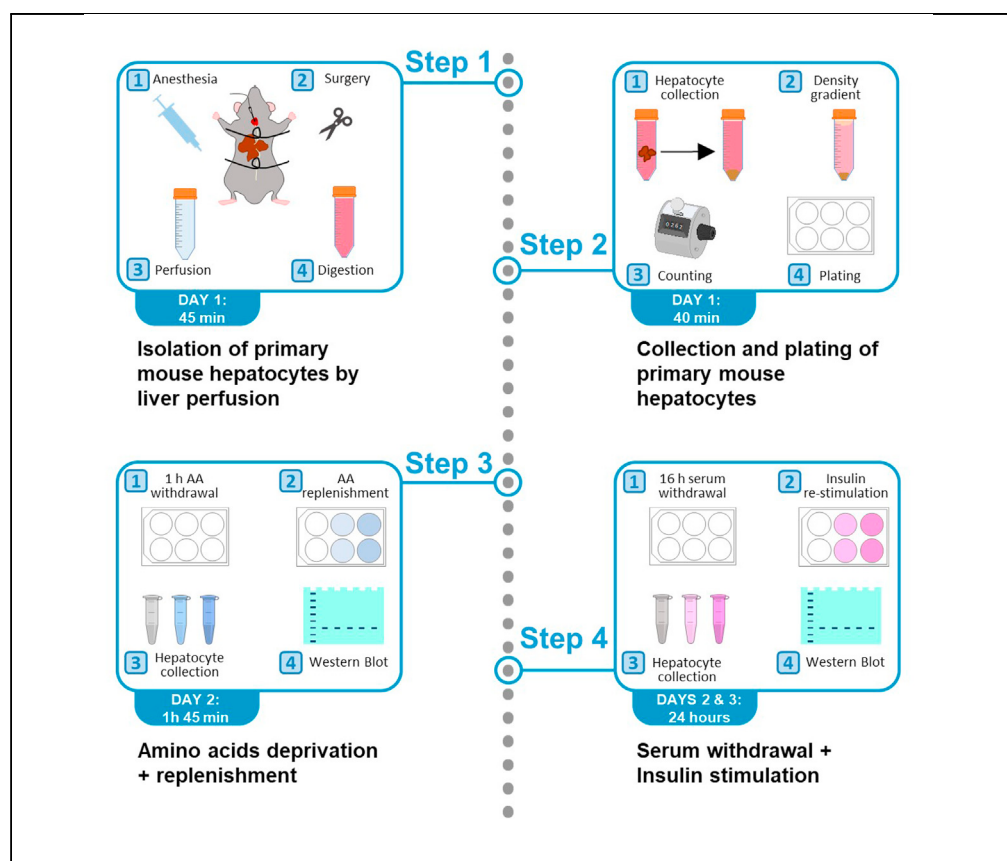


Protocol

Protocol for the assessment of mTOR activity in mouse primary hepatocytes



We present a protocol for measuring the activity of the mechanistic target of rapamycin (mTOR) pathway in ex vivo isolated mouse primary hepatocytes. It can be used as a tool for genetic, pharmacological, metabolomic, and signal transduction procedures. We discuss critical aspects for improving yield, viability, and modulation of the mTOR pathway. This protocol can be adapted to other signaling cascades and is compatible with multiple readouts.

Ana Belén
Plata-Gómez, María
Crespo, Celia de la
Calle Arregui, Lucía
de Prado-Rivas,
Guadalupe Sabio,
Alejo Efeyan

guadalupe.sabio@cnic.es
(G.S.)
aefeyan@cnic.es (A.E.)
abplata@cnic.es (A.B.P.-
G.)

Highlights

Describes the
purification and
culture of primary
hepatocytes from
mice

Describes the
assessment of
mTORC1 activity
manipulating nutrient
and growth factor
signals

Protocol adaptable to
other signaling
cascades and
compatible with
multiple readouts

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Protocol

Protocol for the assessment of mTOR activity in mouse primary hepatocytes

Ana Belén Plata-Gómez,^{1,3,*} María Crespo,² Celia de la Calle Arregui,¹ Lucía de Prado-Rivas,¹ Guadalupe Sabio,^{2,*} and Alejo Efeyan^{1,4,*}

¹Metabolism and Cell Signaling Laboratory, Spanish National Cancer Research Centre (CNIO), Melchor Fernández Almagro 3, 28029 Madrid, Spain

²Myocardial Pathophysiology, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, Spain

³Technical contact

⁴Lead contact

*Correspondence: guadalupe.sabio@cnic.es (G.S.), aefeyan@cnio.es (A.E.), abplata@cnio.es (A.B.P.-G.)
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SUMMARY

We present a protocol for measuring the activity of the mechanistic target of rapamycin (mTOR) pathway in *ex vivo* isolated mouse primary hepatocytes. It can be used as a tool for genetic, pharmacological, metabolomic, and signal transduction procedures. We discuss critical aspects for improving yield, viability, and modulation of the mTOR pathway. This protocol can be adapted to other signaling cascades and is compatible with multiple readouts.

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

BEFORE YOU BEGIN

Tissue culture room

⌚ Timing: 3 days

1. Prepare the solutions for perfusion and digestion of the liver and the hepatocyte medium.
 - a. **Perfusion Solution:** Mix 500 mL of Hank Balanced Salt Solution 1× (HBSS) without Ca²⁺ without Mg²⁺ without Phenol red with 5 mL 1M HEPES and 5 mL of 0.1M EGTA.
 - b. **Digestion Solution:** Mix 500 mL of Williams E Medium with 5 mL 1M HEPES. Each 50 mL of this solution must be supplemented with 40 mg of Collagenase Type I.

Optional: Aliquot Perfusion Solution and Digestion Solution in 50 mL centrifuge tubes. Each tube contains the volume necessary for the perfusion of two mouse livers.

Note: Prepare envelopes containing 40 mg of Collagenase Type I. Each envelope contains the Collagenase necessary to digest two mouse livers. Immediately before the first step of isolation of primary mouse hepatocytes add the content of one envelope to one 50 mL tube of Digestion Solution and mix well.

⚠ **CRITICAL:** The Collagenase must be added to the Digestion Solution immediately before the start of the isolation procedure. It must be completely dissolved and warmed to obtain the best digestion efficiency.

- c. **Rinsing Solution:** Mix 500 mL of sterile PBS with 5 mL 5,000 U/mL Penicillin-Streptomycin and 500 µL 50 mg/mL Gentamycin.



d. **Gradient Solution:** Mix 22.5 mL Percoll with 2.5 mL HBSS 10× per mouse.

△ **CRITICAL:** Gradient Solution must be prepared immediately before the density-gradient separation.

e. **Hepatocyte Attachment Medium:** Mix 200 mL DMEM high glucose, 200 mL F12 Nutrient mix Ham Medium, 100 mg 0.02% BSA fatty acid free, 255 mg NaHCO₃, 25 mL 100 mM Sodium Pyruvate, 9 mL 1 M HEPES, 5 mL 200 mM L-Glutamine, 5 mL 5,000 U/mL Penicillin-Streptomycin, 500 µL 50 mg/mL Gentamycin and 50 mL fetal bovine serum (FBS). Filter the medium through a 0.22 µm filter.

Store all the solutions at 4°C.

2. Prepare cell culture plates for primary hepatocytes.

- a. Mix 1 mL of rat tail Collagen Type I with 49 mL of Rinsing Solution and homogenize by repeated pipetting up and down.
- b. Add 2 mL of the mix in each well of a 6 multi-well plate. Ensure that the bottom of the well is completely covered.

Note: For the assessment of mTOR activity, 6 multi-well plates yield sufficient whole protein lysates for western blotting (around 250 µg), but 10 cm, 12-well and 24-well plates are also suitable for hepatocyte plating.

- c. Incubate the plates at 37°C during 3 h under sterile conditions inside the cell culture incubator.
- d. Sterilize the plates inside the cell culture hood by UV light exposure for 30 min.
- e. Transfer the plates to a 4°C fridge. Close and cover each plate with parafilm.

Note: Collagen must be aspirated from cell plates before the plating of the primary hepatocytes in step 17.

3. Prepare solutions for amino acid withdrawal and for FBS withdrawal.

- a. Dialyzed FBS (dFBS):
 - i. Take a SnakeSkin Dialysis Tube and cut the required length of membrane from the packaging tube.
 - ii. Close the tube in one end using the dialysis clips or by making a tight knot.

Note: Ensure that the tube is properly closed before pouring the FBS inside.

- iii. Pour 100 mL of FBS inside the tube and close the tube in the open end.
- iv. Place the tube in a beaker with 5L of PBS 1× and put it in a magnetic mixer. Transfer to a 4°C room.

Note: Ensure that the external surface of the whole tube is in contact with the PBS.

- v. Set a slow spin in the magnetic mixer and leave 16 h.
- vi. The following day take out the tube carefully. Discard the PBS 1× and fill the beaker with 5L of fresh cold PBS 1×.
- vii. Return the beaker in the magnetic mixer at 4°C.
- viii. Set a slow spin in the magnetic mixer and leave 16 h.
- ix. The following day take out the tube carefully. Open it by one side and pour the content into a clean beaker.
- x. Filter the dFBS through a 0.22 µm filter inside a cell culture hood.
- xi. Aliquot the dFBS in 15 mL conical tubes to avoid to multiple freeze-thaw cycles and freeze at −20°C.

- b. **Pre-starvation Medium:** Dissolve 3.85 g DMEM/F12 without Amino acids Medium in 450 mL of ddH₂O. Supplement the medium with 255 mg NaHCO₃, 9 mL 1M HEPES and 5 mL 2.5M Glucose. Filter the medium through 0.22 µm filter.
- c. **Amino acid Starvation Medium:** Mix 90 mL of Pre-starvation Medium with 10 mL of dFBS. Supplement the medium with 100 µL of 100 µM insulin.

Note: DMEM/F12 without Amino acids, supplemented with dFBS, is better suited to perform amino acid starvation on primary hepatocytes, as compared to other media (see below; DAY 2 Amino acid deprivation). Moreover, the strength of acute activation of mTORC1 further improves when medium is supplemented with insulin.

Note: Specific metabolite withdrawal procedures can be performed to assess the effect on mTORC1 activity. For example, single amino acid drop-out experiments can be performed following the same procedure but incorporating all other amino acids (except for the one to be deprived of) in the Amino acid Starvation Medium.

- d. Amino acid cocktail:
 - i. Prepare a 50× Essential Amino acid mix stock:
50× AA mix

Amino acid	Amount	Final concentration (stock)	Final concentration (when added to amino acid starvation medium)
C (L-Cystine-2HCl)	325 mg	3,250 mg/L	207.52 µM
F (L-Phenylalanine)	75 mg	750 mg/L	90.81 µM
H (L-Histidine)	75 mg	750 mg/L	71.56 µM
I (L-Isoleucine)	250 mg	2,500 mg/L	381.19 µM
K (L-Lysine-HCl)	200 mg	2,000 mg/L	218.99 µM
L (L-Leucine)	250 mg	2,500 mg/L	381.19 µM
M (L-Methionine)	75 mg	750 mg/L	100.53 µM
Q (L-Glutamine)	1,500 mg	15,000 mg/L	2052.83 µM
R (L-Arginine)	1,000 mg	10,000 mg/L	1148.11 µM
T (L-Threonine)	100 mg	1,000 mg/L	167.89 µM
V (L-Valine)	100 mg	1,000 mg/L	170.72 µM
W (L-Tryptophan)	25 mg	250 mg/L	24.48 µM
Y (L-Tyrosine-2Na-2H ₂ O)	145 mg	1,450 mg/L	160.05 µM
ddH ₂ O	100 mL	N/A	N/A
Total	100 mL	50×	1×

Note: aliquot and store the mix at −20°C.

- ii. Prepare a 100× Non-Essential Amino acid mix stock:
100× AA mix

Amino acid	Amount	Final concentration (stock)	Final concentration (when added to amino acid starvation medium)
D (L-Aspartic Acid)	200 mg	2,000 mg/L	150.26 µM
E (L-Glutamic Acid)	200 mg	2,000 mg/L	135.93 µM
G (Glycine)	100 mg	1,000 mg/L	133.21 µM
N (L-Asparagine)	500 mg	5,000 mg/L	378.44 µM
P (L-Proline)	200 mg	2,000 mg/L	173.72 µM
S (L-Serine)	300 mg	3,000 mg/L	285.47 µM
ddH ₂ O	100 mL	N/A	N/A
Total	100 mL	100×	1×

Note: aliquot and store the mix at -20°C .

- iii. Mix 1000 μL of 100 $\times$ Non-Essential Amino acids and 2000 μL of 50 $\times$ Essential Amino acids in 7 mL of Amino acid Starvation Medium to have a 10 $\times$ amino acid solution.
- e. **FBS withdrawal Medium:** Mix 200 mL DMEM high glucose, 200 mL F12 Nutrient mix Ham Medium, 100 mg 0.02% BSA fatty acid free, 255 mg NaHCO_3 , 25 mL 100 mM Sodium Pyruvate, 9 mL 1M HEPES, 5 mL 200 mM L-Glutamine, 5 mL 5,000 U/mL Penicillin-Streptomycin and 500 μL 50 mg/mL Gentamycin. Filter the medium with 0.22 μm filter.

Note: The composition of the FBS withdrawal Medium is the same as in the Hepatocyte Attachment Medium but without FBS.

4. Prepare Protein Lysis Buffer:

Protein Lysis Buffer		
Reagent	Final concentration	Amount
HEPES-KOH 1M pH 7.4	50 mM	25 mL
NaCl	40 mM	1.175 g
EDTA 0.5 M	2 mM	2 mL
Sodium orthovanadate (NaVO_4) 150 mM *	1,5 mM	500 μL per 50 mL
Sodium Fluoride (NaF) 0.5M	50 mM	50 mL
Sodium Pyrophosphate	10 mM	1.325 g
β -Glycerophosphate disodium salt hydrate 1M *	10 mM	500 μL per 50 mL
Triton X-100	1%	5 mL
Sodium deoxycholate	1%	5 g
Complete Mini Protease Inhibitor Cocktail *	N/A	2 tablets per 50 mL
Sodium dodecyl sulfate (SDS) 10%	0.1 %	5 mL
ddH ₂ O	N/A	408.5 mL
Total	N/A	500 mL

Note: store at -20°C .

Note: Reagents marked with * in the Protein Lysis Buffer Table are to be added immediately before using the Protein Lysis Buffer.

Animal facility

⌚ **Timing:** 10 min

5. Mouse housing:
 - a. All animal procedures carried out were performed according to protocols approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CElyBA) and the Autonomous Community of Madrid (CAM). Protocol number PROEX15/18.
 - b. Mice were housed under specific pathogen-free conditions at 22°C on a regular 12 h light and 12 h dark cycle. 7 to 10-week-old mice were used in this protocol.

Note: Optimal age of mice for isolating primary hepatocytes is 7–10 weeks old. In younger mice the veins are smaller and more fragile, and veins in older mice have increased deposition of visceral fat, making more difficult the surgery process and the cannulation. Cannulation gauge and perfusion speed may need to be optimized for younger and older mice.

6. Preparation of anesthesia: Mix 500 μ L of 100 mg/mL Ketamine, 250 μ L of 20 mg/mL Xylazine and 4.25 mL Saline Solution.
7. Sterilization of the perfusion line: Wash the perfusion tube of the pump with 70% ethanol. Maintain the 70% ethanol solution in the tube for 16 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb (dilution 1:500)	Cell Signaling Technology	Cat#9234; RRID: AB_2269803
P70 S6 Kinase (49D7) Rabbit mAb (dilution 1:500)	Cell Signaling Technology	Cat#2708; RRID: AB_390722
Phospho-S6 Ribosomal Protein (Ser235/236) Antibody (dilution 1:1000)	Cell Signaling Technology	Cat#2211; RRID: AB_331679
S6 Ribosomal protein (5G10) Rabbit mAb (dilution 1:1000)	Cell Signaling Technology	Cat#2217; RRID: AB_331355
Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb (dilution 1:500)	Cell Signaling Technology	Cat#2855
4E-BP1 (53H11) Rabbit mAb (dilution 1:1000)	Cell Signaling Technology	Cat#9644; RRID: AB_2097841
Phospho-Akt (Ser473) (D9E) XP Rabbit mAb (dilution 1:1000)	Cell Signaling Technology	Cat#4060; RRID: AB_2315049
Akt (pan) (C67E7) Rabbit mAb (dilution 1:1000)	Cell Signaling Technology	Cat#4691; RRID: AB_915783
Vinculin (dilution 1:5000)	Sigma-Aldrich	Cat#V9131; RRID: AB_477629
Chemicals, peptides, and recombinant proteins		
Collagen, Type I solution from rat tail	Sigma-Aldrich	Cat#C3867
PBS – Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	Cat#D8537
Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher	Cat#15070063
Gentamycin solution 50 mg/mL	Sigma-Aldrich	Cat#G1397
HBSS (1 $\times$), no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat#14175053
HEPES Buffer 1M	Lonza	Cat#BE17-737E
Ethylene glycol-bis(2-aminoethylether)-N,N',N'-tetraacetic acid	Sigma-Aldrich	Cat#E3889
William's E Medium, no glutamine	Thermo Fisher Scientific	Cat#22551022
Collagenase, Type I	Worthington-biochem	Cat#LS004196
Percoll	Sigma-Aldrich	Cat#GE17-0891-01
HBSS (10 $\times$), no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	Cat#14185052
DMEM, high glucose	Thermo Fisher Scientific	Cat#1965039
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	Cat#21765029
Bovine Serum Albumin essentially fatty acid free	Sigma-Aldrich	Cat#A6003
Sodium bicarbonate (NaHCO ₃)	Merck	Cat#1063291000
Sodium pyruvate solution 100 mM	Sigma-Aldrich	Cat#S8636
L-Glutamine solution 200 mM	Sigma-Aldrich	Cat#G7513
Fetal Bovine Serum (FBS)	Hyclone	Cat#SV30160.03
Dulbecco's MEM (DMEM) F-12 without Amino acids, Glucose, L-Glutamine, Sodium Bicarbonate, HEPES, Sodium	USBiological Life Sciences	Cat#D9807-10
D-(+)-Glucose solution	Sigma-Aldrich	Cat#G8769
C (L-Cystine-2HCl)	Sigma-Aldrich	Cat#C6727
F (L-Phenylalanine)	Sigma-Aldrich	Cat#P5482
H (L-Histidine)	Sigma-Aldrich	Cat#H5659
I (L-Isoleucine)	Sigma-Aldrich	Cat#I7403
K (L-Lysine-HCl)	Sigma-Aldrich	Cat#L8662
L (L-Leucine)	Sigma-Aldrich	Cat#L8912
M (L-Methionine)	Sigma-Aldrich	Cat#M5308
Q (L-Glutamine)	Sigma-Aldrich	Cat#G3126
R (L-Arginine)	Sigma-Aldrich	Cat#A8094
T (L-Threonine)	Sigma-Aldrich	Cat#T8441
V (L-Valine)	Sigma-Aldrich	Cat#V0513
W (L-Tryptophan)	Sigma-Aldrich	Cat#T8941
Y (L-Tyrosine-2Na-2H ₂ O)	Sigma-Aldrich	Cat#T8566

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
D (L-Aspartic Acid)	Sigma-Aldrich	Cat#A8949
E (L-Glutamic Acid)	Sigma-Aldrich	Cat#G1251
G (Glycine)	Sigma-Aldrich	Cat#G7126
N (L-Asparagine)	Sigma-Aldrich	Cat#A0884
P (L-Proline)	Sigma-Aldrich	Cat#P0380
S (L-Serine)	Sigma-Aldrich	Cat#S4500
HEPES	Sigma-Aldrich	Cat#H3375
Sodium chloride	Sigma-Aldrich	Cat#S9625
EDTA 0.5 M, pH 8	Thermo Fisher Scientific	Cat#15575020
Sodium orthovanadate (NaVO ₄)	Sigma-Aldrich	Cat#S6508
Sodium Fluoride (NaF)	Sigma-Aldrich	Cat#S7920
Sodium Pyrophosphate tetra-basic decahydrate	Sigma-Aldrich	Cat#S6422
β-Glycerophosphate disodium salt hydrate	Sigma-Aldrich	Cat#G9422
Triton X-100	Sigma-Aldrich	Cat#T8787
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750
Complete Mini Protease Inhibitor Cocktail	Roche	Cat #11836153001
Sodium dodecyl sulfate (SDS)	Merck	Cat#151-21-3
70% Ethanol	N/A	N/A
Ketamine	Imalgene 1000	N/A
Xylazine	Rompur	N/A
Physiological saline solution	B Braun	Cat #607189.2
Trypan Blue solution	Sigma-Aldrich	Cat#T8154
Insulin human	Sigma-Aldrich	Cat#I2643
SnakeSkin Dialysis Tubing, 3.5K MWCO, 35 mm dry I.D., 35 feet	Thermo Fisher Scientific	Cat #88244
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#23225
Primary mouse hepatocytes	This paper	N/A
Experimental models: Organisms/strains		
C57BL/6J (7–10 weeks)	Jackson Laboratory	Cat#JAX:000664; RRID: IMSR_JAX:000664
Software and algorithms		
ImageJ software	ImageJ	https://imagej.nih.gov/ij/
Other		
6-well Clear Multiwell Plate	Life Sciences	Cat#353046
500 mL Vacuum Filter/Storage Bottle System, 0.22 μm Pore 33.2 cm ² PES Membrane, Sterile, 12/Case	Life Sciences	Cat#431097
Corning Polypropylene Centrifuge Tubes, Sterile (50 mL)	Fisher Scientific	Cat#10509891
Masterflex C/L Analog Variable-Speed Pump with Dual-Channel Pump Head for Microbore Tubing Pump, 10–60 rpm; 12 VDC	Cole-Parmer	Cat#EW-77120-62
Water bath Thermo Haake	Thermo Fisher Scientific	Cat#003–2859
Sterile needles 23G ×1" – Nr. 16 0.6 mm × 25 mm	BD Microlance 3	Cat#300800
Sterile surgical non-absorbable sutures 2/0 USP 90 cm	LorcaMarín	Cat#55184-50U
Intravascular Catheter BD Vialon 22GA 0.9 × 25 mm	Clinimark SL	Cat#381223
ROCKER 3D digital (Shaker)	IKA	Cat#0004001000
Fine Scissors – Sharp-Blunt	Fine Science Tools	Cat#14028-10
Dumont #5 – Fine Forceps	Fine Science Tools	Cat#11254-20
Corning 100 μm Cell Strainer, Yellow, Sterile, Individually Packaged, 50/Case	Life Sciences	Cat#431752
TC20 Automated cell counter	Bio-Rad	Cat#145-0101
Cell Lifter with J-Hook Blade	VWR	Cat#76036-004
Eppendorf 5810 Centrifuge	Merck	Cat#EP5810000320
Eppendorf 5424R Refrigerated Centrifuge	Marshall Scientific	Cat#EPP-5424R
ELMI RM-2L Intelli-mixer Large	ELMI	Cat#ELMI RM-2L
Parafilm	Merck	Cat#P7793

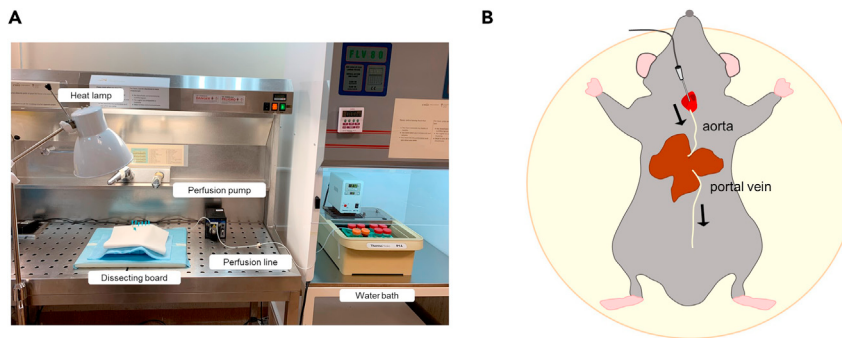


Figure 1. Isolation of primary mouse hepatocytes by liver perfusion

(A) Working area for isolation of primary hepatocytes from mouse. Most relevant materials are indicated.
(B) Cartoon showing the direction of the non-recirculating circuit from aorta artery to portal vein.

STEP-BY-STEP METHOD DETAILS

Day 1: Isolation of primary mouse hepatocytes by liver perfusion

⌚ Timing: 45 min (per mouse)

This major step allows to obtain digested livers from mice. Following anesthesia, two surgical knots are performed in the abdominal and the thoracic cavities of the mouse; the heart is cannulated through the left atrium and the liver is perfused and digested *in situ*.

1. **Preheating:** Warm the Perfusion Solution and the Digestion Solution in the water bath at 37°C. The water bath should be inside a laminar flow hood to assure sterile conditions of the solutions. Keep both solutions inside the bath during the whole isolation procedure.

⚠ **CRITICAL:** Collagenase must be added to the Digestion Solution immediately before the start of the isolation procedure. It must be completely dissolved and warmed before dissection to obtain the best digestion efficiency. We recommend to add the Collagenase before anesthetizing the mouse See [troubleshooting 1](#).

2. **Anesthesia:** Anesthetize the mouse according to your institution's approved animal protocol; for example, through intraperitoneal injection of Ketamine/Xylazine (100 μ L/ 10 g animal weight). Mice lose consciousness within 15 min after the injection.

Note: Pre-warm the anesthesia before injecting the mouse to avoid thermal shock.

Note: Confirm that the mouse is completely anesthetized before continuing with the step 3. Loss of the righting and palpebral reflexes, relaxation in the muscular tone, disappearance of pedal reflex and a decrease in the rate and depth of respiration are common readouts of successful anesthesia. If the mouse still maintains reflex 15 min after the injection, we recommend to additionally inject 50 μ L of Ketamine/Xylazine.

3. **Preparation of the perfusion pump:** Extensively wash the perfusion line with Rinsing Solution to eliminate 70% ethanol. Place one end of the perfusion line inside the Perfusion Solution located inside the water bath. Fill the perfusion line with Perfusion Solution before opening the mouse peritoneal cavity. Connect the outlet end of the perfusion line to the catheter ([Figure 1A](#)).

⚠ **CRITICAL:** Bubbles in the pumping tube can compromise digestion efficiency and viability of the isolated hepatocytes. See [troubleshooting 2](#) for instructions on how to avoid the presence of bubbles.

4. **Positioning:** Immobilize the anesthetized mouse by using needles to attach the upper and lower limbs on a dissecting board. Spray the mouse with 70% ethanol to ease dissection ([Methods video S1 – part 1](#)).

Note: If primary hepatocytes from more than one mouse are going to be isolated on a day, anesthetize the second mouse at this step. The 15 min of anesthesia of the second mouse will fit with the 15 minutes of dissection, surgery, cannulation, perfusion, digestion and collection of the liver from the first mouse.

5. **Dissection:** Using surgical scissors and forceps, open the abdominal cavity and carefully lean the small intestine to expose inferior vena cava and portal vein. With a syringe, add approximately 2 mL of Rising Solution into the abdominal cavity of the mouse to prevent contaminations ([Methods video S1 – part 2](#)).

Note: All the surgical material must be sterilized with 70% ethanol or by dry heating before use.

6. **Surgery:** For stable liver perfusion, perform two surgical knots in the abdominal and the thoracic cavities ([Methods video S1 – part 3](#)):
 - a. Abdominal knot: Take the needle and thread (Sterile surgical non-absorbable sutures) and pass the needle under the inferior cava vein and the left kidney (located at researcher's right side). Pass the needle above the kidney located at your left and tie a loose knot.
 - b. Thoracic knot: Open the thoracic cavity vertically cutting the ribs on both sides. Heart must not be damaged, so slow and gentle scissor movements are needed. Remove the ribs to facilitate posterior cannulation. Pass the needle under the visible aorta artery above the liver and tie a loose knot.

Note: Ensure the thoracic knot is over the end of the catheter. Otherwise, the circuit would be blocked.

7. **Cannulation:** Make an incision in the left atrium of the heart and cannulate.
 - a. Switch on the pump to begin a very slow perfusion (1 mL/min) of the mouse liver with Perfusion Solution. Secure the catheter with two needles.
 - b. Once the mouse is cannulated, tight the thoracic knot to secure the catheter above the liver; this allows the catheter to remain stable during posterior perfusion and digestion.
 - c. Then, tight the abdominal knot below the liver and cut inferior vena cava below the knot. Wait until the portal vein becomes distended, and then cut the portal vein ([Methods video S2](#)). At the end of this step a non-recirculating circuit from aorta artery to portal vein has been created ([Figure 1B](#)).

⚠ **CRITICAL:** Proper cannulation of the aorta is crucial for a successful perfusion. Verify that no bubbles are introduced to avoid an incomplete digestion. See [troubleshooting 2](#).

Note: Cannulation of the inferior cava vein instead of the left atrium for the isolation of primary mouse hepatocytes has been described in other protocols ([Charni-Natan and Goldstein, 2020](#); [Shen et al., 2021](#)).

8. **Liver perfusion:** Perfuse approximately 25 mL of Perfusion Solution at 5 mL/min of speed ([Methods video S3 – part 1](#)).

Note: If the surgery, cannulation and initial perfusion were performed correctly, the liver should turn pale. The Perfusion Solution should leave the circuit through the portal vein.

Optional: Add Rinsing Solution to the abdominal cavity of the mouse regularly to prevent contamination.

Optional: A soft massage of the liver while perfusing, favors posterior liver digestion.

9. **Liver digestion:** Switch on the heat lamp facing the mouse liver. Switch the end of the perfusion line from the Perfusion Solution to the Digestion Solution and perfuse the liver with 25 mL of this solution at 3.5 mL/min ([Methods video S3](#) – part 2).

Note: When switching the perfusion line from the Perfusion Solution to the Digestion Solution, briefly turn off the perfusion pump to avoid bubbles.

Note: The optimal pump speed depends on the diameter of the tube. In this protocol, pump speed was optimized according to the perfusion pump used (Masterflex C/L Analog Variable-Speed Pump with Dual-Channel Pump Head for Microbore Tubing Pump, 10–60 rpm; 12 VDC).

10. **Liver collection:** Once the perfusion with the Digestion Solution has finished, collect the mouse liver carefully. Remove gallbladder and the leftovers of tissue from diaphragm. Transfer the liver to 25 mL of Hepatocyte Attachment Medium in a 50 mL centrifuge tube in ice ([Methods video S3](#) – part 3).

Note: Carefully remove the liver avoiding puncturing/cutting the small intestine to prevent bacterial contamination.

⚠ **CRITICAL:** After digestion, liver texture should be soft and easily disaggregated by shaking the 50 mL centrifuge tube. If the liver has a dense-viscous texture, the perfusion failed and the yield for cell culture will be very low. See [troubleshooting 3](#).

11. **Liver disaggregation:** To favor release of hepatocytes, place the 50 mL tube containing the liver in a shaker (ROCKER 3D digital) for 5 min on ice.

Note: After finishing liver perfusion of one mouse, and before starting the procedure with another mouse, wash the perfusion line of the pump with Rinsing Solution to eliminate the rest of Collagenase from Digestion Solution.

Day 1: Collection and plating of primary mouse hepatocytes

⌚ **Timing:** 35–40 min (plus 6 h for incubation)

This major step allows to culture primary hepatocytes from the digested liver obtained in the previous step. Hepatocytes are filtered and separated by a density gradient; cells are counted and plated at a suitable confluence to perform the signaling experiment in the following step.

Note: All the steps of this section must be performed in a sterile cell culture hood ([Methods video S4](#)).

12. **Hepatocyte filtration:** Filter the mouse liver through a 100 μ m cell strainer transferring the sample to a clean 50 mL centrifuge tube. Favor liver disaggregation by gently smashing the liver with the plunger of a 10 mL syringe on top of the cell strainer. Use 10 mL extra of Hepatocyte Attachment Medium to clean the initial centrifuge tube and recover the possible remaining cells.
13. **Hepatocyte collection:** Centrifuge the filtered hepatocytes at 50 $\times$ g for 5 min at 20°C–25°C ([Figure 2A](#)). Aspirate the supernatant and resuspend the cell pellets with 25 mL of Hepatocyte Attachment Medium.

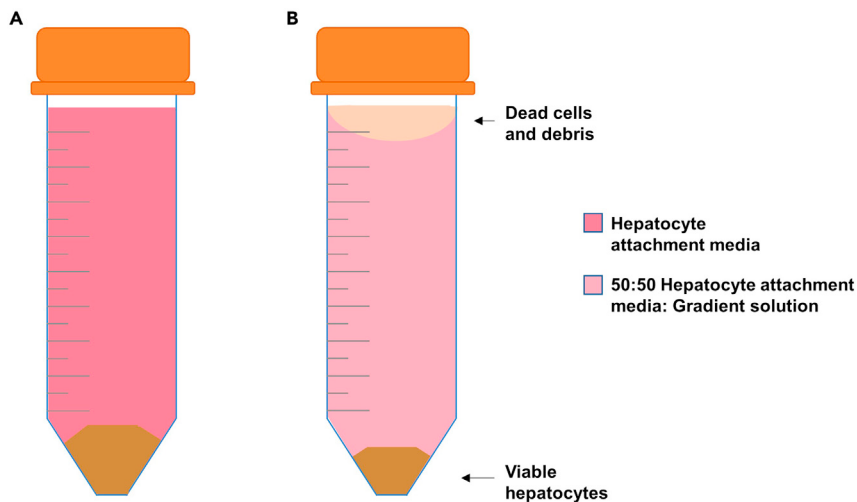


Figure 2. Hepatocyte collection and density-gradient separation

(A) Filtered hepatocytes collected from liver are centrifuged. The cartoon shows the pellet with total cells from liver. (B) The pellet in (A) is resuspended in 50:50 Hepatocyte Attachment Medium: Gradient Solution and centrifuged. The cartoon shows the pellet containing viable hepatocytes; the supernatant is composed by dead cells and debris.

Note: Prepare the Gradient Solution while cells are centrifuging.

14. **Density-gradient separation:** Add 25 mL of Gradient Solution slowly to the resuspended cells without mixing. Homogenize the solution by flipping the centrifuge tube 10 times. Centrifuge at $50 \times g$ for 10 min at 20°C–25°C with deceleration. At the end of the centrifugation, viable hepatocytes will be pelleted and dead cells will remain in the upper fraction of the supernatant (Figure 2B).

△ CRITICAL: Double-check that the deceleration step is set in the centrifuge. Otherwise, the gradient will be lost and the hepatocyte pellet will be contaminated with dead cells.

Note: This step is an improvement over other protocols, in which viable hepatocytes are not separated from dead cells through a density gradient. Percoll-based separation results in higher viability of the isolated hepatocytes.

15. **Hepatocyte clean-up:** Centrifuge the hepatocytes to remove dead cells.
 - a. Aspirate the supernatant very carefully to remove dead cells and resuspend the pellet using 20 mL of Hepatocyte Attachment Medium. Centrifuge the cells at $50 \times g$ for 5 min at 20°C–25°C.
 - b. Aspirate supernatant and resuspend the pellet with Hepatocyte Attachment Medium. In this step the volume for resuspending the hepatocytes depends on the pellet size.

Note: A successful isolation will require 25 mL of Hepatocyte Attachment Medium for resuspending the hepatocyte pellet (Figure 3A). If the yield of isolation is lower, volume can be decreased to 10 mL (Figure 3B) or 5 mL (Figure 3C) of Hepatocyte Attachment Medium.

16. **Assess hepatocyte viability:** Gently homogenize the hepatocyte suspension by pipetting up and down with a 1000 μ L pipette. Stain the hepatocytes with 10 μ L of cell sample with 10 μ L of Trypan Blue staining solution. Count both dead (dark blue) and live (translucent) cells using a TC20 Automated cell counter to calculate the survival rate. Alternatively, a classical hemocytometer could be used. Hepatocyte viability should be around 85%.

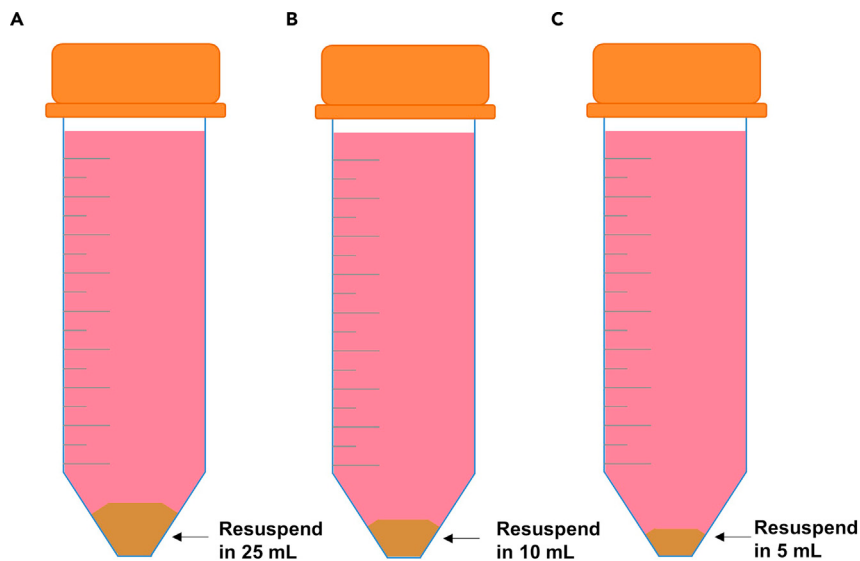


Figure 3. Hepatocyte clean up

The volume of Hepatocyte Attachment Medium for resuspending hepatocytes depends on the size of the pellet obtained in step 15.

- (A) A successful isolation requires a resuspension volume of 25 mL of Hepatocyte Attachment Medium.
- (B) If the yield of isolation is lower, volume can be decreased to 10 mL.
- (C) If the isolation was limited, the volume of resuspension should not exceed 5 mL.

Note: Repeat the staining and cell counting twice or three times on each sample and average the numbers obtained. Variability in sampling can lead you to inaccurate quantification of the hepatocyte concentration and to differences in confluence upon plating.

17. **Hepatocyte plating:** Plate 300,000 live hepatocytes per well in the collagen-coated 6 multi-well plates with 2 mL of Hepatocyte Attachment Medium. Replace the medium after 6 h and culture hepatocytes for another 16 h with Hepatocyte Attachment Medium before the signaling experiment.

⚠ **CRITICAL:** Confluence of hepatocytes must not be higher than 300,000 cells per well, because mTORC1 activity is affected by confluence (Figures 4A and 4B). See [troubleshooting 4](#).

Note: If mTORC1 signaling will be determined in different conditions (e.g., mice of different genotypes, under different treatments, etc.), we strongly recommend to plate all groups in the same plate. If so, upon processing the plate, different conditions would have simultaneous, equal handling (Figures 5A and 5B).

Optional: Add one extra well in the plate as positive control for the signaling experiments. These hepatocytes will be cultured in complete Hepatocyte Attachment Medium during the whole experiment.

Note: Before changing the medium 6 hours after the plating, we recommend to wash each well with Rinsing Solution to prevent potential contaminations.

Day 2: Amino acid deprivation + replenishment of primary mouse hepatocytes

⌚ **Timing:** 1 h 45 min for ON rate; 4 h for OFF rate

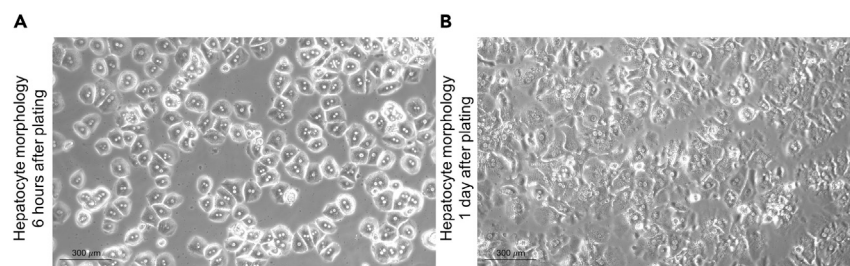


Figure 4. Hepatocyte plating

Liver cells should be plated at a confluence of 300,000 hepatocytes per 3.5 cm well for signaling experiments. (A) Within 6 h after hepatocyte plating, cells attach to the plate and acquire a round shape (Scale bar 300 μ m). (B) 16–20 h after hepatocyte plating, cells have acquired their hexagonal shape (Scale bar 300 μ m).

This major step allows for a time-lapse assessment of nutrient-dependent activation of the mTOR signaling pathway through amino acid deprivation followed by replenishment.

Note: We have previously performed nutrient signaling experiments on other primary cultures of mouse cells: Mouse Embryonic Fibroblasts (de la Calle Arregui et al., 2021) (Ortega-Molina et al., 2021) and naïve B lymphocytes (Ortega-Molina et al., 2019), using standard RPMI without amino acids, supplemented with dFBS. However, this medium resulted suboptimal for the assessment of mTORC1 activity in primary hepatocytes from adult mice: poor acute activation of mTORC1 was observed with this medium and with several iterations aimed to more closely resemble Hepatocyte Attachment Medium (Table 1 and Figure 6, See troubleshooting 5). In contrast, a wide dynamic range of activation/inhibition suitable for the assessment of nutrient-dependent activation of mTORC1 was obtained with Amino acid Starvation Medium (CELL MEDIUM E with insulin in Table 1).

18. **Hepatocyte washing:** The next day, rinse cultured hepatocytes three times with Pre-starvation Medium. Aspirate thoroughly the medium in each wash to remove any leftover of the Hepatocyte Attachment Medium (containing non-dialyzed FBS and amino acids).
19. **Amino acid starvation:** Add to each well 2 mL of Amino acid Starvation Medium. Return the plates to the incubator for 1 h.

Note: During this incubation prepare the Amino acid cocktail.

20. **Amino acid replenishment:** 1 h after incubation of hepatocytes in the Amino acid Starvation Medium, add 200 μ L of Amino acids cocktail, or 200 μ L of Amino acid Starvation Medium (without amino acids) to control, non-replenished hepatocytes. In our experiments, we perform re-stimulation during 1, 2, 5, 10 and 20 min (Figure 5A top).

Note: Timing of plate processing is critical. Start the amino acid re-stimulation with the longest timepoint. In our experiment the longest replenishment is 20 min. 10 min after adding back amino acids in the “20 min” wells, we add back amino acids to the “10 min” wells. 5 min after this condition we add back amino acids to the “5 min” wells. 3 min after this condition we add back amino acids to the “2 min” wells. Finally, 1 min after this condition we add back amino acids to the “1 min” wells.

Optional: In case you have included an additional well for high mTORC1 activity, replace the medium 16 hours after plating, adding to each well 2 mL of Hepatocyte Attachment Medium and incubate for 1 hour.

21. **Hepatocyte collection:** Once the timepoints are finished, place the cell plates on ice.

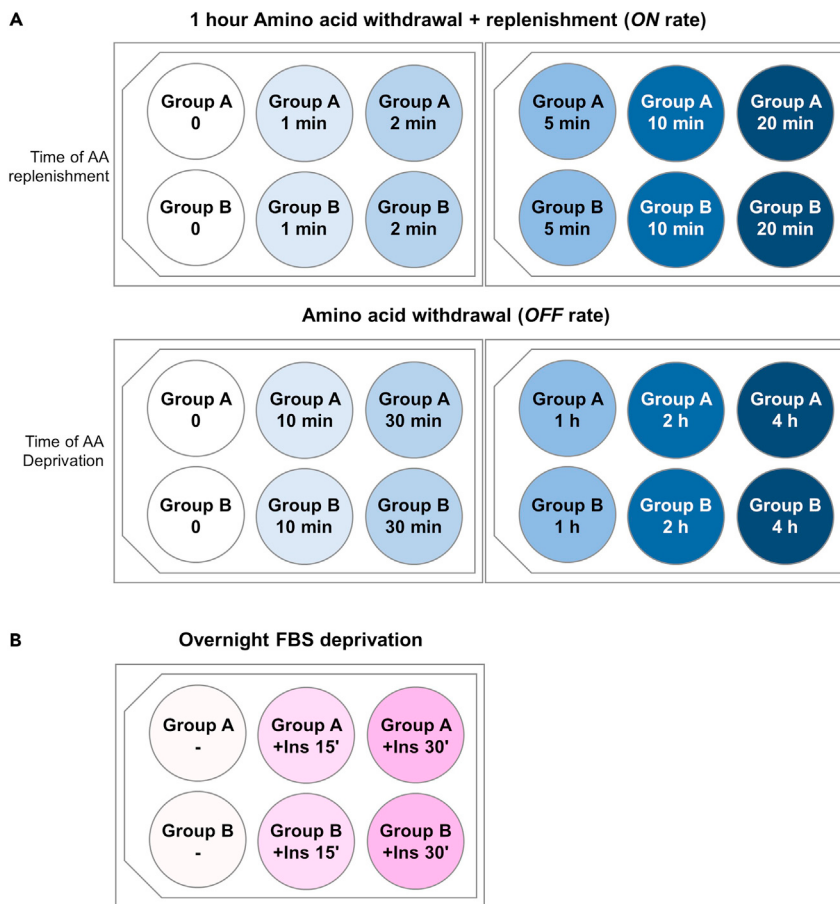


Figure 5. Scheme of hepatocyte plating for signaling experiments

Groups A and B are shown as an example to distribute different experimental groups and to achieve equal handling. (A) Amino acid deprivation of mouse primary hepatocytes. Top: mTOR ON rate. Down: mTOR OFF rate. (B) Serum withdrawal + insulin stimulation of mouse primary hepatocytes.

- Wash twice with cold PBS. Aspirate thoroughly the PBS in each wash for minimal PBS leftover volume.
- Add 150 μ L of cold, freshly prepared Protein Lysis Buffer to each well of the plate.
- Scrape hepatocytes with cell lifters.
- Collect the hepatocytes in 1.5 mL centrifuge tubes.

Note: At this point the cells could be stored at -80°C , or proceed to protein extraction.

22. Protein extraction:

- Incubate the samples at 4°C during 10 min with rotation in the ELMI Intelli Mixer.
- Centrifuge at 4°C during 10 min at 21,130 g.
- Transfer supernatant to a new 1.5 mL centrifuge tube.

23. Protein quantification: Quantify protein concentration with Pierce BCA method or an equivalent method.

Note: Protein concentration from isolated primary hepatocytes should be approximately 1.5 $\mu\text{g}/\mu\text{L}$.

24. Western Blot: Load 8 μg of protein in each lane in a 16% polyacrylamide gel and proceed to run and transfer a standard Western Blot. Probe for levels of Phospho-Thr389-p70 S6 Kinase (1:500), total S6 Kinase (1:500), Phospho-Ser235/236-S6 (1:1000), total S6 (1:1000), Phospho-Thr37/46-4E-BP1 (1:500) and total 4E-BP1 (1:1000) See [troubleshooting 5](#) (Figure 7A).

Table 1. Composition of cell culture media tested to optimize amino acid starvation on primary mouse hepatocytes

Name	Medium	Supplements
CELL MEDIUM A	RPMI R8999-04A	Glucose, Penicillin/Streptomycin, Gentamycin, dFBS
CELL MEDIUM B	RPMI R8999-04A	Glucose, NaHCO ₃ , BSA, Sodium Pyruvate, HEPES, Penicillin/Streptomycin, Gentamycin, dFBS
CELL MEDIUM C	RPMI R8999-04A	Glucose, HEPES, Penicillin/Streptomycin, Gentamycin, dFBS
CELL MEDIUM D	RPMI R8999-04A	Glucose, NaHCO ₃ , Sodium Pyruvate, HEPES, Penicillin/Streptomycin, Gentamycin, dFBS
CELL MEDIUM B with insulin	RPMI R8999-04A	Glucose, NaHCO ₃ , BSA, Sodium Pyruvate, HEPES, Insulin, Penicillin/Streptomycin, Gentamycin, dFBS
CELL MEDIUM E	DMEM/F12 D9807-10	Glucose, NaHCO ₃ , HEPES, dFBS
CELL MEDIUM E with insulin (Amino acid Starvation Medium)	DMEM/F12 D9807-10	Glucose, NaHCO ₃ , HEPES, Insulin, dFBS

Optional: As an alternative to the assessment of the *ON* rate of amino acid-dependent mTOR activation, it is possible to assess the *OFF* rate. In such case, the experiment is performed identically until the step 19. Primary hepatocytes are deprived of all amino acids for 0 min, 10 min, 30 min, 1h, 2h and 4h (Figure 5A down, Figure 7B).

Days 2 and 3: Serum withdrawal + insulin stimulation of primary mouse hepatocytes

⌚ Timing: 24 h

This major step allows to assess growth factor-dependent activation of the mTOR signaling pathway through withdrawal of FBS followed by a 10-min stimulation with insulin.

* Continue from step 17.

25. **Change medium:** 16–24 h after plating, replace the medium (Hepatocyte Attachment Medium).
26. **Hepatocyte washing:** 4 h after changing the medium, rinse cultured hepatocytes three times with FBS withdrawal Medium. Thoroughly aspirate the medium in each wash to remove any leftover of FBS from the Hepatocyte Attachment Medium.
27. **Serum withdrawal:** Add 2 mL of FBS withdrawal Medium to each well. Return the plates to the incubator and leave for 16 h.
28. **Insulin re-stimulation:** After 16 h, add 2 μ L of 100 μ M insulin in the corresponding wells during 15 or 30 min (Figure 5B). 10% FBS can be used instead of insulin for re-stimulation.

Optional: In case you have included an additional well for high mTORC1 activity, replace the medium 20 and 4 hours after plating, adding to each well 2 mL of Hepatocyte Attachment Medium (containing FBS).

29. **Hepatocyte collection:** After 15 or 30 min, place the cell plate on ice.
 - a. Wash twice with cold PBS. Aspirate thoroughly the PBS in each wash for minimal PBS leftover volume.
 - b. Add 150 μ L of Protein Lysis Buffer to each well of the plate.
 - c. Collect the hepatocytes with cell lifters in 1.5 mL centrifuge tubes.

Note: At this point the cells could be stored at -80°C , or proceed to protein extraction.

30. **Protein extraction:**
 - a. Incubate the samples at 4°C during 10 min with rotation in the ELMI Intelli Mixer.
 - b. Centrifuge at 4°C during 10 min at 21,130 g.
 - c. Transfer supernatant to a new 1.5 mL centrifuge tube.
31. **Protein quantification:** Quantify protein concentration with Pierce BCA method.

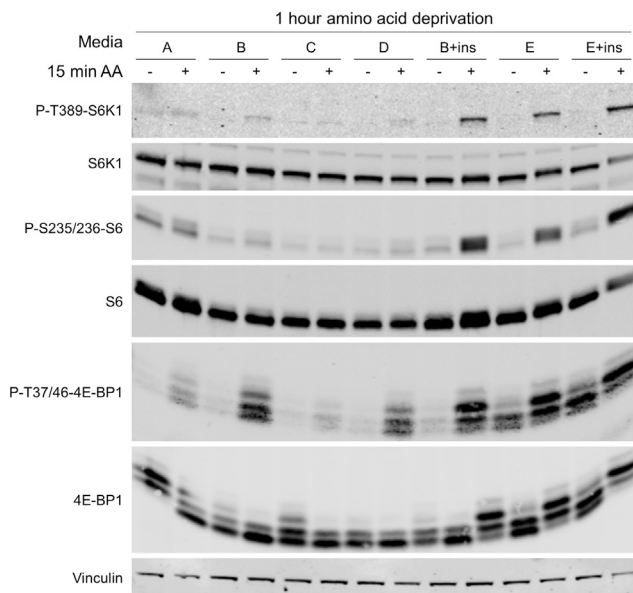


Figure 6. Assessment of mTORC1 activity in the cell culture media tested and sub-optimal to perform amino acid starvation on primary mouse hepatocytes

DMEM/F12 is best suited to perform amino acid starvation on primary hepatocytes when supplemented with insulin (CELL MEDIUM E with Ins, Amino acid Starvation Medium).

32. **Western Blot:** Load 8 μ g of protein in each lane in a 16% polyacrylamide gel and proceed to run and transfer a standard Western Blot. Probe for levels of Phospho-Thr389-p70 S6 Kinase (1:500), total S6 Kinase (1:500), Phospho-Ser235/236-S6 (1:1000), total S6 (1:1000), Phospho-Thr37/46-4E-BP1 (1:500), total 4E-BP1 (1:1000), Phospho-Ser473-Akt (1:1000) and total Akt (1:1000) (Figure 8).

The isolated primary hepatocytes can be alternatively used for other molecular purposes independently of the assessment of mTOR activity. This protocol can be applied to assess the activity of additional signaling pathways, by exposing the primary hepatocytes to different experimental manipulations (chemical, physical and pharmacological). We have previously used this *in vitro* system to measure *ex vivo* cellular respiration (de la Calle Arregui et al., 2021), as well as to evaluate specific proteins through immunofluorescence, gene expression through RT-qPCR or whole transcriptome profiling, and metabolomic analysis.

EXPECTED OUTCOMES

A typical hepatocyte yield from a young C57BL/6 mouse is 25×10^6 cells with a viability of 85%. Yield of hepatocyte isolation in genetically-engineered mice may be lower, particularly if livers have excess fibrous tissue and/or hepatic damage. Hepatocytes adhere to the surface of the well 6 h after plating, looking sparsely plated, at the optimal confluence for performing signaling experiments (Figures 4A and 4B). Mouse primary hepatocytes do not survive in cell culture for more than 3 days, and trypsinization/replating is not feasible. Hence, signaling experiments should be performed one or two days after cell plating.

mTOR signaling will be evaluated by the phosphorylation status of main targets: Phospho-Thr389-p70 S6 Kinase, Phospho-Ser235/236-S6 proteins and Phospho-Thr37/46-4E-BP1. Total protein levels will be used to normalize phosphorylation levels. In the amino acid deprivation protocol of mouse primary hepatocytes (ON rate), time 0, without re-stimulation, mTOR pathway should be off, revealed by a very faint band for Phospho-Thr389-p70-S6 Kinase and Phospho-Ser235/236-S6 (Figure 7A). One, 2, 5, 10 and 20 min after amino acid replenishment, a gradual increase in the signals of Phospho-Thr389-p70-S6 Kinase and Phospho-Ser235/236-S6 should be observed, reaching the maximum levels in the

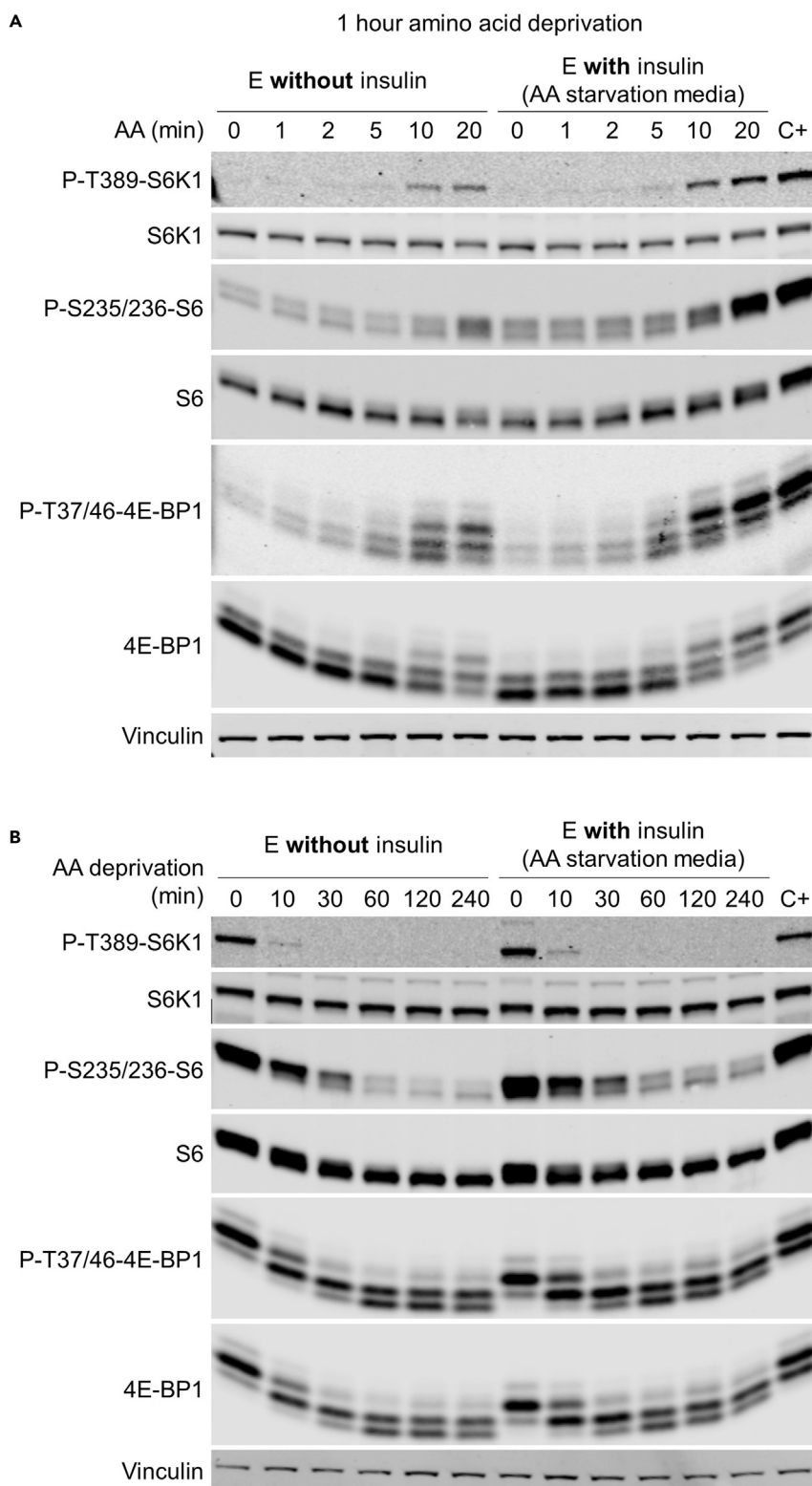


Figure 7. Assessment of mTORC1 activity by amino acid deprivation/stimulation in DMEM/F12 medium without or with insulin

(A) mTORC1 ON rate.

(B) mTORC1 OFF rate.

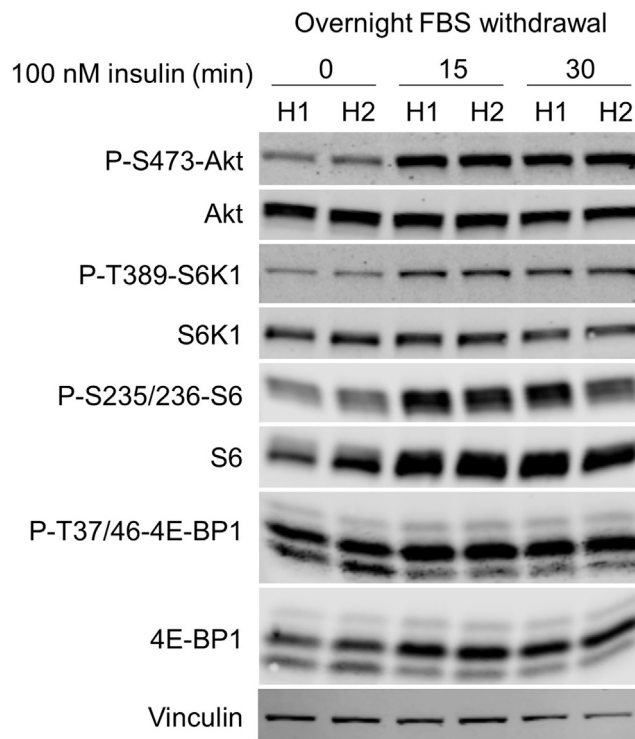


Figure 8. Assessment of mTORC1 activity by serum withdrawal + insulin stimulation

Two different samples of hepatocytes (H1 & H2) are shown for each condition.

20-min timepoint, together with a slight upwards shift. By 10 or 20 min after stimulation, mTORC1 activity should be similar to that of the positive control (C+) of hepatocytes cultured with Hepatocyte Attachment Medium (Figure 7A). During the same 1-to-20-min time lapse, Phospho-Thr37/46-4E-BP1 levels should also increase progressively. In addition to an increase in overall Phospho-Thr37/46-4E-BP1 bands intensity, the ratio of the upper (phosphorylated)-to-lower (unphosphorylated) bands should also gradually increase. This change in upper-to-lower ratio should also be appreciable in the membrane incubated with the antibody raised against total 4E-BP1 (Figure 7A) See [troubleshooting 4 & 5](#).

In the amino acid deprivation protocol of mouse primary hepatocytes (OFF rate), similar results will be observed, with a gradual decrease in the signals of Phospho-Thr389-p70-S6 Kinase and Phospho-Ser235/236-S6, reaching the minimum levels already at 60-min timepoint (Figure 7B). During the same 0-to-240-min time lapse, Phospho-Thr37/46-4E-BP1 levels should also decrease progressively (Figure 7B).

In the FBS withdrawal of mouse primary hepatocytes (time - without re-stimulation) mTOR pathway should be off. 15 and 30 min of insulin stimulation will switch on the mTOR pathway, which should be observed as described for amino acid-replenishment (Figure 8).

QUANTIFICATION AND STATISTICAL ANALYSIS

Western Blot bands can be quantified using ImageJ software. The intensities of the phosphorylation levels of p70 S6 Kinase and Akt should be made relative to the total p70 S6 Kinase and total Akt, respectively, or to a loading control, such as β -actin or Vinculin. We discourage quantifying total S6 levels, and we always use another loading control for Phospho-Ser235/236-S6. For the quantification of phosphorylation levels of 4E-BP1, the upper-band-to-lower-band ratio can be quantified.

LIMITATIONS

If hepatocyte yield is low or cellular density is too low or too high, downstream signaling experiments may be compromised. Similarly, if the viability of the cells is not suitable, signaling may not be reliable.

TROUBLESHOOTING

Problem 1

The cannulation is successful but liver is not properly digested.

Potential solution

Check that Collagenase in the Digestion Solution is completely dissolved. Temperature is a key factor in this process, confirm that the Digestion Solution is at 37°C during the whole perfusion procedure (Figure 1A). We recommend adding the Collagenase before anesthetizing the mouse to allow complete solution.

Problem 2

Bubbles appear in the tube of the perfusion pump before liver perfusion.

Potential solution

To remove bubbles from the tube, halt the perfusion pump before the bubbles reach the catheter connected to the mouse heart. Gently pull the pump tube back from the catheter, switch on the pump and allow Perfusion Solution to drain until all bubbles are eliminated. Before reconnecting the tube to the catheter, fill the catheter with perfusion buffer. It is critical to eliminate all bubbles to successfully perfuse the liver and to guarantee the viability of the resulting isolated hepatocytes.

Problem 3

Final liver texture after digestion is not soft and tissue is not easily disaggregated.

Potential solution

Before starting the digestion step, verify the perfusion flow ensuring that the Perfusion Solution is leaving the circuit through the portal vein.

Problem 4

High variability in mTOR signaling is observed between replicate samples.

Potential solution

Maintaining identical hepatocyte confluence across the wells is critical when performing these signaling experiments. Hepatocytes should not exceed 300,000 per 3.5 cm well (Figures 4A and 4B). We recommend to repeat the counting of dead and viable cells with Trypan Blue twice or three times per sample. In addition, simultaneous processing of all wells belonging to the same condition (e.g., time 1 min) is important for minimizing the potential of producing technical artifacts, particularly with the early time points. Replenishment of amino acids should be performed as fast as possible, as cells are removed from the incubators and transiently exposed to atmospheric concentration of CO₂ and to room/hood temperature.

Problem 5

mTOR signaling in hepatocytes does not reach high levels of activation upon stimulation.

Potential solution

The levels of activation of mTOR pathway after 20 min of amino acid replenishment should be similar to those of the positive control hepatocytes (cultured with Hepatocyte Attachment Medium). (Figures 7A and 7B). We have seen that supplementation of the Amino acid Starvation Medium with insulin is important for robust mTOR activation after amino acid replenishment without undermining nutrient-dependent control on mTORC1 activity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Alejo Efeyan; aefeyan@cniio.es

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any data set or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100918>.

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AUTHOR CONTRIBUTIONS

A.B.P.-G. performed and optimized the experiments with support from M.C., C.d.I.C.A., and L.d.P.-R. A.B.P.-G. wrote the manuscript and M.C., G.S., and A.E. edited the manuscript. A.E. and G.S. conceived and supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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