Standard Operating Procedure

Procedure	In Vitro Kinase Assay with [γ-^{32}P] - ATP
Department	
Location	
SOP Prepared By:	

Section 1: Purpose

Kinase assays are used to detect the activity of specific kinases from cells. Kinases are a group of enzymes that modifies a target substrate by phosphorylation. In this assay, the kinase removes the radioactively labeled phosphate group from [γ-^{32}P] - ATP and adds it to the target substrate. Kinase activity is measured by running an SDS PAGE (gel electrophoresis for proteins) and visualizing the results with an imaging technique which quantifies the amount of radiolabeled substrate.

Given kinases' essential role in cell metabolism, signaling, etc, this assay can be applied virtually to any biological research that wishes to investigate enzymatic activity within a cellular process. Some studies include measuring yeast kinases to understand chromosomal segregation[1] and measuring protein kinase R (PKR) activity[2], an important modulator of chronic metabolic inflammation associated to obesity, diabetes, and cardiovascular disease.

Section 2: Personal Protective Equipment and Survey Equipment

PPE:

- Lab coat
- Nitrile Gloves
- Heat resistant gloves
- Safety Glasses
- Closed-toe shoes

Other Equipment:

- Geiger counter with pancake probe
- Personal chest dosimeter
- Personal finger dosimeter

Section 3: Radioactive Material

ATP, [γ-^{32}P],
Supplier: Perkin Elmer
Starting activity: 10 uCi/uL
Typical use quantities: 100 uCi - 1mCi per experiment

- Radioactive material exposure (RAM) time on benchtop: ~30 minutes

Activity used per experiment: _____
RAM handling time: _____
Frequency of experiment: _____

Section 4: Potential Hazards

- ^{32}P is a high-energy beta emitter and has a half-life of 14.29 days. ^{32}P can present a substantial skin and eye dose hazard.
- Heat block may get as hot as 100^{o} Celsius.
- Possible acid burns (if using acid to terminate kinase reaction).
- TEMED, a chemical used in preparation of the SDS gel, is harmful if swallowed or inhaled and can cause severe skin burns and eye damage.

Any other hazards (potential for airborne release of radioactive material, chemical hazards, etc.)?

Section 5: Safety Precautions

The following precautions should be taken while handling ^{32}P:

- Designate area for handling ^{32}P and clearly label all containers and equipment. Equipment used in procedure:
  - Pipette
  - Centrifuge
  - Heat block
  - SDS PAGE gel box
  - Gel Dryer
  - Cassette with phosphor screen
  - Phosphorimager
- Any samples, stock, or equipment containing ^{32}P should be used behind Plexiglas shielding.
- Use filtering pipette tips to prevent contamination of pipette.
- Line all RAM countertops with absorbent sheets.
- Survey all areas and equipment where RAM is used with a Geiger counter before and after the procedure.
- Keep Geiger counter on when working with ^{32}P directly.
- Survey hands, body, and face with Geiger counter after conducting the procedure.
- Minimize exposure by keeping a hot hand (holding tubes with radioactive material) and cold hand (pipetting, etc.) as much as possible.
- Conduct all non-radioactive steps of the procedure before moving to the RAM corner.
- Minimize the time of contact with ^{32}P by setting up bench space before starting procedure.
- Use heat resistant gloves when heat block reaches high temperatures (100^{o} C). The heat block should not be touched when light is on. Ensure that Eppendorf tube lids are tightly sealed and carefully remove tubes from heat block.

Consult the Safety Data Sheet (SDS) on the acid used to terminate the reaction. However, most acids will

be used at low concentrations.

Section 6: Procedure

**indicates step must be performed in a designated RAM area and behind shielding.*

A. Preparation of Samples (in uL amounts)

1) Obtain the protein kinase of interest from stock solution or cell extraction.
    a) For cell extraction, a specific antibody is needed to bind to and pull down the kinase. Purify cell extracts using an appropriate phosphatase inhibitor.
2) Thaw and add the appropriate concentration of kinase buffer to the kinase in an Eppendorf tube.
3) Add the peptide substrate (normally recombinant protein) to the tube.
    a) For assays using different substrate concentrations, perform this step after the addition of ATP and distribution to sample tubes (steps 4 and 5). Add substrate separately to each 1.5mL tube in the RAM area and mix well with a vortex or by pipetting.
4) *Bring mix to designated radioactive area. Add appropriate volumes of [γ-^{32}P] - ATP and cold ATP to the mix. Mix well pipetting up and down in the tube.
5) *Distribute the mix to 1.5 mL Eppendorf tubes with a designated and labeled pipette. Pulse centrifuge to pull down liquid to the bottom of the tube to minimize exposure.
6) *Incubate samples on the heat block at 30 degrees C for one hour.
7) *Terminate the kinase reaction with the addition of 2X Laemmli (SDS) sample buffer or acid (TCA). Pulse centrifuge to pull down liquid to the bottom of the tube to minimize exposure.
8) *Boil samples on the heat block at 100 degrees C for 10-15 minutes.
9) *Remove samples from heat block onto centrifuge rack behind shielding to cool.

B. SDS PAGE

10) Prepare 7% polyacrylamide gel and running buffer before conducting assay.
    a) +SDS gel ingredients: acrylamide, Tris, SDS, dH2O, ammonium persulphate, TEMED
11) *Load samples into the wells of the gel and run.
12) *Discard running buffer in a designated RAM sink.

C. Blotting via Gel Dryer

13) *Transfer the gel to a membrane using the gel dryer vacuum system (references gel drying). The gel dryer is sealed shut during the vacuuming process, so no extra Plexiglass shielding is necessary.

D. Phosphorimaging

14) *Wrap the (dry) membrane with saran wrap or a plastic casing.
15) *Place filter paper in an exposure cassette that contains a phosphor screen. Close the cassette to allow the radiation from the membrane to produce an image on the screen. The time it takes to produce the image will vary.
16) Take the screen from the cassette and place on the phosphorimager. Close top and analyze readout with scanning software.
17) When finished, blank screen by exposing to visible light or a light box.
18) *Discard of membrane in RAM solid waste container.

Section 7: Spills/Incidents/Clean Ups

- For spills onto lined countertops, carefully discard of the absorbent lining into the solid RAM waste box. Check the countertop with Geiger counter afterwards.
- Check the centrifuge for possible leakage. If contaminated, clean the rotor of the centrifuge - use an effective cleaner for radioactive material. Check again with Geiger counter, and keep cleaning until counts are at background level.
- For extra caution, place the SDS PAGE box in a secondary containment (casserole dish) to avoid buffer spills onto countertop.
- For possible leakage of radioactivity in the phosphorimager, clean with an effective cleaner and check with Geiger counter. (Normally, the membrane is dry and wrapped in saran wrap, which contains the radioactivity and is never transferred to the screen.)
- The phosphorimager is labeled with a radioactive sticker but should never be contaminated, as the phosphor screen contains no radioactive material. Survey with a Geiger counter after use to ensure the phosphor screen did not contaminate the scanner.
- For major spills or any personal contamination, contact Radiation Safety Services for proper instructions and guidance. Try and contain the spill and check yourself and the area for radioactivity.
- For small spills onto lined countertops, carefully discard of the absorbent lining into the solid RAM waste box. Check the countertop with Geiger counter afterwards. Document the spill and cleanup procedure (https://www.ehs.harvard.edu/node/7589) used with other radiation records and notify radiation_protection@harvard.edu.
- At any point you may call Radiation Safety Services for assistance.

Section 8: Transportation, Storage, and Disposal

- Store [γ-^{32}P] - ATP stock in a locked 4 degrees Celsius fridge in a locked acrylic box. Keep key/passcode in a safe place.
- If transportation of samples containing ^{32}P is necessary, place samples in an acrylic container as a secondary containment.
- When using a pipette, keep the lid of the solid RAM waste container slightly open to quickly discard of pipette tips.
- Buffer from SDS PAGE may be discarded in the designated sink as long the amount of ^{32}P is below sink disposal limit of 10 uCi per day.
- Small volumes of possibly radioactive liquid may be poured onto paper towels inside the solid RAM waste container.
- Store phosphorimaging cassettes and blank screens in a safe area away from radioactive material.
- Dispose of gels and membranes in the solid RAM waste container. If the gel still has some liquid, discard in a RAM waste container with sawdust.
- Radioactive waste should be tagged and separated by isotope.

Section 9: References

- Harvard EHS website: https://www.ehs.harvard.edu/services/radiation-protection
- PerkinElmer 32P Handling Precautions: http://www.perkinelmer.com/lab-

solutions/resources/docs/TCH_Phosphorus32.pdf

- Kinase Assay of LRRK2 in vitro: https://www.jove.com/video/3495/assaying-the-kinase-activity-of-lrrk2-in-vitro
- In vitro kinase assay (materials): http://www.openwetware.org/wiki/Griffin:In_Vitro_Kinase_Assay
- Radioactive In vitro Kinase Assays: http://www.perkinelmer.com/lab-products-and-services/application-support-knowledgebase/radiometric/radioactive-in-vitro-kinase-assays.html
- Kinase buffer: https://www.neb.com/products/b6022-nebuffer-for-protein-kinases-pk#tabselect0
- SDS Page: http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6040.pdf
- Western blot technique: https://www.jove.com/video/3495/assaying-the-kinase-activity-of-lrrk2-in-vitro
- MSDS TEMED: http://www.bio-rad.com/webroot/web/pdf/WWMSDS/LSGC/USA/USA_USA_1610801.pdf
- Gel Drying: Chapter 19 “Drying Gels”, from *Methods in Molecular Biology*, Vol 32 *Basic Protein and Peptide Protocols*
- Phosphorimaging: http://www.ispybio.com/search/protocols/CPMB.Ch.App3.Techniques.pdf pages 8-9