CHAPTER 3
MATERIALS AND METHODS
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3.1 MOLECULAR METHODS

3.1.1. Polymerase Chain Reaction (PCR)

Programmable Thermal Cycler (DNA Engine, BioRad, USA) was used for performing one step RT-PCR and PCR. The reaction was carried out using SS III one Step RT-PCR with Platinum Taq DNA polymerase (Sigma Aldrich, USA) along with high fidelity Pfu polymerase (Promega, USA) wherever required.

3.1.1.1 cDNA synthesis and PCR amplification for cloning

1. CHIKV mRNA (kind Gift from Dr. Sudhanshu Vrati, National Institute of Immunology, New Delhi) was used as template to amplify all nine genes of CHIKV using gene specific primers.

2. A 25 µl reaction was performed to amplify each gene for cloning. The PCR reaction mix was prepared as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Reaction Mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Template RNA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Sense primer</td>
<td>0.4 pmol</td>
</tr>
<tr>
<td>Anti-sense primer</td>
<td>0.4 pmol</td>
</tr>
<tr>
<td>SuperScript III RT/Platinum Taq</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>Variable</td>
</tr>
</tbody>
</table>

3. The thermal cycler was programmed for the PCR reaction as follows:
<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. cDNA synthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT cycle</td>
<td>50 °C</td>
<td>30 min</td>
</tr>
<tr>
<td><strong>2. PCR Amplification:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 Initial Denaturation</td>
<td>94 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>2.2 Amplification - 30 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>20 sec*</td>
</tr>
<tr>
<td>Annealing</td>
<td>54 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>1 min/kb</td>
</tr>
<tr>
<td>2.3 Final Extension</td>
<td>68 °C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

* After 2\textsuperscript{nd} cycle of amplification, paused the PCR cycle and added 0.3 \textit{Pfu} µl DNA polymerase to reaction mix and continued the reaction cycle.

### 3.1.1.2 DNA Agarose Gel Electrophoresis

1. Amplified PCR products were electrophoresed on 1.2 % (w/v) agarose gel prepared in 1X TAE buffer (Appendix A.1) containing 0.5 µg/ml Ethidium bromide (EtBr, Appendix B).
2. Each sample was mixed with 6X sample loading dye (Appendix A.1) before loading into the well.
3. DNA molecular weight marker (Genei, India) of 1 kb or 100 bp was used as a reference.
4. Samples were electrophoresed at 100 V till the dye front reached upto 3/4th of the gel length and visualized under UV Gel Doc (BIORAD).

### 3.1.1.3 Gene Amplification by PCR for Cloning

1. CHIKV structural genes cloned in TOPO sequencing vector were used as template for amplification using gene specific primers.
2. Initially, an analytical reaction of 20 µl was performed to standardize the PCR conditions.
3. Following this, a preparative reaction of 100 µl was performed to get sufficient amount of amplified DNA product for cloning.
4. The PCR reaction mixture was prepared as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>1X</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>dNTPs (10 mM mix)</td>
<td>0.25 mM each</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5 pmol</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5 pmol</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 - 10 ng/reaction</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase</td>
<td>6 U/reaction</td>
</tr>
<tr>
<td><em>Pfu</em> DNA polymerase</td>
<td>1 U/reaction</td>
</tr>
</tbody>
</table>

5. The thermal cycler was programmed for the PCR reaction as follows:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial Denaturation</td>
<td>95 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>2. Amplification: 30 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C (variable)</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min/kb</td>
</tr>
<tr>
<td>3. Final Extension</td>
<td>72 °C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

6. The PCR products were analysed by agarose gel electrophoresis as discussed in section 3.1.1.2. The DNA molecular weight markers used for reference of 1 kb or 100 bp were (Fermentas, USA).

### 3.1.1.4 Colony PCR Screening

To screen positive colonies after transformation Colony PCR was performed using gene or vector specific primers.

1. A reaction mixture of 10 µl was prepared as discussed in section 3.1.2.
2. Picked a single colony per reaction, from the LB agar plate (Appendix A.2) containing cells transformed with ligation mixture/plasmid and resuspended in PCR reaction mixture.

3. The thermal cycler was programmed for the following cycle:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>25 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 °C (variable)</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min/kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

4. The PCR products were analysed by agarose gel electrophoresis as described in section 3.1.1.2. DNA molecular weight markers used.

3.1.2. Purification of Amplified PCR Products

The PCR obtained after amplification were purified using GenElute PCR clean-up kit (Sigma USA) as per manufacturer’s protocol. This procedure removes excess primers, nucleotides, DNA polymerase and salts from the amplified product.

1. Placed a spin column was into a 2 ml collection tube. To prepare the column for purification, added 500 µl of column preparation solution to the column. Centrifuged the column for 1 min at 12,000 rpm (Eppendorf Centrifuge 5415 R, Standard Rotor F45-24-11, 45° fixed angle, 8.5 cm radius). Discarded the flow through.

2. Mixed binding buffer (five times the volume of the PCR product) with the PCR product and transferred the mixture to the prepared binding column. Centrifuged for 1 min at 12,000 rpm (Eppendorf Centrifuge 5415 R, Standard Rotor F45-24-11, 45° fixed angle, 8.5 cm radius) and discarded the flow through.

3. Washed the spin column twice with membrane wash solution (700 µl each) and centrifuged for 1 min at 12,000 rpm to remove the solution from the column. A free spin was given to remove any residual wash solution.
4. Transferred the spin column to a fresh 2 ml collection tube.
5. Added 35 µl of Nuclease free water (NFW) to the center of the membrane. Incubated it at RT for 1 min followed by centrifugation to finally elute the DNA.
6. Step 5 was repeated with 35 µl of nuclease free water.
7. Purified DNA was stored at -20 °C.

3.1.3. **Restriction Endonuclease Digestion**

Restriction Digestion of amplified PCR products and/or plasmids with Restriction Endonucleases (RE) was executed to either create the compatible ends in plasmids and inserts enabling them to ligate or for confirmation of recombinant clones.

3.1.3.1 **Double Digestion with Restriction Endonucleases**

1. To clone in multiple cloning site (MCS) based vectors, both insert and plasmid were treated with same combination of restriction enzymes (Fast digest enzymes, Fermentas).
2. A 100 µl enzymatic reaction was set as following:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X fast digest buffer</td>
<td>1 X</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>Insert/plasmid DNA</td>
<td>1 µg*</td>
</tr>
<tr>
<td>Restriction Endonuclease 1</td>
<td>5 U/µg of DNA</td>
</tr>
<tr>
<td>Restriction Endonuclease 2</td>
<td>5 U/µg of DNA</td>
</tr>
</tbody>
</table>

*For confirmation of cloning the reaction is 20 ul and plasmid DNA concentration is 500 ng.*

3. The reaction mixture was incubated at 37 °C for 2 h. After digestion, the DNA samples were purified using PCR cleanup kit (Sigma, USA) according to manufacturer’s protocol (Section 3.1.2).
3.1.3.2 Digestion with Bsa I Enzyme

1. To clone in stuffer based vector (pCAK/pLTA), the plasmid was digested with Bsa I (New England Biolabs; NEB, UK) enzyme.

2. Enzymatic reaction carried out is as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB buffer 4</td>
<td>1 X</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5 µg</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>1 X</td>
</tr>
<tr>
<td>Bsa I</td>
<td>6U/µg</td>
</tr>
</tbody>
</table>

3. The reaction was carried out at 37 °C for 4 h.

   *Since Bsa I has a half-life of 1 h, enzyme was added to the reaction mixture after every 1 h of incubation.*

4. After completion of digestion reaction, the complete reaction mixture was loaded on 1% agarose gel and electrophoresed at 100 V to separate the vector backbone and stuffer fragment completely.

5. The vector backbone was excised and gel purified from agarose gel using Gel Extraction kit (Sigma, USA) according to manufacturer’s protocol.

3.1.4. De-phosphorylation using Calf Intestinal Alkaline Phosphatase (CIP)

To remove 5’ phosphate group from linearized vector dephosphorylation was performed, to prevent self-ligation and recircularization.

1. The reaction components are as follows (100 µl):

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB buffer 3</td>
<td>1 X</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>Template DNA</td>
<td>500 ng</td>
</tr>
<tr>
<td>CIP (NEB, UK)</td>
<td>0.5 U/µg of DNA</td>
</tr>
</tbody>
</table>
2. Incubated the reaction mix for 1 h at 37 °C.
3. The DNA was purified using PCR cleanup kit (Sigma, USA) according to manufacturer’s protocol (Section 3.1.2).

3.1.5. T4 DNA Polymerase Reaction

To clone CHIKV genes in pCAK/pLTA vector, PCR products were treated with T4 DNA polymerase to produce compatible ends to Bsa I digested vector.

1. A 100 µl reaction mix was prepared as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X T4 DNA polymerase buffer</td>
<td>1 X</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 µg</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>7.5 U/µg</td>
</tr>
</tbody>
</table>

2. Incubated the reaction mix at 11 °C for 20 min. Heat inactivated the enzyme at 75 °C for 10 min.
3. Purified the reaction mixture using PCR cleanup Kit (Sigma, USA) as described in section 3.1.2.

3.1.6. Ligation Reaction

1. For each ligation reaction, 50 ng vector was used at a molar ratio of 1:5 (vector: insert).
2. Following components were added to a ligation reaction of 20 µl final volume.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector DNA</td>
<td>~50 ng</td>
</tr>
<tr>
<td>Insert DNA</td>
<td>~250 ng</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>T4 DNA ligase buffer (10X)</td>
<td>1X</td>
</tr>
<tr>
<td>T4 DNA ligase (Fermentas)</td>
<td>5 U</td>
</tr>
</tbody>
</table>
3. The reaction mixture was incubated at 22 °C for an hour followed by 4 °C for another 1 h. The mixture was used directly for transformation in E. coli DH5α cells.

### 3.2 CLONING IN SEQUENCING AND BACTERIAL EXPRESSION SYSTEM

#### 3.2.1 Bacterial Strains

Two bacterial strains of *E. coli* were used in this study. (i) DH5α strain, which was used for transformation of ligation mixture, for purification of plasmid DNA and (ii) BL21 λ(DE3) strain, which was used for protein expression studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Invitrogen</td>
<td>φ80d lacZΔM15, recA1, endA1, gyrAB, thi-1, hsdR17(rK- mK+), supE44, relA1, deoR, Δ(lacZYA-argF) U169, phoA</td>
</tr>
<tr>
<td><em>E. coli</em> BL21</td>
<td>Invitrogen</td>
<td>F-, ompT, hsdSB(rB, mB+), dcm, gal, λ(DE3)</td>
</tr>
</tbody>
</table>

#### 3.2.2 Sequencing Vector System

To clone and characterize individual genes of CHIKV TOPO 2.1 TA cloning system (Invitrogen, Life Technologies, USA) was used. This vector system enables ligase free cloning of gene into the vector. The reaction for cloning in TOPO vector (Figure 3.1) was prepared as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>For cloning in chemically competent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh PCR product obtained after</td>
<td></td>
</tr>
<tr>
<td>One-strep PCR</td>
<td>4 µl</td>
</tr>
<tr>
<td>Salt Solution</td>
<td>1 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Variable</td>
</tr>
<tr>
<td>TOPO Vector</td>
<td>1 µl</td>
</tr>
<tr>
<td><strong>Total Reaction volume</strong></td>
<td><strong>6 µl</strong></td>
</tr>
</tbody>
</table>

1. Mixed the reaction gently followed by incubation at room temperature (RT) for 30 mins.
2. This mixture was then used for transformation in *E. coli* DH5α cells.
Figure 3.1 Vector Map of 2.1 TOPO vector used for cloning CHIKV genes

Table 3.1: Characteristic features of sequencing vector

<table>
<thead>
<tr>
<th>Features</th>
<th>pCR 2.1 TOPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>3.9 kb</td>
</tr>
<tr>
<td>Origin of replication</td>
<td>pUC</td>
</tr>
<tr>
<td>Promoter</td>
<td>Lac</td>
</tr>
<tr>
<td>Resistance</td>
<td>Ampicillin and Kanamycin</td>
</tr>
</tbody>
</table>
3.2.3 Expression Vectors

The expression of desired proteins was achieved by cloning the gene of interest in different bacterial expression vectors.

Table 3.2: Characteristic features of bacterial expression vectors

<table>
<thead>
<tr>
<th></th>
<th>pGEX-4T3</th>
<th>pCAK</th>
<th>pLTA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>4.9 kb</td>
<td>5.5 kb</td>
<td>5.6 kb</td>
</tr>
<tr>
<td><strong>Origin of replication</strong></td>
<td>pBR322</td>
<td>ColA</td>
<td>ColE1</td>
</tr>
<tr>
<td><strong>Tag</strong></td>
<td>GST (N terminal)</td>
<td>Strep (N terminal)</td>
<td>His (N Terminal)</td>
</tr>
<tr>
<td><strong>Promoter</strong></td>
<td>Lac</td>
<td>Ara</td>
<td>Tet</td>
</tr>
<tr>
<td><strong>Resistance</strong></td>
<td>Ampicillin</td>
<td>Kanamycin</td>
<td>Ampicillin</td>
</tr>
</tbody>
</table>

Figure 3.2: Vector maps of pGEX-4T3, pCAKExp10, pLTAExp20 bacterial expression vectors

In steps 3.2.4 to 3.2.10 Bacterial cells were grown in NBS bench top refrigerated incubator shaker (model: Innova 42, Stroke/Orbit 2.54 cm). All the centrifugation steps involving large culture volumes were carried out in Hermel 383K (45° fixed angle, 9.6 cm radius). Small culture volumes were centrifuged in Eppendorf Centrifuge 5415 R (Standard Rotor F45-24-11, 45° fixed angle, 8.5 cm radius).
3.2.4 Preparation of Competent Cells

3.2.4.1 Preparation of Chemically Competent Bacterial Cells
1. Inoculated a single isolated colony from LB agar plate (Appendix A.2) in 5 ml LB broth (Appendix A.2) and incubated at 37 °C overnight with shaking at 220 rpm.
2. Next day transferred 4 ml of primary (overnight) culture a flask containing 400 ml of LB broth. Incubated the culture (secondary) at 37 °C with shaking at 220 rpm until the O.D. reached 0.4-0.5.
3. When the O.D.₆₀₀ reached 0.4-0.5, arrested the growth of the culture by incubating it on ice for 20 min.
4. To harvest the cells, the secondary culture was centrifuged at 4 °C for 5 min at 6000 rpm. Discarded the supernatant and resuspended the pellet in first in 200 ml (half the culture volume) of ice-cold 50 mM CaCl₂ (Appendix A.1). After incubation centrifuged the culture and 4 °C for 5 min at 6000 rpm.
5. Resuspended the pellet in 40 ml (one fifth of the culture volume) of ice-cold 50 mM CaCl₂ gently and centrifuged at 4 °C for 5 min at 6,000 rpm and discarded the supernatant.
6. Resuspended the pellet was in 4 ml of 50 mM CaCl₂ containing 20% glycerol.
7. The cells were then aliquoted and stored at -80 °C.

3.2.4.2 Preparation of Electrocompetent bacterial cells
1. Inoculated a single colony of *E. coli* DH5α cells from LB agar plate (Appendix A.2) was in 5 ml 2X YT broth (Appendix A.2). Incubated the primary culture at 37 °C overnight with shaking at 220 rpm.
2. Following overnight incubation, 4 ml of primary culture was transferred to a flask containing 400 ml of 2X YT broth (Appendix A.2). The secondary culture was then incubated at 37 °C with continuous shaking at 220 rpm to grow until the O.D.₆₀₀ reached 0.7-0.8.
3. The growth of the secondary culture was then arrested by keeping it on ice for 30 min.
4. Centrifuged the chilled secondary culture at 4 °C for 5 min at 6,000 rpm to harvest the bacterial cells. Discarded the supernatant.
5. Resuspended the pellet in 160 ml (for 200 ml culture) of ice cold autoclaved distilled water gently and centrifuged at 4 °C for 5 min at 6,000 rpm. Discarded the supernatant and again
resuspended the pellet in ice cold 10% glycerol (Appendix A.1, 8 ml 10% glycerol for 100 ml culture) followed by centrifugation at 4 °C for 5 min at 6,000 rpm.

6. Resuspended the cells in 0.8 ml of 10% glycerol (Appendix A.1).
7. Finally the electrocompetent bacterial cells were aliquoted and stored at -80 °C.

3.2.5 Transformation in Chemically Competent Bacterial Cells
1. To 40 µl chemically competent cells added 1 µl of plasmid DNA (1 ng/ul) or 4 µl for ligation mix (generated in section 3.1.6) in a fresh 1.5 ml tube and the mixture was kept on ice for 20 min.
2. Subjected the transformation mixture to heat shock treatment at 42 °C for 1 min followed by chilling for 2 min in ice.
3. Added 200 µl of antibiotic free LB media (Appendix A.2) to the transformation mixture and incubated it at 37 °C for 1 h with shaking at 220 rpm.
4. Following incubation spread plated 100 µl of cells on LB agar (Appendix A.2) containing appropriate antibiotic for the selection of the transformed plasmid. Incubated the plate at 37 °C overnight.

3.2.6 Isolation of Plasmid DNA
3.2.6.1 Alkaline Lysis Method
1. Inoculated a single bacterial colony from LB agar plate (Appendix A.2) in 5 ml LB broth (Appendix A.2) containing appropriate antibiotic selection and incubated at 37 °C overnight with shaking at 220 rpm.
2. Harvested 3 ml of cells from the overnight grown culture at RT for 5 min at 6,000 rpm. Discarded the supernatant and resuspended the cell pellet in 200 µl of resuspension buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose, 100 µg/ml RNase A and 4 mg/ml lysozyme) gently.
3. Added 400 µl of freshly prepared alkaline SDS solution (Appendix A.1). The contents of the tube were mixed gently by inversion to ensure bacterial cell lysis and then incubated on ice for 5 min.
4. Added 300 µl of ice cold potassium acetate solution (Appendix A.1) to neutralize the lysis solution and precipitated the chromosomal DNA along with proteins. Invert mixed the
suspension gently and incubated on ice for 10 min. Centrifuged at 4 °C for 15 min at 12,000 rpm.
5. Collected the supernatant carefully in a fresh tube and added 500 µl of isopropanol. Invert mixed and incubated on ice for 5 min to precipitate the plasmid DNA.
6. Centrifuged the solution at 4 °C for 15 min at 12,000 rpm. Discarded the supernatant. Washed the pellet with 70% ethanol.
7. The pellet was air dried completely after decanting ethanol and resuspended in 50 µl of nuclease free water.
8. The plasmid DNA was stored at -20 °C.

3.2.6.2 Plasmid DNA Isolation using Miniprep Kit

Plasmid DNA was purified using plasmid Miniprep kit (Sigma, USA). This kit is based on the principle of alkaline lysis but uses a column support which allows binding of DNA to silica column, followed by its washing and purification. Each step was performed according to manufacturer protocol. All the steps were carried out at RT.
1. Pelleted 3 ml of overnight grown culture at 12,000 rpm. Discarded the supernatant.
2. Added 200 µl of the resuspension solution containing RNase to the bacterial cell pellet. Vortexed till the cells were resuspended homogenously.
3. Lysed the resuspended cells by adding 200 µl of lysis solution. Mixed the contents by gentle inversion 6-8 times for less than 5 min.
4. Precipitated the cell debris with 350 µl of neutralization solution. Gently invert mixed 4-6 times. Centrifuged for 10 min at 12,000 rpm to pellet the cell debris.
5. In the meantime, to prepare the miniprep binding column added 500 µl of column preparation solution into the spin column and centrifuged it at 12,000 rpm for 1 min. Discarded the flow through.
6. To this column, loaded the supernatant and centrifuged for 1 min at 12,000 rpm. Discarded the flow through.
7. Washed the column twice with 700 µl wash solution followed by a free spin for removing additional wash solution from the column.
8. To elute the plasmid DNA from the column, transferred the column to a fresh 1.5 ml microcentrifuge tube and added 70 µl of nuclease free water (NFW) to the center of column.
Incubated at RT for 1 min and centrifuged for 1 min at 12000 rpm. Stored the plasmid DNA at -20 °C.

3.2.7 Storage of Recombinant Plasmids

The recombinant plasmid containing the gene of interest where transformed in chemically competent *E. coli* DH5α cells. Plasmid DNA isolated from transformed cells using plasmid isolation kit (Sigma, USA) was stored at -20 °C. The cells containing recombinant plasmids were stored in 20% glycerol at -80 °C.

3.2.8 Overexpression, Solubilization and Purification of Recombinant Proteins

To carry out expression studies, gene of interest was first transformed in *E. coli* λ BL21 (DE3) cells competent cells. Transformation protocol has been described in section 3.2.5. The colonies obtained after transformation were then tested for expression. For growth of liquid cultures NBS bench top refrigerated incubator shaker (model: Innova 42, Stroke/Orbit 2.54 cm) was used.

3.2.8.1 Screening Bacterial Cells for Protein Expression

1. In a sterile culture tube containing 5 ml of LB broth (Appendix A.2) supplemented with appropriate antibiotic, inoculated a single isolated colony and incubated at 37 °C overnight with shaking at 220 rpm.
2. To 50 ml of LB broth (Appendix A.2) supplemented with appropriate antibiotic added primary culture (grown overnight) in a ratio of 1: 50 such that the OD₆₀₀ was ~0.1.
3. Allowed the secondary culture to grow at 37 °C for ~2 h with shaking at 220 rpm until its OD₆₀₀ reached 0.3 - 0.4.
4. At OD₆₀₀ = 0.3 - 0.4, aliquoted 1 ml culture as uninduced culture. Induced the remaining secondary culture with suitable inducer [1 mM IPTG (Appendix A.1) for pGEX-4T3, 0.5% arabinose (Appendix A.1) for pCAK and 50 ng/ml Anhydrotetracycline (Appendix A.1) for pLTA].
5. Incubated both induced and uninduced culture at 25 °C for 4 h with continuous shaking at 220 rpm.
6. After four hours of incubation, transferred 1 ml of induced culture in a 1.5 ml microcentrifuge tube for expression analysis. The remaining culture was transferred in 50 ml centrifuged tube. The cells were centrifuged at 4 °C for 5 min at 6,000 rpm. The supernatant was discarded and the pellet was stored at -80 °C.

7. Centrifuged the 1 ml aliquots of uninduced and induced culture at were 4 °C for 5 min at 6,000 rpm. Discarded the supernatant completely and resuspended the pellet in 400 µl of 1X sample buffer (Appendix A.1). Analyzed the samples for protein expression by SDS-PAGE.

### 3.2.8.2 Lysis of Induced Bacterial Cultures

The induced cultures were lysed with lysis buffer (IBA GmbH, Germany) according to the manufacturer’s protocol.

1. Resuspended the frozen pellets of 50 ml culture in 1.25 ml lysis buffer (IBA GmbH, Germany). Added 12.5 µl of protease inhibitor cocktail (100X; Clontech, USA) and 2.5 µl of lysozyme (1 µg/ml) and incubated on ice for 30 min with intermittent vortexing.

2. Following cell lysis, added 2.5 µl of DNase (20 ng/ml) to the suspension and incubated again for 15 min on ice.

3. Added 0.85 µl RNase (6 ng/ml) and incubation on ice for another 1 h.

4. Cellular protein extract was cleared from the cell debris by centrifugation at 4 °C for 40 min at 12,000 rpm and analysed by 10% SDS-PAGE for presence of protein of interest in cellular protein extract.

### 3.2.8.3 Solubilization of proteins present in incusion bodies

Solubilization of proteins obtained in inclusion bodies was achieved by treatment with sarkosyl.

1. Pellet obtained after cell lysis (discussed in section 3.2.8.2) was resuspended in solubilization buffer (SB) containing 10% sarkosyl (Appendix A.1). For 500 gm pellet 2 ml of SB was used.

2. Incubated the cell suspension at 16 °C/175 rpm overnight.

3. Next day, centrifuged the cell suspension at 13000 rpm for 10 min at 4 °C to recover the supernatant from cell debris.

4. Analysed the cell pellet and supernatant fraction on SDS-PAGE.
For purification of proteins, supernatant was diluted to 1% final concentration of sarkosyl with 1X PBS (Phosphate Buffer Saline). The sample was further dialysed in 1X PBS buffer for 20 hours with six changes of buffer at 4 °C. Cellulose acetate dialysis tubing with cut off of 12 kDa (Sigma Aldrich) was used for dialysis.

3.2.8.4 Purification of Fusion Proteins using Gravity Flow Columns
1. For purification of GST tagged proteins, Glutathione-superflow resin [Clontech, USA] was used whereas for Strep tagged protein, Strep-Tactin Sepharose [IBA GmbH, Germany] was used. The column was equilibrated with 2 Column bed Volume (CV) Buffer W (Wash buffer; Appendix A.1).

*Binding capacity of protein is dependent and normally lies between 50-100 nmol recombinant protein per ml of the bed volume.*

2. Loaded supernatant of cleared lysates on to the column. Incubated the column for 10-15 min at 4 °C.
3. After the cell lysates had entered the column completely washed it 5 times with 1 CV of Buffer W.
4. This was followed by elution where with 0.5 CVs of Buffer E (Elution buffer; Appendix A.1) was used and the eluate was collected in 0.5 CV fractions. This step was repeated 5 more times. The purification profile of the proteins was then analyzed on SDS-PAGE

3.2.9 Protein Expression Analysis
3.2.9.1 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples with sample buffer (final concentration 1X, Appendix A.1) were boiled at 100°C for 5 min and separated by 10% SDS-PAGE in 1X Tris-glycine buffer (Appendix A.1) according to the protocol described by Laemmlili. The Miniprotean SDS-PAGE apparatus (BioRad, USA) was used and assembled according to manufacturer’s protocol. All reagents used for preparation of gel are mentioned in appendix.

1. Mixed the reagents for a resolving gel with 10% acrylamide by gentle swirling and poured the solution immediately in the gap between the two glass plates such that it reaches three-
fourth of the plate length. Filled remaining one-fourth space with distilled water to prevent oxidation of the top surface.

2. Allowed the gel mix to set at RT for 20-25 min for making the resolving gel.

3. After this, discarded the water layer and added stacking gel with 5% acrylamide prepared using the reagents in appendix. Immediately inserted a comb to form the wells in such a manner that no bubble is introduced. Allowed the gel to polymerize for 15 min.

4. Once the gel was polymerized, removed the comb and rinsed the wells with distilled water. Placed the plates with gel in buffer tank containing 1X running buffer (Appendix A.1).

5. Boiled the protein samples at 100°C for 10 min and loaded into the wells. For reference Unstained protein ladder (10 - 200 kDa, Fermantas, USA) or prestained protein ladder (10 - 170 kDa, Fermantas, USA) was used to estimate protein molecular weight.

6. Electrophoresed the protein samples at 100 V till the sample front reached the end.

7. Remove the gel from plates and visualized the protein bands staining with coomassie brilliant blue stain (Appendix A.1). The background stain was removed from gel by incubating it in destaining solution (Appendix A.1).

### 3.2.9.2 Western Blot analysis

Protein samples were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore, USA) using BioRad Western blotting apparatus (BioRad, USA).

1. The PVDF membrane was charged as per manufacturer’s protocol. Briefly, the membrane was treated with methanol for 15 sec, followed by washing with MilliQ water for 2 min and finally incubated it in 1X transfer buffer (Appendix A.1) for 5 min.

2. For electro blotting arranged the transfer cassette by placing the sponge on negative side of the cassette. Then, placed a pre-wet 3 MM Whatman filter on it followed by gel, charged membrane, another Whatman filter and sponge.

3. Electroblotted the proteins onto PVDF membrane for 2 h at 100 mA.

4. Disassembled the apparatus after transfer and removed the PVDF membrane carefully. Placed it in blocking buffer (5% BSA in PBS; Appendix) for 1 h at RT to prevent non-specific background binding of primary and secondary antibodies to the membrane.

5. Washed the membrane one time with PBST for 5 min.
6. Dilute primary antibody in blocking buffer and incubate the membrane for 1.5 h at RT on a rocker. Removed excess of antibody by washing the membrane three times with PBST for 5 min each after incubation. The dilutions used for different antibodies are as follows:

<table>
<thead>
<tr>
<th>Primary Antibody*</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GST IgG</td>
<td>Sigma, USA</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Anti- His IgG</td>
<td>Sigma, USA</td>
<td>1:3000</td>
</tr>
<tr>
<td>Strep-Tactin HRP conjugate</td>
<td>IBA GmbH, Germany</td>
<td>1:4000</td>
</tr>
<tr>
<td>Anti-c-Myc</td>
<td>Sigma, USA</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>Santa Cruz, USA</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

*All the primary antibodies used in this study are monoclonal and raised in mouse. Anti-GST and anti-His mouse IgG were prepared in 1% BSA-PBS solution. Strep-Tactin HRP conjugate was made in biotin blocking buffer (bioting blocking reagent in PBS).

7. Incubate the membrane with secondary antibody [Goat Anti-Mouse conjugated with horseradish peroxidase (HRP)] diluted in PBST (1:2000 dilution, Appendix A.1) for 1.5 h at RT with shaking.

8. Washed the membrane three times for 5 mins each with PBST and finally with PBS. Developed the blot with 10 ml of 3, 3’-diaminobenzidine (DAB; 0.05 %, Appendix A.1 as substrate for HRP and hydrogen peroxide (0.1%) as reaction catalyst. Stopped the reaction with distilled water as the bands appeared on the blot.

3.2.10 Assays for Protein-Protein Interaction Analysis

ELISA and Pull down assays are two efficient methods for analysis of binary interaction between two proteins. In the current study, GST pull down assay was used to assay viral-viral protein interaction. ELISA and pull down using Strep-tactin resins were employed to study viral-host interactions. All centrifugation steps were done in Eppendorf Centrifuge 5415 R (Standard Rotor F45-24-11, 45° fixed angle, 8.5 cm radius).
3.2.10.1 GST Pull down Assay

1. Mixed together the soluble fraction (lysates) of fusion proteins (one GST and the other Strep tagged) in equal volume (500 μl each) and incubated at 4°C for 2 h with end-over-end mixing.

2. Meanwhile to remove ethanol from resin by centrifuged 500 μl of 50% glutathione resin was centrifuged at 4°C for 5 min at 2,200 rpm and washed with 1X PBS (Appendix A.1) thoroughly to remove the ethanol.

3. Equilibrated the resin with 250 μl of 1X PBS (Appendix A.1).

4. Loaded the protein mix onto the equilibrated resin and again kept at 4°C for 2 h with end-over-end mixing to allow binding of interacting protein complex to the resin.

5. Centrifuged the resin at 4°C for 5 min at 2,200 rpm. To remove nonspecific binding washed the resin 5 times with 2 CV (column volume) of 1X PBS. Collected the supernatant (flow through) obtained to analyse by SDS-PAGE and/or Western blotting.

6. To elute the bound protein complexes added 0.5 CV of 30 mM reduced glutathione in 50 mM Tris-Cl pH 8.0 and incubated it at RT for 15-20 min. The beads were centrifuged at 4°C for 5 mins at 2,200 rpm and eluates were collected. Repeated the step 5 times.

7. The eluates were analysed by western blotting along with flow through.

8. Each elute was analyzed using mouse anti-GST antibody followed by goat anti-mouse HRP conjugated secondary antibody to detect the bait. For prey protein, samples were treated with Strep-Tactin HRP conjugate.

   Presence of prey and bait protein in the same eluate detected with Strep-Tactin HRP conjugate and anti-GST antibody respectively, confirmed the binding of GST fusion protein with the glutathione resin and interaction. Binding of Strep fusion proteins with only GST tag was taken as negative control for the experiment.

3.2.10.2 Enzyme Linked Immunosorbant Assay (ELISA)

In protein interaction ELISA the test proteins were taken as Strep and GST fusions. The Strep fusion protein was coated onto the streptactin microtitre plates and anti-GST monoclonal antibody was used to detect the presence or absence of interaction among selected proteins.
1. Mixed the soluble fraction of Strep fusion protein with binding buffer (Appendix A.1) and coated the protein mix on the streptactin microtitre plate. Incubated the plate overnight at 4°C.

2. Next day, removed the coating solution was removed from the wells and washed the wells three times with 300 µl of PBST (Appendix A.1) to remove unbound proteins from the wells.

3. Blocked the wells with 100 µl of blocking buffer (2% BSA in PBS) for 1 h at RT.

4. Removed the blocking buffer and added lysates of Strep fusion proteins to the wells and incubated for 2 h at 4°C.

5. Washed the wells five times with PBST. Incubated the wells with 100 µl anti-GST primary monoclonal antibody (1:10,000 dilution in 1% BSA) for 1 h at RT.

6. Washed the wells with PBST and incubated with HRP conjugated secondary antibody (1:2,000 dilution in PBST) for 1 h at RT.

7. Following incubation with secondary antibody, washed the plate extensively with PBST and PBS (Appendix A.1) before addition of 100 µl TMB (3,3’,5,5’-tetramethylbenzidine) and incubated at RT for 15-20 min in dark.

8. Allowed the colour to develop, before stopping the reaction with 2N HCl. Absorbance was taken at 450 nm.

   Binding of only Strep fusions and only GST fusions in the absence of the counterpart protein partner with the Strep-tactin plate was taken as negative control. Appearance of blue coloured complex on addition of substrate indicates interaction among test pair proteins.

### 3.3 YEAST TWO-HYBRID SYSTEM

Viral-viral interactions of CHIKV structural proteins (sPs) were identified using Yeast two-hybrid (Y2H) system. Also, viral-host interaction of CHIKV envelope proteins E1 and E2 were screened from Mate & Plate library-human fetal brain (Clontech, USA). The cDNA library was constructed by homologous recombination mediated cloning in pGADT7-Rec plasmid and transformed in Y187 stain of *Saccharomyces cerevisiae*. 
3.3.1 Yeast Strains

For expression of BD and AD fusion proteins, two different strains of *S. cerevisiae* were used, namely, AH109 and Y187. Genotypic features of the two strains have been elaborated in Table 3.3.

<table>
<thead>
<tr>
<th>YEAST STRAIN</th>
<th>SOURCE</th>
<th>GENOTYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH109</td>
<td>Clontech</td>
<td>MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_UAS-GAL1_TATA-HIS3, MEL1, GAL2_UAS-GAL2_TATA-ADE2, URA3 : : MELI_UAS-MELI_TATA-lacZ</td>
</tr>
<tr>
<td>Y187</td>
<td>Clontech</td>
<td>MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1_UAS-GAL1_TATA-LacZ, MELI</td>
</tr>
</tbody>
</table>

3.3.2 Expression plasmids of Y2H system

CHIKV sPs were cloned in two different vectors pGBK7 (bait) and pGAD7 (prey) to carry out Y2H analysis. The pGBK7 vector expresses proteins fused to GAL4 DNA binding domain (BD) with a c-myc tag whereas pGAD7 and the library plasmid pGAD7-Rec expresses proteins with N-terminal GAL4 DNA activation domain (AD) fused to a HA (hemagglutinin) tag (Figure 3.3).
Figure 3.3: Vector maps of pGBK7 (BD; bait), pGADT7 (AD; prey) and pGADT7-Rec (AD-Rec; prey) of Y2H system

Table 3.4 Characteristic features of pGBK7 and pGADT7-Rec vectors

<table>
<thead>
<tr>
<th>Features</th>
<th>pGBK7 (BD) vector</th>
<th>pGADT7 (AD) vector</th>
<th>pGADT7-Rec (AD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>7.3 kb</td>
<td>7.9 kb</td>
<td>8 kb</td>
</tr>
<tr>
<td>Origin of replication</td>
<td>pUC (for E. coli) and 2μ ori (for S. cerevisiae)</td>
<td>pUC (for E. coli) and 2μ ori (for S. cerevisiae)</td>
<td>pUC (for E. coli) and 2μ ori (for S. cerevisiae)</td>
</tr>
<tr>
<td>Promoter</td>
<td>ADH1 promoter</td>
<td>ADH1 promoter</td>
<td>ADH1 promoter</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Kanamycin (Kan')</td>
<td>Kanamycin (Kan')</td>
<td>Ampicillin (Amp')</td>
</tr>
<tr>
<td>Nutritional</td>
<td>TRP1</td>
<td>LEU2</td>
<td>LEU2</td>
</tr>
<tr>
<td>Tag</td>
<td>c-myc</td>
<td>HA</td>
<td>HA</td>
</tr>
</tbody>
</table>
3.3.3 Control Vectors

3.3.3.1 Positive Control

The plasmids pGBKT7-p53 and pGADT7-T (Clontech, USA) encoding the known interacting proteins tumor suppressor protein p53 and Simian Virus 40 (SV40) large T-antigen as GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) fusions, respectively, were used as positive control.

3.3.3.2 Negative Control

The plasmids pGBKT7-Lam and pGADT7-T which encode the non-interacting human Lamin C protein and SV40 large T-antigen as GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) fusions, respectively, served as negative control.

3.3.4 Preparation of Yeast Competent Cells

1. Prepared a primary culture by inoculating 5 ml of YPD (Appendix A.2) or SD broth (Appendix A.2) with 1-2 colonies (each of 2-3 diameter). Incubated the primary culture at 30 ºC for 16-18 h at 220 rpm.
2. Added appropriate amount of primary culture to a flask containing 100 ml of YPD broth such that the initial OD$_{600}$ was 0.2-0.3.
3. Incubated the secondary culture at 30 ºC for ~3 h with shaking at 220 rpm until its OD$_{600}$ reached 0.4-0.6.
4. The culture was then centrifuged at room temperature (RT) for 5 mins at 6,000 rpm. Discarded the supernatant and resuspended the cell pellet in 50 ml (half the culture volume) of autoclaved distilled water.
5. Centrifuged the cell suspension again at RT for 5 min at 6,000 rpm. Discarded the supernatant and resuspended the cell pellet in 0.5 ml (for 100 ml culture) of freshly prepared sterile 1X TE/LiAc solution (Appendix A.1).

3.3.5 Lithium Acetate Transformation of Yeast

1. Added 0.1 μg of plasmid DNA and 0.1 mg of herring testes carrier DNA to a fresh 1.5 ml tube and mixed them. *Activated Herring testes carrier DNA by heating at 100 ºC for 10 min prior to its usage.*
2. Added 0.1 ml of competent yeast cells followed by 0.6 ml of sterile PEG/LiAc solution (Appendix A.1) to the tube containing the plasmid DNA and mixed intermittently by vortexing.

3. Incubated the transformation mixture at 30 °C for 30 min at 220 rpm.

4. After incubation, added 70 µl DMSO and mixed by gentle inversion.

5. Heat shock treatment was given to cells at 42 °C for 15 min in water bath followed by chilling for 1-2 min in ice.

6. Pelleted down the cells subsequently at RT for 5 sec at 12,000 rpm. Discarded the supernatant and resuspended the cells were in 0.5 ml of sterile 1X TE buffer (Appendix A.1).

7. Plated 0.1 ml on SD/-Trp (Appendix A.1) for selection of BD fusions or SD/-Lu (Appendix A.1) for selection of AD fusions transformants.

8. Incubated the plates at 30 °C for 3-5 days (until the colonies appeared).

3.3.6 Preparation of Yeast Protein Extracts

1. Prepared a primary culture by inoculating 10 ml of SD broth (Appendix A.1) with 1-2 colonies (each of 2-3 diameter) and incubated it at 30 °C for 16-18 h with shaking at 220 rpm.

2. Next day, added appropriate amount of primary culture to a flask containing 100 ml of YPD broth (Appendix A.1) such that the OD<sub>600</sub> was 0.2-0.3.

3. Incubated the secondary culture at 30 °C for ~3 h with shaking at 220 rpm until its OD<sub>600</sub> reached 0.4-0.6.

4. Following incubation, the cultures were quickly poured into pre-chilled 50 ml tubes and centrifuged at 4 °C for 5 min at 6,000 rpm. Discarded the supernatant and resuspended the cells in 50 ml (half the culture volume) of ice cold autoclaved distilled water.

5. Centrifuged the cell suspension at 4 °C for 5 min at 6,000 and discarded the supernatant.

   The pellets were either used directly for protein extraction following 2-3 freeze-thaw cycles or freezeed immediately on dry ice or in liquid nitrogen and stored at -80 °C until further use.

6. Quickly resuspended cells pellets in prewarmed (60 °C) complete cracking buffer (Appendix A.1). Per 7.5 OD<sub>600</sub> units of cells, 100 µl of complete cracking buffer was used (1 OD<sub>600</sub> units = OD<sub>600</sub> X volume of secondary culture).
Since the initial excess PMS in the complete cracking buffer degrades quickly, added additional aliquot of the 100X PMSF stock solution to the samples after 15 min and approximately every 7 min thereafter until right before loading on gel or when they are placed on dry ice or are safely stored at -80°C.

7. Transferred each cell suspension to a 1.5 ml tube containing 80 µl of glass beads per 7.5 OD₆₀₀ units of cells and heated at 70 °C for 10 min followed by vigorous vortexing for 1 min.

8. Centrifuged the cell suspension at 4 °C for 5 min at 12,000 rpm and the supernatant (first supernatant) was collected in a fresh 1.5 ml tube kept on ice.

9. Incubated the cell pellet at 100 °C for 3-5 min in a boiling water bath followed by vigorous vortexing for 1 min. (If no supernatant was obtained, 50-100 µl of cracking buffer was added)

10. Centrifuged the sample again at 4 °C for 5 min at 12,000 rpm and the supernatant was collected and pooled with the corresponding first supernatant.

11. The samples were either stored at -80 °C until further used or analysed by SDS-PAGE.

3.3.7 Autoactivation Analysis

Autoactivation refers to activation of HIS3 reporter gene by BD or AD fusion proteins in the absence of the partner protein (AD or BD fusion respectively). Autoactivation by CHIKV sPs as BD or AD fusion was tested by selecting the BD fusion AH109 transformants on SD/-Trp/-His media (Appendix A.2) and AD fusion Y187 transformants on SD/-Leu/-His media (Appendix A.2). Empty BD and AD vector were taken as negative controls.

3.3.8 Y2H Screening of Protein-Protein Interactions

For interaction analysis AH109 and Y187 yeast cells transformed with BD and AD fusion constructs respectively, were with each other.

3.3.8.1 Yeast Mating

1. Inoculated loopful of Y187 and AH109 cells transformed with BD and AD fusion constructs respectively, in 500 µl of YPDA media taken in a 1.5 ml microcentrifuge tube.

2. Resuspended the cells by vortexing and incubated overnight at 30 °C at 220 rpm.

3. Next day, plated 100 µl of the mated culture on SD/-Trp/-Leu media to ensure successful
mating.

4. Finally, screened the interacting partners among CHIKV sPs on SD/-Trp/-Leu/-His media.

### 3.3.8.2 Yeast two-hybrid Library Screening

1. Prepared a primary culture by inoculating 50 ml of SD -Trp broth (Appendix A.2) with 1-2 colonies (each of 2-3 diameter) of yeast strain AH109 transformed with recombinant pGBK7T plasmid (BD-TrE1 or BD-TrE2; bait strains) and incubated at 30 ºC for 16-18 h with shaking at 220 rpm.

2. Next day, centrifuged the culture at RT for 5 min at 6,000 rpm. Discarded the supernatant resuspended the cell pellet in 4 ml SD -Trp broth (cell density >1X10^8 cells/ ml).

3. Thawed a vial (1 ml) of human fetal brain cDNA library pretransformed in yeast strain Y187 (library strain; Clontech, USA) at RT in a water bath.

*Before screening of interactors for TrE1 and TrE2 proteins by yeast mating, 10 μl of the library was used for Library Titration*.

4. Transferred contents of the vial to a 2 L flask containing 45 ml of 2X YPD broth (Appendix A.2) supplemented with 50 μg/ml kanamycin along with 4 ml of bait strain (from step 2) and incubated at 30 ºC for 20-24 h with shaking at 50 rpm for mating.

5. After 24 h incubation, centrifuged the cell culture at RT for 10 min at 6,000 rpm. Discarded the supernatant resuspended pellet in 10 ml of 0.5X YPD broth (Appendix A.2) supplemented with 50 μg/ml kanamycin.

6. Following this 250 μl each of mated culture was plated on the forty SD/-Trp/-Leu/-His plates (150 mm; Appendix A.2) supplemented with X-α-Gal and incubated at 30 ºC for 3-5 days (until blue coloured colonies appeared).

*The mated culture (100 μl) was also plated on SD/-Trp, SD/-Leu (Appendix A.2) and SD/-Trp/-Leu (Appendix A.2) plates for determining the mating efficiency*.

7. The positive clones (blue coloured colonies) were then streaked and amplified on SD/-Trp/-Leu/-His plates.

8. Well isolated blue coloured colonies were restreaked on SD/-Trp/-Leu/-His/-Ade plates (Appendix A.2) supplemented with X-α-Gal and incubated at 30 ºC for a period of one week for obtaining cells with high stringency selection.
**Library Titration**- Mixed the library vial by gentle vortexing. In a 1.5 ml microcentrifuge tube, transferred 10 µl of library to 1 ml of 1X YPDA broth (Appendix A.2). The mixture obtained (Dilution A \([1:10^2]\)) was vortexed gently. To a 1.5 ml microcentrifuge tube containing 1 ml of 1X YPDA broth (Dilution B \([1:10^4]\)), transferred 10 µl of Dilution A. Both dilution A and B were plated (100 µl each) on SD/-Leu plates and incubated at 30 °C for 3-5 days. Numbers of colonies were counted to determine the titer (cfu/ml). The library titer was calculated as follows:

\[
\text{No. colonies/plating volume (ml) x dilution factor} = \text{cfu/ml (library)}
\]

# Mating Efficiency (percentage of diploids) =

\[
\left(\frac{\text{No. of cfu/ml of diploid (colonies on SD/-Trp/-Leu plates)}}{\text{No. of cfu per ml of limiting partner (colonies on SD/-Leu plates)}}\right) \times 100.
\]

*In this case limiting partner is prey library.*

**3.3.9 Yeast Colony PCR and Elimination of Multiple Library Plasmids**

The library co-transformants may contain more than one library (pGADT7-Rec/AD/prey) plasmid which may lead to incorrect analysis of putative positive clones. Yeast colony PCR was performed using vector specific primers to identify diploid yeast cells (co-transformants) carrying multiple plasmids. The PCR reaction mix and amplification programme used are same as bacterial colony PCR and have been described in section (section 3.1.1.4). However in case of yeast colony PCR, for generation of template, the cells were treated with lyticase (Appendix A.1) in order to break the cell wall prior to amplification. Following amplification PCR products were analyzed by agarose gel electrophoresis.

To eliminate single diploid yeast cells carrying multiple prey plasmids (colonies showing multiple bands on colony PCR); re-streaked the cells on SD/-Trp/-Leu plates (Appendix A.2) supplemented with X-α-Gal (Appendix A.1) 2-3 times. Incubated the plates at 30 °C for 3-5 days (until blue or white coloured colonies appeared). A mixture of blue white colonies indicated segregation of plasmids. In case all the plasmids corresponded to interacting proteins, no white colonies were observed even after re-streaking 3 times. The segregation of plasmids in such cases was evaluated by repeated yeast colony PCR after each re-streak.
3.3.10 Restriction Digestion to Eliminate Library Plasmids in Duplicates

In order to eliminate library plasmids in duplicates, the AD/library inserts (following plasmid isolation) were amplified by PCR using vector specific primers. Those PCR products that migrated to same size on electrophoresis were digested with Hae III, a restriction enzyme which frequently cuts the human genome. The fragment sizes were then analyzed by agarose gel electrophoresis and plasmids having same restriction profile were regarded as duplicates. Only one of the two clones was considered for subsequent studies.

3.3.11 Isolation of Library Plasmid DNA from Yeast

1. For isolation of pGADT7-Rec (prey) plasmid, prepared a primary culture by inoculating 5 ml of SD/-Leu broth with 1-2 colonies (each of 2-3 diameter, positive interactor) and incubate it at 30 °C for 16-18 h with shaking at 220 rpm.
2. Next day, centrifuged the overnight grown cells at RT for 5 min at 6,000 rpm. Discarded the supernatant and resuspended the pellet in the residual liquid (~50 μl).
3. Added 10 μl of lyticase solution (Appendix A.1) and mixed the cells thoroughly by vortexing.
4. Incubated the cell suspension at 37 °C for 45 min with shaking at 220 rpm.
5. Added 10 μl of 20% SDS to each tube and vortex vigorously for 1 min to mix.
6. Subsequently, freeze/thawed the samples 2-3 times and vortexed again to ensure complete lysis of the cells.
7. The plasmid DNA was then isolated from lysed cells using plasmid DNA isolation kit described in section 3.2.6.2.
8. Analysed the eluted plasmids on 1% agarose gel.

3.3.12 Transformation of Yeast plasmids in E. coli (DH5 α)

1. Electrocompetent E. coli (DH5α) cells were thawed on ice. Added 5 μl of yeast plasmid DNA isolated in section 3.3.11 to 40 μl cells on ice.
2. Transferred samples to a prechilled cuvette having a 0.1 cm gap and pulsed for 1 sec.
3. Quickly added 200 μl of LB and transferred the cell suspension to 1.5 ml microcentrifuge tube.
4. Incubated it at 37 °C for 1 h with shaking (220 rpm).
5. Plated the cell suspension on LB agar supplemented with ampicillin (100 µg/ml). Incubate plates at 37 °C overnight.

3.3.13 Bacterial Colony PCR and Plasmid DNA isolation for sequencing

The presence of insert in *E. coli* (DH5α) cells transformed with prey plasmids was confirmed by bacterial colony PCR as discussed in section 3.1.1.4. Prey plasmids were isolated from *E. coli* DH5α cells (showing amplification of inserts) using commercially available plasmid DNA isolation kit as described in section 3.2.6.2. These plasmids were then characterized by sequencing.