In vitro Phosphorylation Assay

This assay was first published in Westfall et al., Am. J. Physiol. 280:C324, 2001.

1. **Solutions to prepare one day ahead**
   1. Relaxing Solution:
      - 40 ml of 2.5 M KCl (100 mM)
      - 20 ml of 0.5 M Imidazole (10 mM)
      - 20 ml of 100 mM EGTA (2 mM)
      - 10 ml of 100 mM MgCl₂ (1 mM)
      - 2.42 g Na₂ATP (2 mM)
      - pH to 7.0 with KOH
      - Dilute to 1 liter in volumetric
      - Split into 1-500ml bottle and 2
      - 250 ml bottles
   
   B. Relaxing solution with 0.1% TX-100
      - 4 ml KCl
      - 2 ml Imidazole
      - 2 ml EGTA
      - 2 ml MgCl₂
      - 0.242 g Na₂ATP
      - 1.0 ml of Pierce 10% TX-100
      - pH to 7.0 with KOH
      - Final volume = 100 ml

2. **Other preparations needed one day ahead**
   1. Label 0.5 µl microfuge tubes for phosphorylation gel - usually use 2
coverslips/tube.
   2. Label 0.5 µl microfuge tubes for Western blots - 2 coverslips/tube.
   3. Check to make sure there are enough micropipets - 1/sample.
   4. Label 1.5 ml tubes needed for reagents (e.g. Relaxing solution + Calyculin A;
      Relaxing Solution + Calyculin A + Catalytic subunit; Relaxing solution +
      Calyculin A + ³²P; Relaxing solution + Calyculin A + catalytic subunit + ³²P)
   5. Setup radioactive area - organize shielding; put down blue pads; get liquid and
dry waste containers ready; get ice bucket.
   6. Pour separating gel for SDS-PAGE - store in fridge overnight after overlaying
      with 2% butanol.
   7. Make sure there is enough sample buffer in freezer.

3. **Day of the experiment**
   1. Pour stacking gel and make electrode buffer
   2. Get ³²P-ATP (adenosine 5'-triphosphate tetra(triethylammonium) salt) out of
      freezer (I have been using Dupont NEN NEG002A but now I hear it’s cheaper to
get it through ICN via Fisher) and put into a shielded container where it can be 
thawed inside it’s lead pig.

3. Finish preparing shielding and work area
   1. All work with $^{32}$P-ATP should be shielded - put original radioactive stock in 
      shielded container for thawing and only remove for pipetting. Use 3 
      shields to protect yourself from exposure. Line floor with blue pads to 
      prevent contamination if a spill should occur. Have a dry waste container 
      (shielded container with plastic bag liner) and a liquid waste container 
      (shielded container with 500-1000 ml beaker for collecting liquid waste). 
      These containers should be different from the waste collected for the lab, 
      since those containers are not easily accessible during the assay.
   2. Fill ice bucket (it will become contaminated so use bucket designated for 
      radioactive work) with ice. Put 3 beakers on ice. Pour RS into 1-250 ml 
      beakers and keep ice cold. Pour RS alone into a 100 ml beaker and 
      RS+TX-100 into a second 100 ml beaker and keep ice cold.
   3. Get aerosol resistant tips ready - need 1000 µl, 100 µl and 10 µl tips.
   4. **MAKE SURE YOU MONITOR YOUR LAB COAT, SLEEVES, 
      HANDS, and SHOES EVERY TIME YOU LEAVE THE WORK 
      AREA ONCE YOU HAVE RADIOACTIVE STOCK OPENED.**

4. Prepare reagents for assay
   1. RS* = 8 ml relaxing solution + 48 mg dithiothreitol
   2. RS* + Catalytic subunit = 2.5 ml RS* added to catalytic subunit bottle 
      (Sigma P2645; 2500 U).
   3. RS*+ Calyculin A + Catalytic subunit 
      Calyculin A stock: Dissolve 1-10 µg vial of Calyculin A (Calbiochem 
      208851; mw = 1009.2 mg/mM) with 400 µl 100% ethanol in dark to get 25 ng/µl Calyculin A. 
      Keep vial covered with foil when out of the dark. Calyculin A is a phosphodiesterase 2A 
      inhibitor needed to prevent dephosphorylation during the assay. Although cells are 
      permeabilized, I found that there was a significant amount of dephosphorylation occurring 
      without adding Cal A.
      Tube #1: In wrapped foil tube: 1.5 ml RS* + 3 µl Calyculin A (50 nM)
      Tube #2: Wrap the RS*+Cat. subunit tube with foil and add 5 µl calyculin A 
      Note: These are suggested volumes that can be adjusted if a smaller vial of cat. subunit of 
      activity is used (e.g. 500 U rather than 2500 U) and/or fewer samples of one or the other 
      treatment group are being used.

5. Preparation of radioactive stock solutions:
   1. Calculate the amount of $^{32}$P-ATP needed based on the calibration date and 
      decay table provided by the manufacturer. Each coverslip requires 10 uCi 
      of $^{32}$P-ATP and I found that 75 µl of volume per coverslip was ideal for 
      this assay. Higher volumes often leaked off of the coverslip and then 
      radioactivity is lost and that coverslip can no longer be included for study. 
      Example: For 1000 µl of RS*+Cal. A, 133.3 µCi of $^{32}$P-ATP should be
added. Again, the exact volume depends on the date used relative to the calibration date.

2. RS*+Calyculin A + 32P-ATP
   Add calculated vol of $^{32}$P-ATP to above solution.
   An example: Add 133.3 µCi $^{32}$P-ATP (10 mCi/ml on calibration date would be 13.33 µl) to 1000 µl RS*+Calyculin A.

3. RS*+Calyculin A + Cat. subunit - see calculation above except you would use the stock containing the catalytic subunit.

4. Fill out radiation notebook - you can calculate that roughly 10% of your waste will be in the dry waste and 90% will be in the liquid waste based on what I obtained in my studies.

5. Thaw sample buffer, retrieve good pair of forceps for handling coverslips, and get two timers - one timer is for radioactive assay, the other timer will be used for permeabilization of cells in the tissue culture hood.

F. The assay

1. You will be able to do 1 six-well plate about every 5 minutes - the assay is 10 minutes long so you can do 2 six-well plates over a 20 min. time span. For example, you can start the assay on plate 1, then start the assay on plate 2 when plate 1 is 5 min into the assay, stop the assay on plate 1 and scrape cells into sample buffer, then at 15 min after initially starting you will stop the assay in plate 2 and scrape these cells. You may not be able to scrape all of the coverslips from plate 1 before it is necessary to stop the assay on plate 2. It is reasonable to expect you can no more than 8 plates a day, with 6 plates being the most reasonable. The first time you do this assay I would do no more than 4 plates.

1. In a second ice bucket you will need to have relaxing solution (RS) and RS+TX-100. This bucket will not be radioactive and should be placed in or close to the tissue culture hood. Remove 2 six-well plates from incubator. Aspirate off media, add 2 ml of ice-cold RS, aspirate, add 2 ml of ice-cold RS+TX-100 and start the timer for 30 seconds as you work. After the 30 sec, aspirate and rinse each well 3x with RS alone. Make sure you keep the RS ice cold. Aspirate off about half of the RS solution.

2. Take six-well plates to radioactive area and aspirate off the remaining RS solution with a 1 ml pipette. Aspirate around the edge of each coverslip to remove any excess liquid with a 100 µl pipette. This is time consuming at first but you can build speed with practice. When sufficient solution is removed, pipette 75 ul of the desired solution onto each coverslip (e.g. RS*+CalA+$^{32}$P-ATP or RS*+CalA+Cat.subunit+$^{32}$P-ATP). Start timer as you work and aim for adding solution to each coverslip at 20 sec intervals. It may be best to start with 30 or 40 sec intervals to give extra time should there be mistakes. This is a timed 10 min assay so it is not acceptable to
add the 75 µl without regard for time. Time is not so critical for 30 min assays (I observed a slight increase in TnI phosphorylation over 30 min but more variable MyBPC phosphorylation at the 30 min time point, regardless of how precise the time interval was).

3. After 10 min, add 2 ml of ice-cold RS in 20 sec intervals (or whatever interval used above).

4. After adding RS to each well, begin picking up each coverslip individually with forceps, dipping in the 100 ml RS beaker 3 times. Then wipe away excess solution on a kimwiipe, add 10 µl of sample buffer to coverslip and scrape into pre-labeled 0.5 ml microfuge tubes. Wrap tubes with parafilm and place on ice. Repeat for each coverslip in plate until finished.

5. When experiment is entirely finished, boil samples for 2 min, sonicate for 10 min. While doing this, the gel can be prepared for loading. Aim to have the gel loaded by 1 pm at the latest.

6. Load gel. Dispose of all gel tips in dry radioactive waste container and load gel within it’s plastic container to minimize exposure to radioactivity. Run gel for about 4 hours - stop just before dye front comes off.

7. Clean up the radioactive work area and put dry and liquid waste in appropriate long-term storage containers. There should be little radioactivity detectable in the area, except in the waste.

8. Remove gel from plates, scan gel apparatus to make sure it is not hot, and then remove the very bottom portion of the gel (around dye front to get rid of unbound radioactivity) for placement in dry radioactive waste. Promptly fix and silver stain the gel using the Daiichi protocol. Dry down gel overnight. Unlike our other gels, these steps should be completed on the same day to maximize detection of radioactive bands.

4. **The next day** - wipe down gel to remove excess glycerol. Cut away any excess mylar/cellophane, scan gel, place gel in Kapak, and then heat seal. Scan all waste material and if radioactive, dispose of in dry waste. Tape down gel in phosphorimage cassette (make sure it is flat so it won’t wrinkle when the cassette is added), add cassette, and lock down. If the gel is not in a Kapak bag you are likely to ruin the phosphorimage cassette which is ruined with ANY exposure to moisture. Expose the cassette for 4 days, develop in phosphorimager and save image on zip disk.