3. Methods

3.1 Vertebrate-Cell Culture Methods

3.1.1 Sterilisation of the equipments and solutions

All the vessels, materials, solutions and medias were sterilised before usage. Sterilisation was achieved by autoclaving at 121°C, 2 bars for 20 min or baking at 180°C over night. Solutions which contain heat unstable substances such as aminoacids and proteins, were filtered through a sterile filter with a 0.4µm pore size. Culture vessels were supplied as already sterile.

3.1.2 Cell culture medias and buffers

Basis of the cell culture medias or cell culture solutions is high quality Ampuwa-Water, which is produced by several times filtering. All the medias and buffers which were used for culturing follow as in table (Table 3.1.):

Table 3.1 Cell culture medias and buffers

PBS	140 mM NaCl	8g
	2.7 mM KCl	0.2g
	10 mM $Na_2HPO4 \times 2H_2O$	1.44g
	1.5 mM KH_2PO_4	0.2g
	pH 7.4	ad 1l
McCoy	McCoy 5A	12g
	26mM $NaHCO_3$	2.2g
	Glutamin	10ml
	pH 7.4	ad 1l
MCDB 402	MCDB 402	
	With Inositol and Phosphate Phenolret	for 1l
	pH 7.4	ad 1l
RPMI	RPMI	
	Glutamin	2mM
	26mM $NaHCO_3$	2.2g
	pH 7.4	ad 1l
10xTrypsin/ EDTA (Fa. Gibco)	Trypsin	0.5g
	EDTA	0.2g
	NaCl	0.85g
	H_2O	ad 100ml

Before usage in cell culture all the medias and buffers were pre-warmed at 37°C and CO_2-content equilibrated by incubating in culture incubator.

3.1.3 Cultivation of AKR-2B mouse fibroblasts

AKR-2B cell line is an adherent growing, subcloned Mouse fibroblasts cell line. The cells were cultured in McCoy containing 5% (v/v) Hyclone-Calf serum (CS) at 37°C, 5% (v/v) CO_2 and humidified atmosphere. For subculturing, culture media was sucked away, cells were washed by PBS and treated with Trypsin/ EDTA for 30s, relieve of the cells were was followed by microscopy. When all the cells were completely relieved, they were taken into McCoy + 5% (v/v) CS, counted and seeded into a new culture vessel. Subcultures were maintained at a density of 3500 cells / cm^2 splitted every 2 days in McCoy +containing 5% (v/v) CS (Hyclone) without a medium change. For all experiments subcultures were seeded at a density of 0.1 x 10^5/ml.

3.1.4 Cultivation of THP.1 human monocytic tumor cells

Human monocytic tumor cells (THP.1) were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum and supplemented with 100 units of penicillin and 10µg/ml streptomycin and L-glutamine in 5% (v/v) CO_2 at 37°C. Subcultures were maintained as 0.1-1x 10^6 cells/ml, splited 1:2 to 1:3 every 3-4 days and seeded out approximately at a density of 0.5 x 10^6.

3.1.5 Cell counting by Casy1

The number of viable cells was determined by using the Casy-1 system based on the coulter counter principle. 100µl cell suspension diluted in 10ml PBS and automatically determined as triplicate in the Casy1 system.

3.1.6 Cryoconservation

For long term storage, cells were frozen at a density of 4 x 10^6/ml in CS or FCS 10% DMSO. First 2 h all cells were kept in -20°C then removed to the –80°C for 2 days, afterwards were taken into the liquid nitrogen tanks.

By thawing, cells were taken into the pre-warmed media and centrifuged at 110x g to precipitate the DMSO. Supernatant was thrown away and cells were resuspended in culture medias and taken to the culture flasks.

3.1.7 Induction of apoptosis

To induce apoptosis cells were washed twice by PBS and further cultured in either MCDB-402 media without serum (Life technologies, Eggenstein, Germany) or in McCoy supplemented with 10µM Anisomycin. To reconstitute the in vitro complex formation cells were incubated with dATP, Cytochrome c and $MgCl_2$. To induce ER. stress cells were incubated with A23187 (ionophore) and Thapsigargin.

Amount of the substances used for stimulation, are follows as in table:

Tab.3.2. Final concentrations of the substances used for apoptotic stimuli

Substances	**Concentration**
dATP	2 mM
Cytochrom c	200 µg
$MgCl_2$	5 mM
Anisomycin	10 µM
A23187	2.5 µM
Thapsigargin	0.5 µM

3.1.8 Transfection of AKR 2B-mouse fibroblasts by electroporation

18-24h pre-transfection, 2d old sub confluent cells were splited and seeded in to tissue culture dishes (16 cm/4 x 10^6). For transfection 2 dishes were splited by trypsinization and taken into 40ml PBS + 2.5% (v/v) CS, counted and centrifuged 5 min at 110xg. After centrifugation sedimented cells were resuspended in Hyperosmosis Buffer at a density of $2x10^6$/ml and transferred into 1.5 ml eppis as 1ml and further centrifuged 5 min at 110xg. Sedimented cells were resuspended once again in 0.5 ml Hyperosmosis Buffer. 400 µl of this suspension and 10 µg of plasmid DNA mixed and transferred into the special-cuvettes for electroporation. Electroporation was performed by using a Pulser Apparatus. Cells were transfected by one electrical pulse at 220V and 600µF capacity. cells were then transferred into 6x well plates and further cultured in pre-warmed McCoy without phenol red, containing 5% (v/v) CS. 24 h post transfection cells were stimulated for apoptosis by using MCDB +10µM Anisomycin for 6h time kinetic. Stimulated cells were washed with PBS.

Hyperosmosis Buffer: For 1 l stock

(f.c. 0.3 mM) 3 ml KH_2PO_4 (0.1M stock: 1.36g/100 ml)

(f.c.0.85 mM) 8.5 ml 0.1 M K_2HPO_4 (0.1 M stock: 1.74g/100 ml)

(f.c.25 mM) 25 ml 1 M KCl (1 M stock: 7.46 g/100 ml)

6.1 g myo-Inositol

with Inositol Osmolarity should adjusted to 90-100 mOsmol (app. 6-7g)

3.1.9 Preparation of cytosolic extracts and total cell lysates

Stock cultures of AKR-2B mouse fibroblasts were propagated in antibiotic-free McCoy-5A medium with 5% Hyclone calf serum for less than 3 months to minimize fluctuations. Cultures were seeded at a density of 5 x 10^3 cells/cm^2 either into 14.5 cm plastic dishes or 3.5 cm 6 x well plates and grown for 5 days without medium change. For preparing cytosolic extracts all steps were performed at 4°C. Cells grown on 14.5cm culture dishes were rinsed in 25ml Buffer A , scraped with a rubber policeman and disrupted with a tight fitting Dounce homogenization.

Table 3.3. All buffers which are used for preparation of the total cell lysates

Buffer A(Extraction Buffer) :	5mM $MgCl_2$ 2mM EGTA 50mM HEPES pH 7.0
PMSF-Stock Solution	1mM PMSF in EtOH
7x Inhibitor mix EDTA-free	1 Tablet in 1ml Extractions-Buffer
Lysis Buffer:	50mM Tris pH 6.8 2% SDS 1mM Na_3VO_4

Homogenate was clarified by centrifugation at 100000 x g for 25 min and the supernatant was supplemented with 1µM PMSF and 1x Protease Inhibitor mix - EDTA free and immediately used. Total cell lysates were prepared by resuspending cells in a Lysis buffer, shaking 5 min /1000 on a plate shaker and further treatment of 6 % of Benzonase, (see Table3.3.for buffers).

3.1.10 Immunostaining of cells for fluorescence microscopy

For immunostaining cells were grown on cover slips to sub confluency and treated with 10µM of Anisomycin in the presence and/or absence of MCDB for 4h. Then the media was sucked away and washed 2x with PBS/Mg/Ca. Fixation of the cells was achieved by 20 min incubation in Acetic acid : Methanol (1:3) at -20°C. After fixation cells were incubated for 5 min. at RT in 0.1% Triton in PBS/Mg/Ca incubation and then washed 2x with PBS/Mg/Ca containing 0.2% Gelatin. First Antibody was prepared as (1:100 = 1µg/ml) in PBS/Mg/Ca containing 0.2% fat free BSA and incubated for 1h at 37°C in darkness. After first antibody incubation, cells were washed again 2x with PBS/Mg/Ca containing 0.2% Gelatin and incubated with secondary antibody again for 1h at 37°C in darkness. Secondary antibody (Rabbit Cy3 red fluorescence conjugated) was prepared as (1:1500) dilution in

PBS/Mg^{+2}/Ca^{+2} containing 0.2% fat free BSA. After secondary antibody incubation, cells were washed 2x with PBS/Mg^{+2}/Ca^{+2} containing 0.2% Gelatin. Then cover slips were taken onto the cut edges in the presence of 2.5% DABCO and kept for drying overnight at RT in a dark place keep. Next day slides were used for fluorescence microscopy.

Table 3.4. All buffers which are used for immunostaining

PBS : PBS was prepared as described in section 3.2.1

10x Ca^{+2}/Mg+2
For 1 l: 1.32 g $CaCl_2$ x $2H_2O$
+ 1.0 g $MgCl_2$ x $6H_2O$

Acetic acid : Methanol (1:3)
50 ml: 12.5 ml aceticacid + 37.5 ml Methanol

0.1% Triton in PBS/Mg^{+2}/Ca^{+2}: 100 ml: 100 µl Triton to 100 ml PBS/Mg^{+2}/Ca^{+2}

0.2% Gelatine in PBS/Mg^{+2}/Ca^{+2}: 100 ml: 0.2 g Gelatine to100 ml PBS/Mg^{+2}/Ca^{+2}, warmed up 37°C in Mikrowave owen.

0.2% BSA in PBS/Mg^{+2}/Ca^{+2} : 100 ml: 0.2g BSA to 100 ml PBS/Mg^{+2}/Ca^{+2}

3. 1. 11 Fluorescence microscopy

For the fluorescence microscopy a Leica *DM IRB* type of microscope was used. It is equipped with I3 and N.21 filters from Leica, which are providing ultraviolet and red fluorescence lights respectively. Immunostaining of Caspase-3p17 was observed in cells by red fluorescent lightning. A table of summary of corresponding filters are follows as below:

Tab.3. 5. Filters of Fluorescence microscope

Filterblock	Sharpening filter	Reflection mirror	Blockfilter
A	BP 340-380	RSP400	LP425
I3	BP 450-490	RSP510	LP515
N.21	BP 515-560	RSP580	LP590

in nm; BP=Bandpassfilter, LP=Longpassfilter, RSP=Reflections-Shortpassfilter

3.2 Working with E.Coli

Growth, Maintenace and Preservation of Bacterial Strains

3.2.1 Growth of bacterias for plasmid preparation

Bacterial cultures were prepared in either LB- or selective media. Selective media is supplemented with appropriate antibiotics. All cultures were prepared from a single colony in sterile culture flask or for small scale in 2ml culture tubes. Growth of bacterias were maintained at 37 ^{o}C attaching the tubes or flasks to a rotary shaking platform (250rpm) or a rotating wheel (100 rpm) for 7 h or overnight. These cultures can be used for plasmid preparation or can be streaked on to an agar-plate for subcloning.

Table 3.6. Bacterial culture medias

LB-Media:	0.5% Yeast-Extract	5g
	1% Trypton	10g
	1% NaCl	10g
		H_2O ad 1l
	Autoclaving	
LB-Agarplate:	0,5% Yeast-Extract	5g
	1% Trypton	10g
	1% NaCl	10g
	1.5% Agar	15g
		H_2O ad 1l
	Autoclaving	
Selection Media:	LB media suplemented with 70 mg/l Ampicillin or 34mg/l Chloramphenicol or both together.	

3.2.2 Growth and induction of bacterias for expression of a target gene

A starter culture which is containing the recombinant target gene was prepared. For this a sterilee loop of cells taken from a glycerol stocks. For small scales 3 ml of appropriate media (containing antibiotics) was inoculated in a culture tube and starting OD_{600} was adjusted as 0.05. Then starter culture was incubated at 37 ^{o}C with shaking at 250 rpm to OD_{600} of approxiamately 0.5 and then the entire 3ml culture was added to 100 ml LB media containing antibiotics. The 100 ml culture was shaked at the desired temperature (at 37 ^{o}C) until the OD_{600} was approximately 0.5-1.0 (during growth the OD_{600} was monitored by removing and measuring 1 ml aliquots). Just prior to induction the 100 ml culture was splitted into 2x 50 ml cultures. For plasmids having the T7*lac* promoter, induction was performed by addition of sterile 1mM (f.c.) of IPTG to one of the 50 ml cultures. The other 50 ml culture was served as a noninduced control. Induction was performed by incubation with shaking at 30 ^{o}C for the

appropriate amount of time (this time depends on expression profile of the gene which is determined by Coomassie staining after induction). During induction every hour 1ml aliquot was removed and OD_{600} was determined. All aliquots from induction step and the aliquot of noninduced control were assesed for the expression of target gene by analysis of total cell protein on an SDS-PAGE and followed by Coomassie blue staining. The various parameters of growth, induction and localization were established on a small scale, these were applied to larger scale cultures (as 1-2 l) for production of target proteins.

3.2.3 Storage of the bacterial strains

For long term storage a single colony was inoculated into a flask containing 50 ml LB media supplemented with an appropriate antibiotic at 37°C (OD_{600}= 0.5-0.6) overnight. Next day the culture was centrifuged at 500x g for 10min and then suspended in LB-Glycerol (V:V) transferred in to a sterilee vial. The glycerinated cultures were stored at -20°C.

3.2.4 Transformation of *E.coli* by plasmid DNA

Competent cells

For transformation chemically competent cells from Novagen, either NovaBlue for plasmide multiplication or BL21(DE3) for expression were used.

NovaBlue single competent cells

NovaBlue is a K-12 strain ideally suited as an initial cloning host due to its high transformation efficiency, blue/white screening capabilitiy (with appropriate plasmids) and *recA endA* mutations, which result in high yields of quality plasmid DNA.The DE3 lysogen of NovaBlue is potentially useful as astringent host due to the peresence of the *lacI^{l} repressor* encoded by the F episome. thus Novablue cells were used to multiply the plasmid copies.

Genotype of NovaBlue singles : *endA1, hsdR17* (r_{K12}-m_{k12}+), supE44*thi-1*, *recA1, gyrA96,* relA1, lac, [F',lac-proA+B+, lacI^{q}ZΔM15:: Tn*10*(tet^{R})]

BL21(DE3) pLysS single competent cells

BL21 is a B strain, the most widely used host backround for protein expression and has the advanTaqe of being deficient in both *Ion* and *ompT protease* that can degrade proteins during purification. pLysS plasmid providing additional stability to the BL21 cells. Cells carrying this plasmid accumulate lower levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase. This plasmid confer resistance to chloramphenicol and compatible with the vectors such as pET, for cloning target genes.

Genotype of Bl21(DE3) pLysS singles : F^{-} *ompT hsdS$_B$ (r_B^- m_B^-) gal dcm* (DE3) pLysS (Cm^{R})

3.2.4 Transformation of the competent *E.coli* cells

Appropriate number of competent cells were removed from -70 ^{o}C and immediately placed on ice. Cells were allowed to thaw on ice 2-5 min and were then flicked to resuspension and aliquoted into each prechilled tubes as 20µl. 1µl of either ligation reaction or purified plasmid DNA was added directly to the cells and stirred gently to mix and kept on ice for 5min. After ice incubation, cells were heated at 42 ^{o}C in a water bath exactly for 30 sec. and then once again kept on ice for 2 min. 80 –200µl of SOC medium added to the each transformation reaction. For NovaBlue cells transformation tubes were shaked at 37 ^{o}C (250 rpm) for 30 min prior to plating on selective media. For strains other than NovaBlue transformation tubes were sahaked 37 ^{o}C for 1hr. and then plated.

3.3 Proteinchemistry Methods

3.3.1 Bradford protein determinataion

With Bradford test concentration of protein samples that are not containing SDS and reducing agents can be determined. 96 well of microtiterplate was used for the assay. Each well adjusted with 100µl H_2O, 10µl sample or standard protein solutions and with 100µl Bradford-Reagent. As Standard-Protein Solutions, a serial dilutions of BSA from 0.25mg/ml to 0.0125mg/ml in sample buffer were prepared and used. The colour alterations were measured as densitometrically by using ELISA-Reader Test Wave at 630nm and Reference at 405nm. Protein amount of samples were determined from standart values.

Bradfordreagent:	0.06% (w/v) Coomassie-G250 in 1.6% (v/v) Perchloricacid

3.3.2 Redingbaugh protein determination

With this method concentration of protein samples in Laemmli-Buffer that are not containing β-Mercapoethanol can be determined. In microtiterplate each well was adjusted with 200µl mixture of Solutions A and B (49:1) supplemented with 10µl sample or standard-protein solution. As Standard-Protein Solution a serial of dilutions from 2.5mg/ml to 0.125mg/ ml BSA in sample buffer were prepared and used. Microtiterplate was incubated at 54°C for 45 min and afterwards colour alterations were measured as densiometrically by using ELISA-Reader Test Wave at 550 nm and Reference at 630 nm.Protein amount of samples were determined from standart values.

Table 3.7. Solution for Redinbaugh protein determination

SolutionA:	1.35% (w/v) $NaHCO_3$	6.75g	
		0.85% (w/v) NaOH	2.9g
		1% (w/v) Bicinchonicinacid	5g
		0.57% (w/v) KNa-Tartrat	285g
			ad 500ml
Solution B:		2.3% (w/v) $CuSO_4 \times 5H_2O$	

3.3.3 SDS-Polyacrylamid-Gelelektrophoresis (from Laemmli [130])

For electrophoretical seperations of proteins method of Laemmli was used [130].

3.3.3.1 Minigels

Polyacrylamidgels were prepared in Miniprotean Apparatus of Biorad with a thickness of 0,75mm. Miniprotean Apparatus was constructed according to the manufacturer instruction. Gels were prepared according to the table (Tab 3.8.) below and poured between glass plates. After seperating gel had been filled, concentarting gel was poured carefully without mixing the two phases. Then the appropriate comb was inserted immediately, being careful not to allow air bubles to become trapped under the teeth. The acrylamide was allowed to polymerization around 30 min, and attached to the electrophoresis tank which is filled up with 1x electrophoresis buffer for SDS-PAGE, the comb was removed carefully . Then the samples and the molecularweight marker were loaded and run at a volTaqe of 100V and 200V for concentrating and seperating gels respectively.

3.3.3.2 Midigel for 2D-Gelelektrophoresis

Polyacrylamidgels were prepared with a thickness of 0,75 mm in Midi-Apparatus which was made by workshop of the institute for 2D-Gelelektrophoresis. Midiapparatus consist of 2 glassplates (14cm x 12cm) that are seperated by using spacers and fixed with clamps. The lower part of the plates were sealed with 2% (w/v) Agarose. Gels were prepared according to the table 3.8. below and poured between glass plates (Tab 3.8.) .

After seperating gel had been filled, concentarting gel was poured carefully without mixing the two phases till 5mm down. to the starting point of plates. Only one teeht was inserted in to the gel for the molecularweight marker. After 30 min polymerization gels were attached to the apapratus. Molecularweight marker loaded as 30µl and the gel stripes from the first dimension were placed between the glass plates through the gel and fixed with 1% (w/v) Agarose in 1x SDS Reservoir Buffer. Electrophoresis tank was filled up with 1x Reservoir

Buffer for SDS-PAGE and run at a voltaqe of 100V and 200V for concentrating and seperating gels respectively.

Tab.3.8. Contents of SDS-PAGE for midigels

	Concentrating Gel 4X	**Seperating Gel 4X**		
	3%	**10%**	**12,5%**	**15%**
Acrylamidsolution 30,8%T, 1,6%C	0.5ml	5.0ml	6.25ml	7.5ml
Lower buffer	--	3.8ml	3.8ml	3.8ml
Upper buffer	1.25ml	--	--	--
H_2O	3.2ml	3.7ml	1.975ml	1.2ml
Gycerin	--	2.5ml	2.5ml	2.5ml
TEMED	6µl	12µl	12µl	12µl
40% (w/v) APS	12µl	18µl	18µl	18µl
Totalvolums	5ml	15ml	15ml	15ml

Table 3.9. Solutions and buffers for SDS-Gel preparation

Acrylamidsolution 30.8%T, 1.6%C	30% (w/v) Acrylamid 0.8% (w/v) Methylenbisacrylamid
Lowerbuffer	1,5M Tris-Cl pH 8.8 1.% (w/v) SDS
Upperbuffer	0.5M Tris-Cl pH 6.7 10% (w/v) SDS
10 x Reservoir (Run) Buffer	2M Glycin 1.5% SDS 0.25M Tris

3.3.4 2D-Gelelektrophoresis

To seperate proteins by their molecularsizes and isoelectrical points 2D-Gel Electrophoresis was used. Isoelectrical focusing was achieved by using ready made immobilized dry strips (pH 3-10 linear Ammersham-Pharmacia).

Rehydration of gel stripes:

Ready made IPG strips must be rehydrated prior to IEF. Samples can be applied by including it in the rehydration solution. Up to 1mg of sample per strip can be diluted into or redissolved

rehydration solution just prior to IEF. Vacuum concentrated or lyophilized protein samples were dissolved in 450 µl rehydration buffer and applied to the reservoir slots of IPG rehydration/reswelling tray carefully. Airbubles were removed, IPG strips were placed on the surface of sample and each strip was covered with 1.5-3ml IPG Cover Fluid to minimize the evaporation and the urea crystallization. Rehydration was achieved by overnight incubation.

Rehydrationbuffer:	8M Urea	2,4g
	Thiourea	0,76g
	1% (w/v) CHAPS	50mg
	1% (w/v) DTT	50mg
	Ampholyte, pH3-10	26µl

adjusted to 5ml with clean millipore H_2O.

Isoelektrical focusing:

After rehydration the IPG strips were removed from reswelling tray and rinsed in a deionized water and transferred to the electrophoresis unit for first dimension IEF. The strips were placed with the pointed (acidic) end at the top of the tray near the anode (red electrode) and the blunt end at the bottom of the tray near the cathode (black electrode). The IPG stripes were aligned so that the the anodic gel edges were lined up. Then the moistened electrode strips (paper) were placed across the cathodic and anodic ends of aligned strips as the electrode strips at least partially contacted the gel surface of each strip. To prevent drying of the IPG strip, strips were fully covered with IPG cover fluid. Isoelectric focusing was achieved in two steps as first 1h at 500V, 2mA, 5W and then overnight at 3500V, 2mA, 5W. The electrophoresis tank was cooled down 20°C by using a circulating pump.

IPG strip equilibration:

The equilibration Step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration solution contains buffer, urea, glycerin, reductant, SDS and dye. Equilibration introduces reagents essential for the second- dimension separation. The second dimension gel must be ready for use prior to IPG strip equilibration. For equilibration the stripes were incubated 15 min with Buffer A and B on a shaking platform. After equllibration the stripes were washed carefully with H_2O and placed on to a filter paper to drain and to remove the excess of buffers. Once the equilibrated IPG strip have drained at least 3 minutes, they were placed on the SDS gel so that IPG strip is in full direct contact with the SDS gel. To remove any bubles, the plastic backing of the IPG strip was

stroaked gently with a pair of forceps. The stripes were sealed and fixed with 2% (w/v) Agarose in 1x Reservoir buffer. SDS-PAGE was performed as indicated in 3.3.4.2.

Table 3.10. Buffers and solutions for 2D-Gel preparations

Equilibration solutions:		
	6M Urea	36g
	30% (v/v) Glycerin	30ml
	4% (w/v) SDS	4g
	5mM Tris-Cl pH 6.8	10ml [50mM]
	adjusted to 100ml with millipore H_2O	
Buffer A:	Equilibration solution 3,5mg/ml DTT	
Buffer B:	Equilibration solution 45mg/ml Iodacetamid Bromphenolblue	

3.3.5 Coomassie Blue staining

To visualize the proteins on polyacryilamide gel, gels were satined with Coomassie Blue which is a relatively simple and quantitative method. Coomassie Blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined. Gels were incubated with satining solution for 20-30 min on a shaking platform at R.T. After staining gels were destained with first and second destainer respectively until the bands become visible and clear.

Table 3.11. Preparations of Comassie stain and destainers

Coomassie Stain:	0.2% (w/v) Coomassie Briliant Blue R250 10% (v/v) Acetic acid 50% (v/v) Methanol
Destainer 1	10% (v/v) Acetic acid 50% (v/v) Methanol
Destainer 2	5% (v/v) Acetic acid 10% (v/v) Isopropanol

3.3.6 Western- and affinity-blotting

To detect an individual protein from a mixture, proteins were seperated electrophoretically by SDS-PAGE, (as indicated in part SDS PAGE) and the individual protein bands were then transferred to nitrocellulose membrane.Transferring was conrolled by using a prestained molecular weight marker such as SDS-7B from Sigma or Prestained Benchmark from Gibco

BRL. Specific antibody was then used to probe for any of the bands it might bind to, and the nonbound antibody was washed away. The bound antibody was then detected by the addition of a secondary antibody against the immunoglobulin species contained in the first antibody. This secondary antibody was labeled with an enzyme, and the specific protein band can then be visualized by the addition of an enzyme substrate containing a colour developer.

Blotting:

For blotting semi-dry blotting procedure was used. Appropriate size of nitrocellulose membrane BAS 85 and Blottingpaper GB003, from Fa. Schleicher & Schuell, were cut and soaked in Transferbuffer. Afterwards membrane and the gel were placed on to the *Graphite Cathode plate* of *Semi-Dry* apparatus as in the following order, and then covered with *Anode plate* .

- 6 pieces wet Whatmanpaper
- SDS-Gel
- Wet Nitrocellulose Membrane
- 6 pieces Whatmanpaper

All air bubles between the papers or membrane or gel were removed by using a glassrod. Blotting was performed under a constant currency at $1cm^2 \cong 0.8mA$ for 1hr. When it was necessary to use a PVDF-Membrane, prior to transferring it was completely pre-soaked with methanol and Whatmanpapers were washed for transfer buffer for PVDF Membrane.

The detection:

After transferring, membranes were blocked with a blocking buffer in 5% milk powder in TBS-0.1% Tween 20 or 2% BSA in Tris/NaCl/0.2% Triton-x100 in the container, 1hr at RT on a shaker; or O.N. at 4 ^{o}C on a rocker. Then membranes were incubated with first antibody for 1hr at RT or O.N. at 4°C. First antibody dilutions were prepared in 2% BSA in Tris/NaCl 0.2% Triton-x100 or 2%-BSA-TBS/Tween 20 supplemented with 0.03% (w/v) Sodiumazide and stored at 4°C for further use. After first antibody incubation membranes were washed 6 times for 5 min. with 0.5%-BSA- in Tris-NaCl-0.2% Triton-x100 or in TBS-0.1 % Tween 20. Appropriate dilution of peroxidase conjugated secondary antibody dilution was prepared in 2% (w/v) BSA-in Tris/NaCl 0.2% Triton-x100 or 2%-BSA-TBS/Tween 20. Membranes were incubated for 1hr with secondary antibody. After secondary antibody incubation membranes were washed again 6 times for 5 min with 0.5%-BSA- in Tris-NaCl-0.2% Triton-x100 or in TBS-0.1 % Tween 20 .

For Affinity blotting Peroxidase conjugated Avidin was used and the dilution was prepared in in 2%-BSA-in Tris/NaCl 0.2% Triton-x100. After 1hr incubation at R.T. membranes were washed 10 times 5 min with 0.5%-BSA- in Tris-NaCl-0.2% Triton-x100.

Table3.12. Buffers for Western blotting and solutions for ECL.

Transferbuffer for Nitrocellulose:	50mM CAPS-Buffer 1mM 3-Mercaptopropionicacid pH 10 10% Methanol (given freshly) 0.2% SDS (given freshly)	11.1g 87µl H_2O ad 1l
Transferbuffer for PVDF-Membranes:	50mM CAPS-Buffer 1mM 3-Mercaptopropionacid pH 10 30% Methanol (given freshly) 0.2% SDS (given freshly)	11.1g 87µl H_2O ad 1l
Tris-NaCl Buffer for Western Blot:	50mM Tris 150mM NaCl 0.2% NP 40 pH 7.5	6.06g 8.77g 2ml H_2O ad 1l
10x TBS/ + 0.1% Tween20	250 ml 1 M Tris, pH 8.0 (f.c. = 250 mM) 150 ml 5 M NaCl / 43.8 g NaCl (f.c. = 750 mM) 4.2 ml 3 M KCl / 932 mg KCl (f.c. = 12.5 mM) with Millipore-H2O ad to 1 l	
5% Milkpowder-Solution	5% Milkpowder in 1x TBS/ + 0.1% Tween20 (freshly prepared)	
2%-BSA-Solution:	2% BSA in Tris-NaCl Buffer for Western-Blot (freshly prepared)	
0.5%-BSA-Solution:	0.5% BSA in Tris-NaCl Buffer for Western-Blot (freshly prepared)	

Enhanced Chemiluminescence (ECL):

Membranes were incubated in darkroom at Redlight 1min with 5ml Luminol-Reaction solution and H_2O_2-Solution (solutions must be freshly prepared see table 3.13 for all the solutions). After draining with paper towels, membranes were palced in to plasticfolio and exposed with Rontgenfilms first 1 min. and then adjusted for the later trials. After exposure films were developed at a developing maschine. Stock solutions of lumonol and p-Cumaracid were aliquoted and stored in a dark place at -20°C .

Table 3.13. Slotuions for the Enhanced Chemie Luminescence

Luminol-Stock Solution:	250mM Luminol in DMSO	224mg ad 5ml
p-Cumaracid-Stock Solution:	90mM in DMSO	74.2mg ad 5ml
Luminol-Reaction Solution:	Luminol-Stock Solution p-Cumarsäure-Stock Solution 0.1M Tris-Cl, pH 8.5	500µl 220µl ad 50ml
H_2O_2-RectionSolution:	H_2O_2 (37%) 0.1M Tris-Cl, pH 8.5	20µl ad 40ml

3.3.7 Activity assay of caspases

To determine the caspase activities either in cytosolic extracts or in chromotographic fractions corresponding tetrapeptide substrates were used. In general, the substrates are N-blocked tetrapeptides containing a reporter group at the C terminus which is adjacent to the specificity determining Aspartate and is released by the active protease. The most commonly used reporter groups are *p*-nitroanilide (pNA, spectrophotometric detection by absorbance at 405 nm). The quantification and the specific activity was calculated using *p*-Nitroanilide release as a standard. The amount of activity was calculated as in *pmol*, incubation time taken as in *min* and the amount of protein used for the assay as in *mg* or as *ml*. Tofind the activity of thesamples expressed as pmol substrate/min, the below formula was used:

Activty (pmol/min)= (OD/min)xconversion factor(µM/OD)x assay vol (µL)

Conversion factor of 50 was used in the assays which is supplied from manufacturers instruction of the caspase substrates.

Specific activity was considered as the difference between the activities with and without specific inhibitor. So that the rest of the activity from an inhibition assay was considered as contaminating activity.

3.4.1 Activity assay

To determine the caspase activities from the fractions of gel filtration chromatography, fractions were incubated with either 100 or 50 µM (f.c.) of the respective substrates. Minimum 75-100µg amount of protein from cytosolic extracts or 50-100 µl fractions from Gel Filtration Chromotography were supplemented with 10 µl of concentrated substrate and adjusted in microtiter plates to the volume of 100 µl with or without BufferA. After shortly

shaking, the enzymatic reaction was incubated at 37°C for 30–45min. Whereas to determine the recombinant caspase activities, recombinant caspase was incubated for 1 or 5 min with the respective substrates. Absorbance was densiometrically measured at 405 nm (Testwave) and 630 nm (Reference wave) by ELISA-Reader. The quantification and the specific activity was calculated from the above indicated formula using p-Nitroanilide release as a standard.

Buffer A:	50mM HEPES, pH 7.0 5mM EGTA 2mM $MgCl_2$
p-Nitroanilide-Solution	50µM p-Nitroanilide in Buffer A

3.3.8 Chromatography

Individual proteins such as Caspases can be purified from a protein mixture like cytosolicextracts by using chromatography techniques. Here gel filtration chromatography were used to seperate caspases or caspase containing complexes.

Chromatographic separations were carried out using an inert pump model L 6210 from Merck-Hitachi. For all seperations samples were injected by using a syringe into a sample loop 1-2 ml which is then flushed through with solvent to inject the sample on to the coloumns. To monitor the seperations absorption of proteins were measured by a UV detector at 280 nm and saved in computer by using a software programm. Fractions were collected automatically. In order to prevent air bubles all the solutions and buffers were filtered using a 0.45µm filters and de-gassed. In order to prevent back pressure increase due to particulate fouling, the samples were freed of precipitate and suspended solids, by using centrifugation or filtration.

To detect the caspases from the fractions aliquots of individual fractions were used for caspase activity assay and western or affinity blotting .

3.3.8.1 Gel filtration chromatography

To seperate caspase activating complexes gel filtration chromatography was used. For seperation 20 mg protein of cytosolic extract was applied on to a HiPrep Sephacryl-S-300 coloumn (1.6 x 60 cm Amersham Pharmacia) with a flow rate of 0.8 ml/min in bufferA. Fractions were collected as 1 ml . For a better resolution of high molecular weight complexes 10 mg protein of cytosolic extract was applied on to a Superose 6 HR 10/30 coloumn (Amersham Pharmacia). Seperation was performed at flow rate of 0.5 ml/min in bufferA. Fractions were collected as 0.25 ml. Both Sephacryl-S-300 and Superose 6 HR 10/30

coloumns were calibrated by using a gelfiltration calibration kit (Amersham Pharmacia) showing the expected resolution. Prior to all gel filtration chromatography cytosolic extracts were perpared in buffer A.

Buffer A: 50mM HEPES, pH 7.0
5mM EGTA
2mM $MgCl_2$

3.3.8.2 Purification of recombinant target proteins by immobilized metal affinity chromatography

Immobilized metal affinity chromotography (IMAC) was introduced in 1975 as a group-specific affinity technique for seperating proteins [131]. The principle is based on the reversible interaction between various amino acid side chains and immobilized metal ions. Depending on the immobilized metal ion, different side chains can be involved in the adsorption process. Most notably, histidine, cysteine and tyrptophan metal cahins have been implicated in protein binding to immobilized transition metal ions and zinc [131].

Preparation of Extracts with Bug Buster™ Reagent and Benzonase® Nuclease [132].

After the induction of bacterial cultures for expression of target protein, cells were harvested by centrifugation at 10000 x g for 10 min. and used for further extraction of proteins. As much as supernatant was removed from the pellet and the pellet was allowed to drain. Then the cell pellet was completely resuspended at +4 ^{o}C with BugBuster reagent by pipetting or gentle vortexing .Bug buster reagent was used as 5ml/g of wet cell paste, and supplemented with 1µl of Benzonase per ml of reagent. Then the suspension was incubated on shaking platform or rotating mixer at a slow setting for 10-20 min. at+4 ^{o}C. Next the insoluble cell debris was remove by centrifugation at 16 000 xg for 20 min. at 4^{o}C and the pellet was saved for further analysis. The soluble fraction prepared by Bug Buster reagent contains the soluble periplasmic and cytoplasmic proteins and the insoluble fraction contains inclusion bodies and other macromolecular complexes. The soluble extract can be loaded directly onto protein purification resins.The soluble and insoluble fractions were analyzed by SDS-PAGE and Coomassie staining side by side.

Purification of target proteins with talon® metal affinity resin [133].

TALON® Resins are durable, cobalt-based IMAC resins designed to purify recombinant polyhistidine-Taqged proteins.These resins are compatible with many commonly used reagents and allow protein purification under native or denaturing conditions [134]. They can be used with allprokaryotic and eukaryotic expression systems in a variety of formats,

including small- (or-mini) scale batch screening, large-scale batch preparations, and methods using gravity-flow coloumns and spin coloumns .

Before starting to purification, TALON® Metal Affinity Resin was equillibrated with extraction wash buffer pH 7.0 . Equilibration was achieved by 3-4 times washing with 10 Bed Volume of resin by centrifugation at 700 x g for 5 min. After the equilibration the soluble extract was added to the metal resin (*usually 2 ml resin enough per mg of anticipated polyhistidine-Taqged protein)*. After gently agitation at R.T. 20 min (to allow the Taq bind to resin) on an overhead shaker the suspension was centrifuged at 700 x g for 5 min. and then as much as supernatant was removed. The suspension was washed again with 10 BV (Bed Volume) of 1x Ex-Wash buffer, gently agitated for 10 min on overhead shaker and centrifuged 700 x g for 5 min. The supernatant was removed and discarded. Washing and agitating steps were repeated once again. After last centrifugation step the resin was resuspended with 1 BV of Ex-wash buffer and transferred to the gravity coloumn and resin was allowed to settle down. Then the end cap of the coloumn was removed and the buffer was allowed to drain. Coloumn was washed once with 5 BV of Ex-Wash (Extraction-Wash) buffer. Washing was repeated by 2 BV Intermediate Buffer and the fractions were started to collect (for small scale purifications fractions were collected as 0.5 ml, whereas for large scale as 1ml). as last step the His-tagged bound protein was eluted by using 1 BV of Elution Buffer. After collection of the all fractions, the amount of the protein were determined by using *Bradford* method.

Table.3.14. Buffers for metal affinity chromatography

10 X (0.5 M)Phosphate Buffer (pH: 7.0) :	0.5 M Na_2HPO_4 * H_2O (500 ml) 0.5 M NaH_2PO_4 * $2H_2O$ (500 ml)
By using these two buffers pH adjusted at 7.0	(*300 ml Na_2PO_4 + ? NaH_2PO_4 up to ph: 7.0*)
1x Extraction-Wash Buffer (pH:7.0) :	50mM Phosphate Buffer, 300mM NaCl.
1x Elution Buffer (pH: 7.0) :	50mM Phospahte Buffer, 300mM NaCl, 150mMImidazole
1x Intermediate Elution Buffer (pH: 7.0) :	50mM Phospahte Buffer, 300mM NaCl, 15 mM Imidazole.
10x (1.5 M) Imidazole pH: 7.0, stock	

Analysis of the quality of the purified fractions was achieved by SDS-PAGE and Coomassie staining. 20µl aliquot from each fraction was supplemented 7µl of Laemmli Sample Buffer, loaded as 13µl to each slot.

3.4 Isolation and Purification of Nucleic acids

3.4. 1 Standard methods for purification of DNA

The below methods for purification of DNA are standart protocols used in molecularbiology and parts of the following methods in this chapter.

Extraction with phenol/chloroform

Purification of nucleic acids can often be carried out simply by extracting the aqueos solutions of nucleic acids with phenol and/or chloroform. Such extarctions are used whenever it is necesary to inactivate and remove enzymes that are used in one step of a cloning operation before proceeding to the next. The standart way is to extract the DNA once with phenol and once with a 1:1 mixture of phenol and chloroform.

DNA samples were extracted with an equal volume of phenol (phenol equilibrated with buffer and containig 0.1% hydroxyquinoline and 0.2% β-mercaptoethanol, pH: 8.0) and after shaking centrifuged at R.T. 5 min at 13000x g. Upperphase was transferred in to a new eppi and extracted 2 x with Chloroform/Isoamylalkohol (24:1 v/v) and centrifuged as before. After chlorophorm/isoamylalcohol extraction DNA was recovered by precipitation with ethanol as described in below.

Precipitation with ethanol

The most widely used method for concentrating DNA is precipitation with ethanol. To precipitate of DNA, which is allowed to form at low temperatures (-20 ^{o} C or less) in the presence of moderate concentrations of monovalent cations, is recovered by centrifugation and redissolved in an appropriate buffer at the desired concentarions. 2V of ice-cold ethanol and 1/10 V of 3M Sodiumacetat (pH 5.2) solution (f.c. 0.3M in DNA solution) were added to DNA solution mixed well and incubated in –20 ^{o}C 30-60 min. Afterwards to precipitate the DNA it was centrifuged at 4°C at 13000x g minimum for 15 min and then pellet was washed with 500µl 70% (v/v) Ethanol by centrifugation for 5 min. Pellet was dried by using a vacuum concentrator and dissolved with an appropriate volume of H_2O or TE Buffer

3.4.2 Plasmid preparation from *E. coli* with LiCl

For preculturing a single colony from the original plate or 20-30 µl aliquot from the stemculture was picked and added to 2 ml LB-Media supplemented with the appropriate antibiotic and allowed for growing on shaker at 37°C for 7 h. Then 2ml of this preculture was used for mainculture, taken into 2x 50 ml culture flasks containing LB-Media (supplemented with the appropriate antibiotic) and growed on shaker at 37°C O.N.

Following day both cultures were transferred into 50 ml falcons and centrifuged for 10 min 13 000 g at RT , the supernatant was discarded. One of the 50 ml falcon's pellet was used for glycerin stock culture and the second one used for plasmid preparation. The pellet from 50 ml culture was resuspended in 2ml ice-cold Solution 1 and incubated 10 min on ice. Then 4 ml of solution 2 was added, rinsed well and incubated 10 min on ice. Next 3 ml Solution 3 was added and rinsed well and incubated 10 min on ice. Then 9 ml 5M LiCl added and once again rinsed, incubated 10 min on ice and centrifuged 10 min at 13 000 g at RT. After centrifugation supernatant was transferred through a paper filter into a new falcon tube. 9 ml isopropanol was added and incubated 10 min on ice and 10 min centrifugated at 13 000 g at RT. The pellet was dried in Vacum dryer 5-10 min. After drying the pellet was resuspended in 1ml of solution 1and incubated 45 min at 37°C . Then the resuspension was transferred in 2 Eppis as 500 µl and extracted with cold phenol and as last plasmid DNA was precipitated by adding 60% isopropanol and 10% 3 M NaAc, pH 5.2, resulting pellet was dissolved in EB-Puffer (QIAgen Plasmidprep-Kit = 10 mM Tris, pH8.0) and the concentration was determined by UV-Spektrophotometer (240-320 nm.) as described in part.

Table 3.15. Solutions for the plasmid prep

Solution 1:	5 ml 1M Tris-Cl, pH 8.0	(f.c. = 50 mM)
	2 ml 0.5 M EDTA, pH 8.0	(f.c. = 10 mM)
	1 ml 10 mg/ml RNase	(f.c. = 100 µg/ml)
	92 ml Millipore-H_2O	
	Storage at 4°C	
Solution 2:	0.8 g NaOH	(f.c. = 200 mM)
	10 ml 10% SDS	(f.c. = 1 %)
	Adjust to 100 ml with Millipore-H_2O.	
	Storage at R.T.	
Solution3:	25.61 g KAc, pH 5.2	(f.c. = 26 M)
	adjust the pH with Aceticacid	
	Adjust to 100 ml with Millipore-H_2O.	
	Storage at 4°C	

Table 3.15. continued

Solution 4:	5 M LiCl (Sigma) 21.2 g in 100 ml Millipore-H2O. **Storage at R.T.**
3 M NaAc, pH 5,2:	3M NaAc x 3 H_2O 204.05 g + 400 ml H_2O Adjust pH to 5.2 with Acetic acid, fill up to 500 ml with H_2O. **Storage at R.T.**
0.5 M EDTA:	93.05 g x 2 H2O EDTA + 400 ml H2O + 10 g NaOH-Pellets Adjust pH to 8.0, fill up to 500 ml with H_2O. **Storage at R.T.**
10% SDS:	50 g SDS in 450 ml H2O , warm up at 68°C adjust pH 7.2, fill up to 500 ml with H_2O.

3.5 Analysis of Nucleic acids

3.5.1 Seperating DNA by agarose gel electrophoresis

The standart method used to separate, identify and purify DNA fragments is electrophoresis through agarose gels. To prepare the analytical agarose gels, gels were prepared as horizantal with a size of 10cm x 7.5cm and/or 7.5cm x 5.5cm. The concentration of agarose in gels depending on the molecular sizes of DNA fragments. A table is given below which is indicating the concentration of agarose gels and seperation range.

Table 3.14. The concentration of agarose gels and seperation range.

Amount of Agarose in Gel %	Efficient Range of Seperation of Linear DNA Molecules (kb)
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

The most convenient method of visualizing DNA in agrose gels is by use of the fluorescent dye ethidium bromide. Ethidium bromide can be used to detect both single- and double stranded nucleic acids. Agarose was prepared in 1x TAE Buffer bolied and precooled to 50°C. Ethidium bromide was added to a final concentration of 0.5µg/ml (from a stock solution of 5mg/ml in water) to the cooled agarose. After the seal was set, the warm agarose solution poured in to the mold which was containing a comb. After the gel was completely set, and gel mounted in the electrophoresis tank which is containing 1x TAE buffer as it is fully covering the gel and the comb was removed. Then samples are mixed with loading buffer 20-50% (v/v) and were loaded in to the slots of the submerged gel. A molecularweight marker (as described in part…) around 100-150 ng was loaded additionally in one slot for comparing the bands of samples. For seperation of DNA a cuurency of 5V/cm was applied. At the end of the run gel was examined directly under UV illumination and photographed by using a Video-camera system.

Table 3.16. Buffers and agarose contents of the agarose gel

50x TAE:	2M Tris Aceticacid EDTA pH 8.0	121.1g 28.6ml 9.3g H_2O ad 500ml
1% Agarose:	1% Agarose (w/v) in 1 x TAE boiled and cooled to 50°C	
3% Agarose:	1% Agarose (w/v) 2% Agarose Low-Melting-Grade (w/v) in 1 x TAE boiled and cooled to 50°C	
DNA-Loading buffer:	50% 1 x TAE (v/v) 50% Glycerin (v/v) 0.05% Bromphenolblau (w/v) 0.05% Xylencyanol (w/v)	

3.5.2 DNA-sequencing

For DNA-Sequencing, necessary PCR products or plasmids were sent to a SEQ LAB. Seguencing reaction was prepared total in 7 µl, containing 15 µM of appropriate primers forward and/ or reverse. For sequencing of plasmids total 0.6 µg plasmid DNA was used whereas 200 ng for PCR products. Sequencing was carried out up to 300 bp or as extended up to 500 bp.

3.5.3.Quantitation of nucleicacids

Two methods are widely used to measure the amount of DNA/RNA in a preparation. If the sample is pure (i.e., without significant amounts or contaminants such as protein, phenol, agarose or other nucleic acids), spectrophotometric measurement of the amount of UV irradiation adsorbed by the basis simple and accurate. If the amount of DNA/RNA is very small or if the sample contains significant quantities ofimpurities, the amount of nucleic acid can be estimated from the intensity of fluorescence emitted by ethidium bromide.

Spectrophotometric determination:

For quantitating the amount of DNA or RNA, readings was taken at wavelengths between 240- 320 nm. The reading at 260 nm allows calculation of concentration of the nucleic acids in samples. The ratio between the readings at 260 nm and 280 nm (OD_{260} /OD_{280}) provides an estimate for purity of the nucleic acids. Pure preparations of DNA and RNA have OD_{260} /OD_{280} of 1.8 and 2.0 respectively. If there is no contamination of protein or phenol, the OD_{260} /OD_{280} will be significantly less than the values given above, and accurate quantition of the amount of nucleic acid will not be possible. To determine the concentrations first the measurement of adsorbtion at 260nm was taken and then compared and calculated according to the following values:

Double-stranded DNA:	An OD_{260nm} = 1 corresponds to 50 µg/ml
Single-stranded DNA and RNA:	An OD_{260nm} = 1 corresponds 40 µg/ml
Oligonucleotides:	An OD_{260nm} = 1 corresponds 20 µg/ml

Ethidium Bromide Fluorescent Quantition of the Amount of Double-stranded DNA

Sometimes there is not sufficient DNA (< 250 ng/ml) to assay spectrophotometrically, or the DNA may be heavily contaminated with other UV-absorbing substances that impede accurate analysis. A rapid way to estimate the amount of DNA in such samples is to utilize the UV-induced fluorescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of DNA in sample can be estimated by comparing the fluorescent yield of the sample with that of a series of standards. As little as 1-5 ng of DNA can be detected by this method.

Gels can be visualized by using a video-camera sytem and saved in computer. By using computer programmes such as „Image Quant“ (from Molecular Dynamics) individual bands can be correlated by the bands of the standards.

3.6 In vitro Amplification of DNA by the Polymerase Chain Reaction (Standard-Protocol)

3.6.1 PCR standart protocol

The Polymerase-Chain Reaction is a method which is cabaple of amplifying as little as single molecule of DNA. [135,136]. Components of a typical PCR are oligonucleotides, buffers, *Taq* DNA polymerase and deoxyribonucleoside triphosphates and template. A typical PCR consist of denaturation, annealing and polymerization steps. Typical conditions for denaturination, annealing and polymerization are as follows:

Tab. 3.17. Typical PCR cycles

Cycle	Denaturation	Annealing	Polymerization
1st. cycle	5 min at 94°C	2 min at 50°C	3 min at 72°C
Subsequent cycles	1 min at 94°C	2 min at 50°C	3 min at 72°C
Last cycle	1 min at 94°C	2 min at 50°C	10 min at 72°C

The temperatures chosen for annealing of the oligonucleotide primers to the target DNA is a compromise. Timing of the individual steps depends on the length of a target sequence. the number of amplification cycles depends on the concentration of target DNA in the reaction mixture. The times given above are usual for a target sequence approxiamately 500 nucleotides in length. Polymerase chain reactions can be automated with a thermal cycler.

Oligonucleotides:

Oligonucleotides used for the priming polymerase chain reaction should be at least 16 nucleotides and preferably 20-24 nucleotides in length. Usually oligonucleotides are used at concentration of 1µM in polymerase chain reaction. This is usually sufficient for at least 30 cycles of amplification.

Buffers:

The satndard buffers for polymerase chain reaction contains 50 mM KCl, 10 mM Tris Cl (pH 8.3 *at R.T.*) and 1.5mM $MgCl_2$. In particular theconcentration of Mg^{+2} should be optimized whenever a new combination of target and primers is first used or when the concentration of dNTPs or primers is altered. Recommended setting up a set of reactions follows : containing fixed concentrations of Tris.Cl (10 mM) and KCl (50 mM) and varying concentrations of $MgCl_2$ (0.05-5 mM in 0.5 mM increments).

***Taq* DNA polymerase :**

Two forms of *Taq* DNA polymerase are available: The native enzyme purified from *Thermus aquaticus* and a genetically engineered form of the enzyme synthesized in *E.coli* (AmpliTaq^{TM}). Both form of the polymerase carry a 5'→3' polymerization-depentent exonuclease activity. Approximately 2 units of either of the enzymes are required to catalyze a typical polymerase chain reaction.

Deoxyribonucleoside triphosphates :

dNTPs are used at saturating concentrations (200 µM for each dNTP). A stock solution of dNTPs (50 mM) should be adjusted to pH 7.0 with 1 N naOH to ensure that the pH of the final reaction does not fall below 7.1.

Target sequences (template) :

DNA containing the target sequences can be added to the polymerase chain reaction mixture in a single- or double-stranded form.The concentration of the target sequences in the template DNA obviously varies according to the circumstances. It is worthwhile setting up a series of control reactions that contain decreasing amounts of known target sequences (1ng, 0.1 ng, 0.0001 ng etc.) to check that the amplification reaction is working at the required sensitivity.

3.6.2 PCR Protocol for Pfu *Taq* Polmerase

PCR was used for two different approaches in this study. First in vitro amplification of template DNA for cloning : To amplificate the template DNA for cloning usually PCR reaction prepared in a 100 µl volume. For all PCR reactions usually plasmid DNA was used as template. A thermostable *Taq* polymerase, *Pfu Turbo*® DNA polymerase was used for amplification of the template for high-fidelity PCR. *Pfu Turbo*® DNA polymerase exhibits lowest error (Error rate: 1.3 x 10^{-6}) compared to normal *Taq* polymerase (Error rate: 8.0x10^{-6}). A mastermix was prepared in case of more than one reactions and pipetted in equal volumes to the PCR tubes. The steps of the PCR programme, components and their concentration in amplification reaction for *Pfu Turbo*® *Taq* polymerase follows as in Table 3.17 and 3.18.

Tab. 3.18. PCR cycles for *Pfu Turbo*® *Taq* polymerase [137]

Cycle	Denaturation	Annealing	Polymerization
1st. cycle	30 sec at 94^{o}C	-	-
25- 30 cycle	30 sec at 94^{o}C	1 min at 60^{o}C	2 min at 68^{o}C
Last cycle	4 ^{o}C ∞		

Tab. 3.19. Contents of the PCR for *Pfu Turbo® Taq* polymerase [137]

Component	Vol./reaction	Final concentration
10x Buffer	10.00 µl	1x
Primer F (15µM)	2.36 µl	125 ng
Primer R (15µM)	2.36 µl	125 ng
Template (20-100ng)	variable	0.2-1 ng/µl
2.5 mM dNTP mix	2.00 µl	0.5 mM
PfuTurbo® Taq Polymerase (2.5U/µl)	2.00 µl	5U/100µl V
st dH_2O	variable	
Total volume	**100 µl**	-

10x Buffer : 200 mM Tris-HCl pH 8.8,
20mM $MgSO_4$; 100 mM KCl,
100 mM$(NH_4)_2SO_4$; 1% Triton®- X-100,
1mg/ml BSA (nuclease-free)

3.6.3 PCR protocol for Hot Star*Taq* polymerase

The second aim of using PCR was, to check the transformants of E. coli for (+) clones which are having the desired plasmid. For this approach an other *Taq* polymerase, *Hot StarTaq*™ polymerase was used. It is a modified form of the recombinat 94-kDa *Taq* DNA polymerase from QIAGEN. Hot Star Taq DNA polymerase is provided in an inactive state with no polymerase activity at ambient temperatures. This prevents the formation of misprimed products and primer-dimers at low temperatures. HotStarTaq DNA polymerase is activated by a 15 min, 95°C incubation step, providing high PCR specificity. HotStarTaq DNA Polymerase eliminating the extra handling steps and contamination risks associated with conventional hot-start methods [138].

Tab. 3.20. Typical cycles of the PCR for HotStar*Taq* DNA Polymerase [138].

Cycle	Denaturation	Annealing*	Polymerization
1st. cycle	15 min at 95°C	-	-
25- 30 cycle	30 sec at 94°C	1 min at 60°C	1 min at 72°C (1 min/kb)
Last cycle	-	-	10 min 72°C + 4 °C ∞

Annealing*: approximately 5°C below T_m of primers

The steps of the PCR programme, components and their concentration in amplification reaction for HotStarTaq DNA Polymerase are follows as in Table 3.19. and 3.20, [138].

Tab. 3.21. Contents of the PCR for HotStar*Taq* DNA Polymerase [138].

Component	**Vol./reaction**	**Final concentration**
10x Buffer	10.00 µl	1x
Primer F (15µM)	2.36 µl	0.3 µM
Primer R (15µM)	2.36 µl	0.3 µM
Template (20-100ng)	variable	0.2-1 ng/µl
10 mM dNTP mix	2.00 µl	0.2 mM of each
HotStarTaq DNA Polymerase (5U/µl)	0.5.00 µl	2.5 U/100µl V
st dH_2O	variable	
Total volume	**100 µl**	-
10x Buffer : 20 mM Tris-HCl pH 8.7, 15mM $MgCl_2$, 100 mM KCl, $(NH_4)_2SO_4$		

3.6.1 Amplification by Dovetail PCR cloning kit

Dovetail ™ PCR Product Cloning Kit provides a versatile and efficient method for cloning virtually any PCR amplified DNA fragment in to a desired vector.A major advantage of this approach lies in fact that it is independent of whether the PCR products are blunt-ended or contain one or more extra nucleotides at their 3' end. The underlying idea of this method is the use of three well characterized typeIIS restriction endonucleases, *Bpi*I , *Eco*3 1I and *Esp*3I. These enzymes recognize a hexanucleotide targets in DNA and cleave aside from their recognition sites producing four-base 5'-overhangs. Therefore by selecting an appropriate flanking sequence in the 5' section of a primer, the sequence of the tetranucleotide overhang produced upon cleavage with one of the three Dovetail ™ enzymes can be selected at will to match cloning sites of restriction endonucleases. In addition, the restriction endonuclease cleavage remove its own recognition sequence from the resulting DNA such that only the desired portion of the PCR product and the four-base 5'- overhangs are present [139].

Cloning of the PCR product was achieved by introduction of a uniqe restriction endonuclease site at the 5' end of each amplification primer folowed by digestion of the PCR fragment with typeIIS restriction endonucleases, *Bpi*I , *Eco*3 1I and *Esp*3I to form the desired sticky ends for cloning.

3.7.Site-directed mutagenesis [140].

3.7.1 Site directed mutagenesis by using two internal mutagenesis primer

In vitro site directed mutagenesis is an invaluble tecnique to make point mutations, switch amino acids, and delete or insert single or multiple aminoacids. The Quick Change site-directed mutagenesis method was performed using Pfu Turbo^{TM} *Taq* DNA polymerase and a thermal cycler. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by using Pfu Turbo^{TM} *Taq* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product was treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA [140]. DNA isolated from almost all *E. coli* strains is dam methylated and susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations was then transformed into E.coli as described in part 3.2.2. The amplification reaction was carried out as descriped before in Table 3.21. for Pfu Turbo *Taq* DNA polymerase and amplification cycles are follows as in below:

After amplification, 1µl of *Dpn* I (10U /µl) was added to 50 µl reaction and incubated 1hr. at 37^{o}C .

Tab. 3.22. PCR cycles for site directed mutagenesis [140].

Cycle	Denaturation	Annealing	Polymerization
1st. cycle	30 sec at 95^{o}C	-	-
12- 18 cycle	30 sec at 95^{o}C	1 min at 55^{o}C	2 min (/kb of plasmid length) at 68^{o}C
+ 4 ^{o}C ∞			

3.7.2 Site-directed mutagenesis by overlap extension

Overlap extension was originally devised as a way of introducing mutations in the center of PCR-generated sequence segments in order to make PCR mutagenesis. By using one external one internal proimers two separate fragments are amplified from target gene. The first fragment is amplified with primers a and b (product AB): primer b introduces asequence change at the right end of the product AB. The second fragment (product CD) is amplified with primers c and d, with primer c introducing the same mutation, but into the left end of the product CD. These two products now share a segment identical sequence called the overlap

region. When these intermediate products are mixed together , melted and reannealed, the top strand of AB can anneal to the bottom strand of CD in such a way that two strands act as primers on one another. By using two external primers extension of this overlap by *Pfu Taq polymerase* creates the full-length mutant insert AD, which has the desired mutation at an arbitrary distance from either end, PCR cycles are performed as indicated in table 3.22., for reaction contents see table 3.18.

Tab. 3.23. PCR cycles for overlap extension site directed mutagenesis .

Cycle	Denaturation	Annealing	Polymerization
1st. cycle	30 sec at 94°C	-	-
30 cycle	30 sec at 94°C	1 min at 56°C	2 min (/kb of plasmid length) at 68°C
+ 4 °C ∞			

3.8 Enzymatic Modification of Nucleic acids

3.8.1 Digestion with a single restrictionendonucleases

For cloning into a uniqe restriction sites the vectors and inserts should be digested with the same restriction endonucleases. For vector preparation two different restriction endonucleases leading to 4 base 5'-overhangs, that are complemetary to digested PCR product sticky 5'-overhangs were used. Each restriction enzyme has a set of optimal reaction conditions, which are given on the information sheet supplied by the manufacturer. The major variables are the temperature of incubation and the composition of the buffer. Usually each enzyme supplied with its own buffer by the manufacturer. To digest either isolated plasmid DNA or PCR product, DNA was incubated with the desired restriction enzyme and in its own buffer (typically 1U enzyme defined as the amount required to digest 1µg DNA). Digestion was achieved incubation at 37° C 1h or O.N. Digested DNA was checked by agarose gel electrophoresis.

3.8.2 5'-Dephosphorylation of DNA

Usually after digestion the vector (plasmid DNA) must be dephosphorylated to prevent the self ligation. For this, the terminal 5' phosphates can be removed from DNA by treatment either with calf intestinal (CIP) alkaline phosphatase or bacterial alkaline phosphatase (BAP). Calf intestinal alkaline phophatase has an advantage that it can be completely inactivated by heating to 68°C in SDS.

For dephosphorylation 1-5µg vector-DNA was used. Dephosphorylation was achieved in 50µl reaction by adjusting the salt concentration using a 10xCIP Buffer which is supplied by manufacturers. Alkaline phosphatase was added at a concentration of 0.01 U/ 1 pmol 5' ends of DNA. The reaction was kept at 37°C for 40 min. After dephosporylation phenol purification and ethanol precipitation was performed. The contents of reaction and the 10X Buffer were follows as :

Dephosphorylation:	10x CIP-Buffer	5µl
	Alkalische Phosphatase (CIP)	2µl
	Vector DNA (1-5µg)	xµl
		H_2O ad 50µl
10x CIP-Buffer:	500mM Tris-Cl, pH 8.5	
	1mM EDTA	

3.8.3 Ligation of DNA fragments

After preparation of vector and DNA fragment, ligation was performed by using a Clonables™ Ligation/Transformation Kit which is including a 2x Ligation premix. Ligation reaction was carried out in the presence of a 2X ligation premix which is containing a bacterial T4-Ligase. DNA fragment and vector were added in a molar ratio of 3:1. For Blunt-End-Ligation, DNA fragment has to be phosphorylated and vector has to be dephosphorylated.

For 10µl ligation reaction 100 ng Vector DNA and appropriate insert DNA were used, reaction was adjusted with 2x Ligation premix and incubated at 16°C either O.N. or 1h. After ligation, 1-2µl of Ligation reaction was used for transformation of 50µl *E.coli* as described in 3.2.4.

Ligationreaction:	2x Ligation premix	5µl
	Insert-DNA (10-50pmol)	xµl
	Vector-DNA (100ng)	yµl
	H_2O ad 10µl	