S•Tag™ Thrombin Purification Kit

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About the Kit

S•Tag™ Thrombin Purification Kit 69232-3

Description

The S•Tag™ Thrombin Purification Kit is designed for rapid affinity purification of S•Tag fusion proteins produced from many Novagen vectors. S-protein Agarose specifically retains S•Tag™ fusion proteins (1). When fusion proteins are expressed from vectors that also encode a thrombin cleavage site (LeuValProArg↓GlySer) between the S•Tag sequence and the cloning region, the target protein can be released from S-protein Agarose simply by digestion with thrombin. This kit uses a unique strategy that employs Biotinylated Thrombin, which enables simple and specific removal of the enzyme after digestion with Streptavidin Agarose. The standard protocol calls for batch-wise binding to S-protein Agarose, washing, treatment with Biotinylated Thrombin, and capture with Streptavidin Agarose, leaving the purified protein in solution. Under these native conditions, homogeneous target proteins lacking the S•Tag peptide are recovered and the S-protein Agarose is not reused.

As an alternative, fusion proteins can be eluted from S-protein Agarose under conditions that disrupt the S•Tag:S-protein interaction (e.g., 3 M guanidine thiocyanate, 0.2 M citrate, pH 2, or 3 M magnesium chloride), such that the S•Tag peptide remains attached to the protein and the S-protein Agarose can be recycled. If the target protein accumulates in cells as inclusion bodies, the insoluble fraction can be prepared and dissolved in 6 M urea. Proteins are bound to the S-protein Agarose in the presence of 2 M urea and eluted either with Biotinylated Thrombin digestion in the presence of urea or with the partially denaturing conditions mentioned above.

Binding can be performed in a column or batch mode. The capacity of S-protein Agarose will vary somewhat based on the size and folding characteristics of a given target protein. The resin is tested under native conditions using S•Tag β-galactosidase and shows a minimum binding capacity of 2000 µg/ml of slurry. The amount of cell extract to use for purification also depends on the expression level of the target protein.

Components

The S•Tag Thrombin Purification Kit contains enough affinity resin, Biotinylated Thrombin and Streptavidin Agarose to purify up to 1 mg target protein under standard conditions.

- 2 ml S-protein Agarose (50% slurry in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide)
- 3 x 5 ml 10X Bind/Wash Buffer (200 mM Tris-HCl pH 7.5, 1.5 M NaCl, 1% Triton X-100)
- 3 ml 10X Thrombin Cleavage Buffer (200 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 25 mM $CaCl_2$)
- 50 U Biotinylated Thrombin
- 2 x 0.4 ml Streptavidin Agarose (50% slurry in phosphate buffer, pH 7.5, 0.02% sodium azide)
- 2 ea Spin Filters, 5 ml capacity

Storage

Store Biotinylated Thrombin at –20°C. Store other components at 4°C.

Related products/available separately	Size	Cat. No.
S-protein	2 ml	69704-3
	5 x 2 ml	69704-4
Biotinylated Thrombin	50 U	69672-3
Streptavidin Agarose	5 ml	69203-3
Spin Filters, 5-ml capacity	2 ea/pkg	69074-3
Perfect Protein™ Markers, 15–150 kDa	100 lanes	69149-3
Perfect Protein Markers. 10–225 kDa	100 lanes	69079-3
Perfect Protein Western Markers	25 lanes	69959-3
S•Tag™ rEK Purification Kit	1 kit	69065-3
S•Tag Rapid Assay Kit	100 assays	69212-3
S•Tag Western Blot Kits		
AP (colorimetric)	25 blots	69213-3
AP LumiBlot™ (chemiluminescent)	25 blots	69099-3
HRP LumiBlot™ (chemiluminescent)	25 blots	69058-3
S-protein AP Conjugate	50 ml	69598-3
S-protein HRP Conjugate	50 ml	69047-3
Thrombin, Restriction Grade	50 U	69671-3

Preparation of Cell Extracts

The purification procedure begins with a cell culture (bacterial, insect, or mammalian) that has been induced or otherwise treated for target protein production. S•Tag™ fusion proteins contained in reticulocyte lysate extracts, such as Single Tube Protein™ System 3 transcription/translation reactions, can be used directly, beginning with binding. The target protein must be expressed from a vector containing the S•Tag peptide sequence. Included here are brief protocols for the preparation of bacterial and insect cell extracts (using the pET and BacVector™ Systems, respectively). The procedure for insect cell extracts can also be used for mammalian cell extracts with minor modifications. For a detailed description of the S•Tag vectors and protein expression considerations, please refer to the technical literature that accompanies the pET, pCITE®, and BacVector® Systems.

Bacterial cell extracts (pET System)

Induction of λDE3 Lysogens

After a target plasmid is established in an expression host, e.g., BL21(DE3), HMS174(DE3), NovaBlue(DE3) or in one of these strains containing pLysS or pLysE, expression of the target DNA is induced by the addition of IPTG to a growing culture. For pET constructions carrying the "plain" T7 promoter (e.g., pSCREEN™), a final concentration of 0.4 mM IPTG is recommended, whereas 1 mM IPTG is recommended with vectors having the T7lac promoter (e.g., pET-30 and pET-32). Examples of induction protocols are presented below.

Pick a single colony from a freshly streaked plate and inoculate 50 ml LB medium containing the appropriate antibiotic in a 250 ml Erlenmeyer flask. (For good aeration, add medium up to only 20% of the total flask volume.)

Note: Include 34 µg chloramphenicol/ml LB medium if the cells carry pLysS or pLysE.

Alternatively, inoculate a single colony or a few µl from a glycerol stock into 2 ml LB medium containing the appropriate antibiotic. Incubate with shaking at 37°C until the OD_{600} reaches 0.6–1.0. Store the culture at 4°C overnight. The following morning, collect the cells by centrifugation (30 s in a microcentrifuge). Resuspend the cells in 2 ml fresh medium and use this to inoculate 50 ml medium.

1. Incubate with shaking at 37°C until the OD600 reaches 0.4–1 (0.6 recommended; about 3 h).
2. Remove samples for the uninduced control. Add IPTG from a 100 mM stock to a final concentration of 0.4 mM (T7 promoter) or 1 mM (T7lac promoter) and continue the incubation for 2–3 h.

It is often useful to determine where in the cell the target protein is accumulated. Simple methods for analyzing crude cell fractions are presented below.

Total cell protein

The expression of target genes may be quickly assessed by analysis of total cell protein on an SDS-polyacrylamide gel followed by Coomassie blue staining. Collect induced cells by centrifugation, resuspend in 1/10 culture volume of 10 mM Tris-HCl pH 8.0, 2 mM EDTA, remove a sample and add to it an equal volume of 2X SDS sample buffer. Heat to 70°C for 5 min and load 5–20 µl on an SDS-polyacrylamide gel. The proper amount of material to load depends on the cell density at time of harvest and the expression level of the target protein. Usually, an amount equivalent to 15 µl of a culture with an OD_{600} of 1.5 (3 µl of sample by the above method) gives the proper band intensities with Coomassie blue staining. Much less protein (~1/500 of this amount) is required for Western blot or dot blot analysis with the S-protein HRP or AP Conjugate.

Soluble and insoluble fractions

Many target proteins are expressed in both soluble and insoluble forms. Crude soluble and insoluble fractions can be prepared by the following protocol. This protocol will work with any of the pET host strains; in principle the lysozyme addition could be omitted with strains having pLysS or pLysE. It should be noted that in addition to (or instead of) gel analysis, S•Tag fusion proteins can be rapidly quantified in crude cell extracts using the S•Tag Rapid Assay Kit. This assay can be used with samples containing up to 1% SDS (which is diluted during the assay procedure), thus it is compatible with analysis of material extracted from insoluble fractions.

1. Collect induced cells (50 ml culture) by centrifugation at 5000 x g for 5 min. Discard the supernatant and resuspend the cell pellet in 1/10 culture volume (5 ml) of 50 mM Tris-HCl pH 8.0, 2 mM EDTA.
2. Add lysozyme to a concentration of 100 µg/ml; use a 10 mg/ml stock freshly prepared in the buffer used in Step 1. Then add 1/10 volume (0.5 ml) 1% Triton X-100. Incubate at 30°C for 15 min.
3. Place the tube in an ice bath and sonicate with a microtip to shear the DNA. The solution should lose viscosity after one or two 10 sec pulses at a high output setting.

4. Centrifuge at 12,000 x g for 15 min at 4°C. The supernatant contains soluble proteins; add an equal volume of 2X SDS sample buffer to a sample for gel analysis. The pellet is the insoluble fraction; resuspend in 1X SDS sample buffer for gel analysis. An amount of sample corresponding to 30 µl of the original culture volume is usually sufficient for proteins to be visualized by Coomassie blue staining. A 50 to 500–fold dilution provides sufficient protein for detection by the S-protein HRP or AP Conjugate in most cases.

Cell extract preparation

1. Prepare 25 ml 1X Bind/Wash Buffer by diluting 2.5 ml of the supplied 10X stock with 22.5 ml deionized water, and chill on ice. If the above test for solubility shows the target protein in the insoluble fraction, prepare 5 ml/sample of 1X Bind/Wash Buffer containing 6 M urea.
2. Harvest the induced cells by centrifugation at 5000 x g for 5 min. Decant the supernatant and allow the cell pellet to drain as completely as possible. Resuspend the cells in 10 ml ice-cold 1X Bind/Wash Buffer (the amount of Bind/Wash Buffer can be scaled up or down proportionally; this is for cells from a 100 ml culture induced at an OD_{600} of 0.6 and harvested 2 h post-induction). If resuspension is difficult, a Dounce homogenizer, blender or sonicator can be used to break up the cell pellet.
3. With the sample in a tube on ice or in a salt-ice bath, sonicate. The conditions are not specified here because results depend on the type of sonicator probe used, the power setting, and the shape and size of the vessel holding the cells. Avoid long sonication times where the sample could heat up; instead, break up the sonication into bursts with cooling in between. Sonicate until the sample is no longer viscous. If the DNA is not sheared by sonication, the extract will be so viscous that it will be difficult to work with.
4. Centrifuge the lysate at 39,000 x g for 20 min to remove debris. If the target protein is in the supernatant remaining after centrifugation, filter the supernatant through a 0.45 micron membrane to prevent clogging of the resin (syringe-end filters are convenient for this purpose). The sample is now ready for binding to S-protein Agarose (see page 7).
5. If the target protein is in the insoluble fraction (pellet from Step 4), decant the supernatant and resuspend the pellet in 10 ml 1X Bind/Wash Buffer. Sonicate or process with a Dounce homogenizer to resuspend the inclusion bodies.
6. Centrifuge at 12–20,000 x g for 15 min to collect the washed inclusion bodies. Decant the supernatant completely and resuspend the pellet in 3.3 ml of 1X Bind/Wash Buffer containing 6 M urea.
7. Incubate on ice for 1 h to dissolve the protein. Dilute 3–fold with 1X Bind/Wash Buffer (not containing urea) to bring the urea concentration to 2 M. Remove any remaining insoluble material by centrifugation at 39,000 x g for 20 min. Filter the supernatant through a 0.45 micron membrane and proceed to binding.

   Note: this procedure works well with proteins that remain soluble when diluted to 2 M urea; otherwise the target protein may precipitate during purification. This will vary with each protein and must be tested empirically.

Insect cell extracts

The purification procedure starts with cells, or medium, produced from a baculovirus infection. The first issue to address is the identification of the most appropriate time for harvest. This should be determined in a time-course experiment, and by monitoring both the amounts and integrity of the product.

In initial studies, do not freeze either the cells or medium until it is determined that it is safe to do so (i.e., it produces no untoward effects on the target protein). Since cellular proteases may be present in the extracts, do the initial steps at 4 °C. Protease inhibitors may be added if necessary; however, *the use of serine protease inhibitors should be avoided when planning to use thrombin for elution of the target protein, because residual inhibitor could inactivate the thrombin*. To eliminate possible degradation by a baculovirus-encoded protease, we recommend the use of BacVector-3000 Triple Cut Virus DNA (which lacks the viral cathepsin gene) to prepare baculovirus recombinants.

Monitor the distribution of protein, yields and integrity of the protein at all steps. The use of S·Tag™ detection products is particularly advantageous for such assays. Once the preliminary studies have been undertaken, develop a streamlined protocol for protein purification.

If it is known that the protein is secreted into the medium, determine the distribution between cells and medium. It may be necessary to process both samples. The use of serum-free medium facilitates the purification of proteins secreted into the medium.

- Small amounts of culture medium may be used directly for S•Tag affinity purification following a centrifugation step to remove particulates. It is advisable when dealing with large volumes of culture medium to concentrate the medium with a size exclusion filter to reduce the volume, and remove media components and other low molecular weight products. Addition of extra buffer of choice and reconcentration will help further in removing such materials. To avoid

clogging of the filter, it is important to rigorously remove particulate materials by high speed centrifugation beforehand, provided that the target product is not pelleted at the same time.

- Cell extracts may be prepared by a variety of procedures and in a buffer of choice. First try a simple freeze-thaw in a buffer lacking detergent (e.g., PBS). Usually once is enough to obtain cell disruption, but check by examination in a microscope and repeat as necessary, followed by centrifugation to remove particulate products.
- For proteins that remain in the particulate phase, subsequently extract the pellet with a buffer that contains a mild detergent. DNA is usually not a problem when processing cells late in the baculovirus infection cycle, but if the sample is viscous, briefly vortex to shear the DNA, or sonicate to reduce viscosity. [Be aware that sonication may be deleterious to some proteins and structures. Avoid heating by keeping the samples on ice during sonication.]
- The use of detergent will liberate some membrane proteins, which may prove difficult to remove at subsequent steps, depending on the protein. A common lysis buffer is 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 (1X Bind/Wash Buffer), which contains protease inhibitors. Use a ratio of 1 ml lysis buffer per 1 x 10^8 cells (or less if desired). Incubate the mixture on ice for 45 minutes. After centrifugation, determine the distribution of the protein. Alternatively, to avoid nuclear lysis, substitute 1% NP40 for the Triton X-100. Centrifuge at low speed to pellet nuclei and insoluble proteins.
- If the protein remains in the particulate phase, try addition of 1% deoxycholate to the detergent mixture and repeat the extraction. Also try high salt extraction (various salts and concentrations up to 2 M, depending on the salt), and determine the effects of different buffers at different pH values on solubilizing the protein.
- For proteins that may be bound to nucleic acids, try the appropriate nuclease treatment, followed by salt extraction. For nuclear proteins, try using 1% NP40 to solubilize plasma membrane and cytosolic proteins.
- If all else fails and the protein remains insoluble, after all the soluble products have been removed it may be necessary to resort to a harsher denaturation protocol (e.g., 6 M urea). After incubation in 6 M urea, dilute to 2 M urea and centrifuge or filter before proceeding to the binding step.

Purification

Binding

1. Gently suspend the S-protein Agarose by inversion and add 2 ml of the slurry (equivalent to 1 ml settled resin) to the desired amount of soluble protein extract prepared above. If binding from partially denatured protein in 2 M urea, add an equal volume of 4 M urea in 1X Bind/Wash Buffer to 2 ml of resin prior to adding it to the protein extract. The resin is most conveniently transferred with a 1 ml wide-mouth pipet tip. Mix thoroughly and incubate at room temperature on an orbital shaker for 30 min. Do not shake vigorously as this will tend to denature protein.
2. Centrifuge the entire volume at 500 x g for 10 min and carefully decant supernatant.
3. Resuspend the S-protein Agarose, which now contains bound S•Tag fusion protein, in 5 ml 1X Bind/Wash Buffer (include 2 M urea if using partially denatured protein). Mix by gently vortexing or by repeated inversion (avoid vigorous vortexing).
4. Repeat steps 2 and 3 twice more to wash away unbound proteins. Remove the final supernatant and elute the target protein either with Biotinylated Thrombin or using GuSCN, pH, or $MgCl_2$ as described below.

Elution with Biotinylated Thrombin

1. Prepare 15 ml 1X Thrombin Cleavage Buffer by diluting 1.5 ml of the 10X stock (supplied) in 13.5 ml deionized water (remember to add 2 M urea if purifying proteins from solubilized inclusion bodies).
2. Resuspend the washed S-protein Agarose containing the bound target protein in 5 ml 1X Thrombin Cleavage Buffer from Step 1. Centrifuge at 500 x g for 10 min and carefully remove the supernatant. Resuspend and centrifuge again to fully equilibrate the resin in 1X Thrombin Cleavage Buffer. Remove as much supernatant as possible.
3. Resuspend the washed, equilibrated agarose pellet in a final volume of 2 ml 1X Thrombin Cleavage Buffer (plus 2 M urea if necessary). Add 25 units Biotinylated Thrombin and incubate for up to 2 h at room temperature on an orbital shaker. The target protein released from the agarose no longer contains the S•Tag peptide. The Biotinylated Thrombin is removed with Streptavidin Agarose.

   Note: Biotinylated Thrombin is fully active in the presence of 2 M urea, if used in the buffer. The recommended cleavage conditions (25 U, 2 h) are optimal for a variety of proteins; however, if secondary cleavage is observed with a particular protein, less enzyme can be used and/or the incubation period varied.
4. Thoroughly resuspend the Streptavidin Agarose by inversion. Add 800 ml of the slurry to the cleavage reaction see note below if using urea in the buffers). Mix thoroughly and incubate for 10 min at room temperature on an orbital shaker.

   Note: the Streptavidin Agarose, in its supplied buffer, can be added directly to the Biotinylated Thrombin/target protein/S-protein Agarose mixture without the need for preequilibration If urea has been included in the procedure, first bring the urea concentration in the Streptavidin Agarose slurry to 2 M by adding an appropriate volume from a concentrated stock solution. If desired, the Strepavidin Agarose can be pre-equilibrated in 1X Thrombin Cleavage Buffer to avoid the addition of other components (phosphate, azide) in the supplied storage buffer. If not using a Spin Filter to remove the agarose, proceed to step 7.
5. Transfer the entire reaction to a Spin Filter which has been placed in a collection tube (included in the Spin Filter package). Centrifuge at 500 x g for 5 min.
6. Without removing the filtrate in the lower chamber, add 1.25 ml 1X Thrombin Cleavage Buffer (plus urea, if necessary) to the “cake” of resin in the upper chamber, and centrifuge at 500 x g for 5 min. The clear filtrate contains the purified target protein, which can be used directly in many applications. See “Processing the sample after elution” for suggested procedures for concentration and changing the buffer.
7. **Optional:** If a spin filter is not used, centrifuge at 500 x g for 5 min and transfer the supernatant, which contains the target protein, to a fresh tube. Wash the agarose pellet with an additional 1–2 ml 1X Thrombin Cleavage Buffer, centrifuge and pool the second supernatant with the previous supernatant.

Elution with GuSCN, pH, or $MgCl_2$

1. Resuspend the washed resin containing the bound target protein in 1.5X settled resin volumes of one of the following elution buffers:

   0.2 M citrate, pH 2*
   or 3 M magnesium chloride
   or 1X Bind/Wash Buffer containing 3 M guanidine thiocyanate
   Incubate for 10 min at room temperature; mix gently every few minutes to keep the resin suspended.
   **Note: To make this buffer, prepare a 2 M stock of citric acid, if necessary adjust the pH to 2.0 with 10 M KOH, and dilute to 0.2 M.*
2. Transfer the entire reaction to a Spin Filter which has been placed in a collection tube (included in the Spin Filter package). Centrifuge at 500 x g for 5 min.
3. Without removing the filtrate, add 1.25 ml elution buffer to the "cake" of resin in the upper chamber, and centrifuge at 500 x g for 5 min. The clear filtrate contains the purified target protein.
4. **Optional:** If a spin filter is not used, centrifuge at 500 x g for 5 min and transfer the supernatant, which contains the target protein, to a fresh tube. Wash the agarose pellet with an additional 1–2 ml elution buffer, centrifuge and pool the second supernatant with the previous supernatant.
5. Change the buffer in the eluted sample by one of the methods described in the next section. Note that some proteins may precipitate when the buffer is changed.
6. The S-protein Agarose may be recycled by washing 3 more times with elution buffer, then 3 times with 1X Bind/Wash Buffer. Store at 4°C in 1X Bind/Wash Buffer containing 0.02% sodium azide or other preservative.

Processing the sample after elution

The buffer of the purified sample may be changed or the sample concentrated by one of several methods. Note that, depending on the solubility characteristics of target protein, changing the buffer may result in precipitation. Three alternative procedures are:

1. Dialyze into the buffer of choice. After dialysis, the sample may be concentrated by sprinkling solid polyethylene glycol (15,000-20,000 molecular weight) or Sephadex G-50 (Pharmacia) on the dialysis tubing. Use dialysis tubing with an exclusion limit of 6,000 MW or less, and leave the solid in contact with the tubing until the desired volume is reached, replacing it with fresh solid as necessary.
2. Use plastic disposable microconcentrator units (e.g. Centricon; Amicon) as directed by the manufacturer to both desalt and concentrate the sample by ultrafiltration.
3. Desalt the sample by gel filtration on Sephadex (G-10, G-25, G-50; Pharmacia) or Bio-Gel (P6DG, P-10, P-30; Bio-Rad).

Reference

1. Kim, J.-S., and Raines, R.T. (1993) *Protein Science* **2,** 348-356.