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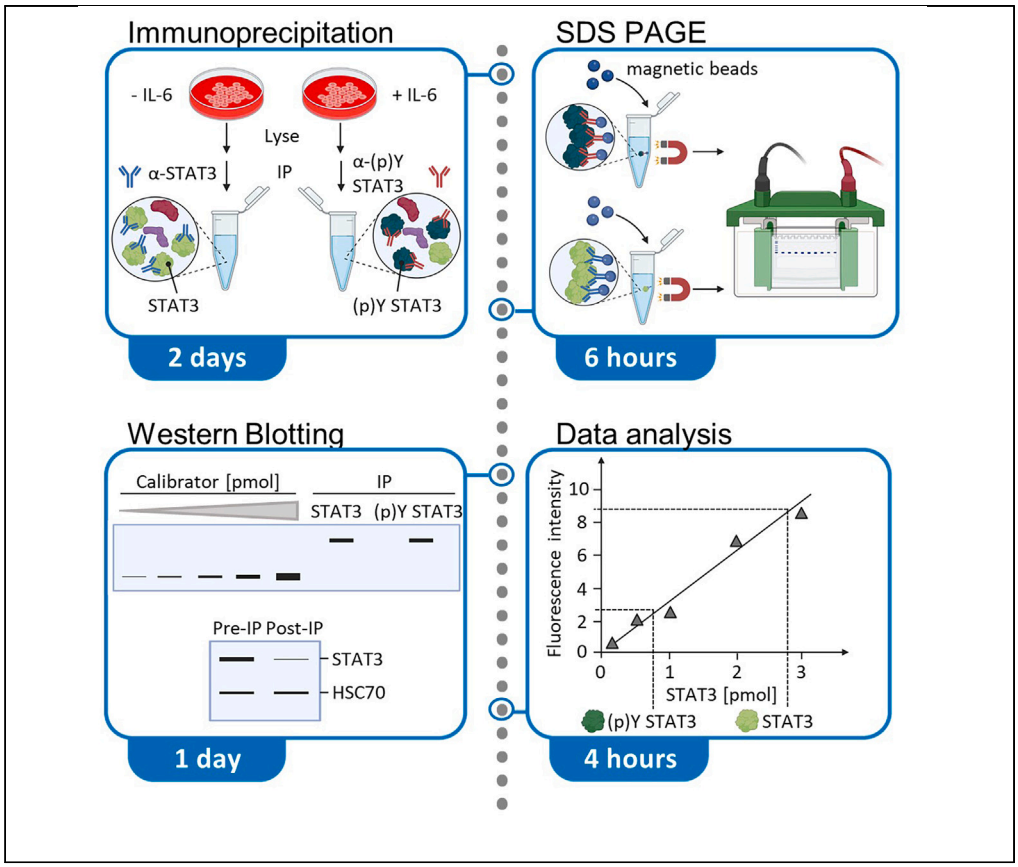
Quantification of total and phosphorylated STAT3 by calibrated western blotting



Quantification of intracellular proteins is essential to understand signaling. Here, we describe quantification of the expression and phosphorylation of the transcription factor STAT3. We present isolation of total and phosphorylated STAT3 from cell lysates by immunoprecipitation, followed by SDS-PAGE and western blot together with known amounts of a calibrator protein that shares an epitope with the precipitated proteins. Finally, we explain how to relate the amount of precipitated protein to the amount of calibrator protein considering the efficiency of immunoprecipitation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Quantification of total and unphosphorylated STAT3

Immunoprecipitation of total and phosphorylated STAT3 with correction for IP efficiency

SDS-PAGE and western blot with unphosphorylated STAT3 calibrator proteins

Optimized for quantification of STAT3 but can be easily expanded to other proteins

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Quantification of total and phosphorylated STAT3 by calibrated western blotting

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SUMMARY

Quantification of intracellular proteins is essential to understand signaling. Here, we describe quantification of the expression and phosphorylation of the transcription factor STAT3. We present isolation of total and phosphorylated STAT3 from cell lysates by immunoprecipitation, followed by SDS-PAGE and western blot together with known amounts of a calibrator protein that shares an epitope with the precipitated proteins. Finally, we explain how to relate the amount of precipitated protein to the amount of calibrator protein considering the efficiency of immunoprecipitation.

For complete details on the use and execution of this protocol, please refer to Dittrich et al. (2012)[1] and Reeh et al. (2019).[2]

BEFORE YOU BEGIN

Molecular biology, biochemistry, and systems biology methods are utilized to study cellular signaling, with quantitative experimental data being essential for understanding mechanisms of signaling.[3] Absolute quantification of intracellular signaling proteins and membrane-bound receptors is fraught with different problems and requires specific approaches. Here, we describe a method to quantify expression and phosphorylation of intracellular signaling proteins.

Quantification of intracellular signaling proteins can be performed, e.g., with sophisticated microarrays or mass spectrometry approaches. Without doubt these approaches allow precise high throughput quantification of the expression and activation of signaling proteins. However, they rely on specific machinery and know-how and are expensive. Alternatively, the quantitative calibrated western blot approach as described here allows accurate and cost-efficient quantification of intracellular proteins without the need of specific machinery.

This protocol describes how to quantify both phosphorylated and unphosphorylated signaling proteins using recombinant unphosphorylated calibrator proteins. First, a defined number of cells is lysed and the proteins of interest (here either STAT3 or tyrosine phosphorylated ((p)Y) STAT3 are isolated by immunoprecipitation (IP). IP guarantees that the precipitates only contain total STAT3 or (p)Y STAT3, respectively.

The quantities of the proteins in both precipitates are then related to the same unphosphorylated calibrator protein of known concentration. To do so, the precipitated proteins together with defined

amounts of the recombinant calibrator protein are separated by SDS-PAGE, blotted onto a membrane, and stained with a detection antibody that recognizes an epitope which is present in all three protein species (STAT3, (p)Y STAT3, and calibrator STAT3). Based on the strength of the immunodetection signal of the calibrator protein, the concentration of the protein of interest is calculated. Note, that the results obtained must be corrected for the efficiency of the IPs. To determine the efficiencies of the IPs, the amounts of STAT3 and (p)Y STAT3 in aliquots of the lysate before (Pre) and after (Post) IP are monitored in a semi-quantitative western blot.

In the following, STAT3 will serve as example for the quantification of intracellular signaling proteins. Information on how to adopt this protocol to other proteins are given in the Notes sections. This protocol does not include detailed descriptions of SDS-PAGE and western blot. Any established protocol for these techniques can be used.

Before you begin, prepare all buffers and media needed according to the materials and equipment section. While it is preferable to have all buffers freshly prepared, for convenience, some may be stored long-term, as indicated in the materials and equipment section.

Institutional permissions

Before starting work, inform yourself about the safe handling of the chemicals and cells described here and about the local protection and disposal regulations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HSC70 (1:1,000)	StressMarq	Cat# SMC-104B
STAT3 (1:1,000 in western blot, 1 µg per IP)	Cell Signaling Technology	Cat# 9139
(p)Y705 STAT3 (1:1,000 in western blot, 1 µg per IP)	Cell Signaling Technology	Cat# 9145
IRDye 800CW Goat anti-Rabbit IgG (1:10,000)	LI-COR	Cat# 926-32211
IRDye 680RD Goat anti-Mouse IgG (1:10,000)	LI-COR	Cat# 926-68070
Chemicals, peptides, and recombinant proteins		
AEBSF hydrochloride	Carl Roth	Cat# 2931
Aminocaproic acid ($C_6H_{13}NO_2$)	Thermo Fisher Scientific	Cat# A14719
Aprotinin	Sigma-Aldrich	Cat# A1153
β-Mercaptoethanol	Carl Roth	Cat# 4227
Bovine serum albumin (BSA), pH 7	Serva Electrophoresis	Cat# 11930
Bromophenol blue	Sigma-Aldrich	Cat# 114391
DMEM	Gibco	Cat# 21068028
EDTA	Carl Roth	Cat# 8043.1
Fetal calf serum (FCS)	Gibco	Cat# 10270-106
Glycerol	ITW Reagents	Cat# A3739
Glycine	Carl Roth	Cat# 3908.1
Hydrochloric acid (HCl)	Carl Roth	Cat# 4625
IL-6	PeproTech	Cat# 200-06
Soluble IL-6 receptor (sIL-6R)	PeproTech	Cat# 200-06RC
Leupeptin	Sigma-Aldrich	Cat# L2884
Methanol	Carl Roth	Cat# 4627
IGEPAL CO-630	Sigma-Aldrich	Cat# 542334
Penicillin (50,000 U/mL) /streptomycin (50 mg/mL)	Roche	Cat# 15140-122
Pepstatin A	Sigma-Aldrich	Cat# P4265
Potassium chloride (KCl)	Carl Roth	Cat# 6781
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth	Cat# 3904
Recombinant calibrator protein (STAT3 AA 670–769)	Abnova	Cat# 16145965
Sodium chloride (NaCl)	ITW Reagents	Cat# 131659.1214

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium dodecyl sulfate (SDS)	Carl Roth	Cat# CN 30
Sodium fluoride (NaF)	Sigma-Aldrich	Cat# 106449
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2\ H_2O$)	Carl Roth	Cat# 4984
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich	Cat# 450243
TRIS	VWR	Cat# 0497
Experimental models: Cell lines		
HEK293	ATCC	Cat# CRL-1573
Software and algorithms		
Excel or another spreadsheet program	Microsoft	
Image Studio or another image analysis software	LI-COR	
Other		
Dynabeads Protein G for immunoprecipitation	Thermo Fisher Scientific	Cat# 10004D
Nitrocellulose Blotting Membrane Premium 0.2 µm	Amersham Protran	Cat# 10600004
Cell culture equipment including incubator, sterile hood, centrifuge, vacuum pump	Any company	
Neubauer chamber or other equipment to determine cell count	Any company	
Overhead shaker for reaction tubes	Any company	
Magnetic rack for 1.5 mL reaction tubes	Thermo Fisher Scientific	Cat# 12321D
Equipment for SDS-PAGE and western blot	Any company	
Imager for fluorescent western blot	Any company	

MATERIALS AND EQUIPMENT

All chemicals were used in the quality *pro analysis*.

4× Laemmli buffer		
Reagent	Final concentration	Amount
TRIS/HCl (1 M, pH 6.8)	250 mM	25 mL
Glycerol (85%)	40%	47.2 mL
beta-Mercaptoethanol	20%	20 mL
SDS	8%	8 g
Bromophenol Blue	0.02%	20 mg
ddH_2O		ad 100 mL
Total	**N/A**	**100 mL**

Note: Laemmli buffer can be prepared in larger volumes and stored in aliquots at −20°C for up to one year. Store aliquots in use at 20°C–22°C for up to one month.

Note: Dilute 4× Laemmli buffer to 2× Laemmli buffer in ddH2O upon warming to 37°C and gently mixing to ensure all components have been dissolved.

⚠ CRITICAL: SDS can cause skin, eye, and respiratory irritation. Avoid inhaling dusts, substance contact, and keep away from heat and sources of ignition. Personal protective equipment must be used when handling this compound.

PBS		
Reagent	Final concentration	Amount
NaCl	200 mM	11.69 g
KCl	2,5 mM	0.186 g
$Na_2HPO_4 \times 2\ H_2O$	8 mM	1.42 g
KH_2PO_4	1.5 mM	0.2 g
ddH_2O		ad 1 L
Total	**N/A**	**1 L**

Note: Adjust pH to 7.4 with HCl before adjusting to final volume with ddH_2O.

Note: Autoclave PBS directly after preparation and subsequently store the sterile solution at 20°C–22°C for up to one year.

RIPA lysis buffer		
Reagent	Final concentration	Amount
TRIS/HCl (1 M, pH 7.4)	50 mM	50 mL
NaCl	100 mM	5.8 g
EDTA (0.5 M)	1 mM	2 mL
Nonidet P-40	0.5%	5 mL
Glycerol (85%)	15%	172 mL
ddH_2O		ad 1 L
Total	**N/A**	**1 L**
add inhibitors right before use		
Na_3VO_4 (0.1 M)	1 mM	100 µL
NaF (0.5 M)	10 mM	20 µL
AEBSF (0.1 M)	0.8 mM	80 µL
Aprotinin (25 mg/mL)	5 µg/mL	2 µL
Leupeptin (5 mg/mL)	5 µg/mL	10 µL
Pepstatin A (1,5 mg/mL)	3 µg/mL	3.5 µL
RIPA lysis buffer		10 mL
Total	**N/A**	**10 mL**

Note: RIPA lysis buffer without inhibitors can be prepared in larger volumes and stored at 4°C for up to one year. Store phosphatase inhibitors (Na_3VO_4 and NaF) at 4°C and protease inhibitors (AEBSF, Aprotinin, Leupeptin, Pepstatin) at −20°C for up to one year. Add inhibitors immediately before use. Keep RIPA lysis buffer on ice after adding inhibitors. Do not store the buffer after adding protease and phosphatase inhibitors.

RIPA wash buffer		
Reagent	Final concentration	Amount
TRIS/HCl (1 M, pH 7.4)	50 mM	50 mL
NaCl	100 mM	5.8 g
EDTA (0.5 M)	1 mM	2 mL
Nonidet P-40	0.5%	1 mL
ddH_2O		ad 1 L
Total	**N/A**	**1 L**

Note: Store at 4°C for up to one year.

Note: RIPA wash buffer does not contain glycerol as glycerol reduces binding of the magnetic beads to the magnetic rack.

SDS running buffer		
Reagent	Final concentration	Amount
TRIS	250 mM	3 g
Glycine	1.92 M	14.4 g
SDS	0.1%	1 g
ddH_2O		ad 1 L
Total	**N/A**	**1 L**

Note: To prevent precipitation of SDS store SDS running buffer at 20°C–22°C for up to 6 months.

Note: SDS is a detergent. Do not shake the mixture to avoid formation of foam.

⚠ CRITICAL: SDS can cause skin, eye, and respiratory irritation. Avoid inhalation or contact, and keep away from heat and sources of ignition. Personal protective equipment must be used when handling this compound.

TBS-N		
Reagent	Final concentration	Amount
TRIS	20 mM	2.42 g
NaCl	140 mM	8 g
IGEPAL CO-360	0.1%	1 mL
ddH_2O		ad 1 L
Total	**N/A**	**1 L**

Note: Adjust pH to 7.6 with HCl before adjusting to final volume with ddH_2O. Store at 20°C–22°C for up to one year.

⚠ CRITICAL: HCl can cause severe skin burn and eye damage. Avoid contact. Personal protective equipment must be used when handling this compound.

Note: IGEPAL CO-360 substitutes Nonidet P-40.

- 10% BSA: 5 g BSA in 50 mL TBS-N.

Note: Store at 4°C for up to 2 weeks.

Note: BSA dissolves very poorly at 20°C–22°C. Excessive stirring produces foam. Cooling the BSA suspension at – 20°C for 5 min shortens the dissolving time enormously and no excessive stirring is necessary.

STEP-BY-STEP METHOD DETAILS

Day 1: Cell culture

Timing: 24 h

In this step a defined number of cells is seeded on three cell culture dishes. Cells on one dish (control) will be used to determine the exact number of cells at the time-point of cell lysis. The cells on the other two dishes will be used to quantify STAT3 (unstimulated) and (p)Y STAT3 (stimulated), respectively (Figure 1).

1. Seed 10^6 cells/cell culture dish on three 10 cm cell culture dishes in standard medium and incubate at 37°C, 10% CO_2 in a humidified atmosphere for 24 h.

⚠ CRITICAL: Always use a certified sterile hood and sterilised equipment and solutions, and follow aseptic technique when working with cells.

⚠ CRITICAL: Depending on the strength of expression of the protein of interest, the efficiency of the IP, and the linear range of the calibrator proteins, the number of cells analyzed needs to be adjusted in preliminary experiments. The amount of the precipitated

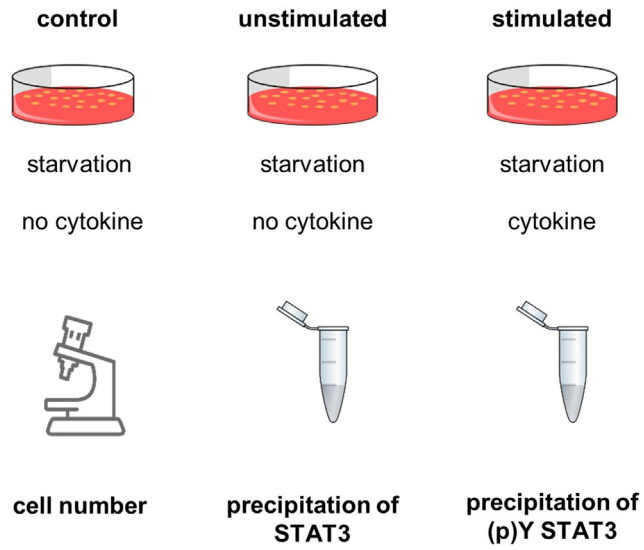

Figure 1. Schematic overview of the experimental set up to quantify STAT3 and (p)Y STAT3
The figure was generated using bioRender (https://biorender.com).

proteins of interest should always be within the linear range of the calibration curve. Avoid any extrapolation as the linear range of quantitative western blot is restricted.

Note: For HEK293 cells standard medium refers to DMEM plus 10% FCS plus Pen/Strep. For other cell types adjust medium and supplements.

Day 2: Stimulation, cell lysis and immunoprecipitation

Timing: 24 h

Timing: 40 min (for step 7)

Timing: 20 h (for steps 8 and 9)

Next, the cells will be starved and subsequently cells on dish 1) will be counted to determine the cell number, cells on dish 2) will be left untreated and cells on dish 3) will be stimulated with cytokines (e.g., IL-6) to induce phosphorylation of STAT3. The cells on dish 2) and dish 3) are then lysed, and target proteins of interest will be isolated from the cell lysates by IP.

2. Remove cell culture medium from all three cell culture dishes by vacuum aspiration and gently wash cells once with sterile PBS.
3. Replace PBS on each dish with 4 mL FCS-free cell culture medium warmed up to 37°C and starve cells at 37°C, 10% CO_2 in a humidified atmosphere for 2 h.

Note: Since washing and starving the cells might affect cell number and/or receptor expression, and thus the strength of cytokine-induced STAT3 phosphorylation, it is necessary to treat the cells on all three cell culture dishes identically.

Note: Cells are starved to reduce basal signaling induced by FCS in the medium. Starvation time needs to be adjusted for your cell type of choice to find the best trade-off between reduced basal STAT3 activation and reduced responsiveness to stimulation caused by e.g., receptor internalization or cell stress.

4. Determination of number of cells.
   a. Remove starvation medium from the first cell culture dish (Figure 1, control) by vacuum aspiration.
   b. Add 1 mL trypsin to the cells on this dish.
   c. Incubate cells at 37°C for 5 min.
   d. Stop the reaction with 5 mL medium + FCS.
   e. Resuspend the cells by pipetting up and down.
   f. Count the number of cells in the suspension e.g., with a Neubauer chamber.
   g. Discard cells after counting.

   Note: Make sure that all cells on the cell culture dish are detached, so that the determined cell number/dish is correct.

   Note: Make sure to log the number of cells/dish and not the number of cells/mL.

5. Stimulate cells on one of the two remaining cell culture dishes with cytokine (e.g., 20 ng/mL IL-6 + 200 ng/mL sIL-6R) by directly adding the cytokine solution into the serum-free medium on the cell culture dish to induce STAT3 phosphorylation (Figure 1, stimulated). Keep the cells on the second cell culture dish unstimulated (Figure 1, unstimulated).
6. Incubate cells at 37°C, 10% CO_2 in a humidified atmosphere for 15 min.

   Note: Stimulation of cells expressing membrane-bound IL-6 receptor (IL-6R) with ≥20 ng/mL IL-6 for 15 min induces strong phosphorylation of STAT3. However, most cells, e.g., HEK293 cells, do not express membrane-bound IL-6R. For cells that do not express membrane-bound IL-6R, add 200 ng/mL sIL-6R to 20 ng/mL IL-6.[2] Pre-mix IL-6 and sIL-6R before stimulation to allow formation of an IL-6:sIL-6R complex.

   Note: It is not necessary to quantify the amount of (p)Y STAT3 for each time point and each dose of IL-6/sIL-6R of interest, as this is very expensive and time-consuming. Alternatively, you can quantify the amount of (p)Y STAT3 at one dose and time of stimulation (reference sample). With this knowledge you can convert semi-quantitative STAT3 phosphorylation kinetics from relative to absolute values by following the rule of proportion.[1]

7. Cell lysis.
   a. Remove the starvation medium from the two remaining cell culture dishes containing stimulated and unstimulated cells (Figure 1) by vacuum aspiration.
   b. Gently wash cells with ice-cold PBS.
   c. Place cell culture dishes on ice.
   d. Add 300 µL RIPA lysis buffer (supplemented with phosphatase and protease inhibitors) per dish.
   e. Detach cells from the cell culture dish surface with a rubber scraper.
   f. Transfer cell suspensions to 1.5 mL reaction tubes.
   g. Incubate lysates on ice for 20 min. Vortex lysates every 10 min rigorously. Pre-cool the table top centrifuge during incubation to 4°C.
   h. Remove cell debris by centrifugation at 4°C, 13.800 × g for 15 min in a table top centrifuge.
   i. Transfer the supernatants to fresh 1.5 mL reaction tubes.
   j. Discard the pellets.

   Note: Keep all buffers and samples on ice to avoid protein degradation and dephosphorylation.

   Note: If the cells are detaching very easy from the cell culture dish do not wash them with PBS before addition of RIPA lysis buffer as the number of cells on the cell culture dish could be reduced.

A certain unknown percentage of the proteins of interest will not be isolated during the following IP and will thus reduce the amount of quantified protein. To correct for this, the Pre-IP samples, i.e., input samples, are essential to determine the efficiency of the IPs as described in the quantification and statistical analysis section.

8. Preparation of Pre-IP samples.
   a. Transfer 15 μL of the lysates from unstimulated and stimulated cells, respectively, into fresh 1.5 mL reaction tubes.
   b. Add 5 μL 4× Laemmli buffer to each lysate.
   c. Denature the Pre-IP samples for 5 min at 95°C.
   d. Store the samples at 20°C–22°C until the next day. They are now ready for SDS-PAGE.

   Alternatives: You can also bypass the determination of IP efficiency in some cases. To do so, you spike known increasing amounts of calibrator protein into the samples before IP. In this case, the efficiencies of the IP of the protein of interest and of the IP of the calibrator protein are identical and no correction for efficiency of IP needs to be done. Additionally, protein of interest and spiked calibrator protein are detected within the same lane of the western blot, which reduces the influence of background signals on the quantification. However, this approach is only possible if the antibody used for IP binds both protein of interest and calibrator protein. This is not the case when (p)Y STAT3 is analyzed and an unphosphorylated STAT3 calibrator protein is used. In addition, you must be able to distinguish your protein of interest and the calibrator protein on the basis of different protein masses and the associated different mobility in SDS-PAGE.

9. IP of proteins of interest.
   a. Add 1 μg of anti-STAT3 and anti-(p)Y STAT3 antibody to the lysates of unstimulated and stimulated cells, respectively.
   b. Incubate the lysates for 16–20 h at 4°C on an overhead shaker.

   ⚠ CRITICAL: Antibodies with very high affinity to their epitopes should be chosen to precipitate proteins of interest. These antibodies should almost completely precipitate the protein of interest from the lysate. With the antibodies listed in the key resources table we achieve efficiencies above 90% for IPs of STAT3 and (p)Y STAT3.

Day 3: SDS-PAGE and western blot

Timing: 28 h

Timing: 4.5 h (for step 10)

Timing: 1 h (for step 11)

Timing: 30 min (for step 12)

Timing: 15 min (for step 13)

Timing: 20 min (for step 14)

Timing: 1.5 h (for step 15)

Timing: 2 h (for step 16)

Timing: 16 h (for step 17)

On this day, IPs are completed. Then, the precipitated proteins are separated by SDS-PAGE along with a serial dilution of the calibrator protein. Finally, the proteins are transferred to a nitrocellulose membrane and stained with specific antibodies that bind with the same affinity to the calibrator protein and the protein of interest. In addition, the IP efficiencies are determined.

10. Incubation of lysates with Protein G Dynabeads.
    a. Washing of Protein G Dynabeads.
        i. Spin down and vortex Protein G Dynabeads to resuspend them in the storage buffer into a homogeneous bead slurry.
        ii. Transfer 10 µL of Protein G Dynabeads/IP into a fresh 1.5 mL reaction tube.
        iii. Add 1 mL of ice-cold RIPA wash buffer and vortex for 5 s.
        iv. Spin down Protein G Dynabeads to ensure that no beads remain on the inner lid of the reaction tube.
        v. Isolate the Protein G Dynabeads by placing the reaction tube on a magnetic rack until all beads are bound to the magnet (app. 15 s).
        vi. Carefully remove the supernatant by gentle vacuum aspiration with the tube still on the magnetic rack.
        vii. Resuspend the Protein G Dynabeads in 10 µL RIPA wash buffer/IP.
    b. Add 10 µL of the Protein G Dynabeads to the lysates of unstimulated and stimulated cells, respectively. Make sure no beads remain on the wall of the tube.
    c. Incubate the lysates at 4°C on an overhead shaker for 4 h.

Note: Use a 10 µL pipette tip placed on the end of the vacuum aspirator to carefully remove all buffer from the beads without sucking up the beads. Alternatively, you can remove the supernatant with a pipette. Make sure that you do not touch the beads bound to the magnet with large pipette tips.

Note: During the 4 h incubation time in step 10 prepare SDS gels (step 11) and dilute calibrator proteins (step 12).

Note: Dynabeads are coupled to either Protein A or Protein G. The choice of Protein A or Protein G depends on the host and isotype of the antibody used for IP. Protein G generally binds to mouse and rat IgGs with higher affinity than Protein A, while Protein A binds to rabbit IgGs with higher affinity than Protein G. Consult the bead manufacturer to determine which beads are more suitable to isolate your IP antibody.

Note: Reducing agents and chelators such as DTT and EDTA might reduce the binding capacity of the beads. We, however, even observe high IP efficiencies when cells are lysed in RIPA lysis buffer containing 1 mM EDTA.

Alternatives: Isolation of precipitates with Protein A/G agarose or sepharose is also possible. Usage of magnetic beads has the advantage that precipitates are nicely visible and no centrifugation steps are necessary.

11. Casting of SDS gels.

Prepare two discontinuous SDS polyacrylamide gels with 10% separating gel and 4% stacking gel and a minimum of 10 sample pockets.

Note: Many types of SDS-PAGE chambers are available, so the exact procedure is not described here.

Note: It is not necessary to use pre-casted commercial gels for quantitative western blots. To obtain good results of SDS-PAGE and western blot, however, always freshly prepare SDS gels right before use.

Note: The acrylamide concentration of the separation gel must be chosen according to the molecular weight of the proteins of interest and the calibrator proteins. STAT3 has a molecular weight of 86 kDa and the STAT3 calibrator protein used here of 35 kDa.

12. Preparation of the dilution series of the calibrator protein.
    a. Dilute 0.1, 0.5, 1, 2, and 3 pmol of the calibrator protein in 15 µL RIPA lysis buffer, respectively.
    b. Add 5 µL 4× Laemmli buffer to each dilution.
    c. Denature the samples for 5 min at 95°C.
    d. Store the samples at 20°C–22°C. They are now ready for SDS-PAGE.

Note: We describe calibrator proteins consisting of a fragment of STAT3. Thus, it is necessary to calculate the number of calibrator proteins in pmol instead of the mass of the calibrator proteins.

Note: Recombinant calibrator proteins can be either bought commercially or expressed at site, e.g., in *E.coli*. For proper quantification, it is important that 1) the epitope of the detection antibody is present in both the calibrator protein and the protein of interest, 2) the molecular weight of the standard protein is known, and 3) the concentration of the calibrator protein is given.

13. Preparation of Post-IP samples.
    a. Place the lysates - now containing complexes of protein of interest, IP antibody and Protein G Dynabeads - on a magnetic rack until all beads are bound to the magnet (app. 15 s).
    b. Transfer 15 µL lysate from the reaction tubes containing lysates from unstimulated or stimulated cells, respectively, into fresh 1.5 mL reaction tubes.
    c. Add 5 µL 4× Laemmli buffer to each lysate.
    d. Denature the Post-IP samples for 5 min at 95°C.
    e. Store samples at 20°C–22°C. They are now ready for SDS-PAGE.

Note: This control is necessary to determine the efficiency of IP. A certain unknown percentage of the proteins of interest will not be isolated in the IP and will thus reduce the amount of quantified protein. The Post-IP sample contains the non-isolated protein of interest and is thus essential to determine the IP efficiency as described in the quantification and statistical analysis section.

14. Isolation of precipitated proteins.
    a. Leave the remaining lysates - containing complexes of protein of interest, IP antibody and Protein G Dynabeads - on a magnetic rack.
    b. Carefully remove the supernatant by gentle vacuum aspiration with the tube still on the magnetic rack.
    c. Wash the Protein G Dynabeads three times with 1 mL ice-cold RIPA wash buffer. Do not vortex the lysates at this point, as this would destroy the immune complexes, but slowly pipette the sample up and down several times. Make sure to also remove RIPA wash buffer from the inner side of the lid.
    d. After removing the RIPA wash buffer, wait two additional minutes after the last wash step to remove any remaining RIPA wash buffer that accumulates at the bottom of the reaction tube. Otherwise, the volume of the IP will be too large to load it onto the SDS-PAGE.

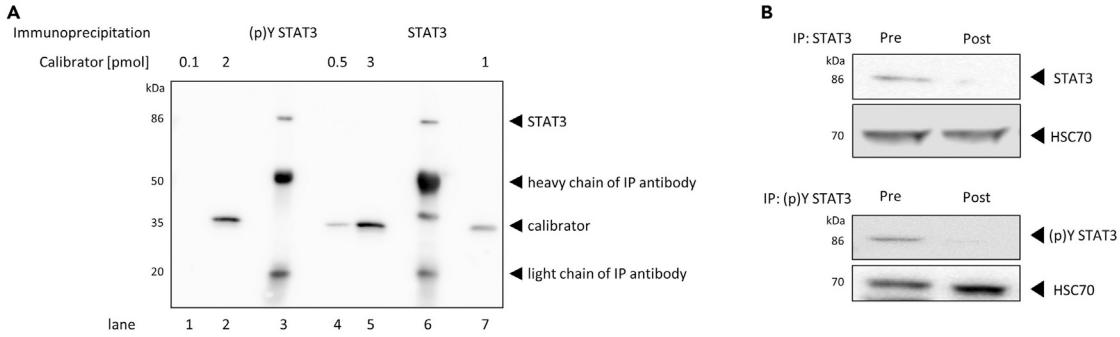

Figure 2. Quantification of STAT3 and p(Y) STAT3 by calibrated western blot

(A) HEK293 cells stably expressing membrane-bound IL-6 receptor were either left untreated or stimulated with 20 ng/mL IL-6 for 15 min. Subsequently, cells were lysed and STAT3 and (p)Y STAT3 were precipitated using specific antibodies against STAT3 and (p)Y STAT3, respectively. Precipitates together with recombinant STAT3 calibrator proteins were analyzed by western blot using a specific antibody against STAT3.

(B) Before (Pre) and after (Post) IP aliquots of the lysates were taken and analyzed by western blot using specific antibodies against STAT3, (p)Y STAT3, and HSC70. HSC70 serves as loading control. Figure 2 is modified from[1] with permission of Royal Society of Chemistry.

e. Remove the reaction tubes from the magnetic rack and resuspend the Protein G Dynabeads in 15 µL 2× Laemmli buffer. Make sure no beads remain on the wall of the tube.
f. Denature the precipitated proteins for 5 min at 95°C.
g. Store samples at 20°C–22°C. They are now ready for SDS-PAGE.

Note: Quantification results are best if precipitates are not stored at this point but directly loaded onto the SDS-PAGE.

15. SDS-PAGE.
    a. Load precipitated STAT3 and precipitated (p)Y STAT3 together with the dilutions of the calibrator protein onto one SDS gel. This gel is from now on called quantification gel (Figure 2A).

Note: Randomized loading of the samples disrupts lane correlation i.e., the influence of experimental artefacts on adjacent samples.

Note: Keep the reaction tubes with the precipitated proteins in the magnetic rack while loading the samples to avoid loading the magnetic beads onto the SDS gel.

Note: Sometimes the lanes that include precipitated proteins widen during the SDS-PAGE. To avoid affecting other samples, load 10 µL of 2× Laemmli buffer in the lanes next to the lanes containing the precipitated proteins.

    b. Load Pre- and Post-IP samples for STAT3 and (p)Y STAT3 on the second gel. Separate the pairs of STAT3 and (p)Y STAT3 samples with a marker. This gel is from now on called efficiency gel (Figure 2B).

Note: The marker between the STAT3 and phosphorylated STAT3 Pre- and Post-IP pairs, respectively, is necessary as you will cut this membrane in two parts after western blot.

    c. Run the SDS-PAGE.
16. Western blot and blocking of membrane.
    a. Blot the two SDS gels onto nitrocellulose membranes following an established western blot protocol.

Note: Any established western blot protocol can be used here.

Note: For detection of proteins with fluorophore-coupled secondary antibodies nitrocellulose membrane is preferred against PVDF membrane as it results in less background staining.

b. Block free binding sides on nitrocellulose membranes with 10% BSA at 20°C–22°C for 1 h.

Note: If you observe high background when detecting the membranes, you can also use commercially available blocking reagents. Avoid using milk powder as blocking reagent when detecting phosphorylated proteins.

17. Staining of proteins of interest and calibrator proteins with specific antibodies.
    a. Cut the membrane that contains the Pre-IP and Post-IP samples (efficiency gel) vertically along the marker in two parts.

Note: To cut the membrane, carefully transfer it to a clean glass plate without touching the membrane with your fingers. Use a clean scalpel to cut and avoid drying the membrane.

b. Incubate the three membrane parts with primary antibodies for 16–20 h at 4°C on an orbital shaker.
    i. Quantification membrane: anti-STAT3 antibody (1:1,000 in TBS-N).
    ii. STAT3 IP efficiency membrane: anti-STAT3 and anti-HSC70 antibodies (each 1:1,000 in TBS-N).
    iii. (p)Y STAT3 IP efficiency membrane: anti-(p)Y STAT3 and anti-HSC70 antibodies (each 1:1,000 in TBS-N).

⚠ CRITICAL: The antibody used to stain proteins on the quantification membrane detects STAT3, (p)Y STAT3 and the calibrator protein with the same affinity. If you adjust this protocol to another protein of interest, choose a detection antibody that detects an epitope that is present on all three variants of your protein of interest and that is not changed by posttranslational modification of the protein of interest upon stimulation with cytokines.

⚠ CRITICAL: If you buy sets of GST-tagged recombinant proteins and (polyclonal) antibodies for detection, make sure to test whether the detection antibody (also) detects GST by staining pure GST or any other GST-tagged protein with this antibody. In case the antibody detects GST, you must replace the detection antibody.

Note: Staining of HSC70 on the two IP efficiency membranes serves as loading control. You can also stain any other housekeeping protein, that is not affected by stimulation with the respective cytokine (Step 5) here.

Note: To avoid stripping artefacts you can mix the anti-STAT3 or anti-(p)Y STAT3 antibodies with the anti-HSC70 antibodies as the stained proteins differ in size. To stain two proteins of similar size together you have to use secondary antibodies coupled to different fluorophores.

Day 4: Detection of the stained proteins

Timing: 3 h

On this day the membranes will be incubated with secondary antibodies to allow detection of the stained proteins. Finally, results will be quantified and absolute numbers of STAT3 and (p)Y STAT3 will be calculated.

18. Detection.
    a. Wash the membranes three times in TBS-N at 20°C–22°C on an orbital shaker for 10 min.
    b. Incubate the three membrane parts with fluorophore-coupled secondary antibodies at 20°C–22°C on an orbital shaker in the dark for 30 min.
        i. Quantification membrane: anti-mouse antibody (1:10,000 in TBS-N).
        ii. STAT3 IP efficiency membrane: anti-mouse antibody (1:10,000 in TBS-N).
        iii. (p)Y STAT3 IP efficiency membrane: anti-mouse and anti-rabbit antibodies (1:10,000 in TBS-N).

⚠ CRITICAL: Fluorophore-coupled secondary antibodies are light-sensitive. Thus, membranes should be incubated e.g., in dark boxes with lids.

Alternatives: Detection of western blots with horseradish peroxidase (HRP)-coupled secondary antibodies is possible. However, the linear range of enzymatic detection methods is smaller than that of fluorescence-based detection methods. In both cases you must titrate the calibration curve and the number of cells analyzed in preliminary experiments, to guarantee that the standard curve is linear and samples are in the range of the standard curve.

    c. Wash the membranes three times in TBS-N at 20°C–22°C on an orbital shaker in the dark for 10 min.
    d. Detect fluorescence using a fluorescence scanner.

⚠ CRITICAL: Make sure to work in the linear range of the fluorescence scanner and avoid saturated signals and thus overexposure. In case of saturation of the fluorescence intensity reduce the detector gain. If you are using HRP-coupled secondary antibodies, reduce the exposure time when signals are saturated.

    e. Calculate the amount of STAT3 and (p)Y STAT3 as described in the quantification and statistical analysis section.

EXPECTED OUTCOMES

An exemplary result of the quantification of STAT3 and IL-6-induced (p)Y STAT3 is shown in Figure 2 (modified from[1]). Figure 2A depicts the quantification membrane. Samples were loaded in a randomized order to reduce the influence of experimental artefacts, such as unequal transfer of proteins during western blot in neighboring lanes. Both STAT3 and (p)Y STAT3 have been successfully precipitated (lanes 3 and 6). In addition to the 86 kDa STAT3, two other proteins with approximate sizes of 50 kDa and 25 kDa are stained in lanes 3 and 6. These correspond to the heavy and the light chains of the IP antibodies that were isolated with the Protein G Dynabeads along with the precipitated proteins and that were denatured during sample preparation. Note, that the heavy and light chain of the IP antibodies are not recognized by the specific anti-STAT3 detection antibody, but by the polyclonal fluorophore-coupled secondary antibody. In lanes 1, 2, 4, 5 and 7 five different amounts of the calibrator protein are analyzed. Note, that the amount of precipitated STAT3 and (p)Y STAT3 is within the linear range of the calibrator proteins.

Figure 2B depicts the two parts of the efficiency membrane. The amount of the housekeeping protein HSC70 is not affected by the IPs, indicating that the same volume of the lysates was analyzed before and after IP. Notably, precipitation of both STAT3 and (p)Y STAT3 is near 100%. However, the residual amount of protein of interest after IP that has not been precipitated needs to be considered, when calculating the results.

Table 1. Exemplary fluorescence intensities of different amounts of calibrator protein

Calibrator protein [pmol]	Fluorescence intensity [a.u.]
0.1	8,05E+05
0.5	1,51E+07
1.0	2,82E+07
2.0	6,96E+07
3.0	8,73E+07

Recombinant STAT3 calibrator proteins were analyzed by western blot using a specific antibody against STAT3 and a fluorophore-coupled secondary antibody(Figure 2A). Quantification of fluorescence intensity was done by using Image Studio software.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Quantify the fluorescence intensity of the stained proteins on all three membrane parts using a image quantification Software (e.g., Image Studio).

⚠ CRITICAL: For absolute quantification you must use minimally processed raw image files from a western blot scanner. It is not possible to quantify x-ray films or file formats that do not contain raw data such as .pdf or .jpeg. Some programs lose image information when pictures are e.g., rotated. Always refer to the user manual of the image quantification software for proper quantification and background subtraction.

2. Calibration curve.
   a. Plot the fluorescence intensity of the stained calibrator protein against the number of recombinant calibrator proteins [pmol] (Table 1; Figure 3).
   b. Calculate a linear calibration curve with the equation:

$$Fluorescence\ Intensity\ =\ a\ STAT3\ [pmol] + b \qquad \text{(Equation 1)}$$

where a is the slope and b is the y-intercept of the calibration curve (Figure 3).

Note: Perform analysis of the results and linear regression in Excel or any other spreadsheet program.

3. Calculate efficiency of IPs (Table 2).
   a. Divide the fluorescence intensity of stained STAT3 and (p)Y STAT3 in Pre- and Post-IP samples, respectively, by the fluorescence intensity of stained HSC70 for each sample to normalize for unequal loading.

Table 2. Exemplary calculation of IP efficiency

Sample	Fluorescence intensity STAT3	Fluorescence intensity HSC70	Fluorescence intensity STAT3 normalized by fluorescence intensity HSC70	% Of total STAT3	STAT3 IP efficiency
Pre-IP STAT3	4,83E+05	8,95E+05	5,40E-01	100	90%
Post-IP STAT3	5,11E+04	9,57E+05	5,34E-02	10	
Sample	**Fluorescence intensity (p)Y STAT3**	**Fluorescence intensity HSC70**	**Fluorescence intensity (p)Y STAT3 normalized by fluorescence intensity HSC70**	**% of total (p)Y STAT3**	**(p)Y STAT3 IP Efficiency**
Pre-IP (p)Y STAT3	1,85E+05	7,89E+05	2,34E-01	100	92%
Post-IP (p)Y STAT3	1,39E+04	7,16E+05	1,94E-02	8	

HEK293 cells stably expressing membrane-bound IL-6 receptor were either left untreated or stimulated with 20 ng/mL IL-6 for 15 min. Subsequently, cells were lysed and STAT3 and (p)Y STAT3 were precipitated using specific antibodies against STAT3 and (p)Y STAT3, respectively. Before (Pre-) and after (Post-) IP aliquots of the lysates were taken and analyzed by western blot using specific antibodies against STAT3, (p)Y STAT3, and HSC70. HSC70 is used as loading control (Figure 2B). Quantification of fluorescence intensities was done using Image studio software. Table 2 summarizes the calculation of IP efficiencies.

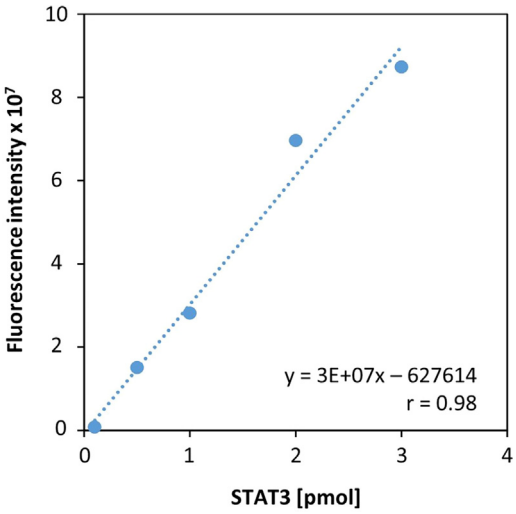

Figure 3. Calibration curve
Recombinant STAT3 calibrator proteins were analyzed by western blot using a specific antibody against STAT3 and a fluorophore-coupled secondary antibody (Figure 2A). Quantification was done by using Image Studio software (Table 1). The equation displayed is the result of a linear regression. R is the correlation coefficient. Figure 3 is modified from[1] with permission of Royal Society of Chemistry.

b. Set normalized Pre-IP samples to 100% and calculate the percentage of residual STAT3 and (p) Y STAT3 in the Post-IP samples (x %).
c. Calculate the efficiency of each IP with the equation.

$$Efficiency = 100\,\% - x\,\% \quad \text{(Equation 2)}$$

4. Calculation of the amount of STAT3 and (p)Y STAT3 (Table 3).
   a. Calculate the number of STAT3 and (p)Y STAT3 in [pmol]/lysate based on the fluorescence intensity of stained precipitated STAT3 and (p)Y STAT3 and Equation 1 (Figure 3).
   b. Divide this number by the total number of cells analyzed (as determined in Step 4 on day 2) to calculate STAT3 and p(Y) STAT3 in [pmol]/cell. Here, we exemplarily use 4.5E+06 cells/dish.
   c. Divide this number by 1E+12 to calculate the number of STAT3 and (p)Y STAT3 in [mol]/cell.
   d. Multiply this number with the Avogadro constant (6.02214E+23) to calculate the number of STAT3 and (p)Y STAT3/cell.
   e. Correct this number by the efficiency of the respective IP with the equation:

$$Corrected\ number\ of\ proteins = \frac{Number\ of\ proteins\ x\ 100}{Efficiency\ of\ IP\ [\%]} \quad \text{(Equation 3)}$$

   f. Calculate the concentration of STAT3 and (p)Y STAT3 in a cell [nM] based on the mean cell volume (0.5 pL for HEK293 cells).
      i. Divide the result from Step 4e., which gives the number of STAT3 and p(Y) STAT3 in one cell, by 5E+13 to calculate number of STAT3 and (p)Y STAT3 in 1 L of cells.

Table 3. Exemplary quantification of STAT3

Sample	Fluorescence intensity	STAT3 [pmol]/lysate	STAT3 [pmol]/cell	STAT3 [mol]/cell	STAT3/cell	STAT3/cell corrected by IP-efficiency	STAT3 [nM]
IP STAT3	1.76E+07	0.61	1.35E-07	1.36E-19	81,633	90,704	300
Sample	Fluorescence intensity	(p)Y STAT3 [pmol]/lysate	(p)Y STAT3 [pmol]/cell	(p)Y STAT3 [mol]/cell	(p)YSTAT3/ cell	(p)Y STAT3/cell corrected by IP-Efficiency	(p)Y STAT3 [nM]
IP (p)Y STAT3	1.72E+07	0.59	1.32E-07	1.36E-19	78,957	85,823	286

This table summarizes the calculation of the concentration of STAT3 and (p)Y STAT3 in a cell based on the raw data presented in Figure 2A, the calibration curve presented in Figure 3 and efficiencies of IP presented in Table 2.

ii. Divide this number by the Avogadro constant (6.02214E+23) to calculate the concentration of STAT3 and (p)YSTAT3 in [M].
iii. Multiply this number by 1E0+09 to calculate the concentration of STAT3 and p(Y)STAT3 in [nM].

Note: You can estimate the cell volume from the mean diameter of freshly trypsinised cells. As these cells are round, the volume of a cell is calculated by calculating the volume of a sphere ($V = 4/3 \times \pi \times r^3$, where r is the radius of the cell). Alternatively, confocal pictures of the cells can be taken with a confocal microscope. Based on a z-stack of the cell you can use the voxel counter plugin for ImageJ to calculate the volume of the cell.

LIMITATIONS

The expression and posttranslational modification of proteins involved in JAK/STAT signaling differ strongly between different cell types and stimuli.[3] Thus, it is mandatory to repeat the quantification for each experimental system to be analyzed. Additionally, calibrated western blot is not applicable to determine the distribution of STAT3 within a cell population as in calibrated western blot whole cell lysates are analyzed.[4] As a remedy, quantification of signaling proteins in single cells can also be performed by flow cytometry as discussed in Miri et al.[5]

The protocol described here is optimized for quantification of intracellular proteins. Quantification of membrane-bound proteins such as cytokine receptors with this approach is in principle also possible. However, the results will greatly overrate the number of available receptors as with the approach described here, not only receptors at the cell surface but also internalized receptors and receptors that are currently produced and still stick in the secretory pathway will be quantified.

TROUBLESHOOTING

Problem 1

Low efficiency of IP when new antibodies are tested.

Potential solution 1

We advise you to reach for IP efficiencies higher than 70%. As some antibodies only bind to denatured proteins, it might increase the efficiency of the IP when 0.5%–2% SDS are added to the lysates before IP.

Problem 2

Results differ strongly between independent experiments.

Potential solution 2

Western blot is prone to errors such as unequal background or staining. These make quantification of stained membranes difficult. As a remedy, we only include results of those independent replicates in the final result, when no obvious staining artefacts (such as unequal background, air bubbles, spots) occurred, the Pearson correlation coefficient (r value) of the calibration curve is acceptable (i.e., $r > 0.9$) and the signal intensity of the stained proteins of interest is in the linear range of the calibration curve.

Problem 3

STAT3 is not strongly phosphorylated.

Potential solution 3

Phosphorylation of STAT3 depends on the dose and length of cytokine stimulation. Make sure to quantify STAT3 phosphorylation under conditions where STAT3 is highly phosphorylated.

Additionally, ensure high viability and responsiveness to cytokine stimulation. In our experience, cells should be about 80% confluent at the time of stimulation to meet this goals.

Problem 4

More than 100% of STAT3 is phosphorylated.

Potential solution 4

If more STAT3 is phosphorylated than STAT3 is expressed, an experimental error occurred. First, optimize the IP conditions, because when IP efficiency is low (i.e., lower than 70%) correcting for the efficiency will still lead to debatable results. Second, never extrapolate the calibration curve as the linear range of western blot is very small.

Problem 5

Treatment affects cell number.

Potential solution 5

Always control whether stimulation affects cell number (i.e., induces apoptosis or proliferation). In this case you have to count the number of treated cells before lysis instead of counting cells on the control cell culture dish.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and/or reagents should be directed to and will be fulfilled by the lead contact, Dr. Anna Dittrich, Institute of Biology, Department of Systems Biology, Otto-von-Guericke University Magdeburg, Universitätsplatz 2, Building 28, 39106 Magdeburg, E-Mail: anna.dittrich@ovgu.de.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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The graphical abstract was created using BioRender (https://biorender.com).

Figures 2 and 3 were reproduced from Dittrich et al.[1] with permission from the Royal Society of Chemistry.

AUTHOR CONTRIBUTIONS

Conceptualization, A.D.; investigation, A.D., N.K., N.M.; visualization, A.D., N.K., writing – original draft, N.K.; writing – review and editing, A.D., N.K., N.M.; supervision, A.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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