Mtor KInase Assays

[Adopted from Ikenoue et al., Methods Enzymol. 452: 165-180 (2009)]

If you plan to do less than 5 reactions, a 6-cm plate of cells is enough. If you plan to do more than 5 reactions, use a 10-cm plate.

1. Subconfluent cells (10-cm plate) are lysed in 1 ml of CHAPS lysis buffer.

2. After collecting the lysates, 30 µl is saved for Western analysis as input controls. The remaining lysates are used for immunoprecipitation (IP).

3. For endogenous raptor or rictor IP: Add 4 ul of antibody in the lysate → incubate for 1 hr at 4 °C with rocking → add 60 ul of Protein G (50% slurry) → Incubate for 1 hr at 4 °C with rocking.

   For Flag-mTOR IP: 60 ul of a 50% slurry of M2-beads are added and incubated for 1 hr at 4 °C with rocking.

4. Immunoprecipitates are centrifuged at 9,000 rpm for 1 min and then washed with CHAPS wash buffer 3 times. For mTORC1 kinase assay, add a wash with CHAPS wash buffer with 500mM NaCl to remove PRAS40.

5. The immunoprecipitates are washed once in kinase wash buffer. Resuspend the immunoprecipitates in kinase wash buffer and make 10-12 aliquots (~2.5 ul beads per reaction). Spin, and remove buffer completely with a syringe fitted with a 25G needle. The beads are subjected to kinase assays as follows.

6. mTORC1 kinase assay:
   - mTORC1 kinase buffer
   - 250 µM ATP
   - 100 ng GST-S6K1
   - Total volume: 20 µL
   - Incubate at 30 °C for 30 min in a thermomixer at speed 12x100/min.
   [In order to examine the effect of a regulator protein (e.g., FKBP38, FKBP12), add the protein to the beads 15 min prior to adding reaction buffer.]

7. mTORC2 kinase assay:
   - mTORC2 kinase buffer
   - 500 µM ATP
   - 250 ng His-Akt
   - Total volume: 20 µL
   - Incubate at 37 °C for 30 min in a thermomixer at speed 12x100/min.

8. Add 20 ul 2x SDS sample buffer to stop the reaction. Samples are boiled for 5 min and then subjected to Western analysis using anti-pT389-S6K1 or anti-pS473-Akt antibody.
NOTE: If you are doing the kinase assay with a substrate for which phospho-specific antibody is not available, add 0.5 - 1µCi \( ^{32} \text{P} \)-ATP to each kinase reaction, and use autoradiography following SDS-PAGE to detect the phosphorylated product.

**CHAPS lysis buffer:**
40 mM HEPES, pH 7.4,
120 mM NaCl,
2 mM EDTA,
0.3% CHAPS,
10 mM pyrophosphate,
10 mM glycerophosphate,
50 mM NaF,
Protein inhibitor cocktail (PIC, from Sigma) at 1:100

**CHAPS wash buffer:**
Same as CHAPS lysis buffer but without PIC.

**CHAPS wash buffer for mTORC1:**
Same as CHAPS washing buffer plus 500 mM NaCl.

**Kinase wash buffer:**
25 mM HEPES-KOH, pH 7.4
20 mM KCl

**mTORC1 kinase buffer:**
25mM HEPES (pH 7.4)
50 mM KCl
10 mM MgCl\(_2\)

**mTORC2 kinase buffer:**
25mM HEPES (pH 7.4)
100 mM potassium acetate
1 mM MgCl\(_2\)