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USES FOR POSITIVE CONTROL PROTEIN SUBSTRATE KIT – BIS300

The positive control protein substrate kit can be used in two ways.

- **The kit provides an unbiotinylated Avitag'd protein that can be placed in a biotin ligase reaction to verify biotinylation has occurred. Varying buffer conditions that are necessary for your protein of interest can be inserted to test the ability of the biotin ligase to function.**
- **The proteins within the kit can be used to determine the extent of biotinylation of proteins in an ELISA format (BRTA assay)**

Reagents:

AviTag Positive Control Substrate Kit Reagents
Fully Biotinylated MBP-AviTag fusion protein (50 µl, 1.0 mg/ml)
Un-Biotinylated MBP-AviTag fusion (100 µl, 1 mg/ml)

Reagents Not Provided With Kit
Streptavidin-Alkaline Phosphatase (Molecular Probes, Eugene, OR) 2 mg/ml
Biotin ligase (BirA500 or BirABulk, Avidity, LLC)

Biotin Ligase reaction:

See protocol-Biotin ligase with purified proteins

BRTA Determination of the Extent of Biotinylation:

This procedure follows a typical ELISA protocol. The extent of biotinylation of the test protein is measured by comparison with known quantities of fully biotinylated C-terminal AviTag Maltose Binding Protein (MBP-AviTag protein). 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. Extraneous biotin is removed when the samples are washed after adsorption, eliminating exhaustive dialysis steps. The disadvantage of the BRTA is that some test proteins may not bind well to the plastic of the 96-well plate.

Solutions (Not Provided**):**

PBS: 138 mM NaCl, 2.6 mM KCl in 10 mM potassium phosphate (pH7.4)
PBST: PBS plus 0.05% Tween 20
Blocking Solution: PBS plus 40 µg/ml BSA
Dilution Buffer: PBS plus 0.15 µg/ml BSA
40 µg/ml Streptavidin-Alkaline Phosphatase in PBS

Developing solution for alkaline phosphatase: p-nitrophenyl phosphate in diethanolamine buffer (BIO-RAD, Hercules, CA)

Equipment:

Corning Costar Corp, (Corning, NY) 96-well polystyrene, high binding, flat bottom microtiter plates
Biokinetics Reader EL312e (Bio-Tek Instruments)

Procedure:

1. For the standard curve, coat the wells of a 96-well polystyrene microtiter plate with 1 to10 ng (1 ng increments) and 20 to 45 ng (5 ng increments) of fully biotinylated MBP-AviTag fusion protein using dilution buffer to bring the volume to 50 µl.

2. Coat wells of a 96-well polystyrene microtiter plate with 1.5 to 45 ng of the protein of interest. The proportion of contaminants in the test protein samples must be determined to accurately assess the degree of biotinylation.

3. 45 ng of un-biotinylated MBP-AviTag should be added to one well as a negative control. Wells containing dilution buffer alone are used to blank the plate reader.

4. Allow the proteins to adsorb to the plate for at least 1 hr at room temperature with gentle rotational shaking.

5. Wash the plate four times with PBST.

6. Incubate plate with 300 µl of blocking solution for 1 hr at room temperature to block nonspecific binding.

7. Rinse four times with PBST.

8. Pipet 50 µl of streptavidin-alkaline phosphatase solution into each well and incubate with gentle shaking at least 1 hr at room temperature.

9. Wash 4 times with PBST.

10. Wash the plate twice with TBS (10 mM Tris, pH 7.5, 150 mM NaCl).

11. Add 200 µl of the developing solution.

12. Develop the plate with 200 µl of Developing Solution. Monitor absorption at 405 nm every minute to assure the linearity of the alkaline phosphatase reaction. Terminate the reaction by adding 50 µl of 2 M KOH.