Gene cloning and recombinant protein production

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Learning objectives

Student can be able to-

1. Describe the principle and methods of gene cloning and recombinant protein production.
2. Clone gene (DNA) into the plasmid vector.
2. Express recombinant protein using bacteria or yeast cell.

Recombinant DNA

- Plasmid DNA
- Restriction enzymes

Gene cloning

4 stages

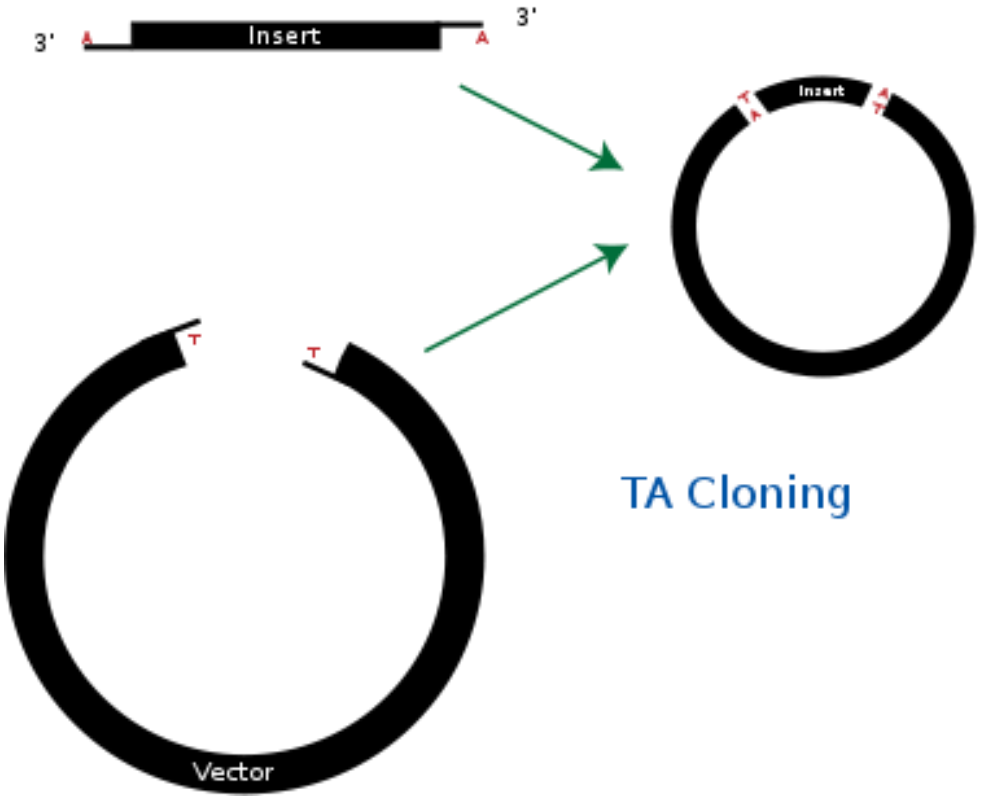
1. Digestion of DNA and cloning vector DNA
2. Insertion of DNA into cloning vector
3. Transformation
4. Manipulation of the cloned DNA: sequencing, gene expression

Applications

1. **DNA or gene cloning-** **isolation of a DNA sequence of interest to obtain multiple copies of it *in vitro.***
2. **Protein expression-** **produce recombinant protein encoded by interested gene.**

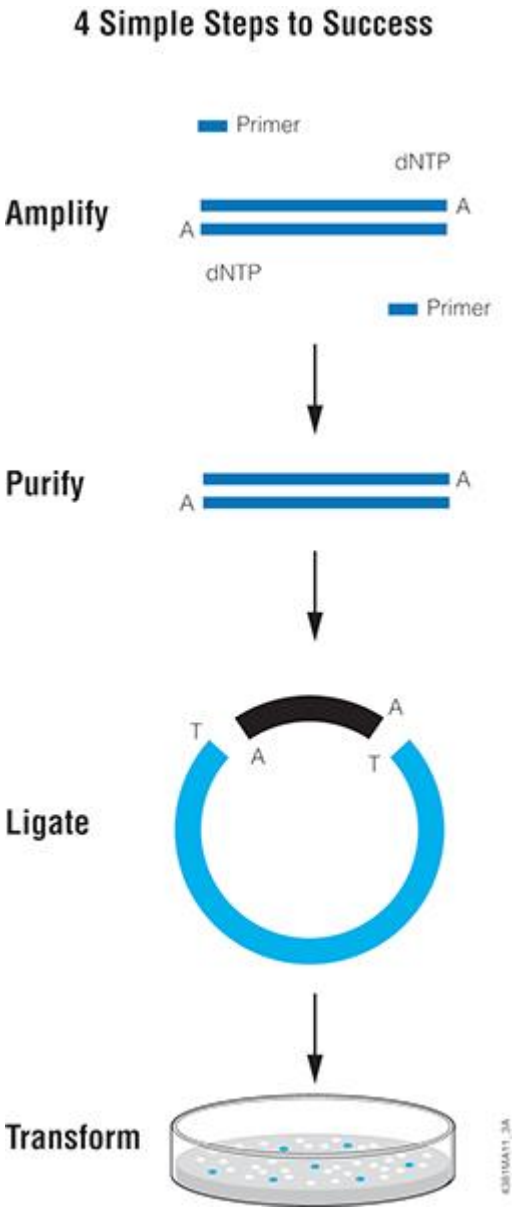
DNA or gene cloning

- Using TA plasmid vector

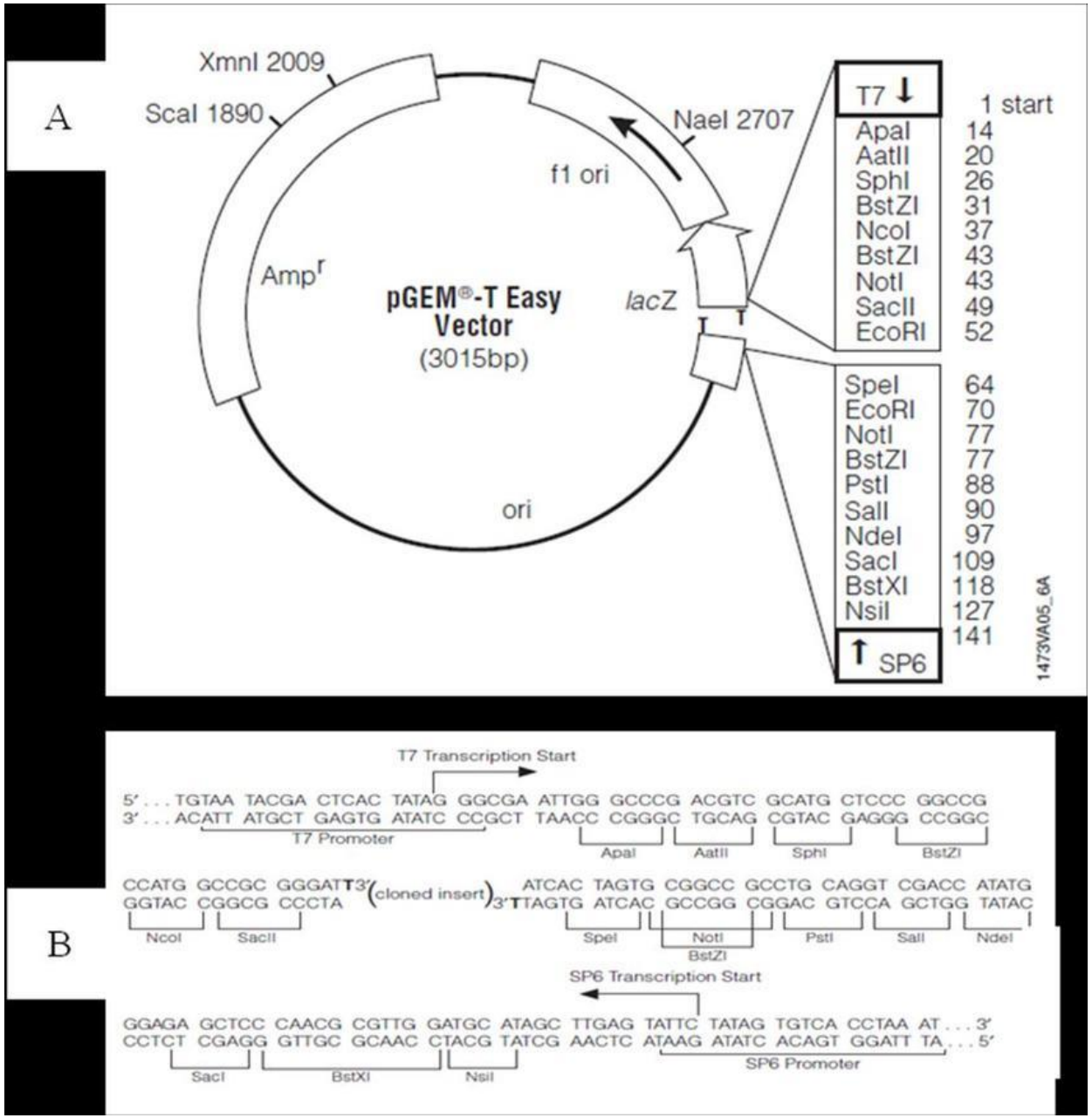


Procedures

1. Identify target gene or sequence
    - Isolate the sequence- PCR
    - Modify the sequence
2. Ligate to the plasmid vector
3. Transform to bacteria cell
4. Replicate (culture bacteria cell)
5. Plasmid extraction
6. DNA sequence analysis



pGEM®-T Easy Vector Systems



Cloning PCR Products with pGEM®-T and pGEM®-T Easy Vectors

Cloning PCR Products with pGEM®-T and pGEM®-T Easy Vectors

Ligation Using 2X Rapid Ligation Buffer

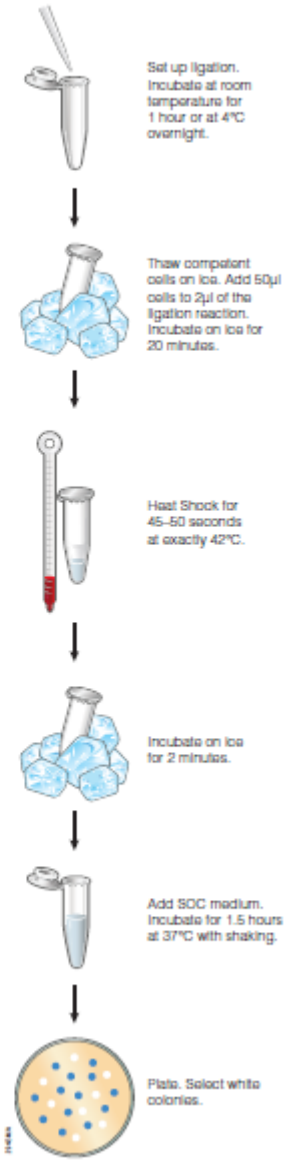
1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.
2. Set up ligation reactions as described below. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Use 0.5ml tubes known to have low DNA-binding capacity.

Reagents	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Deionized water to a final volume of	10µl	10µl	10µl

3. Mix the reactions by pipetting. Incubate the reactions 1 hour at room temperature. Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

Transformation of JM109 High Efficiency Competent Cells

1. Prepare LB/ampicillin/IPTG/X-Gal plates.
2. Centrifuge the ligation reactions briefly. Add 2µl of each ligation reaction to a sterile 1.5ml tube on ice. Prepare a control tube with 0.1ng of uncut plasmid.
3. Place the JM109 High Efficiency Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by gently flicking the tube.
4. Carefully transfer 50µl of cells to the ligation reaction tubes from Step 2. Use 100µl of cells for the uncut DNA control tube. Gently flick the tubes and incubate on ice for 20 minutes.
5. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tubes to ice for 2 minutes.
6. Add 950µl room temperature SOC medium to the ligation reaction transformations and 900µl to the uncut DNA control tube. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
7. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the uncut DNA control, a 1:10 dilution with SOC is recommended.
8. Incubate plates overnight at 37°C. Select white colonies.



Ligation of plasmid vector and insert DNA

- Vector:insert ratio calculation
  - Mostly 1:1 or 1:3 molar ratio work well
  - Calculation for ng of insert =

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

Example

$$\frac{100 \text{ ng vector} \times 1 \text{ kb insert}}{6 \text{ kb size of vector}} \times \frac{3}{1} = 50 \text{ ng}$$

- T4 DNA ligate

Transformation of DNA to bacteria

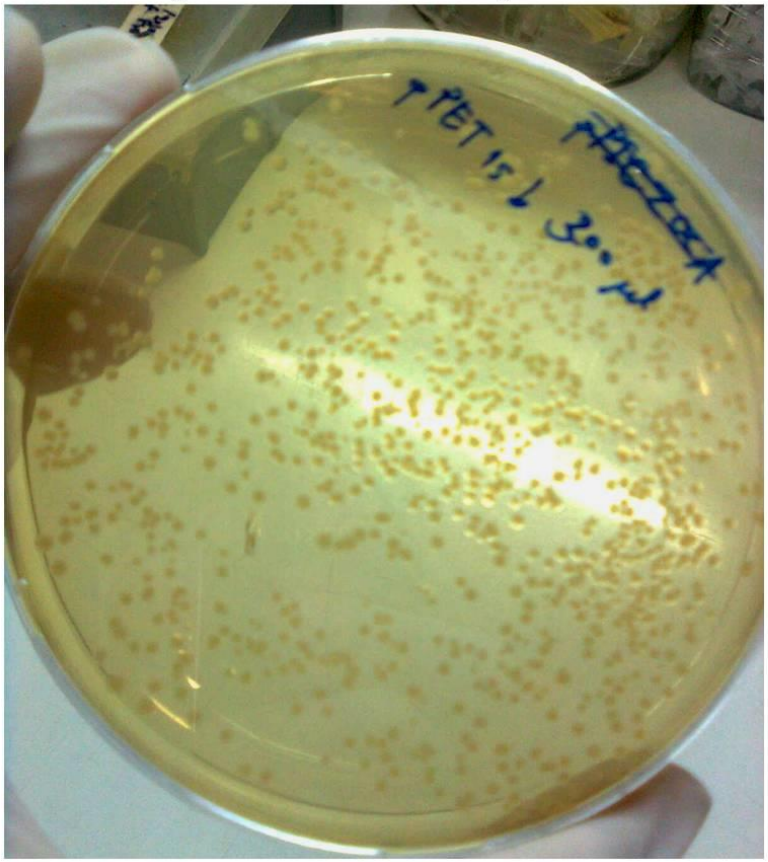
1. Preparation of competent cell
2. Transformation of competent cells- heat shock or electroporation.
3. Selection of transformants.

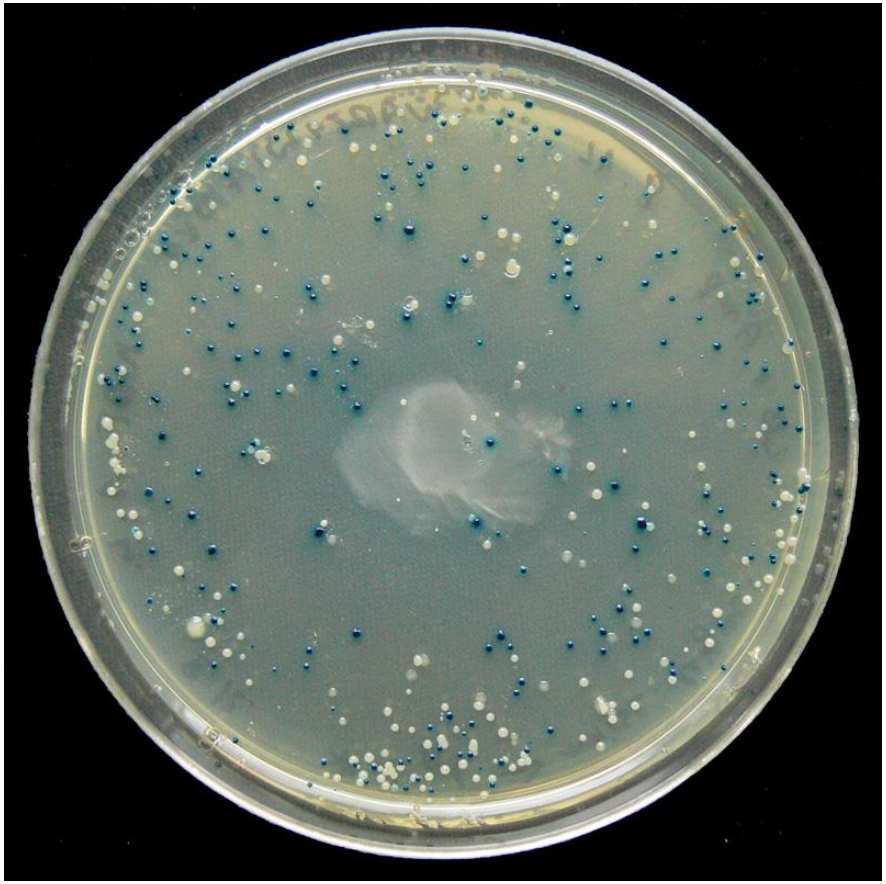
Selection of transformants

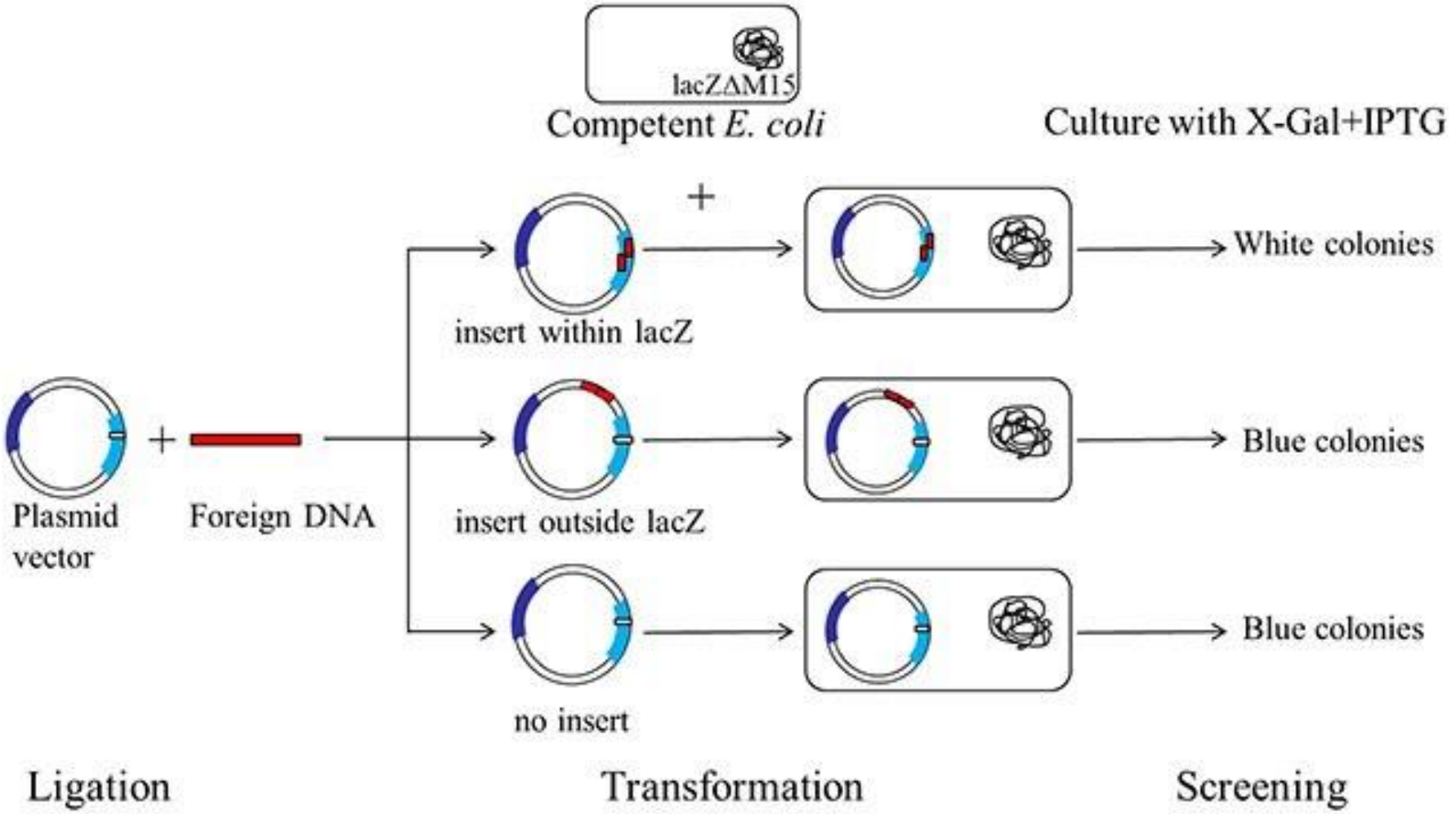
- Antibiotic selection : in this case use amplicillin
- Colony PCR
- DNA Sequencing

Selection the positive transformants by antibiotic or blue-white colony selection

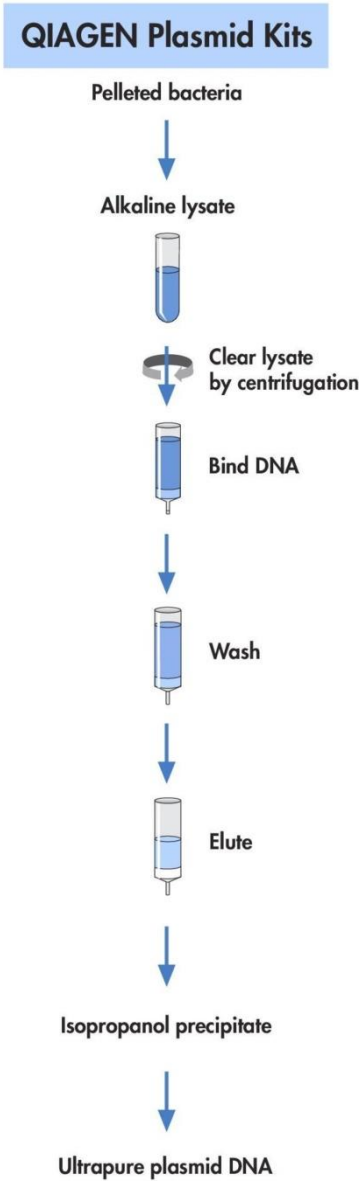
pPET 15b-circular plasmid






lacZΔM15
Competent *E. coli*
Culture with X-Gal+IPTG
+
White colonies
insert within lacZ
Plasmid vector
+
Foreign DNA
insert outside lacZ
Blue colonies
no insert
Blue colonies
Ligation
Transformation
Screening

Plasmid extraction and purification



O. viverrrini tetraspanin 3 sequence

TM1

ATGGTCTCCCTCAGCTGTGGCTACAAGTGTTTGCAATGCATGTTAGTCGTCTTCAATGTG 60

M V S L S C G Y K C L Q C M L V V F N V

TM1 Cleavage site

GTTGTCATCTGTTGTGGAATTGCGTTGATAGTTGTTGGTAGCATCGCCCAAGTCCAGCTG 120

V V I C C G I A L I V V G S I A Q V Q L

TM2

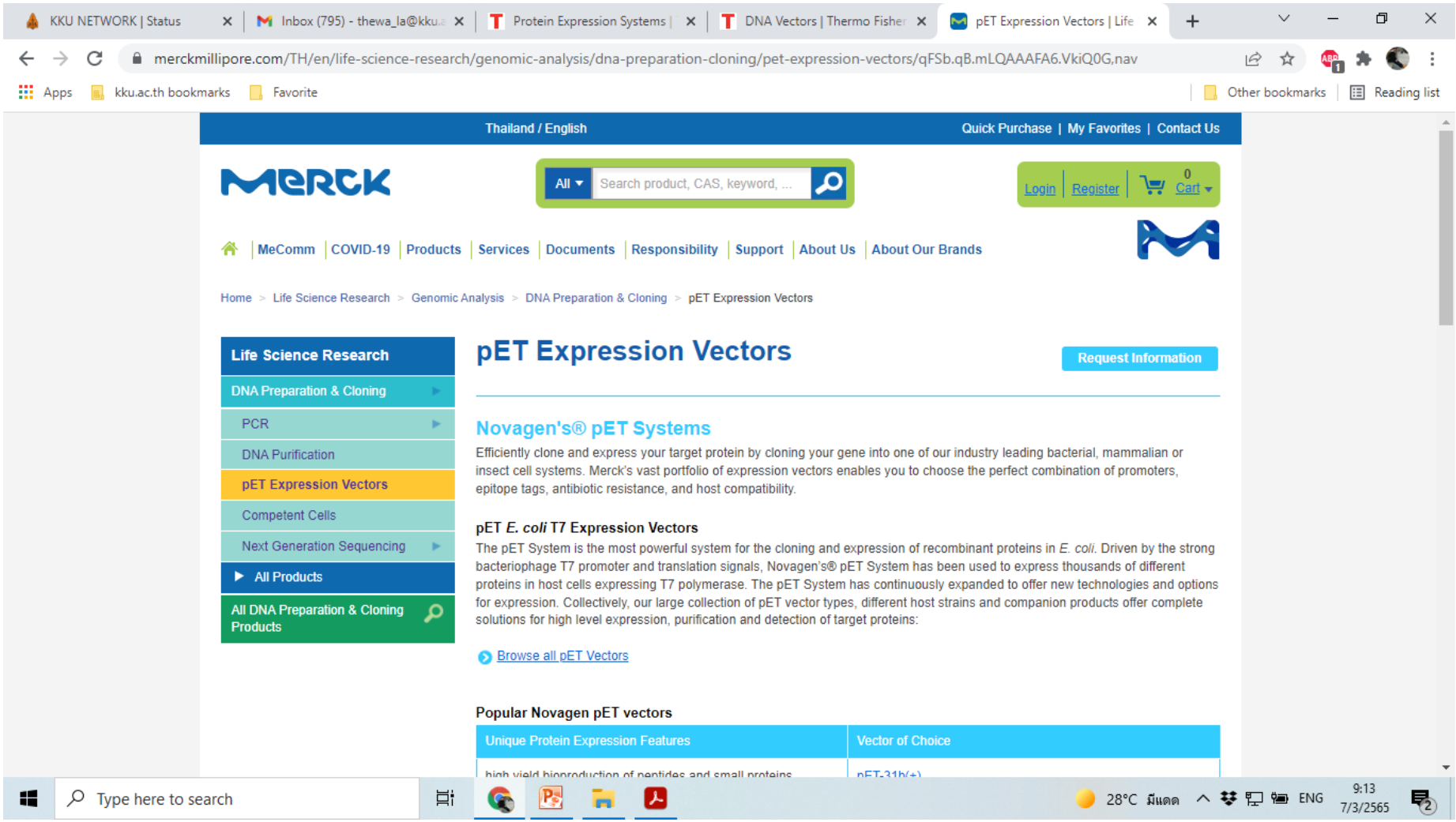
AAGACATACTTATCCAGCGAAGATGCTCAGCTTATGGCCTTCGTCATCTTTATAATAGCT 180

K T Y L S S E D A Q L M A F V I F I I A

TM2

Protein expression

- *In vivo* expression of recombinant protein.
- Express cloned gene products in prokaryotic and eukaryotic system.
- Recombinant protein is used for studies of protein structure and function, protein-protein and protein-DNA interactions, antibody production and mutagenesis.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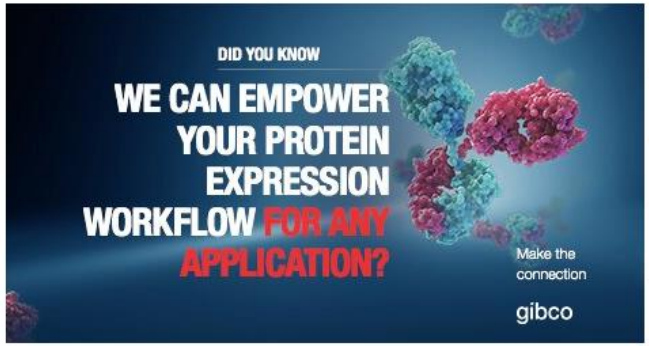


- Mammalian
- Insect
- Bacterial
- Cell culture plastics
- Technical resources

Recombinant protein expression technology enables study of gene regulation and protein structure and function. Utilization of recombinant protein expression can also vary widely—from investigation of function *in vivo* to large-scale production for biotherapeutic drug discovery and structural studies. Using the right protein expression system for your specific application is critical to success. Consider protein solubility, functionality, purification speed, and yield when choosing an expression system. We offer a wide selection of superior mammalian, insect, yeast, bacterial, algal, and cell-free protein expression systems to suit your research needs, backed by trusted brands like Gibco and Invitrogen.

Give Feedback

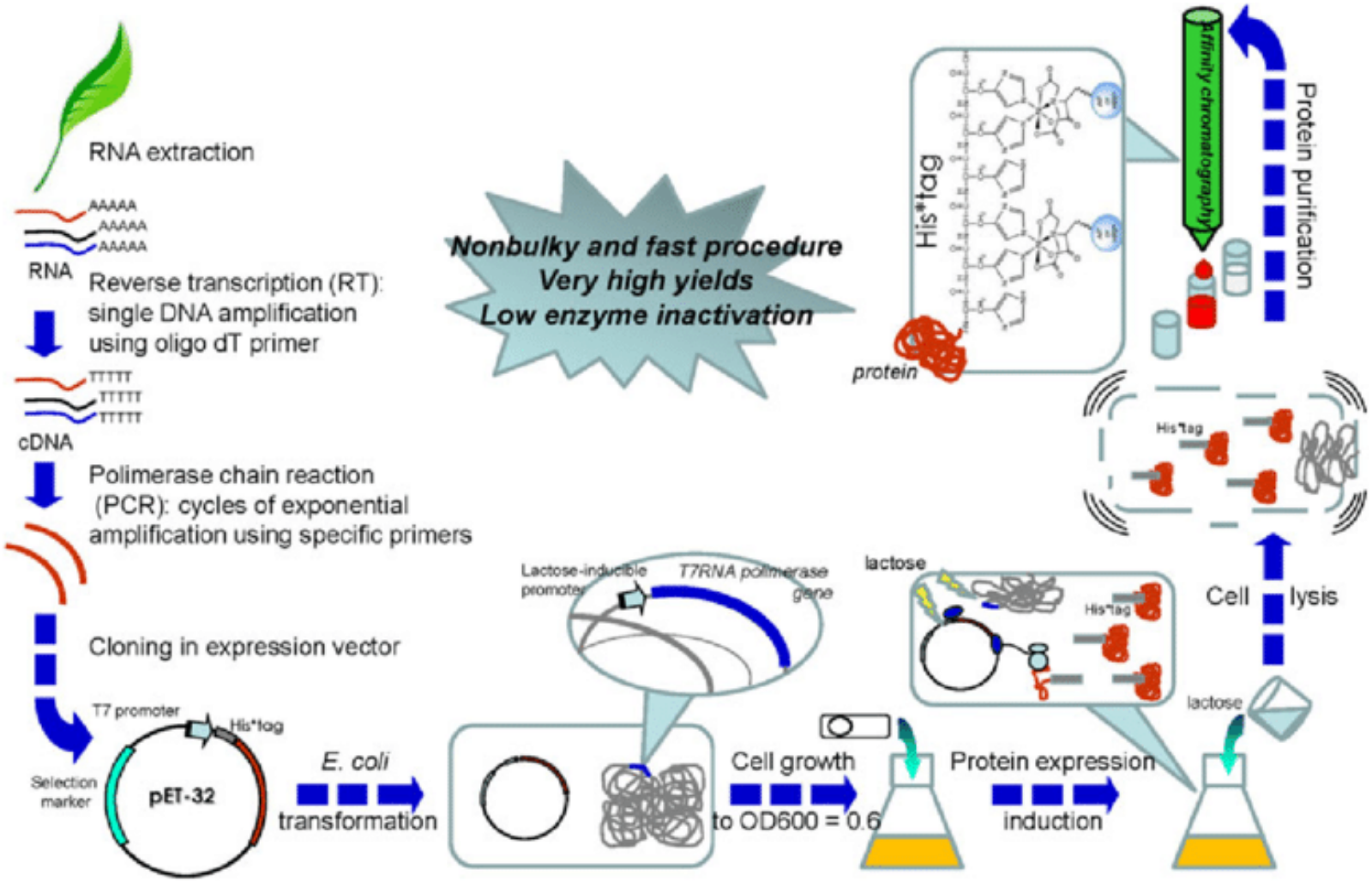
Prokaryotic system

- Expression of protein in *E. coli*
- Advantages: inexpensive, high yield, easy to regulate
- Problems: expression of eukaryote gene products using prokaryotic system if the protein need post-translation modification for activity, protein produce in insoluble form.

Identify target gene or sequence

- Screening
- Transcriptomic
- Proteomic

Procedures


RNA extraction
AAAAA
AAAAA
AAAAA
RNA
Reverse transcription (RT): single DNA amplification using oligo dT primer
TTTTT
TTTTT
TTTTT
cDNA
Polimerase chain reaction (PCR): cycles of exponential amplification using specific primers
Cloning in expression vector
T7 promoter
His*tag
Selection marker
pET-32
E. coli
transformation
Nonbulky and fast procedure
Very high yields
Low enzyme inactivation
Lactose-inducible promoter
T7RNA polimerase gene
Cell growth
to OD600 = 0.6
lactose
His*tag
Protein expression
induction
lactose
Cell lysis
His*tag
His*tag
protein
affinity chromatography
Protein purification

International Journal of Infectious Diseases (2008) 12, e49–e59

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Asparaginyl endopeptidase from the carcinogenic liver fluke, *Opisthorchis viverrini*, and its potential for serodiagnosis

Thewarach Laha [a,*], Jittiyawadee Sripa [a], Banchob Sripa [b], Mark Pearson [c], Leon Tribolet [c], Sasithorn Kaewkes [a], Paiboon Sithithaworn [a], Paul J. Brindley [d], Alex Loukas [c]

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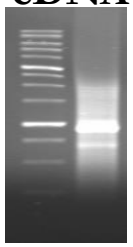
[c] *Division of Infectious Diseases, Queensland Institute of Medical Research, Brisbane, Queensland, Australia*
[d] *Department of Microbiology, Immunology and Tropical Medicine, George Washington University Medical Center, Washington, DC, USA*

Approaches

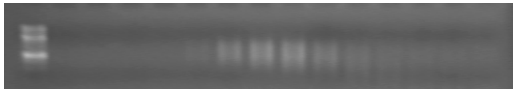
cDNA library of adult of O.V.

Adult worm

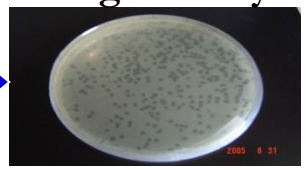
cDNA



cDNA size fractionate



Phage library



Screened with rabbit hyperimmune sera

Screened with OV positive human sera

Collected positive clones

Sequencing analysis

Produce the recombinant protein

Functional assay

Application

- Ag for serodiagnosis
- Vaccine trial

Map OV Haemoglobinase

```
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 181 TGTATATCACGCGTATCAAATCATGAAGCGCAACAACATTTCGACGGAGCAAATAATTAC 240
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       S  G  S  M  F  Q  D  I  L  P  S  D  I  G  I  Y  V  T  T  A
 661 GGCTAACAGTGAGGAATCCAGCTGGGCTACTTTCTGTCGCGACACAATCATTGGCACTTG 720
       A  N  S  E  E  S  S  W  A  T  F  C  R  D  T  I  I  G  T  C
 721 TCTGGCGGACGAATACTCGTACAACTGGCTCACGGACTCTGAGCATCACGATCTGTCGCA 780
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 781 TCGCACACTGGATGATCAGTTCCAATCGGTGAAACAGAATACCAAGCAAAGTCACGTATC 840
       R  T  L  D  D  Q  F  Q  S  V  K  Q  N  T  K  Q  S  H  V  S
 841 GAGATTCGGGGAACTGCCTCAGGTACTTCATAGCCATCCGTCACGCTGGGCACATTTGGT 900
       R  F  G  E  L  P  Q  V  L  H  S  H  P  S  R  W  A  H  L  V
 901 CACCATGGTCCGACGAATGATGAAAGCCGAAACCGAGGAAGAACATGAATTGGCATCCCG 960
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1201 TGAAGCACTTGTTCAATCTGTTCATGAAGTCTGTTCCTAGTGGACGCTGTCAACATTTGA 1260
       E  A  L  V  Q  S  V  H  E  V  C  S  *
1261 TGGAACAACCTTAATTCGAAAAATGCATAAGTCCTGCAGTGG 1302
```

OV-TSP1

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SQCLLGAYFI ILLVIFTSQV TGATLGYVFR EEIMQHVEQQ
MFEGVEEYSM LRDQRENPSP FMDNIHRVLQ CCGVNGYTDY
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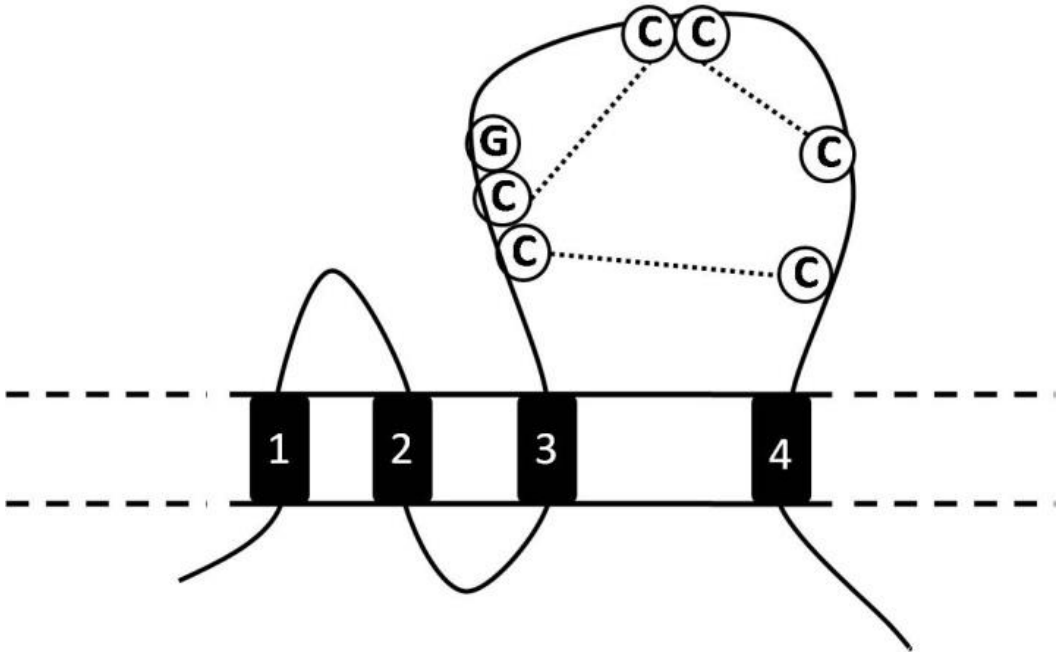
VDYAI



Schematic illustration of the structural design of OvTSP1

247 aa Predicted Large external loop = 105-203 (Underlined)Expression region: Tyr (Y)107- GlT (E)193 (Green) = 87 aa

Frame 1

MMGCVQCLRILLVVFNFLVVLVGLVVLGFSVYVSQEPEAQDIIRASGHYVAVQIALYALMGVGGITLITA

LFGCCGAYHESQCLLGAYFIILLVIFTSQVTGATLGYVFREEIMQHVEQQMFEGVEEYSMLRDQRENPSP

FMDNIHRVLQCCGVNGYTDYRDRIPVTCCDRRKSNCNELQLTPDEVYTEGCKEKYKRFFKDKLIVFFLIA

VSIAAFEIFCLLFSMVLCCAIRQYHSDYYGVDYAIAT*

```
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      R  ?  S  R  ?  ?  *  V  P  A  N  Q  N  R  F  E  M  M  G  C
  61 GTCCAATGCTTGCGGATTTTATTAGTTGTGTTCAACTTTCTCGTTGTGCTGGTCGGTTTG 120
      V  Q  C  L  R  I  L  L  V  V  F  N  F  L  V  V  L  V  G  L
 121 GTGGTTCTAGGGTTTTCCGTATACGTCTCTCAAGAACCTGAGGCACAAGATATCATACGG 180
      V  V  L  G  F  S  V  Y  V  S  Q  E  P  E  A  Q  D  I  I  R
 181 GCGTCCGGACACTATGTGGCTGTTCAGATCGCTCTCTACGCTCTCATGGGAGTCGGCGGT 240
      A  S  G  H  Y  V  A  V  Q  I  A  L  Y  A  L  M  G  V  G  G
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      I  T  L  I  T  A  L  F  G  C  C  G  A  Y  H  E  S  Q  C  L
 301 CTCGGTGCATACTTCATAATACTGTTGGTAATATTCACCTCTCAAGTTACCGGAGCTACT 360
      L  G  A  Y  F  I  I  L  L  V  I  F  T  S  Q  V  T  G  A  T
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      L  G  Y  V  F  R  E  E  I  M  Q  H  V  E  Q  Q  M  F  E  G
 421 GTGGAGGAATACTCAATGCTTCGCGATCAACGTGAGAATCCGTCGCCCTTCATGGACAAC 480
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 781 ATAGCAACTTAAGTGACCCGTAGTTGCCTGCACACCAACTACTGCTTCGTTTTGATTCAA 840
         I  A  T  *  V  T  R  S  C  L  H  T  N  Y  C  F  V  L  I  Q
```

Approaches

- cDNA library screening
- PCR
  - Specific oligoprimer designed from targeted DNA sequence
  - Degenerate primer designed from target protein sequence

Ligation of plasmid vector and insert DNA

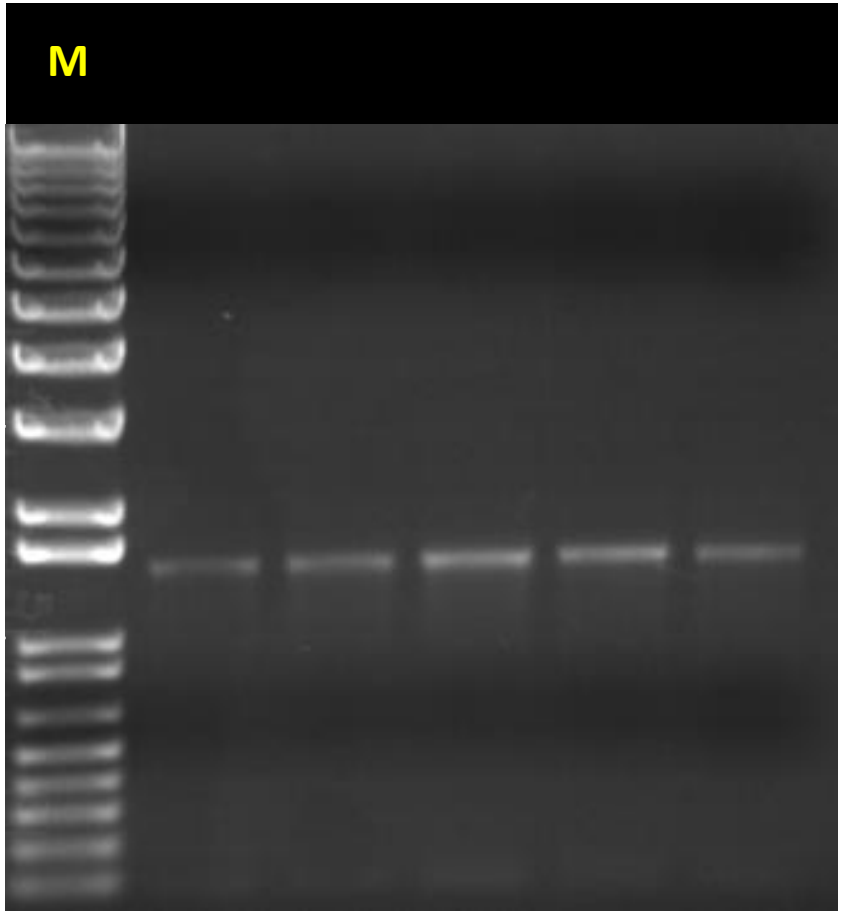
- Insert DNA preparation
- Vector preparation
- Ligation reaction

Insert DNA preparation

- Generate restriction sites at both ends by PCR

- Results:
  - Correct PCR products
  - Correct size: Gel electrophoresis
  - Correct sequence: DNA sequencing

PCR checking



1.6 kb

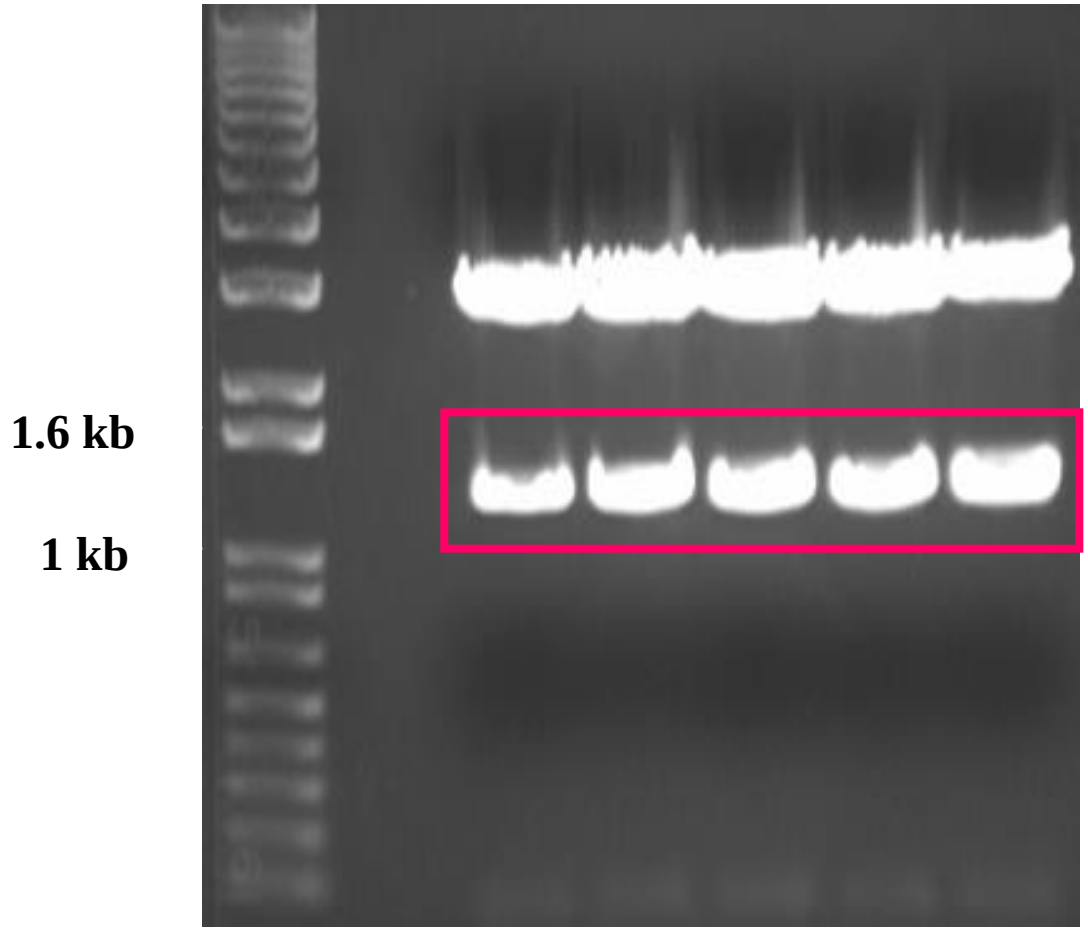
1 kb

Expected size of insert

Insert DNA preparation

1. Direct cloning with PCR product
   - Digested with restriction enzymes
   - Separate through agarose gel electrophoresis
   - Purify
2. If direct clone is difficult, clone into T-vector
   - Clone PCR product into vector
   - Digested with restriction enzymes
   - Separate through agarose gel electrophoresis
   - Purify

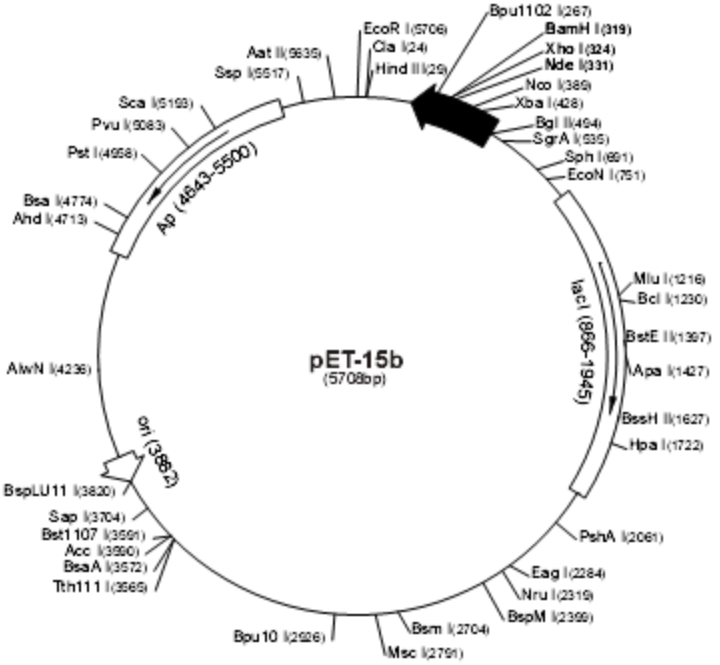
Double digestion of LGM_pGEM-T with *Nde* I and *Xho* I

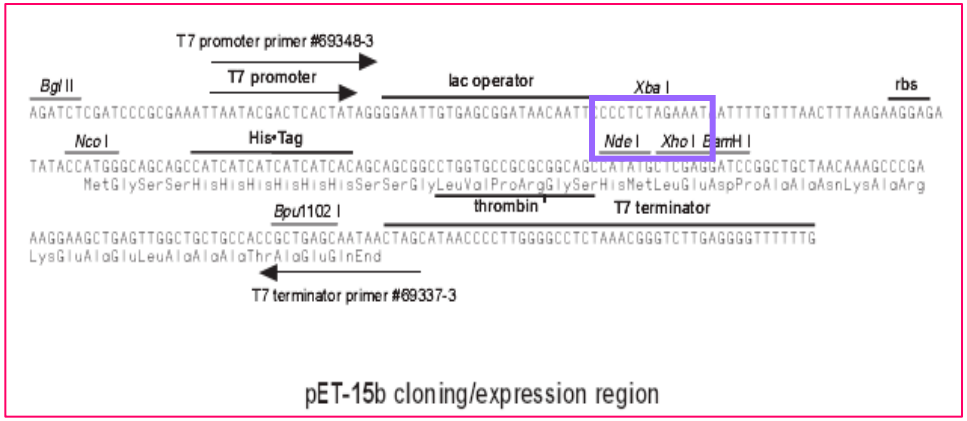


Vector preparation

- Digest with restriction enzymes
- Separate through agarose gel
- Purify
- Concentrate

Ligate LGM gene into pET-15b vector





Ligation of plasmid vector and insert DNA

- Vector:insert ratio calculation
  - Mostly 1:1 or 1:3 molar ratio work well
  - Calculation for ng of insert =

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

Example

$$\frac{100 \text{ ng vector} \times 1 \text{ kb insert}}{6 \text{ kb size of vector}} \times \frac{3}{1} = 50 \text{ ng}$$

- T4 DNA ligate

Transformation of DNA to bacteria

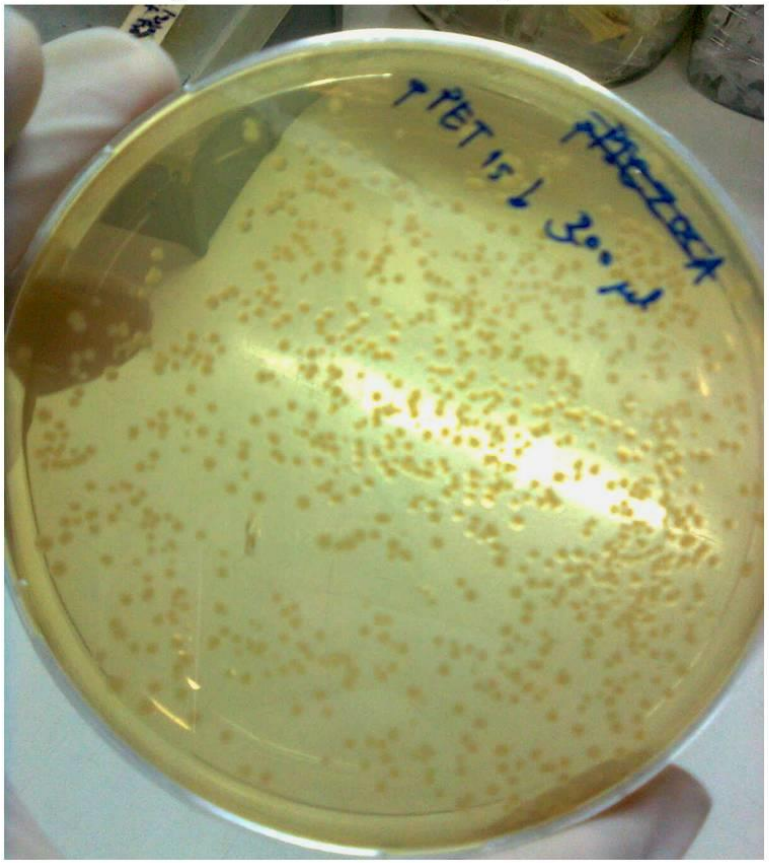
1. Preparation of competent cell
2. Transformation of competent cells
3. Selection of transformants

Selection of transformants

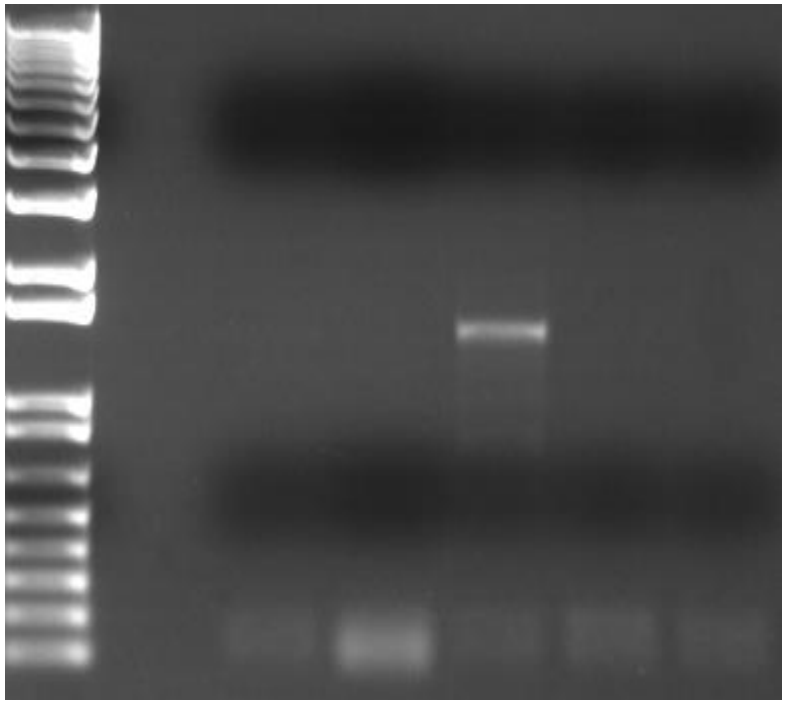
- Antibiotic selection : in this case use amplicillin
- Colony PCR
- DNA Sequencing

Selection the positive transformants by antibiotic or blue-white colony selection

pPET 15b-circular plasmid



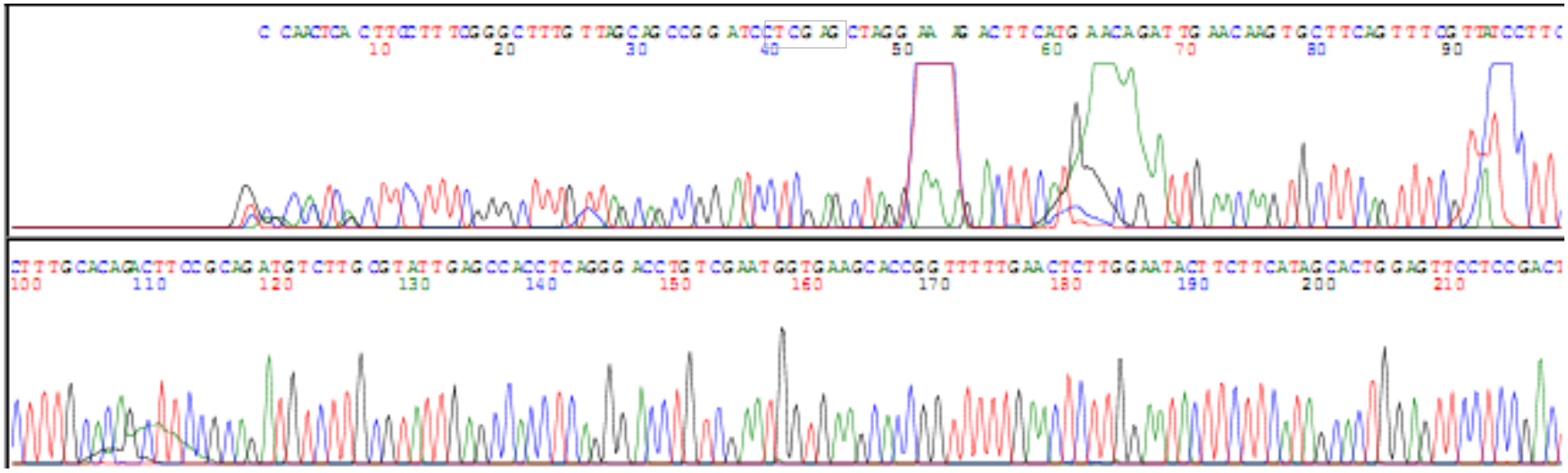
Selection the positive transformants by Colony PCR



lane 1 = 1 Kb marker
lane 2 = -
lane 3 = colony 1
lane 4 = colony 2
lane 5 = colony 3
lane 6 = colony 4
lane 7 = colony 5

ACGCGCGCATATGGCATGGTTAGGCGCTGTT

ACGGCGCCCTCGAGCTAGGAACAGACTTCATGAAC



Prokaryotic expression protocol

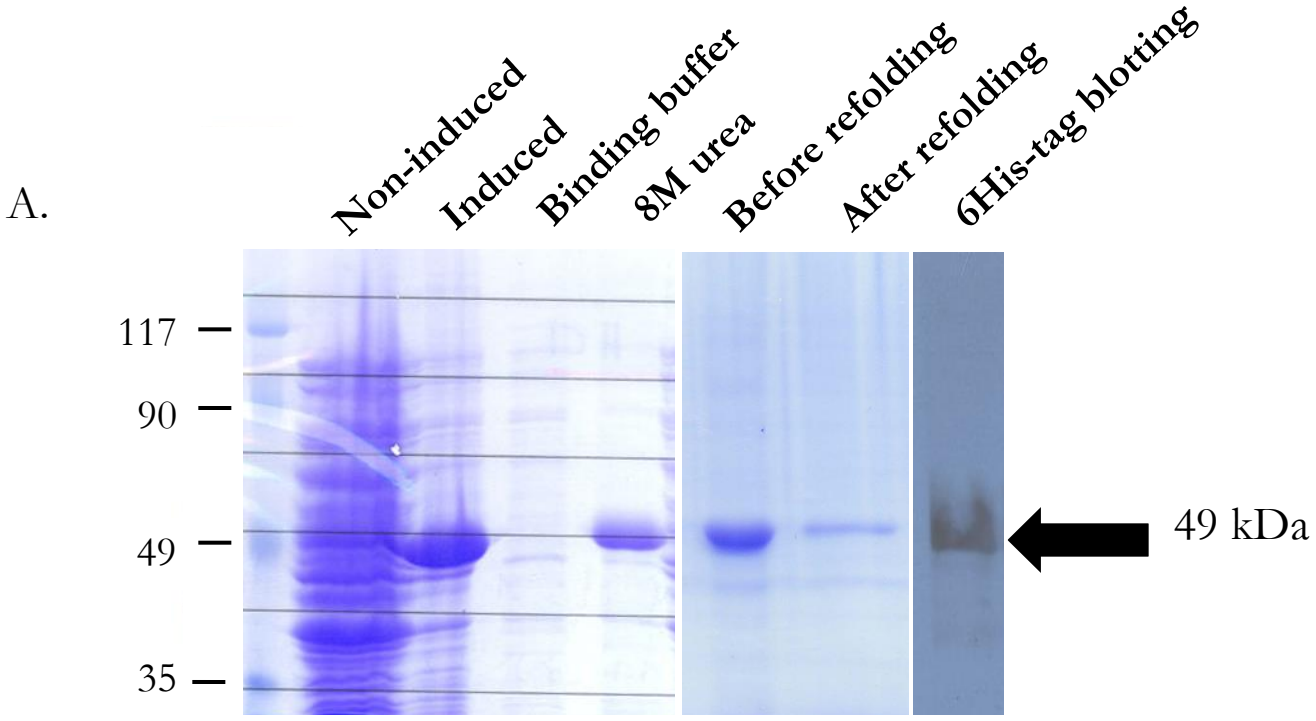
Materials

- LB broth containing antibiotics (100 ug/ml amplicillin)
- IPTG (100mM stock)

1. Transform recombinant plasmid into bacterial
2. Pick colonies and grow in LB amp 50 ml at 37 C for overnight
3. Inoculate 1-2 ml into new LB amp and grow at 37 C to A600 of 0.6
4. Add 100 mM IPTG to culture to final concentration of 0.4 – 1 mM (in 100 ml add 1 ml of 100 mM IPTG)

5. Incubate at 37 C for various time points (1, 2, 3, 4, 6 h and overnight)
6. Analyze expression protein
    - in pellet
    - in supernatant

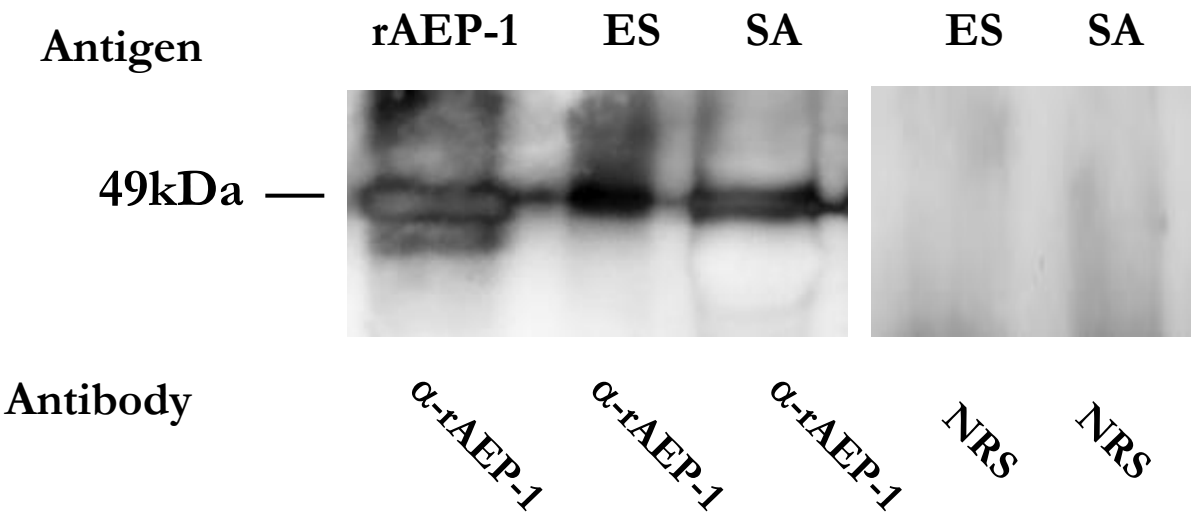
Production of OV rAEP-1 in *E.coli*



Applications

- Antibody production for immunological assay
- Functional assay of protein
- Immunodiagnosis antigen
- Vaccine antigen

Ov-AEP-1 is secreted by adult *O. viverrini*



rAEP-1 = Recombinant *O.V.* asparaginyl endopeptidase

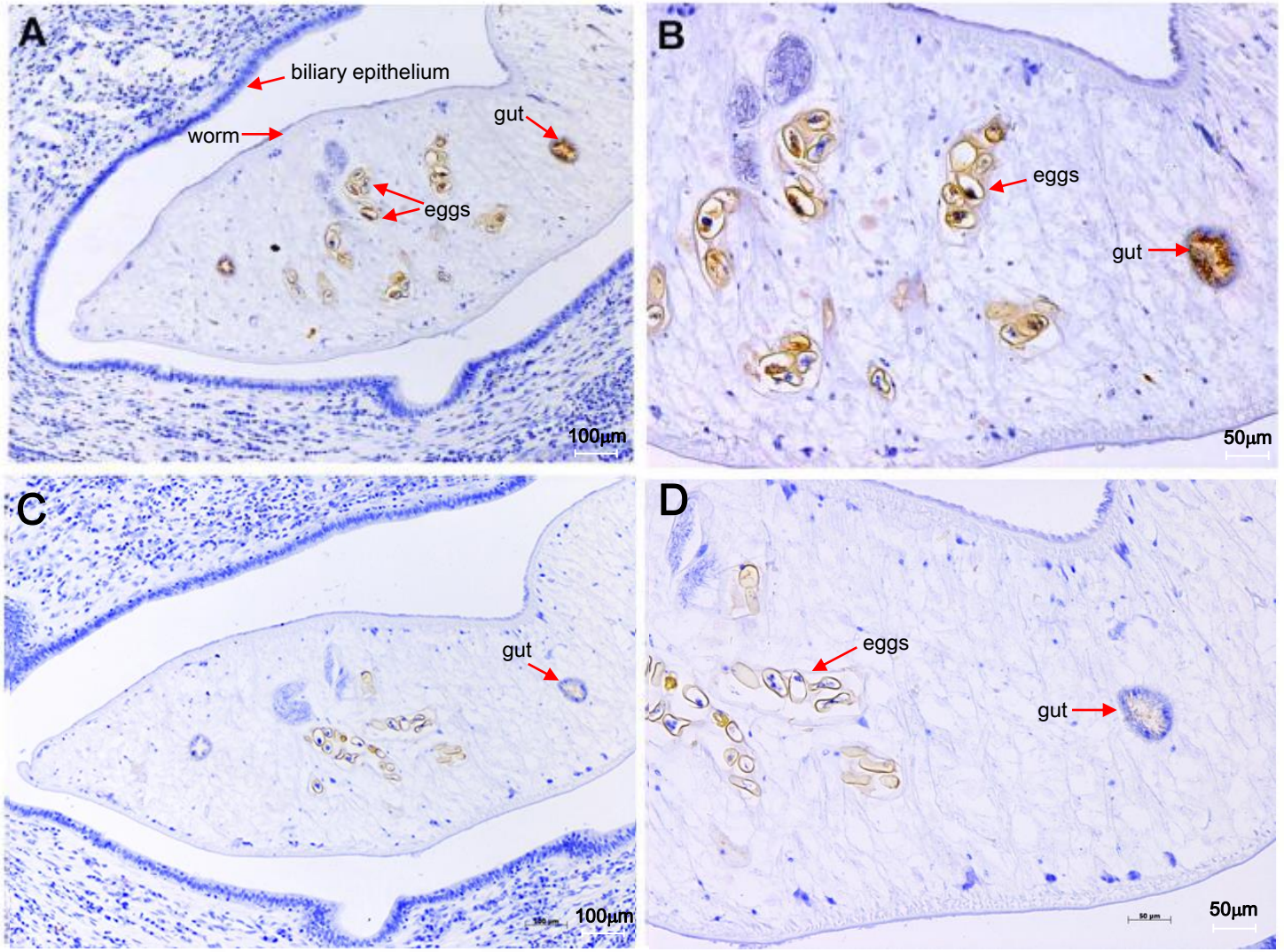
ES = Excretory-Secretory antigen

SA = Somatic antigen

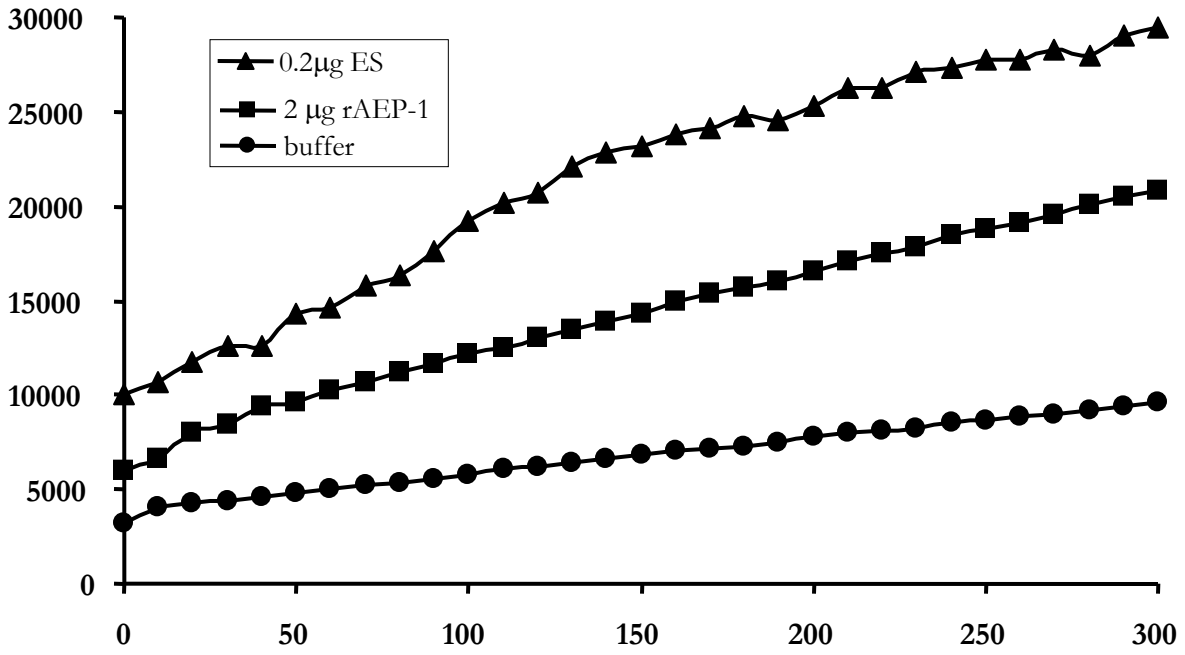
α-rAEP-1 = IgG from the rabbit serum immunized with rAEP-1

NRS = Normal rabbit serum (Negative control)

Asparaginyl endopeptidase expressed in the parasite gut and in eggs



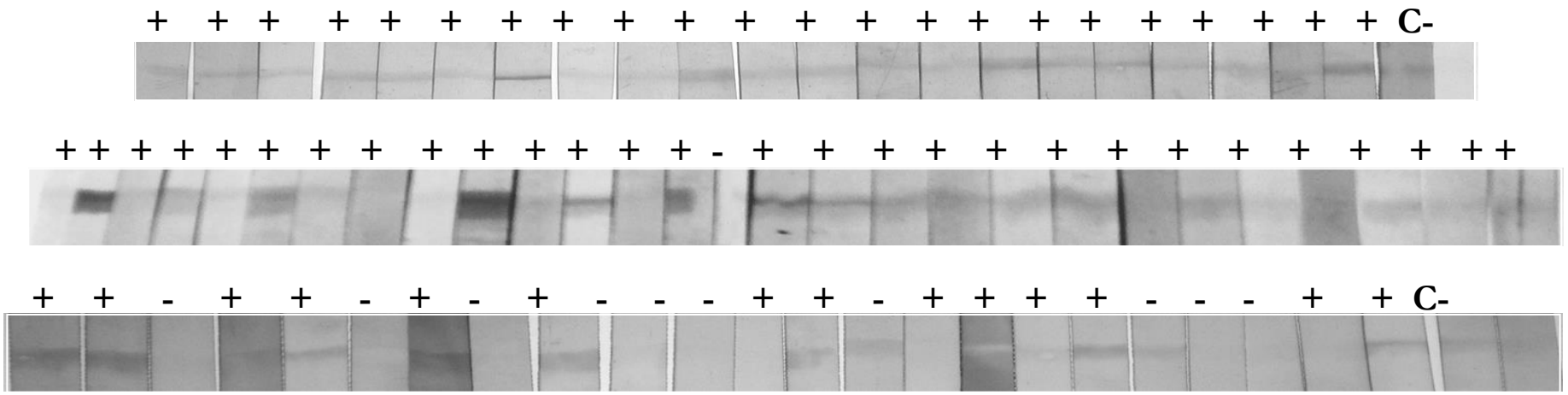
Detection of AEP catalytic activity in refolded recombinant *Ov*-AEP-1 and *O. viverrini* ES products



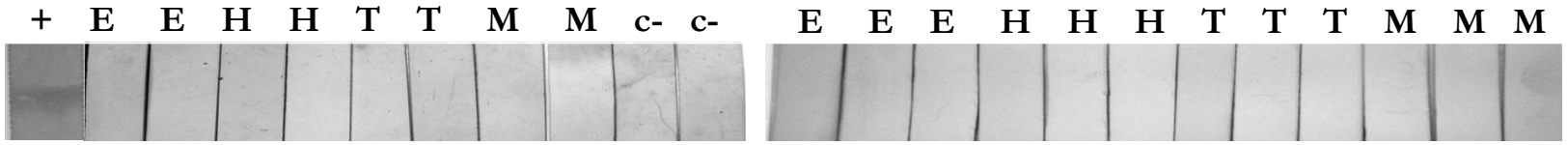
Recombinant *Ov*-AEP-1 was recognized by serum antibodies from opisthorchiasis patients

- The sensitivity = 85% (64/75).
- The specificity = 100% (Hookworm, minute intestinal fluke, *Taenia* and Echinostome).
- The positive and negative predictive values are 100% and 67% respectively.

A.



B.



Development of immunodiagnostic tool using recombinant OV-AEP1 as a specific Ag

Eukaryotic system

- **Yeast; *Saccharomyces cerevisiae, Pichia Pastoris***

- ***Baculovirus-infected cells;* insect cell (Sf9) or** mammalian cells (HeLa)

Commercial kit for protein expression

thermofisher.com/th/en/home/life-science/protein-biology/protein-expression/yeast-protein-expression.html

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- Pichia Expression Systems

Gibco Protein Expression System Tool

Gibco Protein Expression Protocol Calculator

Protein Expression Essentials

Protein Expression Optimization

Bacterial Protein Expression

Mammalian Protein Expression

Scalable recombinant protein expression in *Pichia pastoris*

Yeast strains have proven to be extremely useful for the expression and analysis of eukaryotic proteins, and are ideally suited for large-scale production.

Features:

- Suitable for many posttranslational modifications with well-characterized genetics
- Grows quickly in defined medium
- Easy to use and less expensive than mammalian cells
- Adapts well to fermentation

Protein vector selection tool

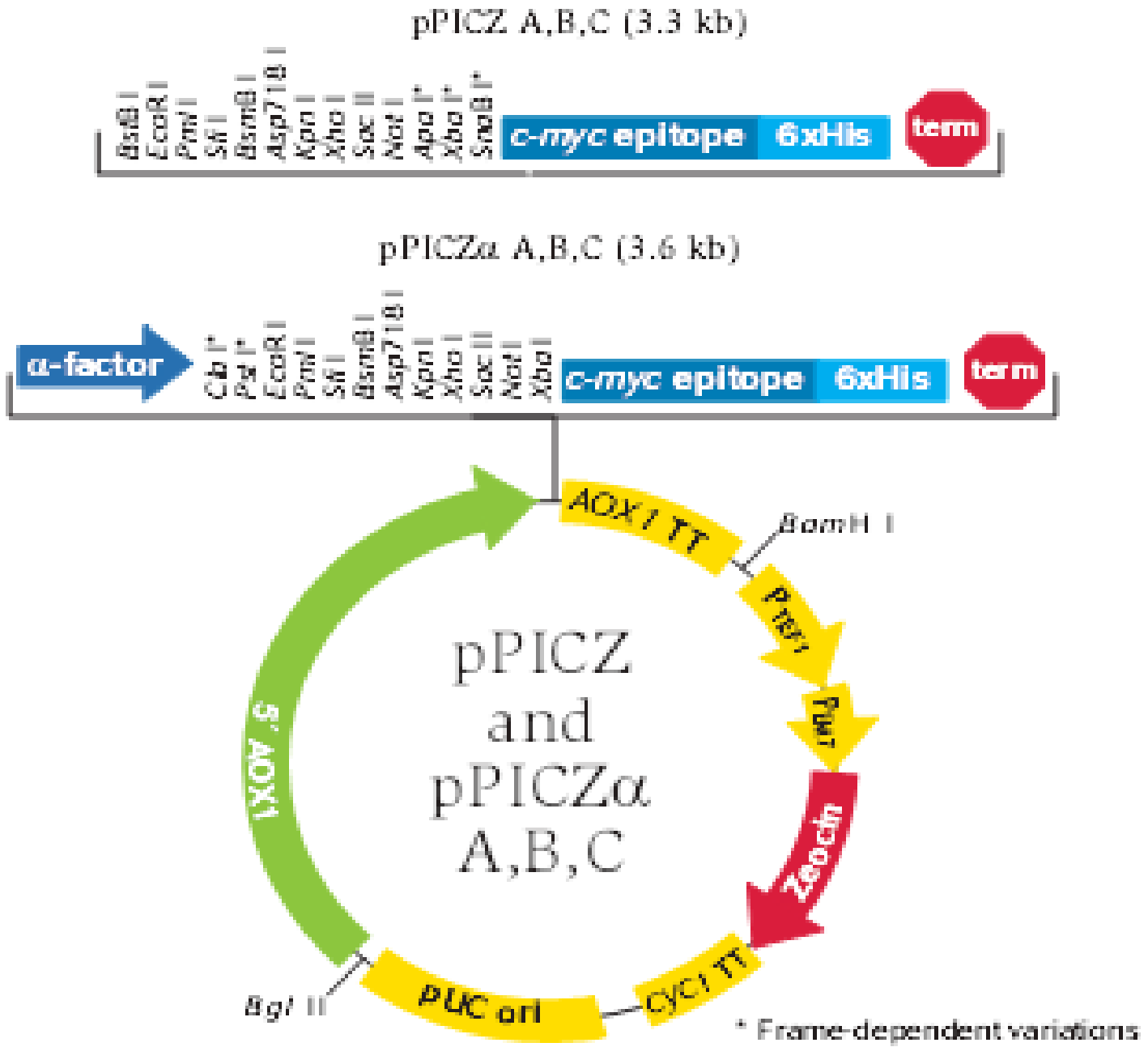
Give Feedback

Yeast expression methods

Expression of LGM gene in yeast

The full-length of LGM gene was clone into pPIC ZαA expression vector and transformed into competent *Pichia pastoris* strain X-33

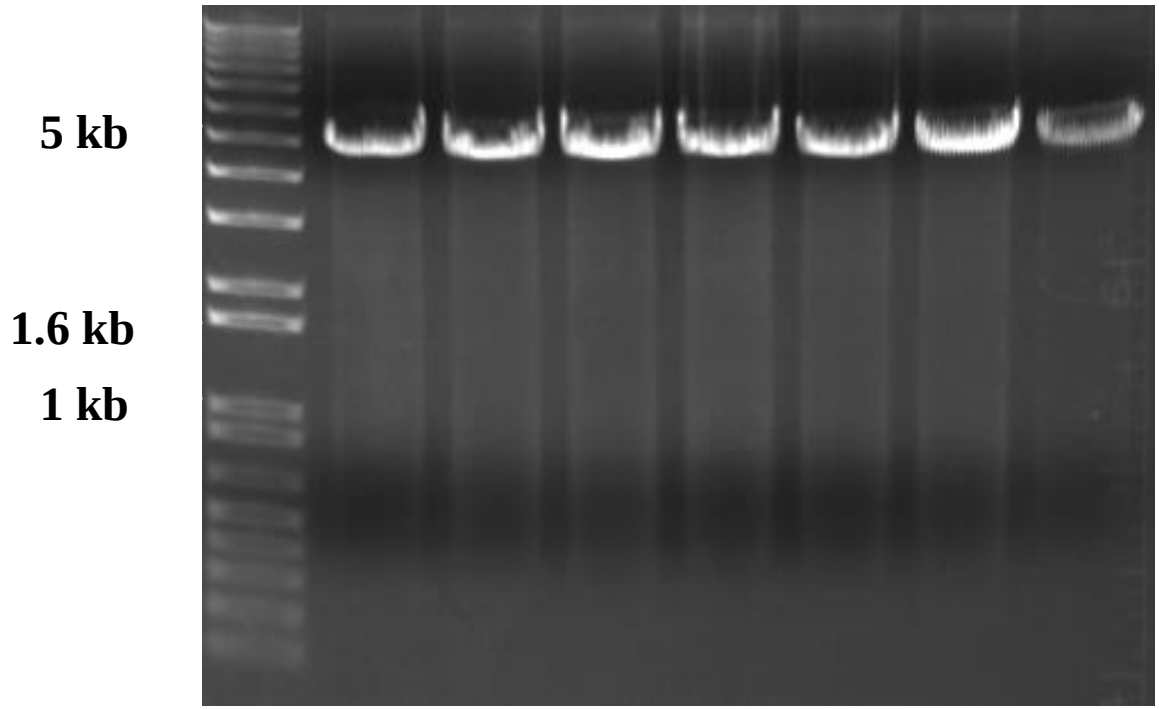
1. 5′OVLGM: 5′- gCg CgC gAA TTC gCA Tgg TTA ggC gCT gTT
2. 3′OVLGM: 5′- CgC gCg gCg gCC gCg gAA CAg ACT TCA TgA AC
3. 5′AOX1: 5′- gAC Tgg TTC CAA TTg ACA AgC
4. 3′AOX1: 5′- gCA AAT ggC ATT CTg ACA TCC


pPICZ A,B,C (3.3 kb)
BstB I
EcoR I
Pme I
Sfi I
BsmB I
Asp718 I
Kpn I
Xho I
Sac II
Not I
Apa I*
Xba I*
SnaB I*
c-myc epitope
6xHis
term
pPICZα A,B,C (3.6 kb)
α-factor
Cla I*
Pst I*
EcoR I
Pme I
Sfi I
BsmB I
Asp718 I
Kpn I
Xho I
Sac II
Not I
Xba I
c-myc epitope
6xHis
term
AOX1 TT
BamH I
P TEF1
PuvI
Zeocin
CYC1 TT
pUC ori
Bgl II
5' AOX1
pPICZ
and
pPICZα
A,B,C
* Frame-dependent variations

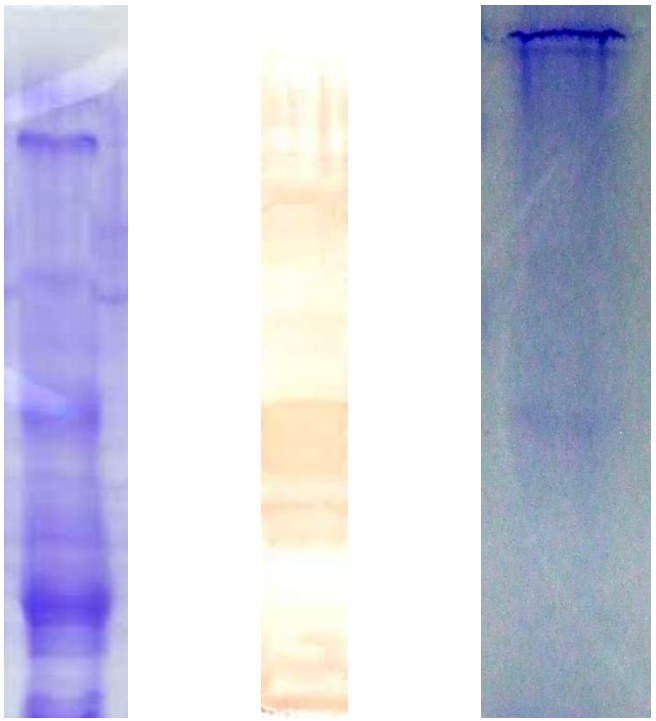
Protocol

- Produce recombinant plasmid clone
- Transfect into yeast cell
- Induction ; methanol induction
- Collect protein from supernatant
- Purify

Linearize the recombinant plasmid for transformation into *Pichia pastoris*



Production of recombinant *OV*-AEP-1 in *Pichia pastoris*



47 kDa

Example work

Parasitology Research (2019) 118:3419–3427
https://doi.org/10.1007/s00436-019-06488-3

HELMINTHOLOGY - ORIGINAL PAPER

Check for updates

Recombinant *Opisthorchis viverrini* tetraspanin expressed in *Pichia pastoris* as a potential vaccine candidate for opisthorchiasis

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Tetraspanins (TSPs) are transmembrane proteins containing four transmembrane domains interspersed by a small extracellular loop (SEL) and a large extracellular loop (LEL). In platyhelminths, TSPs are distributed throughout the tegumental membranes and have also been found in secreted extracellular vesicles (EVs) (Chaiyadet et al. 2015; Cwiklinski et al. 2015; Nowacki et al. 2015; Sotillo et al. 2016; Zhu et al. 2016). TSPs from *O. viverrini* (*Ov*-TSPs) have been found in both EVs and the tegument of adult flukes (Chaiyadet et al. 2017a; Chaiyadet et al. 2015; Piratae et al. 2012) and are essential for maintaining the integrity of the adult fluke tegument (Chaiyadet et al. 2017b; Piratae et al. 2012).

Despite substantial effort and many publications focusing on antigen discovery and efficacy assessment in animal models, there is still no vaccine available for any human helminth infection. Previous studies describing experimental vaccines against opisthorchiasis in animal models revealed partial protection with crude somatic antigen (Jittimanee et al. 2012) and irradiated larval stage (Papatpremsiri et al. 2016). Several vaccine candidate molecules have been identified from transcriptomics (Laha et al. 2007) and proteomics (Mulvenna et al. 2010) studies of the *O. viverrini* secretome; however, *Ov*-TSP is the only subunit vaccine tested thus far (Chaiyadet et al. 2019). Noteworthy, a TSP from *S. mansoni* is a promising vaccine candidate against schistosomiasis (Tran et al. 2006) and has completed a phase 1 clinical trial (Tebeje et al. 2016). Moreover, we showed that antibodies to an *O. viverrini* TSP were able to block the uptake of fluke EVs by human cholangiocytes (Chaiyadet et al. 2015), the cells that line the bile ducts and are intimately exposed to *O. viverrini* during chronic human infections.

In this study, we have produced the large extracellular loop of *O. viverrini* tetraspanin-2 (r*Ov*-LEL-TSP-2) as a recombinant protein in the yeast *Pichia pastoris* and evaluated its immunogenicity and efficacy in the hamster challenge model of opisthorchiasis.

Materials and methods

Production and purification of recombinant *Ov*-LEL-TSP-2

cDNA sequence corresponding to the LEL of *Ov-tsp-2* was obtained by PCR as previously described (Chaiyadet et al. 2017a) using specific primers that included *Eco*R I and *Not* I restriction enzyme sites (indicated with bold and underlined type). The primers used were *Ov*-TSP2-EC2F (5′ ACGC**GAATTC**CGCGATAAGATCCCCGG-3′) and *Ov*-TSP2-EC2R (5′ACGC**GCGGCCGC**CTGG ATGAACTCTTCGAC-3′). The resulting amplicon was cloned into the plasmid pPICZαA resulting in pPICZα-*Ov-lel-tsp-2*, and the recombinant protein was produced in *P. pastoris* X33 strain following the manufacturer's instructions with slight modifications. Briefly, pPICZα-*Ov-lel-tsp-2* plasmid was linearized with *Sac* I restriction enzyme and transfected into *P. pastoris* X33 using electroporation (MicroPulser Electroporator, BioRad, USA). Selected transfected colonies containing pPICZα-*Ov-lel-tsp-2* were inoculated in YPD culture medium containing Zeocin (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 100 μg/ml Zeocin), and grown overnight at 28 °C on a shaking incubator. The culture was inoculated into BMGY (1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) d-biotin, and 1% (v/v) glycerol) containing 100 mM potassium phosphate, pH 6.0, and incubated for 24 h at 28 °C with shaking. To induce expression of r*Ov*-LEL-TSP-2, the cell pellet was resuspended in 200 ml of BMMY medium (100 mM potassium phosphate, pH 6.0, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base, 4×10^{-5} % (w/v) d-biotin, and 1% (v/v) methanol), incubated with shaking at 28 °C for 72 h at 250 rpm, and 100% methanol was added to a final concentration of 1% every 24 h. The supernatant containing r*Ov*-TSP-2 was collected and concentrated by Amicon ultra-15 centrifuge filter unit, 3 kDa MWCO (Merck, Germany). Lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM Imidazole, pH 8.0) was added to the supernatant prior to purification with a nickel–nitrilotriacetic acid (Ni-NTA) affinity column (Thermo Fisher Scientific, USA). The purified protein was dialyzed against 20 mM HEPES using an Amicon ultra-15 centrifuge filter unit, 3 kDa MWCO. The concentration of the purified r*Ov*-LEL-TSP-2 protein was measured by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

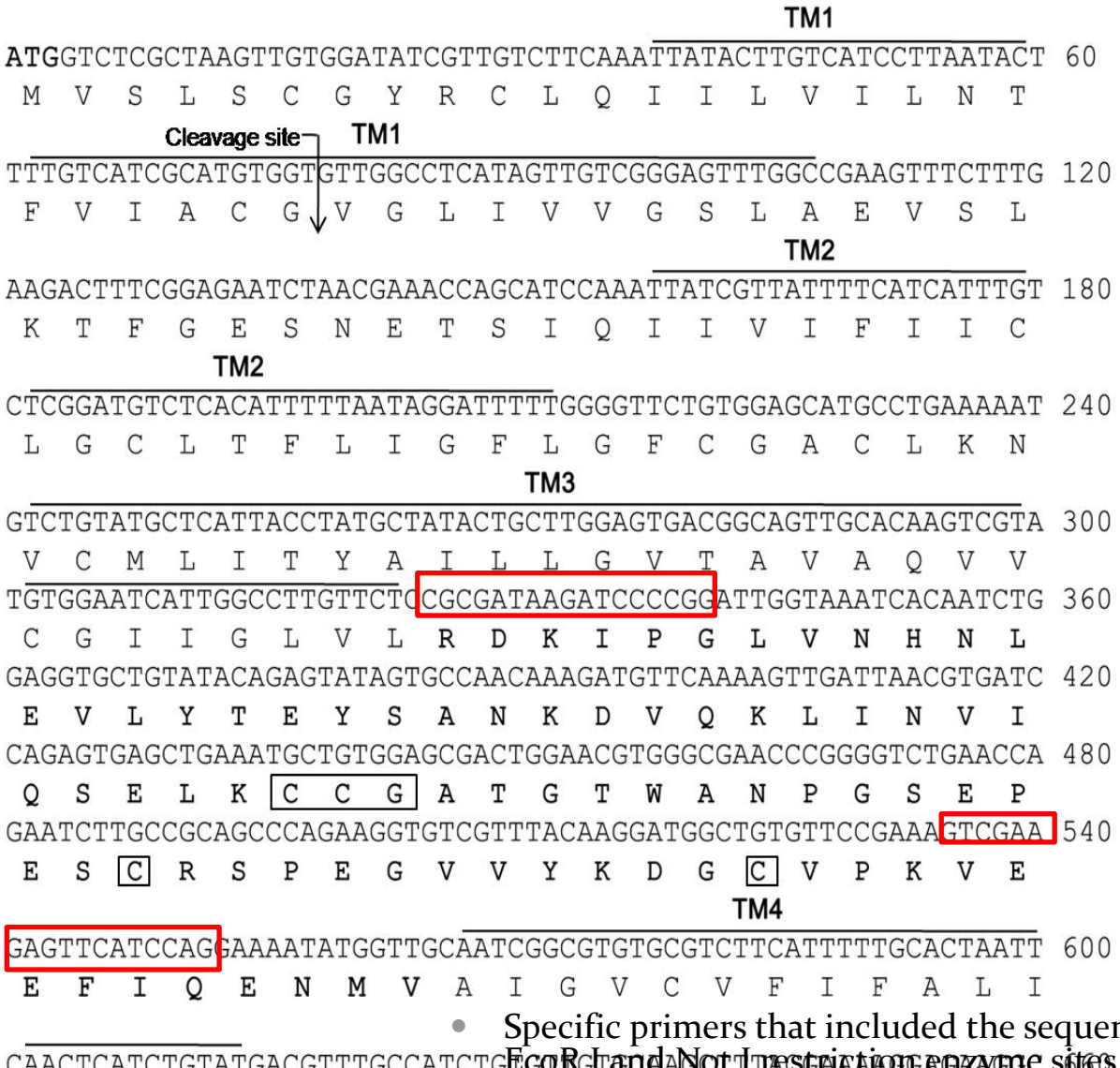
Immunoblot analysis of the recombinant *Ov*-LEL-TSP-2 protein

Purified r*Ov*-LEL-TSP-2 protein was separated on a 15% SDS–PAGE gel and transferred onto a nitrocellulose membrane (Mini Trans-Blot Cell, Bio-Rad). The membrane was blocked with 5% skim in PBS containing 0.1% Tween-20 (PBST) for 2 h, at room temperature and slight shaking. The membrane was, then, washed three times with PBST and incubated with rabbit anti-*Ov*-LEL-TSP-2 antiserum as previously described (Chaiyadet et al. 2017a) (diluted at 1:2,000 in PBST) overnight at 4 °C. Finally, the probed membrane was washed three times with PBST and incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:1,000 in PBST) at room temperature for 2 h. The colorimetric signal was developed using LuminataTM Forte Western HRP Substrate (Millipore, USA).

Preparation of *O. viverrini* metacercariae

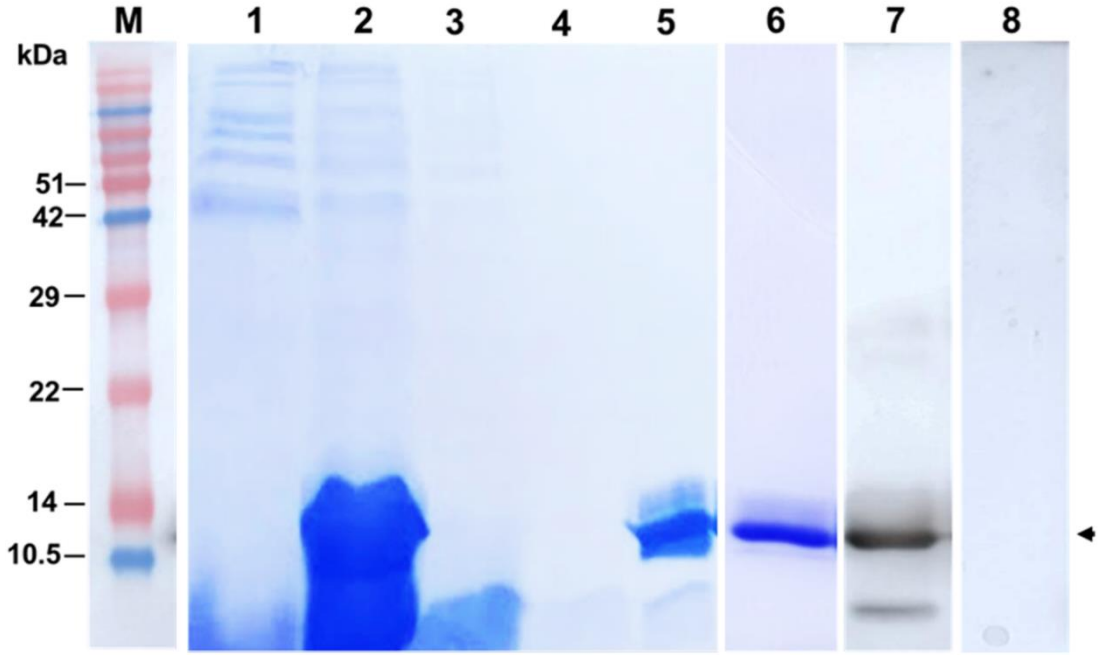
O. viverrini metacercariae were collected as previously described (Laha et al. 2007). Briefly, naturally infected cyprinid

- Specific primers that included the sequences for the EcoR I and Not I restriction enzyme sites (indicated with bold and underlined type), Ov-TSP2-EC2F (5'ACGC**GAATTC**CGCGATAAGATCCCCGG-3') and Ov-TSP2-EC2R (5'ACG**CGCGGCCGC**CTGGATGAACTCTTC GAC-3').



- Specific primers that included the sequences for the EcoR I and Not I restriction enzyme sites (indicated with bold and underlined type), Ov-TSP2-EC2F (5'ACGC**GAATTC**CGCGATAAGATCCCCGG-3') and Ov-TSP2-EC2R (5'ACG**CGCGGCCGC**CTGGATGAACTCTTC GAC-3').

SDS-PAGE and western blot analysis of rOv-TSP-2 expressed in *Pichia pastosis*



Protein marker (lane M), pre-induction supernatant (lane 1), methanol-induction supernatant (lane 2), flow through (lane 3), wash (lane 4), eluted protein (lane 5), purified protein in 20 mM HEPES (lane 6), western blot of rOv-LEL-TSP-2 probed with rabbit anti-Ov-TSP-2 serum (lane 7), western blot of 20 mM HEPES buffer probed with rabbit anti-Ov-TSP-2 serum-negative control (lane 8). The molecular weight of rOv-LEL-TSP-2 protein is approximately 12 kDa.