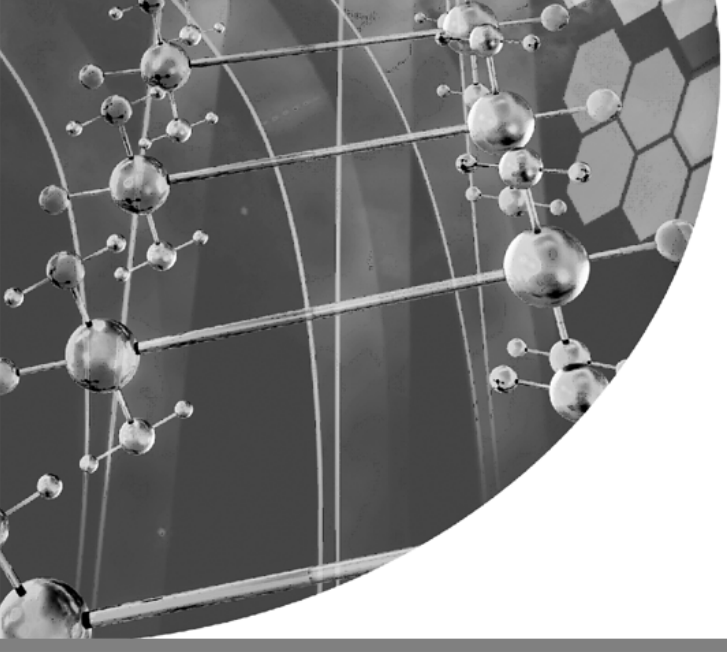

5 PRIME

RTS™ 9000 *E. coli* HY Kit Manual

For cell-free expression of functionally active protein from circular and linear templates

RTS 9000 *E. coli* HY Kit Manual, October, 2009

Contents

Product specifications

The RTS 9000 *E. coli* HY Kit in conjunction with the Eppendorf® Thermomixer Comfort is designed for protein expression in the scale of 5–50 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 Kit and RTS 500 Proteomaster *E. coli* HY Kit. The RTS 9000 *E. coli* HY 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 *E. coli* HY Kit. Proteins in the molecular weight range from 15–120 kDa have been successfully produced. For a current list of expressed proteins, visit www.5PRIME.com.

Product description

The RTS 9000 *E. coli* HY Kit provides the components and procedures necessary for a single 10 ml reaction of cell-free protein expression.

Product limitations

The RTS 9000 *E. coli* HY Kit is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Materials supplied

RTS 9000 *E. coli* HY Kit

Bottle	Contents and function	No. included (for 10 ml reaction)
Ordering number		2401900
E.coli Lysate; 9000 E.coli (Bottle 1, red cap)	→ Enhanced lysate from *E. coli*; stabilized and lyophilized → Contains components for transcription and translation	1 bottle
Reaction Mix; 9000 E.coli (Bottle 2, green cap)	→ Substrate mix to prepare 11 ml reaction solution; stabilized and lyophilized	1 bottle
Feeding Mix; 9000 E.coli (Bottle 3, white cap)	→ Substrate mix to prepare 109 ml feeding solution; lyophilized	1 bottle
Amino Acid Mix w/o Meth.; 9000 E.coli (Bottle 4, white cap)	→ Amino acid mix without Methionine; lyophilized	1 bottle
Methionine; 9000 E.coli (Bottle 5, white cap)	→ Methionine; lyophilized	1 bottle
Reconstitution Buffer; 9000 E.coli (Bottle 6, white cap)	→ 60 ml buffer solution for the reconstitution of lyophilizates in bottles 1–5	2 bottles

Control Vector GFP; 9000 E.coli (Bottle 7, colorless cap)	→ 50 µg lyophilized GFP (Green Fluorescent Protein) expression vector with C-terminal His_6-tag for the positive control reaction	1 bottle
8 RTS 9000 Reaction Device	→ Disposable three-chamber device for CECF protein expression → Device to be used with Eppendorf® Thermomixer Comfort and RTS ProteoMaster Instrument	1 device with one Reaction Compartment
9 Filling Syringe for Feeding Mix; 9000 E.coli	→ Disposable plastic syringe including tube for filling and emptying the RTS 9000 Reaction Device Feeding compartment	1 × 50 ml syringe, including tube
10 Filling/Removal Pasteur Pipette; 9000 E.coli	→ Disposable Pasteur pipets for filling and emptying the RTS 9000 Reaction Device Reaction Compartment	2

Note: Bottles 1, 2, 3, 4, and 5 contain <25% dithiothreitol.

Additional materials

To perform the protocols described in this manual, the following additional materials must be provided by the user:

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

- Eppendorf® Thermomixer Comfort (the kit can also be used with the RTS ProteoMaster Instrument)
- Pipets 100–1,000 µl, 1,000–5,000 µl, graduated 10 ml
- Eppendorf reaction vial (1–1.5 ml)
- Pipet 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, luminometer, or SDS PAGE equipment
- Spatula or screwdriver
- 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 (5 PRIME)

To avoid contamination, all materials should be RNase-free.

For convenience, additional materials to be supplied by the user are listed at the beginning of the protocol for which they are required.

Shipping and storage conditions

The RTS 9000 *E. coli* HY Kit is shipped on dry ice. The RTS 9000 *E. coli* HY Kit and components should be stored in the dark at –15 to –25°C and are stable until the expiration date printed on the label. Avoid repeated freezing and thawing.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of 5 PRIME products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Bottles 1, 2, 3, 4, and 5 contain <25% dithiothreitol. 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 of in waste water in accordance with local regulations. In case of eye contact, flush eyes with water. In case of skin contact, wash with water. In case of ingestion, seek medical advice.

Additional safety information is available from www.5PRIME.com in material safety data sheets (MSDSs) for 5 PRIME products and 5 PRIME product components.

Quality assurance

5 PRIME products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing:** each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing:** each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from www.5 PRIME.com. Certificate of analysis sheets for 5 PRIME products and 5 PRIME product components can be obtained on request.

Product warranty

5 PRIME is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

5 PRIME guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use.

This warranty is in place of any other warranty or guarantee, expressed or implied, instituted by law or otherwise. 5 PRIME provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall 5 PRIME be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by 5 PRIME.

Protocols

Product principle

Introduction

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 byproducts diffuse through the same membrane into the Feeding Compartment. Protein is expressed for up to 24 hours, yielding up to 50 mg of control GFP protein in a single compartment of the RTS 9000 Reaction Device (Figure 1).

Note: This kit cannot introduce post-translational glycosylation, phosphorylation, multiple disulfide bond formation, or signal sequence cleavage.

Advantages of the system include:

- fast
    - kit components mixed in minutes
    - up to 50 mg protein expressed in 24 hours
- flexible
    - easy modification of expression conditions
    - express toxic proteins
    - expression independent of codon usage (due to supplementation of all tRNAs)
    - incorporation of Selenomethionine is possible
    - labeled amino acids can be incorporated to aid nuclear magnetic resonance (NMR) analysis
- reliable
    - good reproducibility ensured by strict quality control

Description of procedure

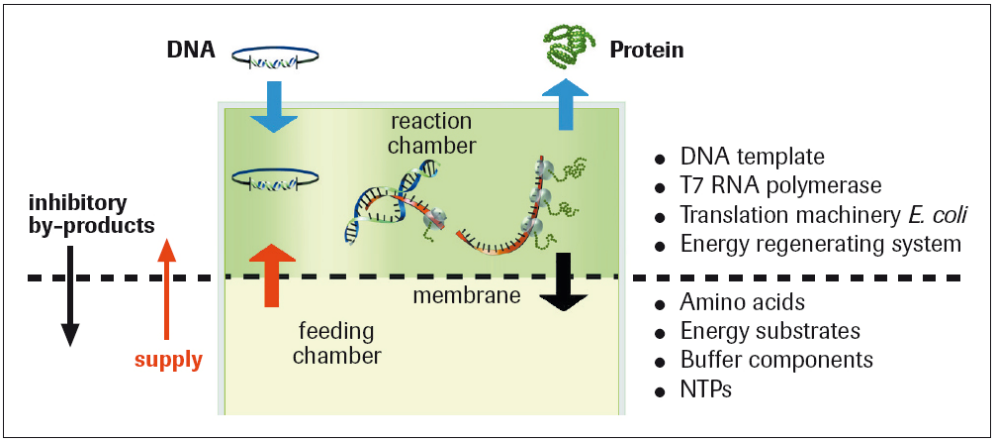

Figure 1. Schematic illustration of CECF protein expression.

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

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 Protocol 1 page 12. 5 PRIME recommends pIVEX vectors, which are optimized for *in vitro* protein expression with the RTS *E. coli* Kits.

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 begin to translate the 5'-end of the nascent mRNA. This system is, therefore, much more productive compared with the use of isolated mRNA.

CECF protein synthesis

During coupled transcription and translation energy components, nucleotides, and amino acids are consumed, and reaction byproducts and other 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 (e.g. NDPs, NMPs, PPi, P, DNA and RNA fragments) and with the replenishment of fresh substrate components (Amino acids, energy substrates, nucleotides) through the semi-permeable

membrane of the RTS 9000 Reaction Device, which separates the Reaction Compartment from the Feeding Compartment, see Figure 2.

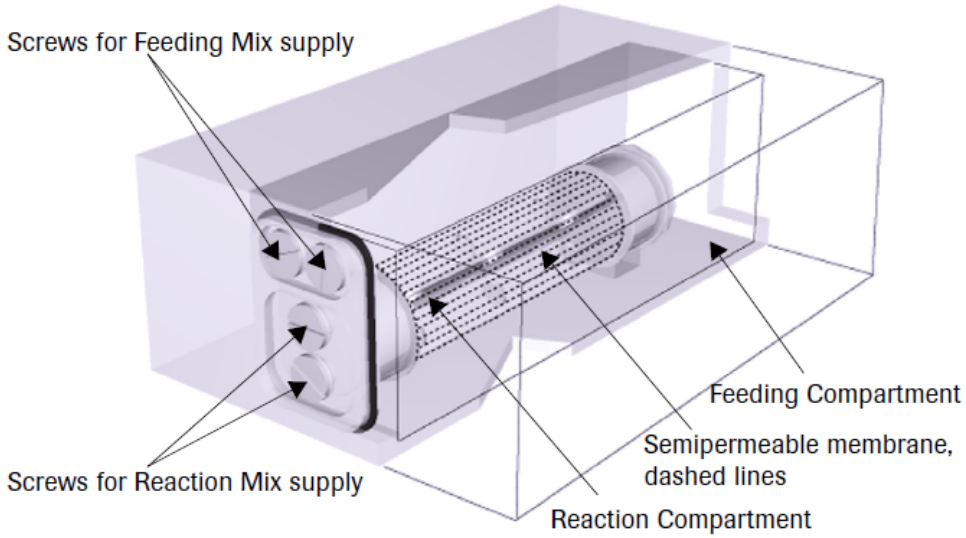

Figure 2. RTS 9000 E. coli HY Reaction Device.

Protocol 1: Preparation of DNA templates for *in vitro* expression

Expression vectors

Any vector used with the RTS must include the following elements and structural features:

- Target gene must be under control of T7 promoter that is located downstream from an RBS (ribosome binding site) sequence
- Distance between T7 promoter and start ATG should not exceed 100 base pairs
- Distance between the RBS sequence and the start ATG should not be more than 5–8 base pairs
- T7 terminator sequence must be present at the 3' end of the gene

General recommendations

The pIVEX vector family has been developed and optimized for use in the RTS. 5 PRIME recommends cloning target genes into a pIVEX vector prior to expression. Testing of the expression vectors can be easily performed using the RTS 100 *E. coli* HY Kit.

Maps of some of the available pIVEX vectors are shown schematically in Figure 3. For more information, visit www.5PRIME.com/RTS.aspx.

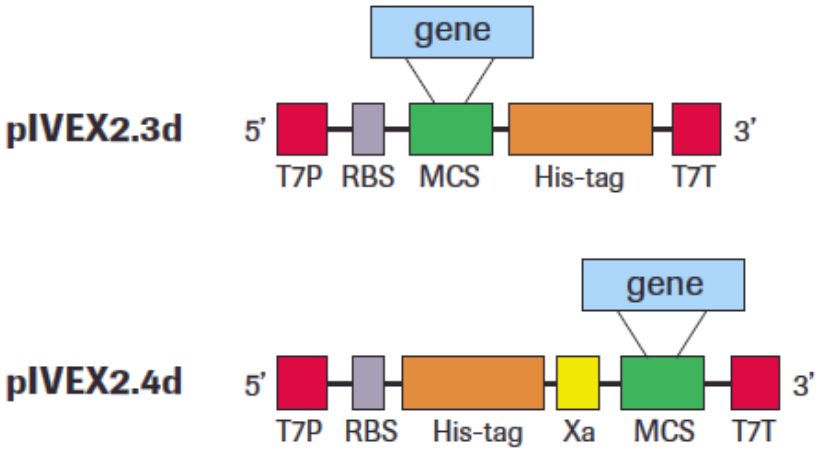

Figure 3. Functional elements of cloning vectors.

***T7P**: T7 Promoter; **RBS**: Ribosome binding site; **His-tag**: C- or N-terminal tag position ; **Xa**: Factor Xa restriction protease cleavage site; **MCS**: Multiple cloning site for the insertion of the target gene; **T7T**: T7 Terminator.*

Purity of the plasmid preparation

Plasmids obtained from commercially available DNA preparation kits (e.g. PerfectPrep EndoFree Plasmid Maxi Kit, 5 PRIME) are usually pure enough to be used as template in the RTS. If DNA is not pure enough (OD260/280 <1.7), use phenol extraction to remove traces of RNase from the preparation, which may enhance its performance in the expression reaction.

Improving success rate

The transcription reaction produces an unique mRNA molecule from every individual gene, inserted into an expression vector. Therefore, it is difficult to predict interactions between coding sequences of the target gene and the 5'-untranslated region, which may potentially interfere with translation. 5 PRIME recommends cloning the gene of interest into more than one expression vector. In particular, N-terminal extensions of the open reading frame have been shown to improve expression yields.

Protocol 2: Standard protein synthesis reaction

Before starting

- For new proteins, 5 PRIME strongly recommends to first optimize expression conditions using the RTS 100 and RTS 500 *E. coli* HY Kits

Equipment and reagents required

- DNA template: Prepare and purify the DNA template as described in Protocol 1
- Eppendorf® Thermomixer Comfort (the RTS ProteoMaster Instrument can also be used)
- Calibrated pipets
- RNase-free plastic and glassware

Reagent notes

- All required reagents are supplied with the kit
- Do not combine reagents from kits with different lot numbers
- For reconstitution of bottles 1–5 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 1–4 immediately before use. This can be done at room temperature (15–25°C)

Procedure

1. Reconstitute the reaction components according to Table 1.

Table 1. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E.coli* Lysate; 9000 E.coli (Bottle 1, red cap)	Reconstitute the lyophilizate with 5.2 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 9000 E.coli (Bottle 2, green cap)	Reconstitute the lyophilizate with 2.2 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 8
3	Feeding Mix; 9000 E.coli (Bottle 3, white cap)	Reconstitute the lyophilizate with 80 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 7
4	Amino Acid Mix w/o Meth.; 9000 E.coli (Bottle 4, white cap)	Reconstitute the lyophilizate with 30 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solutions 7 and 8
5	Methionine; 9000 E.coli (Bottle 5, white cap)	Reconstitute the lyophilizate with 3.6 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 9000 E.coli (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, and 5

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

2. Prepare the working solutions according to Table 2.

Table 2. Working solutions

Solution	Content	Reconstitution procedure	For use in
7	Feeding Solution	Add 26 ml reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml reconstituted Methionine (solution 5) to Feeding Mix (solution 3). Mix by gentle shaking. Total volume of Feeding Solution (solution 3) is 109 ml.	Running an experiment, page 16
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 reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl 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 page 18). 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 (do not vortex). Total volume of reaction solution can be up to 10.9 ml. Do not vortex!	Running an experiment, page 16

Running an experiment

Before starting

- Before loading the Reaction Compartment of the RTS 9000 Reaction Device, the screws for the Reaction Mix supply and for the Feeding Mix supply must be completely removed in order to balance out any pressure differences that have built up during shipping and storage. The screws can be removed with a screwdriver or a spatula
- The reaction compartment of the RTS 9000 Reaction Device must be filled first

Procedure

1. Fill the Reaction Solution (solution 8) through the circular opening using a disposable Pasteur pipet (Figure 4).

   Allow the air to escape through the openings by tipping the device slightly. It is not necessary to remove air bubbles from the reaction compartment.

Figure 4. ***Filling the reaction compartment***

2. Close the screws of the Reaction Mix supply securely.
3. Open the feeding compartment and fill with approximately 100 ml Feeding Solution (solution 7) through the circular opening using a 50 ml disposable syringe equipped with a tube (Figure 5).

   The tube is supplemented in order to easily and securely remove the Feeding Solution from bottle 3.

   Allow the air to escape through the opening by tipping the device slightly. Larger air bubbles should be avoided.

Figure 5. Filling the feeding compartment.

4. Close the screws of the Feeding Mix supply securely.
5. Insert the filled reaction device into the Eppendorf® Thermomixer Comfort (or RTS ProteoMaster Instrument) (Figure 6).
6. Follow the instructions in the instrument manual to set the parameters to a shaking speed of 900 rpm at 30°C and a reaction time of 24 h (optional).

Figure 6. Starting the run.

7. Start the run.

   For more information regarding the parameters, see 'Points to consider' below.
8. Stop the run.
9. Remove the RTS 9000 Reaction Device from the Eppendorf® Thermomixer Comfort.
10. 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.
11. Carefully remove all of the Reaction Solution through the opening using the second Pasteur pipet.
12. Store the Reaction Solution containing the expressed protein at –15 to –25°C or at 2–8°C until purification or further processing.

Points to consider

Shaking speed: Shaking is essential to guarantee homogeneous distribution of components and to accelerate the exchange through the membrane. 5 PRIME recommends a shaking speed of 900 rpm.

Temperature: The optimal 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. However, for unstable proteins the optimum yield of soluble active protein may be produced at shorter reaction times.

GFP batch control reaction

Procedure

1. Reconstitute bottles 1, 2, 3, 4, and 5 according to Table 1, page 15.
2. Briefly centrifuge down the contents of vial 7. Reconstitute the lyophilized Control Vector GFP with 50 µl sterile DNase- and RNase-free water (5 PRIME). 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 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 thermostatic device (e.g. Eppendorf® Thermomixer) at 30°C for 24 hours.
6. The fluorescence of GFP (excitation wavelength 395 nm, emission at 504 nm) can be observed using an UV lamp (360 nm).
7. Optional: Run 1 µl of the reaction on a SDS-polyacrylamide gel. Run a known GFP standard in a separate lane. 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 (5 PRIME).

Oxidation of GFP

GFP needs molecular oxygen to post-translationally form the fluorophore (Coxon & Bestor, 1995).

The yield of correctly folded fluorescent GFP is increased by storing the Reaction Mix for 72 hours at 2–8°C.

Protocol 3: Incorporation of Selenomethionine

Selenomethionine is used for X-ray studies of proteins. It is usually added to the fermentation medium of growing cells and is incorporated into the protein *in vivo*. Incorporation into the protein is normally incomplete because the Selenomethionine is metabolized and is contaminated with the naturally occurring Methionine. Therefore, the labeled protein molecules differ in molecular weight and Selenomethionine content, which can potentially complicate the crystallization process.

Methionine is supplied separately from all other kit components in the RTS 9000 *E. coli* HY Kit. Therefore the kit is highly suited for the substitution of Methionine by Selenomethionine.

The use of RTS 9000 *E. coli* HY Kit also reduces the amount of Selenomethionine required to express sufficient protein for further studies.

Before starting

- Selenomethionine is highly toxic. Ensure appropriate safety precautions are taken when handling the reagent
- Selenomethionine is not supplied with this kit, but is available in crystalline form from several suppliers
- Prepare a working solution of Selenomethionine according to Table 3

Table 3. Preparation of a Selenomethionine working solution

Solution	Contents	Preparation of working solution	For use in
5a	Seleno-methionine	→ **Dissolve 14 mg D,L-Selenomethionine and 2 mg DTT in 3.6 ml Reconstitution Buffer (bottle 6) at 30°C.** Selenomethionine is sensitive to air oxidation. DTT is only required if the solution is to be stored for >1 day. The solution can be frozen and thawed up to 10 times.	Step 2 Solutions 7 and 8

Procedure

1. Reconstitute the reaction components according to Table 4.

Table 4. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E.coli* Lysate; 9000 E.coli (Bottle 1, red cap)	Reconstitute the lyophilizate with 5.2 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 9000 E.coli (Bottle 2, green cap)	Reconstitute the lyophilizate with 2.2 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 8
3	Feeding Mix; 9000 E.coli (Bottle 3, white cap)	Reconstitute the lyophilizate with 80 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 7
4	Amino Acid Mix w/o Met; 9000 E.coli (Bottle 4, white cap)	Reconstitute the lyophilizate with 30 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 9000 E.coli (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, and 5

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

2. Prepare the working solutions according to Table 5.

Table 5. Working solutions

Solution	Content	Reconstitution procedure	For use in
7	Feeding Solution	Add 26 ml reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml Selenomethionine (solution 5a) to Feeding Mix (solution 3). Mix by gentle shaking.	Running an experiment, page 16
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 reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl 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).	Running an experiment, page 16

3. Proceed with the protocol, see 'Running an experiment', page 16.

Protocol 4: Production of labeled proteins for NMR spectroscopy

There has been increased interest in determining protein structures via NMR since the available hardware and software have improved.

In *E. coli*, labeled protein for NMR experiments is usually produced by adding ^{15}N-ammonium chloride and ^{13}C-labeled glucose to the medium of growing cells, with the result being protein with uniformly ^{15}N and/or ^{13}C-labeled amino acids. However, selective incorporation of single, labeled amino acids *in vivo* is difficult or impossible.

In the RTS 9000 *E. coli* HY Kit, the amino acids are provided separately from all other reagents necessary to drive the reaction (e.g., reaction mix, feeding mix). This kit design facilitates an easy exchange of the amino acids with other desired amino acid mixtures, allowing the following applications:

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

Labeled amino acids are not supplied with this kit, but available in crystalline form from several suppliers.

Preparing amino acid mixtures

For the specific incorporation of selected amino acids, an amino acid mixture must be prepared according to the experimental requirements. The RTS Amino Acid Sampler (5 PRIME) provides appropriate stock solutions of each individual unlabeled 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 (Protocol 3, page 19) can be followed, using the Amino Acid Mix without Methionine provided in this kit.

Procedure

1. Reconstitute the reaction components according to Table 6.

Table 6. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E. coli* Lysate; 9000 E.coli (Bottle 1, red cap)	Reconstitute the lyophilizate with 5.2 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 9000 E.coli (Bottle 2, green cap)	Reconstitute the lyophilizate with 2.2 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 8
3	Feeding Mix; 9000 E.coli (Bottle 3, white cap)	Reconstitute the lyophilizate with 80 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 7
4a	Amino Acids Labeling Mix	Make an 8.4 mM stock solution of all amino acids in 30 ml Reconstitution Buffer (bottle 6). Check the certificate of analysis received from the supplier. Ensure 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).	Step 2 Solutions 7 and 8
5b	DTT	Make a fresh 40 mM stock solution in 3.3 ml Reconstitution Buffer (bottle 6).	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 9000 E.coli (Bottle 6, white cap)	→ Ready-to-use solution → The solution is stable at 2–8°C, but can also be stored at 15–25°C	Solutions 1, 2, 3, 4, and 5

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

2. Prepare the working solutions according to Table 7.

Table 7. Working solutions

Solution	Content	Reconstitution procedure	For use in
7	Feeding Solution	Add 26 ml reconstituted Amino Acid Labeling Mix (solution 4a) and 3 ml DTT (solution 5b) to Feeding Mix (solution 3). Mix by gentle shaking.	Running an experiment, page 16
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 reconstituted Amino Acid Labeling Mix (solution 4a) and 300 µl DTT (solution 5b). 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).	Running an experiment, page 16

3. Proceed with the protocol, see 'Running an experiment', page 16.

Protocol 5: Addition of supplements

Depending on the nature of the target protein, it may be necessary to add chemicals (e.g. detergents) or supplements (e.g. chaperones) in order to produce soluble and functional protein. 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 (keep in mind that the addition of high-molecular-weight components to the feeding solution is ineffective if the molecular weight is >5000 Da since the cut-off of the membrane separating the reaction from the feeding compartment is 10 kDa). Note that components with a molecular weight <5,000 Da will not be retained in the Reaction Compartment.

For efficient optimization of reaction conditions 5 PRIME recommends to study the effects (yield, solubility) of additional chemicals or supplements in the RTS 100 *E. coli* HY Kit. If a positive effect is observed in the 50 µl reaction volume of the RTS 100 HY Kit, the conditions can be applied in the 1 ml reaction volume of the RTS 500 *E. coli* HY Kit, and finally to the RTS 9000 *E. coli* HY Kit.

Procedure

1. Reconstitute the reaction components according to Table 8.

Table 8. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E.coli* Lysate; 9000 E.coli (Bottle 1, red cap)	Reconstitute the lyophilizate with 3.2 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 9000 E.coli (Bottle 2, green cap)	Reconstitute the lyophilizate with 2.2 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 8
3	Feeding Mix; 9000 E.coli (Bottle 3, white cap)	Reconstitute the lyophilizate with 60 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solution 7
4	Amino Acid Mix w/o Meth.; 9000 E.coli (Bottle 4, white cap)	Reconstitute the lyophilizate with 30 ml Reconstitution Buffer (bottle 6), mix by shaking.	Step 2 Solutions 7 and 8
5	Methionine; 9000 E.coli (Bottle 5, white cap)	Reconstitute the lyophilizate with 3.6 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 9000 E.coli (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, and 5
S	Supplement	Prepare a stock solution of the particular chemical or supplement using Reconstitution Buffer (bottle 6).	Step 2 Solutions 7 or 8

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

2. Prepare the working solutions according to Table 9.

Table 9. Working solutions

Solution	Content	Reconstitution procedure	For use in
7	Feeding Solution	Add 26 ml reconstituted Amino Acid Mix without Methionine (solution 4) and 3 ml reconstituted Methionine (solution 5) to Feeding Mix (solution 3). Add 20 ml solution S or Reconstitution Buffer (bottle 6). Mix by gentle shaking.	Running an experiment, page 16
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 reconstituted Amino Acid Mix without Methionine (solution 4) and 300 µl reconstituted Methionine (solution 5). Add 2 ml solution S or Reconstitution Buffer (bottle 6). 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).	Running an experiment, page 16

3. Proceed with the protocol, see 'Running an experiment', page 16.

Supporting information

Typical results

Expression kinetics

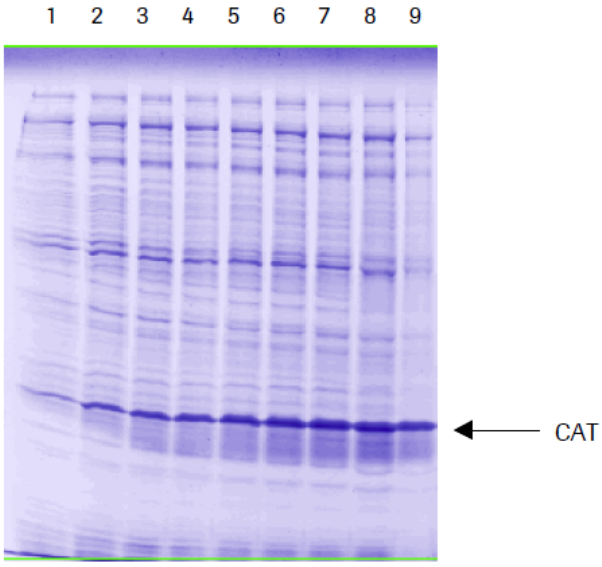

Figure 7. Kinetics of synthesis of chloramphenicol acetyl transferase (CAT) using the RTS 9000 E. coli HY Kit and the RTS ProteoMaster.

Samples were taken from the reaction chamber at various time points, diluted (1:60), and 5 µl were analyzed using SDS-PAGE and colloidal Coomassie staining. ***Lanes 1****: 0 h;* ***2****: 1 h;* ***3****: 2 h;* ***4****: 3 h;* ***5****: 4 h;* ***6****: 5 h;* ***7****: 6 h;* ***8****: 7 h;* ***9****: 23 h.*

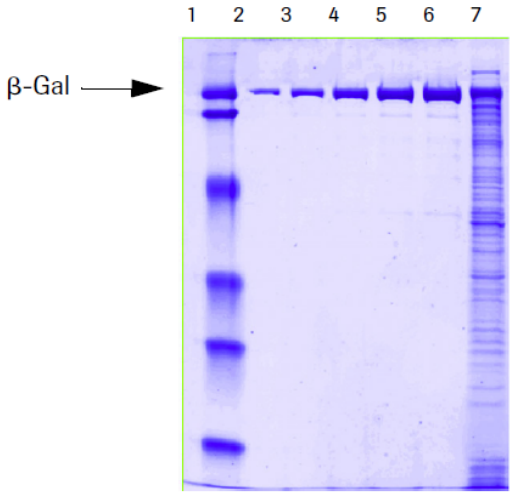

Figure 8. Expression of β-Galactosidase, monitored on colloidal Coomassie-stained SDS-PAGE gel (dilution 1:60, sample amount 5 µl),

Lanes 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.*

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Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

General problems

5 PRIME recommends that conditions for protein expression are first optimized using the RTS 100 and RTS 500 *E. coli* HY Kits. The reaction parameters can then be applied to the RTS 9000 *E. coli* HY Kit.

Observation	**White precipitate in the Feeding/Reaction Solution after the run**
Possible cause	Insoluble salts are formed
Resolving	The expression is not usually affected by the precipitate.

Observation	**No expression using the GFP control reaction**
Possible cause	Kit expired
Resolving	Order a new kit.
Possible cause	Kit has not been stored at –15 to –25°C
Resolving	Order a new kit.
Possible cause	Contamination with RNases
Resolving	Repeat experiment and ensure to work RNase-free at every step.
Possible cause	Incorrect handling
Resolving	Repeat experiment exactly according to the instructions.

Possible cause	A kit component is inactive or degraded
Resolving	Contact 5 PRIME technical services.

Problems expressing the target protein, although the GFP control reaction works

Observation	**Sufficient protein expression, but low yield of active protein**
Possible cause	Incorrect folding of protein due to dependence on cofactors
Resolving	Add necessary cofactors.

Possible cause	Necessity of disulfide bond formation
Resolving	Proteins with more than three disulfide bonds cannot be expressed in a functional form in the RTS *E. coli* system. For proteins with up to three disulfide bonds, allow oxidation after the reaction for the formation of disulfide bonds [1, 2]

Possible cause	Dependence on secondary modifications
Resolving	The *E. coli* lysate cannot introduce post-translational modifications, such as glycosylation, phosphorylation, or signal sequence cleavage.

Possible cause	Dependence on chaperones
Resolving	Add chaperones [3].

Observation	Product appears in the pellet after centrifugation
Possible cause	Aggregation
Resolving	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).

Observation	Low expression yield
Possible cause	Expression time too short
Resolving	Extend expression time.
Possible cause	The tag interferes with protein folding
Resolving	Use different pIVEX vectors to prepare the expression template.

Observation	No expression of the target gene, but normal expression of GFP
Possible cause	Cloning error
Resolving	Check the sequence.
Possible cause	Low purity of DNA template
Resolving	Ensure the absorbance ratio 260 nm/280 nm is at least 1.7. Perform a phenol extraction if purity is low. Make a new plasmid preparation.

Possible cause	Contamination with RNases
Resolving	Repeat experiment and be sure to work RNase-free at every step.

Possible cause	No initiation of translation due to strong secondary structures of the mRNA
Resolving	Use different pIVEX vectors to prepare the expression template. Try to express the protein as an N-terminally tagged protein.

Possible cause	Expressed protein interferes with the translation or transcription process
Resolving	Express gene of interest together with GFP. If GFP expression is inhibited, the target protein cannot be expressed with the kit.

Observation	**Several product bands on SDS-PAGE or product smaller than expected**
Possible cause	Proteolytic degradation
Resolving	Add protease inhibitors to reaction. For example: Dissolve 1 tablet of Complete Mini, EDTA-free (Roche) for a 1 ml reaction mix. Use up to 10 mM EGTA.

Possible cause	Internal initiation site
Resolving	Eliminate the corresponding Methionine by point mutation.

Possible cause	Premature termination of translation
Resolving	Check sequence of target gene for incorrect reading frame or mutation that produces a stop codon. Search for strong secondary structures in the mRNA and eliminate them by using conservative mutations.

Ordering information

Product	Size	Order/ref. no.
RTS *E. coli* LinTempGenSet, His- tag	96 reactions	2401000
RTS 100 *E. coli* HY Kit	24 reactions	2401100
RTS 100 *E. coli* HY Kit	96 reactions	2401110
RTS 500 ProteoMaster™ *E. coli* HY Kit	5 reactions	2401500
RTS 500 *E. coli* HY Kit	5 reactions	2401510
RTS 9000 *E. coli* HY Kit	1 reaction	2401900
RTS pIVEX His_6-Tag 2^{nd} Gen. Vector Set	2 vectors, 10 µg each	2401010
RTS Wheat Germ LinTempGenSet, His_6-tag	96 reactions	2402000
RTS pIVEX Wheat Germ His_6-tag Vector Set	10 µg each	2402010
RTS 100 Wheat Germ CECF Kit	24 reactions	2402100
RTS 500 Wheat Germ CECF Kit	5 reactions	2402500
RTS GroE Supplement	For 5 RTS 500 reactions	2401030
RTS DnaK Supplement	1 set	2401020
RTS Amino Acid Sampler	For 5 RTS 500 reactions	2401530
Water, Molecular Biology grade	10 x 50 ml	2500010
Anti·His Antibody Selector Kit (Mouse Tetra·His Antibody, Mouse Penta·His Antibody, Mouse RGS·His Antibody)	3 µg each; lyophilized, for 30 ml working solution	2400300
Penta·His Antibody, BSA free	100 µg; lyophilized, for 1,000 ml working solution	2400320
Penta·His HRP Conjugate Kit	for 250 ml working solution	2400410
RGS·His Antibody	100 µg	2400330
RGS·His Antibody, BSA-free	100 µg	2400340
Tetra·His Antibody, BSA free	100 µg; lyophilized, for 1,000 ml working solution	2400310
PerfectPrep Spin Mini Kit	50 Preps	2300100
PerfectPrep EndoFree Plasmid Maxi Kit	10 Preps	2300120

5 PRIME distributors

A complete list of 5 PRIME distributors is available from www.5PRIME.com.