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Enzyme linked immunosorbent assays (ELISAs) for mouse IL-10, IL-6, IL-1β and TNF-α

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1 Acquire the necessary reagents and antibodies.

1.1 **Salts (all available from Sigma-Aldrich):**

34.56 g Na2HPO4

192 g NaCl

5.76 g of KH2PO4
4.8 g of KCl
2.23 g NaN3
0.12 g MgCl2.6H2O

Other reagents for solutions (all available from Sigma-Aldrich):
10 ml Tween 20
25 g BSA
14.6 ml di-ethanolamine
37 % HCL (about 10 ml)

1.2 Streptavadin alkaline phosphatase and phosphatase substrate:
BD Streptavidin-alkaline phosphatase (SAv-AKP) was obtained from Ascendis Medical (#BD/554065). Phosphatase substrate was obtained from Sigma-Aldrich (#P4744-1G).

1.3 Protein-binding 96 well plates:
Nunc Maxisorp 96 well Immuno Plates (available from Thermo Fisher).

1.4 Antibodies & recombinant cytokines:
We utilise antibodies from R&D systems (a biotechne brand) - in South Africa, Whitehead Scientific supplies these antibodies.

IL-10
Capture antibody: Mouse IL-10 Antibody (#MAB417).
Detection antibody: Mouse IL-10 Biotinylated Antibody (#BAF417).
Recombinant cytokine: Recombinant Mouse IL-10 (#417-ML).

IL-6
Capture antibody: Mouse IL-6 Antibody (#MAB406).
Detection antibody: Mouse IL-6 Biotinylated Antibody (#BAF406).
Recombinant cytokine: Recombinant Mouse IL-6 (#406-ML).

IL-1β
Capture antibody: Mouse IL-1β/IL-1F2 Antibody (#MAB401).
Detection antibody: Mouse IL-1β/IL-1F2 Biotinylated Antibody (#BAF401).
Recombinant cytokine: Recombinant Mouse IL-1β/IL-1F2 (#401-ML).

TNF-α
Capture antibody: Human/Mouse TNF-α Antibody (#AF-410-NA).
Detection antibody: Mouse TNF-α Biotinylated Antibody (#BAF410).
Recombinant cytokine: Recombinant Mouse TNF-α aa 80-235 (#410-ML).

2 Prepare the solutions you will require (these volumes will be sufficient for about 25 x 96 well plates).

2.1 1.2 L Phosphate buffered saline (PBS) pH 7.4 (20X)

Add the following reagents in 500 mL diH2O IN ORDER:

34.56 g of Na2HPO4
192 g of NaCl
5.76 g of KH2PO4
4.8 g of KCl

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Make up to 1.2 L with diH_2O and store at room temperature.

2.2 1.5 L Phosphate buffered saline (pH 7.4) (1X)

Dilute 75 ml of 20X PBS with 1425 ml diH_2O.

2.3 1 L Washing Buffer (20x)

Add the following to 990 ml 20X PBS:

10ml Tween 20
2 g NaN3*

*If horse radish peroxidase is utilised instead of alkaline phosphatase, NaN3 must be omitted from solutions.

2.4 500 ml Blocking Buffer

Add the following to 300 ml 1X PBS:

20g BSA
0,1g NaN3

Make up to 500 ml with 1X PBS and store at 4 °C.

2.5 500 ml Dilution Buffer

Add the following to 300 ml 1X PBS:

5 g BSA
0.1 g NaN3

Make up to 500 ml with 1X PBS and store at 4 °C.

2.6 150 ml Substrate Buffer

Add the following to 100 ml diH2O:

0,03 g NaN3
14.6 ml di-ethanolamine
0.12 g MgCl2.6H2O

Adjust pH to 9.8 with 37 % HCL.
Make up to 150 ml with diH2O and store at 4 °C.

3 Prepare stock solutions of antibodies and recombinant cytokines.

3.1 Coating antibodies:

1. Reconstitute 100 µg of the primary antibody in 1000 µl autoclaved 1X PBS (100 µg/ml).
2. Label 20 x 500 µl microcentrifuge tubes with name of the cytokine, 1st antibody, concentration and aliquot volume (eg. TNF-α, 1st AB, 100 µg/ml, 50 µl).
3. Pipette 50 µl of the reconstituted antibody solution into each microcentrifuge tube.
4. Place in labelled freezer box and store aliquots at -80 °C.

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3.2 Detection (biotinylated) antibodies:

First stock:
1. Reconstitute 50 µg of the secondary/biotinylated antibody in 500 µl of 1X PBS. Label 10 x 500 µl microcentrifuge tubes with name of the cytokine, 2nd antibody, concentration and aliquot volume (eg. IL-6, 2nd AB, 100 µg/ml, 50 µl).
2. Pipette 50 µl of the reconstituted antibody solution into each microcentrifuge tube.

Second stock:
1. Add 1 x 50 µl aliquot of stock 1 to 450 µl 1X PBS.
2. Label 10 x 500 µl microcentrifuge tubes with name of the cytokine, 2nd antibody, concentration and aliquot volume (eg. IL-6, 2nd AB, 10 µg/ml, 50 µl).
3. Pipette 50 µl of the reconstituted antibody solution into each microcentrifuge tube.
4. Place in labelled freezer box and store aliquots at -80 °C.

3.3 Recombinants:

1. First stock: Prepare a 10 µg/ml solution by adding the appropriate volume of dilution buffer to the antibody (the volume of dilution buffer required will vary depending on the amount of cytokine purchased). Divide into 100 µl aliquots.
2. Second stock: 500 ng/ml, 20 x 100 µl aliquots (add 1900 µl dilution buffer to stock 1 aliquot).
3. Third stock: 100 ng/ml, 5 x 100 µl aliquots (add 400 µl dilution buffer to stock 2 aliquot).
4. Final stock: 10 ng/ml, 10 x 100 µl aliquots (add 400 µl culture media or dilution buffer to stock 3 aliquot).

4 Coat the 96-well plate with coating antibody.

4.1 For IL-10, IL-6 and IL-1β:

Prepare 5100 µl of approx 3 µg/ml coating antibody by adding 3 x 50 µl aliquots of the coating antibody stock solution (100 µg/ml) to 4950 µl of 1X PBS.

For TNFα:
Prepare 5100 µl of approx 1 µg/ml coating antibody by adding 1 x 50 µl aliquots of the coating antibody stock solution (100 µg/ml) to 5050 µl of 1X PBS.

For all:
Add 50 µl of the newly prepared coating antibody solution to each well of a Nunc Maxisorp 96 well Immuno Plates. Cover the plate with parafilm or foil and leave overnight at 4 °C.

5 Wash 4 times with 1X washing buffer.

5.1

1. Prepare 1L of 1X washing buffer by adding 50 ml of 20X washing buffer to 950 ml of diH_2O.
2. Fill a plastic squeeze wash bottle with 1X washing buffer.
3. Remove the foil from the plate and shake the coating antibody out of the plate by inverting the plate and shaking/flicking it over a wash basin.
4. Fill each well of the plate with washing buffer using the squeeze wash bottle.
5. Remove the wash buffer by inverting the plate and shaking/flicking it over a wash basin.
6. Repeat this washing process another 3 times.
7. After the final wash, try to remove as much of the washing buffer from the wells as possible by shaking/flicking the plate, and then hit the plate (upside down) on a clean towel/thickly layered

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tissue paper until you have managed to remove practically all fluid from the wells.

6 Add blocking buffer to all wells to prevent non-specific antibody binding.

6.1 Add 200 µl of blocking buffer to each well.

Cover with parafilm/foil and leave overnight at 4 °C (in the fridge)/or incubate at 37 °C for 2 hr. (Plates may be blocked and left at 4 °C for up to 1 week).

7 Wash 4 times with 1X washing buffer.

7.1 1. Prepare 1L of 1X washing buffer by adding 50 ml of 20X washing buffer to 950 ml of diH_2O.
2. Fill a plastic squeeze wash bottle with 1X washing buffer.
3. Remove the foil from the plate and shake the coating antibody out of the plate by inverting the plate and shaking/flicking it over a wash basin.
4. Fill each well of the plate with washing buffer using the squeeze wash bottle.
5. Remove the wash buffer by inverting the plate and shaking/flicking it over a wash basin.
6. Repeat this washing process another 3 times.
7. After the final wash, try to remove as much of the washing buffer from the wells as possible by shaking/flicking the plate, and then hit the plate (upside down) on a clean towel/thickly layered tissue paper until you have managed to remove practically all fluid from the wells.

8 Prepare serial dilutions of recombinant cytokines to produce "known concentration" standard curves.

8.1 1. Prepare 250 µl of 4 ng/ml recombinant cytokine by adding 150 µl dilution buffer/culture media to 100 µl of 10 ng/ml recombinant cytokine stock.
2. Pipette 50 µl of the 4 ng/ml recombinant cytokine into well A1, A2, B1 and B2.
3. Add 50 µl of dilution buffer/culture media to wells A2 and B2.
4. Add 50 µl of dilution buffer to wells A3-A12 and B3-B12.
5. Perform two-fold serial dilution from A2 to A12. Using a pipette set to 50 µl, mix well A2 by drawing up and then expelling 50 µl of the solution in the well about 3 to 5 times.
6. Draw up 50 µl of the solution in well A2 and add it to well A3. Again, mix the solution as described above, using a
pipette set to 50 µl.
7. Now add 50 µl of the solution in well A3 to well A4.
8. Repeat this diluting/mixing process through to well A11. Dispose of the last 50 µl that you draw up of
well A11. This will create a standard curve, through serial dilution, of 4 ng/ml to 3.9 pg/ml, with a dilution buffer/culture medium blank in well A12.
9. Repeat this whole process in row B, to produce the standard curve in duplicate.

9 Add samples.

9.1 50 µl of experimental samples should be added into wells reserved for samples.

If you have not run samples from a specific experimental set-up before, it is wise to dilute samples threefold over three wells (ie. in one well you will have the undiluted sample, in an adjacent well

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you will have a sample which you have diluted threefold, and in a well next to that you will have a sample that you have diluted ninefold).

Cover plate with parafilm/foil and incubate overnight at 4 °C or for 2hr at 37 °C.

10 Wash 4 times with 1X washing buffer.

10.1 1. Prepare 1L of 1X washing buffer by adding 50 ml of 20X washing buffer to 950 ml of diH_2O.
2. Fill a plastic squeeze wash bottle with 1X washing buffer.
3. Remove the foil from the plate and shake the coating antibody out of the plate by inverting the plate and shaking/flicking it over a wash basin.
4. Fill each well of the plate with washing buffer using the squeeze wash bottle.
5. Remove the wash buffer by inverting the plate and shaking/flicking it over a wash basin.
6. Repeat this washing process another 3 times.
7. After the final wash, try to remove as much of the washing buffer from the wells as possible by shaking/flicking the plate, and then hit the plate (upside down) on a clean towel/thickly layered tissue paper until you have managed to remove practically all fluid from the wells.

11 Add the detection (biotinylated) antibody.

11.1 **For IL-10, IL-6, IL-1β and TNF-α:**

Prepare 5100 µl of approx 0.3 µg/ml coating antibody by adding 3 x 50 µl aliquots of the coating antibody stock solution (10 µg/ml) to 4950 µl of dilution buffer.

Add 50 µl of the newly prepared detection antibody solution to each well. Cover the plate with parafilm or foil and and incubate for 1 hr at 37 °C.

12 Wash 4 times with 1X washing buffer.

12.1 1. Prepare 1L of 1X washing buffer by adding 50 ml of 20X washing buffer to 950 ml of diH_2O.
2. Fill a plastic squeeze wash bottle with 1X washing buffer.
3. Remove the foil from the plate and shake the coating antibody out of the plate by inverting the plate and shaking/flicking it over a wash basin.
4. Fill each well of the plate with washing buffer using the squeeze wash bottle.
5. Remove the wash buffer by inverting the plate and shaking/flicking it over a wash basin.
6. Repeat this washing process another 3 times.
7. After the final wash, try to remove as much of the washing buffer from the wells as possible by shaking/flicking the plate, and then hit the plate (upside down) on a clean towel/thickly layered tissue paper until you have managed to remove practically all fluid from the wells.

13 Add the streptavadin alkaline phosphatase.

13.1 Prepare 6000 µl of 1:1000 streptavadin alkaline phosphatase by adding 6 µl of 1 mg/ml streptavadin alkaline phosphatase to 5994 µl dilution buffer.

Add 50 µl of the 1:1000 streptavadin alkaline phophatase solution to each well. Incubate at 1 hr at 37

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°C.

14 Wash 4 times with 1X washing buffer.

14.1 1. Prepare 1L of 1X washing buffer by adding 50 ml of 20X washing buffer to 950 ml of diH_2O.
2. Fill a plastic squeeze wash bottle with 1X washing buffer.
3. Remove the foil from the plate and shake the coating antibody out of the plate by inverting the plate and shaking/flicking it over a wash basin.
4. Fill each well of the plate with washing buffer using the squeeze wash bottle.
5. Remove the wash buffer by inverting the plate and shaking/flicking it over a wash basin.
6. Repeat this washing process another 3 times.
7. After the final wash, try to remove as much of the washing buffer from the wells as possible by shaking/flicking the plate, and then hit the plate (upside down) on a clean towel/thickly layered tissue paper until you have managed to remove practically all fluid from the wells.

15 Add phosphatase substrate.

15.1 Prepare 6000 µl of 1 mg/ml phosphatase substrate by adding 0.006 g of phosphatase substrate to 6 ml substrate buffer.

Add 50 µl of the 1 mg/ml phophatase substrate solution to each well. Cover plate and allow colour to develop for 30-45 min.

16 Read absorbance on a 96-well plate spectrophotometer.

16.1 Absorbance should be measured 405 nm with reference wavelength of 492 nm.

17 Plot a 4-parameter or 5-parameter standard curve and estimate cytokine concentration in your experimental samples.

17.1 First, subtract the absorbance of the blanks from all samples (the software on most modern 96 well plate spectrophotometers is able to do this for you).

Next, plot a 4 parameter or 5 parameter curve using your duplicate recombinant cytokine standards (the software on most modern 96 well plate spectrophotometers is also able to do this for you).

Once you have the standard curve you can utilise it' formula to estimate the cytokine concentrations of your unknown samples (the software on most modern 96 well spectrophotometers is also able to do this for you).

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