RTS™ 500 *E. coli* Disulfide Kit Manual

For cell-free expression of disulfide-bonded proteins

RTS 500 *E. coli* Disulfide Kit Manual, April, 2015

Contents

Product specifications

The RTS 500 *E. coli* Disulfide Kit is used for the expression of disulfide-bonded proteins in a reaction volume of 1 ml.

Product description

The RTS 500 *E. coli* Disulfide Kit provides the components and procedures necessary for five coupled transcription/translation reactions under disulfide-bond–forming conditions.

Product limitations

The RTS 500 *E. coli* Disulfide 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 500 *E. coli* Disulfide Kit	Contents and function	No. vials per kit
Ordering number	BR1400501	
E. coli Lysate; 500 E.coli Dis. (Vial 1, red cap)	→ Stabilized lysate from *E. coli* → Contains components for transcription and translation → Contains optimized amounts of GroE chaperones	5
Reaction Mix; 500 E.coli Dis. (Vial 2, green cap)	→ Stabilized substrate mix to prepare reaction solution → Contains a redox system and a disulfide isomerase for formation of disulfide bonds	5
Amino Acids; 500 E.coli Dis. (Vial 3, brown cap)	→ Stabilized amino acid mix without Methionine to prepare reaction and feeding solutions	5
Methionine; 500 E.coli Dis. (Vial 4, yellow cap)	→ Stabilized Methionine to prepare reaction and feeding solutions	5
Lysate Activator; 500 E.coli Dis. (Vial 5, pink cap)	→ Solution required for efficient formation and rearrangement of disulfide bonds	5
Feeding Mix; 500 E.coli Dis. (bottle 6, blue cap)	→ Stabilized substrate mix to prepare feeding solution → Contains a redox system for the formation of disulfide bonds	5
RNase-DNase-free Water; 500 E.coli Dis. (bottle 7, colorless cap)	→ Water to complete the reaction and feeding solutions	1
Control Vector UK; 500 E.coli Dis. (Vial 8, white cap)	→ 50 µl liquid (±25 µg), pIVEX 2.3 UK encoding mouse urokinase	1
Reaction Device; 500 E.coli Dis.	→ 5 disposable two-chamber devices, for continuous exchange cell-free (CECF) protein expression reactions → Devices are compatible with the Eppendorf® Thermomixer Comfort or RTS ProteoMaster™ Instrument → Devices are not compatible with the RTS 500 Instrument	5

Note: Reagents for each reaction are packed separately to avoid repeated freeze–thaw cycles.

Additional materials

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

- Eppendorf Thermomixer Comfort or RTS ProteoMaster Instrument
- Pipets: 200–1,000 µl, 1–10 ml, 10–200 ml, 1 ml, and 10 ml
- Pipet tips autoclaved at 121°C for 20 minutes
- Eppendorf reaction tubes
- Other than the template, no additional reagents are required for the protocols. However, biotechrabbit recommends following the workflow as described on page 9. This workflow requires several additional reagents

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 *E. coli* Disulfide Kit is shipped at on dry ice.

The RTS 500 *E. coli* Disulfide Kit and components should be stored at –70°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 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.

No bottles contain hazardous substances in reportable quantities. Observe the usual precautions when handling chemicals. After use, reagents can be discarded in wastewater in accordance with local regulations. If reagent contacts your eyes, flush eyes with water. If reagent contacts your skin, wash off with water. If you swallow a reagent, seek medical advice.

Note: 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

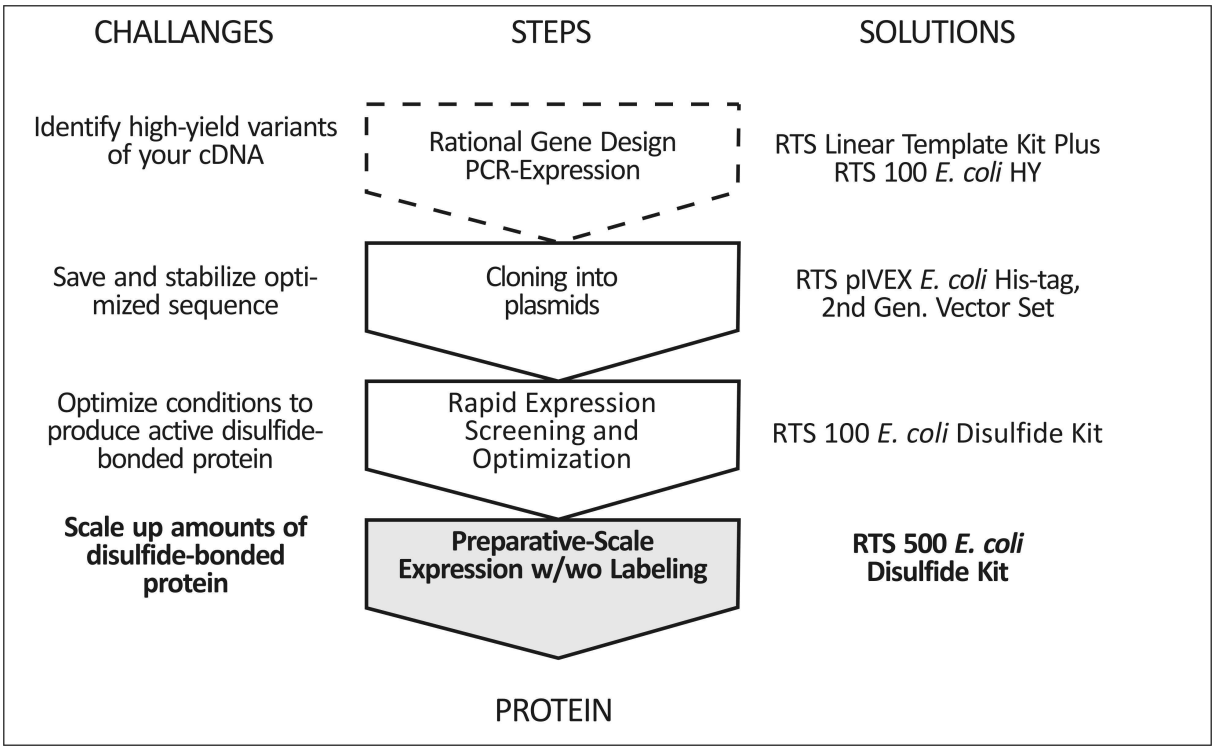
The RTS 500 *E. coli* Disulfide Kit allows expression of preparative amounts (reaction size 1 ml) of disulfide-bonded protein from circular templates within 24 hours.

The Rapid Translation System (RTS) workflow (Figure 1) ensures optimal cell-free protein expression and overcomes limitations that often affect traditional *in vitro* systems. Major innovations provided by the workflow include software-based template design, efficient amplification of templates, and continuous exchange cell-free (CECF) systems for both optimization of *in vitro* expression conditions (in RTS 100 small-scale reactions) and high yield *in vitro* expression (in RTS 500 large-scale reactions).

Advantages of the system include:

- rapid, easy, and reliable setup of reactions
- good yields in both small- and large-scale reactions
- a cell-free system
    - only express the protein of interest
    - express toxic proteins
    - expression independent of codon usage, since all tRNA species are present in excess
- functional disulfide-bonded proteins
- versatile – expression conditions can be easily adapted or tailored for X-ray or nuclear magnetic resonance (NMR) analysis

Description of procedure



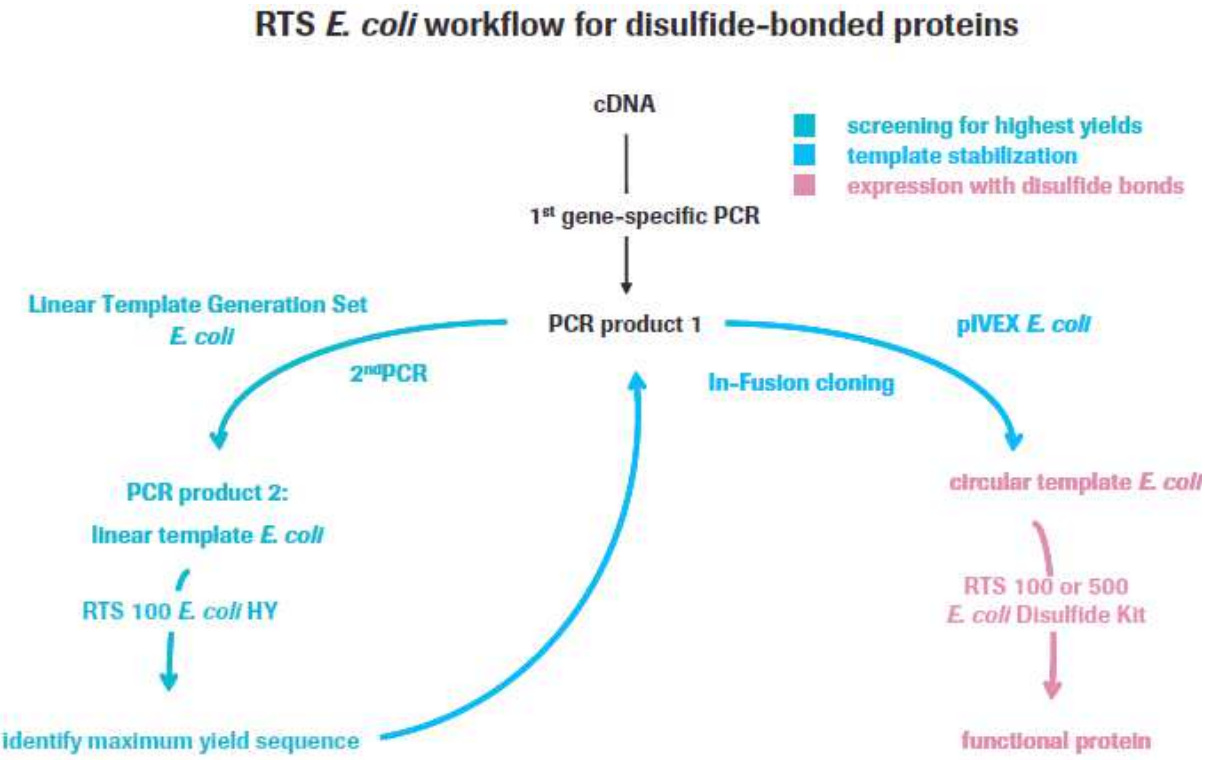


Figure 1. Integration of the RTS 500 *E. coli* Disulfide Kit into the RTS workflow.

Biochemistry

The RTS 500 *E. coli* Disulfide Kit uses an optimized package of *E. coli* lysate, reagents, chaperones and buffer systems to produce maximum yields of active protein. The kit uses several innovative technologies:

- coupled transcription/translation system for simultaneous translation of nascent mRNA
- biochemically enhanced high-yield *E. coli* lysate
- redox buffer to maintain the system under oxidizing conditions, which enhances disulfide bond formation
- disulfide isomerase for the formation and rearrangement of disulfide bonds, which enhances yields of correctly folded proteins
- increased amounts of GroE chaperones, which produce higher yields of soluble, properly folded proteins.

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

CECF principle

The RTS 500 *E. coli* Disulfide Kit uses the CECF principle (Figure 2). Transcription, translation, and disulfide bond formation take place in the 1 ml reaction compartment of the reaction device (Figure 3). Substrates and energy components essential for a sustained reaction are continuously supplied through a semi-permeable membrane. At the same time, potentially inhibitory reaction byproducts are diluted since they diffuse through the membrane into the 10 ml feeding compartment. Protein expression and folding continues for up to 24 hours, yielding as much as several mg/ml of active protein.

Note: The RTS 100 *E. coli* Disulfide Kit uses the same CECF principle as the RTS 500 Kit. Thus, you can use the small-scale (50 µl) reactions in the RTS 100 kit to rapidly determine optimal reaction conditions for your system. These optimized reaction conditions (reaction time, temperature, supplements etc.) may then easily be transferred to large-scale expression reactions (1 ml) in the RTS 500 *E. coli* Disulfide Kit.

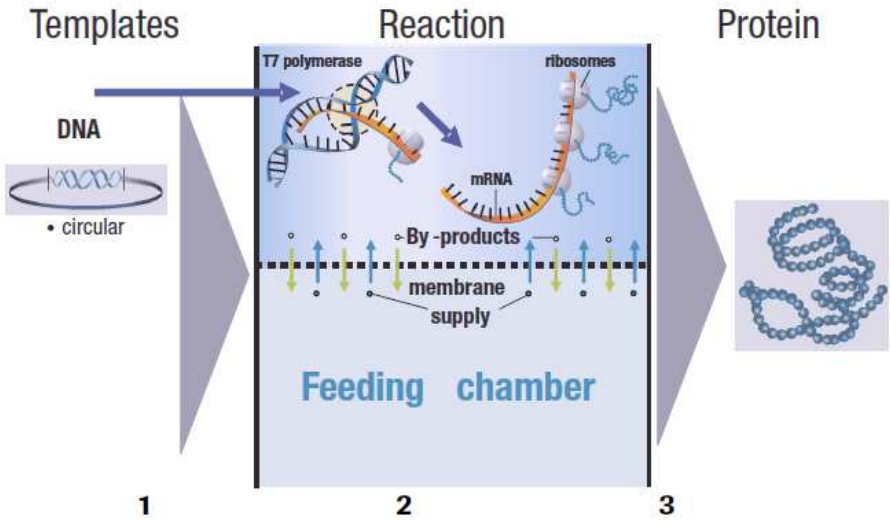


Figure 2. The CECF principle.

CECF procedure

Firstly, DNA with the gene of interest is cloned into a suitable T7-driven expression vector and this is added to the CECF device (Figure 3). The circular DNA template is transcribed by T7 RNA polymerase *in vitro*, followed by translation of the mRNA into protein by the ribosomal machinery of the *E. coli* lysate. The protein is then folded into its native conformation and the disulfide bonds are formed. Finally, the product accumulates in the reaction mix during a run of 6–24 hours.

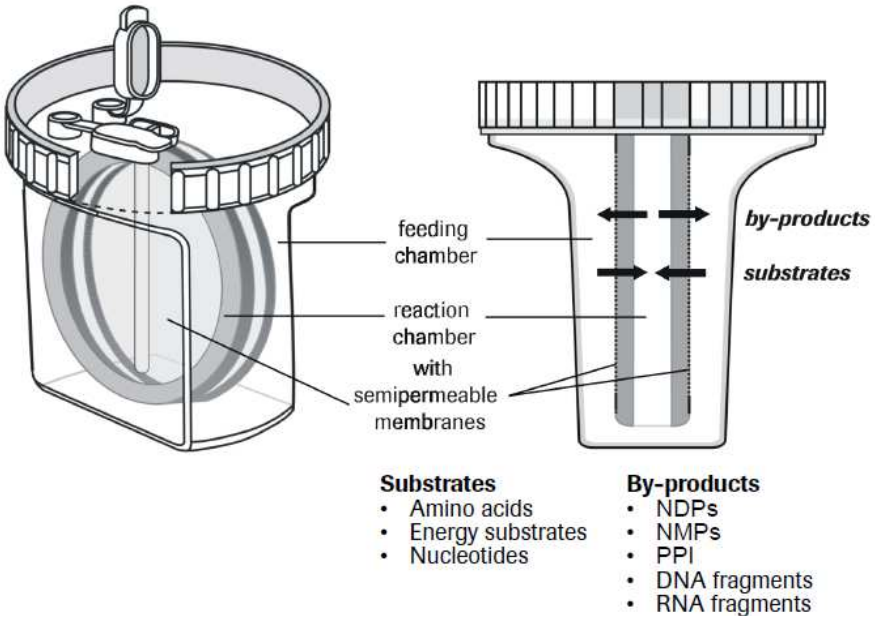


Figure 3. Illustration of the RTS 500 *E. coli* Disulfide device.

Template DNA

biotechrabbit recommends the use of pIVEX vectors, which are optimized for *in vitro* protein expression. The pIX3.0 Vector with cloned PCR product of the RTS Linear Template Kit Plus is also recommended. Other vectors may also be used, providing they are designed for prokaryotic *in vitro* protein expression and contain a T7 promoter and a ribosome binding site. The requirements are described in Expression vectors (Protocol 1), page 13.

Note: Since the RTS 500 *E. coli* Disulfide Kit generally requires long reaction times (6–24 hours) to ensure good yields, proper folding, disulfide formation, and rearrangement, linear templates should not be used due to their relative instability.

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

Expression vectors

Any vector used with the RTS 500 *E. coli* Disulfide Kit 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 (see 'Generation of expression templates', page 14).

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

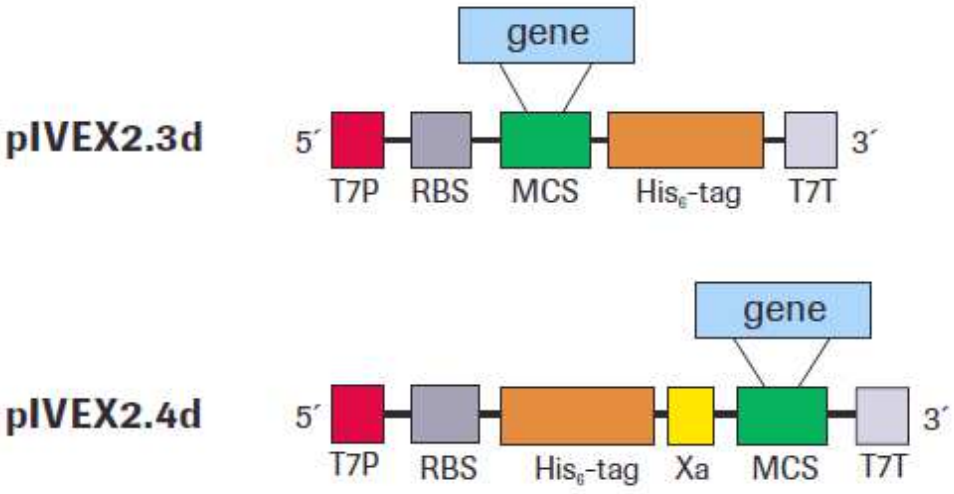


Figure 4. Functional elements of cloning vectors.

T7P: T7 Promoter; **RBS**: Ribosome binding site; **His6-tag**: Tag sequence at C- or N-terminal position; **Xa:** Factor Xa restriction protease cleavage site; **MCS**: Multiple cloning site in three different reading frames for the insertion of the target gene.

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. biotechrabbit recommends a solution containing 0.5–1.5 mg/ml plasmid.

Note: Do not use plasmid DNA purified from agarose gels as such preparations may contain traces of chemicals that inhibit *in vitro* synthesis

Generation of expression templates

Template optimization

For the expression of your gene of interest, you must consider two important characteristics of your template:

- regulatory elements for transcription and translation should be present up- and downstream from the open reading frame (ORF)
- the coding sequence itself should be adapted specifically for the selected expression system

Template generation by cloning

Regulatory elements can easily be added to the gene of interest by inserting the gene into a pIVEX vector using standard cloning techniques. This requires restriction sites compatible with the MCS of the selected pIVEX vector, which must be introduced into the gene, e.g. via the gene-specific primers during a first, gene-specific PCR.

Another method of inserting the product of the first PCR into a pIVEX vector is to use In-Fusion™ technology. The In-Fusion PCR Cloning Kit (Clontech) requires a PCR product with overhangs homologous to the ends of the linearized pIVEX vector. Addition of the In-Fusion enzyme leads to strand displacement. Subsequently, the DNA ends from the PCR product and the vector are brought together and fused in a simple 30-minute incubation step.

Template generation by PCR

Linear templates containing the regulatory elements required for expression can be generated with the RTS Linear Template Kit Plus (visit www.biotechrabbit.com for more information). Before they are used in the RTS 100 E. coli Disulfide Kit, linear templates must be cloned into the RTS pIX3.0 Vector. (Refer also to the RTS Linear Template Kit Plus manual for more Information.)

Optionally, generated linear templates can be directly expressed in a RTS 100 E. coli HY Kit (biotechrabbit) batch reaction. These expression reactions may be useful for screening different variants and selecting the construct that gives the maximum yield. After this, the construct with optimal performance is cloned into the RTS pIX3.0 Vector for expression of the selected sequence in the RTS 100 *E. coli* Disulfide Kit (see above).

Note: Since the RTS 100 *E. coli* Disulfide Kit generally requires long reaction times (6–24 hours) to ensure good yields, proper folding, disulfide formation and rearrangement, linear templates should not be directly used in the kit due to their relative instability.

Protocol 2: Protein synthesis reaction

Before starting

- biotechrabbit strongly recommends that the expression yield of the DNA is first evaluated in small-scale expression reactions using the RTS 100 *E. coli* Disulfide Kit. If the initial yield is low, try the following in the order listed:
    - use a pIVEX vector, which was developed for *in vitro* expression reactions
    - optimize expression reaction conditions by changing the reaction parameters (temperature, time) and adding supplements and chaperones

Equipment and reagents required

- DNA template: Prepare and purify the DNA template as described in Protocol 1.
- 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).
- Calibrated pipets
- RNase-free plastic and glassware

Reagent notes

- Do not combine reagents from different kit lots
- Store solutions from vials 1 to 8 at –70°C or below. Aliquot the reagents according to the requirements of your experiment. The reagents can withstand three freeze–thaw cycles without significant decrease in activity
- Thaw the reagents immediately before use
- Keep reagents and working solutions on ice until they are used
- Only treat as much lysate with Lysate Activator as will be used on that day. Treated lysate cannot be stored
- Turbidity does not affect the activity of lysate (vial 1)
- If a precipitate forms in the Reaction Mix, resuspend it by vortexing or pipetting up and down until the solution is homogenous
- A precipitate may form in the Amino Acid Mix; this can be dissolved at 37°C

Procedure

1. Prepare the working solutions according to Table 1.

Table 1. Working solutions for protein synthesis

Solution	Contents	Preparation of working solution
1	*E. coli* Lysate; 500 E.coli Dis.	➔ Add 22 µl Lysate Activator (vial 5, pink cap) to Lysate (vial 1, red cap) ➔ Mix by rolling or shaking and incubate for 10–20 min at room temperature (15–25°C)
6	Feeding Solution	Add the following components to the Feeding Mix (bottle 6, blue cap) and mix carefully: ➔ 1.54 ml Amino Acid Mix (vial 3, brown cap) ➔ 0.22 ml Methionine (vial 4, yellow cap) ➔ 2.2 ml water (bottle 7, colorless cap) or supplements
2	Reaction Solution	Add the following components to the Reaction Mix (vial 2, green cap) and mix carefully: ➔ 154 µl Amino Acid Mix (vial 3, brown cap) ➔ 22 µl Methionine (vial 4, yellow cap) ➔ 170 µl water (vial 7, colorless cap) or supplements ➔ 550 µl activated lysate (solution 1, above) ➔ 10–15 µg circular DNA template in 50 µl water or TE buffer

Lysate and Reaction Mix may be turbid, but this does not affect the activity.

If a precipitate forms in the Amino Acid Mix, dissolve by incubating at 37°C.

2. Open the colorless lid of the feeding compartment in one reaction device. Insert a 10 ml pipet into the circular opening of the compartment and fill the compartment with 10 ml of Feeding Solution (solution 6).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

3. Close the lid of the compartment securely.
4. Open the red lid of the 1 ml reaction compartment in the reaction device. Insert a 1 ml pipet into the circular opening of the compartment and fill the compartment with 1 ml Reaction Solution (solution 2).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

   Important: Do not use the oval opening to fill the chamber.

5. Close the lid of the compartment securely.

6. Insert the filled reaction device into the Eppendorf Thermomixer Comfort, using the RTS 500 adapter (biotechrabbit).
7. Set the instrument run parameters to 32°C and 800 rpm and start the reaction.

   For more information about setting the parameters, see Points to consider, below.
8. After 6-24 hours remove the reaction device from the incubator.
9. Analyze your protein.

   If necessary, store reaction product frozen or at 0–4°C until it can be purified or further processed.

Points to consider

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. For some proteins, the folding process is slow and may require longer incubation times. However, for unstable proteins the optimum yield of soluble active protein may be produced at shorter reaction times. If your protein may be sensitive to longer incubation times, take samples (e.g. after 4 h, 8 h, 12 h, 24 h) and compare the expression and solubility of the product in each of these samples.

Control reaction with urokinase gene

Procedure

1. Prepare working solutions according to Table 1. Use 10 µg (20 µl) of Control Vector UK (vial 8, white cap) as template in the Reaction Solution.
2. Set the temperature to 32°C, the shaking speed at 800 rpm and time for 24 hours. Start the reaction.
3. To detect the product, load 1.25 µl of the reaction onto an SDS-polyacrylamide gel.
4. Transfer the proteins to a membrane by Western blotting and detect the Urokinase protein using an anti-His_6-tag antibody (e.g. 5 PRIME).

   Note: Due to the His-tag at the C-terminus, the control protein shows little or no activity (in contrast to the untagged protein).

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.

Because Methionine is a separate reagent in the RTS 500 *E. coli* Disulfide Kit, rather than mixed with other reagents, it is easy to substitute Selenomethionine for Methionine in the RTS 500 reactions. Using RTS 500 *E. coli* Disulfide Kit also reduces the amount of Selenomethionine required to generate express enough labeled 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.
- Prepare a working solution of Selenomethionine according to Table 2.

Table 2. Preparation of a Selenomethionine working solutions

Solution	Contents	Preparation of working solution
4a	Selenomethionine	→ Dissolve 25 mg D,L-Selenomethionine in 1.3 ml water (botlle 7, colorless cap) at 30°C. → Add 16 µl of a 1 M DTT solution to the vial. This amount is sufficient for five 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.

Procedure

1. Prepare the working solutions according to Table 3.

Table 3. Preparation of labeled working solutions

Solution	Contents	Preparation of working solution
1	Lysate	➔ Add 22 µl Lysate Activator (vial 5, pink cap) to Lysate (vial 1, red cap) ➔ Mix by rolling or shaking and incubate for 10–20 min at room temperature (15–25°C)
6	Feeding Solution	Add the following components to the Feeding Mix (vial 6, blue cap) and mix carefully: ➔ 1.54 ml Amino Acid Mix (vial 3, brown cap) ➔ 0.22 ml Selenomethionine (solution 4a) ➔ 2.2 ml water (vial 7, colorless cap) or supplements
2	Reaction Solution	Add the following components to the Reaction Mix (vial 2, green cap) and mix carefully: ➔ 154 µl Amino Acid Mix (vial 3, brown cap) ➔ 22 µl Selenomethionine (solution 4a) ➔ 170 µl water (vial 7, colorless cap) or supplements ➔ 550 µl activated lysate (solution 1, above) ➔ 10–15 µg circular DNA template in 50 µl water or TE buffer

2. Open the colorless lid of the feeding compartment in one reaction device. Insert a 10 ml glass pipet into the circular opening of the compartment and fill the compartment with 10 ml of Feeding Solution (solution 6).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

3. Close the lid of the compartment securely.
4. Open the red lid of the 1 ml reaction compartment in the reaction device. Insert a 1 ml pipet into the circular opening of the compartment and fill the compartment with 1 ml Reaction Solution (solution 2).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

   Important: Do not use the oval opening to fill the chamber.

5. Close the lid of the compartment securely.
6. Insert the filled reaction device into the Eppendorf Thermomixer Comfort, using the RTS 500 adapter (biotechrabbit).
7. Set the instrument run parameters to 32°C and 800 rpm and start the reaction.

   For more information about setting the parameters, see Points to consider, below.

8. After 6-24 hours remove the reaction device from the incubator.
9. Analyze your protein.

   If necessary, store reaction product frozen or at 0 to 4°C until it can be purified or further processed.

Points to consider

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. For some proteins, the folding process is slow and may require longer incubation times. However, for unstable proteins the optimum yield of soluble active protein may be produced at shorter reaction times. If your protein may be sensitive to longer incubation times, take samples (e.g. after 4 h, 8 h, 12 h, 24 h) and compare the expression and solubility of the product in each of these samples.

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 been 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 *E. coli* Disulfide Kit, all amino acids are kept in a separate solution, away from the other reagents necessary to drive the reaction (e.g. reaction mix, feeding mix). This design allows the incorporation of single, ^{15}N- and/or ^{13}C-labeled amino acid(s) into a protein by substituting a labeled amino acid mixture for the amino acid solution provided in the kit.

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

Before starting

Prepare the amino acid mixtures required:

- Use the RTS Amino Acid Sampler (biotechrabbit) as the source of unlabeled amino acids. It contains all 20 (unlabeled) amino acids packaged as separate ready-to-use solutions (generally 168 mM each)
- Prepare a 168 mM solution (either in water or in 60 mM Hepes, pH 7.45) of each of the labeled amino acids. (For labeled Tryptophan, Tyrosine or Leucine, see the relevant notes in the *Amino Acid Sampler Manual*)
- Pipet 150 µl of each of the 20 amino acids into a labeling Amino Acid Mix, using a mixture of the labeled amino acids and the unlabeled amino acids from the Amino Acid Sampler. To guarantee complete incorporation of label, use only the labeled form of the ^{15}N and/or ^{13}C-labeled amino acids in the Amino Acid Mix; do not include the corresponding unlabeled form
- Add 60 µl of the DTT (provided with the sampler) to the Amino Acid Mix

  Note: Do NOT add more than 60 µl DTT since DTT can disturb the redox conditions needed for the expression reaction
- Use this Amino Acid Mix for the procedure below

  Note: If Methionine is the only labeled amino acid to be introduced, you can follow the procedure for Selenomethionine labeling (Protocol 3, page 18). Use the Amino Acid Mix without Methionine (provided with the kit) and a 100 mM solution of labeled Methionine (not provided)

Procedure

1. Prepare the working solutions according to Table 4.

Table 4. Preparation of labeled working solutions

Solution	Contents	Preparation of working solution
1	*E. coli* Lysate; 500 E.coli Dis.	➔ Add 22 µl Lysate Activator (vial 5, pink cap) to Lysate (vial 1, red cap) ➔ Mix by rolling or shaking and incubate for 10–20 min at room temperature (15–25°C)
6	Feeding Solution	Add the following components to the Feeding Mix (vial 6, blue cap) and mix carefully: ➔ 2.68 ml labeled Amino Acid Mix (see Before starting, page 21) ➔ 1.28 ml water (vial 7, colorless cap) or supplements
2	Reaction Solution	Add the following components to the Reaction Mix (vial 2, green cap) and mix carefully: ➔ 268 µl labeled Amino Acid Mix (see Before starting, page 21) ➔ 78 µl water (vial 7, colorless cap) or supplements ➔ 550 µl activated lysate (solution 1, above) ➔ 10–15 µg circular DNA template in 50 µl water or TE buffer

2. Open the colorless lid of the feeding compartment in one reaction device. Insert a 10 ml glass pipet into the circular opening of the compartment and fill the compartment with 10 ml of Feeding Solution (solution 6).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

3. Close the lid of the compartment securely.
4. Open the red lid of the 1 ml reaction compartment in the reaction device. Insert a 1 ml pipet into the circular opening of the compartment and fill the compartment with 1 ml Reaction Solution (solution 2).

   Allow air to escape through the oval opening. Air bubbles do not need to be removed from the compartment.

   Important: Do not use the oval opening to fill the chamber.

5. Close the lid of the compartment securely.
6. Insert the filled reaction device into the Eppendorf Thermomixer Comfort, using the RTS 500 adapter (biotechrabbit).
7. Set the instrument run parameters to 32°C and 800 rpm and start the reaction.

   For more information about setting the parameters, see Points to consider, below.

8. After 6-24 hours remove the reaction device from the incubator.
9. Analyze your protein.

   If necessary, store reaction product frozen or at 0 to 4°C until it can be purified or further processed.

Points to consider

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. For some proteins, the folding process is slow and may require longer incubation times. However, for unstable proteins the optimum yield of soluble active protein may be produced at shorter reaction times. If your protein may be sensitive to longer incubation times, take samples (e.g. after 4 h, 8 h, 12 h, 24 h) and compare the expression and solubility of the product in each of these samples.

Supporting information

Typical results

Expression of urokinase (6 disulfide bonds) from the control vector pIVEX 2.3-UK

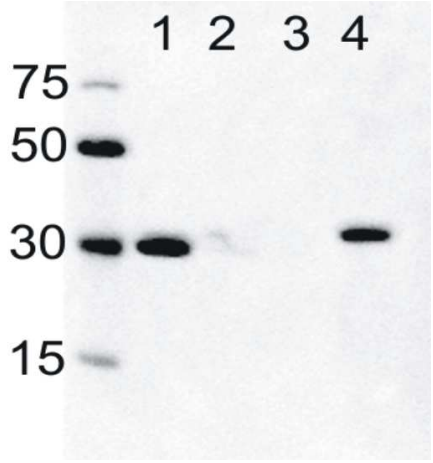


Figure 5. Western blot with anti-His_6 antibody.

Lane 1: Soluble urokinase expressed in the RTS 500 *E. coli* Disulfide Kit; **Lane 2**: Insoluble urokinase (RTS 500 *E. coli* Disulfide Kit); **Lane 3**: Soluble urokinase (RTS 500 *E. coli* HY); **Lane 4**: Insoluble urokinase (RTS 500 *E. coli* HY).

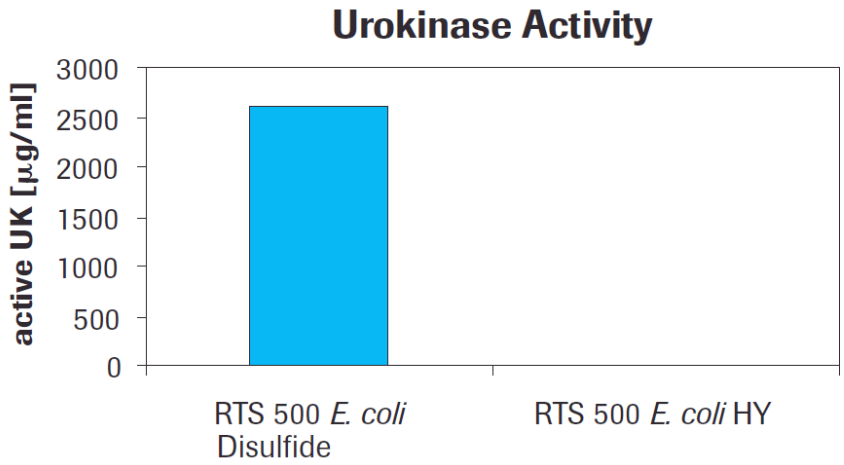


Figure 6. Activity assay for urokinase.

The C-terminal tag, that is present in the control template provided with this kit, inhibits the activity of the urokinase. Hence, this test was done with untagged urokinase.

Expression of single-chain antibody fragments (2 disulfide bonds)

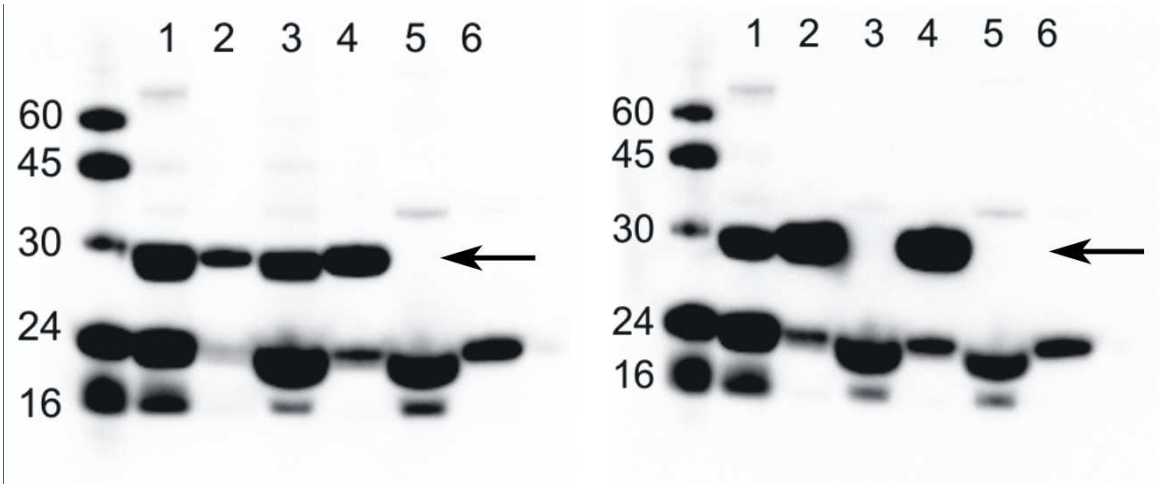


Figure 7. Expression of 2 different scFv fragments with the RTS 500 *E. coli* disulfide kit

The analysis was performed using a Western blot with streptavidin POD; the product is indicated by an arrow. **Lane 1**: Soluble scFv expressed in the RTS 500 *E. coli* Disulfide Kit; **Lane 2**: Insoluble scFv (RTS 500 *E. coli* Disulfide Kit); **Lane 3**: Soluble scFv (RTS 500 *E. coli* HY); **Lane 4**: Insoluble scFv (RTS 500 *E. coli* HY); **Lane 5**: Negative control, soluble (no DNA added); **Lane 6**: Negative control, insoluble (no DNA added). The 19 and 16 kDa bands are components of the lysate; which only interact with streptavidin if the protein is denatured.

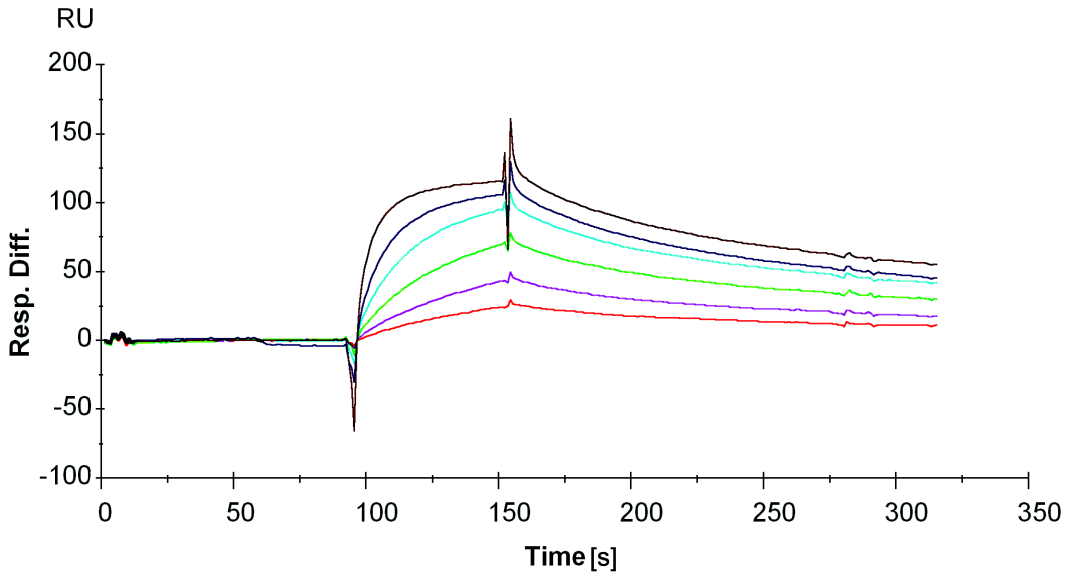


Figure 8. Biacore analysis of scFv fragment expressed with the RTS 500 *E. coli* Disulfide kit.

The expressed scFv specifically binds immobilized cell adhesion protein. Six concentrations (15 nM – 500 nM) of scFv were tested. (Data courtesy of Morphosys AG, Martinsried, Germany).

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	Control protein is not expressed
Possible cause	Kit expired
Resolving	Order a new kit.
Possible cause	Kit has not been stored at –70°C
Resolving	Order a new kit.
Possible cause	Contamination with RNases
Resolving	Repeat experiment and ensure RNases are excluded at every step.

Observation	Expressed control protein is not active
Possible cause	The control protein was cloned with a His6-tag at the C-terminus, which allows easy detection on the Western blot but inhibits the protein activity

Observation	Expression yields in the RTS 500 *E. coli* Disulfide Kit are different to those in the RTS 100 *E. coli* Disulfide Kit
Possible cause	The geometry of the reaction devices in the two kits is different. Also the kits have different ratios of reaction solution to feeding solution and membrane size. Therefore, expression results may vary slightly

Problems with expression of target protein

Observation	Good protein expression, but low yield of active protein
Possible cause	Incorrect folding due to dependence on cofactors
Resolving	Add necessary cofactors.
Possible cause	Incorrect folding due to dependence on secondary modifications
Resolving	The *E. coli* lysate cannot introduce post-translational modifications such as glycosylation, phosphorylation, or signal sequence cleavage.
Possible cause	Incorrect folding due to dependence on chaperones
Resolving	Add chaperones.

Observation	Low expression yield
Possible cause	Expression time too short
Resolving	Extend reaction time.
Possible cause	The epitope tag has a negative influence on the folded protein
Resolving	Try different pIVEX vectors.
Resolving	Introduce different epitope tag sequences via PCR.
Possible cause	Amount of template DNA not optimal
Resolving	Use a small-scale RTS 100 *E.coli* Disulfide Kit (50 µl) to optimize the circular template concentration. Try varying the DNA concentration for a 50 µl reaction from 0.1 µg to 5 µg/50 µl reaction.

Observation	Several product bands on SDS-PAGE or product smaller than expected
Possible cause	Proteolytic degradation
Resolving	Add protease inhibitors to the reaction.
Possible cause	Internal initiation site
Resolving	Eliminate the corresponding Methionine by point mutation.
Possible cause	Premature termination of the translation
Resolving	Check the sequence of the target gene for correct reading frame or a mutation that produces a stop codon.
Resolving	Search for strong secondary structures of the mRNA and eliminate them by using conservative mutations.
Resolving	Increase the amount of unlabeled Methionine during radioactive labeling, or decrease the reaction time.

Observation	Product in the pellet fraction
Possible cause	Aggregation
Resolving	Add or adjust chaperones.
Resolving	Adjust experimental conditions (e.g. time, temperature).
Resolving	Add mild detergents (e.g. up to 1% Brij 35 [v/v], 0.1% Triton® X-100 [v/v], or 0.1% Chaps [w/v] for membrane proteins).

Observation	No expression of target gene, but expression of normal control protein
Possible cause	Cloning error caused by premature termination of translation
Avoiding	Check the sequence of the target gene for correct reading frame or a mutation that produces a stop codon.

Possible cause	Low purity of DNA template
Avoiding	Ensure that the absorbance ratio 260 nm/280 nm is at least 1.7.
Resolving	Perform a phenol extraction if purity is low.
Resolving	Make a new template preparation.

Possible cause	Contamination with RNases
Resolving	Repeat experiment taking care to exclude RNases at every step.

Possible cause	No initiation of translation due to strong secondary structures in the mRNA
Avoiding	Try to express the protein as an N-terminally tagged fusion protein.

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

References

1. Ausubel, U.K., et al. (1993) "Current Protocols In Molecular Biology" John Wiley & Sons Inc., New York.
2. Rudolph, R., et al. (1997) in "Protein Function – A Practical Approach" Creighton, T.E. ed. Oxford University Press Inc. New York, pp 57.
3. Sambrook et al (2001) "Molecular Cloning – A Laboratory Manual" Third Edition, Cold Spring Harbor Laboratory Press, New York.
4. 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.
5. 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.
6. 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.
7. Riek, R., Pervushin, K., Wuethrich, K. (2000). TROSY and CRINEPT:NMR with large molecular and supramolecular structures in solution TIBS, 25, 462.
8. 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.

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