Chapter 2

Co-immunoprecipitation Protocol to Investigate Cytokine Receptor-Associated Proteins, e.g., Janus Kinases or Other Associated Signaling Proteins

Claude Haan and Serge Haan

Abstract

Jak binding to cytokine receptors has been shown to be a complex and tight interaction. When studying loss-of-function or gain-of-function mutants of the Jaks or cytokine receptors it is often necessary to know if a certain mutant still associates correctly in the context of the signaling complex. The standard technique to show interaction of Jaks with cytokine receptors or other signalling molecules is Co-immunoprecipitation. Here we describe our protocol and discuss different pitfalls that can be encountered during the procedure.

Key words: Co-immunoprecipitation, Cytokine receptors, Janus kinases, Discontinuous SDS-PAGE, Western blotting, Immunodetection

1. Introduction

Due to the lack of structural data, the details of the interaction between cytokine receptors and Janus kinases are unknown. The N-termini of the Jaks consisting of the FERM and the SH2-like domain are involved in binding to cytokine receptors (1–8). Since it has been shown that overexpression of proteins (e.g., Janus kinases) can lead to artifacts in binding behavior (8, 9) expression systems which allow close to endogenous expression of Jaks (7, 10) were developed. The described method allows the detection of co-precipitated endogenous proteins with cytokine receptors and includes different techniques. Parts of the immunoprecipitation-, SDS-PAGE-, Western blotting-, or immunodetection-procedures have been changed (compared to the original protocols) and adapted to lead to higher sensitivity of detection of the co-precipitated proteins.

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2. Materials

Please check how to securely work with the chemicals that are described in this protocol, before starting the experiment. Prepare all solutions using ultrapure water and analytical grade reagents and follow all waste disposal regulations diligently. All reagents can be stored at room temperature unless otherwise stated.

2.1. Co-immunoprecipitation

1. Lysis buffer: 0.5% IGEPAL-CA630, 20 mM Tris–HCl, pH 7.5, 130 mM NaCl (see Notes 1 and 2). To prepare 1 L of lysis buffer dissolve 2.4 g Tris and 7.6 g NaCl in 900 ml of water and adjust the pH to 7.5 with HCl (see Note 3). Make up with water to 1 L and add 5 ml of IGEPAL-CA630.
2. Washing buffer: 0.1% to 0.5% IGEPAL-CA630, 20 mM Tris–HCl, pH 7.5, 130 mM NaCl, 1 mM sodium vanadate (see Note 4). Prepare this solution as described in the point before and adjust the detergent concentration as needed.
3. 4× Sample buffer: 125 mM Tris–HCl, pH 6.7, 20% glycerol, 10% 2-mercaptoethanol, 4% SDS. Prepare 1 L of 1 M Tris–HCl, pH 6.7 stock solution by dissolving 121.14 g Tris in 900 ml of water and adjust the pH to 6.7 with HCl. Make up to 1 L. To prepare 50 ml of sample buffer use 6.25 ml of 1 M Tris–HCl, pH 6.7, 10 ml glycerol, 10 ml 20% SDS, and 5 ml 2-mercaptoethanol and make up with water to 50 ml. Add very little bromophenol blue to give the solution a blue color.
4. 2× Sample buffer: Dilute 4× sample buffer with water 1:1.
5. Protease inhibitors: We use complete protease inhibitor mix (Roche) (see Note 5).
6. Protein A sepharose: We use Protein A sepharose CL-B4 (GE Healthcare).

2.2. SDS-PAGE

1. 4× separating gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4% SDS. Dissolve 181.7 g Tris in 800 ml H_2O. Adjust pH to 8.8 with 30% HCl. Make up with water to 1 L and recheck the pH. Add 20 ml of 20% SDS solution.
2. 4× stacking gel buffer: 0.5 M Tris–HCl, pH 6.7, 0.4% SDS. Dissolve 30.3 g Tris in 400 ml H_2O. Adjust pH to 6.7 with 30% HCl. Make up with water to 500 ml and recheck the pH. Add 10 ml of 20% SDS solution.
3. Acrylamide solution 30%, acrylamide-to-bisacrylamide ratio 29:1 (e.g., by Applichem) (see Note 6).
4. *N,N,N′,N′*-tetramethylethylendiamine (TEMED) (see Note 7).
5. Ammonium persulfate (see Note 8).

6. 10× SDS running buffer: 0.25 M Tris-base, 1.92 M glycine, 1% SDS (see Note 9). Dissolve 60.6 g Tris, 288.3 g glycine, and 20 g SDS in 2 L of water. Dilute 10 times before use.

2.3. Western Blotting

1. Anode buffer I: 300 mM Tris-base, 20% methanol. Use 150 ml of a 2 M Tris-base solution and 200 ml methanol and make up to 1 L with water.
2. Anode buffer II: 25 mM Tris-base, 20% methanol. Use 12.5 ml of a 2 M Tris-base solution and 200 ml methanol and make up to 1 L with water.
3. Cathode buffer: 40 mM ε-aminocaproic acid, 20% methanol, 0.01% SDS. Use 40 ml of a 1 M ε-aminocapronic acid solution, 200 ml methanol, and 0.5 ml of a 20% SDS solution and make up to 1 L with water.
4. PVDF membrane (pore size 0,45 μm).
5. Blotting paper (see Note 10) (e.g., Whatman® 3 MM Chr. (0.34 mm thickness)).

2.4. Immunodetection

1. TBS-N: 10 mM Tris–HCl, pH 7.4, 135 mM NaCl, 0.1% IGEPAL CA-630. To prepare 5 L of TBS-N, dissolve 6.1 g Tris and 39.4 g NaCl in 1 L of water and adjust the pH with HCl to 7.5. Add 5 ml of IGEPAL CA-630, mix, and make up with water to 5 L.
2. Blocking buffer: 10% bovine serum albumin (BSA) and 0.01% NaN_3 in TBS-N.
3. pCA-ECL solution: (see Note 11) 100 mM Tris/HCl pH 8.8 (see Note 12), 2.5 mM luminol (IUPAC name: 5-Amino-2,3-dihydro-1,4-phthalazinedione) (see Note 13), 0.2 mM para coumaric acid (pCA, IUPAC name: 3-(4-hydroxyphenyl)-2-propenoic acid), and 2.6 mM hydrogen peroxide (see Note 14). For the pCA-ECL prepare a large batch of ready-to-go solution containing luminol and pCA without the H_2O_2 and keep this at 4°C and in a dark bottle. Prepare 500 ml pCA-ECL by adding 5 ml of 250 mM luminol stock solution (to prepare 250 mM luminol dissolve 1.2 g luminol in 25 ml DMSO) and 1.1 ml of 90 mM pCA stock solution (to prepare 90 mM pCA dissolve 0.15 g of pCA in 10 ml DMSO) in 494 ml 100 mM Tris–HCl, pH 8.8. To activate the pCA-ECL solution for the detection of the Western blots, add 3.5 μl of 30% H_2O_2 (see Note 15) per 10 ml of pCA-ECL solution a few minutes before detection of the Western blot.
4. 4IPBA-ECL solution: 100 mM Tris–HCl, pH 8.8, 1.25 mM luminol, 2 mM 4IPBA (IUPAC name: (4-iodophenyl) boronic acid), 5.3 mM hydrogen peroxide (see Note 16). The 4IPBA-ECL is best prepared fresh from the stocks just before use since longer storage results in increasing background. To prepare

10 ml of 4IBPA-ECL add 50 µl of 250 mM luminol stock, 225 µl of 90 mM 4IPBA stock, and 5 µl of 30% H_2O_2 (to prepare 90 mM 4IPBA stock solution, dissolve 0.22 g of 4-iodophenyl boronic acid in 10 ml DMSO) (see Note 17).

5. Stripping buffer 1 (see Note 18): 2 M glycine, pH 2.5. Add half a teaspoonful of SDS powder (avoid pellets as they take a long time to dissolve and tend to stick to the blot) to 20 ml of buffer when the blot is already covered with buffer. The SDS dissolves during the process (see Note 19). To prepare 2 M glycine, pH 2.5, dissolve 150 g glycine in 800 ml water and adjust the pH to 2.5 using HCl. Make up to 1 L with water and readjust the pH if necessary.
6. Stripping buffer 2 (see Note 20): 60 mM Tris–HCl, pH 6.8, 2% SDS. To prepare 1 L dissolve 7.28 g Tris in 800 ml and adjust the pH to pH 6.8. Add 20 g of SDS and make up with water to 1 L while stirring. Add 80 µl 2-mercaptoethanol per 10 ml of stripping buffer just before use and incubate at 70°C for 20–30 min (see Note 21).

3. Methods

3.1. Cell Lysis and Immunoprecipitation

All steps of cell lysis and immunoprecipitation are performed at 4°C using ice-cold buffers (see Note 22). When showing the interaction between cytokine receptors and Jaks one can either precipitate the receptor and check for co-precipitated Jak or use the inverse approach. However, since cytokine receptors are highly glycosylated, they migrate on SDS-PAGE gels as diffuse bands, while the Janus kinases are visible as sharp bands on Western blots. Probably because it is easier to detect the low levels of co-precipitated protein when this protein does not migrate as a fuzzy band, we have always been more successful in precipitating the cytokine receptor and show the Jak co-precipitation than the other way around. Be sure to validate all antibodies (see Note 23 and Fig. 1) (see Note 24 and Fig. 2) and to include the important controls (see Notes 25, 26 and Fig. 3).

1. Cells are lysed on a 10 cm cell culture dish (placed on ice) (see Note 27) (approx. 5–10 million cells) with 500–1,000 µL of ice-cold lysis buffer containing protease inhibitors. After scraping of the cells from the surface the lysates are transferred to a microfuge tube.
2. Incubate the lysates on ice for 30 min, and then clear the lysates by centrifugation at 12,000×*g* for 15 min in a cooled centrifuge.
3. The cleared lysates are transferred to fresh microfuge tubes and the concentration of the lysates is measured (e.g., by Bradford assay) (see Note 28).

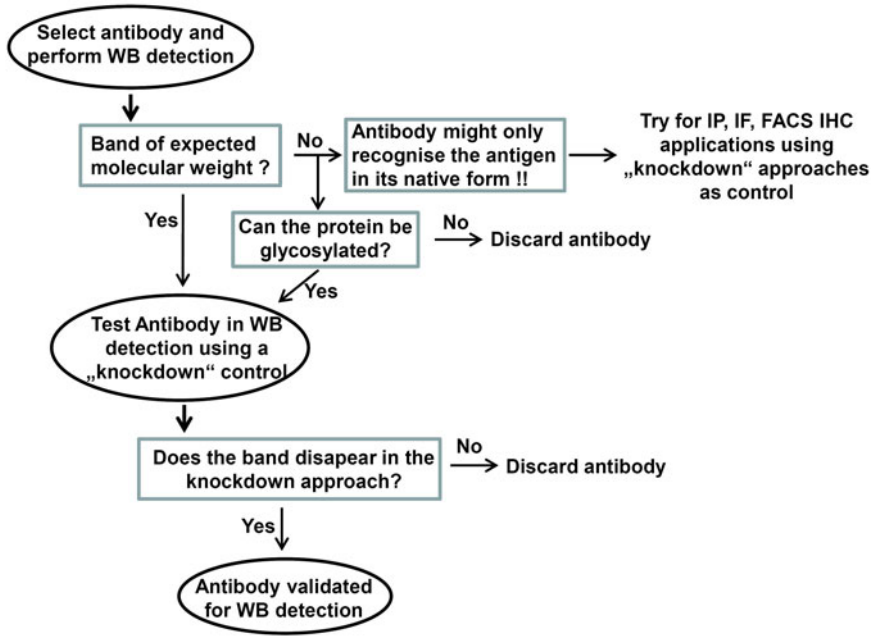

Fig. 1. Possible workflow to test antibody specificity. *WB* Western blot, *IP* immunoprecipitation, *IF* immunofluorescence, *FACS* fluorescence-activated cell sorting, *IHC* immunohistochemistry.

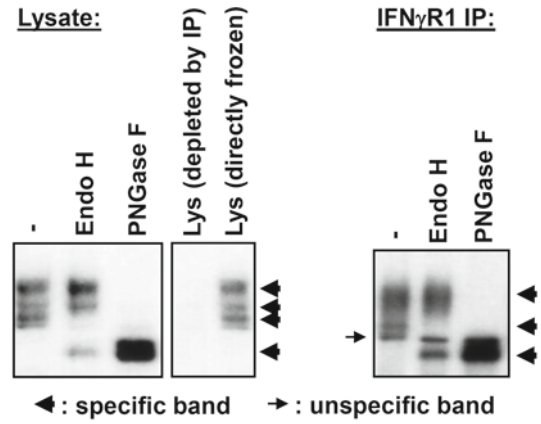

Fig. 2. Lysates and an immunoprecipitation of IFNγR1 were treated with EndoH and PNGaseF (37°C for 1 h following the manufacturer's instructions (New England Biolabs)) to remove the corresponding glycosylation. SDS-PAGE and Western blots were performed and detected with an antibody against IFNγR1. A directly frozen lysate and a lysate depleted by quantitative precipitation of IFNγR1 are also shown as controls. The treatment with EndoH shifts the bands corresponding to the immature ER forms of IFNγR1 towards higher mobility on the Western blot detection. The PNGaseF treatment shifts all glycosylated IFNγR1 forms towards higher mobility. The nonspecific bands are not shifted.

4. Equal amounts of lysate are transferred to fresh microfuge tubes and the volume in each tube is adjusted to 1 ml so that the concentration of protein lysate in each tube is similar. Transfer 90 µl of each lysate to fresh microfuge tubes and add 30 µl of 4× sample buffer before freezing the lysates at −20°C

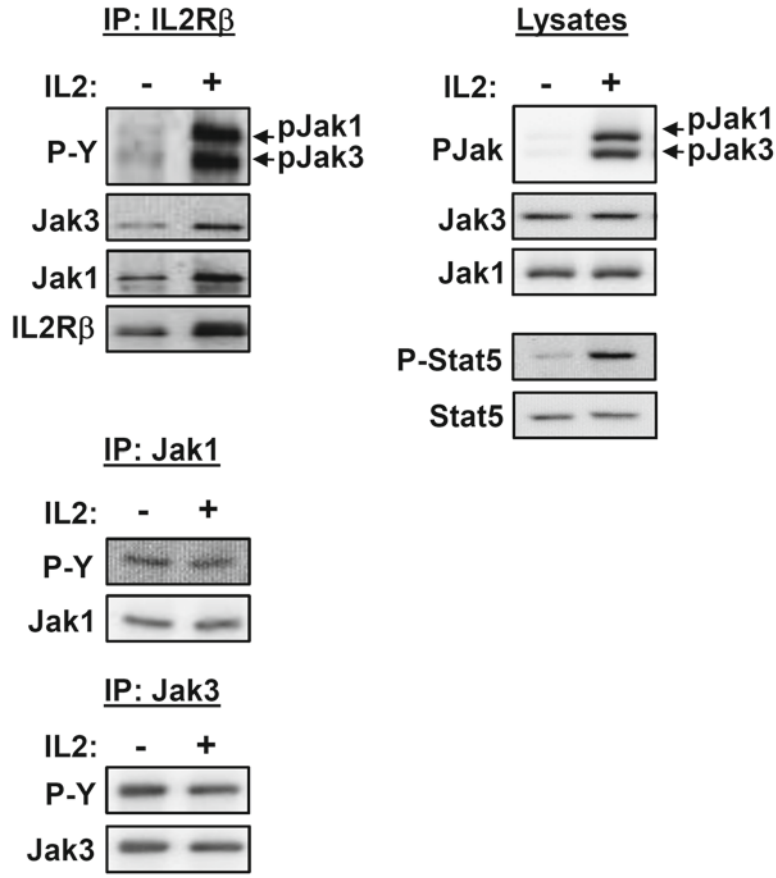

Fig. 3. U4C cells expressing IL2Rβ/γ, Jak1, and Jak3 (10) were stimulated with IL-2. Lysates were prepared and immunoprecipitates with antibodies against IL2Rβ, Jak1, or Jak3 were performed. After SDS-PAGE, Western blots of the lysates and the immunoprecipitates were prepared and detected as indicated. The detection of Jak1 and Jak3 phosphorylation is better from the immunoprecipitates of the IL2Rβ which precipitates the whole signaling complexes than from the immunoprecipitates of Jak1 or Jak3.

(see Note 29). Add 1 μg of precipitating antibody to the remaining lysate and incubate the solution at low speed on an overhead shaker for 2–3 h (or overnight) at 4°C.

5. A protein A sepharose (see Note 30) slurry is prepared in the meantime by equilibrating 2.5 mg protein A sepharose (per IP sample) with 100 μl (per IP sample) lysis buffer for 1 h minimum at 4°C in an overhead shaker (see Notes 31 and 32).
6. 100 μl of protein A sepharose slurry is added to each microfuge tube containing the lysates with the immunocomplexes (see Note 33). The mixture is incubated on the overhead shaker for 1 h at 4°C.
7. The protein A sepharose is collected by centrifugation at 12,000 × *g* for 1 min.

8. The supernatant is discarded (see Note 34) and the pellet containing the sepharose and the immunocomplexes are washed three to five times (see Note 35). 1 ml of washing buffer is added and the immunoprecipitate is re-suspended by vortexing. After a 5-min incubation on ice the sepharose is pelleted by centrifugation at 12,000 × *g* for 1 min. This procedure is repeated 2 to 4 times.
9. After the last washing solution has been removed the sepharose and the immunocomplexes are mixed with 30–50 µl of 2× Sample buffer (depending on the volume of sepharose used, the ratio should roughly be 1:1), vortexed, and stored at –20°C until further use for SDS-PAGE.

3.2. SDS-PAGE

The immunoprecipitates are electrophoretically resolved by mass on a polyacrylamide hydrogel. To achieve better resolution on the gel we recommend discontinuous SDS-PAGE. The sample is first focused on a "stacking gel" and then separated in the second part of the gel, the "separating gel" (see Note 36). There are many types of SDS-PAGE chambers which require slightly different handling when producing the gels or mounting the gel into the electrophoresis chamber, so we do not describe these steps in detail here because they might be specific for our system only (see Note 37).

1. Water, the acrylamide solution, and the 4× separating gel buffer are mixed so that a separating gel of the wanted percentage is achieved (see Note 38). Per 10 ml of solution 5 µl TEMED and 25 µl of 20% APS (see Note 39) are added resulting in final concentrations of both initiators of 0.05% (see Note 40). The mixture is thoroughly mixed and the separating gel is cast between the glass plates. To achieve a level and smooth separating gel surface the polymerizing solution is overlaid with water-saturated isopropanol (see Note 41).
2. When the separating gel is polymerized (see Note 42) remove the isopropanol by inverting and use some water to wash. Remove the water by inverting and aspire the remaining water using a filter paper without touching the gel surface (see Note 43).
3. Water, the acrylamide solution, and the 4× stacking gel buffer are mixed so that a stacking gel of 3% acrylamide is achieved. Per 10 ml of solution add 10 µl TEMED and 30 µl of 20% APS (see Note 44). The mixture is thoroughly mixed and the separating gel is cast between the glass plates. The comb is inserted and the mixture is left to polymerize for 2 h.
4. When the gels are polymerized the comb is removed and the gels mounted in the SDS-PAGE chamber. The running buffer

is filled into the chamber and eventual air bubbles are chased from beneath the gel.

5. The immunoprecipitates and the lysate samples are heat-denatured for 5 min at 95°C.
6. After heating, the samples are centrifuged for 30 s at 13,000 × *g* (see Note 45). They are now ready to be applied to the gel.
7. The samples are loaded by using a Hamilton syringe or a pipette using very thin tips.
8. The electrophoresis apparatus is now connected to the power supply and the gels are run at 25 mA/gel (see Note 46).
9. When the running front has reached the bottom of the gel the run is stopped, the gel is carefully removed from within the plates, and the stacking gel can be discarded.

3.3. Western Blotting

The transfer of the proteins onto the membrane by Western blotting can be performed in different ways (capillary transfer, electrotransfer). Our method uses PVDF membrane and a semidry Western blotting chamber in combination with a 3 buffer system. The assembly of the Western blotting chamber may be deduced from Fig. 4.

1. During the SDS-PAGE run, 10 pieces of filter paper are cut per gel to be transferred (see Note 47). The PVDF membranes are also cut (see Note 48) and are shortly rinsed with methanol and then put into anode buffer II (see Note 49) for equilibration.
2. The SDS-PAGE separating gels are incubated in cathode buffer for 5 min.

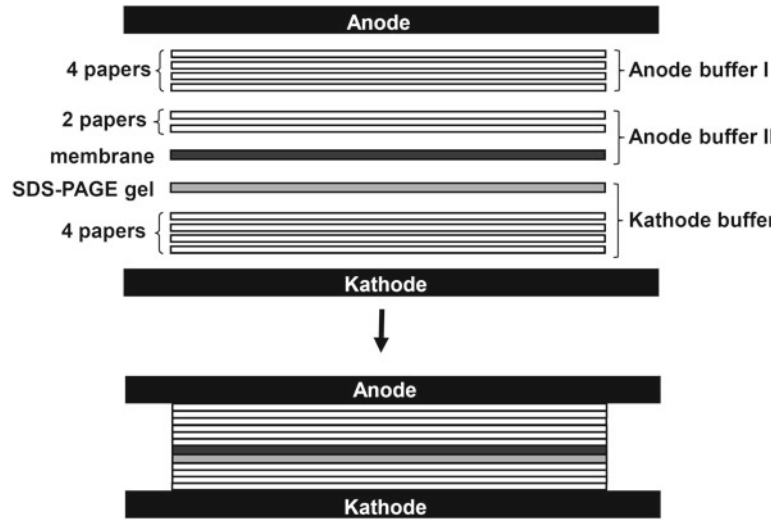

Fig. 4. Typical setup of a semidry Western blotting chamber (3 buffer system).

3. The blotting stack is built now in the Western blotting chamber. We describe it for our chamber which has the cathode as the base of the apparatus (see Note 50) (see also Fig. 4).
4. Filters are incubated in cathode buffer until they are completely wet and are placed on the cathode. Then the gel is placed on the filter papers. After adding the PVDF membrane to the stack two papers incubated with anode buffer II and lastly 4 papers incubated in anode buffer I are added to the stack.
5. Remove eventual residual air bubbles trapped within the stack by rolling a 25 ml pipette (see Note 51) gently over the stack consisting of gel, blotting membrane, and filter papers (move from the middle to the exterior to best chase the air) (see Note 52).
6. Place the top of the chamber carefully (do not displace the stack) on top of the blotting stack and fasten the tightening device according to the make of your blotting chamber (see Notes 53 and 54).
7. Connect the chamber to the power supply and blot for 1 h (see Note 55) at 0.8 mA/cm**2**. The current setting depends on the area of the blotting stack and is kept constant during the run (see Note 56).
8. The blotting membrane is recovered from the stack and immersed in TBS-N buffer in a plastic box (see Note 57). Conserve the blot in TBS-N until proceeding to Western blot detection.

3.4. Immunodetection of Western Blots

1. Block 30 min in blocking buffer (see Note 58).
2. Wash the blot shortly in TBS-N.
3. Add the first antibody solution (1 μg/ml antibody in TBS-N + 0.01–0.1% NaN_3) (see Note 59) for 1 h at room temperature or even better, overnight at 4°C (see Note 60).
4. Wash 3 times with TBS-N.
5. Add the HRP-labeled secondary antibody (1:2,500 to 1:5,000) (mind the species of the first antibody!!) (see Note 61).
6. Wash 3 times in TBS-N.
7. Prepare the ECL solution and add H_2O_2 as described in the materials section. Add the solution to the blot for 1 min before detecting the blot.
8. Place the blot between two sheets of plastic foil (see Note 62) and detect the emitted light with any system available to you (see Notes 63 and 64).
9. The Western blots can be stripped and redetected using the so-called stripping buffers. After stripping (see Subheading 2) the blot is thoroughly washed and the immunodetection is performed again.

4. Notes

1. We have successfully used different detergents for co-immunoprecipitations. The IGEPAL can also be replaced by 1% Brij 96 or 0.5% Triton X100. We recommend testing different detergents.
2. If phosphorylation events need to be investigated the lysis and washing buffers may also be supplemented with 10 mM $MgCl_2$ and 1 mM sodium vanadate.
3. More diluted solutions of HCl may be used when approaching the target pH to avoid abrupt changes in pH.
4. We always use the same detergent in the lysis and the washing buffer.
5. The protease inhibitors can also be added individually. In this case we use 10 mM PMSF, 1 mM benzamidine, 5 μg/ml aprotinin, 3 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM EDTA.
6. We recommend ready-to-use solutions. We have never had problems with these solutions and it avoids working with acrylamide powder, which represents an even greater hazard than the ready-made solutions.
7. The purer the TEMED is the greater the shelf life will be (99% pure is advised). TEMED is subject to oxidation, which causes a gradual loss of TEMED reactivity with time. TEMED is also very hygroscopic and will gradually accumulate water, which will accelerate oxidative decomposition. The latter reason is why we store TEMED at room temperature. If TEMED is stored at 4°C and is opened and handled at room temperature it will more quickly be spoiled due to water condensation. We recommend replacing the TEMED stock half yearly.
8. Ammonium persulfate is also very hygroscopic and additionally decomposes immediately when in contact with water, which results in a rapid loss of reactivity. This is why ammonium persulfate solutions should be prepared fresh daily and the crystalline stock should be replaced half yearly.
9. Never adjust the pH of SDS-running buffer. The added ions might interfere with gel focusing.
10. Blotting paper is alternatively called chromatography paper.
11. We use two self-made ECL solutions, which have been described earlier (11), have good signal intensity, and are more budget friendlier than commercial ECL solutions. The pCA-ECL is a robust product that is easy to handle. The 4IBPA-ECL requires a bit more handling but is interesting in situations where a low background signal has to be achieved. We recommend trying the pCA-ECL (which works just fine for most antibodies) first and switching to 4IPBA-ECL if high background occurs.

12. For both ECL solutions it is important that the pH of the 100 mM Tris–HCl, pH 8.8 is accurate. Often dilutions from 1 M or 1.5 M Tris–HCl, pH 8.6 stocks are performed to get 100 mM Tris–HCl, pH 8.6. pH measurement is only accurate in highly diluted solutions and the pH of a solution shifts to lower pH at dilution. Thus upon dilution of the stock solution the pH might drop well below 8.5. The pH for the HRP/peroxide/luminol reaction is optimal around pH 8.6–8.8; thus make sure to readjust the pH if necessary.
13. Luminol purity grade of 97% is sufficient for this application. However, recrystallization to a higher purity level may still increase the performance of the self-made ECL. We also replace the 30% H_2O_2 stock solution every year.
14. The pCA-ECL has comparable or higher signal intensity compared to the 4IPBA-ECL but shows slightly higher background chemiluminescence. We also obtained higher signal intensity compared to other commercial ECL solutions (11).
15. We replace the 30% H_2O_2 stock solution yearly.
16. The 4IPBA powder is best replaced yearly.
17. The 4IPBA stock solution degrades to boric acid and iodophenol within minutes when it is diluted in the 100 mM Tris–HCl solution. Some people develop headaches when breathing this iodophenol during the detection of the Western blot.
18. We use this stripping buffer when we want to redetect the blot with other phospho-specific antibodies.
19. The solution turns slightly viscous. If the solution is so viscous that the blot does not freely float around, reduce the amount of SDS.
20. We use this buffer to strip blots if we do not plan to redetect with phospho-specific antibodies any more.
21. 2-mercaptoethanol is toxic and volatile. We recommend using tightly sealable boxes (which can be purchased in regular supermarkets) for the stripping procedure if you do not have an oven installed in a hood.
22. It is best to work on ice in a cold room. If no cold room is available it is best to cover the ice container with the samples with a tray so that the samples remain well cooled during the different incubation times.
23. The specificity of the antibody is paramount for the success of the experiment. Different antibodies have to be tested for their suitability in IP and Western blot. Figure 1 provides an overview of the workflow for validating antibodies.
24. Cytokine receptors are heavily glycosylated which results in highly shifted (in comparison to the expected molecular weight), multiple, and diffuse bands in Western blot detections.

Thus it is hard to interpret if the antibody of choice which is used to detect the cytokine receptor is specific. Alternatively or additionally to an siRNA approach to validate the antibody, the receptor can be de-glycosylated by treatment of the lysates with different glycosidases (see Fig. 2). Endoglycosidase H (EndoH) is a recombinant glycosidase which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins but does not release complex oligosaccharides. When proteins are correctly processed through the endoplasmic reticulum (ER) and Golgi, they become resistant to EndoH. Thus treatment of the cytokine receptor immunoprecipitates or of the lysates with EndoH will reveal which protein bands on the Western blot have not yet been processed beyond the ER. PNGaseF (also called N-glycosidase F) is an amidase which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. Thus treatment of lysates or immunoprecipitates with PNGase F will reveal the native polypeptide size of a protein. The molecular mass of the un-glycosylated cytokine receptor band can now also be compared to the theoretical expected value. All Western blot bands that are not shifted upon the two treatments are nonspecific bands.

25. Control samples showing the specificity of the co-immunoprecipitation should also be included. One important control is to perform the IP protocol with lysate without precipitating antibody. This shows if the putatively "co-precipitated" protein is in fact nonspecifically interacting with the sepharose. A lysate in which the co-precipitated protein is knocked down (e.g., by an siRNA approach) should also be performed. This control will reveal whether the detection antibody (the one which is used in the Western blot detection) is unspecifically detecting another protein of similar migration behavior. This seemingly improbable situation is surprisingly common and has occurred several times in our lab. One explanation for this being so common might be that antibody-providing companies often test their antibodies in the presence of excess peptide (against which the antibody has been generated) as a negative control (personal communication of many sales representatives of different companies). Of course using this peptide in any assay (Western blot detection, immunoprecipitation, or immunofluorescence) as a negative control will compete with the specific and the unspecific signal generated by the antibody (and thus is no real control). Obviously antibodies detecting proteins of the right molecular weight in Western blots will be chosen for sale even though there is unrecognized, nonspecific detection of a protein migrating with a similar mobility (only experiments in cells not expressing this protein of choice can yield information about specificity versus unspecificity of the

antibody). Thus we strongly recommend using knockdown experiments to validate the precipitating and the detecting antibodies. Fortunately, for Janus kinases, negative control cell lines lacking each of the Jaks exist. Jak3 expression is restricted to the hematopoietic system (12) and the fibrosarcoma cell lines U4C, γ2A, and U1D lack Jak1, Jak2, or Tyk2, respectively (4, 13, 14). If a protein cannot be knocked down efficiently it is a good idea to verify co-precipitation with more than one detecting antibody. Last, but not least, it is important to perform Western blots of the lysates from which the immunoprecipitates have been generated in order to compare the lysate expression levels of the same proteins detected in the immunoprecipitation. This might not only reveal differences of expression but also possible degradation problems which might occur in the lysates during immunoprecipitation and which might account for unreproducible results.

26. Janus kinase phosphorylation upon cytokine stimulation can also be determined from the co-immunoprecipitates for some robust cytokine receptor antibodies (e.g., IL2Rβ immunoprecipitates; see Fig. 3). This can be of advantage since many antibodies used to precipitate the Jaks (to investigate their phosphorylation) lead to a strong background phosphorylation of the Jaks upon IP so that the cytokine-induced phosphorylation can barely be seen anymore, although it can clearly and specifically be detected from the lysates (see Fig. 3 and (10)). These problems in detecting activated phosphorylated Jaks from Jak immunoprecipitations might result from autoactivation of the Jaks upon aggregation on the sepharose or it might be that the majority of precipitated Jaks do not associate with the cytokine receptor through which the stimulus has occurred (the four Jaks bind to a much greater variety of cytokine receptors). Moreover a proportion of the Jaks which have been precipitated might not have been bound to cytokine receptors present at the cell surface (as some of the cytokine receptors are present in the ER or Golgi as part of their maturation process and in which Jak binding is involved (reviewed in ref. (8)). However, when precipitating the cytokine receptor complex, the associated Jak is sure to be activated after stimulation through this cytokine receptor. This latter situation reflects the lysate situation much better if the signal intensity is strong enough to be detected reproducibly (which is more difficult in this Co-IP situation). Much depends on the quality of the antibodies used in this case.

27. This only applies to adherent cells. If working with non-adherent cells, pellet the wanted amount of cells by centrifugation at 200 rpm for 5 min. Remove the medium and resuspend the cell pellet in ice-cold lysis buffer.

28. Any method available can be used here.
29. The total amount of lysate should not be used for the immunoprecipitation here, since a gel with the lysates should always be run in parallel to the IP to make sure that expression levels of the different proteins investigated by IP can also be checked in the lysate. This also enables you to check if a given treatment (e.g., cytokine stimulation) was successful, for example, by detecting activated signalling proteins in the lysate by Western blot. This 120 μl of lysate is enough to produce 4–5 Western blots.
30. Protein A fixed to magnetic beads can of course also be used. In this case follow the protocol of the magnetic bead provider to precipitate the immunocomplexes.
31. Take care to always prepare protein A sepharose slurry for a few more samples than you actually have.
32. Protein A binds to Fc-fragments of antibodies. Unfortunately it does not bind all isotypes equally well and even binds some very inefficiently. If you use a precipitating antibody isotype that does not bind protein A, you can still check if protein G (which has a different binding specificity for the different isotypes of antibody) is able to precipitate your antibody. If this also does not work a "bridging" antibody can be used. Antibodies recognizing a certain isotype of antibody (this would be the isotype of your precipitating antibody) can be bought from different providers. Such an antibody can be used to saturate the protein A sepharose (3 μg can be used for this). After washing 3 times with lysis buffer this antibody-bound sepharose A can be used to precipitate the immunocomplexes.
33. Before adding the slurry to the samples shorten the pipette tip so that the orifice is widened somewhat. With some tips the opening is so small that sepharose particle aggregates might block the tip, which leads to unequal amounts of sepharose added, which in turn yields unreproducible results.
34. When performing the experiment for the first time you can keep an aliquot of this supernatant to check if the IP was quantitative. This can be checked by directly performing a Western blot of the supernatant or by performing a second IP with this supernatant. In this way the amount of antibody can be optimized if wanted.
35. The strength of the binding of the co-immunoprecipitated protein and the background of the Western blot detection determine the washing steps. If the background of the blot is high more washing steps are needed. However if the co-precipitated signal is weak the washing steps might better be reduced in case the complex is not very stable. In the same line of thought a higher concentration of detergent in the washing

buffer reduces background while less detergent might enhance the signal intensity of the co-precipitated protein. This is why the composition of the washing buffer might need to be optimized. We recommend testing the lysis buffer as washing buffer first as this works for most situations.

36. Acrylamide is toxic and so are PAGE gels. Also polymerization reactions are not accomplished to 100% and some highly toxic monomers are embedded in the gel matrix. Do not touch PAGE gels without wearing gloves.
37. However, special care has to be taken to ascertain the best possible migration behavior of the samples, e.g., some systems have a tendency to accumulate air bubbles under the gel when mounting the gel. Little things like this can have massive repercussions on the resolution of the protein bands in the gel.
38. This depends on the proteins which should be resolved on the gel. 7.5–10% gels are best for most Janus kinases and cytokine receptors.
39. Best prepare the 20% ammonium persulfate shortly before use.
40. As little ammonium persulfate and TEMED as possible should be used. Excess of either can lead to oxidation of sample proteins or changes in buffer pH, which can affect the resolution of the proteins on the gel. Polymerization should occur within 15–20 min but the time one should allow for the polymerization to be complete is about 2 h. If the gelation takes longer than 20 min to start, the inhibitory effects of atmospheric oxygen will begin to appear and the amounts of peroxide and TEMED should be increased. The gels should be cast at room temperature (23–25°C is optimal for polymerization). It is best to have all materials and solutions at room temperature before starting. Oxygen can serve as an inhibitor since it can function as a free radical trap. This is why degassing the solutions used to produce the gel can help if there are problems of reproducibility.
41. This also protects the polymerizing gel from atmospheric oxygen.
42. Usually the gel is polymerized enough after 15–20 min to remove the isopropanol and then to cast the stacking gel on top of the separating gel. The two gels have polymerized for 1–2 h by the time the whole procedure is finished. Thus one does not have to wait for 2 h before casting the stacking gel.
43. This is a precaution because the presence of isopropanol could lead to precipitation of some proteins.
44. Stacking gels have more contact with oxygen than the isopropanol-overlaid separating gel. This is why we use more initiators for stacking gels.

45. This is important since it removes insoluble debris which can lead to poorer resolution of the samples.
46. Depending on the buffer system the gels are run under very different conditions. This only applies to our Laemmli-type system.
47. Always cut the paper so that the paper area exceeds the gel area so that you do not "lose" lanes when the gel slightly extends during handling. This can happen with gels of low percentage.
48. Handle the membrane only with tweezers or gloves and try to touch it as little as possible. Also take care to label your membranes in one way or another so that blots do not get mixed up. We label our membranes in one corner with a regular pen. However one has to take care that the writing does not dissolve when incubating the membrane with methanol.
49. Always wear gloves when handling the aqueous buffers. All buffers contain 20% methanol.
50. Western blot chambers of a different make can have the anode at the base and the cathode as lid. The stack is then built in the inverse order as described here.
51. Plastic 25 ml pipettes can be shortened (to 15–20 cm depending on the size of the blotting chamber) quite easily to fit this purpose. Unshortened pipettes often hit the outer rim of the chamber resulting in inefficient purging of the air.
52. Be careful not to press too much so that you do not purge the buffer from the stack. If the stack is too dry the gel will shrink during the procedure which will have an impact on the aspect of the bands in the Western blot detection and which will also lead to an inefficient transfer.
53. Always try to blot more than 1 gel to ensure that the top is placed flat on the stack surface.
54. If the chamber does not have a tightening mechanism (e.g., screws) to apply some pressure during the blotting procedure it is best to place a weight on top of the chamber. This serves to counteract the possibility that the gaseous reaction products (O_2 and H_2 which form at the anode and at the cathode, respectively) may interfere with the maintenance of a homogenous electric field across the blotting stack during the blotting procedure. If no pressure is applied, over time these gases may increasingly form bubbles between the layers of the blotting stack.
55. For different proteins optimal blotting times might be different. For high-percentage gels the transfer is slower. Small proteins are transferred faster.
56. Also here alternative settings might work as well.
57. The membrane must stay completely wet at all times!

58. 5% skim milk powder can also be used and is best prepared fresh each day.
59. We reuse these antibody solutions for up to 3 months, depending on the quality of the antibody of course. This does not work for all antibodies. Some are too unstable in high dilution to be used for long periods of time. This has to be determined for each antibody.
60. An overnight incubation gives better results, especially for the detection of the co-precipitated proteins.
61. We do not add NaN_3 (it inhibits the HRP) to the secondary antibody solution and also do not reuse the secondary antibody solution.
62. This guarantees that the blot stays wet during the procedure which is important if you want to strip and re-probe the blot again.
63. Depending on the means of light detection the data are more or less fit for quantitation. Quantitation of ECL signals from exposed films by densitometry has a very low linear range and should really be avoided. Quantitation of CCD camera-generated files can be reliable and the range of linear fit is much greater (15). The activity of, e.g., HRP (the enzyme catalyzing the ECL reaction) changes over time during the detection. Thus the linear range is limited as it is in other assays using enzymes (e.g., ELISA) and thus a standard has to be run on every blot to perform an accurate quantitation. This standard can be a series of dilutions of the detected lysate or IP (e.g., a mix of stimulated versus unstimulated lysate) so that at least a relative quantitation can be performed. Care has to be taken at the start of the experiment to ensure that there is enough material to perform the standard. Furthermore there are many ECL providers and they use different reagents so that different chemical reactions lead to the generation of the light and the linearity and duration of light emission varies from product to product. This leads to inter-laboratory differences that are suboptimal for quantitative data comparison. Thus, ECL is unlikely to become the standard method for Western quantitation in the future.
64. The blots can also be detected using fluorescently labeled secondary antibodies. Blocking buffers and washing steps might have to be optimized for this. Fluorescence detection is the method of choice for quantitation of Western blots as the linear range is far greater than that for ECL. Far infrared fluorophores, which do not interfere with auto-fluorescence of biomolecules (in contrast to many "visible-spectrum" fluorophores), are best suited to weak signals such as those associated with the detection of co-precipitated proteins.

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