Kinase assay for TopBP1 activation of ATR D. Cortez 3-19-2012

Protocol to purify Flag-ATR-HA-ATRIP complexes from 293T cells (keep all solutions and cells cold unless otherwise stated; centrifugation can be done in microfuge or in 15ml conical tubes)

293T transfections

Day 1: For each sample, split 293T into 2 x 10cm plates with complete DMEM (7.5%FBS) so that they are roughly 50% confluent. Try using 5 x 10^6 cells per 10cm2 dish approximately 18h prior to transfection.

Day 2 Transfect using PEI protocol using 3 ug of Flag-ATR + 1 ug HA-ATRIP (pDC720) per dish.

So per dish:
Mix 4ug of DNA total in 100ul of DMEM without serum in 1.5ml tube.
Add 24 ul of PEI (1mg/ml solution – see below)
Mix by vortexing
Leave at room temperature 10-15 minutes
Aspirate media off 293T cells and replace with 10 mL of fresh complete DMEM
Add PEI/DNA mixture dropwise while swirling the plate of 293T cells.
Incubate overnight at 37°C.

3. Day after transfection split both dishes into 2 10cm2 dishes if necessary. Harvest 72 hours after transfection.

Lysis and IP

1. Harvest 4 10cm2 dishes of cells expressing Flag-ATR/HA-ATRIP. Wash 2x with PBS. Do not freeze!

2. Resuspend the cells in 1ml ice cold hypotonic buffer. Resuspend by gentle pipetting or swirling of cells.

3. Spin cells at 2500 x g in microfuge for 3 minutes at 4 degrees.

4. Discard supernatant.

5. Resuspend the cells in 400ul ice cold hypotonic buffer and allow to swell on ice 10 minutes.

6. Transfer cells to small dounce homogenizer. Homegenize with ten up and down strokes using type B pestle (this is the tighter one). Transfer cells back to eppendorf tube.

7. Centrifuge cells for 6 minutes at 3300 x g at 4 degrees.

8. At this point you can remove and discard the supernatant. This is the cytoplasmic fraction. The pellet is the nuclei. Flick the pellet several times with your finger to try to loosen it and make it easier to resuspend in the next step.

9. Add 250ul of "High salt buffer" slowly and with mixing. When you do this, the extract will get very viscous very quickly. I recommend adding the buffer dropwise while attempting to mix the tube by gentle shaking or flicking. After all the High salt buffer is added then try to mix by turning end over end several times. Incubate for 30 minutes on ice. Mix the extract during this 30 minutes by swirling every 5 minutes or rotating in cold room.

10. Spin the extract at full speed in a microfuge for 10 minutes. transfer supernatant to a new eppendorf tube. Add 200ul of "No salt buffer" to supernatant; mix by pipetting then spin again for 5 minutes at full speed. Save the supernatant—this is your nuclear extract-- for the HA IP.

11. You can measure the concentration of protein in the nuclear extract but it is likely to be low with this method (approximately 1-2 mg/ml).

12. *Optional:* Pre-clear with 20ul of protein-g agarose beads + 5ug of Mouse IgG by incubating for 20-30 minutes in cold room. Spin in microfuge for 10minutes at full speed. Keep supernatant.

13. IP as follows:
Use 25ul of the HA bead slurry (pipette with cut-off tip). Wash these beads one time in No Salt Buffer. Then use for IP. IP in cold room for 3h in cold room rotating.

14. Wash HA beads 3 times with TGN lysis buffer containing protease and phosphatase inhibitors. I do the wash quickly without any incubation time to help preserve the ATR-ATRIP interaction. Please note that I found it necessary to spin the beads at 10,000 rpm to pellet them adequately. They may be more resistant to pelleting initially because of the higher concentration of glycerol in the lysis buffers. You may find it useful to pellet the beads, remove most of the liquid, then centrifuge again but this time turn the tube so the other side is up so the beads will pellet to the bottom of the tube instead of the side of the tube. Then remove the remainder of the liquid.

15. Wash HA beads 1 time with LiCl wash (500mM LiCl in TGN buffer) – do this step quickly so as to avoid losing the ATR-ATRIP interaction

16. Wash HA beads 2x with Kinase buffer. After the second wash resuspend in 400ul of kinase buffer and aliquot 100ul each into 4 kinase reaction tubes. You may be able to split into as many as 6-8 reactions if the transfection was really good. Spin again and remove supernatant. Ready to do kinase reaction. Keep these on ice until ready to use.

17. Proceed with kinase reactions.

Kinase reactions

Each reaction will be 30ul final containing the following:

0.5-1ug of GST-MCM2 fragment substrate
0.5ul of hot g-32P-ATP
10uM final cold ATP
10pmol of recombinant GST or GST-AAD

Best to set up 2 master mixes as follows:

GST-MCM2 (1mg/ml)	1ul/reaction **vol will change based on concentration
Hot ATP	0.5ul/reaction
Cold ATP (1mM)	0.3ul/reaction
GST (1.8uM)	5.5ul/reaction ** vol will change based on concentration
2x kinase buffer	15ul/reaction
H2O	7.7ul/reaction

Or

GST-MCM2 (1mg/ml)	1ul/reaction **vol will change based on concentration
Hot ATP	0.5ul/reaction
Cold ATP (1mM)	0.3ul/reaction
GST-AAD (3uM)	3.3ul/reaction ** vol will change based on concentration
2x kinase buffer	15ul/reaction
H2O	9.9ul/reaction

Make mixes on ice (add the hot ATP last behind a shield). Be sure to mix well by pipetting.

When ready, add 30ul to each kinase reaction sample.
Mix by gentle flicking of tube
Place at 30 degrees for 20 minutes
Mix every 3-4 minutes by gentle flicking of tube.
Stop after 20 minutes by adding 30ul of 2X sample buffer.

Analyze by running two gels. Blot one 8% gel with anti-Flag/anti-ATR N19 (top) and anti-HA/anti-ATRIP (bottom) to visualize ATR (>220kda) and ATRIP (85kDa)

Stain the other gel 4-12% gradient with coomassie, dry, expose to film.

Reagents

Hypotonic Buffer
20 mM Hepes pH=7.9
1.5 mM $MgCl_2$
10 mM KCl
0.2 mM PMSF*
0.5 mM DTT*

*add just before use

No Salt Buffer
20 mM Hepes pH=7.9
20% glycerol
0.2 mM PMSF*
0.1% Tween 20

High Salt Buffer
20 mM Hepes pH=7.9
25% glycerol
1.5 mM $MgCl_2$
350 mM NaCl
0.2 mM PMSF*
0.5 mM DTT*
1mM NaF*
1mM NaVanadate*
10mM B-glycerolphosphate*
aprotinin*
leuopeptin*

2X Kinase Buffer
Hepes 20mM pH=7.5
NaCl 100mM
B-glycerolphosphate 100mM
MgCl2 20mM
MnCl2 20mM
DTT 2mM

(dilute 1:2 before using for washes)

TGN Buffer
50mM Tris pH=7.5
150mMNaCl

10% Glycerol
1% Tween 20
0.2 mM PMSF*
0.5 mM DTT*
1mM NaF*
1mM NaVanadate*
10mM B-glycerolphosphate*
aprotinin*
leuopeptin*

PEI is linear polyethylenimine (MW 25,000) from Polyscience, Inc. (Cat # 23966). Make a 1 mg/mL solution of PEI in water, neutralize to pH 7.0 with HCl and filter sterilize. Use a hot plate and stir bar to dissolve PEI; it may take 6 – 8 hours to dissolve. It is stable at 4°C for at least 3 months. Keep bulk of aliquots stored at -80°C.