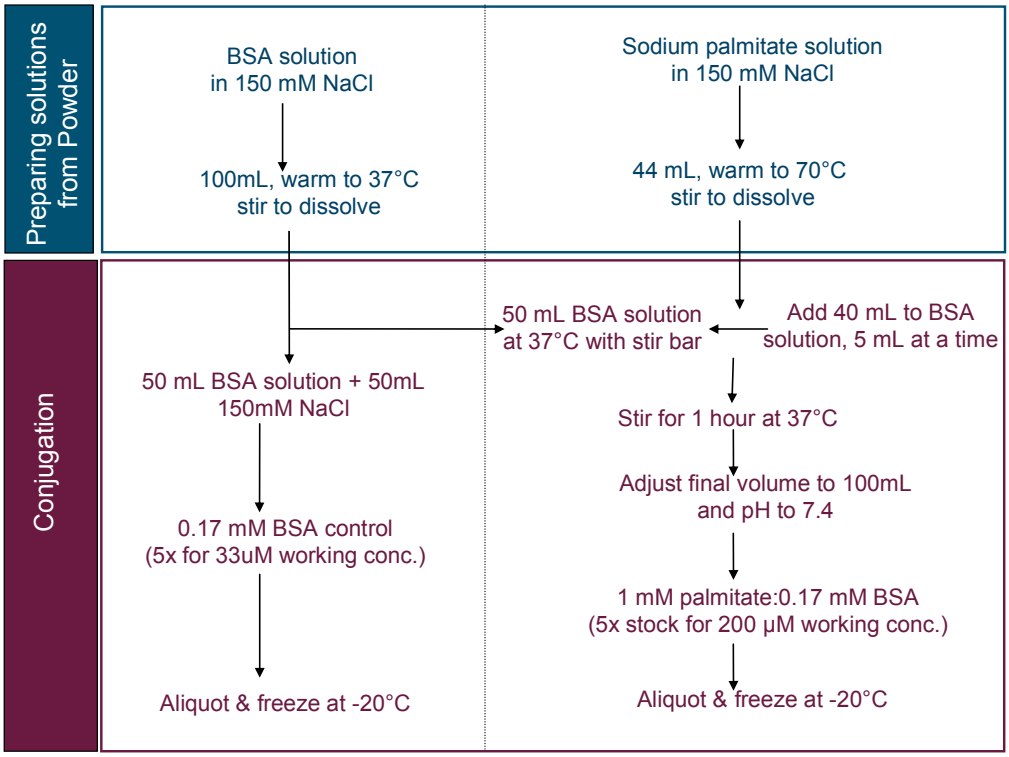
Preparation of Bovine Serum Albumin-Palmitate conjugate for XF24 FAO Assays



Process Flow Chart

I. Materials and reagents

Sodium palmitate (Sigma, CN P9767)
Ultra fatty acid free BSA (Roche, CN 03 117405 001)
Tissue culture grade deionized water (Gibco 1523-0147)
5 M NaCl solution (Sigma, CN S6316)
Glass beakers or Pyrex glass jars, 1 L , 2
Glass beakers, 250 mL, 2
Glass Erlenmeyer flask, 50 mL, 1
Small stir bars, 3
Heated stir plate, 2
Thermometer, 2
150 mL filter unit, 0.22 micron (Corning, CN 430626)
Glass vials, 4 mL (National Scientific, CN B7999-2)
Ice

II. Preparation of 1 mM Sodium Palmitate /0.17 mM BSA Solution (6:1 FA:BSA)

Note: keeping each solution at the specified temperature is critical in this protocol

II.1. Setup prior to BSA and sodium palmitate solution preparation

II.1.1. Warm about 200 mL tap water in each of 2 1 L beakers in 37°C water bath.

II.1.2. Warm 250 mL beaker with stir bar in 37°C incubator.
II.1.3. Make 300 mL 150 mM NaCl by adding 9 mL 5 M stock to 291 mL dH_2O

II.2. BSA solution preparation

Note: ½ of the solution is intended for making aliquots of a BSA vehicle control for the FAO assay. The remaining ½ is conjugated with sodium palmitate.

II.2.1. Weigh out 2.267 g ultra-fatty acid free (FAF) BSA,
II.2.2. Add BSA to 100 mL 150 mM NaCl in 250 mL glass beaker while stirring in a stir plate.
II.2.2. Cover beaker with parafilm and place in one of the 1L water baths pre-warmed to 37°C on a heated stir plate; adjust heat as needed to maintain temperature near 37°C but never more than 40˚C (keep a thermometer in the water bath).
II.2.3. Stir until the BSA is completely dissolved.
II.2.4. Transfer the BSA solution to the upper chamber of 150 mL filter unit in laminar flow hood. Filter with vacuum.
II.2.5. Transfer 50 mL BSA to a pre-warmed 250 mL beaker, cover with parafilm, return to a 37°C water bath and resume stirring.
II.2.6. Dilute the remaining 50 mL BSA solution with 50 mL 150 mM NaCl for 0.17 mM BSA stock.
II.2.7. Aliquot 4 mL/glass vial and freeze at -20°C for use as a BSA control.

II.3. Sodium palmitate solution preparation

II.3.1. Once BSA is stirring in the water bath, weigh 30.6 mg of sodium palmitate, and add it to 44 ml of 150 mM NaCl solution in a 50 mL Erlenmeyer flask.
II.3.2. Cover the flask with parafilm and weight it with a lead ring. Place in the other pre-warmed 1 L water bath on a heated stir plate; heat to 70°C while stirring (thermometer in water bath).
II.3.3. Palmitate solution may appear increasingly cloudy as the temperature reaches 50-60°C but will clarify near 70°C.

II.4. Conjugating Palmitate and BSA

II.4.1. Remove parafilm from both beaker and flask.
II.4.2. Transfer 40 mL hot palmitate solution to the 50 mL of BSA solution stirring at 37°C. Palmitate will precipitate if it is allowed to sit in a pipette – thus transfer 5 mL at a time, 8 times, in a 10 mL pipette, taking up and expelling quickly.
II.4.3. Re-cover beaker with parafilm.
II.4.4. Stir at 37°C for 1 hour, monitoring temperature of the water bath to keep it between 35 and 40˚C. Add ice to water bath to lower temperature if it reaches 40°C.
II.4.5. Measure volume in 100 mL glass graduated cylinder and adjust final volume to 100 ml with 150 mM NaCl for a 1 mM palmitate solution.
II.4.6. Check pH with pH meter and adjust to 7.4. (Should take 5-10 µL of 1 N NaOH.) Note: clean the pH meter afterwards following manufacturer's instructions.)
II.4.7. Aliquot 4 mL (or smaller volume if preferred) per glass vial and freeze at -20°C.

III. Thawing Palmitate-BSA and BSA for FAO assay

III.1. Thaw palmitate-BSA and BSA for control in a 37°C water bath for about 7-10 minutes before loading onto an XF cartridge. Suggestion: if palmitate is thawed immediately before pre-incubating the cell plate, it will be ready to load by the time the medium change is accomplished.

III.2. Palmitate and BSA are known to be stable at -20°C for at least two weeks, and thought to be stable for up to one month.

Note: Specifications of Solutions

1. Palmitate is 1 mM for a 5x dilution to 200 µM final. It is complexed in a 6 to 1 molar ratio with BSA which is 0.17 mM and diluted 5x to 33.3 µM final.
2. The conjugated palmitate can be diluted to a lower concentration with 150 mM NaCl or KHB.
3. This protocol can be adapted to make a 2 mM palmitate solution for 10x injection, or 8x, etc. as desired.