25-OH Vitamin D3 Sandwich ELISA Protocol

Materials

- Clear 96-well plate
- Coating buffer (0.1 M phosphate buffer pH7.4, or 50 mM carbonate buffer pH 9.4)
- Blocking buffer (Assay Buffer) (1% BSA/Wash Buffer)
- Vitamin D releasing buffer as the diluent of biofluids sample
- Wash buffer (Tris-buffered or phosphate-buffered saline with 0.05% Tween 20)
- Reagent reservoirs
- Capture (coating) antibody: Anti-25-OH Vitamin D3 Rabbit/Human Chimeric Monoclonal Antibody (Clone RMH04)
- Detection antibody: Biotinylated Anti-(25-OH Vitamin D3/ RMH04 Complexes), Rabbit Monoclonal Antibody (Clone RM428)
- Streptavidin-HRP
- TMB substrate solution
- Stop solution (2N HCl or 1.8 N H_2SO_4)

Procedure

1. Prepare Coating Solution by diluting the Capture Antibody (RMH04) to 1 ug/mL in Coating buffer.
2. Coat plates with 100 µL per well of Coating Solution. Cover plates, and incubate overnight (12–18 hours) at 2–8 °C.
3. Aspirate wells and wash 2 time with >300 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
4. Block plate with 200 µL per well with Blocking buffer for 1 hour at room temperature.
5. Aspirate wells and wash 3 time with >300 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
6. Prepare sample dilutions in Blocking buffer (Use Vitamin D releasing buffer for biofluids sample). Add detection antibody (Biotin-RM428) to the final concentration of 0.2ug/mL and mix well.
7. Pipette 100 µL of samples and detection antibody mixture into designated wells. Incubate for 30 minutes at room temperature.
8. Aspirate and wash 5 times with >300 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
9. Make working solution of Streptavidin-HRP with Blocking buffer. Refer to manufacturer for dilution recommendations.
10. Add 100 µL of working streptavidin-HRP solution into each well. Incubate for 30 minutes at room temperature.
11. Aspirate and wash 5 times with >300 µL of Wash buffer per well. Following wash, invert and tap on absorbent paper to remove excess liquid.
12. Add 100 µL of TMB substrate solution to each well. Refer to manufacturer for incubation time.
13. Add 100 µL of Stop solution to each well.
14. Measure absorbance at 450 nm within 30 minutes of adding Stop solution.