For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.

Rapid Translation System RTS 500 *E. coli* HY Kit

In vitro protein synthesis system based on *E.coli* lysate

Cat. No. 3 246 817, for two 1 ml reactions
Cat. No. 3 246 949, for five 1 ml reactions

Store this kit at −15°C to −25°C

Instruction Manual

Version 1, June 2001

The Rapid Translation System website:
www.proteinexpression.com

Please refer to “Notice for the purchaser” on page 24.

Roche

1. Preface

1.1 Table of contents

1. 2 Kit contents

Bottle No./ Cap	Label	Contents and function	Cat. No. 3246 817 for two 1 ml reactions	Cat. No. 3246 949 for five 1 ml reactions
1 red cap	*E.coli* Lysate	• stabilized and lyophilized lysate from *E.coli*, • contains components for transcription and translation	2 bottles	5 bottles
2 green cap	Reaction Mix	• stabilized and lyophilized substrate mix to prepare 1,1 ml reaction solution	2 bottles	5 bottles
3 blue cap	Feeding Mix	• stabilized and lyophilized substrate mix to prepare 11 ml feeding solution	2 bottles	5 bottles
4 brown cap	Amino Acid Mix without Methionine	• stabilized and lyophilized amino acid mix, without methionine	2 bottles	5 bottles
5 yellow cap	Methionine	• stabilized and lyophilized methionine	1 bottle	1 bottle
6 white cap	Reconstitution Buffer	• 35 ml buffer solution for the reconstitution of bottles 1 to 5	1 bottle	2 bottles
7 colorless cap	Control Vector GFP	• 50 µg lyophilized plasmid GFP (Green Fluorescent Protein) expression vector with C-terminal His-tag	1 vial	1 vial
8	Reaction Device	• disposable two-chamber device for Continuous Exchange Cell Free (CECF) protein expression • device fits into Rapid Translation System RTS 500 Instrument	2 devices	5 devices

Safety information

Bottles 2, 3, 4 and 5 contain < 25% dithiothreitol, < 20% EDTA. All other bottles contain no hazardous substances in reportable quantities. The usual precautions taken when handling chemicals should be observed. Used reagent can be disposed off in waste water in accordance with local regulations. In case of eye contact flush eyes with water. In case of skin contact wash off with water. In case of ingestion seek medical advice.

Laboratory requirements

To avoid contamination with RNases we recommend using RNase-free materials and wearing gloves. Heat-treated glassware is not required, since solutions can be reconstituted in the bottles provided. An UV lamp (360 nm) is needed for the detection of GFP (control reaction).

Additional equipment required

To perform protein expression with this kit, the following equipment is required:

- Rapid Translation System RTS 500 Instrument, Cat. No. 3 064 859
- Pipettes 10-200 µl, 200-1000 µl, and 10 ml
- Pipette tips autoclaved at 121° C for 20 min

Additional reagents required

Besides the template vector for the protein of interest, no additional reagents are required.

For reconstitution of the control vector (Vial 7) use only deionized DNase- and RNase-free water.

2. Introduction

2.1 Product overview

System components

The Rapid Translation System RTS 500 consists of the RTS 500 Instrument and the RTS 500 *E. coli* HY Kit. The kit contains reagents and reaction devices for 2 (Cat. No. 3246 917) or 5 (Cat. No. 3246 949) coupled transcription/translation reactions and a control vector GFP.

Reaction principle

Transcription and translation take place simultaneously in the upper 1 ml reaction compartment of the reaction device. Substrates and energy components essential for a sustained reaction are continuously supplied via a semi-permeable membrane. At the same time, potentially inhibitory reaction by-products are diluted via diffusion through the same membrane into the 10 ml feeding compartment. Protein is expressed for up to 24 hours yielding up to 6 mg of protein.

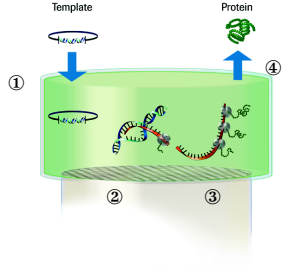

Fig 1: ***Schematic illustration of CECF protein expression.***
Transcription and translation take place simultaneously in the reaction compartment

Step	Description
①	The gene of interest is cloned into a suitable vector and added to the reaction compartment.
②and ③	In a coupled *in vitro* reaction the DNA is first transcribed from the template vector into mRNA by T7 RNA polymerase (2a), followed by translation by the ribosomal machinery present in the *E.coli* lysate (2b).
④	Expressed protein accumulates in the reaction compartment and is harvested after a 4-24 hours.

Application

The system consisting of the RTS 500 *E.coli* HY Kit and the RTS 500 Instrument is designed for protein expression in the scale of 500 to 6000 µg in a 1 ml reaction volume. Proteins in the molecular weight range from 15 to 120 kD have been successfully synthesized. For a current list of expressed proteins, please refer to our website at **www.proteinexpression.com.**

Limitations in post-translational modifications

The RTS 500 *E.coli* HY Kit is not able to introduce the following post-translational modifications:

- Glycosylation
- Phosphorylation
- Disulfide bond formation
- Signal sequence cleavage

Template DNA

The DNA of interest must be cloned into a vector, designed for prokaryotic *in vitro* protein expression and containing a T7 promotor. The requirements are described in chapter 3.5.

We recommend pIVEX vectors, which are optimized for *in vitro* protein expression.

Reaction time

Between 4 and 24 hours

Number of reactions

The kit provides reagents and plastic disposables (devices) for two (Cat. No. 3246 817) or five (Cat. No. 3246 949) 1 ml reactions. Reagents for each reaction are bottled separately except bottle 5 (Methionine) which contains enough reagent for all reactions.

Stability

This product is stable at –15°C to -25°C until the expiration date printed on the label.

Advantages

Feature	Advantage
Fast	• Kit components reconstituted and mixed within minutes • Proteins expressed within 24 hours • RTS 500 Instrument (available separately) for controlled stirring and heating easy to set up and program
Cell-free	• Expression of toxic proteins • Expression independent of codon usage: due to the excess of all t-RNA species • No restrictive safety regulations due to lack of recombinant organisms.
Versatile	• Easy adaptation of expression conditions by changing temperature, adding detergents, chaperones or protease inhibitors • Selenomethionine labeled proteins for x-ray analysis can easily be produced • Labeled amino acids for NMR-analysis of the protein can be introduced • Monitoring of the reaction by taking samples throughout the run
Reliable	• Reproducible results secured by quality control
Complete	• All required components for the expression supplied with the kit
Productive	• Up to 6 mg of protein can be expressed in a 24 hour reaction

2.2 Background Information

Coupled *in vitro* transcription/ translation

Upon addition of the DNA template and T7-RNA-polymerase to the DNA-depleted *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. This makes the system much more productive compared to the addition of isolated mRNA (DeVries & Zubay, 1967).

Continuous Exchange Cell-Free protein synthesis (CECF)

During coupled transcription and translation energy components, nucleotides, and amino acids are consumed, whereas waste products like inorganic pyrophosphate, phosphate, nucleotide mono- and diphosphates, and other degradation products are formed. The accumulation of inhibitory waste components and the depletion of substrates will quickly shut down the reaction.

This is prevented by a continuous removal of waste and by a supply with substrate components through a semi-permeable dialysis membrane, which separates the upper reaction compartment from the lower feeding compartment in the RTS 500 Reaction Device as shown in figure 2.

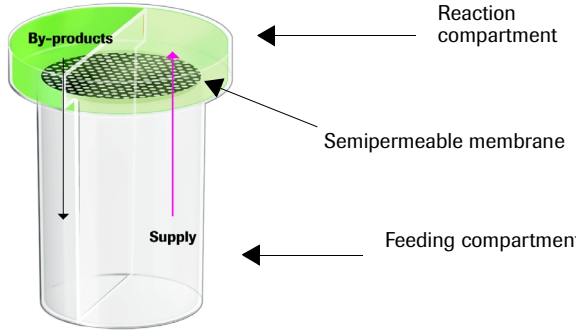

Fig 2: RTS Reaction Device. Principle of *Continuous Exchange Cell-Free* protein synthesis (CECF) as invented by Spirin (1988).

3. Procedures and required materials

3.1 Regular protein synthesis reaction

3.1.1 Before you begin

Solutions

- All required reagents are supplied with the kit.
- Do not combine reagents from different kit lots.
- Reconstitute only the bottles needed for the experiment, except bottle 5 (Methionine) which contains enough methionine for all reactions. It can be frozen and thawed 10 times after reconstitution.
- For reconstitution of bottles 1 to 5 use only Reconstitution Buffer (bottle 6) supplied with the kit.
- For reconstitution of the control vector (bottle 7) use sterile DNase- and RNase-free water.
- Reconstitution Buffer can be thawed at 25°C in a water bath.
- Store the reconstituted control plasmid at –15°C to -25°C
- Reconstitute the lyophilized contents of the bottles 1 to 4 directly prior to use.
- Keep reconstituted reagents and working solutions on ice before use.

DNA template

Prepare and purify as described in the cloning section (chapter 3.5)

Equipment

- Use the Rapid Translation System RTS 500 Instrument from Roche Molecular Biochemicals for optimal reaction performance and convenience (Cat. No. 3 064 859)
- Use only calibrated pipettes.
- Use RNase-free plastic and glassware.
- For precise quantification of the control reaction with GFP, apply the reaction products onto a SDS-polyacrylamid gel. A qualitative estimation can be made using an UV-lamp (360 mm).

3.1.2 Reconstitution of reaction components

Solution	Content	Reconstitution/Preparation of working solution	For use in
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute the lyophilizate with 0.525 ml of Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. DO NOT VORTEX!	• section 3.1.3 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.1.3 • solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.1.3 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, brown cap	Reconstitute the lyophilizate with 3 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking	• section 3.1.3 • solution 7 and 8
5	Methionine Bottle 5, yellow cap	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.1.3 • solution 7 and 8
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 4°C but can also be stored at –20°C	• solution 1, 2, 3, 4,5

Appearance of solutions

With the exception of the *E.coli* lysate all reconstituted lyophilizates should be clear solutions.

3.1.3 Preparation of working solutions

Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 0.3 ml of reconstituted Methionine (solution 5) to solution 3. Mix by rolling or shaking. Total volume of feeding solution (solution 3) is 11 ml.	• section 3.1.4
8	Reaction Solution	To the content of solution 1 (E. coli lysate), add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 30 µl of reconstituted Methionine (solution 5). 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!	• section 3.1.4

3.1.4 Running an experiment

Loading of the reaction compartment

The reaction compartment must be filled first.

- Open both lids of the 1 ml reaction compartment (large diameter).
- Fill with approx. 1 ml Reaction Solution (solution 8) through the circular opening using a 1 ml pipette. Let escape the air through the oval-formed opening by tipping the device slightly. It is not necessary to remove all air bubbles from the reaction compartment.
- Close the lids securely.

Loading of the feeding compartment

- Turn the reaction device upside-down.
- Open the two lids of the feeding compartment (small diameter).
- Fill with approx. 10 ml Feeding Solution (solution 7) through the circular opening. Let escape the air through the oval-formed opening by tipping the device slightly. **Air bubbles should be removed as completely as possible from the feeding compartment.**
- Close the lids securely and turn the reaction device (reaction compartment upright again).

Starting the run

Note: Make sure, that both stir bars are properly located at the bottom of their respective compartment. Otherwise **separate the stir bars by gently tapping the device on the table or with the help of another magnet**.

- Insert the loaded reaction device into the RTS 500 Instrument.
- Set instrument parameters according to the instrument manual.
- Start the run.
- Look through the illuminated window to be sure, that both stir bars are rotating.

continued on next page

Points to consider

Parameter	Guideline
Stirrer speed	Stirring is essential to guarantee homogeneous distribution of components and to avoid membrane clogging. Stirrer speed should be set between 120 and 180 rpm.
Temperature	Optimum temperature for most proteins is 30°C. However, lower temperatures may be used for proteins that tend to aggregate.
Time	Protein synthesis continues for up to 24 hours. For unstable proteins the optimum yield of soluble active protein may be achieved at shorter reaction times.

End of run

- Stop the run.
- Remove the Reaction Device from the RTS 500 Instrument.
- Open both lids from the reaction compartment (large diameter).
- Remove Reaction Solution through the circular opening with a 1 ml pipette.
- Store the Reaction Solution at −15 to −25°C or at 2–8°C until purification or further processing.

3.1.5 GFP control reaction

Reaction procedure for GFP control protein

Step	Procedure
1	Reconstitute bottles 1, 2, 3, 4 and 5 according to chapter 3.1.2
2	Briefly centrifuge down the contents of vial 7. Reconstitute the lyophilized Control Vector GFP with 50 µl sterile DNase- and RNase-free water. The solution (1 µg/µl) is stable at –20° C.
3	Prepare Feeding Solution according to chapter 3.1.2 and 3.1.3
4	Prepare Reaction Solution according to chapter 3.1.2 and 3.1.3. Add 15 µg (15 µl) reconstituted Control Vector GFP (vial 7).
5	Fill the reaction chamber to only **50 %** with **0.5 ml** of Reaction Solution. For explanation see section "Oxidation of GFP" below this table.
6	Fill the feeding chamber with Feeding Solution
7	Start the reaction by setting the following parameters: Temperature: 30° C Stirring speed: 120 rpm Time: 20 hours
8	After completing the run remove the Reaction Solution with a pipette through the round opening and store it in a 2 ml vial for 72 hours at 4° C for maturation of GFP.
9	The fluorescence of GFP (excitation wavelength 395 nm, emission at 504 nm) can be observed using an UV lamp (360 nm).
10	Apply 0,25 – 0,5 µl of the reaction onto SDS-polyacrylamide gels, and put GFP standard (separately available) onto a separate lane.
11	Run the gel and stain with Coomassie blue and quantify the synthesized GFP by comparison with the GFP standard. ***Note:*** The GFP protein can also be detected on Western blots by using an anti-His antibody.

Oxidation of GFP

GFP needs molecular oxygen to post-translationally form the fluorophor (Coxon, A. & Bestor T.H. (1995)). To enhance this conversion to the active form, air is provided during the reaction (and for this control reaction only!) by filling the reaction chamber only to 50% with Reaction Solution.
The yield of properly folded fluorescent GFP is further increased by storing the Reaction Mix after the expression in a 2 ml vial for 72 hours at 4° C.

3.2 Incorporation of Selenomethionine

Caution

Note: Selenomethionine is TOXIC. Please inform yourself about safety rules and information prior to use. In case of eye contact or skin contact flush immediately with water and seek immediately medical advice. DO NOT SWALLOW SELENOMETHIONINE.

Introduction

Selenomethionine is used for x-ray studies of proteins. Usually it is added to the fermentation medium of growing cells, leading to the labeled protein. The incorporation into the protein is normally not complete because of metabolism and impurities of the natural occurring methionine, leading to protein molecules different in molecular weight and selenomethionine content, which can potentially complicate the crystallization process. Because methionine is kept separately from all other ingredients, this kit is designed to easily replace methionine by selenomethionine .

The use of RTS 500 also reduces the amount of selenomethionine required to express sufficient protein.

3.2.1 Reconstitution of reaction components

Before you start

Please read the general instructions in section 3.1.2.

Solution	Content	Reconstitution/Preparation of working solution	For use in
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute the lyophilizate with 0.525 ml of Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. DO NOT VORTEX!	• section 3.2.3 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.3 • solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.3 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, brown cap	Reconstitute the lyophilizate with 3 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.2.3 • solution 7 and 8
-	Methionine Bottle 5, yellow cap	THIS BOTTLE IS NOT REQUIRED FOR SELENOMETHIONINE LABELING	
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 4°C but can also be stored at –20°C	• solution 1, 2, 3, 4,5

Appearance of solutions

With the exception of the *E.coli* lysate all reconstituted lyophilizates should be clear solutions.

3.2.2 Preparation of Selenomethionine Solution

Source of selenomethionine

Selenomethionine is not provided in this kit. Selenomethionine can be obtained in crystalline form from several suppliers (e.g. Fluka)

Solution	Content	Reconstitution/Preparation of working solution	For use in
5a	Selenomethionine	Dissolve 7 mg D,L-Selenomethionine and 1 mg DTT in 1.8 ml Reconstitution buffer (bottle 6) This amount is enough for five 1 ml reactions. ***Note:*** DTT is only added if you want to store the solution for longer than 1 day. The solution can be frozen and thawed up to 10 times.	• section 3.2.3 • solution 7 and 8

3.2.3 Preparation of working solutions

Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 0.3 ml of Selenomethionine solution (solution 5a) to solution 3. Mix by rolling or shaking.	• section 3.1.4
8	Reaction Solution	To the content of solution 1 (E. coli lysate), add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 30 µl of Selenomethionine (solution 5a). Add 10-15 µg of the DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. DO NOT VORTEX!	• section 3.1.4

Reaction set-up

Please refer to section 3.1.4.

Literature

- Cowie, D. B. & Cohen, G. N. (1957). Biosynthesis by *Escherichia coli* of active altered proteins containing selenium instead of sulphur. *Biochim. Biophys. Acta*, **26**, 252-261.
- Hendrickson, W. A., Horton, J. R. & LeMaster, D. M. (1990) Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. *EMBO J.*, **9,** 1665-1672.
- Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J. & Huber, R. (1995) High level biosynthetic substitution of methionine in proteins by its analogues 2-aminohexanoic acid, selenomethionine, telluromethionine and ethionine in *Escherichia coli*. *Eur. J. Biochem.*, **230**, 788-796

.

3.3 Production of labeled Proteins for NMR-Spectroscopy

Introduction

The determination of protein structures via Nuclear Magnetic Resonance (NMR) technologies has gained a lot of interest since the hard- and software could be improved.

Usually ^{15}N- and/or ^{13}C-labeled amino acids are incorporated into proteins by adding ^{15}N-ammonium chloride and ^{13}C-labeled glucose to the medium of growing cells, producing uniformly labeled proteins. Procedures to selectively incorporate single, labeled amino acids are not practical.

In the RTS 500 *E. coli* HY Kit all amino acids are separated from the other reagents necessary to drive the reaction (e.g. reaction mix, feeding mix). By exchanging the amino acids provided in the kit with other amino acid mixtures this design allows the following possibilities:

- incorporation of uniformly labeled amino acids
- specific incorporation of single ^{15}N- and/or ^{13}C-labeled amino acid(s).

3.3.1 Incorporation of uniformly labeled amino acids

Source of ^{15}N- and/or ^{13}C-labeled amino acid

Labeled amino acids are not provided with this kit. For the uniform labeling of proteins, labeled amino acids prepared from algae are normally used. They can be obtained in crystalline form from several suppliers (e.g. Isotec Corp., Silantes GmbH)

3.3.1.1 Reconstitution of reaction components

Before you start Please read the general instructions in section 3.1.2.

Solution	Content	Reconstitution/Preparation of working solution	For use in
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute the lyophilizate with 0.525 ml of Reconstitution Buffer (bottle 6) Mix gently. DO NOT VORTEX!	• section 3.3.1.3 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.3.1.3 • solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with 8.1 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• Section 3.3.1.3 • solution 7
-	Amino Acid Mix without methionine Bottle 4, brown cap	THIS BOTTLE IS NOT REQUIRED FOR INCORPORATION OF UNIFORMLY LABELED AMINO ACIDS	
-	Methionine Bottle 5, yellow cap	THIS BOTTLE IS NOT REQUIRED FOR INCORPORATION OF UNIFORMLY LABELED AMINO ACIDS	
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 4°C but can also be stored at –20°C	• solution 1, 2, 3, 4, 5

Appearance of solutions With the exception of the *E.coli* lysate all reconstituted lyophilizates should be clear solutions.

3.3.1.2 Preparation of amino acid labeling mix

Solution	Content	Reconstitution/Preparation of working solution	For use in
4a	Amino acids labeling mix	Make a 2.1 mM stock solution of all amino acids in 3 ml of Reconstitution buffer (bottle 6). It is necessary to check the certificate of analysis you receive from the provider of the labeled amino acid mixture. Be sure that all amino acids have nearly the same concentration (usually some amino acids are missing or the content is lower compared to others, e.g. asparagine, cysteine, glutamine, tryptophane)	• section 3.3.1.3 • solution 7 and 8
5b	DTT	Make a fresh 40 mM stock solution in 1.5 ml Reconstitution Buffer (bottle 6). This is enough for five 1 ml reactions.	• section 3.3.1.3 • solution 7 and 8

3.3.1.3 Preparation of working solutions

Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	To the content of solution 3 add 2.65 ml of the reconstituted Amino acid labeling mix (solution 4a) and 0.3 ml of DTT-solution (solution 5b). Mix by rolling or shaking	• section 3.1.4
8	Reaction Solution	To the content of solution 1 add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted amino acid labeling mix (solution 4a), and 30 µl of DTT solution (solution 5b). Add 10-15 µg of DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. DO NOT VORTEX!	• section 3.1.4

Reaction set-up Please refer to section 3.1.4.

3.3.2 Specific Incorporation of single ^{15}N-, ^{13}C-labeled amino acid(s)

Preparing amino acid mixtures

For the specific incorporation of selected amino acids an amino acid mixture must be prepared in accordance with the experimental requirements. The Rapid Translation System RTS Amino Acid Sampler (Cat. No. 3 262 154) provides appropriate stock solution of each individual **un**labeled amino acid. Specific amino acid mixtures can be prepared by including the appropriate labeled amino acid(s). Yields obtained after exchanging the amino acids provided in the kit with such adapted amino acid mixtures are in the same range.

Note: If methionine is the only labeled amino acid to be introduced, the procedure for labeling with selenomethionine (section 3.2.1) can be followed, using the Amino Acid Mix without Methionine provided in this kit.

Literature

- Riek, R., Pervushin, K., Wuethrich, K. (2000). TROSY and CRINEPT:NMR with large molecular and supramolecular structures in solution *TIBS* (2000), **25**, 462
- Gardner, K.H., Kay, L. E. (1998): The use of H, C, N multidimensional NMR to study the structure and dynamics of proteins. *Annu Rev Biophys Biomol Struct*, **27**, 357

3.4 Addition of Supplements

Introduction

Depending on the nature of the particular protein of interest it may be necessary to add chemicals (e.g. detergents) or supplements (e.g. chaperones) in order to produce functional proteins. The design of this kit allows the addition of up to 200 µl of supplement solution to the reaction solution and/or 2 ml to the feeding solution (please keep in mind that the addition of high molecular weight components into the feeding solution is useless if the molecular weight is > 5000 Da since the cut-off of the membrane separating the reaction from the feeding compartment is 10 kDa).

For efficient optimization of reaction conditions we generally recommend to study the effects (yield, solubility) of additional chemicals or supplements in the Rapid Translation System RTS 100 *E. coli* HY Kit (Cat. No. 3186148). If a positive effect is observed in the 50 µl reaction volume of the RTS 100, the same conditions can be applied in the 1 ml reaction volume of the RTS 500.

3.4.1 Reconstitution of reaction components

Before you start

Please read the general instructions in section 3.1.2.

Solution	Content	Reconstitution/Preparation of working solution	For use in
1	*E.coli* Lysate Bottle 1, red cap	Reconstitute the lyophilizate with **0.340** ml of Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. DO NOT VORTEX!	• section 3.4.2 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 0.25 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.4.2 • solution 8
3	Feeding Mix Bottle 3, blue cap	Reconstitute the lyophilizate with **6.1** ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.4.2 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, brown cap	Reconstitute the lyophilizate with 3 ml of Reconstitution Buffer (bottle 6), mix by rolling.	• section 3.4.2 • solution 7 and 8
5	Methionine Bottle 5, yellow cap	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.4.2 • solution 7 and 8
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 4°C but can also be stored at –20°C	• solution 1, 2, 3, 4,5
S	Supplement	Make a stock solution of the particular chemical or supplement in Reconstitution Buffer (bottle 6)	section 3.4.2 solution 7 or 8

Appearance of solutions

With the exception of the *E.coli* lysate all reconstituted lyophilizates should be clear solutions.

3.4.2 Preparation of working solutions

Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 0.3 ml of reconstituted Methionine (solution 5) to solution 3. Finally add 2 ml of solution S or Reconstitution buffer (bottle 6). Mix by rolling or shaking. Total volume 11 ml.	• section 3.1.4
8	Reaction Solution	To the content of solution 1 add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 30 µl of reconstituted Methionine (solution 5). Add 10 - 15 µg of DNA template in a maximum volume of 50 µl. Finally add 200 µl of solution S or Reconstitution buffer. Mix carefully by rolling or gentle shaking. Total volume 1.1 ml DO NOT VORTEX!	• section 3.1.4

Reaction set-up

Please refer to section 3.1.4.

3.5 Preparation of DNA Templates

Literature

Basic molecular biology methods may be found in one of the following method books:

- Sambrook et al (1989) "Molecular Cloning: A Laboratory Manual", Second Edition, Cold Spring Harbor Laboratory Press, New York.
- Ausubel, U. K. et al (1993) "Current Protocols In Molecular Biology", John Wiley & sons Inc., New York.

Necessary vector elements

Any vector to be used in combination with the Rapid Translation System RTS must include the following elements and structural features:

- target sequence under control of T7 promotor located downstream of a RBS (ribosomal binding site) sequence
- distance between T7 promotor and start ATG should not exceed 100 base pairs
- distance between the RBS sequence and start ATG should not be more than 5-8 base pairs
- T7 terminator sequence at the 3'-end of the gene

General Recommendations

The pIVEX vector family has been developed and optimized for use in the Rapid Translation System RTS. Therefore, we strongly recommend cloning target genes into a pIVEX vector.

A schematic view of pIVEX vectors is given in Fig. 3. Recent listings of available vectors as well as whole vector sequences and vector maps can be found on www.proteinexpression.com

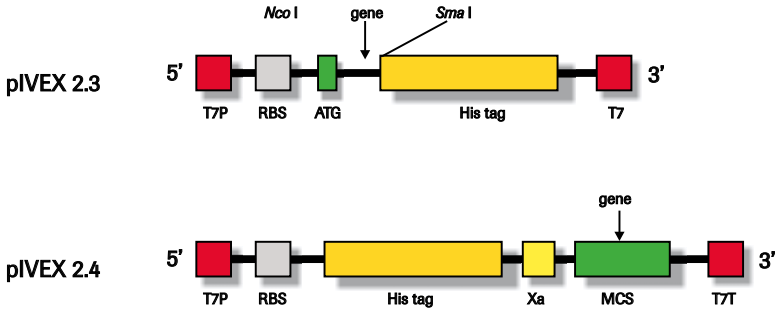

Fig. 3: Functional elements of cloning vectors

Abbreviations:

T7 P	= T7 Promotor
RBS	= Ribosomal binding site
ATG	= Start codon
Nco I, Sma I	= restriction enzyme recognition sites for cloning of the target gene
C-Tag, N-Tag	= C- or N-terminal tag position
Xa	= Factor Xa restriction protease cleavage site
MCS	= Multiple cloning site in three different reading frames for the insertion of the target gene
T7 T	= T7 Terminator

Purity of Plasmid Preparations

The purity of plasmids obtained from commercially available DNA preparation kits is sufficient for the use in the Rapid Translation System. If the DNA purity is not sufficient ($OD_{260/280} < 1.7$), a phenol treatment may be necessary to remove traces of RNase.

Improving success rate

Every gene, inserted into an expression vector, gives after transcription a unique mRNA. It is difficult to predict interactions between coding sequences of the target gene and the 5'-untranslated region, which can potentially interfere with translation. We therefore recommend to clone the gene of interest in more than one expression vector. In particular, N-terminal extensions have proven to show positive impact on expression yield.

4. Typical results

Expression kinetics

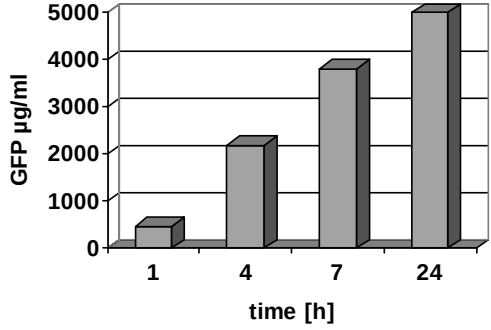

Fig. 4: Synthesis rates of active green fluorescent protein. Samples were taken from the reaction chamber at various time points. Fluorescence was measured after a storage period for 72 hours at 4°C needed for complete oxidation.

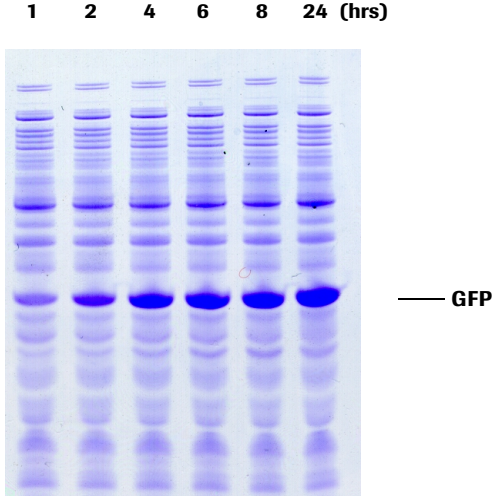

Fig. 5: Expression of GFP, monitored on Coomassie-stained SDS-PAGE gel. Samples were taken from the reaction chamber at various time points.

5. Appendix

5.1 Trouble shooting

5.1.1 General problems

Problem	Possible cause	Recommendation
No or weak stirring during a run	Stir bars stick to each other.	Separate stirring bars by gently tapping the device on the table or with the help of a magnet.
	Stirring speed is set to zero or too low.	Increase stirring speed.
White precipitate in the Feeding/ Reaction Solution after the run	Insoluble salts are formed.	Usually the expression is not affected by the precipitate.
No expression using the GFP control reaction	Kit expired	Order a new kit.
	Kit has not been stored at −15 to −25°C.	Order a new kit.
	Contamination with RNases	Repeat experiment and take care to work RNase-free at each step.
	Air bubbles in the large feeding chamber	Remove any air bubbles from the feeding chamber before starting a run.
	Wrong handling	Repeat the experiment exactly according to the working instructions.
	A kit component is inactive or degraded.	Contact Roche Molecular Biochemicals technical service. For addresses see the last page of this instruction manual.

continued on next page

5.1.2 Problems with the expression of the target protein, while the GFP control reaction works

Oberservation	Potential Reasons	Recommendation
Sufficient protein expression, but low yield of active protein	Incorrect folding of the protein due to: • dependence on cofactors	Add necessary cofactors.
	• necessity of disulfide bond formation	Proteins with more than 3 disulfide bonds, cannot be expressed in a functional form in the RTS *E.coli* system. For protein with up to 3 disulfide bonds, allow oxidation after the reaction for the formation of disulfide bonds (see e.g. Ahmed et al (1975), Odorzinsky & Light (1979)
	• dependence on secondary modifications	The *E.coli* lysate can not introduce post-translational modifications like glycosylation, phosphorylation, or signal sequence cleavage.
	• dependence on chaperones	Add chaperones [see Rudolph (1997)].
Product appears in the pellet after centrifugation	Aggregation	• Add/adjust chaperones. • Change experimental conditions (time, temperature). • Add mild detergents (e.g. up to 0.1% Tween 20 (v/v) , or 0.1% Chaps (w/v) for membrane proteins).
Low expression yield	Expression time too short	Extend expression
	The tag has a negative influence on the folding of the protein.	Try expression in different pIVEX vectors.
Several product bands on SDS-PAGE or product smaller than expected	Proteolytic degradation	Add protease inhibitors to the reaction. For example take 1 tablet of Complete mini EDTA-free for a 1 ml reaction. Use up to 10 mM EGTA.
	Internal initiation site	Replace the corresponding methionine by point mutation.
	Premature termination of the translation	• Check the sequence of the target gene regarding reading frame and mutations which might yield a stop codon. • Search for strong secondary structures of the mRNA and eliminate them by conservative mutations.
No expression of the target gene, but normal expression of GFP	Cloning error	Check the sequence.
	Low purity of plasmid DNA	• Assure that the absorbance ratio 260 nm/280 nm is at least 1.7. • Perform a phenol extraction if purity is low. • Make a new plasmid purification.
	Contamination with RNases	Repeat experiment and take care to work RNase-free at each step.
	No initiation of translation due to strong secondary structures of the mRNA	• Try expression in different pIVEX-vectors. • Try expression as N-terminally tagged fusion protein
	Expressed protein interferes with the translation or transcription process.	Express the gene of interest together with GFP. If GFP expression is inhibited the active protein can not be expressed with the kit.

5.2 References

1 Zubay, G (1973) *Annu. Rev. Genet.* **7,** 267

2 Spirin, A. S. et al (1988) "A continuous cell-free translation system capable of producing polypeptides in high yield" *Science* **242**, 1162

3 Coxon, A. & Bestor, T. H. (1995) *Chemistry & Biology* **2**, 119

4 Sambrook et al (1989) "Molecular Cloning - A Laboratory Manual" Second Edition, Cold Spring Harbor Laboratory Press, New York

5 Ausubel, U. K. et al (1993) "Current Protocols In Molecular Biology" John Wiley & Sons Inc., New York.

6 Ahmed, A. K. et al (1975), *J. Biol. Chem.* **250**, 8477

7 Odorzinsky, T. W. & Light, A. (1979), *J. Biol. Chem.* **254**, 4291

8 Rudolph, R. et al (1997) in "Protein Function – A Practical Approach" Creighton, T. E. ed. Oxford University Press Inc. New York, pp 57 - 99

5.3 Notice for the Purchaser

"The purchase price of this product includes a limited, non-exclusive, non-transferable license under U.S Patent 5.478.730 or its foreign counterparts, assigned to the Institute of Protein Research at the Russian Academy of Science, Pushchino and owned by Roche Diagnostics Corporation ("Roche"), to use only this amount of the product to practice a cell-free expression system achieving continuous production of a polypeptide in the presence of a semi-permeable barrier and related processes described in said patents solely for the internal research and development activities of the purchaser. Further patents regarding improved performance of the reagents are pending. This license expressly excludes any commercial application of the product for resale of polypeptide or commercial offering services related to the product. Further information on purchasing a commercial license may be obtained by contacting Roche Diagnostics Corporation, 9115 Hague Road, P.O. Box 50457 Indianapolis, IN, 46250-0457, USA."

5.4 How to contact Roche Molecular Biochemicals

Three ways to contact us

To contact Roche Molecular Biochemicals for technical assistance, please choose one of the following:

IF you are using...	THEN...
Internet	access our web-site at: http://www.proteinexpression.com, or http:\\biochem.roche.com.
E-mail	Please refer to the address that corresponds to your particular location, on the last page of this instruction manual.
Telephone	Please refer to the address that corresponds to your particular location, on the last page of this instruction manual.

5.5 Related products

Product	Pack Size	Cat. No.
Rapid Translation System RTS 500 Instrument	1 instrument	3064 859
Rapid Translation System RTS 100 *E. coli* HY Kit	24 reactions (50 µl each)	3186 148
Rapid Translation System RTS pIVEX His-tag Vector Set	5 vectors (10 µg each)	3253 538
Rapid Translation System RTS GroE Supplement	5 x 250 µl	3263 690
Rapid Translation System RTS Amino Acid Sampler	For five RTS 500 reactions	3262 154
Restriction Protease Factor Xa	3 x 30 µg 3 x 100 µg 3 x 250 µg	1179 888 1585 924 1179 896
Restriction Protease Factor Xa Cleavage and Removal Kit	Kit I : 3 x 30 µg Kit II: 3 x 100 µg	1644 777 1644 785
rGFP	50 µg	1814 524
Anti-His_6	100 µg	1922 416
Anti-His_6-Peroxidase	50 U	1965 085
Complete Protease Inhibitor Cocktail Tablets, mini, EDTA-free	25 tablets (each tablet for 10 ml extract)	1836 170

E-mail Address	Country	E-mail Address	Country
argentina.biochem@roche.com	Argentina	Raitis@invitros.lv	Latvia
biochem.au@roche.com	Australia	Sakkijha@rdleb.com	Lebanon
Gerhard.Muehlbauer@roche.com	Austria	Gintaras@eksma.lt	Lithuania
biochem.be@roche.com	Belgium	diagnostics@prophac.lu	Luxembourg
Valent@mbox.cit.bg	Bulgaria	Vccl@vol.net.mt	Malta
africhem@camnet.cm	Cameroon	Aiouche.echo@dounia.net.ma	Morocco
biochem.ca@roche.com	Canada	biocheminfo.nl@roche.com	Netherlands
biochem.cn@roche.com	China	biochem.nz@roche.com	New Zealand
Info@medisell.com.cy	Cyprus	bofungwu@linkserve.com.ng	Nigeria
Bm-comp@bm-comp.cz	Czech Republic	biochem.se@roche.com	Norway
dk.biochem@roche.com	Denmark	biochem.pt@roche.com	Portugal
ou.melestrum@neti.ee	Estonia	Topdiag@fx.ro	Romania
pharsc.et@telecom.net.et	Ethiopia	biochem.sg@roche.com	Singapore
helsinki.biochem_diagnostics@roche.com	Finland	roche.diagnostics@siol.net	Slovenia
biochem.fr@roche.com	France	south_africa.bioboffin@roche.com	South Africa
mannheim.biocheminfo@roche.com	Germany	biochem.es@roche.com	Spain
Bm_roche@hotmail.com	India	biochem.se@roche.com	Sweden
h.hajian@tebtech.com	Iran	BiochemInfo.CH@roche.com	Switzerland
tubanegin@istn.irost.com	Iran	Jean-Marie.kindbeiter@roche.com	Tunisia
Dyn@netvision.net.il	Israel	bmuae@emirates.net.ae	United Arab Emirates
it.biochem@roche.com	Italy	uk.biochem@roche.com	United Kingdom
biochemicals@rdj.co.jp	Japan	biochemts.us@roche.com	USA
pharmakp@net2000ke.com	Kenya	Mvalentiner@telcel.net.ve	Venezuela
Bmskorea@chollian.net	Korea	dusica@eunet.yu	Yugoslavia
react@ncc.moc.kw	Kuwait	biochemts.row@roche.com	All other countries

http://biochem.roche.com/pack-insert/3246817a.pdf

0601**R235**.1.**3257100**®R MB GPM

Argentina 541 954 5555; **Australia** (02) 9899 7999; **Austria** (01) 277 87; **Belgium** (02) 247 4930; **Brazil** +55 (11) 3666 3565; **Bulgaria** +35929625408; **Cameroon** 237-370269; **Canada** (450) 086 7050; (800) 361 2070; **Chile** 00 56 (2) 22 33 737 (central) 00 56 (2) 22 32 099 (Exec); **China** 86 21 6427 5586; **Colombia** 0057-1-3412797; **Cyprus** +357-2-311362; **Czech Republic** (0324) 45 54, 58 71-2; **Denmark** +45 363 999 58; **Egypt** 20-2-3619047; **Estonia** 372-7-447600; **Ethiopia** 251-1-552799; **Finland** +358 9 525 333 66; **France** 04 76 76 30 87; **Germany** (0621) 759 8568; **Greece** 3 (01) 67 40 238; **Hong Kong** (852) 2485 7596; **India** +91-22-8379906; **Indonesia** 62 (021) 252 3820 ext. 755; **Iran** +98-21-8072374 / +98-21-8797027; **Israel** 972-6-6380569; **Italy** 039 247 4109-4181; **Japan** 03 3432 3155; **Kenya** +254-2-750112; **Korea** 82-2-3471-6500; **Kuwait** +965-4837859; **Latvia** 371-787828309; **Lebanon** Fax: 00961-1-399667; **Lithuania** 370-2-729715; **Luxembourg** +352-496098; **Malta** Fax: +356-341087; **Morocco** Fax: +212-2-944040; **Malaysia** 60 (03) 755 5039; **Mexico** (5) 227 8967; **Netherlands** (036) 539 4911; **New Zealand** (09) 276 4157; **Nigeria** +234-1-521767; **Norway** (47) 23 373300; **Philippines** (632) 810 7246; **Poland** +48 (22) 22 66 84 305; **Portugal** (01) 4171717; **Republic of Ireland** 1 800 40 90 41; **Romania** +40-1-2123763; **Russia** (49) 621 759 8636 Fax: (49) 621 759 8611; **Saudia Arabia** +966-1-4010364; **Singapore** 0065 272 9200; **Slovenia** +386 61 1363528; **South Africa** (011) 886 2400; **South Korea** 02 569 6902; **Spain** (93) 201 4411; **Sweden** (08) 404 8800; **Switzerland** +41 (41) 799 6161; **Taiwan** (02) 736 7125; **Thailand** 66 (2) 274 07 08 (12 line); **Turkey** 0090 212 216 32 80; **United Arab Emirates** +971-4-694351; **United Kingdom** (0800) 521578; **USA** (800) 428 5433. **Venezuela** Fax: +0058-4810697; **Yugoslavia** +381 11 137163.

The Rapid Translation System website: www.proteinexpression.com

Roche Diagnostics GmbH
Roche Molecular Biochemicals
Sandhofer Strasse 116
D-68305 Mannheim
Germany