Phosphotyrosine peptide enrichment

(Avoid too many froth bubbles during binding, washing, eluting because it will inactivate the antibody)

Antibody prebinding to Agarose G beads
(Follow this only if you do prebinding antibody by yourself) All operation carried out at 4°C unless mentioned

Antibody coupling to protein G agarose:

Add 40 μl beads (80 μl slurry) to 1.4 ml PBS (GIBCO, DBPS, Cat No. 14190), mix, spin. Wash with 1.4 ml PBS.
Add 160-250 μg of pY Ab to beads in a total vol. of 1.4 ml (fill up with PBS).
Rotate overnight in fridge.
Wash 3 x with 1.4 ml PBS each.
Wash once with 1.4 ml IAP buffer.

Immoaffinity Purification (IAP) Detailed Protocol for CST pY100 antibody (beads)

Solutions and Reagents

All operation carried out at 4°C unless mentioned
Note: Prepare solutions with Milli-Q or equivalently purified water. Trifluoroacetic acid should be
of the highest grade. All percentage specifications for solutions are vol/vol.
Provided in kit:
10X IAP buffer. IAP buffer (1X): 50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl.
Dilute 10X IAP buffer 10-fold. Store diluted IAP buffer (1X) at 2-8°C.

Procedure
1. Briefly spin tube with lyophilized peptide to collect all peptide at the bottom.
2. Add 1.4 ml IAP buffer to the lyophilized peptide and let stand for 5 minutes. Peptides will start to dissolve.
3. Further dissolve peptides under gentle shaking for 30 minutes at room temperature, optionally aided by brief sonication in a sonicator waterbath. Note: After dissolving the peptide in IAP buffer, check the pH of the peptide solution by spotting less than 1 μl on pH indicator paper (or on the relevant segment of a multi-panel pH indicator strip).
The pH should be close to neutral, or at least not lower than 5-6. In the rare cases that the pH is more acidic (due to insufficient removal of TFA from the peptide under suboptimal conditions of lyophilization) titrate the peptide solution with a concentrated Tris solution which has not been adjusted for pH. Do not use strong base.
4. Clear solution by centrifugation for 5 minutes at 1,800 x g (the pellet of insoluble matter may at times seem considerable, but this will not pose a problem since most of
the peptide will be dissolved nonetheless). Cool on ice. Wash the phosphotyrosine antibody beads (40μl) once with IAP buffer (1.4ml IAP buffer, mix by invert 5 times). Centrifuge at 1,500g for 30 sec. Remove supernatant.

5. Transfer the peptide solution into the microfuge tube containing phosphotyrosine antibody beads (40 μl; 80 μl slurry).

6. Incubate for 30 minutes (you can go up to 1hr) on a rotator at 4°C.

7. Centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.

Note: If the cells were directly harvested from culture medium without PBS washing, some of the Phenol Red pH indicator will remain (it co-extracts during the Sep-Pak® C18 purification of peptides) and color the peptide solution yellow. This coloration has no effect on the immunoaffinity purification step.

Note: All subsequent wash steps are at 0-4°C.

Note: In all wash steps except the last one, the supernatant should be removed reasonably well, but it does not have to be done to the last few microliters.

8. Add 1 ml of IAP buffer to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.

9. Repeat step 8 two times.

10. Add 1 ml water to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor.

11. Add 1 ml water to the beads, mix by inverting tube 5 times, centrifuge at 1,500 x g for 1 minute and remove supernatant with a 1 ml micropipettor until about 100 – 200 μl is left.

12. Centrifuge at 1,500 x g for 5 seconds to remove fluid from the tube walls, and carefully remove the supernatant to completion with a 200 ml micropipettor (using a gel loading tip can aid in complete removal of the wash)

13. Add 55 μl of 0.15% TFA to the beads, tap the bottom of the tube several times (do not vortex) and let stand at room temperature for 10 minutes.

Note: In this step, phosphopeptides will elute.

14. Tap the bottom of the tube again, centrifuge at 1,500 x g for 1 minute, remove the supernatant and add it to another microcentrifuge tube.

15. Add 45 μl of 0.15% TFA, tap the bottom of the tube a few times, centrifuge at 1,500 x g for 1 minute and combine the supernatant with the first eluate, mix.