



RTS™ 100 Wheat Germ CECF Kit Manual

Eukaryotic cell-free protein synthesis system based on wheat germ lysate

RTS 100 Wheat Germ CECF Kit Manual, April, 2015

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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.

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Product specifications

The RTS 100 Wheat Germ CECF Kit is designed for:

- expression of (mainly eukaryotic) cDNAs that produce insoluble products or are only very poorly expressed in *E. coli* lysates
- rapid parallel protein synthesis reactions
- use with either plasmids or PCR-generated linear DNA templates
- rapid functional testing of PCR-generated mutations
- synthesis of truncated protein variants for epitope or functional domain mapping
- expression from *in vitro*-synthesized RNA
- synthesis of proteins of widely varying size (up to 250 kDa as tested)

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

Product description

The RTS 100 Wheat Germ CECF Kit provides the components and procedures necessary for 24 coupled transcription/translation or translation-only reactions of 50 µl.

Product limitations

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

Materials supplied

RTS 100 Wheat Germ CECF Kit	Contents and function	No. per kit
Ordering number		BR1401001
Wheat Germ Lysate; 100 WG (Vial 1, red cap)	<ul style="list-style-type: none"> → Lysate from wheat germ; stabilized and lyophilized → Contains components for transcription and translation 	3
Reaction Mix; 100 WG (Vial 2, green cap)	<ul style="list-style-type: none"> → Substrate mix to prepare reaction solution; stabilized and lyophilized 	3
Feeding Mix; 100 WG (Vial 3, blue cap)	<ul style="list-style-type: none"> → Feeding mix to prepare feeding solution; lyophilized 	3
Amino Acids; 100 WG (Vial 4, brown cap)	<ul style="list-style-type: none"> → Mix of 19 amino acids (all natural except Methionine) to prepare Reaction Solution; stabilized and lyophilized 	3
Methionine; 100 WG (Vial 5, yellow cap)	<ul style="list-style-type: none"> → Methionine to prepare Reaction Solution; stabilized and lyophilized 	1
Reconstitution Buffer; 100 WG (Vial 6, white cap)	<ul style="list-style-type: none"> → 30 ml buffer solution for the reconstitution of bottles 1, 2, 3, 4, and 5 	1
Control Vector GUS; 100 WG (Vial 7, colorless cap)	<ul style="list-style-type: none"> → 50 µg plasmid, lyophilized → GUS (Glucuronidase) expression vector for control reaction 	1
RTS 100 CECF Device with Film; 100 WG (8)	Module with 3 trays for 8 reactions each	1

Additional materials

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

- Eppendorf® Thermomixer Comfort or RTS ProteoMaster™ instrument
- Pipets: 0–10 µl, 10–200 µl, and 200–1,000 µl
- Pipet tips autoclaved at 121°C for 20 minutes
- Starting cDNA encoding the protein of interest
biotechrabbit recommends cloning the cDNA into the RTS pIVEX Wheat Germ His₆-tag Vector Set (cat. no. BR1401301, biotechrabbit) to form a circular template. Alternatively, for initial screening, convert the cDNA into a linear expression template with the RTS Wheat Germ LinTempGenSet, His₆-tag (cat. no. BR1401201, biotechrabbit). PCR reagents and equipment to either amplify the cDNA for cloning or convert it into a linear expression template will also be required.
Note: The His₆-tag is available in an N- or C-terminal version in the RTS pIVEX Wheat Germ His₆-tag Vector Set and in the RTS Wheat Germ LinTempGenSet, His₆-tag.
- If the reaction is to be performed with an RNA template, biotechrabbit recommends using SP6/T7 Transcription Kit (Roche) to produce mRNA. A vector containing the cDNA of interest downstream from a suitable promoter (e.g. T3, T7, SP6) will also be required.
- For radioactive labeling (optional) use [³⁵S]-Methionine (>1,000 Ci/mmol at 15 mCi/ml).
- For reconstitution of the GUS Control Vector use only deionized DNase- and RNase-free water.

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 Wheat Germ CECF Kit is shipped on dry ice.

Upon delivery, remove the reaction devices from the box. If reaction devices are left in the box until use, they may contain residual CO₂ (from the dry ice used to ship the kit). This residual CO₂ will change the pH of the reaction mixes and may inhibit protein synthesis.

If the kit is to be used shortly after delivery, cut open one side of the protective plastic bag around the device (but do not remove it from the bag completely). Store the device upside down at 15–25°C for at least 24 hours before use.

For long-term storage, devices should be stored at 2–8°C.

The RTS 100 Wheat Germ CECF Kit is stable in dark at –15 to –25°C until the expiration date printed on the label.

Safety information

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

Quality assurance

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

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

Additional quality information is available from www.biotechrabbit.com. Certificate of analysis sheets for biotechrabbit products are available on request.

Product warranty

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

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

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

Protocols

Product principle

Introduction

The RTS Wheat Germ continuous exchange cell-free (CECF) system is a preparative, scalable, eukaryotic cell-free translation system. A two-chamber reaction device based on a microplate allows production of several hundred micrograms protein per milliliter (>400 µg/ml for GUS control protein).

Description of procedure

Transcription and translation take place simultaneously in the 50 µl reaction compartments of the reaction device. Substrates and energy components needed for a sustained reaction are continuously supplied via a semi-permeable membrane. At the same time, potentially inhibitory reaction byproducts are diluted as they diffuse through the same membrane into the 1 ml feeding compartment (Figure 1). In contrast to traditional batch systems, CECF conditions allow protein synthesis to continue for 24 hours. Yields approaching several hundred micrograms of newly synthesized protein are possible in reactions with PCR-generated linear template; yields of almost one milligram are possible if vector template or exogenously added capped mRNA template are used.

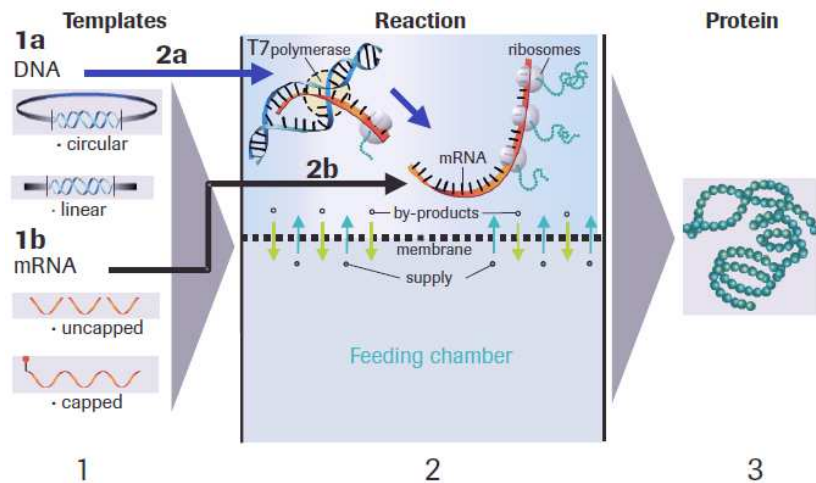


Figure 1. Basic steps of the RTS Wheat Germ CECF system.

1a: If DNA is used as template in the reaction device, the gene of interest is either cloned into a suitable T7-driven pVEX Wheat Germ expression vector, or fused to the 5' and 3' regulatory sequences (UTRs) by PCR using the RTS Wheat Germ LinTempGenSet to produce a linear expression template. **1b:** If mRNA is used as template in the reaction device, it is pre-synthesized in a separate *in vitro* transcription reaction and added to the translation reaction with or without purification. **2:** In a coupled reaction, cDNA is first transcribed into mRNA. Subsequently, the mRNA is translated into protein. **2a:** T7 RNA polymerase transcribes the DNA template *in vitro*. **2b:** The translational machinery of the wheat germ lysate translates the synthesized mRNA *in vitro*. Note: Alternatively pre-synthesized mRNA (1b) can be directly translated into protein by the wheat germ lysate (2b). **3:** Product accumulates in the reaction mix during a run of up to 24 hours.

Template DNA

biotechrabbit recommends using PCR products as expression templates, since this approach allows multiple constructs to be rapidly generated and screened for yield and solubility in parallel. Guidelines on how to design PCR primers to attach T7 transcription/translation regulatory elements to your target gene are provided in the RTS Wheat Germ LinTempGenSet, His₆-tag Manual (biotechrabbit).

For expression scale-up, biotechrabbit recommends cloning cDNA into a pIVEX Wheat Germ plasmid, which contains an enhancer sequence optimized for wheat germ lysates (see page 11). Such a recombinant plasmid will provide a well-characterized, stable template for use in all subsequent experiments that investigate the same gene.

Ready-for-expression ORF clones

To avoid cloning, ready-for-expression clones such as OmicsLink™ Cell-Free ORF Expression Clones (GeneCopoeia Inc.) can be used. OmicsLink Cell-Free Expression Clones contain various tandem tags and identical 5'- and 3'-regulatory elements as pIVEX Wheat Germ vectors. These expression clones are created by using proprietary high-fidelity non-PCR gene transfer technology that minimizes mutations caused by PCR amplification.

Description of OmicsLink Cell Free Expression Clones, cloning technology and expression data of more than 10,000 human full-length protein coding clones using RTS 100 Wheat Germ CECF Kit are available at www.genecopoeia.com.

Note: biotechrabbit does not recommend using other vectors that contain a T7 promoter but lack the wheat germ-optimized expression enhancer sequences present in pIVEX Wheat Germ vectors; use of such vectors in the expression reaction can lead to a greatly reduced synthesis rate. If such vectors must be used, biotechrabbit recommends using them as templates to produce capped mRNA, and then using this mRNA as template in the expression reaction. This will considerably improve synthesis rates (see Protocol 2, page 14).

Coupled *in vitro* transcription/translation

Similar to other *in vitro* expression systems, the RTS 100 Wheat Germ system allows expression of exogenously added mRNA. However, it is more convenient to produce the mRNA in an *in vitro* coupled transcription/translation reaction. When DNA template and T7 RNA polymerase are added to the DNA-free wheat germ lysate, transcription and translation are closely coupled in time and space. Whilst the T7 RNA polymerase transcribes the gene of interest encoded by PCR-generated template or vector, the ribosomes provided in the wheat germ lysate begin to translate mRNA.

Unlike *E. coli* lysates, eukaryotic lysates, such as wheat germ, have very different Mg^{2+} optimums for *in vitro* transcription and translation. Highly efficient protein synthesis, therefore, normally requires separate preparation of mRNA (at high Mg^{2+}) prior to translation (at low Mg^{2+}). Therefore, in contrast to systems based on *E. coli* lysates (e.g. RTS 100 *E. coli* HY Kits), eukaryotic *in vitro* coupled transcription/translation reactions normally (e.g. in batch mode) cannot achieve preparative yields (several micrograms per 50 μ l; hundreds of μ g/ml).

The RTS Wheat Germ system solves this problem by using the two-chamber CECF format, which allows dialysis to adjust the Mg^{2+} from high to low in the course of the same reaction. Consequentially, both transcription and translation take place with much higher efficiencies in the RTS system than in other systems.

Note: Batch (non-CECF) reactions with the RTS Wheat Germ system will produce much lower yields and should, therefore, only be used for labeling expressed protein with radioactive amino acid precursors.

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

Necessary vector elements

Any vector or linear DNA to be used in combination with this kit must contain the target gene under control of the T7 promoter. Template should contain 5' and 3' regulatory untranslated regions that are optimized for wheat germ expression systems. (See information included in the RTS pIVEX Wheat Germ, His₆-tag Vector Set; biotechrabbit)

Some of the available pIVEX vectors are shown schematically in Figure 2. Sequences and maps of these vectors can be downloaded from www.biotechrabbit.com.

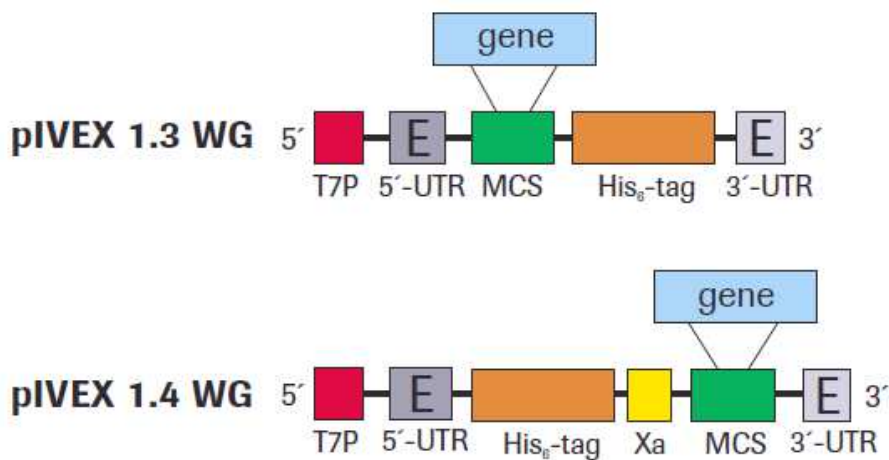


Figure 2. Functional elements of RTS Wheat Germ expression vectors.

T7P: T7 promoter; **UTR:** Untranslated regions (UTRs) containing optimized translation enhancer (E) elements (for details see pack insert for each vector); **His₆-tag:** Tag sequence at C- or N-terminal position; **Xa:** Factor Xa restriction protease cleavage site; **MCS:** Multiple cloning site in three different reading frames for the insertion of the target gene.

Note: Vector DNA purified from agarose gels cannot be used as template for *in vitro* expression, because the purification treatment may introduce chemicals that inhibit protein synthesis.

Cloning procedure

Protocols for cloning the cDNA of interest into RTS pIVEX Wheat Germ vectors are provided in the respective vector manuals. They are based on either standard restriction digestions, or, for the processing of multiple samples in parallel In-Fusion™ cloning.

Ready-for-expression ORF clones

Ready-for-expression clones (OmicsLink Cell Free ORF Expression Clones) for 20,000 human proteins can also be used (www.genecopoeia.com).

Purity of the plasmid preparation

Plasmids obtained from commercially available DNA preparation kits (e.g. GenUP™ Plasmid Kit and GenUP™ Plasmid Plus Kit, cat. no. BR0700201, BR0701201, biotechrabbit) are usually sufficiently pure to be used as template in the Rapid Translation System. If DNA is not sufficiently pure ($OD_{260}/OD_{280} \leq 1.7$), use phenol extraction to increase purity and to remove traces of RNase, which may otherwise inhibit template performance in the expression reaction.

Note: Do not purify DNA fragments from agarose gels, since this treatment may inhibit *in vitro* protein synthesis.

Main applications

For rapid production of expression templates from wild-type or modified DNA sequences, biotechrabbit recommends using PCR instead of subcloning the sequences into vectors. PCR methods permit (for example):

- addition of the T7 promoter and both the 5'- and 3'- regulatory regions (UTRs) to the cDNA of interest
- addition of epitope tag sequences to allow detection with antibodies
- introduction of mutations
- changes in codon usage
- construction of truncated proteins

After identifying suitable linear expression templates they can optionally be subcloned into the RTS pIVEX Wheat Germ vectors. Protocols for cloning the linear expression templates are available in the RTS™ Wheat Germ LinTempGenSet, His₆-tag Manual.

Generation of a linear expression template using a two-step PCR protocol

To incorporate the 5'- and 3'- UTRs and the T7 promoter into a template for subsequent *in vitro* transcription/translation reactions, biotechrabbit recommends using a two-step overlap extension PCR protocol such as the one outlined below. This protocol uses the RTS Wheat Germ LinTempGenSet, His₆-tag (cat. no. BR1401201, biotechrabbit), which provides a DNA that contains these regulatory regions as well as epitope tag sequences.

Equipment and reagents required

- RTS Wheat Germ LinTempGenSet, His₆-tag

Procedure

1. Design a sense primer that contains a 15–20 nucleotide sequence homologous to the cDNA of interest and an additional 20 base sequence that overlaps the chosen tag region (as indicated in the manual for the applicable RTS Wheat Germ LinTempGenSet).

Use the RTS Wheat Germ LinTempGenSet, His₆-tag, which allows either the N- or C-terminal epitope tag to be added to the gene of interest.

2. Design an antisense primer that contains a 15–20 nucleotide sequence homologous to the gene of interest and an additional 20 base sequence that overlaps the chosen tag region (as indicated in the manual for the applicable RTS Wheat Germ LinTempGenSet).
3. Perform the first PCR in a 50 µl reaction volume, using the primers you designed and template DNA that contains the gene of interest.

Note: The detailed protocol is provided in the *RTS Wheat Germ LinTempGenSet, His₆-tag Manual*.

4. Perform a second PCR in a 50 µl reaction volume using 2–4 µl (150–300 ng) of the first PCR reaction as template, plus primers and DNA containing the T7 promoter, enhancer elements for translation in wheat germ and C- or N-terminal epitope tags. (This DNA is supplied with the RTS Wheat Germ LinTempGenSet, His₆-tag.)
5. Purify the PCR product using GenUP™ PCR Cleanup Kit (cat. no. BR0700301, biotechrabbit), determine the concentration of the PCR product on an agarose gel and compare to known amounts of DNA molecular size markers. Unpurified PCR products may be used, but these may give lower expression rates.
6. Use 1–2 µg of the PCR product from the second PCR for a 50 µl *in vitro* protein synthesis reaction.

Note: Do not use agarose gels to purify the PCR product that will be used in the expression reaction, since this purification process may inhibit *in vitro* protein synthesis.

7. Optional: The desired linear template can be cloned into the RTS pIVEX Wheat Germ vector according to the protocols available in the RTS™ Wheat Germ LinTempGenSet, His₆-tag Manual.

Generation of expression templates using *in vitro* transcription

mRNA may be pre-synthesized *in vitro*, e.g. from a T7-driven DNA template, and then added as translation template to the reaction devices of the RTS 100 Wheat Germ CECF Kit. Template mRNA may be either capped or non-capped, but capped forms often perform more efficiently in the translation steps.

Note: For production of non-capped mRNA, biotechrabbit recommends using the Transcription Kit SP6/T7 (Roche). The resulting mRNA should be precipitated with ethanol and resuspended before it is used in translation reactions.

Protocol 2: Protein synthesis reaction

Equipment and reagents required

- Eppendorf® Thermomixer Comfort (or RTS ProteoMaster Instrument) set to 24°C and a shaking speed of 900 rpm for reaction incubation
- Calibrated pipets
- RNase-free plastic and glassware
- SDS-polyacrylamide gel, to check the control reaction with glucuronidase.

A band with a molecular weight of 68 kDa should be visible after the gel is stained with Coomassie® Brilliant Blue

Reagent notes

- Prepare and purify template as detailed in Protocol 1, page 11.
- Do not combine reagents from different kit lots
- Reconstitute the lyophilized reagents or thaw the solutions immediately before use
- Reconstitute only the bottles needed for the experiment
- Reconstitution Buffer can be thawed in a 25°C water bath
- For reconstitution of bottles 1–5, use only Reconstitution Buffer from this kit (bottle 6). For reconstitution of the control plasmid (bottle 7), use sterile DNase- and RNase-free water
- Keep reconstituted reagents and working solutions on ice until use
- Store the reconstituted solutions from bottle 2–5 at –15 to –25°C or at lower temperatures
- Store Bottle 1 (Wheat Germ Lysate) at –80°C after reconstitution. The reagent can withstand three freeze–thaw cycles without significant decrease in activity
- Store the reconstituted plasmid at –15 to –25°C

Procedure

1. Reconstitute the reaction components according to Table 1.

Table 1. Reaction components

Solution	Contents	Reconstitution procedure	For use in
1	Wheat Germ Lysate; 100 WG (Bottle 1, red cap)	Reconstitute the lyophilizate with 0.14 ml Reconstitution Buffer (bottle 6), mix carefully by rolling or gentle shaking. Do not vortex!	Solution 9
2	Reaction Mix; 100 WG (Bottle 2, green cap)	Reconstitute the lyophilizate with 0.14 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Solution 9
3	Feeding Mix; 100 WG (Bottle 3, blue cap)	Reconstitute the lyophilizate with 7.7 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Solution 8
4	Amino Acids; 100 WG (Bottle 4, brown cap)	Reconstitute the lyophilizate with 0.8 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Solutions 8 and 9
5	Methionine; 100 WG (Bottle 5, yellow cap)	Reconstitute the lyophilizate with 2.2 ml Reconstitution Buffer (bottle 6), mix by rolling or shaking.	Solutions 8 and 9
6	Reconstitution Buffer; 100 WG (Bottle 6, white cap)	<ul style="list-style-type: none"> → 30 ml ready-to-use solution → The solution is stable at 4°C, but can also be stored at -20°C 	Solutions 1, 2, 3, 4, and 5
7	Control Vector GUS; 100 WG (Bottle 7, colorless cap)	<ul style="list-style-type: none"> → Briefly pellet the content of the bottle by centrifugation → Reconstitute the lyophilizate with 50 µl sterile water → The solution is stable at -15 to -25°C 	Control reaction with GUS gene, page 17

All reconstituted solutions should be clear, with the exception of the Wheat Germ Lysate, which remains cloudy.

2. Prepare the working solutions according to Table 2.

Table 2. Working solutions

Solution	Content	Reconstitution procedure	For use in
8	Feeding Solution	Mix the following components: → 900 µl Feeding Mix → 80 µl Amino Acids → 20 µl Methionine	Running an experiment, standard reaction, page 16
9	Reaction Solution	Mix the following components carefully by rolling or gentle shaking; do not vortex: → 15 µl Reaction Mix → 4 µl Amino Acids → 1 µl Methionine → 15 µl Wheat Germ Lysate → 15 µl of sterile DNase and RNase-free water containing 2–4 µg circular DNA template <u>or</u> 1–2 µg linear PCR-generated template <u>or</u> 2–4 µg mRNA Note: For multiple parallel reactions, biotechrabbit recommends premixing components 1–4, then adding the appropriate template to the premix	Running an experiment, standard reaction, page 16

Running an experiment, standard reaction

Procedure

1. Place the microplate on the workbench and insert one or more CECF modules into it.
Note: Each CECF module contains two types of compartments. Reaction compartments are labeled with red rings to distinguish them from feeding compartments (which are unlabeled) (Figure 3).
2. Pipet 1 ml Feeding Solution into each of the (unlabeled) feeding compartments to be used.
3. Pipet 50 µl Reaction Solution into each reaction compartment (labeled with a red ring) that is directly above a filled feeding compartment.
4. Carefully close the modules of the microplate with the adhesive film (supplied).

5. Insert the microplate into the Eppendorf Thermomixer Comfort (or RTS ProteoMaster Instrument).
6. Set the instrument parameters to a shaking speed of 900 rpm and a temperature of 24°C.
7. Start the run.
8. After the run (up to 24 h) remove the reactions from the incubator.
9. Store reaction solutions frozen or at 0–4°C until purification or further processing.



Figure 3. RTS 100 Wheat Germ CECF device

Points to consider

Temperature: Optimal temperature for most proteins is 24°C.

Time: Protein synthesis continues for up to 24 hours.

Control reaction with GUS gene

Procedure

1. Reconstitute solutions 1–5 and solution 7 according to Table 1, page 15.
2. Prepare Reaction and Feeding Solutions (solution 8 and 9) according to Table 2, page 16.
3. Add 2 µg (2 µl) of reconstituted GUS Control Vector (bottle 7) to one part Reaction Solution.
4. Set up reactions as per the procedure on page 16.

Note: Include a negative control reaction in the run. Use 2 µl water in place of vector in this control.

5. Start the reaction and incubate for 24 hours at 24°C, shaking the microplate at 900 rpm.
6. Stop the reaction after 24 hours.
7. Apply 1 µl of each reaction to a SDS-polyacrylamide gel.
8. Run the gel and stain with Coomassie Brilliant Blue.

9. A band with a molecular weight of ~68 kDa should be visible in the reaction with the GUS Control Vector, but not in the negative control reaction.

Note: The GUS protein can also be detected on a Western blot using an anti-His₆-tag antibody (e.g. 5 PRIME).

Radioactive labeling

Procedure

1. Reconstitute solutions 1–5 according to Table 1, page 15.
2. To obtain a labeled 3 mM Methionine solution for each labeling reaction mix:

5 µl reconstituted Methionine solution (solution 5)

20 µl L-[³⁵S]-Methionine (e.g. SJ 235, Amersham) 15 mCi/ml

Note: This labeling solution is for autoradiographic detection of labeled samples. If using fluorography to detect labeled products, the amount of label used in Step 2 can be reduced. For fluorography, the labeled 3 mM Methionine solution should contain 3 µl L-[³⁵S]-Methionine, 5 µl reconstituted Methionine solution (solution 5), and 17 µl deionized DNase- and RNase-free water.

3. Prepare Feeding Solution (solution 8) according to Table 2, page 16, but use 20 µl of labeled 3 mM Methionine solution as Methionine source.
4. Prepare Reaction Solution as specified on page 16, but use 1 µl of labeled 3 mM Methionine solution as Methionine source.
5. Start the reaction and incubate for 24 hours at 24°C, shaking the microplate at 900 rpm.
6. Stop the reaction after 24 hours.
7. Apply 2 µl of the labeled samples to a SDS-polyacrylamide gel.
8. After the separation, dry the gel and expose it to a Kodak® X-OMAT AR film for autoradiography (3–20 hours exposure time).

Supporting information

Use the short protocol only if you are familiar with the standard procedures described in this manual.

Short protocol

Reconstitution of reaction components and run of a reaction

1. Reconstitute the following reagents:

Reagent	Reconstitution
Wheat Germ Lysate; 100 WG (Bottle 1, red cap)	Reconstitute with 0.14 ml Reconstitution Buffer. Do not vortex.
Reaction Mix; 100 WG (Bottle 2, green cap)	Reconstitute with 0.14 ml Reconstitution Buffer
Feeding Mix; 100 WG (Bottle 3, blue cap)	Reconstitute with 7.7 ml Reconstitution Buffer
Amino Acids; 100 WG (Bottle 4, brown cap)	Reconstitute with 0.8 ml Reconstitution Buffer
Methionine; 100 WG (Bottle 5, yellow cap)	Reconstitute with 2.2 ml Reconstitution Buffer
Control Vector GUS; 100 WG (Bottle 7, colorless cap)	<ul style="list-style-type: none"> → Pellet the content of the bottle by centrifugation → Reconstitute the lyophilizate with 50 µl sterile DNase- and RNase-free water
Feeding Solution	Mix the following components: <ul style="list-style-type: none"> → 900 µl Feeding Mix → 80 µl Amino Acids → 20 µl Methionine

Continued on the next page

Reaction Solution**Mix the following components:**

- 15 µl Reaction Mix
 - 4 µl Amino Acids
 - 1 µl Methionine
 - 15 µl Wheat Germ Lysate
 - 15 µl of sterile DNase and RNase-free water containing 2–4 µg circular DNA template or 1–2 µg linear PCR-generated template or 2–4 µg mRNA
-

2. Start the reaction and incubate for 24 hours at 24°C, shaking the microplate at 900 rpm.

Typical results

Standard reaction

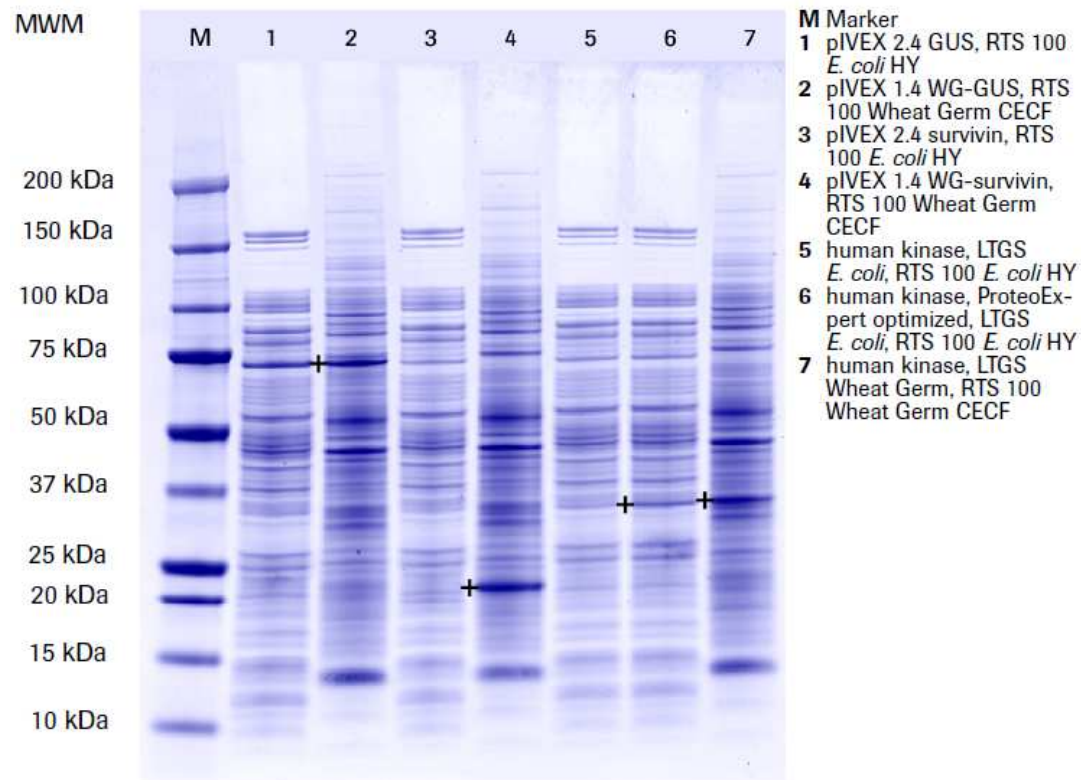


Figure 4. Comparison of protein yields obtained in RTS 100 Wheat Germ CECF vs. RTS 100 *E. coli* HY Kit expression reactions.

At the completion of the reactions, 0.5 μ l of each reaction mixture was analyzed using SDS-PAGE. Gels were stained with Coomassie Brilliant Blue. **Lanes 1–4:** The template in each reaction was a pIVEX vector construct. **Lanes 5–7:** The template in each reaction was a PCR-generated linear template. **GUS:** glucuronidase. **LTGS:** LinTempGenSet. Bands of the expected size are marked with a +.

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

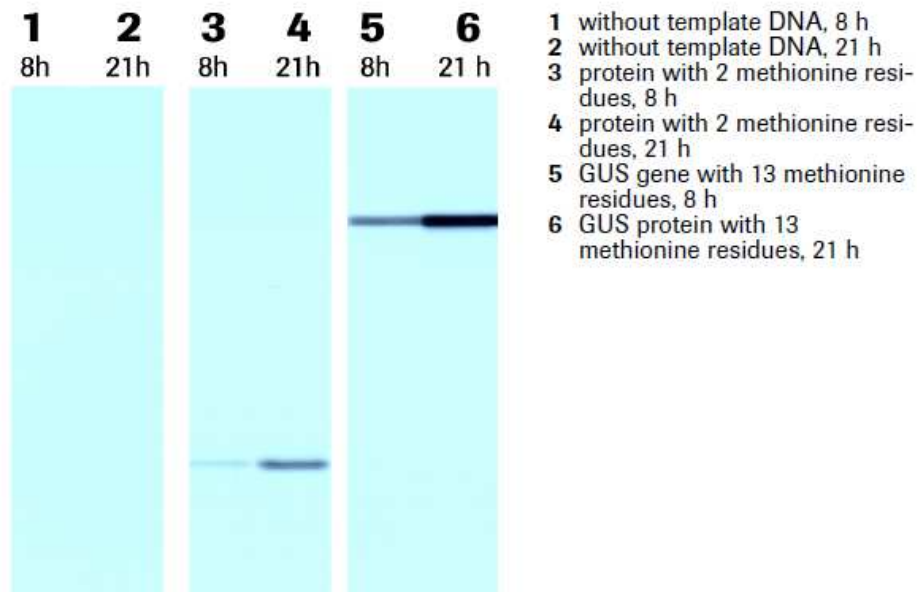


Figure 5. Radioactive labeling reaction.

Radioactive labeling was performed according to procedure on page 18, except the labeled Methionine solution contained only 3 μ l L-[³⁵S]-Methionine. Labeling reactions were run for either 8 or 21 hours. Two different template genes (GUS and another DNA) were used. The GUS control protein had 13 Methionines; the other product contained only 2 Methionines. After labeling, 2 μ l aliquots from the reactions were applied to 10% SDS gels. Labeled products were detected by fluorography (14 hours exposure at -80°C).

References

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Troubleshooting guide

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

General problems

Observation	Control protein is not expressed
Possible cause	Reaction device has not been stored long enough at room temperature (15–25°C)
Resolving	Repeat experiment using correctly stored device.
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 be sure to work RNase-free at every step.

Problems expressing target protein, although GUS control protein works

Observation	Good protein expression, but low yield of active protein
Possible cause	Incorrect folding of the protein due to a requirement for co-factors or disulfide bond formation
Resolving	Add necessary cofactors. Try the RTS 100 <i>E. coli</i> Disulfide Kit for the expression of disulfide bonded proteins (biotechrabbit). Alternatively, after the reaction, oxidize protein to form disulfide bonds [4, 5].
Possible cause	Dependence on other secondary modifications
Avoiding	The RTS Wheat Germ Lysate cannot introduce post-translational modifications such as glycosylation, disulfide bond formation, or signal sequence cleavage.

Observation	Low expression yield
Possible cause	Expression time too short
Resolving	Extend expression time.
Possible cause	Epitope tag interferes with protein folding
Resolving	Use different pIVEX WG vectors to prepare the expression template. Try different epitope tag sequences that are introduced via PCR.
Possible cause	Amount of template DNA not optimal
Resolving	For optimal results, vary the DNA concentration in a 50 μ l reaction between 0.5 μ g and 5 μ g for circular templates, or between 0.2 μ g and 2 μ g for linear templates.

Observation	Several product bands on SDS-PAGE or product smaller than expected
Possible cause	Proteolytic degradation
Resolving	Add protease inhibitors to reaction. For example: Dissolve 1 tablet of Complete Mini, EDTA-free (Roche) in 0.5 ml nuclease-free water and add 2 μ l of this solution to a 50 μ l reaction mix.
Possible cause	Internal initiation site
Resolving	Eliminate the corresponding Methionine by point mutation.
Possible cause	Premature termination of translation
Resolving	Check sequence of target gene for incorrect reading frame or mutation that produces a stop codon. Search for strong secondary structures in the mRNA and eliminate them by using conservative mutations. Increase amount of unlabeled Methionine in radioactive labeling mix, or decrease the reaction time.

Observation	Product in the pellet fraction
Possible cause	Aggregation
Resolving	Add/adjust chaperones [6]. Adjust experimental conditions (time, temperature). Add mild detergents (e.g. up to 0.05% [v/v] Brij 35, Digitonin or Nonidet NP-40 or up to 0.1% Triton® X-100 [v/v] or 0.1% CHAPS for membrane proteins).
Observation	No expression of the target gene, but normal expression of GUS
Possible cause	Cloning error
Resolving	Check the sequence
Possible cause	Low purity of DNA template
Resolving	Ensure the absorbance ratio 260 nm/280 nm is at least 1.7. Perform a phenol extraction if purity is low. Make a new template preparation.
Possible cause	Contamination with RNases
Resolving	Repeat experiment and be sure to work RNase-free at every step.
Possible cause	No initiation of translation due to strong secondary structures of the mRNA
Resolving	Try expressing the protein from a capped mRNA template. Use different pIVEX WG vectors to prepare the expression template. Try to express the protein as an N-terminally tagged protein e.g. in pIVEX 1.4 WG.
Possible cause	Expressed protein interferes with the translation or transcription process
Resolving	Express gene of interest together with GUS. If GUS expression is inhibited, the target protein cannot be expressed with the kit.

Ordering information

Product	Size	Order no.
RTS Linear Template Kit Plus	20 reactions	BR1402401
RTS pIX3.0 Vector	1 vector, 25 µg	BR1402701
RTS 100 <i>E. coli</i> HY Kit	24 reactions	BR1400101
RTS 100 <i>E. coli</i> HY Kit	96 reactions	BR1400102
RTS 500 ProteoMaster <i>E. coli</i> HY Kit	5 reactions	BR1400201
RTS 9000 <i>E. coli</i> HY Kit	1 reaction	BR1400301
RTS 100 <i>E. coli</i> Disulfide Kit	24 reactions	BR1400401
RTS 500 <i>E. coli</i> Disulfide Kit	5 reactions	BR1400501
RTS 100 <i>E. coli</i> Fab Kit	10 reactions	BR1400601
RTS 100 <i>E. coli</i> Fab Kit	96 reactions	BR1400602
RTS pIVEX <i>E. coli</i> 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

