In vitro mTORC1 Kinase Assay for Mammalian Cells Protocol

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[Abstract] Historically, mechanistic target of rapamycin (mTOR) was purified from mammalian cells using mild nonionic detergents such as NP-40 and or Triton-X100 that resulted in dissociation of core regulatory components essential for its native kinase activity. Consequently, these older kinase assays required $MnCl_2$ to artificially enhance the weak phosphotransfer activity observed (Bai *et al.*, 2007; Kim *et al.*, 2002). With the use of the zwitterionic detergent 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), the mTOR complex 1 (mTORC1) containing Regulatory-associated protein of mTOR (Raptor) and Lst8 (also known as GbetaL) can be successfully purified as a complex. This *in vitro* kinase assay allows for purification of mTORC1 that resembles its physiological state and retains kinase activity under physiological $MgCl_2$ concentrations (Sancak *et al.*, 2007). The activity of mTORC1 can be further enhanced through the use of hyperactive mutations within the kinase domain of mTOR or inclusion of GTP-bound RAS enriched in brain (Rheb) that is supplemented into the *in vitro* kinase assays. Rheb is a small-G-protein that binds to and activates mTORC1 to phosphorylate downstream substrates, such as eukaryotic initiation factor 4E-BP1 (4E-BP1) (Burnett *et al.*, 1998), ribosomal protein S6 kinase 1 (S6K1) (Kim *et al.*, 2002), Signal transducer and activator of transcription 3 (STAT3) (Dodd *et al.*, 2015), and proline-rich Akt substrate of 40 kDa (PRAS40) (Dunlop *et al.*, 2009).

Materials and Reagents

1. HEK293E cells
2. Plasmids and vectors
   a. HA-Raptor (Addgene, catalog number: 8513)
   b. myc-mTOR (Addgene, catalog number: 1861)
   c. Rheb (National Center for Biotechnology Information, Gene, catalog number: 6009) cloned into pDEST27 using the gateway cloning system in accordance with manufacturer protocol (Life Technologies, catalog number: 11812-013)
      Note: Currently, it is "Thermo Fisher Scientific, Invitrogen™, catalog number: 11812-013".
   d. GST-4E-BP1/pGEX vectors generated as previously described (Dunlop *et al.*, 2009)
3. Antibodies

   a. Clone 9E10 anti-Myc antibodies (Sigma-Aldrich, catalog number: M5546)
   b. Clone 9B11 anti-Myc antibodies (Cell Signaling Technology, catalog number: 2276)
   c. Anti-HA (Roche Diagnostics, catalog number: 11867431001)
   d. Anti-GST (Merck Millipore Corporation, catalog number: 05-782)
4. Cell culture and transfection
   a. Dulbecco's modified eagle's medium (DMEM)
   b. 10% foetal bovine serum (FBS), EU Approved (South American) (Thermo Fisher Scientific, Gibco™, catalog number: 10270-106)
   c. Penicillin-streptomycin (Thermo Fisher Scientific, Gibco™, catalog number: 15070-063)

   Note: HEK293E cells were cultured in DMEM supplemented with 10% FBS, 1 µg/ml penicillin and 1 µg/ml streptomycin.
5. Insulin (Sigma-Aldrich, catalog number: I9278)
6. Rapamycin (EMD Millipore Corporation, catalog number: 553210)
7. Chemicals of analytical grade
   a. HEPES (Sigma-Aldrich, catalog number: H3375)
   b. EDTA (Sigma-Aldrich, catalog number: 431788)
   c. β-glycerophosphate (disodium salt, pentahydrate) (Sigma-Aldrich, catalog number: 50020)
   d. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
   e. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
   f. Adenine triphosphate (ATP) (Sigma-Aldrich, catalog number: A26209)
   g. Leupeptin (Sigma-Aldrich, catalog number: L5793)
   h. Antipain (Sigma-Aldrich, catalog number: 10791)
   i. Benzamadine (Sigma-Aldrich, catalog number: 12072)
   j. Pepstatin A (Sigma-Aldrich, catalog number: P5318)
   k. Sodium vanadate (Sigma-Aldrich, catalog number: 289361)
   l. Dithiothreitol (Sigma-Aldrich, catalog number: 43815)
   m. Phenylmethylsulfonyl fluoride (Sigma-Aldrich, catalog number: 78830)
   n. Wortmannin (Sigma-Aldrich, catalog number: W1628)
   o. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) (Sigma-Aldrich, catalog number: 226947)
8. mTOR lysis buffer (see Recipes)
9. Low salt mTOR wash buffer (see Recipes)
10. High salt mTOR wash buffer (see Recipes)
11. mTOR wash buffer (see Recipes)
12. 3x mTOR kinase assay buffer (see Recipes)
13. Rheb lysis buffer (see Recipes)

14. Rheb storage buffer (see Recipes)
15. Phosphate buffered saline (see Recipes)
16. mTOR assay start buffer (see Recipes)
17. Protease inhibitors (see Recipes)

Equipment

1. Thermo mixer heating block (Eppendorf AG, model: Eppendorf thermomixer® compact)
2. Refrigerated mini centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Heraeus and Fresco 17 centrifuge)
3. Stuart™ SB2 fixed speed rotator (Bibby Scientific Limited, Stuart Scientific)

Procedure

Note: An overview of the whole procedure can be found in Figure 1.

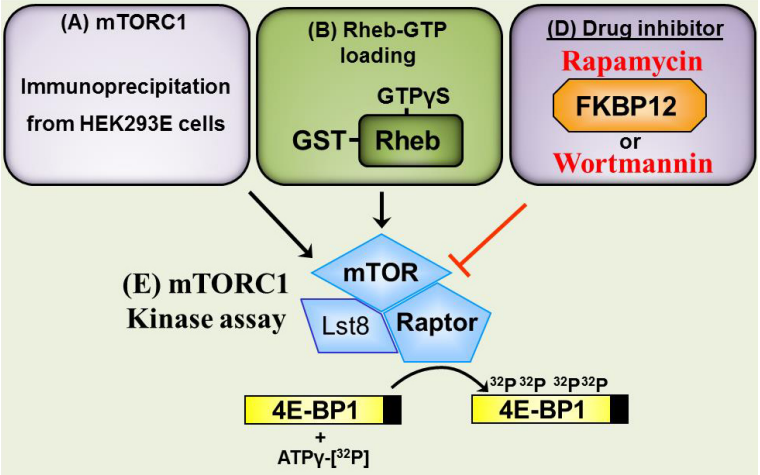

Figure 1. mTOR *in vitro* kinase assay. As described in the "Procedure", preparation of (A) mTORC1 complexes, (B) Rheb-GTP and (D) drug inhibtors (rapamycin/FKBP12 and wortmannin) and incubation of mTORC1 substrate (4E-BP1) within the mTORC1 kinase assay (E).

A. Generating mTOR/raptor complexes from HEK293E cells
   1. HEK293E cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1%, 100 µg/ml penicillin and 100 µg/ml streptomycin.
   2. 75 cm^2 flasks of 80% confluent HEK293E cells were either co-transfected with 5 µg Myc-tagged mTOR and 5 µg HA-tagged Raptor constructs or transfected with a

GST-tagged Rheb construct using the calcium phosphate transfection method (1 x 75 cm^2 flask of HEK293E cells is sufficient for three mTOR kinase assays). Cells were harvested 36 h post-transfection. Cells are treated with 10 µg/ml insulin for 30 min prior to lysis. This dose is sufficient to stimulate mTORC1 signaling and ensure that the active complex is purified as previously demonstrated (Dunlop *et al.,* 2009).

3. Stimulate cells with 100 nM insulin for 15 min then lyse in 1 ml of mTOR lysis buffer supplemented with protease inhibitors and 0.3% CHAPS (w/v).
4. Centrifuge at 16,200 *x g* for 8 min at 4 °C (refrigerated mini centrifuge).
5. Incubate lysates with 3 µl of Myc- or HA-antibodies (the mTORC1 complex can be purified with either HA-raptor or myc-mTOR immunoprecipitation) for 1.5 h at 4 °C with rotation.
6. Make a 50% volume ratio mix of Protein G plus mTOR lysis buffer, add 40 µl to each tube and incubate for 1 h at 4 °C with rotation (Stuart™ SB2 fixed speed rotator).
7. Wash immunoprecipitates with 0.5 ml of the following buffers supplemented with protease inhibitors:
   a. 1x low salt mTOR wash buffer (supplemented with 0.3% w/v CHAPS)
   b. 2x high salt mTOR wash buffer (supplemented with 0.3% w/v CHAPS)
   c. 2x mTOR wash buffer
8. Split immunoprecipitates equally into three Eppendorf tubes for the in vitro kinase assay.

B. Preparing GTP-bound Rheb

Human Rheb (Gene ID: 6009) cloned into pDEST27, which contains an N-terminal GST-tag.

1. Transfect four 75 cm^2 flasks of 80% confluent HEK293E cells with GST-Rheb-pDEST27 (10 µg DNA per flask), using standard calcium phosphate transfection procedures (Schalm *et al.,* 2002).
2. Grow HEK293E cells over-night in the presence of 10% (v/v) FBS till fully confluent (6-7 x 10^6 cells).
3. Lyse HEK293E cells in 1 ml Rheb lysis buffer supplemented with 0.3% (w/v) CHAPS (plus protease inhibitors).

   Note: Reducing agents (such as DTT) should be omitted as this will interfere with GST-purification. Incubate lysates on ice for 30 min to facilitate lysis.
4. Incubate pre-cleared lysates (after centrifugation at 16,200 *x g* for 10 min at 4 °C) with immobilized 40 µl of a 50% volume ratio mix of Rheb lysis buffer and glutathione-sepharose beads, incubate for 2 h at 4 °C with rotation.
5. Wash the glutathione-sepharose beads twice with 0.5 ml Rheb lysis buffer and then once with 0.5 ml Rheb storage buffer supplemented with protease inhibitors.

6. Elute GST-Rheb from the glutathione-sepharose beads in 50 µl Rheb storage buffer supplemented with of 10 mM glutathione (pH readjusted back to 8.0).
7. Incubate eluted GST-Rheb protein at 30 °C for 10 min with either 10 mM EDTA and 1 mM GDP to generate inactive Rheb-GDP, or 10 mM EDTA and 0.1 mM non-hydrolysable GTPγS to generate active Rheb-GTP. To tightly bind the guanine nucleotide to Rheb, add $MgCl_2$ to a final concentration of 20 mM. Incubate on ice until use.

C. Generating dephosphorylated 4E-BP1 protein for positive control substrate
1. Transform BL21 (DE3) pLys bacteria with GST-tagged 4E-BP1/pGEX plasmid (4E-BP1 GeneID, 1978) using standard transformation methods.
2. Grow bacteria until OD_{600} is 0.6-0.8, to induce expression add isopropyl-β D-thiogalactoside (IPTG) to give a final concentration of 0.5 mM and incubate for 3 h at 30 °C. Pellet cells by centrifugation at 1,500 *x g* for 30 min at 4 °C.
3. Lyse bacteria with a freeze/thaw cycle in 10 ml of PBS supplemented with 10 mM EDTA, 0.1% (v/v) Triton and protease inhibitors.
4. Use pulse sonication to shear bacterial DNA [3 x 5 sec cycles on full power (30 µm)]. Centrifuge at 16,200 *x g* for 10 min at 4 °C, then purify GST-4E-BP1 from the bacterial supernatant using glutathione-sepharose beads.
5. Dephosphorylate GST-4E-BP1 protein using 50 U shrimp alkaline phosphatase, washed in PBS, 10 mM EDTA, 0.1% (v/v) Triton X-100. Elute in 10 mM reduced glutathione in PBS (pH 7.6).
6. Desalt the eluent using a HiTrap Desalting Column in accordance with manufacturer protocol.
7. Resolve using SDS-PAGE and stain with Coomassie Brilliant Blue to check the purity and concentration against known bovine serum albumin (BSA) standards.
8. Dephosphorylated 4E-BP1 can be stored at -80 °C in 10 µg/µl aliquots for future use.

D. Preparing FKBP12/rapamycin drug/protein complexes to inhibit mTORC1
1. Human FKBP12 protein can be expressed and purified using the same protocol to generate GST-4E-BP1 above (omitting the dephosphorylation step C5).
2. FKBP12 can also be frozen in 10 µg/µl aliquots at -80 °C for future use.
3. To generate FKBP12/rapamycin complexes: Make up 25 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 20 mM rapamycin and 0.5 µg FKBP12 in 10 µl final volume (dH_2O). Incubate at room temperature in the dark for 5 min, and store on ice until needed.

E. Performing mTOR kinase assays
1. Make up mTOR/Raptor immunoprecipitates in 3x mTOR kinase assay buffer and add 75 ng of Rheb-GTP and or 2 µl FKBP12/rapamycin as required.

2. Incubate for 20 min on ice prior to starting the kinase assay.
3. Add 10 µl of mTOR assay start buffer supplemented with 500 µM ATP (freshly added before use) plus 150 ng of purified GST-4E-BP1 or the test substrate to start the assay. Phospho-4E-BP1 (Thr 36/45) levels are used as a positive control and indicate mTOR kinase activity.
4. Incubate at 30 °C for 30-60 min in thermo mixer heating block, shaking at20 FCS.
5. Stop reaction by adding 4x sample buffer.
6. Analyse samples using SDS-PAGE and western blotting. An example of representative data is shown in Figure 2.

Representative data

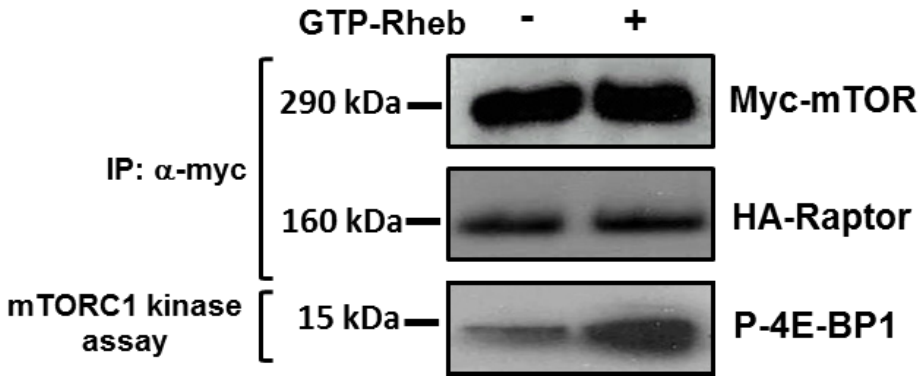

Figure 2. mTORC1 directed phosphorylation of 4E-BP1. Western blotting showing phosphorylation of purified GST-4E-BP1 after in vitro mTORC1 kinase assay performed in the presence and absence of GTP-Rheb. Levels of myc-mTOR and HA-Raptor are shown as controls.

Notes

1. It is essential that Rheb is purified from mammalian cells, rather than from bacteria, as proper folding and post-translational modifications (such as prenylation) is required for its activity to enhance mTORC1.

Recipes

Note: All buffers are stored at 4 °C unless otherwise stated.

1. mTOR lysis buffer
   40 mM HEPES (pH 7.4)
   2 mM EDTA

10 mM β-glycerophosphate

2. Low salt mTOR wash buffer
   40 mM HEPES (pH 7.4)
   150 mM NaCl
   2 mM EDTA
   10 mM β-glycerophosphate
3. High salt mTOR wash buffer
   40 mM HEPES (pH 7.4)
   400 mM NaCl
   2 mM EDTA
   10 mM β-glycerophosphate
4. mTOR wash buffer
   25 mM HEPES (pH 7.4)
   20 mM KCl
5. 3x mTOR kinase assay buffer (aliquoted and stored at -20 °C)
   X3 stock solution: 75 mM HEPES (pH 7.4), 60 mM KCl, 30 mM $MgCl_2$
   Add 1:50 dilution of 0.5 M stock of $MgCl_2$ to 25 mM HEPES (pH 7.4), 20 mM KCl
6. Rheb lysis buffer
   40 mM HEPES (pH 7.4)
   10 mM glycerophosphate
   5 mM $MgCl_2$
7. Rheb storage buffer
   20 mM HEPES (pH 8.0)
   200 mM NaCl
   5 mM $MgCl_2$
8. Phosphate buffered saline
   137 mM NaCl
   10 mM phosphate
   2.7 mM KCl (pH 7.4)
9. mTOR assay start buffer (aliquoted and stored at -20 °C)
   25 mM HEPES (pH 7.4)
   10 mM $MgCl_2$
   140 mM KCl, plus 500 μM adenine triphosphate (ATP) added fresh before use
10. Protease inhibitors (1,000x stock solutions aliquoted and stored at -20 °C)
    10 μM leupeptin
    2 μM antipain
    1 mM benzamidine
    1 μg/ml pepstatin
    100 μM PMSF

1 mM sodium orthovanadate
1 mM dithiothreitol (DTT not used for GST-purifications)

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