2 Materials and Methods

2.1 Materials

2.1.1 Sets of biological components

IBA:

Strep-tag® Starter Kit (2-1101-000)

Qiagen:

Ni-NTA Spin Kit (50), (31314)

Plasmid Maxi Kit (121163)

Plasmid Midi Kit (100), (12145)

QIAprep® Spin Miniprep Kit (250), (27106)

QIAquick® Gel Extraction Kit (50), (28706)

Roche:

Rapid Translation System 100, RTS 100 *E. coli* HY Kit (3 186 156)

Rapid Translation System 500, RTS 500 *E. coli* HY Kit (3 246 949)

2.1.2 Chemicals and simple biological components

Amersham:

[γ ^{32}P]-Adenosine-5'-triphosphate (PB 10218)

Bovine Serum Albumine (BSA, RNase/DNase Free), (27-8914-02)

Cytidine 3'5'-bis[α-^{32}P]-phosphate (PB10208)

L-[^{35}S]-Methionine (SJ 235)

L-[4,5-^{3}H]-Leucine (TRK 510)

L-[4-^{3}H]-Phenylalanie (TRK 204)

L-[U-^{14}C]-Phenylalanine (CFB 70)

Poly(A), (27-4110-01)

Poly(U), (27-4440-02)

Hybond *N*-plus membrane (RPN 203N)

Beckman:

Ready Value (Liquid Scintillation Cocktail), (PN 586602)

Ultracentrifuge tubes (Ultra-Clear) SW40 and SW28, (344060, 344058)

Biorad:

2x Laemmli Sample Buffer (161-0737)

2x Native Sample Buffer (161-0738)

Premixed 10x SDS-Tris-Glycine Buffer (161-0732)

Premixed 10x Tris-Glycine Buffer (161-0734)

Sodium-dodecyl-sulfate (SDS), (161-0301)

Calbiochem:

HEPES, Free Acid, ULTROL® Grade (391338)

Difco:

Bacto™ Agar (214010)

Bacto™ Peptone (211677)

Bacto™ Yeast Extract (212750)

Invitrogen:

Agarose Electrophoresis Grade (15510-019)

Sucrose (15503-022)

TEMED (15524-010)

Fermentas:

2x Loading Dye Solution for RNA electrophoresis (#R 0641)

6x Loading Dye Solution (#R 0611)

GeneRuler™ 100bp DNA Ladder RNA ladder (#SM 0241)

GeneRuler™ 1kb DNA Ladder (#SM 0311)

RNA Ladder, Low Range (#SM 0411)

T4 DNA Ligase, (#EL 0015)

Fluka:

Spermidine trihydrochloride (85578)

Spermine tetrahydrochloride (85605)

Merck:

2-Mercaptoethanol (8.05740.0250)

2-Propanol (1.09634.2500)

Acetic acid glacial 100% (1.00063.2500)

Ammonium acetate (1.01116.1000)

Ammonium chloride (1.01145.1000)

Ammonium peroxodisulfate (APS), (1.01201.1000)

Boric acid (1.00165.1000)

Chloroform (1.02445.1000)

Diethyl ether (1.00926.500)

Ethanol (1.00986.2500)

Ethidinum bromide (1%), (1.11608.0030)

Formamide (1.09684.1000)

Glycerol 100% (1.05819.1000)

Glycine (1.04201.1000)

Hydrochloric acid 32% (1.00319.2500)

Magnesium aceate (1.04936.1000)

Magnesium chloride hexahydrate (1.05833.1000)

Methanol (1.06002.2500)

Potassium acetate (1.04820.1000)

Potassium chloride (1.04936.1000)

Potassium hydroxide solution 1 mol/L (1.09108.1000)

Sodium acetate trihydrate (1.06265.1000)

Sodium carbonate monohydrate (1.06386.1000)

Sodium chloride (1.06404.1000)

Sodium citrate (1.06448)

Sodium hydroxide (1.06498.1000)

Sodium hydroxide solution 1 mol/L (1.09137.1000)

Triplex III GR (EDTA), (1.08418.1000)

Tris(hydroxymethyl) aminomethane (1.08382.1000)

tri-Sodium citrate dihydrate (1.06448.1000)

Urea (1.08487.1000)

Millipore:

Filters 0.45 µm (HAW02500)

New England BioLabs:

Restriction endonucleases with buffers

T4 DNA Ligase (M0202S)

PerkinElmer:

Filter-Count™ (complete LSC-cocktail), (6013149)

Promega:

Steady-Glo® Luciferase Assay System

Qiagen:

Ni-NTA Agarose (100 ml), (30 230)

Roche Pharmaceuticals:

1,4-Dithiothreitol (DTT) (1 583 786)

DNase I, RNase-free (10 776 785 001)

dNTPs: dATP, dCTP, dGTP, dUTP (1 051 440, 1 051 458, 1 051 466, 1 420 470)

Phosphatase, alkaline (AP) from calf intestine (713 023)

Polynucleotide kinase (174 645)

Pyruvate kinase (PK), (109 045)

$tRNA^{bulk}$ from *E. coli* MRE 600 (RNase negative), (109 550)

rGFP 50 µg (11 814 524 001)

Roth:

1,4-Dithioerythritol (DTE), (8814.1)

Ampicillin (K029.2)

IPTG 25g (2316.4)

Phenol (0040.2)

Roti-Mark STANDARD 1ml (T851.1)

Rotiphorese® Gel 30 (37,5:1), (3029.1)

Rotiphorese® Gel 40 (19:1), (3030.1)

Trichloroacetic acid (TCA), (8789.1)

Sartorius:

Nitrocellulose filter (11306)

Schleicher and Schüll:

Glass Fiber Filters ∅23 mm (10 370 021)

Serva:

Aluminiumoxid Alcoa A-305 (12293)

Coomassie® Brilliant Blue G-250 (17524)

Coomassie® Brilliant Blue R-250 (17525)

Sigma:

3-(N-morpholino)propanesulfonic acid (MOPS), (M-1254)

Albumin, bovine (A-7906)

Dextran sulfate (D-6001)

Ficol 400 (F-4375)

Formaldehyde (F-8775)

Lithium potassium acetyl phosphate (A 0262)

Lysozyme (L-6876)

Polyvinylpolypyrolidone (P-6755)

ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (P-4600)

2.1.3 Non-typical laboratory machines

- Wallac 1409 Liquid Scintillation Counter
- RTS ProteoMaster Roche
- PhosphorImager STORM 820 (Molecular Dynamics, Amersham Biosciences) and PhosphorImager cassette BAS 2325 (Fijifilm)
- Luminometer Centro LB 960 (Berthold technologies, Germany)
- Beckmann Coulter DU®640B Spectrophotometer
- Sorvall RC 5B plus centrifufe
- Beckmann L7-55 ultracentrifuge
- Beckmann Coulter Optima™L-90K ultracentrifuge
- New Brunswick Scientific GmbH Innova 4400 incubator shaker
- New Brunswick Scientific GmbH BIOFLO 3000 Batch/Continuous Bioreactor

2.1.4 Bacterial strains of **E. coli**

XL-1 Blue: recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)],

BL21(DE3): F^- dcm ompT hsdS(r_{B-} m_{B-}) gal λ(DE3),

CAN20: derived from *E. coli* K12, which is deficient in five RNases (Zaniewski et al., 1984)

K12: *F´proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)M15 zzf::Tn*10(Tet^R)*/ fhuA2 glnV Δ(lac-proAB) thi-1 Δ(hsdS-mcrB)5*

2.1.5 Plasmids

- Rapid Translation System RTS pIVEX His-tag, 2nd Generation Vector Set (Roche, 3 269 019)
- pET-23c(+), (Novagen, 69747)
- luciferase T7 Control DNA (Promega)

2.1.6 Sequences of in vitro *transcribed mRNAs*

1) **pU-SD/RF2 (Márquez, 2002)**
   5'-GGUUC$(UUC)_{11}$CGUAUGAAACUGGUUCUUGUUCUUCGCGGCUA UCUUUGACUCUGAUUCAAAAAGGGAU-3'
2) **MF-mRNA (Triana-Alonso et al., 1995)**
   5'-GGG$(A_4G)_3$AAAAUGUUC$(A_4G)_3$AAAU-3'
3) **MVF-mRNA with SD (Schäfer et al., 2002)**
   5'-GGGAAAAGGAGGUCACAUAUGGUAUUC$(A_4G)_3$AAAU-3'
4) **MVF-mRNA without SD (Schäfer et al., 2002)**
   5'-GGG$(A_4G)_3$AAAAUGGUAUUC$(A_4G)_3$AAAU-3'
5) **MFV-mRNA (Schäfer et al., 2002)**
   5'-GGG$(A_4G)_3$AAAAUGUUCGUU$(A_4G)_3$AAAU-3'

2.1.7 Antibiotics

Ampicillin in H_2O (Roth, K029.2)
Chloramphenicol in 100% ethanol (Boehringen-Mannheim GmbH, 634433)
Edeine (EDE) in H_2O
Gentamycin (GEN) in H_2O (Sigma-Aldrich, 46305)
Hygromycin B (HYG) in H_2O (Fluka, 56682)
Kanamycin A (KAN) in H_2O (Roth, T832.3)
Kasugamycin (KSG) in H_2O (BioMol, requested synthesis)
Neomycin (NEO) in H_2O (Fluka, 72133)
Novel Ribosomal Inhibitor (NRI, A72310) in H_2O, Abbott Laboratories
Pactamycin (PCT) in 100% ethanol
Paromomycin (PAR) in H_2O
Puromycin in 2% 1N KOH, pH 7.4 (Serva, 33835)
Streptomycin (STR) in H_2O (Fluka, 85880)

Tetracycline (TET) in 100% ethanol (Fluka, 87128)

Thiostrepton (THIO) in 100% DMSO (Sigma, T8902-1G)

Viomycin (VIO) in H_2O

2.1.8 Buffers

2.1.8.1 Electrophoresis solutions

Agarose gel solution	10x TBE Agarose Ethidium Bromide (1%) H_2O	5 ml 0.7 – 2% (w/v) 2 µl ad 50 ml
Coomassie Blue R-250 Staining Solution (0.05%)	Coomassie® Brilliant Blue R-250 Methanol Acetic acid glacial 100%	0.05% (w/v) 50% (w/v) 10% (w/v)
Coomassie Blue R-250 Destaining Solution	Methanol Acetic acid glacial 100%	25% (w/v) 8% (w/v)
TBE (1x)	Tris Boric acid EDTA	89 mM 89 mM 2 mM
APS solution 10%	Ammonium peroxodisulfate	10% (w/v)
Polyacrylamide gel 15% for protein electrophoresis (separating gel)	1.5 M Tris solution, pH 8.8 Rotiphorese® Gel 30 (37,5:1) APS solution 10% TEMED H_2O	3 ml 6 ml 50 µl 10 µl ad 12 ml
Polyacrylamide gel 5% for protein electrophoresis (stacking gel)	0.5 M Tris solution, pH 6.8 Rotiphorese® Gel 30 (37,5:1) APS solution 10% TEMED H_2O	1.25 ml 0.85 ml 75 µl 15 µl ad 5 ml
Tris solution, 0.5 M, pH 6.8	Tris Hydrochloric acid 32%	0.5 M Adjust pH ~6.8
Tris solution, 1.5 M, pH 8.8	Tris Hydrochloric acid 32%	1.5 M Adjust pH ~8.8

Sequencing gel - 10%	Rotiphorese® Gel 40 (19:1) Urea TBE buffer (10x) H_2O	20 ml 36g 8 ml Ad 80 ml

2.1.8.2 Buffers for microbiological methods

Luria-Bertani (LB) medium	Bacto™ Peptone Bacto™ Yeast Extract Sodium chloride (NaCl)	1% (w/v) 0.5% (w/v) 1% (w/v)
Luria-Bertani (LB) solid medium	Bacto™ Peptone Bacto™ Yeast Extract Sodium chloride (NaCl) Bacto™ Agar	1% (w/v) 0.5% (w/v) 1% (w/v) 1.5% (w/v)
Luria-Bertani (LB) medium for competent cells preparation	Bacto™ Peptone Bacto™ Yeast Extract Sodium chloride (NaCl)	1% (w/v) 0.5% (w/v) 0.5% (w/v)
Glycerol stock 50%	Glycerol 100%	50% (w/v)

2.1.8.3 Buffers for molecular methods

Transcription buffer (1x)	Tris-HCl, pH 8.0 Magnesium chloride 1,4-Dithioerythritol (DTE) Spermidine	40 mM 22 mM 5 mM 1 mM
RNA extraction buffer (1x)	Tris-HCl, pH 7.8 Sodium Chloride (NaCl) 1,4-Dithioerythritol (DTE) EDTA Sodium-dodecyl-sulfate (SDS)	10 mM 100 mM 1 mM 1 mM 1% (w/v)
Tris solution, 1 M, pH 7.8	Tris Hydrochloric acid 32%	1 M Adjust pH ~7.8
Tris solution, 1 M, pH 8.0	Tris Hydrochloric acid 32% (HCl)	1 M Adjust pH ~8.0

2.1.8.4 Buffers for studies of ribosomal functional states and ribosomal isolation

Binding buffer ($H_{20}M_{4.5}N_{150}SH_4Spd_2Spm_{0.05}$)	Hepes-KOH, pH 7.5 Magnesium acetate [$Mg(Ac)_2$] Ammonium acetate (NH_4Ac) β-mercaptoethanol Spermidine Spermine	20 mM 4.5 mM 150 mM 4 mM 2 mM 0.05 mM
Tico buffer ($H_{20}M_6N_{30}SH_4$)	Hepes-KOH, pH 7.5 Magnesium acetate [$Mg(Ac)_2$] Ammonium acetate (NH_4Ac) β-mercaptoethanol	20 mM 6 mM 30 mM 4 mM
VD^+ buffer	Tris-HCl, pH 7.8 Magnesium acetate [$Mg(Ac)_2$] Ammonium chloride (NH_4Cl) β-mercaptoethanol	10 mM 10 mM 60 mM 6 mM
Mix I ($H_{100}M_{21}N_{870}SH_{20}Spd_{12}Spm_{0.3}$)	Hepes-KOH, pH 7.5 Magnesium acetate [$Mg(Ac)_2$] Amonium acetate (NH_4Ac) β-mercaptoethanol Spermidine Spermine	100 mM 21 mM 870 mM 20 mM 12 mM 0.3 mM
Mix II / Charging Mix ($H_{80}M_{15}N_{840}SH_{16}Spd_{12}Spm_{0.3}$)	Hepes-KOH, pH 7.5 Magensium acetate [$Mg(Ac)_2$] Ammonum acetate (NH_4Ac) β-mercaptoethanol Spermidine Spermine	80 mM 15 mM 840 mM 16 mM 12 mM 0.3 mM
Mix E (energy mix)	dATP dGTP Acetyl phosphate Potassium hydroxide	45 mM 22.5 mM 75 mM 360 mM

2.1.8.5 Buffers for Northern-blot hybridization

10x MOPS	MOPS Sodium acetate (NaAc) EDTA	0.4 M 0.2 M 10 mM
20x SSC	Sodium chloride (NaCl) Sodium citrate	3 M 0.3 M
50x Denhardt's solution	Ficoll 400 Polyvinylpyrrolidone BSA fraction V	1 g 1 g 1 g fill up to 50 ml
Pre-hybridization buffer	SSC Formamide Denhardt's solution SDS Salomon sperm DNA	5x 50% 5x 1% 100 µg/ml
Hybridization buffer	SSC Formamide Denhardt's solution SDS Dextransulfate (Na-salt, MW 500,000)	5x 50% 5x 1% 5%

2.1.9 Software

Sequence analysis and RNA structure prediction:

- BioEdit (Ibis Therapeutics, USA)
  http://www.mbio.ncsu.edu/BioEdit/bioedit.html
- Chromas lite version 2.01 (Technelysium Pty Ltd, Austria)
  http://www.technelysium.com.au/chromas.html
- GeneRunner
- LaserGene packet version 4.03 (DNASTAR, USA)
- RNAfold
  http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

Gel documentation:

- ImageQuant packet version 5.2 (Molecular Dynamics, Amresham Biosciences)

Molecular structure visualization:

- PDB Viewer version 3.7 (GalaxoSmithKline and Swiss Institute of Bioinformatics)
  http://expasy.org/spdbv
- PyMol version 0.99 (DeLano Scientific LLC, USA)

2.2 Methods

2.2.1 Isolation of plasmid DNA in the range of 20 µg to 500 µg (Qiagen)

Isolation of plasmid DNA was performed with the help of Qiagen protocols and with usage of Qiagen materials (see section 2.1.1). Three different scale of DNA isolation were distinguished: (1) small scale up to 20 µg of DNA (*QIAprep*® *Spin Miniprep Kit*), (2) midi scale up to 100 µg of DNA (*Plasmid Midi Kit*) and (3) maxi scale up to 500 µg of DNA isolation (*Plasmid Maxi Kit*).

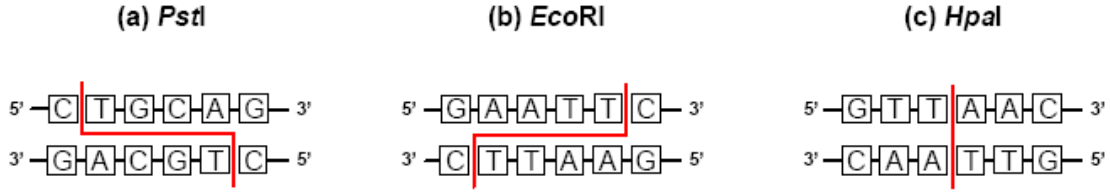
All methods of plasmid DNA isolation base on remarkable selectivity of patented QIAGEN Resin, allows the isolation of ultrapure supercoiled plasmid DNA with high yields. In the first stage the cell are centrifuge to the pellet and then disrupted by lysis buffer containing 200 mM NaOH and 1% SDS (w/v). The hydrolysis of the cell is partial and therefore chromosomal DNA is still bound to the cell membrane. This “trick” is essential for separation between chromosomal and plasmid DNA. After centrifugation the chromosomal DNA is in the pellet as well as the rest of cell components, e.g. cell membrane and cell wall. The supernatant contains small molecules and plasmid. To facilitate the purity of plasmid DNA, the supernatant is applied on the column containing matrix which selectively binds DNA. The eluted DNA is then precipitated with 2-propanol and finally dissolved in EB buffer (10 mM Tris-Cl, pH 8.5).

Concentration of plasmid DNA was quantified by spectroscopic methods. The absorbance of measured sample was detected at A_{260} and A_{280}. The concentration of DNA was based on the equation that 1 A_{260} is equal to 50 µg of double-stranded DNA in 1 ml (1 cm cuvette). Additionally, the purity of samples (contamination with proteins) was verified by absorbance at A_{280}.

2.2.2 Restriction hydrolysis of DNA

Restriction enzymes recognize specific sequence in double-stranded DNA. The sequence is in the range of 4-6 nucleotides and within it DNA can be cut giving as a product three different kinds of ends: (a) cohesive ends with 3’ tail (e.g.

*Pst*I), (b) cohesive ends with 5’ tail (e.g. *EcoR*I) and (c) blunt-ends without any single-stranded tail (e.g. *Hpa*I).



This feature of restriction enzymes has an enormous application in techniques of DNA recombination. DNA fragments coding entire genes can be easily cut out from a genome and inserted into vectors or even into other genomes.

Single reaction with restriction enzyme(s) contains: (1) 1-10 µg of DNA in the form of vector or PCR products, (2) commercial buffer and (3) restriction enzyme(s) in concentration of 10 units per 1 µg of DNA. The reaction is driven in 100 µl of total value for 5 hours at 37°C. In some exceptions the temperature is increased up to 50°C. After incubation, hydrolyzed DNA is analyzed in the agarose gel (see section 2.2.7).

2.2.3 Kinase reaction – labeling DNA or RNA with γ-[^{32}P]-ATP

The kinase reaction is driven by T4 polynucleotide kinase and causes moving of phosphor in the position of γ in ATP to the free 5’-OH end in DNA or RNA**.** The γ-phosphor in ATP is radioactive, so in consequence after reaction, DNA is labeled.

20 pmol of synthetic DNA or 800 pmol of deacylated RNA was taken for kinase reaction. DNA or RNA was incubated with 20-50 µCi of γ-[^{32}P]-ATP in present of 20 units of T4 polynucleotide kinase. The ionic conditions were established with the help of commercial buffer supplied with the enzyme: 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 6 mM β-mercaptoethanol in the final value of 20 µl. All components were incubated at 37°C for 20 min and finally the reaction was stop by adding of 10 µl of 2x RNA Loading Buffer (NEB). The solution was applied on the DNA/RNA sequencing gel. DNA or RNA extracted from the gel

(see section 2.2.8) was purified by phenol extraction and ethanol precipitation (see section 2.2.6).

Pure and labeled RNA had to be immediately diluted with the same kind of cold (non-radioactive) RNA to yield specific radioactivity in the range from 3 000 to 10 000 dpm/pmol. To keep the specific activity relatively high is important due to very fast decay of the 32-phosphor (half-life of this isotope is equal to 14 days). Additionally, to prevent such fast decay the radioactive samples should be kept at -80°C where the degradation of phosphor is slower.

2.2.4 Ligase reaction – insertion of DNA fragments into vectors

Fragments of DNA can be “glue” together with the help of ligase. This reaction allows incorporate DNA to any vector or chromosomal DNA. In the range of this thesis was performed the ligation of DNA fragments with DNA vector (plasmid).

The ligase reactions were done according to manufacture’s guide (Fermentas). The ratio between plasmid and insert has to be 1:6. To the reaction was usually taken 20 – 40 µg of DNA and adequate amount of insert in the molar ratio of 1:6. Two equations which allow convert weight mass of DNA to its molar mass and *vice versa* are listed below:

to convert pmol to µg:

$$pmol \cdot N \cdot \frac{660\,pg}{pmol} \cdot \frac{1mg}{10^6\,pg} = mg$$

to convert µg to pmol:

$$mg \cdot \frac{10^6\,pg}{1mg} \cdot \frac{pmol}{660\,pg} \cdot \frac{1}{N} = pmol$$

where N is the number of nucleotide pairs and 660 pg/pmol is the average MW of a nucleotide pair.

In the final value of 20 µl, DNA fragments and app. 3 units of T4 DNA ligase were incubated at 16°C overnight. The ionic conditions were established by commercial buffer and their final concentrations were: 40 mM Tris-Cl, 10 mM $MgCl_2$, 10 mM DDT, 0.5 mM ATP (pH 7.8 at 20°C).

After ligation, the ions were removed by micro-dialysis. On the surface of water in the Petri-dish, 1 cm-size filter with 0.05 µm pores was placed. The reaction solution was put on the filter as a drop and incubated there for 15 min.

Then, the drop was taken back and placed in the fresh tube. The sample with the ligated DNA was ready for the transformation (see section 2.2.9).

2.2.5 Ligase reaction - labeling of RNA by pCp

In order to label mRNA at 3' end the ligase reaction could be used. The ligation mix contained 120 pmol mRNA, 2 µl cytidine 3'5'-bis[α-^{32}P]-phosphate (pCp, 10 mCi/ml), 1x buffer for ligase (supplied with the enzyme), 0.01% BSA (supplied with the enzyme) and 1 µl T4 RNA ligase in the total volume of 20 µl. The reaction was performed at 5°C for 16 h. Then the mRNA and free pCp were separated in the 10% PAGE with 7M urea.

2.2.6 Extraction and precipitation of DNA or RNA

To the reaction mixture with DNA/RNA was added an equal volume of phenol and the sample was mixed for 2 min by vortex (when the sample is vortexed it should have a milk-like consistence). Both phases, organic (phenol) and water containing DNA/RNA, were separated by centrifugation at 14 000 rpm for 1 min. Aqua phase on the top was gently moved to the fresh tube and the equal amount of chloroform was added. The sample was mixed by vortex for 2 min and both phases were separated. The water phased again was transferred to the fresh tube. In order to precipitate the DNA/RNA, 0.1 volume of 3 M sodium acetate (pH 5.5 for DNA and pH 4.5 for RNA) and 2.5 volume of 100% ethanol were added to the aqua phase. The sample was incubated at –20 °C for one hour (this step could be omitted – it is not critical for purification of DNA/RNA). DNA/RNA was recovered by centrifugation at 14 000 rpm for 30 min at 4°C. Then, 90% of ethanol supernatant was removed and 100 – 300 µl of 70% ethanol was added in order to wash the pellet and remove salt. The sample was centrifuged again in the same conditions but only for 10 min. Then, the whole supernatant was gently removed and the DNA pellet was dried in Vacuum SpeedVac® (about 5-10 min). The dried DNA/RNA was dissolved EB buffer and stored at 4 °C for the short time storage or at -20°C for longer time.

Safety information: Phenol is a hazardous organic solvent. Always use suitable laboratory gloves when handling phenol containing solutions. The phenol wastes should be collected in a special container and removed once per month.

2.2.7 Agarose electrophoresis of DNA or RNA

Agarose electrophoresis of DNA/RNA belongs to the most basic method for DNA/RNA visualizations. The pool of DNA/RNA molecules with different sizes can migrate in the gel with other velocity, so finally molecules are separated according to their length – shorter molecules migrate faster through the gel pores. The molecules were visualized by ethidium bromide, the substance which intercalates between bases in nucleotides. Ethidium bromide gives a signal under the UV-radiation.

This method was used for analysis of plasmid DNA, hydrolyzed DNA and PCR products as well as for the quantitative restriction analysis. According to RNA, agarose electrophoresis was performed for qualitative evaluation of rRNA from 70S ribosomes, 50S and 30S subunits. Agarose electrophoresis was the first step in the northern-blot technique.

For standard agarose electrophoresis of DNA, 0.7-2% concentration of agarose was used. Agarose was added to 1x TBE buffer and dissolved *via* boiling at the temperature of 95°C for 2 min in a microwave. The solution was cooled down to the temperature of 50°C and ethidium bromide was added in the ratio of 2 µl per 50 ml of gel. Then the liquid gel was spilled in the form (electrophoresis chamber). When the gel was solid it could be used for electrophoresis. The samples (about 1 µg of DNA per 4 mm pocket) were applied on the gel, but previously they were mixed with 6x DNA loading buffer (Fermentas). The electrophoresis was driven for about 45 min at 60V (20 mA) in 1x TBE buffer.

If the samples contained RNA, the agarose gel was modified. Due to high ability of RNA to form secondary structure the gel had to contain denaturing conditions, which were done by formaldehyde. Formaldehyde was added to the gel solution just prior to polymerization to the final concentration of 2%. The same concentration of formaldehyde contained running buffer – 1x TBE. The samples before applied on the gel were mixed with 2x RNA loading buffer (Fermentas) containing formaldehyde.

The samples of DNA/RNA in the gel were visualized by the UV-radiation. The length of DNA/RNA was estimated according to the migration of specific markers with known sizes of DNA/RNA.

2.2.8 Elution of DNA or RNA from agarose and polyacrylamide gels

DNA was eluted from agarose gel with the help of Qiagen *QIAquick Gel Extarction Kit* and according to the manufacturer guide. The selected piece of agarose gel with DNA was cut out and incubated in 2 ml tube in buffer QG (100 µl per 100 mg of the gel) at 50°C until the agarose became liquid. This solution was applied on the column and the all was spun for half a minute in the table centrifuge at maximum speed. The column was washed with 750 µl of buffer PE *via* centrifugation in the same conditions like upper. Before the elution of DNA, to enhance the yield and to remove ethanol, the column was dried *via* additional short centrifugation. For DNA elution, 30 µl of buffer EB was used with prior short incubation with a column. DNA was eluted to the fresh tube and the concentration was quantified by spectroscopic method.

The elution of DNA/RNA from polyacrylamide gels was based on the physical disruption of gel. The piece of gel contained DNA/RNA was fragmented and incubated at 4°C in water with overnight vigorous shaking. Then, the sample was centrifuged at 14 000 rpm for one hour in cold room. The DNA/RNA-contained supernatant was phenolyzed again and nucleic acids were precipitated with the help of salt and ethanol.

2.2.9 Transformation of cells with high-voltage method (electroporation)

The electroporation is one of the most efficient methods for transformation of bacterial cells with plasmids. The cells were exposed to short high voltage (~1.5 kV). The process how plasmids get in the cells by high voltage is still not known. It is likely, that electricity causes the holes in the cell membrane which allow the transfer of plasmids.

The conditions for cell transformation were: current - 1.5 kV (for 0.5 mm cuvette), electrical capacity - 40 µF, resistance - 412 Ω, mode - one pulse. To the tube with competent cells (~ 50 µl) were added plasmids (~20 ng) and the solution was transferred into transformation cuvette placed in the ice. The cuvette was put into transformation machine with prior drying with paper towel and the current was

induced. Then immediately 1 ml of cold LB medium was added to the cells. The solution of cells was mixed and moved to the fresh 2 ml tube. The cells were incubated one hour at 37°C without any antibiotic. Finally the cells were plated out on the solid LB medium with an antibiotic as a selection marker. The cells were incubated overnight at 37°C.

2.2.10 Selection of plasmids recombinant by direct amplification DNA from cells colonies (Colony PCR)

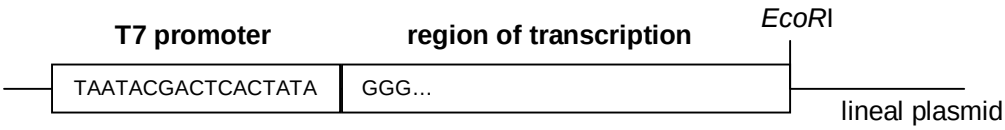
This method was used for selection of high amount of recombinants directly from cells colonies. It based on the standard PCR reaction with primers flanking the region of plasmid where the recombination was taken place. Each colony was picked up from the plate and the part of it was stricken on the fresh LB plate with selection marker and the rest was put directly to the PCR tubes. The pipeting tip was used as a tool for colony transfer. The PCR tube was contained PCR reaction mix: (1) 5 µl of *ReadyMix™ Taq PCR Reaction Mix with* $MgCl_2$, (2) 0.3 µl of forward primer (50µM) and 0.3 µl of reverse primer (50 µM), (3) 4.4 µl of water. The PCR reaction was performed according to the program:

1) pre-denaturation: 5 min at 94°C
2) denaturation: 20 sec at 94°C
3) primer hybridization: 30 sec at 52°C
4) polymerase reaction: 1 min per 1000 bp at 72°C
5) 30 times cycle repetition to step 2
6) final filling of DNA ends: 7-10 min at 72°C
7) storage temp. at 4°C.

The samples after reaction were analysed in 0.7-2% agarose gel.

2.2.11 In vitro *transcription*

The transcription of all mRNAs was performed under T7 polymerase. mRNA was transcribed under T7 promoter (TAATACGACTCACTATA) until *EcoR*I restriction position. Prior to the transcription the plasmid DNA was hydrolyzed to the line form by *EcoR*I, so synthesis of mRNA was stopped when *EcoR*I position was achieved.



Two transcription scales was performed. The analytical preparation was used to verify the potency of plasmid for mRNA synthesis. It was done in 20 µl and all of synthesized mRNA applied on the 0.7-2% agarose gel with formaldehyde (see section 2.2.7). The preparation scale (100-500 µl) was performed for mRNA production for other experiments e.g. translation.

The components needed for transcription were brought to the room temperature prior to mixing in order to avoid nucleotides precipitation caused by spermidine and $MgCl_2$ (Milligan and Uhlenbeck, 1989). The final concentration of all transcription components in 100 µl is listed below:

Amount from the stock	Molar concentration (f.c.)	Component
63 µl		water
10 µl	$T_{40}M_{22}Spd_1DTE_5$	10x transcription buffer
3.75 µl	3.75 mM	ATP-Tris (100 mM)
3.75 µl	3.75 mM	CTP-Tris (100 mM)
3.75 µl	3.75 mM	GTP-Tris (100 mM)
3.75 µl	3.75 mM	UTP-Tris (100 mM)
3.6 µl	100 µg/ml	BSA (RNase free, 2.9 mg/ml)
2.6 µl	1 U/µl	RNasin (40 U/µl)
1.3 µl	5 U/ml	PPase (400 U/µl)
4 µl		linear DNA plasmid (1 µg/µl)
0.5 µl	ca. 25 µg/ml	T7 polymerase

The components of the NTP mix were prepared as 100 mM stock solutions adjusted to pH 5.5-6.0 with 1 M KOH (in order to minimize the spontaneous hydrolysis) and stored at -80 °C before use. The reaction was prepared in the extremely RNase-free condition in order to prevent digestion of nascent mRNA. After mixing of all transcription components the reaction was driven for four hours

at 37°C and it was stop by addition of EDTA to the final concentration of 5 mM or by direct phenol extraction (see section 2.2.6).

2.2.12 Northern-blot hybridization

The Northern-blot hybridization was performed for quantification of GFP-encoding mRNAs (mRNA-GFP) in the samples of RTS reaction. The method based on the fact that ssDNA and mRNA having complementary sequence can hybridize. As a primer the 21-mer of ssDNA was used (CATCTTCTTTAAAATCAATAC) being complementary to the middle sequence of mRNA-GFP. The ssDNA was labeled by γ-^{32}P.

10 µl samples of RTS reaction were withdrawn and phenolyzed by 70% phenol. 5 µl of aqua phase (upper phase) was transferred to the fresh tube and added 5 µl of *2x Loading Dye Solution for RNA electrophoresis*. The samples were applied on 2% agarose gel with 2% formaldehyde and ethidium bromide (see section 2.2.7). The electrophoresis was performed for 1 h at 100V in 1x MOPS buffer. Prior to the transfer of mRNA to the membrane the control UV-radiation photo was made in order to exclude degradation of mRNA during electrophoresis. The gel was washed 3 times for 5 min in water to remove the contamination of formaldehyde.

The mRNAs from agarose gel was transferred (“blotted”) onto the Hybond *N*-plus membrane using capillarity forces made by buffer (10x SSC buffer) going to stack of paper towels. The transfer was performed for 24 h at room temperature. After the transfer the membrane was briefly washed in 2x SSC buffer and placed on the UV lamp for 5 min (the site without RNA being in front of the lamp) in order to crosslink mRNAs. Then the membrane was baked at 80°C for 1 h.

The next step was to hybridize primer ssDNA with corresponding mRNAs. In the first part the membrane was incubated in the prehybridization buffer containing salmon sperm which covered entire membrane and eliminated nonspecific hybydization of ssDNA. The prehybridization was performed at 42°C for 4 h. Then the prehybrydization buffer was removed and the hybridization buffer containing radioactive labeled ssDNA was added and incubated with membrane overnight at 42°C. On the day the membrane was washed in the following order: (1) 15 min with 2x SCC at room temperature, (2) 45 min with 2x SSC containing

0.1% SDS at 60°C, (3) 15 min with 2x SSC containing 0.1% SDS at 60°C. Then the membrane was wrapped with saran and placed into the PhosphorImager cassette for at least 2 h. The scanned PhosphorImager screen was then quantified with the ImageQuant software.

2.2.13 RNA integrity test in 1D-electrophoresis in tube gel

This method was using for checking the quality of ribosomal RNAs.

In the first part a plastic tube (0.5 cm x 12 cm) was filled (~10 cm) with ~2 ml of gel solution with SDS (3.1% Rotiphorese® Gel 40, 0.2% SDS, 1x TBE). For polymerization just prior to the tube filling 40 µl of 10% APS and 1 µl TEMED were added per 2 ml of the gel solution. The upper surface of gel solution was covered with few drops of water to ensure the formation of a flat surface. The gel polymerized for about 2-3 h. The tubes were placed in the electrophoresis chamber and two compartments (upper and bottom) were filled with running buffer (1x TBE, 0.2% SDS). After that the pre-electrophoresis with empty tube gels was performed in the conditions of 1 mA per tube for half an hour.

The amount of 0.5-1 A_{260} of ribosome (RNA) solution mixed with *2x Loading Dye Solution for RNA electrophoresis* in the ratio of 1:1 to the final volume of ~30 µl. The loading solution incubated at 70°C for 5 min and then immediately placed on the ice. The samples were applied on the top of the gel tube and the electrophoresis performed at 1.5-2 mA per tube for about 3 h at 4°C, until the first dye reached the bottom of the tube. Then the gels from tubes were pumping out with help of syringe with water and placed to the photometer cuvette. The gels were scanned in the photometer at 260 nm. This method helped to establish not only the part of damaged RNA but also the degree of cross-contamination of subunits after their isolation.

2.2.14 Preparation of competent cells

The technique for obtaining the competent cells especially for electroporation is based on the repeatedly washing of cell with the distilled water. In the first stage the cell were plated out on the LB plate, incubated overnight at 37°C and finally one colony was transferred to the fresh 10 ml LB medium. The cells were incubated again overnight and finally, on the next day, diluted to the

concentration of 0.05 A_{595} with fresh 2 l LB medium containing the half of sodium chloride than present in standard LB medium. The bacteria were grown until concentration at A_{595} was equal to 0.6-0.7. Then the cells were centrifuged at 5,000 RPM for 15 min at 4°C. The supernatant was discarded and cells pellet were mixed with fresh cold (4°C) water. The procedure repeated three times. After last centrifugation the cells were mixed with 10 ml water contained 10% glycerol and centrifuged at upper condition. Finally the supernatant discarded and cells were resuspended in 6 ml of 10% glycerol. Then, the ready competent cells were aliquitized for 40 µl samples, immediately froze in the liquid nitrogen and keep at -80°C.

2.2.15 Breaking the cells - sonication and French-press

The sonication is a method based on the disruption of cell's walls caused by ultrasounds. The homogenous solution of bacteria keeping on ice was exposed to the ultrasounds 3-5 times for 1 min each round. After sonication the broken cells were centrufugated at 12,000 RPM for half an hour. The supernatant placed to the fresh tube and used for other experiments or eventually froze and kept at -80°C.

The second technique - French-press (microfluidizer) based on the cell's walls disruption cased by high pressure. Cells go through the capillary tube under high pressure (17,000 psi). I have used the the microfuidizer model M-110L which was suitable apparatus for producing high yields in cell disruption with a minimal processing and easy recovery. In this apparatus walls were disrupted by optimized-shear forces that do not simultaneously destroy cell content, such as ribosomes.

For microfluidizer the cells were first homogenously resuspended in Tico buffer (100 g of frozen cells per 700 ml). Then the solution was placed in the feed reservoir and the pump was switched on. The disrupted cells were collected in the continuous flow outlet. After that the solution was centrifuged at 12,000 RPM for half an hour at 4°C. The supernatant was used for other experiments.

2.2.16 Protein in vitro *expression systems*

Rapid Translation System (RTS 100 *E. coli* High Yield Kit; Roche)

The preparation followed the protocol of the manufacturer except that a reaction volume of 10 µl was used instead of the suggested 50 µl. Standard incubation temperature was 30°C. Samples were introduced into ProteoMaster instrument and incubated according to the assay requirements. 1.5 µl was applied to either the SDS gel or to the native gel.

In case of incorporation of hot L-[^{35}S]-Methionine the protocol was modified also according to Roche. The final volume was 25 µl with the concentration of plasmid about 0.2 µg.

Rapid Translation System (RTS 500 *E. coli* High Yield Kit; Roche)

The preparation and incubation followed the RTS 500 Kit protocol. The reaction solution was loaded into the 1 ml reaction compartment of the supplied reaction device, and the feeding solution into the feeding compartment with care avoiding air bubbles. The filled reaction device was introduced into ProteoMaster instrument and incubated at 30°C if not otherwise indicated. Incubation time was up to 12 h for RTS 100 reactions, and up to 40 h for RTS 500 reactions.

For the assays with mutant T7 polymerase we had to constitute our own S30 system derived from *E. coli* BL21. The concentrations were adapted to those published by (Kim and Swartz, 2000) and are summarized in the followed table:

Component	Final concentrations according to (Kim and Swartz, 2000)	Final concentrations in our reaction mix
HEPES-KOH (pH 8.2)	57.2 mM	60 mM
Ammonium acetate	80 mM	80 mM
Potassium glutamate	200 mM	230 mM
Sodium oxalate	2.7 mM	3 mM
DTT	1.76 mM	2 mM
Cycle-AMP	0.67 mM	0.7 mM
Folinic acid	34 µg/ml	35 µg/ml
tRNAs	340 µg/ml	350 µg/ml
NADH	0.33 mM	0.35 mM
Coenzyme A	0.27 mM	0.3 mM
ATP	1.2 mM	1.5 mM
CTP	0.86 mM	1 mM
GTP	0.86 mM	1 mM
UTP	0.86 mM	1 mM
PEG-8000	2% (w/v)	2% (w/v)
Methionine	2 mM	2 mM
19 Amino acids	0.5 mM	2 mM

PEP	33 mM	35 mM
Magnesium acetate	15 mM	12 mM
T7 RNA polymerase	30 µg/ml	100 µg/ml
E. coli S30 cell lysate		4-6 A_{260}
Plasmid DNA		4 µg/60 µl
Rifampicin		10 µg/ml
^{3}H-Leu	1.2 µM	———
^{35}S-Met	———	1.5 µM

Second addition of a mixture of 20 amino acids

The amino-acid mixture was prepared as follows: lyophilized 19 amino acids supplied with the Roche RTS 500 kit were reconstituted by the kit-reconstitution buffer to a volume of 1.5 ml and mixed with the reconstituted methionine solution of 0.9 ml. After 7 h of incubation 2.4 ml was removed from the feeding solution chamber and substituted with this freshly prepared mixture of 20 amino acids.

In case of the RTS 100 we used a reaction volume of 50 µl and added 6 µl amino acid mixture in reconstitution buffer after 8 and 10 h (30 and 20°C incubation temperature, respectively).

Standard poly(U)-dependent poly(Phe) synthesis

Poly(U)-dependent poly(Phe) synthesis is a simple translation reaction where on the template of poly(U)-mRNA the poly(Phe) chain is synthesized. The assay had the same final ionic conditions ($H_{20}M_{4.5}N_{150}SH_4Spd_2Sp_{0.05}$) as described also for complex formation and was performed in the reaction volume of 15 µl. Each incubation mixture contained tight-coupled ribosomes (5 pmol), poly(U)-mRNA (25-50 µg), [^{14}C]-phenylalanine (7 nmol at 10 dpm/pmol), $tRNA^{Phe}$ (40 pmol), ATP (3 mM), GTP (1.5 mM), acetyl-phosphate (5 mM), S-100 fraction (1-3 µl per reaction), and eventually translation inhibitors. The reaction mixture was incubated at 37°C for specified periods of time (up to 60 min), and stopped by hot TCA precipitation as described by (Bommer et al., 1996). Finally, phenylalanine incorporation was expressed as pmols of Phe incorporated per pmol of 70S ribosomes.

Misincorporation assay based on standard poly(Phe) synthesis

Poly(U)-dependent poly(Phe) synthesis was performed as described above expect in addition 100 pmol extra amino acid (among of leucine, lysine, glutamic acid and valine) labeled by tritium (3000 dpm/pmol) and 200 pmol $tRNA^{bulk}$ were included. After incubation and hot TCA precipitation, the radioactivity results from [^{14}C] -phenylalanine and [^{3}H]-X (where X is any amino acid from above list) isotopes could be accurately separated by scintillation counting. Misincorporation is expressed in terms of incorrect amino acid (e.g. leucine) incorporated ber 1000 molecules of phenylalanine polymerized.

2.2.17 Protein in vivo *expression system*

For *in vivo* protein synthesis the BL21(DE3) cell strain was chosen. The plasmid contained T7 promoter was introduced into this kind of strain by electroporation methods. After that 2 ml LB medium with selective antibiotic was inoculated with one colony of plasmid-rich cells. The cells incubated overnight at 37°C and on the next diluted with fresh LB medium contained selective factor to the final concentration of 0.05 A_{595}. The cells incubated at 37°C and measured the A_{595} concentration. When the cells density reached $A_{595} \approx 0.5$ the IPTG was added to the final concentration of 1 mM. Then the cells were incubated for 4 h in the same conditions. Finally the cells were harvested by centrifugation at 5,000 RPM for half an hour at 4°C. The supernatant discarded and cells dissolved in Tico buffer or similar one.

2.2.18 In vitro *binding assays*

All experiments utilized ribosomes (re-associated 70S ribosomes or 30S subunits) prepared according to (Blaha et al., 2000). The MVF-mRNA [G_3ACTCAGAGCTACGGAACG$(GA_4)_5$GAAA**ATG**GTATTCAAAA$(GA_4)_3$TATTCGG] and a corresponding one with a Shine-Dalgarno sequence [G_3AAGAA**AAGGAG**GTCACAT **ATG**GTATTC$A_3(GA_4)_5$TGGACTCAGAGCTAC GGA$_3$TATTCGG] were prepared according to (Schäfer et al., 2002) and leaderless mRNA [AUGUUCUAAU] was purchased from Dharmacon (USA). [^{3}H]-fMet-$tRNA_f^{Met}$ was prepared according to (Rheinberger et al., 1988). mRNAs were labeled at the 3' end by ligation with cytidine 3',5'-bis [α-^{32}P] phosphate, separated

from free pCp's in 15% PAGE with 7M urea, then extracted from gel, precipitated with ethanol and finally resolved in water. 10mM water stocks of kasugamycin were prepared.

P_i complexes were prepared with 5 pmol of re-associated 70S ribosomes or 30S subunit in a volume of 7.5 µl incubated with 6-fold molar excess of [^{32}P]-mRNA, 1.5-fold molar excess of fMet-tRNA$_f^{Met}$ in binding buffer. Ksg was added at the various concentrations as indicated. The reaction was incubated for 15 min at 37 °C, then aliquots of 6 µl were withdrawn and washed through a 0.45 µm nitrocellulose filter with 2 ml binding buffer. Samples were washed repeatedly (3 times for leaderless mRNA and 6 times for the other mRNAs) before transfer to scintillation vials. To each filter, 5 ml of complete Filter-Count™ cocktail for radioactivity counting was added and the isotopes signal was counted in a liquid scintillation counter.

2.2.19 Quantification of GFP and luciferase expression

Quantification of the GFP amount in SDS-PAGE

As standard GFP reporter protein GFP cycle 3 was used. The GFP cycle3 (GFPcyc3) has three point mutations that allow a fast maturation within 3-4 hours, whereas wild-type GFP requires maturation overnight at 4°C (Crameri et al., 1996). It was expressed from DNA plasmid for *in vitro* expression (pIVEX2.2) with T7 promoter and Strep-tag at the N-terminus. The polyacrylamide gels were prepared without SDS addition, the loading and running buffers contained SDS, which is enough for denaturing of the proteins (for buffer components and further details see BIO-RAD Application Guide, cat. No. 161-0993). From each 10 µl reaction (RTS 100), 1.5 µl was mixed with 3.5 µl water and 5 µl sample SDS-buffer, kept at 95°C for 5 minutes, cooled down on ice, and applied to the 15% PAGE, (Laemmli and Favre, 1973). The running conditions for electrophoresis were 75 V for 10 min, 150 V for 3-4 h, which enabled good separation of the GFP protein band from neighboring bands. The SDS gels were stained with Coomassie R-250 (Serva) and scanned by the Personal Densitometer SI (Molecular Dynamic, Sunnyvale, USA). The data were processed using ImageQuant image analysis software, version 5.2. GFP bands were quantified and the total amount of GFP in each lane was determined by comparison with reference bands of known amounts

of GFP. The input variations per lane were normalized by scanning of a well-defined band from the S30 pattern (**ê** in Figure 7), taking into account the respective pixel numbers. 0.3 to 0.7 mg/ml of total GFP were produced in 10 h in the RTS 100 reactions (volume 10 µl).

Fluorometric analysis of GFP

The active GFP present in each reaction sample was calculated by measuring the fluorescence of the GFP at 430–580 nm in the native PAGE (for details see also the above mentioned BIO-RAD information). After a maturation period of at least 8 h at 4°C under the conditions of the reaction mixture 1.5 µl from a 10 µl reaction was mixed together with the native loading buffer and directly applied to the 15% PAGE for analysis under the native conditions (Maniatis et al., 1982). A longer maturation period of up to 30 h did not improve the active fraction of GFP. Native PAGE, loading and running buffers were prepared without SDS. Conditions for electrophoresis were 75 V for 10 min, 150-180 V for 1.5 h. The fluorescence was measured directly in the gel with a FluorImager 595 dual-excitation, laser-induced fluorescence scanner (Amersham Biosciences). The images were analyzed using the ImageQuant software. The reference GFP, commercially available and synthesized *in vivo*, was arbitrarily assigned as 100%, and the relative activity of the newly translated GFP was calculated. On average, the activity of the GFP from the coupled in vitro system was (50±20) % of that of the reference GFP.

Luciferase expression and quantification

The expression of luciferase and GFP as a control was driven for 6 h at 20°C and 30°C in the RTS100 system according to Roche protocol for [^{35}S]-Met incorporation reaction in the volume of 25µl. After reaction, two samples of 2 µl and 1.5 µl from the same tube were taken for measurements of active and total luciferase expression, respectively. The former sample was mixed with $H_{20}M_6K_{150}$ buffer (20 mM HEPES, 6 mM magnesium acetate, 150 mM potassium acetate, pH 7.6 at 0°C) and then added to the solution containing the luciferase substrate. After 45 minutes at room temperature in the dark the lumino units were measured with the luminometer. The second sample (1.5 µl) was applied to a 15%

polyacrylamide protein SDS-gel. The electrophoresis was performed for 4 h at 150V and the gel stained with Coomassie R-250 for checking protein separation and then dried on 3MM Whatman® paper under vacuum at 60°C for 3 h. The dried gel was exposed in a PhosphorImager cassette at room temperature for 2 h, scanned in the PhosphorImager scanner, and the pixels of luciferase expressions were quantified using ImageQuant software. The results were normalized to 2 µl of reaction mixture.

2.2.20 Isolation of ribosomes

Large-scale *E. coli* cells preparation

Large-scale cultures from *E. coli* K12 strain were performed in a 100L fermentor at 37°C. In the first stage 2.5 L of LB medium with cells were prepared from one single colony (with intermediate 10 ml LB medium). 100 L of sterile LB medium with 0.5% glucose was inoculated with 2.5 L of *E. coli* cells. The bacterial growth was followed by determination of optical density at A_{595}. When the culture reached the early logarithmic phase (0.5 A_{595}), the cells were harvested using a continuous flow centrifuge (Padberg, model 41) operating at 2,000 RPM. The cells were shock frozen in liquid nitrogen and stored at -80°C. The yield of the fermentation was about 200-300 g of wet cells per 100 L.

Small-scale isolation of 70S ribosomes from *E. coli* using Alcoa-305

The isolation of ribosomes and the soluble enzyme fraction (S-100) was performed according to the procedure described in (Rheinberger et al., 1988) with slight modifications, using a near *in vivo* ionic condition ($H_{20}M_6N_{30}SH_4$, see: Tico buffer) that ensures to obtain ‘’tight-couple’’ 70S ribosomes (Hapke and Noll, 1976). In a typical preparation about ~300 grams of frozen *E. coli* cells were thawed while re-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 (4 °C). The cell pellet was weighted and a double amount of aluminium oxide (Alcoa-305) was added. This mixture was transferred to a Retsch-Mill, 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 centrifuged at 8,000 rpm for 10 minutes in a GSA rotor (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 (cell debris) 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 resuspended 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. A complete separation of tightly coupled 70S ribosomes from ribosomal subunits was achieved upon two consecutive zonal centrifugation steps of samples containing 6,000-9,000 A_{260} units of crude 70S preparation through a sucrose gradient (6-38% sucrose in Tico buffer). After the first zonal centrifugation (16 hours at 21,000 rpm in a Beckman Ti XV rotor) the fractions containing 70S were pooled and the ribosomes sedimented *via* a centrifugation step (24,000 rpm for 24 hours in a 45 Ti rotor). The sediment was resuspended in a small volume of Tico buffer and applied to a second zonal centrifugation under the same conditions. The resulting tight couple 70S ribosomes were essentially freed from 50S subunits (main contaminant after the first zonal centrifugation). The 70S pellet was resuspended in Tico buffer, aliquotized in 50 µl portions, shock-frozen in liquid nitrogen and stored at -80 °C. The yield of tight couple 70S ribosomes ranged between 10 and 20% of the total A_{260} units initially applied to the zonal.

Large-scale isolation of 30S and 50S ribosomal subunits

The 30S and 50S ribosomal subunits were isolated by zonal centrifugation using a linear sucrose gradient from 0 to 40% in 20 mM Hepes, 1 mM $MgCl_2$, 150 mM NH_4Ac, 4 mM ß-mercaptoethanol (dissociating conditions). For each zonal centrifugation, 3000-6000 A_{260} of 70S ribosomes (tight couple) were used. The centrifugation was performed using a Beckmann zonal rotor Ti15 at 22,000 rpm for 17 h at 4 °C. The gradient was pumped out the rotor using a solution containing 50% of table sugar in water. After the zonal centrifugation two pools were made with fractions containing the 30S and 50S subunits, respectively. The 30S and 50S

subunits were pelleted in 45 Ti rotors (Beckmann) at 35,000 rpm, for 22 h at 4 °C. The pellets were resuspended in 3 ml of Tico buffer or re-associated buffer. In order to eliminate large aggregates, the resuspended material was once more centrifuged in a SS-34 rotor (Sorvall) at 7,000 rpm for 15 min at 4 °C. The concentrations of the 30S and 50S subunits were determined by absorbance at 260 nm and the suspension was divided in small aliquots, frozen in liquid nitrogen and stored at -80 °C. The typical yield starting from 5,000 A_{260} of 70S was 1,000 A_{260} and 1,200 A_{260} of pure 30S and 50S subunits, respectively.

Preparation of re-associated 70S ribosomes

The tight couple ribosomes are derived partially from polysomes and thus contain still some tRNAs and small fragments of mRNA. In order to get a very pure preparation of ribosomes free of these ligands, the ribosomal subunits purified by sucrose gradient were incubated in the presence of high Mg^{2+} concentrations to form 70S re-associated ribosomes (Blaha et al., 2002). Re-associated ribosomes are more efficient in both tRNA binding and in poly(U) dependent poly(Phe) synthesis, as compared to tightly coupled ribosomes. If the subunits contain intact rRNA, 6000 A_{260} of purified 30S and 50S ribosomal subunits at molar ratio of 1:1 of A_{260} were diluted in re-association buffer until 40-140 A_{260}/ml and incubated for 60 min at 40 °C in a water bath with gently agitation. Adaptation buffer was prepared when ribosomal subunits were dissolved in Tico buffer. The final ionic concentration was adjusted to that of the re-association conditions ($H_{20}M_{20}K_{30}SH_4$ pH 7.5). It is important to note that excess of 30S subunits minimise the amount of free 50S subunit, thus it improves the separation of the re-associated 70S ribosomes from the 50S subunits in the following gradient centrifugation (Blaha et al., 2000).

After the first incubation before the samples were applied to the gradient centrifugation, a second incubation was performed for 10 min at 4 °C. Then the particles were subjected to a gradient centrifugation (10-40 % sucrose) in re-association buffer and centrifuged for 17 h at 18000 rpm, 4 °C. in Beckman zonal rotor. The gradient was fractionated and the fractions corresponding to the 70S peak were pooled and centrifuged at 24,000 rpm for 24 h, 4 °C in a 45 Ti rotor in order to pellet the re-associated 70S ribosomes. The use of higher centrifugation

rates is not recommended, because it may lead to pressure-induced dissociation of the ribosomes. The ribosomes were resuspended in re-association buffer ($H_{20}M_{20}K_{30}SH_4$, pH 7.5 at 0°C) and incubated again for 20 min at 40 °C. This additional incubation improves the re-association process. The 70S re-associated ribosomes were clarified by low speed centrifugation and then dialyzed against Tico buffer ($H_{20}M_6N_{30}SH_4$, pH 7.5): 3 times with 100x volume, each time 45 min. The concentration of ribosomes was determined at 260 nm. Small aliquots were prepared, shock frozen in liquid nitrogen and stored at -80 °C.

Quality and functionality determination of the ribosomes preparation

The quality of the ribosome preparation can be tested using three assays: (1) A SW 40 centrifugation is performed (gradient 10-30% sucrose in binding buffer or re-association buffer, 18 h, 18,000 rpm, 4 °C) in order to check the homogeneity of the ribosomal particles (30S and 50S subunits) and the 70S ribosomes (tight couples or re-associated). (2) RNA gels are done in order to check the integrity of the ribosomal RNA. By means of this analysis degradation of the 16S and 23S rRNA can be detected. Both, the analytical sucrose gradient and the RNA gel analysis provide information about the structural integrity of the ribosomal particles. (3) The activity in the poly(U)-dependent poly(Phe) synthesis is the third criterion for estimating the activity of the ribosomes.

2.2.21 Analytical sucrose gradient centrifugation

Sucrose gradient centrifugation is a technique that allows the separation of complexes based on the sedimentation coefficient (S). This technique can be used to separate 70S, 50S and 30S ribosomal subunits.

A sucrose gradient [10-30% (w/v) in binding buffer] was prepared in Ultra-Clear tubes (14 x 95 mm Beckman). The reaction mix (0.5-1 A_{260}) was overlaid on the gradient and centrifugation was performed in SW 40 rotors (Beckmann). In the SW 40 up to 10 A_{260} of pure ribosomes or ribosomal subunits per tube can be loaded. The centrifugation was performed at 18,000 rpm for 18 h, 4 °C. After centrifugation the gradient was fractionated while monitoring the absorbance at 260 nm.

2.2.22 Watanabe assay: site specific binding of tRNA to ribosomes, translocation and puromycin reaction

The functional states of the elongating ribosome (Pi, PRE and POST states) appearing during the elongation cycle were studied using the methodology described by Watanabe (Watanabe, 1972) with some modifications. The Watanabe assay allows a controlled stepwise execution of the partial reactions of the ribosomal elongation cycle (Figure 6). In the first step a 70S-mRNA-tRNA complex is formed, in which the tRNA is located in the ribosomal P-site. If N-acetylated-tRNA (e.g., N-Acetyl-Phe-tRNA^{Phe}) or N-formylated-Met-tRNA is used in the first step, an initiation complex is simulated (Pi-complex, I for inititiation). In a second step the A site can be filled with the corresponding cognate tRNA enzymatically (with EF-Tu) or non-enzymatically (without EF-Tu) forming a pre-translocational complexes (PRE complex). In the third step, PRE-complexes containing tRNAs in P and A sites are translocated to the E and P sites respectively (POST-complexes) upon addition of elongation factor G (EF-G) and GTP. The efficiency of the translocation reaction and /or the binding state of the tRNAs is determined in a fourth step taking advantage of the antibiotic puromycin (analogue of the 3' aminoacylated end of a tRNA). This antibiotic reacts specifically with the P-site bound acyl-tRNA, if the ribosomal A site is free, forming an acyl-puromycin derivative (Allen and Zamecnik, 1962). The puromycin reaction defines the location of a charged tRNA on the ribosome, i.e., if the P-site binds a peptidyl-tRNA, the puromycin reaction will be positive, whereas if the aminoacyl or peptidyl bound tRNA is present at the A site, the puromycin will not react (Traut and Monro, 1964). In any case, after the addition of EF-G that does not affect the binding state, the puromycin reactivity of a P site bound aminoacyl- or peptidyl-tRNA will be positive, while the A site bound species should show a translocation factor-dependent puromycin reaction.

The final ionic condition used in this experimental scheme were $H_{20}M_{4.5}N_{150}SH_4Sd_2Sp_{0.05}$ pH 7.5, the same as the poly(Phe) synthesis. A typical experiment was conducted as follows:

First step: P site binding or Pi complex formation

Pi complexes were prepared with 3-5 pmol of re-associated ribosomes in a volume of 12.5 µl incubated with: 25 µg of homo-polymeric mRNA (e.g., Poly(U)) or 8-fold molar excess of a natural or hetero-polymeric mRNA over ribosomes and 1.5-2-fold excess of labeled N-Acetyl-Phe-tRNA^{Phe} or N-formyl-Met-tRNA$_f^{Met}$, respectively. The first step was incubated for 15 min at 37 °C in a volume of 12.5 µl.

For P-site blockage with deacylated tRNA, programmed ribosomes with specified messengers were primed with 1.5-2-fold of deacylated-tRNA (sometimes 5' labeled with γ-^{32}P) keeping constant the same size aliquots and the ionic binding conditions.

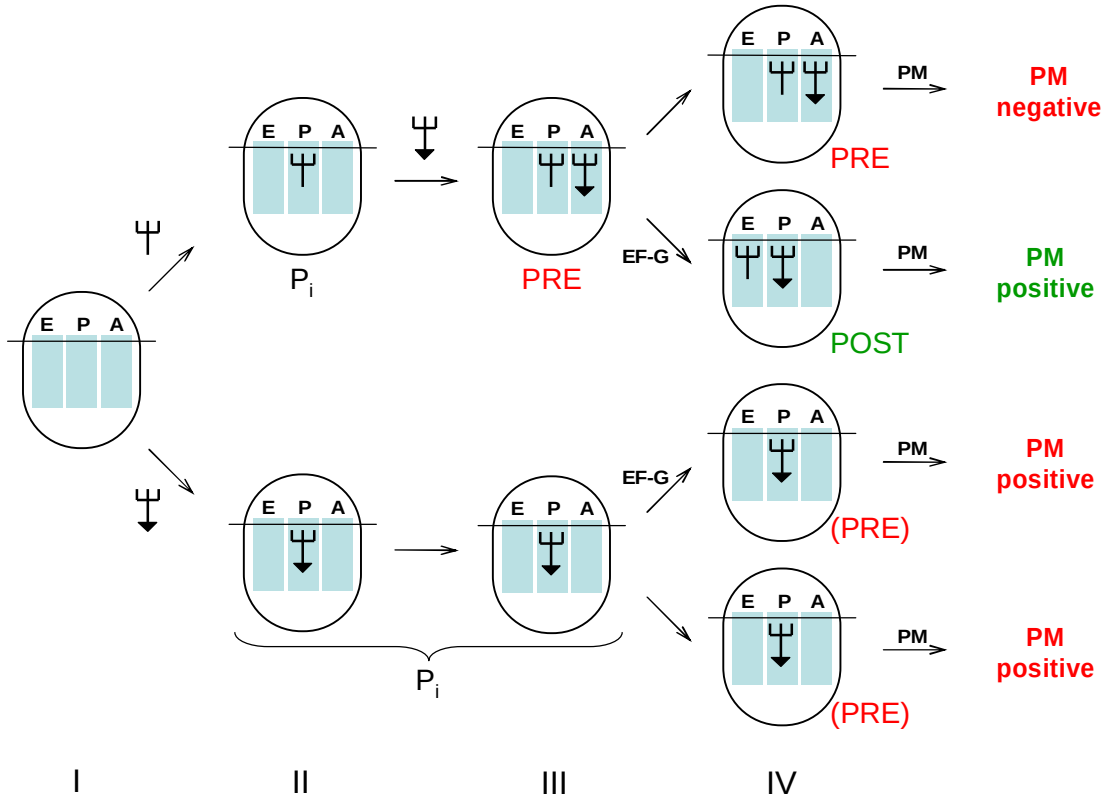


Figure 6: Watanabe assay. General picture of the modified non-enzymatic A-site tRNA binding. (I) 70S programmed ribosome with mRNA. (II) P site is occupied by deacylated tRNA (upper row) or with acetylated tRNA (lower row) - P_i complex formation. (III) Pre-translocational complex formation after addition of acetylated tRNA (upper row). (IV) EF-G dependent translocation (the proper translocation can be only observed after A-site occupation: see upper row). (V) Puromycin reaction.

For a complete set of samples analyzed in all four steps with duplicated binding determinations and puromycin reaction the indicated amounts and the volume was increased up to 10-fold.

Second step: A site binding and/or PRE complex formation

Keeping constant the ionic conditions (binding buffer), the volume of reaction was increased to 25 µl per single determination. Non-enzymatic A site occupation was carried out (PRE complex formation) adding 0.8 to 1.5 molar excess of N-acetyl-aminoacyl-tRNA ([^{3}H] or [^{14}C] labeled) to ribosomes whose P site was pre-occupied with deacylated tRNA in the first step. The mix was incubated for 30 min at 37°C. The binding was measured with double determination by nitrocellulose filtration as described before.

Third step: Translocation reaction

At this step, a GTP-mix (5 µl per aliquot) was added to P_i or PRE complexes maintaining constant the binding ionic conditions ($H_{20}M_{4.5}N_{150}SH_4Sd_2Spm_{0.05}$). Samples were split in 30 µl aliquots and 2.5 µl of EF-G was added to each (0.1-0.4-fold EF-G per ribosome). Control aliquots contained binding buffer instead of EF-G. After the addition of EF-G the aliquots were incubated for 10 min at 37°C.

Fourth step: puromycin reaction

Four aliquots from the binding assay containing EF-G and four without EF-G, were processed in the following way: 2.5 µl of puromycin stock solution in binding buffer (final concentration = 0.7 mM) were added to two aliquots from every group (± EF-G), while the other two received 2.5 µl of binding buffer. After these additions the samples were incubated at 37 °C for 5 min and the reaction was stopped adding 32.5 µl of 0.3 M sodium acetate, pH 5.5, saturated with $MgSO_4$. The amount acyl-puromycin formed was determined by extraction with 1 ml of ethyl acetate. After the addition of the organic solvent, the samples were strongly vortexed for 1 min, left 10 min on ice and centrifuged for 30 seconds at 15,000 x g in order to achieve complete phase separation. 800 µl of the organic phase was withdrawn and counted.

The radioactivity extracted in the controls (minus puromycin) was subtracted from that plus puromycin in order to calculate the amount of acyl-puromycin formed.

A successful puromycin reaction depends critically in the way in which the puromycin solution is prepared and handled. Two basic rules for the preparation of the puromycin stock solution with the maximal activity should be observed:

The pH of the solution must be neutral. Since the puromycin is obtained commercially as hydrocloride, the pH of the solution had to be neutralised adding 1 M KOH (1/100 of the reaction volume).

The puromycin stock solution must be maintained at room temperature (otherwise it precipitates lowering the effective concentration). Under these conditions the stock solution retained its maximum activity for about one hour. However, one can prepare a larger stock of puromycin solution that if aliquotized and shock-frozen in liquid nitrogen keeps its activity for months.

Binding assays without ribosomes were included in all the experiments as standard controls in order to determine the background of radioactivity adsorbed to the filters. This background was normally low (below 10% of the binding signal) and directly proportional to the concentration of the radioactive component in the assay.

Controls without mRNA plus ribosomes were also included when needed (e.g. the test a new heteropolymeric mRNAs).

2.2.23 Preparation of POST state ribosomes

The POST state ribosomes preparation based on the Watanabe reaction described in the section 2.2.22, modified by multiplication of reactions prepared in one batch. The ionic conditions for all reactions were as followed: 20 mM HEPES, pH 7.5, 4.5 mM $Mg(Ac)_2$, 150 mM NH_4Ac, 4 mM β-ME, 2 mM Spermidine, 0.05 mM Sperimine. In the first step P-site was filled (300 pmol of 70S, 2400 pmol mRNA, 600 pmol tRNA in the total volume of 750 μL; reaction time 15' at 37°C). The A-site was filled then by addition of 750 pmol of Ac-aa-tRNA and broadening the reaction volume to 1.5 ml. The reaction was incubated for 30' at 37°C. The final reaction is EF-G dependent translocation. GTP was added to the final concentration of 0.08 mM and EF-G to the concentration of 0.8 molecule per 70S

(the reaction volume should be equal to 2 ml). The translocation reaction was driven for 10' at 37°C.

The solution with prepared POST-state ribosomes was divided for two 1 ml parts and each of them was applied on the 2 ml 10% sucrose cushion (sucrose was solved in the binding buffer; composition see in the section 2.1.8.4). For centrifugation the TLA 100.3 rotor with corresponding tubes was used. The centrifugation conditions were as followed: 40,000 rpm, 18 h, 4°C.

After centrifugation supernatant was immediately discarded and the pellet was dissolve in 500-700 µL Binding buffer. The concentration of POST state ribosome was quantified by spectrophotometric method at A_{260} nm. The sample was divided into small portion containing not more then 30 pmol of POST ribosomes each, immediately frozen by liquid nitrogen and stored at -70°C.

2.2.24 Toeprint

The following mRNA was used: GGCAA**<u>AGGAGGU</u>**AUUAUUA***AUGUUCA AACGAUCAAUCUACGUAUAA***UAAAAGAAAAGAAAAGAAAAGAAAAGAAAAG GA*<u>CAUCACAGAUUAACG</u>*; this contains a Shine-Dalgarno sequence (bold underlined) and codes for MFKSIRYV (bold italic). The mRNA was annealed to a ^{32}P-5'-end-labeled primer (underlined italics) as described in (Hartz et al., 1988) and then used to program ribosomes for PRE and POST complexes. Briefly, 200 pmol reassociated 70S were incubated with 5 pmol mRNA:primer and 400 pmol each of $tRNA_f^{Met}$ and Ac-Phe-$tRNA^{Phe}$ in standard buffer. Aliquots of the reaction mixture with 5 pmol 70S were withdrawn before and after EF-G-dependent translocation reaction and used for toeprinting assays. The remaining posttranslocational mixture of 275 µl was centrifuged through a 1 ml 10% sucrose cushion in standard buffer (65,000 x g for 18 hr). The pellet was resuspended in 90 µl standard buffer and aliquoted into 15 µl portions. 7.5 pmol POST complexes in 15 µl were incubated for 30 min at 37°C with 5 times excess of LepA and 200 times excess GTP (0.1 mM) and used for the toeprinting assay. The end-labeled primer on the mRNA was extended by 100 units of MuMLV reverse transcriptase (Fermentas) in the presence of dNTPs, each 135 µM in standard buffer at 37°C for 15 min. The reaction was stopped by 20 µl of loading buffer (9 M urea, 90 mM TRIS [pH 8.3] at room temperature, 90 mM boric acid, 15 mM EDTA, 0.05%

xylene cyanol, 0.05% bromophenol blue) and heated at 95°C for 5 min. Toeprint reactions were analyzed on 8% urea-PAGE (8 M urea). The gels were quantified using a Molecular Dynamics Storm PhosphorImager.