RTS™ 100 Insect Disulfide Manual

For *in vitro* synthesis of proteins with disulfide bonds and posttranslational modifications using insect-cell lysates

RTS 100 Insect Disulfide Kit

RTS pIX4.0 Insect Vector

RTS 100 Insect Disulfide Kit, April, 2015

Contents

Product specifications

Product description

The RTS 100 Insect Disulfide Kit provides the components and procedures necessary for 10 (cat. no. BR1401401) or 96 (cat. no. BR1401402) transcription/translation reactions of 100 µl. 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	Catalog no.	Size
RTS 100 Insect Disulfide Kit	BR1401401	10 reactions
RTS 100 Insect Disulfide Kit	BR1401402	96 reactions

RTS 100 Insect Disulfide Kit

Kit	No. included (10 reactions)	No. included (96 reactions)
Ordering number	BR1401401	BR1401402
Insect Extract 100 Insect Disulfide	5 x 70 µl (colorless snap-cap)	4 x 924 µl (white screw cap)
Reaction Buffer 100 Insect Disulfide (blue screw-cap)	2 x 50 µl	4 x 264 µl
Energy Mix 100 Insect Disulfide (red screw-cap)	2 x 100 µl	4 x 528 µl
RNase-free Water (colorless screw-cap)	1 x 1.4 ml	2 x 1.4 ml
Positive-Control DNA 100 Insect Disulfide (yellow screw-cap)	1 x 20 µl	1 x 80 µl
Brij-35 Solution 100 Insect Disulfide violet screw cap	1x 60 µl	1 x 576 µl
Microtiter reaction plate, 96 well	–	1
Silicon mat for 96 well plate	–	1

RTS pIX4.0 Insect Vector Ordering number	BR1400901
pIX4.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)

Shipping and storage conditions

The **RTS 100 Insect Disulfide Kit** is shipped on dry ice.
Positive Control DNA and RNase-free Water must be stored at –15 to –25°C upon arrival.

Insect Extract, Reaction Buffer, Energy Mix and Brij-35 Solution must be stored at –70 to – 80°C upon arrival.

Once thawed, Insect Extract should be stored on ice and used within 4 hours. Once thawed, Brij-35 Solution must be stored at +2 to +8°C.

The **RTS pIX4.0 Insect Vector** is shipped on dry ice and must be stored at –15 to –25°C upon arrival.

The RTS 100 Insect Disulfide Kit and components are stable until the expiration date printed on the label. Avoid repeated freezing and thawing.

Safety Information

The components of the kit do not contain more than 1% of a component classified as hazardous and not more than 0,1% of a component classified as carcinogenic.

We however suggest wearing safety glasses, a lab coat and safety gloves.

biotechrabbit expressly excludes any liability for damage or loss which could result from handling, touching or from otherwise coming into contact with these chemicals.

Product Use Limitations

The RTS 100 Insect Disulfide Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Quality Control

Each lot of RTS 100 Insect Disulfide Kit is tested against predetermined specifications to ensure consistent product quality.

Technical Assistance

Our Technical Service is staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology. If you have any questions or experience any difficulties regarding products for cell-free protein synthesis in general, please do not hesitate to contact us.

We encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please contact biotechrabbit Technical Service support@biotechrabbit.com

Protocols

Product principle

Introduction

In vitro translation is a widely used tool for the production of recombinant proteins. Proteins produced by cell-free expression can be used for a wide variety of downstream applications; including activity assays, interaction studies (protein–protein, protein–ligand, and protein–DNA), small-molecule inhibition, and the expression and analysis of open reading frames and expression constructs.

A broad range of eukaryotic proteins require posttranslational modifications such as phosphorylation, glycosylation, or signal peptide cleavage to display full functional activity. Eukaryotic cell-free expression systems provide the possibility to synthesize eukaryotic proteins with posttranslational modifications and are especially useful for expression and analysis of human proteins with native structure and function.

The RTS 100 Insect Disulfide and RTS 100 Insect Membrane Kits, a new eukaryotic cell-free expression system, enables expression of eukaryotic proteins — including membrane proteins — with posttranslational modifications such as intra- and intermolecular disulfide bonds. In contrast to many rabbit-reticulocyte lysate (RRL) -based systems, the insect-cell Extract does not require any additives to display full functionality. In addition, the RTS pIX4.0 Insect Vector (cat. no. BR1400901, biotechrabbit) has been developed for generation of optimal expression templates to be used with the RTS 100 Insect Membrane Kit (cat. no. BR1401501) and RTS 100 Insect Disulfide Kit (cat. no. BR1401401).

Principle and procedure

The RTS 100 Insect Disulfide and RTS 100 Insect Membrane Kits use highly productive insect cell lysates obtained from a *Spodoptera frugiperda* cell line, which contain all translational machinery components (i.e., ribosomes, ribosomal factors, tRNAs, aminoacyl-tRNA synthetases, etc.) required for efficient protein synthesis. In addition, the lysates contain functional organellar membrane fractions, whose activity is required for posttranslational modification of eukaryotic proteins (1), including membrane proteins (Figures 1 and 2). The RTS 100 Insect Disulfide Kit is a coupled transcription/translation system, while the RTS 100 Insect Membrane Kit is a linked transcription–translation system (see flowchart, page 9).

In the *in vitro* transcription reaction of the RTS 100 Insect Membrane Kit mRNA is produced using linearized or circular plasmid DNA or PCR products containing a T7 promoter. Both, the RTS 100 Insect Disulfide Kit and the RTS 100 Insect Membrane Kit use high-quality capped mRNA to express active full-length proteins.

Using the RTS 100 Insect Membrane Kit, up to 40 µg/ml functionally active posttranslationally modified protein can be synthesized within ~3.5 hours. Using the RTS 100 Insect Disulfide Kit, up to 20 µg/ml functionally active disulfide-bonded and posttranslationally modified protein can be synthesized within 4 hours.

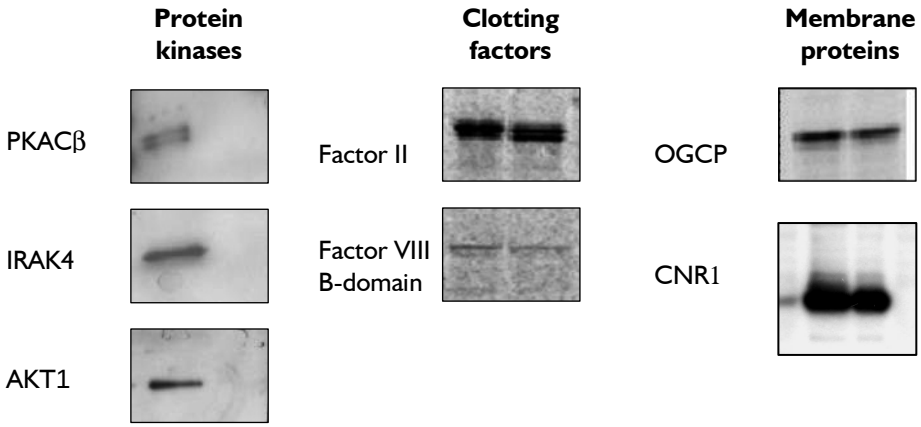


Figure 1. Proteins successfully expressed using the RTS 100 Insect Membrane Kit. 6xHis-tagged protein kinases and cannabinoid receptor (CNR1) were visualized using the Penta·His HRP Conjugate. Clotting factors and OGCP (mitochondrial 2-oxoglutarate/malate carrier) were synthesized in duplicate reactions using ^{14}C-labeled amino acids and visualized using a PhosphorImager®.

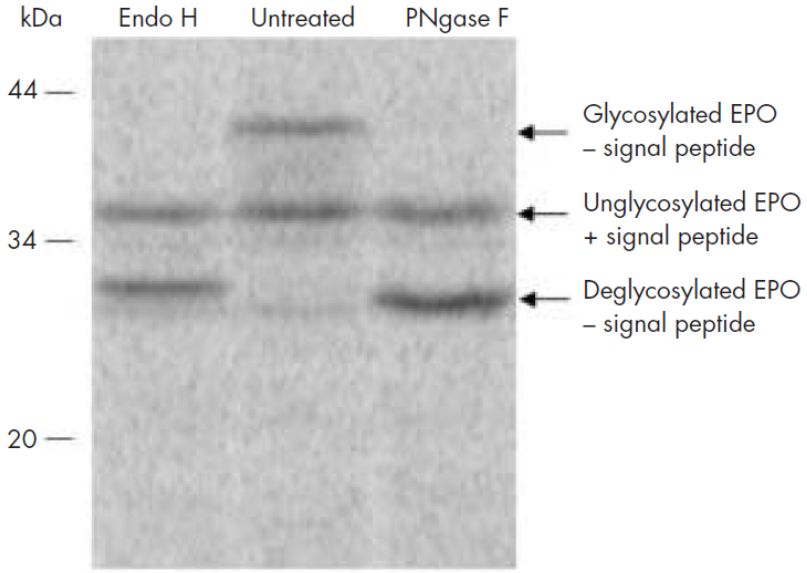
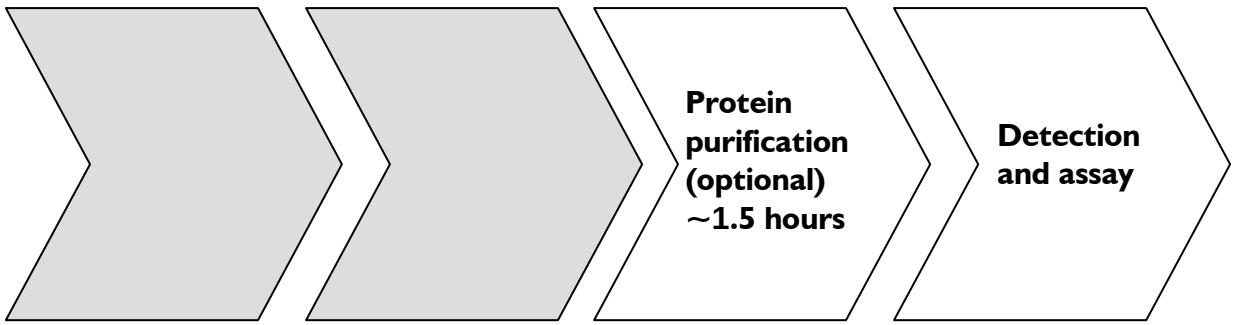


Figure 2. Efficient glycosylation of erythropoietin. The glycoprotein Erythropoietin (EPO) was synthesized using the RTS 100 Insect Membrane Kit in the presence of ^{14}C-labeled amino acids. To remove the glycan moieties from the synthesized glycoproteins, aliquots of the synthesis reactions were incubated either in the presence of endoglycosidase H (Endo H) or peptide N-glycosidase F (PNGase F). After separation by SDS-PAGE, proteins were visualized using a PhosphorImager. Removal of the glycan moieties increases the electrophoretic mobility of the protein compared to the glycosylated form.



RTS pIX4.0 Insect Vector

RTS Linear Template Kit Plus

RTS Linear Template Fab Kit

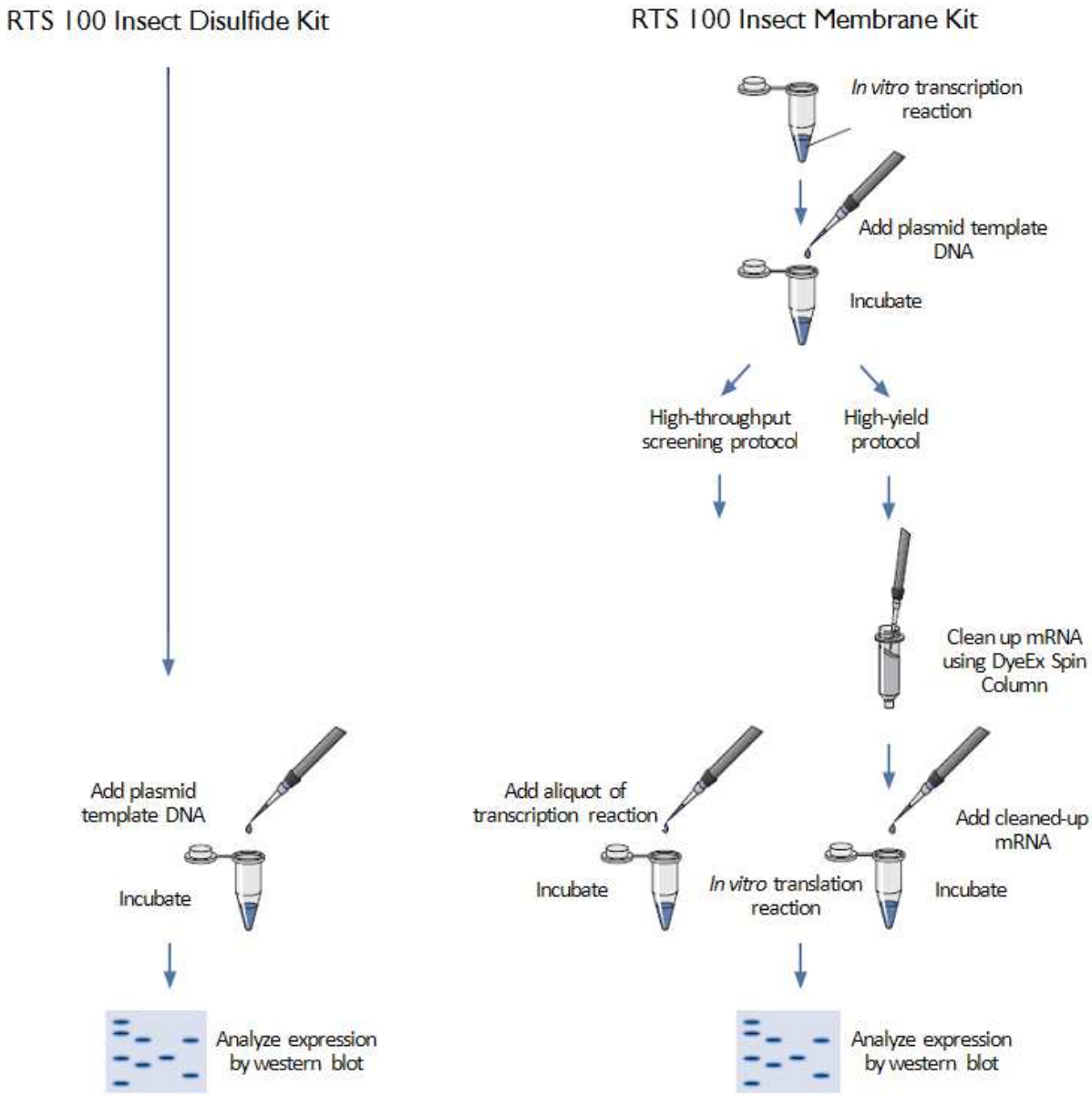
RTS 100 Insect Membrane Kit

Ni-NTA Magnetic Agarose Beads

Penta·His Antibody or Penta·His HRP Conjugate Kit

Strep-tag Antibody

Protein Synthesis Procedure



DNA Templates

The RTS 100 Insect Disulfide Kit can be used to express proteins from a variety of DNA templates. Templates must contain a T7 promoter upstream of the coding sequence. Suitable DNA templates include circular and linearized plasmid DNA, and PCR products generated using the RTS Linear Template Kit Plus (cat. no. BR1402401) or the RTS Linear Template Fab Kit (cat. no. BR1402201).

Minimum template requirements

DNA templates must contain the T7 promoter (Figure 3) for transcription. A stretch of at least 5 base pairs should be placed upstream of the promoter. The sequence of the transcribed mRNA must begin with at least one G. The 5' untranslated region (5'-UTR) must not contain an ATG triplet in any reading frame. Strong secondary structures within the 5'-UTR should be avoided. The translation start codon must be ATG and the translation stop codon must be TAA, TAG, or TGA. When using circular plasmid DNA as template, the plasmid must contain a T7 terminator.

For optimal efficiency of transcription and translation we strongly recommend using the cloning and expression vector RTS pIX4.0 Insect Vector (cat. no. BR1400901), see Figure 9, page 17 or RTS pIX3.0 Vector after cloning of linear templates produced with the RTS Linear template kit plus or RTS Linear Template Fab Kit.

5'...XXXXX**TAATACGACTCACTATA**G... 3'

Figure 3. Sequence of T7 promoter (bold) and transcription start (underlined).

Plasmid DNA

Greatest yields of capped mRNA, and consequently, high protein yields are obtained using template DNA of the highest purity. High-purity plasmid DNA can easily be obtained with the GenUP™ Plasmid Kit and GenUP™ Plasmid Plus Kit, cat. no. BR0700201 and BR0701201, biotechrabbit). DNA prepared using the standard alkaline lysis method described by Sambrook, Fritsch, and Maniatis (2) may be sufficiently pure, but DNA must be free of RNases.

To achieve optimal protein yields we recommend linearization of expression plasmid DNA prior to *in vitro* coupled transcription/translation.

PCR products

If PCR products are added to the cell-free protein synthesis reaction, we recommend that they are generated using the RTS Linear Template Fab Kit (cat. no. BR1402201) or the RTS Linear Template Kit Plus (cat. no. BR1402401). PCR products can be added directly to the protein synthesis reactions without further cleanup. The amount of PCR product added to each 100 µl protein synthesis reaction should be 500 ng. **Note**: XE-solution provided with the Linear Template Kit Plus or RTS Linear Template Fab Kit **should not be added** to the protein synthesis reaction.

The manuals of RTS Linear Template Fab Kit and RTS Linear Template Kit Plus give comprehensive and detailed information on producing PCR products suitable for use as expression constructs with RTS 100 Insect Disulfide Kit.

Identification of optimal constructs in a single day

The PCR-based RTS Linear Template Fab Kit and RTS Linear Template Kit Plus can be used to generate a range of templates that encode target proteins with varying combinations of 6xHis and *Strep*-tag® affinity tags (Figure 4A). The PCR products are generated in a 2-step PCR procedure that uses target-specific primers (supplied by the user) and specially designed tag-encoding primers, which encode regulatory elements that optimize transcription and translation in cell-free expression reactions.

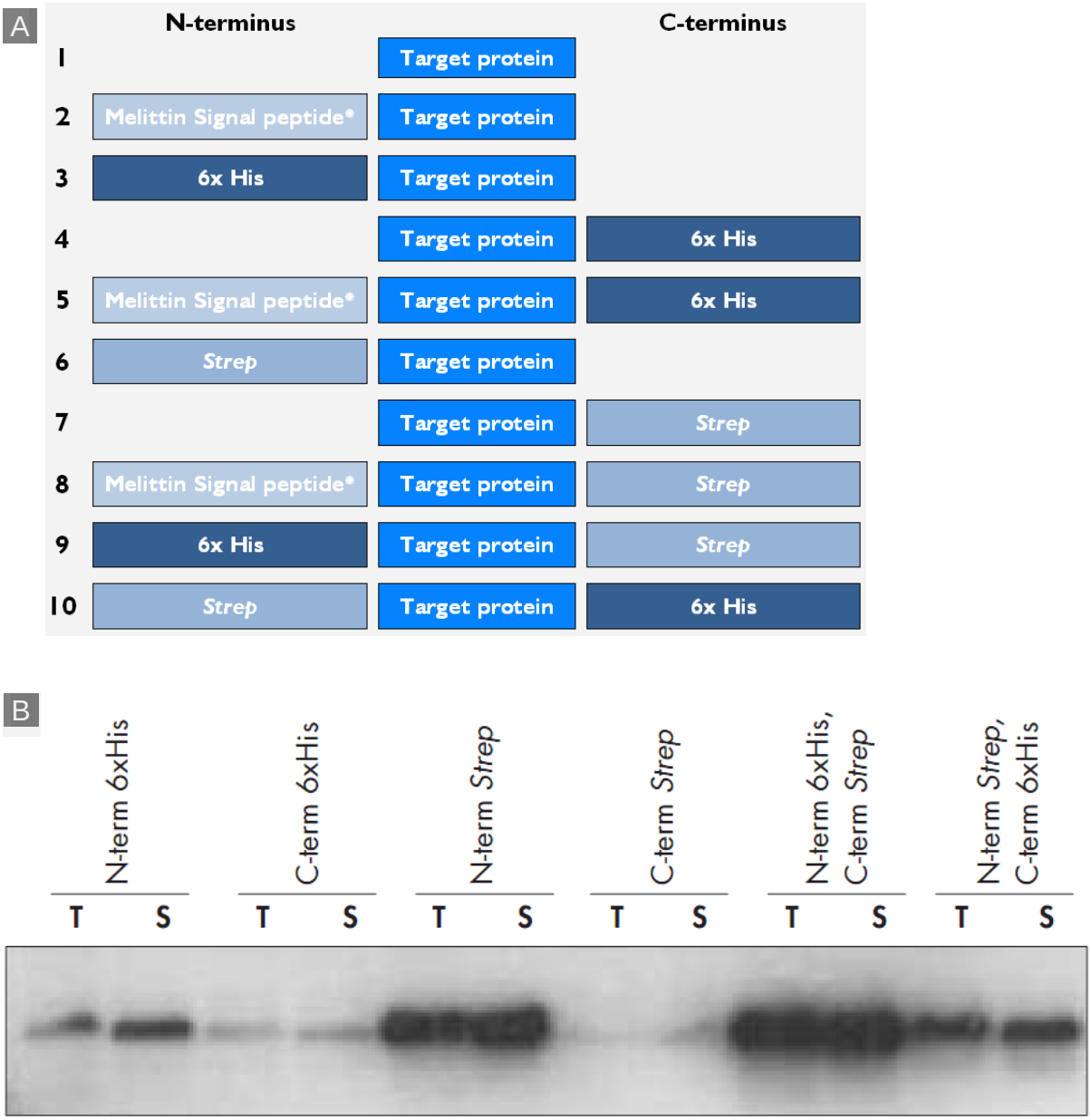


Figure 4. Expression analysis reveals the optimal construct. PCR products generated using the Linear Template Kit Plus and carrying the indicated tag(s) A are added to small-scale IVT reactions.Instead of an N-terminal tag the mellitin signal peptide can be added to direct synthesized proteins into the ER-based microsomes. B TFII variants were synthesized using the RTS 100 Insect Membrane Kit and separated by SDS-PAGE, transferred to a membrane, and visualized using a mixture of Anti·His antibodies and *Strep*-tag antibodies and chemiluminescent detection. **T**: total protein; **S**: soluble fraction.

The screening of such constructs in small-scale cell-free expression (IVT) reactions using the insect-cell based RTS 100 Insect Membrane Kit saves a significant amount of time. The analysis of cell-free expressed proteins by western blotting with tag-specific antibodies enables the identification of the construct or reaction conditions that provide the highest ratio of soluble protein. This procedure also enables a number of different mutation or truncation forms to be tested to find the best-expressing construct (Figure 4B). Expression efficiencies obtained using *in vitro* systems correlate well with those seen in *in vivo* systems, meaning that the best-expressing construct identified in the screening procedure will usually deliver optimal yields in scaled-up *in vivo* expression.

Development of a specialized cell-free expression system for disulfide-bonded proteins and antibody fragments

The existing RTS product line has been the basis for the development of a new cell-free protein expression system especially suited for disulfide-bonded proteins and antibody fragments. To meet the demands of disulfide-bond formation the synthesized proteins are directed via signal peptide into the lumen of endogenous microsomal vesicles derived from the endoplasmic reticulum. A redox buffer system is included in the reaction buffer to maintain a stable oxidizing environment during the whole expression reaction to allow for disulfide-bond formation.

In comparison to the RTS protein synthesis reaction for cytosolic proteins, the expression protocol is optimized to enhance disulfide-bond formation. The expression time is prolonged since disulfide-bonded proteins need time to maturate. Meanwhile, disulfide bonds form and rearrange in the presence of endogenous chaperone-like protein disulfide isomerases (PDI).

Description of procedure

The overall process starts with with prepared plasmid or PCR-based template generation, followed by cell-free protein expression. A final activity assay can be completed within 2 days (Figure). This process can be done either for single tube reactions or in a 96-well, high-throughput format. We recommend testing general handling and expression conditions first using a single reaction before using the high-throughput format. In addition to linear templates (PCR product), plasmid DNA is also a suitable expression template (for detailed requirements see 'Expression templates', page 15).

Using the RTS 100 Insect Disulfide Kit, up to 20 µg/ml biologically active protein can be synthesized within 4 hours. The synthesis reaction can be easily scaled up to several milliliters. The amount of protein synthesized increases linearly with reaction volume.

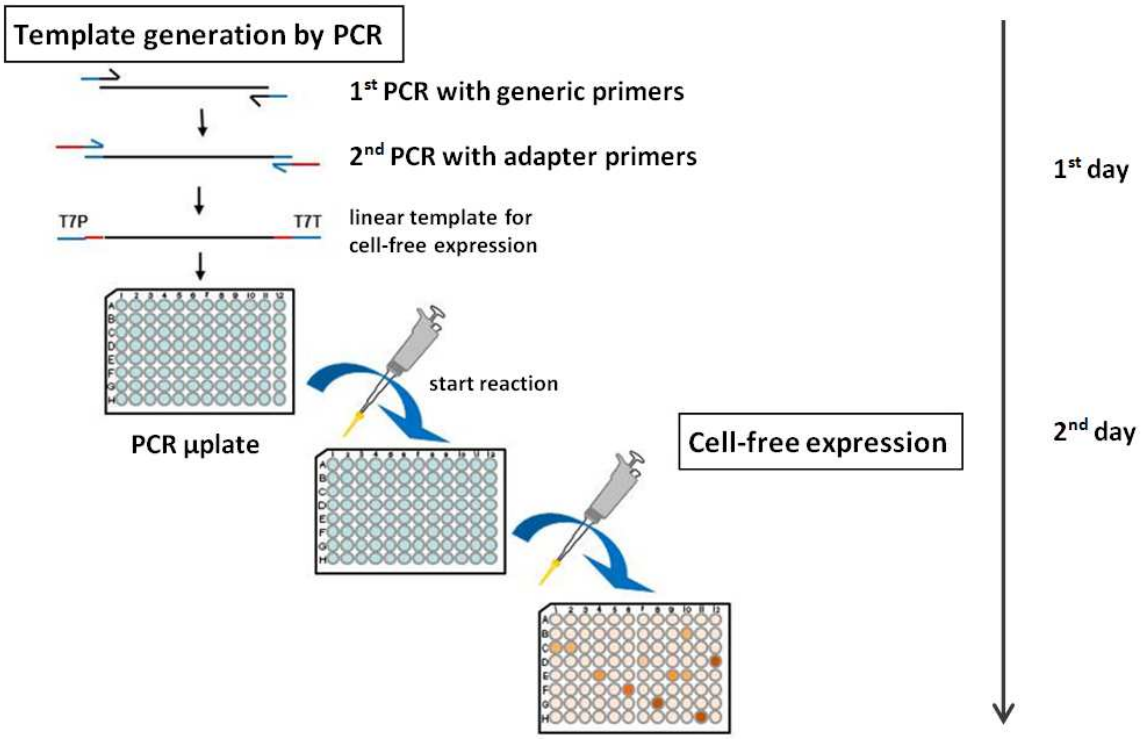


Figure 5. Workflow of template generation, cell-free expression, and subsequent analysis of disulfide-bonded proteins. 2 days are necessary to complete the whole workflow.

Expression of active disulfide-bonded proteins

The RTS 100 Insect Disulfide Kit was developed to generate soluble and fully active disulfide-bonded proteins. Based on the existing RTS 100 Insect Membrane Kit, the Extract was modified accordingly to meet the demands of disulfide-bond formation (see Figures 6 and 7).

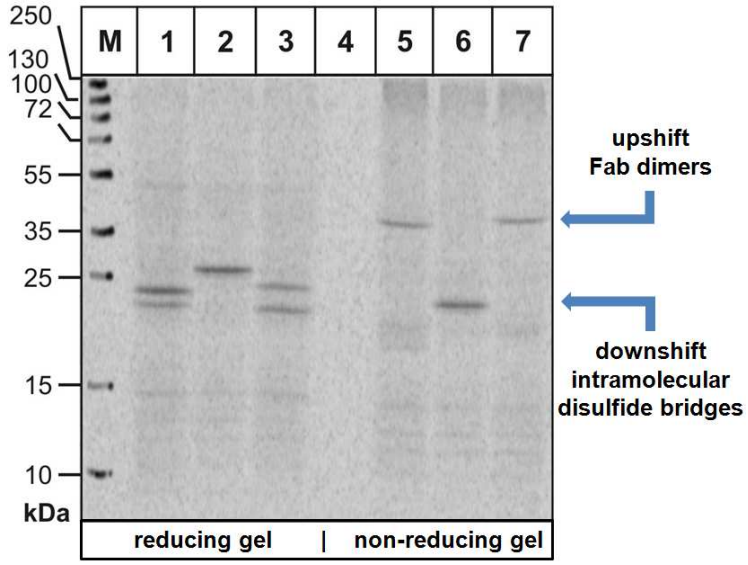


Figure 6: SDS-PAGE analysis of intra- and intermolecular disulfide bridge formation using the Disulfide Insect Kit Anti-lysozyme Fab and anti-CD4 Fab light (L) and heavy (H) chains and anti-lysozyme single-chain antibody were expressed in the RTS 100 Insect Disulfide Kit. The protein bands in the non-reducing gel are shifted to lower apparent MW by intramolecular disulfide bridges and to higher MW by disulfide mediated dimer formation. 1+5: anti-lysozyme Fab (L + H chain coexpression), 2+6: anti-lysozyme scFv, 3+7: anti-CD4 Fab (L + H chains)

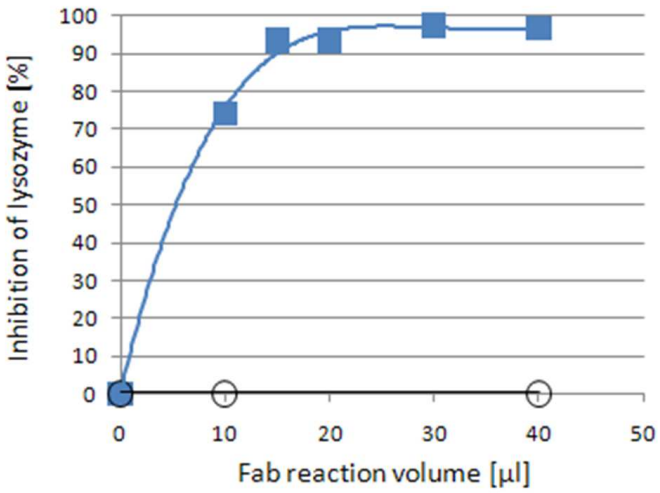


Figure 7: Activity of anti-lysozyme Fab measured by its inhibiting effect on lysozyme Lysozyme activity was measured by lysis of *Micrococcus lysodeikticus* cell suspension monitored by decrease of absorbance at 450 nm light with time. The specific inhibiting effect on lysozyme was measured by incubating different sample volumes of ■ anti-lysozyme Fab reaction with lysozyme and comparing the resulting lysis to control reaction with ○ erythropoietin.

The intramolecular disulfide bonds (two in each Fab light and heavy chain; two in each scFv; (Figure 6) are readily formed. However, the formation of the intermolecular disulfide bond between Fab light and heavy chain ("fifth S-S bridge") plays an essential role in the stabilization of fully active Fab fragments. The RTS 100 Insect Disulfide Kit is also suitable for the expression disulfide-bonded proteins other than antibody fragments.

Expression templates

The RTS 100 Insect Disulfide Kit can be used to express proteins from a variety of DNA templates that contain a T7 promoter upstream of the coding sequence. Further prerequisites of DNA-templates a the translational start point (preferably AUG) and an untranslated region (UTR) downstream of the stop codon (Figure 8). In addition, we have incorporated elements in our expression constructs to increase stability and translational efficiency that are present in many standard expression vectors. Stem-loop structures, recommended for stabilizing the mRNA transcript against nucleases, form at the mRNA 5' and 3' termini, thereby increasing the efficiency of expression. Suitable DNA templates include linear DNA (e.g. PCR products) and supercoiled plasmids. Recommended standard vectors are RTS pIX3.0 Vector with cloned linear templates or RTS pIX4.0 Insect Vector (Figure 9, cat no. BR1400901), which gives slightly higher protein synthesis yield.

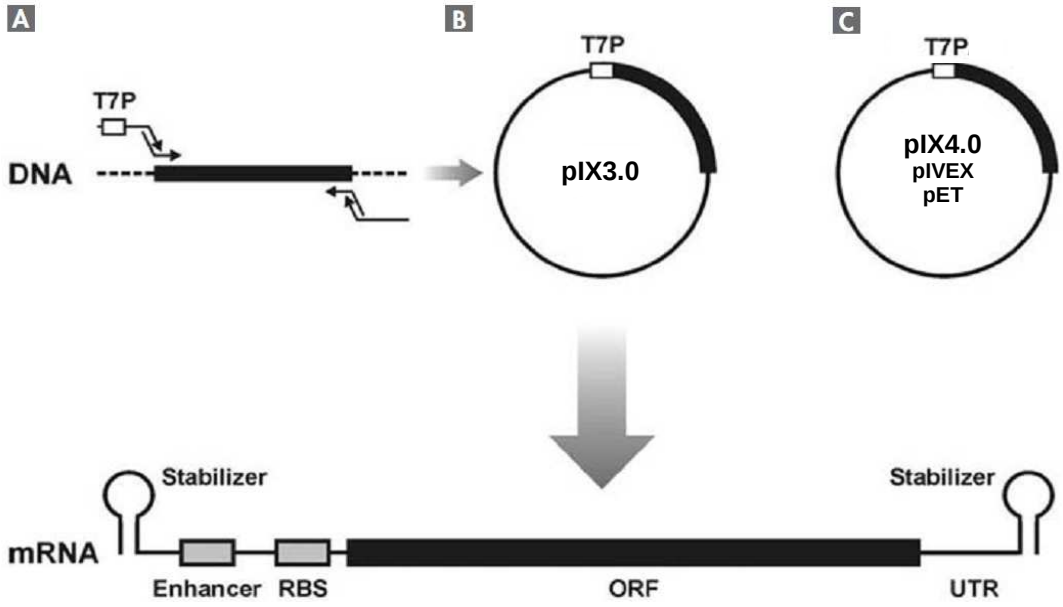


Figure 8. Different types of DNA templates compatible with the RTS 100 E. coli Fab system (top) and optimal organization of an mRNA for efficient cell-free expression (bottom). A Linear template generated with the RTS Linear Template Kit Plus or RTS Linear Template Fab Kit. B Linear template subcloned into vector pIX3.0 C Other vectors containing a T7 or T5 promoter. **T7P**: T7 promoter, **RBS**: ribosome binding site, **ORF**: open reading frame, **UTR**: untranslated region.

Linear template (PCR product)

If PCR products are added to cell-free expression reactions, we recommend that they are generated using the RTS Linear Template Fab Kit (cat. no. BR1402201) or the RTS Linear Template Kit Plus (cat. no. BR1402401). PCR products can be added directly to cell-free expression reactions without further cleanup. The amount of PCR product added to each 100 µl cell-free expression reaction should be 0.5 µg. If PCR products need to be concentrated, we recommend using the GenUP™ PCR Cleanup Kit (cat. no. BR0700301, biotechrabbit). The section 'Generating PCR Products for *In Vitro* Translation' in the RTS Linear Template Kit manuals gives comprehensive and detailed information on producing PCR products suitable for use as expression constructs with RTS protein synthesis kits. The PCR products generated using the RTS Linear Template Kits can be easily and quickly cloned into the RTS pIX3.0 Vector (cat no. BR1402701).

Generation of linear templates with generic primers amplifying a library

If a library with constant 5' and 3' end sequences is used as a template for a series of linear templates with different open reading frames (e.g. antibody fragment library with constant protein backbone and constant DNA end sequences, it may be possible to synthesize only one pair of genespecific primers to generate all templates with different open reading frames using the RTS Linear Template Kits. If a variation of affinity tags is desired (6xHis- or Strep-tag, N- or C-terminal tag), library specific primer pairs acoording to Figure 4 on page 12 can be synthesized.

Choosing a protocol

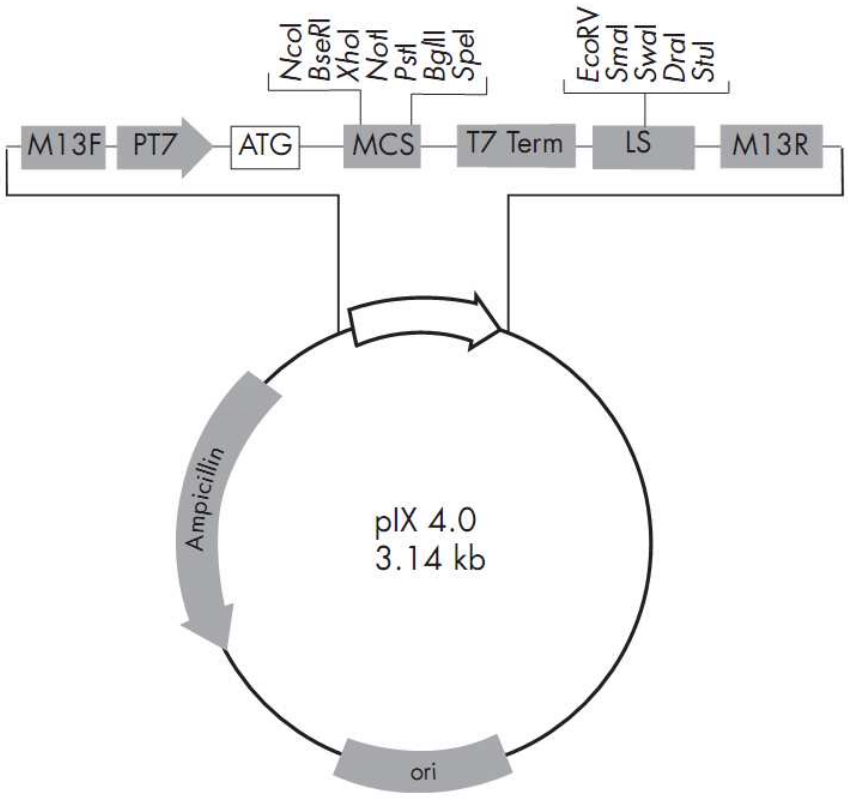
Protocols 1 and 3 are for expression of recombinant disulfide-bonded proteins and protein complexes using linear templates in low- and high-throughput, respectively.

Protocols 2 and 4 are for expression of recombinant disulfide-bonded proteins and protein complexes, using plasmid DNA templates in low- and high-throughput, respectively.

RTS pIX4.0 Insect Vector

The RTS pIX4.0 Insect Vector (cat. no BR1400901) is designed for high-level expression of proteins using the RTS 100 Insect Membrane Kit. This high-copy plasmid has the following features:

- Efficient initiation of translation through a T7 promoter element
- Optimized 3' UTR, including a T7 terminator, and an optimally positioned linearization site
- Multiple cloning site and translational stop codons in all reading frames for convenient preparation of expression constructs
- Optimized 3' UTR combined with T7 terminator for generation of stabilized RNA protected from degradation by exonucleolytic nucleases
- Site for plasmid linearization consisting of multiple restriction sites for blunt end linearization for effective *in vitro* transcription
- β-lactamase gene conferring resistance to ampicillin



*Nco*I *Bse*RI *Xho*I *Not*I *Pst*I *Bgl*II *Spe*I

CC**ATG**GGAGACCCCTCCTCGAGCGGCCGCCTGCAGATCTAAATAATAAGTAATTAACTAGT
GGTACCCTCTGGGGAGGAGCTCGCCGGCGGACGTCTAGATTTATTATTCATTAATTGATCA
Met Gly Asp Pro Ser Ser Ser Gly Arg Leu Gln Ile

Figure 9. The RTS pIX4.0 Insect Vector. **M13F**: M13 forward, **PT7**: T7 promoter, **MCS**: multiple cloning site, **T7 term**: T7 terminator, **LS**: Linearization site, **M13R**: M13 reverse, **ori**: origin of replication.

Designing Primers for Cloning into the RTS pIX4.0 Insect Vector

Comprehensive instructions for cloning protein-coding sequences into the RTS pIX4.0 Insect Vector can be found in Appendix B, pages 34–41.

Protocol 1: Cell-free expression of disulfide proteins using a PCR product as template (single tube format)

- This protocol is suitable for expression of recombinant disulfide-bonded proteins and protein complexes, especially Fab and scFv antibody fragments using linear templates. The RTS 100 Insect Disulfide Kit is developed to perform coupled transcription/translation reactions.
- For template design, it is necessary to refer to the chapter 'DNA templates' on page 10.
- A thermomixer (Eppendorf, Hamburg, Germany) or incubator is required
- All centrifugation steps (except "sample analysis") should be carried out very short (< 5 seconds) and very briefly (< 500 x g)
- The cell-free protein synthesis system is extremely sensitive to nuclease contamination. Wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided as five individual aliquots in single tubes (5 x 70 µl). Once thawed, use Insect Extract within 4 hours.
- Once thawed, store the Brij-35 solution at 4 °C
- Except for the actual transcription/translation incubation, all handling steps should be performed on ice. For protein synthesis reactions, it is important to follow the order of addition for each component given in the protocol and Table 1

Table 1. Pipetting scheme for setup of RTS 100 Insect Disulfide reactions

Reagent	Disulfide protein or Fab synthesis reaction	Positive control reaction	No template control reaction
RNase-free Water	Add to 100 µl	15 µl	35 µl
Reaction Buffer	10 µl	10 µl	10 µl
Insect Extract	35 µl	35 µl	35 µl
Energy Mix	20 µl	20 µl	20 µl
Linear template DNA†	500 ng† (max. 20 µl volume)	-	-
Positive-Control DNA§	-	20 µl	-
Total	**100 µl**	**100 µl**	**100 µl**

† For most linear templates used for protein expression the optimal amount of template is 500 ng per reaction. In some cases an increase up to 1.5 µg per reaction might lead to higher protein yield. For expression of Fab light and heavy chain 250 ng per100 µl reaction for each Fab chain is optimal. In some cases, an increase up to 500 ng/100 µl leads to higher protein yield. Total volume of light and heavy chain linear templates should not exceed 20 µl per 100 µl protein synthesis reaction.

§ Positive-Control DNA is supplied with the kit (yellow screw-cap). The Heavy chain of synthesized anti-CD4 Fab that serves as positive control contains a C-terminal 6x His tag.

1. **Thaw and store Insect Extract, Reaction Buffer and Energy Mix on ice. Thaw RNase-free Water and Positive-Control DNA at room temperature (+15 to +25°C). Mix and centrifuge gently before use. Make sure that the Reaction Buffer is completely thawed and fully dissolved. If a precipitate is visible, warm the Reaction Buffer at +37°C. Mix by vortexing and briefly centrifuge to collect the components at the bottom of the tubes.**
2. **Pipet the components of the transcription/translation reactions in the order shown in Table 1.**

   Use the PCR product from the second PCR step directly, without purification, as a linear template for protein synthesis. For most linear templates, the optimal amount to be used is 500 ng/100 µl reaction. If expressing Fab light and heavy chains from two separate linear templates, use 250 ng/100 µl reaction for each chain. The amount of linear template can be optimized but the total volume of linear templates should not exceed 20 µl/100 µl protein synthesis reaction.
3. **Mix the reactions by briefly vortexing and briefly centrifuge (< 5 seconds and < 500 x g) to collect the reactions at the bottom of the tubes.**
4. **Incubate the cell-free transcription/translation reactions in a Thermomixer (at ~700 rpm) or incubator for 4 h at 25°C.**
5. **Proceed to sample analysis or store samples if necessary at –80°C until analysis.**

Sample analysis

1. Thaw and store Brij- 35 Solution on ice. Before use warm to room temperature. Once thawed, store Brij-35 Solution at +2 to +8°C.
2. After protein synthesis, add 6 µl of Brij-35 Solution to each 100 µl protein synthesis reaction in order to lyse microsomes harboring the synthesized disulfide-bonded protein. If expression reactions have been stored at –80°C prior analysis, thaw samples on ice before lysis.
3. Incubate for 5 min at room temperature with strong shaking (700–900 rpm), thereby avoiding air bubbles (do not vortex).
4. At room temperature, centrifuge the lysis reactions 10 min at 15.000 x g. Transfer the supernatant containing the disulfide-bonded protein to a new reaction tube and store on ice. The synthesized protein now is ready for further analysis. For western blot analysis, use 5–10 µl of the supernatant per gel lane. For ELISA assays, perform serial dilutions of the soluble fraction starting at 1:100.
5. If the insoluble protein fraction is to be analyzed, resuspend the pellet in 100 µl PBS per reaction containing 0.5 % Triton X 100 by vigorously vortexing.

Protocol 2: Cell-free expression of disulfide proteins using a plasmid as template (single tube format)

- This protocol is suitable for expression of recombinant disulfide-bonded proteins and protein complexes, especially Fab and scFv antibody fragments using plasmid DNA. The RTS 100 Insect Disulfide Kit is developed to perform coupled transcription/translation reactions.
- For template design, it is necessary to refer to the chapter 'DNA templates' on page 10.
- A thermomixer (Eppendorf, Hamburg, Germany) or incubator is required
- All centrifugation steps (except "sample analysis") should be carried out very short (< 5 seconds) and very briefly (< 500 x g)
- The cell-free protein synthesis system is extremely sensitive to nuclease contamination. Wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided as five individual aliquots in single tubes (5 x 70 µl). Once thawed, use Insect Extract within 4 hours.
- Once thawed, store the Brij-35 solution at 4 °C
- Except for the actual transcription/translation incubation, all handling steps should be performed on ice. For protein synthesis reactions, it is important to follow the order of addition for each component given in the protocol and Table 2

Table 2. Pipetting scheme for setup of RTS 100 Insect Disulfide reactions

Reagent	Disulfide protein or Fab synthesis reaction	Positive control reaction	No template control reaction
RNase-free Water	25 µl	15 µl	35 µl
Reaction Buffer	10 µl	10 µl	10 µl
Insect Extract	35 µl	35 µl	35 µl
Energy Mix	20 µl	20 µl	20 µl
Plasmid template (75 µg/ml)†	10 µl	-	-
Positive-Control DNA§	-	20 µl	-
Total	**100 µl**	**100 µl**	**100 µl**

† Prepare a plasmid mix containing 0.75 µg of DNA in a volume of 10 µl. For most plasmids, the optimal final concentration is 0.75 µg/100 µl reaction. If expressing Fab heavy and light chains from separate plasmids, the final concentration is 0.375 µg/100 µl reaction each. In some cases, the protein yield can be improved by increasing the final plasmid concentrations up to 0.75 µg/100 µl reaction for each Fab chain.

‡ Positive-Control DNA is supplied with the kit (yellow screw-cap). The Heavy chain of the synthesized anti-CD4 Fab that servers as positive control contains a C-terminal 6x His-tag.

1. **Thaw and store Insect Extract, Reaction Buffer and Energy Mix on ice. Thaw RNase-free Water and Positive-Control DNA at room temperature (+15 to +25°C). Mix and centrifuge gently before use. Make sure that the Reaction Buffer is completely thawed and fully dissolved. If a precipitate is visible, warm the Reaction Buffer at +37°C. Mix by vortexing and briefly centrifuge to collect the components at the bottom of the tubes.**
2. **Pipet the components of the transcription/translation reactions in the order shown in Table 2.**

   For each reaction, prepare a plasmid DNA expression template solution containing 0.75 µg of plasmid in a volume of 10 µl of RNAse-free water. If expressing Fab heavy and light chains from separate plasmids, the final concentration in the transcription/translation reaction is 0.375 µg/100 µl reaction each.
3. **Mix the reactions by briefly vortexing and briefly centrifuge (< 5 seconds and < 500 x g) to collect the reactions at the bottom of the tubes.**
4. **Incubate the cell-free transcription/translation reactions in a Thermomixer (at ~700 rpm) or incubator for 4 h at 25°C.**
5. **Proceed to sample analysis or store samples if necessary at –80°C until analysis.**

Sample analysis

1. Thaw and store Brij- 35 Solution on ice. Before use warm to room temperature. Once thawed, store Brij-35 Solution at +2 to +8°C.
3. After protein synthesis, add 6 µl of Brij-35 Solution to each 100 µl protein synthesis reaction in order to lyse microsomes harboring the synthesized disulfide-bonded protein. If expression reactions have been stored at –80°C prior analysis, thaw samples on ice before lysis.
3. Incubate for 5 min at room temperature with strong shaking (700–900 rpm), thereby avoiding air bubbles (do not vortex).
4. At room temperature, centrifuge the lysis reactions 10 min at 15.000 x g. Transfer the supernatant containing the disulfide-bonded protein to a new reaction tube and store on ice. The synthesized protein now is ready for further analysis. For western blot analysis, use 5–10 µl of the supernatant per gel lane. For ELISA assays, perform serial dilutions of the soluble fraction starting at 1:100.
5. If the insoluble protein fraction is to be analyzed, resuspend the pellet in 100 µl PBS per reaction containing 0.5 % Triton X 100 by vigorously vortexing.

Protocol 3: Cell-free expression of disulfide proteins using a PCR product as template (96-well format)

- This protocol is suitable for expression of recombinant disulfide-bonded proteins and protein complexes, especially Fab and scFv antibody fragments using linear templates. The RTS 100 Insect Disulfide Kit is developed to perform coupled transcription/translation reactions.
- For template design, it is necessary to refer to the chapter 'DNA templates' on page 10.
- A PCR cycler, a stepper pipette and a 96-well plate centrifuge are required
- The cell-free protein synthesis system is extremely sensitive to nuclease contamination. Wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided in four individual bottles (4 x 924 µl). Once thawed, use Insect Extract within 4 hours. The RTS 100 Insect Disulfide Kit (96) can be quartered, use one prefilled bottle to set up a master mix for 24 rxn including a 10 % overfill
- One 96-well plate and silicone mat is provided with the RTS 100 Insect Disulfide Kit (96). If you intend to quartering the Kit (24 rxn each), tape empty wells.
- Additional plates can be ordered at nerbe plus GmbH (#040830140)

Table 3. Pipetting scheme for setup of RTS 100 Insect Disulfide reactions

Reagent	Disulfide protein or Fab synthesis 22 reactions	Positive control reaction	No template control reaction
Prefill 96-well plate with linear template, positive or no template control			
Linear template DNA†,‡	500 ng	-	-
Positive-Control DNA§	-	20 µl	-
RNase-free Water	Add to 20 µl	-	20 µl
	Preparation of master mix (including 10% overfill)		
RNase-free Water	*396 µl*		
Reaction Buffer	*264 µl*		
Insect Extract	*924 µl*		
Energy Mix	*528 µl*		
Master Mix	80 µl	80 µl	80 µl
Total volume per well	**100 µl**	**100 µl**	**100 µl**

† For most linear templates used for protein expression the optimal amount of template is 500 ng per reaction. In some cases an increase up to 1.5 µg per reaction might lead to higher protein yield.

‡ For most linear templates, 250 ng per100 µl reaction for each Fab chain is optimal. In some cases, an increase up to 500 ng/100 µl leads to higher protein yield. Total volume of light and heavy chain linear templates should not exceed 20 µl per 100 µl protein synthesis reaction.

§ Positive-Control DNA is supplied with the kit (yellow screw-cap). The Heavy chain of synthesized anti-CD4 Fab that serves as positive control contains a C-terminal 6x His tag.

1. **Dilute linear expression templates in the provided 96- well plate with RNase-free Water to 500 ng per 20 µl. The filled plate can be stored at +2 to +8°C overnight. Seal tightly.**

   Use the product from the second PCR step directly, without further purification, as a linear template for protein synthesis. For most linear templates, the optimal amount to be used is 500 ng/100 µl reaction. If expressing Fab light and heavy chains from two separate linear templates, use 250 ng/100 µl reaction for each chain. The amount of linear template can be optimized but the total volume of linear templates should not exceed 20 µl/100 µl protein synthesis reaction.

2. **For master mix preparation the RTS Insect Disulfide Kit can be quartered. Use one prefilled bottle of Insect Extract for preparation of a master mix for 24 rxn including a 10 % overfill by addition of Reaction Buffer, RNase-free Water and Energy Mix according to Table 3.**
3. **Thaw and store Insect Extract, Reaction Buffer and Energy Mix on ice. Thaw RNase-free Water and Positive-Control DNA at room temperature (+15 to +25°C). Mix and centrifuge gently before use. Make sure that the Reaction Buffer is completely thawed and fully dissolved. If a precipitate is visible, warm the Reaction Buffer at +37°C.**
4. **Pipet the components of the transcription/translation reactions in the order shown in Table 3 for linear templates (PCR product).**
5. **Mix all components by gently pipetting up and down before use.**
6. **Incubate the master mix for 2 min at 25 °C in a water bath, subsequently dispose the master mix within 15 min in the applicable 96-well plate; use a stepper pipette if possible.**
7. **Incubate the cell-free transcription/translation reactions in a Thermocycler for 4 h at 25°C.**
8. **Proceed to sample analysis or store samples if necessary at –80°C until analysis.**

Sample analysis

1. Thaw and store Brij- 35 Solution on ice. Before use warm to room temperature. Once thawed, store Brij-35 Solution at +2 to +8°C.
2. After protein synthesis, add 6 µl of Brij-35 Solution to each 100 µl protein synthesis reaction in order to lyse microsomes harboring the synthesized disulfide-bonded protein. If expression reactions have been stored at –80°C prior analysis, thaw samples on ice before lysis.
3. Incubate for 5 min at room temperature with strong shaking (700–900 rpm), thereby avoiding air bubbles (do not vortex).
4. At room temperature, centrifuge the lysis reactions 60 min at 4.000x g.
5. Transfer the supernatant containing the disulfide-bonded protein to a new reaction tube and store on ice. The synthesized protein now is ready for further analysis. For western blot analysis, use 5–10 µl of the supernatant per gel lane. For ELISA assays, perform serial dilutions of the soluble fraction starting at 1:100.
6. If the insoluble protein fraction is to be analyzed, resuspend the pellet in 100 µl PBS per reaction containing 0.5 % Triton X 100 by vigorously vortexing.

Protocol 4: Cell-free expression of disulfide proteins using a plasmid as template (96-well format)

- This protocol is suitable for expression of recombinant disulfide-bonded proteins and protein complexes, especially Fab and scFv antibody fragments using plasmid DNA. The RTS 100 Insect Disulfide Kit is developed to perform coupled transcription/translation reactions.
- For template design, it is necessary to refer to the chapter 'DNA templates' on page 10.
- A PCR cycler, a stepper pipette and a 96-well plate centrifuge are required
- The cell-free protein synthesis system is extremely sensitive to nuclease contamination. Wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided in four individual bottles (4 x 924 µl). Once thawed, use Insect Extract within 4 hours. The RTS 100 Insect Disulfide Kit (96) can be quartered, use one prefilled bottle to set up a master mix for 24 rxn including a 10 % overfill
- One 96-well plate and silicone mat is provided with the RTS 100 Insect Disulfide Kit (96). If you intend to quartering the Kit (24 rxn each), tape empty wells.
- Additional plates can be ordered at nerbe plus GmbH (#040830140)

Table 4. Pipetting scheme for setup of RTS 100 Insect Disulfide reactions

Reagent	Disulfide protein or Fab synthesis 22 reactions	Positive control reaction	No template control reaction
Prefill 96-well plate with linear template, positive or no template control			
Plasmid template 37.5 µg/ml†	20 µl	-	-
Positive-Control DNA§	-	20 µl	-
RNase-free Water	-	-	20 µl
	Preparation of master mix (including 10% overfill)		
RNase-free Water	*396 µl*		
Reaction Buffer	*264 µl*		
Insect Extract	*924 µl*		
Energy Mix	*528 µl*		
Master Mix	80 µl	80 µl	80 µl
Total volume per well	**100 µl**	**100 µl**	**100 µl**

† Prepare a plasmid mix containing 0.75 µg of DNA in a volume of 20 µl. For most plasmids, the optimal final concentration is 0.75 µg/100 µl reaction. If expressing Fab heavy and light chains from separate plasmids, the final concentration is 0.375 µg/100 µl reaction each. In some cases, the protein yield can be improved by increasing the final plasmid concentrations up to 0.75 µg/100 µl reaction for each Fab chain.

§ Positive Control DNA is supplied with the kit (yellow screw-cap). The Heavy chain of the synthesized anti-CD4 Fab that servers a positive control contains a C-terminal 6x His tag.

1. **Dilute plasmid templates in the provided 96- well plate with RNase-free Water to 0.75 µg per 20 µl. The filled plate can be stored at +2 to +8°C overnight. Seal tightly.**

   For each reaction, prepare a plasmid DNA expression template solution containing 0.75 µg of plasmid in a volume of 20 µl of RNase-free Water. If expressing Fab heavy and light chains from separate plasmids, the final concentration in the transcription/translation reaction is 0.375 µg/100 µl reaction each.

2. **For master mix preparation the RTS Insect Disulfide Kit can be quartered. Use one prefilled bottle of Insect Extract for preparation of a master mix for 24 rxn including a 10 % overfill by addition of Reaction Buffer, RNase-free Water and Energy Mix according to Table 4.**
3. **Thaw and store Insect Extract, Reaction Buffer and Energy Mix on ice. Thaw RNase-free Water and Positive-Control DNA at room temperature (+15 to +25°C). Mix and centrifuge gently before use. Make sure that the Reaction Buffer is completely thawed and fully dissolved. If a precipitate is visible, warm the Reaction Buffer at +37°C.**
4. **Pipet the components of the transcription/translation reactions in the order shown in Table 4 for plasmid templates.**
5. **Mix all components by gently pipetting up and down before use.**
6. **Incubate the master mix for 2 min at 25 °C in a water bath, subsequently dispose the master mix within 15 min in the applicable 96-well plate; use a stepper pipette if possible.**
7. **Incubate the cell-free transcription/translation reactions in a Thermocycler for 4 h at 25°C.**
8. **Proceed to sample analysis or store samples if necessary at –80°C until analysis.**

Sample analysis

1. Thaw and store Brij- 35 Solution on ice. Before use warm to room temperature. Once thawed, store Brij-35 Solution at +2 to +8°C.
2. After protein synthesis, add 6 µl of Brij-35 Solution to each 100 µl protein synthesis reaction in order to lyse microsomes harboring the synthesized disulfide-bonded protein. If expression reactions have been stored at –80°C prior analysis, thaw samples on ice before lysis.
3. Incubate for 5 min at room temperature with strong shaking (700–900 rpm), thereby avoiding air bubbles (do not vortex).
4. At room temperature, centrifuge the lysis reactions 60 min at 4.000x g.
5. Transfer the supernatant containing the disulfide-bonded protein to a new reaction tube and store on ice. The synthesized protein now is ready for further analysis. For western blot analysis, use 5–10 µl of the supernatant per gel lane. For ELISA assays, perform serial dilutions of the soluble fraction starting at 1:100.
6. If the insoluble protein fraction is to be analyzed, resuspend the pellet in 100 µl PBS per reaction containing 0.5 % Triton X 100 by vigorously vortexing.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in biotechrabbit Technical Services are always happy to answer any questions you may have about either the information and protocol in this manual or molecular biology applications. For technical assistance and more information please contact biotechrabbit Technical Service support@biotechrabbit.com

	Comments and suggestions
No target protein	
a) Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA. Generate PCR products using the RTS Linear Template Kit Plus or RTS Linear Template Fab Kit.
b) DNA template not optimally configured, or error in cloning	Check the sequence. Make sure that the start codon is in the right position for expression (see "Minimum template requirements", page 10). Ensure that the expression construct contains a T7 promoter.
c) *In vitro* transcription or *in vitro* translation is disrupted by expressed protein	Express control protein in the presence of the target protein. If expression of control protein is inhibited, it may not be possible to express the target protein using the Insect Protein Synthesis System.
d) Rigid secondary structures in the mRNA inhibit initiation of translation	Include a 6xHis-tag coding sequence at the 5' end of the protein coding sequence. If the protein to be expressed already contains a tag, move the tag to the opposite terminus.

	Comments and suggestions
Low expression yield	
e) Poor quality or wrong quantity of DNA template	Check the concentration, integrity, and purity of the DNA template. Prepare high-purity plasmid DNA. Generate PCR products using the RTS Linear Template Kit Plus or RTS Linear Template Fab Kit. Increase or reduce the amount of DNA in the *in vitro* transcription reaction.

Appendix A: Incorporating Radioactive Labels into Proteins for Quantification

Protein expressed using the RTS 100 Insect Disulfide Kit can be quantified by incorporating radioactive amino acids (e.g. ^{14}C-leucine or ^{35}S-methionine). ^{14}C is more stable than ^{35}S and its use is recommended for accurate quantification. However, ^{35}S provides a stronger signal. It is recommended that 1,500 pmol ^{14}C-labeled leucine (^{14}C-Leu) is added per 100 µl reaction. A protocol and example calculation of protein yield using the RTS 100 Insect Disulfide Kit (10) is given below.

Equipment and reagents required*

1. Linearized plasmid DNA encoding protein of interest
2. Thermomixer
3. 300 µM ^{14}C-labeled leucine (^{14}C-Leu, 100 dpm/pmol)

Procedure

Labeling with ^{14}C-Leucine

1. **Thaw and store Insect Extract, Reaction Buffer and Energy Mix on ice. Thaw RNase-free Water and Positive-Control DNA at room temperature (+15 to +25°C). Mix and centrifuge gently before use. Make sure that the Reaction Buffer is completely thawed and fully dissolved. If a precipitate is visible, warm the Reaction Buffer at +37°C. Mix by vortexing and briefly centrifuge to collect the components at the bottom of the tubes.**
2. **Pipet the components of the transcription/translation reactions in the order shown in Table 5.**

   For each reaction, prepare a plasmid DNA expression template solution containing 0.75 µg of plasmid in a volume of 10 µl of RNAse-free water. If expressing Fab heavy and light chains from separate plasmids, the final concentration in the transcription/translation reaction is 0.375 µg/100 µl reaction each.
3. **Mix the reactions by briefly vortexing and briefly centrifuge (< 5 seconds and < 500 x g) to collect the reactions at the bottom of the tubes.**
4. **Incubate the cell-free transcription/translation reactions in a Thermomixer (at ~700 rpm) or incubator for 4 h at 25°C.**
5. **Use a 10 µl aliquot of each reaction for quantification of protein synthesis by TCA precipitation (see page 29).**

   Alternatively, the reactions can be separated by SDS-PAGE and analyzed by autoradiography after drying the gel.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and safety glasses. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Table 5. Pipetting scheme for radioactive labeling reactions

Reagent	Disulfide protein or Fab synthesis reaction	Positive control reaction	No template control reaction
RNase-free Water	20 µl	10 µl	30 µl
Reaction Buffer	10 µl	10 µl	10 µl
300 µM ^{14}C-Leu	5 µl	5 µl	5 µl
Insect Extract	35 µl	35 µl	35 µl
Energy Mix	20 µl	20 µl	20 µl
Plasmid template (75 µg/ml)†	10 µl	-	-
Positive-Control DNA§	-	20 µl	-
Total	**100 µl**	**100 µl**	**100 µl**

Labeling with ^{35}S-Methionine

As an alternative to ^{14}C-leucine labeling, *in vitro* translated proteins can be labeled using ^{35}S-methionine (^{35}S-Met). A pipetting scheme for the reactions is given in the table below. Labeling proteins with ^{35}S-methionine gives stronger signals. We recommend using 1 µl of 15 µM ^{35}S-methionine (1000 mCi/mmol) for labeling each reaction.

Table 6. Pipetting scheme for radioactive labeling reactions (^{35}S)

Reagent	Disulfide protein or Fab synthesis reaction	Positive control reaction	No template control reaction
RNase-free Water	24 µl	14 µl	34 µl
Reaction Buffer	10 µl	10 µl	10 µl
15 µM ^{35}S-Met	1 µl	1 µl	1 µl
Insect Extract	35 µl	35 µl	35 µl
Energy Mix	20 µl	20 µl	20 µl
Plasmid template (75 µg/ml)†	10 µl	-	-
Positive-Control DNA§	-	20 µl	-
Total	**100 µl**	**100 µl**	**100 µl**

Determination of protein yield by TCA precipitation and scintillation counting

This protocol can be used for accurate quantification of radioactively-labeled protein yields from RTS 100 Insect Disulfide Kit reactions.

Equipment and reagents required

4. 5% (w/v) trichloroacetic acid (TCA) and acetone
5. Casein acid hydrolysate (e.g. Sigma, cat. no. A 2427)
6. Glass microfibre filters (for example Whatman® GF/C)
7. Vacuum manifold (e.g. Glass Microanalysis Filter Holder, Millipore cat. no. XX1002530 in combination with a vacuum pump)
8. Scintillation cocktail (for example Ready Protein+™; Beckman Coulter, Inc., cat. no. 158727) and scintillation counter

Procedure

1. **Briefly vortex the *in vitro* translation reaction mixture and transfer a 10 µl aliquot to a 10 ml test tube.**
2. **Add 3 ml of 5% TCA solution containing 2% (w/v) casein acid hydrolysate.**
3. **Mix and incubate for 15 min at 90°C.**

   During this step radiolabeled aminoacyl-tRNA as well as peptidyl-tRNA will be hydrolyzed.
4. **Incubate on ice for at least 30 min to precipitate the synthesized proteins.**
5. **Collect the precipitate on a glass microfibre filter by using a vacuum manifold. Before starting wet the filter with a few drops of 5% (w/v) TCA.**
6. **Wash the filter 3 times with 2 ml aliquots of 5% (w/v) TCA.**
7. **Dry the filter by rinsing it 2 times with 3 ml aliquots of acetone.**
8. **Transfer the filter to a scintillation vial and add an appropriate volume of scintillation cocktail.**
9. **Shake the sample gently for 1 h at room temperature.**
10. **Count the sample in a liquid scintillation counter.**
11. **To determine the total radioactivity added to the reactions, vortex the protein synthesis reaction mixture, transfer a 10 µl aliquot onto a filter disc placed in a scintillation vial, add scintillation cocktail and count the sample in a liquid scintillation counter.**

    Note: to determine background protein synthesis, take aliquots from the no-template control reaction and treat them as described in steps 2–10.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and safety glasses. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Calculation of protein synthesis yield

Labeling with ^{14}C-leucine

$$\text{Percentage of } ^{14}\text{C-Leu incorporated} = \frac{\text{counts TCA precipitation (dpm/µl) x 100}}{\text{counts unprecipitated sample (dpm/µl)}}$$

$$\text{Yield (µg/ml)} = \frac{\%^{14}\text{C -Leu incorporated x 0.01 x conc. Leu (µM) x mol. wt. protein (g/mol)}}{\text{Leu residues in protein x 1000}}$$

Example calculations

Template	Luciferase DNA
Molecular weight	61,710 g/mol
Leu residues	51
Met residues	14

Labeling with ^{14}C-leucine

Leucine concentration	100 µM	unlabeled (Reaction Mix)
^{14}C-Leu concentration	15 µM	100 dpm/pmol
Total leucine concentration	115 µM	13.04 dpm/pmol

Measured radioactivity

TCA precipitated sample (10 µl)	2100 dpm = 210 dpm/µl
Total radioactivity (10 µl sample)	15,000 dpm = 1500 dpm/µl

$$\text{Percentage of } ^{14}\text{C-Leu incorporated} = \frac{210 \times 100}{1500} = 14\%$$

$$\text{Yield (µg/ml)} = \frac{14\% \times 0.01 \times 115\ \mu M \times 61{,}710\ g/mol}{51 \times 1000} = 19.48\ \mu g/ml$$

Labeling with ^{35}S-methionine

Proteins labeled with ^{35}S-methionine gives stronger signals than ^{14}C-labeled proteins. A typical commercially available ^{35}S-methionine solution has a specific activity of 1 µCi/pmol = 2.22 x 106 dpm/pmol and a concentration of 15 µM. This example is based on the addition of 1 µl of ^{35}S-methionine solution (= 1 µCi) for a 100 µl *in vitro* translation reaction.

Methionine concentration	100 µM	unlabeled (Reaction Mix)
^{35}S-Met concentration	0.15 µM	2.22 x 10^6 dpm/pmol
Total methionine concentration	100.15 µM	3325 dpm/pmol

Measured radioactivity

TCA precipitated sample (10 µl)	166,500 dpm = 16,650 dpm/µl
Total radioactivity (10 µl sample)	3,333,000 dpm = 333,000 dpm/µl

$$\text{Percentage of } ^{35}\text{S-Met incorporated} = \frac{16{,}650 \times 100}{333{,}000} = 5\%$$

$$\text{Yield (µg/ml)} = \frac{5\% \times 0.01 \times 100.15\ \mu M \times 61{,}710\ g/mol}{14 \times 1000} = 22.07\ \mu g/ml$$

Appendix B: Cloning Expression Sequences into the RTS pIX4.0 Insect Vector

The RTS pIX4.0 Insect Vector (cat. no. BR1400901) has been specially developed to provide high expression rates in insect-cell lysates. It contains the T7 transcription promoter, optimized 5'- and 3'-untranslated regions (UTRs), a multiple cloning site (MCS), the T7 transcription terminator to stabilize mRNA against exonucleolytic digestion, several alternative restriction sites for plasmid linearization with blunt ends for effective run-off transcription, hybridization sequences for sequencing primers (M13 forward, reverse), an ampicillin resistance marker, and a high-copy origin of replication.

Cloning strategies

*Nco*I *Bse*RI *Xho*I *Not*I *Pst*I *Bgl*II *Spe*I

```
CCATGGGAGACCCCTCCTCGAGCGGCCGCCTGCAGATCTAAATAATAAGTAATTAACTAGT
GGTACCCTCTGGGGAGGAGCTCGCCGGCGGACGTCTAGATTTATTATTCATTAATTGATCA
   Met Gly Asp Pro Ser Ser Ser Gly Arg Leu Gln Ile
```

RTS pIX4.0 Insect Vector multiple cloning site

If the NcoI cloning site is used, it should be noted that because the G 3' of the ATG start codon is an absolute requirement for NcoI restriction, sequences that are cloned into the RTS pIX4.0 Insect Vector using the NcoI restriction site will start with an N-terminus Met followed by the amino acid Gly, Glu, Asp, Ala, or Val. If the *Xho*I, *Not*I, *Pst*I, *Bgl*II, or *Spe*I cloning sites are used, it should be noted that because translation commences at the ATG start codon, additional amino acids will be added to the N-terminus of the native protein sequence. If native structure proteins, which are free of vector-encoded amino acids, are required, the BseRI cloning site should be used.

The cloning options can be summarized as follows:

1. Protein sequence starts with an N-Terminus Met followed by the amino acid Gly, Glu, Asp, Ala, or Val. See "Cloning into RTS pIX4.0 Insect Vector via *Nco*I", page 33.
2. Proteins are synthesized free of any additional vector amino acids. See "Cloning into RTS pIX4.0 Insect Vector via *Bse*RI", page 35.
3. Protein contains up to 6 vector-encoded N-terminal amino acids. See "Cloning into RTS pIX4.0 Insect Vector via *Xho*I, *Not*I, *Pst*I, or *Bgl*II", page 37.
4. Protein is synthesized with an N-terminal 6xHis or Strep-tag®. See "PCR-mediated addition of affinity-tag sequences", page 39.
5. Protein is synthesized with a C-terminal 6xHis or Strep-tag. See "PCR-mediated addition of affinity-tag sequences", page 39.
6. Protein contains an intrinsic N-terminal sequence motif (signal sequence/signal peptide) directing the protein to the secretory pathway (e.g. glycoproteins), Appendix C, page 42.

Cloning into RTS pIX4.0 Insect Vector via *Nco*I

For the PCR, a sense primer with the following structure should be synthesized:

5' - XXXXXXCC**ATG**G(M_{20})—3'
*Nco*I

Sense primer

The restriction enzyme recognition sequence is underlined and the translation start codon ATG is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (M_{20}) corresponds to bases 5–24 of the target protein coding sequence.

For the PCR, an antisense primer with the following structure should be synthesized:

5' - XXXXXXAGATCT**TTA**(N_{20})3'
*Bgl*II Stop

Antisense primer

The restriction enzyme recognition sequence is underlined and the translation stop is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (N_{20}) corresponds to the antisense sequence of the last 20 bases of the target protein coding sequence.

Example: Primer design for cloning of FABP using the *Nco*I and *Bgl*II cloning sites

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 5–24 [sense] and the last 20 bases [antisense]) are in bold.

```
     Met Ser Phe Ser Gly  Lys Tyr Gln        Lys Arg Ile Ser  Lys Arg  Ile Stop
5'—ATGA GTTTCTCCGGCAAGTACCAA C...AAGAGAATCAGCAAGAGAATTTGA—3'
3'—TACTCAAAGAGGCCGTTCATGGTTG...T TCTCTTAGTCGTTCTCTTAA ACT—5'
```

Coding sequence of FABP (bold)

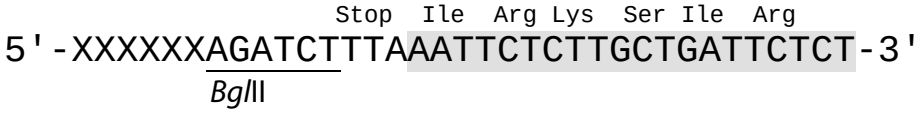
For the PCR the following sense primer is constructed. Native target protein sequence is shaded.

```
                Met Gly  Phe  Ser Gly  Lys Tyr  Gln
5'-XXXXXXCCATGGGTTTCTCCGGCAAGTACCAA-
         NcoI
```

Sense primer

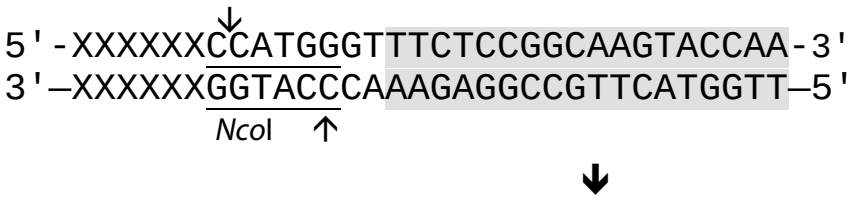
It should be noted that due to the presence of the G 3' of the ATG start codon, the Ser residue in the native sequence will be converted to a Gly.

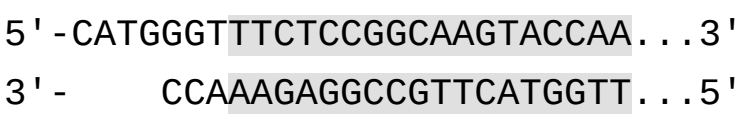
For the PCR the following antisense primer is constructed. Native target protein sequence is shaded.



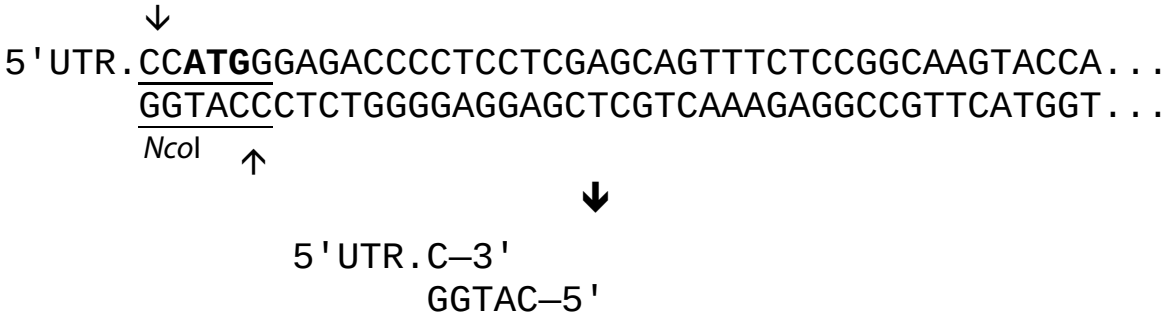
Antisense primer

As a first stage of the cloning procedure, the PCR product is digested with NcoI. Native target protein sequence is shaded.

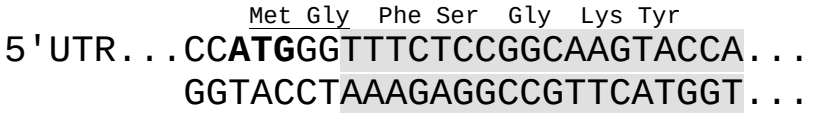




The vector is then digested with *Nco*I.



The digested PCR product and vector are then ligated. Native target protein sequence is shaded. Vector-encoded amino acids are underlined.



Cloning into RTS pIX4.0 Insect Vector via *Bse*RI

For the PCR, a sense primer with the following structure should be synthesized:

5'-XXXXXXGAGGAGGTCTCCC**ATG**(M_{20})—3'
*Bse*RI Met

Sense primer

The restriction enzyme recognition sequence is underlined and the translation start codon ATG is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (M_{20}) corresponds to bases 4–23 of the target protein coding sequence.

For the PCR, an antisense primer with the following structure should be synthesized:

5'-XXXXXXAGATCT**TTA**(N_{20})—3'
*Bgl*II Stop

Antisense primer

The restriction enzyme recognition sequence is underlined and the translation stop is in bold. X can be any base; the presence of this short sequence increases digestion efficiency. (N_{20}) corresponds to the antisense sequence of the last 20 bases of the target protein coding sequence.

A section of the resulting double-stranded PCR product is shown below. It should be noted that *Bse*RI cuts at a staggered site 10 and 8 bases away from its recognition site (arrowed). The recognition site is underlined and the ATG start codon appears in bold.

```
                                    ↓
5' XXXXXXGAGGAGGTCTCCC**ATG**NNN...3'
   XXXXXXCTCCTCCAGAGGGTACNNN...5'
          BseRI          ↑
```

PCR product sequence

A section of the RTS pIX4.0 Insect Vector MCS is shown below. As before, *Bse*RI cuts at a staggered site 10 and 8 bases away from its recognition site (arrowed). In this case the recognition site is located downstream of the ATG start codon (bold). The recognition site is underlined.

```
      ↓
...CC**ATG**GGAGACCCCTCCTC...
...GGTACCCTCTGGGGAGGAG...
    ↑           BseRI
```

RTS pIX4.0 Insect Vector sequence

Example: Primer design for cloning of FABP using the *Bse*RI and *Bgl*II cloning sites

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 4-23 [sense] and the last 20 bases [antisense]) are in bold.

```
     Met  Ser Phe Ser Gly  Lys Tyr Gln        Lys  Arg Ile Ser  Lys Arg Ile Stop
5'—ATG**AGTTTCTCCGGCAAGTACCA**AC...AAGAGAATCAGCAAGAGAATTTGA—3'
3'—TACTCAAAGAGGCCGTTCATGGTTG...T**TCTCTTAGTCGTTCTCTTAA**ACT—5'
```

Coding sequence of FABP

For the PCR the following primers are constructed. Native target protein sequence is shaded.

```
                                  Met  Ser  Phe  Ser Gly Lys Tyr Gln
5'-XXXXXXGAGGAGGTCTCCC**ATG**AGTTTCTCCGGCAAGTACCA-3'
          BseRI
```

Sense primer

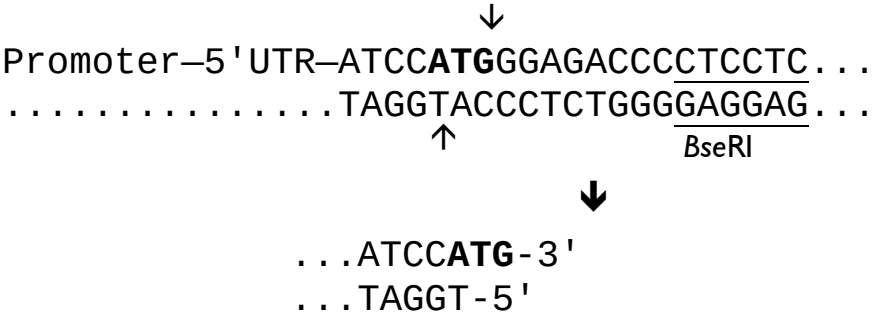
```
                       Stop  Ile Arg  Lys  Ser Ile  Arg
5'-XXXXXXAGATCT**TTA**AATTCTCTTGCTGATTCTCT-3'
          BglII
```

Antisense primer

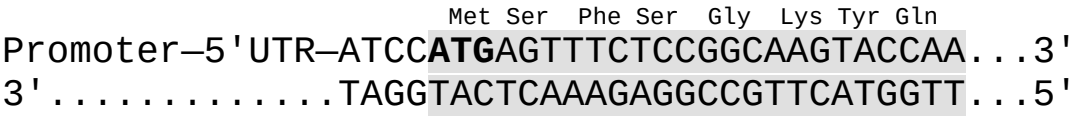
As a first stage of the cloning procedure, the PCR product is digested with BseRI. Native target protein sequence is shaded.

```
                                    ↓
5'-XXXXXXGAGGAGGTCTCCC**ATG**AGTTTCTCCGGCAAGTACCA...3'
3'-XXXXXXCTCCTCCAGAGGGTACTCAAAGAGGCCGTTCATGGT...5'
          BseRI          ↑
                         ⬇

  5'-AGTTTCTCCGGCAAGTACCA...3'
3'-ACTCAAAGAGGCCGTTCATGGT...5'
```

The vector is then digested with *Bse*RI.



The digested PCR product and vector are then ligated. Native target protein sequence is shaded.

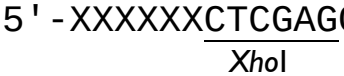


Cloning into RTS pIX4.0 Insect Vector via *Xho*I, *Not*I, *Pst*I, or *Bgl*II

Sequences that are cloned into the RTS pIX4.0 Insect Vector using the *Xho*I, *Not*I, *Pst*I, or *Bgl*II restriction sites will contain up to six vector encoded amino acids (see below). Suggested PCR primers for cloning are given below. Restriction sites are underlined and the translation start codon is in bold. X can be any base, the presence of this short sequence increases digestion efficiency. (M_{20}) corresponds to bases 4–23 of the target protein coding sequence. (N_{20}) corresponds to the final 20 coding bases of the target protein coding sequence.

Cloning into RTS pIX4.0 Insect Vector via *Xho*I

For the PCR, a sense primer with the following structure should be synthesized:



Sense primer

The restriction enzyme recognition sequence is underlined. X can be any base; the presence of this short sequence increases digestion efficiency. (M_{20}) corresponds to bases 4–23 of the target protein coding sequence. The base C between the *Xho*I recognition sequence and the protein coding sequence is required to ensure that the coding sequence codons are shifted into frame.

Example: Primer design for cloning of FABP using the *Xho*I and *Bgl*II cloning sites

The beginning and ends of the protein coding sequence for fatty acid binding protein (FABP) are shown below. The bases that will serve as PCR primer binding sites (bases 4–23 [sense] and the last 20 bases [antisense]) are in bold.

```
     Met Ser Phe Ser Gly Lys  Tyr Gln       Lys Arg  Ile Ser  Lys Arg Ile Stop
5'—ATG**AGTTTCTCCGGCAAGTACCA**AC...AAGAGAATCAGCAAGAGAATTTGA—3'
3'—TACTCAAAGAGGCCGTTCATGGTTG...T**TCTCTTAGTCGTTCTCTTAA**ACT—5'
```

Coding sequence of FABP

For the PCR the following primers are constructed. Native target protein sequence is shaded.

```
                Ser  Ser Ser  Phe  Ser Gly  Lys  Tyr
5'-XXXXXXCTCGAGCAGTTTCTCCGGCAAGTACCA-3'
          XhoI
```

Sense primer

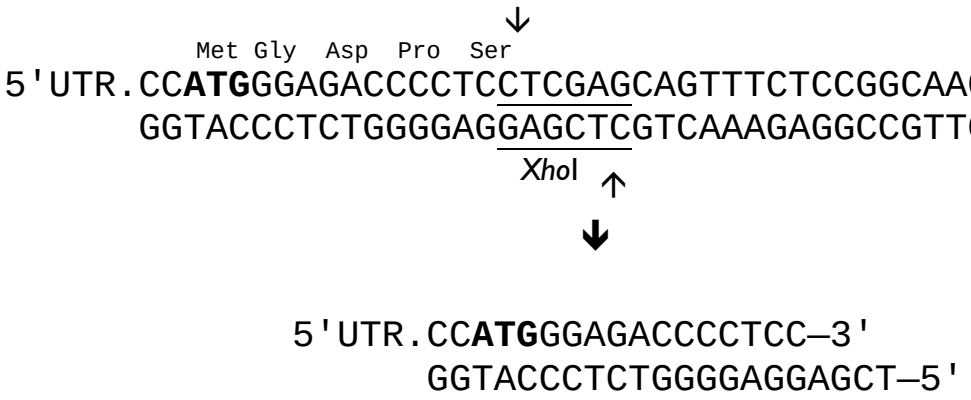
```
                      Stop  Ile Arg  Lys Ser  Ile Arg
5'-XXXXXXAGATCTTTAAATTCTCTTGCTGATTCTCT-3'
          BglII
```

Antisense primer

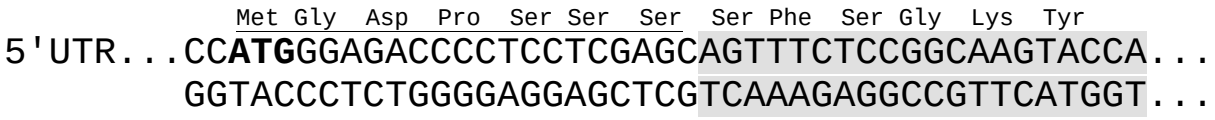
As a first stage of the cloning procedure, the PCR product is digested with XhoI. Native target protein sequence is shaded.

```
          ↓
5'-XXXXXXCTCGAGCAGTTTCTCCGGCAAGTACCA...3
3'-XXXXXXGAGCTCGTCAAAGAGGCCGTTCATGGT...5'
          XhoI  ↑

                ↓

5'-TCGAGCAGTTTCTCCGGCAAGTACCA...3'
3'-    CGTCAAAGAGGCCGTTCATGGT...5'
```

The vector is then digested with *Xho*I.



The digested PCR product and vector are then ligated. Native target protein sequence is shaded. Vector-encoded amino acids are underlined.



PCR-mediated addition of affinity-tag sequences

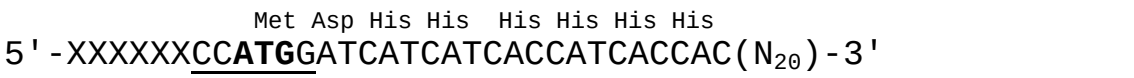
The PCR primers listed below can be used to add affinity-tag coding sequences to expression constructs. Start codons are in bold, and restriction enzyme recognition sites are underlined. If the target protein coding bases lead to the formation of mRNA secondary structures, altering the amino acid codons may improve results.

Sense primers for addition of N-terminal affinity tags

Using the *Nco*I cloning site

N-terminal *Strep*-tag

N-terminal 6xHis tag



Using the *Bse*RI cloning site

N-terminal *Strep*-tag

```
                          Met Trp Ser  His Pro Gln  Phe Glu Lys Ser  Ala
XXXXXXGAGGAGGTCTCCCATGTGGTCTCATCCGCAATTCGAAAAAAGCGCT(N20)
```

N-terminal 6xHis tag

```
                               Met His His  His His His His
5' XXXXXXGAGGAGGTCTCCCATGCATCATCACCATCACCAC(N20)-3'
```

Using the *Xho*I cloning site

N-terminal *Strep*-tag

```
             Ser  Ser  Trp Ser His Pro  Gln Phe  Glu Lys Ser Ala
5'-XXXXXXCTCGAGCTGGTCTCATCCGCAATTCGAAAAAAGCGCT(N20)-3'
```

N-terminal 6xHis tag

```
              Ser Ser His  His His His  His His
5'-XXXXXXCTCGAGCCATCATCACCATCACCAC(N20)-3'
```

Antisense primers for addition of C-terminal affinity tags

Using the *Bgl*II cloning site

C-terminal *Strep*-tag

```
                   Stop  Lys Glu Phe  Gln Pro His  Ser Trp Ala Ser
5'-XXXXXXAGATCTTTATTTTTCGAATTGCGGATGAGACCAAGCGCT(N20)-3'
```

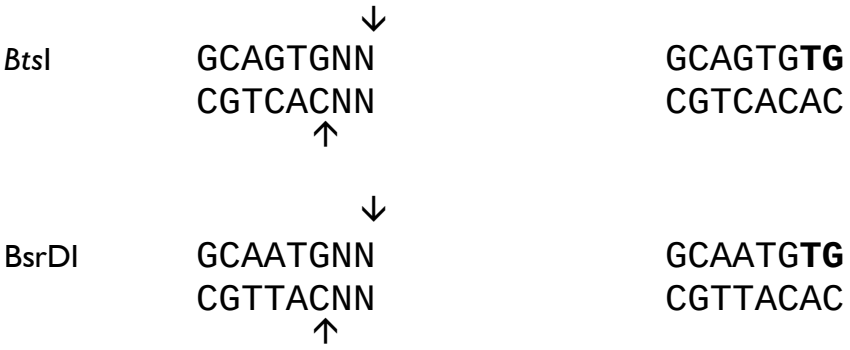
C-terminal 6xHis tag

```
                   Stop  His His  His His His His
5'-XXXXXXAGATCTTTAGTGGTGATGGTGATGATG(N20)-3'
```

Additional cloning options for the RTS pIX4.0 Insect Vector

If the recognition sequence for *Nco*I is present in the target protein's coding sequence, or codon for the N-terminal amino acid cannot commence with a guanine, *Nco*I cannot be used for cloning. In such cases, the recognition sequence for *Bsp*HI (TCATGA) or *Pci*I (ACATGT) can be added to the target gene sequence by PCR; of course these sequences should not appear in the target protein sequence. The *Bsp*HI or *Pci*I restricted insert can be ligated into the *Nco*I restricted vector. Using *Bsp*HI or *Pci*I for restriction dictates that the first base of the N-terminal codon is A or T respectively.

If the recognition sequence for *Bse*RI is present in the target protein's coding sequence, BseRI cannot be used for cloning. In such cases, the recognition sequence for *Bts*I or *Bsr*DI (see below) can be added to the target gene sequence by PCR; of course these sequences should not appear in the target protein sequence.



The *Bts*I or *Bsr*DI restricted insert can be ligated into the BseRI restricted vector if the DNA sense strand contains TG at the position indicated above in bold. The TG motif forms the second and third position of the ATG start codon. Additional restriction enzymes that can be used for restriction of PCR products that can be ligated into a *Bse*RI restricted vector are *Bce*AI, *Bpm*I, *Bpu*EI, *Bsg*I, *Eci*I, or *Mme*I.

Appendix C: Synthesis of Secreted Proteins and Glycoproteins Using the RTS 100 Insect Disulfide Kit

In eukaryotic cells, glycosylated proteins and proteins that are destined for excretion are synthesized by ribosomes associated with the membranes of the endoplasmic reticulum (ER). These classes of proteins are synthesized with an N-terminus containing a so-called signal sequence or signal peptide. The signal peptide usually consists of 13–36 predominantly hydrophobic residues. As the protein is synthesized, the signal sequence is passed through the ER membrane into the lumen of the ER. After the growing protein chain has reached a certain length, the signal peptide is removed by the action of signal peptidases. Protein synthesis continues, and if the protein will be secreted, it ends up completely in the lumen of the ER. In mammalian cells, glycan group attachment to glycoproteins via N-glycosidic linkages occurs predominantly in the lumen of the ER.

The cell lysate in the RTS 100 Insect Disulfide Kit contains microsomal membranes derived from the endoplasmic reticulum of insect cells. To ensure efficient translocation of a glycoprotein or a secreted protein across the membrane of these microsomes, a signal peptide must be present at the N-terminus of the *in vitro* synthesized protein. In such cases, no N-terminal tag sequence should be added to the protein. Addition of C-terminal affinity tags is possible.

Determining the presence of a signal peptide in the protein of interest is an important first step prior to its *in vitro* synthesis. Internet-accessible tools (e.g. SignalP; http://www.cbs.dtu.dk/services/SignalP/) are capable of predicting the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms. These native signal sequences differ widely in their ability to facilitate protein translocation. Moreover, foreign signal sequences (e.g. mammalian sequences) may be less efficient in insect cell lysates. Therefore, substitution of the foreign protein's own signal sequence for a powerful insect melittin signal sequence (see Figure 10) often results in more efficient protein translocation and glycosylation.

PCR-mediated substitution of the N-terminal signal sequence and cloning of the amplification product into the expression vector pIX4.0 can significantly improve the results of cell-free expression reactions using the RTS 100 Insect Membrane Kit.

M K F L V N V A L V F M V V Y I S Y I Y A* D
ATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGAC

* Position of signal peptide cleavage

Figure 10. Coding and amino acid sequence of the mellitin signal peptide.

We recommend inserting this signal peptide coding sequence by generating a sequence-verified synthetic gene, flanked by *Nco*I and *Xho*I sites, and cloning it into *Nco*I/*Xho*I digested pIX4.0 (→ pIX4.0/mellitin). Subsequently, protein coding sequences (lacking endogenous signal sequences) should be amplified by PCR using primers coding for flanking *Xho*I/*Bgl*II or *Xho*I/*Spe*I restriction sites and cloned into the *Xho*I/*Bgl*II or *Xho*I/*Spe*I digested pIX4.0/mellitin vector.

References

1) Merk, H., Gless, C., Maertens, B., Gerrits, M., and Stiege, W. (2012). Cell-free synthesis of functional and endotoxin-free antibody Fab fragments by translocation into microsomes. Biotechniques 53(3),153-160

2) Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning — A laboratory Manual*. 2nd Ed. Cold Spring Harbor. Cold Spring Harbor Laboratory Press.

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