**I. Description**

The S•Tag System is a protein tagging and detection system based on the interaction of the 15aa S•Tag peptide with ribonuclease S-protein. This specific binding is of high affinity (K_d = 10^-9M; F.M. Richards and H.W. Wyckoff (1971) in “The Enzymes”, Vol. IV (Boyer, P.D., Ed.), pp. 647-806, Academic Press, New York) and allows convenient detection and purification of any protein fused with the S•Tag sequence (J.-S. Kim and R.T. Raines (1993) *Protein Science* 2, 348-356). Vectors available for making S•Tag fusions are pET-29a-c(+), pET-30a-c(+), pET-32a-c(+) and pSCREEN™-1b(+) (for T7 RNA polymerase-driven expression in *E. coli*) and pCITE®-4a-c(+) for optimal production of proteins in *vitro* with Novagen’s Red Nova® Lysate.

Whereas neither the S•Tag sequence nor the S-protein alone has enzymatic activity, the two components together form active ribonuclease. Therefore, the assay of ribonuclease activity following the addition of purified S-protein provides a direct measurement of the concentration of S•Tag fusion protein. The S•Tag Rapid Assay Kit is designed for this purpose. In practice, a sample is added to a buffer containing purified S-protein and the ribonuclease substrate poly(C). After a brief 5 minute incubation, the reaction is stopped with trichloroacetic acid. The resulting precipitate is removed by centrifugation. Activity is measured by reading the absorbance of the supernatant at 280nm, which increases as the poly(C) is broken down into acid-soluble nucleotides by the enzyme (S.B. Zimmerman and G. Sandeen (1965) *Anal. Biochem.* 10, 444-449). By comparing the results with a known S-peptide standard included in the kit, the molar concentration of target protein in the sample can be determined. As little as 20fmol of target protein can be detected in a 5 minute incubation with the S•Tag Rapid Assay.

S•Tag fusion proteins also can be quickly detected on blots using the S•Tag Western Blot Kit. Blots are prepared as usual, blocked briefly with gelatin, and then incubated with S-protein alkaline phosphatase conjugate. Target bands are visualized with supplied NBT/BCIP substrates. An optimized protocol allows fully developed blots to be produced in 45 minutes with detection of nanogram amounts of target proteins. The kit also includes a positive control, which is a ready-to-load mixture of S•Tag-containing proteins called the Perfect Protein™ Western Markers. These molecular weight markers are a novel set of seven recombinant proteins with defined sizes at convenient intervals covering 15-150 kilodaltons. Since each protein carries the S•Tag peptide, the markers serve as highly accurate size standards on Western blots. The sizes of the markers are 15,000, 25,000, 35,000, 50,000, 75,000, 100,000 and 150,000 daltons.
II. Kit Components

The **S•Tag Rapid Assay Kit** contains enough components for 100 standard assays.
- 1ml **S•Tag Grade S-protein 50ng/µl**
- 4ml **10X S•Tag Assay Buffer (200mM Tris-HCl pH 7.5, 1M NaCl, 1mg/ml poly(C))**
- 50µl **S•Tag Standard (0.05pmol/µl)**

**Storage**
Store all components at -70°C.

**Additional Materials Needed for S•Tag Rapid Assays**
1. Sterile deionized water (need 0.4ml/sample)
2. Cold (4°C) 25% w/v trichloroacetic acid (TCA) (need 100µl/sample)
3. Microcentrifuge at 4°C.
4. UV spectrophotometer set at 280nm.

The **S•Tag Western Blot Kit** contains enough components for 25 medium sized (10cm x 10cm) Western Blots.
- 50µl **S-protein Alkaline Phosphatase Conjugate**
- 125µl **Perfect Protein™ Western Markers**
- 40ml **10% Gelatin (contains 0.02% azide)**
- 35ml **20% Tween 20 (contains 0.05% azide)**
- 1.5ml **BCIP (42mg/ml in dimethylformamide)**
- 1.5ml **NBT (83mg/ml in 70% dimethylformamide)**
- 20ml **20X Alkaline Phosphatase Buffer**

**Storage**
Store S-protein Alkaline Phosphatase Conjugate, NBT and BCIP at -20°C. Store Perfect Protein Western Markers, 20X Alkaline Phosphatase Buffer and 10% Gelatin at 4°C. Store 20% Tween 20 at room temperature.

**Additional Materials Needed for S•Tag Western Blots**
1. Nitrocellulose or nylon membranes
2. SDS-gel electrophoresis and blotting apparatus, transfer buffer
3. Incubation trays
4. 10X Tris-buffered saline (10X TBS; 100mM Tris-HCl pH 8.0, 1.5M NaCl)

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III. **S•Tag Rapid Assay Protocol**

**A. Assay for in vitro synthesized proteins**

This protocol begins with a protein derived from a standard translation reaction with Red Nova® Lysate programmed with RNA transcripts made from S•Tag vectors. For each set of samples, a blank without added target protein and the S•Tag Standard included in the kit are run in parallel.

1. Assemble the following components in a set of sterile 1.5ml microcentrifuge tubes: This example uses 1 unknown (Tube #3).

<table>
<thead>
<tr>
<th>Component</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>348µl</td>
<td>346µl</td>
<td>348µl</td>
</tr>
<tr>
<td>S•Tag Standard</td>
<td>-</td>
<td>2µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample lysate</td>
<td>-</td>
<td>-</td>
<td>2µl</td>
</tr>
<tr>
<td>Blank lysate*</td>
<td>2µl</td>
<td>2µl</td>
<td>-</td>
</tr>
<tr>
<td>10X S•Tag Assay Buffer</td>
<td>40µl</td>
<td>40µl</td>
<td>40µl</td>
</tr>
<tr>
<td>S•Tag Grade S-protein</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

* The blank lysate should be the no template control translation reaction. Start the reactions by adding the S-protein at timed intervals (e.g. every 20 sec).
2. Incubate the tubes at 37°C for exactly 5 min.
3. Stop the reactions by adding 100µl ice-cold 25% TCA, vortex and place on ice for 5 min.
4. Centrifuge the tubes at 14,000 x g for 10 min.
5. Read the absorbance of the supernatants at 280nm. Zero the spectrophotometer with sample #1.
6. To calculate the amount of S•Tag protein in the sample, use the following equation:
   \[ \text{Tube #3 } A_{280} \times 0.1 \text{ pmol S•Tag Standard/Tube #2 } A_{280} = \text{ pmol/µl} \]
   The mass of target protein can be determined by multiplying the above number by the molecular weight.
   
   For example, if the \( A_{280} \) for the S•Tag Standard (#2) was 0.5 and the sample (#3) was 1.0 and the target protein has a molecular weight of 50,000 daltons, then
   
   \[ 1.0/2\mu l \times 0.1\text{pmol/0.5} = 0.1\text{pmol target protein/µl} \]
   
   \[ 0.1\text{pmol} \times 50\text{ng/pmole} = 5\text{ng/µl} \]

   If a 25µl standard translation reaction was run, then the yield of target protein was 25 x 5ng/µl = 125ng.

B. Assay for proteins in bacterial extracts

This protocol begins with a cell extract prepared from an induced pET-29a-c(+), pET-30a-c(+), pET-32a-c(+) or pSCREEN™ construct. Extracts can be total, soluble or insoluble protein (a recommended protocol for preparing total cell protein samples by SDS extraction is given in the Appendix). In general, samples need to be diluted from 1:10 to 1:500 in water to be in the linear range of the assay. If the target protein is highly expressed, greater dilution may be necessary. Up to 10µl of a 1/100 dilution of 6M urea or guanidine HCl can be added to the assay with little effect. For each set of samples, a blank without added target protein and the S•Tag Standard included in the kit are run in parallel.

1. Assemble the following components in a set of sterile 1.5ml microcentrifuge tubes: This example uses 1 unknown (Tube #8).

<table>
<thead>
<tr>
<th>Component</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>348µl</td>
<td>346µl</td>
<td>348µl</td>
</tr>
<tr>
<td>S•Tag Standard</td>
<td>–</td>
<td>2µl</td>
<td>–</td>
</tr>
<tr>
<td>Sample extract</td>
<td>–</td>
<td>–</td>
<td>2µl</td>
</tr>
<tr>
<td>Blank extract*</td>
<td>2µl</td>
<td>2µl</td>
<td>–</td>
</tr>
<tr>
<td>10X S•Tag Assay Buffer</td>
<td>40µl</td>
<td>40µl</td>
<td>40µl</td>
</tr>
<tr>
<td>S•Tag Grade S-protein</td>
<td>10µl</td>
<td>10µl</td>
<td>10µl</td>
</tr>
</tbody>
</table>

* The blank extract should be prepared from cells that do not contain the target plasmid. Uninduced cells can also be used but may exhibit some activity due to basal expression of the target protein.

Start the reactions by adding the S-protein at timed intervals (e.g. every 20 sec).

2. Incubate the tubes at 37°C for exactly 5 min.
3. Stop the reactions by adding 100µl ice-cold 25% TCA, vortex and place on ice for 5 min.
4. Centrifuge the tubes at 14,000 x g for 10 min.
5. Read the absorbance of the supernatants at 280nm. Zero the spectrophotometer with sample #1. If the absorbance of the sample is greater than 1.5, the assay should be repeated with a more dilute sample to stay within the linear range.
6. To calculate the amount of S•Tag protein in the sample, use the following equation:

\[ \text{Tube #3 } A_{280}/2 \mu l \times 0.1 \text{ pmol S•Tag Standard/Tube #2 } A_{280} = \text{pmol/} \mu l \]

The mass of target protein can be determined by multiplying the above number by the molecular weight.

For example, if the \( A_{280} \) for the S•Tag Standard (#2) was 0.5 and a 1:200 dilution of the sample (#3) was 1.0 and the target protein has a molecular weight of 50,000 daltons, then

\[ 1.0/2 \mu l \times 0.1 \text{ pmol/0.5} \times 200 = 20 \text{ pmol target protein/} \mu l \]

\[ 20 \text{ pmol/} \mu l \times 0.05 \mu g/\text{pmol} = 1 \mu g/ \mu l \]

Notes:

a. The S•Tag Standard is a 20aa peptide with the sequence LysGluThrAlaAlaAlaLysPheGluArgGlnHisMetAspSerSerThrSerAlaAla and molecular mass of 2166 daltons.

b. In general, the level of endogenous RNase in cellular extracts of E. coli and reticulocyte lysates does not result in significant background. This background is negated by including a blank sample prepared from cells that do not contain the target plasmid. In addition, try to keep the buffer composition of the blank as similar to the unknown as possible.

c. When assaying purified proteins, use a buffer containing 10mM Tris-HCl pH 7.5 and 0.1% Triton X-100 to dilute the sample to avoid loss of material on tube surfaces.

d. The above assays are designed for using standard 1ml cuvettes; the assay volume can be scaled down proportionately to accommodate smaller cuvettes (e.g. one-half scale is appropriate for 0.3ml cuvettes).

e. Keep in mind that RNase is being reconstituted in this assay and could contaminate cuvettes used for reading the results. Clean the cuvettes with 0.5N NaOH or a strong quaternary amine detergent to prevent carryover.

IV. S•Tag Western Blot Protocol

1. Run an SDS-polyacrylamide gel of the S•Tag fusion sample. Suggested protocols for sample preparation are given in the Appendix. Load 5\( \mu l \) of the Perfect Protein™ Western Markers to one lane. This lane should give bands at 15,000, 25,000, 35,000, 50,000, 75,000, 100,000 and 150,000 daltons when detected with the kit reagents. A 5\( \mu l \) load contains 10ng of each species, except the 50,000 dalton band, which is 2X the others (20ng). The 150,000 and 15,000 dalton bands may give weaker signals than the other species depending on the percentage gel, membrane support and transfer conditions used. Due to its large size, the 150,000 dalton band may not transfer completely, and is present at a significantly lower molar amount than the others. The 15,000 dalton band may not bind well to the membrane (particularly 0.45\( \mu \) pore size nitrocellulose) due to its small size.

2. Transfer the proteins to nitrocellulose electrophoretically. Any standard device can be used according to the manufacturer’s instructions. The recommended standard transfer buffer is 12mM Tris base, 96mM glycine, pH 8.3, 20% methanol.

3. Remove the nitrocellulose from the blotting apparatus and incubate in TBST + 1% Gelatin at room temperature for 15 minutes to block excess protein binding sites. (TBST = 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% Tween 20). Prepare a 10X stock of TBS and combine 1/10 vol of this solution, 1/10 vol of 10% Gelatin and 1/200 vol of 20% Tween with 0.8 vol sterile water to make TBST + 1% Gelatin. Note that TBST is required for Steps 4 and 5 only.

4. Incubate the membrane with a 1/5,000 dilution of S-Protein Alkaline Phosphatase Conjugate in TBST for 15 min at room temperature.
5. Wash the membrane 4 times with 25ml in TBST at room temperature. This can be done in 1-2 min by adding the wash solution, briefly shaking, and decanting.

6. Develop the color as follows: add 60µl NBT + 60µl BCIP per 15ml 1X AP buffer (diluted with water), then incubate the blot in this solution at room temperature until color develops. Strong purple signals should appear within 2-10 minutes. To stop the reaction, rinse the blot in deionized water, allow to air dry, and store wrapped in plastic at room temperature.

**Dot blot protocol**

1. Induce a culture of the desired pET recombinant and prepare a crude protein extract as described in the Appendix. Make serial dilutions of the extract in 10mM Tris-HCl, 25mM EDTA, pH 8.0 covering a range of 2µg/ml - 200µg/ml protein (make two additional series for the Perfect Protein Western Markers and E. coli lysate made from cells that do not carry the target plasmid (negative control; optional). If you use the Perfect Protein Western Markers, spotting 1µl should give a strong signal when detected with the kit.

2. Spot 1µl samples directly onto nitrocellulose. Allow to air dry for several minutes.

3-6. Proceed exactly as described for Steps 3-6 above for Western blots.

**Appendix: Rapid preparation of total cell extracts for S•Tag analysis**

A complete discussion of cloning target genes in pET vectors is found in the pET Manual, available upon request from Novagen.

After a target plasmid is established in BL21(DE3), HMS174(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-29, pET-30 and pET-32 constructions a final concentration of 1mM IPTG is recommended; use 0.4mM IPTG for pSCREEN™ constructs. An example of an induction protocol is presented below; this can be scaled up or down as required. Note that 1ml of induced culture can produce enough target protein for 1000 S•Tag Rapid Assays and 100 S•Tag Western Blots.

1. Incubate a single colony or a few µl from a glycerol stock into 2ml LB medium containing 30µg/ml kanamycin (substitute 50µg/ml carbenicillin for pET-32 and pSCREEN), plus 34µg chloramphenicol/ml if the cells carry pLysS or pLysE. Use a Falcon 2059 culture tube or equivalent and incubate with shaking at 37°C until the OD$_{600}$ reaches 0.6-1.0. Store the culture at 4°C overnight. A fresh glycerol stock also can be prepared from this culture by adding 0.1 vol 80% sterile glycerol, mixing thoroughly, and storing at -70°C.

2. The following morning, collect the cells (1ml) by centrifugation (30 sec in a microcentrifuge). Wash the cells by resuspending in 1ml fresh media plus kanamycin and centrifuging. Resuspend the cells in 1ml fresh medium plus kanamycin and use this to inoculate 50ml medium in a 250ml Erlenmeyer flask. (For good aeration, add medium up to only 20% of the total flask volume.)

3. Incubate with shaking at 37°C until OD$_{600}$ reaches 0.6-1 (about 3 hours).

4. Remove a 1ml sample for the uninduced control to a sterile culture tube (e.g. Falcon 2059). Keep incubating this sample with shaking along side the induce culture. Add IPTG to 1mM to the remainder of the culture (500µl of 100mM IPTG) and continue the incubation for 2-3hr. Determine the OD$_{600}$ at harvest.

5. Remove 1ml samples of the induced and control cultures to microcentrifuge tubes. Harvest the cells by centrifugation at 12,000 $\times$ g for 30 sec.

6. Remove as much supernatant as possible and resuspend the cell pellet in 100µl 1% SDS. Mix thoroughly and heat at 70°C for 10 min. Intermittent vortex mixing may be needed to homogenize the extract.

7. Centrifuge the extract at 12,000 $\times$ g for 1 min. Remove the supernatant to a fresh tube. Dilute 10 to 100-fold in water for the S•Tag Rapid Assay. For S•Tag Western Blots, add an equal volume of 2X SDS sample buffer to the extract, heat at 70°C for 5 min, cool, and load 1-10µl. A 10µl sample should also be suitable for staining with Coomassie blue.