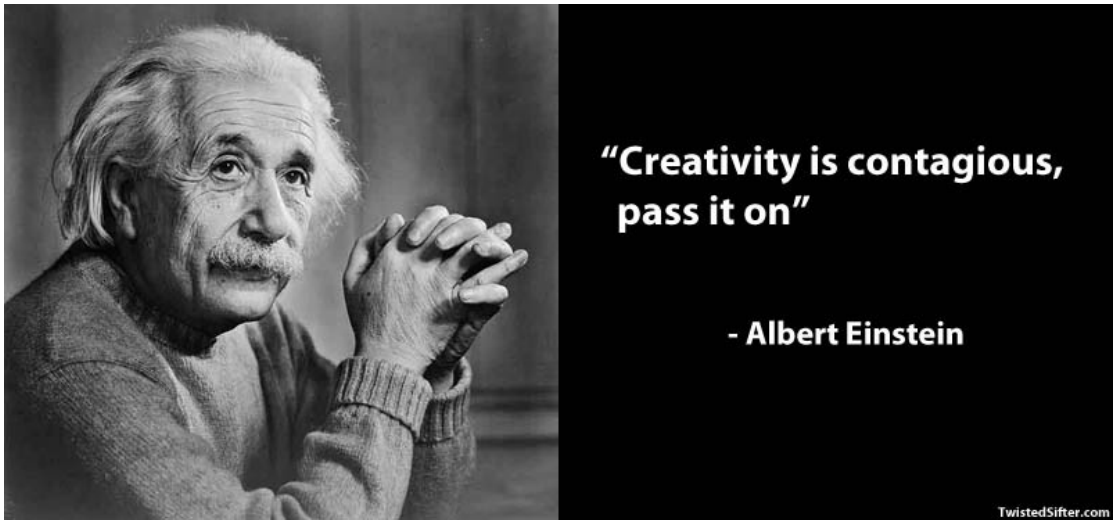

Chapter 4
Development of PCR based assay to monitor the recombinase activity of XisA protein *in vivo*

4.1 Introduction

XisA carries out the excision of *nifD* element by site specific recombination event in the late stages of heterocyst maturation in *Anabaena* PCC 7120. This recombination event has been studied in *E. coli* by cloning the entire 11 kb element including the 11 bp recognition sequence in *Eco*RI site of the pBR322. To this, a transposon mini-mu-lac has been inserted in the 11 kb element to create plasmid pMX25, which has been used to study the excision event on the basis of blue-white selection assay. *xisA* gene expression mediated *nifD* excision was indirectly monitored by loss of *lacZ* gene inserted within the *nifD* element **(Fig. 4.1a)**. (Lammers et al., 1986; Karunakaran et al., 2008) or by transformation method **(Fig. 4.1b)** (Brusca et al., 1989; Brusca et al., 1990). Assay for recombination was based on screening blue-white colony formation in which blue colonies possess pMX25 plasmid while excised plasmid colony is white.

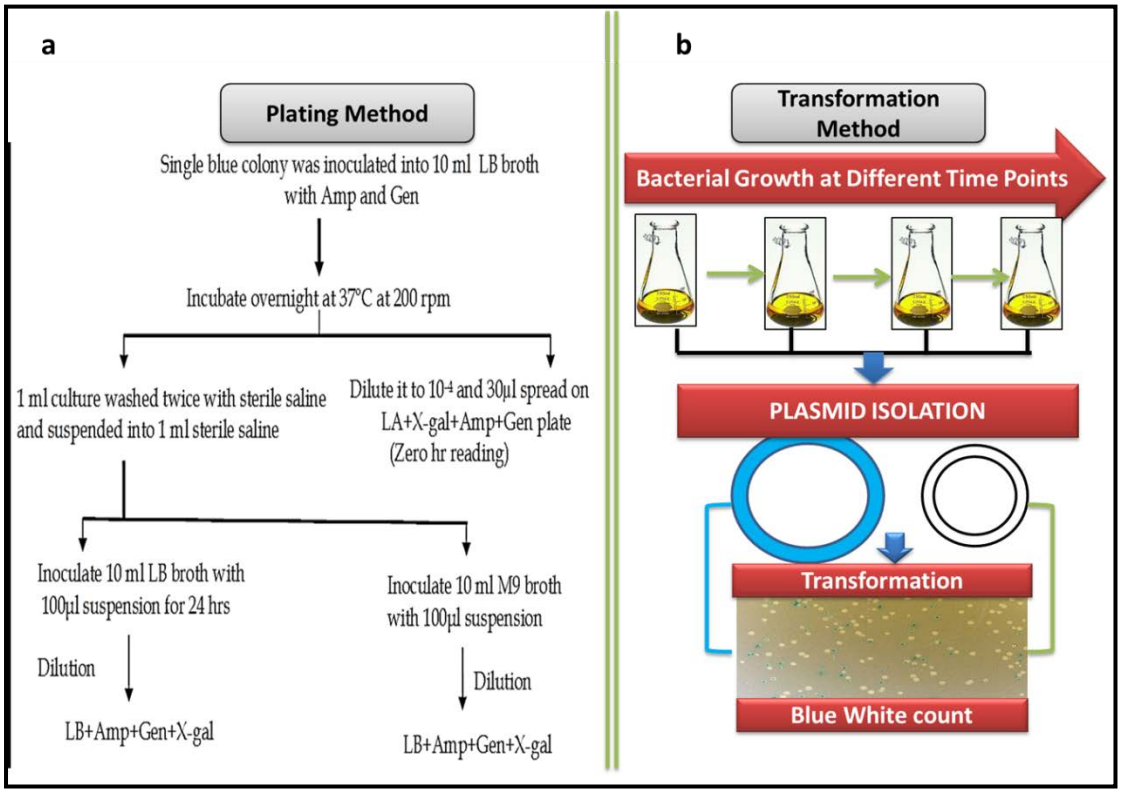

Fig. 4.1 : Earlier rearrangement assay to monitor *nifD* element excision. (A) Plating method (B) Transformation method.

Previous recombinase assays to monitor XisA mediated *nifD* excision were less sensitive, time consuming and were prone to human errors. Since pMX25 is a medium copy plasmid, it is not clear about the number of excised plasmids required for white colony phenotype. Deleted plasmid ΔpMX25 being smaller in size is biased in transformation assay over larger pMX25. Additionally, abortive excision events were postulated for blue-white sectored colonies.

4.1.1 Selection of substrate plasmid

Plasmid pAM461 is a pUC18 based *xisA* deficient plasmid, which contains distal and proximal borders of *nifD* element. Excision of minimal *nifD* element was brought about by expressing *xisA* gene in trans **(Fig. 4.2)** (Brusca et al., 1990).

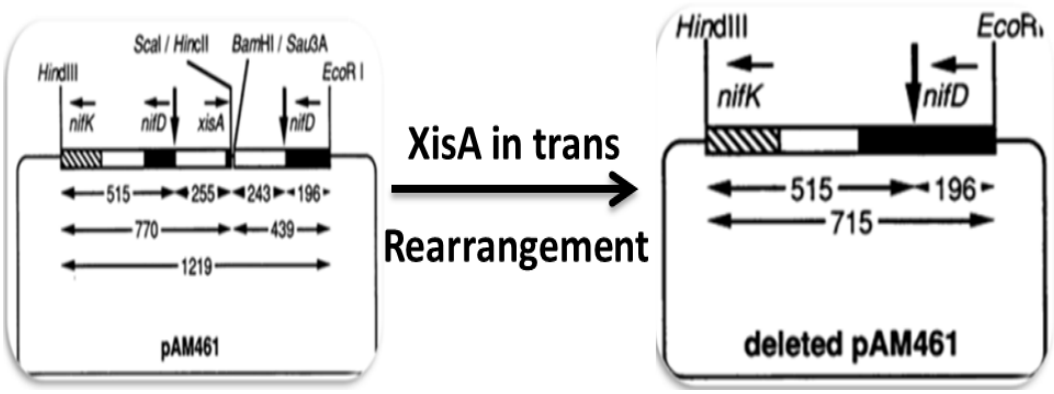

Fig. 4.2 Schematic representation of pAM461 rearrangement (Brusca et al., 1990)

Considering the small size and high copy number of pAM461 compared to pMX25, it was selected as a best fit to develop *in vivo* recombinase assay of XisA protein encoded by p*xisA* in *E. coli.*

4.1.2 Rationale of the present study

Majority of tyrosine recombinases require accessory proteins for their activity. However, no accessory protein has been reported for XisA recombinase activity, which hampered *in vitro* characterization of XisA activity. In such a case, developing a robust *in vivo* recombinase assay will be instrumental to characterize XisA excisase activity. Rationale of the present study was to develop a PCR based assay to monitor the recombinase activity of XisA *in vivo* in *E. coli.* Such a technique will be sensitive and will address the above limitation of the previously reported *nifD* excision assay.

4.2 Materials and Methods

4.2.1 *E. coli* Strains, Plasmids and Oligonucleotide Primers Used in the Study

E. coli strains and plasmid used in the present study are summarized in **Table 4.1** and **Table 4.2,** respectively. Oligonucleotide primers synthesis was out sourced to 1st Base Pvt. ltd (Singapore) and are summarized in **Table 4.3.**

Table 4.1 Plasmids used in the study.

Name	Characteristics	Reference
pMX25	*nifD* element with *lacZ* and Kan^R cloned in pBR322, Amp^R	(Lammers et al., 1986)
pAM461	Contains distal and proximal borders of *nifD* element, Amp_R	(Lammers et al., 1990)
p*xisA*	pET28a (+) containing full length *xisA* gene under pT7 promoter, Kan^R	Chapter 3

Table 4.2 *E. coli* strains used in the study.

Name	Genotype	Reference
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	(Hanahan, 1983)
BL21 DE3	*F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	(Studier and Moffatt, 1986)
BL21 DE3 pLysE	*F- ompT gal dcm lon hsdSB (rB- mB-) λ(DE3) pLysS(cmR)*	(Moffatt and Studier, 1987)
UN1	BL21 DE3 (pET28a + pAM461)	Chapter 3
UN2	BL21 DE3 (p*xisA* + pAM461)	Chapter 3

Table 4.3 Oligonucleotide primers used in the study.

Name	Sequence
C(F)	5'- GTATCTCTCTACGCTTGCTGGTTGG-3'
C(R)	5'- ACCACCCACTACATCGATAACGCC-3'
X(R)	5'- TGCCGTCGGTAGATGAAAGTGGC-3'

4.2.2 Strategy to monitor XisA protein recombinase activity by PCR assay

4.2.2.1 Design of primers to monitor recombination

Plasmid pAM461 (substrate) rearranges *in vivo* in *E. coli* to ΔpAM461 (product) in presence of XisA **(Fig. 4.2)** releasing a 500 bp fragment from recombination cassette. In order to monitor XisA mediated pAM461 recombination, specific unique nucleotide sequences on pAM461 and ΔpAM461were identified. Sequence of antisense primer C(R) complimentary to *nifD* distal region used for substrate specific PCR was 5'-TGCCGTCGGTAGATGAAAGTGGC-3' and that for product specific PCR X(R) complimentary to excised fragment was 5'-ACCACCCACTACATCGATAACGCC-3'. Sequence of the sense primer C(F) complimentary to *nifD* proximal region was 5'- GTATCTCTCTACGCTTGCTGGT TGG-3' and was same for both the reactions **(Fig 4.3)**.

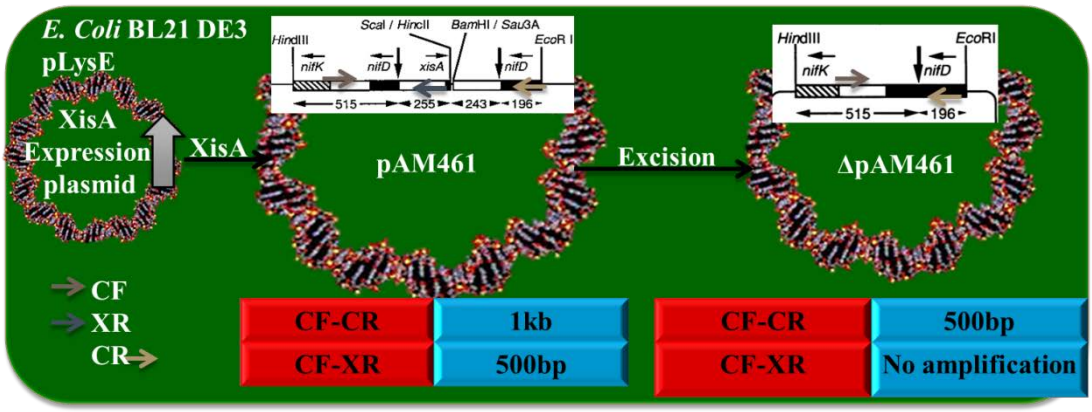

Fig. 4.3 Strategy to design primers for PCR based *in vivo* monitoring of excision by XisA

PCR amplification pAM461 with primer pairs C(F) and X(R) yields 528 bp amplicon (substrate specific PCR) and absolutely no amplification is shown by ΔpAM461 using same primers. ΔpAM461 yields 501 bp amplicons using primer pair C(F) and C(R) (product specific PCR). On the other hand, primers C(F) and C(R) yields 1 kb amplicons with unrearranged pAM461. Primer combination C(F) and X(R) in unrearranged pAM461 would yield 528 bp amplicons **(Fig. 4.4)**.

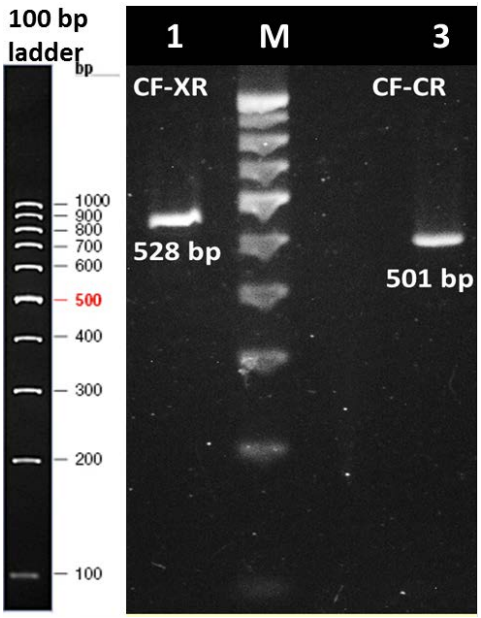

Fig. 4.4 Detection of pAM461 rearrangement using substrate and product specific PCR primers. Lane 1 :Substrate specific PCR using CF-XR primers ; Lane 2: 1 kb ladder; Lane 3: Product specific PCR using CF-CR primers.

4.2.2.2 Design of experiment

XisA recombinase activity encoded under overexpressed condition facilitated by p*xisA* was monitored in *E. coli* BL21 expression host system **(Fig. 4.5).** p*xisA* and pAM461 was co-transferred to either *E. coli* BL21 (DE3) or *E. coli* BL21 (DE3) pLysE as described in section **3.2.4.** Transformed colonies of were screened to detect the presence of rearranged product using product specific colony PCR as described in section **4.2.2.1.** *E. coli* BL21 (DE3) (p*xisA*, pAM461) displayed complete rearrangement of pAM461 at the transformation stage **(Fig. 4.5a)** whereas due to suppression of basal expression of *xisA* gene from p*xisA* in *E. coli* BL21 (DE3) pLysE, substrate (pAM461) was maintained in unrearranged form **(Fig. 4.5b)** as detected from substrate specific colony PCR. This observation suggests *E.coli* BL21 (DE3) as an ideal host to develop the PCR assay.

E. coli BL21 (DE3) pLysE (*pxisA*, pAM461) was grown overnight on LB agar containing appropriate antibiotics. The following day, bacterial biomass was scraped and resuspended in LB broth to give a suspension of OD_{600} = 1. *xisA* expression was induced by adding 0.8 mM IPTG, after which the cells were incubated at 37 ^{o}C in static condition and an aliquot of 10 µl was collected every 5 min. 5 µl of which was

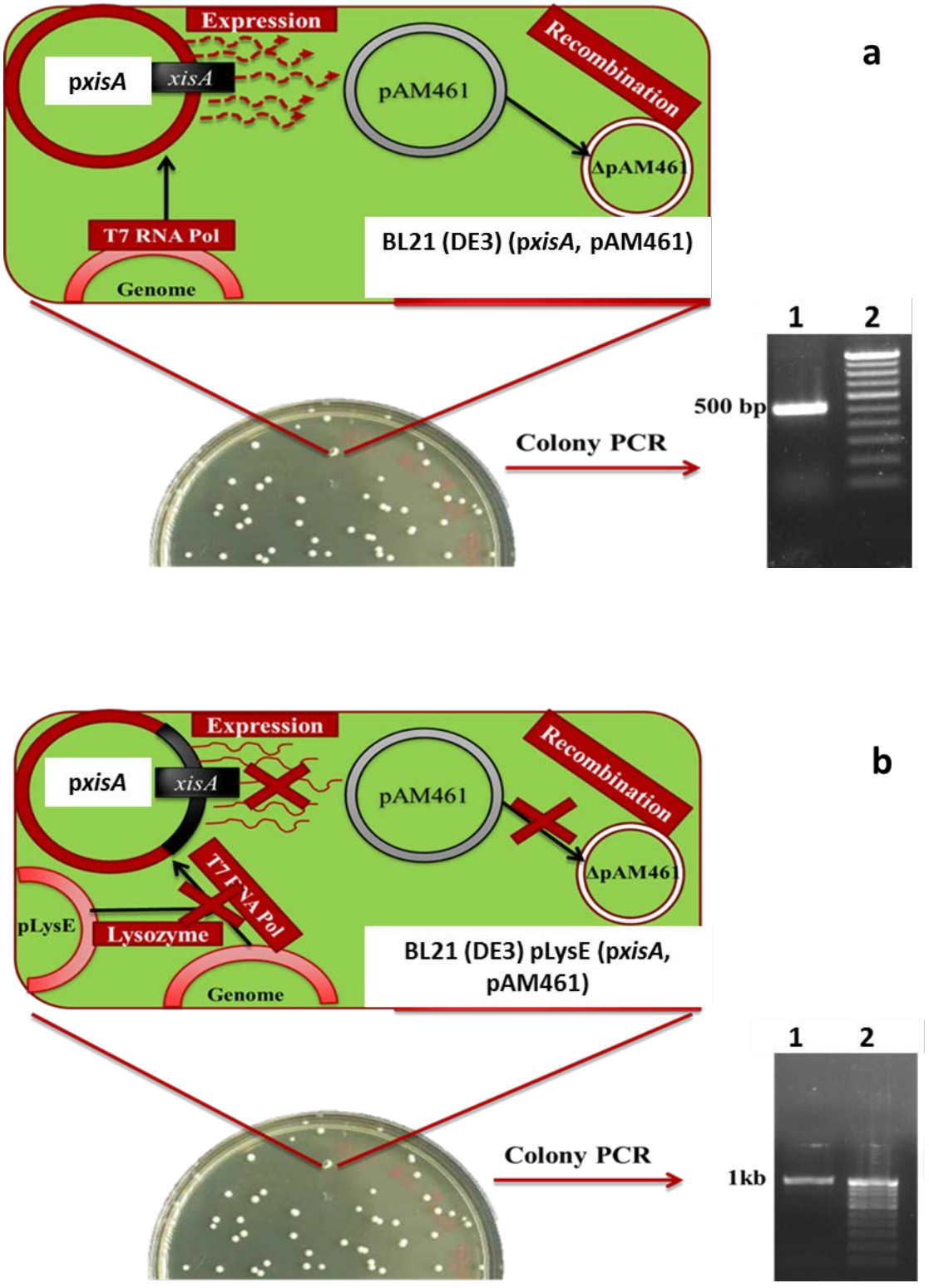

Fig. 4.5 *In vivo* colony PCR detection of pAM461 rearrangement in *E. coli* expression host. (a) Schematic representation of pAM461 rearrangement by leaky expression of *xisA* gene in *E. coli* BL21 (DE3) host. (Inset) Detection of pAM461 rearrangement by product specific colony PCR. Lane 1: 500 bp amplified product of rearranged pAM461; 1 kb ladder. (b) Schematic representation of suppression of *xisA* gene leaky expression preventing pAM461 rearrangement in *E. coli* BL21 (DE3) pLysE host. (Inset) Detection of unrearranged pAM461 by product specific colony PCR. Lane 1: 1 kb amplified product of unrearranged pAM461; Lane 2: 1 kb ladder.

utilized for each substrate specific and product specific PCR **(Fig. 4.6)**. The intensity of the respective amplicons on agarose gel was quantified by measuring the integrated density values (IDV). Excision frequency was calculated using the formula:

$$\textbf{Excision (\%)} = (IDV_{Product}) \times 100 / IDV_{Substrate} + IDV_{Product}$$

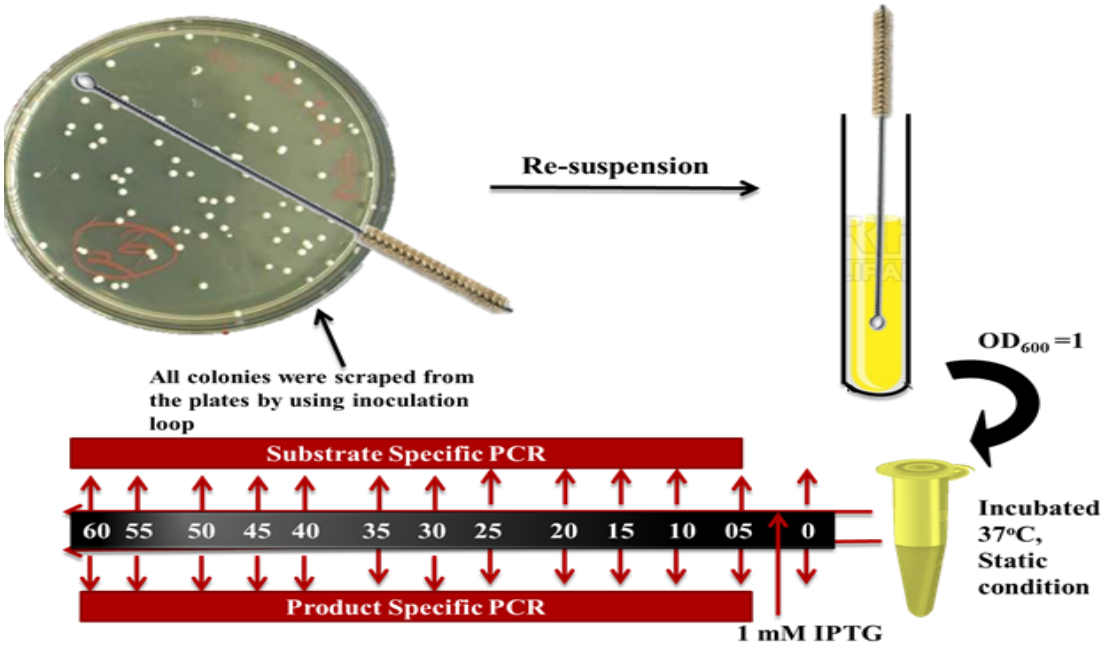

Fig. 4.6 Experimental design and to monitor XisA recombinase activity *in vivo* by PCR based assay.

4.3 Results

4.3.1 Monitoring recombinase activity of XisA protein by PCR assay

A PCR based method was developed to monitor excision frequency of XisA substrate plasmid (pAM461) using substrate and product specific primers **(Fig. 4.7)**. Near 100 % excision was observed in *E. coli* BL21 (DE3) (pAM461, p*xisA* strain within 1 h at 37 ^{o}C on LB under static condition.

4.4 Discussion

Most of the site specific recombinases require accessory protein to carry out recombination. Such a stringent requirement hampers *in vitro* characterization of these recombinases. Most of the functional studies monitoring XisA excisase potential were based on *in vivo* assay that involved calculating blue and white colonies by

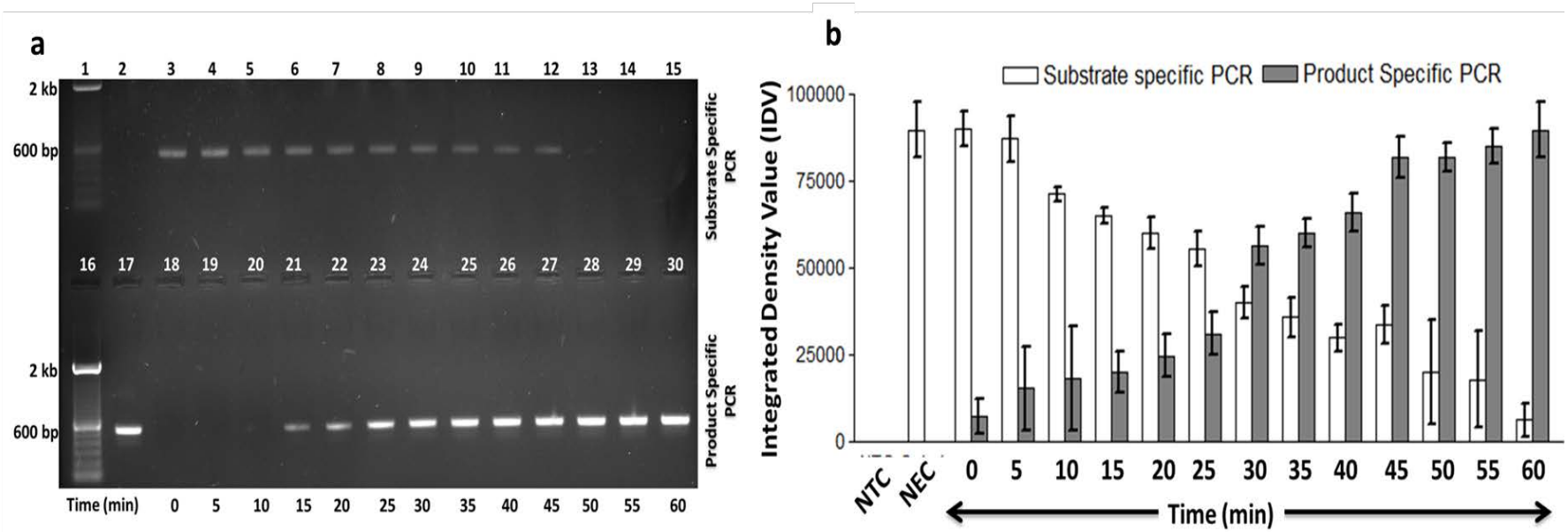

Fig. 4.7 PCR based monitoring of excision. (a) Agarose gel profile of substrate and product specific PCR amplicons obtained at different time points. Lane1 and Lane 16: 100 bp ladder; Lane 2: No template control (NTC); Lane 3-15: substrate specific PCR; Lane 17: No enzyme control (NEC); Lane 18-30: product specific PCR. (c) Quantification of substrate and product specific amplicons by measuring IDV. Values are expressed as Mean ±SEM (N=3).

either plating method (Lammers et al., 1986; Lammers et al., 1990) or transformation method (Lammers et al., 1990). Such rearrangements may lead to abortive excision events resulting in lesser sensitivity of the assay. Additionally, smaller rearranged plasmid have more transformation potential over larger substrate plasmids resulting in inaccurate monitoring of recombination. None of these assays could demonstrate 100 % excision of efficiency of XisA.

In the present study, problems arising from unavailability of accessory proteins to characterize recombinase activity is addressed by developing a very robust *in vivo* PCR based method. Since, PCR is a very sensitive technique, problems arising from in accuracy of previously reported *in vivo* assays are addressed by specifically monitoring the formation of rearranged product as a function of XisA protein expression. This method can be utilized to monitor the rearrangement under leaky or overexpression of *xisA* gene. We also report for the first time, the 100% excision efficiency of XisA protein in the overexpressed state. This method can be optimized to monitor excision frequency of previously uncharacterized recombinase proteins *in vivo*.