Mitochondrial Triglyceride Content Assay.v4

This assay is designed to measure the triglyceride content of isolated mitochondria from skeletal muscle samples using the "Serum Triglyceride Determination Kit" by Sigma (catalog number TR0100). The assay is to be performed using isolated skeletal muscle mitochondria instead of serum samples.

Procedures

Mitochondrial isolation from adult skeletal muscle

1. Pre-cool the centrifuge (Marathon) to 4°C.
2. Euthanize the mouse under anesthesia (Isoflurane-cervical dislocation).
3. Harvest GA muscle (~130 mg). Trim off visible connective tissue and mince the muscle well with a pair of scissors on a piece of parafilm immediately.
4. Transfer the sample to a 50-ml centrifuge tube with 5 mL of ice-cold Isolation Buffer.
5. Add 200 µl of freshly made Nagarse (5 mg/mL) and incubate for exactly 1 min on ice.
6. Homogenize the sample using a Polytron at the lowest speed for 15" x 3 times with at least 10" intervals in an ice-water bath (a beaker containing ice and water)
7. Add 15 ml of ice-cold Isolation Buffer immediately.
8. Centrifuge at 700 g (not rpm) in Marathon centrifuge for 10 min at 4°C.
9. Pouring the supernatant to a 50-mL centrifuge tube. IMPORTANT: Do not try to be aggressive since the pellet may be loose and contains a lot of debris.
10. Centrifuge in at 10,000 g for 10 min at 4°C.
11. Remove supernatant and resuspend the pellet in 15 mL of Suspension Buffer, transfer the suspension to a 15-mL conical tube and centrifuged at 8,000 g for 10 min at 4°C.
12. Resuspend the mitochondrial pellet (which is a streak on the side) in 50 or 100 µL (pending the size of the pellet) of Suspension Buffer and keep on ice.
13. Perform protein assay to determine the protein concentrations.

Mitochondrial Triglyceride Content Determination

1. Prepare Free Glycerol and Triglyceride Reagents according to below procedures.
2. Prepare serial dilutions of Glycerol Standard (stock = 2.5 mg/ml) such that you have concentrations of 0, 0.039063, 0.078125, 0.15625, 0.3125, 0.625, 1.25, and 2.5 mg/ml. Standards can be prepared in ddH20 or in suspension buffer. Preferably use the same mixture of suspension buffer in which your mitochondria have been suspended.
3. Set the spectrophotometer wavelength to 540 nm and absorbance reading to zero with water as reference.
4. Warm Free Glycerol and Triglyceride Reagents to room temperature.
5. Pipette 200 µl of Free Glycerol Reagent into each well.
6. Add 2.5 µl of appropriate glycerol standard to wells in duplicate, add 20 µl of sample to each appropriate well. Make sure that all samples are well mixed in tube, you can do this by pipetting up and down the bulk of the volume in the well.
7. Incubate for 5 min at 37°C.
8. Read and record initial absorbance (IA) for each well at 540 nm. In the FLUOmega software select the MITO TAG protocol.
9. Add 50 µl of reconstituted Triglyceride Reagent to each well and incubate at 37°C for 5 minutes.

10. Read and record final absorbance (FA) for each well at 540 nm. In the FLUOmega software select the MITO TAG protocol.
11. Calculate standard curve for both IA and FA readings.
12. Calculate triglyceride concentrations as follows:
    a. Use FA to calculate total triglyceride
    b. To calculate "true triglyceride content" you can use the following:
        i. Create a corrected standard curve by using corrected absorbance calculated as follows:
        = (FA Standard X - (IA Standard X * F))
        Where F = 0.81/1.01 = 0.80
        ii. Calculate corrected absorbance of samples using the above equation.
        iii. Use the corrected standard curve to calculate sample concentrations using corrected absorbance.
13. Convert sample concentrations to triglyceride per µl of loaded sample by dividing concentration above by 20.
14. Convert concentration to mM/l by multiplying the concentration in mg/ml by 1.129.
15. Convert mM to pM and multiply by reciprocal of protein concentration of sample to calculate pM/µg mitochondrial protein.
    a. E.g., 3mM/l * 1µl/2 µg where 1liter = 1,000,000 µl and 1 mM = 1,000µM is = 0.003 µM triglyceride/µg mitochondrial protein to get µM/µg mitochondrial protein, then multiply by 1,000,000 pM per µM to get pM/µg.

Reagents

Mitochondrial Isolation

Isolation Buffer (1000 ml)

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Sucrose	Sigma S7903	342.3	Powder	51.34 g	150 mM
KCl	P217-500	74.551	1 M	75 ml	75 mM
Tris-base	Fisher	121.14	1 M, pH 7.4	50 ml	50 mM
KH2PO4	P-380-500	136.09	1 M	1 ml	1 mM
MgCl2	M33-500	203.3	1 M	5 ml	5 mM
EGTA	EG:200-651-2	380.34	0.5 M, pH 7.4	2 ml	1 mM
BSA	Sigma A7979		35%	5.71 ml	0.2%

Dissolve in ~800 ml of ddH2O and adjust the pH to 7.4. Add ddH_2O to a final volume of 1000 ml. Filter sterilize and store at 4°C.

Suspension Buffer (1000 ml)

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Sucrose	Sigma S7903	342.3	Powder	85.6 g	250 mM
Tris-base	Fisher	121.14	1 M, pH 7.4	10 ml	10 mM
EGTA	EG:200-651-2	380.34	0.5 M, pH 7.4	0.2 ml	0.1 mM

Dissolve in ~800 ml of ddH2O and adjust the pH to 7.4. Add ddH_2O to a final volume of 1000 ml. Filter sterilize and store at 4°C.

Nagarse (5 mg/ml) 1 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Nagarse	Sigma P8038			5 mg	5 mg/ml

Take the enzyme bottle out of the storage. Warm up the bottle completely. Weigh 5 mg of nagarse. Dissolve nagarse in Isolation Buffer to make a final concentration of 5 mg/ml and keep on ice and use it fresh (This is enough for 5 mice).

Triglyceride Reagent 10 mL

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Triglyceride	T2449			10 ml	

Reagent contains:

250,000 units/L Lipase (microbial)
0.05% Sodium azide, as preservative
Nonreactive stabilizers and fillers

Reconstitute reagent in 10 ml ddH_20 and store at 4°C.

Free Glycerol Reagent 40 mL

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Free Glycerol	F6428			40 ml	

Reagent contains:

0.75 mM ATP
3.75 mM Magnesium salt
0.188 mM 4-Aminoantipyrine
2.11 mM N-Ethyl-N-(3-sulfopropyl) m-anisidine, sodium salt
1,250 units/L Glycerol Kinase (microbial)
2,500 units/L Glycerol Phosphate Oxidase (microbial)
2,500 units/L Peroxidase (horseradish)
Buffer, pH 7.0 ± 0.1
0.05% Sodium azide (as preservative)
Nonreactive stabilizers and fillers

Reconstitute reagent in 40 ml ddH_2O and store at 4°C.