AMPK Assay

Require:

Acetone	Sigma (1L, $18.30) A4206
Aluminum foil	
Ammonium sulfate	Fisher BP212R-1
AMP	Sigma A1752
ATP	Sigma A6144 (alt. use A7699)
Beta-mercaptoethanol	Sigma M6250 (alt. use M7154)
Bio-Rad protein assay dye reagent	Bio-Rad 500-0006 (450mL)
BSA	Sigma A3059
cotton tipped swabs	Fisher ($5.24/pack 100) 14-960-3N
DTT	Sigma D9779 (alt. use D5545)
EDTA	Sigma E1644
Glycerol	Sigma G7893 (alt. use G2289)
Hamilton syringe (500µL)	#81265; Fisher 14-815-284
HEPES	Sigma H7523
liquid nitrogen	
Mannitol	Sigma M9647
MgCl2·6H2O	Sigma M2670
mortar, pestle	Coors 60310; Fisher 12-961B + 12-961-5B
NaCl	Sigma 7653
NaF	Sigma S6521
Nalgene cryotubes (4.5 mL)	Nunc 379146; Fisher 12-565-161N
P^{32}-γATP	ICN 35001X
Phosphoric acid (85%)	Fisher A242-500
Pipettes (25mL)	
polycarbonate centrifuge tube	Beckman #343778; Fisher NC9495303
Polytron PT 2100 and Probe (7.5 mm PT-DA 2107/2EC)	Kinematica
Roche protease inhibitors tablets	Roche 1836153
SAMS peptide	*gift of Don Mcclain (additional: see Upstate)*
Small stir bars (5x2mm)	Sigma Z118788-3EA
spatula	
TLA 120.2 rotor	Beckman, for TL 100 centrifuge
Trizma Base	Sigma T6066
Whatman P81 cellulose (2.5cm circle)	#3698325; Fisher 05-717-2B
+ 32P workspace with: vortexer, magnetic stirrer, incubator (37ºC), pipette (20 µL), pipette tips, tube racks	

Preparation of Buffers

Stock Solutions:

AMP (Mw = 347.2) 10 ml

Dissolve 0.2 g in 10 ml water. Confirm concentration using 1 to 1000 dilution in water at UV = 260 (extinction coefficient = 15400), Dilute with water to give a final concentration of 50 mM

Store at –80˚C

ATP (Mw – 551.1) 10 ml

Dissolve 0.4 g in 10 ml water. Confirm concentration using 1 to 1000 dilution in water at UV = 260 (extinction coefficient = 15400), Dilute with water to give a final concentration of 50 mM.

Store at –80˚C

DTT (Mw = 154.24) 1 ml

Dissolve 0.1542 g into 0.8457 ml of water to give 1M

Store at –80˚C

SAMS peptide

(Mw = 1779.2)

Seq.= HMRSAMSGLHLVKRR

Gift of Don McClain

Obtain as a chloride salt with >95% purity

Dissolve in water to give 20 mM.

Store at –80˚C

Homogenization Buffer	100 ml:	
Mannitol (Mw= 182)	3.6 g	
NaF (Mw = 41.99)	0.21 g	
Tris Base (Mw = 121)	0.12 g	
EDTA (Fw = 372)	0.037 g	
Beta-mercaptoethanol	75 µl	(add after pH adjustment)

Dissolve in 90 ml nanopure water, adjust pH to 7.5 with HCl, Adjust volume to 100 ml. Store indefinitely at 4˚C.

Prior to use add 1 Roche mini-complete protease inhibitor tablet (1 836 153) per 10 ml.

Final concentrations: 200 mM mannitol, 50 mM NaF, 10 mM Tris-Hcl 7.5, 1 mM EDTA, 10 mM bME with protease inhibitors added.

Assay Buffer Stock Solution	100 ml:
HEPES	2.38 g
NaCl	1.16 g
Glycerol	20 ml
EDTA	0.074 g
MgCl2	0.254 g

Dissolve in 90 ml nanopure water, adjust pH to 7.0 with NaOH. Adjust volume to 100 ml. Store indefinitely at 4˚C.

Working Assay Mix (cocktail) (make fresh; 1.0 ml):

AMP	10 µl x 50 mM
DTT	2 µl x 1 M
ATP	10 µl x 50 mM

Assay Buffer Working Solution; 978 µl to give a total of 1000 µl. Contains 0.5mM AMP, 2mM DTT, 0.5mM ATP. To 100µL of required Working Assay Mix add 2 µl of 10 mCi/ml P^{32}-γATP (ICN cat#35001X)

Final assay concentrations: 40mM HEPES, 0.2mM AMP, 80mM NaCl, 8% glycerol, 0.8mM DTT, 5mM MgCl2, 0.2mM ATP, 0.2mM SAMS.

Assay setup:

1. Set centrifuge at 4ºC, set incubator at 37ºC
2. Homogenization buffer: 10mL homogenization buffer stock + 1 Roche tablette
3. Grind ammonium sulfate granules
4. Label tubes and filter papers
5. Cocktail: 10µL AMP (50mM) +2µL DTT (1M) + 10µL ATP (50 mM) + 978µL assay buffer stock + 2µL *ATP / 100µL cocktail
6. Dilute SAMS to 1mM in water
7. Phosphoric acid 1%: 16.5mL phosphoric acid (85%) + 1383.5mL H2O

Preparation of Heart Extract:

1. Preweigh Nalgene cryotubes (Nunc 379146 (4.5 ml))
2. Bring mortar, pestle, spatula and cryotbes to liquid N2 temperature
3. Grind tissue in mortar and transfer as much powder as possible into the respective cryotubes. Keep in liquid N2 until all tissue has been ground.
4. Weigh all cryotubes and calculate amount of ground tissue in each one.
5. Add 9 X the volume (µl/mg)) of Homogenization Buffer
6. Homogenize on ice using polytron (PT 2100; 7 mm Kinematica probe) for 20 s at maximum speed.
7. Transfer a fixed volume (for mouse hearts about 500 µl but can up to a maximum of 1000 µl) into a Beckman polycarbonate centrifuge tube (11 x 34 mm; cat# 343778).
8. Centrifuge in a TLA120.2 rotor, at **37 000 rpm (48 000 g) for 30 min at 4˚C**.
9. Aspirate supernatant (Hamilton 500 µl syringe #81265), and transfer to another 1 ml Beckman centrifuge tube on ice on a magnetic stirrer with a magnetic stir bar (Sigma Z119788-3EA) with 144 mg ammonium sulfate per ml (grind the ammonium sulfate granules ahead of time to increase solubility).
10. Stir for 30 min, remove stir bar and centrifuge for **30 min at 37 000 rpm at 4˚C**.
11. Aspirate and discard supernatant. (Wipe away excess liquid.)
12. Resuspend in homogenization buffer in **10%** of the original volume (~ 50 µl, maximum = 100 µl). Freeze at least 10µL aliquot of each sample for pAMPK and pACC Western Blot.

Assay Protocol:

1. Load assay tubes with cocktail that contains water, SAMS and radioactive ATP. Dilute samples 1:5.
- **With just 1 person a maximum of 20 tubes can be assayed at one time!**
2. Start reaction by adding resuspended ammonium sulfate pellet
3. Incubate for 10 minutes at 37˚C
4. Terminate by pipetting **15µl of incubation onto a 15 x 15 mm** P81 Whatmann Filter paper. Allow **at least 20s for the solution to absorb** into paper. Drop into 150 ml of 1% phosphoric acid to terminate reaction. Use a large beaker (500-500mL).
5. Wash the papers in 6 changes of 150 ml 1% phosphoric acid, for 5 min each, followed by a final wash in 50 ml acetone. Agitate the solution without using a stir-bar.
6. Allow to dry
7. Place papers in vials with 3 ml Ecolite and count in liquid scintillation counter.
- Add two extra vials with 10µL cocktail.

Assay sheet (see next page)

Protein assay:

Standard Procedure for Microtiter Plates

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts DDI water. Filter through a Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for about 2 weeks when kept at room temperature.
2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.
3. Pipet 10 µl of each standard and sample solution into separate microtiter plate wells.
4. Add 200 µl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use a multi-channel pipet to dispense the reagent. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.
5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
6. Measure absorbance at 595 nm.

AMP-activated Protein Kinase – Heart Extract Sheet

Rat #	Empty Tube	Tube + Heart	Heart AMPK	Homogenate µL (9x mg heart)	Amonium Sulfate g (144 x mL sn)
					0.144 x =
					0.144 x =
					0.144 x =
					0.144 x =
					0.144 x =
					0.144 x =

AMP-activated Protein Kinase – Assay Sheet

Tube #		Cocktail (µL)	Water	Homog Buffer	SAMS (1.0mM)	Sample	Start (min)	Stop (min)
1	**1**	10	8		5	2	**0**	**10**
1	**2**	10	8		5	2	**1**	**11**
1	**3**	10	8		5	2	**2**	**12**
ne	**4**	10	8		5	2	**3**	**13**
ne	**5**	10	8		5	2	**4**	**14**
ns1	**6**	10	8		5	2	**5**	**15**
ns1	**7**	10	8		5	2	**6**	**16**
ns1	**8**	10	8		5	2	**7**	**17**
	9	10	8		5	2	**8**	**18**
	10	10	8		5	2	**9**	**19**
	11	10	8		5	2	**0**	**10**
	12	10	8		5	2	**1**	**11**
	13	10	8		5	2	**2**	**12**
	14	10	8		5	2	**3**	**13**
	15	10	8		5	2	**4**	**14**
	16	10	8		5	2	**5**	**15**
	17	10	8		5	2	**6**	**16**
	18	10	8		5	2	**7**	**17**
NS#	**19**	10	13			2	**8**	**18**
NE	**20**	10	8	2	5		**9**	**19**

filter paper: 15µL, >20" adsorption

* Negative controls without enzyme (ne): use 2µL homogenization buffer instead of homogenate.

** Negative controls without SAMS (ns, for each tissue sample): use 5µL H_2O instead of SAMS solution.