T7•Tag® Affinity Purification Kit

Description

T7•Tag Affinity Purification KIt 69025-3

The T7•Tag Affinity Purification Kit is designed for rapid immunoaffinity purification of target proteins that carry the 11aa T7•Tag sequence (*i.e.* the initial 11aa of the T7 gene *10* protein). Purification is based on binding target proteins to T7•Tag monoclonal antibody which is covalently coupled to cross-linked agarose beads, washing away unbound proteins, and eluting at pH 2.2. Capacity will vary somewhat between different target proteins, but the beads are standardized to bind a minimum of 300µg T7•Tag β-galactosidase per ml of settled resin. The beads can be used in either batch or column modes and can be recycled a minimum of five times without loss of binding activity.

The following vectors can be used to produce fusion proteins carrying the T7•Tag peptide (given appropriate insertion of target sequences):

pET-3a-d	pET-21a-d(+)	pGEMEX (Promega)
pET-3xa-c	pET-23a-d(+)	pRSET (Invitrogen)
pET-5a-c	pET-24a-d(+)	
pET-9a-d	pET-28a-c(+)	
pET-11a-d	pTOPE®-1b(+)	
pET-17b	pSCREEN™-1b(+)	
pET-17xb	pEX*lox*®	

Components

- 1ml T7•Tag Antibody Agarose (50% slurry in 1X Bind/Wash Buffer containing 0.02% sodium azide)
- 20ml 10X T7•Tag Bind/Wash Buffer (10X = 42.9mM Na_2HPO_4, 14.7mM KH_2PO_4, 27mM KCl, 1.37M NaCl, 1% Tween-20, 0.02% sodium azide, pH 7.3)
- 20ml 10X T7•Tag Elute Buffer (10X = 1M citric acid pH 2.2)
- 20ml 1X T7•Tag Neutralization Buffer (2M Tris base pH 10.4)
- 1 chromatography column with closures
- Protocol

Available separately:

Product	Size	Cat. #
T7•Tag Antibody Agarose	2ml	69026-3

Procedure

This procedure begins with a cell culture that has been induced for target protein production. The target protein must be expressed in an appropriate vector (listed above) to include the T7•Tag sequence. For a detailed description of the vectors and protein expression considerations, please refer to the technical literature that accompanies Novagen's pET vector systems.

The T7•Tag Affinity Purification Kit is optimized for the purification of soluble proteins under native conditions. The protocol describes column purification, however the method can be adapted to batch-wise purification using the same volumes for equilibration, binding, washing and elution steps. When performing batch purification, do not neutralize the eluted protein fraction until supernatant has been decanted from the resin since the protein can be re-bound by the antibody agarose upon neutralization.

Proteins can be purified under mild denaturing conditions by the addition of urea to a final concentration of up to 2M in the 1X T7•Tag Bind/Wash and T7•Tag Elute Buffers. At a final concentration of 2M urea, the column capacity will be reduced by approximately 67% to around 100µg/ml resin. One option for the purification of insoluble pro-

teins is to solubilize inclusion bodies in a buffer containing 6-8M urea and then dilute solubilized protein fraction to 2M urea with 1X Bind/Wash Buffer, remove insoluble debris by centrifugation, and load the soluble fraction on the T7•Tag Affinity Column. The purification is performed as described below, except the 1X Bind/Wash and 1X Elute buffers are supplemented with 2M urea to maintain protein solubility. For more information on the isolation of inclusion bodies and the handling of insoluble proteins, consult Novagen's pET System Manual (available on request).

Cell Extract Preparation

The following protocol is for 100ml induced culture; it can be scaled up or down proportionately as desired.

1. Prepare 50ml 1X T7•Tag Bind/Wash Buffer by diluting 5ml of the 10X T7•Tag Bind/Wash Buffer supplied with 45ml deionized water. Place 10-15ml of the buffer in a separate tube on ice for cell resuspension. Maintain the remainder of buffer at room temperature.
2. Harvest the cells by centrifugation at 5000 × g for 5 minutes. Decant the supernatant and allow the cell pellet to drain as completely as possible. Resuspend the cells in 10ml ice-cold 1X T7•Tag Bind/Wash Buffer. If resuspension is difficult, a Dounce homogenizer, a 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 clog up the column.
4. Centrifuge the lysate at 39,000 × g for 20 minutes to remove debris. Optimal results are obtained if the post-centrifugation supernatant is filtered through a 0.45 micron membrane to prevent clogging of the resin (syringe-end filters are convenient for this purpose).

Cell Extract Preparation: Insoluble Proteins

The procedure entails isolating inclusion bodies in regular Bind/Wash buffer, and then suspending them in Bind/Wash buffer plus 6M urea to solubilize the protein.

1. Harvest the cells by centrifugation at 5000 × g for 5 minutes. Decant the supernatant and allow the cell pellet to drain as completely as possible. Resuspend the cells in 10ml 1X Bind/Wash buffer **that does not contain urea**.
2. Sonicate briefly to resuspend the pellet thoroughly and to shear the DNA.
3. Centrifuge at 20,000 × g for 15 minutes to collect the inclusion bodies and cellular debris while leaving other proteins in solution.
4. Remove the supernatant and suspend the pellet in 5ml 1X Bind/Wash buffer (without urea). Repeat Step 3. Sonication may be necessary to resuspend the pellet. Sometimes repeating this step several times releases more trapped proteins.
5. Remove the supernatant from the final centrifugation and resuspend the pellet in 10ml 1X T7•Tag Bind/Wash buffer containing 6M urea. Incubate on ice for 1 hour to completely dissolve the protein. Remove insoluble material by centrifugation at 39,000 × g for 20 minutes. Filter the supernatant through a 0.45 micron membrane before binding to the resin.
6. Carry out binding, wash and elution as described below in the presence of 2M urea. Dilute the extract to 2M urea with 1X T7•Tag Bind/Wash buffer and centrifuge at 15,000 × g for 10 minutes. Save the pellet (in case the fusion protein precipitates at the lower urea concentration) and load the supernatant on the beads.

Note: this procedure works well with proteins that remain soluble when diluted to 2M urea; some target proteins may precipitate during purification. This will

vary with each protein and must be tested empirically.

Immunoaffinity Purification: Column Procedure

1. Snap off the lower fitting from the supplied chromatography column and mount on an appropriate support. If desired, "prime" the column with several ml of Bind/Wash Buffer to eliminate air from the bottom frit area.
2. Allow the T7•Tag Antibody Agarose to equilibrate to room temperature. Fully suspend the 50% slurry and transfer the entire contents (2ml) into the provided chromatography column. Allow storage buffer to flow to waste. Note that the storage buffer contains 0.02% sodium azide; gloves are strongly recommended. Do not be concerned if the column runs dry briefly prior to step 3.
3. Equilibrate the resin by washing with ten column volumes (10ml) of 1X T7•Tag Bind/Wash Buffer at room temperature.
4. Reserve an aliquot of the crude sample prepared above for later gel analysis. Bring the cell extract to room temperature and load the column. Collect the unbound protein fraction (flow-through) in a 15ml tube.
5. Wash the column with ten column volumes (10ml) of 1X T7•Tag Bind/Wash Buffer. Label five 1.5ml microcentrifuge tubes and dispense 150µl of 1X T7•Tag Neutralization Buffer into each tube.
6. Prepare 5ml of 1X T7•Tag Elute Buffer by diluting 0.5ml of 10X T7•Tag Elute Buffer with 4.5ml of deionized water.
7. Elute the bound fusion protein with 5 serial 1ml volumes of 1X T7•Tag Elute Buffer into each of the tubes prepared in step 5. Following the elution of each fraction, cap the sample and invert several times to mix the elution and neutralization buffers. Place eluted fractions on ice. The neutralized 1X T7•Tag Elute Buffer is compatible with direct analysis of the fractions using a Coomassie blue dye-binding protein assay and/or SDS-polyacrylamide gel electrophoresis.
8. Wash the column with ten column volumes (10ml) of 1X T7•Tag Bind/Wash Buffer and replace the lower cap to seal the column outlet, fill with 1X T7•Tag Bind/Wash Buffer and replace the upper cap. For long term storage, it is desirable to add sodium azide to a final concentration of 0.02% to prevent bacterial growth. Seal the upper and lower caps with Parafilm (American Can, Inc.), and store at 4°C. **DO NOT FREEZE.**

Immunoaffinity Purification: Batch-wise Purification

1. Gently suspend the 50% slurry of T7•Tag Antibody Agarose by inversion and add the desired amount to the sample extract. The resin is most conveniently transferred with a disposable polyethylene transfer pipet. Mix thoroughly and incubate at room temperature on an orbital shaker for 30 minutes. Do not shake vigorously as this will tend to denature protein.
2. Centrifuge the entire volume at 500 × g for 10 minutes and carefully decant supernatant.
3. Resuspend the beads in 5ml 1X Bind/Wash Buffer. Mix by gently vortexing or by repeated inversion (avoid vigorous vortexing). Add urea to 2M if purifying proteins from solubilized inclusion bodies.
4. Repeat steps 2 and 3 twice more. For proteins that are expressed at low levels (less than 5% of the total protein), more washes may be necessary to remove unbound proteins. Remove the final supernatant and proceed to elution.
5. Elute the bound fusion protein by resuspending the beads in 1.5 volumes of 1X Elute Buffer (plus 2M urea if purifying from solubilized inclusion bodies). Incubate for 10 minutes at room temperature; mix gently every few minutes to keep the resin suspended.
6. Centrifuge at 500 × g for 10 minutes. Recover the supernatant, which contains the target protein.
7. Repeat steps 5 and 6. Pool the second supernatant with the first.
8. Neutralize the recovered eluate pool by adding 150µl 1X Neutralization Buffer per ml total pool volume.

Processing the Sample after Elution

The buffer of the purified sample may be changed or the sample concentrated by one of several methods. 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).

Note: all conditions used to concentrate and/or further process the purified protein should be tested to ensure that the protein remains soluble.