RTS™ 500 ProteoMaster™ *E. coli* HY Kit Manual

For medium-scale cell-free expression of functionally active protein from circular DNA templates

RTS 500 ProteoMaster *E. coli* HY Manual, April, 2015

Contents

Product specifications

The RTS 500 ProteoMaster *E. coli* HY Kit is designed for protein expressions in the scale of 0.5 to 6 mg in a 1 ml reaction volume. Proteins varying in molecular weight from 8.5 to 120 kDa have been successfully synthesized.

Product description

The RTS 500 ProteoMaster *E. coli* HY Kit contains reagents and reaction devices for five coupled transcription/translation reactions and a control vector encoding chloramphenicol acetyltransferase (CAT). The kit provides reagents and disposable devices for five 1 ml reactions. Reagents for each reaction are bottled separately, except bottle 5 (Methionine) which contains sufficient reagent for all reactions.

The kit is designed to be used in combination with the Eppendorf® Thermomixer Comfort. It can also be used with the RTS ProteoMaster Instrument (Roche). It can not be used with the RTS 500 Instrument.

Product limitations

The RTS 500 ProteoMaster *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

Kit	Contents and function	No. included (for 5 x 1 ml reaction)
Ordering number	BR1400201	
E. coli Lysate; 500 PM *E.coli* (Bottle 1, red cap)	→ Lysate from *E. coli*; stabilized and lyophilized → Contains components for transcription and translation	5 bottles
Reaction Mix; 500 PM *E.coli* (Bottle 2, green cap)	→ Substrate mix to prepare 1.1 ml Reaction Solution; stabilized and lyophilized	5 bottles
Feeding Mix; 500 PM *E.coli* (Bottle 3, blue cap)	→ Substrate mix to prepare 11 ml Feeding Solution; stabilized and lyophilized	5 bottles
Amino Acids.; 500 PM *E.coli* (Bottle 4, brown cap)	→ Mix of 19 amino acids without Methionine; stabilized and lyophilized	5 bottles
Methionine; 500 PM *E.coli* (Bottle 5, yellow cap)	→ Methionine; stabilized and lyophilized	1 bottle
Reconstitution Buffer; 500 PM *E.coli* (Bottle 6, white cap)	→ Buffer solution for the reconstitution of bottles 1, 2, 3, 4, and 5	2 bottles
Control Vector CAT; 500 PM *E.coli* (Bottle 7, colorless cap)	→ 25 µg lyophilized plasmid CAT (Chloramphenicol acetyltransferase) expression vector with C-terminal His_6-tag for the control reaction	1 vial
Reaction Device	→ Disposable device for Continuous Exchange Cell-Free (CECF) protein expression → For use in the Eppendorf® Thermomixer Comfort or the RTS ProteoMaster Instrument → Does NOT fit into the RTS 500 Instrument	5 devices

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:

- Eppendorf® Thermomixer Comfort or the RTS ProteoMaster Instrument
- Pipets 10–200 µl, 200–1,000 µl, graduated 10 ml
- Pipet tips autoclaved at 121°C for 20 minutes (RNase-free)
- 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

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 500 ProteoMaster *E. coli* HY Kit is shipped on dry ice.

Upon delivery, remove the reaction devises from the box.

The RTS 500 ProteoMaster *E. coli* HY Kit and components should be stored at –15 to –25°C in the dark 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 biotechrabbit 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. Observe the usual precautions when handling chemicals. After use, reagents can be discarded in wastewater in accordance with local regulations. If reagent gets in your eyes, flush eyes with water. If reagent gets on your skin, wash off with water. If you swallow a reagent, seek medical advice.

Selenomethionine is highly toxic. Users should know how to handle the reagent safely before using it in a labeling experiment.

Quality assurance

biotechrabbit 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.biotechrabbit.com. Certificates of Analysis are available on request.

Product warranty

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

biotechrabbit 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. biotechrabbit provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall biotechrabbit 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 biotechrabbit.

Protocols

Product principle

Introduction

The Rapid Translation System (RTS) technologies have been combined in a family of products designed to work together, overcoming expression limitations of other cell-free systems. Within the RTS product family, the RTS 500 platform is designed for medium-scale protein expression.

The RTS 500 ProteoMaster *E. coli* HY Kit allows expression of up to 6 mg of protein in 24 hours starting from circular DNA templates. Figure 1 shows how the RTS 500 ProteoMaster *E. coli* HY Kit is integrated in the entire RTS workflow and the RTS product portfolio.

Description of procedure

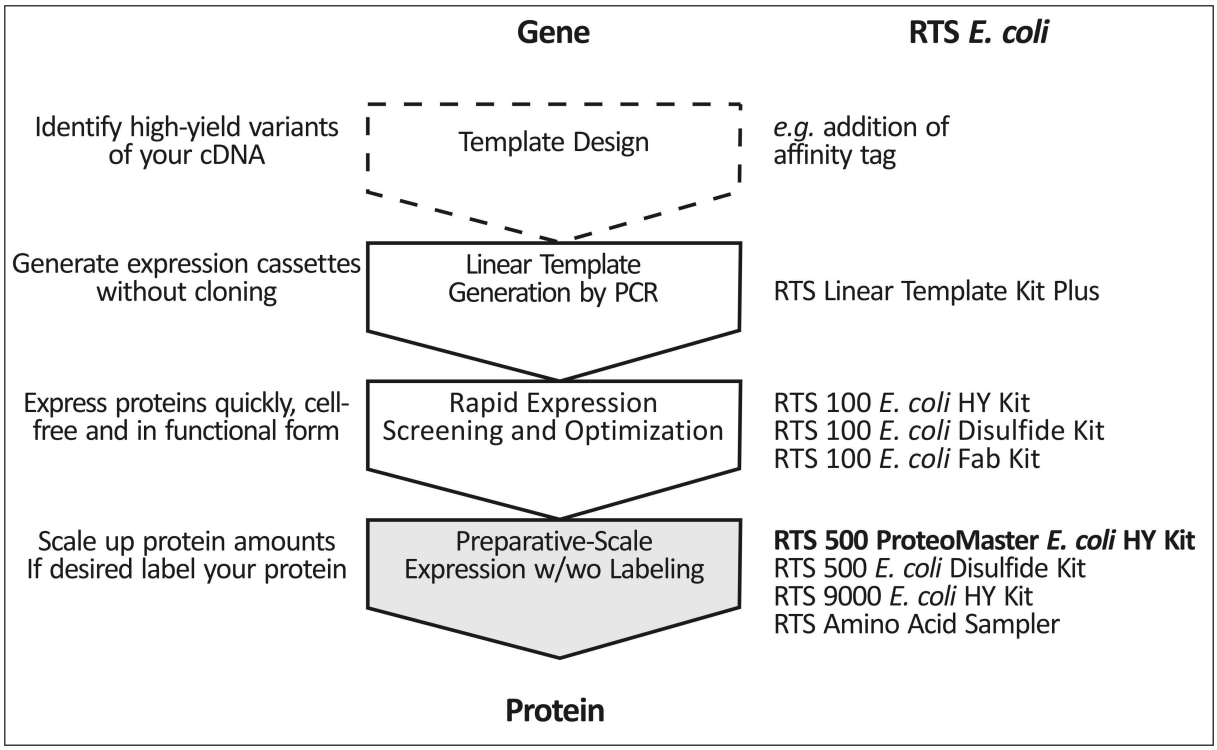

Figure 1. RTS workflow.

For further information about the RTS platforms and other RTS products, visit the RTS web site www.biotechrabbit.com

Reaction principle

RTS uses a coupled transcription/ translation reaction for *in vitro* protein synthesis. Transcription and translation take place simultaneously in the reaction: While the T7-RNA-polymerase transcribes the template gene, the ribosomes provided by the *E. coli* lysate starts to translate the 5'-end of the nascent mRNA (Figure 2). This makes the system highly productive compared to the use of isolated mRNA.

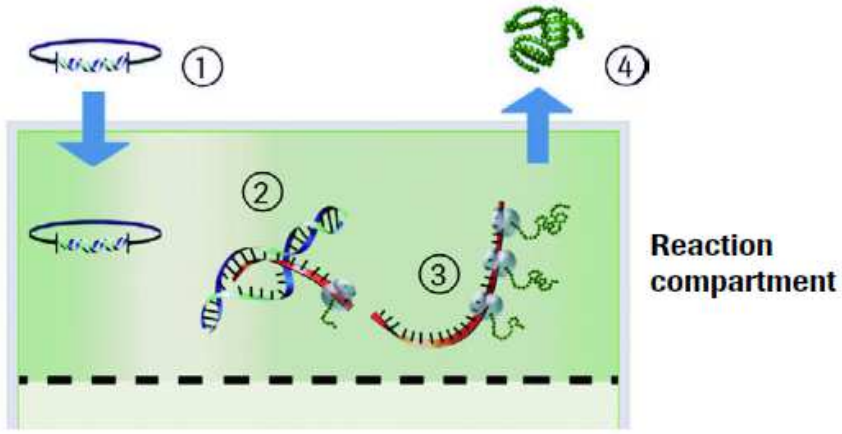

Figure 2. Schematic illustration of the coupled transcription/translation reaction.

Firstly, an expression plasmid carrying the gene of interest is added to the reaction compartment. In a coupled *in vitro* reaction, the DNA is first transcribed from the template vector (1) into mRNA by T7 RNA polymerase (2), followed by translation by the ribosomal machinery present in the *E. coli* lysate (3). Expressed protein accumulates in the reaction compartment and is harvested after a 4–24 hours (4).

To make the coupled transcription/translation reaction highly efficient, the RTS 500 ProteoMaster *E. coli* HY Kit includes a biochemically enhanced High-Yield (HY) *E. coli* lysate and a reaction device based on Continuous Exchange Cell-Free (CECF) principle. The combination of HY and CECF technology dramatically increases the productivity of protein synthesis and allows the expression of up to 6 mg of protein in one RTS 500 ProteoMaster *E. coli* HY reaction.

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

Advantages of the RTS 500 ProteoMaster *E. coli* HY Kit include:

- fast – proteins expressed within 24 hours
- cell-free
  - only the protein of interest is expressed
  - expression of toxic proteins
  - expression is independent of codon usage due to the excess of all t-RNA species
- versatile
  - easy adaptation of expression conditions by changing temperature, adding detergents, chaperones or protease inhibitors
  - proteins can be easily labeled with Selenomethionine for X-ray analysis
  - labeled amino acids for NMR-analysis of the protein can be introduced.

CECF principle

The CECF principle of the RTS 500 ProteoMaster device (Figure 3) is based on the exchange of substrates and byproducts through two semi-permeable membranes. During the coupled transcription/translation reaction, which takes place in the 1 ml reaction compartment, energy components, nucleotides, and amino acids are consumed, whereas reaction byproducts are formed, potentially inhibiting the reaction. Using the CECF principle, substrates essential for a sustained reaction are continuously supplied from the 10 ml feeding chamber into the 1 ml reaction chamber via the two semi-permeable membranes. At the same time, inhibitory byproducts are diluted through the same membranes into the 10 ml feeding compartment. This allows protein expression continue for up to 24 hours.

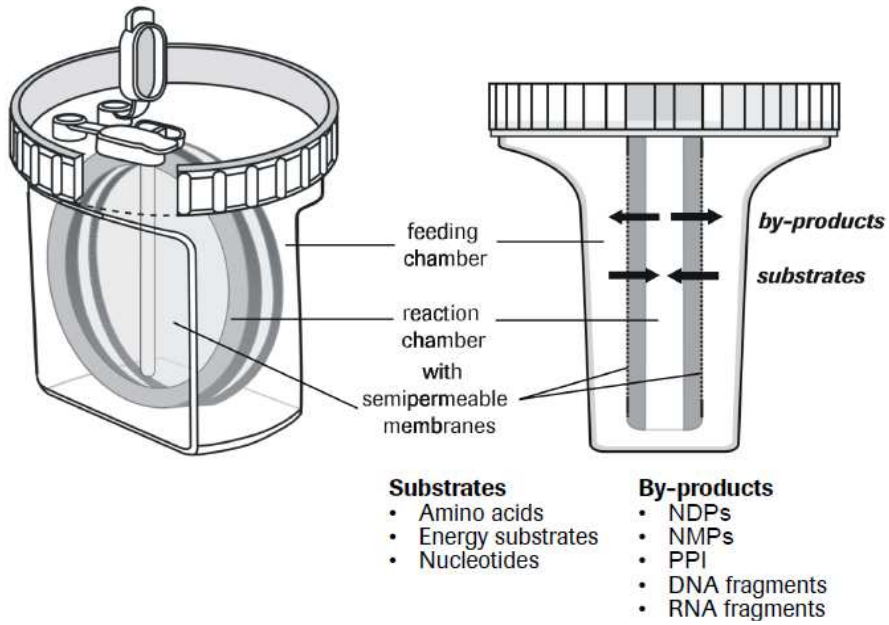

Figure 3. Illustration of the RTS 500 ProteoMaster device.

Template DNA

The DNA of interest must be cloned into a vector, designed for prokaryotic *in vitro* protein expression and containing a T7 promoter. The requirements are described in Protocol 1, page 11. biotechrabbit recommends pIVEX vectors (cat. no. BR1400701), which are optimized for *in vitro* protein expression in RTS. The pIX3.0 Vector (cat. no. BR1402301) with cloned PCR product of the RTS Linear Template Kit Plus (cat. no. BR1402401) is also recommended.

Protocol 1: Preparation of DNA templates

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. biotechrabbit recommends cloning target genes into a pIVEX vector prior to expression.

Maps of some of the available pIVEX vectors are shown schematically in Figure 4. For more information, visit www.biotechrabbit.com

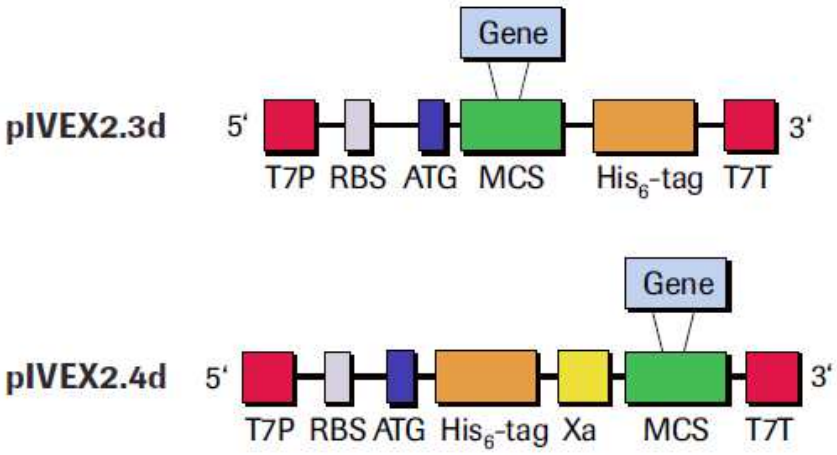

Figure 4. Functional elements of cloning vectors.

T7P: T7 Promoter; **RBS**: Ribosome binding site; **His_6-Tag**: C- or N-terminal tag position; **ATG**: Start codon; **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. GenUP™ Plasmid Kit and GenUP™ Plasmid Plus Kit, cat. no. BR0700201 and BR0701201, biotechrabbit) 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.

Protocol 2: Standard protein synthesis reaction

Before starting

biotechrabbit strongly recommends that the expression yield of sequences is first evaluated by performing a small-scale expression reaction using the RTS 100 *E. coli* HY Kit (BR1400101). In case of low expression yield, take the following steps:

- Generation of optimized PCR templates using the RTS Linear Template Kit Plus (see www.biotechrabbit.com for more information)
- Certification of expression yield using the RTS 100 *E. coli* HY Kit (biotechrabbit)
- Cloning of optimized templates into a plasmid vector for use in a RTS 500 ProteoMaster *E. coli* HY reaction. biotechrabbit recommends cloning the linear templates into the RTS pIX3.0 Vector (biotechrabbit)

Equipment and reagents required

- DNA template: Prepare and purify the DNA template as described in Protocol 1, page 11
- Use the Eppendorf® Thermomixer Comfort in combination with the RTS 500 Adapter (biotechrabbit) or the RTS ProteoMaster Instrument to guarantee optimal reaction performance and to run up to six reactions in parallel. The RTS 500 ProteoMaster *E. coli* HY Kit cannot be used with the RTS 500 Instrument
- Calibrated pipets
- RNase-free plastic and glassware
- For precise quantification of the control reaction with CAT, analyze the reaction products by SDS PAGE

Reagent notes

- All required reagents are supplied with the kit
- Do not combine reagents from different kit lots or other RTS 500 Kits (RTS 500 *E. coli* Disulfide Kit, RTS 500 *E. coli* HY Kit)
- 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 (vial 7) use sterile DNase- and RNase-free water
- Reconstitution Buffer can be thawed in a water bath at 25°C
- Store the reconstituted control plasmid at –15 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

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; 500 PM *E.coli* (Bottle 1, red cap)	Reconstitute the lyophilizate with 0.525 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 500 PM *E.coli* (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.25 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 8
3	Feeding Mix; 500 PM *E.coli* (Bottle 3, blue cap)	Reconstitute the lyophilizate with 8.1 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 7
4	Amino Acids; 500 PM *E.coli* (Bottle 4, brown cap)	Reconstitute the lyophilizate with 3 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
5	Methionine; 500 PM *E.coli* (Bottle 5, yellow cap)	Reconstitute the lyophilizate with 1.8 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 500 PM *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

All reconstituted solutions should be clear, with the exception of the *E. coli* lysate, which remains cloudy.

2. Prepare the working solutions according to Table 2.

Table 2. Working solutions

Solution	Content	Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acids (solution 4) and 0.3 ml of reconstituted Methionine (solution 5) to Feeding Mix (solution 3). Mix by rolling or shaking. Total volume of feeding solution is 11 ml.	Running an experiment, page 15
8	Reaction Solution	To solution 1 (*E. coli* Lysate), add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acids (solution 4) and 30 µl of reconstituted Methionine (solution 5). Optional: To check that pipetting was done correctly, mix carefully by rolling or gentle shaking. Then remove a 50 µl aliquot and follow the instructions in 'CAT control reaction – batch reaction', page 17. Add 10–15 µg of the DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. The total volume of reaction solution is 1.1 ml. Do not vortex!	Running an experiment, page 15

Running an experiment

The reaction compartment must be filled first.

Procedure

1. Open the red lid of the 1 ml reaction compartment.
2. Fill the Reaction Solution (solution 8) through the circular opening using a 1 ml pipet (Figure 5).

   Allow the air to escape through the oval opening. Do not use the oval opening to fill the chamber. It is not necessary to remove air bubbles from the reaction compartment.

   Figure 5. Filling the reaction compartment.
3. Close the lid securely.
4. Open the colorless lid of the feeding compartment.
5. Fill with Feeding Solution (solution 7) through the circular opening using a 10 ml glass pipet (Figure 6).

   Allow the air to escape through the oval opening. It is not necessary to remove air bubbles from the reaction compartment.

   Figure 6. Filling the feeding compartment.
6. Close the lid securely.

7. Insert the filled reaction device (using the RTS 500 Adapter) into the Eppendorf® Thermomixer Comfort (the RTS ProteoMaster Instrument can also be used) (Figure 7).

Figure 7. Starting the run.

8. Follow the instructions in the instrument manual to set the standard parameters to a shaking speed of 800 rpm at 32°C and a reaction time of 20 h (optional).
9. Start the run.

   For more information regarding the parameters, see 'Points to consider' below.
10. Stop the run.
11. Remove the Reaction Device from the Eppendorf® Thermomixer Comfort (or the RTS ProteoMaster Instrument).
12. Open the reaction compartment (red lid).
13. Remove Reaction Solution through the circular opening using a 1 ml pipet (Figure 8).

Figure 8. End of the run.

14. The sample is ready for analysis (e.g., gel electrophoresis, Western blotting).

    If the protein has a molecular weight <12 kDa, also check the Feeding Solution for the protein, as it may have crossed the membrane (10 kDa cut-off).

Points to consider

Shaking speed: Shaking is essential to guarantee homogeneous distribution of components and to accelerate the exchange through the membrane. biotechrabbit recommends a shaking speed of 800 rpm.

Temperature: The optimal temperature for most proteins is 30°C. However, lower temperatures may be used for proteins that tend to aggregate. Please note that for an effective temperature of 30°C in the Reaction Device the Eppendorf® Thermomixer Comfort must be set to 32°C.

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.

CAT control reaction

There are two options for running a control experiment:

- a batch reaction to check if pipetting was carried out correctly
- using the CECF-device to compare the yield of the target protein to a control protein

The control protein, chloramphenicol acetyltransferase (CAT) is expressed in the control experiment.

Batch reaction: Procedure

1. Briefly centrifuge down the contents of vial 7. Reconstitute the lyophilized Control Vector CAT with 25 µl sterile DNase- and RNase-free water (concentration 1 µg/µl). The solution is stable at –15 to –25°C.
2. Take a 50 µl aliquot of your reaction solution (table 2 of Protocol 2, page 14) before adding your DNA template
3. Add 1 µl of the reconstituted CAT vector solution.
4. Run the batch reaction for 2 hours at 30°C in a water bath or thermomixer. Analyze via Coomassie®-stained SDS-gel or Western blot using an anti-His_6 antibody (e.g. 5 PRIME).

CECF reaction: Procedure

1. Reconstitute bottles 1, 2, 3, 4, and 5 according to table 1 of Protocol 2, page 13.
2. Briefly centrifuge down the contents of vial 7. Reconstitute the lyophilized Control Vector CAT with 25 µl sterile DNase- and RNase-free water (concentration 1 µg/µl). The solution is stable at –15 to –25°C.
3. Prepare Feeding Solution according to table 2 of Protocol 2, page 14.
4. Prepare Reaction Solution according to table 2 of Protocol 2.
5. Add 15 µg (15 µl) reconstituted Control Vector CAT (vial 7) to Reaction Solution.
6. Fill the reaction chamber with Reaction Solution according Protocol 2, page 15.
7. Fill the feeding chamber with Feeding Solution according Protocol 2, page 15.

8. Insert the device into the Eppendorf® Thermomixer Comfort according to Protocol 2 (page 16) and start the reaction by setting the following parameters:

   Temperature: 32°C

   Shaking speed: 800 rpm

   Time: 20 hours

   Note: The RTS ProteoMaster Instrument may also be used.
9. After completing the run remove the Reaction Solution with a 1 ml pipet through the circular opening.
10. Apply 0.25–0.5 µl of the reaction onto SDS-polyacrylamide gels. For quantification of the CAT control reaction apply an appropriate protein standard in parallel.
11. Run the gel and stain with Coomassie blue. Quantify the synthesized CAT by comparison with the protein standard.

    Note: The CAT protein can also be detected on Western blots by using an anti-His_6 antibody (e.g. 5 PRIME).

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 500 ProteoMaster *E. coli* HY Kit also reduces the amount of Selenomethionine required to express sufficient protein.

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.

Procedure

1. Reconstitute the reaction components according to Table 3.

Table 3. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E. coli* Lysate; 500 PM *E.coli* (Bottle 1, red cap)	Reconstitute the lyophilizate with 0.525 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 500 PM *E.coli* (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.25 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 8
3	Feeding Mix; 500 PM *E.coli* (Bottle 3, blue cap)	Reconstitute the lyophilizate with 8.1 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 7
4	Amino Acids.; 500 PM *E.coli* (Bottle 4, brown cap)	Reconstitute the lyophilizate with 3 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
5a	Selenomethionine	➔ **Dissolve 28 mg D,L-Selenomethionine and 1 mg DTT in 1.8 ml Reconstitution Buffer (bottle 6) at 30°C.** This amount is sufficient for 5 x 1 ml reactions. 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.	–
6	Reconstitution Buffer; 500 PM *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 5a

All reconstituted solutions should be clear, with the exception of the *E. coli* lysate, which remains cloudy.

2. Prepare the working solutions according to Table 4.

Table 4. Working solutions

Solution	Contents	Preparation of working solution	For use in
7	Feeding Solution	Add 2.65 ml of the reconstituted Amino Acids (solution 4) and 0.3 ml of reconstituted Selenomethionine (solution 5a) to Feeding Mix (solution 3). Mix by rolling or shaking.	Running an experiment, page 15
8	Reaction Solution	To solution 1 (*E. coli* Lysate), add 0.225 ml of the reconstituted Reaction Mix (solution 2), 0.27 ml of the reconstituted Amino Acids (solution 4) and 30 µl of reconstituted 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!	Running an experiment, page 15

3. Proceed with the protocol, see 'Running an experiment', page 15

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 500 ProteoMaster *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 (biotechrabbit) provides appropriate stock solutions of each individual unlabeled amino acid.

Specific amino acid mixtures can be prepared using the RTS Amino Acid Sampler (cat. no. BR1401801, biotechrabbit) 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.

Select those labeled amino acids you want to incorporate and strictly exclude their unlabeled form to guarantee complete incorporation.

Note: If Methionine is the only labeled amino acid to be introduced, the procedure for labeling with Selenomethionine (Protocol 3, page 18) can be followed, using the Amino Acids provided in this kit.

Procedure

1. Reconstitute the reaction components according to Table 5.

Table 5. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	*E. coli* Lysate; 500 PM *E.coli* (Bottle 1, red cap)	Reconstitute the lyophilizate with 0.525 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 500 PM *E.coli* (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.25 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 8
3	Feeding Mix; 500 PM *E.coli* (Bottle 3, blue cap)	Reconstitute the lyophilizate with 8.1 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 7
4a	Amino Acid Labeling Mix	Make a 8.4 mM stock solution of all amino acids in 3 ml of Reconstitution Buffer (bottle 6) or refer to the RTS Amino Acid Sampler Manual in Table 2 in Protocol 2. Note: Check the certificate of analysis supplied with the labeled amino acid mixture. Ensure that all amino acids have almost the same concentration (usually some amino acids are missing or the content is lower compared to others, e.g. asparagine, cysteine, glutamine, tryptophane).	Step 2 Solutions 7 and 8
5b	DTT	Make a fresh 40 mM stock solution in 1.8 ml Reconstitution Buffer (bottle 6) or use DTT ready-to-use solution of the RTS Amino Acid Sampler. This is sufficient for five 1 ml reactions.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 500 PM *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, 4a, and 5b

All reconstituted solutions should be clear, with the exception of the *E. coli* lysate, which remains cloudy.

2. Prepare the working solutions according to Table 6.

Table 6. Working solutions

Solution	Contents	Preparation of working solution	For use in
7	Feeding Solution	To Feeding Mix (solution 3), add 2.65 ml of the prepared Amino Acid Labeling Mix (solution 4a) and 0.3 ml of DTT solution (solution 5b). Mix carefully by rolling or gentle shaking.	Running an experiment, page 15
8	Reaction Solution	To solution 1 (*E. coli* Lysate), 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 the DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. The total volume of reaction solution is 1.1 ml. Do not vortex!	Running an experiment, page 15

3. Proceed with the protocol, see 'Running an experiment', page 15.

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 functional protein (examples in Table 7.). The design of this kit allows the addition of up to 200 µl of supplement solution to the reaction solution and 2 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).

For efficient optimization of reaction conditions biotechrabbit 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, the conditions can be applied in the 1 ml reaction volume of the RTS 500 ProteoMaster *E. coli* HY Kit.

Please also note that alternatively, aggregated proteins can often be reconstituted using reagents exemplarily shown in Table 7, because they are salted out in native conformation in contrast to the formation of inclusion bodies containing denatured protein after overexpression *in vivo*. In these cases after cell-free protein synthesis precipitated protein should be pelleted by centrifugation (e.g. for 10 min at 16,000x g). This will also partially purify the target protein. The protein pellet should then be reconstituted in the reagent by strong shaking for several hours, while avoiding the generation of foam.

Table 7. Examples of chemicals and supplements to improve solubility and yield

Chemical/Supplement	Suggested concentration in reaction and feeding solution	Used for
Brij35	0.05 – 0.2%	membrane proteins, hydrophobic and other proteins
Brij78	0.05 - 0.2%	
DDM (n-Dodecyl β-D-maltoside)	0.05 - 0.2%	
CHAPS	10 mM	
Sorbitol	0.5 M	non-membrane proteins
DnaK supplement (cat. no. BR1401601)	please refer to RTS DnaK Supplement Short Instruction	
GroE supplement (cat. no. BR1401701)	please refer to RTS GroE Supplement Short Instruction	

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; 500 PM *E.coli* (Bottle 1, red cap)	Reconstitute the lyophilizate with **0.34** ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 8
2	Reaction Mix; 500 PM *E.coli* (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.25 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 8
3	Feeding Mix; 500 PM *E.coli* (Bottle 3, blue cap)	Reconstitute the lyophilizate with **6.1** ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solution 7
4	Amino Acids; 500 PM *E.coli* (Bottle 4, brown cap)	Reconstitute the lyophilizate with 3 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
5	Methionine; 500 PM *E.coli* (Bottle 5, yellow cap)	Reconstitute the lyophilizate with 1.8 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Step 2 Solutions 7 and 8
6	Reconstitution Buffer; 500 PM *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, 5 and S
S	Supplement	Make a stock solution of the particular chemical or supplement using Reconstitution Buffer (bottle 6).	Step 2 Solution 7 or 8

All reconstituted solutions should be clear, with the exception of the *E. coli* lysate, which remains cloudy.

2. Prepare the working solutions according to Table 9.

Table 9. Working solutions

Solution	Contents	Preparation of working solution	For use in
7	Feeding Solution	To solution 3, add 2.65 ml reconstituted Amino Acids (solution 4) and 0.3 ml of reconstituted Methionine (solution 5). Finally, add 2 ml of solution S or Reconstitution Buffer (bottle 6). The total volume is 11 ml. Mix carefully by rolling or gentle shaking.	Running an experiment, page 15
8	Reaction Solution	To solution 1 (*E. coli* Lysate), add 0.225 ml reconstituted Reaction Mix (solution 2), 0.27 ml reconstituted Amino Acids (solution 4) and 30 µl reconstituted Methionine (solution 5). Add 0.185 ml solution S or Reconstitution Buffer (bottle 6). Add 10–15 µg DNA template in a maximum volume of 50 µl. Mix carefully by rolling or gentle shaking. The total volume of reaction solution is 1.1 ml. Do not vortex!	Running an experiment, page 15

Proceed with the protocol, see 'Running an experiment', page 15.

Supporting information

Short protocols

Use the short protocols only if you are familiar with the standard procedures described in this manual.

Short protocol for reconstitution of reaction components

General recommendations

- Mix the reconstituted solution in bottle 1 by carefully rolling or shaking. Do not vortex!
- Mix the reconstituted solutions in bottles 2–5 by rolling or shaking

	Addition of Reconstitution Buffer; 500 PM *E.coli* (bottle 6, white cap)	
Bottle	For standard synthesis and control reaction	For incorporation of Selenomethionine
1 *E. coli* Lysate; 500 PM *E.coli* (red cap)	+ 0.525 ml Do not vortex!	+ 0.525 ml Do not vortex!
2 Reaction Mix; 500 PM *E.coli* (green cap)	+ 0.25 ml	+ 0.25 ml
3 Feeding Mix; 500 PM *E.coli* (blue cap)	+ 8.1 ml	+ 8.1 ml
4 Amino Acids; 500 PM *E.coli* (brown cap)	+ 3.0 ml	+ 3.0 ml
5 Methionine; 500 PM *E.coli* (yellow cap)	+ 1.8 ml	Not required

Short protocol for standard and control protein synthesis reaction

Preparation of working solutions and filling device

	For standard reaction	For control reaction
Reaction Solution	Add 0.225 ml reconstituted solution of bottle 2 + 0.270 ml reconstituted solution of bottle 4 + 30 µl reconstituted solution of bottle 5 to the reconstituted solution of bottle 1.	
	Add 10–15 µg template DNA (max. volume 50 µl).	→ Reconstitute the CAT control vector (vial 7) with 25 µl water. → Add 15 µl CAT vector to bottle 1.
	→ Mix by rolling or gentle shaking. Do not vortex!	
	→ Open the red lid of the red reaction chamber of the device.	
	→ Add 1 ml of the prepared reaction solution to the reaction chamber of the device through the circular opening, using a 1 ml pipet.	
Feeding Solution	Add 2.65 ml reconstituted solution of bottle 4 + 0.3 ml reconstituted solution of bottle 5 to the reconstituted solution in bottle 3	
	→ Mix by rolling or shaking.	
	→ Open the colorless lid of the device.	
	→ Add 10 ml of the prepared feeding solution to the feeding chamber of the device through the circular opening, using a 10 ml pipet.	

Running the experiment

- → Insert the device into the Eppendorf® Thermomixer Comfort using the RTS 500 Adapter. Start the reaction by setting the following parameters: Temperature: 32°C; shaking speed: 800 rpm; time: 20 hours.
- → Alternatively, the RTS ProteoMaster Instrument can also be used.

Analyzing the RTS reaction

- → After completing the run remove the reaction solution through the circular opening using a 1 ml pipet.
- → Analyze the protein synthesis reaction by SDS PAGE.

Typical results

Expression kinetics

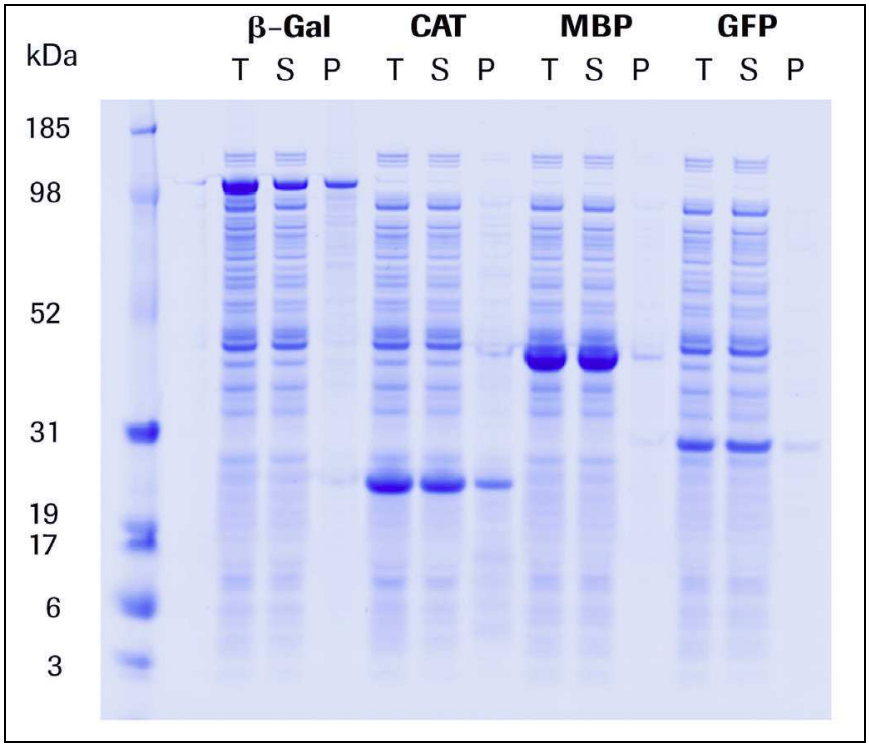

Figure 9. Coomassie-stained gel-analysis of the expression of different proteins.

Samples were taken after 20 hours reaction time in the RTS 500 Proteomaster E. coli HY Kit. **β-Gal**: Beta-galactosidase; **CAT**: chloramphenicol acetyltransferase; **MBP**: Maltose binding protein; **GFP**: Green fluorescent protein (variant); **T**: Total expression; **S**: Supernatant; P: Pellet.

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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

Observation	White precipitate in the feeding solution or reaction solution after the run
Possible cause	Insoluble salts are formed
Resolving	Usually the expression is not affected by the precipitate.

Observation	No expression using the CAT 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 RNases are excluded at every step.
Possible cause	Incorrect handling
Resolving	Repeat experiment according to instructions.
Possible cause	A kit component is inactive or degraded
Resolving	Contact biotechrabbit technical services.

Problems with expression of target protein, whilst the CAT control reaction works

Observation	Sufficient protein expression, but low yield of active protein
Possible cause	Incorrect folding of the protein due to: → dependence on co-factors → necessity of disulfide bonds → dependence on post-translational modifications → dependence on chaperones
Resolving	Add necessary co-factors
Resolving	Proteins with more than three disulfide bonds (up to three disulfide bonds can be formed) may be not expressed in a functional form in the RTS *E. coli* system.
Resolving	Add chaperones.
Resolving	For proteins with up to 3 disulfide bonds, allow oxidation after the reaction for the formation of disulfide bonds.
Resolving	The *E. coli* lysate cannot introduce post-translational modifications such as glycosylation, phosphorylation, or signal sequence cleavage.

Observation	Product appears in the pellet after centrifugation
Possible cause	Aggregation
Resolving	Add/adjust chaperones or cofactors.
Resolving	Change experimental conditions (time, temperature, e.g., lower in steps of 4–14°C).
Resolving	Add mild detergents (e.g., up to 0.2% Brij 35, or 10 mM CHAPS for membrane proteins, see also Table 7 on page 24).

Observation	Low expression yield
Possible cause	Expression time too short
Resolving	Extend expression time.
Possible cause	Suboptimal expression template
Resolving	Try expression in different pIVEX vectors.

Observation	Several product bands on SDS-PAGE or product smaller than expected
Possible cause	Proteolytic degradation
Resolving	Add protease inhibitors to the reaction. For example, use 1 tablet of Complete mini EDTA-free (Roche) for a 1 ml reaction. Use up to 10 mM EGTA.
Possible cause	Internal initiation site
Resolving	Replace the corresponding Methionine by point mutation.
Possible cause	Premature termination of the translation
Resolving	Check the sequence of the target gene regarding reading frame and mutations which might yield a stop codon.
Resolving	Search for strong secondary structures of the mRNA and eliminate them by conservative mutations.

Observation	No protein band of the target protein, but normal expression of control protein
Possible cause	Cloning error
Resolving	Check the sequence.
Possible cause	Low purity of plasmid DNA
Resolving	Ensure that the absorbance ratio 260 nm/280 nm is at least 1.7.
Resolving	Perform a phenol extraction.
Resolving	Make a new plasmid preparation.
Possible cause	Contamination with RNases
Resolving	Repeat experiment and ensure to work RNase-free at every step.
Possible cause	No initiation of translation due to strong secondary structures of the mRNA
Resolving	Try expression in different pIVEX vectors.
Resolving	Try expression as N-terminally tagged fusion protein.
Possible cause	Expressed protein interferes with the translation or transcription process
Resolving	Express the protein of interest together with control protein. If control protein expression is inhibited, the active protein can not be expressed using RTS.

Ordering information

Product	Size	Order no.
RTS Linear Template Kit Plus	20 reactions	BR1402401
RTS pIX3.0 Vector	1 vector, 25 µg	BR1402701
RTS 100 *E. coli* HY Kit	24 reactions	BR1400101
RTS 100 *E. coli* HY Kit	96 reactions	BR1400102
RTS 500 ProteoMaster *E. coli* HY Kit	5 reactions	BR1400201
RTS 9000 *E. coli* HY Kit	1 reaction	BR1400301
RTS 100 *E. coli* Disulfide Kit	24 reactions	BR1400401
RTS 500 *E. coli* Disulfide Kit	5 reactions	BR1400501
RTS 100 *E. coli* Fab Kit	10 reactions	BR1400601
RTS 100 *E. coli* Fab Kit	96 reactions	BR1400602
RTS pIVEX *E. coli* His-tag, 2nd Gen. Vector Set	2 vectors, 10 µg each	BR1400701
RTS Wheat Germ LinTempGenSet, His6-tag	96 reactions	BR1401201
RTS pIVEX Wheat Germ His6-tag Vector Set	2 vectors, 10 µg each	BR1401301
RTS 100 Wheat Germ CECF Kit	24 reactions	BR1401001
RTS 500 Wheat Germ CECF Kit	5 reactions	BR1401101
RTS 500 Adapter	1 adapter	BR1401901
RTS GroE Supplement	For five RTS 500 reactions	BR1401701
RTS DnaK Supplement	For five RTS 500 reactions	BR1401601
RTS Amino Acid Sampler	1 set	BR1401801
RTS 100 Insect Membrane Kit	5 reactions	BR1401501
RTS 100 Insect Membrane Kit	20 reactions	BR1401502
RTS 100 Insect Disulfide Kit	10 reactions	BR1401401
RTS 100 Insect Disulfide Kit	96 reactions	BR1401402
RTS Linear Template Fab Kit	96 reactions	BR1402201
RTS pIX4.0 Insect Vector	1 vector, 25 µg	BR1400901