

New Tools for Cell-Free Protein Production of Eukaryotic Proteins: New RTS 100 and 500 Wheat Germ CECF Kits

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Introduction

For the expression of eukaryotic proteins, cell-based and cell-free *E. coli* systems are often not suitable. Proteins that are physiologically tolerated by a living cell cannot be synthesized. In addition, these systems produce misfolded polypeptide aggregates in many cases. While these issues can be addressed in the Rapid Translation System (RTS) *E. coli* to a certain extent by optimization of the expression template sequence via the ProteoExpert Software and the use of chaperons and detergents, the required effort is often a bottleneck, especially for high-throughput applications. With the new RTS 100 and RTS 500 Wheat Germ CECF Kit, the expression of soluble proteins, particularly eukaryotic targets, is highly successful and overcomes the limitations that often restrict the use of *E. coli*-based systems. The RTS Wheat Germ CECF Kits combine several new technologies for efficient and optimized protein expression: the Continuous Exchange Cell-Free (CECF) principle [1], a combined transcription-translation reaction, and highly efficient eukaryotic wheat germ extract.

The major advantage of the system is that PCR-generated linear templates can be conveniently used with the RTS 100 Wheat Germ CECF Kit. In combination with the newly developed CECF device (Figure 1a), the use of robotic systems for the processing of many genes under high-throughput conditions is possible. Depending on the type of protein, several hundred µg/ml can be expressed from PCR-generated linear templates in one reaction within 24 hours. Using the RTS Wheat Germ Vectors, up to 1 mg protein per milliliter synthesis reaction can be produced in the RTS 500 Wheat Germ CECF Kit.

Materials and Methods

Preparation of linear template DNA

To amplify linear templates for expression in RTS Wheat Germ CECF Kits, linear expression units were prepared by a two-step PCR, using

- the Expand High Fidelity PCR System (with gene-specific primers designed as described in the pack insert) for the first step, and
- the RTS Wheat Germ Linear Template Generation Set (LTGS), His₆-tag for the second step.

Primers for the second PCR and DNA fragments for the introduction of upstream and downstream regulatory elements necessary for eukaryotic translation are provided with the Linear Template Generation Set.

Twenty cycles were performed as follows: 1 minute 94°C, 1 minute 50°C, 1 minute/kb 72°C. The concentration of the PCR product was determined using agarose gel electrophoresis. Resulting PCR expression templates contained all upstream and downstream regulatory elements that are necessary for efficient expression in wheat germ lysate. They were routinely used for the transcription/translation reaction without further purification.

Construction of expression vectors

All genes were cloned in pIVEX 1.3 WG (C-terminal His₆-tag) and/or pIVEX 1.4 WG (N-terminal His₆-tag) expression vectors that included all necessary regulatory elements for template expression. Amplified templates were cloned, either using In-Fusion technology (BD Bioscience Clontech) or by simple recloning into the *Nco*I/*Sma*I, *Nco*I/*Xho*I or *Nde*I/*Xho*I restriction sites from pIVEX 2.3 or pIVEX 2.4 *E. coli* expression vectors. pIVEX 1.3 WG and pIVEX 2.3, and pIVEX 1.4 WG and pIVEX 2.4 have identical multiple cloning sites (www.proteinexpression.com).

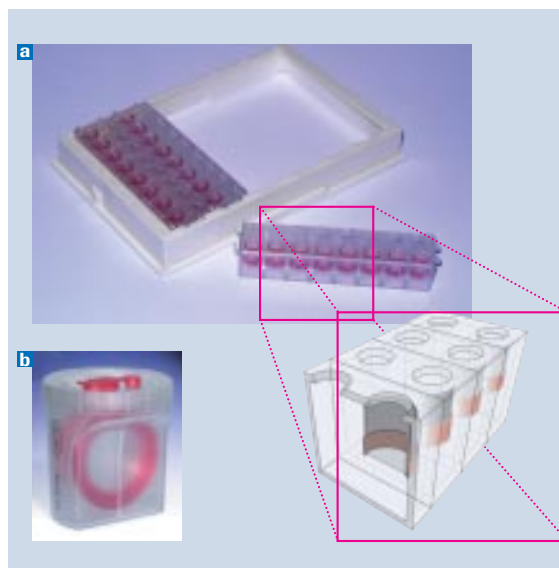


Figure 1:
CECF Devices.
(a) CECF device of the RTS 100 Wheat Germ CECF Kit.
(b) CECF device of the RTS 500 Wheat Germ CECF Kit.

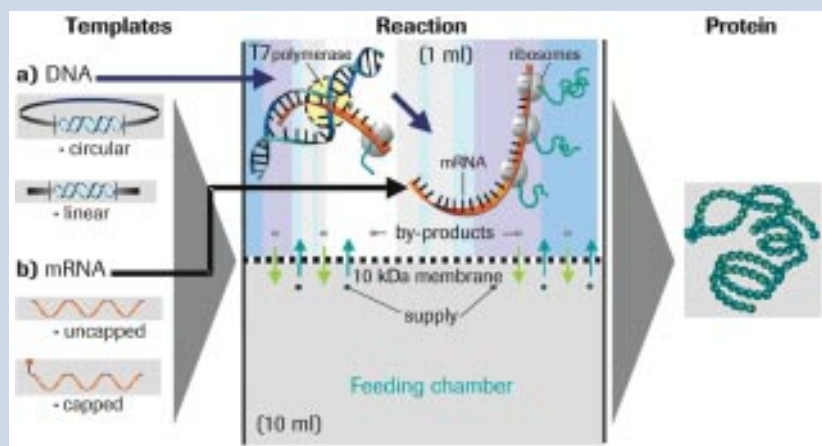


Figure 2: The continuous exchange cell-free (CECF) reaction principle. (a) Possible templates for RTS *E. coli* and RTS Wheat Germ (b) Templates only suitable for the RTS Wheat Germ System.

***In vitro* protein synthesis and affinity purification of reaction products**

All expression vectors were expressed in RTS 100 or RTS 500 Wheat Germ CECF Kits according to the pack insert. The reactions were incubated for 24 hours at 24°C in the RTS ProteoMaster Instrument. The reaction mix included 40 µg/ml plasmid or 20 µg/ml PCR-generated expression units. For the RTS 500 Wheat Germ reaction format, 60 µg/ml vector was used. Routinely, 0.5 µl of the expression reaction products was analyzed on SDS polyacrylamide gels and either stained with Coomassie Brilliant Blue (CBB) or transferred to nitrocellulose membranes. His₆-tagged proteins were subsequently detected with anti-His₆ peroxidase-conjugated antibody and the signals were visualized using LumiLight^{PLUS} Western Blotting

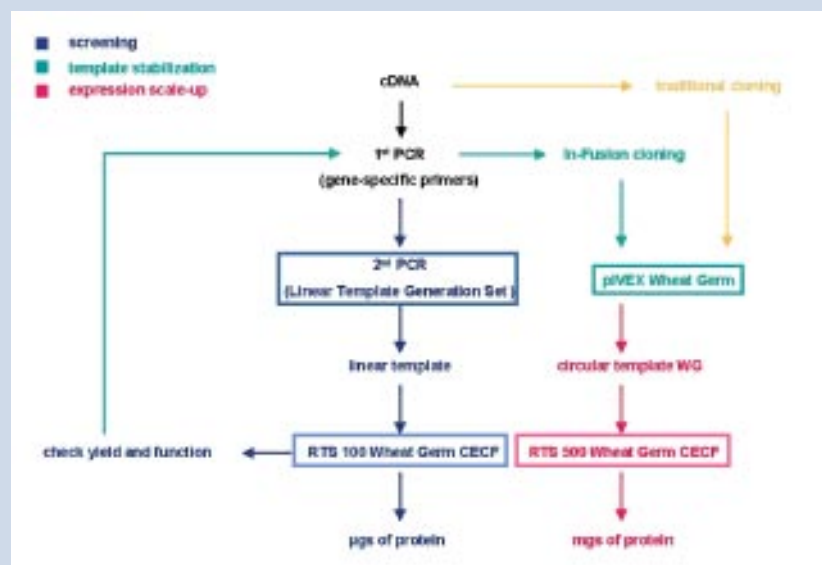


Figure 3: RTS Wheat Germ Workflow.

Substrate and the Lumilmager Software. The amount of protein after CBB staining was estimated by a comparison to known standards applied on the same gel.

Affinity purification of His₆-tagged proteins was performed using an affinity resin (Qiagen). All purification steps were performed according to the manufacturer's instructions in 1-ml Mobicols columns (MoBiTec GmbH).

Using the RTS Wheat Germ Kits

The RTS Wheat Germ CECF is a scalable eukaryotic cell-free translation system. It comprises a two-chamber reaction format in a special CECF device (Figure 1). Transcription and translation take place simultaneously in the reaction compartments of the reaction device. Substrates and energy components are continuously supplied via a semipermeable membrane (Figure 2). At the same time, potentially inhibitory by-products are diluted via diffusion through the same membrane into the feeding compartment.

In contrast to traditional batch systems, protein synthesis under CECF conditions continues for 24 hours, leading to substantially higher yields compared with methods that do not employ the CECF technology. Samples are incubated in the ProteoMaster Instrument.

The RTS 100 Wheat Germ CECF Kit contains sufficient reagents to perform 24 50-µl transcription/translation reactions. The RTS 500 Wheat Germ CECF Kit – an up-scaled version of the RTS 100 Wheat Germ CECF Kit – provides reagents for five cell-free reactions of 1 ml volume. Both kits contain in separate vials wheat germ lysate, reaction mix, feeding mix, amino acids (without methionine), methionine, reconstitution buffer, and glucuronidase (GUS) expression vector to be used for the control reaction, as well as reaction devices and spare caps for reconstitution of the feeding mix (bottle 3 in the kit).

Results and Applications

RTS Wheat Germ Kits workflow

Linear PCR-generated templates can be used to rapidly and conveniently screen for expression ability with the RTS 100 Wheat Germ Kit (Figure 3). To produce a template for expression scale-up, or to make sure a well-characterized (*i.e.*, sequenced) and stable template is used in subsequent experiments, cloning into a RTS pIVEX vector is generally recommended. Traditional cloning via restriction sites or PCR cloning techniques are time-saving alternatives for template stabilization. Resulting expression units include all necessary regulatory elements for an efficient translation in wheat germ extract.

One of the major advantages of cell-free over cell-based expression systems is the possibility of using PCR-generated templates. If gene-specific primers for the first-step PCR are designed according to the package insert, the resulting PCR product can be used to generate functional linear expression templates for both the RTS 100 *E. coli* HY Kit (using RTS *E. coli* Linear Template Generation Sets) or the RTS 100 Wheat Germ CECF Kit (using RTS Wheat Germ Linear Template Generation Sets). Moreover, the design of these kits allows easy switching from any expression system to both RTS platforms (Wheat Germ and *E. coli*), or between the two.

Protein synthesis in the RTS Wheat Germ CECF Kits

To evaluate the RTS Wheat Germ CECF Kits, we initially tried to synthesize proteins that were not expressed or were insoluble in *E. coli* lysate. Twenty four candidate proteins of eukaryotic and prokaryotic origin were selected and expression templates were prepared using the RTS Wheat Germ Linear Template Generation Set, His₆-tag. Figure 4 illustrates an example of different proteins expressed in the RTS 100 Wheat Germ CECF Kit. The overall success rate for protein expression was close to 100%.

Figure 5 shows that after cloning into pVEX Wheat Germ vectors, β -glucuronidase, survivin, human kinase and nuclear receptor proteins were efficiently synthesized in the RTS 100 Wheat Germ CECF Kit. Genes of interest were cloned in pVEX WG 1.3 and pVEX WG 1.4 to obtain His₆-tagged proteins (both C- or N-terminally tagged). Plasmid DNA was prepared by conventional methods and 2 μ g of each DNA was incubated in the cell-free reaction. Protein expression was performed and evaluated as described under Materials and Methods. The amount of proteins synthesized in the RTS 100 Wheat Germ CECF Kit was estimated as several hundred microgram expressed per milliliter synthesis reaction.

Affinity purification of His₆-tagged proteins

The newly synthesized His₆-tagged proteins were successfully purified with an anti-His₆ affinity resin. The concentration of imidazole in the washing buffer was varied to improve purity of the eluted reaction product. The proteins were eluted using 500 mM imidazole and analyzed on an SDS polyacrylamide gel (for β -glucuronidase, see Figure 6).

RTS 100 versus RTS 500 Wheat Germ Kits

To compare protein productivity in the RTS 100 WG CECF Kit with that in the RTS 500 WG CECF Kit, β -glucuronidase, survivin, human kinase, and nuclear receptor proteins were synthesized using both kits. The reactions were incubated

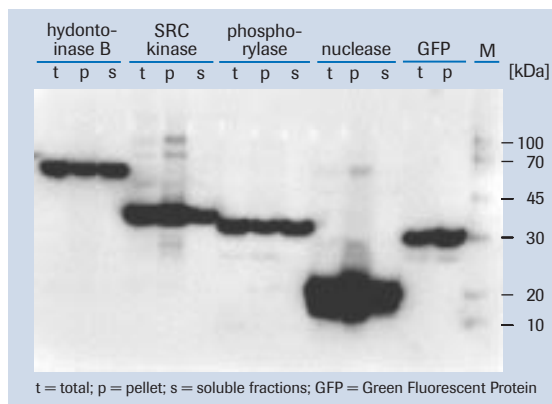


Figure 4: Western blot analysis of cell-free protein synthesis reactions. Different proteins were expressed using the RTS 100 Wheat Germ CECF Kit.

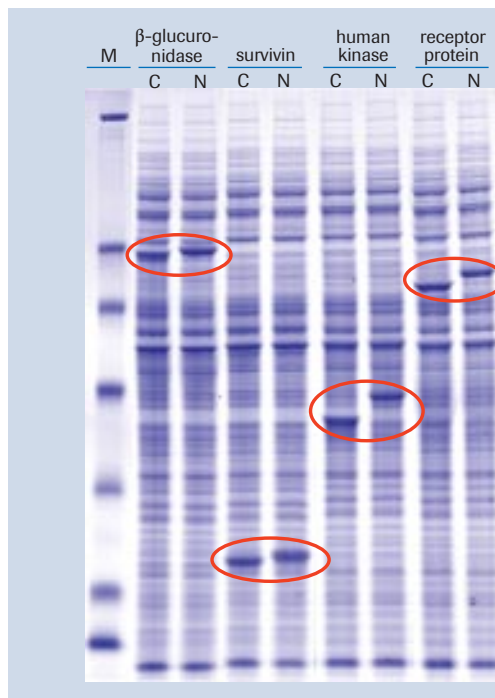


Figure 5: Cell-free protein synthesis. Different His₆-tagged proteins were expressed using the RTS Wheat Germ Kit. Expression reactions were analyzed using SDS-PAGE. The gel was stained with Coomassie Brilliant Blue (N=N-terminal tag, C=C-terminal tag).

for 24 hours and aliquots were taken after 3, 6, 12, and 24 hours. Reaction products were analyzed on an SDS polyacrylamid gel. For all tested samples, increasing the reaction time to 24 hours resulted in an increased amount of protein. Approximately 1 mg per milliliter of reaction was produced after 24 hours. The results were confirmed by western blotting (data not shown). Figure 7 shows that for survivin, newly synthesized protein was accumulated in both reaction formats with similar efficiency. As an estimate, the RTS 500 reaction format yielded more protein (calculated per ml of reaction) than the RTS 100 format. This may be due to the different design of the CECF device. The exchange surface of the dialysis membrane in RTS 500 CECF device is larger – it contains two membranes – compared with the RTS 100 CECF device (Figure 1b).

Summary

Wheat germ extract-based cell-free protein synthesis in the RTS 100 format is the ideal technology for rapid

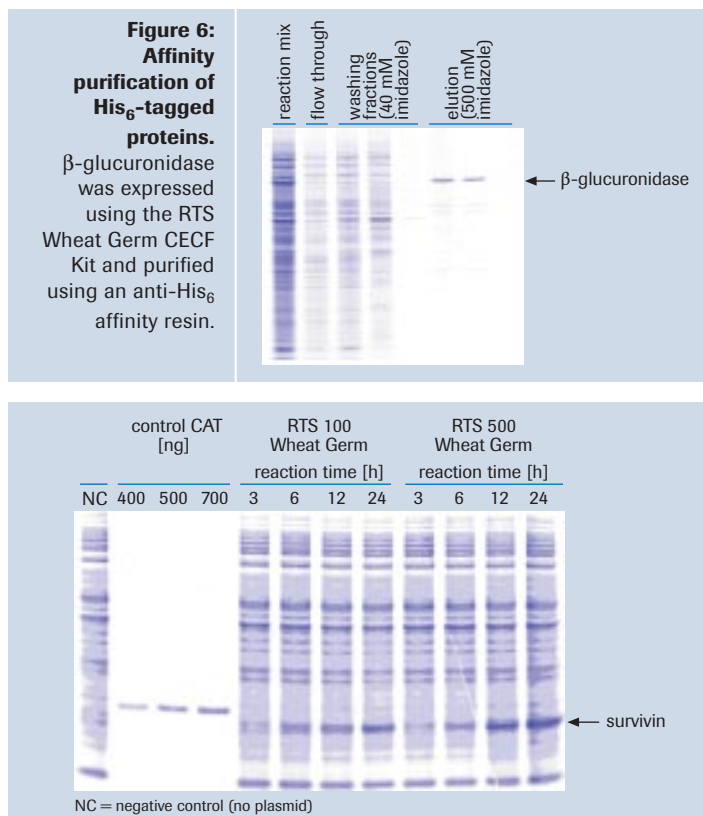


Figure 7: Protein productivity. SDS PAGE analysis of survivin synthesis in the RTS 100 and RTS 500 Wheat Germ CECF Kits is shown.

generation of high amounts of protein (up to 30 μ g per 50- μ l reaction depending on the protein) and for activity and solubility screening. This PCR-based *in vitro* protein expression method has an advantage over cell-based expression systems as it avoids time-consuming cloning procedures and the generation of nonfunctional protein molecules in bacterial host cells. It is also suitable for the

rapid expression and identification of active protein homologs or their fragments from gene families. The system can be used for protein characterization and supports protein-protein interaction determination. In the larger scale expression reaction format using the RTS 500 Wheat Germ CECF Kit, expression of up to 1 mg of protein (depending on the type of protein) per milliliter of synthesis reaction is possible.

In summary, this inexpensive cell-free wheat germ translation system shortens the route from gene to protein characterization from days to a few hours in comparison to traditional cell-based systems. It can be easily adapted to various applications such as activity screening of proteins, or kinetic or inhibition studies. In this article, we have shown that the RTS Wheat Germ CECF Kits enable high success rates for expression and solubility, particularly for eukaryotic target proteins. ■

References

1. Spirin AS *et al.* (1988) *Science* 242:1162-1164
2. Hoffmann M *et al.* (2004) *Biotechnol Annu Rev* 10:1-30



Product	Pack Size	Cat. No.
RTS 500 Wheat Germ CECF Kit	5 x 1 ml reactions	04 492 838 001
RTS 100 Wheat Germ CECF Kit	24 x 50 μ l reactions	03 728 811 001
RTS Wheat Germ Linear Template Generation Set, His₆-tag	96 reactions	03 728 790 001
RTS pIVEX Wheat Germ His₆-tag Vector Set	2 vectors, 10 μ g each	03 728 803 001
RTS ProteoMaster Instrument	1 instrument	03 265 650 001

Production of Active Disulfide-Bonded Proteins in Milligram Amounts: The New RTS 500 *E. coli* Disulfide Kit

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Introduction

The synthesis of soluble and correctly folded disulfide-bonded proteins in large amounts is still a challenge in the field of protein expression. The major problem is that the cytoplasm – the “volume” in which the protein is usually

synthesized within a cell – maintains reducing conditions that impede the formation of disulfide bonds. Consequently, the protein aggregates and has to be refolded – a tedious procedure which gives only limited yields and therefore has to be repeated several times.