Ca^{2+} Challenge Assay for Mitochondria Isolated from Adult Skeletal Muscle.v1.4

This assay determines if isolated mitochondria from adult skeletal muscle are susceptible to Ca^{2+}-induced mitochondrial permeability transition based on a method published previously (Csukly et al., 2006; Fontaine et al. 1998)

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 clean of 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 µl of Suspension Buffer and keep on ice.
13. Perform protein assay to determine the protein concentrations.

Ca^{2+} challenge assay

1. Open the BMG FLUOmega software and chose the existing Calcium Green Protocol.
2. Use a 15 ml tube with at least 10 ml of $CaCl_2$ (312 µM) and prime the auto-injector. The auto-injector will inject 4 µl into the 100 µl well.
3. Prepare 1 ml of Mitochondrial Challenge Buffer SR by adding 10 µl of Na-Succinate (0.5 M), 5 µl of Rotenone (200 µM). Keep at room temperature and protect it from light.
4. Prepare 1 ml of Mitochondrial Challenge Buffer GM by adding 10 µl of Glutamate (0.5 M), 10 µl of Malate (0.25 M). Keep at room temperature.
5. Prepare Calcium Green 5N (100 µM) by adding 2 µl of stock (1 mM) to 18 µl Mitochondrial Challenge Buffer. Store at room temperature wrapped by aluminum foil.
6. Add 100 µl Mitochondrial Challenge Buffer SR and 15 µg of mitochondria to a well of Falcon 96 plate. Use the same well for measurements.
7. And 1 µl of Calcium Green 5N stock (100 µM). Incubate in the FLUOmega for 5 min.
8. Start the measurement. The machine will add calcium pulses (83-nmol/mg protein) every 2 minutes for 8 min. Additional cycles will need to be run after the first ends.
9. Continue measurements until a plateau of fluorescence is achieved.
10. Repeat step 6-9 using Mitochondrial Challenge Buffer GM.
11. Prime the FLUOmega with ddH2O 5 times and turn off the machine.

Reagents

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 ddH2O 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 ddH2O to a final volume of 1000 ml. Filter sterilize and store at 4°C.

Mitochondrial Challenge Buffer (100ml)

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
MOPS	Sigma M1254	209.3	100 mM	10 ml	10 mM
Sucrose	Sigma S7903	342.3	20% (584 mM)	42.8 ml	250 mM
Pi-Tris	Sigma 93348	219.1	100 mM	10 ml	10 mM
EGTA	EG:200-651-2	380.34	0.5 M, pH 7.4	10 µl	0.05 mM

Dissolve in ~80 ml of ddH2O and adjust the pH to 7.4. Add ddH2O to a final volume of 100 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 1 ml of 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).

$CaCl_2$ Stock Solution (100 mM) 100 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
$CaCl_2$	Sigma C3881	147		1.47 g	100 mM

Weigh CaCl2 and add 80 ml of ddH2O and completely dissolve it. Add ddH2O to a final volume of 100 ml and filter sterilize the solution and store at room temperature.

$CaCl_2$ (312 µM) 500 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.

$CaCl_2$	Sigma C3881	147	100 mM	1.56 ml	312 μM

Adding 1.56 ml of CaCl2 Stock Solution to 498 ml of ddH2O. Store at room temperature.

Succinate (0.5 M) 10 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Na-Succinate dibasic	Sigma S2378	270.14		1.35 g	0.5 M

Weigh 1.35 g Na succinate dibasic and add ddH2O to 10 ml and completely dissolve it. Filter sterilize the solution and store in 0.5 ml aliquots at -20°C (pH is around 7.1).

Rotenone Stock Solution (2 mM)

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
Rotenone	Sigma R8875	394.42		4.7 mg	2 mM

Dissolve 4.7 mg of rotenone in 6 ml of absolute (100%) ethanol. Mix well for complete dissolution after overnight. Store in 1 ml aliquots at -20°C. Before use, dilute 1:10 to 200 μM in Mitochondrial Challenge Buffer and store in ice.

Glutamate (0.5 M) 100 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
L-Glutamic acid	Sigma G1251	147.13		7.36 g	0.5 M

Weigh 7.36 g L-Glutamic acid and dissolve in ~75 ml ddH2O. Adjust the pH to 7.3 with 10 N NaOH (takes about 10 ml) and add ddH2O to 100 ml. Filter sterilize the solution and store in 10-ml and 1-ml aliquots at -20°C.

Malate (0.25 M) 100 ml

Reagent	Catalog#	MW/FW	Stock C.	Quantity	Final C.
L-(-)-Malic acid	Sigma M1000	134.09		3.35 g	0.25 M

Weigh 3.35 g L-(-)-Malic acid and dissolve in ~75 ml ddH2O. Adjust the pH to 7.3 with 10 N NaOH (takes about several ml) and add ddH2O to 100 ml. Filter sterilize the solution and store in 10-ml and 1-ml aliquots at -20°C.

Calcium Green 5N (1 mM)

Dissolve the dye in powder of 500 μg (Invitrogen C3737, MW 1192.19) in 419 μl in anhydrous dimethylsulfoxide (DMSO) to get the final concentration of 1 mM. Please protect against light all the time. Store the dye in 5-μl aliquot at -20°C.

References

1. Csukly et al. Muscle denervation promotes opening of the permeability transition pore and increases the expression of cyclophilin D. J Physiol (Lond) (2006) vol. 574 (Pt 1) pp. 319-27

2. Fontaine et al. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation By electron flow through the respiratory chain complex i. The Journal of biological chemistry (1998) vol. 273 (20) pp. 12662-8