UC Davis MMPC-Live Protocol
Lipocalin-2 ELISA

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Summary:

Fecal lipocalin-2 (Lcn2) is a sensitive marker for gastrointestinal tract inflammation.

Reagents and Materials:

Reagent/Material	*Vendor*	*Stock Number*
Mouse Lipocalin-2/NGAL DuoSet ELISA	R&D Systems	DY1857-05
StartingBlock (PBS) Blocking Buffer	Thermo Fisher	37538
1-Step Ultra TMB-ELISA	Thermo Fisher	34029
Clear Polystyrene Microplates	R&D Systems (or equivalent)	DY990
Microplate sealing films		
Concentrated Sulfuric Acid (~36 N)		
20x PBS		
Tween-20 Detergent		
12 well multichannel pipets		
Microplate Shaker		
Microplate Reader		

Protocol:

1. Prepare all solutions and dilute all reagents

Standard Curve

1. Dilute 5 µl of reconstituted Lipocalin-2 Standard into 495 µl of Blocking/Dilution Buffer. This will give a concentration of 1000 pg/ml for the first standard.
2. Make six more 2.5-fold dilutions (200 µl of the previous dilution and 300 µl of Blocking/Dilution Buffer) to create a seven point standard curve. The concentrations for the standards are 1000, 400, 160, 64, 26, 10 & 4 pg/ml.
3. Freeze remaining standards at -80°C in 25 µl aliquots for future use.

Plate Preparation

1. Dilute 83 µl of the reconstituted Capture Antibody in 10 ml PBS without carrier protein (for a full plate, reduce amount accordingly if less wells are used). Immediately coat a 96-well microplate (R&D Systems, Catalog # DY990, or equivalent) with 100 µl per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Discard capture antibody by inverting plate over a wash tub and shaking out liquid. Remove remaining liquid by striking the plate several times on paper towels on the bench. Wash with once Wash Buffer. Wash by filling each well with Wash Buffer (300 µl) using a multichannel pipet. Remove wash by inverting plate over a wash tub and shaking out liquid. Remove remaining liquid by striking the plate several times on paper towels on the bench.
3. Block plates by adding 300 µl of Blocking/Dilution Buffer to each well. Incubate at room temperature for a minimum of 1 hour.
4. Wash three times as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µl of sample or standards in Blocking/Dilution Buffer, per well. Cover with an adhesive strip and incubate 1 hours at room temperature with vortexing at ~750 rpm.
2. Immediately before washing dilute 166.7 µl of reconstituted Detection Antibody in 9.8 ml Blocking/Dilution Buffer (for a full plate).
3. Discard extracts and standards by inverting plate over a wash tub and shaking out liquid. Remove remaining liquid by striking the plate several times on paper towels on the bench. Wash with Wash Buffer, repeating the process for a total of three washes. Wash by filling each well with Wash Buffer (300 µl) using a multichannel pipet. Remove wash by inverting plate over a wash tub and shaking out liquid. Remove remaining liquid by striking the plate several times on paper towels on the bench. Complete removal of liquid at each step is essential for good performance.
4. Add 100 µl of the Detection Antibody, diluted in Blocking/Dilution Buffer, to each well. Cover with a new adhesive strip and incubate 1 hours at room temperature with vortexing at ~750 rpm.
5. Immediately before washing dilute 250 µl of Streptavidin-HRP concentrate in 9.8 ml Blocking/Dilution Buffer
6. Discard detection antibody and wash plate three times, as in step 7 of Assay Procedure.
7. Add 100 µl of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 30 minutes at room temperature with vortexing at ~750 rpm. Avoid placing the plate in direct light.
8. Discard Streptavidin-HRP and wash plate three times, as in step 7 of Assay Procedure.
9. Add 100 µl of TMB Substrate Solution to each well. Incubate for 15 minutes at room temperature. Cover plate in with foil to avoid excessive exposure to direct light.
10. Add 50 µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well immediately, using a microplate reader, at 450 nm (TMB) and 570 nm (reference).

Concentration Calculation

1. Subtract the absorbance at 570nm from the absorbance at 450 nm for each well. This give a reference-corrected absorbance.
2. Subtract the mean of the blank sample reference-corrected absorbances from the reference-corrected absorbance of each well. This give a blank-corrected optical density (OD).
3. Calculate the mean of blank-corrected optical density (OD) of standard duplicates, reference serum and the samples.
4. Using Graph Pad Prism (easy) or R (nplr package, free). Fit a 5-parameter logistic curve (5PLC) to the standards.
5. Interpolate the concentration of samples from this standard curve.
6. Multiply the interpolated concentrations by any dilution factor (if samples were diluted prior to assay) to get the actual lipocalin concentration.

Reagent Preparation:

Reagent Dilutions

Rat Anti-Mouse Lipocalin-2 ***Capture Antibody***: Reconstitute each vial with 0.5 mL of PBS. This will yield a stock concentration of 480 µg/ml. Working concentration is 4 µg/ml

Biotinylated Rat Anti-Mouse Lipocalin-2 ***Detection Antibody***: Reconstitute each vial with 1.0 mL of Blocking/Dilution Buffer. This will yield a stock concentration of 30 µg/ml. Working concentration is 500 ng/ml

Streptavidin-HRP: 2.0 mL of streptavidin conjugated to horseradish-peroxidase. Dilute to the working concentration specified on the vial label using Blocking/Dilution Buffer. This is a 40x stock.

Recombinant Mouse ***Lipocalin-2 Standard***: Reconstitute each vial with 0.5 mL of Blocking/Dilution Buffer. This gives a solution that is 100 ng/ml

Solutions

Wash Buffer: 0.05% Tween® 20 in 1x PBS

Blocking/Dilution Buffer: StartingBlock (PBS) Blocking Buffer (Thermo Cat# 37538)

TMB Substrate Solution: 1-Step Ultra TMB-ELISA (Thermo Cat# 34029)

Stop Solution: 2 N Sulfuric Acid (5 ml of concentrated sulfuric acid in 40 ml of MilliQ H_20).

Caution: Concentrated sulfuric acid is extremely corrosive and water reactive. Always add the acid to water. Work in the chemical safety hood, wearing a lab coat, splash-proof apron, gloves and goggles & face shield.