CYTOKINE ELISA

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BACKGROUND AND NOTES

- This protocol outlines a sandwich-ELISA for the detection of cytokine/chemokine concentrations in a cell supernatant. Samples can be diluted or used as is for ELISA analysis
- During all coating steps, timing is not critical. However, incubation with sample, detecting antibodies, and avidin incubation times are critical. Many reagents are light-sensitive so cover plate with plastic sticker and wrap in tin foil
- Many washing steps are included; these can be done manually but the risk of pipetting error makes this task fallible. If a plate washer is available, it is strongly recommended to use it. Volume error from pipetting can have significant effects.

EQUIPMENT

- Equipment:
  - 96-well plate [NUNC clear plastic strips or any appropriate plate]
  - Multi-channel pipette [capable of dispensing 50 – 200 μL]
  - Plate-reader [capable of measuring absorbance at 450nm]
- Reagents:
  - Assay diluent
    - add 50 mL fetal bovine serum in 450 mL PBS for 10% v/v FBS; filter sterilize and store at 4^{o}C immediately after use
  - Wash buffer
    - 0.5% v/v Tween 20 in PBS
  - Capture antibodies (cAb)
  - Cytokine of interest for standard
    - Lyophilized cytokines **must** be resuspended in PBS + 0.1% w/v bovine serum albumin and store at -80^{o}C; do not handle cytokines at room temperature for extended periods of time to prevent risk of denaturing; create 10μg/mL stock solutions
  - Detection antibody (dAb)
  - STRP-avidin conjugated to horseradish peroxidase
  - TMB substrate solution
  - 2N H_2SO_4

PROTOCOL

Day 1:

1) Dilute cAb in PBS (**not** assay diluent) to a final concentration of 1μg/mL in 100 μL per well. Incubate overnight (or over weekend) in 4^{o}C
    a. Easiest to prepare antibody dilution in a Falcon tube, quickly mix, and pour into a plastic boat; use a multi-channel pipette to dispense 100 μL into each well **directly** into the bottom of the well

Day 2:

1) Dump and tap dry ELISA plate
2) Add 300 μL of assay diluent into each well, ensuring each well is **completely full**; volume can be adjusted to fill the well; incubate at room temperature for 2 hours or overnight at 4^{o}C
3) Wash 3x with wash buffer
4) Prepare standard curve
    a. Suspend lyophilized cytokines in 0.1% bovine serum albumin (BSA) + PBS to any desired concentration. This is absolutely crucial as the BSA will prevent cytokine adherence to the walls of the container. In this protocol, this stock solution is made to 200 μg/mL. Since the standard curve will have concentrations in pg, create working aliquots by diluting this stock to a concentration of 10 μg/mL. Complete this entire step over ice, briefly centrifuge to collect the solution at the bottom of the container, and store immediately at -80^{o}C. Prolonged exposure to room temperatures will inevitably denature the cytokines.
    b. Prepare 9 Eppendorf tubes. Dilute the working aliquot (10 μg/mL) 100x in Assay Diluent. The final concentration will be 100 ng/mL. Depending on the volumes required for the standard curve, a standard for a single plate only requires 2 μL of working aliquot diluted in 200 μL Assay Diluent. Vortex briefly to mix well.
    c. In one Eppendorf tube, aliquot 980 μL Assay Diluent. Dilute the 100 ng/mL solution to 2,000 pg/mL by diluting 5x in the 980 μL Assay Diluent. This will be the top concentration of the standard curve.
    d. In the remaining Eppendorf tubes, aliquot 500 μL Assay Diluent into each tube. To create a linear standard curve, dilute 1x repeatedly. Ensure the solution is well mixed by either pipetting up and down or vortexing briefly. The last Eppendorf tube should be reserved for a blank (i.e. Assay Diluent only). Avoid harsh vortexing as mechanical disruption of cytokines is possible. The standard curve will thus have the following concentrations:
        i. 2,000 pg/mL
        ii. 1,000 pg/mL
        iii. 500 pg/mL
        iv. 250 pg/mL
        v. 125 pg/mL
        vi. 62.5 pg/mL
        vii. 31.25 pg/mL
5) Add samples to ELISA plate
    a. If supernatants need to be diluted prior to ELISA, dilute in assay diluent
6) Incubate for 2 hours at room temperature
7) Wash 3x with wash buffer
8) Dilute dAb to a final concentration of 1 μg/mL in assay diluent and add 100 μL of antibody solution to each well, ejecting directly to the bottom of the well
9) Incubate for 2 hours at room temperature
10) Wash 3x with wash buffer

11) Dilute STRP-Avidin HRP 1/250 in assay diluent; STRP-Avidin is light sensitive so cover all tubes and plate with tinfoil
12) Add 100 μL diluted STRP-Avidin into each well
13) Incubate for 25 minutes; meanwhile, warm TMB substrate to room temperature
14) Wash 5x with wash buffer
15) Add 100 μL of TMB substrate to each well; no dilution is required
    a. If the cytokine of interest is present, the well should turn blue
16) Monitor the change in the standard curve; when the standard curve turns into a nice gradient, immediately add 50 μL of H_2SO_4 to stop the reaction. Do not let the wells turn deep blue or purple. Addition of H_2SO_4 will turn the blue wells yellow.
17) Measure absorbance at 450nm within 20-30 min; wrap in tinfoil when plate is not in use

Disposal:

- Excess reagents can be flushed down the sink with plenty of water; discard used plates or strips in garbage

RESULTS

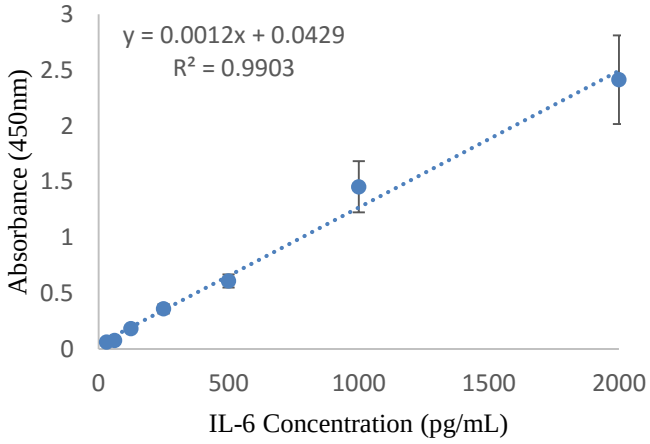

Figure 1 Example IL-6 ELISA Standard Curve