

**Protocol for 0.25 mL plasma or serum**

1. Combine 0.25 mL of plasma (adjusted with approximately 3  $\mu$ L of acetic acid to pH 4) and 0.25 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
  - i. Upper organic phase – ethyl acetate phase (lipoproteins)
  - ii. Interphase – proteins
  - iii. Lower phase – aqueous phase
2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 0.75 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac or under nitrogen or argon gas (b).
5. Saponification Step (to cleave fatty acid from glycerol backbone): Dissolve the dried residues (b) in 0.5 mL of 20% KOH solution (for preparation see 14,15-DHET measurement in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).
6. Dilute 0.5 mL of the aqueous solution (c) with 0.75 mL of H<sub>2</sub>O. Adjust the pH using 20% formic acid (33  $\mu$ L) to pH~5.5. Add ethyl acetate (1 part aqueous solution (c) + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2000 rpm for ten minutes at 22°C. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Collect the upper phase containing saponified lipids.
7. Dry the pooled ethyl acetate upper phase (d) and dry in a Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20  $\mu$ L of ethanol, then add 280  $\mu$ L of 1X Sample Dilution Buffer, pH 7.4. (*Please note that the 10X Sample Dilution Buffer that is supplied with the ELISA kit must be diluted 10-fold*). This provides enough sample for triplicate measurements.
8. When calculating the concentration, consider any dilution factors. In this case, a final volume of 0.3 mL was obtained from a sample containing 0.25 mL plasma (a difference of 1.2).
9. Perform the ELISA for 14,15-DHET (according to the instructions of the manufacturer).