UC Davis MMPC-Live Protocol
Calprotectin ELISA

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Summary:

S100A8 and S100A9 form Ca2+-dependent heterodimer/heterotetramer complexes called calprotectin. Fecal calprotectin is a sensitive marker for gastrointestinal tract inflammation.

Reagents and Materials:

Reagent/Material	*Vendor*	*Stock Number*
Mouse S100A8/S100A9 Heterodimer DuoSet ELISA	R&D Systems	DY8596-05
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		
Tris free base		
Brij 35 detergent		
Hydrochloric acid		
Sodium Chloride		
Calcium Chloride		
12 well multichannel pipets		
Microplate Shaker		
Microplate Reader		
pH Meter		

Protocol:

1. Prepare all solutions and dilute all reagents

Standard Curve

1. Dilute 42.8 µl of reconstituted Heterodimer Standard into 457 µl of Reagent Diluent. This will give a concentration of 24 µg/ml for the first standard.
2. Make six more 2.5-fold dilutions (200 µl of the previous dilution and 300 µl of Reagent Diluent) to create a seven point standard curve. The concentrations for the standards are 24000, 9600, 3840, 1536, 614, 246 & 98 ng/ml.
3. Freeze remaining standards at -80°C in 50 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. Repeat the wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 µl of sample or standards in Reagent Diluent, 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 Reagent Diluent (for a full plate).
3. 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 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 Reagent Diluent, 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 10 ml Reagent Diluent
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 calprotectin concentration.

Reagent Preparation:

Reagent Dilutions

Rat Anti-Mouse S100A8/S100A9 Heterodimer ***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 Goat Anti-Mouse S100A8/S100A9 Heterodimer ***Detection Antibody***: Reconstitute each vial with 1.0 ml of Reagent Diluent. This will yield a stock concentration of 24 µg/ml. Working concentration is 400 ng/ml.

Streptavidin-HRP: 2.0 ml of streptavidin conjugated to horseradish-peroxidase. This is a 40x stock. Dilute to the working concentration specified on the vial label using Reagent Diluent.

Recombinant Mouse S100A8/S100A9 Heterodimer ***Standard***: Reconstitute each vial with 0.5 ml of deionized or distilled water. This gives a solution that is 280 ng/ml

Solutions

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

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

Reagent Diluent: 50 mM Tris, 10 mM $CaCl_2$, 0.15 M NaCl,0.05% Brij® 35, pH 7.45-7.55

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.