

# STP3<sup>®</sup> Kits

## About the Kits

Introductory Single Tube Protein System <sup>®</sup> 3, T7	10 rxn	70205-3
Single Tube Protein System 3, T7	50 rxn	70192-3
Single Tube Protein System 3, SP6	50 rxn	70207-3

## Description

The Single Tube Protein System 3 (STP3) is designed for efficient *in vitro* production of proteins directly from supercoiled or linear DNA templates. The method is based on a linked reaction in which transcription by a bacteriophage RNA polymerase is directly followed by translation in an optimized rabbit reticulocyte lysate. Unlike other commercial kits, the patented STP3 method inherently provides an optimal amount of RNA template for translation, which results in superior protein yield and consistency between samples.

Any DNA coding sequence inserted downstream from a T7 or SP6 promoter can be quickly expressed as protein simply by adding it to the linked reaction. Suitable templates can be supercoiled or linearized plasmid DNA. Compared with standard methods for translation of synthetic RNA templates, several tedious manipulations are avoided, including restriction enzyme digestion, linearized plasmid DNA purification, and RNA purification, which normally require four to six hours to perform. Performance can be further enhanced using the Novagen pCITE<sup>®</sup> or pT7Blue-2 vectors containing eukaryotic translation enhancer sequences.

In the standard STP3 reaction, the DNA template (typically 0.5 µg plasmid) is transcribed at 30°C for 15 min, followed by the addition of translation mix and continued incubation for 60 min. All components are premixed such that the only reagents to be added are template, water, and a choice of unlabeled methionine (included) or <sup>35</sup>S-methionine (not included).

The standard kit has enough reagents to perform 50 standard 50-µl reactions or 100 small-scale 25-µl reactions. An introductory kit for 10 standard reactions is also available. A positive control DNA containing the *E. coli* β-galactosidase gene is included with the kits to monitor performance. The translation product of the STP3 control reaction can be measured by standard incorporation assays, nonradioactive S•Tag<sup>™</sup> Rapid Assay or FRETWorks<sup>™</sup> S•Tag Assay, S•Tag Western Blot, or direct measurement of β-galactosidase activity with the BetaFluor<sup>™</sup> β-Gal Assay Kit. Careful quality control ensures that the Single Tube Protein System 3 provides the highest activity, lowest background, and most consistent performance available.

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## Components

### Introductory Single Tube Protein System® 3, T7

Kits contain enough components for 10 standard 50 µl reactions.

- 1 × 80 µl STP3® T7 Transcription Mix
- 1 × 300 µl STP3 Translation Mix
- 100 µl 625 µM Methionine
- 1.5 ml Nuclease-free Water
- 50 µg STP3 T7 Control DNA, 0.5 µg/µl

### Single Tube Protein System 3, T7 or SP6

Kits contain enough components for 50 standard 50 µl reactions.

- 5 × 80 µl STP3 T7 Transcription Mix or STP3 SP6 Transcription Mix
- 5 × 300 µl STP3 Translation Mix
- 100 µl 625 µM Methionine
- 1.5 ml Nuclease-free Water
- 50 µg STP3 T7 or SP6 Control DNA, 0.5 µg/µl

## Storage

Store all components of STP3 kits at -70°C.

## DNA Templates

The STP3 System will function with a variety of DNA templates containing T7 or SP6 promoters upstream from coding sequences, including supercoiled plasmids and linear DNA.

Although a number of different T7 promoter-containing vectors can be used successfully for *in vitro* protein production, optimal performance in the STP3, T7 system is observed for most target genes when they are cloned downstream from the translation enhancer present in Novagen's pCITE® vectors. These plasmids carry a segment of the encephalomyocarditis virus (EMC) 5' non-coding region that functions as an internal entry point for initiation of translation by eukaryotic ribosomes (1). This Cap-Independent Translation Enhancer (CITE) (also known as the IRES, for internal ribosome entry sequence) dramatically increases (typically by 5–10-fold) the *in vitro* translation efficiency of synthetic RNA by rabbit reticulocyte lysates (2). In addition, the CITE sequence results in very high stringency for the correct AUG initiation codon (3). In the pCITE plasmids, the CITE sequence is located immediately downstream from a T7 promoter and is followed by a region of cloning sites for insertion of target sequences. The pCITE-4a-c(+) vectors also contain an optional amino-terminal S•Tag™ sequence, which enables affinity purification, Western blot analysis and accurate, sensitive, nonradioactive assay of any target protein.

Another vector, the Novagen pT7Blue-2 Vector, is also designed for optimal performance in the STP3 System. This plasmid contains a *Xenopus* β-globin 5'-UTR translation enhancer element downstream of a T7 promoter and upstream of an ideal consensus translation initiation site (4). In addition, the vector provides blue/white screening, an S•Tag™ sequence, and is available as a Perfectly Blunt® Vector prepared as a blunt-ended dephosphorylated plasmid ready for cloning.

## The Procedure

The STP3<sup>®</sup> Transcription Mix and STP3 Translation Mix are 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. Set up the transcription reaction first. After the transcription reaction is finished, proceed directly to the translation reaction (or store the translation components to the transcription reaction, the transcription reaction can also be added to the translation components assembled in a second tube without affecting results. Control reactions are described on page 4.

### Transcription reaction

The standard transcription reaction volume is 10 µl, which can be scaled up or down proportionately. (In the translation step, keep the volume of transcription reaction at 20% of the final reaction volume.) The transcription and translation mixes are supplied as aliquots containing enough reagent for 10 standard reactions each.

1. Stir STP3 Transcription Mix gently with pipet tip to ensure proper mixing. Assemble the following components in the order given at room temperature in a 0.5- or 1.5-ml microcentrifuge tube:
 

8 µl	STP3 T7 or SP6 Transcription Mix
x µl	Nuclease-free Water
y µl	DNA template (0.5 µg plasmid free of RNase, Mg <sup>2+</sup> and salts)
<hr style="width: 100%;"/>	
10 µl	Total volume
2. The Transcription Mix or DNA can be added to start the reaction. Mix by stirring with pipet tip and incubate at 30°C for 15 min. For non-CITE<sup>®</sup> plasmid template, the transcription incubation should not exceed 15 min.

*Note:* Transcription reactions can be allowed to proceed up to 30 min without loss of performance. Completed transcription reactions can be stored at -20°C for up to one week prior to translation.

### Translation reaction

The standard translation reaction is 50 µl, 10 µl of which comes from the transcription reaction. The other 40 µl comes from the addition of the following thawed components directly to the transcription reaction.

1. Add the following in the order given:
 

x µl	Nuclease-free Water
y µl	2 µl of 625 µM Methionine (supplied) <i>or</i> 4 µl (40 µCi) <sup>35</sup> S-methionine (not supplied)
<hr style="width: 100%;"/>	
30 µl	STP3 Translation Mix
40 µl	Total added volume

*Tip:* You may also prepare a master mix of STP3 Translation Mix, methionine and water and then initiate the translation reaction by adding 40 µl of the master mix directly to the 10 µl transcription reaction.

2. Gently stir with pipet tip and incubate at 30°C for 60 min.

*The quality and age of the <sup>35</sup>S-methionine label can dramatically affect incorporation levels. See Radioactive Label, page 6.*

## Control reactions

1. Always include a blank reaction without added DNA to measure the small amount of background incorporation of labeled amino acid, or to serve as a negative control for S•Tag™ nonradioactive assays or for Western blot analysis.
2. A positive control reaction with the supplied STP3® Control DNA can be done to verify performance. The reaction is performed exactly as described above, except that 0.5 µg (1 µl) Control DNA is used as the template for the transcription reaction. The size of the translation product from the STP3 T7 Control DNA is 119 kDa (T7) and the STP3 SP6 control DNA is 116 kDa.

## Analysis of translation products

Translation products from STP3 reactions can be analyzed with isotopic detection (incorporation assays, gel fluorography) or non-isotopic detection (Western blotting and protein assays). Numerous Novagen vectors (pET, pTriEx™, pETBlue™, pBAC™) contain fusion tags (GST•Tag™, His•Tag®, Nus•Tag™, S•Tag, T7•Tag®) for the purpose of non isotopic detection (Western blotting) and affinity purification (visit [www.novagen.com](http://www.novagen.com) for appropriate Technical Bulletins). It is important to note that HRP detection is not recommended with STP3 reactions because the lysate contains endogenous peroxidases that may interfere. Also note that STP3 reactions are not compatible with IMAC methods (i.e., His•Bind® resins).

The 119 kDa (T7) or 116 kDa (SP6) translation product of the STP3 control reaction contains an S•Tag sequence and can be assayed with non-radioactive S•Tag Rapid Assay (Cat. No.69212), FRETWorks™ S•Tag Assay (Cat. No. 70724) or BetaFluor™ β-Gal Assay (Cat. No. 70979) in addition to Western blotting with S•Tag conjugates. The S•Tag sequence is derived from the first 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)

3. After incubation, transfer 2 µl reaction to 1.5-ml tube containing 98 µl 1 N NaOH/2% H<sub>2</sub>O<sub>2</sub>. Mix and incubate at 37°C for 10 min. This step hydrolyzes charged tRNAs and removes the red color that may quench scintillation counting.
4. Add 0.9 ml 25% trichloroacetic acid (TCA)/2% casamino acids. Vortex, and leave on ice for 10 min.
5. Filter samples on Whatman GF/A or equivalent filters (presoaked with 5% TCA) under vacuum. Wash 3 times with 3 ml cold 5% TCA. Rinse filters with acetone (optional). Aspirate excess moisture from filter.
6. Allow to air dry. Count in the presence of an appropriate scintillant.
7. To determine total cpm in the reaction, add another 2 µl sample to 18 µl deionized water and spot 2 µl of this dilution directly onto a dry filter. Allow to air dry and count with the other samples.

### Calculations

$$\% \text{ incorporation} = \text{net cpm incorporated} / \text{total cpm in reaction} \times 100\%$$

$$\text{fold stimulation} = \text{cpm incorporated with DNA} / \text{cpm incorporated in blank}$$

Example: 100,000 cpm were incorporated/2 µl of a test sample, 2,000 cpm were incorporated/2 µl with no added DNA, and 80,000 cpm were observed on the filter with the diluted reaction spotted directly without TCA precipitation. Corrected for dilution, this gives 800,000 input cpm/2 µl.

$$\% \text{ incorporation} = (100,000 - 2,000) / 800,000 \times 100\% = 12.2\%$$

$$\text{fold stimulation} = 100,000 / 2,000 = 50$$

## Gel analysis and fluorography (radioactive amino acid)

1. After the incubation, add 1  $\mu$ l 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. Remove 5  $\mu$ l of the reaction and mix with 20  $\mu$ l 2X SDS sample buffer (0.01% bromophenol blue, 150 mM DTT, 20% glycerol, 4% SDS, 125mM Tris-HCl, pH 6.8).
3. Heat at 85°C for 3 min, cool to room temperature, and load 5–10  $\mu$ l on an SDS polyacrylamide gel.
4. Run the gel after loading the desired amount of cooled sample. Using the STP3 Control DNA under the above reaction conditions, 15  $\mu$ l 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 STP3 T7 Control DNA is 119 kDa; with the STP3 SP6 Control DNA, it is 116 kDa).

After electrophoresis, fix the proteins by immersing the gel in 10% TCA for 5 min, rinse briefly with water, dry under vacuum and expose to X-ray film at room temperature. For greater sensitivity, the gel can be processed for fluorography with EN<sup>3</sup>HANCE™ (NEN) or Amplify™ (Amersham) according to the manufacturer's instructions and exposed with intensifying screens at –70°C.

## Optimization and Troubleshooting

### DNA sample

For the STP3<sup>®</sup> reaction to proceed, any type of DNA template must be substantially free of contaminating RNase. Although the transcription mix contains an RNase inhibitor, excess amounts of RNase will greatly affect the amount and quality of the translation products. Plasmid DNA isolated with Mobius™ or UltraMobius™ Plasmid Kits are essentially RNase free. However plasmid DNA isolated with SpinPrep™ Plasmid Kits or other protocols may require an additional phenol:CIAA extraction to eliminate RNases. Extract the sample 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 (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) or water. Salts or Mg<sup>2+</sup> (see below) in the DNA template may affect the STP3 reaction.

*Note:* Addition of Pellet Paint<sup>®</sup> Co-Precipitant to the DNA facilitates recovery in the precipitation step without affecting performance in the STP3 reaction.

The translation efficiency of transcripts is usually further enhanced by including a translation termination codon following the coding sequence. Natural termination at a stop codon appears to be more efficient than “run-off” translation from truncated templates. Most pET and pCITE<sup>®</sup> vectors have translation stop codons in all three reading frames between the multiple cloning region and T7 transcription terminator.

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 (5). The antisense RNA will base pair with desired transcripts and effectively inhibit translation in the STP3 reaction. If an enzyme that produces 3' overhangs cannot be avoided, the DNA can be treated with Klenow DNA polymerase in the presence of 25  $\mu$ M nNTPs (5 min, 25°C, 1 U/ $\mu$ g DNA) to flush the ends prior to use. Commonly used restriction enzymes that produce 3' overhangs include *Pst* I, *Kpn* I, *Sac* I, *Sac* II, *Bst*X I, *Nsi* I, *Apa* I, and *Aat* II.

Base pairings in 5' untranslated regions can dramatically affect translation efficiency (6). If the RNA secondary structure blocks binding to the ribosome, translation can be severely inhibited. If a target gene contains any upstream sequences that are not normally translated, this type of inhibition can occur.

## Magnesium concentration

The STP3<sup>®</sup> translation reaction is carried out in the presence of 1 mM magnesium acetate (in addition to that present endogenously in the lysate), which has been balanced with chelators to optimize the translation of most templates. Additional Mg<sup>2+</sup> may reduce the amount of protein produced. Therefore, templates should not contain appreciable levels of Mg<sup>2+</sup> or other compounds (e.g., spermidine, NTPs) that may affect the “free” Mg<sup>2+</sup> concentration.

## Radioactive label

To achieve higher incorporation it is possible to increase the amount of label added up to 10 µl (100 µCi). The quality of label can also dramatically affect incorporation levels. <sup>35</sup>S-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 STP3 Translation Mix is supplemented with high-grade calf liver 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 may cause 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 lysate with tRNA from the same organism from which the DNA is derived.

## References

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