CYTOKINE ELISA

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BACKGROUND

- This protocol outlines the steps necessary to test for cytokine/chemokine concentrations in a cell supernatant. Prior to performing the ELISA, macrophages should be stimulated (for example with LPS or bacteria) for a set period of time and cell supernatants should be collected and frozen at -80˚C. Please see [Cytokine Production] for more details.

NOTES

- The 96-well plate must be coated with cAb the night before the ELISA is to be performed.
- The greatest amount of error produced in any ELISA is due to pipetting error or during the wash steps. I suggest continuously checking multichannel pipettors to ensure each tip is picking up the same amount of solution as well as using an automated plate washer for wash steps.
- At all points in the protocol, when you are not doing anything to the plate it should be wrapped in tin foil. Many of the reagents and reactions are light-sensitive.
- Wear gloves and a lab coat for each step of the procedure.

EQUIPMENT

- 96-well plate [NUNC clear plastic plate with removable 8-well strips]
- Multi-channel pipettor [capable of dispensing 50 – 200µL]
- Plate reader [for measuring absorbance]
- Reagents:
    - Assay diluent [10% FCS in PBS, filter-sterilized]
    - Wash buffer [0.5% Tween 20 in 10X PBS]
    - cAb for cytokine of interest [diluted in PBS to 1µg/mL]
    - Standard for cytokine of interest [10µg/mL stock solution]
    - dAb for cytokine of interest [diluted in assay diluent to 1µg/mL]
    - STRP-Avidin Horeseradish peroxidase [1/250 dilution in assay diluent]
    - TMB substrate solution [no dilution]
    - 2N H_2SO_4

PROTOCOL

Day 1:

1) Dilute cAb (capture antibody) in PBS (NOT assay diluent) to a final concentration of 1µg/ml in 100 µl/well. Incubate overnight (or over weekend) in the fridge.

Day 2:

1) Add 300 µl of assay diluent per well, ensuring that each well is **completely full**, cover and incubate at room temperature for 2 hours (or overnight in 4 ˚C).
2) Add samples and standards in Assay Diluent and incubate 2 hours.
    a. To make standards – From the stock solution (10 µg/ml) make a 1/100 dilution (e.g. 2 µl in 200 ul), vortex briefly. (Concentration = 100 ng/ml)
    b. Fill one eppendorf with 980 ul of Assay Diluent (Label #1) and 6 eppendorfs with 500 µl of Assay diluent. (Label 2-6)
    c. Add 20 µl from your 100ng/ml stock to the 980 µl in eppendorf #1. Vortex briefly.
    d. Take 500 µl from #1 and add to #2, vortex briefly. Changing the pipette tip each time, take 500 µl from #2 and add to #3, etc, until you have finished the series. Pipette 100 µl of each standard into the corresponding wells.
    e. Make dilutions of samples in assay buffer. It is often easiest to add the assay buffer to the well of the ELISA plate and then add the sample (e.g. 80 µl of assay diluent + 20 µl sample = 1/5 dilution)
3) Wash 3 times in wash buffer (see recipe below).
4) Add 100 µl of a 1/500 dilution of detecting antibody in assay diluent (e.g. stock concentration is 500 µg/ml and the final concentration should be 1 µg/ml). Incubate 2 hours at room temp.
5) Wash 3 times with Wash buffer.
6) Add 1/250 diluted STRP-Avidin HRP in Assay Diluent at 100µL/well for 20-30min
7) Wash 5 times with Wash buffer.
8) Add 100µL/well of TMB substrate (no dilution)
9) Wait until you get a good gradient in standard rows
10) Add 50µL/well of 2N H_2SO_4 to TMB
11) Read using plate reader within 20-30min (Keep in Dark)

DISPOSAL

- Any excess reagents can be flushed down the sink with plenty of running water.
- When the plate has been used the wells should be emptied in the sink and flushed with plenty of water and the plate can be thrown in the garbage.