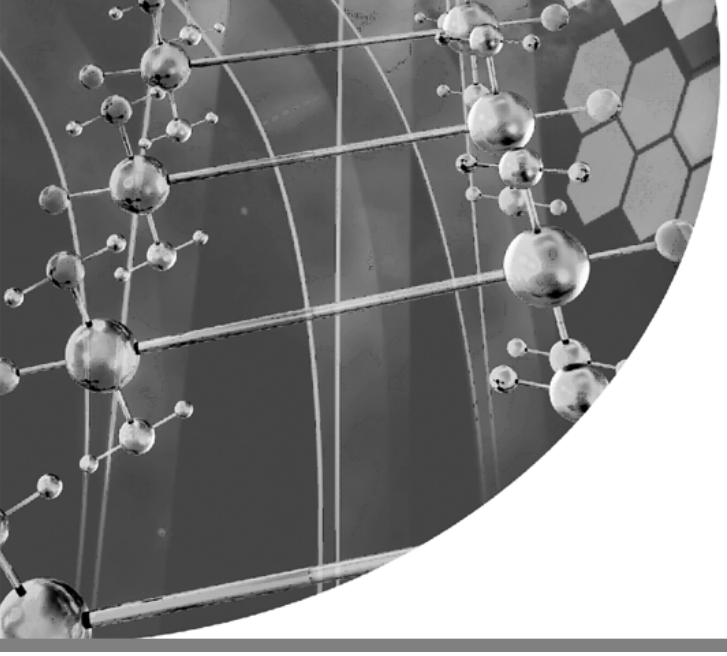

RTS 100 *E. coli* HY Kit Manual

For cell-free expression of functionally active protein from circular and linear templates

RTS 100 *E. coli* HY Kit Manual, September, 2009

Contents

Product specifications

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

- rapid parallel protein synthesis reactions
- compatibility with PCR generated templates and plasmids
- rapid optimization of expression constructs
- rapid functional testing of PCR-generated mutations
- expression of toxic gene products
- synthesis of truncated gene products from PCR-generated or restriction enzyme digested DNA, for epitope or functional domain mapping
- successful synthesis of proteins in the molecular weight range from 10–120 kDa
- Incorporation of radiolabeled Methionine into the protein

Product description

The RTS 100 *E. coli* HY Kit provides the components and procedures necessary for 24 (cat. no. 2401100) or 96 (cat. no. 2401110) coupled transcription/translation reactions of 50 µl.

Product limitations

The RTS 100 *E. coli* HY Kit is developed, designed, and sold for research purposes only. It is not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of the materials described in this text.

Materials supplied

Kit	Catalog no.	Size
RTS 100 *E. coli* HY Kit	2401100	24 reactions
RTS 100 *E. coli* HY Kit	2401110	96 reactions

RTS 100 *E. coli* HY Kit

Kit	Contents and function	No. included (24 reactions)	No. included (96 reactions)
Ordering number		2401100	2401110
E. coli Lysate; 100 E.coli (Bottle 1, red cap)	→ Lysate from E. coli; stabilized and lyophilized → Contains components for transcription and translation	1 bottle	4 bottles
Reaction Mix; 100 E.coli (Bottle 2, green cap)	→ Substrate mix to prepare reaction solution; stabilized and lyophilized	1 bottle	4 bottles
Amino Acids; 100 E.coli (Bottle 3, brown cap)	→ Mix of 19 amino acids to prepare Reaction Solution; stabilized and lyophilized	1 bottle	4 bottles
Methionine; 100 E.coli (Bottle 4, yellow cap)	→ Methionine to prepare Reaction Solution; stabilized and lyophilized	1 bottle	1 bottle
Reconstitution Buffer; 100 E.coli Buffer (Bottle 5, white cap)	→ Buffer solution for the reconstitution of bottles 1, 2, 3, and 4	1 bottle	4 bottles
Control Vector GFP; 100 E.coli (Bottle 6, colorless cap)	→ 50 µg lyophilized plasmid GFP (green fluorescent protein) expression vector with C-terminal His_6-tag for the control reaction	1 bottle	1 bottle
Reaction Tubes + Caps; 100 E.coli or Microplate + Adhesive Films; 100 E.coli (7)	→ Reaction tubes or microplate	24 x 200 µl reaction tubes	Module with 12 strips (8 wells)

Additional materials

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

- Eppendorf Thermomixer Comfort. Also used with legacy RTS ProteoMaster Instrument (Roche). Alternatively, a water bath or heater adjustable to 30°C can be used. Optimal results are obtained if the reaction is shaken during incubation.
- Pipets 0–10 µl, 10–200 µl, 200–1,000 µl,
- Pipette tips autoclaved at 121°C for 20 min (RNase free)
- Besides the template vector coding for the protein of interest, pIVEX cloning vectors or the RTS *E. coli* LinTempGenSet plus PCR reagents to create a linear DNA expression construct are needed. N- and C-terminal epitope tags, as well as protein fusions can be added to the gene of interest by using the RTS *E. coli* LinTempGenSet. For the latest product information visit www.5PRIME.com
- $^{[35[}$S-Methionine (>1,000 Ci/mmol at 15 mCi/ml) is required for optional radioactive labeling
- Deionized DNase- and RNase-free water is required for reconstitution of the GFP Control Vector
- A GFP standard protein (e.g. r-GFP from Roche Molecular Biochemicals) is available for comparison of GFP (control reaction) on SDS polyacrylamide gels.
- Acetone is required for precipitating samples prior to SDS-gel electrophoresis.

For convenience, additional materials to be supplied by the user are listed at the beginning of the protocol for which they are required.

Shipping and storage conditions

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

The RTS 100 *E. coli* HY Kit and components should be stored in the dark at –15 to –25°C and are stable until the expiration date printed on the label. Avoid repeated freezing and thawing.

Safety information

All due care and attention should be exercised in the handling of this product. We recommend all users of 5 PRIME products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines. Specifically, always wear a suitable lab coat, disposable gloves, and protective goggles when working with chemicals.

Additional safety information is available from www.5PRIME.com in material safety data sheets (MSDSs) for 5 PRIME products and 5 PRIME product components.

Quality assurance

5 PRIME products are manufactured using quality chemicals and materials that meet our high standards. All product components are subjected to rigorous quality assurance testing process:

- **Component testing:** each component is tested to ensure the composition and quality meet stated specifications.
- **Performance testing:** each product is tested to ensure it meets the stated performance specification.

Additional quality information is available from 5 PRIME (www.5PRIME.com). Certificates of Analysis are available on request.

Product warranty

5 PRIME is committed to providing products that improve the speed, ease-of-use and quality of enabling technologies.

5 PRIME guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use.

This warranty is in place of any other warranty or guarantee, expressed or implied, instituted by law or otherwise. 5 PRIME provides no other warranties of any kind, expressed or implied, including warranties of merchantability and fitness for a particular purpose. Under no circumstance shall 5 PRIME be responsible for any direct, indirect, consequential or incidental damages or loss arising from the use, misuse, results of use or inability to use its products, even if the possibility of such loss, damage or expense was known by 5 PRIME.

Protocols

Product principle

Introduction

The Rapid Translation System (RTS) workflow (Figure 1) combines a series of new technologies for efficient and optimized protein expression. They overcome the limitations that often restrict the use of cell-free systems. These innovations include software-based template optimization, generation of stable expression templates without cloning, optimization of *in vitro* expression conditions and high yield *in vitro* expression using the continuous exchange cell free principle (CECF) and an optimized lysate biochemistry.

The RTS 100 *E. coli* HY Kit allows the expression of up to 20 µg functionally active protein in a 50 µl reaction from circular and linear templates within 6 hours. It is therefore suited for the rapid testing and optimization of expression constructs as well as for the screening of many expressions in parallel.

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

Advantages of the system include:

- fast – express 2–20 µg protein within 6 hours
- convenient
    - protein expression reaction preparation takes less than 30 minutes
    - no fermentation and therefore no safety regulations for recombinant organisms
- ability to express multiple proteins in parallel
- flexible
    - alter reaction conditions to overcome insolubility or folding problems
    - express toxic proteins
    - incorporate radiolabeled Methionine
- reliable
    - kits are tested to yield at least 300 µg/ml GFP
    - lyophilized bacterial lysate offers unprecedented stability
    - good reproducibility

Description of procedure

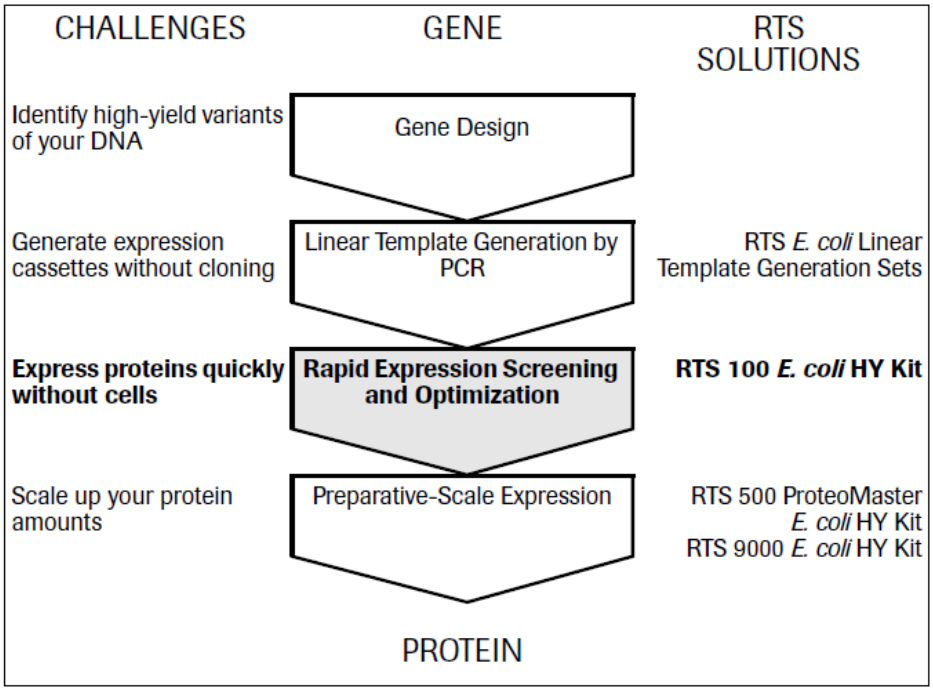

Figure 1. Integration of the RTS 100 E. coli HY Kit into the RTS workflow.

Reaction principle

RTS uses a coupled transcription/translation reaction for *in vitro* protein synthesis. Transcription and translation take place simultaneously in the reaction: While the T7-RNA-polymerase transcribes the template gene, the ribosomes provided by the *E. coli* lysate start to translate the 5'-end of the nascent mRNA (Figure 2). This makes the system highly productive compared with the use of isolated mRNA (1). The *E. coli* lysate is prepared with some modifications according to the method of Zubay (2), resulting in the biochemically enhanced High-Yield (HY) *E. coli* lysate (3, 4). An *E. coli* strain with low exonuclease activity was selected and growth conditions were optimized to allow optimum protein expression from linear (PCR-generated) and plasmid templates.

Regulatory elements, such as T7 promoter, ribosomal binding site and T7 terminator are added to the gene of interest by PCR or cloning using a pIVEX vector. The resulting DNA template is then added to the Reaction Solution.

In a coupled *in vitro* reaction, the template DNA is first transcribed into mRNA by T7 RNA polymerase, followed by translation into protein by the ribosomal machinery present in the *E. coli* lysate. The expressed protein accumulates during the reaction and is harvested after 1–6 hours. The reaction will reach 90% completion after 4 hours.

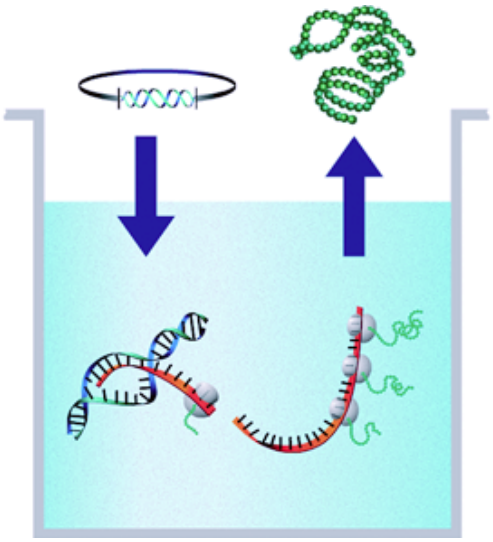

Figure 2. Schematic illustration of the coupled transcription/translation reaction.

Firstly, an expression plasmid or linear template DNA carrying the gene of interest is added to the reaction compartment. In a coupled *in vitro* reaction, the DNA is first transcribed from the template vector into mRNA by T7 RNA polymerase, followed by translation by the ribosomal machinery present in the *E. coli* lysate. Expressed protein accumulates in the reaction compartment and is harvested after a 4–24 hours.

Template DNA

Linear and circular expression templates are suited. Use pIVEX vectors, the RTS *E. coli* LinTempGenSet or a simple one-step PCR to attach T7 transcription/ translation regulatory elements to the target gene.

Other vectors, designed for prokaryotic *in vitro* protein expression and containing a T7 promoter as well as a ribosomal binding site may also be used. The requirements are described in Protocol 1, ‘Use of expression vectors’, page 14.

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

Template optimization

The expression yield of a given gene is strongly influenced by its mRNA structure. Interactions between the coding sequence and the 5'-untranslated region can interfere with translation initiation and therefore have an impact on translation efficiency. In the case of low expression yields, 5 PRIME recommends that the sequence is optimized. For example, silent mutations in the first few codons can be introduced using the RTS *E. coli* LinTempGenSet and may help to increase expression and protein yield.

Generation of expression templates by PCR: Principal applications

The use of PCR protocols instead of subcloning allows rapid expression from new or modified DNA-templates:

- generation of a DNA expression template by an overlap extension (OLE) PCR protocol (by addition of a T7 promoter, a prokaryotic ribosomal binding site and other regulatory elements to the gene of interest)
- addition of Tag sequences for detection with antibodies and purification
- optimization of the gene sequence for expression (see Protocol 1, 'Use of expression vectors', page 14)
- introduction of mutations
- change of codon usage
- truncation of proteins
- domain screening

Generation of a linear DNA expression template by overlap extension PCR

The RTS *E. coli* LinTempGenSets are recommended to generate a linear DNA template for the *in vitro* expression via an overlap extension PCR protocol. They provide a DNA for the introduction via an OLE PCR of the regulatory elements, as well as different N- or C-terminal tags.

Procedure

1. Select the appropriate RTS *E. coli* LinTempGenSet depending on the kind of tag or fusion protein you wish to add to the gene of interest.
2. Design a gene-specific sense primer that contains 15–20 nucleotides homologous to the gene of interest and an additional 20 bases overlapping the chosen tag region, as indicated in the respective RTS *E. coli* LinTempGenSet manual.
3. Design a gene-specific antisense primer that contains 15–20 nucleotides homologous to the gene of interest and an additional 20 bases overlapping

the chosen tag region, as indicated in the respective RTS *E. coli* LinTempGenSet package insert. (For an example, see 'Typical results', page 21.)

4. Perform 20 cycles of a 50 µl PCR using these primers and the template DNA containing the gene of interest as indicated in the respective RTS *E. coli* LinTempGenSet manual.
5. Perform 30 cycles of a second 50 µl PCR using 100 ng of the first PCR product as template together with the primers and DNA supplied with the RTS *E. coli* LinTempGenSet containing regulatory elements for prokaryotic expression and C- or N-terminal epitope tags.
6. Determine the concentration of the PCR product densitometrically on an agarose gel by comparison to DNA molecular weight markers.
7. Use 100 ng PCR product for a 50 µl *in vitro* protein synthesis reaction.

Note: Use the PCRExtract Mini Kit (5 PRIME) to remove potentially inhibitory primer-dimers. Do not purify the PCR product using agarose gels because this treatment inhibits *in vitro* protein synthesis.

Addition of a C-terminal epitope tag sequence by a one-step PCR protocol

Standard PCR reactions may be used to introduce epitope tags or mutations into the already cloned gene of interest.

Procedure

1. Design a sense primer that is located upstream of a T7 promoter sequence in a prokaryotic expression plasmid with the gene of interest.

   This plasmid has to contain a T7 promoter sequence and the necessary regulatory elements as detailed in 'Template optimization', page 11.
2. Design an antisense primer that contains the T7 terminator sequence, a spacer region of at least 25 nucleotides distance to the stop codon followed by the tag sequence and a 15–20 base-pair sequence homologous to the gene of interest.
3. Perform a PCR using these primers and follow steps 4 and 5 of the procedure on page 11.
4. Use 500 ng of the PCR product for a 50 µl *in vitro* protein synthesis reaction.
5. Protocol for a 50 µl PCR. Combine the following:

   37 µl H_2O

   5 µl 10× PCR buffer with $MgCl_2$ (final conc. 1.5 mM) from the Expand High Fidelity PCR System (Roche)

   1.25 µl dNTP-Mix (dNTP 10 mM each)

   2.5 µl sense primer (10 pmol/µl)

   2.5 µl antisense primer (10 pmol/µl)

   0.9 µl enzyme from Expand High Fidelity PCR System

1 µl of template DNA (1 ng/µl)

Run the following PCR cycles:

4 min/94°C

+ 20x (1 min/94°C + 1 min/72°C)

+ 4°C.

Use of expression vectors

Necessary vector elements

Any vector or linear DNA to be used in combination with RTS must include the following elements and structural features:

- target gene under control of T7 promoter located downstream of a RBS (ribosomal binding site) sequence
- distance between T7 promoter and start ATG should not exceed 100 base pairs
- distance between the RBS sequence and start ATG should not be more than 5–8 base pairs
- T7 terminator sequence at the 3'-end of the gene

General recommendations

The pIVEX vector family has been developed and optimized for use in the RTS. 5 PRIME recommends cloning target genes into a pIVEX vector prior to expression (see 'Generation of expression templates', page 11).

Maps of some of the available pIVEX vectors are shown schematically in Figure 3. For more information, visit www.5PRIME.com/RTS.aspx.

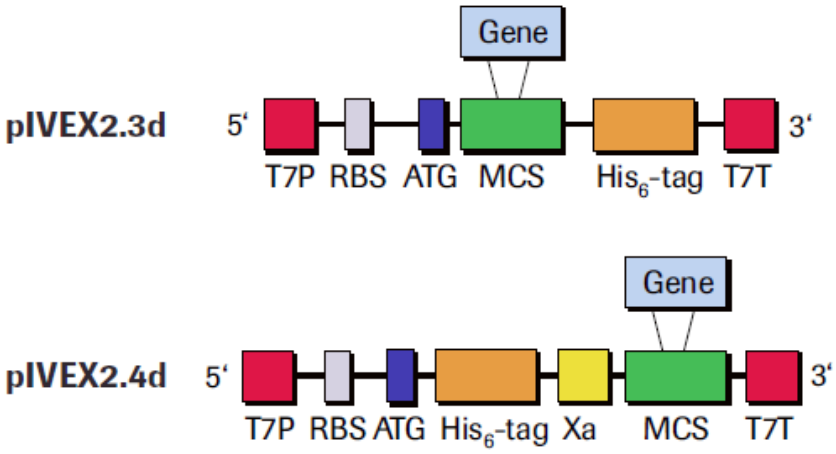

Figure 3. Functional elements of cloning vectors.

***T7P**: T7 Promoter; **RBS**: Ribosome binding site; **ATG**: Start codon; **Xa:** Factor Xa restriction protease cleavage site; **MCS**: Multiple cloning site for the insertion of the target gene; **T7T**: T7 Terminator.*

Purity of the plasmid preparation

Plasmids obtained from commercially available DNA preparation kits (e.g. PerfectPrep EndoFree Plasmid Maxi Kit, 5 PRIME) are usually pure enough to be used as template in the RTS. If DNA is not pure enough (OD260/280 <1.7), use phenol extraction to remove traces of RNase from the preparation, which may enhance its performance in the expression reaction.

Note: do not purify DNA-fragments from agarose gels, as this treatment inhibits *in vitro* protein synthesis.

Protocol 2: Protein synthesis reaction

Equipment and reagents required

- DNA template: Prepare and purify the DNA template as described in Protocol 1.
- Use the Eppendorf® Thermomixer Comfort or a water bath or an incubator, adjusted to 30°C. Shaking of the reaction solution during the reaction results in higher yields. The RTS ProteoMaster Instrument can also be used.
- Calibrated pipets
- UV lamp (360 nm) for the detection of GFP (control reaction)
- RNase-free plastic and glassware

Reagent notes

- Do not combine reagents from different kit lots
- Reconstitute only the bottles needed for the experiment
- For reconstitution of bottles 1 to 4, use only the Reconstitution Buffer supplied with the kit (bottle 5). For reconstitution of the plasmid (bottle 6), use sterile DNase- and RNase-free water (5 PRIME)
- Store reconstituted solutions of bottles 1 to 4 at –15 to –25°C. The reagents can withstand two freeze–thaw cycles without significant decrease in activity
- Reconstitution Buffer can be thawed in a water bath at 25°C
- Store reconstituted plasmid at –15 to –25°C.
- Reconstitute the lyophilized reagents or thaw solutions immediately before use
- Keep reagents and working solutions on ice

Procedure

1. Reconstitute the reaction components according to Table 1.

Table 1. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	E. coli Lysate; 100 E.coli (Bottle 1, red cap)	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer (bottle 5), mix carefully by rolling or gentle shaking. Do not vortex!	Step 2 Solution 7
2	Reaction Mix; 100 E.coli (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.30 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	Step 2 Solution 7
3	Amino Acids; 100 E.coli (Bottle 3, brown cap)	Reconstitute the lyophilizate with 0.36 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	Step 2 Solution 7
4	Methionine; 100 E.coli (Bottle 4, yellow cap)	Reconstitute the lyophilizate with 0.33 ml of Reconstitution Buffer (bottle 5), mix by rolling or shaking.	Step 2 Solution 7
5	Reconstitution Buffer; 100 E.coli (Bottle 5, white cap)	→ 1.6 ml → Ready-to-use solution → The solution is stable at 2–8°C, but can also be stored at –15 to –25°C	Solutions 1, 2, 3, and 4
6	Control Vector GFP; 100 E.coli (Bottle 6, colorless cap)	→ Briefly centrifuge down the contents of the bottle → Reconstitute the lyophilizate with 50 µl of sterile DNase- and RNase-free water (5 PRIME) → The solution is stable at –15 to –25°C	GFP control reaction, see page 17

All reconstituted solutions should be clear, with the exception of the *E. coli* lysate, which remains cloudy.

2. Prepare the working solution according to Table 2.

Table 2. Working solution

Solution	Contents	Preparation of working solution for one 50 µl reaction	For use in
7	Reaction Solution	Into one of the reaction tubes supplied, pipet the following components: ➔ 12 µl E. coli Lysate ➔ 10 µl Reaction Mix ➔ 12 µl Amino Acids ➔ 1 µl Methionine ➔ 5 µl Reconstitution Buffer ➔ 10 µl DNA in water or TE buffer Template amount: ➔ 0.5 µg circular template ➔ 0.5 µg linear template derived from one-step PCR ➔ 0.1 µg linear template derived from two-step PCR ➔ A premix of solutions 1–5 without DNA is recommended for multiple parallel reactions ➔ Mix carefully by rolling or gentle shaking; do not vortex!	Running an experiment, see page 17

Running an experiment, standard reaction

1. Close the reaction tubes, or the modules of the microplate, with the supplied caps or adhesive film, respectively.
2. Place the loaded reaction tubes into the Eppendorf Thermomixer Comfort, an incubator, a water bath, or the ProteoMaster Instrument at 30°C.

   Note: If using modules, place the loaded modules within the frame of the microplate into the Eppendorf Thermomixer Comfort, ProteoMaster Instrument, or an incubator at 30°C. A water bath is unsuitable for modules.
3. After 4–6 hours remove the reactions from the Eppendorf Thermomixer Comfort, the ProteoMaster instrument, incubator or water bath.
4. Store Reaction Solution at –25°C or at 0–4°C until purification or further processing.
5. See 'Supporting information', page 20 for sample preparation for SDS-PAGE.

Points to consider

Temperature: The optimal temperature for most proteins is 30°C. However, lower temperatures may be used for proteins that tend to aggregate.

Time: Protein synthesis continues for up to 6 hours. However, the reaction is 90% complete after 4 hours.

GFP control reaction

1. Reconstitute bottles 1 to 4 and bottle 6 according to Table 1, page 15.
2. Prepare Reaction Solution in one of the reaction tubes according to Table 2, page 16.
3. Add 1 µg (1 µl) of reconstituted Control Vector GFP (bottle 6) and 9 µl water (5 PRIME) or TE-buffer.
4. Start the reaction: Temperature: 30°C; Time: 6 hours.
5. After the run, store the Reaction Solution in a 2 ml vial for 24 hours at 4°C for maturation of GFP.
6. The fluorescence of GFP (excitation wavelength 395 nm, emission wavelength 504 nm) can be observed using an UV lamp (360 nm).
7. Apply 2–5 µl of the reaction onto SDS-polyacrylamide gels.
8. Run the gel, and then stain with Coomassie® Blue.

   Note: The GFP protein can also be detected on Western blots by using an anti-His_6 antibody (5 PRIME).

Oxidation of GFP

GFP needs molecular oxygen to form the fluorophore post-translationally (5). The yield of properly-folded fluorescent GFP is further increased by storing the Reaction Solution after the expression for 24 hours at 2–8°C.

After 24 hours of storing at 2–8°C the maturation is almost completed.

Solubility of GFP

A remarkable fraction of the synthesized GFP will be in the pellet fraction. This is caused by the nature of the GFP gene used in the control reaction. GFP expression and detection via UV are for qualitative purpose only and should not be used to quantify the expression yield.

Radioactive labeling

1. Reconstitute bottles 1 to 4 and bottle 6 according to Table 1, page 15.
2. Dilute 10 µl of the reconstituted Methionine solution (Table 1) with 990 µl of nuclease-free water (5 PRIME) to yield a 1 mM Methionine solution.
3. For one radioactive reaction prepare the following Reaction Solution in one of the reaction tubes:

   6 µl *E. coli* Lysate

   5 µl Reaction Mix

   6 µl Amino Acids

   1.25 µl 1 mM Methionine solution (see Note)

   2 µl of a L-[^{35}S]Methionine (SJ 235 Amersham) 15 mCi/ml

   2.5 µl Reconstitution Buffer

   0.25 µg of plasmid DNA or 0.05–0.25 µg of linear template in 2.25 µl of water (5 PRIME) or TE buffer

   Note: Addition of unlabeled Methionine to the labeling reaction is required to prevent premature termination for larger proteins or proteins with many Methionine residues.
4. Start the reaction: Temperature: 30°C; Time: Stop the reaction after 60 min.

   Note: The Methionine added in this reaction is used up after 1 hour. For most applications the yield achieved with this protocol is sufficient. If more radioactive protein is required please adapt conditions.
5. Apply 2–5 µl of the reaction samples onto SDS-polyacrylamide gels.

   Note: For optimum results precipitate the proteins before applying onto SDS-polyacrylamide gels (see ‘Supporting information’, page 20).
6. After the separation, dry the gel and apply it onto Kodak X-OMAT AR films for autoradiography (3–20 hours exposure).

Supporting information

Short protocol: Reconstitution of reaction components and running a reaction

Table 3. Short protocol steps

Step	Contents	Reconstitution procedure
1	E. coli Lysate; 100 E.coli (Bottle 1, red cap)	Reconstitute with 0.36 ml Reconstitution Buffer. Do not vortex!
2	Reaction Mix; 100 E.coli (Bottle 2, green cap)	Reconstitute with 0.30 ml Reconstitution Buffer
3	Amino Acids; 100 E.coli (Bottle 3, brown cap)	Reconstitute the lyophilizate with 0.36 ml Reconstitution Buffer
4	Methionine; 100 E.coli (Bottle 4, yellow cap)	Reconstitute the lyophilizate with 0.33 ml Reconstitution Buffer
5	Control Vector GFP; 100 E.coli (Bottle 6, colorless cap)	→ Briefly centrifuge down the contents of the bottle → Reconstitute the lyophilizate with 50 µl of sterile DNase- and RNase- free water
6	Reaction Solution	Mix the following components: → 12 µl E. coli Lysate → 10 µl Reaction Mix → 12 µl Amino Acids → 1 µl Methionine → 5 µl Reconstitution Buffer → 10 µl DNA in water (5 PRIME) or TE-buffer (0.5 µg circular template or 0.1–0.5 µg linear template)
7	Start the reaction	Incubate for 4–6 hours at 30°C

Optimizing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Precipitation of proteins prior to SDS-PAGE

The Reaction Solution contains polymers that interfere with the separation of proteins with an apparent molecular weight between 20 and 30 kDa. 5 PRIME recommends that samples are precipitated with acetone prior to the addition of SDS-PAGE sample buffer.

Procedure

1. To a 5 µl sample of Reaction Solution add 50 µl of –20°C cold acetone, mix and incubate on ice for 5 min.
2. Centrifuge for 5 min at 10,000 rpm.
3. Discard the supernatant and air dry the pellet for 10 min (Speedvac may be used).
4. Dissolve the pellet in 20 µl of SDS-PAGE sample buffer, heat for 5 min at 95°C and apply 5–20 µl onto a SDS gel.

To distinguish between the soluble and pellet fractions of the protein of interest, add the following steps:

5. Centrifuge a 5 µl sample of the Reaction Solution for 5 min at 10,000 rpm.
6. Pipet the supernatant into a separate tube.
7. Add 50 µl ice-cold acetone to each tube and proceed as described above.

Typical results

Yield from various template generation methods

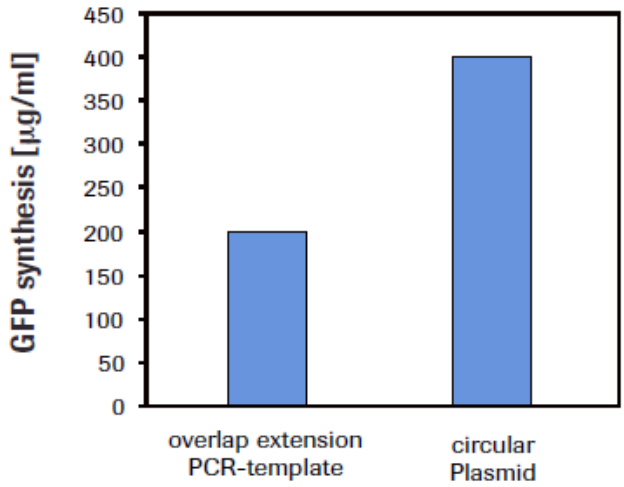

Figure 4. A GFP coding DNA template (generated by overlap extension PCR or by cloning into the pIVEX 2.3 expression plasmid) was used for the in vitro protein synthesis reactions. The overlap extension PCR product was generated using the RTS E. coli LinTempGenSet with C-terminal His_6-Tag primers for the second PCR and the following primers for the first PCR:

Sense primer

5'CTTTAAGAAGGAGATATACC ATGACTAGCAAAGGA 3´

Antisense primer

5'TGATGATGAGAACCCCCCCC GGGTTTGTATAGTTCATC 3´

Up to 200 µg/ml of protein from overlap extension PCR, and up to 400 µg/ml from plasmid template were obtained in a 4-hour reaction.

Application: Optimization of protein expression using the RTS workflow

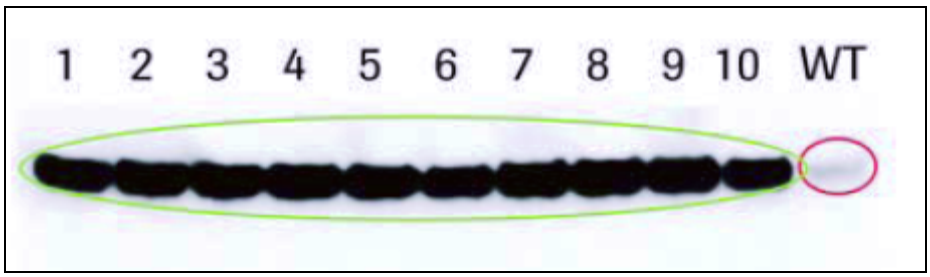

Figure 5. Optimization of the expression of human p58 (function unknown).

Linear templates were generated with the RTS LinTempGenSet, His_6-tag.The protein was expressed with the RTS 100 E. coli HY Kit. The figure shows western blot analysis (anti-His_6) of wild type (small ellipse) and optimized (large ellipse) gene variants.

Application: Radioactive labeling with L-[^{35}S]Methionine

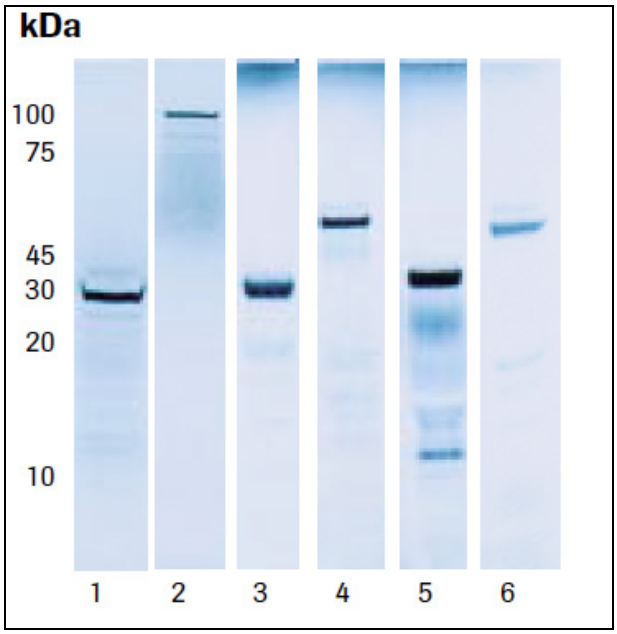

Figure 6. Autoradiography of in vitro synthesized proteins from different species labeled with L-[^{35}S]Methionine.

***Lane 1**: GFP (A. victoria); **Lane 2**: β-Galactosidase (E. coli); **Lane 3**: Fc gamma receptor (human); **Lane 4**: Rec. Plasminogen activator (human); **Lane 5**: Single chain antibody fragment (mouse); **Lane 6**: Rhodanese (bovine).*

The indicated genes were cloned into pIVEX2.3 vectors and used for in vitro expression without further purification. After 30 minutes at 30°C, 2 µl samples were separated on 10% SDS polyacrylamide gels. After drying, the gels were applied to Kodak X-OMAT AR film for autoradiography (3-hour exposure time).

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Troubleshooting guide

The following troubleshooting recommendations are designed to address unexpected or undesired results. To ensure optimal use, follow the guidelines and recommendations in the manual.

The control reaction

Observation	**No control protein visible**
Possible cause	Kit expired
Resolving	Order a new kit.
Possible cause	Kit has not been stored at –15 to –25°C
Resolving	Order a new kit.
Possible cause	Contamination with RNases
Resolving	Repeat experiment and ensure to work RNase-free at every step.

Expression of the target protein

Observation	No protein band of the target protein, but normal expression of control protein
Possible cause	Protein concentration too low
Resolving	Load the maximum amount of sample on the gel
Possible cause	Protein is insoluble
Resolving	Analyze the supernatant and pellet
Possible cause	Tag is hidden
Resolving	Try different tag position or protein-specific antibody. If the protein is His_6-tagged, try a different anti-histidine antibody (e.g. anti-Penta-Histidine antibody; 5 PRIME).
Resolving	Check protein on a Coomassie-stained gel in addition to the western blot.
Possible cause	Cloning error
Resolving	Check the sequence.
Possible cause	Low purity of plasmid DNA
Resolving	Ensure that the absorbance ratio 260 nm/280 nm is at least 1.7.
Resolving	Perform a phenol extraction.
Resolving	Make a new plasmid preparation.

Possible cause	Contamination with RNases
Resolving	Repeat experiment and ensure to work RNase-free at every step.

Possible cause	No initiation of translation due to strong secondary structures of the mRNA
Resolving	Introduce different N- or C-terminal tags using the RTS *E. coli* LinTempGenSets (His_6-tag, HA-tag, MBP-fusion).

Possible cause	Expressed protein interferes with the translation or transcription process
Resolving	Express the protein of interest together with control protein. If control protein expression is inhibited, the active protein can not be expressed using RTS.

Observation	**Low expression yield**
Possible cause	Expression time too short
Resolving	Extend expression time.

Possible cause	The tag has a negative influence on the folding of the protein
Resolving	Introduce different N- or C-terminal tags using the RTS *E. coli* LinTempGenSets (His_6-tag, HA-tag, MBP-fusion).

Possible cause	Amount of template DNA not optimal
Resolving	Vary (increase) DNA concentration to get optimum results.

Observation	Sufficient protein expression, but low yield of active protein
Possible cause	Incorrect folding of the protein due to: → dependence on co-factors → necessity of disulfide bonds → dependence on post-translational modifications → dependence on chaperones
Resolving	Add necessary co-factors
Resolving	Proteins with more than a few disulfide bonds (it could be shown, that three disulfide bonds can be formed) may be not expressed in a functional form in the RTS *E. coli* system.
Resolving	Add chaperones (8).
Resolving	For proteins with up to 3 disulfide bonds, allow oxidation after the reaction for the formation of disulfide bonds (2, 3).
Resolving	The *E. coli* lysate cannot introduce post-translational modifications such as glycosylation, phosphorylation, or signal sequence cleavage.
Resolving	Use RTS GroE Supplement for proteins in the range between 20 and 30 kDa.

Observation	Product appears in the pellet after centrifugation
Possible cause	Aggregation
Resolving	Add/adjust chaperones or cofactors.
Resolving	Change experimental conditions (time, temperature, e.g., lower in steps of 4–14°C).
Resolving	Add mild detergents (e.g., Triton® X-100, Tween® 20, Brij-58, CHAPS; for further information, see reference 9).
Resolving	Use RTS pIVEX MBP fusion vector or RTS *E. coli* LinTempGenSet, MBP-fusion to gain solubility.

Observation	Several product bands on SDS-PAGE or product smaller than expected
Possible cause	Proteolytic degradation
Resolving	Dissolve 1 tablet of Complete mini EDTA-free (Roche) in 0.5 ml nuclease-free water (5 PRIME) and add 2 µl for a 50 µl reaction.
Possible cause	Internal initiation site
Resolving	Check sequence for internal Methionine codons (ATG), look for RBS-like sequence (variants of `AAGGAG´) 5 to 12 nucleotides upstream of the ATG. Mutate the RBS-like sequence, if possible replace the corresponding Methionine by point mutation.
Possible cause	Premature termination of the translation
Resolving	Check the sequence of the target gene regarding reading frame and mutations which might yield a stop codon.
Resolving	Search for strong secondary structures of the mRNA and eliminate them by conservative mutations.

Ordering information

Product	Size	Order/ref. no.
RTS *E. coli* LinTempGenSet, His_6- tag	96 reactions	2401000
RTS 100 *E. coli* HY Kit	24 reactions	2401100
RTS 100 *E. coli* HY Kit	96 reactions	2401110
RTS 500 ProteoMaster *E. coli* HY Kit	5 reactions	2401500
RTS 500 *E. coli* HY Kit	5 reactions	2401510
RTS 9000 *E. coli* HY Kit	1 reaction	2401900
RTS 100 *E. coli* Disulfide Kit	24 x 50 µl reactions	2401120
RTS 500 *E. coli* Disulfide Kit	5 x 1 ml reactions	2401520
RTS pIVEX His_6-Tag 2nd Generation Vector Set	2 vectors, 10 µg each	2401010
RTS Wheat Germ LinTempGenSet, His_6-tag	96 reactions	2402000
RTS pIVEX Wheat Germ His_6-tag Vector Set	10 µg each	2402010
RTS 100 Wheat Germ CECF Kit	24 reactions	2402100
RTS 500 Wheat Germ CECF Kit	5 x 1 ml reactions	2402500
RTS GroE Supplement	For 5 RTS 500 reactions	2401030
RTS DnaK Supplement	1 set	2401020
RTS Amino Acid Sampler	For 5 RTS 500 reactions	2401530
Water, Molecular Biology grade	10 x 50 ml	2500010
Anti·His Antibody Selector Kit (Mouse Tetra·His Antibody, Mouse Penta·His Antibody, Mouse RGS·His Antibody)	3 µg each; lyophilized, for 30 ml working solution	2400300
Penta·His Antibody, BSA free	100 µg; lyophilized, for 1,000 ml working solution	2400320
Penta·His HRP Conjugate Kit	for 250 ml working solution	2400410
RGS·His Antibody	100 µg	2400330
RGS·His Antibody, BSA-free	100 µg	2400340

Tetra·His Antibody, BSA free	100 µg; lyophilized, for 1,000 ml working solution	2400310
PerfectPrep Spin Mini Kit	50 Preps	2300100
PerfectPrep EndoFree Plasmid Maxi Kit	10 Preps	2300120

5 PRIME distributors

A complete list of 5 PRIME distributors is available from www.5PRIME.com.