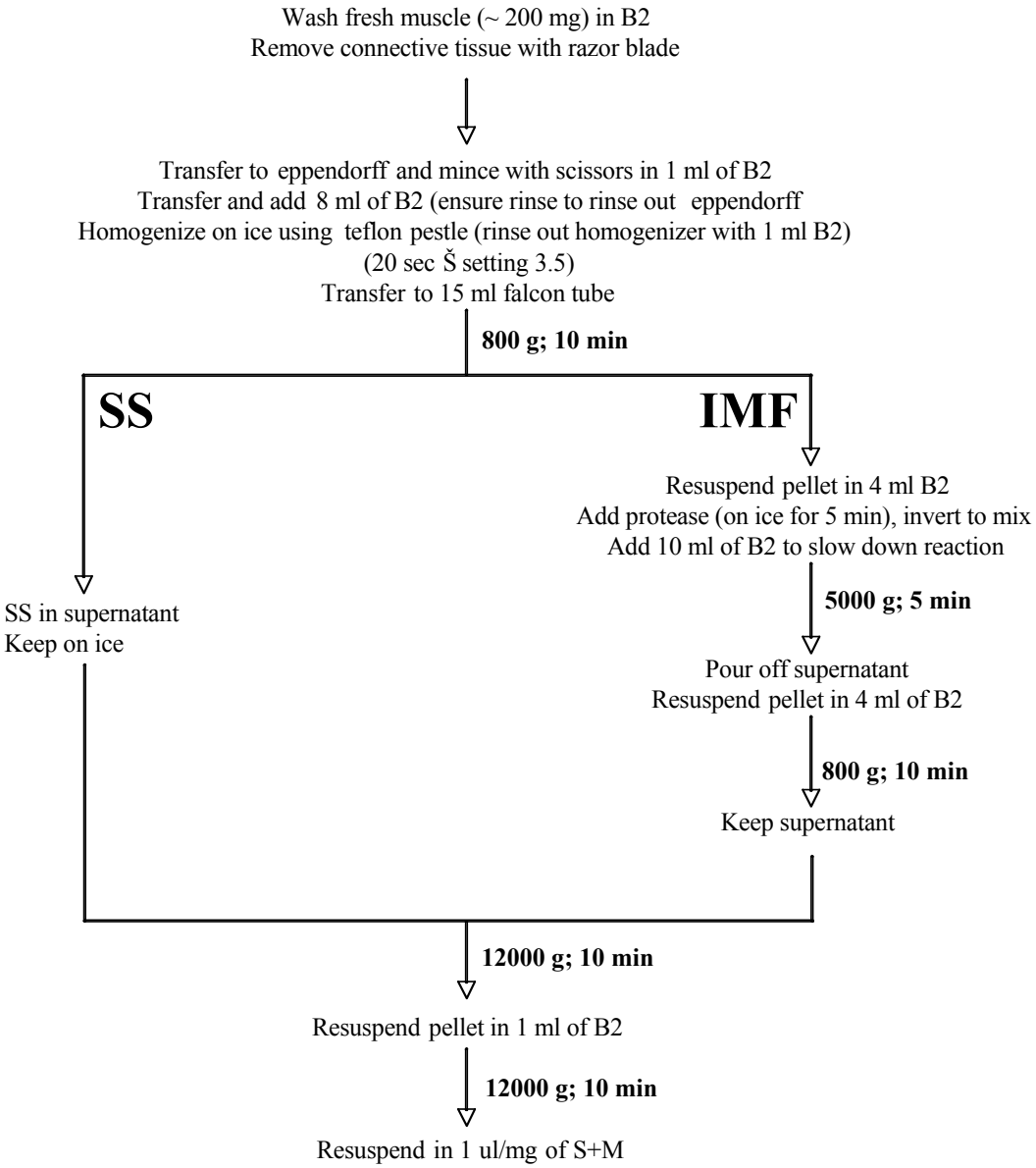
Mitochondrial Isolation



MITOCHONDRIAL ISOLATION BUFFERS

1. Buffer 1 (pH 7.4)

	1 L
50 mM Tris HCl	7.88 g
5 mM $MgSO4*7H_2O$	1.23 g
5 mM EDTA	1.86 g
100 mM KCl	7.45 g

Add 800 ml of distilled H_2O, pH to 7.4 with NaOH. Adjust to final volume. Store in glass at 4˚C, shelf like 6 months.

2. Buffer 2 (pH 7.4)

	500 mL
ATP	0.303 g
Buffer 1	500 mL

Check pH (should be 7.4). Store in glass at 4˚C, shelf life 6 months.

3. S+M (pH 7.4)

	50 mL
220 mM sucrose	3.76 g
70 mM mannitol	637.7 mg
10 mM Tris HCl	78.8 mg
1 mM EDTA	18.61 mg

Add 40 ml of distilled H_2O, pH to 7.4. Adjust to final volume. Store in glass at 4˚C, shelf life 6 months.

4. Protease

Protease (Sigma P-5380)	1 mg
Buffer 2	100 µl

Use 0.025 µl/mg. Make fresh daily.

Palmitate Oxidation

Isolate mitochondria according to standard protocol;

1. Place a 1.5 ml eppendorfs (without cap) in 20 ml glass vial; make 3 vials for each sample and blanks

   (Sample blanks: 1000 µl S-MKR + 0 mito + 750 µl label)

2. Add **900 µl S-MKR (pre-gassed for 20 min) and 100 µl mitochondria** sample to the base of the vial

3. Place **500 µl benzethonium hydroxide** into the small eppendorfs, and seal the system with a rubber stopper

4. If using SSO to inhibit FAT/CD36 during oxidation: add 1 µl of either SSO or DMSO to the vials (3 vials for each sample with SSO, 3 with DMSO); cap and incubate at 37˚C in shaking water bath for 30 minutes*** **see Notes**

5. If not using SSO (or after 30 minute incubation), **add 750 µl of label** to samples and blank (or MKR for label blank) through cap at 20 sec intervals; incubate at 37˚C in shaking water bath for 30 minutes

6. Stop reaction by adding **500 µl PCA** through cap at 20 sec intervals using a 1 ml syringe (aim for bottom of vial)

7. Using a Hamilton syringe, **remove 500 µl** of reaction medium for determination of **acid soluble product**; keep original vials for **CO_2 production** measurements

ACID SOLUBLE PRODUCT

- Place 600 µl reaction medium to **3 ml of chloroform:methanol** in 15 ml flat-bottom tubes; cap and shake at RT for 15 min
- Add **1.2 ml of 2 M KCl:HCl** solution to the tubes and shake at RT for 15 min
- Pellet out the precipitate at **5000 g (6500 rpm) for 15 min**
- Pipet **1 ml of the aqueous top layer** to a scintillation vial (do in duplicates – results in 6 scintillation vials per samples)
- Add **5 ml scintillation cocktail** to the vials, allow to sit in the dark for 12 hours and then count

CO_2 PRODUCTION

- Using a third syringe, add **1 ml of sulfuric acid** to the base of original vial to release CO_2 by shifting pH; swirl gently
- Allow to **incubate** in the dark for 1 hour
- **Remove eppendorfs** from the vials and place into scintillation vials
- Add **10 ml scintillation cocktail** to the vials, allow to sit in the dark for at least 12 hours and then count (1 vial per sample)
- Use 750 µl (in triplicates) of left-over S-MKR for SA measurements

Notes: Technique for SSO/DMSO

- Pre-incubate mitos in buffer and SSO/DMSO for 30 minutes, then spin down mitos (10 000g) and wash twice with 1 ml of S+M
- Resuspend mitos in original volume (100 µl), add buffer and eppendorfs with benzothonium, cap and start reaction

Palmitate Oxidation Solutions

Modified Krebs Ringer (MKR)

	In 1 L H_2O
• NaCl (115 mM)	6.721 g
• KCl (2.6 mM)	0.194 g
• KH_2PO_4 (1.2 mM)	0.163 g
• $NaHCO_3$ (10 mM)	0.840 g
• Hepes (10 mM)	2.383 g

Can be stored at 4˚C for ~ 2 months.

Supplemented MKR (S-MKR)

	In 20 ml MKR
• ATP (5 mM)	60.25 mg
• NAD+ (1 mM)	14.40 mg
• Cytochrome C (25 uM)	6.19 mg
• CoA (0.1 mM)	1.62 mg
• L-malate (0.5 mM)	1.38 mg
• L-carnitine (0.5 mM)	125 ul of 40 mM solution

Store a 4˚C; make daily.

Palmitate-BSA complex (10X stock solution)

Note: when working with new ^{14}C-palm label, evaporate toluene under N_2 and reconstitute in original volume for 100% ethanol

- Dissolve 23.1 mg of cold palmitate in 10 ml of 100% ethanol
- Add 10 μCi of ^{14}C-palmitate to 10 ml of cold palmitate
- Add 10 ml of 13.5 mM KOH to palmitate mixture to form soap
- Evaporate ethanol from palmitate soap mixture for 2 hours at 45˚C under N_2 (volume should be decrease from 20 ml to 10 ml)
- Add 1 g BSA and 0.5 ml of 100 mM $CaCl_2$ to 40 ml of MKR
- Using a Pasteur pipette, slowly add palmitate soap mixture to 40 ml of MKR while stirring
- Total volume is 50 ml; aliquot and freeze at –20˚C
- Dilute 10-fold before using (1 ml palmitate-BSA + 9 ml MKR = 10 ml); 12 vials

2:1 Chloroform:Methanol:

200 ml chloroform + 100 ml ethanol

1 M Sulfuric Acid:

28 ml to 500 ml H_2O

2 M KCl : HCl:

74.33 g KCl
83 ml HCl
Fill up to 500 ml with H_2O

Perchloric Acid (PCA): use as 70%

100 mM $CaCl_2$:

147 mg in 10 ml H_2O

13.5 mM KOH:

20 mg in 20 ml H_2O

CALCULATION

Total palmitate oxidation (nmol/hour/mg protein) = Acid Soluble + CO_2 counts

Acid Soluble:

1. Prior to calculating from the DPM printout have to account for 2 dilution factors

    - DPM / (acid soluble sample / total volume)
      where the acid soluble sample is 600 µl and the total volume is 2250 µl
    - Corrected DPM / (volume counted / total volume)
      where the counted sample is 1000 µl and the total volume is 2800 µl (600µl sample+1000µl methanol+1200 µl KCL-HCL)

note: do not need to correct for chloroform

2. Subtract the blank from the corrected DPM

3. Multiply this number by 2 to get DPM/hour (reaction ran for 30 minutes)

4. Divide this number by specific activity (SA)

    SA – DPM / nmol amount of the amount of hot and cold palmitate loaded

5. To account for protein loaded, divide number calculated in step 4 by the amount of protein loaded (mg)

CO_2 Counts:

6. Prior to calculating from the DPM printout have to account for 1 dilution factor

    - DPM / ((total volume – volume removed for acid soluble) / total volume) where the total volume is 2250 µl and the volume removed for acid soluble counts is 600 µl

7. Subtract the blank from the corrected DPM

8. Multiply this number by 2 to get DPM/hour (reaction ran for 30 minutes)

9. Divide this number by specific activity (SA)

    SA – DPM / 135 which accounts for the amount of hot and cold palmitate loaded

10. To account for protein loaded, divide number calculated in step 4 by the amount of protein loaded (mg)

CPTI ASSAY

REFERENCE: McGarry et al., Biochem J. 214:21-28, 1983

PREPARATION:

1) Turn ON water bath; set at 37°C
2) Let aliquots for assay mixture thaw
3) Prepare 4 sets of eppendorffs for each condition being tested in duplicates (ie: Blank, max activity and 3 different [M-CoA])
4) First set (PREP) should have 28.5 µl of water or a combination of water and effector adding up to 28.5 µl
5) Second set (BUT/H_2O) should have 200 µl of saturated butanol and 100 µl of water
6) Third set (25 H_2O) should have 25 µl of water
7) fourth set (15 H_2O) should have 15 µl of water

ASSAY MIXTURE: Multiply volume by number of samples (x 2 for dupl.) + 2
In a test tube, put the following chemicals

117 mM Tris-HCl……..	20.5 µl of 1 M solution
0.28 mM Reduced glutathione……..	5 µl of 5 mM solution
4.4 mM ATP…….	5 µl of 80 mM solution
2.22 mM KCN……..	5 µl of 40 mM solution
Rotenone……...	5 µl of 0.8g/L solution
0.5% BSA...……..	5 µl of 10% BSA
300 µM Palmitoyl-CoA……	10 µl of 3 mM solution
5 mM carnitine…….	5 µl of 100 mM solution
1 µCi hot carnitine……	1 µl of 1mCi/ml stock

Total final volume: 61.5 µl of assay mixture + 28.5 µl H_2O + 10 µl mitos
NOTE: if volume is large enough, pH mixture before adding labelled carnitine; should be ~ 7.0

ASSAY:

1) Pipet 61.5 ul of assay mixture in each PREP eppendorff (volume = 90 µl)
2) Incubate the assay mixture at 37˚C for 5 min before starting the reaction
3) Add 10 µl of mitochondrial suspension to each eppendorff at 15 sec intervals to start reaction
4) Add 10 µl of mitochondrial suspension buffer to blanks instead of mitos
5) Quickly vortex each eppendorff for 2-3 sec and let sit in water bath
6) After 6 min, add 60 µl of ice cold HCl (1M) (again at 15 sec intervals) and put eppendorff on ice
7) The reaction is linear for 8 min

PALMITOYL[^{3}H]CARNITINE EXTRACTION:

1) When the reaction is stopped, add 200 µl of butanol saturated with water to PREP
2) Vortex and centrifuge (5 min @ 1000g)
3) Take the butanol phase (upper) and transfer to 'But/H_20' eppendorffs
4) Vortex and centrifuge again
5) Transfer 250 µl of butanol phase to '25 H_2O' eppendorffs
6) Vortex and centrigure again
7) Transfer 150 µl of butanol phase to '15 H_2O'
8) Vortex and centrifuge again
9) Tranfer 100 µl of butanol phase to 7ml scintillation vial and add 5 ml of 'liquid scintillation cocktail'
10) Count the labeled palmitoylcarnitine (5 min per sample)
11) Measure the specific activity by adding 20 µl of assay mixture directly to 5 ml of LSC (do 2 of these; multiply by 5 for specific activity of 100 µl)

CHEMICALS:

1 M Tris-HCl (pH 7.4)

31.5 g Tris HCl	MW 157.6
6 g Tris base	MW 121.1
1.09 g $MgCl_2$	MW 203.3
1.52 g KCl	MW 74.55
Total volume	250 ml

pH weekly; store at 4˚C

Make several milliliters of the following chemicals; aliquot in 100 µl and store at -20˚C

5 mM Reduced glutathione	1.54 mg/ml	MW 307.3
80 mM ATP	48.42 mg/ml	MW 605.2
40 mM KCN	2.61 mg/ml	MW 65.12
Rotenone	0.8 mg/ml (in ethanol an dH_2O 50:50)	
3 mM Palmitoyl-CoA	10 mg/3.215ml	MW 1038
100 mM L-carnitine	19.77 mg/ml	MW 197.7
10% BSA	1 g/ml	

1 M HCl

Saturated butanol
Add ~ 30 ml of distilled water and 100 ml of butanol in a sep funnel. Mix and wait for separation of both phases. The upper phase is butanol saturated with water and the lower one is the excess of water, which must be removed.

CALCULATION

1. Subtract the blank from you counts per minute (CPM) average for each sample

2. To calculate a rate, divide CPM by 6 (because the reaction ran for 6 min)

3. Specific activity (SA) is calculated as follows:

$$SA = \frac{CPM \times 5}{\text{Hot carnitine (nmol)} + \text{cold carnitine (nmol)}}$$

where – the CPM by 5 (because you only counted 20 µl of buffer, but counted 100 µl for each sample)
– Hot carnitine concentration is 0.01205 nmol
– Cold carnitine concentration is 500 nmol

4. Activity is calculated as follows;

activity #1 (nmol/min) = rate (CPM/min) / SA

activity #2 (µmol/min/ml) = activity #1 (1000 / 10)

where – the 1000 converts to µmol, and 10 accounts for the amount of mitochondria used in assay

activity (µmol/min/g ww) = activity #2 (100 / yield)

where – $\text{yield} = \frac{CS_{TS} - CS_{EM}}{CS_{HOM}} * 100\%$

see citrate synthase (CS) methods below for more detail, but this accounts for the amount of mitochondria isolated, and enables CPTI expression per g wet weight muscle

CITRATE SYNTHASE

This reaction involves the measurement of citrate synthase activity by linking the release of CoASH to the colormetric agent DTNB 5,5-dithiobis-2-nitrobenzoate. Changes in absorbance are followed at 412 nm.

Oxaloacetate + acetyl CoA $\longrightarrow$ CoASH + Citrate

CoA-SH + DTNB $_{\text{pale yellow}}$ $\longrightarrow$ CoA + DTNB $_{\text{dark yellow}}$

PREPARATION OF BUFFERS

100mM TRIS buffer (pH 8.3)
Dissolve 3.0275g in 250 ml of distilled water. Use HCl to adjust pH to 8.3.
Store in refrigerator.

1mM DTNB (Sigma D-8130) Make fresh every 2 weeks
Dissolve 3.96 mg of DTNB in 10 ml of 100mM TRIS buffer (pH 8.3). Store in opaque bottle in refrigerator.

10mM Oxaloacetate (Sigma O-4126) Prepare fresh daily
Dissolve 13.2mg in 10ml of 100mM TRIS buffer, pH 8.3.

3mM Acetyl CoA (Sigma A-2897) Make fresh daily.
Dissolve 3.1mg Acetyl CoA in 1ml of distilled water. Make stock and store at –20°C.

Homogenizing Solution
Add 1.36g of 0.1M KH_2PO_4 and 50 mg of BSA to ~ 80 ml H_2O. Adjust pH to 7.3 with KOH and top to 100 ml with H_2O. Store in fridge for 4-6 months.

PROCEDURE

1. Chip a muscle 6-10 mg wet mass piece and weigh in cryovial

2. Add 100 µl of homogenizing solution/mg wet mass and homogenize until muscle is fully broken up; immediately freeze sample in liquid N_2.

3. Ts and Em are diluted 20 X (5 µl mitos + 95 µl S+M)

4. MH and Ts go through 2 sets or freeze/thaw cycles

5. Prepare spec set up as follows:

ON/IDLE VISIBLE light source
TEMP controller at 37°C
MEMU SETUP: Kinetics; 412 nm, 20 sec intervals, 5min run; 0 and 1.5 abs limits, multi cell sampling device; 6 cells

7. Prepare the following in quartz cuvettes in duplicates:

Reagent	Total Muscle (MH)	Total Suspension (ts)	Extra mitochondrial (em)
Tris buffer (µl)	150	150	160
DTNB (µl)	25	25	25
Acetyl-CoA (µl)	40	40	40
M. homogenate (µl)	10	10	10
Triton (10%) (µl)	10	10	--

8. Use water as blank

9. Insert 6 cuvettes, add 15 µl of **OAA** and stir thoroughly, close lid and push **RUN**

CALCULATIONS:

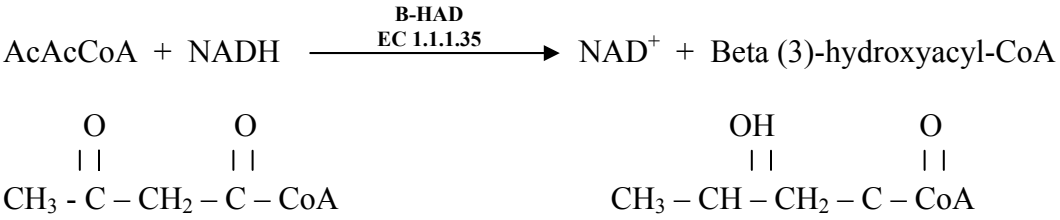
$$\text{CS Activity} = \frac{\text{Abs/min x Total Volume}}{\text{13.6 x Sample Volume}} \times \text{dilution factor (101)} = \mu\text{mol/min/g}$$

Change in absorbance per minute = 0.172
Total volume (in cuvette) = 250 µl
Sample volume (muscle homogenate) = 10 µl
Dilution factor = 100

$$\text{CS Activity} = \frac{0.172 \times 250\ \mu\text{l}}{13.6 \times 10\ \mu\text{l}} \times 100 = 31.93\ \mu\text{mol/min/g}$$

BETA(3)-HYDROXYACYL-COA DEHYDROGENASE

PRINCIPLE:



B-HAD converts the 4 carbon AcetoAcetyl-CoA (name for this fatty acyl-CoA) to a 4 carbon alcohol in the reverse order to what occurs in the B-oxidation pathway.

SOLUTIONS:

Tris – HCl, (1M pH 7.0, fridge) ……..……3.94 g / 25 ml

EDTA (200 mM, fridge)…….. 0.744 g / 10 ml

Triton x-100 (10%, fridge)……. 0.5 ml triton + 2ml ethanol + 2.5 ml H_2O

NADH (5mM, fresh each day)…….. 17.74 mg / 5 ml

Acetoacetyl-CoA** (5 mM, fresh each day)…….…….. 5 mg / 1.13 ml H_2O

**Sigma A1625 (expensive at 10 mg / $188, so weigh out only what you need; 10 µl per cuvette)

ASSAY MIXTURE:

Tubes	**~ 50**	**~ 100**	**~ 200**
Tris-HCl	1.25 ml	2.5 ml	5 ml
EDTA	0.25 ml	0.5 ml	1 ml
NADH*	1.25 ml	2.5 ml	5 ml
H_2O	Up to 25 ml	50 ml	100 ml

* this can be adjusted up or down – you want an initial absorbance of ~1-1.5 after the homogenate has been added.

METHOD:

1. Pipette into each cuvette: 215 µl assay mixture
   25 µl homogenate
   5 µl triton

2. Stir and let sit in spectrophotometer for 5 min.

3. Record slope for 2 min (at 340 nm, 25, 30 or 37^0 C) – this is blank (often no drift at all, and only run this on every 5-10 samples).

4. Add 5 µl AcAcCoA, stir and record decrease in NADH for 3 min. Usually linear from ~1 min on. This assay needs serious stirring – cover with parafilm and invert cuvette (esp. narrow cuvettes) 5-10 times.

Additional points – above assumes a homogenization ditution of 1 mg wet muscle / 100 µl homog. solution. Can increase enzyme slope (activity) by decreasing dilution (i.e. 1 mg / 50 µl). May also alter slope by increasing or decreasing 25 µl homogenate in cuvette.

CALCULATION:

Activity is the slope/min after the addition of the AcAcCoA (i.e. 0.0553) minus the slope of the blank (often 0 or 0.0010).

i.e. Homogenate DF = 101
Cuvette TV = 250 µl
Cuvette SV = 25 µl
Abs/min (blank) = 0.0010
Abs/min (sample) = 0.0553
Ext. coeff. (340 nm) = 6.22 µmol/cm^2

$$\text{B-HAD} = \frac{(0.0553 - 0.0010) \times 250}{6.22 \times 25} \times 101$$

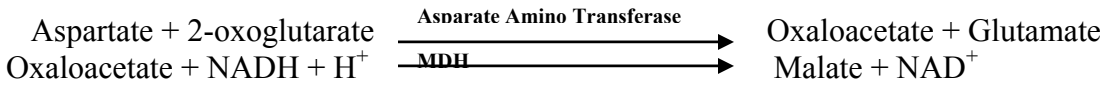
= 8.82 µmol/g wm/min (or mmol/kg wet muscle)

REFERENCE:

Bergmeyer, H.U. *Methods of Enzymatic Analysis.* New York: Academic. 1974. Vol. 1. p. 474.

ASPARTATE AMINO TRANSFERASE

This assay measures maximal aspartate amino transferase activity by pushing the Rx to the right with the addition of excess aspartate and oxoglutarate, and linking the production of the product oxaloacetate to the malate dehydrogenase Rx and the reduction of NADH. Changes in absorbance are followed at 340 nm.



PREPARATION OF BUFFERS

100mM Stock phosphate buffer (pH 7.4)
Dissolve 0.15 g KH_2P0_4 and 1.58 g K_2HP0_4 in 100 ml of distilled water. Use HCl to adjust pH to 7.4. Final concentration is 75 mM.
Store in refrigerator for 6 months.

800 mM Stock 2-Oxoglutarate (pH 7.4)
Dissolve 3.80 g of oxoglutarate in 25 ml of distilled water. Final concentration is 60 mM.
Store in refrigerator for 4 weeks.

4.5 M Stock Aspartate (pH 7.4)
Dissolve 15.3 g in 25 ml of distilled water. Adjust pH with NaOH. Final concentration is 400 mM. Store in refrigerator for 6 months.

NADH, Make fresh daily
Dissolve 7 mg NADH in 800 µl of distilled water.

MDH, Make fresh daily
Dilute MDH (Roche, 127 256) stock 10 times. Final concentration is 6 U.

Homogenizing Solution (pH 7.3)
Add 1.36g of 0.1M KH_2PO_4 and 50 mg of BSA to ~ 80 ml H_2O. Adjust pH to 7.3 with KOH and top to 100 ml with H_2O. Store in fridge for 4-6 months.

PROCEDURE

1. Chip a muscle 6-10 mg wet mass piece and weigh in cryovial.

2. Add 100 µl of homogenizing solution/mg wet mass and homogenize until muscle is fully broken up; immediately freeze sample in liquid N_2. This homogenate is common to the citrate synthase and β-HAD assays.

3. Ts and Em are diluted 20 X (5 µl mitos + 95 µl S+M)

4. MH and Ts go through 2 sets or freeze/thaw cycles

5. Prepare DU70 set up as follows:

ON/IDLE VISIBLE light source
TEMP controller at 25°C TIME DRIVE
MEMU SETUP: Kinetics; 340 nm, 20 sec intervals, 5min run; 0 and 4 abs limits, multi cell sampling device; 6 cells

6. Prepare the following in quartz cuvettes in duplicates:

Reagent	Total Muscle (MH)	Total Suspension (ts)	Extra mitochondrial (em)
Phos buffer (µl)	300	300	310
10% Titon-x (µl)	10	10	--
2-oxoglutarate (µl)	30	30	30
NADH (µl)	5	5	5
MDH (µl)	10	10	10
Sample (µl)	10	10	10

When doing this assay on just plain muscle homogenate – would be best to calculate how many cuvettes needed and make up one large buffer with the 5 ingredients above – then just add sample.

7. Use water as blank

8. Insert 6 cuvettes, and stir thoroughly, close lid and push **RUN**

9. After 1 min, add 35 µl of **ASPARTAT**E to initiate reaction

10. When reaction complete, select Tabulation press COPY to print

CALCULATIONS:

$$\text{mAspAT Activity} = \left(\frac{\text{Abs/min – baseline Abs/min) x Total Volume}}{\text{6.22 x Sample Volume}}\right) \times \text{dilution factor}$$

Dilution factor = 101 for homogenate and 10 for mitochondria

mAspAT Activity = µmol/min/g or mmol/min/kg

WESTERN BLOTTING – GENERAL PROCEDURE

WESTERN
PURIFYING MITOCHONDRIA FOR WESTERNS:

1. Make 60% Percoll: 1.336 ml of 5X SMEA + 4 ml of Percoll + 1.334 ml of H_2O (the density should be 1.1 g/ml)

5X SMEA Buffer (pH 7.0)
- 1.25 M sucrose
- 25 mM MOPS
- 5 mM EDTA
- 25 mM NaN_3

2. Isolate mitos following std protocol

3. In each prep, add 1 ml of 60% Percoll mix on top of mitos in eppendorf

4. Spin tubes at 20 000g for 1 h (set decceleration to '0')

5. Remove middle mito layer, and place in eppendorf

6. Spin at 20 000g for 5h (again, dec at '0')

7. Remove mito layer

8. Store at -80

Notes:
- The first 1 hour spin must be done with fresh mitos but can freeze mitos after taking the mitochondrial layer off (before second spin)

-Try to do a large dilution for the protein assay (60X) b/c Percoll can interfere with protein assay so the less the better.

SDS-PAGE:

- Prepare running gel and stacking gel (do not add APS & Temed to stacking gel until ready to use)
- Assemble clear stand with green gel holder and grey pad
- Pour running gel up to 1 cm below comb
- Add dH_2O to cover gel and tap on bench to ensure no bubbles have formed

	Stacking	Running				
		5%	6%	8%	10%	12%
dH_2O	6.8 ml	11.4	10.6 ml	9.4 ml	8 ml	6.7 ml
1.5M Tris-HCl, pH 8.8	***	5 ml	5 ml	5 ml	5 ml	5 ml
1M Tris-HCl, pH 6.8	1.25 ml	***	***	***	***	***
30 % Acrylamide	1.70 ml	3.4 ml	4 ml	5.3 ml	6.7 ml	8 ml
10 % SDS	100 µl	200 µl	200 µl	200 µl	200 µl	200 µl
10 % APS	100 µl	200 µl	200 µl	200 µl	200 µl	200 µl
Temed	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl

- Turn on heat block to 95ºC
- Thaw samples on ice
- Wait 30 minutes for gel to set
- Soak up water layer using blotting paper
- Using a pipette, add stacking gel up to top of glass plate
- Immediately insert the comb diagonally
- Wait ~ 20-30 minutes until gel has set

SAMPLE PREPARATION:

- Prepare samples in Laemmeli's buffer (see below)
- Mix quantities determined from protein assay in centrifuge tubes, vortex
- Boil for 5 min @ 95ºC using heat block
- Centrifuge

4X LAEMMELI'S BUFFER:

Chemicals	Quantities
Glycerol	40 ml
SDS	8.2 g
0.5 M Tris-HCl (pH 6.8)	50 ml
1 % Bromophenol Blue	500 µl
Complete to… pH to 6.8	100 ml

NOTE: Before use, add 31 mg DTT to 500 µl of 4X Laemmeli's buffer

ELECTROPHORESIS:

- After polymerisation, place gel plates in white holder and slide holder into white reservoir (use thick clear plastic plate if not running 2 gels)
- Carefully remove comb to expose the sample wells, then fill reservoir with 1X running buffer and rinse wells out with running buffer using 5 ml syringe

- Pour cold running buffer to fill tank ~1/2
- Add 5 µl of MW marker to end well and load samples
- Load blank wells with water; load wells R to L
- Run the gels @ 160 V for 1hr or until samples have run off bottom of the gel

SETUP OF TRANFER:

- Carefully separate glass plates; using scalpel blade, carefully cut down sides of gel and cut top right hand corner for gel orientation
- Label PVDF transfer membrane with name of gel top right corner
- Pre-soak PVDF membrane 1 min in methanol, 1 min in H_2O, 15-20 min in transfer buffer to equilibrate
- Pre-soak Whatman paper & cotton blot pad in transfer buffer
- Assemble immuno-blot sandwich under transfer buffer at all times:
    - 1 x cotton blot pad
    - 2 x Whatman
    - Gel (cut at left corner)
    - PVDF membrane (cut corner on cut gel corner)
    - 2 x Whatman
    - 1 x cotton blot pad

There should be no air bubbles between gel and membrane; Use rolling tube to smooth membrane and layers but be careful not to stretch gel

- Put stirrer in bucket (between black and red)
- Put bucket in low plastic container
- Insert sandwich (white side top, black facing black)
- Insert ice block
- Run transfer @ 100 V for 1 h

After transfer, carefull remove membrane (with forceps) and place protein side up in a clean plastic container; you should be able to visualize the major bands

From this point on, each protein will have different conditions for obtaining optimal results; refer to **Antibody Conditions** for your protein of interest.

DETECTION USING CHEMILUMINESCENCE (Image station in Bonen Lab)

While washing secondary antibody, go to Bonen lab to make sure the image station is free; After wash is complete, take with you the following:

- ECL reagent & 9 ml of H_2O
- Pipette with 500 µl tips

- Forceps
- Blotting paper
- Plastic petri dish for ECL reagent
- Spare plastic petri dish for your membranes
- Membranes in wash buffer

Settings for Genesnap Program:

- Chemisample (top right drag)
- No light
- Medium sensitivity
- No filter
- Iris fully open (1.2)
- Magnification (31; 111)

Detection and Photos:

- Combine 0.5 ml of each ECL solution with 9 ml of H_2O
- Blot membrane and place in petri dish containing ECL mixture for 1 min
- Blot membrane, wrap in saran wrap and mark the MW with fluorescent pen
- Put wrapped membrane in imager
- Click green button to have real time image
- Adjust magnification, focus and position of container
- Click off red button
- Click camera icon and choose time and images (5 images, 1 min exposure)
- Close imager
- Image comes up as black; click on control panel to view histogram
- Switch red bars to get optimal image of lanes and reduce background
- Ensure lanes are not saturated
- Save best image and print
- Close all images

Quantification:

- Use Gene tool software

WESTERN BLOTTING – PREPARATION OF BUFFERS

10 X Running Buffer:

	500 ml	1 L	2 L
Tris	15 g	30 g	60 g
Glycine	72 g	144 g	288 g
SDS	5 g	10 g	20 g

1 X Running Buffer: 10 X Running buffer 100 ml
dH_2O 900 ml

---

10 X Western Transfer Buffer:

	500 ml	1 L
Glycine	52.5 g	105 g
Tris Base	22.5 g	45 g

1 X Western Transfer Buffer : 10 X Tranfer buffer 80 ml
Methanol 160 ml
dH_2O 560 ml

---

10 X TBS (Tris Buffered Saline):

	500 ml	1 L
Tris	12.1 g	24.2 g
NaCl	40 g	80 g
Adjust to pH 7.6 with HCl		

1 X TBST:

	1 L	2 L
10 X TBS	100 ml	200 ml
dH_2O	900 ml	1800 ml
Tween 20	500 µl	1 ml

Note: Ensure solution is stirring when adding Tween

WESTERN – ANTIBODIES

NOTE: Unless indicated otherwise, washes are 1 x 15 min @ RT + 3 x 5 min @ RT

FAT/CD36

MW: 88 kDa

Blocking: 7.5% BSA for 1 hour
- 1.5 g BSA in 20 ml TBST

1ºAb: 1:20 000; 7.5 % BSA (overnight)
- 4.5 g BSA in 60 ml TBST
- 3 µl Ab to 60 ml (freeze 40 ml; use other 20 ml)

2ºAb 1:20 000 mouse;
- 1 µl Ab to 20 ml TBST

FABPpm

MW: 43 kDa

Blocking: 7.5% BSA for 1 hour
- 1.5 g BSA in 20 ml TBST

1ºAb: 1:30 000; 7.5 % BSA (overnight)
- 4.5 g BSA in 60 ml TBST
- 2 µl Ab to 60 ml (freeze 40 ml; use other 20 ml)

2ºAb 1:3 000 rabbit;
- 3.33 µl Ab to 10 ml TBST

FATP-4

MW: 65 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:5 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 2 µl Ab to 10 ml

2ºAb 1:4 000 goat;

- 2.5 µl Ab to 10 ml TBST

Na/K ATP

MW: 112 kDa

Blocking: 7.5% BSA for 1 hour

- 750 mg BSA in 10 ml TBST

1ºAb: 1:500; 7.5 % BSA (overnight)

- 750 mg BSA in 10 ml TBST
- 20 µl Ab to 10 ml

2ºAb 1:4 000 rabbit;

- 2.5 µl Ab to 10 ml TBST

Cox-IV

MW: 22 kDa

Blocking: 10% NFM for 1 hour

- 2 g NFM in 20 ml TBST

1ºAb: 1:30 000; 10 % NFM (overnight)

- 3 g NFM in 30 ml TBST
- 1 µl Ab to 30 ml (freeze 20 ml; use other 10 ml)

2ºAb 1:5 000 mouse

- 2 µl Ab in 10 ml TBST

SERCA1

MW: 110 kDa

Blocking: 10% NFM for 1 hour

- 2 g NFM in 20 ml TBST

1ºAb: 1:5 000; 5 % NFM (overnight)

- 1 g NFM in 20 ml TBST
- 4 µl Ab to 20 ml (freeze 10 ml; use other 10 ml)

2ºAb 1:4 000 mouse

- 2.5 µl Ab in 10 ml TBST

Hormone Sensitive Lipase (HSL)

MW: 55 kDA

Blocking: 5% non-fat milk in TBST for 1 hour
- 1g non-fat milk in 20 ml TBST

1°Ab: 1:3 000, 2.5% BSA (overnight)
- 0.5g BSA in 10ml TBST
- 3.33 µl Ab to 10 ml

2ºAb: 1:2 000 chicken,
- 5 µl Ab to 10 ml TBST

GLUT-4

MW: 40

Blocking: 7.5% BSA in TBST for at least 2 hours
- 750 mg BSA in 10 ml TBST

1ºAb: 1:4 000, 7.5% BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 2.5 µl Ab to 10 ml

2ºAb: 1:4 000 rabbit HRP
- 2.5 µl Ab to 10 ml

Cav-3

MW: 18

Blocking: 10% NFM in TBST for at least 2 hours
- 1.0 g NFM in 10 ml TBST

1ºAb: 1:3000 10% NFM (overnight)
- 1.5 g NFM in 15 ml TBST

- 5 µl Ab to 15 ml

2ºAb: 1:3000 mouse HRP
- 5 µl Ab to 15 ml

PGC1α

MW: 90 kDa

Blocking: 10% NFM for 1 hour
- 1 g NFM in 20 ml TBST

1ºAb: 1:1 000; 10 % BSA (overnight)
- 1 g BSA in 10 ml TBST
- 10 µl Ab to 10 ml

2ºAb 1:1 000 rabbit;
- 1 µl Ab to 10 ml TBST

PGC1β

MW: 112 kDa

Blocking: 7.5% BSA for 1 hour
- 1.5 g BSA in 20 ml TBST

1ºAb: 1:3 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 3.33 µl Ab to 10 ml

2ºAb 1:1 000 mouse;
- 10 µl Ab to 10 ml TBST

PPARγ

MW: 55 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:5 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 2 µl Ab to 10 ml

2ºAb 1:1 000 mouse;
- 10 µl Ab to 10 ml TBST

PPARα

MW: 55 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:5 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 2 µl Ab to 10 ml

2ºAb 1:1 000 mouse;
- 10 µl Ab to 10 ml TBST

PPARβ

MW: 55 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:5 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 2 µl Ab to 10 ml

2ºAb 1:1 000 mouse;
- 10 µl Ab to 10 ml TBST

tFAM

MW: 30 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:1 000; 7.5% BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 10 µl Ab to 20 ml

2ºAb 1:1 000 goat

- 10 µl Ab in 10 ml TBST

NRF-1

MW: 53 kDA

Blocking: 7.5% non-fat milk in TBST for 1 hour

- 750 mg non-fat milk in 20 ml TBST

1^{o}Ab: 1: 500, 7.5% BSA (overnight)

- 750 mg BSA in 10ml TBST
- 20 µl Ab to 10 ml

2ºAb: 1:500 mouse,

- 20 µl Ab to 10 ml TBST

NRF-2

MW: 57 kDa

Blocking: 7.5% BSA in TBST for at least 2 hours

- 750 mg BSA in 10 ml TBST

1ºAb: 1:500, 7.5% BSA (overnight)

- 750 mg BSA in 10 ml TBST
- 20 µl Ab to 10 ml

2ºAb: 1:500 rabbit

- 20 µl Ab to 10 ml TBST

ERRα

MW: 58 kDa

Blocking: 7.5% BSA in TBST for at least 2 hours

- 750 mg BSA in 10 ml TBST

1ºAb: 1:500 7.5% BSA (overnight)

- 750 mg BSA in 10 ml TBST
- 20 µl Ab to 10 ml

2ºAb: 1:1 000 mouse

- 10 µl Ab to 10 ml TBST

MFN-1

MW: 87 kDa

Blocking: 7.5% BSA for 1 hour

- 1.5 g BSA in 20 ml TBST

1ºAb: 1:1 000; 7.5 % BSA (overnight)

- 4.5 g BSA in 60 ml TBST
- 3 µl Ab to 60 ml (freeze 40 ml; use other 20 ml)

2ºAb 1:20 000 mouse;

- 1 µl Ab to 20 ml TBST

MFN-2

MW: 110 kDa

Blocking: 7.5% BSA for 1 hour

- 1.5 g BSA in 20 ml TBST

1ºAb: 1:1 000; 7.5 % BSA (overnight)

- 750 mg BSA in 10 ml TBST
- 10 µl Ab to 10 ml

2ºAb 1:1 000 mouse

- 10 µl Ab to 10 ml TBST

OPA-1

MW: 112 kDa

Blocking: 7.5% BSA for 1 hour

- 750 mg BSA in 10 ml TBST

1ºAb: 1:500; 7.5 % BSA (overnight)

- 750 mg BSA in 10 ml TBST
- 20 µl Ab to 10 ml

2ºAb 1:500 mouse
- 20 µl Ab to 10 ml TBST

DRP-1

MW: 82 kDa

Blocking: 7.5% BSA for 1 hour
- 750 mg BSA in 10 ml TBST

1ºAb: 1:1 000; 7.5 % BSA (overnight)
- 750 mg BSA in 10 ml TBST
- 10 µl Ab to 10 ml

2ºAb 1:1 000 mouse
- 10 µl Ab to 10 ml TBST