BRTA (Biotinylation Reaction Titration Assay):

This is an ELISA-like reaction using BIS-300 kit components to examine the extent of biotinylation of an AviTag'd protein-of-interest versus a known amount of biotinylated standard. The BRTA procedure follows a typical ELISA protocol. The standard protein and unknown proteins are adsorbed to the wells of a 96-well plate at known protein quantities. The biotin associated with the AviTag™ is detected by its interaction with streptavidin-conjugated alkaline phosphatase. After adsorption, extraneous biotin is removed by washing the wells, eliminating the need for dialysis steps. NOTE: Some test proteins may not bind efficiently to the plastic of the 96-well plate.

Reagents needed:

- Fully biotinylated MBP-AviTag fusion protein standard (1mg/ml biotinylated MBP-AviTag™; BIS-300 kit component; Avidity, LLC)
- Un-biotinylated MBP-AviTag fusion protein (1mg/ml unbiotinylated MBP-AviTag™; BIS-300 kit component; Avidity, LLC)
- Streptavidin-Alkaline Phosphatase conjugate (Molecular Probes or other)

Solutions:

- PBS: 138mM NaCl, 2.6mM KCl in 10mM potassium phosphate (pH 7.4)
- PBST: PBS plus 0.05% Tween 20
- TBS: 10mM Tris, pH 7.5, 150mM NaCl
- Blocking Solution: PBS plus 40µg/mL BSA
- Dilution Buffer: PBS plus 0.15µg/mL BSA
- Streptavidin-Alkaline Phosphatase conjugate solution: 40µg/mL in PBS
- Development Solution for alkaline phosphatase: p-nitrophenyl phosphate in diethanolamine buffer (Bio-Rad Laboratories)
- Stop Buffer: 2M KOH

Equipment /materials:

- 96-well polystyrene, high binding, flat bottom micro-titer plates (Corning Costar Corp.)
- Biokinetics Reader EL312e plate reader (Bio-Tek Instruments) or other.

Protocol:

1. Use one 96-well polystyrene micro-titer plate for all of the following:

- Standard curve: coat ten (10) wells with 50µL each of 1 to 10ng (in 1ng increments) fully biotinylated MBP-AviTag™ fusion protein diluted in Dilution Buffer. Coat seven (7) additional wells with 50µL each of 15 to 45ng (in 5ng increments) fully biotinylated MBP-AviTag™ fusion protein diluted in Dilution Buffer.
- Biotinylated sample: coat new wells on the same plate as done above with 1 to 45ng of the biotinylated protein-of-interest in Dilution Buffer. The degree of purity of the test protein samples must be determined to accurately assess the degree of biotinylation. For example, if the protein-of-interest is only 80% of the total protein in the sample, this must be accounted for when making the dilutions.
- Negative control/blanks: add 45ng of the un-biotinylated MBP-AviTag fusion protein in Dilution Buffer to a single well as a negative control. Also add wells containing Dilution Buffer alone to act as blanks for the plate reader.

2. Allow the proteins to adsorb to the plate for at least 1 hour at room temperature with gentle rotational shaking.
3. Shake the liquid out of the wells over a sink and wash the plate wells four times with PBST.
4. Incubate plate with 300µL of Blocking Solution in all the assay wells for 1 hour at room temperature with gentle rotational shaking to block non-specific binding.
5. Shake the liquid out of the wells over a sink and wash the plate wells four times with PBST.
6. Pipette 50µL of the 40µg/mL streptavidin-alkaline phosphatase conjugate solution into each assay well and incubate with gentle rotational shaking for at least 1 hour at room temperature.
7. Shake the liquid out of the wells over a sink and wash the plate wells four times with PBST.
8. Wash the plate twice with TBS.
9. Add 200µL of the Development Solution and monitor absorption in the plate reader at 405nm every minute to assure the linearity of the alkaline phosphatase reaction. Terminate the reaction by adding 50µL of 2M KOH.