S•Tag Rapid Assay

The S•Tag rapid assay is based on the reconstitution of ribonucleolytic (RNase S) activity. A sample containing the target protein is added to a buffer containing purified S-protein and the ribonuclease substrate poly(C). After a brief incubation the reaction is stopped with trichloroacetic acid and the resulting precipitate 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 (3). In the presence of excess S-protein the level of RNase S activity is directly proportional to the amount of recombinant protein present in the sample.

Typical assay profiles are shown in Figure 1. A linear signal was obtained during a 5 minute incubation at 37°C. In this experiment, 2µl of crude translation mix and 2µl of a 1/100 dilution of *E. coli* lysed with 1% SDS were used as samples. Appropriate blanks serve as controls for any endogenous RNase activity, which appears to be minimal under the conditions of the Rapid Assay. By comparing the absorbance profile of a known standard (provided in the kit), the molar concentration of the S•Tag target protein can be calculated.

Unlike amino acid incorporation assays (both radioactive and non-radioactive), S•Tag Rapid Assay is independent of protein size, amino acid composition and endogenous amino acid pool size. It, therefore, represents a much more accurate method for measuring *in vitro* translation efficiencies. The method also is extremely versatile for measuring protein expression in cells, since it can be applied to both soluble and insoluble proteins. Up to 10µl of a 1/100 dilution of 6M urea or guanidine HCl can be added to the assay with little effect (data not shown). Multiple samples can easily be screened for expression levels by preparing crude extracts of whole cells in 1% SDS. Since the assay will detect as little as 20fmol target protein in a 5 minute incubation, even poorly expressed proteins can be measured with a high degree of accuracy. Typically, 1ml of induced culture provides enough material for over 1000 S•Tag Rapid Assays or 100 S•Tag Western Blots.

S•Tag Western Blot

Western blotting of S•Tag proteins provides a second means of detection and allows visualization of protein integrity. The S•Tag Western Blot Kit is based on the interaction between the S•Tag sequence and S-protein:biotin or enzyme conjugates. Colorimetric substrates are included which allow as little as 250pg of target protein to be visualized using a 30 minute protocol. Figure 2 shows a time course of induction of β-galactosidase and a recombinant antibody cloned in pET-30b(+). The S•Tag western blot shows high specificity for target proteins (and their amino-terminal-containing breakdown products) with very low background staining of other *E. coli* proteins. The gels also contain Novagen's Perfect Protein™ markers, a set of 7 proteins with precise molecular weights at convenient intervals (15, 25, 35, 50, 75, 100, and 150kd). The markers are known amounts of defined recombinant proteins containing the S•Tag and thus serve as precise internal standards for S•Tag western blots.

S•Tag Affinity Purification

The high affinity interaction between S-protein and S•Tag also can be applied to purification of target proteins. The S•Tag Purification Kit contains S-protein immobilized on agarose beads to achieve rapid,

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S-Peptide in Protein Fusion Systems: History and Significance

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Ribonuclease A (RNase A) catalyzes the cleavage of RNA. Almost forty years ago, Fred Richards and coworkers discovered that the protease subtilisin prefers to cleave a single peptide bond in native RNase A (1). The product of this cleavage, ribonuclease S (RNase S), consists of two tightly-associated fragments: S-peptide (residues 1–20) and S-protein (residues 21–124). Although neither fragment alone has any ribonuclease activity, RNase S has enzymatic activity similar to that of intact RNase A. Richards and Harold Wyckoff determined the structure of crystalline RNase S by x-ray diffraction analysis (2).

The S-peptide fragment of RNase A has played an important role in the history of biochemistry. Before molecular biologists were able to use recombinant DNA technology to explore protein structure-function relationships, organic chemists synthesized analogs of S-peptide and studied their complexes with S-protein. These studies provided much information on the role of individual residues in RNase S. Most significantly, Chris Anfinsen and coworkers found that only residues 1–15 of S-peptide were necessary to form a fully functional complex with S-protein (3). This shorter fragment is called "S15" (or "S•Tag™" by Novagen) and its complex with S-protein is called "RNase S."

The detection, immobilization, and purification of proteins is idiosyncratic and can be problematic. Fortunately, these processes can be generalized by using recombinant DNA technology to produce fusion proteins in which target proteins are fused to carrier polypeptides (4). The affinity of the carrier for a specific ligand enables the facile detection, immobilization

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one-step purification of up to 1mg of target protein from crude extracts. Several purification strategies are available depending on the application. For purification of soluble proteins under native conditions, target proteins bound to the matrix can be eluted by cleavage with biotinylated thrombin, leaving the S•Tag peptide behind. The biotinylated thrombin is then quantitatively removed with streptavidin-agarose. Figure 3 shows an example of the purification achieved under these conditions.

If retention of the S•Tag fusion is desired, proteins can be eluted with 2M sodium thiocyanate. Target proteins found in inclusion bodies can also be purified by solubilization in 6M urea followed by column binding and washing in 2M urea and elution with thiocyanate

The S•Tag purification strategy is recommended for small to medium scale applications. For producing target proteins on a larger scale, a more economical strategy takes advantage of the His•Tag® fusion peptide also encoded by these vectors for His•Bind® metal chelation chromatography. His•Tag proteins can be purified under a wide range of either native or denaturing conditions. The His•Bind affinity resin has a very high capacity for target proteins; up to 20mg of fusion protein can be bound on a single 2.5ml column. The method is based on the interaction between immobilized nickel cations and the His•Tag oligohistidine domain, and elution is achieved with imidazole. In practice, this strategy produces an equivalent amount of pure protein for usually less than 1/20 the cost of affinity methods based on immobilized protein ligands such as antibodies or avidin.

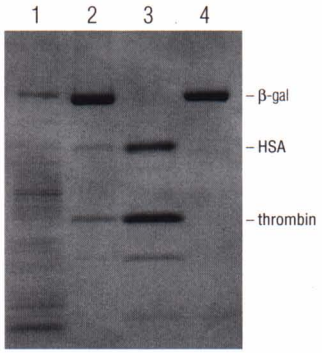

Figure 3. S•Tag purification.

S•Tag β-galactosidase from pET-29b(+) was purified from a crude soluble fraction under native conditions. Samples were analyzed by SDS-PAGE and Coomassie blue staining.

1. Crude extract
2. Protein released with Biotinylated Thrombin
3. Thrombin standard (HSA added as stabilizer)
4. Protein after biotinylated thrombin removal

The availability of two independent affinity strategies (S•Tag and His•Tag) also allows a dual affinity approach to purify target proteins "from both ends," effectively eliminating all non-full length products and producing exquisitely pure preparations (2).

Summary

The S•Tag System provides a unique set of research tools suitable to quantification, detection and purification of expressed proteins. Perhaps its most unique feature is the reconstitution of enzymic activity that is easily assayed with high sensitivity. The small size, low cost and specificity of the S-protein may provide additional advantages over other enzyme-based detection methods in a variety of applications, which we continue to investigate.

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and purification of a fusion protein. The most sensitive detection methods rely on the catalytic activity of an enzymic carrier. Unfortunately, enzymes are relatively large, and are, therefore, more likely than simple peptides to perturb a target protein or to be immunogenic.

The wealth of information that has been accumulated on RNase S suggested to us that this non-covalent complex may provide a useful carrier and ligand for a fusion protein system. We have drawn on this wealth to develop a fusion protein system in which S•Tag is the carrier and S-protein is the ligand (5). The S•Tag carrier combines a small size (15 amino acid residues) with a high sensitivity of detection (10ng of a typical fusion protein in solution or 1pg in a polyacrylamide gel, both without antibodies or radioactivity). S•Tag has several additional properties that are desirable in a carrier. For example, S•Tag [which is composed of 7 charged polar, 3 uncharged polar, and 5 nonpolar residues] is an excessively soluble peptide with little structure and net charge at neutral pH. The S•Tag carrier is therefore unlikely to interfere with the proper folding or function of a fused target protein. Also, the topology of RNase S is such that target proteins fused to either terminus of S•Tag allow binding to S-protein. Finally, the affinity between S•Tag and S-protein can be fine-tuned by rational mutagenesis (5). Together, these properties make S•Tag an extremely useful and versatile carrier in protein fusion systems.

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