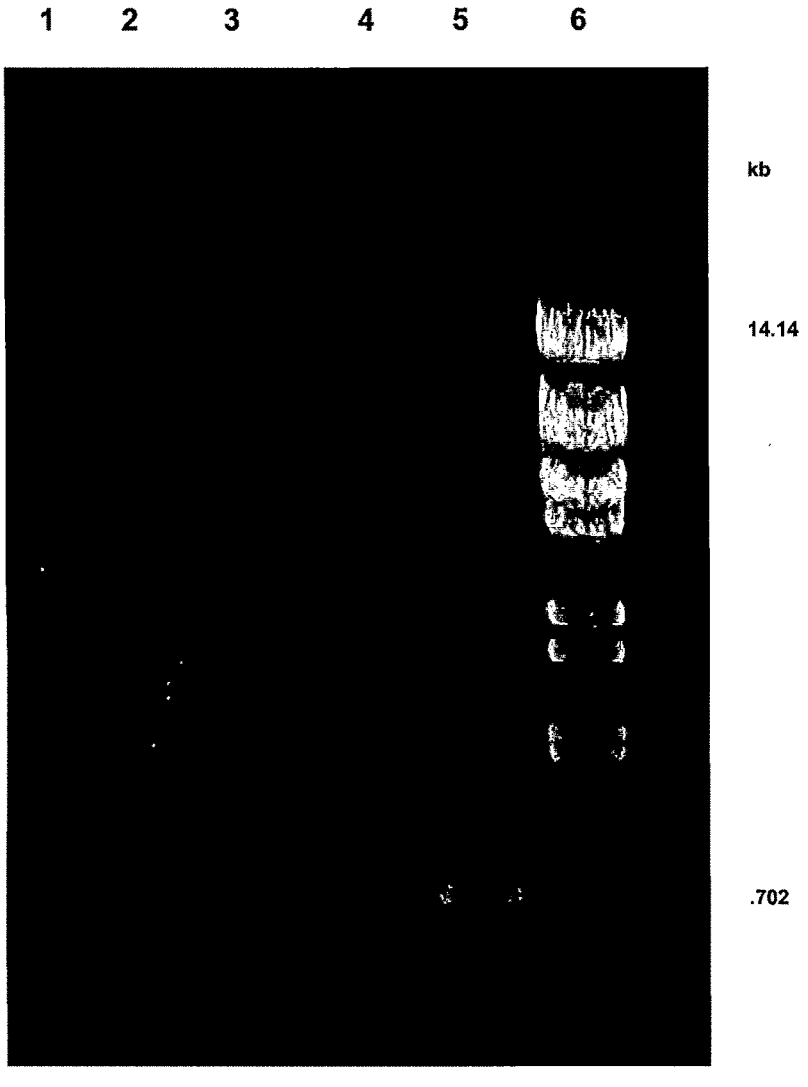
4.5.3 RT-PCR amplification of GFP from total cell RNA of transformants

pA15S(2)GFP and pA15S(1)GFP transformants were further confirmed by RT-PCR amplification of GFP mRNA from the total RNA. Total cell RNA was extracted from log phase cells **(Section 2.5.4)** and 1μg of it was used for RT-PCR. Primer MR09 was used for reverse transcription of GFP mRNA and MR09 and MR10 **(Section 2.1.2)** were used for the amplification of GFP cDNA **(Section 2.4.5)**.

Presence of about 750 bp band in the transformants **[Fig. IV(n), lane 2,3]** as compared to the untransformed cells **[Fig. IV(n), lane 4]** reaffirmed the stable integration and transcription of GFP in the transformed cells.

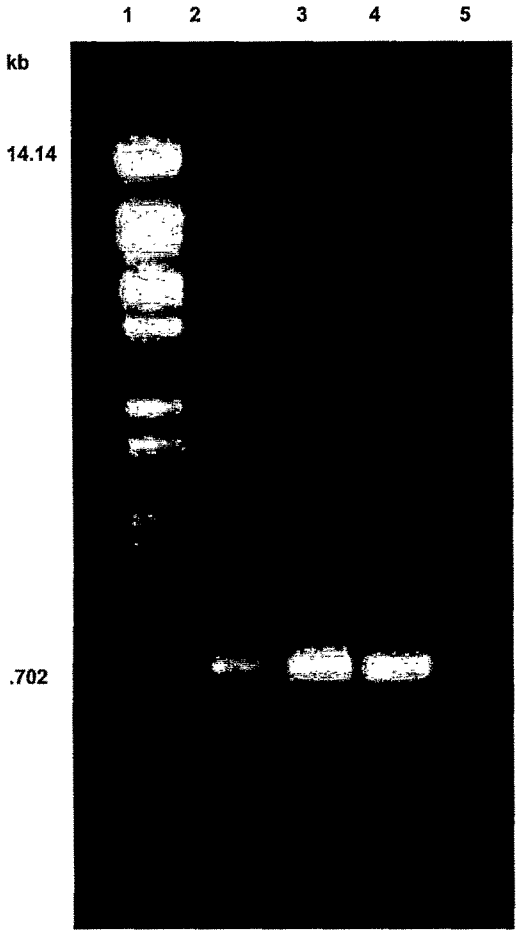


4.5.1 Fig. IV(I) : Relative Flouroscence of Ax2 cells transformed with various GFP constructs and their growth medium. Fluorescence was recorded at Ex: 488 nm and Em: 507 nm. Growth medium was TCA precipitated (10% final concentration) and ten fold concentrated.



4.5.2 Fig. IV(m) - PCR amplification of genomic DNA from Ax2 cells transformed with various GFP constructs (0.8% agarose gel)

Lane 1: AX2
Lane 2: AX2[S(1)GFP]
Lane 3: AX2[S(2)GFP]
Lane 4: AX2[A15GFP]
Lane 5: pA15GFP
Lane 6: lambda DNA digested with BstEII



4.5.3 Fig. IV(n) - RT-PCR amplification of total RNA from Ax2 cells transformed with various GFP constructs(0.8% agarose gel)

Lane 1: lambda DNA digested with BstEII
Lane 2: pA15GFP
Lane 3: AX2[A15GFP]
Lane 4: AX2[S(2)GFP]
Lane 5: AX2[S(1)GFP]

4.6 Discussion

The fast and easy *in vivo* detection predestines the GFP for its use as a reporter for promoter activities. We used GFP as a reporter molecule to confirm the activity of secretion signal and also to evaluate the expression potential of *D. discoideum* for production of recombinant proteins.

GFP was cloned in pA15S(2) construct that puts GFP in-frame. Transformants grew at rates comparable to Ax-2 cells and went through the developmental cycle normally upon starvation. Spores as well as glycerol stock of the transformants retained the G418 resistance on revival in the absence of the antibiotic indicating the stability of the integrating plasmid over the generations. Transformants were highly resistance to the G418- growing upto 75 μg/ml concentration. No difference in the transformation efficiency as well as in growth and development pattern was observed with different GFP constructs.

Growth medium of *D. discoideum* i.e. HL-5 shows green fluorescence under fluorescence microscope. To eliminate the medium interference, cells were washed and suspended in KK2 buffer for observation. Cells were observed under fluorescence microscope (blue filters) assuming that even if most of the expressed protein was secreted out, some would be remaining inside the cells making them fluorescent. No significant fluorescence was detectable in the pA15(2)GFP transformants where as pA15GFP transformants (positive control) appeared bright green. Medium was TCA precipitated (10%) to remove most of the interfering small molecules. Ten fold concentrated TCA precipitate of medium was checked for secretion of GFP at Ex: 488 nm and Em: 507 nm using spectrofluorimeter. No significant absorbance as compared to negative control was observed indicating low expression/secretion of GFP. As discussed in section 4.1.4, although GFP is a convenient marker for gene expression studies, it's signals are detectable for highly transcribed/ translated messages and are used in all-or-none type of expression assays. The non-linearity of fluorescent signals of GFP might possibly be responsible for the insignificant fluorescence observed.

Codon bias is considered to be one of the potential factors affecting the product yield in recombinant protein production. It has been observed that organisms do not use all codons for one amino acid at the same frequency and codon preferences vary among different organisms depending on their genome composition. The general consensus from literature on heterologous gene expression and codon use is that optimizing codon sequences increases expression levels in organisms. *D. discoideum* genome has an AT content of > 75% and codon usage is highly biased towards AT-rich codons. GC rich sequences are known to interfere with *D. discoideum*'s translational machinary by causing ribosomal instability. In pA15S(2)GFP, GFP is preceded by part of the MCS containing stretch of GC sequences recognized by restriction enzymes. Presence of these GC sequences might be responsible for low level of transcription or slow rate of translation of GFP. Therefore pA15S(1)GFP was constructed in which PCR amplified GFP was cloned in the first RE site (*SacI*) of MCS of pA15S(1) to avoid most of the in between GC rich RE sites.

Fluorescence was undetectable in the pA15S(1)GFP transformed cells. Ten fold concentrated TCA precipitate of the growth medium of pA15S(1)GFP showed marginal increase in fluorescence (at Ex: 488nm & Em: 507nm) as compared to Ax-2 medium and pA15GFP transformants where GFP fluorescence is cytoplasmic indicating expression and secretion of GFP. As discussed earlier, GFP is a poor quantitative marker and this might be the reason for the marginal increase in fluorescence observed in the medium of pA15S(1)GFP transformants. Bright fluorescence in pA15GFP cells and less GFP in pA15S(1)GFP medium is expected since in pA15GFP transformants GFP is confined in a small cell volume whereas in case of pA15S(1)GFP transformants, expressed GFP is getting diluted in the growth medium. Therefore GFP needs to be purified from the medium using Ni^{2+} nitriloacetic acid metal - affinity column for proper quantitation.

PCR amplification of GFP sequence from the genomic DNA of the transformants verified the presence and integration of GFP in the genome of the cells indicating the integrative nature of pA15 backbone. RT-PCR amplification of

GFP from the total cell RNA further reaffirmed the presence and transcription of GFP in the transformants.

Using GFP as a reporter molecule, expression and secretory potential of the secretory vector has been illustrated. Although no significant fluorescence was detectable in the transformed cells, growth medium of pA15S(1)GFP transformed cells showed marginal fluorescence as compared to untransformed cells. Amplification of GFP gene from genomic DNA and mRNA from total RNA of transfomants further reaffirmed the stable integration and transcription of GFP in the cells transformed with GFP constructs.