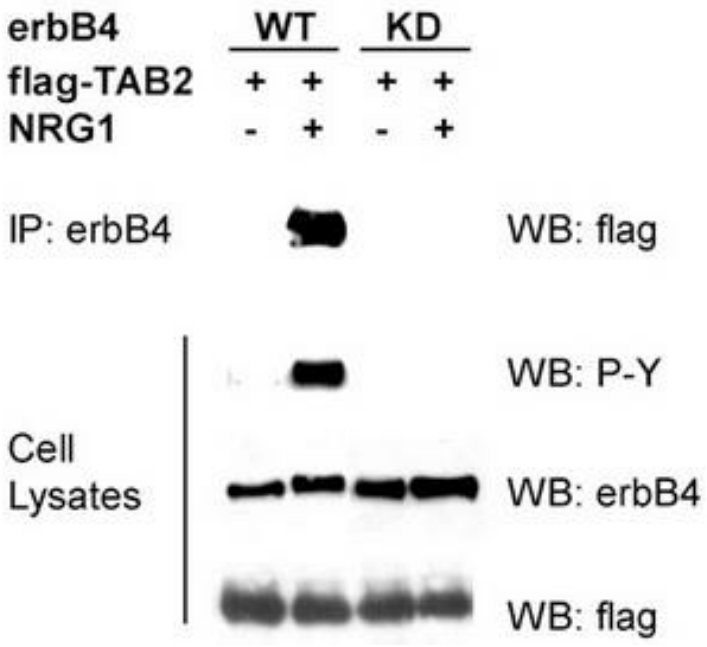
Advanced Cell Biology and Biotechnology

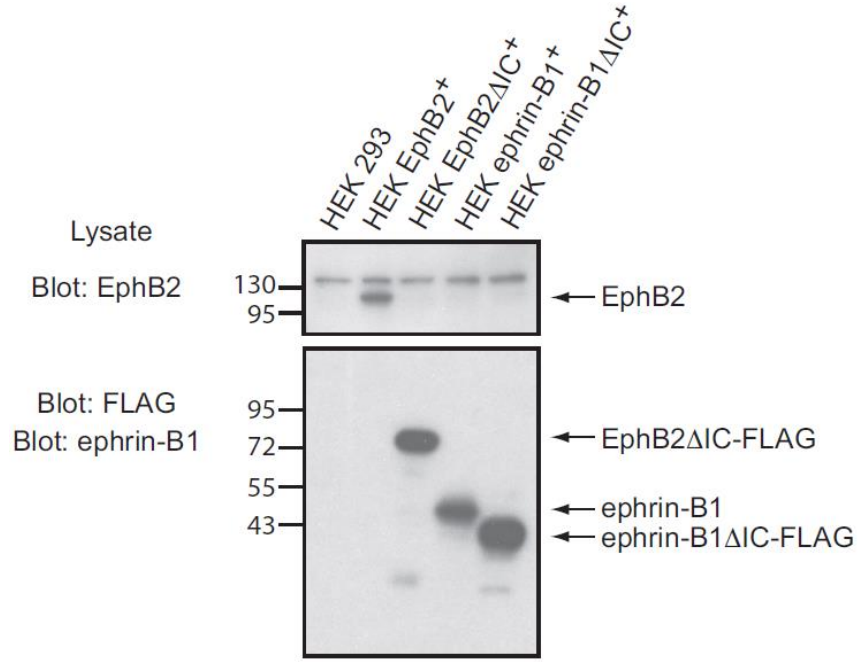
# ACBB 2021/22

# Course objective

The main objective of the course is to develop the ability to **critically analyse** and **interpret** the results of the scientific literature & to be able to apply this knowledge to afford new scientific questions.

- Protein expression
- Protein-protein interactions
- Protein post-translational regulation


erbB4
WT
KD
flag-TAB2
+ + + +
NRG1
- + - +
IP: erbB4
WB: flag
WB: P-Y
Cell Lysates
WB: erbB4
WB: flag


HEK 293
HEK EphB2+
HEK EphB2ΔIC+
HEK ephrin-B1+
HEK ephrin-B1ΔIC+
Lysate
Blot: EphB2
130
95
EphB2
Blot: FLAG
Blot: ephrin-B1
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72
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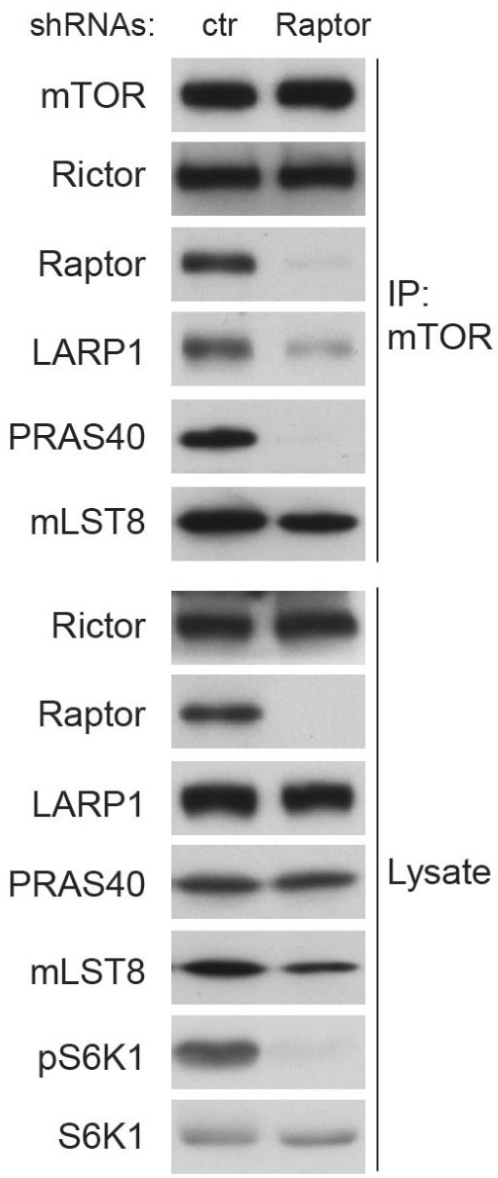
43
EphB2ΔIC-FLAG
ephrin-B1
ephrin-B1ΔIC-FLAG


shRNAs:
ctr
Raptor
mTOR
Rictor
Raptor
LARP1
PRAS40
mLST8
IP: mTOR
Rictor
Raptor
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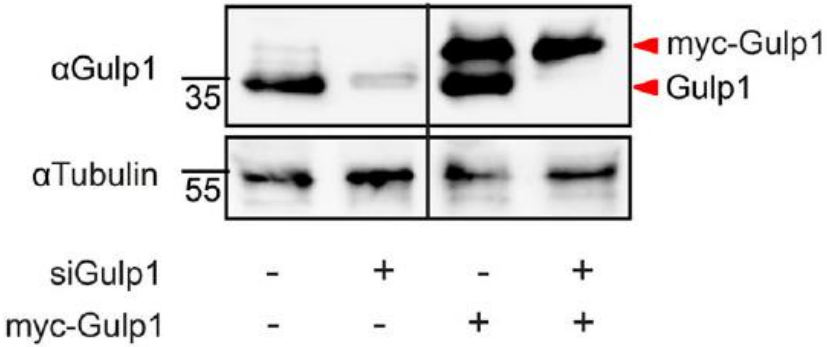
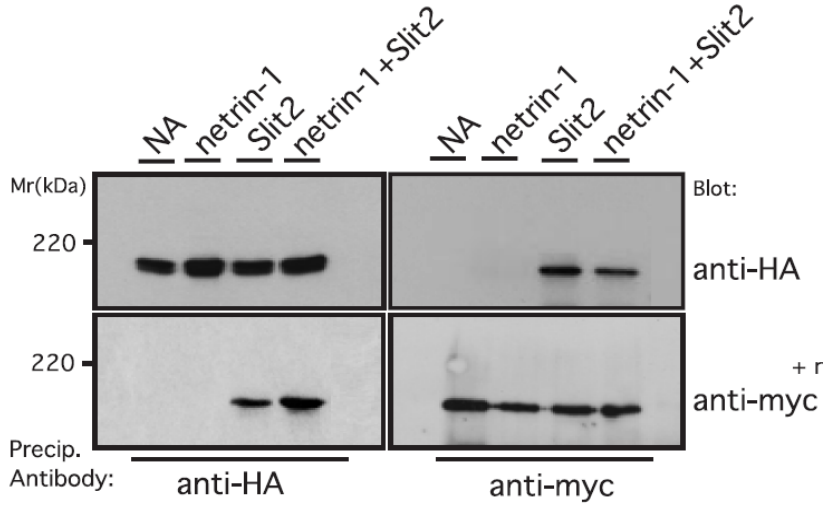
PRAS40
mLST8
pS6K1
S6K1
Lysate


αGulp1
35
myc-Gulp1
Gulp1
αTubulin
55
siGulp1
- + - +
myc-Gulp1
- - + +


NA
netrin-1
Slit2
netrin-1+Slit2
NA
netrin-1
Slit2
netrin-1+Slit2
Mr(kDa)
220
220
Blot:
anti-HA
anti-myc
+ r
Precip. Antibody:
anti-HA
anti-myc

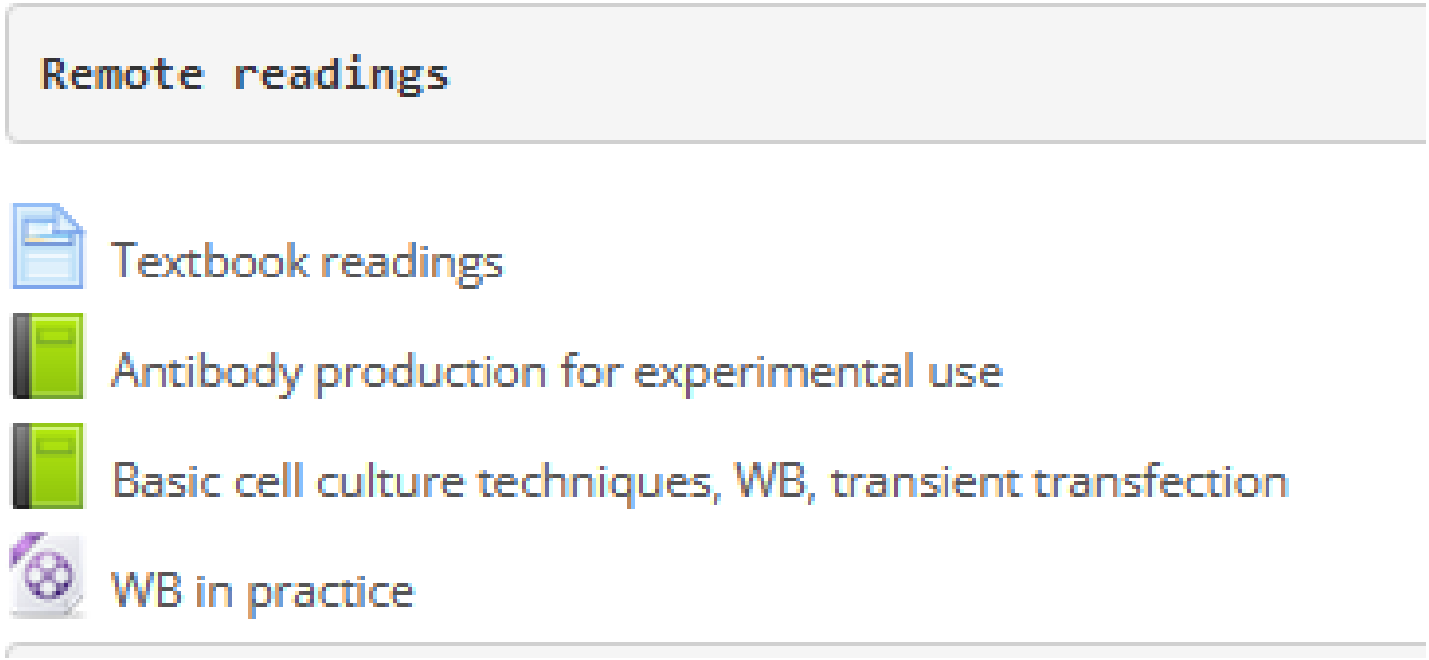
# Protein expression analysis

Western blotting, is a widely used technique to detect **protein expression** in biological samples (cell or tissue extracts) through antibody binding to the specific protein of interest.

The **precise binding** that occurs between an **antibody** and its **target protein** epitope allows detection of specific amino acid sequences within a protein.

**Antibody specificity** is necessary to obtain reliable results.

**Monoclonal** and **polyclonal antibody** characteristics and production are presented in our moodle page (prof. Perroteau's section).




https://www.citeab.com/

-a very good tool to find information and references about antibodies and lab reagents.

# Post-translational modification

Antibodies can also detect specific **post-translational modifications** of a protein.

**Phospho-specific antibodies** can be used to identify components of specific signaling pathways and to study changes in phosphorylation events in various biological contexts.

Antibodies specific to other modifications have been developed, to monitor changes in **acetylation, methylation, and ubiquitination** status of a protein.

Western blotting part I: protein isolation  (3’ 27’’)
https://www.youtube.com/watch?v=GJJGNOdhP8w

Western blotting part II: polyacrylamide gel electrophoresis (6’ 25’’)
https://www.youtube.com/watch?v=JcN0EkcHrKk

Western blotting part III: transfer, blotting and visualization (4’ 23’’)
https://www.youtube.com/watch?v=IoVzpL_heFo


Western blot videos


Watch the videos about western blot and answer the questions

The following videos are on JoVe, you can see them only if you have UNITO login/password:

SDS-PAGE (7’ 29’’)
https://www-jove-com.bibliopass.unito.it/v/5058/separating-protein-with-sds-page

WESTERN BLOT (8’ 47’’)
https://www-jove-com.bibliopass.unito.it/v/5065/the-western-blot

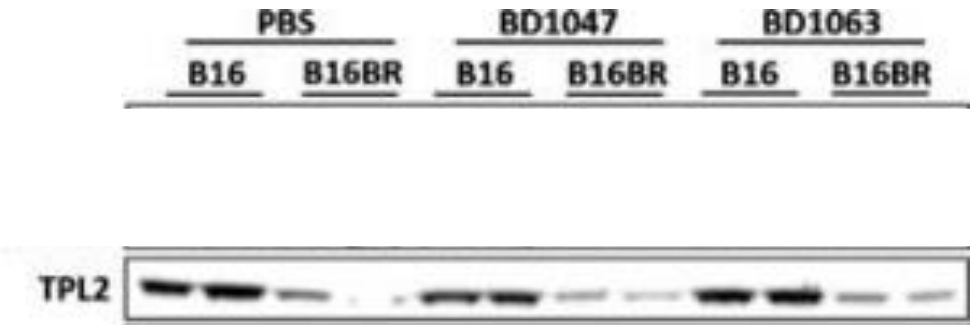
# Control of Your Experiment

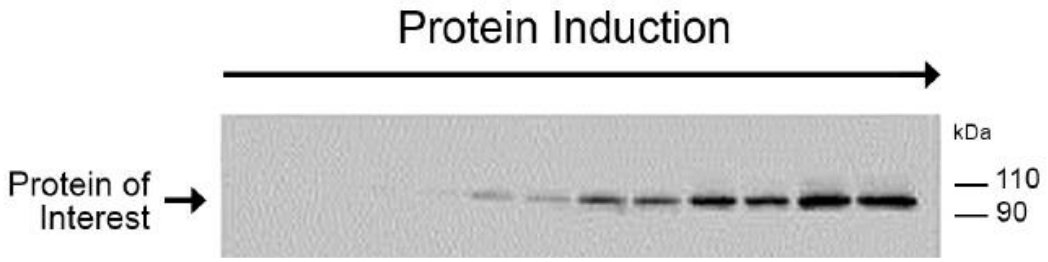
In any experiment you have to include the appropriate controls.

- when possible, negative and positive controls for the protein (or the protein modification) detected by the primary antibody
- loading control
- mock control
- ….

# Loading controls are used to:

- ensure equal loading of a gel
- ensure integrity of the sample
- quantitatively compare samples (sometime it is not possible to obtain equal loading of a gel)



# Loading controls are used to:

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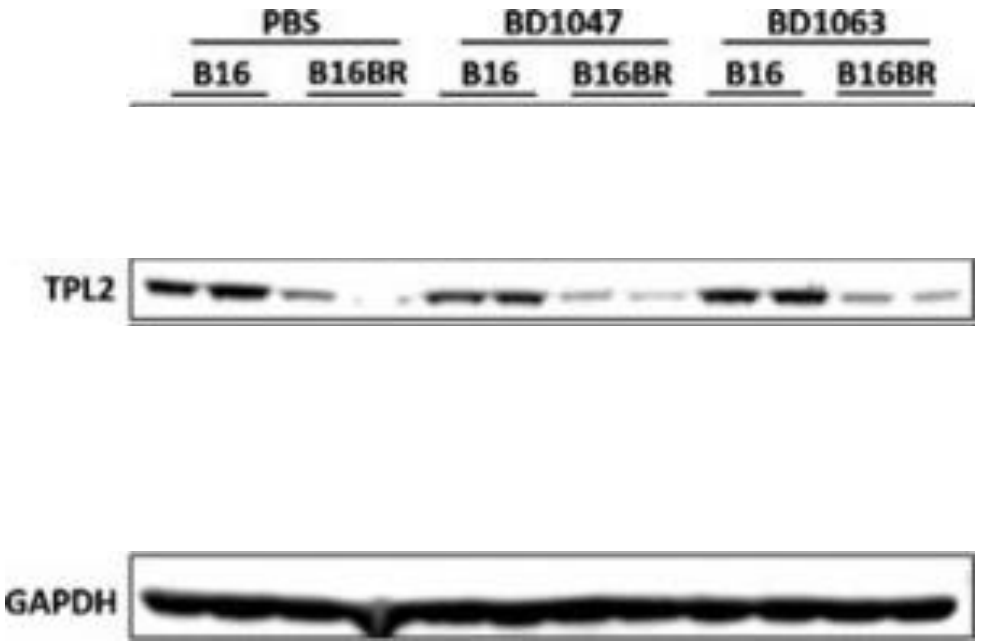
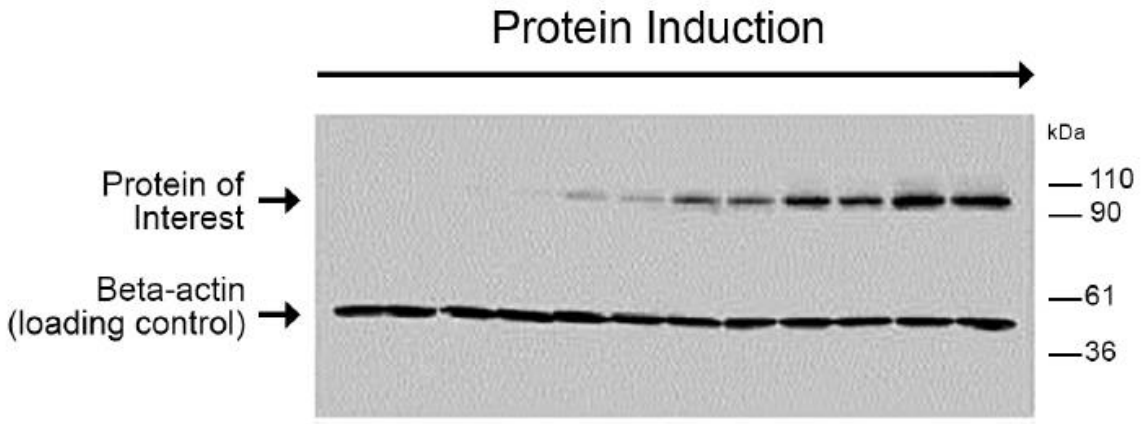
Proteins expressed at the same level in many cell lines and tissues, such as β-actin and GAPDH, are often used as loading controls to compare total protein levels in multiple samples.



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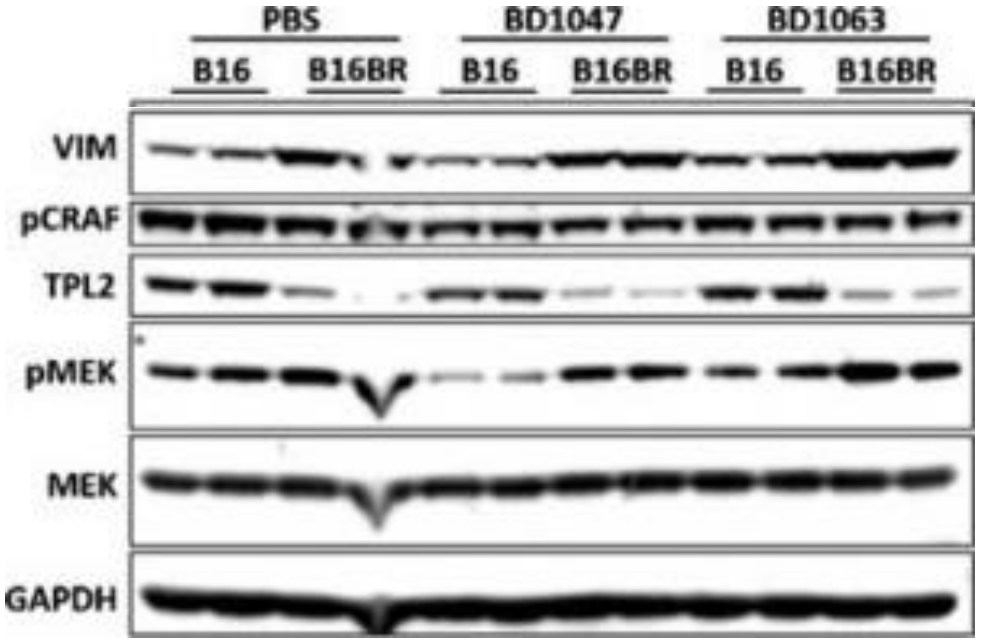
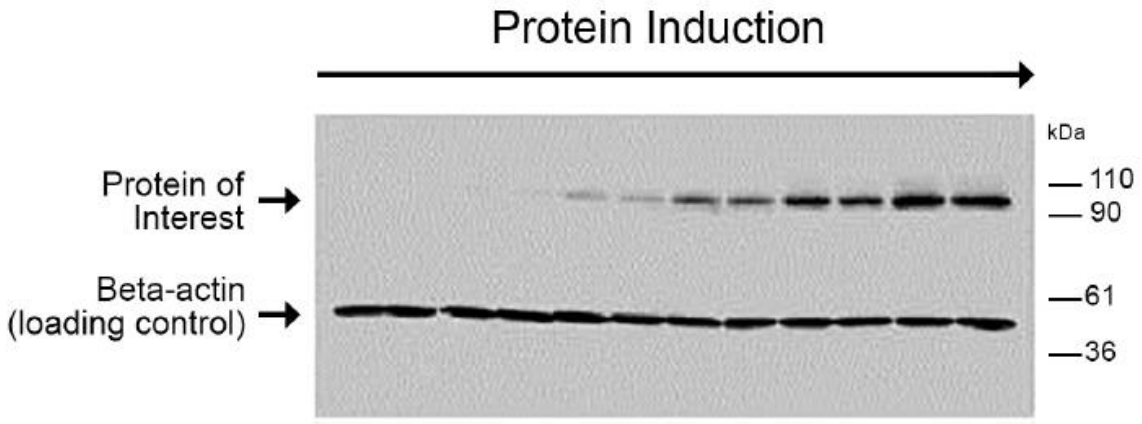
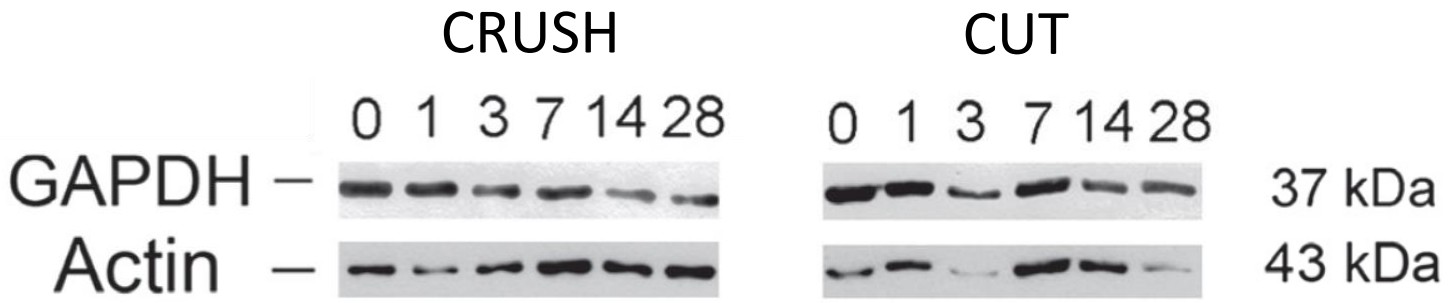


# Loading controls are used to:

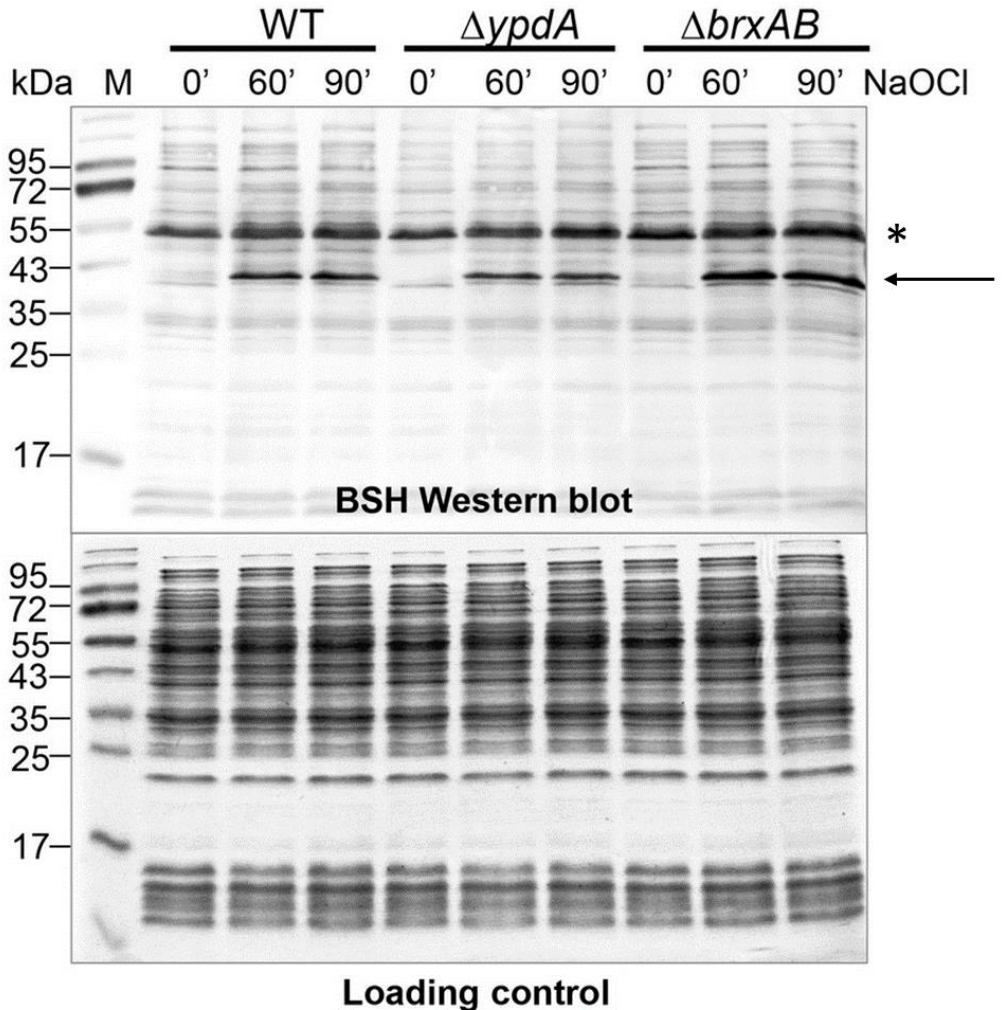
Proteins expressed at the same level in many cell lines and tissues, such as β-actin, α-tubulin, and GAPDH, are often used as loading controls to compare total protein levels in multiple samples.

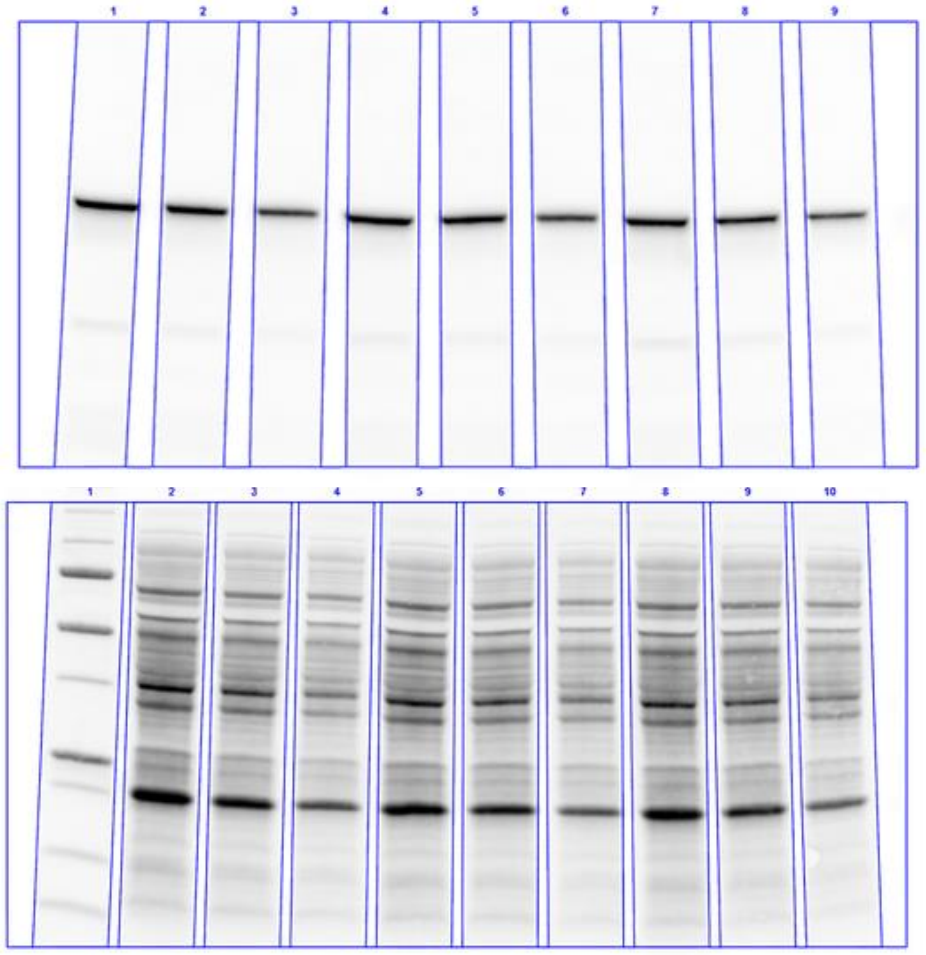
Nevertheless, sometimes housekeeping genes are affected by the treatment!!!



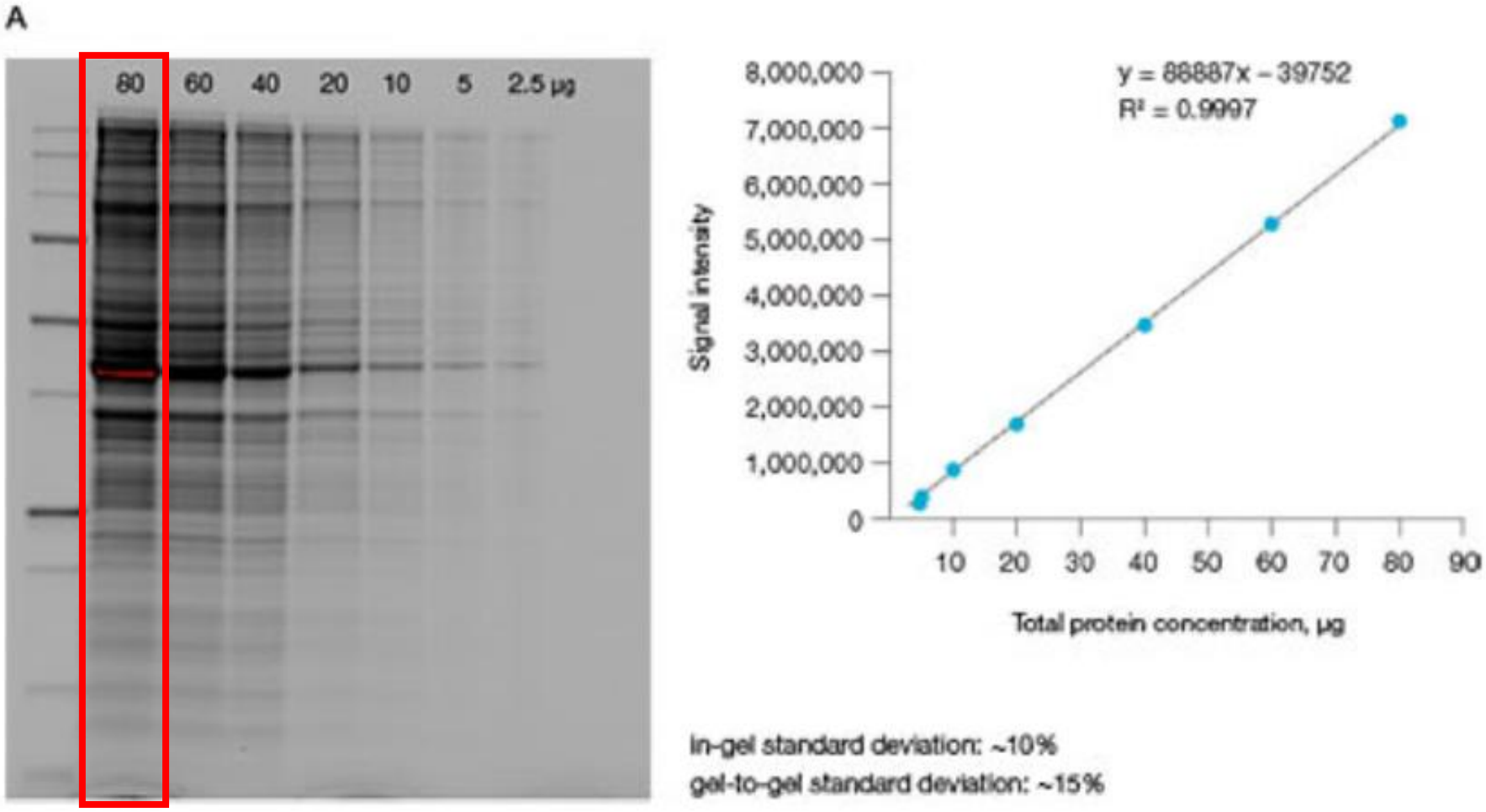

Which alternative could be used???

**Stain-free technology** utilizes the modification of **tryptophan** residues.
The trihalo compound modifies tryptophan residues in the protein by a covalent modification, generating a fluorescence signal. The signal is visualized by UV excitation.
Proteins that lack tryptophan residues, such as aprotinin, are not detected using this technology, but a single tryptophan residue is sufficient for signal activation.

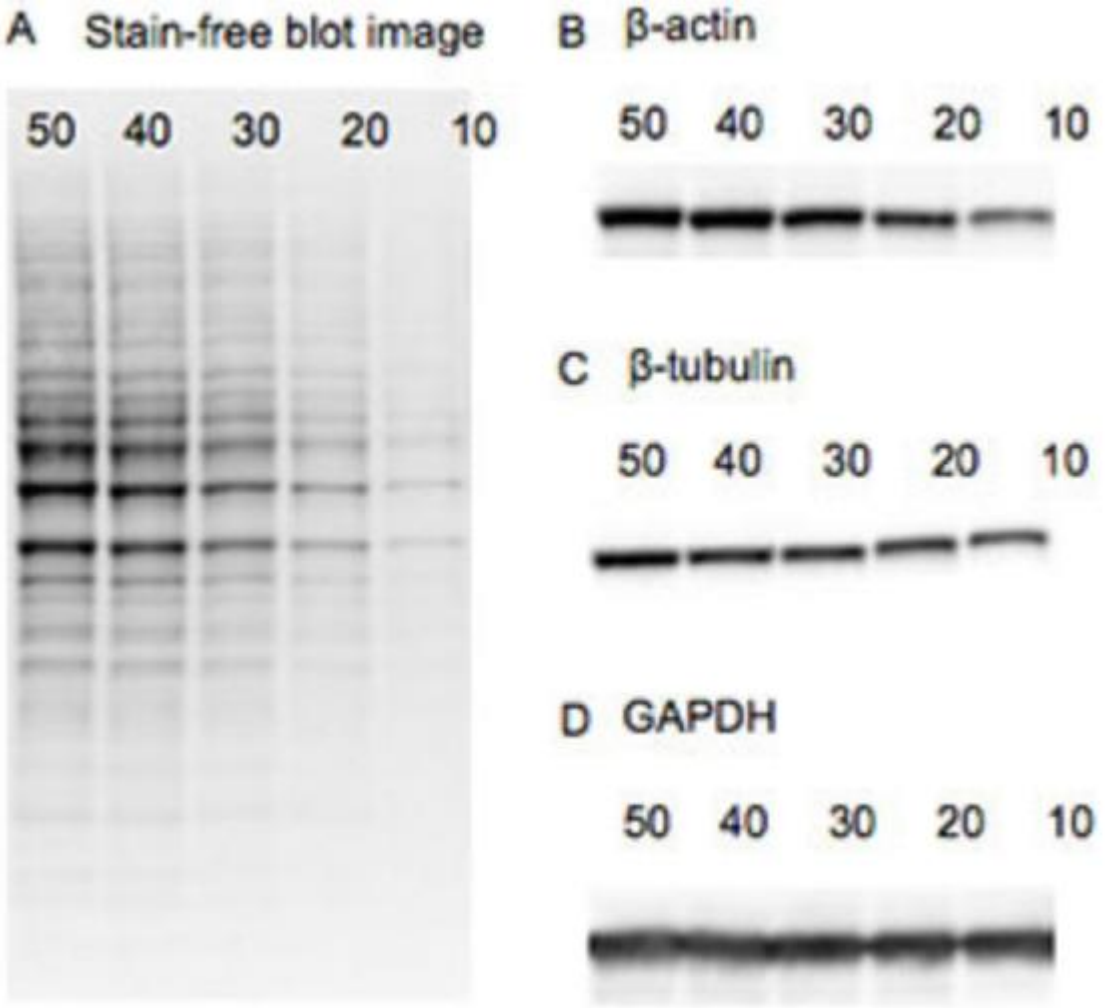


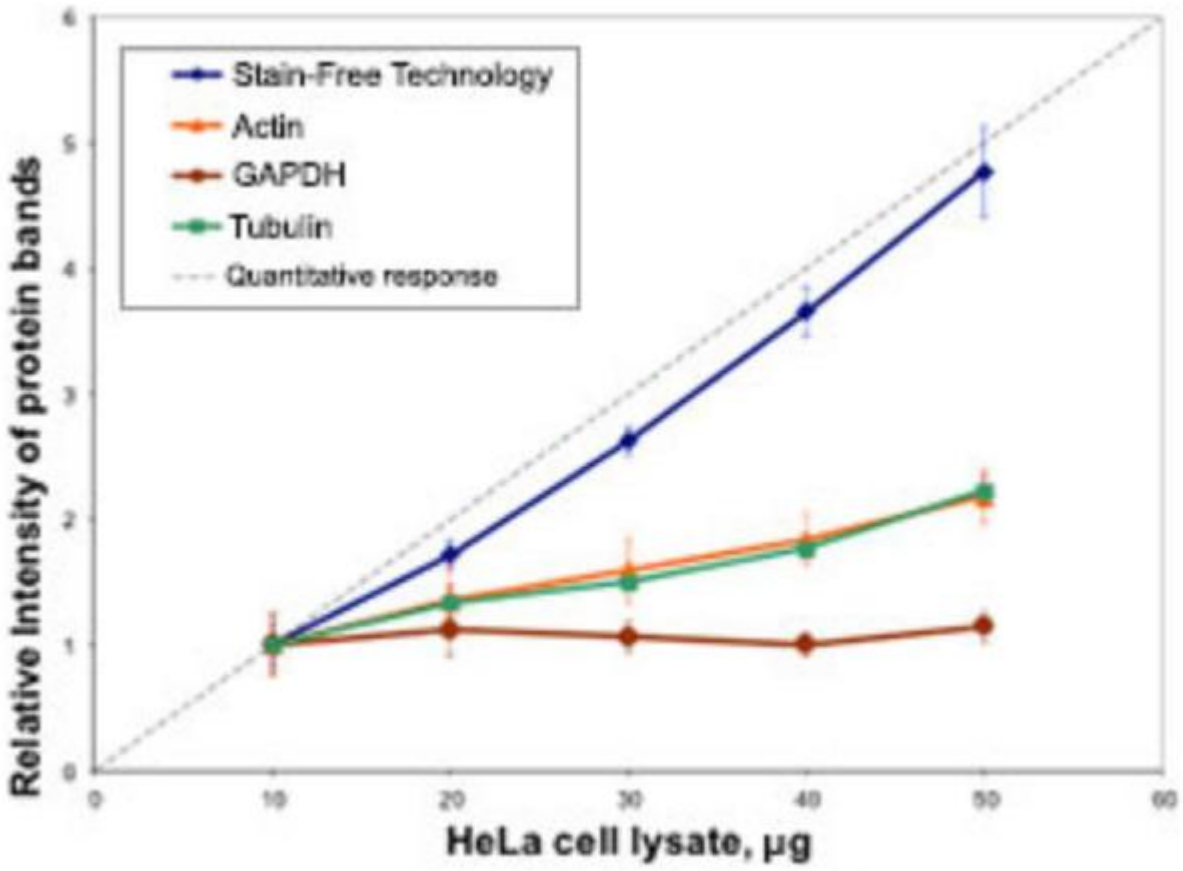



**Stain-free technology** has a good **linear dynamic range** for protein quantitation, defined as “the range through which the signal intensity on a blot proportionally increases with the increase in protein load”.



1-the linear dynamic range for protein quantitation is better for stain-free-technology;
2-the expression of a single loading control protein can be affected by the treatment, but it is highly unlikely that all proteins are affected by the treatment!

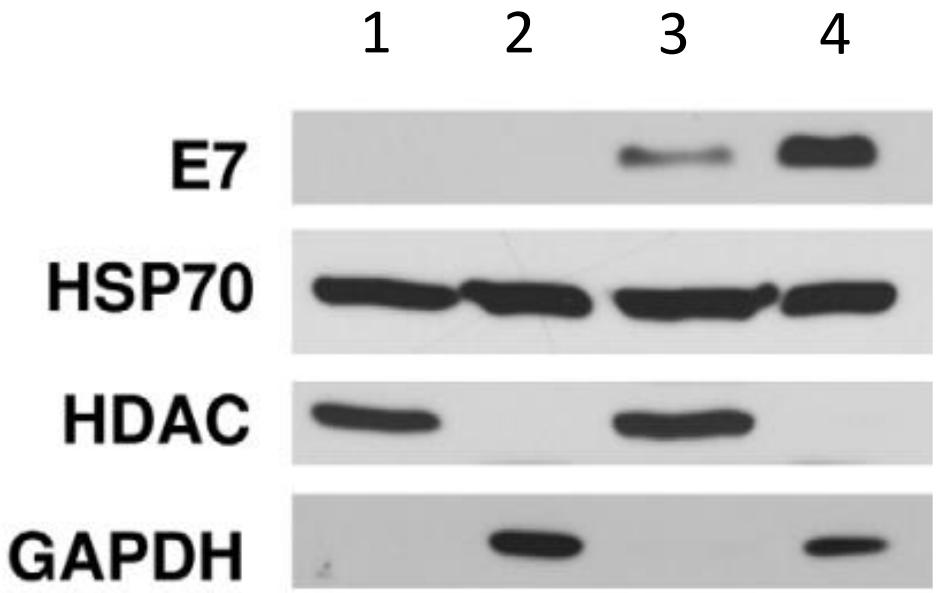


**Cellular fractionation markers** should be used when you are preparing **nuclear** and **cytoplasmic extracts** to confirm that the lysate was prepared appropriately.

Common **nuclear markers** are: Histone H3, Lamin A, PARP1, HDAC2.

Common **cytoplasmic markers** are: MEK1/2, GAPDH, β-actin, α/β-tubulin.



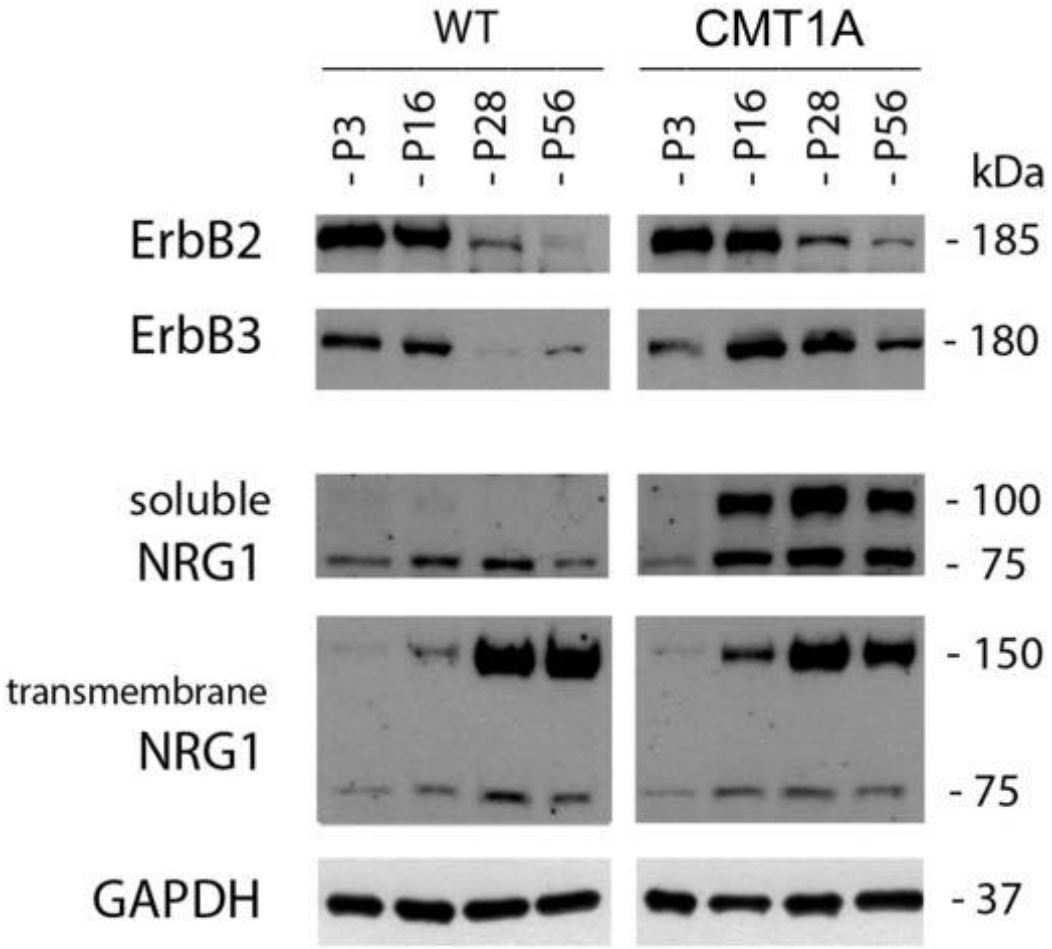

E7 is the protein of interest

HSP70 is used as total loading control

HDAC2 is used as nuclear marker

GAPDH is used as cytoplasmic marker

| Subcellular Localization | Loading Control | Molecular Weight (kDa) |
|---|---|---|
| Cytoplasm/Whole Cell | Alpha-tubulin | 55 |
| | Beta-actin | 43 |
| | GAPDH | 37 |
| | Cyclophilin B | 21 |
| Membrane | CD44 | 82 |
| | Beta-catenin | 86 |
| | Sodium Potassium ATPase Alpha 1 | 112 |
| Mitochondria | HSP60 | 60 |
| | COX IV | 17 |
| Nucleus | Lamin B1 | 66 |
| | HDAC1 | 60 |
| | Histone H3 | 17 |
| Serum | Transferrin | 77 |




*Western blot analysis of proteins extracted from WT and CMT1A sciatic nerves at different time points after birth (P3, P16, P28, P56).*

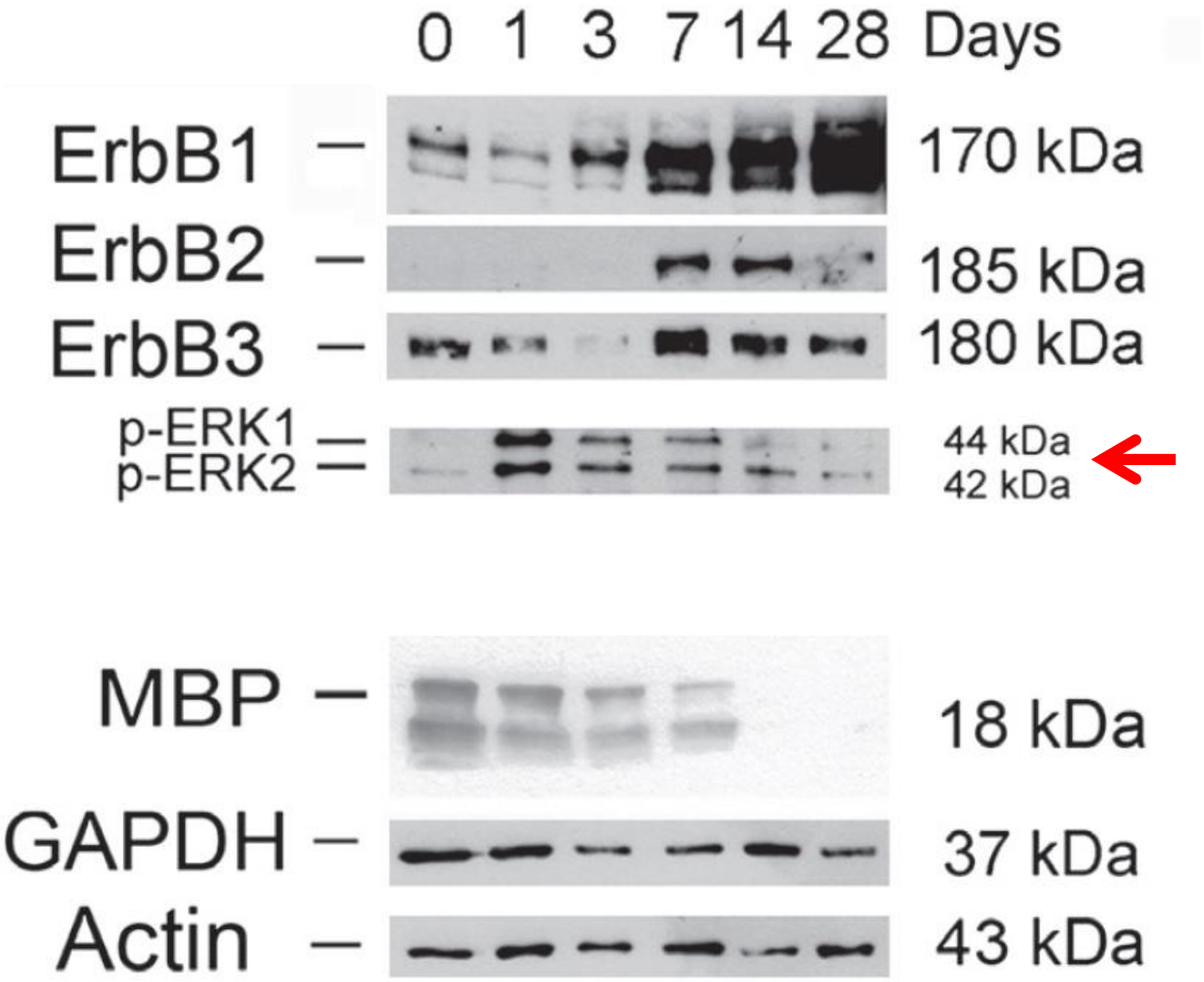
Rat sciatic nerves
WT: wild type
CMT1A: hereditary demyelinating neuropathy
P=post-natal day

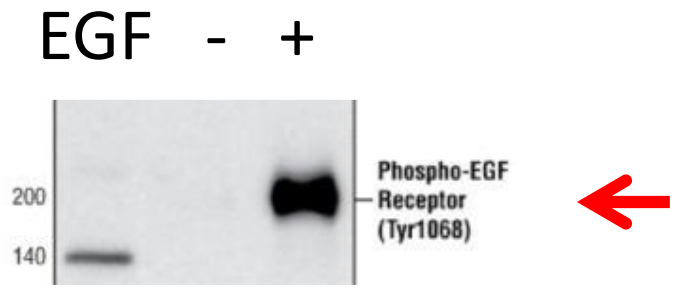
www.wooclap.com/ACBB111021

- How many experimental models were tested?
- How many samples were analysed?
- Which are the experimental time points?
- Which proteins were analysed?
- To which control can you normalize the protein expression?
- **Which results do you observe?**

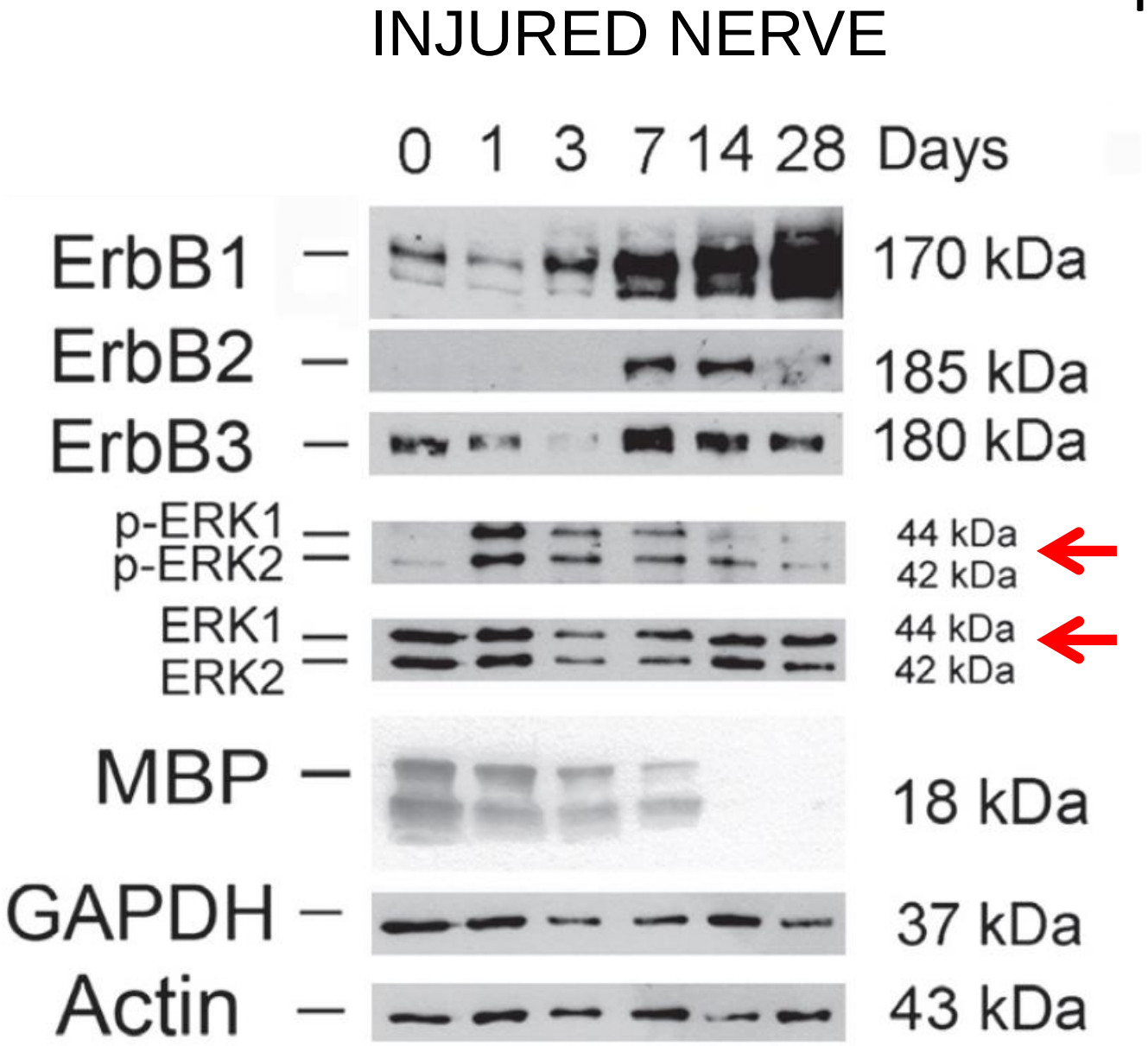
# Phosphorylation loading control

## INJURED NERVE

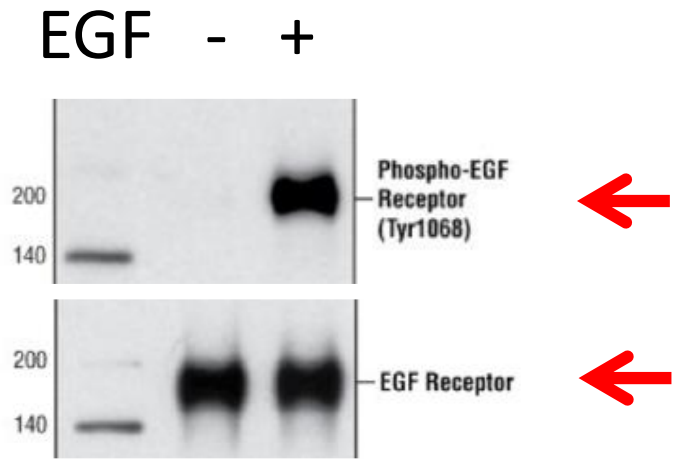


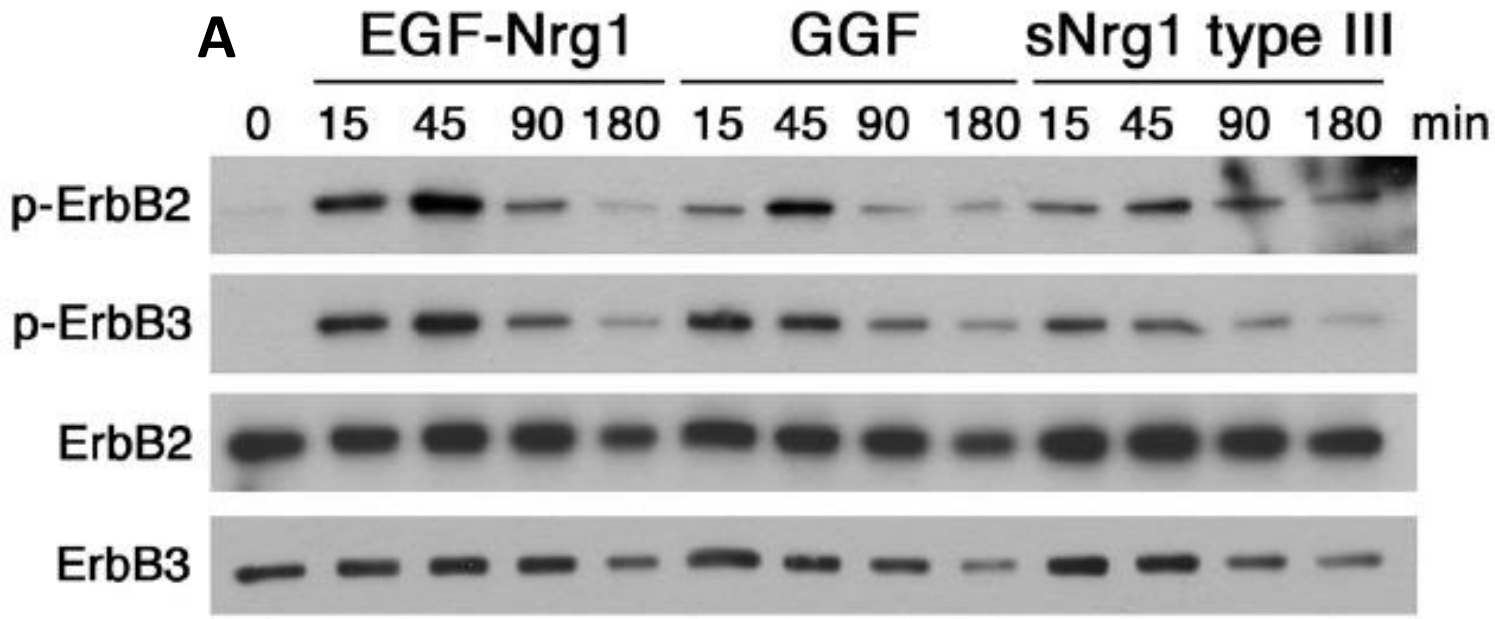



# Phosphorylation loading control




Blot with antibodies against the **protein of interest** (ERK1, ERK2) is used as **loading control** of enzymatic modification of the protein, such as **phosphorylation** (pERK1, pERK2).






- Different treatment types
- Different treatment times

***A.*** *Schwann cells were treated with EGF-Nrg1, GGF, or sNrg1 type III at 1 nM and cell lysates were prepared at indicated times. Activation states of ErbB2 and ErbB3 were determined by immunoblotting using phospho-ErbB2 and phosphor-ErbB3 antibodies.*

- Which are the ligands used?
- Which is the treatment dose?
- How many cell plates do you need for this experiment?
- Which are the experimental time points?
- Which are the proteins analysed by western blot?
- To which loading control protein phosphorylation can be normalized?
- **Which is the best time point to appreciate a stronger protein phosphorylation?**

# IMMUNOPRECIPITATION (IP)

IP is an **affinity purification technique** used to enrich for a protein of interest for further experiments such as western blot and Co-IP, kinase assay, mass spectrometry or structure-function studies.

IP is used to isolate a single protein (the target antigen of the antibody) to investigate its identity, structure, expression, activation or modification.

# Co-IMMUNOPRECIPITATION (Co-IP)

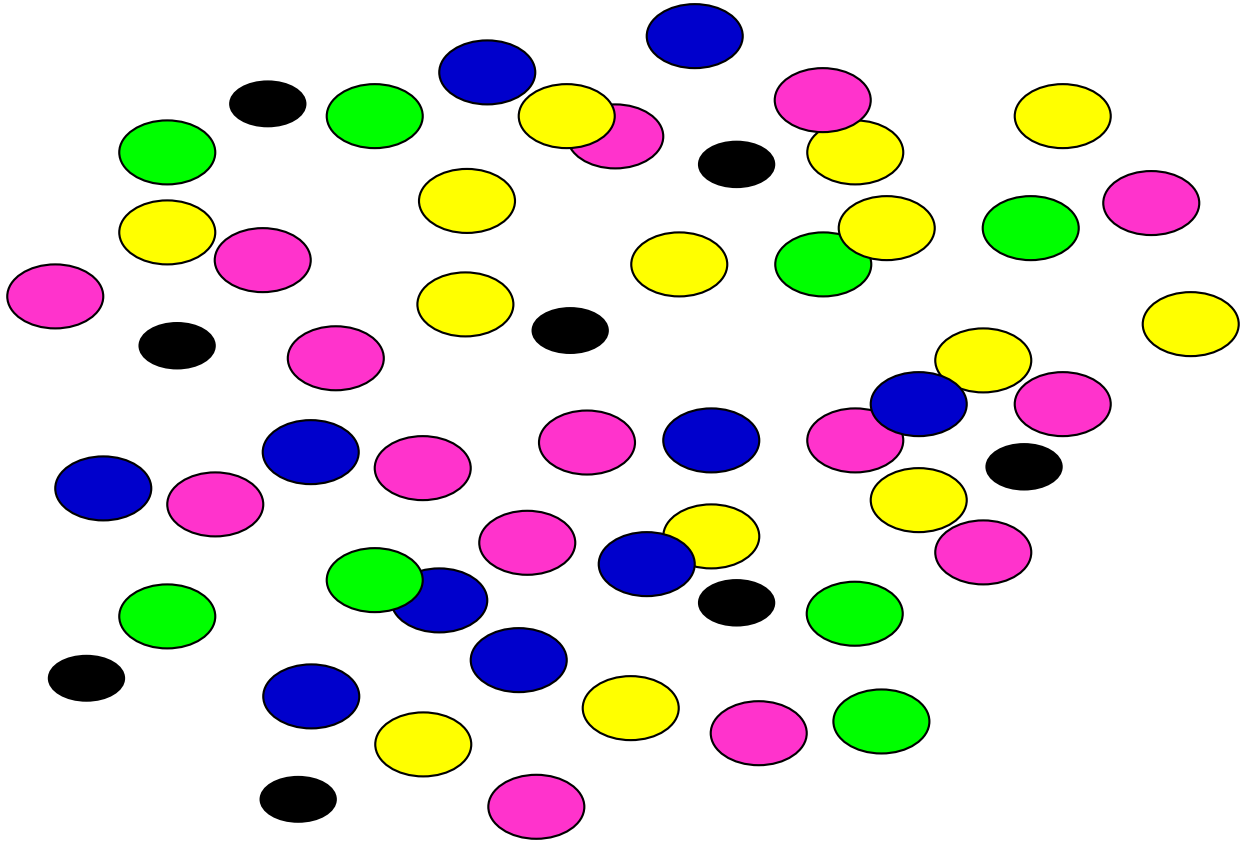
Co-IP is conducted in essentially the same manner as IP, except that the following western blot analysis focuses not only on the antigen protein precipitated by the antibody, but rather on its binding partner(s), which co-precipitate with the target protein.

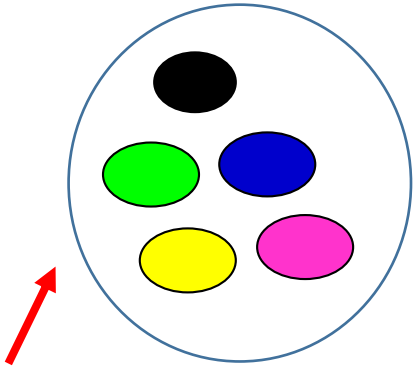
The hypothesis (that must verified) is that associated proteins that co-precipitate with the antigen protein are related (positively or negatively) to its cellular function.

https://www-jove-com.bibliopass.unito.it/v/5695/co-immunoprecipitation-and-pull-down-assays

# IMMUNOPRECIPITATION

- cell culture or tissue ->  total protein extract

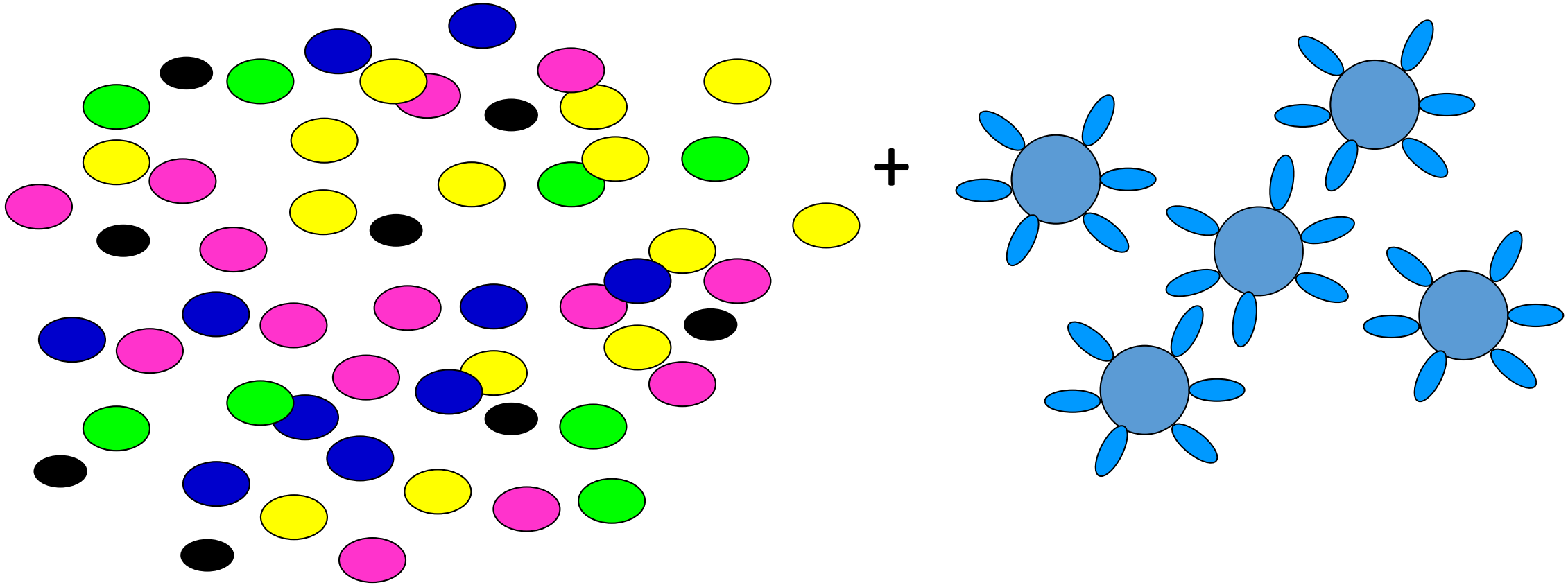




-> **save a small aliquot** as control for the western blotting (called «cell lysate», or «input»)

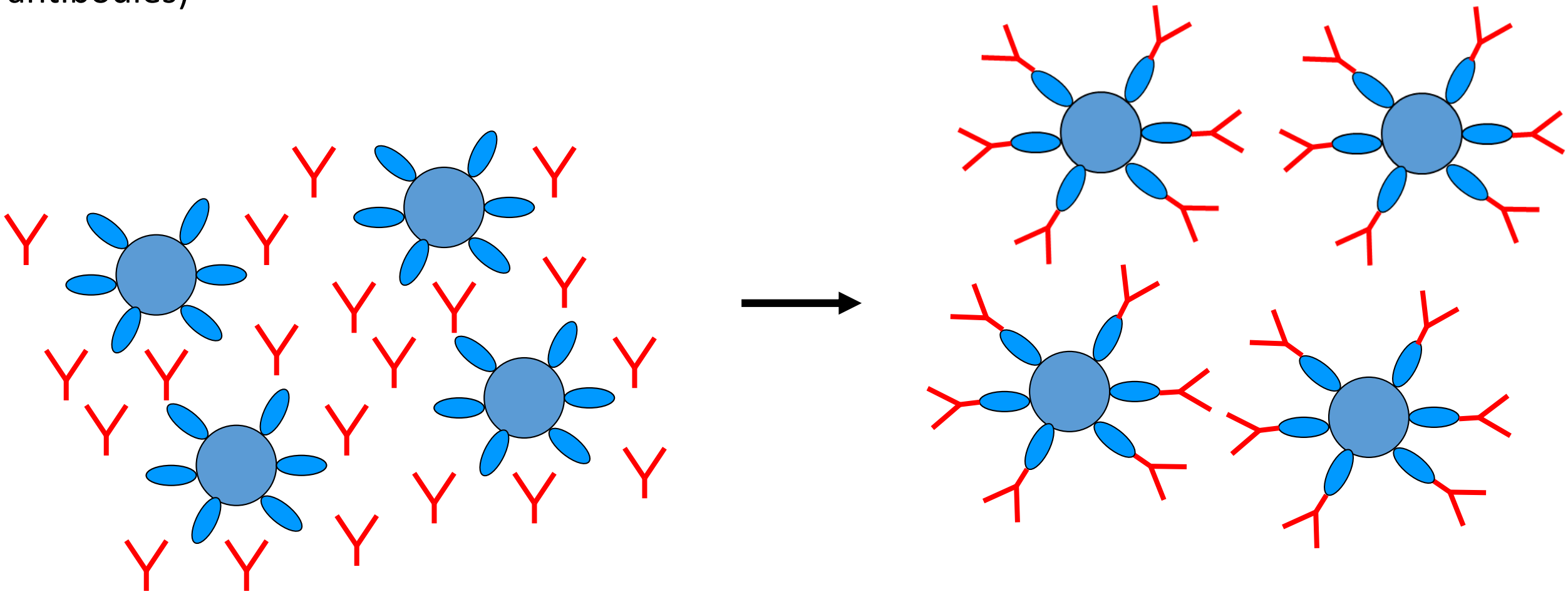
# PRECLEARING

- mix total protein extract with **sepharose beads-protein**
- spin
- save surnatant
- discard sepharose-beads + bound-proteins



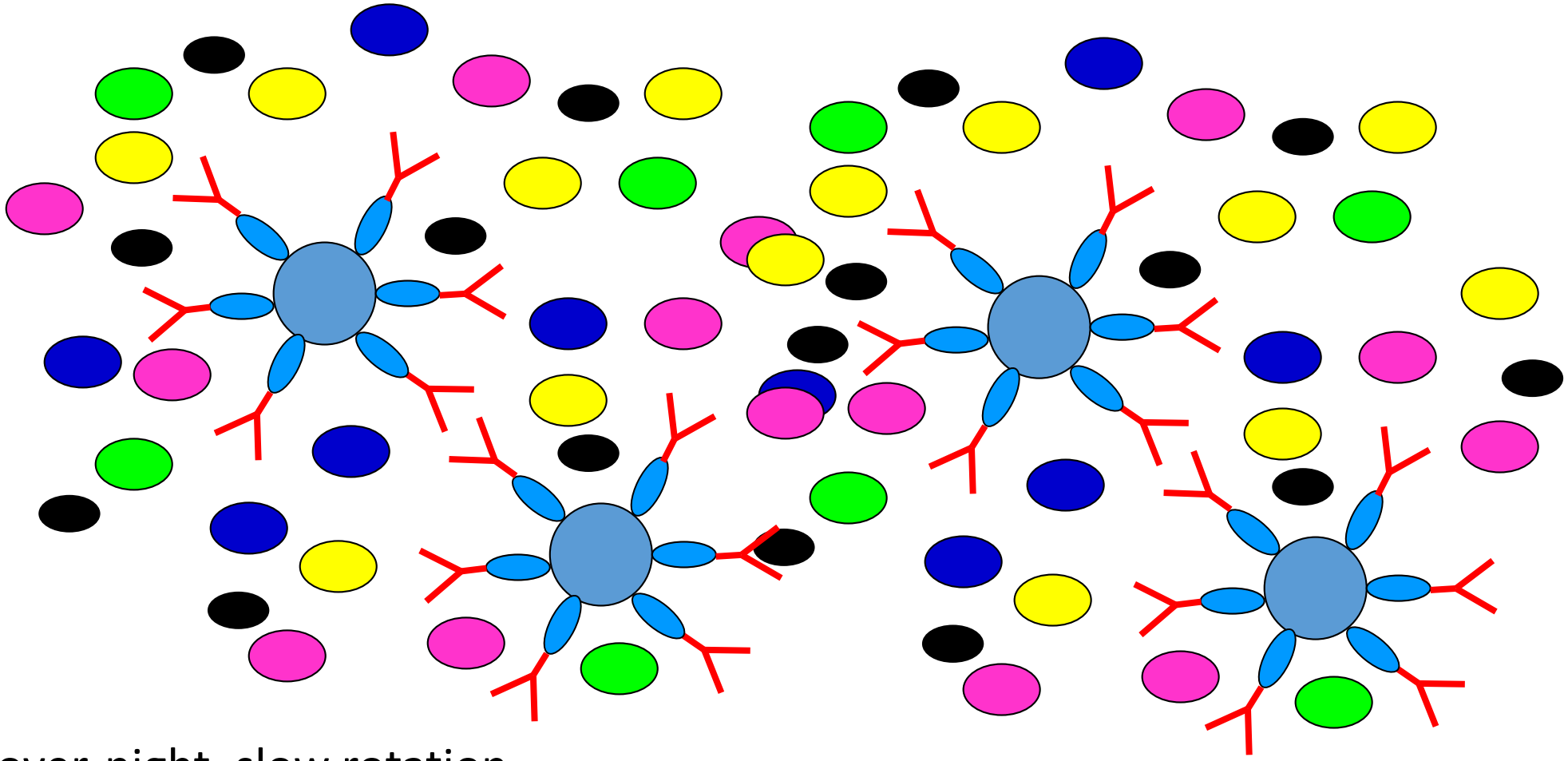
# IMMUNOPRECIPITATION

- mix **antibody** + **sepharose beads**-**protein A** (protein A, bound to sepharose beads, binds antibodies)



# IMMUNOPRECIPITATION

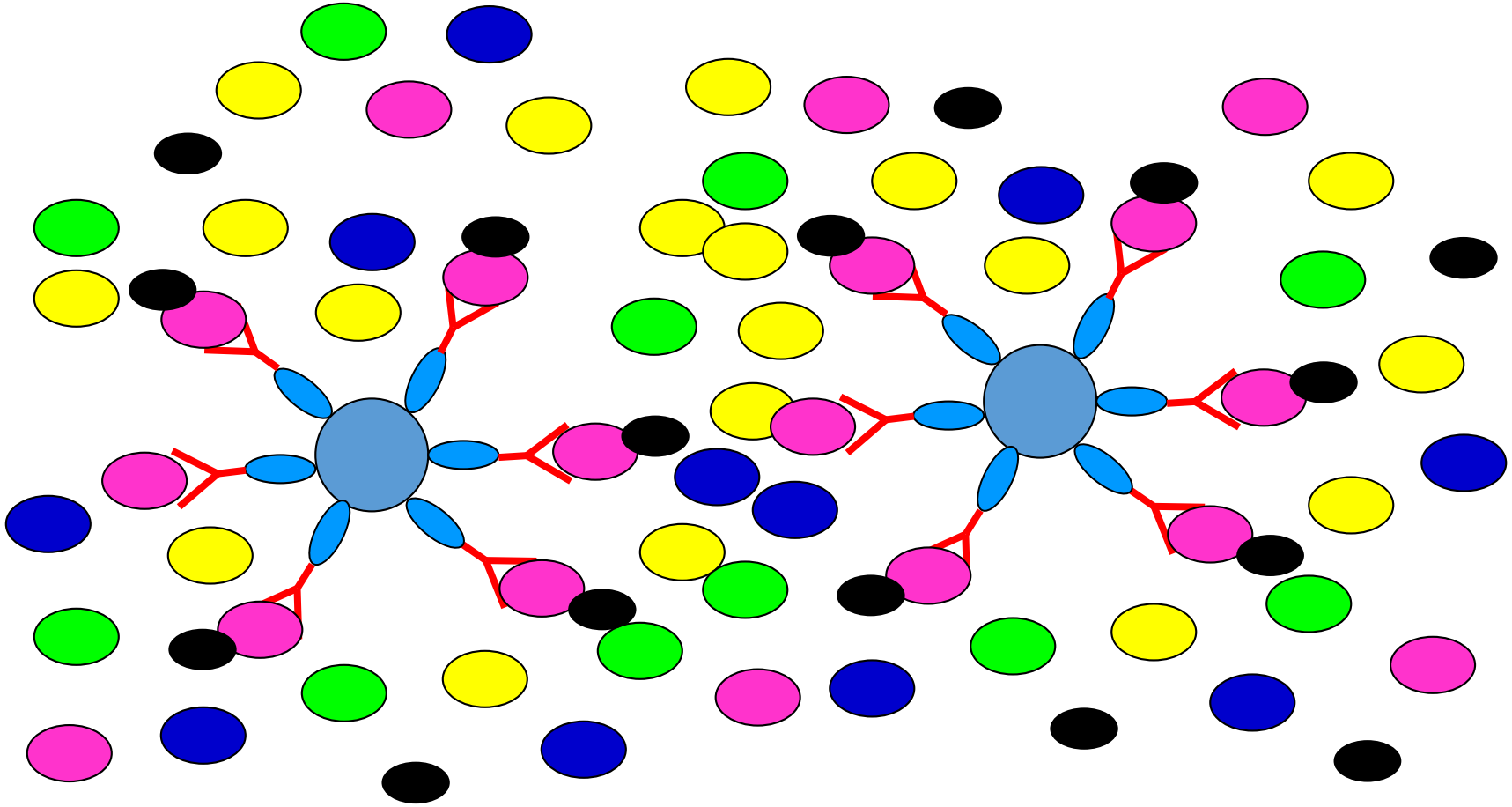
- mix total protein extract + **antibody** + **sepharose beads**-**protein A**



- @ 4°C over-night, slow rotation

# IMMUNOPRECIPITATION – Co-IMMUNOPRECIPITATION

- **protein A** (bound to **sepharose beads**) binds **antibodies**,
- **antibodies** bind **specific protein** (and **proteins bound** to specific proteins)



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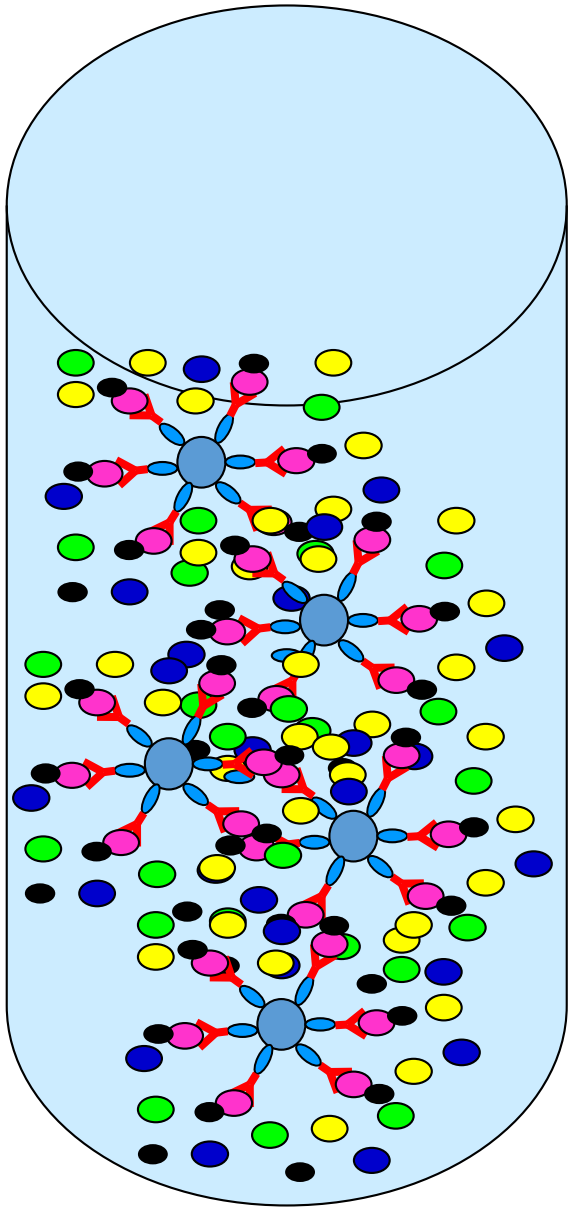
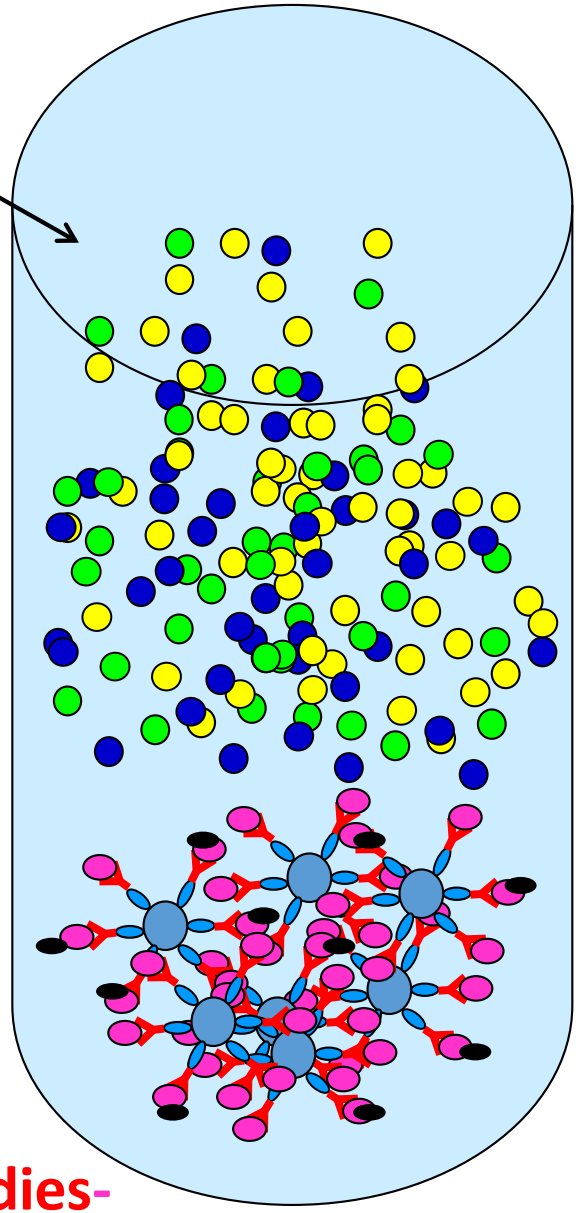


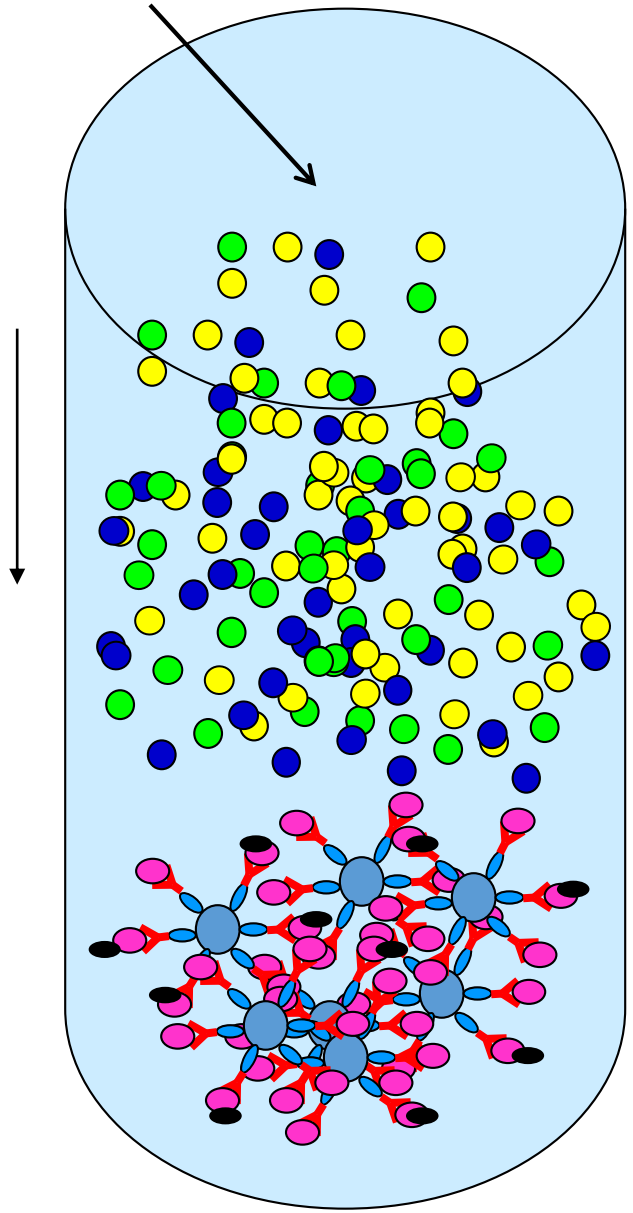
The surnatant contains: **proteins not bound to antibody**



- spin 3000 rpm, 1 min, @ 4°C

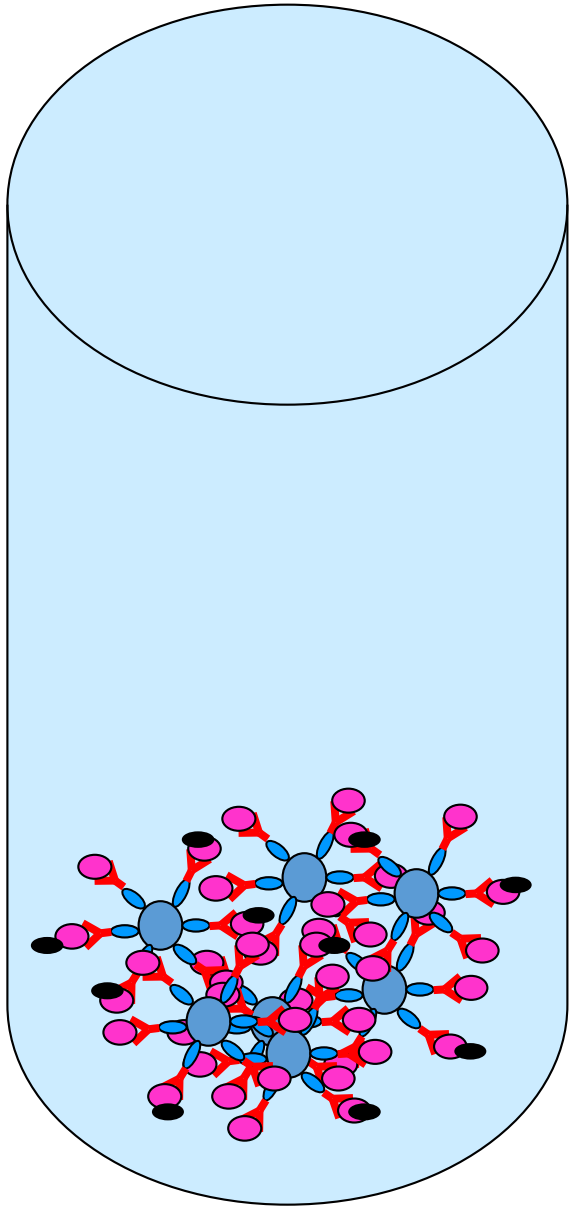
The pellet contains: **sepharose beads -protein A-antibodies-specific protein-proteins bound** to specific proteins

The surnatant contains: **proteins not bound to antibody**

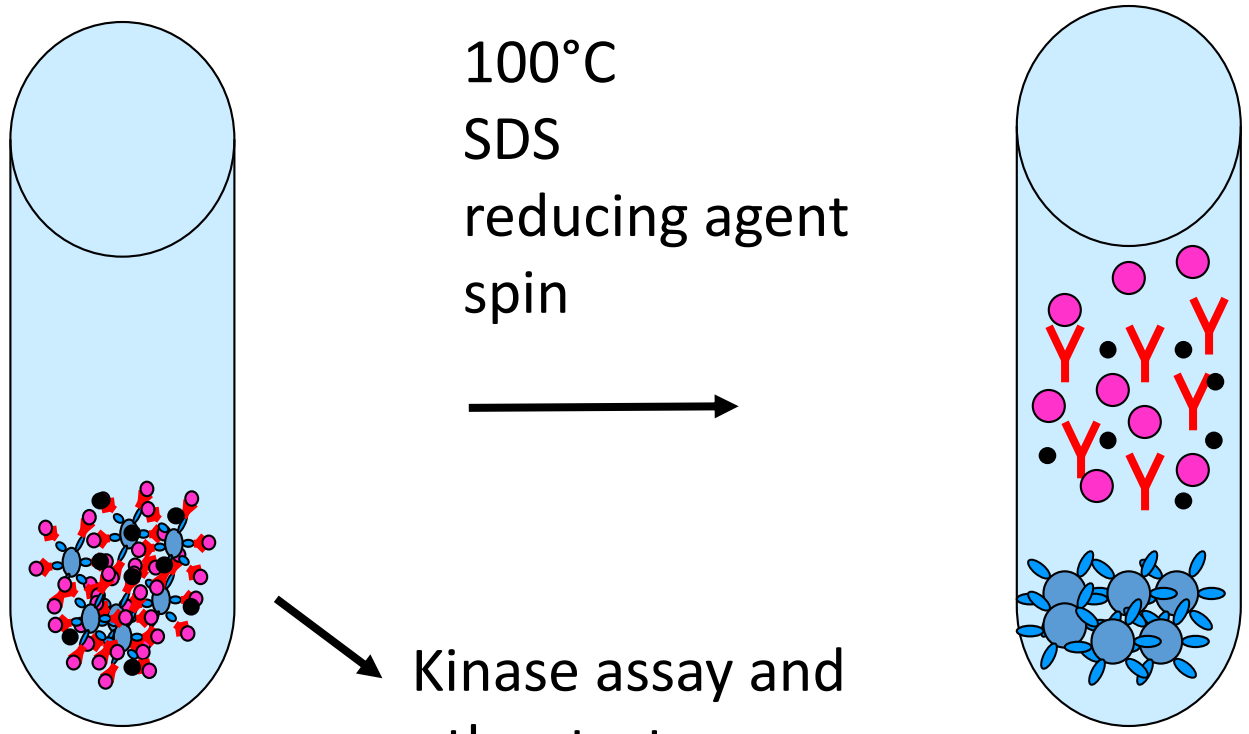


Discard the surnatant & wash the pellet 3 times to eliminate all unbound proteins

The pellet contains: **sepharose beads -protein A-antibodies-specific protein-proteins bound** to specific proteins



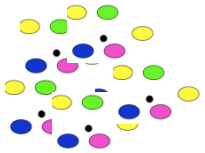
•add SDS (which denatures proteins), β-mercapto-ethanol (which reduces disulfide bridges)
• boil 4 minutes @ 100 °C
→ **sepharose beads-protein A** detaches from the **antibody** which detaches from **protein**
• spin 3 minutes at room temperature
• **sepharose beads-protein A** form a pellet
• in the surnatant you will find the **antibody** and the **protein** (and **any proteins bounded** to the specific protein)

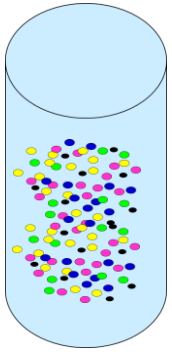


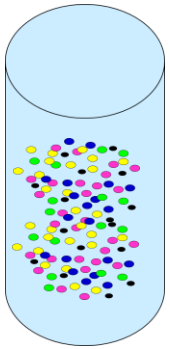

• the solution containing **antibodies** and **proteins** and **bounded proteins** is analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel run:
Western blot analysis

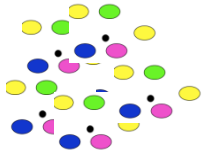
cell lysate

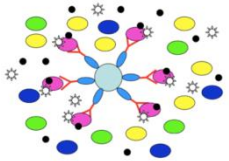




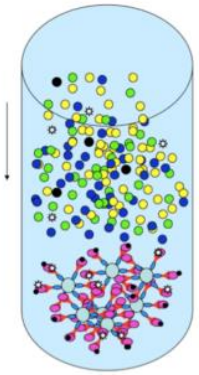


cell lysate/input

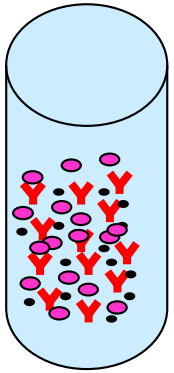




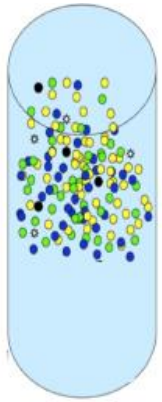
cell lysate +
antibody + beads



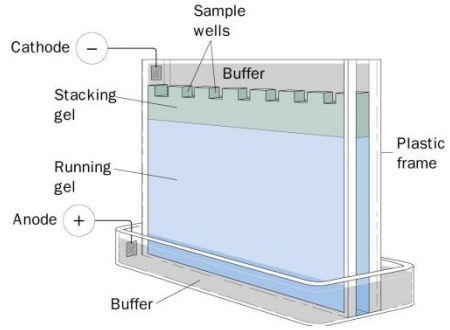
immunoprecipitation



immunoprecipitated
proteins (IP)

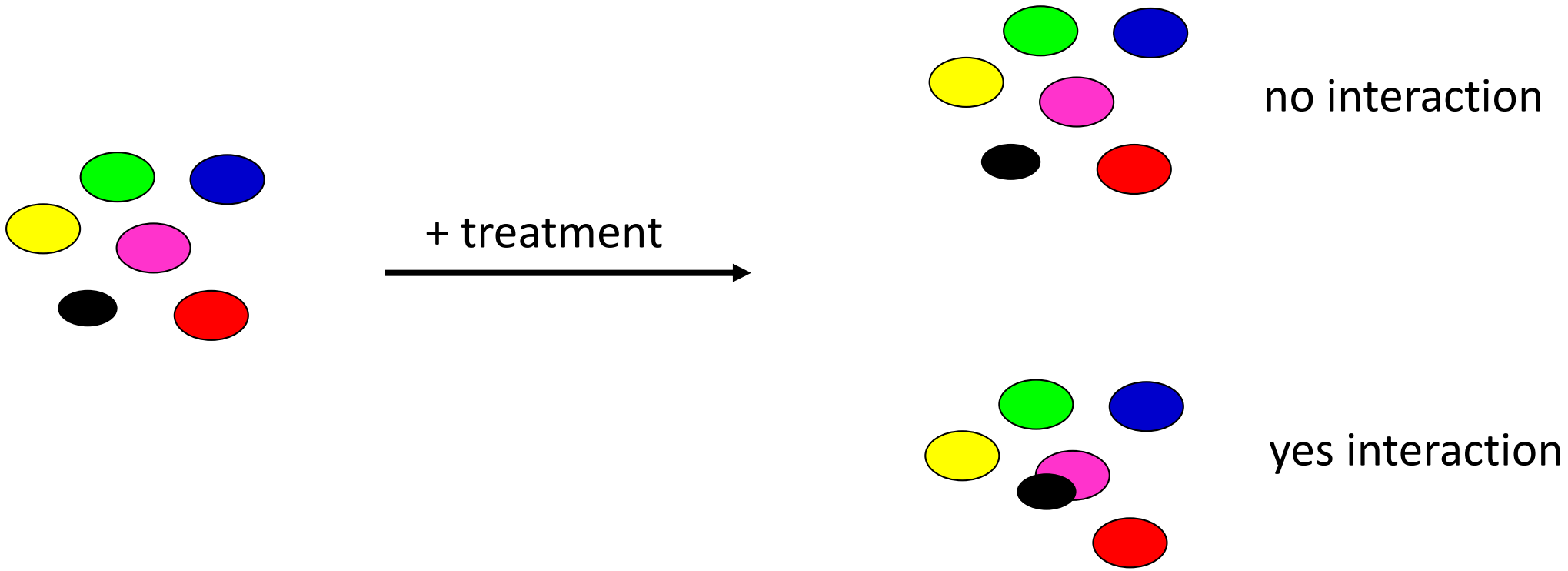


surnatant
(NOT precipitated proteins)



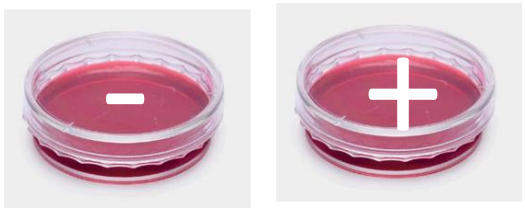
Example of a scientific question:

Do protein pink and black interact following a specific treatment?

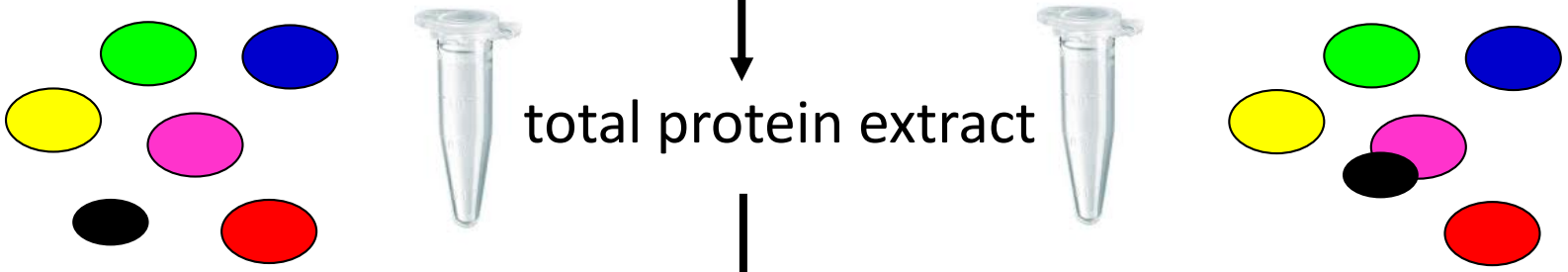



-How many samples do you need to test this hypothesis?

what is a mock control?




mock control  treatment



total protein extract

IP α

Boil, SDS-PAGE

WB α ?

WB α ?