RTS™ 100 *E. coli* Disulfide Kit Manual

For cell-free expression of disulfide-bonded proteins

RTS™ 100 *E. coli* Disulfide Kit, 21.03.2025

For Research Purposes Only. Proteins expressed using the RTS, and data derived therefrom that would enable the expression of such proteins (collectively, “Expressed Proteins”), may be used only for the internal research of the purchaser of this system. Expressed Proteins may not be sold or transferred to any third party without the written consent of biotechrabbit GmbH.

The purchase price of this product includes a limited, non-exclusive, non-transferable license under U.S. patents 6.168.931 and 6.337.191 and their foreign counterparts, exclusively licensed by a member of the biotechrabbit GmbH.

The continuous-exchange cell-free (CECF) technology applied in the RTS 100 Wheat Germ CECF, RTS 500 Wheat Germ CECF, RTS 100 *E. coli* Disulfide, RTS 500 *E. coli* Disulfide, RTS 500 ProteoMaster *E. coli* HY and RTS 9000 *E. coli* HY products is based on patented technology (U.S. Patent 5,478,730). The purchase price of this product includes practicing a cell-free expression achieving continuous production of a polypeptide in the presence of a semi-permeable barrier and related processes described in said patents.

Contents

Product specifications

The RTS 100 *E. coli* Disulfide Kit is designed for:

- rapid parallel synthesis of disulfide-bonded proteins
- rapid optimization of expression conditions (chaperones, detergents, temperature reaction time, etc.)
- rapid functional testing of different constructs
- expression of toxic gene products
- successful synthesis of 10–120 kDa proteins.

Product description

The RTS 100 *E. coli* Disulfide Kit provides the components and procedures necessary for 24 coupled transcription–translation reactions of 50 µl under disulfide-bond–forming conditions.

Product limitations

The RTS 100 *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 100 *E. coli* Disulfide Kit	Contents and function	No. vials per kit
Ordering number	BR1400401	
E. coli Lysate; 100 *E. coli* Dis. (Vial 1, red cap)	→ Stabilized lysate from *E. coli* → Contains components for transcription and translation → Contains optimized amounts of GroE chaperones	1
Reaction Mix; 100 *E. coli* Dis. (Vial 2, green cap)	→ Stabilized substrate mix to prepare 24 x 50 µl reaction solution → Contains a redox system and a disulfide isomerase for formation of disulfide bonds	1
Amino Acids; 100 *E. coli* Dis. (Vial 3, brown cap)	→ Stabilized amino acid mix without Methionine to prepare 24 x 50 µl reaction solution and 24 x 1 ml feeding mix	4
Methionine; 100 *E. coli* Dis. (Vial 4, yellow cap)	→ Stabilized Methionine to prepare 24 x 50 µl reaction solution and 24 x 1 ml feeding mix	1
Lysate Activator; 100 *E. coli* Dis. (Vial 5, violet cap)	→ Solution required for efficient formation and rearrangement of disulfide bonds	1
Feeding Mix; 100 *E. coli* Dis. (bottle 6, blue cap)	→ Stabilized substrate mix to prepare 24 x 1 ml feeding solution → Contains a redox system for the formation of disulfide bonds	1
RNase-DNase-free Water (Vial 7, colorless cap)	→ Water to complete the reaction and feeding solutions	6
Control Vector UK; 100 *E. coli* Dis. (Vial 8, white cap)	→ 12 µl liquid (6 µg), pIVEX 2.3 UK encoding mouse urokinase	1
Reaction Devices + Film; 100 *E. coli* Dis.	→ 3 disposable two-chamber devices, each for eight continuous exchange cell-free (CECF) protein expression reactions → Devices are compatible with the Eppendorf® ThermoMixer® C (or Eppendorf® Thermomixer Comfort) → Includes adhesive film and mounting frame	3

Additional materials

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

- The kit is designed to be used in combination with the Eppendorf® ThermoMixer® C (or Eppendorf® Thermomixer Comfort).
- Pipets: 1–10 µl, 10–200 µl, 200–1,000 µl
- 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

RTS 100 *E. coli* Disulfide Kit is shipped on dry ice.

RTS 100 *E. coli* Disulfide Kit and components should be stored at –70ºC and are stable until the expiration date printed on the label. Avoid repeated freezing and thawing. The adhesive films can be stored dry at room temperature (15–25ºC).

Ensure that the adhesive film and devices are at room temperature (15–25ºC) before use.

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.

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. Certificate of analysis sheets for biotechrabbit products and biotechrabbit product components can be obtained 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

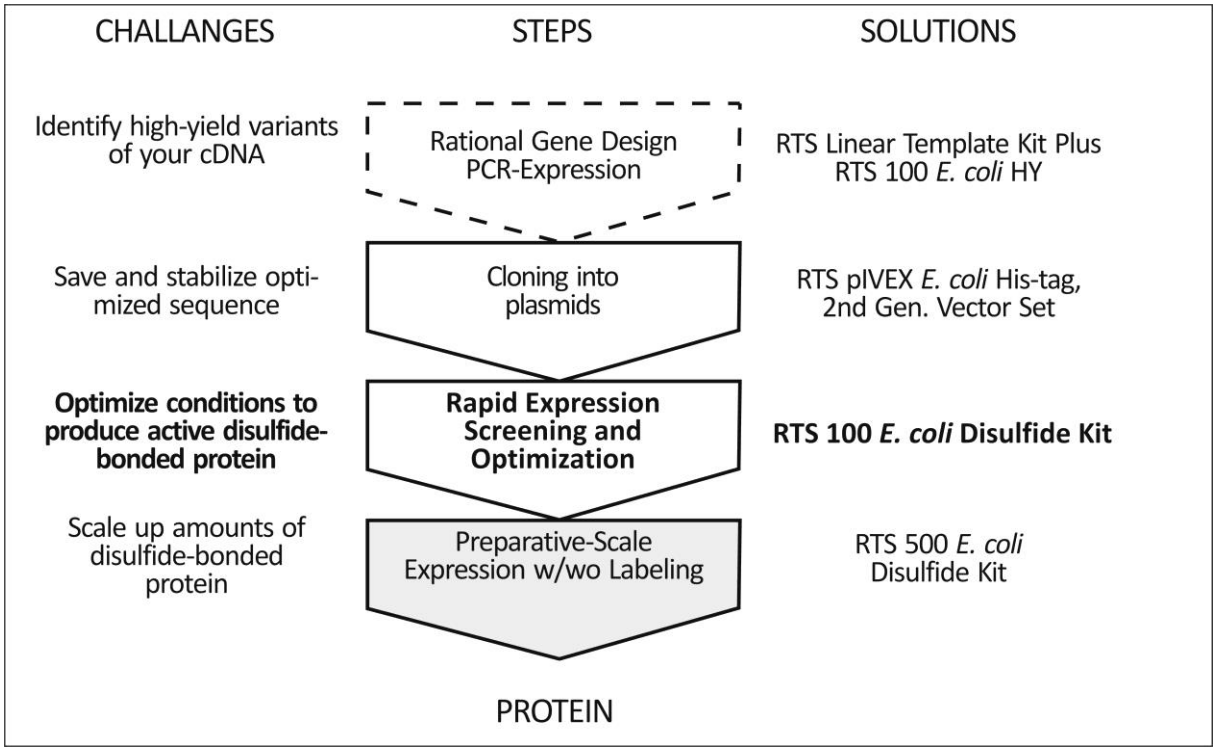
The RTS 100 *E. coli* Disulfide Kit allows expression of analytical amounts (reaction size 50 µl) of disulfide-bonded protein from circular templates within 24 hours. It is suitable for rapid testing and optimization of expression constructs as well as for parallel screening of many reactions for functional expressed proteins.

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



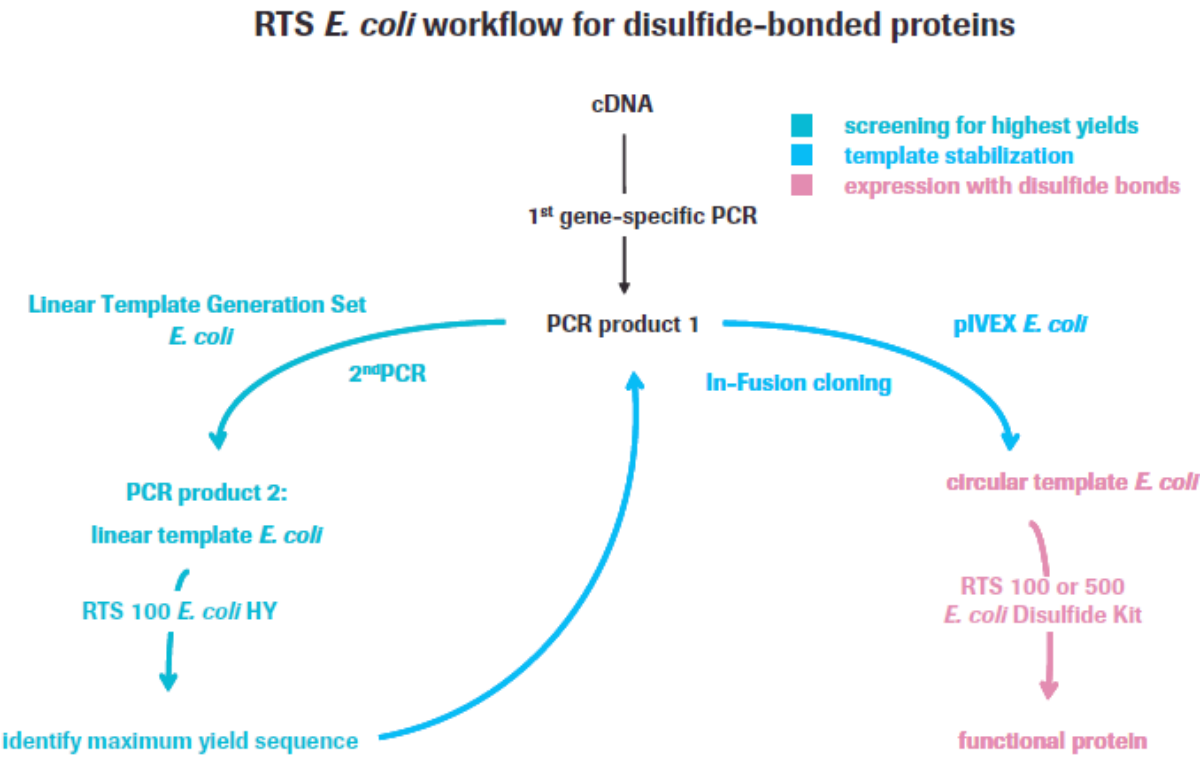


Figure 1. Integration of the RTS 100 *E. coli* Disulfide Kit into the RTS workflow.

Biochemistry

The RTS 100 *E. coli* Disulfide Kit uses an optimized formulation of lysate, reagents, chaperones, and buffer systems to produce maximum yields of active protein in a continuous exchange cell-free (CECF) system. 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 100 *E. coli* Disulfide Kit uses the CECF (continuous exchange cell-free) principle (Figure 2). Transcription, translation and disulfide bond formation take place in the 50 µl reaction compartment of the reaction device. 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 1 ml feeding compartment. Protein expression and folding continues for up to 24 hours, yielding as much as several mg/ml of active protein.

Optimized reaction conditions (reaction time, temperature, supplements etc.) may easily be transferred to large-scale expression reactions (1 ml) in the RTS 500 *E. coli* Disulfide Kit (cat. no. BR1400501), which uses the same CECF technology.

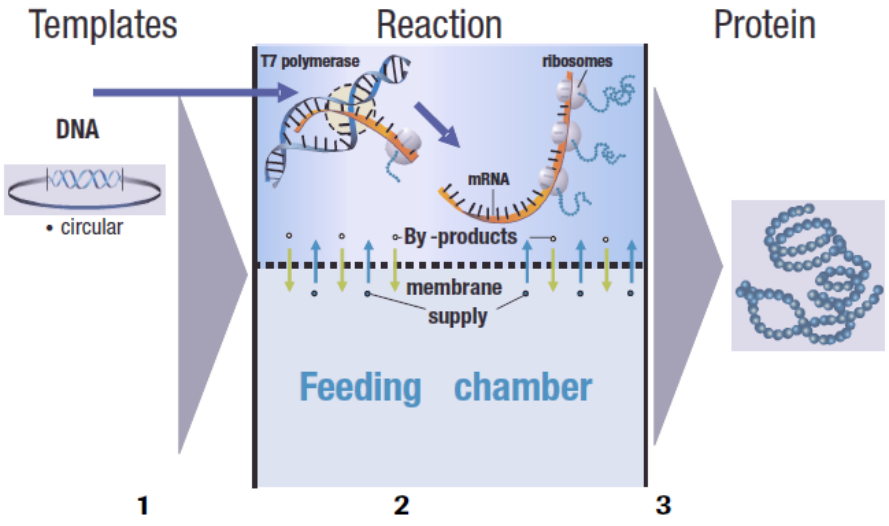


Figure 2. The CECF principle.

CECF procedure

Firstly, DNA with the gene of interest is cloned into a suitable T7-driven expression vector and this is added to the reaction device (1). A 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 correctly (2). Finally, the product accumulates in the reaction mix during a run of 6–24 hours (3).

Template DNA

biotechrabbit recommends the use of pIVEX vectors (cat. no. BR1400701), which are optimized for *in vitro* protein expression. The pIX3.0 Vector (cat. no. BR1402701) with cloned PCR product of the RTS Linear Template Kit Plus (cat. no. BR1402401) 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 12.

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 used due to their relative instability.

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

Expression vectors

Any vector used with the RTS 100 *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 a 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 exceed 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 13).

Maps of some of the available pIVEX vectors are shown schematically in Figure 3. For more information, visit www.biotechrabbit.com, cat. no BR1400701.

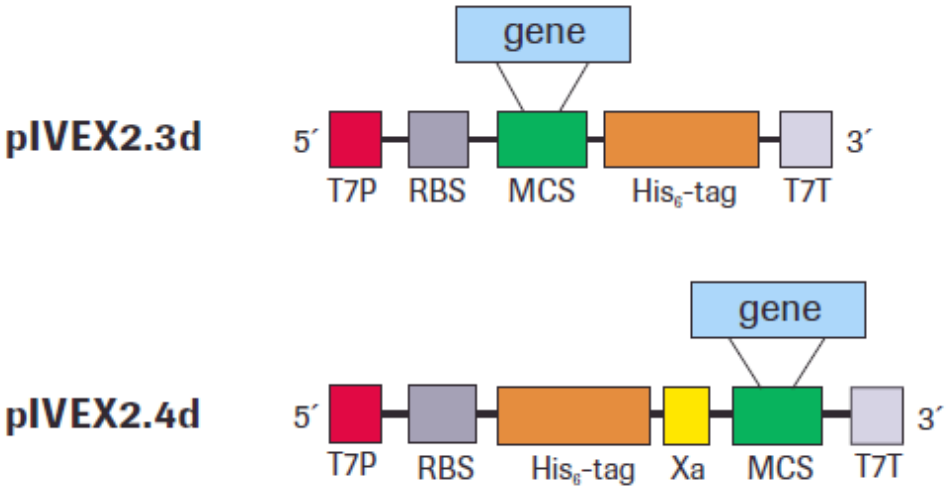


Figure 3. Functional elements of cloning vectors.

T7P: T7 Promoter; **RBS**: Ribosome binding site; **His_6-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, cat. no. BR0700201) 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, cat. no. BR1402401). 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 (cat. no. BR1400101) 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 a proven 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

Equipment and reagents required

- The kit is designed to be used in combination with the Eppendorf® ThermoMixer® C (or Eppendorf® Thermomixer Comfort).
- DNA template: Prepare and purify the DNA template as described in Protocol 1
- 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
- To improve the reproducibility of the results, we recommend washing the reaction device with RNase-DNase-free Water immediately before using it. Remove the wash water from both chambers but do not allow membrane to dry
- Ensure that the adhesive film and the devices are equilibrated to room temperature (15–25°C) before sealing the devices with the film

Procedure

1. Prepare the working solutions according to Table 1.

Table 1. Working solutions for protein synthesis

Solution	Contents	Preparation of working solution
1	Lysate	→ Add 1 µl Lysate Activator (vial 5, violet cap) to 25 µl 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	Mix the following components: → 640 µl Feeding Mix (bottle 6, blue cap) → 140 µl Amino Acid Mix (vial 3, brown cap) → 20 µl Methionine (vial 4, yellow cap) → 200 µl water (vial 7, colorless cap) or supplements
2	Reaction Solution	Mix the following components carefully: → 7 µl Reaction Mix (vial 2, green cap – mix solution before use) → 7 µl Amino Acid Mix (vial 3, brown cap) → 1 µl Methionine (vial 4, yellow cap) → 25 µl activated lysate (solution 1, above) → 5 µl water (vial 7, colorless cap) or supplements → 0.5 µg circular DNA template in 5 µl water or TE buffer Note: A premix of all solutions except DNA template and water is recommended for multiple parallel reactions.

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.

Note: If all 24 reactions are to be run without supplements, the feeding solution for all reactions can be prepared at once and stored frozen. To prepare the feeding solution for 24 reactions, add the following components directly to the Feeding Mix (vial 6, blue cap):

3.720 ml Amino Acid Mix (vial 3, brown cap)

530 µl Methionine (vial 4, yellow cap)

5.313 ml water (vial 7, colorless cap)

2. Rinse the appropriate number of reaction chambers (marked with red ring) of the reaction device (Figure 4) with RNase-DNase-free Water. Remove water from chambers. Do not allow the membrane to dry.

3. Fill one of the feeding compartments in the CECF device with the feeding solution.

   Remove air bubbles under the membrane of the reaction chamber by carefully tapping the device.

4. Add the reaction solution to the reaction compartment that is directly above the filled feeding compartment.

   Note: The reaction compartment is marked with a red lid, which distinguishes it from the feeding compartment (unlabeled).

5. Carefully cover all compartments of the reaction device with the adhesive film (supplied).

   Check the seal before putting the devices into the instrument.

6. Insert the device into the plastic frame supplied with the kit and place it into the Eppendorf® ThermoMixer® C (or Eppendorf® Thermomixer Comfort).
7. Set the instrument to 32°C and 900 rpm and start the reaction.
8. After 6–24 hours remove the reaction devices from the incubator.
9. Analyze the protein.

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

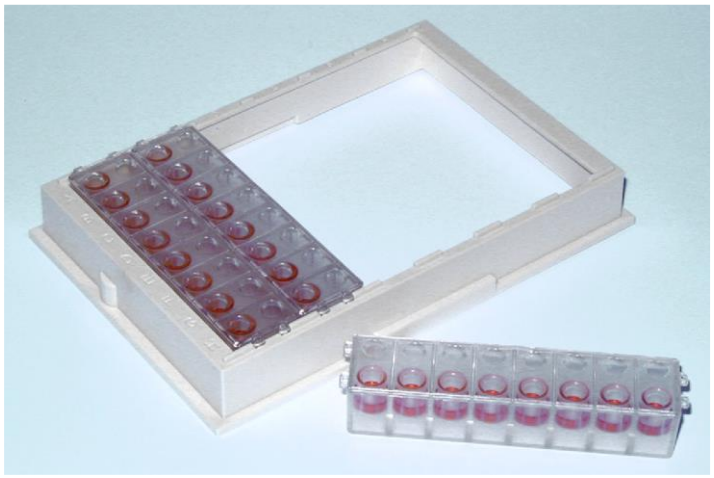


Figure 4. RTS 100 CECF device

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® C (or 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 0.5 µg (1 µl) of Control Vector UK (vial 8, white cap) as template in the Reaction Solution.

   Also prepare a negative control reaction using water in place of the control vector.
2. Set the temperature to 32°C, the shaking speed at 900 rpm, and time for 24 hours. Start the reaction.
3. To detect the product, load 1.25 µl of the reaction onto a 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.

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

Radioactive labeling

Procedure

1. For one labeling reaction, mix:

   5 µl Methionine solution (vial 4, yellow cap).

   20 µl L-[^{35}S]Methionine [15 mCi/ml]

   Note: If fluorography is used to detect the product instead of autoradiography, reduce the amount of L-[^{35}S]Methionine to 3 µl and add 17 µl deionized RNase-DNase-free Water.
2. Treat Lysate with Lysate Activator as described in Table 1.
3. Prepare Feeding Solution as described in Table 1. Use 20 µl of labeled Methionine solution in place of unlabeled Methionine.
4. Prepare Reaction Solution as described in Table 1. Use 1 µl of labeled Methionine solution in place of unlabeled Methionine.
5. Set the following parameters, then start the reaction:

   Temperature: 32°C

   Shaking speed: 900 rpm

   Time: 24 hours
6. Load 2–5 µl of the reaction onto SDS-polyacrylamide gels.
7. After the separation, dry the gel and expose it to autoradiography films (3–20 h exposure).

Supporting information

Short protocol

Protein synthesis using the RTS 100 *E. coli* Disulfide Kit

Step	Action
1 Treat the Lysate	Thoroughly mix the following components: → 25 µl Lysate (vial 1, red cap) → 1 µl Lysate Activator (vial 5, violet cap) → Incubate for 10–20 min at room temperature (15–25°C)
2 Mix and add Feeding Solution	Thoroughly mix the following components: → 640 µl Feeding Mix (bottle 6, blue cap) → 140 µl Amino Acid Mix (vial 3, brown cap) → 20 µl Methionine (vial 4, yellow cap) → 200 µl water (bottle 7, colorless cap) or supplements Rinse appropriate number of reaction devices with RNase-DNase-free Water. Remove water, but do not allow membrane to dry. Add 1 ml prepared Feeding Solution to the larger feeding chamber in the device.
3 Mix and add Reaction Solution	Thoroughly mix the following components: → 7 µl Reaction Mix (vial 2, green cap – vortex solution before use) → 7 µl Amino Acid Mix (vial 3, brown cap) → 1 µl Methionine (vial 4, yellow cap) → 25 µl activated lysate (from step 1, above) → 5 µl water (bottle 7, colorless cap) or supplements → 0.5 µg circular DNA template in 5 µl water or TE buffer Add 50 µl prepared Reaction Solution to the reaction chamber of the device (marked with a red ring).
4 Start the reaction	Seal the device with adhesive film. Insert the device into the Eppendorf® ThermoMixer® C (or Eppendorf® Thermomixer Comfort). Incubate for up to 24 hours at 32°C with shaking at 900 rpm.

Typical results

Expression of urokinase from the control vector pIVEX 2.3-UK

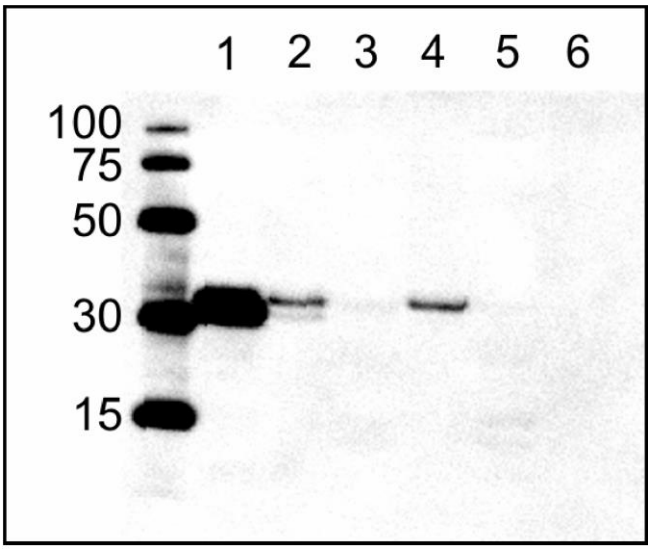


Figure 5. Western blot analyzed using anti-His_6 antibody.

Lane 1: Soluble urokinase expressed using the RTS 100 *E. coli* Disulfide Kit; **Lane 2**: Insoluble urokinase (RTS 100 *E. coli* Disulfide Kit); **Lane 3**: Soluble urokinase (RTS 100 *E. coli* HY Kit); **Lane 4**: Insoluble urokinase (RTS 100 *E. coli* HY Kit); **Lane 5**: Negative control, soluble (no DNA added); **Lane 6**: Negative control, insoluble (no DNA added).

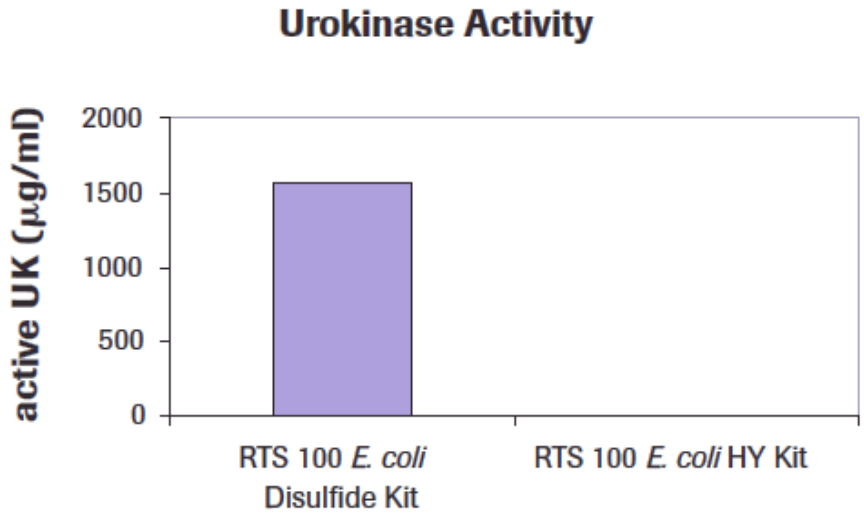


Figure 6. Activity assay for urokinase.

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 performed using untagged urokinase.

Expression of a single-chain antibody

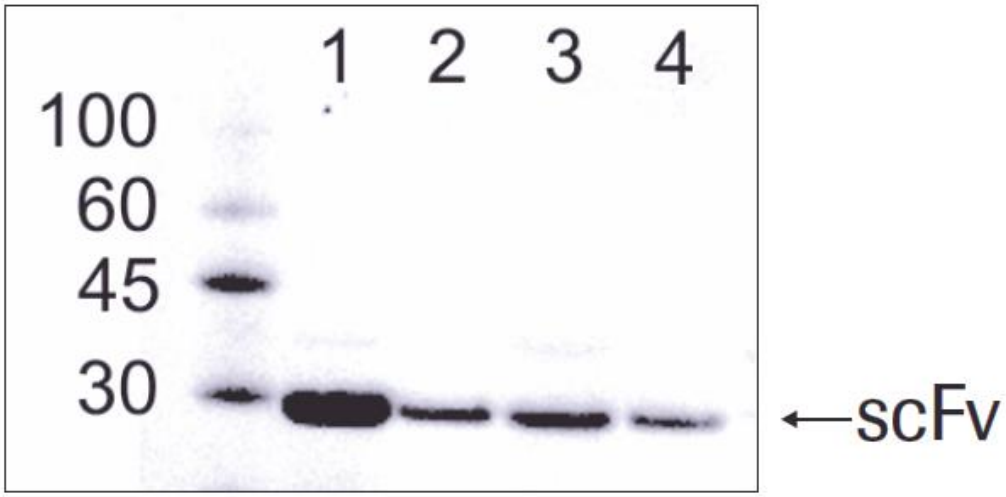


Figure 7. Western blot of a single-chain antibody against a cell-adhesion protein.

Bands for the scFv (single-chain variable fragment) are marked with an arrow. **Lane 1**: Soluble scFv expressed using the RTS 100 *E. coli* Disulfide Kit; **Lane 2**: Insoluble scFv (RTS 100 *E. coli* Disulfide Kit); **Lane 3**: Soluble scFv (RTS 100 *E. coli* HY Kit); **Lane 4**: Insoluble scFv (RTS 100 *E. coli* HY Kit).

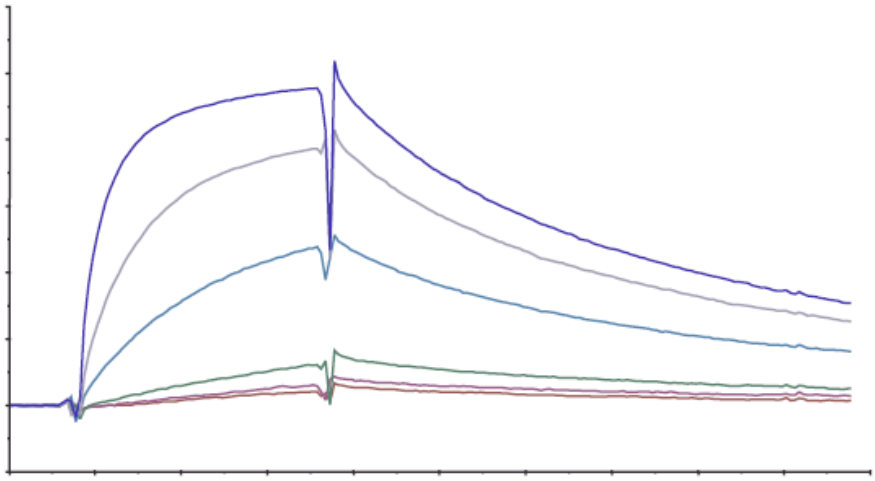


Figure 8. Biacore analysis of a scFv against a cell adhesion protein.

The scFv expressed in the RTS 100 *E. coli* Disulfide Kit shows full activity in Biacore experiments. The binding kinetics of six different dilutions of the scFv was measured. The same scFv is not functional after expression in the RTS 100 *E. coli* HY Kit (data not shown). (Data courtesy of MorphoSys AG, Martinsried, Germany.)

Time dependence of the production of active proteins

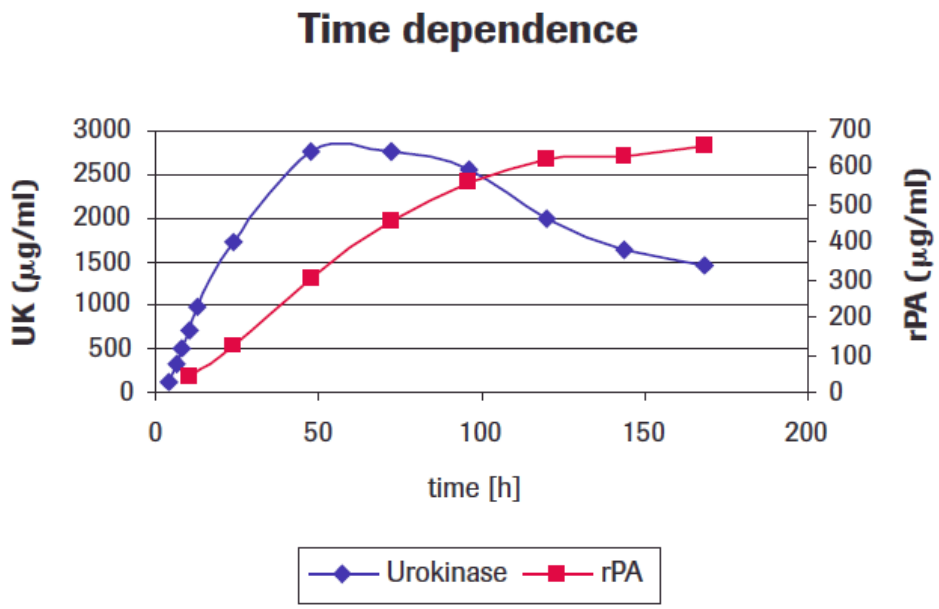


Figure 9. Kinetics for the expression of two proteins where folding is the rate-determining step.

The expression of urokinase and recombinant plasminogen activator is essentially complete after a few hours have elapsed. However, folding continues for several more hours, leading to a dramatic increase in active proteins over time. Note: For a majority of proteins, expression and folding times are under 24 hours.

References

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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	Control protein is not expressed
Possible cause	Kit expired
Resolving	Order a new kit.
Possible cause	Kit has not been stored at –70°C or below
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 His_6-tag at the C-terminus, which allows easy detection on the Western blot but inhibits the protein activity

Problems with expression of target protein even though control reaction works

Observation	Low expression yield
Possible cause	Sequence not optimized for cell-free expression
Resolving	Follow the RTS workflow and optimize sequence.
Possible cause	Vector not ideal for cell-free expression
Resolving	Reclone the open reading frame into a pIVEX vector.
Possible cause	Expression time too short
Resolving	Extend reaction time.
Possible cause	The epitope tag has a negative influence on protein folding
Resolving	Try different pIVEX vectors.
Resolving	Introduce different epitope tag sequences via PCR.
Possible cause	Amount of template DNA not optimal
Resolving	To optimize a 50 µl reaction with circular templates, try varying the DNA concentration between 0.1 µg and 5 µg.

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	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	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	Cloning error caused a premature termination of the translation
Resolving	Check the sequence of the target gene for correct in 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	No expression of target gene, but normal expression of control protein
Possible cause	Cloning error caused a 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 e.g. in pIVEX 2.4.
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.

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 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 Kit	24 reactions	BR1402501
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 5 reactions of 1 ml	BR1401701
RTS DnaK Supplement	For 5 reactions of 1 ml	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 Linear Template Fab Kit	96 reactions	BR1402201
RTS pIX4.0 Insect Vector	1 vector, 25 µg	BR1400901