Chapter 3

Materials and Methods
3.1 Materials and their sources

3.1.1 Vectors

pET 28b(+) plasmid vectors were obtained from Novagen (Merck K GaA, Darmstadt, Germany) and pGEM-T Easy vector was obtained from Promega Life Sciences, Madison, WI, USA

3.1.2 Primers

Primers for purpose of PCR amplification of the various genes from the genomic DNA template were procured from Sigma genosys (Sigma Inc.). Primers were designed based on the sequences of *Plasmodium falciparum* available in the nucleotide database “PlasmoDB” (Table 3.1).

**Table 3.1: Primers used for Cloning**

<table>
<thead>
<tr>
<th>SER. No</th>
<th>PRIMER NAME</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PfD66 20FN1</td>
<td>5'CAAGCTTATGCCAAAGTGGAGGATCTT 3'</td>
</tr>
<tr>
<td>2.</td>
<td>PfD66 20RN1</td>
<td>5'CCTCGAGTTAATTTTTCAATTGGTCA3'</td>
</tr>
<tr>
<td>3.</td>
<td>PfD T1F</td>
<td>5'CAAGCTTGCTTATGCATTGCCTA3'</td>
</tr>
<tr>
<td>4.</td>
<td>PfD T1R</td>
<td>5'CCTCGAGTTAATTTTTCAATTGGTCA3'</td>
</tr>
<tr>
<td>5.</td>
<td>PfD T2F</td>
<td>5'CAAGCTTATGTCTTCACAAGTAgAAACT3'</td>
</tr>
<tr>
<td>6.</td>
<td>PfD T2R</td>
<td>5'CCTCGAGTTAATTTTTCAATTGGTCA3'</td>
</tr>
</tbody>
</table>
3.1.3 Standard markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kb DNA ladder</td>
<td>Invitrogen life technologies (Carlsbad, USA)</td>
</tr>
<tr>
<td>Protein marker and Pre-stained protein marker</td>
<td>MBI Fermentas</td>
</tr>
</tbody>
</table>

3.1.4 Restriction Endonucleases and DNA Modifying Enzymes

<table>
<thead>
<tr>
<th>Restriction enzyme and DNA modifying enzymes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction enzymes</td>
<td>New England Biolabs Inc. MA, USA</td>
</tr>
<tr>
<td>Calf intestinal alkaline phosphatise (CIAP), T4 kinase, T4 DNA Polymerase, Klenow and T4 DNA Ligase</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>Proteinase K and lysozyme</td>
<td>Sigma, USA</td>
</tr>
</tbody>
</table>

3.1.5 Standard Media

All the media were made in milli Q water and sterilized by autoclaving for 20 min at 15 lb/sq. in. pressure unless otherwise indicated.
MATERIALS AND METHODS

3.1.5.1 LB Medium (Luria Bertani Medium)

Per Liter:
- Bacto-tryptone 10 g
- Bacto-yeast extract 5 g
- NaCl 5 g
- MilliQ water 950 ml

The media was dissolved in a 500ml of MilliQ water, the pH adjusted to 7.5 with 5 N NaOH and the volume was finally made up to 1000 ml with MilliQ water. Agar at a concentration of 1.5 % was added whenever required.

3.1.5.2 RPMI Medium

Per Liter:
- RPMI 1 pack
- HEPES 5.96 g
- NaHCO₃ 2.00 g
- Triple distilled milliQ 980 ml

The medium was filtered through 0.25 μm filter and 10 % FCS was added at the time of use to make complete RPMI.

3.1.6 Nucleotides, Radionucleotides

Deoxynucleoside-5'-Triphosphate, 100 mM (dATP, dCTP, dGTP, dTTP)

Nucleoside-5'-Triphosphate, 100 mM (ATP, CTP, GTP, UTP)

Radionucleotide (Amersham Biosciences, Freiburg)

[α-³²P]-dATP 3000 Ci/mmol; 10 Ci/l

[γ³²P]-ATP 6000 Ci/mmol; 10 Ci/l
MATERIALS AND METHODS

3.1.6 Bacterial Strains

**Table 3.4: Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>E. coli Rosetta (DE3) pLysS</td>
<td>Novagen, Darmstadt</td>
</tr>
</tbody>
</table>

3.1.7 *Plasmodium* strains

**Table 3.5: Plasmodium Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>MR4 (USA)</td>
</tr>
</tbody>
</table>

3.1.8 Reaction sets (Kits)

QIAprep Spin Miniprep Kit Qiagen, Hilden

Qiaprep Filter Plasmid Midi Kit Qiagen, Hilden

QIAquick Gel Extraction Kit Qiagen, Hilden

QIAquick PCR Purification Kit Qiagen, Hilden

3.1.9 Special materials

Falcon tubes (5, 15, 50 ml) Greiner, Kremsmünster

Nylon membrane Hybond-(N+) Qiagen, Hilden

Protein A Sepharose Amersham Biosciences, Freiburg
MATERIALS AND METHODS

Reaction tubes (0.5; 1.5; 2 ml) Eppendorf, Hamburg

Sterile filter (0.2; 0.45 μm) Millipore, France

X-ray film BioMax MR Kodak, USA

3.1.10 Immuno-Chemicals and Other Consumables

Nitrocellulose membrane for western blotting was procured from Amersham Pharmacia Biotech (Uppasala, Sweden). Alkaline phosphatase as well as HRP conjugated anti-mouse IgG was from Sigma chemical company St. Louis, USA. Flourescein conjugated anti-mouse IgG was obtained from Sigma (St. Louis, USA).

3.1.11 Chemicals, Media Components, Kits and Other Consumables

Chemicals used in the present investigation and their sources are listed in Table 3.1.

Table 3.6: Chemicals and their Source

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Ammonia E</td>
<td>Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>Ammonium sulfate E</td>
<td>Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>Ammonium per sulphate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Agarose DNA grade</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Agarose DNA grade low melting point</td>
<td>GIBCO BRL</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Bacto Agar</td>
<td>Difco, Becton, Dickinson Company, Maryland, USA</td>
</tr>
<tr>
<td>Bacto Peptone</td>
<td>Difco, Becton, Dickinson Company, Maryland, USA</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Citric acid anhydrous</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Dulbecco's modified eagle medium</td>
<td>GIBCO Invitrogen Corporation, Grand Island, New York</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>GIBCO Invitrogen Corporation, Grand Island, New York</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Bengal Chemicals and Pharmaceuticals Ltd., Calcutta, India</td>
</tr>
<tr>
<td>Fetal Calf serum</td>
<td>GIBCO Invitrogen Corporation, Grand Island, New York</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>Merck (India) Ltd. Mumbai</td>
</tr>
<tr>
<td>Formamide E</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Lipofectamine reagent</td>
<td>Invitrogen Corporation</td>
</tr>
<tr>
<td>Luria Bertani medium</td>
<td>Difco, Becton, Dickinson Company, Maryland, USA</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
</tbody>
</table>
### MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Substance</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol E</td>
<td>Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>PMSF</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>di-Potassium hydrogen phosphate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Potassium Dihydrogen Orthophosphate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Propan-2-ol</td>
<td>Merck (India) Ltd., Mumbai</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>di-Sodium hydrogen phosphate</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Sulfuric acid E</td>
<td>Merck (India) Ltd. Mumbai</td>
</tr>
<tr>
<td>TEMED</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma Chemical Co., St. Louis, USA</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>(GIBCO BRL)</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

3.1.12 Antibiotics and Substrates

Table 3.7: Antibiotics and Substrates

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stock solution</th>
<th>Final concentration in use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/ml</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25 mg/ml</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>IPTG</td>
<td>1M</td>
<td>1 mM</td>
</tr>
<tr>
<td>X-gal</td>
<td>40 mg/ml</td>
<td>40 μg/ml</td>
</tr>
</tbody>
</table>

3.1.13 Reagents and Buffers

All reagents and buffers for DNA and protein work were prepared in MilliQ grade water and sterilized by autoclaving for 15 min at 15 lb/sq. in. pressure unless otherwise mentioned.

3.1.14.1 Commonly Used Buffers

1. Phosphate buffered saline (PBS)

   Per Liter:  
   \[
   \begin{align*}
   \text{NaCl} & = 5.8 \text{ g} \\
   \text{Na}_2\text{HPO}_4 & = 5.3 \text{ g} \\
   \text{NaH}_2\text{PO}_4 & = 1.63 \text{ g} \\
   \text{pH} & = 7.4
   \end{align*}
   \]

2. 1X TE Buffer

   | Tris (pH 8.0) | 10 mM |
   | EDTA (0.5 M)  | 1 mM  |
MATERIALS AND METHODS

3.1.14.2 Buffers for Isolation of Genomic DNA

1. Lysis buffer: Tris (pH 8.0) 10 mM
   EDTA 1 mM
   SDS 0.5%
   NaCl 100 mM
   Proteinase K 200μg/ml

2. Proteinase K: 20 mg/ml in MilliQ water

3. Phenol: Chloroform: Isoamylalcohol

Solution contains 25 parts of buffered phenol, 24 parts of chloroform mixed with 1 part of isoamyl alcohol. The solution is stored in a glass container at 4 °C.

3.1.14.3 Solutions for Preparation of Chemically Competent Cells

1. Solution I  CaCl₂ 50 mM
2. Solution II  CaCl₂ 50 mM
   Glycerol 20 %

3.1.14.4 Buffers for Electrophoresis

1. Tris Acetate EDTA (TAE) Buffer:

   Per Liter: Tris base 242 g
   EDTA (0.5 M) 100 ml
   Glacial acetic acid 57.1 ml
MATERIALS AND METHODS

Milli Q water to 1000 ml pH 8.3

2. 10X TBE Buffer

Per Liter:  
- Tris base 108 g
- Boric Acid 55 g
- EDTA (0.5 M) 40 ml
- MilliQ water to 1000 ml

3. 10X Tris-Glycine buffer

Per Liter  
- Tris base 30.3 g
- Glycine 144.1 g
- SDS 10 g

3.1.14.5 Buffers for Plasmid Isolation

1. Solution I:  
- Glucose 50 mM
- Tris-HCl [pH 8.0] 25 mM
- EDTA 10 mM
- RNAse 10 μg/ml

2. Solution II:  
- NaOH 0.2 M
- SDS 1 %

3. Solution III:  
- Sodium Acetate 3 M
MATERIALS AND METHODS

3.1.14.6 Buffers for Gel Loading

1. Laemmli 4X Sample Buffer (180)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>22.4 ml</td>
</tr>
<tr>
<td>βME</td>
<td>10 ml</td>
</tr>
<tr>
<td>Tris (1 M), pH 6.8</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.02 g</td>
</tr>
</tbody>
</table>

2. 6X dye for agarose gel electrophoresis

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromophenol blue</td>
<td>0.25 %</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40 %</td>
</tr>
</tbody>
</table>

Dye is prepared in 6X TE buffer

3.1.14.7 Reagents for SDS-PAGE

1. Acrylamide 30%

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.2 g</td>
</tr>
<tr>
<td>N, N'-methylenebisacrylamide</td>
<td>0.8 g</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

The solution was stirred to dissolve the acrylamide. The volume was made up to 100 ml and the solution was filtered through Whatman filter paper no. 1 before use.

2. Ammonium per sulfate (APS) 10 %

3. SDS 20 %
MATERIALS AND METHODS

4. Buffer for resolving gel  
Tris-HCl (pH 8.8)  
1.5 M

5. Buffer for stacking gel  
Tris-HCl (pH 6.8)  
1 M

6. Coomassie Blue staining solution

- Brilliant blue (R250)  
0.1 %

- Acetic acid  
10 %

- Methanol  
40 %

The solution was filtered through Whatmann filter paper no. 1 before use.

7. Destaining Solution  
Methanol  
40 %

- Acetic acid  
10 %

3.1.14.8 Reagents for Western Blot analysis and ELISA

1. Transfer Buffer  
Tris  
0.25 M

- Glycine  
0.019 M

- Methanol  
20 %

- SDS  
0.1 %

2. Blocking Buffer  
2 % milk in PBS

3. Wash Buffer  
0.05 % Tween-20 in PBS

4. Developing buffer (HRP)  
DAB  
10 mg

- PBS  
10 ml
### MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>10 ( \mu \text{l} )</td>
</tr>
<tr>
<td>( \text{Na}_2\text{CO}_3 )</td>
<td>0.015 M</td>
</tr>
<tr>
<td>( \text{NaHCO}_3 )</td>
<td>0.035 M</td>
</tr>
<tr>
<td>pH 9.6</td>
<td></td>
</tr>
</tbody>
</table>

#### 3.1.14.9 Buffers for Protein Purification using \( \text{Ni}^{2+}\)-NTA

1. **Sonication buffer**
   - Tris-HCl 50 mM
   - NaCl 300 mM
   - pH 7.4

2. **Lysis buffer**
   - Urea 8 M
   - Tris-HCl pH 8.0 20 mM
   - NaCl 300 mM

4. **Elution Buffer**
   - Imidazole 10 mM-1M
   - Tris-HCl, pH 8.0 20 mM
MATERIALS AND METHODS

3.2 Methods

3.2.1 Collection of blood

O\textsuperscript{+VE} blood was collected in 10% citrate-phosphate-dextrose buffer and centrifuged at 800Xg for 5 minutes. Plasma with buffy coat was removed to obtain packed erythrocytes. These erythrocytes were stored at 4°C for 3-4 weeks.

3.2.2 Parasite culture

*Plasmodium falciparum* 3D7 strain and field isolates were cultured using methods described by Trager and Jensen (Trager and Jensen, 1976). Parasite cultures were maintained under mixed gas (5% CO\textsubscript{2}, 5% O\textsubscript{2} and 90% N\textsubscript{2}) at 37°C in O\textsuperscript{+VE} erythrocytes and RPMI-1640 supplemented with 10% in O\textsuperscript{+VE} human sera and gentamycin (10μg/ml). The cultures were maintained at 4% hematocrit and 5-10% parasitemia. Parasite stage and parasitemia was checked by making a thin smear on a glass slide, fixing with methanol and staining with 5-10% geimsa stain (sigma).

3.2.3 Sorbitol synchronization of parasite

*Plasmodium falciparum* 3D7 cultures were synchronized using the method described by Lambros and Vanderberg (Lambros and Vanderberg, 1979). Parasite culture with majority at early ring stage was centrifuged at 500Xg for 5 mins. After washing the culture once with incomplete RPMI-1640, it was incubated with 7-10 pellet volumes of 5% sorbitol at room temperature for 5-7 minutes with intermittent mixing. Sorbitol lyses the mature trophozoite and schizonts, leaving the rings unaffected. After incubation with 5% sorbitol, it was centrifuged at 500Xg for 5 mins. The cultures were then washed once with incomplete RPMI-1640 at a hematocrit of 4%. This was further allowed to grow at 37°C under mixed gas.
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3.2.4 Genomic DNA Isolation

*Plasmodium falciparum* was grown in culture at 4% hematocrit in complete media having 5.8 ml of 3.6% sodium bicarbonate solution, 200 μl of gentamycin sulfate and 10 ml of O'ne human serum per 100 ml of RPMI 1640 solution. Parasites were grown until the parasitemia reached about 8-10%. Mixed stage parasites were harvested when the culture predominantly contained 8-10% trophozoites. RBC were pelleted down and given a wash with 1X PBS, followed by lysis with 5% saponin. Parasite was resuspended in lysis buffer (10 mM Tris-Cl pH-8.0, 1 mM EDTA pH-8.0, 0.5% SDS, 200 μg/ml proteinase K) and incubated at 37°C for 3 hrs with intermittent shaking. RNase (Sigma) was added at a final concentration of 10 μg/ml and incubation was continued for another 1 hr at 37°C. After lysis of the parasite, equal volume of phenol:chloroform mixture (24:1) was added, mixed well and centrifuged at 8000 Xg for 10 minutes. The genomic DNA was precipitated from upper aqueous layer by the addition of 1/10 volume of sodium acetate (pH-5.2) and equal volume of isopropanol. After 30 minutes of incubation at -20°C, the genomic DNA was pelleted by centrifugation at 8000 xg for 30 minutes at 4°C. Genomic DNA thus isolated was washed with 70% ethanol and air dried. The concentration of the genomic DNA was measured spectrophotometrically by measuring the absorbance at 260 nm. Two aliquots of the genomic DNA were checked on 1% agarose gel for integrity and purity.

3.2.5 RNA Isolation and cDNA preparation

*Plasmodium falciparum* was grown in tissue culture at 4% hematocrit using culture conditions as mentioned above. Parasites were harvested, when the parasitemia of the mixed culture reached 15-18%. Total RNA was isolated from the mixed culture plate, using an RNeasy kit from Qiagen (GmbH, Germany), according to the manufacturer’s protocol. The extracted RNA was then analysed on a 1% agarose gel and it’s O.D. at 260nm was
MATERIALS AND METHODS

determined to estimate RNA concentration. Two aliquots of the total RNA were checked on 1% agarose gel to check purity. The isolated RNA was then used for the preparation of cDNA using a cDNA synthesis kit (Superscript first-strand synthesis system from Invitrogen, Carlsbad, CA, USA).

3.2.6 Spectrophotometric estimation of nucleic acid

The quantity and purity of nucleic acids in solution was determined by measuring the absorbance at 260 and 280 nm. The concentration of the nucleic acid was calculated by taking 1 OD 260 = 50μg/ml for DNA, 40μg/ml for RNA and 33μg/ml for single stranded oligonucleotides. The A260/A280 ratio revealed the purity of the nucleic acid preparation.

3.2.7 PCR Amplification

PCR amplification of the PfD66 gene was done from genomic DNA using the following PCR conditions:- 95°C for 5 min (1 cycle), [95°C for 1 min; 54°C for 1 min; 68°C for 2 min] (30 cycles), 68°C for 12 min (1 cycle). PCR product was then checked on a 1% agarose gel. These PCR fragment was first gel purified using Qiagen PCR/gel extraction kits.

3.2.8 Purification of DNA fragment from agarose gel

DNA samples were resolved by agarose gel electrophoresis on a 1% agarose gel. The desired fragment was identified using standard molecular weight marker (1kb ladder) and purified using the Qiaquick Gel extraction Kit (Qiagen). The agarose gel containing the desired DNA fragment was excised using a surgical blade and dissolved in 3 volumes of QG buffer, as supplied with the Qiaquick gel extraction kit, (Qiagen GmbH, Hilden, Germany). The excised gel piece was dissolved by heating to 50°C for 20 minutes. The mixture was discarded and the column was washed twice with buffer PE. The purified DNA fragment was eluted with 50μl of elution buffer.
MATERIALS AND METHODS

3.2.9 Cloning in pGEM- T Easy vector

These amplified products were then cloned into the pGEM–T Easy vector (Promega, Madison, WI, USA) using TA cloning method. A 10μl ligation reaction was set up with 10ng vector DNA, 5μl of ligation buffer (2X), 1μl of T4 DNA ligase (10U) and 4μl of gel purified PCR fragment having about 400ng of insert DNA. Ligation reaction was incubated at 16°C for 16 hrs and was transformed into DH5α competent cells.

3.2.10 Preparation of chemically competent cells

A single colony was inoculated in 5ml LB medium. The culture was allowed to grow overnight at 37°C with continuous shaking at 200rpm. The overnight grown culture was diluted 100 fold in 100ml LB medium and grown at 37°C with shaking. When OD_{600} reached 0.4-0.5, the culture was chilled in keeping on ice. The culture was centrifuged at 5000rpm for 10 min at 4°C. The supernatant was discarded and pellet was suspended in 5ml ice cold solution I (100mM CaCl₂) by gently pipetting it up and down. 10ml additional chilled solution I was added and pellet was resuspended completely. The cells were incubated in solution I over ice for 1 hr. The cell suspension was then centrifuged at 5000rpm at 4°C for 10 minutes. The cell pellet hence obtained was suspended in 3ml solution II (100mM CaCl₂ and 20% glycerol). Aliquots were stored in pre-chilled micro tubes at -70°C.

3.2.11 Transformation

Ligation mixture (10μl) was mixed with 100μl of chemically competent DH5α E. Coli cells and kept on ice for 20 minutes. This mixture was incubated at 42°C for 90 seconds for heat shock treatment and was transferred immediately on ice for 2 minutes. About 1 ml sterile LB media was added in sterile conditions to the above transformed mixture. The mixture was allowed to grow at 37°C, 200rpm for 45-60 minutes. The transformed mixture after the
incubation period was centrifuged to pellet the cells at 5000rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100μl of LB media and plated on LB agar plate supplemented with appropriate antibiotic. The plate was kept at 37°C for overnight incubation and the transformants appeared as well separated colonies. These colonies were picked up for further analysis by mini prep plasmid preparation.

3.2.12 Screening of transformants
Individual colonies from the plate were inoculated in 5ml LB media containing appropriate antibiotic and allowed to grow overnight at 37°C at 200rpm in the incubator shaker. Simultaneously a master plate was also made by patching individual colonies on a separate LB plate supplemented with appropriate antibiotic. The clones were first screened by blue white screening. The DNA of white clones was isolated and the restriction digestion of the DNA was done to further confirm cloning into pGEM –T Easy vector. The positive clones obtained after this screening were sequenced using the services of TCGA. After the sequence analysis and confirmation, these sequences were submitted to NCBI/GenBank. The accession numbers obtained are: GenBank EF612437 (PF14_0563)

3.2.13 Subcloning into pET vector
The positive clones in pGEMT vector were digested with the restriction endonuclease and the digestion mixture was separated on a 1% agarose gel. The fragment corresponding to the gene was excised and the DNA was eluted using the gel extraction kit. The insert was then ligated into pET28b vectors obtained from Novagen (Madison, WI, USA) for the purpose of expression of encoded proteins. The ligation mixtures were transformed in DH5α cells and the recombinants were screened by preparing the plasmid DNA followed by restriction
digestion. pET 28 was selected as the expression system as it contains the histidine tags at both the N-terminal and C-terminal.

3.2.14 Expression and purification of PF14_0563

Clones having the PF14_0563- pET 28b recombinant construct were confirmed by restriction digestion. This construct was then transformed into BL21 (DE3) plysS E. coli cells for the purpose of expression. The transformed BL21 cells were then grown to appropriate density (O.D. \_600 = 0.6 O.D). 2% ethanol was added to the culture about ½ hour prior to induction for the purpose of inducing heat shock proteins. The protein expression was induced by using 1mM IPTG (Isopropyl β-D Thiogalactopyranoside). The helicase protein PF14_0563 was purified to homogeneity using Ni-NTA affinity chromatography. In order to purify the protein, bacterial cells were harvested and subjected to three freeze/thaw cycles at -70°C. The harvested cells were then suspended in protein lysis buffer having (20mM Tris-HCL pH 8.0, 250mM NaCl, 1% Triton, 0.5% Tween 20 and protease cocktail inhibitor). After centrifugation at 4°C, the soluble bacterial lysate were allowed to bind to pre-equilibrated Ni-NTA from Qiagen (GmbH, Germany) in binding buffer having 250mM NaCl, 20mM Tris-HCL pH 8.0 in the presence of 10mM imidazole and protease cocktail inhibitor from sigma (St. Louis, MO, USA). This bound protein was then washed twice with 10mM and 20mM imidazole and the bound protein was finally eluted using 200mM imidazole in buffer having 20mM Tris-HCL pH 8.0, 250Mm NaCl, 10% glycerol and protease cocktail inhibitor. This eluted protein was further checked for purity and dialyzed against 20mM Tris and 20mM NaCl. The purity of the protein was again assessed through silver staining. This purified protein was used for all the subsequent activity assays. The 1731 base pair genomic sequence codes for a protein of the size of ~66kDa [hence the protein was named Plasmodium falciparum Dbp5 68kDa in size (PfD66)].
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3.2.15 Polyacrylamide Gel Electrophoresis of proteins (PAGE)

PAGE was performed according to the protocol of Laemmli (1970). Gels were prepared as outlined in the table and the electrophoresis was performed in the presence of 0.1% SDS. The protein samples were prepared by mixing with equal volume of 2x sample buffer (100mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue). The samples were denatured by boiling in a water bath for 5 minutes and loaded on a gel. The gels were electrophoresed at a constant voltage of 100V. Gels were stained by 0.25% coomassie blue R-250, 50% methanol and 10% acetic acid as described by Laemmli (1970).

3.2.16 Silver staining

Silver staining was done using a slight modification of the standard protocol (Sambrook et al., 1989). After the separation of the proteins on SDS-PAGE, the gel was fixed overnight with gentle shaking in a fixing solution (methanol: acetic acid: water 50:5:45). The gel was subsequently washed two times in water and soaked in 0.02% solution of sodium thiosulphate for 2 minutes. The gel was again washed two times in water and then soaked in 0.1% silver nitrate solution for 30 minutes at 4°C with gentle shaking. The gel was washed once with water and developed by soaking in a solution of 2% sodium carbonate and 0.02% formaldehyde. After a few minutes the quencher solution (1% acetic acid) was added and the gel was subsequently washed in water.

3.2.17 Raising antibodies against the PfD66 in mice

Polyclonal antibodies were raised against PfD66 in BALB/c mice. 20μg of purified recombinant protein was formulated in complete Freund’s adjuvant and injected in mice intraperitoneally. Priming was followed by two booster immunizations with recombinant proteins formulated in
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Freund’s incomplete adjuvant on days 28 and 69. Bleeds were collected on days 0, 14, 42, 84 and are referred to as prebleed, first bleed, second bleed and third bleed respectively. For collection of sera, blood was allowed to coagulate by incubation at 37°C for 1 hr and overnight at 4°C. After centrifugation at 3000xg for 5 min, sera was transferred to a fresh tube and stored at -70°C till further use. The endpoint titres of antibodies against each protein were determined using ELISA. 100ng of recombinant protein was coated on to ELISA plate (Nunc) and incubated overnight at 4°C. ELISA plates were washed once with PBS and blocked with 300μl of 2% BSA in PBS at 37°C for 2 hrs. Serial dilutions of sera were prepared in 1% BSA in PBS. The wells were washed once with PBST and further incubated with 100μl of various dilutions of sera for 1 hr at 37°C. The wells were washed thrice with PBST and incubated for 1 hr at 37°C with anti-mouse IgG goat antibodies conjugated with horseradish peroxidase (1:2000). The wells were washed thrice with PBST and developed using O-phenylene diamine (OPD-1mg/ml) as substrate. O.D. was recorded at 492nm using ELISA reader (Molecular Devices).

3.2.18 IgG purification using protein A sepharose column

IgG was purified from polyclonal antibody raised against PfD66 protein using protein A sepharose column. Protein A sepharose was washed with water for at least 30 minutes and the column was packed with beads. Column was washed with 10 column volumes of 100mM Tris HCl buffer pH 8.0 (0.135M NaCl, 100mM Tris HCl). Serum was centrifuged at 13000 rpm for 15 minutes prior to loading on the column. Serum was passed through the column 2 times to maximise binding. The flow through serum was collected and saved. The column was washed with 10 column volumes of 10mM Tris HCl pH8.0 and the antibody was eluted off the protein A beads with 100mM glycine pH 3.0. the eluted IgG was collected into eppendorf tubes containing a 15% final fraction volume of 2.0 M Tris-HCl pH 8.0. Fractions of the tube were checked in SDS-PAGE followed by coomassie blue staining to confirm purity.
3.2.19 Western blotting

Western blotting was performed according to Towbin et al. (1979). The Mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from the gel onto nitrocellulose membrane. Electrobblotting was performed in the presence of 39 mM glycine, 48mM Tris base, 0.037% SDS and 20% methanol at a constant voltage of 50V for 2 hours at 4°C. The membrane was rinsed briefly in TBST (10mM Tris pH 7.5, 150mM NaCl and 0.05% Tween-20) and then incubated in blocking solution (3% BSA in TBST) for 1 hour with gentle shaking at 37°C. The blocking solution was replaced with primary antibody solution (with appropriate dilution in TBST containing 1% BSA) and the incubation was continued at 37°C for additional 1 hour with gentle shaking. Thereafter, the blots were washed thrice with TBST for 5 minute each. After washing the blots were incubated with alkaline-phosphatase or horseradish peroxidise (HRP) conjugated secondary antibody solution (1: 3000 dilution in TBST containing 1% BSA) at 37°C for 1 hour. The blots were washed as described above. In the case of alkaline phosphatase conjugated secondary antibody, the reaction was developed using 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 5mM MgCl2 containing 150μg/ml of NBT (Nitroblue Tetrazolium) and 75μg/ml BCIP (Bromo-Chloro-Indolyl phosphate). The reaction was terminated by rising the blot in 10mM EDTA, pH 8.0. When HRP conjugated anti-rabbit IgG was used, the blots were developed using a solution of DAB (Diaminobenzidene) (Sigma) dissolved in PBS (1X) until the color developed. The reaction was stopped by rinsing the blot in water.

3.2.20 Immunofluorescence assay

A thin smear of late stage parasitized red blood cells of different developmental stages were prepared and was fixed in acetone for 5 minutes followed by chilled methanol for 15 seconds at room temperature. For the purpose of blocking the fixed slides were dried and incubated in 10% fetal calf serum in PBS in a humid chamber at 37°C for 2hr. The slides were washed with PBS
and incubated with purified IgG of anti-PfD66 antibodies at 1:200 dilutions in PBS containing 10% fetal calf serum for 1 hour at 37°C. The slides were then washed four times with PBS for 15 min each and then incubated for 1 hr at 37°C with secondary antibody (fluorescein isothiocyanate-conjugated- anti-mouse IgG (Sigma) diluted 1:100 in PBS containing 10% fetal calf serum) after washing the slides were incubated in 4',6'- di-amidino-2-phenylindole-dihydrochloride (DAPI) (2μg/ml in PBS) for nuclear staining. The slides were washed thrice with PBST (PBS, 0.5% Tween 20) for 10 minutes each and twice with PBS for 10 min each and mounted with antifade reagent Fluroguard purchased from BioRad (Hercules, CA, USA) and viewed under oil immersion. Confocal images were collected using a Bio-Rad 2100 laser-scanning microscope attached to a Nikon 2000U microscope.

3.2.21 Helicase Assay

a. Preparation of the helicase substrate: The duplex DNA substrate used consisted of a $^{32}$P-labelled 47-mer DNA oligodeoxynucleotide (5'- (T)$_{15}$GTTTTCCAGTCACGAC(T)$_{15}$-3') annealed to M13mp19 phage ssDNA to create a partial duplex. At both the 5' - and 3' -ends, this oligodeoxynucleotide contains 15 basepairs of non-complementary region. Ten nanograms of this oligodeoxynucleotide was labelled at 5'-end with T4 polynucleotide kinase (5U) in 50 mM Tris–HCl, pH 8.0, 10 mM MgCl$_2$, 5 mM DTT, 0.1 mM EDTA, 0.1 mM spermidine and 1.85 MBq of $[\gamma-^{32}$P]ATP (specific activity 222 TBq/mmol). The labelled oligodeoxynucleotide was then annealed with 2.5 μg of single-stranded circular M13mp19 (+) DNA in 20 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 100 mM NaCl and 1 mM DTT by heating at 95 °C for 1 min, transferring immediately to 65 °C for 2 min and then cooling slowly to room temperature. Non-hybridized oligodeoxynucleotide was removed by gel filtration through a 1 ml Sepharose 4B column (Pharmacia, Sweden).
b. **Enzyme Concentration Curve:** The helicase assay measures the unwinding of $^{32}$P-labelled DNA fragment from a partially duplex DNA molecule. The reaction mixture (10 µl) containing 20 mM Tris–HCl (pH 8.0), 8 mM DTT, 1.0 mM MgCl$_2$, 1.0 mM ATP, 10 mM KCl, 4% (w/v) sucrose, 80 µg/ml BSA, $^{32}$P-labelled helicase substrate ($\sim$1000 cpm) and the helicase fraction to be assayed was incubated at 37 °C for 60 min (unless otherwise indicated). The reaction was terminated by the addition of 0.3% SDS, 10 mM EDTA, 5% glycerol and 0.03% bromophenol blue. After further incubation at 37 °C for 5 min, the substrate and products were separated by electrophoresis on a 12% non-denaturing polyacrylamide gel.

c. **Polyacrylamide Gel Electrophoresis for DNA Unwinding Assay:**
The products were separated by 12% native gel polyacrylamide gel (8cm x 12cm) electrophoresis in 1x TBE. Gels were prepared as outlined in the table below and the electrophoresis was performed in the presence of 1x TBE. The samples were prepared by mixing with 2µl loading buffer. The boiled reaction samples were kept in boiling water for 5 minutes and loaded on to a gel after addition of loading buffer. The boiled reactions were kept in boiling water for 5 minutes and loaded on to a gel after addition of loading buffer. The electrophoresis was performed at a constant voltage of 100V. Gels were fixed in fixing solution (10% methanol, 10% acetic acid) for 5 minutes, transferred to 3 MM Whatman paper, dried in the gel dryer and autographed. The DNA unwinding was quantitated by excising the bands from the dried gels and counting in Beckman Ready safe liquid scintillation fluid. One unit of helicase activity is defined as the amount of enzyme 1% of the DNA helicase substrate in 1 min at 37°C (60% in one hour reaction)
Table 3.8: Composition of 12% resolving to analyse DNA unwinding assay

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Resolving Gel (10ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide stock solution (30%)</td>
<td>4.0ml</td>
</tr>
<tr>
<td>10X TBE</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Water</td>
<td>4.9ml</td>
</tr>
<tr>
<td>Ammonium per sulphate</td>
<td>0.1ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.004ml</td>
</tr>
</tbody>
</table>

3.2.22 ATPase Assay

The hydrolysis of ATP catalyzed by purified PfD66 was assayed by measuring the formation of inorganic phosphate from $[\gamma^{32}P]$ ATP. The reactions conditions were identical to those described for the helicase reaction, except that the $^{32}$P-labelled helicase substrate was replaced by a mixture of $[\gamma^{32}P]$ ATP (specific activity 222 TBq/mmol) and cold ATP (1 mM). The reaction was performed for 2 h at 37 °C both in the presence of 100 ng of M13 mp19 ssDNA (D), and RNA (R) followed by thin layer chromatography. It was observed that enzyme displayed RNA dependent ATPase activity, it hydrolysed more ATP in presence of increasing concentrations of RNA.

3.2.23 Determination of direction of unwinding:

DNA is a bipolar molecule, so motor proteins that walk along nucleic acids require a sense of the direction in which they are moving. Helicases typically bind to a strand of DNA and unwind it in a defined direction. To determine the direction of translocation of PfD66, two different direction-specific DNA duplex substrates were prepared, one specific for the 3’ to 5’ direction and the other specific for the 5’ to 3’ direction. The release of radiolabeled DNA from the substrates indicates movement of the enzyme in the 3’ to 5’ or 5’ to 3’ direction.
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a. **Preparation of the direction specific substrates:** The direction specific substrates were prepared using the M13mp19 ssDNA and the 32mer oligodeoxynucleotide (5_TTCGAGCTCCTACCCCGGATCTCTAGAGT-3_) (RNT-32) having a Smal site in the middle.

b. **Preparation of the 5'-3' direction substrate:** The 32-mer oligodeoxynucleotide was first labelled at 5'-OH end with 50 μCurie [-32P]dATP with PNK for 1 hour at 37°C. It was then annealed with M13mp19 ssDNA in 40mM Tris-HCl (pH 7.5), 10mM MgCl2, 50mM NaCl and 1mM DTT. The annealed substrate was digested with Smal and purified by gel filtration through 1ml of Sepharose 4B. Various fractions of this substrate are shown in Fig. 10a.

c. **Preparation of the 3'-5' directionality substrate:** The oligodeoxynucleotide 32-mer was first annealed to M13mp19 ssDNA and then labelled at 3'-OH end in 40mM Tris–HCl (pH 7.5), 10mM MgCl2, 50mM NaCl and 1mM DTT with 50 μCurie [-32P]dCTP and 5 units of DNA polymerase I (large fragment) at 23 °C for 20 min. The incubation was continued for an additional 20 min at 23 °C after increasing the dCTP to 50mM using unlabelled dCTP. This was digested with Smal and purified by gel filtration through 1ml sepharose 4B. Various fractions of this substrate are shown in Fig. 10b.

d. **Determination of direction:** For determining the direction of translocation the direction specific substrates and the same assay as described above was used with different concentration of purified protein.

3.2.24 NTP requirement

Most of the helicases use ATP as the energy source for the purpose of unwinding nucleic acid, but some may use NTP other than ATP. Therefore the NTP requirement assay was performed to check the NTP requirement of purified PfD66.
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a. **NTP requirement assay:** The similar assay was used and the unwinding of the duplex DNA was observed in the presence of 1mM concentrations of different NTPs. The helicase assay was performed by using 10 μM of enzyme in the same way as described earlier.

3.2.25 **Cation requirement**

Most of the enzymes which are involved in ATP hydrolysis require a cofactor for their activity. Helicases mostly use Mg $^{2+}$ as a cofactor for their activity. In order to check which are the other cofactors are, that the enzyme can utilise for ATP hydrolysis, Ion requirement assay was done.

a. **Ion requirement assay:** NT-47 was used as the oligodeoxynucleotide for performing ion requirement assay. Unwinding of the duplex substrate was ascertained in the presence of 1mM of different ions to check for the enzyme activity. Helicase Assay was done in the same way as mentioned earlier by using 10 μM of the purified protein.

3.2.26. **Assay of Inhibitor for helicase activity**

A variety of DNA intercalating agents have been reported to contain antihelicase activities, therefore, the effects of various kinds of these agents on DNA unwinding activity of PfD66 was studied. The effect of DNA- intercalating compounds on helicase activity, different compounds were added to the reaction mixture prior to the addition of the enzyme. The effect of these compounds on the DNA unwinding activity of PfD66 was initially tested by including 50μM of each compound separately in the standard helicase assay. The compound which inhibited was included in the helicase reaction at final concentration ranging from 0.1 to 10μM.
3.2.27 Assay of Inhibitor for ATPase activity

A variety of DNA interacting agents have been reported to contain anti-helicase and anti-ATPase activities, therefore the effects of various kinds of these agents on ATPase activity of PfD66 was also studied. For studying the effect of DNA interacting compounds on ssDNA-dependent ATPase activity, different compounds were added to the reaction mixture prior to the addition of the enzyme. The effect of these compounds on the ssDNA-dependent ATPase activity of PfD66 was initially tested by including 50μl of each compound separately in the standard ATPase assay. The compounds which inhibited ssDNA-dependent ATPase activity were investigated further for the kinetics of inhibition. For this, each inhibitor was included in the ATPase reaction at final concentration ranging from 0.1 to 10μM.

3.2.28 Preparation of truncated derivatives of PfD66

In order to check the activity contributed by various domains, serial truncations were made using the two sets of primers mentioned in the table 3.1.2 above. The target DNA was denatured for 5 min initially and then 35 cycles of PCR were performed. PCR conditions for amplification are as follows: 95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min followed by 72 °C for 10 min. The resulting clones were verified by sequencing and the fragments were sub-cloned in the protein expression vector pET-28a obtained from Novagen (Madison, WI, USA). The truncated proteins were purified by the same procedure as described for full-length protein.

3.2.29 In vitro RNA binding assay

The RNA binding assay was done by using the same method as described previously with slight modifications (Cheng et al., 2005). For these equal amounts (1 μg) of BSA, PfD66 and
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Truncated derivatives of PfD66 (PfDT1 and PfDT2) were dot-blotted on pre-charged PVDF membrane. This membrane was blocked for 1 h at room temperature in blocking buffer (25 mM NaCl, 10 mM MgCl2, 10 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 3% BSA). The 13 mer RNA oligonucleotide used for the preparation of RNA helicase substrate was labeled at the 5'-end with 1.85 MBq of \( ^{32}P \) ATP (specific activity 222 TBq/mmol) using T4 polynucleotide kinase (NEB, England) and purified using Sepharose 4B (Pharmacia, Sweden) column chromatography. After blocking the membrane was incubated for 2 h in binding buffer (50 mM NaCl, 10 mM MgCl2, 10 mM HEPES, 0.1 mM EDTA, 1 mM DTT, 1.5% BSA) containing 10 pmol of \( ^{32}P \)-labeled RNA substrate. After binding, the membrane was washed thrice with binding buffer and exposed for autoradiography. The spots obtained were quantitated by densitometry. To check for equal loading of proteins, equal amounts (1 \( \mu \)g) of PfD66, PfDT1 and PfDT2 were dot-blotted on another precharged PVDF membrane. This membrane was blocked with blocking buffer (1% BSA in Tris buffered saline) for 1 h at room temperature and probed for a further 1 h with alkaline phosphatase conjugated anti-his antibody (Sigma Chemical Co) (St. Louis, MO, USA) in same buffer. The blot was washed and developed using standard protocol.