Data Sheet
ELISAs
Protocols

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ASSAY PROCESS

Assays to screen for the presence of target proteins are determined using an appropriate ELISA kit, or by developing a sandwich ELISA. Two antibodies specific to two separate epitopes on the target protein (matched-antibody pairs) are utilized in a sandwich ELISA; plates are pre-coated with a one of these antibodies (capture antibody) to immobilize the target, while the second matched pair antibody is either conjugated to a reporter enzyme, or is bound by a third antibody conjugated to an enzyme and specific to the host species of the second matched pair antibody. For an indirect ELISA, the target is immobilized directly to the plate, then bound by a primary antibody specific for the target. This is followed by binding of a secondary antibody coupled to a reporter enzyme, and is specific to the host species of the primary antibody. Addition of a substrate results in a colorimetric read-out. The concentration of target protein can be determined by comparing the absorbance to a standard curve with known concentrations of control protein carried out on the same assay plate.

Sample Preparation

If membrane preparation is required, frozen tissue or washed cells are homogenized in 20 volumes of cold lysis buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM EDTA, protease inhibitor cocktail). After a low-speed spin (100 x g for 3 minutes) to remove large tissue chunks (tissue homogenates), the homogenate is centrifuged at 17,000 x g for 10 minutes at 4 °C to pellet the membranes. The pellet is resuspended in fresh buffer and centrifuged at the same speed for a second time, again at 4 °C. The pellet is then resuspended into buffer (15 ml) containing 10% sucrose as a cryoprotectant, divided into 1 ml aliquots and stored at -80 °C. A sample of the homogenate is analyzed for protein content using the Pierce® BCA assay.

On the day of the assay, membrane preparations or purified proteins are diluted to the appropriate concentration in coating buffer (50 mM carbonate-bicarbonate buffer) immediately before setting up the ELISA plate.

Target Protein Immobilization or Capture

This stage is required to immobilize the target protein onto to the ELISA plate in the case of the indirect ELISA. The target is added to the plate (200 µl per well), with at least 6 concentrations of a protein standard and blank negative control wells. The plate is then sealed and incubated at 4 °C overnight.

For a sandwich ELISA, 200 µl of target per well and at least 6 concentrations of a protein standard and blank negative control wells is added to the plate. It is captured by the first of the matched pair of antibodies which is pre-coated onto the plate. The plate is incubated for 2 h at RT with gentle agitation.

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Blocking Step

The unbound target protein is removed from the plate, which is then washed x3 with 200 µl PBST (phosphate buffered saline, 0.05% Tween 20). The unbound sites are blocked by adding 200 µl 10% goat serum in PBS. The plate is incubated at RT for 1 h with gentle agitation.

Antibody Binding Steps

For the indirect ELISA, the blocking solution is removed and the plate is washed x3 with 200 µl PBST. The primary antibody specific for the target protein is added to the plate (200 µl) and incubated for 2 h at RT with gentle agitation. Unbound antibody is removed, and the plate is washed x3 with 200 µl PBST. The secondary antibody conjugated to a reporter enzyme is added to the wells (200 µl) and incubated for 2 h at RT.

For the sandwich ELISA, the blocking solution is removed, and the plate is washed x3 with 200 µl PBST. The second matched antibody pair is added followed by incubation for 2 h at RT with gentle agitation. Unbound antibody is removed, and the plate is washed x3 with 200 µl PBST. The third antibody conjugated to a reporter enzyme diluted in PBS is added (200 µl). The plate is incubated for 2 h at RT with gentle agitation.

Substrate Addition and Reaction Termination

For both the indirect ELISA and the sandwich ELISA, the unbound antibodies are removed from the plate which is then washed x3 with 200 µl PBST. The substrate solution is prepared immediately before use, and 100 µl added to each well. The plate is incubated for 15 minutes at RT with gentle agitation, or until the desired colour develops. The reaction is then stopped with 100 µl 2M sulphuric acid (or 8M acetic acid with 1M sulphuric acid)

Absorbance Measurement

The plate is read in a plate reader at 450 nm (or at the appropriate wavelength specified by the manufacturer for the colorimetric readout) at RT with gentle shaking. Data is exported to Microsoft Excel for analysis.

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DATA ANALYSIS

Data output for each plate comprises a standard curve for a positive control protein with a ‘best fit’ line for each concentration of protein plotted against the measured absorbance. The absorbances measured for each of the wells containing target protein are compared to the standard curve, and the concentration in each sample calculated.

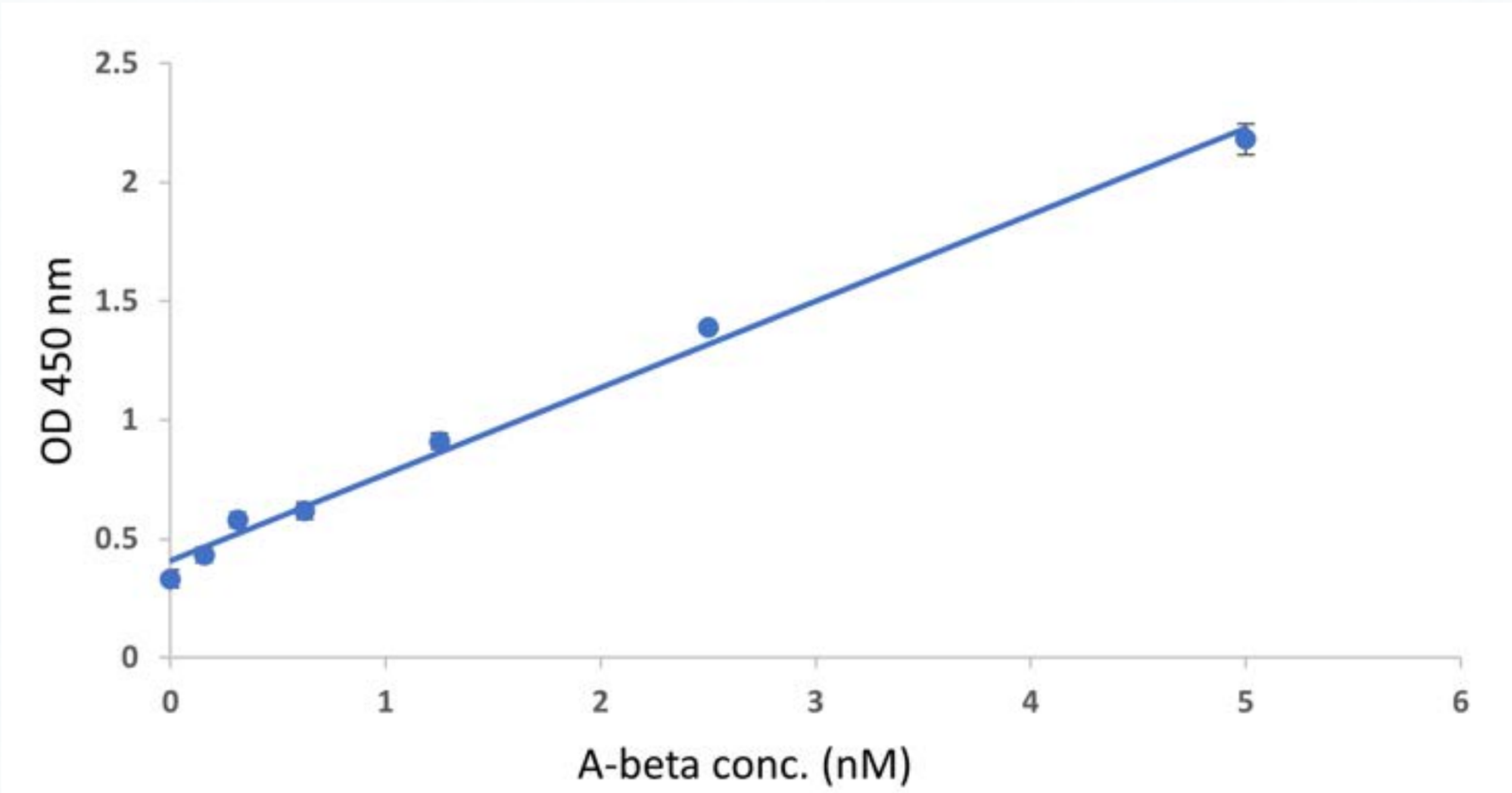

Figure 1: **Example dilution series generated for A-beta fibrils using an indirect ELISA.** Limit of detection (LOD) = 0.53 nM. Limit of quantitation (LOQ) = 1.78 nM. LOD = 3σ/s and LOQ = 10σ/s, where σ is the standard deviation of the blanks and s is the slope.

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