

Preparation of Bovine Serum Albumin (BSA)-Conjugated Palmitate

A substrate for measuring fatty acid oxidation [FAO] in the XF Analyzer

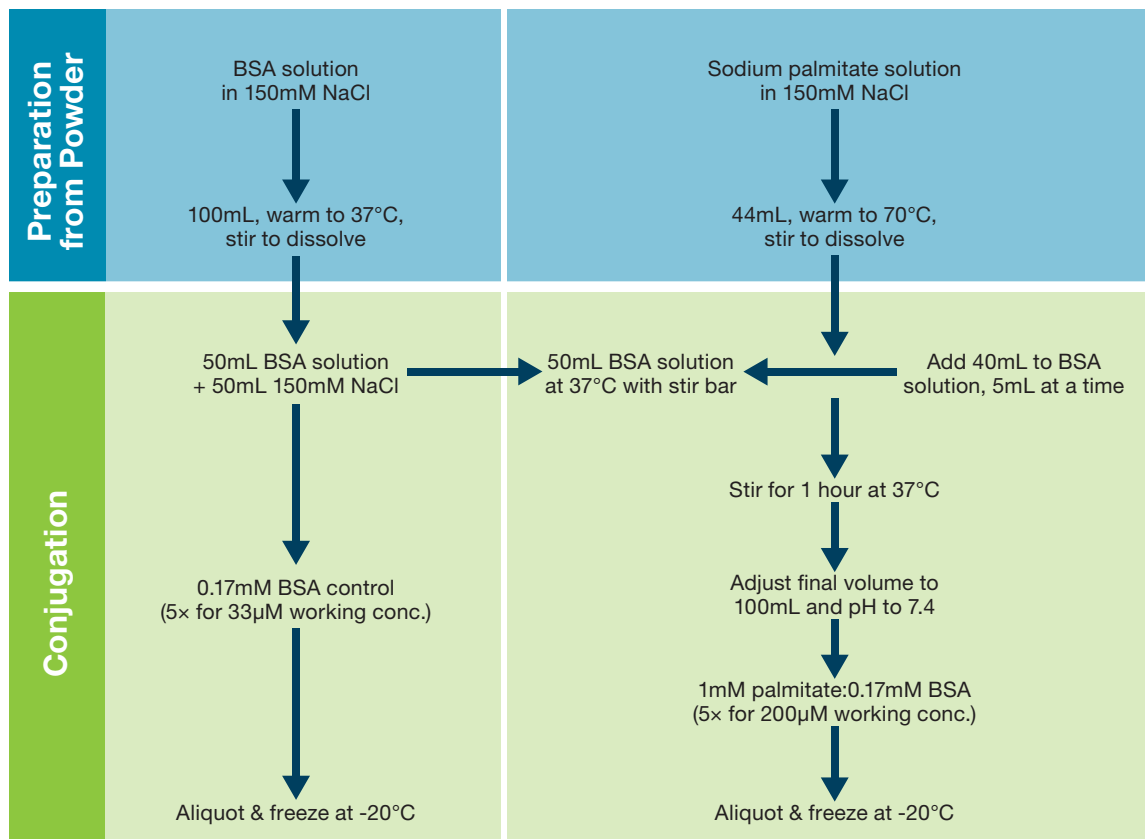
Introduction

Palmitate is the most prominent saturated fatty acid utilized in the body. However the utilization of Palmitate in cell-based assays is challenging due to its low solubility in aqueous solutions. Bovine Serum Albumin (BSA) has been used as a carrier, and stabilizing agent, for insoluble fatty acids. Because Palmitate conjugation to BSA creates an aqueous-soluble reagent that can be absorbed and utilized by cells, it is well-suited for use in cell-based-assays. This protocol describes the preparation required for BSA-Conjugated Palmitate.

The time required to complete this protocol is approximately 3 hours. This protocol may be reasonably scaled up or scaled down without affecting conjugate yield or quality.

References to FAO protocols using the XF Analyzer on page 4.

Protocol Flow Chart



Materials

Reagents and Consumables

Product	Company	Cat #
Sodium Palmitate	Sigma	P9767
Ultra Fatty Acid Free BSA	Roche Applied Science	03117405001
TC grade deionized water	Invitrogen	15230-147
NaCl solution (5M)	Sigma	S6316

Other Equipment

- Glass beaker, 1 liter, or shallow glass dish (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 (1)
- Lead ring weight (1)
- Glass vials with screw caps, 4 mL

Note: Palmitate-BSA will readily adhere to plastic vials. Use glass vials only.

- Standard lab scale
- Ice

Protocol

I. Preparation of Sodium Palmitate BSA Solution

1. 1 mM Sodium Palmitate/0.17 mM BSA Solution (6:1 molar ratio Palmitate:BSA)

Note: Maintaining specified temperatures for all solutions is critical to achieving quality conjugation

2. Warm 200 mL of tap water in two separate 1L beakers, held in a 37°C water bath
3. Warm a 250 mL beaker with a stir bar in 37°C incubator
4. Prepare 300 mL of a 150mM NaCl solution by adding 9 mL of 5M stock to 291 mL dH₂O

II. Preparing BSA Solution (100 mL)

Note: 1/2 of the prepared BSA solution is conjugated with Sodium Palmitate, the remaining 1/2 is used to prepare aliquots for vehicle control in subsequent assays.

1. Weigh out 2.267 g Ultra Fatty Acid Free BSA
2. Add 100mL of 150 mM NaCl solution to the pre-warmed 250 mL glass beaker with a stir bar, and place on a stir plate
3. Add the BSA to the beaker while stirring constantly
4. Place a pre-warmed 1L beaker with tap water on a heated stir plate, and place a thermometer in the water bath
5. Cover the beaker containing the BSA solution with parafilm, and place it in the 1L beaker/water bath
6. Adjust the heat as needed to maintain the temperature at approx 37°C but not higher than 40°C
7. Stir until the BSA is completely dissolved
8. Transfer the BSA solution to the upper chamber of a 150 mL filter unit in a laminar flow hood
9. Filter with a vacuum
10. Transfer 50 mL of the filtered BSA to a pre-warmed 250 mL beaker, cover it with parafilm, and place in the 1L beaker/water bath, resuming stirring
11. Dilute the remaining filtered BSA solution with 50 mL of 150 mM NaCl solution, to make 0.17 mM stock
12. Aliquot into 4 mL glass vials and freeze at -20°C for later use as a vehicle control

III. Preparing Palmitate Solution

1. While the BSA is being stirred in the water bath, weigh out 30.6 mg of the Sodium Palmitate, and add it to 44 ml of 150 mM NaCl solution in a 50mL Erlenmeyer flask with a stir bar
2. Cover the flask with parafilm and weight it with a lead ring.
3. Place the other pre-warmed 1L beaker with tap water on a heated stir plate, and place the thermometer in the water bath
4. Place the Palmitate flask in the beaker/water bath, and heat to 70°C while stirring

Note: The Palmitate solution may become cloudy between 50-60°C. It will become clear as it reaches near 70°C.

IV. Conjugating Palmitate and BSA

1. Remove the parafilm from both the beaker and the flask
2. Transfer 40mL of the 70°C Palmitate solution to the BSA solution while stirring at 37°C

Note: The Palmitate will precipitate if it is allowed to sit in a pipette – transfer 5 mL at a time in a 10mL pipette, aspirating and dispensing quickly

3. Cover the beaker with parafilm
4. Stir at 37°C for 1 hour, monitoring the temperature of the water bath to keep between 35°C and 40°C

Note: Add ice to the water bath to lower the temperature if it approaches 40°C

5. Adjust the final volume to 100 mL in a glass graduated cylinder with 150 mM NaCl

Note: This creates a 1 mM Palmitate-conjugate working solution

6. Check the pH and adjust it to 7.4 with 1N NaOH
7. Prepare 4mL aliquots in glass vials and freeze at -20°C

Note: Palmitate-BSA is stable for at least two months when stored properly at -20°C

V. Thawing Palmitate-BSA and BSA Vehicle Control

1. Thaw the Palmitate-BSA and BSA vehicle control in 37°C for 7-10 minutes prior to loading
2. The Palmitate-BSA and BSA vehicle control should be loaded into two ports per well, and administered via simultaneous injection, to achieve the desired concentrations in the assay

References for FAO assays using the XF Analyzer

1. Measuring fatty acid oxidation in muscle cells: A sensitive non-radiometric real-time assay of FAO
Seahorse Bioscience Application Note
2. Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells
Pike L S, Smift A L, Croteau N J, Ferrick D A and Wu M; *Biochim Biophys Acta*. 2010 Nov 16; Epub ahead of print.
3. SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells
Nasrin N, Wu X, Fortier E, Feng Y, Bare O C, Chen S, Ren X, Wu Z, Streeper R S and Bordone L; *J Biol Chem*. 2010 Oct 15; 285(42):31995-32002. Epub 2010 Aug 6.
4. Mechanism of action of metformin on insulin sensitization: Selective Fatty Acid Oxidation (FAO) over glucose oxidation via inhibition of complex I and activation of amp-activated protein kinase in C2C12 myocytes
M Wu*, A Smift, D Chen, R Winer M Moran, G Hardie, O Shirihai, D Ferrick; 16th European Congress on Obesity ECO 2008, T3.PS.2
5. Advances in measuring cellular bioenergetics using extracellular flux
Ferrick D A, Neilson A and Beeson C; *Drug Discov Today*. 2008 Mar 1; 13(5-6):268-274. Epub 2008 Mar 18.

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