RTS™ Linear Template Kit Plus Manual

For the generation of linear expression templates by PCR

and easy cloning of PCR products into the pIX3.0 Vector

RTS Linear Template Kit Plus

RTS pIX3.0 Vector

RTS Linear Template Kit Plus, April, 2015

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

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

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

Contents

Materials supplied

RTS Linear Template Kit Plus	
Ordering number	**BR1402401**
Number of reactions	**20 reactions**
Strep Sense Primer (yellow screw-cap)	40 µl
Strep Antisense Primer (brown screw-cap)	40 µl
6xHis Sense Primer (yellow screw-cap)	40 µl
6xHis Antisense Primer (brown screw-cap)	40 µl
No tag Sense Primer (yellow screw-cap)	40 µl
No tag Antisense Primer (brown screw-cap)	40 µl
No tag Sense Primer Signal Peptide (yellow screw-cap)	40 µl
Positive-Control DNA (PCR) (white screw-cap)	10 µl
Positive-Control Sense Primer (white screw-cap)	15 µl
Positive-Control Antisense Primer (white screw-cap)	15 µl
XE-Solution (green screw-cap)	40 µl

RTS pIX3.0 Vector	
Ordering number	**BR1402701**
pIX3.0 Vector (white screw-cap)	25 µg (0.5 µg/µl)

Shipping and storage conditions

The RTS Linear Template Kit Plus is shipped on dry ice and should be stored immediately upon receipt at –20°C in a constant-temperature freezer upon arrival. When stored under the above conditions and handled correctly, all kits are stable until the expiration date printed on the label. Avoid repeated freezing and thawing.

Product limitations

RTS Linear Template Kit Plus and RTS 100 *E. coli* HY Kits and RTS 100 Insect cell-based Kits are developed, designed, and sold for research purposes only. They are 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 many of the materials described in this text.

All due care and attention should be exercised in the handling of the products. We recommend all users of *E. coli* cell-free protein synthesis kit products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

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

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

Proteins such as enzymes, antibodies, hormones, and structural elements play essential roles in nearly all biological processes. Therefore, great efforts have been made to develop technologies for the production of proteins using recombinant technology. Modern protein engineering methods, which include cloning of DNA sequences and the *in vivo* expression of genes, allow production of specific proteins in large amounts and also production of proteins with improved or altered biological activities.

Several factors must be carefully considered when producing recombinant proteins using *in vivo* expression methods. Cells must be transformed with an expression construct (e.g., plasmid DNA), and transformants containing the correct construct must be selected and cultivated. Overexpression of proteins that are toxic to the host cells can be difficult. Cell lysis and procedures used for purification of protein from whole cell lysates can be complicated; problems may arise because of aggregation or degradation of proteins within the cell.

In most cases these limitations can be overcome by the use of cell-free protein biosynthesis systems (also termed *in vitro* translation [IVT] systems), which are often seen as a very attractive alternative to classical *in vivo* expression systems. Cell-free expression generates proteins by coupled or successive transcription and translation in cell-free extracts of prokaryotic or eukaryotic cells. The advantages of cell-free expression systems include time savings, the possibility to produce proteins that are toxic or have modified or isotope-labeled amino acids, a high protein yield per unit volume, and the ability to adapt reaction conditions to the requirements of the synthesized protein (for example, the inclusion of cofactors). Compared with current cloning techniques, another important advantage offered by a cell-free expression system is the possibility of using PCR products as templates for protein synthesis. This greatly accelerates the protein production process, because no cloning steps are required. Moreover, there is no need for any specialized equipment: only an incubator, pipette, and reaction tubes are needed.

Proteins produced by cell-free expression can be used for the same wide variety of downstream applications as in-vivo produced proteins, including activity assays, structural and functional analyses, protein–protein interaction studies, and the expression and analysis of open reading frames.

The RTS System

biotechrabbit's Rapid Translation System (RTS) is a scalable *in vitro* protein expression platform that produces large amounts of protein for characterization studies, functional assays, or structural analysis. Specific applications can easily be adapted with a truly "open" system based on bacterial as well as eukaryotic cell lysates. Expression of target proteins can be performed either using RTS expression vectors or using PCR-based generation of Linear Template in a screening manner with RTS Linear Template Kits thereby avoiding cloning procedures. PCR products generated with the RTS Linear Template Kit Plus can be applied in the *E. coli*-based RTS 100 *E. coli* HY Kit (cat. no. BR1400101) or in the insect cell-based RTS 100 Insect Membrane Kits (cat. no. BR1401501) and RTS 100 Insect Disulfide Kits (cat. no. BR1401401).

With Linear Templates synthesis can be scaled up to approx. 0.5 mg protein in 1 ml synthesis scale, while upscaling with Expression Vectors synthesis is possible up to 50 mg protein in 10 ml synthesis scale using our patented technology (continuous-exchange, cell-free) offering much higher levels of protein expression compared to other *in vitro* T7 RNA polymerase-based transcription/translation systems.

RTS Platform - Complementary Prokaryotic and Eukaryotic Systems

The Rapid Translation System is designed to cover a broad range of individual proteins by a combined family of pro- and eukaryotic expression systems, eliminating expression limitations of previously available cell-free systems. Synthesis with outstanding high yields of up to 6 mg/ml is realized by RTS *E. coli*, which in most cases is the first choice for expression.

As an alternative, expression of proteins with complex requirements can be achieved using eukaryotic systems which benefit of chaperone systems suited for eukaryotic proteins. Another advantage of eukaryotic RTS is the absence of bacterial endotoxins avoiding laborious purification of proteins for cell-based assays.

Moreover, RTS Wheat Germ combines high synthesis yields up to 1 mg/ml and high success rate independent on codon usage and especially for large proteins which tend to fragmentation in other systems. RTS Insect benefits of endogenous ER based membrane vesicles suited for signal peptide directed translocation of glycosylated proteins and membrane proteins into natural membranes. This system yields protein synthesis up to 50 μg/ml.

In the Rapid Translation System a unique set of innovative technologies is combined to a powerful new protein expression approach. The entire workflow (Figures 1 and 2) has been analyzed to help reduce bottlenecks and accelerate the protein expression process. The different modules of the Rapid Translation System contribute to a new and revolutionary expression concept. A comparison of pro– and eukaryotic RTS Kit for screening and scaled-up protein synthesis is shown in Figure 3.

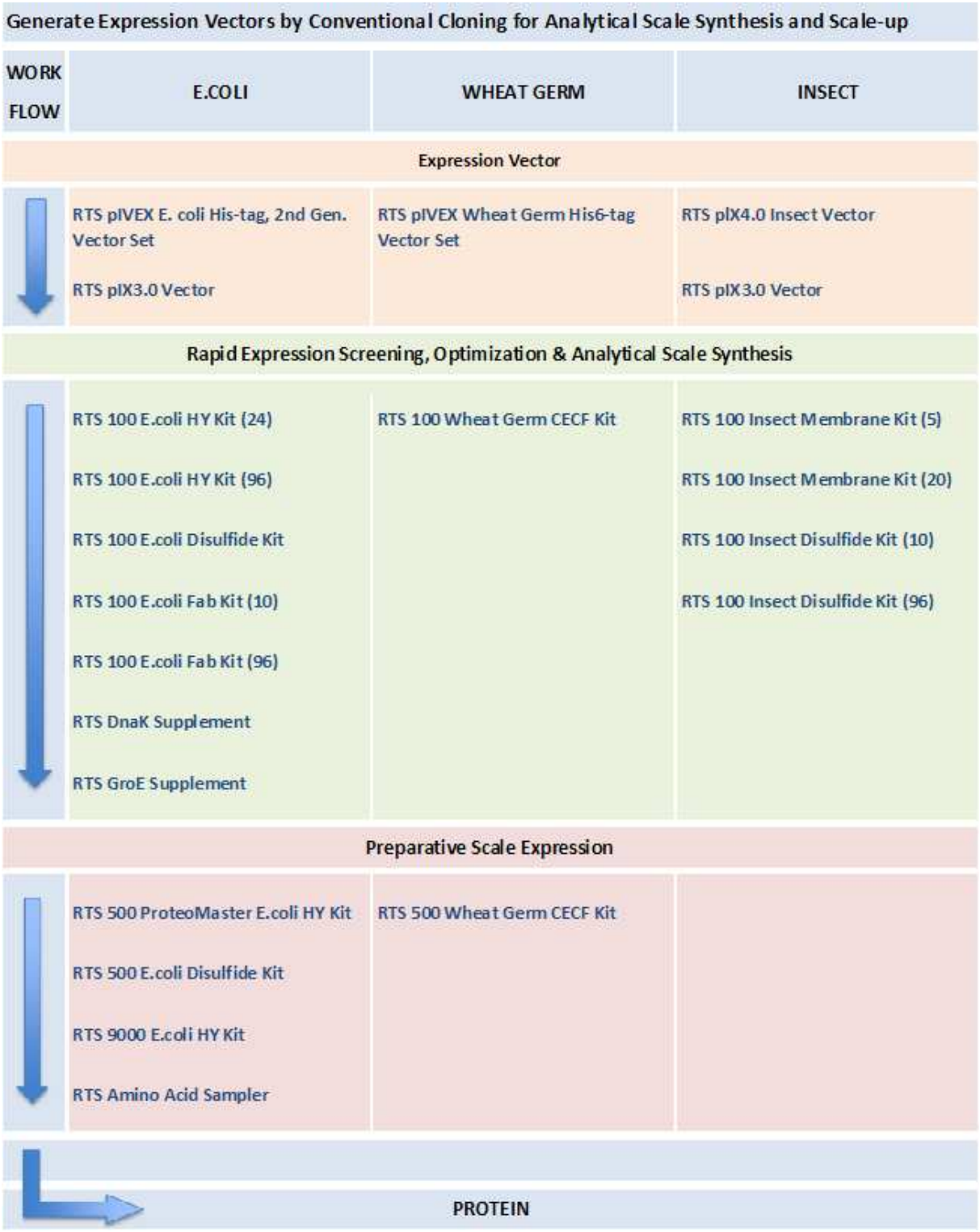


Figure 1. Workflow of RTS System starting with expression vectors.

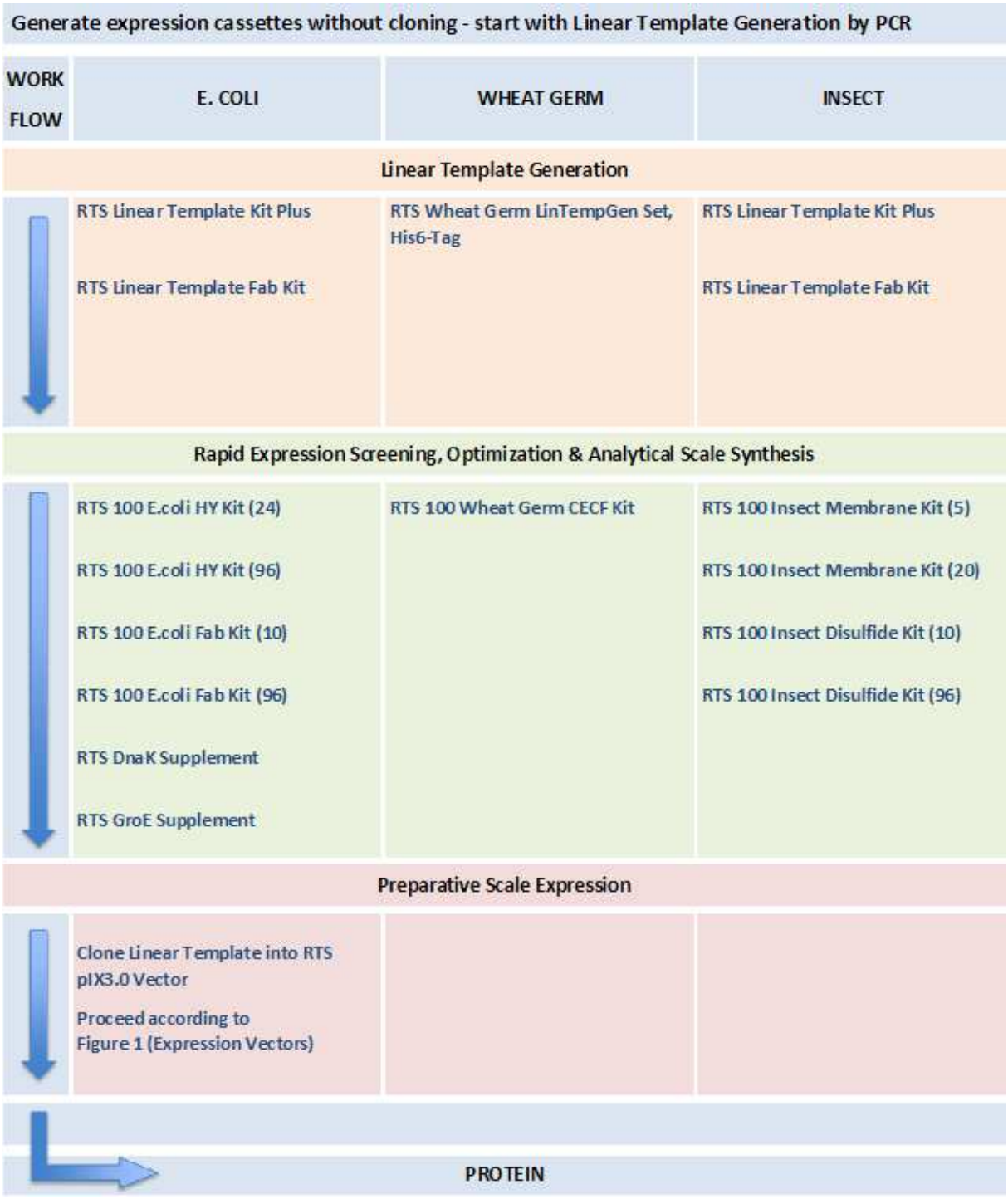


Figure 2. Workflow of RTS System starting with linear templates.

System	Application	Mode	Kit	Expected Yield/Reaction	Linear Templates	Available Reactions	CECF	Eppendorf Thermomixer?
E. coli	Expression and functionality optimization, disulfide bridges, Fab antibodies, supplements for enhanced solubility/functionality, membrane proteins in detergents, scale-up, purification, NMR, X-ray	Screening	RTS 100 E. coli HY	up to 20 µg/50 µl	Yes	24 /96	NO	recommended
			RTS 100 E. coli Disulfide	up to 80 µg/50 µl	No	24	Yes	required
			RTS 100 E. coli Fab	up to 30 µg/140 µl	Yes	10 / 96	No	recommended
		Scale-Up	RTS 500 ProteoMaster E.coli HY	up to 6 mg/1 ml	No	5	Yes	required
			RTS 500 E.coli Disulfide	up to 2.5 mg/1 ml	No	5	Yes	required
			RTS 9000 E.coli HY	up to 50 mg/10 ml	No	1	Yes	required
Wheat Germ	Screening, expression of non-optimized native eukaryotic sequences and large eukaryotic proteins, scale-up, purification, X-ray	Screening	RTS 100 Wheat Germ CECF	up to 50 µg/50 µl	Yes	24	Yes	required
		Scale-Up	RTS 500 Wheat Germ CECF	up to 1 mg/1 ml	No	5	Yes	required
Insect	Screening, membrane proteins in natural membranes, glycosylation, disulfide bridges, Fab antibodies	Screening	RTS 100 Insect Membrane	up to 2 µg/50 µl	Yes	5 / 20	No	recommended
			RTS 100 Insect Disulfide	up to 2 µg/100 µl	Yes	10 / 96	No	recommended

Figure 3. Comparison of pro- and eukaryotic RTS kits for screening and upscaling.

Identification of optimal constructs in a single day

The PCR-based RTS Linear Template Kit Plus can be used to generate a range of transcription templates that encode target proteins with varying combinations of 6xHis and Strep-tag affinity tags and additionally the option to encode a signal peptide (Figure 4). The Melittin signal peptide originates from honey bee and is ideally suited to insert proteins into membraneous vesicles contained in the Insect lysate (1); it may be used to overcome limited production of secreted-, posttranslationally modified-, membrane-, or disulfide- bonded proteins due to inefficient signal peptides. Please note, that the Melittin signal sequence can only be used in combination with the RTS 100 Insect Membrane Kit or RTS 100 Insect Disulfide Kit (www.biotechrabbit.com). 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. Having identified the optimal expression template, optionally, the PCR product can be easily cloned into the RTS pIX3.0 Vector.

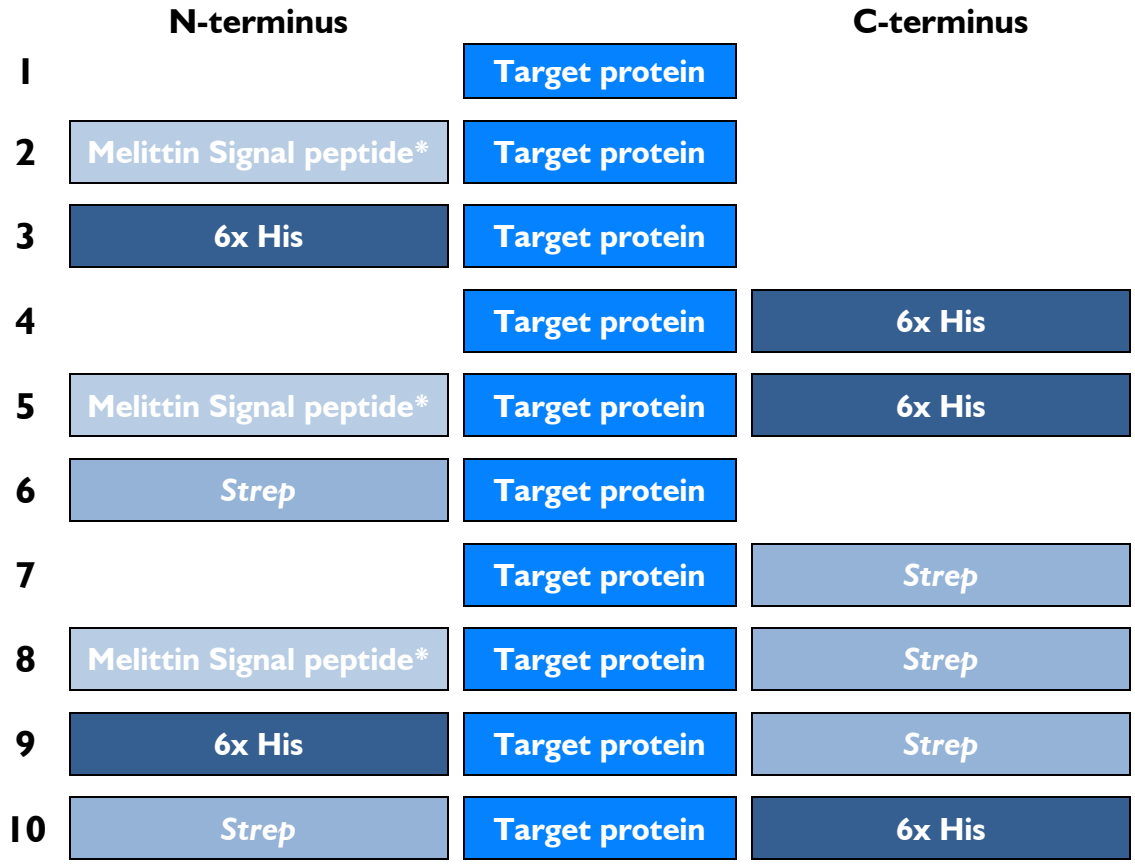


Figure 4. The variety of constructs whose expression can be tested using the Linear Template and Protein Synthesis Kits.

* only for use in combination with RTS 100 Insect Membrane Kit or RTS 100 Insect Disulfide Kit

The screening of such constructs in small-scale cell-free expression (IVT) reactions using *E. coli*-based protein synthesis kits (or the insect-cell based kits) 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. 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.

To meet the special requirements of high throughput expression of antibody fragments (and especially Fab fragments), the application of biotechrabbit RTS Linear Template Fab Kit (cat. no. BR1402201) in combination with the RTS 100 *E. coli* Fab Kit (cat. no. BR1400601) or RTS 100 Insect Disulfide Kit (cat. no. BR1401401) is recommended.

Principle and Procedure

The RTS Linear Template Kit Plus uses a two-step PCR process to generate linear DNA templates for *in vitro* translation systems. Using specially designed primers, coding DNA sequence is both amplified and supplemented with regulatory elements required for optimal transcription and translation in cell-free expression systems. Specially designed 5' untranslated regions (UTRs) on the sense adapter primer sequences reduce the formation of secondary structure in the translation initiation region, one of the commonest causes of low expression rates. A His- or Strep-tag II can be added to either terminus, greatly simplifying protein purification and detection. The N-terminal Strep-tag construct also contains a Factor Xa Protease cleavage site, for easy tag removal. The fast procedure enables researchers to discover the optimal template structure within a single working day (Figure 5).

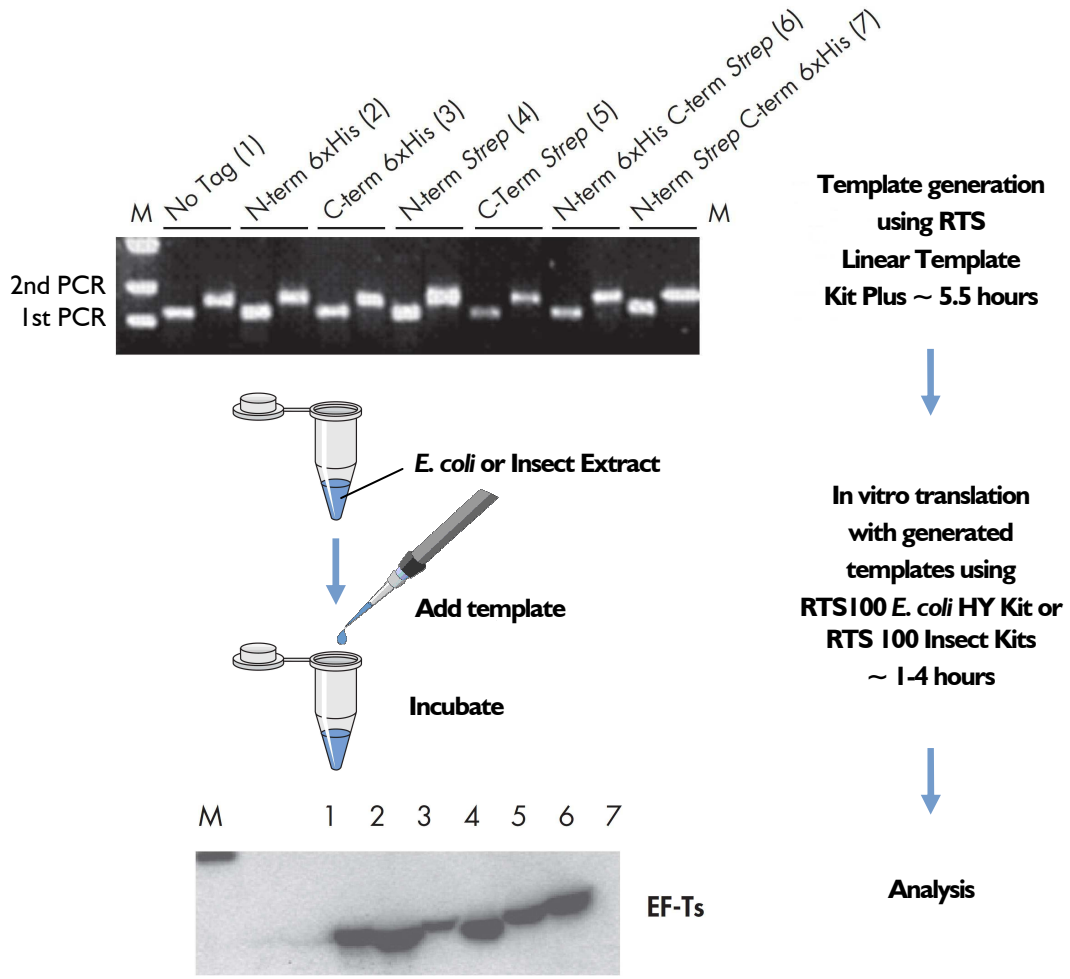


Figure 5. Identification of the optimal expression construct maximizes yields in large-scale in vitro reactions or *in vivo* expression. The western blot was probed with anti-His- and Strep-tag antibodies. Therefore, the untagged protein is not detected.

The RTS pIX3.0 Vector (see Appendix on page 27) has been developed to enable easy cloning of PCR products generated using the RTS Linear Template Kit Plus. Its multiple cloning site (MCS) is compatible with restriction sites in the sense and

antisense adapter primers supplied with the RTS Linear Template Kit Plus (see page 28). Once cloned into pIX3.0, expression constructs can be used to generate larger amounts of protein in large-scale protein synthesis reactions. Alternatively, the vector can be used to transform *E. coli* cells for conventional *in vivo* expression.

Generating PCR Products for Use in *In Vitro* Translation Reactions

The RTS Linear Template Kit Plus uses a two-step procedure to generate PCR products suitable for *in vitro* translation in RTS 100 *E. coli* HY Kits, RTS 100 Insect Membrane Kits and RTS 100 Insect Disulfide Kits. In the first step, defined 5'-tails are added to PCR products using gene-specific primers. The 5'-tails serve as hybridization sites for primers used in a second PCR, in which DNA is amplified using adapter primers that code for regulatory elements required for optimal expression in prokaryotic-cell extracts. These elements include a T7 promoter, ribosomal binding site, and T7 terminator. The resulting PCR products contain multiple cloning sites that are compatible for cloning into the Vector pIX3.0 (Figure 6).

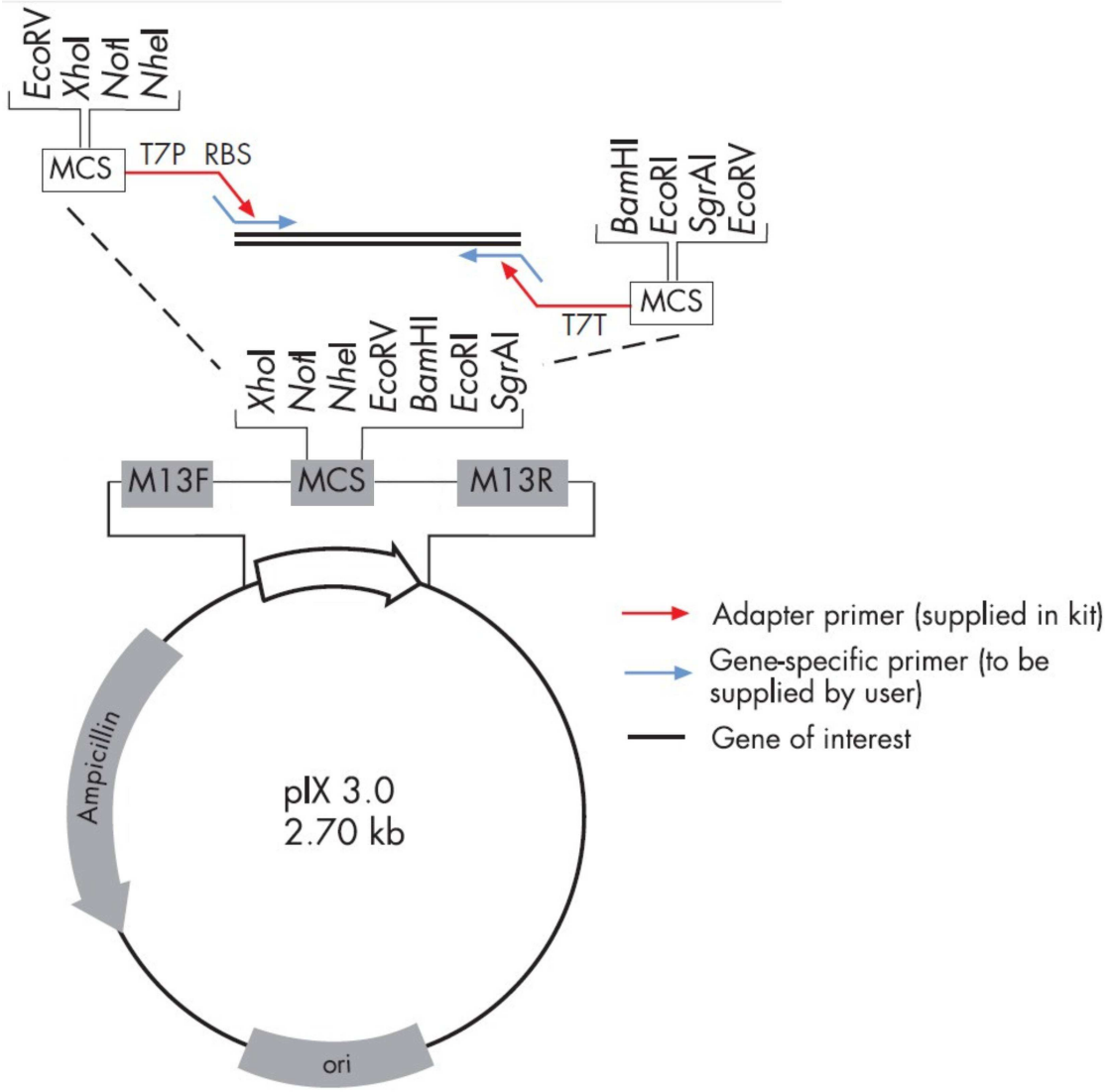


Figure 6. Straightforward cloning into vector RTS pIX3.0 for expression scale-up. T7P: T7promoter; **RBS**: Ribosome binding site; **T7T**: T7 terminator; **MCS**: multiple cloning site.

Adapter primers that encode N- or C-terminal affinity tags are contained in the kit. Addition of affinity tags to constructs greatly facilitates purification and detection of expressed proteins. Different combinations of adapter primers can be used to generate singly or doubly tagged proteins with a His- or Strep-tag at either terminus (Figure 4). In addition to the Strep-tag II epitope, the N-Terminal Strep-tag adapter primer encodes a Factor Xa Protease Cleavage site between the tag and the body of the target protein.

The kit contains enough reagents for 20 two-step PCRs, with each reaction yielding enough expression template for 3–4 *in vitro* translation reactions. The final PCR product can be added to the *in vitro* translation reaction without any further purification steps. The user must provide a DNA template encoding the protein of interest, and two gene-specific PCR primers.

Plasmid DNA, genomic DNA mixtures, or cDNA mixtures can be used as a template for the first PCR. Alternatively, cDNA can be generated by reverse transcription PCR (RT-PCR) using a gene-specific antisense primer (with defined 5' tail sequence, see Table 1, page 18), total RNA, and a reverse transcriptase (e.g. MMuLV Reverse Transcriptase, cat. no. BR0400201). Rules for the design of the gene-specific PCR primers containing the relevant 5' tails are given on pages 16-18.

For optimal expression using PCR products in *in vitro* translation reactions, XE-Solution is provided. XE-Solution is added to *in vitro* translation reactions where it protects linear DNA from degradation by exonucleolytic nucleases.

Strategy for designing gene-specific primers

Prerequisites for successful PCR include the design of optimal gene-specific primer pairs, the use of appropriate primer concentrations, and the correct storage of primer solutions. Primers, for best results, should be HPLC-purified.

The final PCR product added to the *in vitro* translation reaction is generated by a two-step PCR procedure. In the first PCR, primers must be designed that are not only specific for the protein of interest, but also provide 5' tails that will act as hybridization sites for adapter primers used in the second PCR (see Figure 6 on page 15 and Tables 1 and 2 on pages 18 and 19). The first step in designing primers is to decide whether an affinity tag should be attached to the protein and at which terminus. Use the 5'-end sequence information in Table 1 and the information below to design forward (sense) and reverse (antisense) primers for protein constructs with an affinity tag at the respective terminus.

Length

The length of the gene-specific sequence should be 17–20 bases (see Table 1 on page 18). This may be reduced or increased in some cases to give primers suitable for the annealing temperature of 50°C.

Melting temperature (T_m)

The optimal melting temperature (Tm) for primers used with the RTS Linear Template Kit Plus is 55°C. The optimal annealing temperature is 5°C below Tm.

Simplified formula for estimating melting temperature (T_m):

$T_m = 2°C \times (A+T) + 4°C \times (G+C)$

Whenever possible, design primer pairs with similar T_m values.

Table 1. Sequences of Gene-Specific Primers Required for First-Round PCR.

Desired feature(s)	Gene-specific sense primer	Gene-specific antisense primer
No tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG = start codon)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
N-terminal 6xHis tag	5'-ACC CAC GCG CAT GTC GTA AAA AGC ACC CAA + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
C-terminal 6xHis tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG = start codon)	5'-TG GTG ATG GTG GTG ACC CCA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal *Strep*-tag	5'-AAA AGC GCT GAA AAC CTG ATC GAA GGC CGT + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
C-terminal *Strep*-tag	5'-AGAAGGAGATAAACA + **ATG** + 17 nt target sequence (ATG = start codon)	5'-GG ATG AGA CCA GGC AGA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal 6xHis tag and C-terminal *Strep*-tag	5'-ACC CAC GCG CAT GTC GTA AAA AGC ACC CAA + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-GG ATG AGA CCA GGC AGA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
N-terminal *Strep*-tag and C-terminal 6xHis tag	5'-AAA AGC GCT GAA AAC CTG ATC GAA GGC CGT + 17 nt target sequence (no ATG necessary but ensure that downstream codons are cloned in frame)	5'-TG GTG ATG GTG GTG ACC CCA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
Melittin signal peptide without C-terminal tag	5'-GTA TAC ATT TCT TAC ATC TAT GCG GAC + 20 nt sense strand of target sequence	5'-CTTGGTTAGTTAGTTA + **TTA** + 20 nt target sequence (TTA = stop codon)
Melittin signal peptide and C-terminal *Strep*-tag	5'-GTA TAC ATT TCT TAC ATC TAT GCG GAC + 20 nt sense strand of target sequence	5'-GG ATG AGA CCA GGC AGA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)
Melittin signal peptide and C-terminal 6xHis tag	5'-GTA TAC ATT TCT TAC ATC TAT GCG GAC + 20 nt sense strand of target sequence	5'-TG GTG ATG GTG GTG ACC CCA + 20 nt target sequence (ensure that a stop codon from target sequence does not prevent tag expression)

Table 2. Sense and Antisense Primer Pairs Required for Second-Round PCR.

Desired feature(s)	N-terminus sense primer* (yellow screw-cap)	C-terminus antisense primer* (brown screw-cap)
No tag	No tag Sense Primer	No tag Antisense Primer
N-terminal 6xHis tag	6xHis tag Sense Primer	No tag Antisense Primer
C-terminal 6xHis tag	No tag Sense Primer	6xHis tag Antisense Primer
N-terminal Strep-tag	Strep-tag Sense Primer†	No tag Antisense Primer
C-terminal *Strep*-tag	No tag Sense Primer	Strep-tag Antisense Primer
N-terminal 6xHis tag and C-terminal *Strep*-tag	6xHis tag Sense Primer	Strep-tag Antisense Primer
N-terminal *Strep*-tag and C-terminal 6xHis tag	Strep-tag Sense Primer†	6xHis tag Antisense Primer
Melittin signal peptide without C-terminal tag	N-term. Signal Sense Primer	No tag Antisense Primer
Melittin signal peptide and C-terminal *Strep*-tag	N-term. Signal Sense Primer	Strep-tag Antisense Primer
Melittin signal peptide and C-terminal 6xHis tag	N-term. Signal Sense Primer	6xHis tag Antisense Primer

* Nucleotide sequences of primers can be found in the Appendix on page 27.

† In addition to the Strep-tag II epitope, the Strep-tag Sense Primer encodes a Factor Xa Protease cleavage site between the tag and the body of the target protein.

Positive control for the two-step PCR procedure

The functionality of the kit and the PCR procedure is checked by performing a two-step positive-control PCR. The first positive-control PCR should contain RTS Positive-Control DNA (PCR) (white screw-cap), Positive-Control Sense Primer (white screw-cap), and Positive-Control Antisense Primer (white screw-cap). Products from this PCR should then be amplified using the No tag Sense Primer (yellow screw-cap) and 6xHis tag Antisense Primer (brown screw-cap). The final PCR product encodes the 32 kDa elongation factor EF-Ts with a C-terminal 6xHis tag.

Protocol: Two-Step PCR Procedure for Generating an Expression Template

This protocol is made up of two separate PCR procedures. In the first PCR, protein-specific sequence is used as a template. The primers used in this first PCR add sequences that will serve as hybridization sites in a second round of PCR (see Figure 6). In the second PCR, adapter primers (see Table 2) are used to add sequence that encodes regulatory elements required for efficient expression and optional affinity tags.

Important points before starting

- Decide which DNA polymerase you want to use. The kit is adapted to HotStar HiFidelity DNA Polymerase (Qiagen, cat. no. 202602). However, other DNA polymerases may also give satisfactory results (e.g. Hot Start *Taq* DNA Polymerase, biotechrabbit cat. no. BR0200101). If a non-hot-start DNA polymerase is used, preheat the thermocycler to the polymerases denaturing temperature. Subsequently put the PCR tubes in the thermocycler and start the temperature program immediately.
- Wear gloves for all working steps in order to protect the reaction components from contaminating DNA and nucleases.
- Use DNase- and RNase-free filter pipet tips.
- Avoid using DNA templates that already contain promoter and terminator elements of the phage T7 gene 10. To avoid contamination of the second PCR with these elements, remove them from the protein-coding sequence using restriction enzymes. Separate the reaction products by agarose gel electrophoresis and purify the target sequence band from the agarose gel using a gel extraction kit (e.g. GenUP™ Gel Extraction Kit, cat. no. BR0700401). Use the purified target sequence DNA for the first PCR.
- When using HotStar HiFidelity DNA Polymerase, to amplify PCR products <2 kb, use values marked with a ● and for PCR products >2 kb, use the values marked with a ▲.
- The optimal Mg^{2+} concentration should be determined empirically, but in most cases the standard concentration provided in the PCR buffer of the purchased enzyme will produce satisfactory results.
- Lyophilized primers should be dissolved in a small volume of TE buffer (10 mM Tris·HCl; 1 mM EDTA, pH 8.0) to make a concentrated stock solution.
- Prepare small aliquots of primer working solutions (10 μM) to avoid repeated thawing and freezing. Store all primer solutions at –20°C.
- It is recommended to use a thermocycler with heated lid.
- All experimental procedures – if not stated otherwise – should be performed on ice.

Procedure: First PCR using gene-specific primers

Please note, that the protocol is an example for **HotStar HiFidelity DNA Polymerase**. When using another enzyme, please follow the instructions of the corresponding DNA polymerase.

1. **Thaw 5x HotStar HiFidelity PCR Buffer (contains dNTPs)†, primer solutions, and, if required, 25 mM $MgSO_4$. Mix the solutions thoroughly before use.**
2. **Prepare a master mix according to Table 3.**
3. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

Table 3. Reaction composition (Example for HotStar HiFidelity DNA Polymerase)

Component	Volume/reaction	Final concentration
Master Mix		
5x HotStar HiFidelity PCR Buffer (contains dNTPs)†	5 µl	1x
Sense primer	0.75 — 2.5 µl	0.3 — 1 µM
Antisense primer	0.75 — 2.5 µl	0.3 — 1 µM
HotStar HiFidelity DNA Polymerase (2.5 units/µl)‡	● 0.5 µl ▲ 1 µl	● 1.25 U ▲ 2.5 U
RNase-free water	Variable	—
Template DNA		
Template DNA (added in step 4)	Variable	50–500 ng genomic DNA 1–100 ng cDNA 0.5–5.0 ng plasmid DNA
Total volume	**25 µl**	

† Contains optimized concentration of dNTPs, 7.5 mM $MgSO_4$, and Factor SB.

‡ Dependent on expected PCR product length. In general, use ● 0.5 µl HotStar HiFidelity DNA Polymerase when amplifying PCR products <2 kb and ▲ 1 µl enzyme when amplifying PCR products >2 kb.

4. **Add template DNA to the individual tubes containing the master mix.**
   For the positive control reaction use 1 µl of a 1 in 10 dilution of Positive-Control DNA (PCR) (white screw-cap) template. The resulting PCR fragment will have a length of 880 bp.
5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 25 µl mineral oil.**
6. **Program the thermal cycler according to the manufacturer's instructions.**
   Each PCR program must start with an initial heat-activation step at 95°C for 5 min.

Table 4. Cycling protocol 1st PCR (Example for HotStar HiFidelity DNA Polymerase)

	Time	Temp.	Comments
Initial activation step	5 min	95°C	HotStar HiFidelity DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	15 s	94°C	
Annealing	1 min	50°C	Approximately 5°C below T_m of primers.
Extension	1 min/kb (PCR products of 1–2 kb) 2 min/kb (PCR products >2kb)	72°C 68°C	
Number of cycles	30–45 cycles		The number of cycles is dependent on the origin of the template DNA (see footnote)
Final extension	10 min	72°C	

* Use 30 cycles if amplifying PCR products from plasmid DNA and 40–45 cycles when amplifying PCR products from genomic DNA or cDNA.

7. **Place the PCR tubes in the thermal cycler and start the cycling program.**
   After amplification, samples can be stored overnight at 2–8°C or at -20°C for longer storage.
8. **Analyze 1 µl PCR product on a 0.8–1.5% agarose gel.**
   The product of the first PCR should be the dominant band. Use 1 µl (approximately 100 ng DNA) of the first PCR product as template for the second PCR.

Procedure: Second PCR using adapter primers

Please note, that the protocol is an example for **HotStar HiFidelity DNA Polymerase**. When using another enzyme, please follow the instructions of the corresponding DNA polymerase.

1. **Thaw 5x HotStar HiFidelity PCR Buffer (contains dNTPs)†, primer solutions, and, if required, 25 mM $MgSO_4$. Mix the solutions thoroughly before use.**
2. **Prepare a master mix according to Table 5.**
3. **Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes.**

Table 5. Reaction composition (Example for HotStar HiFidelity DNA Polymerase)

Component	Volume/reaction	Final concentration
Master Mix		
5x HotStar HiFidelity PCR Buffer (contains dNTPs)†	5 µl	1x
Sense Adapter Primer*	2 µl	
Antisense Adapter Primer*	2 µl	
HotStar HiFidelity DNA Polymerase (2.5 units/µl)‡	● 0.5 µl ▲ 1 µl	● 1.25 U ▲ 2.5 U
RNase-free water	Variable	—
Template DNA		
Template DNA (added in step 4)	1–2 µl product from first PCR (~100 ng)	
Total volume	**25 µl**	**—**

† Contains optimized concentration of dNTPs, 7.5 mM $MgSO_4$, and Factor SB.

‡ Dependent on expected PCR product length. In general, use ● 0.5 µl HotStar HiFidelity DNA Polymerase when amplifying PCR products <2 kb and ▲ 1 µl enzyme when amplifying PCR products >2 kb.

* For possible adapter primer combinations see Table 2, page 19.

4. **Add template DNA (1–2 µl of the first PCR, ~100 ng) to the individual tubes containing the master mix.**
5. **When using a thermal cycler with a heated lid, do not use mineral oil. Proceed directly to step 6. Otherwise, overlay with approximately 25 µl mineral oil.**
6. **Program the thermal cycler according to the manufacturer's instructions.**

   Each PCR program must start with an initial heat-activation step at 95°C for 5 min.

Table 6. Cycling protocol 2nd PCR (Example for HotStar HiFidelity DNA Polymerase)

	Time	Temp.	Comments
Initial activation step	5 min	95°C	HotStar HiFidelity DNA Polymerase is activated by this heating step.
3-step cycling			
Denaturation	15 s	94°C	
Annealing	1 min	50°C	Approximately 5°C below T_m of primers.
Extension	1 min/kb (PCR products of 1–2 kb)	72°C	
	2 min/kb (PCR products >2kb)	68°C	
Number of cycles	30 cycles		
Final extension	10 min	72°C	

7. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

8. **Analyze 1 µl of the first and second PCR on a 0.8–1.5% agarose gel.**

   The introduction of regulatory elements and affinity-tag sequences in the second PCR adds approximately 160–200 bp to the first PCR product.

9. **Determine the yield of the second PCR by comparing the product band to the molecular weight marker bands.**

   0.7 µg DNA (~7 µl of the second PCR) is required for a 50 µl *in vitro* translation reaction using RTS 100 *E. coli* HY Kits. 0.5 µg (~5 µl of the second PCR) is required for a 50 µl *in vitro* translation reaction using RTS 100 Insect Membrane Kits and 0.5 µg for a 100 µl *in vitro* translation reaction using RTS 100 Insect Disulfide Kits.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in biotechrabbit Technical Service are always happy to answer any questions you may have about either the information or protocol(s) in this handbook. Many of the PCR-related problems in this guide are comprehensively covered in the instruction manuals of the corresponding DNA polymerase (e.g. HotStar HiFidelity DNA Polymerase PCR Handbook).

		Comments and suggestions
First and second PCR		
Little or no product		
a)	DNA polymerase not activated	Check if PCR was started with an initial incubation step (typically some minutes at > 90°C).
b)	Pipetting error or missing reagent	Repeat the PCR. Check the concentrations and storage conditions of reagents, including primers and dNTP mix.
c)	Insufficient starting template	Increase amount of starting template used in PCR.
d)	Primer concentration not optimal	Recalculate primer concentration.
e)	Extension time too short	Increase extension time in increments of 1min. Ensure that the extension time for large DNA fragments fits to the applied DNA polymerase.
f)	Enzyme concentration too low	Some DNA polymerases require increased concentrations for larger DNA fragments.
g)	Insufficient number of cycles	Increase the number of cycles in steps of 5 cycles.
h)	Problems with starting template	Check the concentration, storage conditions, and quality of starting template. If necessary, make new serial dilutions of template nucleic acid from stock solutions. Repeat PCR using the new stock solutions.
i)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM.
j)	Primer design not optimal	Review primer design, see pages 16-18.
k)	Incorrect dNTP concentration	Ensure that the dNTP concentration fits to the applied DNA polymerase. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.

Product is multi-banded

a)	PCR cycling conditions not optimal	Review primer design, see pages 16-18.
b)	Enzyme concentration too low	Some DNA polymerases require increased concentrations for larger DNA fragments.
c)	Extension time too short	Increase extension time in increments of 1min. Ensure that the extension time for large DNA fragments fits to the applied DNA polymerase.
d)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM.
e)	Primer concentration not optimal or primers degraded	Recalculate primer concentration. Particularly when performing highly sensitive PCR, check for possible degradation of the primers on a denaturing polyacrylamide gel.
f)	Primer design not optimal	Review primer design, see pages 16-18.
g)	Incorrect dNTP concentration	Ensure that the dNTP concentration fits to the applied DNA polymerase. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.

Product is smeared

a)	Insufficient starting template	Increase amount of starting template used in PCR.
b)	Extension time too short	Increase extension time in increments of 1 min. Ensure that the extension time for large DNA fragments fits to the applied DNA polymerase.
c)	Mg^{2+} concentration not optimal	Perform PCR with different final concentrations of Mg^{2+} from 1.5 to 5 mM.
d)	dNTP concentration not optimal	Ensure that the dNTP concentration fits to the applied DNA polymerase. Increase dNTP concentration in increments of 50 µM of each dNTP. Do not exceed a concentration of 500 µM of each dNTP since this might lower PCR fidelity.
e)	Primer design not optimal	Review primer design, see pages 16-18.

References

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

2) 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 53,153-160

Appendix: Adapter Primer Sequences and Multiple Cloning Sites

No tag Sense Primer

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggT
TTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA-3'

H_2N — Protein

Strep-tag Sense Primer

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggT
TTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**TggTCTCATCC
gCAATTCgAAAAAAgCgCTgAAAACCTgATCgAAggCCgT-3'

H_2N — MWSHPQFEKSAENLIEGR* — Protein

* Factor Xa cleavage site

6xHis tag Sense Primer

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggT
TTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**AAACATCATCA
CCATCACCACTCgACCCACgCgCATgTCgTAAAAAgCACCCAA-3'

H_2N—MKHHHHHHSTHAHAHVVKSTQ — Protein

No tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTAgAggCCC
CAAggggTACAgATCTTggTTAgTTAgTTA**TTA**-3'

Protein — Stop — COOH

Strep-tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTAgAggCCC
CAAggggTACAgATCTTggTTAgTTAgTTA**TTA**TTTTTCgAATTgCggATgAgACCAggC
AgA-3'

Protein — SAWSHPQFEK — Stop — COOH

6xHis tag Antisense Primer

5'-ATgATATCACCggTgAATTCggATCCAAAAAACCCCTCAAgACCCgTTTAgAggCCC
CAAggggTACAgATCTTggTTAgTTAgTTA**TTA**ATgATggTgATggTggTgACCCCA–3'

Protein — WGHHHHHH — Stop — COOH

No tag Sense Primer Signal Peptide

5'-ATgATATCTCgAgCggCCgCTAgCTAATACgACTCACTATAgggAgACCACAACggT
TTCCCTCTAgAAATAATTTTgTTTAACTTTAAgAAggAgATAAACA**ATg**AAATTCTTAgT
CAACgTTgCCCTTgTTTTTATggTCgTATACATTTCTTACATCTATGCGGAC-3'

H_2N —MKFLVNVALVFMVVYISYIYA*D— Protein

* signal peptide cleavage site

Underlined sequence hybridizes to 5' tails of gene-specific primers.

Sequencing primers

M13 forward (–20)	gTAAAACgACggCCAgT
M13 reverse (–21)	CAggAAACAgCTATgAC

Cloning PCR Products Generated with the RTS Linear Template Kit Plus into the RTS pIX3.0 Vector

The pIX3.0 vector is designed for cloning of PCR products generated by the RTS Linear Template Kit Plus. The PCR products contain multiple cloning sites that are compatible with the multiple cloning site of the pIX3.0 vector.

RTS Sense Primer Multiple Cloning Site

*Eco*RV *Xho*I *Not*I *Nhe*I

```
5'-ATGATATCTCGAGCGGCCGCTAGCT-3'
3'-TACTATAGAGCTCGCCGGCGATCGA-5'
```

RTS Antisense Primer Multiple Cloning Site

BamHI *EcoRI* *SgrAI/AgeI* *EcoRV*

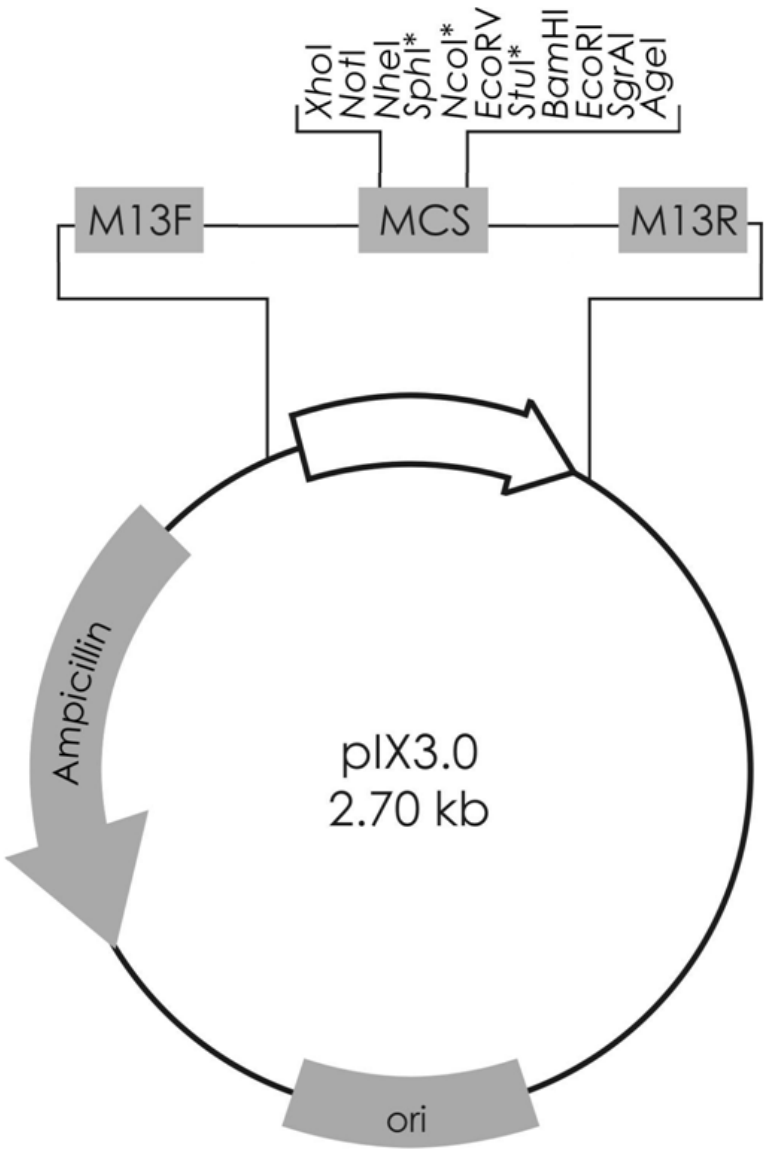
```
5'-TGGATCCGAATTCTCCGGTGATATCAT-3'
3'-ACCTAGGCTTAAGTGGCCACTATAGTA-5'
```

RTS pIX3.0 Vector Multiple Cloning Site

XhoI *NotI* *NheI* *EcoRV* *BamHI* *EcoRI* *SgrAI/AgeI*

```
5'-CTCGAGCGGCCGCTAGCATGCCATGGATATCAGGCCTGGATCCGAATTCACCGGTG-3'
3'-GAGCTCGCCGGCGATCGTACGGTACCTATAGTCCGGACCTAGGCTTAAGTGGCCAC-5'
```

Figure 7. The RTS pIX3.0 Vector.



* As long as they do not have a restriction site within the PCR insert, these restriction enzymes can be added directly to the vector–insert ligation reaction to minimize the number of false positive clones (religations).

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