

For life science research only. Not for use in diagnostic procedures. FOR *IN VITRO* USE ONLY.



RTS 500 Wheat Germ CECF Kit

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

Cat. No. 04 492 838 001

For 5 synthesis reactions of 1 ml each

Store this kit at -15 to -25°C or below.

Instruction Manual

Version August 2008

**The Rapid Translation System website:
www.proteinexpression.com**

www.roche-applied-science.com

1. Preface

1.1 Table of Contents

1.	Preface	2
1.1	Table of Contents	2
2.	Introduction	5
2.1	Product overview	5
2.2	Background information	8
3.	Procedures and required materials	9
3.1	Preparation of DNA for <i>in vitro</i> expression	9
3.1.1	Use of expression vectors	9
3.1.2	Generation of expression templates by PCR	10
3.1.3	Generation of expression templates by <i>in vitro</i> transcription	12
3.2	Protein synthesis reaction	12
3.2.1	Before you begin	12
3.2.2	Reconstitution of reaction components	13
3.2.3	Preparation of working solutions	14
3.2.4	Running an experiment (standard reaction)	14
3.2.5	Control reaction with GUS gene	16
4.	Typical results	17
4.1	Standard reaction with pIVEX-WG vector templates	17
5.	Appendix	18
5.1	Troubleshooting	18
5.1.1	General problems	18
5.1.2	Problems in expressing target protein, but GUS control reaction works	18
5.2	References	20
5.3	Notice for the purchaser	20
5.4	Related products	20
6.	Quick reference procedure	22

1. Preface

Kit contents

Each kit contains enough reagents for 5 coupled transcription-translation or translation-only reactions of 1 ml.

Vial /cap	Label	Contents and function
1 red	Wheat Germ Lysate	<ul style="list-style-type: none">lysate from wheat germ, stabilized and lyophilizedcontains components for transcription and translationfive bottles
2 green	Reaction Mix	<ul style="list-style-type: none">substrate mix to prepare reaction solution, stabilized and lyophilizedfive bottles
3 black	Feeding Mix	<ul style="list-style-type: none">feeding mix to prepare 10 ml of feeding solution; stabilized powderfive bottles
4 brown	Amino Acids	<ul style="list-style-type: none">mix of 19 amino acids (all natural except methionine) to prepare reaction solution, stabilized and lyophilizedfive bottles
5 yellow	Methionine	<ul style="list-style-type: none">methionine to prepare reaction solution, stabilized and lyophilizedone bottle contains enough reagent for all reactions
6 white	Reconstitution Buffer	<ul style="list-style-type: none">buffer solution for the reconstitution of bottles 1, 2, 3, 4, and 5contains 41 mltwo bottles
7 colorless	Control Vector Glucuronidase	<ul style="list-style-type: none">60 µg plasmid, lyophilizedGUS (Glucuronidase) expression vector to be used for the control reactionone bottle
8	Reaction Devices	<ul style="list-style-type: none">disposable device for Continuous Exchange Cell-Free (CECF) protein expressionfor use in the RTS ProteoMaster Instrument or Eppendorf Thermomixer comfortfive devices
9	Five spare caps for bottle 3 (Feeding Mix)	<ul style="list-style-type: none">to be used during reconstitution of Feeding Mix (bottle 3)

1. Preface, continued

Safety information

Observe the usual precautions when handling chemicals. After use, reagent can be discarded in wastewater in accordance with local regulations. If reagent gets in your eyes, flush eyes with water. If reagent get on your skin, wash off with water. If you swallow a reagent, seek medical advice.

Laboratory requirements

To avoid contamination with RNases we recommend using RNase-free materials and wearing gloves. Heat-treated glassware is not required.

Additional equipment required

To use this kit for protein expression, you will need the following equipment:

- RTS ProteoMaster Instrument or Eppendorf Thermomixer comfort
- Pipettes 1-10 μ l, 10-200 μ l, and 200-1000 μ l
- Pipette tips autoclaved at 121°C for 20 min
- Eppendorf reaction tubes
- Template DNA

Important notes:

- This kit contains special reaction devices and can therefore **not** be used in the RTS 500 Instrument.
 - It is important that you remove the reaction devices from the kit box after you receive the kit. If you leave the reaction devices in the box until you use them, they may contain residual CO₂ (from the dry ice used to ship the kit). This residual CO₂ will change the pH of your reaction mixes and may inhibit protein synthesis. Store devices at room temperature for at least one day before use.
-

Additional reagents required

- Starting cDNA encoding the protein of interest.
 - We recommend cloning the cDNA into **RTS pIVEX WG** vectors. You will also need PCR reagents and equipment to amplify the cDNA for either cloning or generating a linear expression cassette to be used directly in RTS 500 Wheat Germ reactions. Different vectors and PCR sets are also available for fusing different types of epitope tags to the N- or C-terminus of the gene of interest (*e.g.*, pIVEX WG His₆-tag Vector Set*; please visit www.proteinexpression.com for the most recent information on tag types available).
 - If the reaction is to be performed with an RNA template, we recommend using Cap Scribe kits from Roche Applied Science (*e.g.*, T7 Cap Scribe) to produce capped mRNA. In this case, you will also need a vector that contains the cDNA of interest downstream from a suitable promoter (*e.g.*, T3, T7, SP6).
 - For reconstitution of the Glucuronidase Control Vector use only deionized DNase- and RNase-free water.
-

2. Introduction

2.1 Product overview

Reaction principle

The RTS Wheat Germ CECF (continuous exchange cell-free) system is a preparative, scalable eukaryotic cell-free translation system. A two-chamber reaction device (Fig. 1) allows production of several hundred micrograms protein per ml (600 µg/ml for GUS control protein). Transcription and translation take place simultaneously in the 1 ml reaction compartment of the reaction device. Substrates and energy components needed for a sustained reaction are continuously supplied via a semipermeable membrane. At the same time, potentially inhibitory reaction by-products are diluted since they diffuse through the same membrane into the 10 ml feeding compartment (Fig. 2). In contrast to traditional batch systems, CECF conditions allow protein synthesis to continue for 24 hours. Yields approaching several hundred µg of newly synthesized protein are possible in reactions with PCR-generated linear template; yields of nearly a milligram are possible if vector template or exogenously added capped mRNA template is used.

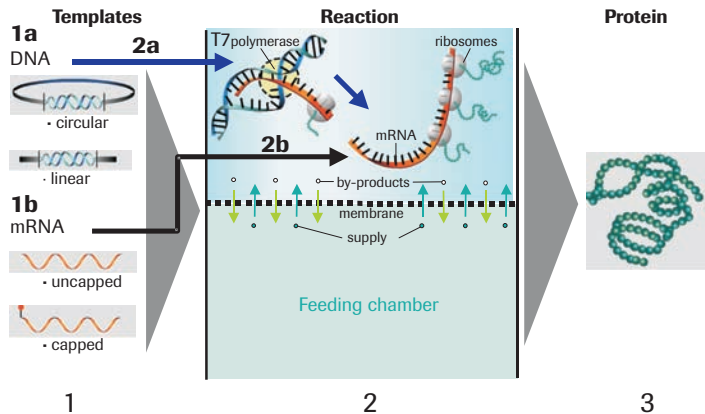
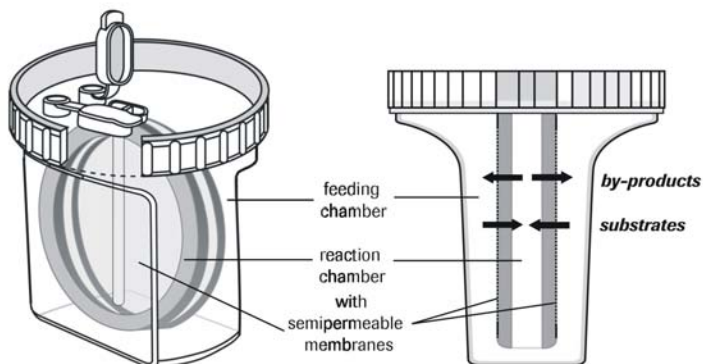


Fig 1: Basic steps

2.1 Product overview, continued

Basic steps shown in figure 1

Step	Description
1a	If DNA is to be used as template in the reaction device, the gene of interest is either cloned into a suitable T7-driven expression vector (see chapter 3.1.1.), or fused to promoter and terminator sequences by PCR (see chapter 3.1.2) to produce a linear template.
1b	If mRNA is to be used as template in the reaction device, it should be pre-synthesized in a separate <i>in vitro</i> transcription reaction and added to the translation reaction with or without further purification (see chapter 3.1.3).
2	In a coupled reaction, cDNA is first transcribed into mRNA. Subsequently, the mRNA is translated into protein. T7 RNA polymerase transcribes the DNA template <i>in vitro</i> (2a). The translational machinery of the wheat germ lysate translates the synthesized mRNA <i>in vitro</i> (2b). Note: Alternatively, pre-synthesized mRNA (see 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.



Substrates

- Amino acids
- Energy substrates
- Nucleotides

By-products

- QGSv#
- QMSv
- SS#
- DNA-fragments
- RNA-fragments

Fig. 2. Schematic of the RTS 500 Wheat Germ CECF Device and illustration of the CECF principle invented by A. Spirin (1988).

2.1 Product overview, continued

Application	<p>The RTS 500 Wheat Germ Kit CECF is designed for</p> <ul style="list-style-type: none">• Expression of (mainly eukaryotic) cDNAs which produce insoluble products or are only very poorly expressed in <i>E. coli</i> lysates• Rapid parallel protein synthesis reactions• Use with either plasmids or PCR-generated linear DNA templates.• Synthesis of truncated protein variants for epitope analysis or functional domain mapping.• Expression from <i>in vitro</i> synthesized RNA.• Synthesis of proteins of widely varying size (up to 220 kD as tested).
RTS Wheat Germ or RTS <i>E. coli</i>?	<p>If you are not sure which system (RTS <i>E. coli</i> or RTS Wheat Germ) is better suited for your application, please consult the RTS system selection guide available at www.proteinexpression.com</p>
Important note: Common limitations of RTS <i>E. coli</i> and RTS Wheat Germ lysates	<p>The RTS <i>E. coli</i> lysate system and the RTS Wheat Germ system (in the absence of further modification) cannot perform the following post-translational modifications on the expressed proteins:</p> <ul style="list-style-type: none">• Glycosylation• Disulfide bond formation• Signal sequence cleavage
Template DNA	<p>We highly recommend that you first evaluate the expression yield of your protein of interest in a small scale expression reaction, using the RTS 100 Wheat Germ CECF Kit. We generally recommend using PCR products as expression templates for this stage because this approach allows constructs to be rapidly generated and screened for yield and solubility. Guidelines for designing PCR primers that will attach T7 transcription/translation regulatory elements to your target gene are provided in the pack insert of the RTS Wheat Germ Linear Template Generation Set, His₆-tag*.</p> <p>For expression scale-up, we recommend cloning your cDNA into a pVEX WG plasmid, which contains an enhancer sequence that has been optimized for wheat germ lysates (see 3.1.1). Such a recombinant plasmid will provide a well-characterized, stable template for use in all subsequent experiments that investigate the same gene.</p> <p>Note: We do not recommend using other vectors that contain a T7 promoter but lack the wheat germ-optimized expression enhancer sequences present in pVEX WG vectors; use of such vectors can lead to a greatly reduced synthesis rate.</p>
Reaction time	<p>Up to 24 hours</p>
Number of reactions	<p>The kit provides reagents and reaction devices for 5 × 1 ml reactions.</p>
Stability	<p>This product is stable at –15 to –25°C for 18 months (until the expiration date printed on the label).</p>
Advantages	<p>Please see www.proteinexpression.com for details on the advantages of the RTS 500 Wheat Germ CECF Kit.</p>

2.2 Background information

Coupled *in vitro* transcription/translation

Similar to other *in vitro* expression systems, the RTS 500 Wheat Germ system allows expression of exogenously added mRNA.

However, it is more convenient to produce 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. While the T7 RNA polymerase transcribes the gene of interest (encoded by PCR-generated template or vector), the ribosomes in the Wheat Germ lysate start to translate mRNA.

Unlike *E. coli* lysates, eukaryotic lysates like 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 cannot achieve preparative yields (several μ g per 50 μ l) in the same reaction (e.g., in batch mode).

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 reactions with wheat germ lysates give much lower yields and are recommended only for labeling expressed protein with radiolabeled amino acid precursors.

3. Procedures and required materials

3.1 Preparation of DNA for *in vitro* expression

Literature

Basic molecular biology methods are described in the following books:

- Sambrook *et al* (2001) "Molecular Cloning – A Laboratory Manual," Third Edition, Cold Spring Harbor Laboratory Press, New York
 - Ausubel, U. K. *et al* (1993) "Current Protocols In Molecular Biology," John Wiley & Sons Inc., New York
-

3.1.1 Use of expression vectors

Required vector elements

- Any vector or linear DNA used with this kit must contain the target gene under control of the T7 promoter.
 - Optional, but strongly recommended for enhancing expression: The template should contain wheat-germ optimized 5' - and 3' regulatory untranslated regions (for details see product information for RTS pIVEX WG, His₆-tag Vector Set*.
-

General recommendations

The pIVEX-WG vector family has been developed and optimized for use in the RTS Wheat Germ system. Some of the available pIVEX vectors are shown schematically in Fig. 3. Sequences and maps of these vectors can be downloaded from the RTS special interest website (http://www.roche-applied-science.com/sis/proteinexpression/products/rts_lineartemplates_vectors.htm#expression)

Cloning procedure

Protocols for cloning your cDNA into pIVEX WG vectors are provided in the vector pack inserts. These are based on either standard restriction digestions, or, for the processing of multiple samples in parallel, In-Fusion^{TM 1)} Cloning, a technology available from BD Biosciences Clontech Inc.

Purity of the plasmid preparation

Plasmids obtained from commercially available DNA preparation kits (*e.g.* Genopure Plasmid Midi* and Maxi Kits* are usually pure enough to be used as template in the Rapid Translation System. If DNA is not pure enough ($OD_{260/280} \leq 1.7$), use phenol extraction to increase purity and to remove traces of RNase, which may enhance template performance in the expression reaction.

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

3.1.1 Use of expression vectors, continued

Recommended expression vectors

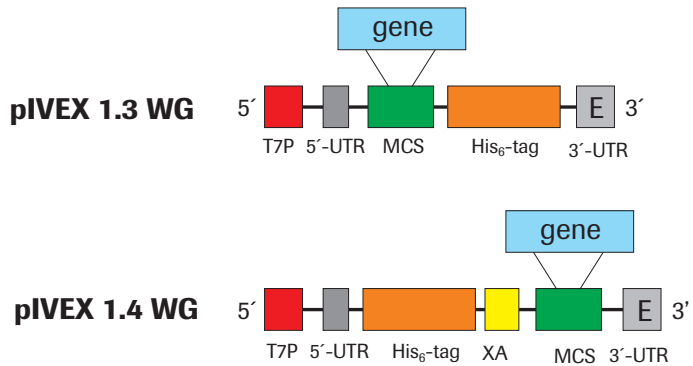


Fig. 3: Functional elements of RTS Wheat Germ expression vectors

Abbreviations

T7 P	=	T7 promoter
UTR	=	Untranslated regions (UTRs) containing optimized translation enhancer (E) elements (for details see pack insert for each vector)
His ₆ -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

3.1.2 Generation of expression templates by PCR

Principle applications

For rapid production of expression templates from wild-type or modified DNA sequences, we recommend using PCR instead of subcloning the sequences into vectors. PCR methods permit you to *e.g.*,

- add the T7 promoter and both the 5' - and 3' - translation enhancer elements to the cDNA of interest
- add epitope tag sequences to allow detection with antibodies
- introduce mutations
- change codon usage
- make truncated proteins

Generation of a linear DNA-expression template by a 2-step PCR protocol

To incorporate the 5' - and 3' - translation enhancers and T7 RNA polymerase regulatory regions into a template for subsequent *in vitro* transcription/translation reactions, we recommend using a two-step overlap-extension PCR protocol like the one described below. This protocol uses the **RTS Wheat Germ Linear Template Generation Sets**, which include a DNA that contains these regulatory regions as well as epitope tag sequences.

3.1.2 Generation of expression templates by PCR, continued

Step	Action
1	<p>Select the appropriate RTS Wheat Germ Linear Template Generation Set. Each allows a different N- or C-terminal epitope tag, as well as protein fusions to be added to the gene of interest. For the most recent information on these sets, visit the RTS special interest website (www.proteinexpression.com).</p> <p>Design a sense primer that contains (1) a 15 – 20 nucleotide sequence homologous to the cDNA of interest and (2) an additional 20 base sequence that overlaps the chosen tag region (as indicated in the package insert for the applicable RTS Wheat Germ Linear Template Generation Set.)</p> <p>Design an antisense primer that contains (1) a 15 – 20 nucleotide sequence homologous to the gene of interest and (2) an additional 20 base sequence that overlaps the chosen tag region (as indicated in the package insert for the applicable RTS Wheat Germ Linear Template Generation Set.)</p>
2	<p>Perform the first PCR reaction in a 50 µl reaction volume, using the primers you designed and template DNA that contains the gene of interest.</p> <p>Note: The detailed protocol is given in the package insert for the applicable RTS Wheat Germ Linear Template Generation Set.</p>
3	<p>Perform a second PCR reaction in a 500 µl reaction volume, using ~200-1000 ng of the first PCR reaction as template, together with primers and DNA containing enhancer elements for translation in wheat germ and C- or N-terminal epitope tags. (This DNA is supplied with the RTS Wheat Germ Linear Template Generation Set).</p>
4	<p>Purify the PCR product using Roche High Pure PCR Product Purification Kit, determine the concentration of the PCR product on an agarose gel and compare to known amounts of DNA molecular size markers.</p> <p>You may use unpurified PCR products but these may give lower expression rates.</p>
5	<p>Use 20–40 µg of the PCR product from the second PCR for a 1 ml <i>in vitro</i> protein synthesis reaction.</p>
<p>Important note: Do not use agarose gels to purify the PCR products to be used in the expression reaction, since this treatment may inhibit <i>in vitro</i> protein synthesis.</p>	

3.1.3 Generation of expression templates by *in vitro* transcription

Principle

mRNA may be pre-synthesized *in vitro*, e.g from a T7-driven DNA template, and then added as translation templates to the reaction devices of the **RTS 500 Wheat Germ CECF Kit**. Template mRNA may be either capped or non-capped, but capped forms often perform more efficiently in the translation steps.

Generation of a capped mRNA by *in vitro* transcription

For *in vitro* transcription an 80 μ l portion of capped mRNA is usually sufficient for one RTS 500 Wheat Germ translation (1 ml) reaction. This 80 μ l portion can be diluted to a volume of 300 μ l with RNase-free water and then added directly to the translation reaction.

Note: For production of non-capped mRNA we recommend using the **Transcription Kit SP6/T7***. The resulting mRNA should be precipitated with ethanol and resuspended before it is used in translation reactions.

3.2 Protein synthesis reaction

3.2.1 Before you begin

Kit reagents

- Do not combine reagents from different kit lots.
- Reconstitute the lyophilized reagents or thaw the solutions immediately before using them.
- Reconstitute only the bottles needed for the current experiment.
- Reconstitution Buffer may be thawed in a 25°C water bath.
- For reconstitution of bottles 1 to 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 you use them.
- Store the reconstituted solutions from bottles 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.

DNA template

Prepare and purify DNA template as detailed in section 3.1.

Equipment

Important note: Remove reaction devices from the kit box and store them at room temperature at least one day before use.

- Set the RTS Proteomaster Instrument to 24°C and a shaking speed of 900 rpm for incubation of this reaction.
- Use only calibrated pipettes.
- Use RNase-free plastic and glassware.
- To check the control reaction with glucuronidase, apply the reaction products to an SDS-polyacrylamide gel. A band with an apparent molecular weight of 68 kDa should be visible after the gel is stained with Coomassie Brilliant Blue.

3.2.2 Reconstitution of reaction components

Solution	Bottle no. / cap	Reconstitution procedure	For use in
1	Wheat Germ Lysate Bottle 1, red	Reconstitute the lyophilizate with 0.35 ml of Reconstitution Buffer (bottle 6), then mix carefully by rolling or gently shaking. DO NOT VORTEX!	<ul style="list-style-type: none"> • section 3.2.3 • solution 9
2	Reaction Mix Bottle 2, green	Reconstitute the lyophilizate with 0.35 ml of Reconstitution Buffer (bottle 6), then mix carefully by rolling or gently shaking.	<ul style="list-style-type: none"> • section 3.2.3 • solution 9
3	Feeding Mix Bottle 3, black	Reconstitute the lyophilizate with 9.55 ml of Reconstitution Buffer (bottle 6), then mix by rolling or gently shaking. Important: Before mixing, replace the original black lid (contains drying reagent!) with one of the spare black lids supplied in the kit (Item 9).	<ul style="list-style-type: none"> • section 3.2.3 • solution 8
4	Amino Acids Bottle 4, brown	Reconstitute the lyophilizate with 3.0 ml of Reconstitution Buffer (bottle 6), then mix by rolling or gently shaking.	<ul style="list-style-type: none"> • section 3.2.3 • solution 8 and 9
5	Methionine Bottle 5, yellow	Reconstitute the lyophilizate with 1.8 ml of Reconstitution Buffer (bottle 6), then mix by rolling or gently shaking.	<ul style="list-style-type: none"> • section 3.2.3 • solution 8 and 9
6	Reconstitution Buffer Bottle 6, white	<ul style="list-style-type: none"> • 41 ml × 2 bottles • Ready-to-use solution • The solution is stable at 4°C but can also be stored at –20°C 	solution 1, 2, 3, 4, 5
7	Control vector GUS Bottle 7, colorless	<ul style="list-style-type: none"> • Briefly pellet the contents of the bottle by centrifugation. • Reconstitute the lyophilizate with 50 µl of sterile DNase- and RNase-free water. • The solution is stable at –20°C 	Section 3.2.5

Appearance of solutions

With the exception of the Wheat Germ lysate, all reconstituted lyophilizates should be clear solutions. The Wheat Germ lysate remains cloudy.

3.2.3 Preparation of working solutions

Solution	Contents	Preparation of working solution for a 1 ml reaction	For use in
8	Feeding Solution	Mix the following components directly in bottle 3: <ul style="list-style-type: none">• 9.555 ml Feeding Mix• 400 µl Amino Acids• 45 µl Methionine	section 3.2.4
9	Reaction Solution	Mix the following components carefully by rolling or gentle shaking: DO NOT VORTEX! <ul style="list-style-type: none">• 300 µl Wheat Germ Lysate• 300 µl Reaction Mix• 40 µl Amino Acids• 4.5 µl Methionine• 55.5 µl Reconstitution Buffer• 300 µl of sterile DNase- and RNase-free water containing<ul style="list-style-type: none">• EITHER 60 µg of the circular DNA template• OR 20–40 µg of linear PCR generated template• OR 40–160 µg of mRNA Note: For multiple parallel reactions, we recommend premixing components 1–5, then adding the appropriate DNA to the premix.	section 3.2.4

3.2.4 Running an experiment (standard reaction)

Standard procedure

1. Filling the reaction compartment

The reaction compartment must be filled first, as follows:

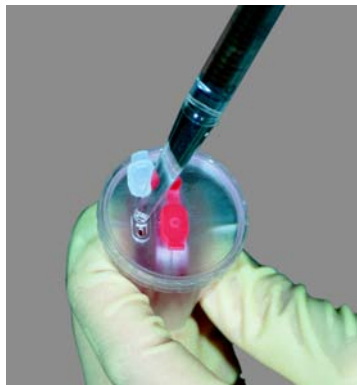
- Open the red lid of the 1 ml reaction compartment.
- Using a 1 ml pipette, pipette 1 ml of Reaction Solution (solution 9) through the **circular** opening. Let displaced air escape the chamber through the oval opening. **DO NOT USE THE OVAL OPENING TO FILL THE CHAMBER.** It is not necessary to remove air bubbles from the reaction compartment.
- Close the lid of the compartment securely.



3.2.4 Running an experiment, continued

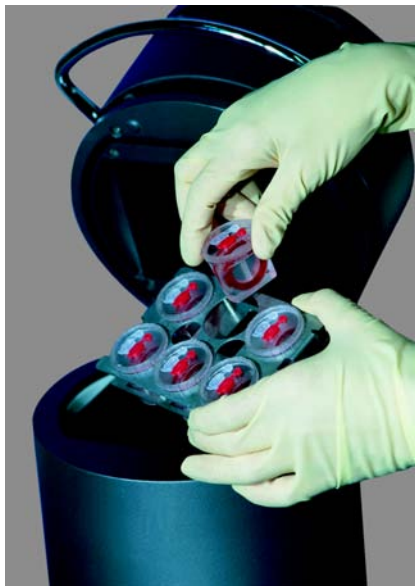
2. Filling the feeding compartment

- Open the colorless lid of the feeding compartment.
- Using a 10 ml glass pipette, pipette 10 ml Feeding Solution (solution 8) through the **circular** opening. Let displaced air escape through the **OVAL** opening. It is not necessary to remove air bubbles from the compartment.
- Close the lid of the compartment securely.



3. Starting the run

- Insert the filled reaction device into the RTS ProteoMaster Instrument, using the appropriate steel insert (provided with the instrument).
- Follow the instructions in the instrument manual to set the run parameters to:
 - shaking speed: 900 rpm
 - temperature: 24°C
 - reaction time: 24 h (optional).
- Start the run.
Note: For more information about setting the parameters, see **points to consider** below.



End of run

- After the run (up to 24 hours) remove the reactions from the incubator.
 - Store reaction solutions either frozen or at 0°C to 4°C until purification or further processing.
-

3.2.4 Running an experiment, continued

Points to consider

Parameter	Guideline
Temperature	Optimal temperature for most proteins is 24°C. However, lower temperatures may be used for proteins that tend to aggregate.
Time	Protein synthesis continues for up to 24 hours.

3.2.5 Control reaction with GUS gene

Reaction procedure

Step	Action
1	Reconstitute bottles 1–5 and bottle 7 according to section 3.2.2.
2	Prepare Reaction and Feeding Solutions according to sections 3.2.2 and 3.2.3. Add 60 µg (in 300 µl in water) of reconstituted Control Vector GUS (bottle 7) to the Reaction Solution. Set up reactions as described in Section 3.2.4. Note: Include a negative control reaction in the run. Use 300 µl water in place of vector in this control.
3	Start the reaction and incubate for 24 h at 24°C, while shaking the reaction chambers at 900 rpm.
4	Apply 0.5 or 1 µl of each reaction to an SDS-polyacrylamide gel.
5	Run the gel, then stain with Coomassie Blue. Result: A band with an apparent 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-His-tag antibody.

4. Typical results

4.1 Standard reaction with pIVEX-WG vector templates

Expression of proteins from pIVEX vector templates

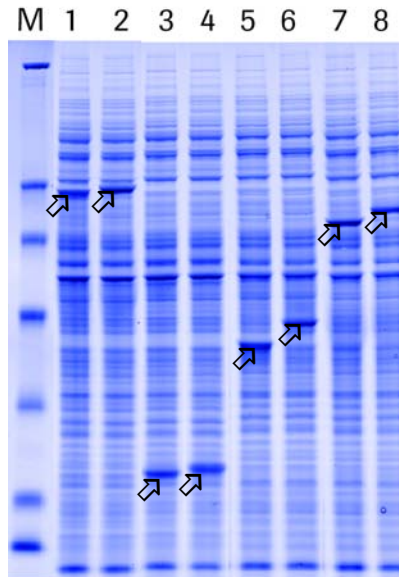


Fig. 4. Different open reading frames were cloned into pIVEX 1.3 WG (C-terminal tag) and pIVEX 1.4 WG (N-terminal tag) vectors. Resulting vectors were expressed with the RTS 500 Wheat Germ CECF Kit. After the run, 0.5 μ l of each expression reaction was analyzed by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. M: Marker proteins. 1, 2: GUS. 3, 4: survivin. 5,6: human kinase. 7,8: receptor protein. Lanes 1, 3, 5, 7: C-terminal tag; lanes 2, 4, 6, 8: N-terminal tag. Bands of the expected size are marked with an arrow (↗).

5. Appendix

5.1 Troubleshooting

5.1.1 General problems

Problem	Possible Cause	Recommendation
Control protein is not expressed	Kit expired	Order a new kit.
	Kit has not been stored at -15 to -25°C	Order a new kit.
	Contamination with RNases	Repeat experiment and be sure to exclude RNases at each step.
	A kit component may be bad	Contact Roche Applied Science.

5.1.2 Problems in expressing target protein, but GUS control reaction works

Problem	Possible Cause	Recommendation
Good protein expression, but low yield of active protein	Incorrect folding of the protein due to: <ul style="list-style-type: none">• Requirement for cofactors• Requirement for disulfide bond formation.• Dependence on secondary modifications	<ul style="list-style-type: none">• Add necessary cofactors.• After the reaction, oxidize product to form disulfide bonds [see e.g. Ahmed et al (1975), Odorzinsky & Light (1979)].• No solution. The RTS Wheat Germ lysate can not introduce post-translational modifications such as glycosylation, disulfide bond formation or signal sequence cleavage.
Product in the pellet fraction	Aggregation	<ul style="list-style-type: none">• Add/adjust chaperones (cf. Rudolph <i>et al.</i>)• Adjust experimental conditions (time, temperature).• Add mild detergents (e.g. up to 0.05 % Nonidet NP-40 [v/v] for membrane proteins).
Low expression yield	Expression time too short	Extend expression time.
	The epitope tag interferes with the folding of the protein.	<ul style="list-style-type: none">• Try different pIVEX WG vectors.• Try/compare different epitope tag sequences that are introduced via PCR.
	Amount of template DNA not optimal	To get optimal results, vary the DNA concentration in a 1 ml reaction between $40\ \mu\text{g}$ and $100\ \mu\text{g}$ for circular templates, or between $10\ \mu\text{g}$ and $30\ \mu\text{g}$ for linear templates.

continued on next page

5.1 Troubleshooting, continued

Problem	Possible Cause	Recommendation
Several product bands on SDS-PAGE or product smaller than expected	Proteolytic degradation	Add protease inhibitors to the reaction. <i>Example:</i> Dissolve 1 tablet of Complete mini EDTA-free* in 0.5 ml nuclease-free water and add 40 μ l to a 1 ml reaction.
	Internal initiation site	Eliminate the corresponding methionine by point mutation.
	Premature termination of the translation	<ul style="list-style-type: none"> • Check the sequence of the target gene for incorrect reading frame or a mutation that produces a stop codon. • Search for strong secondary structures in the mRNA and eliminate them by using conservative mutations. • Increase the amount of unlabeled methionine during radioactive labeling, or decrease the reaction time.
No expression of the target gene, but normal expression of GUS	Cloning error	Check the sequence.
	Impure template DNA	<ul style="list-style-type: none"> • Make sure that 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.
	Contamination with RNases	Repeat experiment and be careful to exclude RNases at each step.
	No initiation of translation due to strong secondary structures in the mRNA	<ul style="list-style-type: none"> • Try expressing the protein from a capped mRNA template (prepared with the T7 Cap Scribe kit) • Try using different pVEX-WG vectors to prepare the expression template. • Try to express the protein as an N-terminally tagged fusion protein <i>e.g.</i> in pVEX-WG 1.4.
	Expressed protein interferes with the translation or transcription process.	Express the gene of interest together with GUS. If GUS expression is inhibited, the active protein cannot be expressed with the kit.

5.2 References

- 1 Spirin, A. S. *et al.* (1988) "A continuous cell-free translation system capable of producing polypeptides in high yield" *Science* **242**, 1162.
- 2 Sambrook *et al.* (2001) "Molecular Cloning – A Laboratory Manual," Third Edition, Cold Spring Harbor Laboratory Press, New York.
- 3 Ausubel, U. K. *et al.* (1993) "Current Protocols In Molecular Biology," John Wiley & Sons Inc., New York
- 4 Ahmed, A. K. *et al.* (1975), *J. Biol. Chem.* **250**, 8477.
- 5 Odorzinsky, T. W. & Light, A. (1979), *J. Biol. Chem.* **254**, 4291.
- 6 Rudolph, R. *et al.* (1997) in "Protein Function – A Practical Approach," Creighton, T. E. ed., Oxford University Press Inc., New York, pp 57 - 99.

5.3 Notice for the purchaser

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 Roche Diagnostics.

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 Roche Group.

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 exclusively licensed by a member of the Roche Group from the Institute of Protein Research at the Russian Academy of Sciences, Pushchino, Russia. The purchase price of this product includes a limited, non-exclusive, non-transferable license under U.S. Patent 5,478,730 or its foreign counterparts, to use only this amount of the product to practice 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 solely for the internal research and development activities of the purchaser.

5.4 Related products

Product	Pack Size	Cat. No.
RTS Wheat Germ products		
RTS 100 Wheat Germ CECF Kit ^{2,3,4}	24× 50 µl reactions	03 728 811 001
Linear Template Generation by PCR		
RTS Wheat Germ Linear Template Generation Set, His ₆ -tag	96 reactions	03 728 790 001
Vectors		
RTS pIVEX Wheat Germ His ₆ -tag Vector Set	2 vectors, 10 µg each	03 728 803 001

Product	Pack Size	Cat. No.
RTS <i>E. coli</i> products		
RTS pIVEX His ₆ -tag 2 nd Generation Vector Set	2 vectors, 10 µg each	03 269 019 001
RTS 100 <i>E. coli</i> HY Kit	24 reactions 96 reactions	03 186 148 001 03 186 156 001
RTS 500 ProteoMaster <i>E. coli</i> HY Kit ¹	5 reactions	03 335 461 001
RTS AviTag <i>E. coli</i> Biotinylation Kit, Plasmid	For 96 reactions (RTS 100) or 5 reactions (RTS 500)	03 514 919 001
Other Reagents		
Expand™ High Fidelity PCR-System	10× 250 units	11 759 078 001
High Pure PCR Product Purification Kit	1 kit	11 732 668 001
Agarose MP	500 g	11 388 991 001
Anti-His ₆ -Peroxidase	50 U	11 965 085 001
Genopure Plasmid Midi Kit	1 kit (20 preparations)	03 143 414 001
Genopure Plasmid Maxi Kit	1 kit (10 preparations)	03 143 422 001
Transcription Kit SP6/T7	1 kit (2× 20 reactions)	10 999 644 001
Complete Mini, EDTA-free	25 tablets	11 836 170 001

¹ For use with the RTS ProteoMaster Instrument only

- ④ The template optimization service, previously offered by Roche Applied Science with its ProteoExpert software tool, is now available from Biomax Informatics AG. For more information please go to www.biomax.com.
- ④ Alternatively to the RTS ProteoMaster Instrument from Roche Applied Science all RTS kits can be incubated on the Eppendorf Thermomixer comfort (Thermomixer R in the U. S. A.). Please refer to our RTS Application Note 15 on the RTS website, www.proteinexpression.com.

GENOPURE, RTS, PIVEX, PROTEOMASTER, EXPAND, and HIGH PURE are trademarks of Roche.

AviTag is a trademark of Avidity LLC.

¹) In-Fusion is a trademark of BD Biosciences Clontech, Palo Alto, CA.

Changes to Previous Version

Editorial changes

6. Quick reference procedure

Reconstitution of reaction components and starting the run

Important Note: Remove reaction devices from the kit box after receiving and store them at room temperature at least one day before use.

Step	Action																		
1	Reconstitute the following reagents: <table border="1"> <thead> <tr> <th>Reagent</th> <th>Reconstitution</th> </tr> </thead> <tbody> <tr> <td>A Wheat Germ Lysate Bottle 1, red</td> <td>Reconstitute with 0.35 ml of Reconstitution Buffer Do not vortex!</td> </tr> <tr> <td>B Reaction Mix Bottle 2, green</td> <td>Reconstitute with 0.35 ml of Reconstitution Buffer.</td> </tr> <tr> <td>C Feeding Mix Bottle 3, black</td> <td>Reconstitute with 9.55 ml of Reconstitution Buffer. Replace the original lid with the spare black lid supplied in the kit.</td> </tr> <tr> <td>D Amino Acids Bottle 4, brown</td> <td>Reconstitute with 3.0 ml of Reconstitution Buffer.</td> </tr> <tr> <td>E Methionine Bottle 5, yellow</td> <td>Reconstitute with 1.8 ml of Reconstitution Buffer.</td> </tr> <tr> <td>F Control vector GUS Bottle 7, colorless</td> <td>Briefly pellet the contents of the bottle by centrifugation. <ul style="list-style-type: none"> Reconstitute the lyophilizate with 60 µl of sterile, DNase- and RNase-free water. </td> </tr> <tr> <td>G Reaction Solution</td> <td>Mix the following components: <ul style="list-style-type: none"> 300 µl Wheat Germ Lysate 300 µl Reaction Mix 40 µl Amino Acids 4.5 µl Methionine 55.5 µl Reconstitution Buffer 300 µl water containing <ul style="list-style-type: none"> <i>EITHER</i> 60 µg of the circular DNA template <i>OR</i> 20-40 µg of linear template in 300 µl water </td> </tr> <tr> <td>H Feeding Solution</td> <td>Mix the following components directly in a feeding buffer bottle: <ul style="list-style-type: none"> 9.555 ml Feeding Mix 400 µl Amino Acids 45 µl Methionine </td> </tr> </tbody> </table>	Reagent	Reconstitution	A Wheat Germ Lysate Bottle 1, red	Reconstitute with 0.35 ml of Reconstitution Buffer Do not vortex!	B Reaction Mix Bottle 2, green	Reconstitute with 0.35 ml of Reconstitution Buffer.	C Feeding Mix Bottle 3, black	Reconstitute with 9.55 ml of Reconstitution Buffer. Replace the original lid with the spare black lid supplied in the kit.	D Amino Acids Bottle 4, brown	Reconstitute with 3.0 ml of Reconstitution Buffer.	E Methionine Bottle 5, yellow	Reconstitute with 1.8 ml of Reconstitution Buffer.	F Control vector GUS Bottle 7, colorless	Briefly pellet the contents of the bottle by centrifugation. <ul style="list-style-type: none"> Reconstitute the lyophilizate with 60 µl of sterile, DNase- and RNase-free water. 	G Reaction Solution	Mix the following components: <ul style="list-style-type: none"> 300 µl Wheat Germ Lysate 300 µl Reaction Mix 40 µl Amino Acids 4.5 µl Methionine 55.5 µl Reconstitution Buffer 300 µl water containing <ul style="list-style-type: none"> <i>EITHER</i> 60 µg of the circular DNA template <i>OR</i> 20-40 µg of linear template in 300 µl water 	H Feeding Solution	Mix the following components directly in a feeding buffer bottle: <ul style="list-style-type: none"> 9.555 ml Feeding Mix 400 µl Amino Acids 45 µl Methionine
Reagent	Reconstitution																		
A Wheat Germ Lysate Bottle 1, red	Reconstitute with 0.35 ml of Reconstitution Buffer Do not vortex!																		
B Reaction Mix Bottle 2, green	Reconstitute with 0.35 ml of Reconstitution Buffer.																		
C Feeding Mix Bottle 3, black	Reconstitute with 9.55 ml of Reconstitution Buffer. Replace the original lid with the spare black lid supplied in the kit.																		
D Amino Acids Bottle 4, brown	Reconstitute with 3.0 ml of Reconstitution Buffer.																		
E Methionine Bottle 5, yellow	Reconstitute with 1.8 ml of Reconstitution Buffer.																		
F Control vector GUS Bottle 7, colorless	Briefly pellet the contents of the bottle by centrifugation. <ul style="list-style-type: none"> Reconstitute the lyophilizate with 60 µl of sterile, DNase- and RNase-free water. 																		
G Reaction Solution	Mix the following components: <ul style="list-style-type: none"> 300 µl Wheat Germ Lysate 300 µl Reaction Mix 40 µl Amino Acids 4.5 µl Methionine 55.5 µl Reconstitution Buffer 300 µl water containing <ul style="list-style-type: none"> <i>EITHER</i> 60 µg of the circular DNA template <i>OR</i> 20-40 µg of linear template in 300 µl water 																		
H Feeding Solution	Mix the following components directly in a feeding buffer bottle: <ul style="list-style-type: none"> 9.555 ml Feeding Mix 400 µl Amino Acids 45 µl Methionine 																		
2	Start the reaction and incubate for 24 hours at 24°C, shaking the reactions at 900 rpm.																		

Contact and Support

If you have questions or experience problems with this or any Roche Applied Science (RAS) product, please contact our Technical Support staff. Our scientists commit themselves to providing rapid and effective help.

We also want you to contact us if you have suggestions for enhancing RAS product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to RAS and the world-wide research community.

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site at:**

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed.

On the Roche Applied Science home page select **Printed Materials** to find:

- in-depth Technical Manuals
- Lab FAQs: Protocols and references for life science research
- our quarterly Biochemica Newsletter
- Material Safety Data Sheets
- Pack Inserts and Product Instructions

or to request hard copies of printed materials.

