

EcoPro[™] T7 System

About the Kits

EcoPro T7 Introductory System	10 rxn	70888-3
EcoPro T7 System	50 rxn	70876-3

Description

The EcoPro coupled transcription/translation system is designed for efficient *in vitro* synthesis of full length proteins directly from supercoiled and linear DNA templates. The EcoPro Extracts are prepared with a proprietary fractionation process* that produces higher protein expression levels and predominantly full-length protein, with a marked reduction in the secondary products that are commonly observed with other prokaryotic *in vitro* expression systems [1]. The EcoPro T7 System can direct the efficient expression of full-length protein from T7 or *E. coli* promoters. The EcoPro T7 System uses an S30 extract prepared from an *E. coli* host strain deficient in protease and nuclease activity and combined with reaction buffer and an amino acid mixture, which reduces the number of pipetting steps required for reaction assembly. In the standard reaction, the DNA template (typically 2–4 µg plasmid) is combined with the EcoPro Extract, Methionine (or ³⁵S-methionine, not provided) and water and incubated at 37°C for 60 minutes.

The standard EcoPro T7 System kit has enough reagents to perform 50 standard 50 µl reactions. The introductory kits have enough reagents for 10 standard reactions. The EcoPro Control DNA is a dual T7/*E. coli* promoter construct encoding an S•Tag[™] β-glucuronidase fusion protein. The expressed protein is easily detected by a variety of methods including ³⁵S-methionine incorporation or S•Tag[™] Western blot [2] or homogeneous non-isotopic assay with the FRETWorks[™] S•Tag Assay Kit [3].

* patent pending

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Components

Introductory EcoPro™ T7 System

- 1 × 350 µl EcoPro T7 Extract
- 100 µl 5 mM Methionine
- 1.5 ml Nuclease-free Water
- 10 µg EcoPro Control DNA, 1.0 µg/µl

EcoPro T7 System

- 5 × 350 µl EcoPro T7 Extract
- 100 µl 5 mM Methionine
- 1.5 ml Nuclease-free Water
- 10 µg EcoPro Control DNA, 1.0 µg/µl

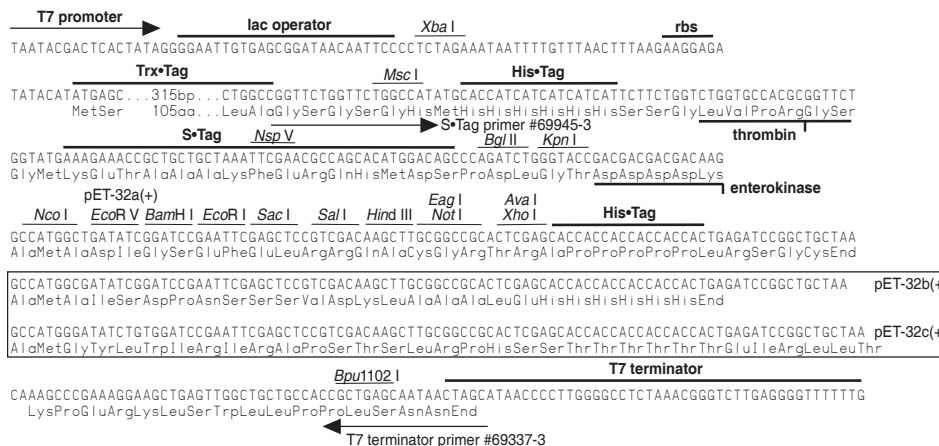
Storage

Store the 8-Cap Strips and Aluminum Plate Sealers at room temperature. Store all other components of the EcoPro System kits at -70°C.

DNA Templates

The EcoPro T7 System will function with DNA templates containing T7 or *E. coli* promoters upstream from coding sequences, including supercoiled plasmids and linear DNA. For a given vector or primer and insert combination, the type of template used (supercoiled, linear, etc.) may affect the efficiency of the reaction. It is necessary that any template be free of RNase, Mg²⁺ and salts.

A number of different T7 or *E. coli* promoter-containing vectors can be used successfully for in vitro protein production. When using the EcoPro T7 System with plasmid templates not containing a T7 promoter, optimal performance is achieved with the use of strong, supercoiling insensitive *E. coli* promoters such as *tac* or *lac*. An example of a T7 promoter construct known to function efficiently in the EcoPro System is shown below:



Coupled Transcription/Translation Reaction

The EcoPro Extract is provided in 10 reaction aliquots. Determine the number of aliquots required for the experiment and transfer the reagents from the -70°C freezer to a container of ice (avoid thawing and re-freezing the mixes because they lose activity with each freeze-thaw cycle). Thaw all of the reagents on ice, except the Nuclease-free Water, which can be thawed at room temperature. The reagent tubes can be spun briefly in a microcentrifuge to collect all solution at the bottom. All of the components necessary for the coupled transcription and translation reactions are provided in the EcoPro Extract, with the exception of methionine, DNA template and water.

Selective inhibition of *E. coli* RNA polymerase

Rifampicin (*rif*) functions as a selective inhibitor of *E. coli* RNA polymerase and can be added to EcoPro reactions to eliminate expression from *E. coli* promoters. When working with T7 promoter-based plasmid constructs, the inclusion of *rif* can eliminate translation products arising from antibiotic markers or other *E. coli* promoter-driven genes present on the plasmid. For example, β -lactamase is widely used as a selectable marker for the maintenance of expression vectors in host cells through antibiotic selection. When vectors containing β -lactamase are used with the EcoPro T7 System, an abundant translation product will be produced with a molecular weight of 31.5 kDa. The inclusion of *rif* in the EcoPro reaction will eliminate the majority (> 98% when used at the recommended level) of *E. coli* promoter-driven expression, simplifying the interpretation of gels and improving the expression level of target proteins. Rifampicin (Calbiochem Cat. No. 557303) can be prepared as a 25 mg/ml stock solution in methanol and stored at -20°C . Before use, dilute the rifampicin stock solution in water to a final concentration of 1.25 mg/ml. If precipitation occurs, adjust the pH to 7.0 with KOH to fully solubilize the rifampicin. For a standard 50- μl reaction, 2 μl *rif* is added (50 ng/ μl final concentration). While some publications describe lower effective concentrations of *rif*, we observe only partial inhibition of *E. coli* promoter driven expression levels unless the final concentration is 50 ng/ μl .

Standard reaction

The standard translation reaction volume is 50 μl , of which 35 μl is the EcoPro™ Extract. The other 15 μl comes from the addition of labeled or unlabeled Methionine, DNA template and water.

1. Stir EcoPro Extract gently with pipet tip to ensure proper mixing. Assemble the following components in the order listed below in 0.5- or 1.5-ml microcentrifuge tube at room temperature:

Tip: You may also prepare a master mix of EcoPro Extract, methionine and water and then initiate the transcription/translation reaction by adding 2–4 μg template to the reaction.

x μl	Nuclease-free Water
y μl	2 μl 5 mM Methionine (supplied) <u>or</u> 4 μl (40 μCi) ^{35}S -methionine (not supplied)
z μl	2–4 μg DNA template
(2 μl)	1.25 mg/ml rifampicin (optional, use only in EcoPro T7 reaction)
35 μl	EcoPro Extract
50 μl	Total added volume

The quality and age of the ^{35}S -methionine label can dramatically affect incorporation levels. See Radioactive Label, page 6.

2. Gently mix with pipet tip and incubate at 37°C for 60 min.

Control reactions

1. Always include blank reaction without added template DNA to measure the small amount of background incorporation of labeled amino acid, or to serve as a negative control for S•Tag assays and Western blot analysis.
2. A positive control reaction with the supplied EcoPro Control DNA can be done to verify performance. The reaction is performed exactly as described above, using 2 μg Control DNA as the template. The size of the encoded protein is 73.5 kDa.

Analysis of translation products

Translation products from EcoPro reactions can be analyzed with isotopic detection (incorporation assays, gel fluorography) or non-isotopic detection (Western blotting and protein assays). Novagen vectors contain various fusion tags (GST•Tag™, His•Tag®, Nus•Tag™, S•Tag™, T7•Tag®) for the purpose of Western blot detection and affinity purification (visit www.novagen.com for appropriate User Protocols). The 73.5-kDa S•Tag β -glucuronidase EcoPro control protein can be assayed with the S•Tag Rapid Assay (Cat. No.69212), or FRETWorks™ S•Tag Assay (Cat. No. 70724), in addition to Western blotting with S-protein AP or HRP conjugates. The S•Tag sequence is derived from the N-terminal 15 amino acids of bovine pancreatic RNase A and binds with high affinity to S-protein (the carboxy terminal 104 aa fragment of RNase A) to reconstitute an enzymatically active RNase molecule. This strong, specific binding provides the

basis for detection of fusion protein with the S•Tag™ Western Blot Kit. The reconstituted enzymatic activity is the basis for quantitative assays of expressed fusion protein with the S•Tag Rapid Assay Kit or FRETWorks™ S•Tag Assay Kit.

Incorporation assay (radioactive amino acid)

1. After reaction incubation, transfer 2 µl each reaction to 1.5-ml tube containing 98 µl 1 N NaOH/2% H₂O₂. Mix and incubate at 37°C for 10 min. This step hydrolyzes charged tRNAs and reduces the assay background levels.
2. Add 0.9 ml 25% trichloroacetic acid (TCA)/2% caseamino acids. Vortex. Incubate on ice for 10 min.
3. Filter 0.5 ml each sample on Whatman GF/A or equivalent filters (pre-wetted with 5% TCA) excess liquid from filter.
4. Allow to air dry and count in the presence of an appropriate scintillant.
5. To determine total cpm in the reaction, spot another 5 µl each reaction directly on dry filter. Allow to air-dry and count with other samples.

Calculations

Net cpm incorporated/µl = (cpm on TCA filter) – (cpm on minus DNA filter)

Total cpm in reaction/µl = (cpm observed on directly spotted reaction)/5

% incorporation = net cpm incorporated per µl / (total cpm in reaction per µl) X 100%

fold stimulation = (cpm on TCA filter) / (cpm on minus DNA filter)

Example: 100,000 cpm were observed in the TCA precipitated sample, 2,000 cpm were observed in the TCA precipitate from the reaction with no added DNA, and 4,000,000 cpm were observed on the filter with 5 µl of the reaction spotted directly, corresponding to 800,000 input cpm/µl.

% incorporation = (100,000 – 2,000)/800,000 × 100% = 12.2%

fold stimulation = 100,000/2,000 = 50 X

Gel analysis and fluorography (radioactive amino acid)

1. Optional: After incubation, add 1 µl 10 mg/ml RNase A and incubate at room temperature for 5 min (this can be done after removing the sample for TCA). This step effectively stops the reaction and removes charged tRNAs, which can appear on protein gels.
2. Transfer 5 µl each reaction into a tube.
3. Add 20 µl 100% acetone. Vortex.
4. Centrifuge 3–5 min at 14,000 × g.
5. Remove supernatant. Resuspend pellet in 20–30 µl 1X SDS loading buffer.
6. Heat at 70–99°C for 5 min. Cool to room temperature. Load 8 µl on an SDS polyacrylamide gel.
7. Load 5–10 µl per well on an SDS polyacrylamide gel.

Note: Load less per well when using ³⁵S-labeled product or signal will be too intense.

8. Run gel after loading desired amount of cooled sample. Using EcoPro™ Control DNA under the above reaction conditions, samples should produce intense bands in an overnight exposure without fluorography, or in about 2 h with fluorography. The full-length translation product of the EcoPro Control DNA is 73.5 kDa.
9. After electrophoresis, fix proteins by immersing gel in 10% TCA for 5 min. Rinse briefly with water. Dry under vacuum. Expose to X-ray film at room temperature. For greater sensitivity, the gel can be processed for fluorography with EN3HANCE™ (NEN) or Amplify™ (Amersham) according to manufacturer instructions and exposed with intensifying screens at –70°C.

Expected Yield

Yield will vary depending on the type of template, template preparation, type of promoter, and use of rifampicin. As much as 28 µg full-length active β-galactosidase (116 kDa) was produced from an EcoPro T7 reaction in 60 min. Other yields include 17 µg active CAT and 3.2 µg active Fluc. Yield may be increased by extending the incubation time up to 90 min at 37°C or 90–120 min at 30°C.

Optimization and Troubleshooting

DNA template

For the EcoPro™ reaction to proceed, all DNA templates used must be substantially free of contaminating RNase, Mg²⁺, and salts. Plasmids isolated using the Mobius™ or UltraMobius™ Plasmid Kit are essentially RNase-free and can be used directly in the EcoPro reaction without further purification. Plasmid DNA prepared with SpinPrep™ Plasmid Kits or other protocols using RNase A should be extracted twice with TE-buffered phenol:CIAA (1:1; CIAA is 24 parts chloroform, 1 part isoamyl alcohol) and once with CIAA prior to precipitation with ethanol in the presence of 0.3 M NaOAc. DNA pellets should be rinsed with 70% ethanol, air dried, and dissolved in RNase-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or water. SpinPrep™ Plasmid Kits are not recommended for low-copy plasmid sample preparation. Use Mobius™ Plasmid Kits with low-copy plasmids to generate sufficient DNA for the EcoPro Reaction.

Addition of Pellet Paint® Co-Precipitant to the DNA facilitates recovery in the precipitation step without affecting performance in the EcoPro reaction.

If linearized plasmids are used as templates, we recommend using restriction enzymes that leave either blunt ends or 5' overhangs. Templates containing 3' overhangs can cause aberrant transcription from the non-coding strand by T7 RNA polymerase and other phage polymerases [4]. The antisense RNA will base pair with desired transcripts and potentially inhibit translation in the EcoPro™ reaction. If an enzyme that produces 3' overhangs cannot be avoided, the DNA can be treated with the Klenow fragment of DNA polymerase I in the presence of 25 μM dNTPs (5 min, 25°C, 1 U/μg DNA) to blunt the ends prior to use. Commonly used restriction enzymes that produce 3' overhangs include *Pst* I, *Kpn* I, *Sac* I, *Sac* II, *BstX* I, *Nsi* I, *Apa* I, and *Aat* II.

If extraneous translation products appear or the reaction appears weak, a template DNA titration series should be performed to determine the optimum amount of DNA for that specific template. For any given construct, the optimal amount of DNA may differ for supercoiled plasmid and linearized plasmid.

Radioactive label

To achieve higher incorporation, increase the amount of label added up to 10 μl (100 μCi). The quality of label can also dramatically affect incorporation levels. ³⁵S-methionine can rapidly oxidize to form a sulfoxide that inhibits translation. Purchase isotope preserved with DTT or 2-mercaptoethanol (NEN Cat. No. NEG-009A or Amersham Cat. No. SJ1015 or SJ1515), dispense into single-use aliquots, and store at -70°C. The use of fresh label (less than 2 weeks old) can dramatically improve translation performance.

If translation is very efficient and a high percentage of label is incorporated, the methionine pool may be depleted to the extent that incomplete translation products are produced. In this case, the reaction can be supplemented with unlabeled methionine after the initial labeling period as a cold "chase" to complete the polypeptide chains.

tRNA

The EcoPro Extract is supplemented with *E. coli* tRNA to enable efficient translation of a wide variety of templates. However, occasionally a given ORF may encode a high proportion of one or two amino acids and cause depletion of particular tRNA pools and the appearance of incomplete translation products. In addition, ORFs from some heterologous species code for nonstandard amino acids that are not found in the lysate pool. In these cases it may be helpful to supplement the reaction with tRNA from the same organism from which the DNA is derived.

References

1. Ambuel, Y., M. Handley, and S. Hayes, *Greater yields of full-length proteins using EcoPro™ T7 transcription/translation system*. in *Novations*, 2003. **16**: p. 3–6.
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4. Schenborn, E.T. and R. Mierendorf, *A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure*. *Nucleic Acids Res*, 1985. **13**(17): p. 6223-36.