Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, enzymes and instruments suppliers

Amersham Biosciences, England

L-^{35}S-Methionine

L-^{14}C-phenylalanine

Spermidine

Spermine

FluorImager 595

Storm™ imaging system

Personal Densitometer™ SI

Storage Phosphor Screens

ImageQuant™ TL (software)

Beckman, Germany

Ready Value (scintillation liquid)

Ultracentrifuge tubes Ultra-Clean

BioRad, USA

Ammonium persulfate

SDS (sodium-dodecylsulphate)

Qiagen, Germany

Qiagen Maxi Prep Tip 500

Qiagen Midi Prep Tip 100

Qiagen Mini Prep

Qiaquick PCR Purification kit

Quiquick Nucleotide Removal kit

Difco BD, USA

Bacto agar

Bactotryptone, Yeast extract

EMD Biosciences, Germany

HEPES

Gibco-BRL, USA

Agarose (ultra-pure)

Urea (ultra-pure)

Sucrose (ultra-pure)

TEMED

Merck, Germany

All chemicals used in the laboratory and not extra listed here, and all essential amino acids

Bromphenol blue

Etidium bromide (1%)

Urea

Tris

Xylen cyanol

Microfluidics, USA

Microfluidizer® Processor M-110L

New England BioLabs, USA

Restriction endonucleases

Reaction buffers

T4 DNA ligase, 6 U/µl

Packard Instrument Company, USA

Filter Count (scintillation liquid)

Pharmacia, USA

BSA (DNase and RNase free, 2.6 mg/ml)

Long polyuridine (poly(U)

Sephadex

Roche, Germany

Adenosine-5'-triphosphate

Alkaline phosphatase (CIP, calf intestine phosphatase) 20 U/µl

Ampicillin

dNTPs

Guanosine-5'-triphosphate

Lysozyme

T4 Polynucleotide kinase

Poly-uridine (poly(U)

Phosphoenolpyruvic acid

Pyruvate kinase 10 mg/ml

RTS 100 *E. coli* HY Kit

RTS 500 *E. coli* HY Kit

RTS ProteoMaster Instrument

T4 DNA polymerase, 1U/µl

$tRNA^{bulk}$ (from *E. coli*)

Roth, Germany

Rotiphenol

Rotiphorese 30 (27,5:1)

Sartorius GmbH, Germany

Nitrocellulose filters (Nr. 11306)

Schleicher and Schuell, Germany

Selecta glass filter (Nr. 6)

Serva, Germany

Acrylamide

Alcoa A-305 Aluminium oxide

Bis-acrylamide

Sigma-Aldrich, USA

NTPs-Tris

$tRNA_f^{Met}$

Whatman Ltd., England

Paper Filters

2.1.2 Bacterial Strains and Plasmids

The new Rosetta™ (DE3) strain is derived from *lacZY* mutant of BL21 (*lon* and *ompT* proteases deletion), to enable precise control of expression levels by adjusting the concentration of IPTG. This strain is designed to alleviate codon bias when expressing eukaryotic proteins in *E. coli*.

Plasmid pET23c(+): The pET-23c(+) vector carries an N-terminal T7•Tag® sequence plus an optional C-terminal His•Tag® sequence. This vector has the "plain" T7 promoter instead of the T7*lac* promoter and the *lacI* gene is absent.

Plasmid pIVEX2.2GFPcyc3: this is an In Vitro EXpression vector that has a N-terminal *Strep*-tag prior to GFPcyc3 gene sequence. This plasmid was utilised as a general control for the study of *in vitro* expression system and for the comparison of the expression of some given proteins in this system. GFPcyc3 is a Green Fluorescence Protein that carries three point mutations, which allow fast maturation of the fluorophore in comparison to the wild type GFP.

2.2 Media

2.2.1 Luria-Bertani (LB) medium

Bacto-Tryptone/Peptone	10 g
Yeast Extract	5 g
NaCl	5 g
1M NaOH	1 ml
H_2O	ad 1 L
	pH 7.4

Media has to be sterilized via autoclaving. It is used for liquid overnight culture in order to obtain cells for isolation of given plasmid or for small scale

ribosome and S30 extract isolation. Cells are grown in the presence of suitable antibiotic.

2.2.2 L-Agar Plate

Agar added to LB 15 g/L

Media is sterilized *via* autoclaving, stirred well and distributed in 15-20 ml aliquots onto sterile Petry dishes while media is still liquid (when required, antibiotics were added just before the pouring to the plates).

2.2.3 Antibiotic Solution

Ampicillin 100 mg/ml

A measured amount of ampicillin powder was weighted and the amount of water was added necessary for the concentration of 100 mg/ml. After complete salvation the clear solution of ampicillin was filtrated through a sterile filter and small aliquots were prepared and stored at – 20°C.

2.3 Buffers

2.3.1 Acrylamide and staining solutions

Solution	Substance	Measure
30% AA/BAA (37.5:1) gel stock solution for protein gel	Acrylamide	300 g
	Bis-acrylamide	8 g
Agarose gel solution	10X TBE	5 ml
	Agarose	0.8-2% w/v
	Etidium Bromide (1%)	1.5 µl
	MQ-H_2O	ad 50 ml
APS solution 10%	Ammonium persulfate	10% w/v
Coomassie blue staining solution	Coomassie blue R-250	0.25% w/v
	Methanol	50% w/v
	Glacial Acetic acid	10% w/v
Distaining solution for Coomassie gels	Methanol	25% v/v
	Glacial acetic acid	8% v/v
	H_2O	67% v/v

Agarose gel sample buffer (5X) (for DNA)	EDTA Ficoll 400 Bromphenol blue Xylencyanol	50 mM 10% 0.25% w/v 0.25% w/v
TBE (10X)	Tris (base) Boric acid EDTA MQ-H_2O	108 g 55 g 7.4 g ad 1 L
Etidium bromide staining solution	Etidium bromide 1% MQ-H_2O	30 µl 300 ml
SDS-PAGE protein sample buffer (for protein)	SDS Tris-HCl (pH 6.8) Glycine ß-mercaptoethanol Bromphenol blue	2% w/v 90 mM 10% w/v 29 mM 0.1% w/v
SDS-PAAG separation buffer (pH 8.8); 1.5 M Tris	Tris (base) MQ-H_2O	180 g ad 1 L
SDS-PAAG stacking buffer (pH 6.8); 0.5 M Tris	Tris (base) MQ-H_2O	60 g ad 1 L
Tris-Glycine 5x running buffer; (Protein SDS-PAGE)	Tris (base) Glycin SDS MQ-H_2O	30 g 144 g 2 g ad 1L
SDS-PAAG separating gel for proteins; (15% acrylamide)	1.5 M Tris-HCl, pH 8.8 30% Acrylamide, 37.5:1 10% SDS 10% APS TEMED MQ-H_2O	2.5 ml 5.0 ml 0.1 ml 50 µl 10 µl ad 10 ml
SDS-PAAG stacking gel for proteins; (5% acrylamide)	1.5 M Tris-HCl, pH 6.8 30% Acrylamide, 37.5:1 10% SDS 10% APS TEMED MQ-H_2O	1.25 ml 0.85 ml 50 µl 15 µl 5 µl ad 5 ml

2.3.2 Buffers for microbiological and molecular methods

Solution	Substance	Measure
P1 (Suspending agent)	Tris-HCl, pH 8.0 EDTA	50 mM 10 mM
P2 (Cell-lysis)	NaOH SDS	200 mM 1% w/v
P3 (Neutralization buffer)	Potassium acetate, pH 5.5	3 M
QBT (Equilibration)	MOPS-KOH pH 7.0 NaCl Ethanol	50 mM 1000 mM 15% v/v
QC (Washing buffer)	MOPS-KOH pH 7.0 NaCl Ethanol	50 mM 1250 mM 15% v/v

QF (Elution buffer)	Tris-HCl, pH 8.5	50 mM
	NaCl	1250 mM
	Ethanol	15% v/v
TE buffer	Tris-HCl, pH 8.0	10 mM
	EDTA	1 mM
10 x Klenow buffer	Tris-HCl, pH 7.6	500 mM
	$MgCl_2$	100 mM
	DTT	10 mM
	BSA (DNase free)	500 µg/ml
10 x Ligation buffer	Tris-HCl, pH 7.6	660 mM
	$MgCl_2$	50 mM
	DTT	50 mM
	Spermidine	5 mM
	BSA (DNase free)	500 µg/ml
10X dephosphorylation buffer	Tris-HCl pH 7.6	500 mM
(for dephosphorylation of	$MgCl_2$	10 mM
5'phosphorylated ends of DNA or RNA)	$ZnCl_2$	10 mM
	EDTA	5 mM
Glycerol storage solution	Tris-HCl, pH 8.0	25 mM
	$MgSO_4$	100 mM
	Glycerol	66% v/v

2.3.3 Buffers for the functional studies and ribosome preparation

Solution	Substance	Measure
Binding buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$)	MgAc	4.5 mM
	NH_4Ac	150 mM
	ß-mercaptoethanol	4 mM
	Spermidine	2 mM
	Spermine	0.05 mM
Tico-Buffer	Hepes-KOH, pH 7.5	20 mM
($H_{20}M_6N_{30}SH_4$)	MgAc	6 mM
	NH_4Ac	30 mM
	ß-mercaptoethanol	4 mM
Mix I	Hepes-KOH pH 7,5	100 mM
$H_{100}M_{21}N_{870}SH_{20}Spd_{12}Spm_{0,3}$	MgAc	21 mM
(For poly(U) dependent poly(Phe)	NH_4Ac	870 mM
synthesis assay)	ß-mercaptoethanol	20 mM
	Spermidine	12 mM
	Spermine	0,3 mM
Mix II/Charging Mix	Hepes-KOH pH 7.5	80 mM
$H_{80}M_{15}N_{840}SH_{16}Spd_{12}Spm_{0.3}$	MgAc	15 mM
(For poly(U) dependent poly(Phe)	NH_4Ac	840 mM
synthesis assay and RF2 system)	ß-mercaptoethanol	16 mM
	Spermidine	12 mM
	Spermine	0.3 mM
Mix E	ATP	45 mM
$ATP_{45}GTP_{22.5}(AcPO_4)_{75}$	GTP	22.5 mM

energy mix/charging mix (for poly(U) dependent poly(Phe) synthesis)	Acetyl phosphate KOH	75 mM 360 mM
10X buffer for RNase assay (Binding Buffer 10X)	HEPES-KOH pH 7.5 MgAc NH_4Ac ß-mercaptoethanol Spermidine Spermine	200 mM 45 mM 1500 mM 40 mM 20 mM 0.5 mM

2.3.4 Components of a batch **in vitro** *system for coupled transcription-translation*

Component	Final concentration	Components	Final concentration
HEPES-KOH (pH 8.2)	60 mM	GTP	1 mM
Ammonium acetate	80 mM	UTP	1 mM
Potassium glutamate	230 mM	PEG-8000	2% (w/v)
Sodium oxalate	3 mM	Methionine	2 mM
DTT	2 mM	Amino acids (19)	2 mM
Cycle-AMP	0.7 mM	PEP	35 mM
Folinic acid	35 µg/ml	Magnesium acetate	12 mM
tRNA	350 µg/ml	T7 RNA polymerase	100 µg/ml
NADH	0.35 mM	*E. coli* S30 extract	4-6 A_{260}
Coenzyme A	0.3 mM	Plasmid DNA	4 µg/60 µl
ATP	1.5 mM	Rifampicin	10 µg/ml
CTP	1 mM		

2.4 Analytic Methods

2.4.1 Photometric measurements

Spectrophotometric analysis of the amount of DNA or RNA

The concentrations of oligonucleotides, DNA and RNA were determined by absorption measurement in a Hitachi U-3000 spectrophotometer. The measurements were performed at 260 or 280 nm. The reading at 260 nm allows estimation of the concentration of nucleic acid in a sample, whereas the ratio between the reading at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acid.

A_{260}/A_{280} = 1.8 for pure DNA*

A_{260}/A_{280} = 2.0 for pure RNA*

* These coefficients are valid for high molecular weight species (Berger, 1987). In the case of oligonucleotides (less than 100 bases), the base composition can have a significant effect in the extinction coefficient. This means that the A_{260}/A_{280} ratio will change with the sequence. Extreme cases are A rich sequences (A_{260}/A_{280} ratio larger than 2.2), and C rich sequences (A_{260}/A_{280} ratio smaller than 1.5).

Conversion factors used for DNA and RNA quantification are:

1 bp in double stranded DNA	660 g/mol
1 A_{260} unit of double stranded DNA	50 µg
1 A_{260} unit single stranded DNA or RNA (>100 bases)	40 µg
1 A_{260} unit of single stranded DNA (less then 25 bases)	20 µg
1 A_{260} unit of single stranded DNA (30-80 bases)	30 µg

Determination of ribosome and nucleic acid concentration

The concentrations of 70S ribosomes and 30S and 50S subunits and tRNAs were determined by photometry at 260 nm, using the following relationships:

70S	1 A_{260}/ ml = 24 pmol/ml
50S	1 A_{260} /ml = 36 pmol/ml
30S	1 A_{260} /ml = 72 pmol/ml
tRNA	1 A_{260} /ml = 1500 pmol/ml

Fluorometric analysis of GFP

Native PAGE with GFP in a native buffer was performed for 2.5 hours in a 15% protein polyacrylamide gel. Fluorescence was measured directly from the gel on a FluorImager™ 595 dual-excitation, laser-induced fluorescence scanner. The images were analysed using the ImageQuant program where the relative intensities of the GFP band from expression system were compared with that one of the GFP with known concentration (marker).

2.4.2 Radioactivity Measurements

The radioactivity measurements were performed using a liquid scintillation counter Wallac Rack Beta model 1209 or a Rack Beta model 1409. The treatment of the samples before measurements was done according to their physical nature and contents of an isotope.

Liquid samples

Radioactive liquid samples with a volume of 1 ml or less were put to the scintillation vials (plastic, 20 ml maximal volume), then 5 ml of Ready Value (Beckman) or Filter Count (Packard Bioscience), were applied. After quick and vigorous shaking (10 sec), the samples were immediately counted.

Quantification of amounts of [^{35}S]-Met labelled proteins in SDS-PAAG

Dried SDS protein gels were stored in Storage Phosphor Screens for 24-48 hours. Storage phosphor screens retain energy from beta particles, X-rays, and gamma rays. The lower limit of detection for a 1 h exposure is less than 2 dpm/mm^2 for ^{14}C (200 and 100 µ only). The lower limit of detection for ^{32}P is typically 5–10 times lower than the limit for ^{14}C. Storage phosphor screens are used in conjunction with a storage phosphor system such as PhosphorImager™ or Storm™.

The electrical signal is digitised to permit image display and analysis. Scan control software and ImageQuant Image Analysis Software are available for Windows and are included with purchase of the Storm system.

Cold trichloroacetic acid (TCA) precipitation assays

From each sample (normally two per assay) a 10 µl aliquot was delivered into a glass test-tube (8 x 10 cm) containing 20 µl of precipitation carrier solution (1% BSA). Two ml of ice cold TCA (10% w/v) were immediately added and mixed for 1-2 seconds. The polypeptide chains were precipitated at

90°C water bath for 15 min, cooled down on ice and the precipitation mix was then filtered through glass fibre filters. The filters were washed three times with 2 ml of cold TCA (10%) and once with 2 ml diethyl ether/ethanol (1:1 v/v). The radioactivity adsorbed on the filter was measured after application of 5 ml of ReadyValue scintillation solution and vigorous shacking.

2.4.3 Agarose gel electrophoresis of DNA and RNA

This technique was used for analysis of plasmid DNA after analytical and preparative isolations from cells, as well as for restriction reaction and polymerase chain reaction (PCR), product analysis, and for the qualitative evaluation of rRNAs from 70S ribosomes, 50S and 30S ribosomal subunits.

The samples were prepared according to the type and the expected size of the nucleic acids to be separated. Plasmid DNA samples were prepared in a total volume of 6-10 µl in 5X agarose gel sample buffer including SDS (2% final concentration), in cases where the preparation was not highly purified (e.g. miniprep DNA). The amount of DNA was 0.2-1 µg per lane if intact plasmid preparations were analysed, or 1.5-2 µg if fragments of 600 bp or less were expected. For rRNA analysis, 0.05 A_{260} units of the corresponding ribosomal particles were incubated 2 min at 65°C in 5X agarose gel sample buffer containing SDS (2% final concentration), and kept 10 min at room temperature before loading onto the gel.

The percentage of agarose used depended on the expected size of the nucleic acid to be separated: 0.8% for rRNA and 3 kb plasmids, and 1.5-2% for restriction fragments of 600 bp or less. The buffer system used were TBE (for minipreps and restriction analysis).

The agarose solution was heated in a microwave oven for not more than one minute. The solution was cooled to room temperature, 1.5 µl of etidium bromide was added and the mixture was poured into the gel chambers:

a) Mini-gels: 9 x 7 x 0.7 cm (total volume = 50 ml) with 14 or 28 wells (10 μl maximal sample capacity). Used mainly in routine checking of plasmid isolation, minipreps and restriction analysis. Run at 50-60 V.

b) Regular gels: 14 x 8 x 0.7 cm (total gel volume = 80 ml) with 14 or 28 sample wells (20 μl maximal sample capacity). Used in the analysis of small DNA fragments and rRNA. Run at 60-100 V.

The length of the nucleic acid molecules was estimated according to the migration of standards of known molecular weights.

2.5 Preparative methods

2.5.1 S30-extract preparation from E. coli

A large amount of fresh or frozen *E. coli* cells collected on a *lag* stage of growth was suspended in two volumes of Tico buffer. Suspension was centrifuged in a pre-weighted chilled GSA tubes for 15 min with 10,000g at 4°C. All the following steps were carried out in a cold room. The pellets (cell debris) then were weighted and dissolved in Tico buffer in proportion as 1 ml per g. Such a suspension should easily go through the tip of 10 ml glass pipette. When this condition was reached, everything was applied to the Microfluidizer apparatus, which was washed and equilibrated with Tico buffer prior. Within this apparatus cells are pushed under high pressure (18 atm), through a tiny channel; which leads to their breakage. Then, collected suspension was centrifuged and resulting supernatant was transferred to the clean tubes and centrifuged again. These two steps of centrifugation were carried out in HB4 rotor with 16,000 g for 45 min at 4°C. After final centrifugation from the clear supernatant fraction fresh aliquots were prepared, shock-frozen and stored at –80°C. A small amount enough for double determination was kept in order to measure A_{260} of S30-exstract.

2.5.2 Preparation of 70S ribosomes

In a typical preparation ~300 grams of frozen *E. coli* cells were thawed via suspending in ~600 ml (double amount), of Tico buffer, and recovered by centrifugation at 8,000 rpm for 15 minutes in a Sorvall GSA rotor at 4°C. The cell pellet was weighted, and a double amount of aluminium oxide (Alcoa-305), was added. This mixture was transferred to a cooled mortar, and the cells were ground for about 40 minutes. After addition of Tico buffer (1.5 ml per gram of cell), the cells were further mixed for 10 minutes. The homogenate was then twice centrifuged at 8,000 rpm for 10 minutes in a GSA rotor at 4°C in order to remove the Alcoa and the unbroken cells. The supernatant was centrifuged at 16,000 rpm (30,000 x g), for 45 minutes in a SA-600 rotor. The pellet was discarded and the supernatant (S-30), containing ribosomes and soluble enzymes was further centrifuged at 22,000 rpm (30,000 x g), during 17-20 hours in a 45 Ti rotor in order to sediment the 70S ribosomes. The pellet was suspended in Tico buffer and again centrifuged in a SA-600 rotor at 8,000 rpm during 10 minutes in order to eliminate the non-dissolved aggregates. The ribosomes in suspension (crude 70S) were then shock-frozen in liquid nitrogen in aliquots containing 6,000-9,000 A_{260} units and stored at – 80°C.

The yield of crude 70S ranged between 300 and 400 A_{260} units per g of wet cells processed.

2.5.3 Extraction of small RNAs from the cell (from the Ph. D. thesis of Ralf Jünemann)

A 30 ml overnight cell culture was centrifuged for 5 min with 5,000 rpm in HB4 rotor at 4°C. The cell pellet was washed with 10 ml Cell Wash buffer and centrifuged again. The resulting pellet was suspended in 1.5 ml of Cell Wash buffer and transferred to 2 ml pre-weighted tubes. Another centrifugation step carried out in a table centrifuge for 5 min with 5,000 rpm at 4°C to estimate the weight of cell pellets, to which 1 ml of $H_{20}M_{10}$ buffer per 0.25 g cell pellet was applied. When suspended well, one volume of 70% phenol (RNase free) was

added to each sample tube and vigorously shacking during 45 min at 4°C. Under such a treatment RNA goes into water phase while DNA and proteins go to the organic phase. It is important to vortex solution strongly and continuously; otherwise the output of small RNAs will be very poor. After the next centrifugation at 14,000 rpm at 4°C for 30 min in a table centrifuge water phase was removed to fresh tubes and the remaining phenol was extracted by adding one volume of chloroform - isoamyl alcohol (24:1) followed another centrifugation step. To the water phase from the last step 0.65 volumes of 5M NaCl were added, and samples were kept on ice for 30 min. During this incubation large RNAs like ribosomal precipitate, and small RNAs like tRNAs are still in solution. After a final centrifugation in a table centrifuge at 4°C with 14,000 rpm for 30 min, the supernatant containing tRNAs was kept, while the pellet was discarded. The small RNAs from the supernatant were precipitated by standard ethanol precipitation technique. The resulting pellets of RNAs were dissolved in water and small aliquots were shock-frozen and stored at –80°C. One small volume is kept for A_{260} measurements.

2.6 Genetic methods

2.6.1 Preparation of **E. coli** *competent cells for electroporation*

The *Escherichia coli* strain XL1-Blue strain allows blue-white colour screening for recombinant plasmids and is an excellent host strain for routine cloning applications using plasmid or lambda vectors. The XL1-Blue genotype is as follows: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q*Z.M15* Tn*10* (Tetr)]. (Genes listed signify mutant alleles. Genes on the F′ episome, however, are wild type unless indicated otherwise). This cell strain is tetracycline resistant and was used for preparation of competent cells.

One-litre culture of the *E. coli* strain of interest was grown to an OD_{600} of 0.5-1.0. The cells were chilled on ice, and then centrifuged, at 4,000 rpm for 15 min at 4°C in a GSA rotor to obtain pellets, which were suspended in 1 volume of pre-chilled sterile MQ-H_2O. Bacterial cells were repeatedly centrifuged and

washed in decreasing volumes of cold MQ-H_2O: two washes with 0.5 volume, one wash with 0.02 volume and final resuspension in 2-3 ml filter sterilised 10% (v/v) glycerol. Finally, from the cell suspension in 40 µl aliquots were prepared, shock frozen in a dry ice-ethanol bath and stored at –80°C.

The competence of the cells was defined by transformation using the plasmid pIVEX2.2GFPcyc3; using 10 ng of plasmid plus 40 µl of competent cells (processed according to the standard protocol using LB/ampicillin plates). The transformation efficiency was determined to be in the range of 0.5-1.5 x 10^6 transformants per µg of plasmid DNA. This level of competence was considered to be sufficient for further experiments.

2.6.2. Cloning strategies

T7 RNA polymerase expression system

In the following section some rudimentary description of the T7 polymerase expression system is given. For a more detailed understanding refer to (Studier *et al.*, 1990). A vast number of diverse vectors and strains are available for use with the pET expression system, and can be accessed through the Novagen web site:

http://www.merckbiosciences.co.uk/g.asp?f=NVG/home.html

The bacteriophage T7 RNA polymerase

The T7 RNA polymerase expression system relies on the fact that the RNA polymerase of bacteriophage T7 has a very high specificity for its own promoter. Furthermore, there are no known *E. coli* sequences that resemble this region. The bacteriophage T7 RNA polymerase initiates rapidly and transcribes about five times faster than an *E. coli* RNA polymerase. Efficient transcription termination signals for T7 RNA polymerase are very rare.

The T7 RNA polymerase can be delivered to a cell by either induction or infection. For convenience the former was preferred (as seen in Figure 2.6.1-1).

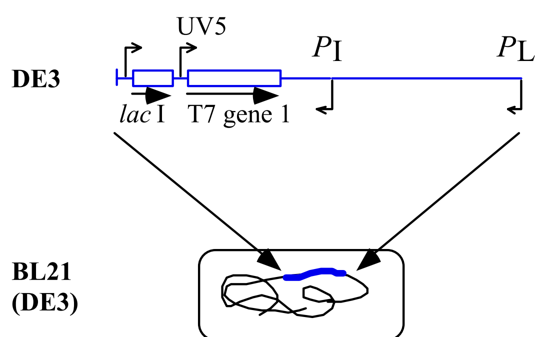


Figure 2.6.1-1 Formation of lysogenic BL21(DE3). The *E. coli* expression strain BL21(DE3) contains the *gene 1* encoding T7 RNA polymerase under control of an IPTG-inducible *lac*UV5 promoter.

pET vectors and expression

The plasmid vectors for expression using the T7 RNA polymerase are termed pET vectors (plasmid for expression by T7 RNA polymerase). This study utilizes pET23c(+). In pET23c(+), genes of interest are cloned directionally between an *Nde* I or *Xba* I and a *Xho* I site, placing the gene under the control of the T7 promoter.

It is important to note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the vector map (Figure 2.6.1-2). The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer.

pET23c(+) sequence landmarks

Name	Position on the plasmid
T7 promoter	303-319
T7 transcription start	302
T7•Tag coding sequence	207-239
Multiple cloning sites (*BamH* I – *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
pBR322 origin	1450
bla coding sequence	2211-3068
f1 origin	3200-3655

Xho I(158)
Not I(166)
Eag I(166)
Hind III(173)
Sal I(179)
Hinc II(181)
Sac I(190)
EcoR I(192)
BamH I(198)
Sty I(57)
Bpu1102 I(80)
Nhe I(231)
Nde I(238)
Xba I(276)
Bgl II(334)
Msc I(359)
Dsa I(360)
Bpu10 I(494)
Bbs I(506)
Bsg I(548)
Eco47 III(642)
NgoA IV(3525)
Drd I(3429)
Dra III(3424)
f1 origin (3200-3655)
pET-23c(+)
(3664bp)
BsaH I(2818)
Sca I(2761)
Pvu I(2651)
Pst I(2526)
Bgl I(2401)
Ahd I(2281)
Ap (2211-3068)
Pvu II(979)
Tth111 I(1133)
Bst1107 I(1159)
ori (1450)
Sap I(1272)
Afl III(1388)
BspLU11 I(1388)
AlwN I(1804)

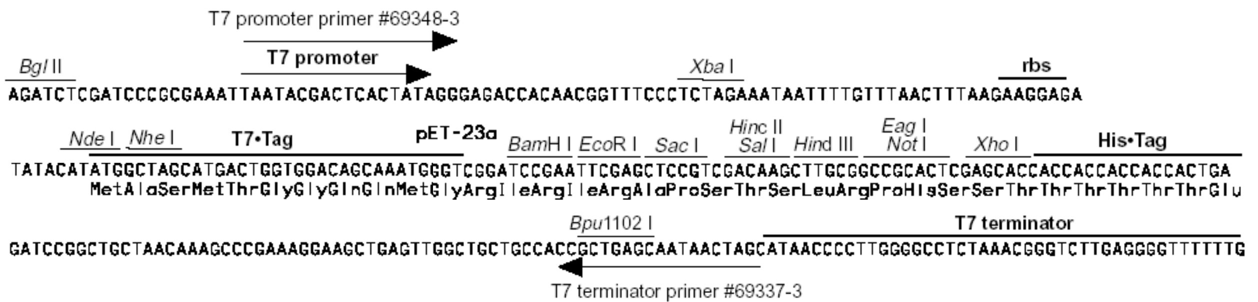
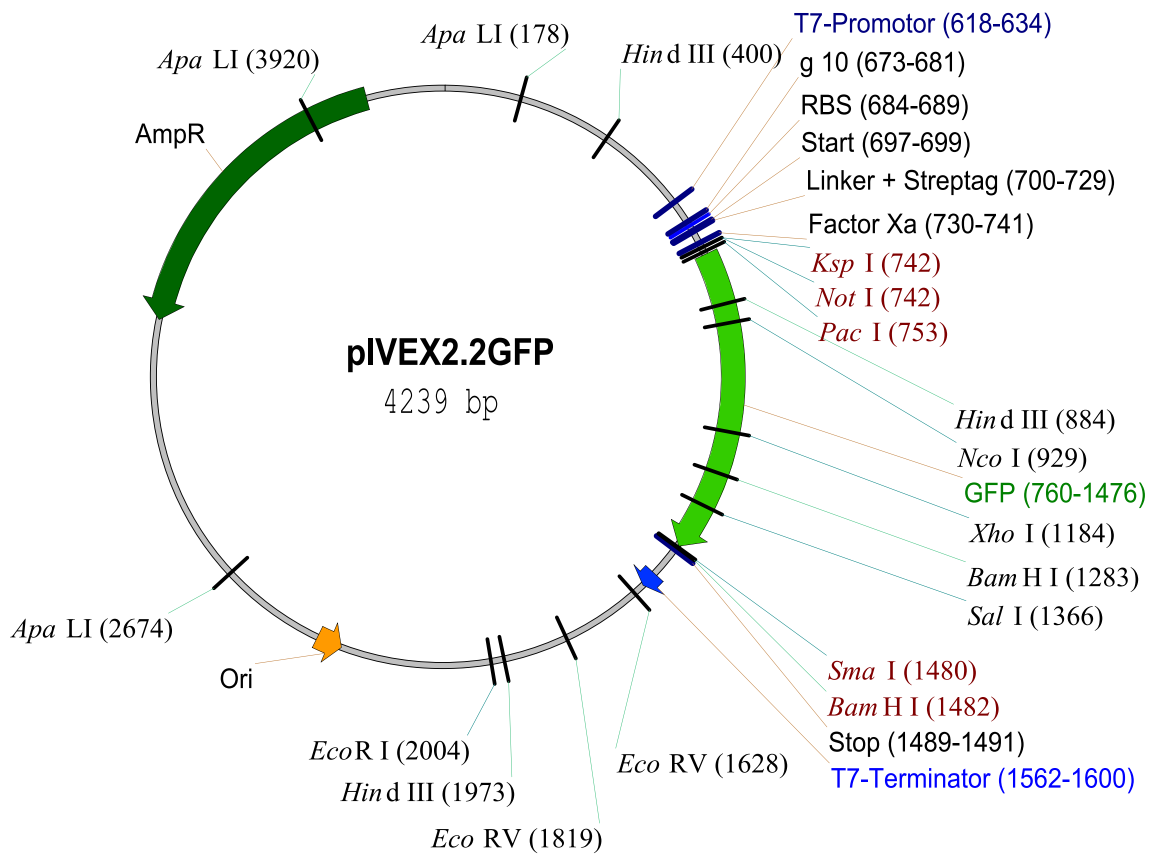


Figure 2.6.1-2 Cloning/expression region of the DNA plasmid pET-23c(+).

pIVEX vector and expression

Plasmid pIVEX2.2GFPcyc3 was utilised as a general control for the study of *in vitro* expression system and for the comparison of the expression of some given proteins in this system. GFPcyc3 is a Green Fluorescence Protein that carries three point mutations, which allow fast maturation of the fluorophore in comparison to the wild type GFP. The gene is introduced on the plasmid directly between *Pac* I and *Sma* I restriction sites.

The plasmid contains (i) a gene providing ampicillin resistance; (ii) the strong T7 promoter sequence (Studier and Moffatt, 1986); (iii) the T7 *gene* 10 translational enhancer (Olins *et al.*, 1988); (iv) the prokaryotic Shine-Dalgarno sequence (Shine and Dalgarno, 1974) as a binding site for prokaryotic ribosomes with an optimum distance to the start AUG codon (Chen *et al.*, 1994); (v) the T7 terminator that stops transcription and prevents 3'-terminal exonucleolytic degradation of the mRNA; (vi) *Strep*-tag® II (Voss and Skerra, 1997) at the N-terminal to allow purification of the expressed protein; (vii) restriction protease factor Xa cleavage site (see Figure 2.6.1-3 below).

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                           T7-Promotor
601   TCTCGATCCC GCGAAATTAA TACGACTCAC TATAGGGAGA CCACAACGGT TTCCCTCTAG
      AGAGCTAGGG CGCTTTAATT ATGCTGAGTG ATATCCCTCT GGTGTTGCCA AAGGGAGATC


                      g10 ε        SD-Sequ.     NcoI    Linker Streptag
661   AAATAATTTT GTTTAACTTT AAGAAGGAGA TATACCATGA CCAGCTGGAG CCACCCGCAG
      TTTATTAAAA CAAATTGAAA TTCTTCCTCT ATATGGTACT GGTCGACCTC GGTGGGCGTC
                                            MetT hrSerTrpSe rHisProGln


                           KspINotI     PacI            NcoI         SalI    XhoI
SacI
721   TTCGAAAAAA TCGAAGGCCG CGGCCGCTTA ATTAAAACCA TGGCAGTCGA CTCGAGCGAG
      AAGCTTTTTT AGCTTCCGGC GCCGGCGAAT TAATTTTGGT ACCGTCAGCT GAGCTCGCTC
      PheGluLysI leGluGlyAr gGlyArgLeu IleLysThrM etAlaValAs pSerSerGlu


          PstI   SmaI  BamHI
781   CTCTGCAGCC CGGGATCCGGC TGCTAACAA AGCCCGAAAG GAAGCTGAGT TGGCTGCTGC
      GAGACGTCGG GCCCTAGGCCG ACGATTGTT TCGGGCTTTC CTTCGACTCA ACCGACGACG
      LeuCysSerP roGlySerGly Cys***
```

Figure 2.6.1-3 Map of the pIVEX2.2GFPcyc3 expression vector. On the map GFPcyc3 insert is shown in green with markings of its position on the plasmid in brackets. Below the linker of the pIVEX2.2 vector is represented with corresponding markings; sites used for insertion of GFPcyc3 are underlined.

2.6.3 Vector construction and protein overexpression

Plasmid constructs

The pET23c(+) expression system was chosen to compare the efficiency of *E. coli* translation elongation factors (EFs) expression in the coupled transcription-translation system. The elongation factor EF-Tu (for temperature instability), is usually fragmented in the *in vitro* systems, unlike EF-Ts (temperature stable). Table 2.6.3-1 summarises the plasmids used in this study.

Table 2.6.3-1 Plasmid constructs

Plasmid	Description	Source
pET23c(+)	T7 pol promoter	Novagen
pET23c(+)_EF-Tu	EF-Tu gene in pET23c(+)	This work
pET23c(+)_EF-Ts	EF-Ts gene in pET23c(+)	This work
pIVEX2.2GFPcyc3	GFPcyc3 gene in pIVEX2.2	///////
pIVEX2.3GFPwt	GFPwt gene in pIVEX2.3	///////

Polymerase chain reaction

Plasmid DNA containing an EF-Tu or EF-Ts gene was used as a template for PCR with the appropriate sets of forward and reverse primer pairs (as seen in Table 2.6.3-2).

Table 2.6.3-2 PCR primers

Forward Primer	Sequence (5'-3')*
Tu_NdeI	CGTCACTTTAAGAAGAGATATA**CATATG**TCTAAAGAAAAGTTTGACC
Ts_NdeI	CGTCACTTTAAGAAGAGATATA**CATATG**GCTGAAATTACCGC
Reverse Primer	**Sequence (5'-3')***
EF-Tu-XhoI	GT**CTCGAG**GCTCAGAACTTTTGCTACAA
EF-Ts-XhoI	GT**CTCGAG**AGACTGCTTGGACATCG

*The restriction sites are in bold and underlined type

PCR conditions

The optimal reaction conditions (incubation times and temperatures, concentration of the enzyme, template DNA, Mg^{2+} ions) depend on the template/primer pair and must be determined individually.

To increase fidelity of the PCR reaction use a thermo stable DNA-polymerase with 3'-5'-exonuclease activity like Pwo or the Expand™ High Fidelity PCR System.

For a standard PCR reaction in a total volume of 50 µl use 20 pmoles of each forward and reverse primers together with 100 ng of the template DNA, 400 pmoles of each dNTP, 2.6 units of Expand™ enzyme and 5 µl 10x Expand HF buffer with 15 mM Mg^{2+}.

To avoid unspecific products, reactions were cycled 25 times with an extension time of 90 sec at 72°C. Conveniently an annealing temperature of 50°C was employed successfully for each set of appropriate primer pairs listed in Table 2.6.3-2. Homogeneous PCR products of the expected size were analysed by 1% agarose gel electrophoresis (see Figure 2.6.3-1).

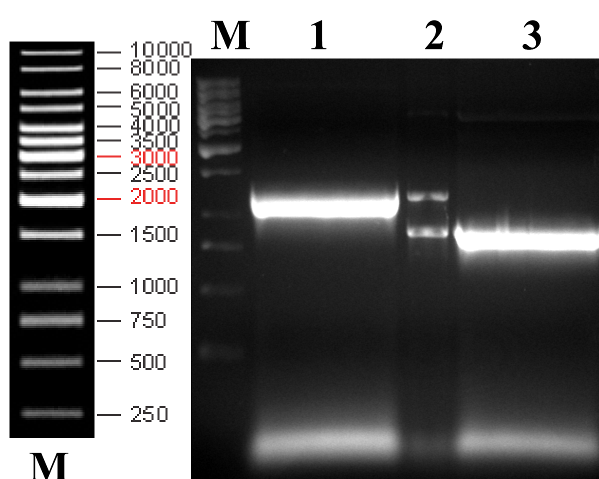

Figure 2.6.3-1 Gel electrophoresis of PCR products. PCR reaction products were analysed by electrophoresis through a 1.0 % agarose gel. Lane M contains GeneRuler™ 1kb DNA Ladder (bp). PCR products were prepared to produce approximately 1185 bp products of *Nde* I-EF-Tu- *Xho* I (Lane 1), and 852 bp *Nde* I-EF-Ts- *Xho* I (Lane 3). Lane 2 contains both EFs.

Cloning of PCR products

PCR products were digested with *Nde* I/*Xho* I and gel purified prior to ligation into *Nde* I/*Xho* I-digested and purified vector pET23c(+). Ligations were transformed into XL1-Blue cells and clones were analysed by restriction digestion for insert containing vectors (Figure 2.6.3-2).

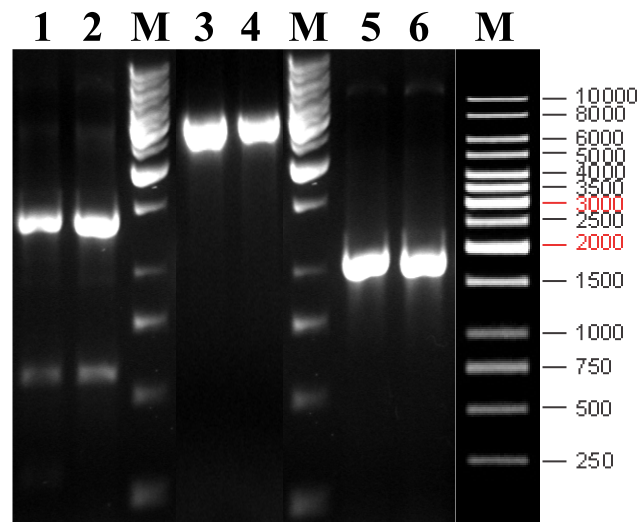

Figure 2.6.3-2 Restriction analysis of clones. Lanes 1 and 2 show *Nde* I/*Xho* I-digested EF-Tu (1.2 kb), lanes 5 and 6 show *Nde* I/*Xho* I-digested EF-Ts (0.8 kb). Pre-ligation pET23c(+) vector (3.6 kb) is shown in lanes 3 and 4. All restriction products were analysed on 1.0 % agarose gel. Lane M contains GeneRuler™ 1kb DNA Ladder; relevant marker sizes are indicated in bp.

Positive clones were sequenced in both directions to verify the absence of polymerase-introduced mutations and used in experiments discussed below.

2.7 *In vitro* protein synthesis systems based on *E. coli* lysate

Transcription and translation take place simultaneously in a reaction mix. Expression of genes behind a T7 promoter is possible from either circular or linear DNA template. Upon addition of the DNA template and T7 RNA polymerase to the DNA-free *E. coli* lysate transcription and translation are closely coupled in time and space: While the T7 RNA polymerase transcribes the template gene, the ribosomes provided by the *E. coli* lysate start to translate the 5'-end of the nascent mRNA.

The *E. coli* lysate is prepared with some modifications according to the method of Zubay (Zubay, 1973). An *E. coli* strain with the lowest protease activity was selected and growth conditions were optimised to allow optimum protein expression from linear (PCR-generated) and plasmid templates.

2.7.1 Batch system

Based on the Kim and Swartz system (Kim and Swartz, 1999; Kim and Swartz, 2000) the substances discussed in section 2.3.4 were used to prepare HEPES salts (HSS) and Small compounds (SCS) stock solutions. Along with substances displayed in Table 2.7.1-1 below solutions were pipetted together into LM-mix in corresponding volumes. The LM-mix is stabled for two to three weeks and can be prepared for multiple reactions assay in advance, shock frozen and stored at – 80°C. This LM-mix is pipetted into the Master-mix alone with essential salts and enzymes (see Figure 2.7.1-1, Master-mix preparation).

Table 2.7.1-1 Pipetting scheme for the LM-mix preparation

Substance	Volume
HSS	400 µl
SCS	80 µl
ATP, 100 mM	50 µl
CTP, 100 mM	40 µl
GTP, 100 mM	40 µl
UTP, 100 mM	40 µl
H_2O	70 µl
aa C, 55 mM	40 µl
aa DY, 55 mM	40 µl
PEP, 670 mM	200 µl

Final volume of 1000 µl was divided into 150µl aliquots and stored at – 80°C for up to three weeks.

The Master-mix contains among the rest [^{35}S]-Met and T7 RNA polymerase.

Master Mix Preparation

Sample	1:10 in H_2O		100 mM	10 mM, ph 7.6			240 µg/ml
	[^{35}S]-Met	**H_2O**	**Mg acet.**	**HEPES-KOH**	**LM-mix**	**T7 RNAP**	**Rifampicin**
Volume	**1µl**	4 µl	6 µl	4 µl	12.5 µl	1 µl	**1.5 µl**
1. DNA - 2. DNA - 3. DNA + 4. DNA +							
Volume x 5	5 µl	20 µl	30 µl	20 µl	62.5 µl	5 µl	7.5 µl

[^{35}S]-Met in H_2O	
[^{35}S]-Met	8.0 µl
H_2O	72.0 µl
	80.0 µl

Preparation of the Reaction Mix

Component	40% PEG	Master mix	DNA template	S30 extract
Volume	2.5 µl	30 µl	5 µl	12.5 µl
	VORTEX !!!			

Figure 2.7.1-1 Preparation of Master- and Reaction-mixes. The [^{35}S]-Met was diluted accordingly.

2.7.2 RTS 100 High Yield E. coli *Kit*

Here we scaled down the Roche batch system to 10 µl volume, where conditions could be easily altered for optimization; Roche is suggesting a volume of 50 µl.

According to the protocol we reconstituted the contents of the kit with a supplied Reconstitution buffer (Table 2.7.2-1). The reaction mixes were prepared according to the Table 2.7.2-2 and 10 µl were distributed to separate vials, DNA template was added after this. For radioactive labelling of elongation factors EF-Tu and EF-Ts the reactions were prepared according to the Table 2.7.2-3. Standard incubation temperature was 30°C. Samples were introduced into ProteoMaster instrument and incubated according to the assay requirements.

Table 2.7.2-1 Reconstitution of reaction components (RTS 100 HY E. coli *Kit)*

Solution	Reconstitution procedure
1. *E. coli* Lysate	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer, mix carefully by rolling or gentle shaking. DO NOT VORTEX!
2. Reaction Mix	Reconstitute the with 0.30 ml of Reconstitution Buffer, mix by rolling or shaking
3. Amino Acids	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer, mix by rolling or shaking.
4. Methionine	Reconstitute the lyophilizate with 0.33 ml of Reconstitution Buffer, mix by rolling or shaking.
5. Reconstitution Buffer	1.6 ml; Ready-to-use solution; stable at 2–8°C, can also be stored at – 15°C to – 25°C.
6. Control vector GFP	Briefly centrifuge down the content of the bottle and reconstitute the lyophilizate with 50 µl of sterile DNase- and RNase- free water The solution is stable at –15°C to –25°C

Table 2.7.2-2 Preparation of working solutions (RTS 100 HY E. coli *Kit)*

Contents	Preparation of working solution for one 50 µl reaction
Reaction Solution	Into one of the supplied reaction tubes pipette the following components: 1. 12 µl *E. coli* Lysate 2. 10 µl Reaction Mix 3. 12 µl Amino Acids 4. 1 µl Methionine 5. 5 µl Reconstitution Buffer 6. 0.5 µg of circular DNA template or 0.1–0.5 µg of linear template in 10 µl of water or TE-buffer. Mix carefully by rolling or gentle shaking. DO NOT VORTEX!

Table 2.7.2-3 Radioactive labelling (RTS 100 HY E. coli *Kit)*

Step	Action
1	Reconstitute bottles 1 to 4 and bottle 6 according to Table 2.4.5.2-1
2	Dilute 10 µl of the reconstituted Methionine solution of 3.2.2 with 990 µl of nuclease-free water to yield a 1 mM Methionine solution.
3	For one radioactive reaction prepare the following Reaction Solution in one of the reaction tubes: 1. 6 µl *E. coli* Lysate 2. 5 µl Reaction Mix 3. 6 µl Amino Acids 4. 1.25 µl 1 mM Methionine solution (see Note) 5. 2 µl of a L-[^{35}S]-Methionine (SJ 235 Amersham) 15 mCi/ml 6. 2.5 µl Reconstitution Buffer 7. 0.25 µg of the plasmid DNA or 0.05–0.25 µg of linear template in 2.25 µl of water or TE-buffer.
4	The reaction temperature: 30°C
5	Stop the reaction after 60 min.
6	Apply 2–5 µl of the reaction samples onto SDS-polyacrylamide gels. Note: For optimum results precipitate the proteins with cold acetone before applying onto SDS-polyacrylamide gels (see in the appendix section).
7	After the separation, dry the gel and place into Storage Phosphor Screens for autoradiography (3–20 hrs exposition time).

Note: Addition of unlabeled methionine to the labelling reaction is required to prevent premature termination for larger proteins or proteins with many methionine residues.

2.7.3 RTS 500 High Yield E. coli *Kit*

The principle of RTS 500 reaction is that transcription and translation take place simultaneously in the 1 ml reaction compartment, and substrates and energy components essential for the reaction are continuously supplied *via* a semipermeable membrane from the 10 ml feeding compartment (Figure 2.7.3-1). Through the same membrane potentially inhibitory reaction by-products are diluted *via* diffusion from reaction compartment into feeding compartment (RTS 500 HY *E. coli* Kit). The reaction device is supplied together with the kit.

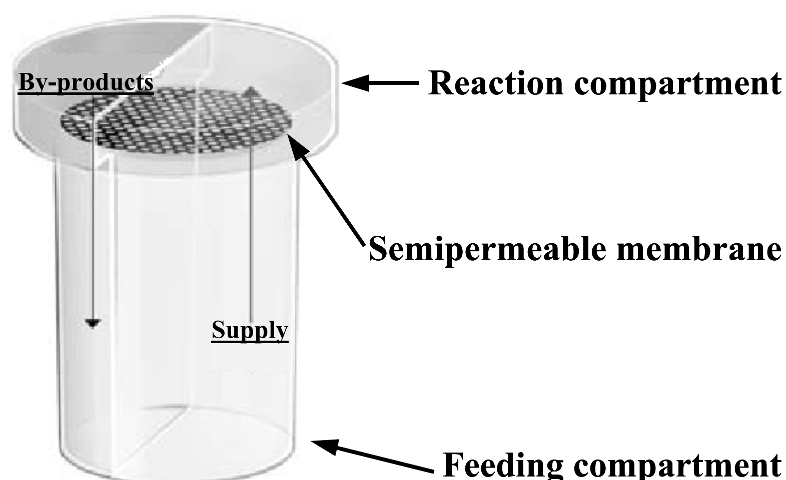

Figure 2.7.3-1 Reaction device for RTS 500. Principles of continuous exchange cell-free protein synthesis as suggested by Spirin (1988). Essential compartments and membrane are indicated. Arrows within the device indicate direction of the flow.

Lyophilised reagents provided with RTS 500 Kit were reconstituted according to the Kit directions (Table 2.7.3-1).

Table 2.7.3-1 Reconstitution of RTS 500 reaction components (RTS 500 HY E. coli *Kit)*

Content	Reconstitution/Preparation of working solution
E. coli Lysate	Reconstitute the lyophilizate with 0.525 ml of Reconstitution Buffer, mix carefully by rolling or gentle shaking. DO NOT VORTEX!
Reaction Mix	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer mix by rolling or shaking.
Feeding Mix	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer, mix by rolling or shaking.
Amino Acid Mix	Reconstitute the lyophilizate with without Methionine 3 ml of Reconstitution Buffer, mix by rolling or shaking.
Methionine	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer, mix by rolling or shaking.
Reconstitution Buffer	Ready-to-use solution stable at 2-8°C, but can also be stored at –15 to – 25°C.

The working solutions were prepared from reconstituted reagent according to the RTS 500 Kit protocol (Table 2.7.3-2). The following Reaction solution was loaded into 1 ml reaction compartment of the supplied reaction device. The

Feeding solution was loaded into feeding compartment with care avoiding air bubbles. The loaded reaction device was introduced into ProteoMaster instrument and incubated at 30°C if not otherwise indicated.

Table 2.7.3-2 Preparation of working solution for RTS 500 (RTS 500 HY E. coli *Kit)*

Content	Reconstitution/Preparation of working solution
Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine and 0.3 ml of reconstituted Methionine to Feeding Solution 3. Mix by rolling or shaking. Total volume of Feeding Solution is 11 ml.
Reaction Solution	To the content of *E. coli* lysate, add 0.225 ml of the reconstituted Reaction Mix, 0.27 ml of the reconstituted Amino Acid Mix without Methionine and 30 µl of reconstituted Methionine. Add 10–15 µg of the DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. Total volume of reaction solution is 1.1 ml. DO NOT VORTEX.

After stop of the reactions with GFP, 5 to 10 µl were taken for the SDS-PAAG electrophoretic analysis of a total fraction and the rest of the reactions was stored at + 4°C overnight to gain the active form. The native PAAG electrophoretic analyses were performed for 24-48 hours (see Chapter Result, section 3.4 for details).