CHAPTER III

CONSTRUCTION OF A SECRETORY EXPRESSION VECTOR FOR *DICTYOSTELIUM DISCOIDEUM*

3.1 Introduction

The objective of this work is to make an expression vector for *D. discoideum* which has a secretory signal, purification tag, a tag removal site and multiple cloning site as desired features. One of the desired features of an expression vector is to have a secretion signal. Important modifications in protein structure take place (e.g. folding) during secretion which are required for proper secretion and extremely crucial for the activity of a protein. Secretion is also known to be beneficial during purification of the desired protein since the cells are not required to be lysed - a process during which proteases are activated leading to degradation of the recombinant proteins. Secretion also helps in scaling up of the culture since the cells can be continuously grown in the media and proteins can be harvested from continuous culture. Considering all these important features and benefits of secretion, our objective was to make a secretory expression vector for *D. discoideum* in all three reading frames. Prespore antigen (PsA) secretory sequence of *Dictyostelium discoideum* which leads to efficient secretion of the concerned protein outside the cell i.e. in the medium (Dittrich *et al.*,1994; Heikoop *et al.*,1998), was planned to be incorporated in the vector to secret out the recombinant proteins.

The construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest simplifies the purification of the recombinant protein from the medium by using affinity chromatography methods specific for the tags used. Expression of the six consecutive histidine residues (6 X *His*) tagged proteins has been carried out successfully in bacteria, mammalian cells, insect cells using baculovirus vectors, yeasts and plants (Coligan *et al.*, 1995). Proteins which have been tagged with 6 X *His* can be purified selectively with metal-affinity chromatography matrices (Ni^{2+}·nitriloacetic acid metal-affinity column) (Bertani *et al.*, 1999). Considering this important aspect of *His* tag, it was decided to be incorporated in the secretory vector downstream to the signal sequence.

One of the most important factors affecting the 3D structure and hence the functionality of a protein is its amino acid sequence. Heterologous sequences (e.g. His tag) present upstream to the protein sequence might affect its folding resulting into altered activity. Therefore tags should be removed after purifying the protein

from the medium. Hence thrombin cleavage site was incorporated into the secretory vector for removal of His tag. Thrombin cleavage site is a stretch of amino acids recognised and cleaved by thrombin.

In order to insert the gene for the recombinant protein in the secretory vector, compatible cloning sites are required. Multiple cloning sites (MCS) provide restriction sites for the insertion by blunt or sticky end cloning of any gene of interest in frame with the start codon and tag sequence.

Therefore in addition to selection marker like G418, strong *Dictyostelium* promoter, origin of replication for bacteria along with its marker already present in the backbone of the plasmid, additional important desired features of the secretory expression vector are:

1. Secretory signal sequence (Prespore antigen leader sequence)
2. Protein purification tag (Histidine tag)
3. Cleavage site to remove purification tag from the protein (thrombin cleavage site)
4. Multiple cloning site (MCS) to clone the gene of interest.

The plasmid backbone from pA15GFP **(section 2.1.5.4)**, an integrating vector of *D. discoideum* was used for constructing the secretory vector. Its expression cassette consists of the strong, constitutive actin 15 promoter, actin 6 Tn5 neomycin resistance cassette which confers G418 resistance, the bacterial ampicillin resistance gene, a high-copy-number *E.coli* plasmid origin of replication (Ori) and *D. discoideum* polyadenylation and termination signals (Fey *et al.*,1995).

Genes that confer drug resistance are generally used as selectable marker in vectors used in expression. The drug resistance cassette has dramatic effect on the transformation efficiency and the expression level of the exogenous gene. In *D. discoideum,* three selectable markers which convey resistance to the drugs G418, hygromycin B, and blasticidin S have been developed (Nellen *et al.,* 1984; Egelhoff *et al.,* 1989; Sutoch *et al.*, 1993). The gene conferring resistance to G418 and blasticidin S can be used as selectable markers in integrating or extrachromosomal vectors while the hygromycin resistance has been shown to work in

cassette exist as high copy number, tandem repeats in chromosomes. This is probably formed by insertion and replication of the plasmid (Nellen and Firtel, 1985; Barth *et al.,* 1998). The G418-resistance gene encodes a phosphotransferase which inactivates G418 (Reiss *et al.,* 1984). Vectors with G418 cassettes consistently yield large number of transformants with high expression levels of the exogenous gene. Moreover, the high expression level is not affected by the method of transformation (Pang *et al.,* 1999). Considering these aspects G418 resistance cassette was selected for the proposed secretory vector.

3.2 Annealing of complementary strands having signal sequence

To add the above mentioned important additional features to pA15GFP to convert it into a secretory expression vector, two complementary strands of DNA (HP1 and HP2) having *D. discoideum* secretory sequence, histidine tag, thrombin cleavage site and BssHII site to clone the MCS, were synthesised **(section 3.2.1)**. 132 nucleotide long two complementary DNA strands (HP1 and HP2) were annealed by mixing in equimolar concentration (100 pmoles) in a total volume of 20 µl 1X annealing buffer, incubated at 90°C for six minutes and cooled to room temperature **(section 2.3.4) [Fig. III(a)]**. Annealed DNA was phosphorylated with Polynucleotide kinase **(section 2.3.6)** and used for further cloning experiment.

3.3 Subcloning of annealed DNA into the designed vector

After phosphorylation of annealed complementary strands HP1:HP2, next step was to insert it into the *Dictyostelium* expression cassette (pA15GFP). pA15GFP was double digested with *ClaI* and *XhoI*, larger fragment (5.85 kb) was separated on 0.8% agarose gel, eluted from the gel and purified **(section 2.3.8)**. Annealed DNA was also digested with *ClaI-XhoI* and ligated **(section 2.3.10)** to the larger fragment (5.85 kb) of pA15GFP. No transformants were obtained in several attempt. It has been suggested that many a times sites present at the end of a sequence are not recognised properly by the restriction enzymes. Therefore it was decided to first clone the sequence in a vector without digesting it i.e. blunt end ligation resulting into the desired flanking sequences around the RE sites (*ClaI* and *XhoI*) compatible for the ligation with the backbone of pA15GFP.

HP1

5' ATC GAT ATC GAT ATG AAA TTT CAA CAT ACT TTT ATT

GCT TTA TTA TCA TTA TTA ACT TAT GCT ATT GCT CAT

CAT CAT CAT CAT CAT CAT TTA GTT CCA AGA GGT TCA

GGC GGC GCG CGC CTC GAG CTC GAG 3'

HP2

5' CTC GAG CTC GAG GCG CGC GGC GCC TGA ACC TCT

TGG AAC TAA ATG ATG ATG ATG ATG ATG ATG AGC

ATT AGC ATA AGT TAA TAA TGA TAA TAA AGC AAT AAA

AGT ATG TTG AAA TTT CAT ATC GAT ATC GAT 3'


ClaI


Prespore Antigen Secretory signal sequence.


Histidine tag for purification of recombinant protein.

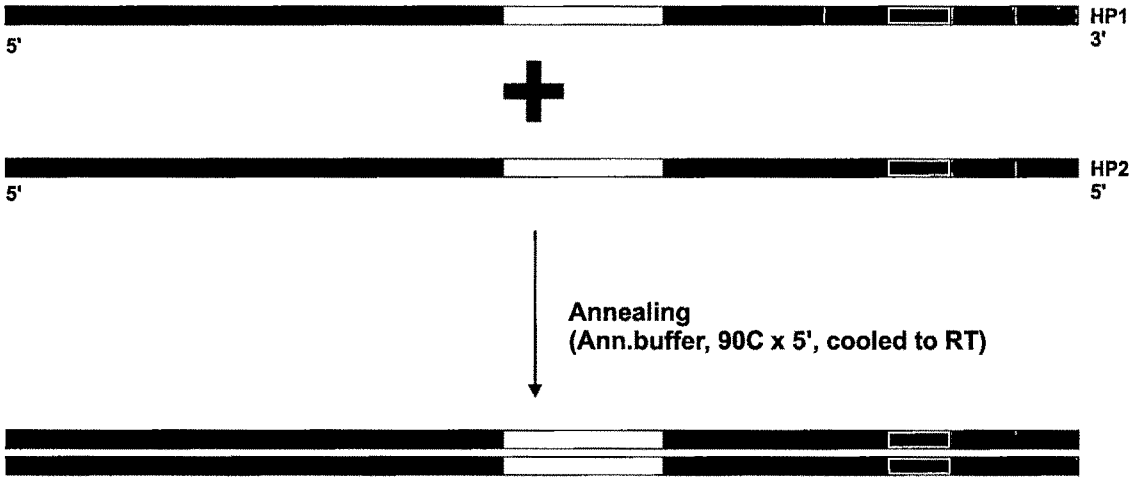

Thrombine cleavage site for removal of His tag from recombinant protein.


KasI/NarI site


BssHII site for insertion of multiple cloning site.


XhoI

3.2.1 Sequence of HP1 and HP2



ClaI

Prespore Antigen Secretory signal sequence.

Histidine tag for purification of recombinant protein.

Thrombine cleavage site for removal of His tag from recombinant protein.

KasI/NarI site

BssHII site for insertion of multiple cloning site.

XhoI

3.2 Fig. III(a) - Schematic summary of annealing of HP1 and HP2

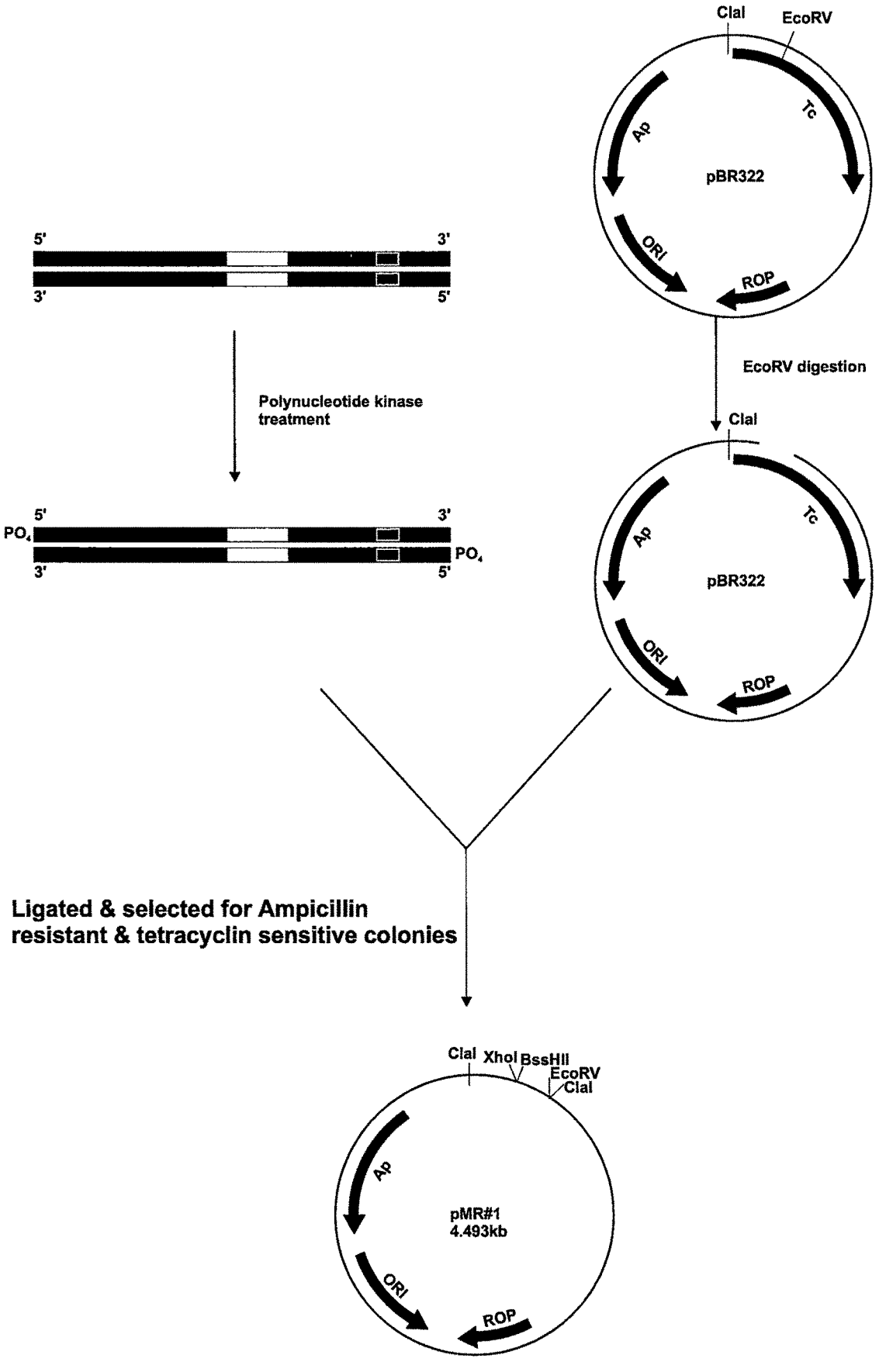
pBR322 **(section 2.1.5.1)** is an *E.coli* plasmid cloning vector containing ampicillin (amp) and tetracycline (tet) as selection markers. Any insertion in either of these genes will result into loss of resistance towards the respective antibiotic. This criteria was exploited for insertion of the annealed HP1:HP2 sequence into this plasmid. *EcoRV* site is present in the tetracycline gene and generates blunt ends in pBR322. pBR322 was digested with *EcoRV* **[Fig III(c), lane 2]** . Annealed DNA (HP1:HP2) was ligated **(section 2.3.10)** to pBR322 linearized with *EcoRV*. The new vector formed was henceforth named as pMR#1 (4.493 kb) **[Fig. III(b)].**

E. coli cells were transformed with pMR#1 **(section 2.3.1.1)** and amp^+ but tet^- clones were screened for the insert. Transformants were first selected on ampicillin plates and then replica plated on tetracyclin plates. 110 amp^+ clones were obtained, out of which 20 were tet^-. These 20 clones were further confirmed for the insert by RE digestion.

3.3.1 Confirmation of clone of pMR#1 by RE digestion (section 2.3.5) [Fig.III(c)]:

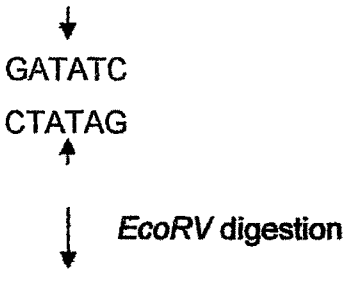
i) *XhoI: XhoI* site is present in HP1:HP2 and absent in pBR322. Therefore linearization of pMR#1 with *XhoI* confirmed the presence of insert in pMR#1 **[Fig. III(c), lane 3]**.

ii) *BssHII: BssHII* site is present in HP1:HP2 and absent in pBR322. Therefore linearization of pMR#1 with *BssHII* confirmed the presence of insert in pMR#1 **[Fig. III(c), lane 4]**.



3.3 Fig. III(b) - Schematic summary of construction of pMR#1

iii) *EcoRV*: *EcoRV* recognises the DNA sequence



GAT ATC
CTA TAG

ClaI site (ATCGAT) on ligation with digested *EcoRV* site generates a new *EcoRV* site:

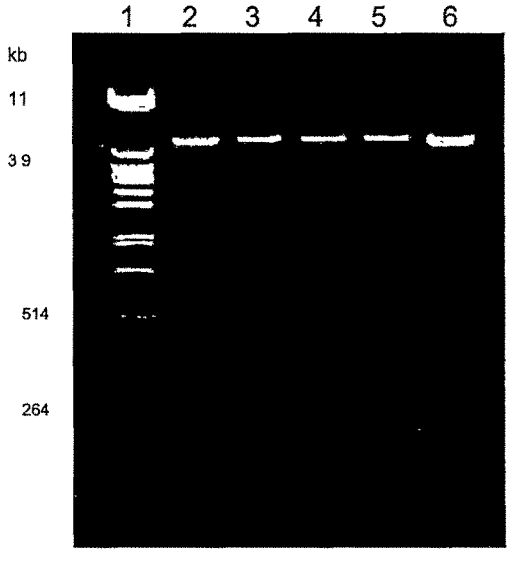
GAT ATCGAT —— ATC
CTA TAGCTA —— TAG
EcoRV

GAT —— ATCGAT ATC
CTA —— TAGCTA TAG
EcoRV

Insertion of HP1:HP2 into the *EcoRV* site of pBR322 (blunt end ligation) will result into loss of *EcoRV* site from the plasmid, but at the same time the *ClaI* site present at the 5' end of the HP1:HP2 will create a new *EcoRV*

Therfore linearization of pMR#1 with *EcoRV* confirmed the presence of insert in pMR#1 **[Fig. III(c), lane 5]**.

iv) *ClaI*: one *ClaI* site is present in HP1:HP2 and one in pBR322. Release of two fragments of 4.19 kb and 294 bp from pMR#1 on digestion with *ClaI* confirmed the presence of insert in between the two sites of pMR#1 **[Fig III(c), lane 6]**.



3.3 Fig. III(c) – Construction and RE digestion pattern of pMR#1 on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pBR322 digested with *EcoRV*
Lane 3: pMR#1 digested with *XhoI*
Lane 4: pMR#1 digested with *BssHII*
Lane 5: pMR#1 digested with *EcoRV*
Lane 6: pMR#1 digested with *ClaI*

3.4 Modification of Multiple Cloning Site(MCS) in pBluescript II KS(pBSKS)

Our next objective was to incorporate MCS in the secretory vector since in order to clone the gene of interest, cloning sites are required. pBSKS is a bacterial plasmid having MCS which has many blunt as well as sticky restriction sites **(section 2.1.5.2)**. MCS is flanked on either ends by a single RE site (*BssHII*) thus making it possible to extract and reclone with a single step digestion. Therefore to enhance the cloning capacity of the secretory vector, MCS from pBSKS was decided to be incorporated in the proposed secretory vector.

Since *ClaI* and *XhoI* sites were present in the annealed DNA which were to be used for its final subcloning into pA15GFP, it was important to remove these sites from the MCS. So pBSKS was digested with *ClaI* and *XhoI* **[Fig. III(e), lane 2,3]**, end filled with Klenow enzyme **(section 2.3.9.1)** and self ligated. The new vector formed was henceforth named as pMR#2 (2.951 kb) **[Fig. III(d)]** .

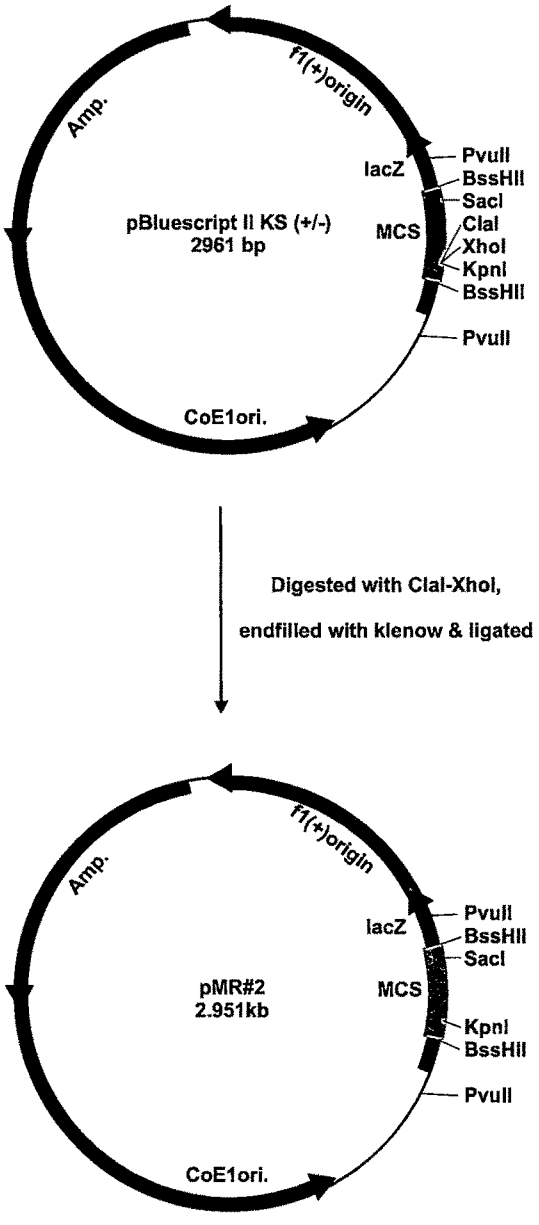
E. coli cells were transformed with pMR#2 **(section 2.3.1.1)**. 162 amp^+ clones were obtained out of which 12 clones were screened for loss of *ClaI* and *XhoI* sites. Since *Klenow* also act as an exonuclease, loss of additional nucleotides from either end could have been possible. However from the following RE digestions it has been inferred that only the desired sites have been deleted from pBSKS.

3.4.1 Confirmation of clone of pMR#2 by RE digestion (section 2.3.5) [Fig. III(e)]:

i) *XhoI*: *XhoI* site has been altered by *Klenow* in pMR#2. Therefore no digestion of pMR#2 with *XhoI* confirmed the loss of site from pMR#2 **[Fig. III(e), lane 6]**.

ii) *ClaI*: *ClaI* site has been altered by *Klenow* in pMR#2. Therefore no digestion of pMR#2 with *ClaI* confirmed the loss of site from pMR#2 **[Fig. III(e), lane 7]**.

iii) *SalI*: *SalI* site is present between *ClaI* and *XhoI* site of pBSKS. No digestion of pMR#2 with *SalI* confirmed the loss of site from pMR#2 **[Fig. III(e), lane 8]**.



3.4 Fig. III(d) - Schematic summary of construction of pMR#2

iv) *SacI*: linearization of pMR#2 with *SacI* confirmed the intact site in the backbone of plasmid **[Fig. III(e), lane 9]**.

3.5 Subcloning of modified MCS from pMR#2 into pMR#1

After confirming the deletion of *ClaI* and *XhoI* sites from the MCS, pMR#2 was digested with *BssHII* to release the modified MCS of 160 bp. MCS was eluted from the gel, purified **(section 2.3.8)** and ligated **(section 2.3.10)** to *BssHII* digested and dephosphorylated pMR#1**(section 2.3.7)**. The new vector formed was henceforth named as pMR#3 (4.663 kb) **[Fig. III(f))]**.

E. coli cells were transformed with pMR#3 **(section 2.3.1.1)**. 103 amp$^+$ Clones were obtained and 24 were screened for the insert.

3.5.1 Confirmation of clone of pMR#3 by RE digestion (section 2.3.5) [Fig.III(g)]:

i) *BssHII*: *BssHII* site was used for cloning of modified MCS in pMR#3. Release of two fragments of 4.493 kb and 160 bp from pMR#3 on digestion with *BssHII* confirmed the presence of MCS in pMR#3 **[Fig. III(g), lane 2]**.

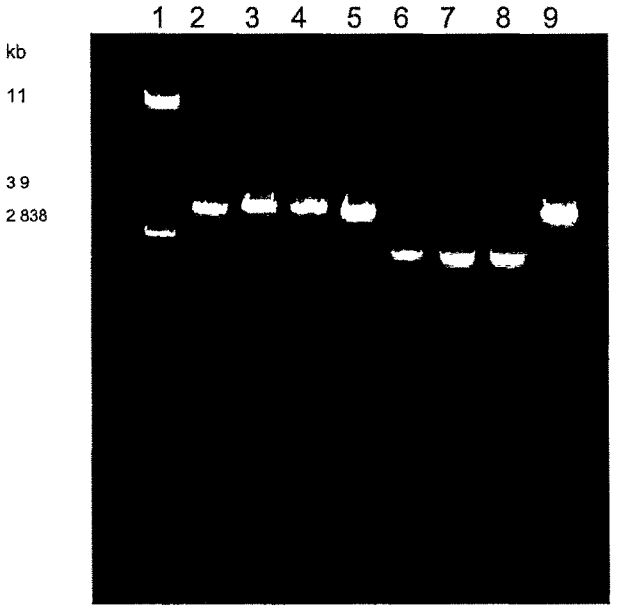
ii) *KpnI*: *KpnI* is present in MCS. Therefore linearization of pMR#3 with *KpnI* confirmed the presence of MCS in pMR#3 **[Fig. III(g), lane 3]**.

iii) *NotI*: *NotI* is present in MCS. Therefore linearization of pMR#3 with *NotI* confirmed the presence of MCS in pMR#3 **[Fig. III(g), lane 4]**.

iv) *XbaI*: *XbaI* is present in MCS. Therefore linearization of pMR#3 with *XbaI* confirmed the presence of MCS in pMR#3 **[Fig. III(g), lane 5]**.

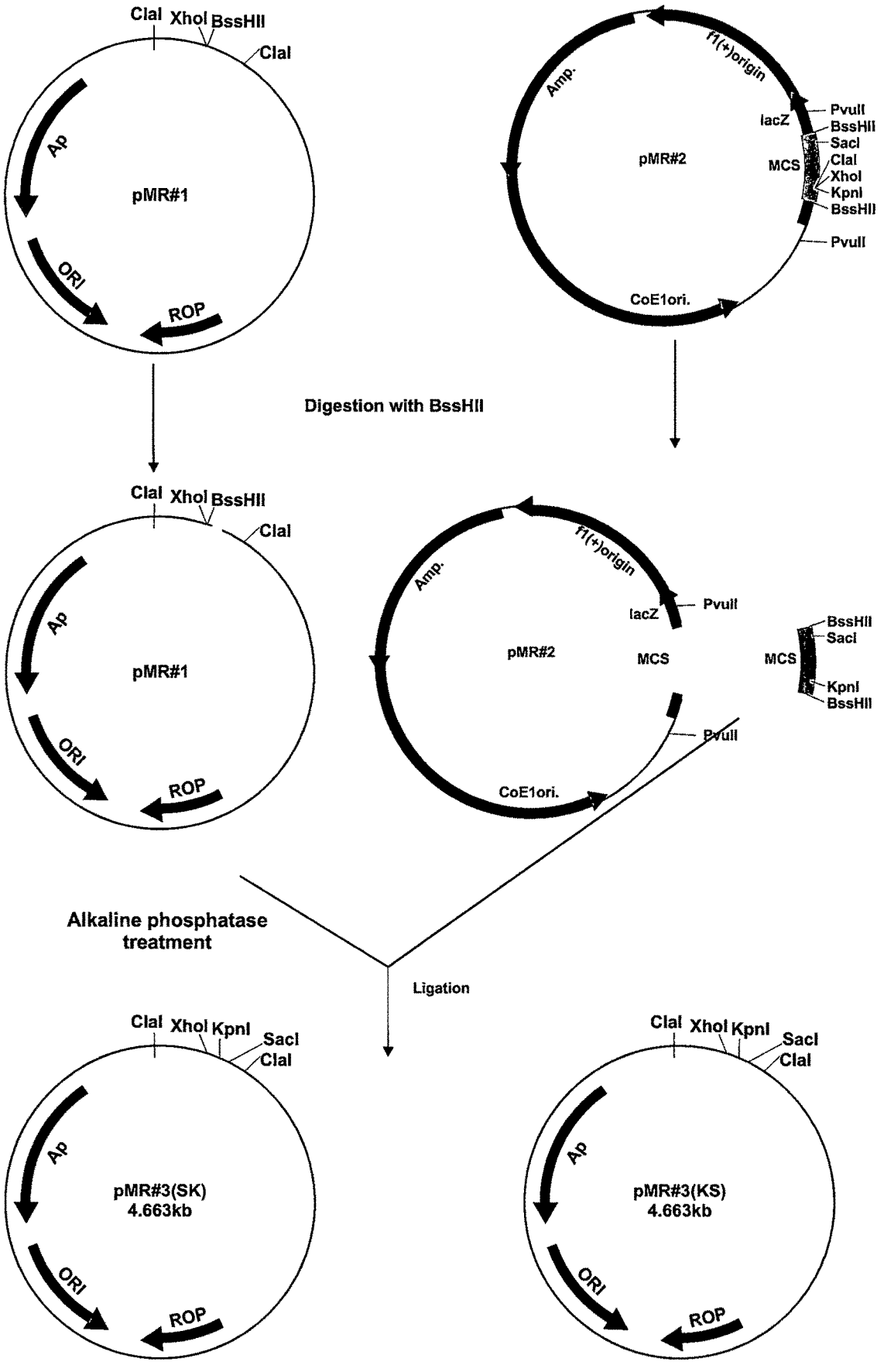
v) *SacI* : one *SacI* site is present at the starting of MCS and another is present at the 3`end of HP1:HP2 i.e. near *XhoI* site. Therefore release of two fragments of 4.5 kb and 130 bp from pMR#3 on digestion with *SacI* confirmed SK orientation of MCS in pMR#3 **[Fig. III(g), lane 6]**.

vi) *ClaI*: one *ClaI* site is present in pMR#1 and another is present at the 5`end of HP1:HP2. Therefore release of two fragments of 4.2 kb and 452 bp from pMR#3 on digestion with *ClaI* confirmed the presence of HP1:HP2 and MCS in between the two *ClaI* sites of pMR#3 **[Fig. III(g), lane 7]**.

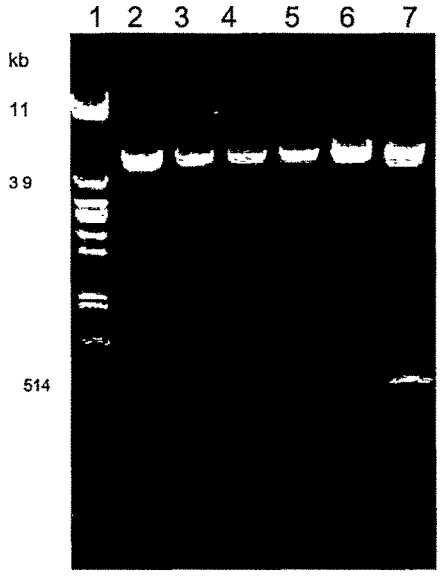


3.4 Fig. III(e) - Construction and RE digestion pattern of pMR#2 on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pBSKS digested with *XhoI*
Lane 3: pBSKS digested with *ClaI*
Lane 4: pBSKS digested with *SalI*
Lane 5: pBSKS digested with *KpnI*
Lane 6: pMR#2 digested with *XhoI*
Lane 7: pMR#2 digested with *ClaI*
Lane 8: pMR#2 digested with *SalI*
Lane 9: pMR#2 digested with *KpnI*



3.5 Fig. III(f) - Schematic summary of construction of pMR#3



3.5 Fig. III(g) Construction and RE digestion pattern of pMR#3 on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#3 digested with *BssHII*
Lane 3: pMR#3 digested with *KpnI*
Lane 4: pMR#3 digested with *NotI*
Lane 5: pMR#3 digested with *XbaI*
Lane 6: pMR#3 digested with *SacI*
Lane 7: pMR#3 digested with *ClaI*

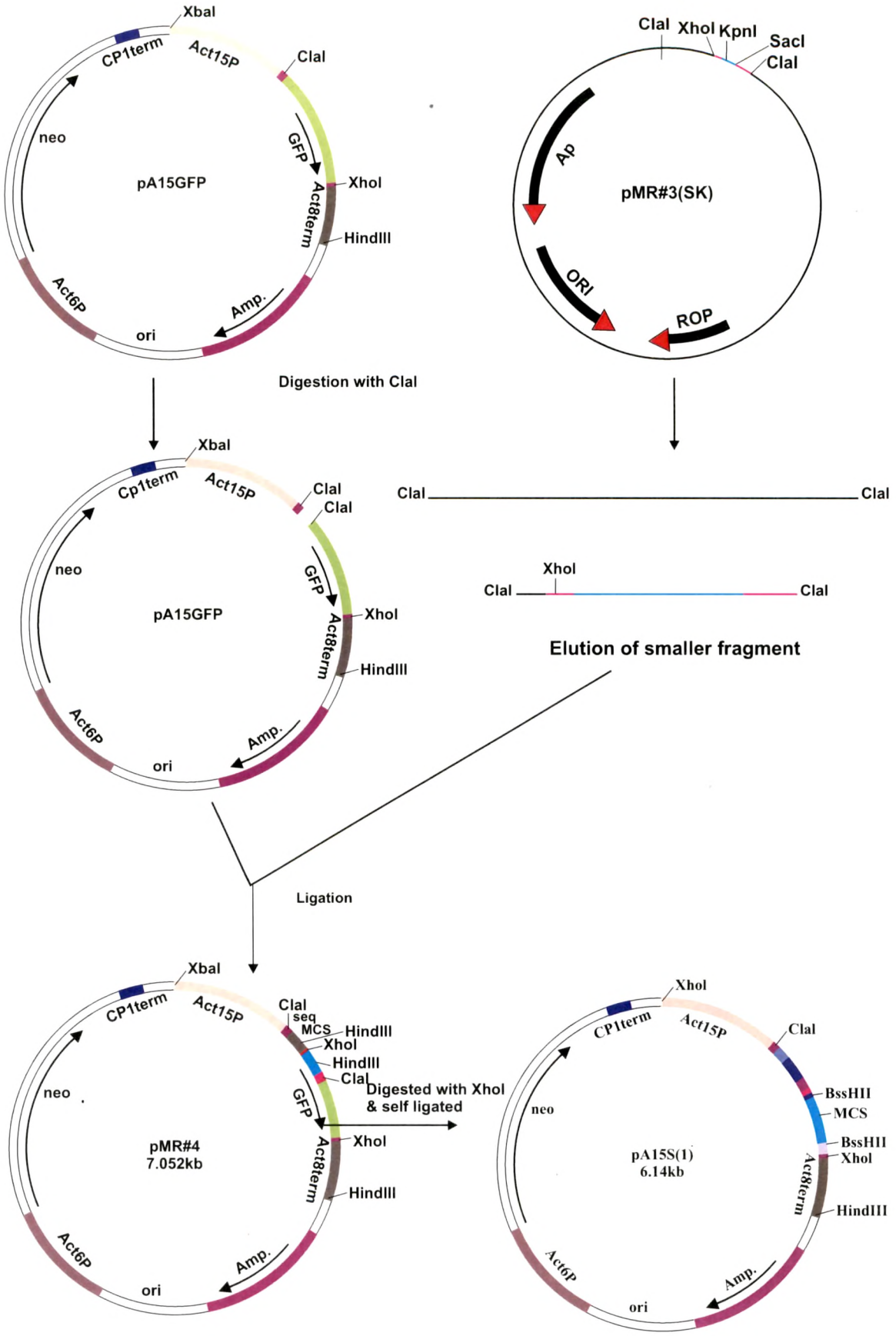
3.6 Subcloning of desired features including MCS from pMR#3 into pA15GFP

Finally in order to clone the fragment from pMR#3 flanked by *ClaI* and *XhoI* sites containing secretory signal, His tag, thrombin cleavage site and MCS into the expression cassette of *D. discoideum*, pA15GFP was double digested with *ClaI* and *XhoI*, and the larger fragment (5.85 kb) was eluted from gel and purified **(section 2.3.8)**. pMR#3 was double digested with *ClaI* and *XhoI* giving three fragments. Middle fragment (292 bp) having Secretory sequence, His tag, thrombin cleavage site and MCS was gel eluted, purified **(section 2.3.8)** and attempted for ligation **(section 2.3.10)** to the larger fragment of pA15GFP. Inspite of several attempts no recombinant (as judged by the absence of clones) was obtained. Therefore it was decided to modify the cloning strategy as follows: in pMR#3, Secretory sequence, His tag, thrombin cleavage site and MCS were flanked by *ClaI* sites on both ends (one in pBR322 and other at 5`of secretory sequence). Instead of ligating the fragment created by digestion with two enzymes (*ClaI* and *XhoI*), it might be fruitful to ligate the slightly larger fragment created by digestion with single enzyme, *ClaI*.

pMR#3 was digested with *ClaI* **[Fig. III(i), lane 2]**, smaller fragment (452 bp) was eluted from gel, purified **(section 2.3.8)** and ligated **(section 2.3.10)** to *ClaI* digested pA15GFP **[Fig.III(i), lane 3]**. The new vector formed was henceforth named as pMR#4 (7.052 kb) **[Fig. III(h)]**.

E. coli cells were transformed with pMR#4 **(section 2.3.1.1)**. 28 amp^+ clones were obtained and all were screened for the insert. Release of 452 bp fragment on digestion with *ClaI* confirmed the presence of insert **[Fig.III(i), lane 4]**.

The resulting vector had 912 bp extra sequence (between *XhoI* and 2^{nd} *ClaI* site) from pBR322 and GFP from pA15GFP. To remove the extra DNA from the plasmid, it was digested with *XhoI* (one site at the end of secretory sequence and another at the end of GFP) **[Fig. III(i), lane 5]** and self ligated. The new vector formed was henceforth named as **pA15S(1)** (6.14 kb) **[Fig. III(h)]**. *E. coli* cells were transformed with pA15S(1) **(section 2.3.1.1)**. 105 amp^+ clones were obtained and 12 were screened for removal of extra sequence from pMR#4.



3.6 Fig. III(h) - Schematic summary of construction of pA15S(1)

3.6.1 Confirmation of clone of pA15S(1) by RE digestion (section 2.3.1.1) [Fig. III(i)]:

i) *XhoI*: linearization of pA15S(1) with *XhoI* confirmed the removal of extra sequences from pA15S(1) **[Fig. III(i), lane 6]**.

ii) *BstXI*: *BstXI* is present in the MCS. Linearization of pA15S(1) on digestion with *BstXI* confirmed the presence of MCS in pA15S(1) **[Fig. III(i), lane 7]**.

iii) *SacII*: *SacII* is present in the MCS. Linearization of pA15S(1) on digestion with *SacII* confirmed the presence of MCS in pA15S(1) linearization confirmed presence of MCS **[Fig. III(i), lane 8]**.

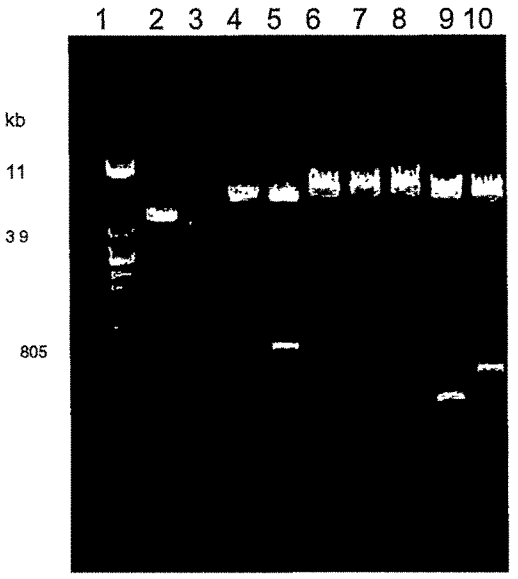
iv) *XbaI*: release of two fragments of 5.7 kb and 395 bp from pA15S(1) on digestion with *XbaI* confirmed the removal of extra sequences from pA15S(1) **[Fig. III(i), lane 9]**.

v) *HindIII*: release of two fragments of 5.3 kb and 773 bp from pA15S(1) on digestion with *HindIII* confirmed the removal of extra sequences from pA15S(1) **[Fig. III(i), lane 10]**.

In order to confirm the sequence and orientation of HP1:HP2 and MCS in pA15S(1), HP1:HP2 along with MCS was sequenced **(section 2.3.14)** from pA15S(1) using primer MR01 **(section 2.1.2).** From the sequencing data **[Fig. III(I)]**. it was confirmed that pA15S(1) has the introduced features in the desired orientation i.e. a secretory signal (PsA), purification tag (His tag), a tag removal site (thrombin cleavage site) and multiple cloning site.

3.7 Shift in reading frame

Expression vectors are made in all three reading frames so that insertion of any gene in these three should provide at least one construct in reading frame for proper expression. When RE acts on a particular DNA sequence, it generates either overhangs or blunt ends. These overhangs can be removed (loss of nucleotides) or end filled (gain of nucleotides). Addition or removal of nucleotides leads to shift in reading frame. This strategy was used for changing the reading frame of pA15S(1). *Klenow* enzyme has both the activities: it acts as an exonuclease on 3` overhangs and can do end filling at 5` overhangs.

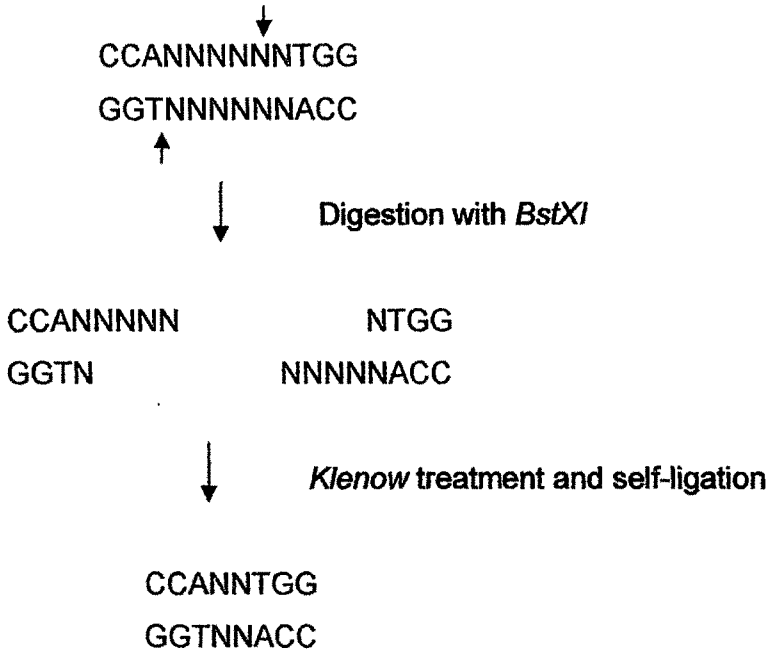


3.6 Fig. III(i) - Construction and RE digestion pattern of pA15S(1) on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pMR#3 digested with *ClaI*
Lane 3: pA15GFP digested with *ClaI*
Lane 4: pMR#4 digested with *ClaI*
Lane 5: pMR#4 digested with *XhoI*
Lane 6: pA15S(1) digested with *XhoI*
Lane 7: pA15S(1) digested with *BstEXI*
Lane 8: pA15S(1) digested with *SacII*
Lane 9: pA15S(1) digested with *XbaI*
Lane 10: pA15S(1) digested with *HindIII*

3.7.1 Shift in reading frame by two nucleotides

BstXI digestion – pA15S(1) was digested with *BstXI* generating a 3` overhang of four nucleotides **[Fig. III(j), lane 2]** and blunted with *Klenow* **(section 2.3.9.2)** and self ligated resulting into loss of 4 nucleotides and frame shift by two nucleotides. The new vector formed was henceforth named as **pA15S(2)**.



E. coli cells were transformed with pA15S(2) **(section 2.3.1.1)**. Clones (amp^+) were obtained and 12 were screened for loss of *BstXI* site. *Klenow* also acts as an exonuclease, presence of *SacI* and *NotI* as confirmed by RE digestion indicates no damage to the backbone of the plasmid.

3.7.1.1 Confirmation of clone of pA15S(2) by RE digestion (section 2.3.5) [Fig. III(j)]:

i) *BstXI*: no digestion of pA15S(2) with *BstXI* confirmed loss of site from pA15S(2) **[Fig. III(j), lane 3]**.

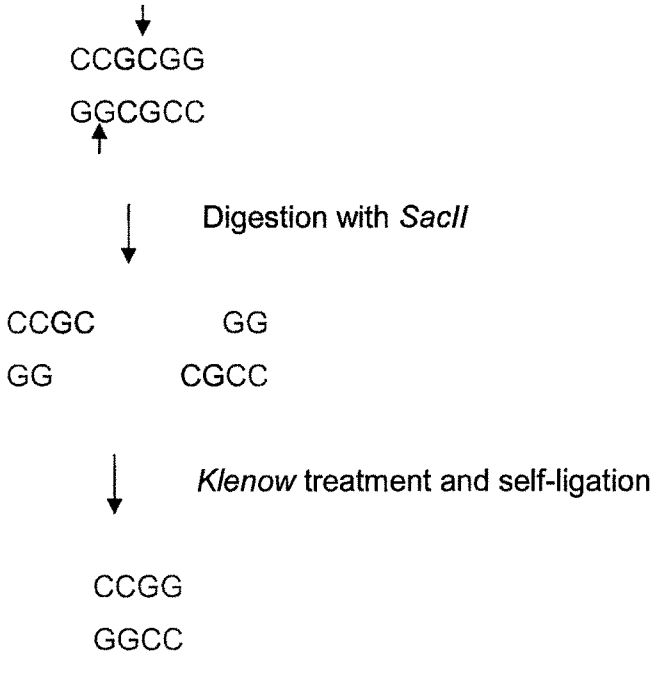
ii) *SacI*: one *SacI* is present in the MCS (5' to *BstXI* site) and one in pA15 backbone. Release of two fragments on digestion with *SacI* confirmed no damage to the backbone of the plasmid **[Fig. III(j), lane 4]**.

iii) *NotI*: *NotI* is present 3' to *BstXI* site. Linearization of pA15S(2) confirmed no damage to the backbone of the plasmid [**Fig. III(j), lane 5**].

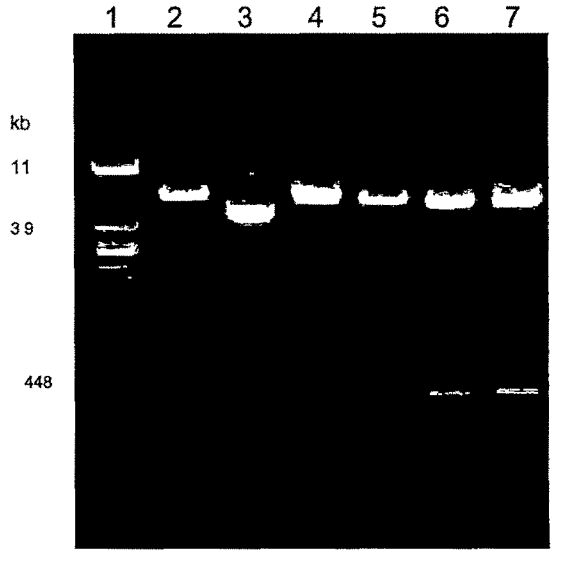
iv) *XbaI*: one *XbaI* is present in the MCS and one in pA15 backbone. Release of two fragments (5.7 kb and 395 bp) on digestion with *XbaI* confirmed no damage to the backbone of the plasmid [**Fig. III(j), lane 6**].

3.7.2 Shift in reading frame by one nucleotide

SacII digestion – pA15S(1) was digested with *SacII* generating a 3` overhang of two nucleotides [**Fig. III(k), lane 2**] and blunted with *Klenow* **(section 2.3.9.2)** and self ligated resulting into loss of 2 nucleotides and frame shift by one nucleotide. The new vector formed was henceforth named as **pA15S(3)**.



E. coli cells were transformed with pA15S(3). Clones (amp^+) were obtained and 12 were screened for the loss of *SacII* site. Since *Klenow* acts as an exonuclease, presence of *SacI* and *NotI* as confirmed by RE digestion indicates no damage to the backbone of the plasmid.



3.7.1 Fig. III(j) Construction and RE digestion pattern of pA15S(2) on 0.8% agarose gel

Lane 1: lambda DNA digested with *PstI*
Lane 2: pA15S(1) digested with *BstXI*
Lane 3: pA15S(2) digested with *BstXI*
Lane 4: pA15S(2) digested with *SacI*
Lane 5: pA15S(2) digested with *NotI*
Lane 6: pA15S(2) digested with *XbaI*
Lane 7: pA15S(1) digested with *XbaI*

3.7.2.1 Confirmation of clone of pA15S(3) by RE digestion (section 2.3.5) [Fig. III(k)]:

i) *SacII*: no digestion of pA15S(3) with *SacII* confirmed loss site from pA15S(3) **[Fig. III(k), lane 3]**.

ii) *SacI*: one *SacI* is present in the MCS (5' to *SacII* site) and one in pA15 backbone. Release of two fragments on digestion with *SacI* confirmed no damage to the backbone of the plasmid **[Fig. III(k), lane 4]**.

iii) *NotI*: *NotI* is present 3' to *SacII* site. linearization of pA15S(2) confirmed no damage to the backbone of the plasmid **[Fig. III(k), lane 5]**.

iv) *XbaI*: one *XbaI* is present in the MCS and one in pA15 backbone. Release of two fragments (5.7 kb and 395 bp) on digestion with *XbaI* confirmed no damage to the backbone of the plasmid **[Fig. III(k), lane 6]**.

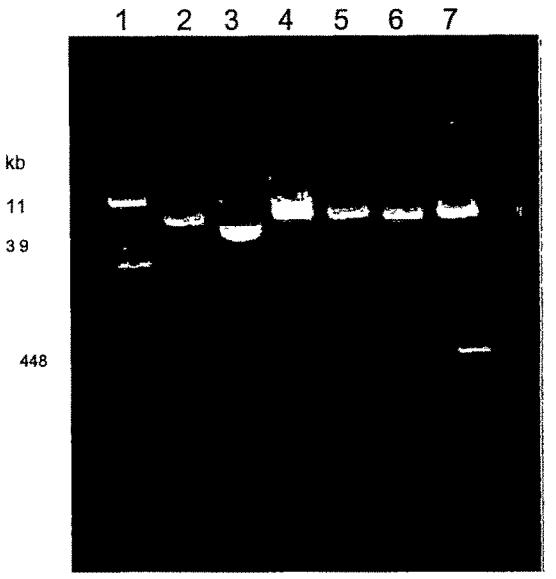
3.8 Sequencing of secretory vectors

In order to confirm the proper deletion and shift in reading frame in pA15S(2) and pA15S(3) due to Klenow exonuclease activity, HP1:HP2 along with MCS was sequenced **(section 2.3.14)** from pA15S(2) and pA15S(3) using primer MR01 **(section 2.1.2).** From the sequencing data vectors appear to have proper deletion and shift in reading frame **[Fig. III(m) and Fig. III(n)]**.

3.9 Discussion

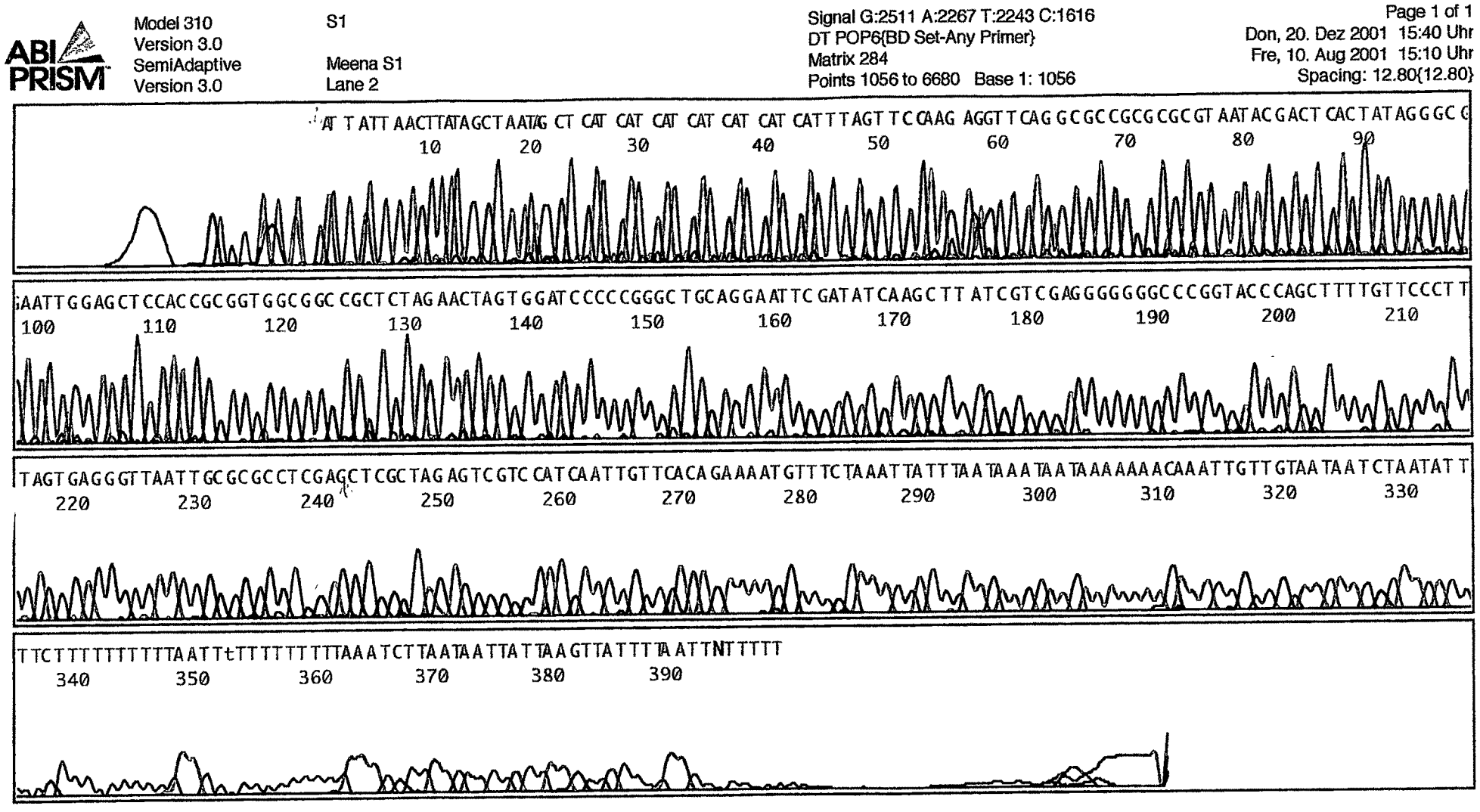
Our aim was to design and construct an expression vector for *Dictyostelium discoedium* which can be used for expression and secretion of virtually any gene. It should also have sequences for purification of recombinant protein from medium and sites to remove this tag from the recombinant protein. pA15GFP, an integrating vector of *Dictyostelium discoedium* having actin 15 promoter was used for construction of the secretory vector since actin 15 promoter is one of the strongest promoters and remains active throughout the vegetative phase and early developmental phase of *Dictyostelium* life cycle.

Synthetic DNA having prespore antigen secretory sequence, histidine purification tag, thrombin cleavage site for removal of tag was annealed and used further for constructing the secretory vector. Initially we tried to clone the annealed

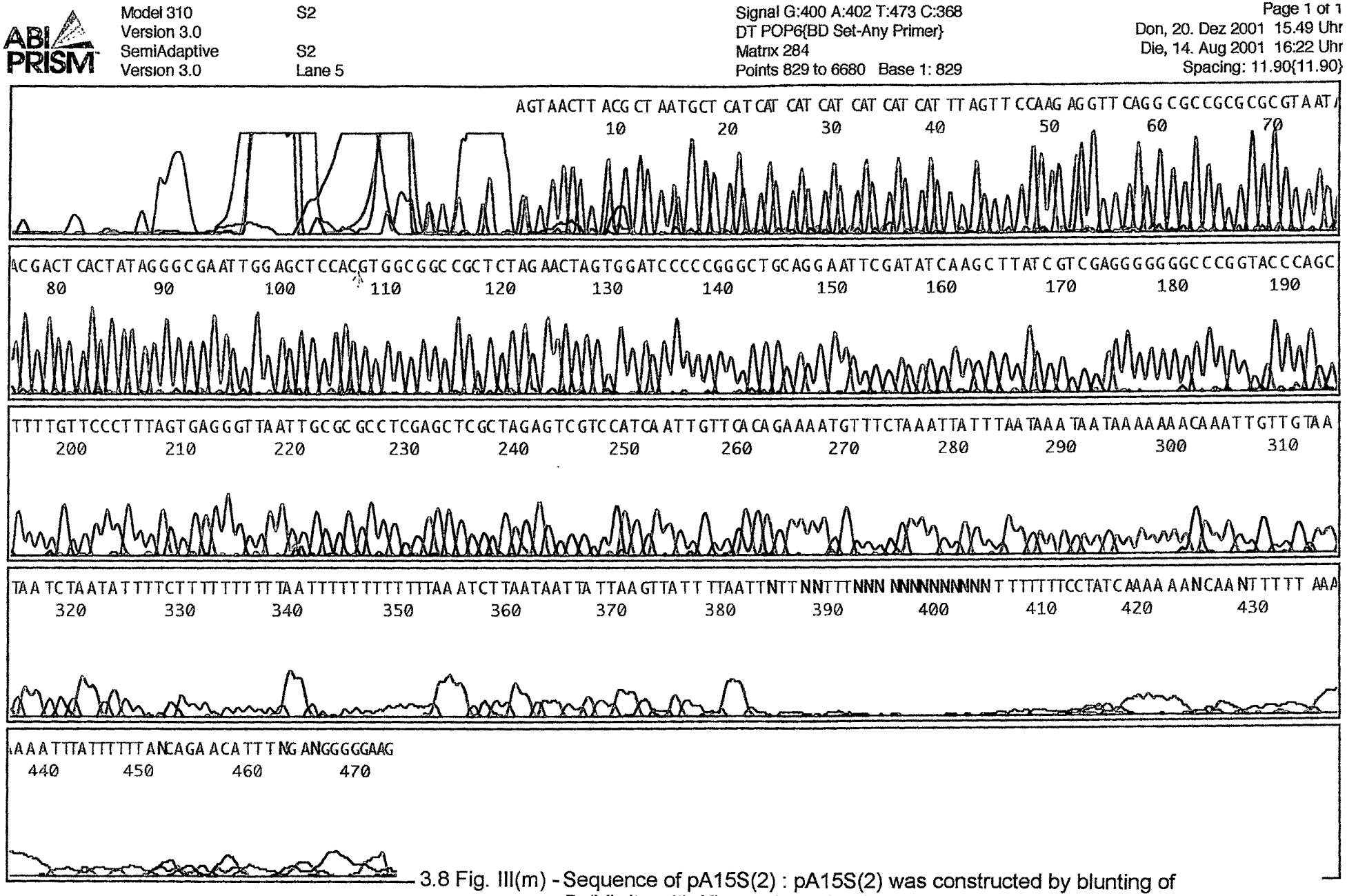


3.7.2 Fig. III(k) - Construction and RE digestion pattern of pA15S(3) on 0.8% agarose gel

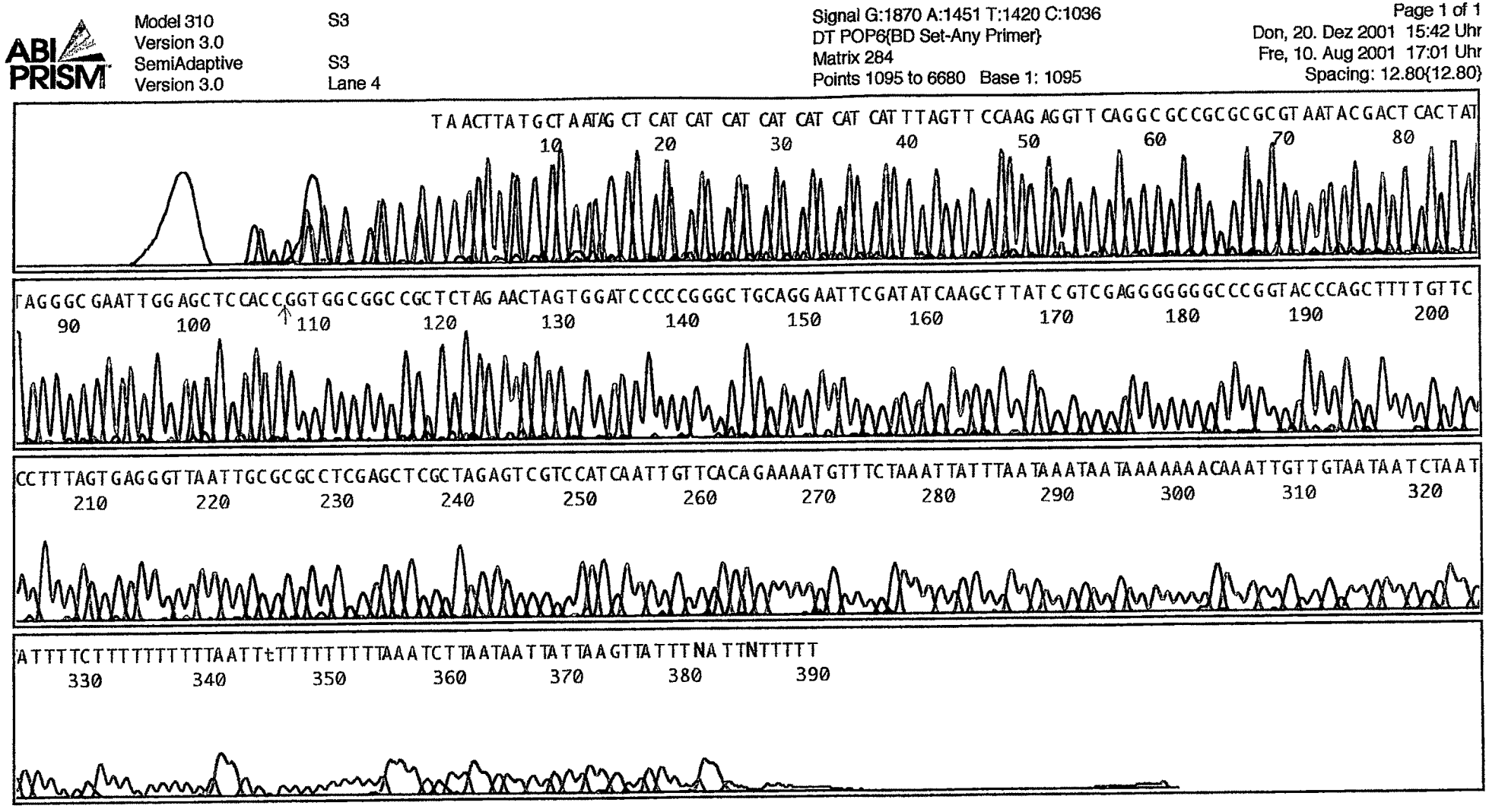
Lane 1: lambda DNA digested with *PstI*
Lane 2: pA15S(1) digested with *SacII*
Lane 3: pA15S(3) digested with *SacII*
Lane 4: pA15S(3) digested with *SacI*
Lane 5: pA15S(3) digested with *NotI*
Lane 6: pA15S(3) digested with *XbaI*
Lane 7: pA15S(1) digested with *XbaI*



3.8 Fig. III(I) - Sequence of pA15S(1) : HP1:HP2 along with MCS was sequenced from pA15S(1) and was found to be inserted in pA15S(1) with no mutation (1 - 242).



3.8 Fig. III(m) - Sequence of pA15S(2) : pA15S(2) was constructed by blunting of *BstXI* site with Klenow in pA15S(1) and sequencing confirmed the proper deletion of four nucleotides after site 107.



3.8 Fig. III(n) - Sequence of pA15S(3) : pA15S(3) was constructed by blunting of *SacII* site with Klenow in pA15S(1) and sequencing confirmed the proper deletion of two nucleotides after site 107.

DNA directly into pA15GFP but no transformants were obtained. Considering the fact that many a times terminal sites are not recognised properly by the restriction enzymes due to inefficient binding at the ends of the DNA strands, *EcoRV* site (blunt end) in tetracycline gene of pBR322 was used for cloning. This strategy helped in two ways- cloning of annealed DNA without any digestion and easy selection of tetracycline sensitive clones due to insertion in tetracycline gene. Direct cloning of synthetic DNA into pBR322 was not successful but when the DNA was phosphorylated with polynucleotide kinase, it was successfully cloned into pBR322 (pMR#1). This might be due to dephosphorylated state of synthetic DNA.

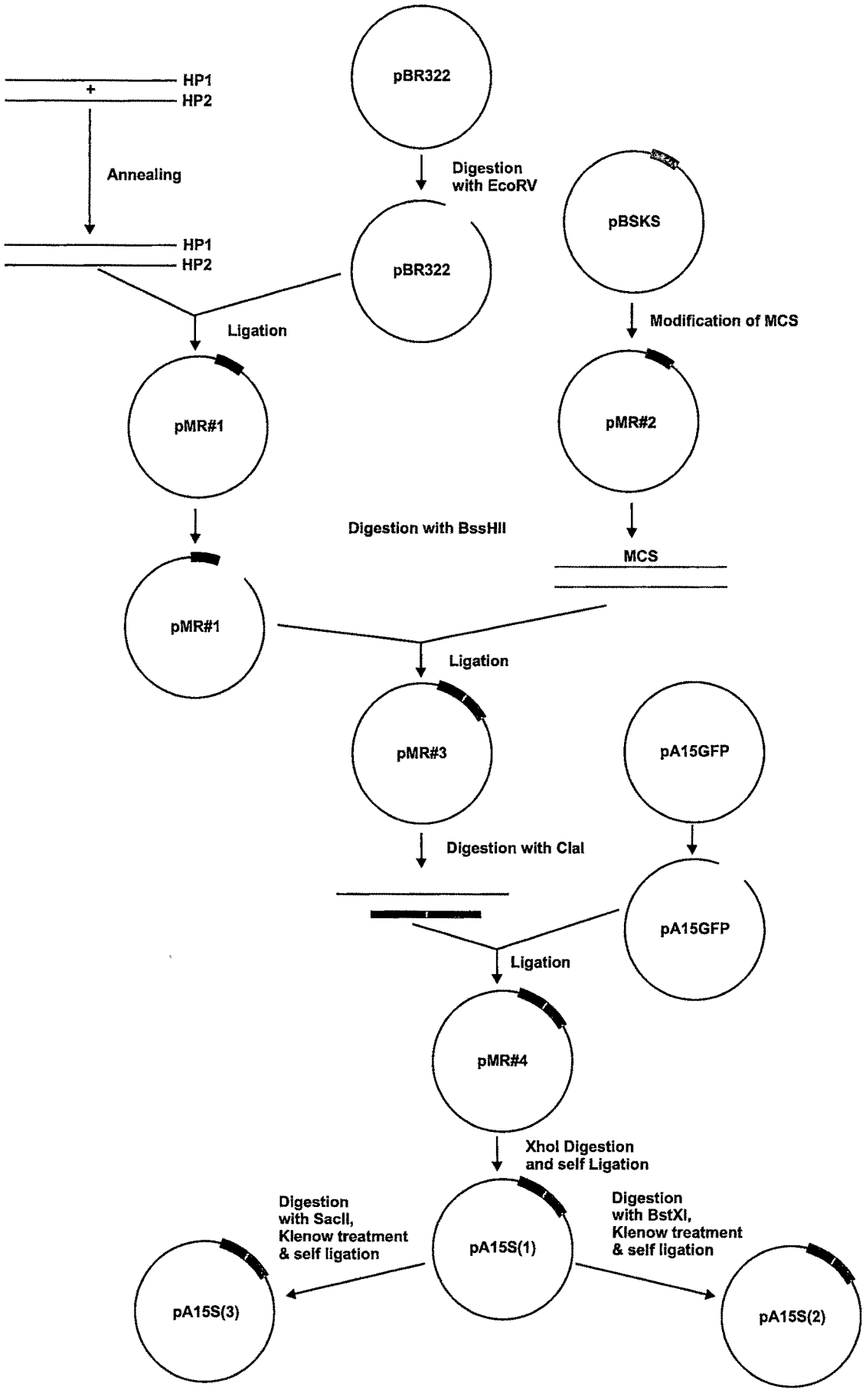
For enhancing the cloning capacity of the secretory vector, MCS from pBSKS was subcloned downstream to the secretory sequence. Prior to subcloning, MCS was modified to suit our cloning strategy i.e. removal of *ClaI-XhoI* sites.

While cloning the DNA fragment containing the secretory sequence, His tag, thrombin cleavage site and MCS from pMR#3 into pA15GFP, when vector and insert were digested with *ClaI* at 5' end and *XhoI* at 3' end, cross ligation did not work out. Therefore the cloning strategy was changed- single enzyme i.e. *ClaI* was used to cut the vector as well as insert at both the ends (5' and 3') and then cross ligated. Recombinants (pMR#4) were obtained with this strategy. Therefore cross ligation between vector and insert digested with single enzyme at both the ends is much more efficient as compared to vector and insert cut with two different enzymes at their ends.

To make the secretory vector in all three reading frames so that at least one frame will fit with the reading frame of the gene of interest, pA15S(1) was digested with *SacII* and *BstXI* and 3' overhangs were removed with *Klenow* resulting into loss of nucleotides and hence shifting the reading frame. Many clones showing loss of *SacII/BstXI* sites were obtained. Molecular weight and restriction digestion pattern of plasmids from all the clones were similar, but on sequencing some clones showed removal of extra nucleotides though most of them had intended nucleotides removed. Therefore sequencing of modified DNA becomes essential before using it for cloning the gene for recombinant protein.

With series of experiments we have generated three constructs [pA15S(1), pA15S(2), pA15S(3)] which have the secretory sequence, His tag, thrombin cleavage site and MCS as desired features and these three constructs represent three reading frames. These experiments have been schematically summerized in **Fig. III(o)**. Therefore insertion of a gene in these three constructs should provide at least one construct in reading frame for proper expression.

The secretory vectors thus constructed were used for exploring the expression potential of *Dictysteolium discoideum* by expressing Green fluorescent protein, human DNaseI and Proteinase K as discussed in chapter IV, V and VI respectively.



3.9 Fig. III(o) - Schematic summary of construction of the pA15S(1), pA15S(2) and pA15S(3)