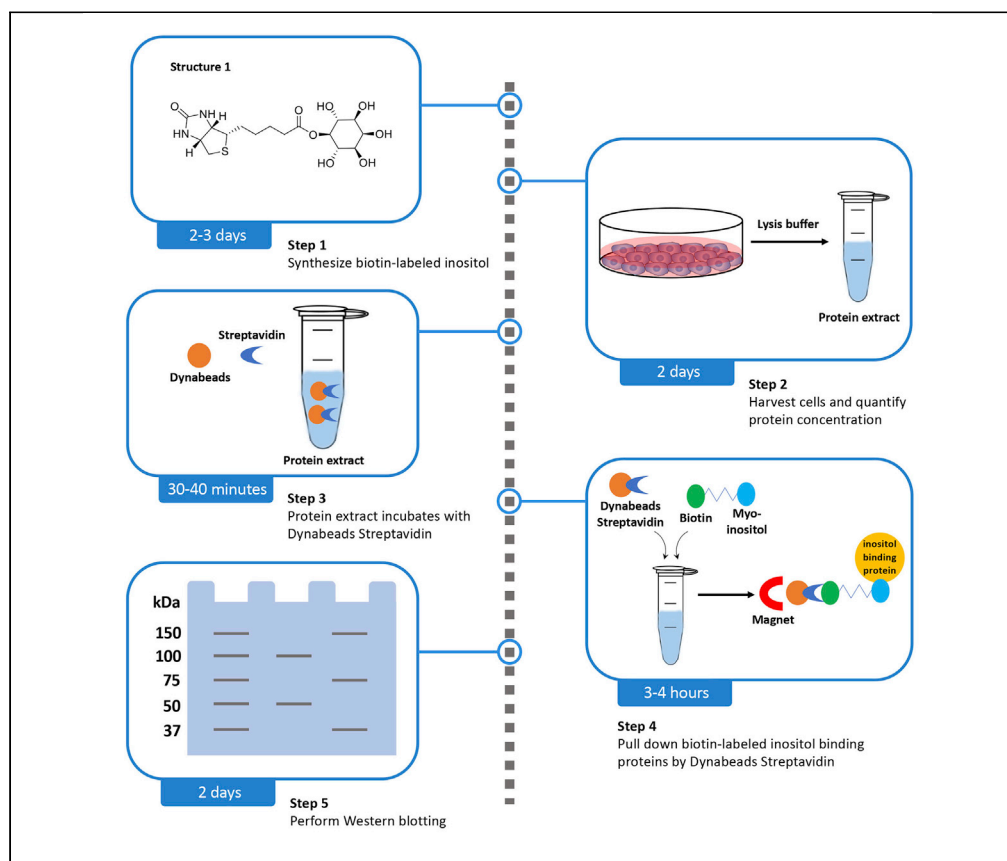


Protocol

Identification of myo-inositol-binding proteins by using the biotin pull-down strategy in cultured cells



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Highlights

A detailed protocol for performing biotin-labeled myo-inositol pull-down assay

Synthesizing and verifying biotin-conjugated myo-inositol moiety

Identifying myo-inositol-binding proteins via biotin-streptavidin approach

Using unlabeled myo-inositol for validating specific myo-inositol-binding proteins

Metabolites are not only substrates in metabolic reactions, but they also serve as signaling molecules to regulate diverse biological functions. Identification of the binding proteins for the metabolites helps in the understanding of their functions beyond the classic metabolic pathways in which they are involved. We provide the protocol for synthesizing the biotin-labeled myo-inositol, which is used to identify its binding proteins by using biotin pull-down assay, given there is no available tool for the rapid screening of inositol-binding proteins in cells and *in vitro* systems. Biotin-labeled inositol probe therefore provides a tool to identify inositol's sensors.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

Identification of myo-inositol-binding proteins by using the biotin pull-down strategy in cultured cells

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SUMMARY

Metabolites are not only substrates in metabolic reactions, but they also serve as signaling molecules to regulate diverse biological functions. Identification of the binding proteins for the metabolites helps in the understanding of their functions beyond the classic metabolic pathways in which they are involved. We provide the protocol for synthesizing the biotin-labeled myo-inositol, which is used to identify its binding proteins by using biotin pull-down assay, given there is no available tool for the rapid screening of inositol-binding proteins in cells and *in vitro* systems. Biotin-labeled inositol probe therefore provides a tool to identify inositol's sensors.

For complete details on the use and execution of this protocol, please refer to Hsu et al. (2021).

BEFORE YOU BEGIN

⌚ Timing: 2–3 days for synthesis of biotin-labeled inositol

⌚ Timing: 2–3 days for establishment of the culture of PC3, HEK293T cell lines, and MEFs

Sensing proteins of distinct metabolites that orchestrate diverse biological processes has been demonstrated in the past five years. For example, lactate binds to MAVS to inhibit retinoic-acid-inducible gene I (RIG-I)-like receptors (RLR)-mediated interferon production (Zhang et al., 2019). α -ketoglutarate (α -KG) generated from the TCA cycle directly activates the key enzymes involved in epigenetics regulation for cell differentiation and stem cell maintenance (Carey et al., 2015). (dihydro-) ceramide synthase 6 (CerS6)-derived sphingolipids interact with Mff and promotes mitochondrial fragmentation in obesity (Hammerschmidt et al., 2019). Moreover, myo-inositol restricts mitochondrial fission via direct binding to AMPK (Hsu et al., 2021). These studies highlight the signaling roles of unique metabolites that go beyond their classic modes, target proteins and pathways.

Biotin-streptavidin system is a protein-ligand interaction used in a number of applications including protein or nucleic acid purification and detection. To identify the sensors of specific metabolites such as inositol, biotin-conjugated inositol might be synthesized as a tool to pull down its interacting



proteins. A hydrophobic straight chain as a linker with 5-carbon aminohexanoic is frequently applied between biotin and inositol (Hsu et al., 2021). Linkers are flexible molecules used to link biotin and peptide of interest together. Herein, we describe a protocol for biotin-conjugated inositol production to demonstrate that AMPK $\alpha\beta\gamma$, the sensor of inositol, binds to inositol both *in vivo* and *in vitro*. Dynabeads MyOne Streptavidin T1 available at Invitrogen (catalog numbers 65601 and 65602), which contains magnetic beads in phosphate buffered saline (PBS), pH 7.4 with 0.1% (W/V) bovine serum albumin (BSA), is a main reagent used to pull down biotin-labeled inositol.

The protocol below enumerates the specific steps by using PC3 cells, a prostate cancer cell line, as a model. However, it is important to note that we also performed this protocol in HEK293T and mouse embryonic fibroblasts (MEFs) for *in vivo* binding assay. The recombinant proteins like AMPK α , AMPK β and AMPK γ are purified from *E. coli* using the strain of BL21 competent cells for *in vitro* binding assay.

1. Synthesis of biotin-labeled inositol.
 - a. A solution of inositol, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) in dry dichloromethane (DCM) need to be prepared. The materials are listed in the [key resources table](#).
 - b. Inositol used in this protocol is myo-inositol, which is available in Sigma (catalog number I7508).
2. Establishment of the culture of PC3, HEK293T cell lines, and MEFs.
 - a. Culture PC3 cells in RPMI-1640 medium supplied with 10% (V/V) FBS, 2 mM glutamine.
 - b. Culture HEK293T cell line and MEFs in Dulbecco's Modified Eagle's Medium (DMEM) medium supplied with 10% FBS, 2 mM glutamine.
 - c. Passage Cells every 2–3 days using 0.25% (W/V) trypsin-EDTA.

△ CRITICAL: At least 1 mg of protein concentration needs to be extracted from cells for each experimental group. Cells could be cultured in 15 cm dish with 90% confluency followed by protein extraction.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
AMPK α (polyclone): 1:1000 dilution	Cell Signaling Technology	Cat#2532; RRID: AB_330331
AMPK β (polyclone): 1:1000 dilution	Cell Signaling Technology	Cat#12063; RRID: AB_2797812
AMPK γ (polyclone): 1:1000 dilution	Cell Signaling Technology	Cat#4187; RRID: AB_10695248
GST (clone B-14): 1:2000 dilution	Santa Cruz Biotechnology	Cat#sc-138; RRID: AB_627677
HRP-coupled antibodies to rabbit: 1:10000 dilution	Thermo Fisher Scientific	Cat#31480; RRID: AB_228457
Chemicals, peptides, and recombinant proteins		
L-glutamine	Fisher Scientific	Cat#SH30034.02
Tris Base	Fisher Scientific	Cat#BP152-500
Glycine	Fisher Scientific	Cat#BP381-5
Sodium chloride	VWR	Cat#BDH9286
Sodium deoxycholate monohydrate	Fisher Scientific	Cat#AAB2075914
EDTA	Fisher Scientific	Cat#AC118432500
Sodium Dodecyl Sulfate	Thermo Fisher Scientific	Cat#BP2436-1
Nonidet P-40 Substitute	VWR	Cat#97064-732
HEPES	Fisher Scientific	Cat#AAA1477730
Magnesium chloride	MP Biomedicals	Cat#MP021914215
Dithiothreitol	Thermo Fisher Scientific	Cat#FERR0861
EGTA	Millipore/Sigma-Aldrich	Cat#32-462-625GM

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Potassium Chloride	Fisher Scientific	Cat#BP366-500
Potassium phosphate	Fisher Scientific	Cat#AC447372500
Sodium phosphate monohydrate	Fisher Scientific	Cat#56-754-9250GM
Myo-inositol	Sigma-Aldrich	Cat#I7508
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	Thermo Fisher Scientific	Cat#22980
4-dimethylaminopyridine	Thermo Fisher Scientific	Cat#AC148270050
Dichloromethane	Sigma-Aldrich	Cat#270997
Dynabeads MyOne Streptavidin T1	Thermo Fisher Scientific	Cat#65602
Dulbecco's Modified Eagle's Medium	Thermo Fisher Scientific	Cat#SH30243FS
RPMI-1640 Medium	Thermo Fisher Scientific	Cat#11875119
Biotin	Sigma-Aldrich	Cat#B4639
Experimental models: Cell lines		
Human: PC3	ATCC	Cat#CRL-1435
Human: HEK293T	ATCC	Cat#CRL-3216
Mouse: mouse embryonic fibroblasts	ATCC	Cat#SCRC-1008
Experimental models: Organisms/Strains		
BL21 chemically competent cells	Lab prepared or Thermo Fisher Scientific	Cat#C600003
Recombinant DNA		
pGEX6P-1-AMPK α 1	This paper	N/A
pGEX6P-1-AMPK β 1	This paper	N/A
pGEX6P-1-AMPK γ 1	This paper	N/A
pGEX6P-1	This paper	N/A

MATERIALS AND EQUIPMENT

The buffers used in this protocol are listing as follows. Lysis buffer was used for extracting protein from cells. Binding buffer was used for pulling down the target proteins of Dynabeads MyOne Streptavidin T1/biotin-labeled inositol complex. Wash buffer was used for washing beads after incubating with biotin-labeled inositol and protein extracts.

Lysis buffer		
Reagent	Final concentration	Amount
Tris-HCl pH7.4 (500 mM)	50 mM	5 mL
150 mM NaCl (1.5 M)	150 mM	5 mL
SDS (10%)	0.1% (W/V)	5 mL
Sodium deoxycholate	0.5% (W/V)	0.25 g
Nonidet P-40 (NP-40)	1% (V/V)	0.5 mL
EDTA (500 mM)	1 mM	0.1 mL
ddH ₂ O	n/a	To 50 mL
Total	n/a	50 mL

Binding buffer		
Reagent	Final concentration	Amount
HEPES pH7.4 (500 mM)	50 mM	5 mL
MgCl ₂ (100 mM)	10 mM	5 mL
EGTA (1 mM)	0.1 mM	5 mL
Dithiothreitol (10 mM)	0.1 mM	0.5 mL
ddH ₂ O	n/a	To 50 mL
Total	n/a	50 mL

Wash buffer		
Reagent	Final concentration	Amount
HEPES pH7.4 (500 mM)	10 mM	1 mL
MgCl ₂ (100 mM)	10 mM	5 mL
EDTA (50 mM)	0.5 mM	0.5 mL
Dithiothreitol (10 mM)	0.1 mM	0.5 mL
NP-40	0.1% (V/V)	0.05 mL
ddH ₂ O	n/a	To 50 mL
Total	n/a	50 mL

Lab prepared PBS buffer		
Reagent	Final concentration	Amount
NaCl	13.68 mM	0.8 g
KCl	0.26 mM	20 mg
KH ₂ PO ₄	0.176 mM	24 mg
NaH ₂ PO ₄	1.2 mM	144 mg
ddH ₂ O		To 1,000 mL
pH 7.4		
Total	n/a	1,000 mL

STEP-BY-STEP METHOD DETAILS

Synthesize biotin-labeled myoinositol

⌚ Timing: 3 days

A series of biotin-labeled inositol moieties with connecting linkers between biotin and myo-inositol are designed and synthesized. The procedure for the direct conjugation between biotin and myo-inositol is described as below. Based on the NMR studies including ¹H NMR, ¹³C NMR, 2D COSY, and 2D HMBC spectra (Figures 1A–1D) and the reactivity of each hydroxyl group in inositol, the chemical structure is proposed as shown as Scheme 1 below.

1. Seal and heat the suspension of 180 mg of inositol (1 mmol), 258 mg biotin methyl (1 mmol) and 11 mg of sodium methoxide (0.2 mmol) in dry *N,N*-Dimethylformamide (DMF) (6 mL) in a 10–20 mL microwave vial with a magnetic stir bar in microwave irradiation (Biotage Initiator Classic).
 - a. Irradiate the reaction mixture at a required ceiling temperature (180°C) using maximum power (250 W) for the stipulated time (30 min).
 - b. Cool to 50°C with gas jet cooling.
2. Transfer the reaction mixture to a round-bottomed flask (20 mL).

Note: Around 4 mL of the solvent is lost under reduced pressure.

- a. Add 3 mL of DCM.

Note: White solid is precipitated from the solvent).

- b. After filtering and washing with DCM, 310 mg of wax solid is obtained after dry.

Note: The yield is 76% from HPLC.

3. Dissolve the crude product (50 mg) in 10% water/acetonitrile (1.0 mL) and carefully deposit a thin line across a pencil line on preparative thin layer chromatography.

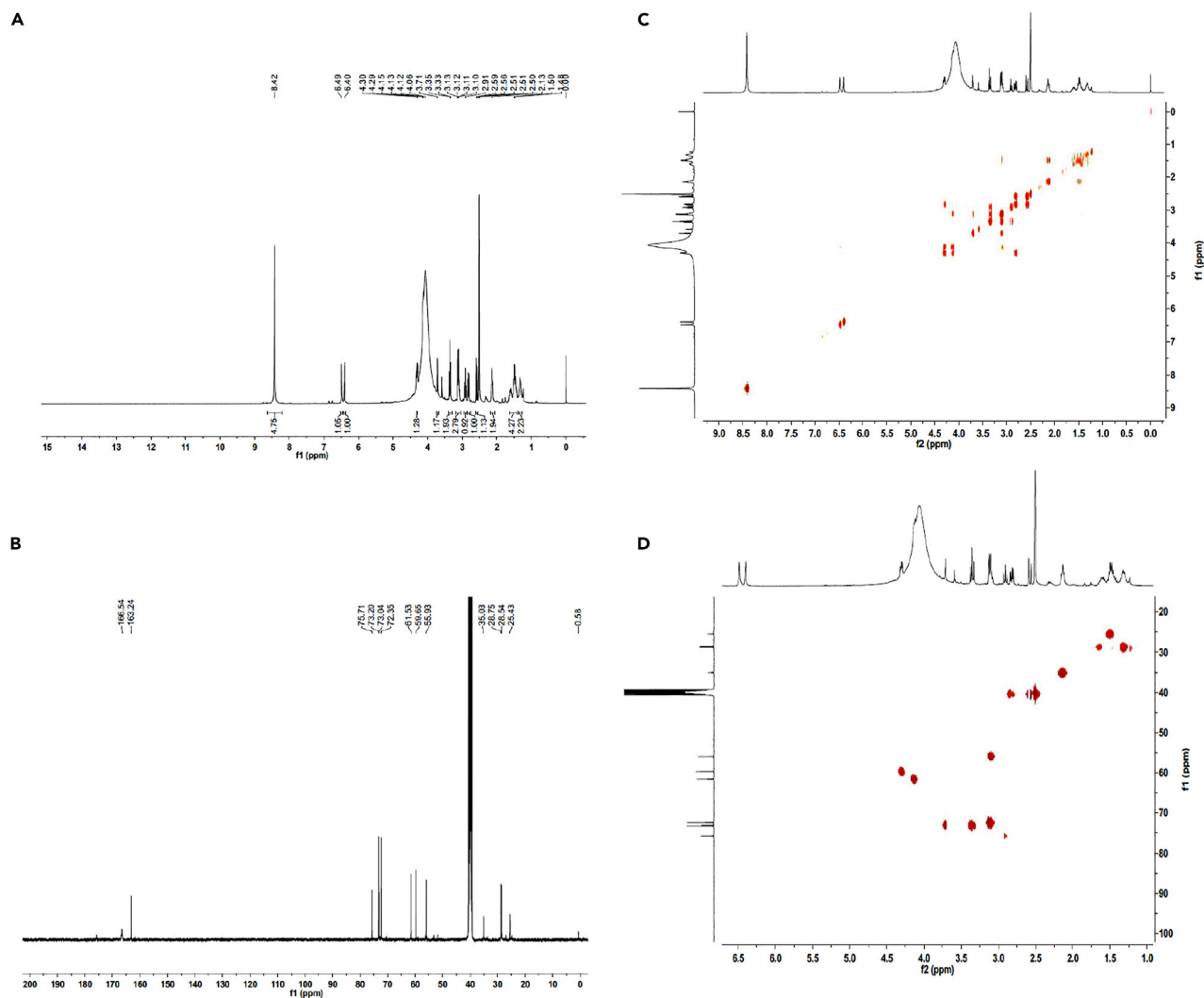


Figure 1. NMR spectra

- (A) ^1H NMR spectrum.
 (B) ^{13}C NMR spectrum.
 (C) COSY NMR spectrum.
 (D) HMBC NMR spectrum.

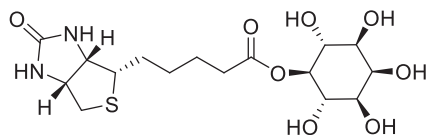
Note: PTLC, silica gel 60, 20 × 20 cm with 0.5 mm.

- a. After drying, place PTLC plate in the chamber with 10% water/acetonitrile.
- b. Run the crude product on PTLC plate by 10% water/acetonitrile.

△ CRITICAL: Seal the top of chamber.

Note: A typical run takes 40 min to 1 h.

4. Take out PLTC plate from chamber and cut down a thin line of the stain from an edge of the plate.
 - a. Use the iodine fuming technique to mark the line of the targeted product.
 - b. Scrape off the band.
 - c. Sonicate the silica gel in acetonitrile (10 mL).



Scheme 1. The structure of biotin-labeled myo-inositol

d. Filter silica gel through the filter paper to concentrate the product.

Note: 32 mg of compound after dry (purity > 98% from HPLC, 10% water/acetonitrile, $R_f = 0.18$).

5. NMR studies including ^1H NMR, ^{13}C NMR, 2D COSY, and 2D HMBC spectra are shown in Figure 1. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.42 (s, 5H), 6.49 (s, 1H), 6.40 (s, 1H), 4.32–4.29 (m, 1H), 3.71 (d, $J = 2.6$ Hz, 1H), 3.36 (t, $J = 9.3$ Hz, 2H), 3.12–3.10 (m, 3H), 2.91 (t, $J = 9.1$ Hz, 1H), 2.84–2.77 (m, 1H), 2.58 (d, $J = 12.4$ Hz, 1H), 2.13 (t, $J = 6.8$ Hz, 2H), 1.67–1.40 (m, 4H), 1.37–1.26 (m, 2H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ 166.54, 163.24, 75.71, 73.20, 73.04, 72.35, 61.53, 59.65, 55.93, 35.03, 28.75, 28.54, 25.43.

Harvest protein extracts

⌚ Timing: 2 days

To harvest protein extracts and quantify protein concentration for cell-based binding assay, PC3 cells are cultured in RPMI-1640 medium supplied with 10% (V/V) FBS and 2 mM glutamine. PC3 cells are harvested from 15 cm dishes once cell confluency reached 90%. Protein concentration is determined using the Bio-Rad Protein Assay Dye Reagent Concentrate (Catalog number #5000006). At least 1 mg of protein lysates for each experimental group is used in biotin pull-down assay including beads control, biotin pull-down control and biotin-labeled inositol pull-down groups.

6. Use lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 0.1% (W/V) SDS, 0.5% sodium deoxycholate, 1% NP-40 and 1 mM EDTA) to harvest cells. After scratching cells from dishes, cell lysates are incubated on ice for 20 min.

Note: Store lysis buffer at 4°C for 1 year.

7. Centrifuge cell lysates at 4°C for 20 min using 16,800 g (Sorvall™ Legend™ Micro 21R Microcentrifuge, Cat#75002445; 24 × 1.5/2.0 mL Rotor, Cat#75003424; VWR 1.5 mL Microcentrifuge Tube, Cat#10025-724).
8. Collect the supernatant in fresh tubes, placed on ice and then protein extract is subjected to Bio-Rad Protein Assay to quantify protein concentration according to manufacturer's instructions (<https://www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT33.pdf>).

Note: Store Bio-Rad Protein Assay Dye Reagent Concentrate (Catalog number #5000006) at 4°C for 1 year.

9. R-squared value for linear regression in the standard curve, which needs to reach close to 0.997–0.999, should be performed to ensure accurate quantification of protein concentration.

⏸ Pause point: Once protein extracts are prepared, cell lysates can be stored at –80°C, providing a potential pause point in this step.

Pre-clear protein extracts

⌚ Timing: 30–40 min

To perform samples pre-clearing, Dynabeads MyOne Streptavidin T1 available in Thermo Fisher Scientific (catalog number 65602) are washed by lab prepared PBS and binding buffer in advance. Pre-clearing the samples with plain beads is a necessary step to reduce non-specific binding and minimize co-purification of off-target molecules before interacting complex is assembled.

10. Prepare 30 μL of Dynabeads MyOne Streptavidin T1 for each experimental group. Calculate total volume of beads including extra one sample in case beads lose during washing and then wash beads by lab prepared PBS for 3 times.

Note: Store lab prepared PBS at 4°C for 1 year.

11. Use 30 μL X (the number of sample + 1) of lysis buffer to resuspend washed beads and aliquot 30 μL of beads in fresh tubes.
12. Add 1 mg of the protein extracts in each 1.5 mL tube with beads and then use the binding buffer to fill up the rest of volume until 500–800 μL , so that each sample has the same volume for pre-clearing step.

Note: Store binding buffer at 4°C for 1 year.

13. Incubate protein extracts with Dynabeads MyOne Streptavidin T1 for 30 min at 4°C in a rotating wheel at 20 rpm with vertical 90° position (Labnet Labroller Rotator II, Cat#H5000).
14. Use a magnetic rack (1.5 mL microcentrifuge tubes SureBeads magnetic racks, Cat#1614916) to separate protein extracts from beads, followed by collecting pre-clearing protein extracts in fresh tubes put on ice.

Biotin pull-down assay in cell-based protocol

⌚ Timing: 2 days

To perform biotin pull-down assay in cell-based protocol, Dynabeads MyOne Streptavidin T1 is incubated with biotin or biotin-labeled inositol, followed by adding protein extracts.

15. Prepare 30 μL of Dynabeads MyOne Streptavidin T1 for each experimental group. Calculate total volume of beads the total number of sample including extra one sample in case beads lose during washing. Wash total beads by using lab prepared PBS for 3 times, respectively.
16. Use 30 μL X (the number of sample + 1) of Lab prepared PBS to resuspend washed beads and aliquot 30 μL of beads in fresh tubes.
17. Add 25 μM –40 μM of biotin or biotin-labeled inositol in each tube with beads and then use lab prepared PBS to fill up the rest of volume until 500 μL .
18. Incubate biotin or biotin-labeled inositol with Dynabeads MyOne Streptavidin T1 for 30 min at 25°C in a rotating wheel.

Note: Dynabeads stably bind to biotin at 25°C.

19. Use a magnetic rack to collect beads that bind with biotin or biotin-inositol, followed by washing beads by using lab prepared PBS for 3 times.
20. Add pre-clearing 1 mg of protein extracts to incubate with the beads binding with biotin or biotin-labeled inositol in each experimental group for 2–3 h at 4°C in a rotating wheel.

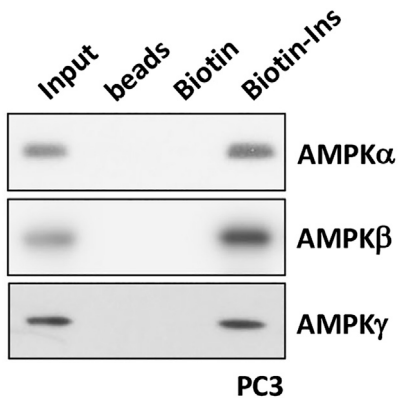


Figure 2. AMPK $\alpha\beta\gamma$ binds to biotin-labeled inositol

Beads only, 25 μ M of biotin or 25 μ M of biotin-labeled inositol (Biotin-Ins 1) incubating with cell lysates from PC3 cells was pulled down by Dynabeads Streptavidin, following by immunoblotting using specific AMPK α , AMPK β and AMPK γ antibodies (Scheme 1).

21. Spin down each sample using 2,400 g to make sure no beads left on the cap of tubes and then use a magnetic rack to separate beads and protein extracts.
22. After removing protein extracts, wash the beads by lysis buffer for 30 s and subsequently by lab prepared PBS 30 s each time for 3 times at 4°C.
23. After removing lab prepared PBS, add 30 μ L of 2 times SDS loading dye in each sample, followed by boiling each sample at 95°C for 5 min.

Note: 2 times SDS loading dye was prepared from 5 times SDS loading dye (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β -mercaptoethanol and 0.02% bromophenol blue) by dilution.

Note: Store SDS loading dye at -20°C for 1 year.

24. Perform Western blotting by loading 10 μ g of protein extracts as input and 30 μ L of each experimental sample.
 - a. Run protein extracts in the 12% SDS-PAGE.
 - b. Transfer protein in the SDS-PAGE to PVDF membrane using Mini Trans-Blot Electrophoretic Transfer Cell from BioRad (Cat#1703930) under 90 voltage for 3 h.
 - c. Conduct PVDF membrane blocking by 3% (W/V) skim milk PBST (lab prepared PBS + 1% Tween 20) for 1 h.
 - d. Incubate with primary antibodies such as AMPK α , AMPK β and AMPK γ antibodies for over 12 h at 4°C.
 - e. After membranes are washed by PBST 7 min each time for 3 times, incubate with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Cat#111-035-003 and Cat#115-035-003) for 1 h at 25°C.
 - f. After membranes are washed by PBST 7 min each time for 3 times, incubate with ECL western blotting substrate (Thermo Fisher Scientific, Cat#32106) with each membrane for 2 min at 25°C, following by visualizing signal on the autoradiography films.
25. Immunoblotting of biotin pull-down assay in cell-based protocol by using AMPK α , AMPK β and AMPK γ specific antibody is shown in Figures 2 and 3A.

Biotin pull-down assay *in vitro* protocol

© Timing: 2 days

To perform biotin pull-down assay *in vitro* protocol, Dynabeads MyOne Streptavidin T1 is incubated with biotin or biotin-labeled inositol, followed by adding recombinant proteins. Pre-clearing the samples with plain beads is still necessary to reduce non-specific binding before binding complex is assembled.

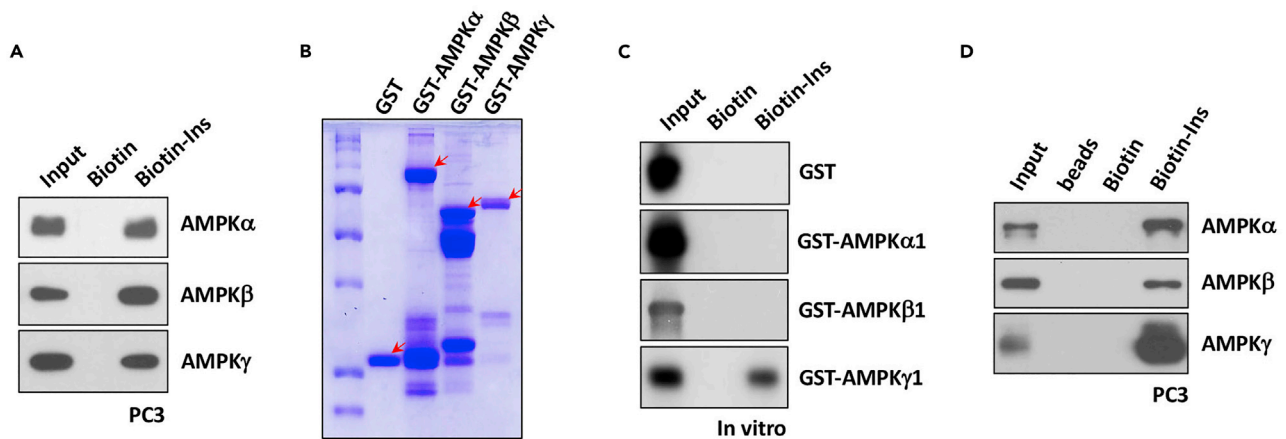


Figure 3. Biotin-labeled inositol (Biotin-Ins) binds to AMPK $\alpha\beta\gamma$ complex in cells and binds to AMPK γ subunit *in vitro*

(A) The cell lysates from PC3 cells were incubated with 40 μ M of biotin or 40 μ M of biotin-labeled inositol, and inositol interacting proteins were pulled down by streptavidin beads and subjected to immunoblotting using specific AMPK α , AMPK β and AMPK γ antibodies.
 (B) The purity of the recombinant proteins of GST, GST-AMPK α , GST-AMPK β and GST-AMPK γ were resolved by SDS-PAGE, followed by Coomassie blue staining.
 (C) 200 ng of GST, GST-AMPK α 1, GST-AMPK β 1 or GST-AMPK γ 1 incubated with 40 μ M of biotin or biotin-labeled inositol (Biotin-Ins 1) for 3 h, followed by pull-down with streptavidin beads. The interaction was determined by immunoblotting with GST antibody.
 (D) The cell lysates from PC3 cells treated with 25 μ M of biotin or biotin-labeled inositol (Biotin-Ins 1) treatment for 1 h were incubated with streptavidin beads and subjected to immunoblotting with indicated antibodies.

26. Express the full-length recombinant proteins of GST, GST-AMPK α 1, GST-AMPK β 1 or GST-AMPK γ 1 in *Escherichia coli* BL21 and purify recombinant proteins by using glutathione-sepharose beads (GE Healthcare, Cat#17-0756-01). Dilute purified proteins by using 10 mM Tris pH 8.0 buffer. The purity of the recombinant proteins is assessed by SDS-PAGE, followed by Coomassie blue staining (Figure 3B).
27. Prepare 30 μ L of Dynabeads MyOne Streptavidin T1 for each experimental group. Wash beads by using lab prepared PBS 30 s each time for 3 times, respectively.
28. Use 30 μ L X (the number of sample + 1) of binding buffer to resuspend washed beads and aliquot 30 μ L of beads in fresh tubes.
29. Add 100–200 ng of recombinant proteins in each tube with beads and then use binding buffer to fill up the rest of volume until 500 μ L.
30. Incubate 1 mg of recombinant proteins with Dynabeads MyOne Streptavidin T1 for 30 min at 4°C in a rotating wheel.
31. Use a magnetic rack to separate 1 mg of protein extracts from beads, and then collect pre-clearing recombinant proteins in fresh tubes put on ice.
32. Prepare 30 μ L of new Dynabeads MyOne Streptavidin T1 for each experimental group after washing with lab prepared PBS 30 s each time for 3 times.
33. Add 25–40 μ M of biotin or biotin-labeled inositol in each tube with beads and use lab prepared PBS to fill up the rest of volume until 500 μ L.
34. Incubate biotin or biotin-labeled inositol with Dynabeads MyOne Streptavidin T1 for 30 min at 25°C in a rotating wheel.

Note: Dynabeads stably bind to biotin at 25°C.

35. Use a magnetic rack to collect beads that binds with biotin or biotin-inositol and then wash beads by lab prepared PBS 30 s each time for 3 times.
36. Add pre-clearing 100–200 ng of recombinant proteins to incubate with the beads binding with biotin or biotin-labeled inositol in each experimental group, followed by incubating with beads for 1–2 h at 4°C in a rotating wheel.

37. Spin down each sample to make sure no beads left on the cap of tubes and then use a magnetic rack to separate beads and protein extracts.
38. After beads incubate with recombinant proteins, wash beads by wash buffer 30 s each time for 3 times and lab prepared PBS 30 s each time for 3 times at 4°C.

Note: Store wash buffer at 4°C for 1 year.

39. After removing lab prepared PBS, add 30 µL of 2 times SDS loading dye in each sample, followed by boiling each sample at 95°C for 5 min.

Note: 2 times SDS loading dye was prepared from 5 times SDS loading dye (250 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol blue) by dilution.

40. Conduct Western blotting by loading 10 ng of recombinant proteins as input and 30 µL of each experimental sample.
 - a. Run recombinant proteins with SDS loading dye were run in the 12% SDS-PAGE.
 - b. Transfer protein in the SDS-PAGE to PVDF membrane using Mini Trans-Blot Electrophoretic Transfer Cell from Bio-Rad (Cat#1703930) under 90 voltage for 3 h.
 - c. Conduct membrane blocking by using 3% skim milk for 1 h.
 - d. Incubate with primary GST antibody for 3 h at 25°C.
 - e. After membranes are washed by PBST (PBS+1% Tween 20) 7 min each time for 3 times, incubate with HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Cat#115-035-003) for 1 h at 25°C.
 - f. After membranes are washed by PBST (PBS+1% Tween 20) 7 min each time for 3 times, incubate ECL western blotting substrate (Thermo Fisher Scientific, Cat#32106) with each membrane for 2 min at 25°C, following by visualizing signal on the autoradiography films.
41. Immunoblotting of biotin pull-down assay *in vitro* protocol by using GST antibody is shown in [Figure 3C](#).

Biotin pull-down assay in cell-based protocol upon treatment

⌚ **Timing: 2 days**

To perform biotin pull-down assay in cell-based protocol upon biotin and biotin-labeled inositol treatment, biotin-labeled inositol treatment could also be taken up by cells, followed by detecting inositol-binding proteins using biotin pull-down assay.

42. Treat 25–40 µM of biotin or biotin-labeled inositol for 1–2 h in 90% confluency of PC3 cells cultured in RPMI-1640 medium supplied with 10% FBS, 2 mM glutamine in 15 cm dishes.
43. Harvest cells by using lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and 1 mM EDTA). After scratching cells from dishes, incubate cell lysates on ice for 20 min.
44. Centrifuge cell lysates for 20 min at full speed (16,800 g). Collect supernatant in fresh tubes placed on ice and cell lysates are subjected to Bio-Rad Protein Assay for quantifying protein concentration.
45. Prepare 30 µL of Dynabeads MyOne Streptavidin T1 for each experimental group after washing by lab prepared PBS 30 s each time for 3 times.
46. Add 1 mg of protein extracts from biotin or biotin-inositol treated cells in 30 µL of Dynabeads MyOne Streptavidin T1 and then use the lysis buffer to fill up the rest of volume until 500 µL.
47. Incubate Protein extracts with beads for 2–3 h at 4°C in a rotating wheel.
48. After beads incubate with protein extracts, wash beads by lysis buffer 30 s each time for 3 times and lab prepared PBS 30 s each time for 3 times.

49. Conduct Western blotting by loading 10 μg of protein extracts as input and 30 μL of each experimental sample.
50. Immunoblotting upon biotin or biotin-labeled inositol treatment in PC3 cells by using AMPK α , AMPK β and AMPK γ specific antibodies is shown in [Figure 3D](#).

Competition biotin pull-down assay by unlabeled myo-inositol

⌚ Timing: 2 days

To perform biotin pull-down assay in cell-based protocol or *in vitro* protocol, unlabeled myo-inositol could be used as a competitor to determine whether target proteins specifically bind to biotin-labeled inositol.

51. After 25–40 μM of biotin or biotin-labeled inositol binds to Dynabeads MyOne Streptavidin T1, add 1 mg of protein extracts or 100–200 ng of recombinant proteins in each tube with beads and use binding buffer to fill up the rest of volume until 500 μL .
52. Add 2.5–4 mM of unlabeled myo-inositol to the beads binding to biotin-labeled inositol and then incubate with pre-clearing protein extracts/recombinant proteins.
53. Incubate beads binding with biotin-labeled inositol and unlabeled myo-inositol together for 1–2 h at 4°C in a rotating wheel like other experimental groups.
54. After beads bind to protein extracts or recombinant proteins, wash beads by lysis buffer, wash buffer and lab prepared PBS.

Note: For beads binding to protein extract, wash beads by lysis buffer 30 s each time for 3 times and lab prepared PBS 30 s each time for 3 times.

Note: For beads binding to recombinant proteins, wash beads by wash buffer 30 s each time for 3 times and lab prepared PBS 30 s each time for 3 times at 4°C.

55. After removing lab prepared PBS, add 30 μL of 2 times SDS loading dye in each sample, followed by boiling each sample at 95°C for 5 min.
56. Each sample from biotin pull-down assay is subjected to immunoblotting by using specific AMPK α , AMPK β , AMPK γ or GST antibody shown in [Figures 4A](#) and [4B](#).

EXPECTED OUTCOMES

In this protocol, biotin pull-down assay can be conducted in *in vitro* systems and cell-based assays upon biotin or biotin-labeled inositol treatment. After samples pre-clearing, non-specific binding or off-target protein binding can be reduced. We used AMPK as an example, which is a sensor of inositol ([Hsu et al., 2021](#)), to demonstrate that biotin-labeled inositol could specifically bind to AMPK complex in a cell-based model. In addition, we can distinguish the specific isoform of AMPK binding to inositol from AMPK complex through *in vitro* biotin pull-down assay in this protocol. The results reveal that biotin-labeled inositol selectively binds to AMPK γ subunit, but not AMPK α and AMPK β subunits, *in vitro* ([Figure 3C](#)).

In cell-based protocol upon biotin or biotin-labeled inositol treatment, biotin-labeled inositol can be expectedly be taken up by cells and will not be converted to downstream metabolites of phosphatidylinositol cycle in 1–2 h, allowing us to identify inositol's sensors by mimicking dynamics of inositol uptake by cells. Additionally, high protein concentration is critical for conducting biotin-labeled inositol pull-down assay. 1 mg of protein extracts is an ideal concentration for performing biotin pull-down assay in cell-based protocol. Given that binding between metabolites and their target proteins may not be as strong as protein-protein interaction, increase of protein concentration can substantially improve the sensitivity of detection. In order to further confirm the specificity of

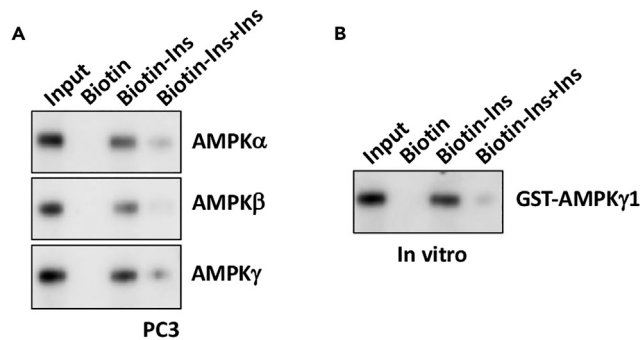


Figure 4. Unlabeled myo-inositol abolishes biotin-labeled inositol binding to its target proteins

(A) The cell lysates from PC3 cells were incubated with 40 μ M of biotin, 40 μ M of biotin-labeled inositol (Biotin-Ins) or 4 mM of unlabeled myo-inositol (Ins); and inositol interacting proteins were pulled down by streptavidin beads and subjected to immunoblotting using specific AMPK α , AMPK β and AMPK γ antibodies.

(B) 200 ng of GST-AMPK γ 1 was incubated with 40 μ M of biotin, 40 μ M of biotin-labeled inositol (Biotin-Ins) or 4 mM of unlabeled myo-inositol (Ins) for 2 h, followed by pull-down with streptavidin beads. The interaction was determined by immunoblotting with GST antibody.

biotin-labeled inositol-binding proteins, we use unlabeled inositol to compete with biotin-labeled inositol. Blocking the binding between biotin-labeled inositol and target proteins by unlabeled inositol indicates specific interaction between inositol and its target proteins.

LIMITATIONS

In this protocol, we use myo-inositol to conjugate with biotin as a tool in order to identify potential inositol-binding proteins. However, there are still some limitations in using biotin-labeled inositol to identify its binding proteins, although biotin-streptavidin system is a common strategy applied to demonstrate protein-ligand interaction. First, it remains unclear whether other isomeric forms of inositol such as *scyllo*-inositol, *epi*-inositol, *D-chiro*-inositol, *neo*-inositol and *muco*-inositol also exhibit the same binding affinity as myo-inositol. Second, although we find that [Scheme 1](#) has good binding affinity with AMPK, whether longer linker with increased carbon number on biotin alkyl chain could improve the binding affinity is also not clear. Last, it is unclear whether different isomers of biotin-labeled inositol contribute to AMPK binding, as they are also identified in the NMR spectra despite in trace quantities.

TROUBLESHOOTING

Problem 1

Biotin control shows the non-specific binding after biotin pull-down by Dynabeads MyOne Streptavidin T1 (steps 3 and 4).

Potential solution

- The concentration of biotin can be reduced to below 10 μ M.
- Increasing the percentage of NP-40 from 0.1% to 1% or adding 0.02%–0.1% of SDS in washing buffer can be implemented.
- Unlabeled inositol can be used to compete with biotin-labeled inositol to confirm proteins binding to biotin-labeled inositol or biotin, thereby clarifying whether binding proteins can bind to both of biotin and biotin-labeled inositol.
- Ensuring the procedure of pre-clearing has been completely performed.

Problem 2

Weak or non-binding proteins appear in the biotin-labeled inositol group after biotin pull-down by Dynabeads MyOne Streptavidin T1 (steps 3 and 4).

Potential solution

- NP-40 can be removed from the wash buffer.
- Lab prepared PBS buffer is used as the wash buffer.
- Protein concentration can be increased for biotin pull-down using Dynabeads MyOne Streptavidin T1.

Problem 3

Unlabeled inositol could not compete with biotin-labeled inositol (step 4).

Potential solution

- The concentration of unlabeled inositol can be increased for biotin pull-down using Dynabeads MyOne Streptavidin T1.
- The linker between biotin and inositol can be changed by modifying the number of carbons.

Problem 4

Weak or non-binding proteins upon biotin-labeled inositol treatment appear in the biotin-labeled inositol group after biotin pull-down by Dynabeads MyOne Streptavidin T1 (step 2).

Potential solution

- The time of biotin-labeled inositol treatment in cells can be reduced to 1 h, thereby preventing biotin-labeled inositol from converting to downstream metabolites.
- The concentration of biotin-labeled inositol treatment can be increased, followed by harvesting protein extracts and biotin pull-down using Dynabeads MyOne Streptavidin T1.

Problem 5

GST only recombinant protein control shows the non-specific binding after biotin pull-down by Dynabeads MyOne Streptavidin T1 (steps 3 and 4).

Potential solution

- The concentration of recombinant proteins can be reduced to below 100 ng.
- Increasing the percentage of NP-40 from 0.1% to 1% or adding 0.02%–0.1% of SDS in the wash buffer can be implemented.
- Unlabeled inositol can be used to compete with biotin-labeled inositol to confirm GST-fusion recombinant proteins binding to biotin-labeled inositol or biotin, thereby clarifying whether GST-fusion recombinant proteins can bind to both of biotin and biotin-labeled inositol.
- Ensuring the procedure of pre-clearing has been completely performed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hui-Kuan Lin (hulin@wakehealth.edu).

Materials availability

The plasmids, cell lines and antibodies generated in this study have not been deposited to any repositories yet. These materials will be available upon request.

Data and code availability

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

C.-C.H. and H.-K.L. designed experiments and wrote the manuscript. C.-C.H. performed experiments and analyzed the data for all figures. Z.-G.X., Z.-Z.C., and H.-Y.L. provided technical and editing support, comments and suggestions, especially for the design, synthesis, and characterization of biotin-labeled compound (Scheme 1).

DECLARATION OF INTERESTS

The authors declare no competing interests.

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