Chapter 3: Materials and Methods

3.1 Materials

3.1.1 *E. coli strains*

Strain	Genotype	Application	Source
DH5α	F^- (φ80d *lac* ZM15) Δ(*lac*ZYA - *arg*F) U169 *deo*R *ace*A1 *end* A1 *hsd* R17 (r-m+) *sup*E44λ-*thi*-1 *gyr* A96 *rel*A1	Plating and growth of plasmids, permits α-complementation with amino terminus of β-galactosidase encoded in pUC vectors	GIBCO BRL, USA

3.1.2 Plasmodium strains

Strain	Source
Plasmodium falciparum	MR4 (USA)
Plasmodium berghei	MR4 (USA)

3.1.3 Standard markers

Marker	Source
1 Kb DNA ladder	Invitrogen life technologies (Carlsbad, USA)
Protein marker	MBI Fermentas
Pre-stained protein marker	MBI Fermentas

3.1.4 Restriction Endonucleases and DNA Modifying Enzymes

Restriction endonucleases, Calf intestinal alkaline phosphatase, T4 kinase, T4 DNA Polymerase, Klenow (DNA polymerase I large fragment) and T4 DNA Ligase used in all routine cloning and transformation experiments were procured from New England Biolabs Inc. MA, USA (NEB). Taq polymerase for PCR amplification was an in-house preparation. Analytical and molecular biology products including

antibiotics, enzymes like proteinase K and lysozyme were purchased from Sigma, USA.

3.1.5 Standard Media

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

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; the pH adjusted to 7.5 with 5 N NaOH and the volume 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
	$NaHCO_3$	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.

3.1.5.3 CPD

Per Liter:		
	Sodium Citrate	100 mM
	Citric Acid	15 mM
	Sodium Phosphate	16 mM
	Dextrose	32.44 g

3.1.6 Molecular Biology Kits

Commercially available kits for preparation of plasmid DNA, Endofree Plasmid Giga Kit, QIA filter Plasmid Maxi kit, QIA filter Plasmid midi kit, QIA prep spin miniprep kit, QIA quick PCR Purification Kit, QIA quick Gel Extraction Kit, were obtained from Qiagen GmbH, Hilden, Germany.

3.1.7 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. X-ray (ready-pack) films for autoradiography were procured from Sigma (St. Louis, USA). Flourescein conjugated anti-mouse IgG was obtained from Sigma St. Louis, USA).

3.1.8 Primers

Primers for PCR amplification of the various genes from the genomic DNA and sequencing of the constructs were procured from Integrated DNA Technologies, Inc. (Coralville, USA).

Table 1: Sequences of the primers synthesized for the amplification.

PRIMER	SEQUENCE
T7 primer	5′-GTA ATA CGA CTC ACT ATA GGG -3′
SP6 primer	5'-ATA AGA TAT CAC AGT GGA TTT A-3′
Fal1 forward	5′-ATG GTT GCC ATA AAA GAA ATG -3′
Fal1 reverse	5′-TTA CAA GAT AGG ATA GAA GAC -3′
Fal2 forward	5′-ATG GAT TAC AAC ATG GAT ATA -3′
Fal2 reverse	5′-TTA TTC AAT TAA TGG AAT GAA -3′

3.1.9 Chemicals, Media Components, Kits and Other Consumables

Chemicals used in the present investigation and their sources are listed in Table 2-2.

Table 2-2-List of chemicals and bio-chemicals used in the present study

Chemical	Source

Acrylamide	Sigma Chemical Co., St. Louis, USA
Ammonia E	Merck (India) Ltd., Mumbai
Ammonium sulfate E	Merck (India) Ltd., Mumbai
Ammonium per sulfate	Sigma Chemical Co., St. Louis, USA
Agarose DNA grade	Sigma Chemical Co., St. Louis, USA
Agarose DNA grade low melting point	GIBCO BRL
Ampicillin	Sigma Chemical Co., St. Louis, USA
Bacto Agar	Difco, Becton, Dickinson Company, Maryland, USA
Bacto Peptone	Difco, Becton, Dickinson Company, Maryland, USA
BCIP/NBT	Promega Life Science, Madison,WI, USA
Boric acid	Sigma Chemical Co., St. Louis, USA
Bovine serum albumin	Sigma Chemical Co., St. Louis, USA
Bromophenol blue	Sigma Chemical Co., St. Louis, USA
Calcium chloride	Sigma Chemical Co., St. Louis, USA
Citric acid anhydrous	Sigma Chemical Co., St. Louis, USA
Dextrose E	Sigma Chemical Co., St. Louis, USA
Dulbecco's modified eagle medium	GIBCO Invitrogen Corporation, Grand Island, New York
Dimethylsulfoxide	Sigma Chemical Co., St. Louis, USA
Dithiothreitol	Sigma Chemical Co., St. Louis, USA
EDTA	Sigma Chemical Co., St. Louis, USA
Ethidium bromide	GIBCO Invitrogen Corporation, Grand Island, New York
Ethanol	Bengal Chemicals and Pharmaceuticals Ltd., Calcutta, India
Fetal Calf serum	GIBCO Invitrogen Corporation, Grand Island, New York
Formaldehyde	Merck (India) Ltd. Mumbai

Formamide E	Sigma Chemical Co., St. Louis, USA
Kanamycin	Sigma Chemical Co., St. Louis, USA
Glycerol	Sigma Chemical Co., St. Louis, USA
Glycine	Sigma Chemical Co., St. Louis, USA
Imidazole	Sigma Chemical Co., St. Louis, USA
Lipofectamine reagent	Invitrogen Corporation
Luria Bertani medium	Difco, Becton, Dickinson Company, Maryland, USA
β-Mercaptoethanol	Sigma Chemical Co., St. Louis, USA
Methanol E	Merck (India) Ltd. , Mumbai
PMSF	Sigma Chemical Co., St. Louis, USA
di-Potassium hydrogen phosphate	Sigma Chemical Co., St. Louis, USA
Potassium Dihydrogen Orthophosphate	Sigma Chemical Co., St. Louis, USA
Propan-2-ol	Merck (India) Ltd. , Mumbai
Sodium bicarbonate	Sigma Chemical Co., St. Louis, USA
Sodium chloride	Sigma Chemical Co., St. Louis, USA
Sodium hydroxide	Sigma Chemical Co., St. Louis, USA
Sodium phosphate	Sigma Chemical Co., St. Louis, USA
di-Sodium hydrogen phosphate	Sigma Chemical Co., St. Louis, USA
SDS	Sigma Chemical Co., St. Louis, USA
Sulfuric acid E	Merck (India) Ltd. Mumbai
TEMED	Sigma Chemical Co., St. Louis, USA
Trypsin EDTA	Invitrogen Corporation
Tris-HCl	Sigma Chemical Co., St. Louis, USA
Triton X-100	Sigma Chemical Co., St. Louis, USA
Tween-20	Sigma Chemical Co., St. Louis, USA
T4 DNA ligase	(GIBCO BRL)

3.1.10 Antibiotics and Substrates

Reagent	Stock solution	Final concentration in use
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	25 mg/ml	25 µg/ml
IPTG	1M	1 mM
X-gal	40 mg/ml	40 µg/ml

3.1.11 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.

3.1.11.1 Commonly Used Buffers

1. Phosphate buffered saline (PBS)

Per Liter:	NaCl	5.8 g
	Na_2HPO_4	5.3 g
	NaH_2PO_4	1.63 g
	pH	7.4

2. 1X TE Buffer	Tris (pH 8.0)	10 mM
	EDTA (0.5 M)	1 mM

3.1.11.2 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	200 µg/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.

3.1.11.3 Solutions for Preparation of Chemically Competent Cells

1. Solution I	$CaCl_2$	50 mM
2. Solution II	$CaCl_2$	50 mM
	Glycerol	20 %

3.1.11.4 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
	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

3.1.11.5 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

3.1.11.6 Buffers for Gel Loading

1. Laemmli 4X Sample Buffer (180)

Per 50 ml	Glycerol	22.4 ml
	βME	10 ml
	Tris (1 M), pH 6.8	1.4 ml
	SDS	6.0 g
	Bromophenol blue	0.02 g

6X dye for agarose gel electrophoresis

	Bromophenol blue	0.25 %
	Sucrose	40 %

Dye is prepared in 6X TE buffer

3.1.11.7 Reagents for SDS-PAGE

1. Acrylamide 30%	Acrylamide	29.2 g
	N,N'-methylenebisacrylamide	0.8 g
	MilliQ water	50 ml

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 %
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 %

3.1.11.8 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
	H_2O_2	10 µl
5. Coating buffer	Na_2CO_3	0.015 M
	$NaHCO_3$	0.035 M
	pH 9.6	

3.1.11.9 Buffers for Protein Purification using 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
3. *Buffers for refolding*		
i. Urea Buffer	Urea	8 M
	Tris-HCl pH 8.0	20 mM
ii. TB	Tris-HCl pH 8.0	20 mM
4. Elution Buffer	Imidazole	10 mM-1M
	Tris-HCl, pH 8.0	20 mM

3.2 Methods

3.2.1 Complete Media Preparation

RPMI powder sealed in a packet was opened and dissolved in 1000 ml of glass distilled water to make 1litre of RPMI medium. For 100ml of complete media, RPMI-HEPES was supplemented with 10 % of heat inactivated O^+ve human serum, 5.8 ml of 3.6 % Sodium bicarbonate and 20 µg/ml of gentamicin. In case of *P. berghei* cultures fetal calf serum was used instead of human serum.

Incomplete media=Complete media – Human serum.

3.2.2 *P. falciparum* Strains used for the Experiments

Parasite strains used for the present study were 3D7, 3D7-GFP +His. 3D7 was obtained from MR4 and 3D7-GFP +His is a kind gift from Dr.Alan Cowman, Walter Eliza Institute, Melbourne, Australia.

3.2.3 Thawing of *P. falciparum* Culture Frozen Stock

Frozen vial of parasite culture was thawed at 37 °C in circulating water bath for 1 min and immediately transferred to sterile tube. An equal volume of thawing solution was added drop by drop with gentle mixing of sample to the sterile tube. The mixture was centrifuged at 1200 rpm for 2 min at room temperature. The supernatant was discarded and 1 ml of thawing solution was added to the pellet by gently mixing the sample. The mixture was again centrifuged at same speed and time as indicated above. This step of centrifugation and addition of thawing solution was continued till there was no lysis of RBC. Fresh complete media of 5 ml with 2 % hematocrit and 10 % human serum was added to the pellet and mixed thoroughly. The parasite culture was transferred to 6 well microtitre plate and kept in a humid chamber, which was flushed with gas mixture (90 % N2, 5 % CO2 and 5 % O2) (SMS Multitech, India). The chamber was incubated at 37 °C for the growth of the parasite.

3.2.3a Maintenance of *P. falciparum* Culture (Trager and Jensen, 1976)

The media was changed after every 48 h of incubation. Fresh prewarmed complete media was added to the culture plate and mixed thoroughly. A small

quantity of sample was taken out for making thick and thin smears to determine parasite growth after staining with Geimsa stain. The plate was re-incubated at 37 °C in a humid chamber.

3.2.3b Subculture and Dilution of Parasite Culture

The parasite culture should be diluted or subcultured, once the culture reached 5-10 % parasitemia. Before dilution, the culture in the plate was thoroughly mixed to make sure that no RBC is stuck to the bottom of the plate. After wards, the culture was diluted by removing appropriate amount of culture media and the same amount of complete media with 4 % hematocrit was added. In case of transgenic lines i.e., 3D7-GFP+His, an appropriate amount of WR99210 was added to the culture before incubation.

3.2.3c Synchronization of *Plasmodium falciparum* by Sorbitol Treatment (Lambros *et al.*, 1979)

The culture was harvested at about 10 % parasiteamia with majority at ring stage by centrifuging at 1600 rpm for 5 min at RT. To the cell pellet 5 vol of 5 % sorbitol solution was added and mixed gently. This solution was incubated at 37 °C for 5 min and centrifuged at 1600 rpm for 5 min. The supernatant was carefully discarded with out disturbing the pellet. Prewarmed complete media was added to the pellet. The culture was mixed thoroughly and incubated at 37 °C for the growth of parasite. Repeat the sorbitol treatment once again after one cycle of growth to ensure synchronization.

3.2.3d Isolation of Purified Parasite from Infected Blood by Saponin Lysis

The culture for saponin lysis was harvested by centrifuging at 1600 rpm for 5 min at RT. To the pellet, 1.5 volumes of 0.15 % saponin was added and mixed gently. The solution was incubated in ice for 10 min and centrifuged at 2000 rpm for 10 min. The supernatant was discarded without disturbing the pellet. The pellet was washed with ice-cold 1XPBS till the red colour of the supernatant disappeared. Parasite pellet was stored at –70 °C till further use.

3.2.3e Preparation of Frozen Stocks of Parasite Culture

Once the parasitemia reaches 5 % ring stage, it was harvested by centrifuging at 1500 rpm for 5 min. To the pellet, 1.5 volumes of freezing mixture was added and dispensed in freezing vial (1ml per vial) and transferred immediately to liquid nitrogen.

3.2.4 Maintenance of *Plasmodium berghei*

The most frequently used strains of *P. berghei* are: K173, ANKA, NK65, SP11 and LUKA. In our present studies, we have used ANKA strain of *P. berghei* and this strain can be maintained 1. ***in vitro*** and 2.**rodent host**.

1. **Maintenance of *P. berghei in vitro*:** Blood was collected from the infected mice in to heparinized syringe or the frozen stock was thawed to set up initial culture. The blood which was collected from the mice should be passed through the CF11 column to remove white blood cells. The initial culture was established at 8-12 % hematocrit and parasitemia of 0.5-2 %. Culture was maintained at 37 °C under constant flow of 5 % CO2 in 10 % O2 and 85 % N2 in T75 flask. The culture was constantly stirred at 50 rpm and once every 24 h was stirred at 400 rpm to assist in the rupture of the accumulated schizonts. The medium was changed daily twice by stopping the magnetic stirrer, allowing the RBC to sediment, so that the old medium was removed and fresh medium was added.

2. **Maintenance of *P. berghei* in mice:** BALB/c mice were used for the maintenance of *P. berghei.* To infect mice, frozen stabilate or heparinised blood was used. Routinely, 100 µl of blood 10^5 parasitized red blood cells was injected intraperitoneally into mice, so that the parasitemia will reach 10 % within 7 days of injection.

3.2.4a Monitoring Parasitemia

In case of *in vitro* cultures, smears were made from the T75 flasks just before changing of the medium. But in the case of infected rodents, the tail was held tight and a small incision was made at the tip and a small drop of blood was collected on the glass slide. Smear was made with the help of other slide, dried in the stream of warm air, fixed in methanol for 30 s and stained in Geimsa for 10-15 min. The slide

was observed under oil immersion and the parasitemia was estimated by counting number of infected and uninfected RBC.

3.2.4b Cryopreservation of *P. berghei* Parasite Culture

Blood from *P. berghei* infected mice was collected into heparinised syringe (final heparin concentration-30 µg/ml), transferred in to 15 ml falcon and was placed immediately on ice. Equal volumes of 20 % DMSO (Dimethyl sulfoxide) made in blood stage culture medium was added in drop wise over a period not less than 10 min to the heparinized blood. Numerous aliquots (0.1 ml) of the final mixture were made in cryotubes. The samples were frozen in a controlled manner to –80 °C. Subsequently, the cryotubes were transferred to the liquid nitrogen and can be stored indefinitely.

3.2.5 Cloning in pGEMT

Gene of interest was PCR amplified from the genomic DNA using gene specific primers. The PCR reaction was set up using following reagents

Chromosomal DNA : 100 ng

10X PCR buffer : 5 µl

dNTPs (1.25mM) : 8 µl

Forward primer : 50 pMoles

Backward pri : 50 pMoles

Taq enzyme : 1 unit

The reaction volume was set to 50 µl using MQ water.

The PCR reaction was run on 1 % agarose gel and the amplified product was gel extracted by using gel extraction kit (Novagen) as per manufacturer's instructions. The eluted fragment was cloned in pGEMT vector (Promega). The ligation in pGEMT was setup as follows.

pGEMT Vector : 1 µl

Insert : X µg

2X buffer : 5 µl

Ligase : 1 µl

The reaction volume was set to 10 µl using MQ water.

The ligation mixture was incubated at 4 °C O/N.

3.2.6 Preparation of Chemically Competent Cells

An overnight seed culture of *E. coli* was prepared in LB by incubating at 37 °C under shaking conditions. The seed culture was diluted fifty fold using fresh LB medium and allowed to grow at 37 °C with shaking till OD reached to 0.5-0.8 at 600 nm. The cells were pellet down by centrifugation at 4000 rpm for 30 min at 4 °C. The media was discarded and the cells were washed with chilled 50 mM calcium chloride. The cells were suspended in 50 mM Calcium chloride and incubated on ice for 1hr to make them competent. The competent cells were pelleted and resuspended in chilled $1/12.5^{th}$ volume of freshly prepared and sterile 50 mM calcium chloride containing 20 % glycerol. These competent cells were stored at –70 °C for several months.

3.2.7 Transformation into Chemically Competent *E. coli* Cells

Ligation mixture was mixed with Chemical competent *E. coli* cells (JM109) and incubated for 30 min on ice. Heat shock was given at 42 °C for 90 sec and immediately transferred onto the ice for 2 min. Appropriate amount of LB broth was added to the transformants and incubated at 37 °C with shaking for 1 h. The cells were plated on LB plate containing appropriate antibiotic for the selection of the transformants. When cloning in pGEMT, the plate was spread with 40 µl of 40 % X-gal and 40 µl of 0.1 mM IPTG for the selection of blue- white colonies.

3.2.8 Isolation of Plasmid from *E. coli*

Plasmid was isolated by alkali-lysis method. Culture for plasmid was inoculated in LB broth containing appropriate antibiotic and incubated O/N at 37 °C under shaking conditions. The cells were harvested by centrifugation at 6000 rpm for 10 min and suspended in 250 µl of P1 buffer. To the same tube 250 µl of P2 buffer was added, mixed gently and incubated for 5 min at room temperature. Later, P3 was added and incubated on ice for 15 min. The precipitate was pellet down by centrifugation at 13 K for 15 min at RT. Supernatant was carefully separated into another fresh eppendorf and phenol extraction was done to remove protein contamination. The DNA was precipitated by adding 0.7 volumes of isopropanol and pellet was recovered by centrifugation at maximum speed for 15 min. The plasmid DNA pellet was washed with 70 % ethanol, air-dried and dissolved in TE buffer.

3.2.9 Polyacrylamide Gel Electrophoresis of Proteins

PAGE was performed according to the protocol of Laemmli (1970). Gels were prepared and run in the presence of 0.1 % SDS (denaturing). Protein samples were prepared by mixing with equal volume of 2 X sample buffer (100 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 4 % β-mercaptoethanol, 0.01 % bromophenol blue). Samples were kept in boiling water for 5 min and loaded on the gel. Gels were run at 50 V till the proteins was stacked properly and thereafter gels were run at a constant voltage of 100 V. Gels were either electro blotted onto the nitrocellulose membrane (Hybond-C, Amersham, England) or stained with Coomassie blue R-250, 50 % methanol and 10 % acetic acid as described by Laemmli (1970).

3.2.10 Western Blotting

Western blotting was done according to Towbin *et al.* (1979). Mini Trans-blot Electrophoretic Cell (Bio-Rad) was used to transfer the proteins from gel onto nitrocellulose membrane. The apparatus for electroblotting was assembled according to the manufacturer's instructions. Electroblotting was performed in the presence of 39 mM glycine, 48 mM Tris base, 0.037 % SDS and 20 % methanol at a constant voltage of 50 V for 2 h at 4 °C. Transfer of proteins onto the membrane was checked by staining with Ponceau S stain (0.2 % w/v Ponceau-S, 3 % w/v tri-chloroacetic acid, 3 % (w/v) sulfosalicylic acid). The membrane was rinsed briefly in TBST (10 mM Tris, 150 mM NaCl, pH 7.5 and 0.05 % Tween-20) and then incubated in blocking solution (3 % BSA in TBST) for 1 h with gentle shaking at 37 °C. The blocking solution was replaced with primary antibody solution (1:2000 dilution of Fal2 and HRPII in TBST containing 1 % BSA) and incubation was continued at 37 °C for another 1 h with gentle shaking. Thereafter, the blots were washed thrice with TBST for 5 min each. After washing, blots were incubated with HRP conjugated secondary antibody solution (1:30,000 dilution in TBST containing 1 % BSA) at 37 °C for 1 h. The blots were washed as described above. The colour of protein-antibody complex was developed by using H_2O_2 and DAB. Alternatively, the blots were also developed by using ECL kit (Amersham Pharmacia).

3.2.11 dsRNA Preparation

Individual DNA fragments coding for *falcipain 1*, *falcipain 2* and *Aminopeptidae-N* (*Spodoptera litura)* are PCR amplified from pGEMT by using T7 and SP6 primers. The amplified products were PCR purified using PCR purification kit (Qiagen) as per manufacturer's instructions. The purified DNA fragments were used to generate sense as well as antisense RNA using T7 and SP6 polymerase.

Reaction mixture is as follows:

DNA : 5 µg

5Xtranscription buffer: 20 µl

rNTPs (2.5mM) : 8 µl

T7/SP6 : 3 µl each

RNase IN : 1 µl

The reaction was set to 100 µl using MilliQ water and incubated for 2 h at 37°C.

The tubes in which the reaction was setup were kept at –20 °C for 15 min to the polymerase activity. DNase I treatment was given by incubating at 37 °C to remove the DNA template contamination. To make dsRNA, the sense and antisense strands were mixed, heated to 65 °C and annealed by cooling slowly to 25 °C over several hours. Individual ssRNA and dsRNA were analyzed on 1 % agarose gel.

3.2.12 Preparation α-[^{32}P] Labeled dsRNA

The labeled dsRNA was prepared in the same way as discussed above. But instead of normal rUTP, α [^{32}P rUTP] was used in the rNTP mix. The internally labeled sense and anti sense RNA was annealed together to make labeled dsRNA. RNaseT1 digestion was done to eliminate ssRNA contamination.

3.2.13 Treatment of *P. falciparum* Culture with dsRNA

The parasites were synchronized as described in section 3.2.3c. For the analysis of the effects of dsRNAs, the synchronized cultures were adjusted to 5 % hematocrit with 1 % infected red blood cells, and 1ml of these cultures was treated in 24 well culture plates in triplicates in serum free medium for 30 min with intermittent mixing.

Subsequently human serum was added to these cultures to a final concentration of 10 % and parasites were maintained for 48 h. For microscopic analysis, smears were made from each well, fixed in methanol and stained with Geimsa.

3.2.14 Hypoxanthine Uptake Assay

To assess the effects of various dsRNA/inhibitors on parasite metabolic activity, a ^{3}[H]- hypoxanthine uptake assay was performed as described by Rosenthal *et al.* (1996). Parasite cultures (1 % parasitemia) of 100 µl volume at early ring stage were taken in 96 well microtitre plate in triplicates. DsRNA was added to each of these wells and incubated for 24 h. Once the parasite reaches trophozoite stage, ^{3}[H]-hypoxanthine (Dupont-NEN) was added (1.2 µCi), and cultures were maintained for additional 24 h. Cultures of mature parasite were frozen and thawed to lyse the infected RBCs. Lysed cultures were harvested on glass fibre filters, which were subsequently, washed with water, dried with ethanol and counted on a scintillation counter.

3.2.15 Treatment of *P. falciparum* cultures with Labeled dsRNA

The parasites were treated in the same way as described in the section 3.2.13. But instead of maintaining culture for 48 h, they were removed after 24 h and analyses for low molecular weight RNA (LMW) formation.

3.2.16 Isolation of RNA from Parasite and Fractionation on Agarose Gel

Culture from which RNA to be isolated was pellet down by centrifuging at 1500 rpm for 5 min. The pellet was taken into DEPC treated eppendorfs and saponin lysed as described in the section 3.2.3d. The parasites obtained after saponin lysis were washed with DEPC treated 1X PBS. Total RNA was isolated from the parasites using RNA isolation kit (Qiagen) as per manufacturer's instructions. Total RNA obtained was mixed with 6X RNA loading dye, heated at 65 °C for 10 min and quenched in ice for 5 min. The agarose gel for running of total RNA was made by dissolving 1.2 gms of agarose in 1X TBE and boiled. Once the temperature comes below 50 °C, 0.5 ml of 1 M GITC was added to 1X TBE containing agarose and poured on to the casting tray. After solidification, comb was carefully removed and

the gel along with the tray was transferred in to the buffer tank containing 1X TBE. The gel was run at 60-70 volts till the dye reaches 3/4th of total gel size.

3.2.17 Northern Blotting

Once the total RNA was properly resolved, the gel was washed thrice each for 15 min in denaturation solution (7.5 mM NaOH). The transfer was done over night in denaturation solution by using capillary transfer method. Briefly in this method, the gel was stacked between six pieces of 3 mm Whatmann sheet on either side with nylon membrane (Amersham N+ hybond) present above the gel. Filters papers were cut as per the exact size of the gel and placed above the Whatmann sheets. For proper transfer to take place, a heavy weight generally a thick book or glass bottle with uniform bottom was placed on the top of the filter sheets. After the transfer, position of the wells on the membrane was properly marked and the membrane was allowed to dry. RNA transferred on to the membrane was cross-linked using UV cross linker (Startagene) and washed in 6X SSC for 5 min. The membrane was dried properly, wrapped in saran wrap and stored at 4 °C till further use.

3.2.18 Preparation of Random Labeled Probe

The DNA probe for Northern hybridization was made by using random labeling kit (Gibco BRL). The DNA to be labeled was either gel-extracted fragment or PCR purified product, which was diluted in 20 µl of water in such a way that the amount of DNA was around 50-100 ng. This DNA was boiled, quenched in ice and was used in random labeling reaction. The reaction for random labeling was set as follows,

Random buffer: 15 µl
dATP : 2 µl
dTTP : 2 µl
dGTP : 2 µl
Taq Klenow : 1 µl
SDW : 7 µl

To the 28 µl reaction, 20 µl of diluted DNA along with 2 µl of α-[^{32}P] CTP was added and incubated at RT for 1 h. Later, the probe was purified by using nucleotide removal column (Qiagen) as per manufacturer's instructions.

3.2.19 Northern Hybridization

The blot for Northern hybridization was incubated in prehybridization solution for 2-3 h under constant rotation at 52 °C. Afterwards; the random labeled probe was added to the prehybridization solution and incubated for 12 h at 52 °C under constant rotation. Next, the blot was washed once in wash buffer I (3X SSC, 0.5 % SDS) and wash buffer II (2X SSC, 0.5 % SDS) at 52 °C for 15 min each. The last wash was given in 2X SSC to remove SDS and then the blot was dried and exposed to X-ray film (Kodak). The cassette was stored at –70 °C.

3.2.20 Analysis of Globin Hydrolysis in *P. falciparum* Parasites

To determine the effects of *falcipain* dsRNA or E-64 on hemoglobin degradation in malaria parasite, *P. falciparum* cultures were treated with this inhibitor/dsRNA for 24 h. After treatment, parasite infected erythrocytes were collected by centrifugation and lysed with saponin as described in the section 3.2.3d. The lysed samples were washed three times with ice cold PBS to remove erythrocyte cytoplasmic contents. The washed parasite pellet were solubilized in SDS sample buffer containing β-mercaptoethanol, boiled and fractionated on 15 % SDS-PAGE. The gels were stained with coomasie blue.

3.2.21 Extraction of Low Molecular Weight (LMW) RNA (25nt)

The dsRNA/siRNA treated cultures were lysed with saponin and the parasites obtained were washed with cold 1X PBS two to three times to remove erythrocyte cytosol contamination. Total RNA was obtained by using trizol based RNA extraction method. Initially, parasites were dissolved in 500 µl of trizol and were frozen at –70 °C. Later for extraction of RNA, tubes were thawed at 37 °C, 200 µl of chloroform was added and centrifuged at 13 K for 20 min at RT. The aqueous layer was carefully recovered and to this equal volume of Phenol/Chloroform was added and centrifuged at high speed for 10min to remove protein and lipid contamination. Total RNA was precipitated by using 3 volumes of absolute ethanol and 0.1 vol of sodium acetate. The pellet was washed once with 70 % ethanol and after drying was dissolved in DEPC treated water. The solution was heated to 65 °C to disrupt any association of the 25 nt RNA with larger RNA and DNA molecules and this treatment also quickens

the dissolving of the pellet. The tube was placed on the ice and PEG (MW 8000) to a final concentration of 5 % and NaCl (final conc. 0.5 M) was added to the solution and incubated on ice for further 30 min. The precipitate, which is high molecular weight nucleic acid, was pellet down by centrifugation at 13 K for 10 min. Three volumes of absolute ethanol was added to the supernatant of PEG precipitate and incubated at –20 °C for at least 2 h. The pellet was obtained by centrifugation at 13 K for 10 min. This pellet comprises mainly tRNA, small rRNA and 25 nt RNA.

3.2.22 Northern Blotting for LMW RNA

The sample was dissolved in formamide by heating at 65 °C for 5min and placed on ice immediately. To the sample 1/3 volume of 4X loading dye (2X TBE 40 % sucrose and 0.1 % bromophenol blue) was added. To run LMW RNA sample, 15 % polyacrylamide denaturation gel (15 % polyacrylamide 19:1, 8 M urea and 0.5X TBE) was used. The gel was run in 0.5X TBE at 100-500 V until BPB was almost at the bottom. The gel was carefully separated, placed on a prewetted (0.5X TBE) piece of nylon membrane (N+ hybond Amersham) and electroblotted for 45 min at about 100 V in 0.5X TBE. After blotting, membrane was placed on several layers of filter paper soaked on 20X SSC to equilibrate the membrane. After about 30 min, the membrane was fixed by UV cross linker (Stratagene). Membrane was stored in dark until further use.

If α-[^{32}P] UTP labeled dsRNA was used instead of normal dsRNA, after running of LMW RNA, the gel was dried on vacuum dryer and exposed to X-ray film for the presence of 25 nt small RNA species.

3.2.23 Preparation of Probe for Northern hybridization of LMW RNA

The probe was prepared by *in vitro* transcribing the DNA in the presence of ^{32}P labeled rUTP. The single stranded labeled RNA was hydrolyzed using 0.2 M sodium carbonate (80 mM $NaHCO_3$, 120 mM Na_2CO_3). For hydrolysis, to 20 µl of *in vitro* transcribed RNA, 300 µl of carbonate buffer was added and incubated at 60 °C for as long as it takes to reduce the ssRNA to an average size of 50 nt. Time was calculated by using the below indicated formula.

$T= (L_i-L_f)/ (k.L_i.L_f)$

Where

T= time

L_i=Initial length of the probe in Kb

L_f = final length of the probe in Kb

K= rate constant =0.11 Kb/min.

3.2.24 Northern Hybridization

The membrane carrying LMW RNA was incubated in prehybridization solution (50 % formamide, 7 % SDS, 50 mM $NaHPO_4/NaH2PO_4$ (pH 7.0), 0.3 M NaCl, 5X Denhardt solutions, 100 µg/ml sheared denatured salmon sperm DNA) for 5-6 h at 40 °C. For hybridization, the probe prepared as described in the section 3.2.23, was added to the prehybridization solution and incubated overnight at 40 °C under constant rotation. After O/N incubation, the blot was washed at 50 °C in 2 X SSC/0.2 % SDS, dried and exposed to X-ray film (Kodak).

3.2.25 *Plasmodium falciparum* Extract Preparation and Degradation of mRNA *in vitro*

The intact parasites treated with dsRNA as well as controls were collected by saponin lysis and washed thrice with ice cold PBS to remove erythrocyte cytoplasmic components. The washed parasite pellets were lysed by sonication in 200 µl of lysis buffer (100 mM Phosphate acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate and 5 mM dithiothreitol, pH 7.4). The lysates were centrifuged at 13000 rpm for 30 min at 4 °C, and supernatants were flash frozen and stored at –70 °C. The supernatants were used for *in vitro* assay. Typically, the reaction mixtures (50 µl) contained 25 µl of parasite extract and 50000 cpm of synthetic ^{32}P labeled mRNA as a substrate in *in vitro* assay buffer (20 mM HEPES, pH 7.3, 110 mM KoAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1mM DTT and 1mM ATP. The reaction mixtures were incubated for different time periods at 37 °C. Reactions were quenched by addition of 10 volumes 2X PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl and 2 % w/v SDS). Protein K was added to a final concentration of 465 µg/ml. reactions were analyzed by electrophoresis in a 0.8 % agarose gel containing GITC. Radiolabeled mRNA degradation was detected by exposing the agraose gel dried under vacuum to X-ray film (Kodak).

3.2.26 siRNA Design and Synthesis

Different siRNAs, fal 2, fal 1, BP 1, BP 2, AP-N, and GFP were designed for *falcipain 2*, *falcipain 1*, *bergheipain 1*, *bergheipain 2*, *aminopeptidase-N* and *green fluorescence protein* genes respectively, by using software developed by Elbhashir *et al.* (2002). All siRNA were obtained in annealed and lyophilized form from Dharmocon research (Colorado). These siRNAs were suspended in RNase free water at concentration of 5 µg/µl.

3.2.27 Treatment of *P. falciparum* Cultures with siRNAs

Synchronized *P. falciparum* cultures were adjusted to 4 % hematocrit with 1 % infected erythrocytes, and 0.5 ml of these cultures were treated with siRNAs in 24 well microtire plates in triplicates in serum free medium for 30 min with intermittent mixing. Subsequently, human serum was added to these cultures at a final concentration of 10 % and parasites were maintained for 48 h. Microscopic analysis and hypoxanthine assay was done as described in the previous section.

3.2.28 Indirect Immunofluorescence Assay

Indirect immunofluorescence assays were performed on 3D7 parasite lines. Thin smears of *P. falciparum* infected erythrocytes were made and fixed with mixture of methanol/ acetone. Slides were blocked in blocking buffer (1X PBS, 10 % FCS) for 2 h at 37 °C. After blocking, slides were incubated with primary antibody of appropriate dilution (mice anti Falcipain2, 1:200; rabbit anti MSP_{42}, 1:200; rabbit anti PfEMP1, 1;100) made in blocking buffer for 1 h. Slides were washed with 1X PBS for 1 h and incubated with appropriate secondary antibody conjugated to fluorescence dye (dilution 1:100) for 1 h. Later, the slides were stained with DAPI (4′, 6′-diamino-2-phenylindole) for 30 min at 37 °C at final concentration of 2 µg/ml and then washed twice in 1X PBS–Tween 0.05 %, once in 1X PBS and mounted on a cover slip in the presence of anti fade. The slides were viewed in confocal microscopy (Nikon).

3.2.29 Fractionation of Infected Erythrocytes and Western Blotting

Infected erythrocytes were fractionated by permeabilisation with streptolysin O (SLO) and saponin. Hemolytic activity of bacterially expressed recombinant streptolysin O was checked by incubating the RBCs with different concentration of

enzyme. Fraction showing considerable amount hemolytic activity was used for further experiments. Parasites (2X 10^7) were incubated in RPMI containing 3-4 hemolytic units of SLO for 10min at 37 °C. Supernatant obtained after spinning was suspended in Laemmli sample buffer, pellet was washed and subsequently resuspended in Laemmli sample buffer. For saponin lysis, $2X10^7$ parasites were incubated in 1.5 volumes of 0.15 % saponin for 10 min on ice, centrifuged and the supernatant was suspended in protein loading dye. The pellet was washed in 1X PBS and subsequently resuspended in the protein loading dye. The samples were boiled and separated on SDS-PAGE, transferred to nitrocellulose membrane and visualized by ECL using mouse anti Falcipain anti serum (1:2000).

3.2.30 Fluorescence Microscopy

P. falciparum culture carrying GFP constructs were synchronized by sorbitol treatments consecutively twice with 4 h gap between each treatment. These synchronized parasites were treated with siRNA at ring stage and allowed to grow until early stage schizonts. Samples were taken hourly during the process of schizogony and merozoite release. SiRNA treated control and treated parasites were cultured in DAPI for 30 min at 37 °C at final concentration (2 µg/ml) prior to imaging. Fluorescence from DAPI and GFP was observed and captured from live cells within 30 min of mounting the sample in culture medium under coverslip on a glass slide using confocal microscopy (Nikon, Japan).

3.2.31 Immuno Electron Microscopy

P. falciparum infected erythrocytes were fixed in 1 % gluteraldehyde, in 0.1 M phosphate buffer, pH 7.4 for 30 min at RT, followed by washing with 1X PBS and suspended in 1X PBS. Samples were dehydrated in 70 % ethanol and embedded in L.R. white resin and polymerized at 37 °C for 2 days. Thin sections were incubated in Falcipain 2 specific antibodies (1:100 diuluted in 1X PBS, 1 % BSA), washed thoroughly and incubated with gold labeled secondary antibodies. Sections were stained with 2.5 % uranyl acetate and visualized under electron microscope (Morgagni Transmission Electron Microscope, FEI Germany).

3.2.32 Injection of siRNA into *P.berghei* Infected Mice

Female BALB/c, of 4-6 weeks age and weighing 20-25 gms were used. Mice were infected by intravenous injection of 10^5 parasitized RBC. After 8-10 h, desired amount of siRNA were suspended in 200 µl of PBS and injected intravenously into infected mice in three replicates. The injections were repeated after 24 h and 48 h. Control mice were injected with GFP siRNA or PBS. Blood was collected from mice 24 h after the last injection. Blood smears stained with Geimsa stain were analyzed for parasite morphology and total parasitemia was counted.

3.2.33 Isolation of Parasites and Analysis of Globin Hydrolysis

Blood collected from siRNA treated and control nice was passed through CF11 column to remove white blood cells. Erythrocytes were lysed by 0.1 % saponin at 37 °C for 15 min. The parasite pellets were collected after centrifugation and washed three times with PBS to remove erythrocyte cytoplasmic content. The parasite pellets were supended in SDS sample buffer with β-mercaptoethanol, boiled and separated on 15 % SDS-PAGE. The gel was stained with Coomassie blue R-250.

3.2.34 Isolation and Detection of Low Molecular Weight RNA

For the siRNA uptake assay and to detect the formation of low molecular weight RNA, mice were first infected with 10^5 parasitised erythrocytes. After parasitemia has reached 2 %, ^{33}P labeled or unlabeled siRNAs were injected intravenously into these mice. After 12-14 h, blood was collected and processed for the extraction of total RNA. Low molecular weight RNA was extracted as described in the section 3.2.21.