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

Rapid Translation System RTS 9000 *E.coli* HY Kit

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

Cat. No. 3 290 395
For 1 × 10 ml reaction

Cat. No. 3 290 468
For 3 × 10 ml reactions

Store this kit at −15 to −25°C

Instruction Manual

Version 1, November 2001

The Rapid Translation System website:
www.proteinexpression.com

Please refer to “Notice for the purchaser” on page 26.

Roche

1. Preface

1.2 Kit contents

Safety information

Bottles 2, 3, 4, and 5 contain < 25% dithiothreitol, and < 10% EDTA. Other bottles do not contain hazardous substances in significant quantities. The standard precautions for handling chemicals should be observed with the RTS 9000 *E.coli* HY Kit. Used reagents 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.

Pos. resp. Bottle No./ Cap	Label	Contents and function	Cat. No. **3 290 395** for 10 ml reaction format	Cat. No. **3 290 468** for 3 × 10 ml reaction format
1 red cap	*E.coli* Lysate	Enhanced *E.coli* lysate (lyophilized) Contains components for transcription and translation	1 Bottle	3 Bottles
2 green cap	Reaction Mix	Substrate mix to prepare 11 ml reaction solution (lyophilized)	1 Bottle	3 Bottles
3 white cap	Feeding Mix	Substrate mix to prepare 109 ml feeding solution (lyophilized)	1 Bottle	3 Bottles
4 white cap	Amino Acid Mix without Methionine	Amino acid mix, without methionine (lyophilized)	1 Bottle	3 Bottles
5 white cap	Methionine	Methionine (lyophilized)	1 Bottle	3 Bottles
6 white cap	Reconstitution Buffer	60 ml buffer solution for the reconstitution of lyophilizates in bottles 1 to 5	2 Bottles	6 Bottles
7 colorless cap	Control Vector GFP	50 µg lyophilized GFP (Green Fluorescent Protein) expression plasmid with C-terminal His-6-tag, for positive control	1 Vial	3 Vials
8	RTS 9000 Reaction Device	• Disposable three-chamber device for Continuous Exchange Cell Free (CECF) protein expression • device to be used with Rapid Translation System RTS ProteoMaster	1 device with one Reaction Compartment	1 device with three Reaction Compartments
9	Filling syringe for Feeding Mix	• Disposable plastic syringe including tube for filling and emptying the RTS 9000 Reaction Device feeding compartment	1 × 50 ml syringe incl. tube	3 × 50 ml syringe incl. tube
10	Filling/ Removal pasteur pipette	• Disposable Pasteur pipettes for filling and emptying the RTS 9000 Reaction Device Reaction Compartment	2	6

Laboratory requirements

To avoid contamination with RNases we recommend using RNase-free materials and the wearing of gloves. Additional glassware is not required, since all solutions can be reconstituted in the supplied bottles.

Additional equipment required

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

- Rapid Translation System RTS ProteoMaster, Cat. No. 3 265 650
- Pipettes 100 µl – 1000 µl, 1000 µl – 5000 µl, graduated 10 ml
- Eppendorf reaction vial (1-1.5 ml)
- Pipette tips autoclaved at 121°C for 20 min
- Glass graduated cylinder 50 ml, 100 ml
- Glassware autoclaved at 121°C for 20 min
- optional for control reaction: UV lamp or luminometer or SDS PAGE equipment
- Spatula or screw-driver

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 9000 consists of the RTS ProteoMaster and the RTS 9000 *E.coli* HY Kit. The kit contains reagents and reaction devices for a single 10 ml reaction (Cat. No. 3 290 395) or 3 separate 10 ml reactions (Cat. No. 3 290 468) for large scale, cell free protein expression.

Reaction principle

In the RTS 9000 Reaction Device, transcription and translation take place simultaneously in a central 10 ml Reaction Compartment Substrates and energy components essential for sustained protein expression are continuously supplied from the Feeding Compartment (100 ml) across a semi-permeable membrane. As the reaction proceeds, inhibitory reaction by-products diffuse through the same membrane into the Feeding Compartment. Protein is expressed for up to 24 hours yielding up to 50 mg of control CAT protein in a single compartment of the RTS 9000 Reaction Device.

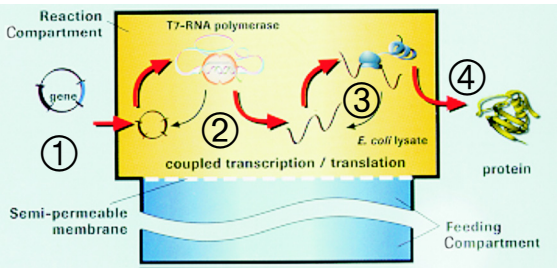


Fig 1: ***Schematic illustration of CECF protein expression.***
Transcription and translation take place simultaneously in the Reaction Compartment.

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

2.1 Product overview, continued

Application	The system consisting of the RTS 9000 *E.coli* HY Kit and the RTS ProteoMaster is designed for protein expression in the scale of 5 to 50 mg in a 10 ml reaction volume. The RTS 9000 is a scale-up device and is fully compatible with the RTS 100 *E.coli* HY Kits and RTS 500 *E.coli* HY Kits. The RTS 9000 Kit is primarily designed to express proteins in amounts sufficient for diagnostic screening trials, for NMR/X-ray analysis or even toxicological studies. Proteins, which are expressed in the RTS 500 *E.coli* HY Kit in only minimal quantities, can be produced in quantities sufficient for antigen production and/or functional testing with the RTS 9000. Proteins in the molecular weight range from 15 to 120 kD have been successfully produced with the RTS *E.coli* Kits. For a current list of expressed proteins, please refer to our website at: **www.proteinexpression.com**
Limitations in post-translational modifications	The RTS 9000 *E.coli* HY Kit is not able to produce proteins with the following post-translational modifications: • Glycosylation • Phosphorylation • Multiple disulfide bonds • 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 promoter. Detailed recommendations are given in chapter 3.5. We recommend RTS pIVEX vectors, which are optimized for *in vitro* protein expression with the RTS *E.coli* Kits.
Reaction time	Between 4 and 24 hours
Number of reactions	The kit provides reagents and plastic disposables for one (Cat. No. 3 290 395) or three (Cat. No. 3 290 468) 10 ml reactions. Reagents for each reaction are bottled separately.
Stability	This product is stable at −15°C to −25°C until the expiration date printed on the label.

2.1 Product overview, continued

Advantages

Feature	Advantage
Fast	• Kit components reconstituted and mixed within minutes • Proteins expressed within 24 hours • RTS ProteoMaster (available separately) for controlled shaking and heating easy to set up and program
Cell-free	• Expression of toxic proteins • Expression independent of codon usage: due to supplementation of all t-RNAs • Minimal safety regulations due to absence of recombinant organisms.
Versatility	• Easy modification of expression conditions by alteration of temperature, addition of detergents, chaperones or protease inhibitors • Selenomethionine labeled proteins for X-ray analysis can easily be produced • Labeled amino acids can be introduced to aid NMR-analysis of your protein • Progression of the synthesis reaction can be easily monitored by taking samples at desired time points.
Reliability	Reproducible results ensured by strict quality control
Complete	All required components for protein expression are supplied in the kit.
High productivity	Up to 50 mg of protein can be expressed in a 24 hour reaction.

2.2 Background information

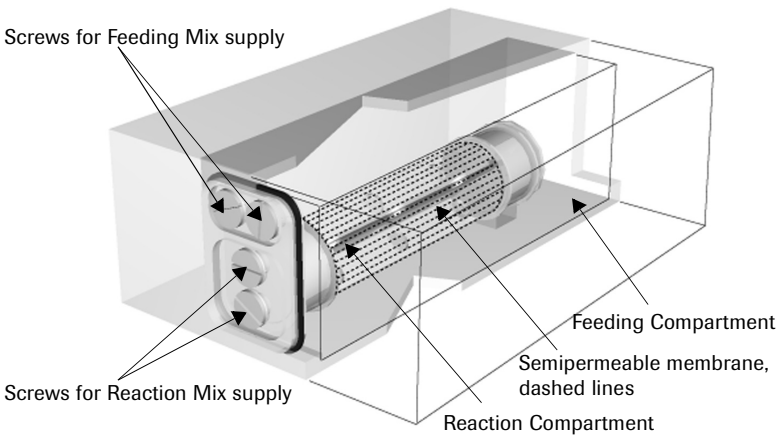
Coupled *in vitro* transcription/ translation

Upon addition of the DNA template to the DNA-depleted *E.coli* lysate containing T7-RNA-polymerase, transcription and translation occur simultaneously: The T7-RNA-polymerase transcribes the template gene, and the ribosomes supplied by the *E.coli* lysate start to translate the 5'-end of the nascent mRNA. This system is thus much more productive compared to the use 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, and reaction by-products other and degradation products such as inorganic pyrophosphate, phosphate, nucleotide mono- and diphosphates 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 with the replenishment of fresh substrate components through the semi-permeable membrane of the RTS 9000 Reaction Device, which separates the Reaction Compartment from the Feeding Compartment as shown in figure 2.



Supply components
- Amino acids
- Energy substrates
- Nucleotides

Waste components
- NDPs
- NMPs
- PP_i
- P
- DNA-fragments
- RNA-fragments

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 Standard protein synthesis reaction

3.1.1 Before you begin

Solutions

- For new proteins, it is highly recommended to first optimize expression conditions using the RTS 100 and RTS 500 *E.coli* HY Kits.
- All required reagents are supplied with the kit.
- Do not combine reagents from kits with different lot numbers.
- For reconstitution of bottles 1 to 5 use only Reconstitution Buffer (bottle 6) supplied.
- Reconstitution Buffer can be thawed at 25°C in a water bath.
- Reconstitute the lyophilized contents of bottles 1 to 4 just prior to use. This can be done at room temperature.

DNA template

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

Equipment

- Use the Rapid Translation System RTS ProteoMaster from Roche Molecular Biochemicals for optimal reaction performance and convenience (Cat. No. 3 265 650)
- Use only calibrated pipettes.
- Use RNase-free plastic and glassware.

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 5.2 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 2.2 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.1.3 • solution 8
3	Feeding Mix Bottle 3, white cap	Reconstitute the lyophilizate with 80 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.1.3 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, white cap	Reconstitute the lyophilizate with 30 ml of Reconstitution Buffer (bottle 6), mix by shaking	• section 3.1.3 • solution 7 and 8
5	Methionine Bottle 5, white cap	Reconstitute the lyophilizate with 3.6 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 2–8°C but can also be stored at 15 to 25°C	• solutions 1, 2, 3, 4, 5

Appearance of solutions

Reconstitution of the *E.coli* lysate will result in a slightly turbid, yellowish solution. The Feeding mix may also be slightly turbid, this does not impair the performance. Reconstitution of all other lyophilizates should result in clear solutions.

3.1.3 Preparation of working solutions

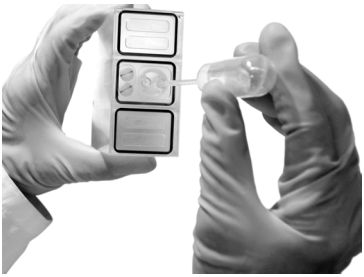
Solution	Content	Reconstitution/Preparation of working solution	For use in
7	Feeding Solution	Add 26 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml of reconstituted Methionine (solution 5) to solution 3. Mix by gentle shaking. Total volume of Feeding Solution (solution 3) is 109 ml.	section 3.1.4
8	Reaction Solution	To the content of solution 1 (*E.coli* lysate), add the total amount of the reconstituted Reaction Mix (2.2 ml solution 2), 2.7 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl of reconstituted Methionine (solution 5). Optional: Remove into a separate Eppendorf vial 500 µl of reaction solution for a GFP positive control reaction *before starting the reaction by adding the DNA template (*see chapter 3.1.5). Add 120–180 µg of the DNA template in a maximum volume of 500 µl. to the remaining reaction solution. Mix carefully by rolling or gentle shaking. Total volume of reaction solution can be up to 10.9 ml DO NOT VORTEX!	section 3.1.4

3.1.4 Running an experiment

Loading of the Reaction Compartment

The Reaction Compartment of the RTS 9000 Reaction Device must be filled first.

- Open both large diameter screws of the Reaction Compartment (s. Fig. 2) using a spatula or screw-driver.
- Fill the amount Reaction Solution (solution 8) through the circular opening using one the disposable Pasteur pipettes. Let the air escape through the openings by tipping the device slightly. It is not necessary to remove all air bubbles from the Reaction Compartment.
- Close the screws securely.



Loading of the Feeding Compartment

- Open the two screws of the Feeding Compartment (see Fig. 2) using a spatula or screw-driver
- Fill with approx. 100 ml Feeding Solution (solution 7) through the circular opening using the 50 ml disposable synringe equipped with a tube. The tube is supplemented in order to easily and securely remove the Feeding Solution from bottle 3. Let the air escape through the opening by tipping the device slightly. Larger air bubbles should be avoided.
- Close the screws securely.

Starting the run

- Insert the loaded reaction device into the RTS ProteoMaster.
  Note: Please take care of the proper orientation of the device in the RTS ProteoMaster Instrument. The temperature sensor of the instrument has to fit into the cavity at the bottom of the reaction device.
- Set instrument parameters according to your previous optimization experiments and using the instrument manual.
- Start the run.

3.1.4 Running an experiment, continued

Points to consider

Parameter	Guideline
Shaking Speed	Shaking is essential to guarantee homogeneous distribution of components and to avoid membrane clogging. Shaking speed should be set around 900 rpm.
Temperature	Optimum temperature for the synthesis of 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 highest yield of soluble active protein may be obtained with shorter reaction .

End of run

- Stop the RTS ProteoMaster.
- Remove the RTS 9000 Reaction Device from the RTS ProteoMaster.
- Put the Reaction Device vertically on the lab bench with the screws of the Reaction Compartment pointing upwards. Open both screws of the Reaction Compartment.
- Carefully remove all the Reaction Solution through the opening by using the second Pasteur Pipette.
- Store the Reaction Solution containing the expressed protein at −15 to −25°C or at 2–8°C until purification or further processing.

3.1.5 GFP batch control reaction (optional)

Reaction procedure for GFP control protein

Step	Action
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 15 to 25°C.
3	Transfer an aliquot of 500 µl reaction mix to an Eppendorf vial before starting your protein synthesis
4	Add 7.5 µl of reconstituted control vector to the reaction mix in the Eppendorf vial and mix it gently
5	Incubate the Eppendorf vial in a water bath or thermostated device (e.g. Eppendorf thermomixer) at 30°C for 24 h
6	The fluorescence of GFP (excitation wavelength 395 nm, emission at 504 nm) can be observed using an UV lamp (360 nm).
7	Optionally apply 1 µl of the reaction onto SDS-polyacrylamide gel, and put GFP standard (separately available) onto a separate lane.
8	Run the gel and stain with Coomassie blue. ***Note***: The GFP protein can also be detected on Western blot 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)
The yield of correctly folded fluorescent GFP is increased by storing the Reaction Mix for 72 hours at 2–8° C.

3.2 Incorporation of selenomethionine

Caution

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 labeling of proteins for X-ray crystallography studies. It is usually added to the fermentation medium of growing *E.coli* cells, leading to the labeling of all synthesized protein. The level of specific selenomethionine incorporation into the protein does not usually approach 100%, because of metabolism and the presence of natural methionine, leading to protein molecules different in molecular weight and selenomethionine content, which can potentially complicate the crystallization process. In the RTS 9000 *E.coli* HY Kit, methionine is supplied separately from all other ingredients, and is thus ideal for substitution of methionine by selenomethionine .

The use of the RTS 9000 also reduces the total amount of selenomethionine required to label sufficient protein for further studies.

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 5.2 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 2.2 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.2.3 • solution 8
3	Feeding Mix Bottle 3, white label	Reconstitute the lyophilizate with 80 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.2.3 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, white label	Reconstitute the lyophilizate with 30 ml of Reconstitution Buffer (bottle 6), mix by shaking	• section 3.2.3 • solution 7 and 8
-	Methionine Bottle 5, white label	THIS BOTTLE IS NOT REQUIRED FOR SELENOMETHIONINE LABELING	
6	Reconstitution Buffer Bottle 6, white cap	• Ready-to-use solution • The solution is stable at 2-8°C but can also be stored at 15 to 25°C	solutions 1, 2, 3, 4, 5

Appearance of solutions

E.coli lysate reconstitutes to a slightly turbid, yellowish solution, feeding mix may also be slightly turbid, this does not impair the performance. All other 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 14 mg D,L-Selenomethionine and 2 mg DTT in 3.6 ml Reconstitution buffer (bottle 6) ***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 26 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml of selenomethione (solution 5a) to solution 3. Mix by gentle shaking.	section 3.1.4
8	Reaction Solution	To the content of solution 1 (*E.coli* lysate), add the total amount of the reconstituted Reaction Mix (2.2 ml solution 2), 2.7 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl of Selenomethionine (solution 5a). Add 120–180 µg of the DNA template in a maximum volume of 500 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 large protein structures via Nuclear Magnetic Resonance (NMR) technologies has recently gained a lot of interest because of major improvements in the available hard- and software.

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 practicle due to metabolism.

In the RTS 9000 *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 substituting 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 5.2 ml of Reconstitution Buffer (bottle 6), mix carefully by gentle shaking. DO NOT VORTEX!	• section 3.3.1.3 • solution 8
2	Reaction Mix Bottle 2, green cap	Reconstitute the lyophilizate with 2.2 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.3.1.3 • solution 8
3	Feeding Mix Bottle 3, white cap	Reconstitute the lyophilizate with 80 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.3.1.3 • solution 7
-	Amino Acid Mix without methionine Bottle 4, white cap	THIS BOTTLE IS NOT REQUIRED FOR INCORPORATION OF UNIFORMLY LABELED AMINO ACIDS	
-	Methionine Bottle 5, white 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 *E.coli* lysate reconstitutes to a slightly turbid, yellowish solution, feeding mix may also be slightly turbid, this does not impair the performance. All other 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 8.4 mM stock solution of all amino acids in 30 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, tryptophan)	• section 3.3.1.3 • solution 7 and 8
5b	DTT	Make a fresh 40 mM stock solution in 3.3 ml Reconstitution Buffer (bottle 6)..	• 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 26 ml of the reconstituted amino acid labeling mix (solution 4a) and 3 ml of DTT-solution (solution 5b). Mix by gentle shaking.	section 3.1.4
8	Reaction Solution	To the content of solution 1 (*E.coli* lysate), add the total amount of the reconstituted Reaction Mix (2.2 ml solution 2), 2.7 ml of the reconstituted Amino Acid labeling mix (solution 4a) and 300 µl of DTT solution (solution 5b). Add 120–180 µg of the DNA template in a maximum volume of 500 µl. Mix carefully by 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 solutions of each individual **un**labeled amino acid. Specific amino acid mixtures can be prepared by including or excluding the appropriate amino acid(s). Yields obtained after exchanging the amino acids provided in the kit with such adapted amino acid mixtures should be in the same range as when using the standard amino acid mixture.

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 soluble and functional proteins. The design of this kit allows the addition of up to 2 ml of supplement solution to the reaction solution and/or 20 ml to the feeding solution (since the cut-off of the membrane separating the reaction from the Feeding Compartment is 10 kD please note that components with a molecular weight $<$5000 D will not be retained in the Reaction Compartment).

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. 3 186 148). 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 (Cat. Nos. 3 246 817, 3 246 949) and finally to the RTS 9000.

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 3.2 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 2.2 ml of Reconstitution Buffer (bottle 6), mix by rolling or shaking.	• section 3.4.2 • solution 8
3	Feeding Mix Bottle 3, white cap	Reconstitute the lyophilizate with 60 ml of Reconstitution Buffer (bottle 6), mix by shaking.	• section 3.4.2 • solution 7
4	Amino Acid Mix without Methionine Bottle 4, white cap	Reconstitute the lyophilizate with 30 ml of Reconstitution Buffer (bottle 6), mix by shaking	• section 3.2.3 • solution 7 and 8
5	Methionine Bottle 5, white cap	Reconstitute the lyophilizate with 3.6 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 2–8°C but can also be stored at −15 to −25°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 26 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml of reconstituted Methionine (solution 5) to solution 3. Finally add 20 ml of solution S or Reconstitution Buffer (bottle 6). Mix by gentle shaking.	section 3.1.4
8	Reaction Solution	To the content of solution 1 (*E.coli* lysate), add the total amount of the reconstituted Reaction Mix (2.5 ml solution 2), 2.7 ml of the reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl of reconstituted Methionine (solution 5). Add 2 ml of solution S or Reconstitution buffer (bottle 6). Finally add 120–180 µg of the DNA template in a maximum volume of 500 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.

3.5 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 promoter located downstream of a RBS (ribosomal binding site) sequence
- distance between T7 promoter 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 must be included at the 3'-end of the gene

General Recommendations The pIVEX vector family has been developed and optimized for use with the *E.coli* Rapid Translation System Kits. Therefore, we strongly recommend cloning target genes into a pIVEX vector. Testing of the expression vectors can be easily performed in the the Rapid Translation System RTS 100 *E.coli* HY Kit (Cat. No. 3 186 148). 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

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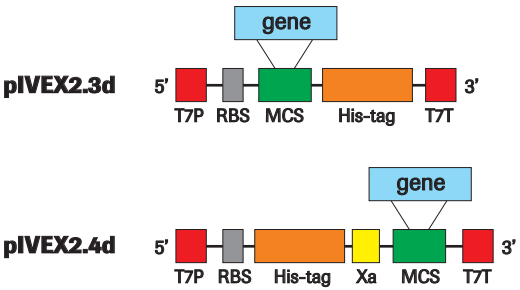

Fig. 3: Functional elements of cloning vectors
Abbreviations:

T7 P	= T7 Promoter
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

The transcription reaction produces a unique mRNA molecule from every individual gene, inserted into an expression vector. It is thus 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 improve expression yields.

4. Typical results

Expression kinetics

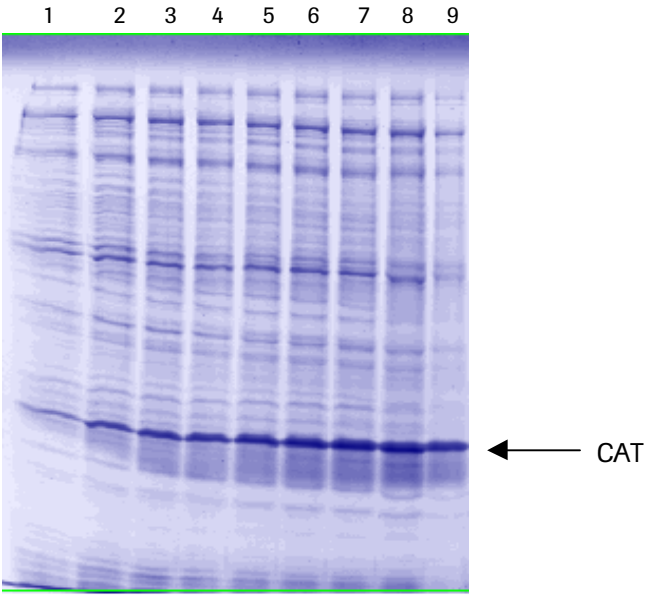

Fig. 4: Kinetics of synthesis of chloramphenicol acetyl transferase (CAT) using the RTS 9000 *E.coli* HY Kit and the RTS ProteoMaster. Samples were taken from the reaction chamber at various time points, diluted (1:60), and 5 µl were subjected to SDS-PAGE analysis and colloidal Coomassie-staining. Lanes from left to right; 1. 0h, 2. 1h, 3. 2h, 4. 3h, 5. 4h, 6. 5h, 7. 6h, 8. 7h, 9. 23h

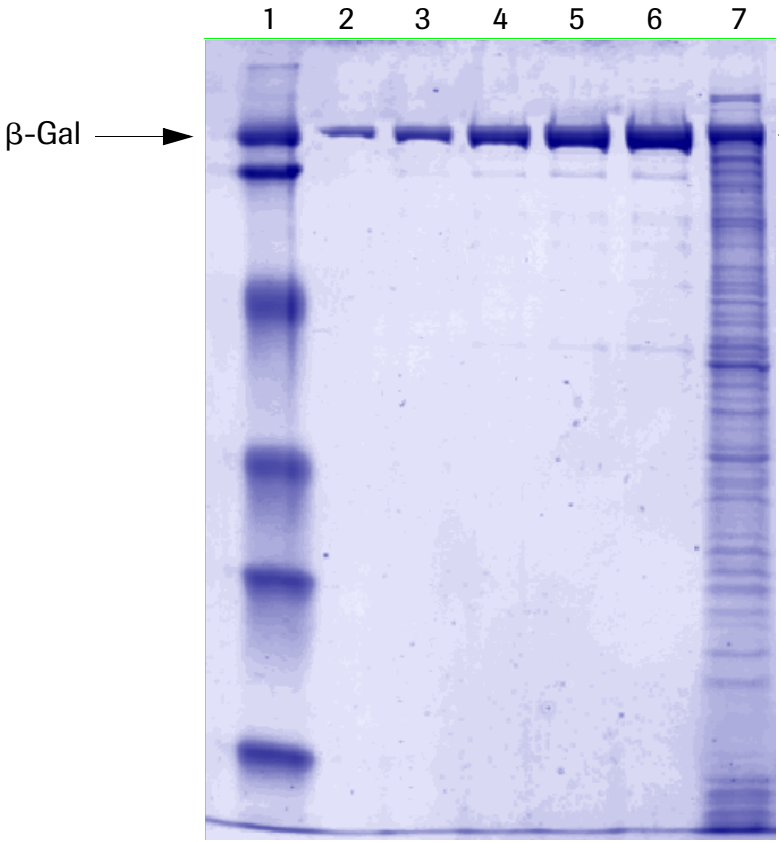

Fig. 5: Expression of β-Galactosidase, monitored on colloidal Coomassie-stained SDS-PAGE gel (dilution 1:60, sample amount 5 µl), lanes from left to right: 1 molecular weight standard; 2.-6. β-Gal reference 0.1 µg, 0.2 µg, 0.4 µg, 0.6 µg, 0.8 µg, 7. RTS 9000 expression experiment 24 h, 30°C, total protein

5. Appendix

5.1 Trouble shooting

Generally: It is recommended to first optimize conditions for protein expression using the RTS 100 and RTS 500 *E.coli* HY Kits and then transfer reaction parameters to the RTS 9000 *E.coli* HY Kit

5.1.1 General problems

Problem	Possible cause	Recommendation
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 correct temperature (−15 to −25°C).	Order a new kit.
	Contamination with RNases	Repeat experiment and take care to work RNase-free at each step.
	Incorrect 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.

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 proteins 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, 0.1% CHAPS, 0.05% C12E10, 0.05% Brij-58, or 2 mM n-octylglycoside 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.

continued on next page

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

Oberservation	Potential Reasons	Recommendation
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 Applied Science

Three ways to contact us

To contact Roche Applied Science 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://www.roche-applied-science.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 ProteoMaster Instrument	1 instrument	3 265 650.
Rapid Translation System RTS 500 Instrument	1 instrument	3 064 859
Rapid Translation System RTS 500 HY	5 reactions	3 246 949
Rapid Translation System RTS 500 HY	2 reactions	3 246 817
Rapid Translation System RTS 100 *E.coli* HY Kit	24 reactions (50 µl each)	3 186 148
Rapid Translation System RTS pIVEX His-tag Vector Set	5 vectors (10 µg each)	3 253 538
Rapid Translation System RTS GroE Supplement	5 × 250 µl	3 263 690
Rapid Translation System RTS Amino Acid Sampler	For five RTS 500 reactions	3 262 154
Restriction Protease Factor Xa	3 × 30 µg 3 × 100 µg 3 × 250 µg	1 179 888 1 585 924 1 179 896
Restriction Protease Factor Xa Cleavage and Removal Kit	Kit I : 3 × 30 µg Kit II: 3 × 100 µg	1 644 777 1 644 785
rGFP	50 µg	1 814 524
Anti-His6	100 µg	1 922 416
Anti-His6-Peroxidase	50 U	1 965 085
Complete Protease Inhibitor Cocktail Tablets, mini, EDTA-free	25 tablets (each tablet for 10 ml extract)	1 836 170

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