Subcellular Phosphatase Assay Entered by Millie Shah
Janes Lab Protocols 04/01/2017

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I. Subcellular Fractionation of Adherent Cell Lines

1. Plate cells at a defined density so that the cell concentration at the time of lysis is similar across wells.
   - *The fractionation requires the cells to be adherent enough to withstand reasonable amounts of shear stress from plate rocking.*
   - *To increase the adhesion of cells that cannot withstand these forces, consider pre-coating the plate with 0.02% gelatin.*
   - *The procedure is compatible with 24-well plates and larger culture formats. Most phosphatases can be measured reliably from 12-well culture formats depending on the cell line. Yields decrease considerably in a 96-well format and is not recommended for this protocol.*
2. Stimulate as needed, aspirate culture media and wash once with ice-cold PBS on ice.
3. Aspirate the PBS wash and add the appropriate amount of saponin extraction (SE) buffer.
   - *See table I for the amount of SE buffer to use for different plate sizes.*
4. Tilt the plate gently to make sure the SE buffer is distributed equally across the plate.
5. Place on a platform rocker (Boekel Rocker II 260350 25 RPM) at 4°C for 30 minutes.
6. Tilt the plate gently to distribute the SE lysis buffer across the well and collect at the bottom of the well on ice.
7. Pipette the SE fraction into a microcentrifuge tube or 96-well storage plate and keep at 4°C until the rest of the fractionation procedure is complete.
8. Add the appropriate volume of saponin wash buffer to each well and incubate on a platform rocker on ice for 5 minutes.
   - *See table I for the amount of wash buffer to use for different plate sizes*
9. Aspirate wash buffer and repeat two more times.
10. After the last wash, add the appropriate volume of NP40 extract (NE) buffer to each well.
    - *See table I for amount of SE buffer to use for different plate sizes*
11. Tilt the plate gently to make sure the NE buffer is distributed equally across the plate.
12. Place on a platform rocker on ice for 5 minutes.
13. Tilt the plate gently to distribute the NE lysis buffer across the well and collect at the bottom of the well on ice.
14. Pipette NE fraction into a microcentrifuge tube or 96-well storage plate and store both SE and NE fractions at –80°C.
    - *Extracts are reasonably stable during long-term storage and repeated freeze-thawing, provided that the extracts are prepared with fresh reducing agents*

II. Subcellular Phosphatase (PPase) Activity Assay

1. Dilute the appropriate amount of phosphorylated substrate in PBS pH 7.4.
   - *The total mass of phosphorylated protein added to each well is determined for each phosphosubstrate preparation as the linear max of the ELISA titration curve. Typical amounts of protein range from 16 ng/well to 500 ng/well.*
2. Add 100 μL of diluted phosphorylated substrate to each well of a 96-well EIA/RIA flat-bottom plate (Corning #29442-322).
3. Cover the plate with an ELISA plate sealer and incubate overnight at 4°C.
4. Decant phospho-substrate dilution from 96-well plate and wash each well with 200 μL TBS + 0.1% Tween-20 (TBS-T).
5. Repeat wash two more times.
6. Remove the last wash and forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.

7. Block the 96-well plate with 100 μL per well of 5% (w/v) BSA in TBS-T, cover the plate with an ELISA sealer, and incubate on a platform shaker for one hour.
8. During the plate blocking, thaw the PPase extracts and move them to ice after thawing. Also, pre-heat the Jitterbug shaker to 30°C.
9. Prepare fresh SE and NE buffers to dilute the extracts. For NE buffer, make both buffer containing NP40 and salt and buffer without NP40 and salt.
10. Prepare extract dilutions on ice.
    - *Dilutions are best achieved by preparing diluted extracts in a 96-well format on ice.*
    - *Each plate must include PBS blanks, PPase extraction buffer controls, and a 5–6 point standard curve of twofold serially diluted PPase extract.*
    - *Extracts should be diluted to the middle of dynamic range of each assay determined previously through extract titration curves.*
11. After blocking, decant the blocking solution and wash the plate with 200 μL TBS-T.
12. Repeat wash two more times.
13. After the last wash, forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.
14. Using a multichannel pipette, add 85 μL of diluted extract to each well keeping the EIA/RIA plate at room temperature.
    - *Do this quickly because drying of the wells before addition of extract will prevent accurate PPase measurements.*
15. Cover the plate with an ELISA plate sealer and incubate the plate on a pre-heated Jitterbug (30°C) for 30–90 minutes on mix setting = 1 (550 RPM).
16. Stop the reaction by adding 85 μL of 2× PPase inhibitor mix with a multichannel pipette.
    - *Add the 2x PPase inhibitor mix with roughly the same timing as the diluted PPase extract to ensure that all wells have the same duration of dephosphorylation.*
17. Decant the terminated PPase reactions and wash with 200 μL of 1× PPase inhibitor mix.
18. Repeat wash with 1x PPase inhibitor mix two more times.
19. Wash with 200 μL TBS-T, decant and forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.
20. Add 50 μL of primary antibody solution diluted in blocking buffer to each well on the EIA/RIA plate.
    - *For optimized antibodies and dilutions see Table II.*
21. Cover the plate with an ELISA plate sealer and incubate on a platform rocker for one hour at room temperature.
22. Decant the primary antibody solution and wash with 200 μL TBS-T.
23. Repeat wash two more times.
24. After the last wash, forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.
25. Add 50 μL biotinylated secondary antibody solution (Jackson ImmunoResearch #111-066-144) diluted 1:10,000 in blocking buffer to each well on the EIA/RIA plate.
26. Cover the plate with an ELISA plate sealer and incubate on a platform rocker for one hour at room temperature.
27. Decant the secondary antibody solution and wash with 200 μL TBS-T.
28. Repeat wash two more times.
29. After the last wash, forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.
30. Add 50 μL streptavidin-HRP diluted 1:200 in blocking buffer to each well. Cover with an ELISA plate sealer and aluminum foil and incubate on platform rocker for 30 minutes at room temperature.
    - *While the strepavidin-HRP is incubating, equilibrate ELISA Reagent A and Reagent B to room temperature. Do not mix the reagents until just before the ELISA reaction.*
31. Decant the strepavidin-HRP solution and wash with 200 μL TBS-T.
32. Repeat wash two more times.

33. After the last wash, forcefully knock out excess liquid by banging the EIA/RIA plate upside down on a paper towel.
34. Add 100 μL ELISA substrate reagent (R&D Systems #DY999; 1:1 mix of Reagent A and Reagent B) to each well on the EIA/RIA plate.
35. Cover the plate with aluminum foil and incubate on a platform rocker for 10–20 minutes depending on the protein substrate.
    - *For optimized ELISA reaction times see Table II.*
36. Add 50 μL 1 M H_2SO_4 to each well, tapping the plate to ensure that the chromogenic reaction is fully stopped.
37. Read the plate on the Optima microplate reader at A_{450} (ELISA signal) and A_{540} (correction for plate background).

III. Total Protein Quantification in Subcellular Extracts

1. Dilute BSA in either SE or NE buffer to a final concentration of 50 mg/mL. Create a 12-point standard curve in the corresponding extraction buffer using twofold serial dilutions starting with 50 mg/mL BSA solution.
2. Add 10 μL of twofold standard dilutions or undiluted extract to a clear-bottom, black walled 96-well plate.
    - *Be sure to include 2–4 wells of buffer-only blanks.*
3. Activate incomplete phthaldialdehyde reagent (Sigma #P7914) with a 1:500 dilution of 2-mercaptoethanol.
4. Using a multichannel, add 50 μL of activated o-pthalaldehyde to each well and incubate on an orbital shaker for 2 minutes at room temperature.
5. Read plate on Optima plate reader (fluorescence λ_{ex} = 355 nm, λ_{em} = 440 nm).
6. Export data to Excel and regress the extract samples against the linear BSA standard curve to quantify total protein.

IV. Quantitative Analysis of PPase ELISA data

1. Export the plate readings to Excel and subtract the A_{540} signal from the A_{450} signal for each well.
2. Invert and normalize the measured signals on the plate by subtracting the (A_{450}–A_{540}) value of each well from the average (A_{450}–A_{540}) value of all the PPase extract buffer control wells; then, divide by the average (A_{450}–A_{540}) value of all the PPase extract buffer control wells.
3. Build a model that relates the serially diluted PPase extracts ("*X*") to the inverted A_{450}–A_{540} value ("*Y*") via the four-parameter logistic (4-PL) function:

$$Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^B} + D$$ where *A* is the minimum, *D* is the maximum, *C* is the EC_{50}, and *B* is a Hill coefficient.

4. Calculate the sum-of-squared error (*SSE*) between the 4-PL prediction of the inverted and normalized A_{450}-A_{540} value ("Y_{pred}") and the measured inverted A_{450}–A_{540} value ("Y_{meas}") across all points on the serial dilution:

$$SSE = \sum_i (Y_{i,pred} - Y_{i,meas})^2$$ where *i* is the i^{th} point on the serial dilution.

5. Use the Solver add-in in Excel and perform a nonlinear least-squares curve fit that minimizes *SSE* by changing *A*, *B*, *C*, and *D*.
    - *The Solver add-in is not installed by default in Excel; depending on the version of Excel there are different ways to install the add-in (e.g., Tools>Add-ins)*
    - *The default gradient-descent algorithm should be sufficient to solve the minimization problem.*
6. Calculate the corrected relative level of PPase activity ("*X*") given the inverted and normalized A_{450}–A_{540} signal ("*Y*") by using the rearranged version of the 4-PL function with the parameters that were optimized with the Excel Solver:

$$X = C\sqrt[B]{\frac{(A - D)}{(Y - D)} - 1}$$

7. Normalize these corrected measures of relative PPase activity to total protein concentration of each sample determined in Section III.
8. These corrected measures of relative PPase activity can then be scaled to untreated or zero-minute conditions as needed.
   - *Be sure to perform coefficient scalings that maintain the error in biological replicates for the untreated or zero-minute conditions, rather than variable scalings that set the reference conditon to one exactly (see Valcu and Valcu, Nat Methods 2011 for details).*

Buffer recipes

- **Saponin Extraction Buffer (SE)** (make fresh):

50 μL	1 M HEPES (pH 7.5)
50 μL	1% (w/v) saponin
2 μL	10 mg/mL aprotinin
2 μL	10 mg/mL leupeptin
1 μL	1 mg/mL pepstatin
1 μL	2 M $MgCl_2$
1 μL	1 M DTT (made fresh weekly)
3.5 μL	2-mercaptoethanol
5 μL	1 M glucose
3 μL	Hexokinase stock: 5 mg/mL Hexokinase (Sigma #H5000) + 15 μM PMSF
881.5 μL	ddH_2O
1 mL total volume	

- **NP40 Extraction Buffer (NE)** (make fresh):

50 μL	1 M HEPES (pH 7.5)
10 μL	10% (w/v) NP40
30 μL	5 M NaCl
2 μL	10 mg/mL aprotinin
2 μL	10 mg/mL leupeptin
1 μL	1 mg/mL pepstatin
1 μL	2 M $MgCl_2$
1 μL	1 M DTT (made fresh weekly)
3.5 μL	2-mercaptoethanol
5 μL	1 M glucose
3 μL	Hexokinase stock: 5 mg/mL Hexokinase (Sigma #H5000) + 15 μM PMSF
891.5 μL	ddH_2O
1 mL total volume	

- **2x PPase Inhibitor mix** (can be stored at 4°C)

4.46 g	NaPP tetrabasic decahydrate (20 mM)
1.26 g	NaF (60 mM)
50 mL	10× TBS

  Volume to 500 mL with ddH_2O
  Before use, add 1:500 dilution of 0.2 mM activated Na_3VO_4 (0.4 μM).

- **1x PPase Inhibitor mix** (make fresh)
  Dilute one part 2× PPase Inhibitor mix (containing Na_3VO_4) with one part 1x TBS and add 1:1000 dilution of Tween-20.

Table I: extraction and wash buffer scaling for different plate sizes

Plate Size	Volume of SE or NE Buffer (uL)	Volume of Wash Buffer (mL)
10 cm	400 uL	5 mL
6-well	200 uL	2 mL
12-well	100 uL	1 mL
24-well	50 uL	0.5 mL

Table II: primary antibody dilutions and ELISA reaction times

Phosphosubtrate	Antibody	Dilution	ELISA Rxn Time (mins)
pERK2	CST 4370	1:1000	10
pp38α	CST 4511	1:1000	15-20
pJNK1	CST 9251	1:100	10
pMK2	CST 3007	1:1000	10
pCREB	CST 9198	1:1000	10
pSTAT1	CST 9167	1:5000	10