RTS™ 100 Insect Membrane Manual

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

RTS 100 Insect Membrane Kit

RTS pIX4.0 Insect Vector

RTS 100 Insect Membrane Kit, November, 2016

Contents

Product specifications

Product description

The RTS 100 Insect Membrane Kit provides the components and procedures necessary for 5 (cat. no. BR1401501) or 20 (cat. no. BR1401502) transcription reactions of 25 µl and translation reactions of 50 µl.

Materials supplied

RTS 100 Insect Membrane Kit Cat. no.	20 x 50 µl reactions BR1401502	5 x 50 µl reactions BR1401501
Insect Extract 100 Insect Membrane (colorless snap-cap)	4 x 100 µl	1 x 100 µl
Reaction Buffer 100 Insect Membrane (blue screw-cap)	1 x 100 µl	1 x 25 µl
Energy Mix 100 Insect Membrane (red screw-cap)	1 x 100 µl	1 x 25 µl
RNase-DNase-free Water (colorless screw-cap)	1 x 1.9 ml	1 x 1.9 ml
Positive Control DNA 100 Insect Membrane (violet screw-cap)	1 x 25 µl	1 x 25 µl
5x Transcription Buffer 100 Insect Membrane (yellow screw-cap)	1 x 100 µl	1 x 25 µl
5x NTP Mix 100 Insect Membrane (green screw-cap)	1 x 100 µl	1 x 25 µl
20x Enzyme Mix 100 Insect Membrane (orange screw-cap)	1 x 25 µl	1 x 6.25 µl
DyeEx® 2.0 Spin Columns	20	5

RTS pIX4.0 Insect Vector Cat. no.	BR1400901
pIX4.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)

Shipping and storage conditions

The **RTS 100 Insect Membrane Kit** is shipped on dry ice.
5x Transcription Buffer, 5x NTP Mix, 20x Enzyme Mix, Positive Control DNA and RNase-DNase-free Water must be stored at –20°C upon arrival.

Insect Extract and Reaction Buffer must be stored at –70°C upon arrival.

Once thawed, Insect Extract should be stored on ice and used within 4 hours.

The **RTS pIX4.0 Insect Vector** is shipped on dry ice and must be stored at –20°C upon arrival.

DyeEx 2.0 Spin Columns should be stored dry at room temperature (15–25°C). For longer storage, these kits can be stored at 2–8°C. Do not freeze.

The RTS 100 Insect Membrane 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

RTS 100 Insect Membrane Kits are 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 Membrane 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 Membrane Kit, a new eukaryotic cell-free expression system, enables expression of eukaryotic proteins — including membrane proteins — with posttranslational modifications. 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 Membrane Kit uses 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). It is a linked transcription–translation system (see flowchart, page 8).

In the *in vitro* transcription reaction, high-quality capped mRNA is produced using linearized or circular plasmid DNA or PCR products containing a T7 promoter. In the linked cell-free expression reaction, the capped mRNA is used as template 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.

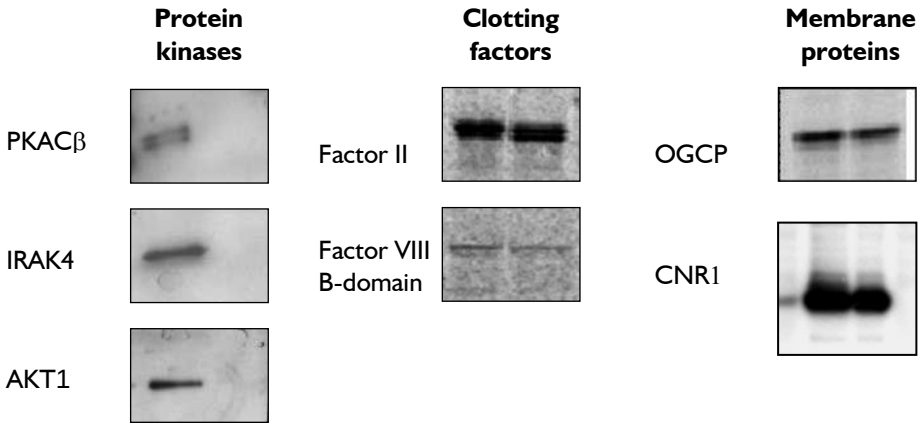


Figure 1. Proteins successfully expressed using the RTS 100 Insect Membrane Kit. 6xHis-tagged protein kinases and cannabinoid receptor (CNR1) were visualized using the Penta·His HRP Conjugate. Clotting factors and OGCP (mitochondrial 2-oxoglutarate/malate carrier) were synthesized in duplicate reactions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

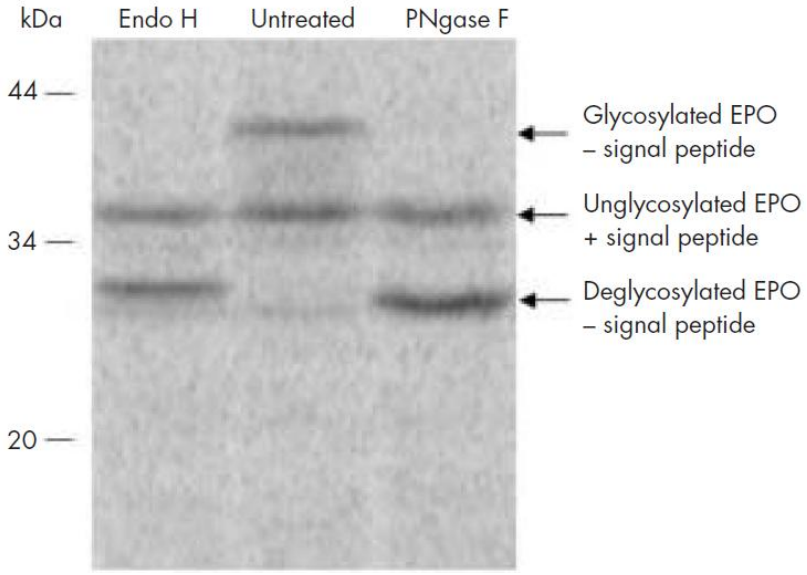
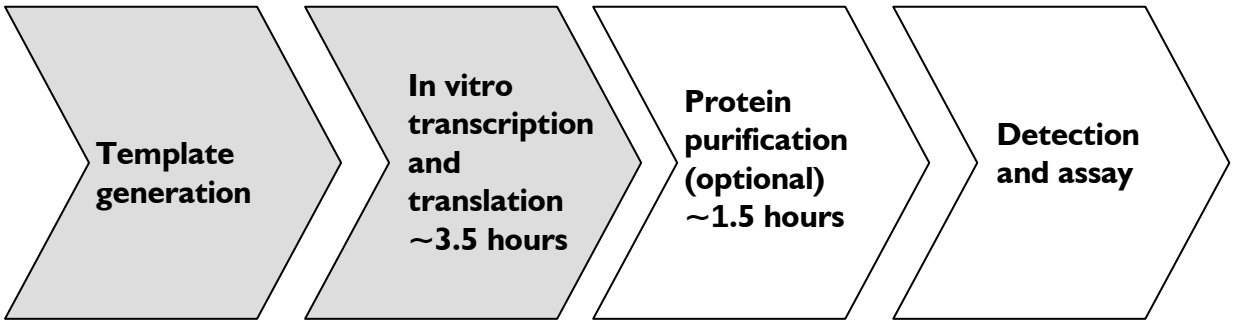


Figure 2. Efficient glycosylation of erythropoietin. The glycoprotein Erythropoietin (EPO) was synthesized using the RTS 100 Insect Membrane Kit in the presence of ^{14}C-labeled amino acids. To remove the glycan moieties from the synthesized glycoproteins, aliquots of the synthesis reactions were incubated 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

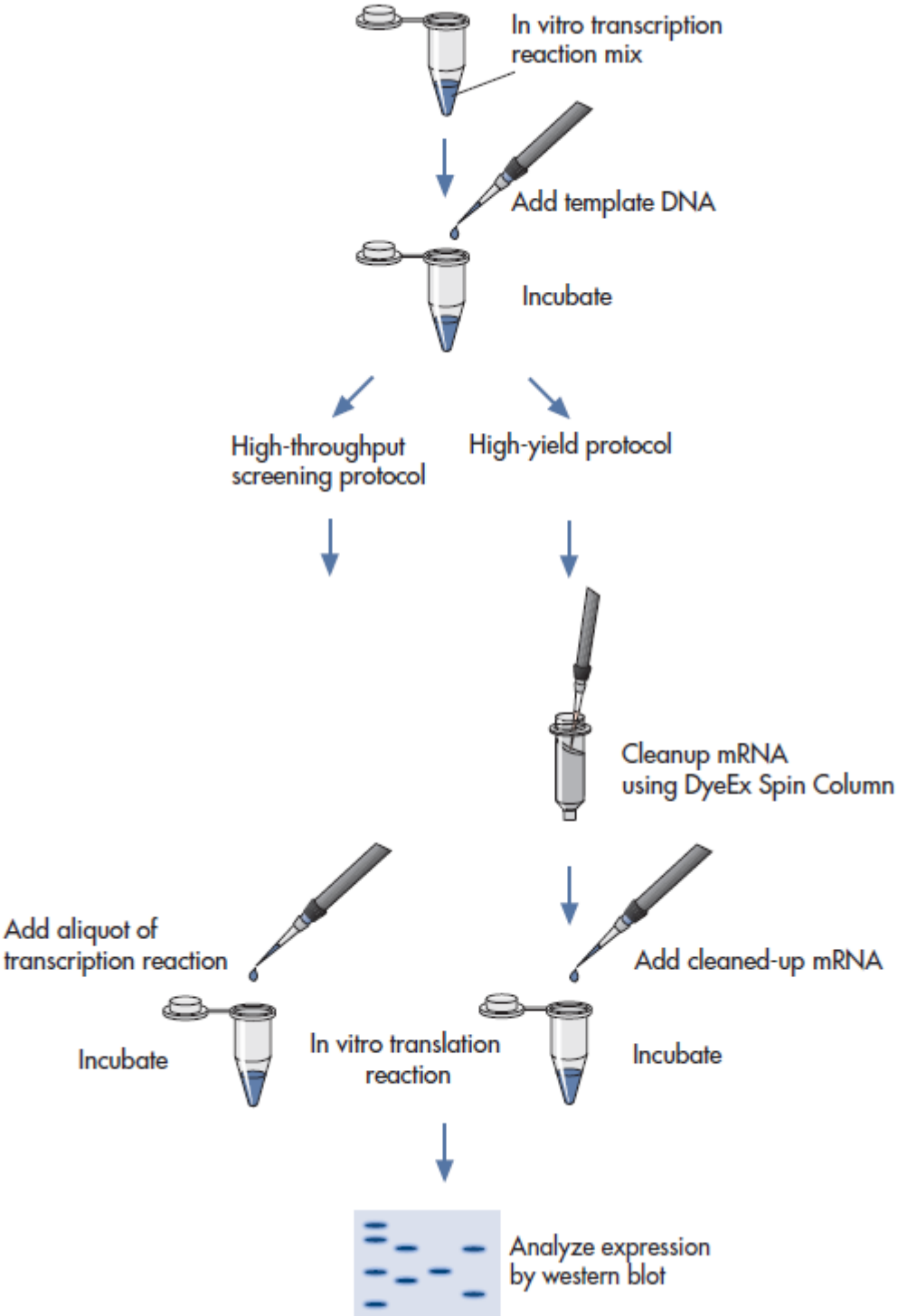
RTS 100 Insect Membrane Kit

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 Membrane 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 5, page 13.

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* transcription. The plasmid DNA must be linearized using a restriction enzyme that cuts downstream of the insert to be transcribed. We recommend using restriction enzymes that produce blunt ends. Restriction enzymes that produce 3' overhangs should be avoided.

Following digestion, linearized DNA can be cleaned up, for example using the GenUP™ PCR Cleanup Kit (cat. no. BR0700301, biotechrabbit).

For linearized DNA encoding mRNAs of less than 1500 bases, •1 µg linearized DNA template should be added to each 25 µl *in vitro* transcription reaction at a concentration of 0.2 µg/µl. In some cases, the yield of protein synthesis can be improved by increasing the DNA amount to 1.5 µg or decreasing the amount to 0.5 µg per 25 µl transcription reaction. Alternatively, the volume of cleaned up mRNA (see Table 3 on page 19) added to the translation reaction can be lowered to 6 µl or increased to 20 µl.

For linearized DNA encoding mRNAs of greater than 1500 bases, 1.5 µg linearized DNA template should be added to each 25 µl *in vitro* transcription reaction at a concentration of 0.3 µg/µl. In some cases, the yield of protein synthesis can be improved by increasing the DNA amount to 2.0 µg or decreasing the amount to 1.0 µg per 25 µl transcription reaction. Alternatively, the volume of cleaned up mRNA (see Table 3 on page 19) added to the translation reaction can be lowered to 6 µl or increased to 20 µl.

If using circular plasmid DNA,•1.5 µg should be added to each 25 µl *in vitro* transcription reaction.

PCR products

If PCR products are added to the *in vitro* transcription reaction, we recommend that they are generated using the RTS Linear Template Kit Plus (cat. no. BR1402401) or the RTS Linear Template Fab Kit (cat. no. BR1402201). PCR products can be added directly to the *in vitro* transcription reactions without further cleanup. The amount of PCR product added to each 25 µl *in vitro* transcription reaction should be 500 ng. **Note**: XE-solution provided with the Linear Template Kit Plus **should not be added** to the *in vitro* transcription reaction.

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

Identification of optimal constructs in a single day

The PCR-based RTS Linear Template Kit Plus and the Linear Template Fab Kit can be used to generate a range of transcription 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.

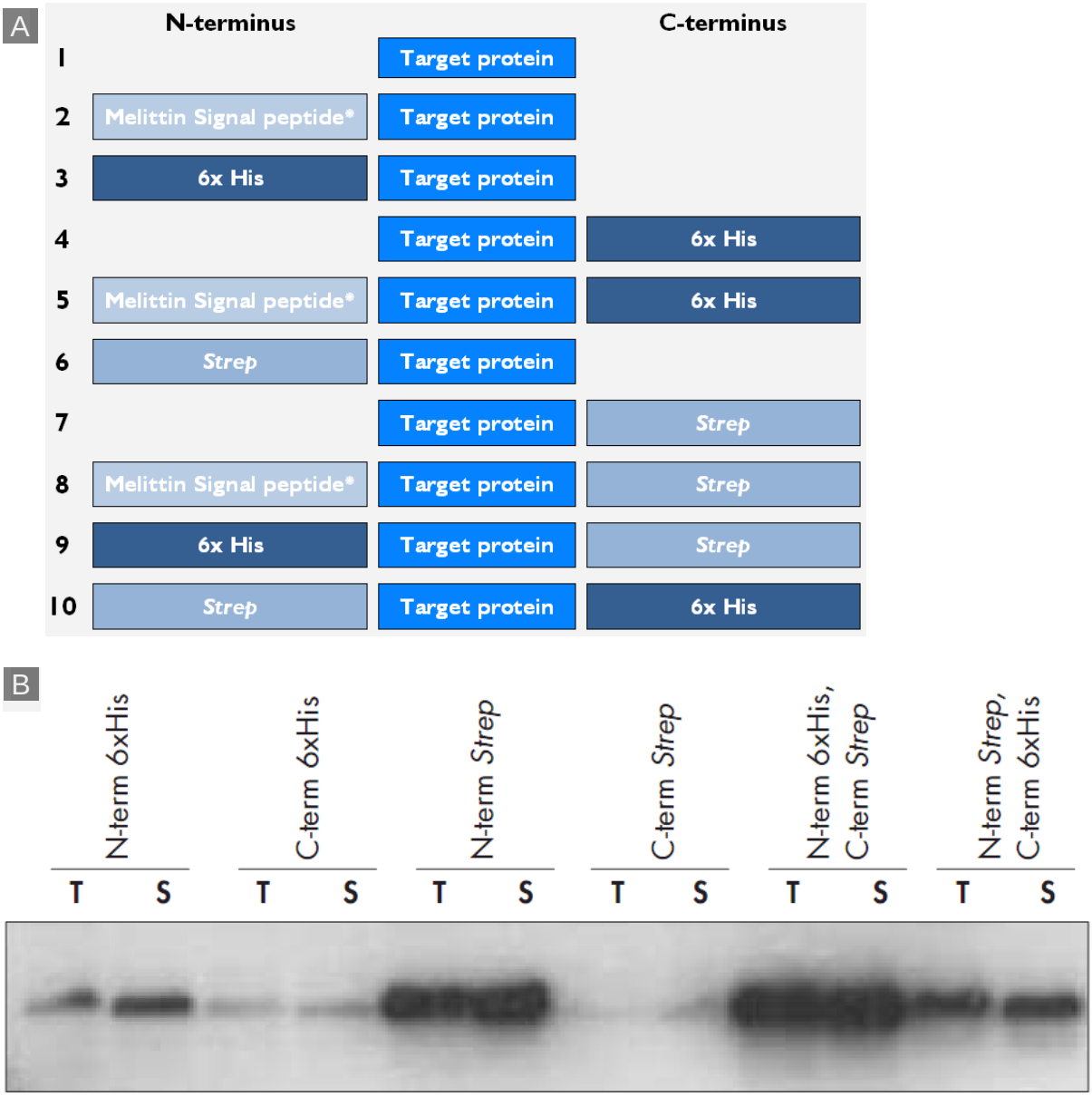


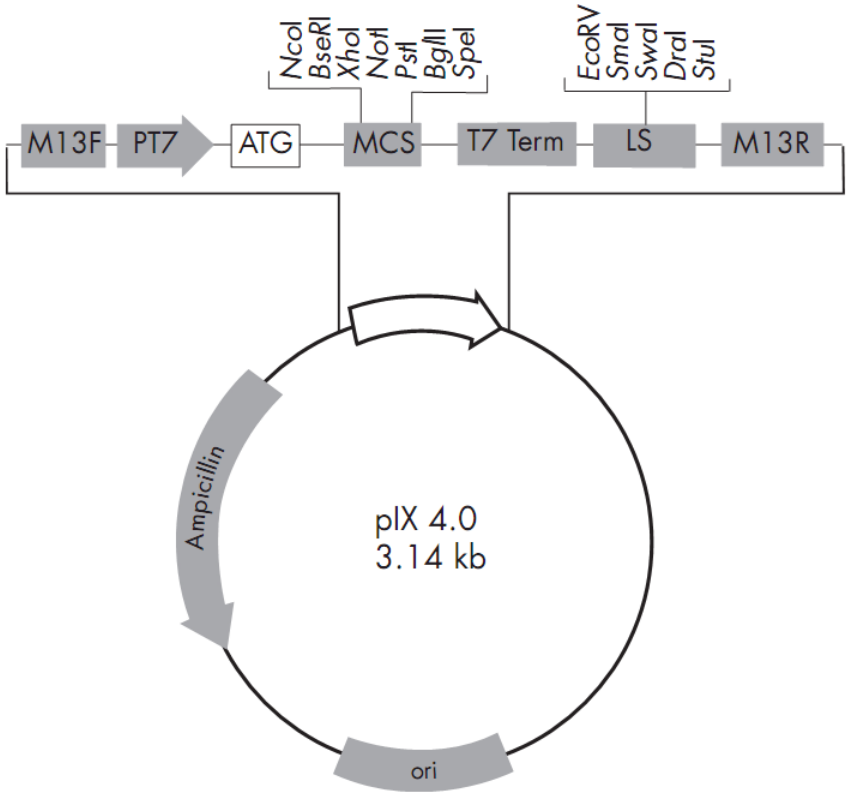
Figure 4. Expression analysis reveals the optimal construct. PCR products generated using the Linear Template Kit Plus and carrying the indicated tag(s) A are added to small-scale IVT reactions.Instead of an N-terminal tag the mellitin signal peptide can be added to direct synthesized proteins into the 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.

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

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

Figure 5. 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 E pages 34–44.

Protocol 1: *In vitro* transcription reaction

This protocol is suitable for the *in vitro* synthesis of recombinant proteins with posttranslational modifications using the RTS 100 Insect Membrane Kit (cat. no. BR1401501and BR1401502). Templates suitable for use as expression constructs can be generated using the RTS Linear Template Kit Plus (cat. no. BR1402401) or the RTS Linear Template Fab Kit (cat. no. BR1402201).

The protocol is divided into two sections, *in vitro* transcription and *in vitro* translation. Using the Positive Control DNA template, the high-yield protocol delivers up to 40 µg active luciferase per ml reaction. A faster high-throughput version of the translation protocol is provided in Appendix A, page 22. In this protocol, the transcription reaction is added directly to the translation reaction without cleanup: however, protein yields are lower.

Equipment and reagents required

- DNA (linearized or circular plasmid, or PCR product) encoding the protein of interest. The plasmid must contain a T7 promoter (see page 10)
- Thermomixer (Eppendorf, Hamburg, Germany)

Important points before starting

- The *in vitro* translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided as 4 individual aliquots in single tubes. Once thawed, store Insect Extract on ice and use within 4 hours. Refreeze the extracts in liquid nitrogen. Do not refreeze and thaw more than four times.
- The recommended incubation temperature for transcription is 37°C, the incubation temperature for protein synthesis is 27°C.
- We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.
- For multiple reactions, prepare a master mix without template, transfer aliquots to reaction tubes, and initiate protein synthesis by adding the template.
- Except for the actual transcription-translation incubation, all handling steps should be carried out on ice.
- To determine the background level of protein synthesis, always include a no template control reaction (without template) in your experiment.

Procedure

In vitro transcription reaction

1. **Thaw and store 5x Transcription Buffer, 5x NTP Mix, 20x Enzyme Mix, RNase-DNase-free Water, and Positive Control DNA on ice.**

   Before use, gently vortex and briefly centrifuge each tube to ensure homogeneity of solutions. **Note**: If the 5x Transcription Buffer shows precipitates after thawing, warm vials to 37°C and vortex until precipitates are dissolved.

2. **Pipet together the components of the three transcription reactions shown in Table 1 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes at room temperature (15–25°C). Label each tube clearly.**

   It is important to add the reaction components in the order shown in Table 1.

3. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
4. **Incubate the *in vitro* transcription reactions for 120 min at 37°C.**
5. **Centrifuge the reactions for 1 min at 12,000 x g at room temperature (15–25°C). Pipet the reaction supernatants into clean DNase- and RNase-free 1.5 ml microcentrifuge tubes and keep at room temperature (15–25°C). Label each tube clearly.**

Table 1. Pipetting Scheme for Transcription Reactions

Reagent	Target protein template reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	8.75 µl	8.75 µl	13.75 µl
5x Transcription Buffer	5 µl	5 µl	5 µl
5x NTP Mix	5 µl	5 µl	5 µl
20x Enzyme Mix	1.25 µl	1.25 µl	1.25 µl
Positive Control DNA	—	5 µl	—
DNA	5 µl*	—	—
Total	**25 µl**	**25 µl**	**25 µl**

* For **linearized DNA encoding mRNAs of less than 1500 bases**, 1 µg linearized DNA template should be added to each 25 µl *in vitro* transcription reaction at a concentration of 0.2 µg/µl.

For **linearized DNA encoding mRNAs of greater than 1500 bases**, 1.5 µg linearized DNA template should be added to each 25 µl *in vitro* transcription reaction at a concentration of 0.3 µg/µl.

For circular plasmid DNA, 1.5 µg template should be added to each 25 µl reaction.

For PCR products, 500 ng template should added to each 25 µl reaction.

6. **For the high-throughput screening protocol, proceed using the protocol in Appendix A on page 22. For the high-yield protocol proceed with step 7 on page 17.**

   We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

Protocol 2: Purification of messenger RNA

In this part of the protocol, mRNA is cleaned up using a DyeEx gel-filtration spin column before addition to the translation reaction.

Important note before starting

All centrifugation steps are performed at 750 x g in a conventional microcentrifuge. The appropriate speed for individual centrifuges can be calculated as follows: rpm = 1000 x (750/1.12 r) (r = radius of rotor in mm).

Table 2. Examples of Suitable Microcentrifuges and the Corresponding Speeds

Microcentrifuge	Speed corresponding to 750 x g
Eppendorf® Centrifuge 5415C	3000 rpm
Eppendorf Centrifuge 5417C	2700 rpm
Heraeus Biofuge 15	2800 rpm
Hettich Mikro 24-48	2630 rpm
Beckman GS15R	2100 rpm
Hettich Mikro EBA12	2700 rpm

Procedure

7. **Gently vortex the DyeEx spin column to resuspend the resin.**
8. **Loosen the cap of the column a quarter turn.**
   This is necessary to avoid a vacuum developing inside the spin column.
9. **Snap off the bottom closure of the spin column (Figure 6), and place the spin column into the DNase- and RNase-free 2 ml microcentrifuge tube (provided).**

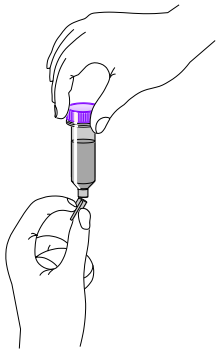


Figure 6. Snapping off the bottom closure of the DyeEx 2.0 spin column (do not screw).

10. **Centrifuge for 3 min at the calculated speed (see Table 2).**

11. **Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the 20 µl *in vitro* transcription reaction to the gel bed (Figure 7).**

    Pipet the *in vitro* transcription reaction directly onto the center of the slanted gel-bed surface (Figure 7). Do not allow the reaction mixture or the pipet tip to touch the sides of the column.

    The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip. It is not necessary to replace the lid on the column.

12. **Centrifuge for 3 min at the calculated speed.**

13. **Remove the spin column from the microcentrifuge tube. Keep the eluate at room temperature and proceed immediately with the *in vitro* translation reaction.**

    The eluate contains the purified RNA.

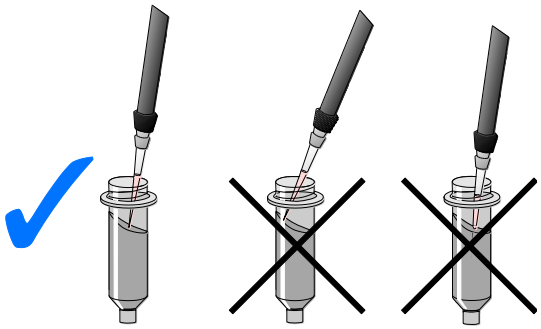


Figure 7. Instructions for sample application to the DyeEx 2.0 spin column.

14. **Thaw and store Reaction Buffer, Insect Extract, Energy Mix, and RNase-DNase-free Water on ice.**

15. **Pipet together the components of the three translation reactions shown in Table 3 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.**

    It is important to add the reaction components in the order shown in Table 3. **Note**: if template mRNA has been stored overnight at –20°C, thaw at room temperature (15–25°C) and vortex briefly before adding to the translation reaction.

Protocol 3: Protein synthesis reaction

Table 3. Pipetting scheme for high-yield *in vitro* translation reactions

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	Add to 50 µl	Add to 50 µl	Add to 50 µl
Reaction Buffer	5 µl	5 µl	5 µl
Insect Extract	20 µl	20 µl	20 µl
Energy Mix	5 µl	5 µl	5 µl
Template mRNA*	12 µl	12 µl	-
Total	**50 µl**	**50 µl**	**50 µl**

* From step 13. Use an aliquot of the DyeEx spin column flow-through from the respective transcription reaction.

16. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
17. **Incubate the *in vitro* translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
18. **Proceed with sample analysis.**

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 by 0.5 µg per 25 µl reaction. Alternatively titrate the volume of cleaned up mRNA added to the *in vitro* translation reaction between 6 and 20 µl, if using the high-yield protocol.
f) GC-rich mRNA	Denature the template mRNA prior to *in vitro* translation at 65°C for 3 min and immediately cool in an ice-water bath. This may increase the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure.
g) mRNA has precipitated	Ensure that mRNA is kept at room temperature (15–25°C) after transcription and after cleanup. If stored on ice, mRNA may precipitate, resulting in lower yields. If template mRNA has been stored overnight at –20°C, thaw at room temperature (15–25°C) and vortex briefly before adding to the translation reaction.

Appendix A: *In vitro* Translation Reaction Using the High-Throughput Screening Protocol

1. **Without further processing, use 5 µl of each *in vitro* transcription reaction for an *in vitro* translation reaction.**
2. **Thaw and store Reaction Buffer, Insect Extract, Energy Mix, and RNase-DNase-free Water on ice.**
3. **Pipet together the components of the three translation reactions shown in Table 4 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.**
4. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
5. **Incubate the *in vitro* translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
6. **Proceed with sample analysis.**

Table 4. Pipetting scheme for high-throughput screening *in vitro* translation reactions

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	Add to 50 µl	Add to 50 µl	Add to 50 µl
Reaction Buffer	5 µl	5 µl	5 µl
Insect Extract	20 µl	20 µl	20 µl
Energy Mix	5 µl	5 µl	5 µl
Template mRNA*	5 µl	5 µl	-
Total	**50 µl**	**50 µl**	**50 µl**

* From step 5 of the transcription protocol on page 15. Use an aliquot of the respective transcription reaction.

Appendix B: Upscaling Protein Synthesis Using the RTS 100 Insect Membrane Kit

This protocol is suitable for upscaling *in vitro* synthesis of recombinant proteins with posttranslational modifications using the RTS 100 Insect Membrane Kit in a 900 µl translation reaction volume. If smaller reactions are desired, scale down the volumes of the reaction components accordingly.

The protocol is divided into two sections, *in vitro* transcription and *in vitro* translation. Using the Positive Control DNA template, the high-yield protocol delivers up to 40 µg active luciferase per ml reaction.

Equipment and reagents required

- DNA (linearized or circular plasmid, or PCR product) encoding the protein of interest. The plasmid must contain a T7 promoter (see page 10).
- Thermomixer (Eppendorf, Hamburg, Germany)

Important points before starting

- The *in vitro* translation system is extremely sensitive to nuclease contamination. Always wear gloves and use RNase- and DNase-free reaction tubes and filter pipet tips.
- Insect Extract is provided as 4 individual aliquots in single tubes. Once thawed, store Insect extract on ice and use within 4 hours. Do not refreeze and thaw more than four times. Refreeze the extracts in liquid nitrogen.
- The recommended incubation temperature for transcription is 37°C, the incubation temperature for protein synthesis is 27°C.
- We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

Procedure

In vitro transcription reaction

1. **Thaw and store 5x Transcription Buffer, 5x NTP Mix, 20x Enzyme Mix, RNase-DNase-free Water, and Positive Control DNA on ice.**

   Before use, gently vortex and briefly centrifuge each tube to ensure homogeneity of solutions.

2. **Pipet together the components of the three transcription reactions shown in Table 5 in two DNase- and RNase-free 1.5 ml microcentrifuge tubes at room temperature (15–25°C). Label each tube clearly.**

   It is important to add the reaction components in the order shown in Table 5.

Table 5. Pipetting Scheme for Transcription Reactions

Reagent	Target protein template reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	157.5 µl	8.75 µl	13.75 µl
5x Transcription Buffer	90 µl	5 µl	5 µl
5x NTP Mix	90 µl	5 µl	5 µl
20x Enzyme Mix	22.5 µl	1.25 µl	1.25 µl
Positive Control DNA	—	5 µl	—
DNA	90 µl*	—	—
Total	**450 µl**	**25 µl**	**25 µl**

* **For linearized DNA encoding mRNAs of less than 1500 bases**, 18 µg linearized DNA template should be added to each 450 µl *in vitro* transcription reaction at a concentration of 0.2 µg/µl.

For linearized DNA encoding mRNAs of greater than 1500 bases, 27 µg linearized DNA template should be added to each 450 µl *in vitro* transcription reaction at a concentration of 0.3 µg/µl.

For **circular plasmid DNA**, 27 µg template should be added to each 450 µl reaction.

For **PCR products**, 9 µg template should added to each 450 µl reaction.

3. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
4. **Incubate the *in vitro* transcription reactions for 120 min at 37°C.**
5. **Centrifuge the reactions for 1 min at 12,000 x g at room temperature (15–25°C). Pipet the reaction supernatants into clean DNase- and RNase-free 1.5 ml microcentrifuge tubes and keep at room temperature (15–25°C). Label each tube clearly.**

   We recommend performing the entire synthesis reaction in a single day. Overnight storage of the transcription reaction is not recommended. However, DyeEx purified RNA can be stored overnight at –20°C.

Purification of messenger RNA

In this part of the protocol, mRNA is cleaned up using DyeEx gel-filtration spin columns before being pooled and added to the translation reaction.

Important note before starting

All centrifugation steps are performed at 750 x g in a conventional microcentrifuge. The appropriate speed for individual centrifuges can be calculated as follows:
rpm = 1000 x $\sqrt{}$ (750/1.12 r) (r = radius of rotor in mm).

Table 6. Examples of suitable microcentrifuges

Microcentrifuge	Speed
Eppendorf® Centrifuge 5415C	3000 rpm
Eppendorf Centrifuge 5417C	2700 rpm
Heraeus Biofuge 15	2800 rpm
Hettich Mikro 24-48	2630 rpm
Beckman GS15R	2100 rpm
Hettich Mikro EBA12	2700

1. **Gently vortex 20 DyeEx spin columns to resuspend the resin.**
2. **Loosen the caps of the columns a quarter turn.**
   This is necessary to avoid a vacuum developing inside the spin column.
3. **Snap off the bottom closure of the spin columns (Figure 8), and place the spin columns into a DNase- and RNase-free 2 ml microcentrifuge tube.**

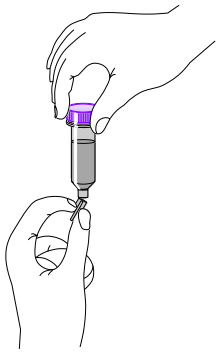


Figure 8. Snapping off the bottom closure of the DyeEx 2.0 spin column (do not screw).

4. **Centrifuge for 3 min at the calculated speed (see Table 6).**
5. **Carefully transfer the spin columns to clean centrifuge tubes. Slowly apply 20 µl *in vitro* transcription reaction to the gel bed (Figure 9) of each spin column.**

   Pipet the *in vitro* transcription reaction directly onto the center of the slanted gel-bed surface (Figure 9). Do not allow the reaction mixture or the pipet tip to touch the sides of the column.

   The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip. It is not necessary to replace the lid on the column.
6. **Centrifuge for 3 min at the calculated speed.**
7. **Remove the spin columns from their microcentrifuge tubes. Pool and keep the eluates at room temperature and proceed immediately with the *in vitro* translation reaction.**

   The eluates contain the purified RNA.

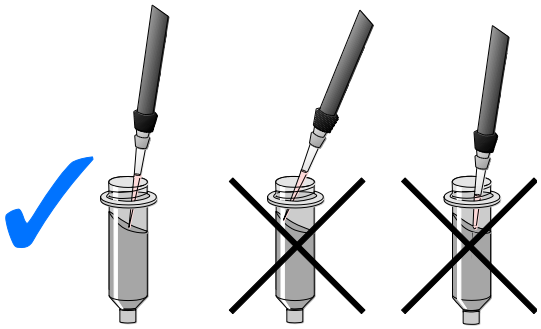


Figure 9. Instructions for sample application to the DyeEx 2.0 spin column.

8. **Thaw and store Reaction Buffer, Insect Extract, Energy Mix, and RNase-DNase-free Water on ice.**
9. **Pipet together the components of the three translation reactions shown in Table 7 in three DNase- and RNase-free 1.5 ml microcentrifuge tubes. Label each tube clearly.**

   It is important to add the reaction components in the order shown in Table 7. **Note**: if template mRNA has been stored overnight at –20°C, thaw at room temperature (15–25°C) and vortex briefly before adding to the translation reaction.

Table 7. Pipetting scheme for upscaling *in vitro* translation reactions

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	144 µl	8 µl	20 µl
Reaction Buffer	90 µl	5 µl	5 µl
Insect Extract	360 µl	20 µl	20 µl
Energy Mix	90 µl	5 µl	5 µl
Template mRNA*	216 µl	12 µl	-
Total	**900 µl**	**50 µl**	**50 µl**

* From step 7. Use an aliquot of the DyeEx spin column flow-through from the respective transcription reaction.

10. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
11. **Incubate the *in vitro* translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
12. **Proceed with protein analysis or purification.**

Appendix C: Analyzing the Luciferase Positive-Control Translation Reaction

The positive-control reaction is performed using a DNA template that encodes a 6xHis-tagged luciferase. Luciferase is a monomeric protein with a molecular weight of 61 kDa, which is found in the cells of bioluminescent organisms and catalyzes the oxidation of luciferin and ATP, producing light. Only full-length luciferase is active.

For a luciferase activity assay using a commercially available kit (e.g., Promega Luciferase Assay Reagent, cat. no. E1483), use 10 µl crude luciferase translation reaction.

For western blot analysis, load 2 µl (chemiluminescent detection) or 8 µl (chromogenic detection) crude luciferase translation reaction onto a 12% SDS-PAGE gel, transfer the protein from the gel onto nitrocellulose, and detect the luciferase protein with an anti-His6 antibody (e.g. 5 PRIME) or an anti-luciferase antibody (e.g., Monoclonal Anti-Luciferase Antibody, Sigma, cat. no. L2164).

For quantification of expressed luciferase using incorporation of radioactively-labeled amino acids, see Appendix D.

Appendix D: Incorporating Radioactive Labels into Proteins for Quantification

Protein expressed using the RTS 100 Insect Membrane 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 50 µl reaction. A protocol and example calculation of protein yield is given below.

Equipment and reagents required*

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

Procedure

Labeling with ^{14}C-Leucine

1. **Perform a transcription reaction (see protocol on page 15).**
2. **Thaw Reaction Buffer (blue screw-cap) and Energy Mix (red screw-cap) on ice. Thaw RNase-DNase-free Water (colorless screw-cap) at room temperature (15–25°C).**
3. **For each reaction to be performed, thaw 20 µl of Insect Extract (colorless snap-cap) on ice.**
4. **Set up the three reactions detailed in Table 8.**
   It is important to follow the order of addition given in the table.
5. **Mix all components by vortexing and briefly centrifuge to collect the reactions at the bottom of the tubes.**
6. **Incubate the *in vitro* translation reactions in a Thermomixer for 90 min at 27°C and 500 rpm.**
7. **Use a 10 µl aliquot of each reaction for quantification of protein synthesis by TCA precipitation (see page 31).**
   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 8. Pipetting scheme for radioactive labeling reactions

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	10 µl	10 µl	15 µl
Reaction Buffer	5 µl	5 µl	5 µl
300 µM ^{14}C-Leu	5 µl	5 µl	5 µl
Insect Extract	20 µl	20 µl	20 µl
Energy Mix	5 µl	5 µl	5 µl
Template mRNA*	5 µl	5 µl	-
Total	**50 µl**	**50 µl**	**50 µl**

* From *in vitro* transcription step 5, page 15. Use an aliquot of the respective transcription reaction.

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

Reagent	Target protein synthesis reaction	Positive control reaction	No template control reaction
RNase-DNase-free Water	14 µl	14 µl	19 µl
Reaction Buffer	5 µl	5 µl	5 µl
15 µM ^{35}S-Met	1 µl	1 µl	1 µl
Insect Extract	20 µl	20 µl	20 µl
Energy Mix	5 µl	5 µl	5 µl
Template mRNA*	5 µl	5 µl	-
Total	**50 µl**	**50 µl**	**50 µl**

* From *in vitro* transcription step 5, page 15. Use an aliquot of the respective transcription reaction.

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 Membrane Kit reactions.

Equipment and reagents required

- 5% (w/v) trichloroacetic acid (TCA) and acetone
- Casein acid hydrolysate (e.g., Sigma, cat. no. A 2427)
- Glass microfibre filters (for example Whatman® GF/C)
- Vacuum manifold (e.g., Glass Microanalysis Filter Holder, Millipore cat. no. XX1002530 in combination with a vacuum pump)
- 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 control DNA
Molecular weight	61,710 g/mol
Leu residues	51
Met residues	14

Labeling with ^{14}C-leucine

Leucine concentration	200 µM	unlabeled (Reaction Mix)
^{14}C-Leu concentration	30 µM	100 dpm/pmol
Total leucine concentration	230 µM	13.04 dpm/pmol

Measured radioactivity

TCA precipitated sample (10 µl)	2100 dpm = 210 dpm/µl
Total radioactivity (10 µl sample)	30,000 dpm = 3000 dpm/µl

$$\text{Percentage of } ^{14}\text{C-Leu incorporated} = \frac{210 \text{ x } 100}{3000} = 7\%$$

$$\text{Yield (µg/ml)} = \frac{7\% \text{ x } 0.01 \text{ x } 230 \text{ µM x } 61{,}710 \text{ g/mol}}{51 \text{ x } 1000} = 19.48 \text{ µg/ml}$$

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 50 µl *in vitro* translation reaction.

Methionine concentration	200 µM	unlabeled (Reaction Mix)
^{35}S-Met concentration	0.3 µM	2.22 x 10^6 dpm/pmol
Total methionine concentration	200.3 µM	3325 dpm/pmol

Measured radioactivity

TCA precipitated sample (10 µl) 166,500 dpm = 16,650 dpm/µl

Total radioactivity (10 µl sample) 6,66,000 dpm = 666,000 dpm/µl

$$\text{Percentage of } ^{35}\text{S-Met incorporated} = \frac{16{,}650 \times 100}{666{,}000} = 2.5\%$$

$$\text{Yield (µg/ml)} = \frac{2.5\% \times 0.01 \times 200.3\ \mu M \times 61{,}710\ g/mol}{14 \times 1000} = 22.07\ \mu g/ml$$

Appendix E: 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:

- 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 35.
- Proteins are synthesized free of any additional vector amino acids. See “Cloning into RTS pIX4.0 Insect Vector via *Bse*RI”, page 37.
- 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, *Bgl*II”, page 39.
- Protein is synthesized with an N-terminal 6xHis or Strep-tag®. See “PCR-mediated addition of affinity-tag sequences”, page 41.
- Protein is synthesized with a C-terminal 6xHis or Strep-tag. See “PCR-mediated addition of affinity-tag sequences”, page 41.
- Protein contains an intrinsic N-terminal sequence motif (signal sequence/signal peptide) directing the protein to the secretory pathway (e.g., glycoproteins), Appendix F, page 43.

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)

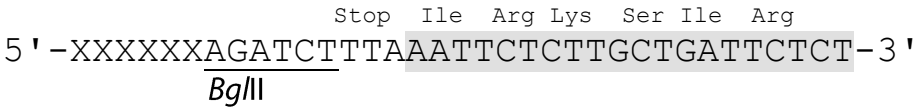
For the PCR the following sense primer is constructed. Native target protein sequence is shaded.

```
                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.

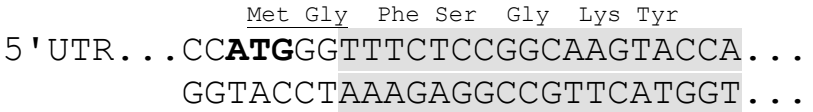


Antisense primer

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'–XXXXXX**GAGGAG**GTCTCC**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'–XXXXXX**AGATCT**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' XXXXXXGAGGAGGTCTCCCATGNNN...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.

```
      ↓
...CCATGGGAGACCCCTCCTC...
...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'—ATGAGTTTCTCCGGCAAGTACCAAC...AAGAGAATCAGCAAGAGAATTTGA—3'
3'—TACTCAAAGAGGCCGTTCATGGTTG...TTCTCTTAGTCGTTCTCTTAAACT—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'-XXXXXXGAGGAGGTCTCCCATGAGTTTCTCCGGCAAGTACCA-3'
          BseRI
```

Sense primer

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

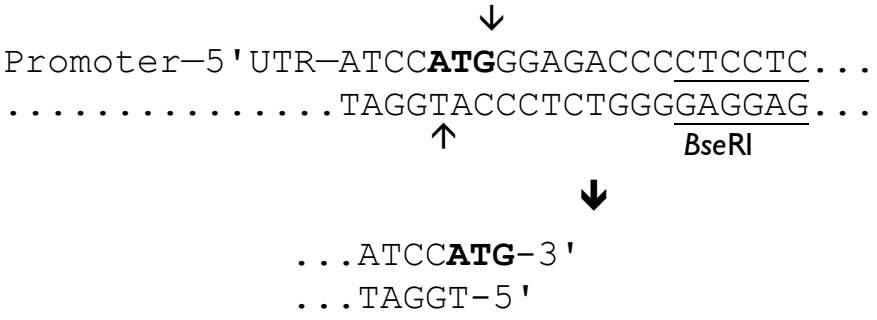
Antisense primer

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

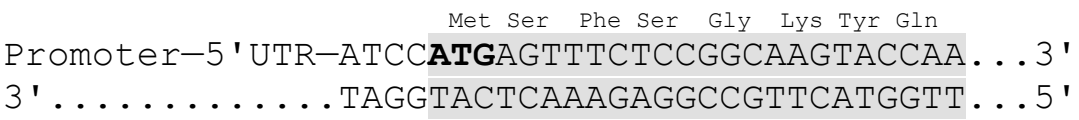
```
                                  ↓
5'-XXXXXXGAGGAGGTCTCCCATGAGTTTCTCCGGCAAGTACCA...3'
3'-XXXXXXCTCCTCCAGAGGGTACTCAAAGAGGCCGTTCATGGT...5'
          BseRI                   ↑
                                  ⬇

  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:

5'-XXXXXXCTCGAGC(M_{20})-3'

*Xho*I

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'—ATGAGTTTCTCCGGCAAGTACCAAC...AAGAGAATCAGCAAGAGAATTTGA—3'
3'—TACTCAAAGAGGCCGTTCATGGTTG...TTCTCTTAGTCGTTCTCTTAAACT—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
```

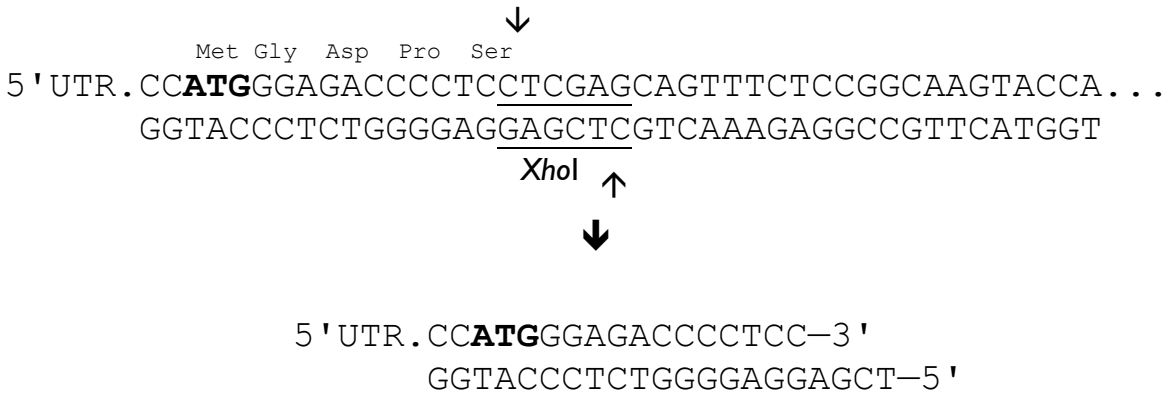
Antisense primer

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

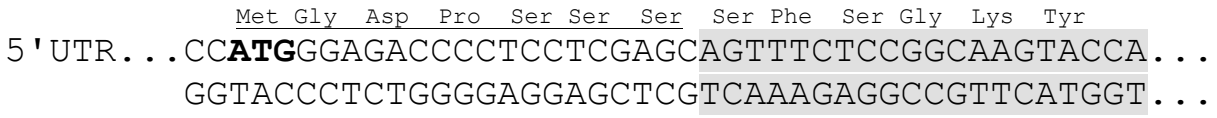
```
5'-XXXXXXCTCGAGCAGTTTCTCCGGCAAGTACCA...3
3'-XXXXXXGAGCTCGTCAAAGAGGCCGTTCATGGT...5'
          XhoI
```

```
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

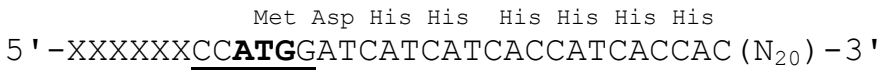
The PCR primers listed below can be used to add affinity-tag coding sequences to expression constructs. Start codons are in bold, and restriction enzyme recognition sites are underlined. If the target protein coding bases lead to the formation of mRNA secondary structures, altering the amino acid codons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

	↓	
*Bts*I	GCAGTGNN	GCAGTG**TG**
	CGTCACNN	CGTCACAC
	↑	
	↓	
BsrDI	GCAATGNN	GCAATG**TG**
	CGTTACNN	CGTTACAC
	↑	

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 F: Synthesis of Secreted Proteins and Glycoproteins Using the RTS 100 Insect Membrane 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 Membrane 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