IP-Kinase Assay

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[Abstract] Immunoprecipitation (IP)- Kinase assays are an invaluable tool to assess the activation status of intracellular signaling cascades within a specific cellular state and also to confirm the enzymatic activity of a specific kinase towards a putative substrate of interest. Intracellular signal transduction cascades play an important role in modulating the localization of transcription factors and thus impact the cellular transcriptome. This in turn regulates key cell fate decisions including cell survival, apoptosis, proliferation, and differentiation. Here we describe an *in vitro* non-radioactive method to assess kinase activity towards a specific substrate. In this protocol we outline the method for Akt, however the basic protocol may be applied to any kinase and putative substrate of interest.

Materials and Reagents

1. Recombinantly produced substrate of interest
   For this study, full-length murine Oct4 was cloned into a mammalian expression vector containing a T3 promoter sequence and a carboxy-terminal 6X His – TEV – 3X FLAG epitope tage.
2. TnT Coupled Reticulocyte Lysate Systems (Promega Corporation, catalog number: L5010)
3. Expression vector (with Sp6, T3, or T7 promoter sequence) containing protein of interest (Test Substrate) wild-type, putative mutant, empty vector control. You will also need a vector containing a previously confirmed (and published) target substrate if one is not commercially available to use as a control for the kinase assay.
4. Transcend tRNA (Promega Corporation, catalog number: L5061)
5. RNaseOUT (Life Technologies, catalog number: 10777019)
6. Cell line which exhibits activity for kinase of interest
7. SDS polyacrylamide gel
8. Polyvinyl difluoride membrane (PVDF) (Bio-Rad Laboratories, catalog number: 162-0177)
9. Primary Antibody to Test and Control Substrates
   a. Akt (total) (Cell Signaling Technology, catalog number: 4685)
   b. Phospho-Akt Substrate Antibody (Cell Signaling Technology, catalog number: 10001)

   c. Gsk3 (total) (Cell Signaling Technology, catalog number: 5676)
10. Kinase agonist (if required) to augment kinase activity
    This protocol uses Ro-31-8220 (Sigma-Aldrich, catalog number: R136-5MG) as an Akt agonist
11. Non-radioactive Akt Kinase Assay Kit (Cell Signaling Technology, catalog number: 9840)
    a. Immobilized Phospho-Akt (Ser473) (D9E) Rabbit mAb (Bead Conjugate)
    b. Phospho-GSK-3 (Ser21/9) (37F11) Rabbit mAb
    c. GSK-3 Fusion Protein at 0.5 mg/ml
    d. 10 mM ATP (50 µl)
12. Bead conjugated primary antibody or primary antibody and Protein A/G Agarose (Pierce Antibodies)
13. Phenyl methlsulfonyl fluoride (PMSF) (Sigma-Aldrich)
14. 1x Cell Lysis Buffer (see Recipes)
15. 1x Kinase Buffer (see Recipes)
16. 3x SDS Sample Buffer (see Recipes)

Equipment

1. Mini-Western/Transfer Apparatus (Bio-Rad Laboratories)
2. 10 cm plates
3. Cell scraper
4. 1.5 ml microfuge tube
5. Refrigerated microfuge
6. Microfuge tube rotator
7. Heat blocks set to 30 and 95 °C

Procedure

A. *In vitro* transcription/translation
   1. Prepare the recombinantly produced substrate of interest using the TnT Coupled Reticulocyte Lysate System. Wild-type, kinase mutant, and empty vector control will be required.
   2. Following the manufacturer's recommended protocol proceed to set up the following reaction mixes for each sample. All reagents should be kept on ice until step C3.

Component	Volume (µl)
TnT Lysate	25
TnT Reaction Buffer	2
T3 RNA Polymerase*	1
Amino Acids-Met	0.5
Amino Acids-Leu	0.5
RNaseOUT	1
Transcend tRNA	1
H_2O	14
DNA (0.2 µg/µl for 1 µg total)	5

**This kit requires that the protein of interest be cloned in an expression vector containing an SP6, T3, or T7 promoter sequence. The appropriate polymerase must be selected based on vector utilized.*

3. Incubate the reactions assembled in Step A2 for 90 min at 30 °C.
4. Typical yields from coupled transcription/translation are from 50-500 ng/µl.
5. Analyze the product by Western blot. Combine 5 µl of the reaction with 20 µl of 1x SDS loading buffer. Denature at 95 °C for 3 min and load 10 µl onto an SDS polyacrylamide gel. Transfer gel to PVDF membrane. Detect using a primary antibody raised against the protein of interest and/or epitope tag contained in the selected vector to confirm expression.
6. Store the remaining *in vitro* transcription/translation reaction at -20 °C until further use in step D.

B. Cell lysate preparation
1. Culture actively growing 10T1/2 Fibroblasts to 75% confluence. Four 10 cm plates will be sufficient for assay of control, wild-type, and putative Akt mutant substrates.
2. Culture the cells in the presence of 10 µM Ro-31-8220 at 37 °C for one hour to increase Akt activation.
3. Aspirate media and quickly rinse the cells twice with ice cold PBS, aspirating between each wash.
4. Lyse cells with complete 1x Cell Lysis Buffer supplemented with 1 mM PMSF. Use 0.5 ml per 10 cm plate. Incubate on ice for 5 min.
5. Remove cells from plate with cell scraper. Place lysate in 1.5 ml microfuge tube on ice for 30 min gently vortexing two times (setting 6) for 10 sec each at 10 and 20 min. Sonication is not necessary.

6. Centrifuge the lysate at 10,000 *x g* for 10 min at 4 °C. Transfer the supernatant to a fresh tube. Store lysate at -80 °C until use.

C. Immunoprecipitation

1. Each experiment will require eight immunoprecipitations; four with cell lysate and four mock immunoprecipitations with 1x Cell Lysis Buffer. For each set of four immunoprecipitations, one will be a positive control for kinase activity, employing a previously confirmed (in the literature) substrate. The remaining three will be used for the recombinantly produced test substrate (wild-type, putative kinase mutant, and empty vector). The mock immunoprecipitation is a negative control used to ensure that the kinase activity emanates from the cell lysate and not other reagents used during this protocol.
2. Add 20 µl of immobilized antibody-bead slurry to 200 µl of lysate (or 1x Cell Lysis Buffer) in a 1.5 ml microfuge tube. Incubate overnight at 4 °C with end-over-end rotation. Proceed to step C6. Immobilized phospho-Akt (Ser473) is included in the Non-Radioactive Akt-kinase Assay Kit.
3. Alternatively, add primary antibody to 200 µl of lysate. The exact amount may need to be titrated. Approximately 1 µg of an affinity-purified antibody is generally sufficient.
4. Incubate overnight at 4 °C with end-over-end rotation.
5. Add prepared Protein A/G Agarose beads (25 µl of 50% slurry). Incubate at 4 °C with end-over-end rotation for 2 h.
6. Centrifuge the immunoprecipitate at 10,000 *x g* for 30 sec at 4 °C. Aspirate off supernatant and wash the pellet (on ice) two times for 3 min each with 500 µl 1x Cell Lysis Buffer.
7. Wash the pellet (on ice) two times for 3 min with 500 µl 1x Kinase Buffer.

D. On-bead non-radioactive *in vitro* kinase assay

1. Resuspend the final pellet in 50 µl of 1x Kinase Buffer.
2. Add 1 µl of 10 mM ATP (from the Non-radioactive Akt Kinase Assay Kit) and 1 or 2 µl of kinase substrate generated in step A6.
3. Incubate for 30 min at 30 °C.
4. To terminate the reaction add 25 µl of 3x SDS Sample Buffer. Vortex gently, then microfuge at 10,000 *x g* to collect.
5. Store at -80 °C or proceed directly to analyze by Western.

E. Western analysis

1. Heat each required sample at 95 °C for 3 min.

a. Properly controlled experiments should contain Westerns showing:
   i. Kinase (total and activated form) in the absence and presence of agonist to confirm that the kinase is active.
   ii. Control IP-kinase assays using a previously confirmed kinase substrate. Duplicate Westerns for the mock and kinase exposed samples should be run for incubation with antibodies directed to both the total and phosphorylated form of the protein.
   iii. Test IP-kinase assays showing empty vector, wild-type, and putative kinase mutant form of the substrate of interest for mock and kinase exposed samples. Duplicate Westerns should be run as above. If an antibody directed to the phosphorylated form of the protein of interest is not commercially available, a phospho-kinase substrate antibody may be used in its place since it will only detect substrates when they are phosphorylated.

F. Interpretation
1. A putative substrate is confirmed when:
   a. Kinase activity is confirmed in step E1a.i.
   b. Phosphorylation of the control substrate is confirmed in step E1a.ii kinase exposed sample, but not in the mock exposed sample.
   c. Phosphorylation of putative substrate is confirmed in wild-type but not the putative kinase mutant form of the protein. No expression of total protein or phospho-protein should be observed for the empty vector control.

Recipes

1. 1x Cell Lysis Buffer
   25 mM Tris (pH 7.5)
   150 mM NaCl
   1 mM EDTA
   1 mM EGTA
   1% Triton
   2.5 mM $Na_4P_2O_7$
   1 mM β-Glycerophosphate
   1 mM Na_3VO_4
   1 µg/µl Leupeptin
   Stored at 4 °C for 1-2 weeks only
2. 1x Kinase Buffer

25 mM Tris (pH 7.5)
5 mM β-Glycerophosphate
2 mM DTT
0.1 mM Na_3VO_4
10 mM $MgCl_2$
Stored at -20 °C
May be stored at 4 °C for 1-2 weeks only

3. 3x SDS Sample Buffer
   187.5 mM Tris (pH 6.8)
   6% w/v SDS
   30% glycerol
   150 mM DTT
   0.03% w/v bromophenol blue
   Aliquot and store at -20 °C
   Add DTT fresh before each use

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References

1. Campbell, P. A. and Rudnicki, M. A. (2013). Oct4 interaction with Hmgb2 regulates Akt signaling and pluripotency. *Stem Cells* 31(6): 1107-1120.

