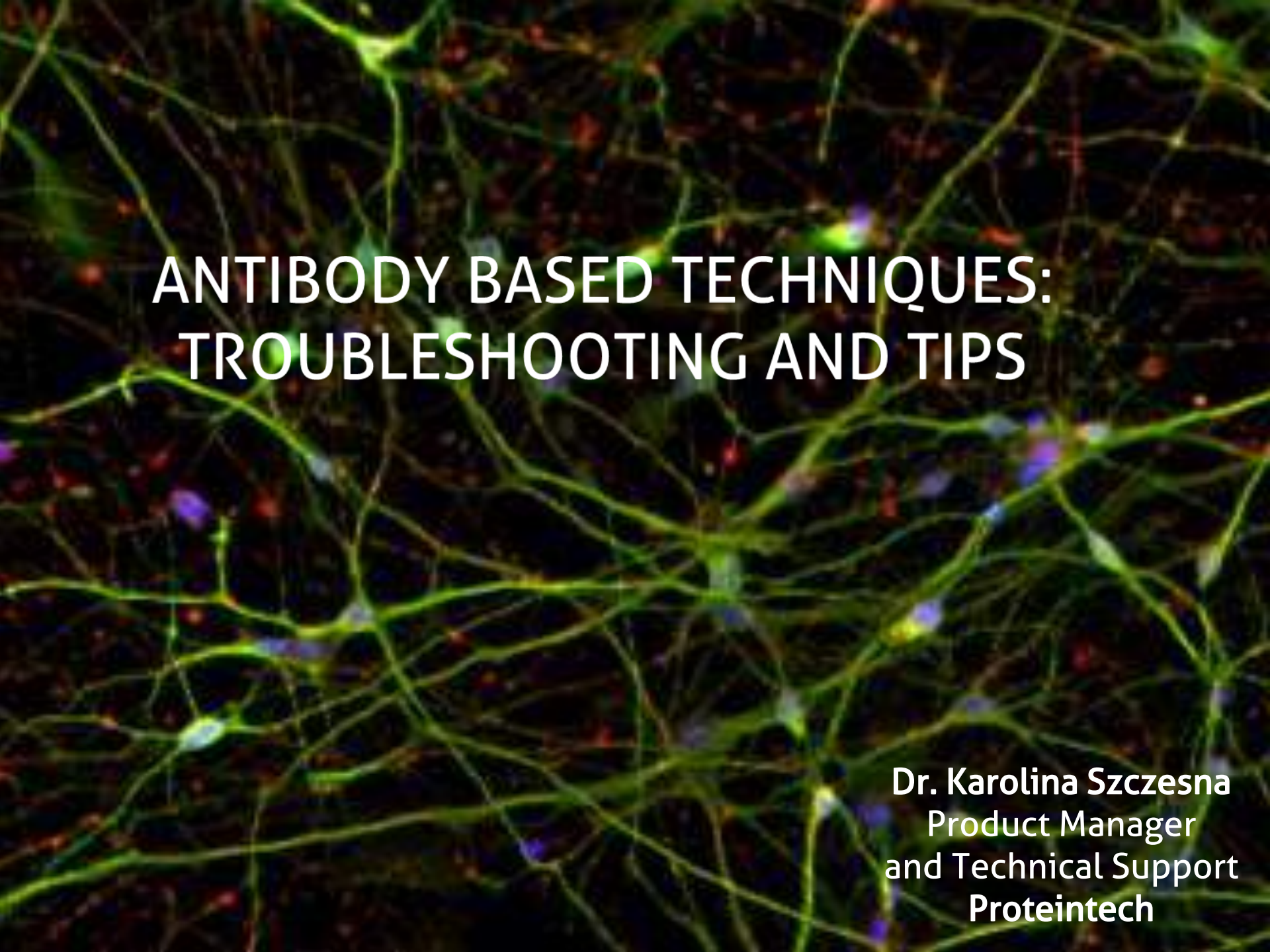

ANTIBODY BASED TECHNIQUES: TROUBLESHOOTING AND TIPS

Dr. Karolina Szczesna
Product Manager
and Technical Support
Proteintech

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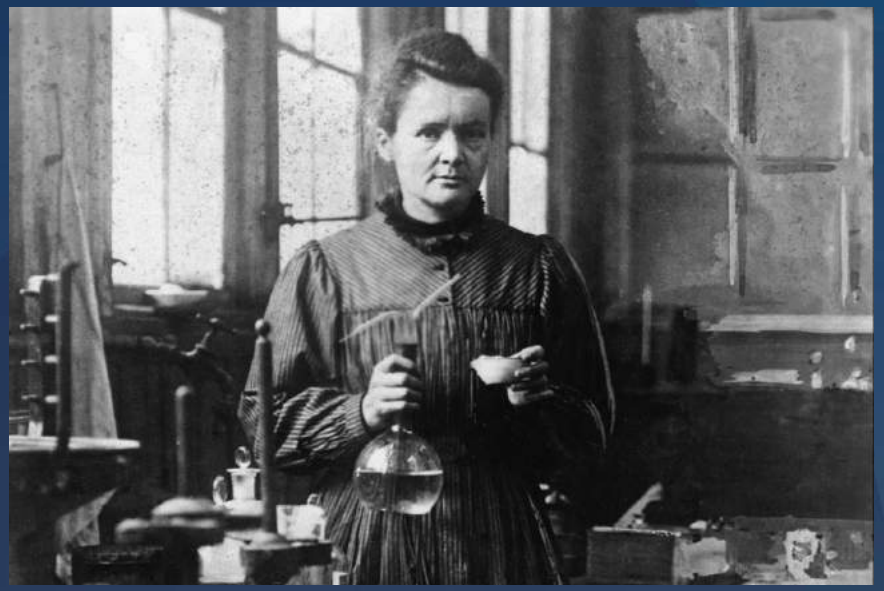
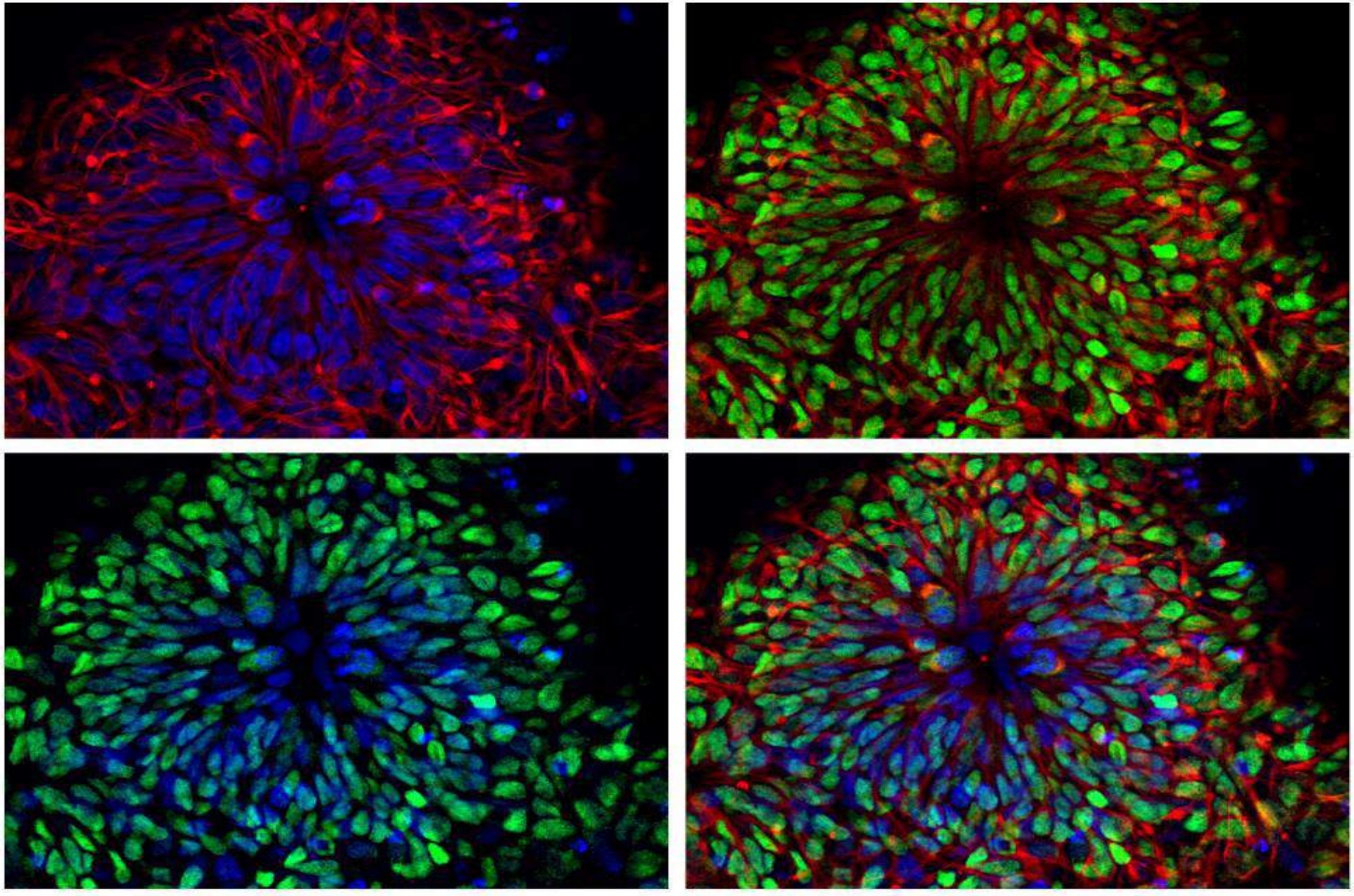
"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less".

Marie Skłodowska-Curie
(1867 – 1934)
a Polish physicist and chemist



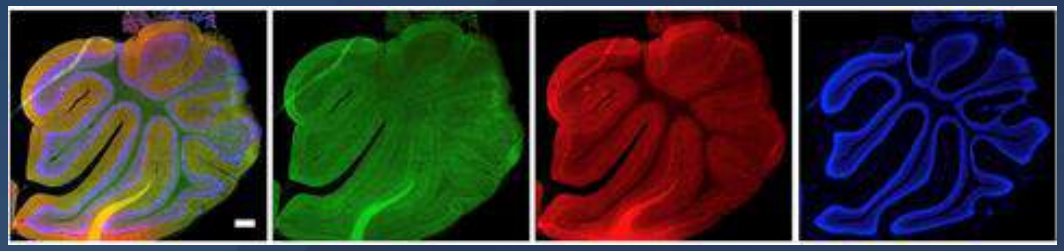
Bachelor in Pharmaceutical Chemistry at the Jagiellonian University in Kraków, Poland
Master in Biotechnology at the University of Amsterdam, The Netherlands



M.Curie network - an International PhD student at the University of Barcelona, Spain
M.Curie network - a self-directed researcher and project management, Biotalentum, Budapest, Hungary

Main research focus related to Central Nervous System and Oncological Disease Models
12 PubMed Publications Szczesna K

Szczesna et al., 2018 May CellReports: Inhibition of Gsk3b Reduces Nfkb1 Signaling and Rescues Synaptic Activity to Improve the Rett Syndrome Phenotype in Mecp2-Knockout Mice



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- Fusion Proteins

...and offer Customer Antibody Production Service

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Recombinant proteins – **HUMANKINE**

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Made from human HEK293 cells

Native post-translational modifications and folding

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OVERVIEW

- IHC? ICC? IF? - What is the difference?
- How to optimize IHC and ICC / IF staining
- Why WBs aren't working
- Helpful resources

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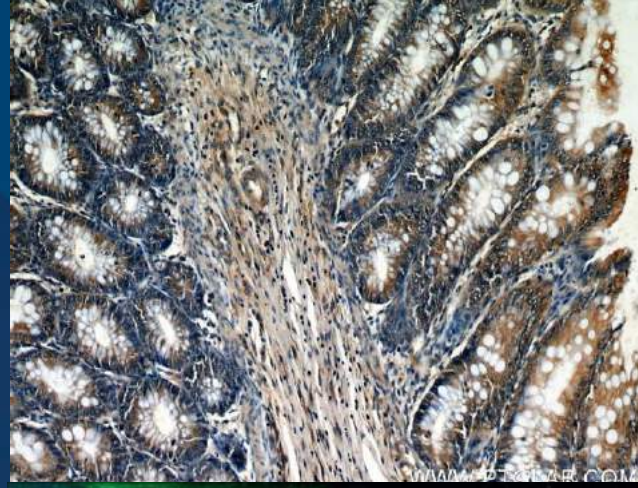
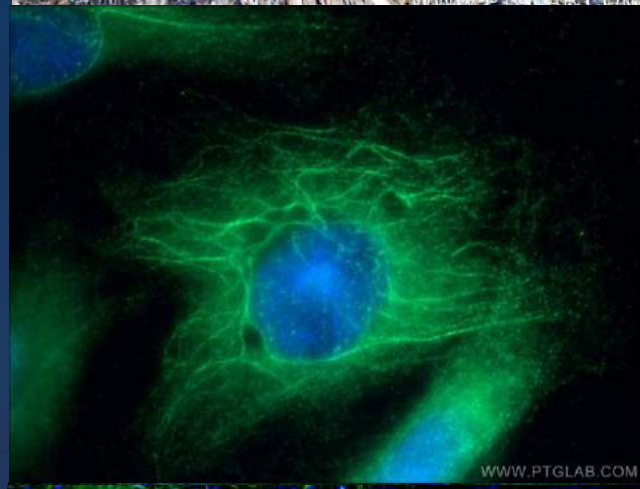
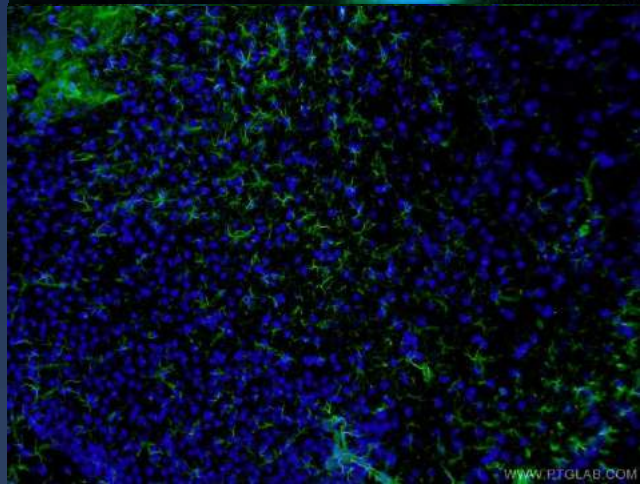
IHC? ICC? IF?

WHAT IS THE DIFFERENCE?


CASP1-antibody-22915-1-AP



F8 antibody 21485-1-AP



GFAP antibody 60190-1-Ig

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IHC - Immunohistochemistry
ICC - Immunocytochemistry
IF - Immunofluorescence

- widely used technique in biological research / clinical diagnostics
- all utilize antibodies to provide visual details about the location, expression, and pattern of the target protein

IHC, ICC and IF - main differences

Sample Type : tissue vs cells

- IHC tissue sections: paraffin embedded or frozen
- ICC / IF samples may be from tissue culture cell lines (adherent or in suspension) or directly from a human or animal source

IHC vs ICC / IF

- ICC samples a shorter fixation

↓

fixatives (e.g.; formaldehyde) can mask epitopes, reduce antigen-antibody binding

TIP: Perform an antigen retrieval to restore tissue antigenicity.

ANTIGEN RETRIEVAL

Heat-induced Epitope Retrieval (HIER)

- Citrate buffer / Tris-EDTA buffer

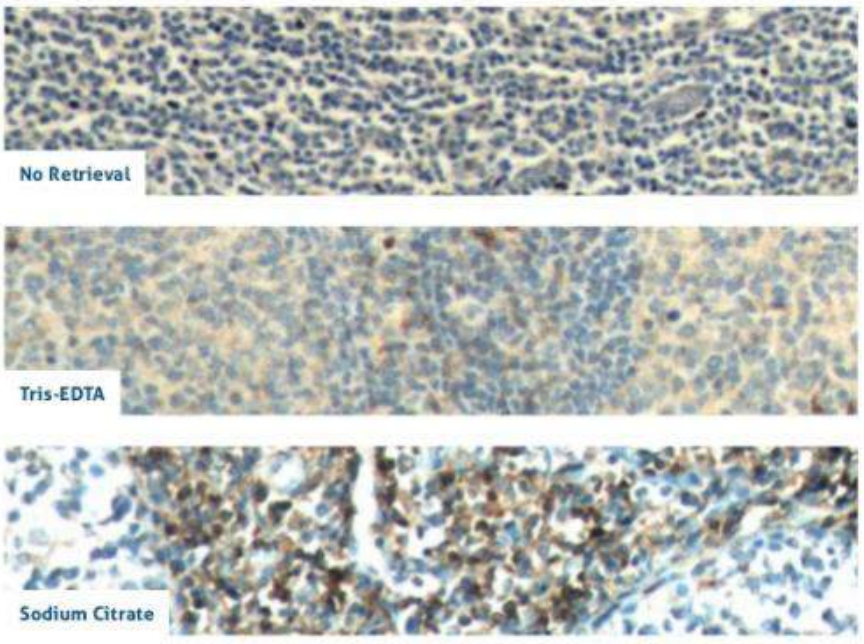
*EDTA buffer for antibodies against phospho-tyrosines

Proteolytic-induced Epitope Retrieval (PIER)

- Enzymes: trypsin, proteinase K, pepsin, protease

Heat-induced Epitope Retrieval (HIER)

*optimal conditions always have to be determined by each laboratory and in accordance with the specific product information



CD3 gamma antibody (60347-1-AP) on paraffin tonsillitis tissue slides.

IHC, ICC and IF - main differences

Labelling Method: chromogenic vs fluorescent

- IHC / ICC: chromogenic reagents detect antigens
- IF: fluorochrome reagents emit light at a specific wavelength

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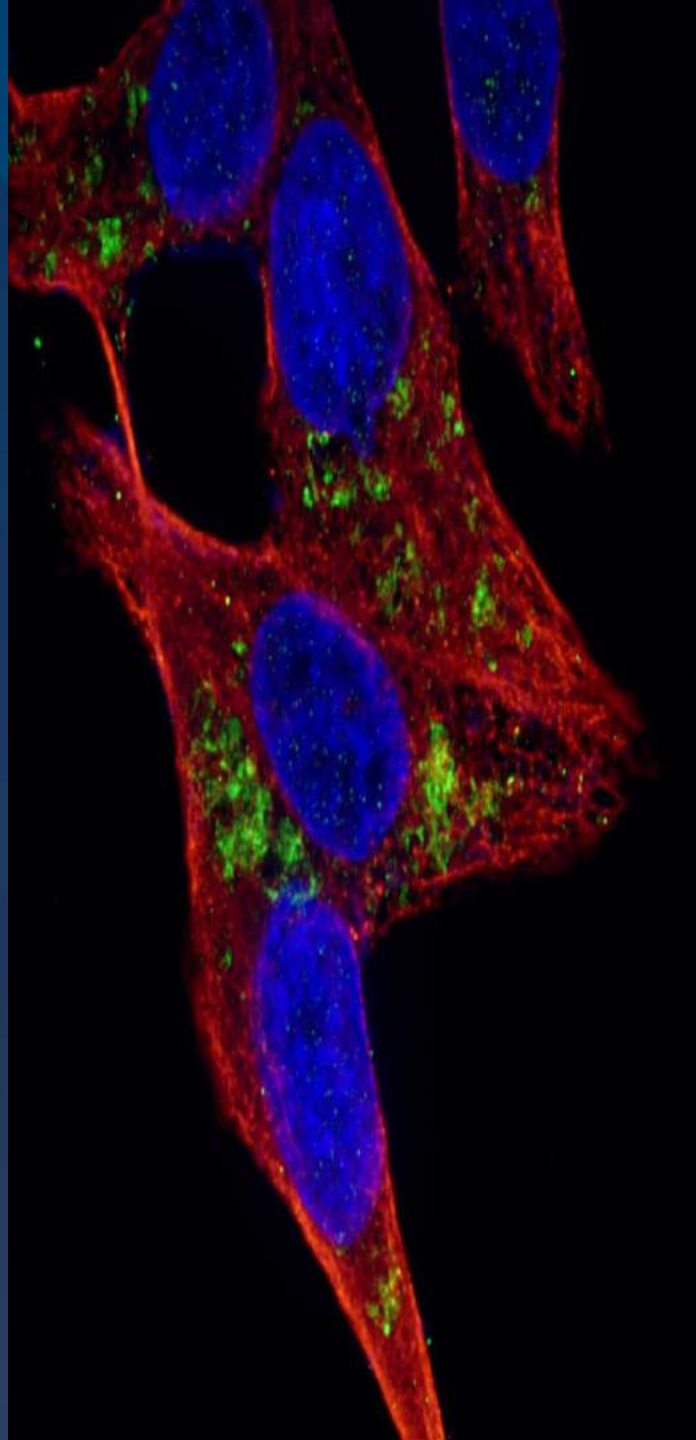
HOW TO OPTIMIZE IHC AND ICC / IF STAINING



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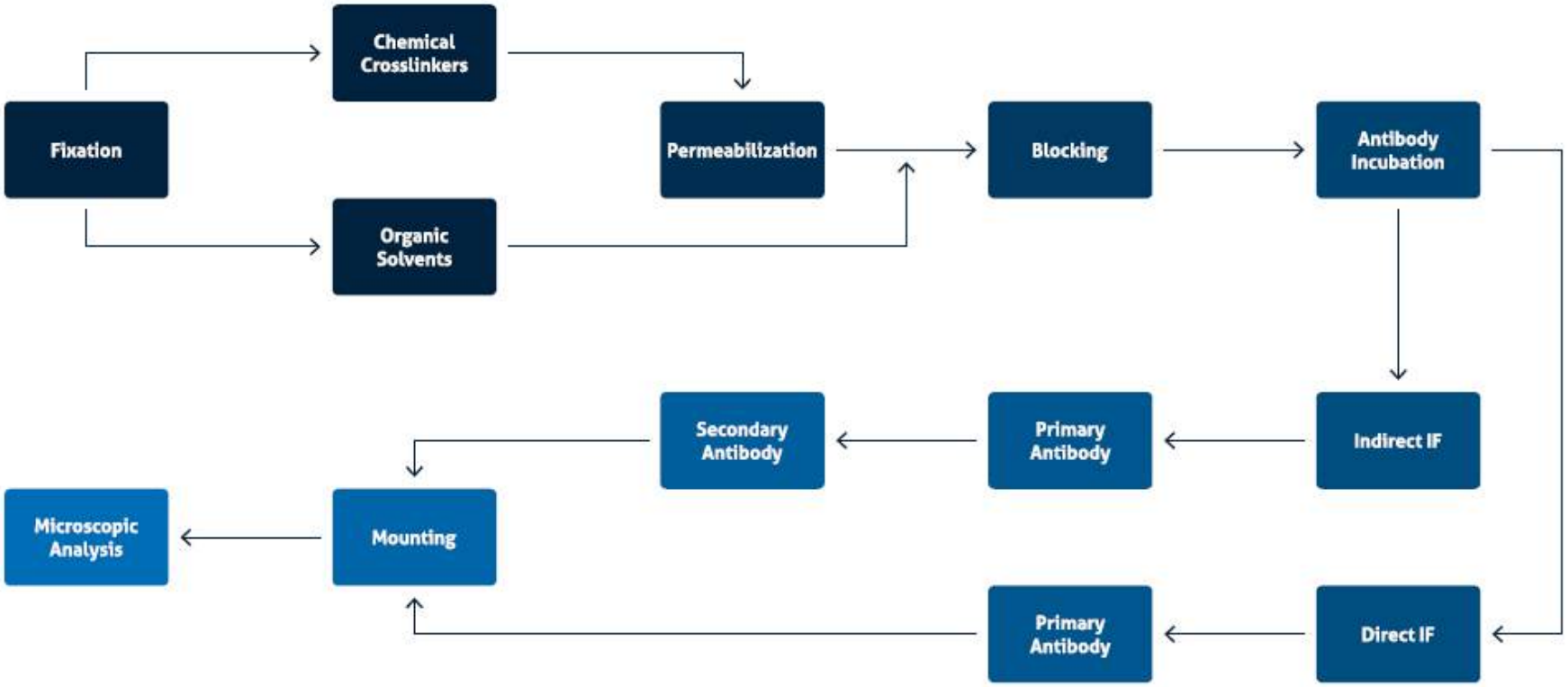
THE MAIN STEPS OF IF



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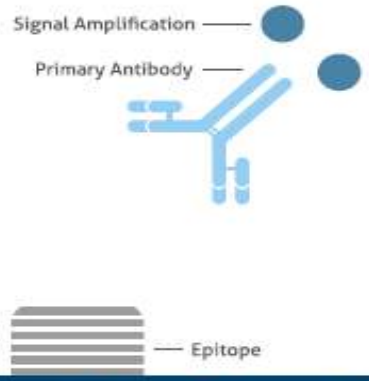
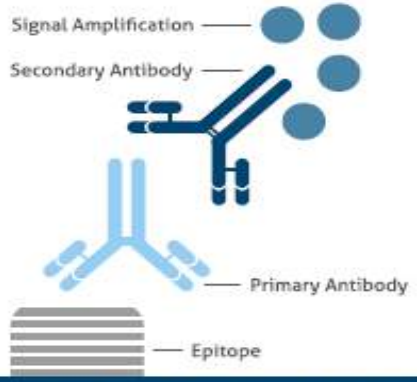
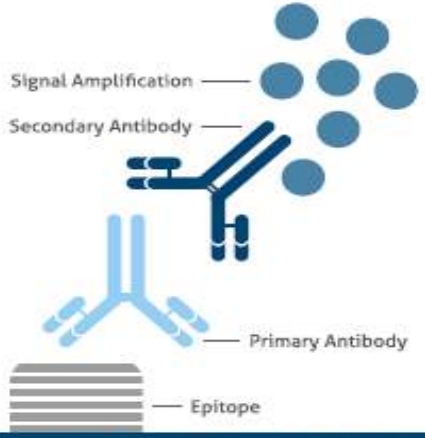
VISUALIZATION

Different Detection Systems & Signal Amplification

Expression level of protein of interest		
High	**Medium**	**Low**
		
Direct-labeled Primary Antibody	**Secondary Antibody Labeled**	**Secondary Antibody Labeled, Plus Enhancer**

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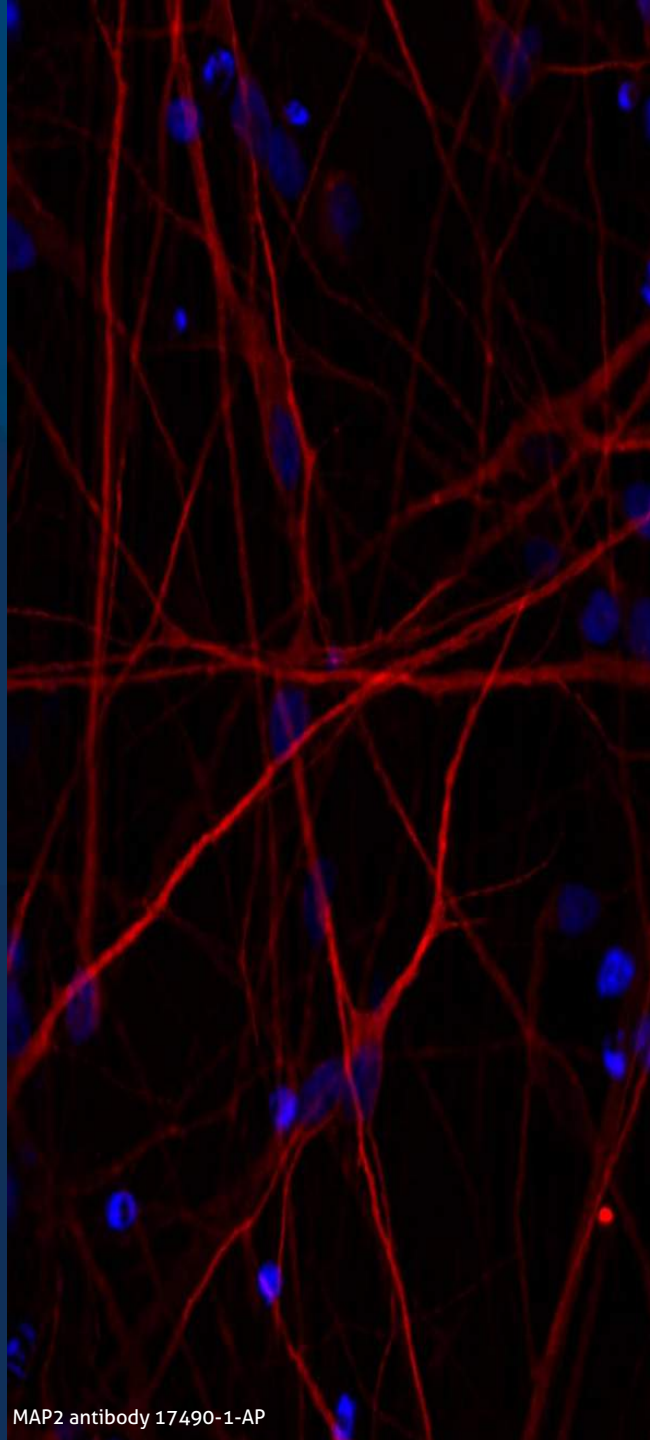
IMMUNOFLUORESCENCE STAINING

CONSIDERATIONS BEFORE STARTING IF



MAP2 antibody 17490-1-AP

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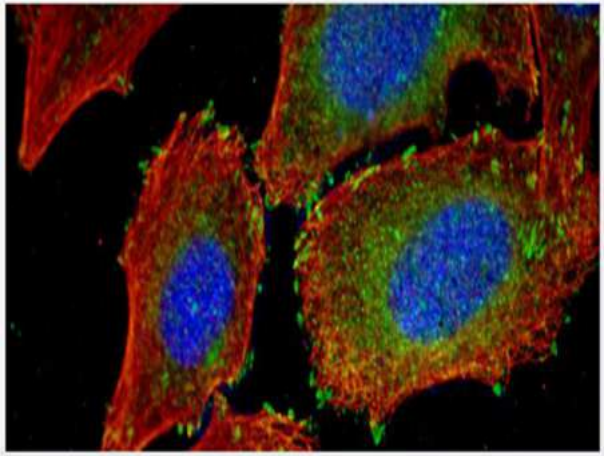
USE THE APPROPRIATE FIXATIVE FOR YOUR SAMPLE

Most Commonly Used Fixatives

Type of fixative	Name	Advantage	Disadvantage
Organic solvent	Methanol	Cellular architecture is conserved	Damaging to several epitopes. Lipid components become lost.
Organic solvent	Acetone	Gentle for epitopes	Lipid components become lost.
Chemical crosslinker	Paraformaldehyde (PFA)	Cellular morphology conserved	Cross-linking of epitopes, autofluorescence.

HepG2 cells fixed with -20°C Ethanol.



HepG2 cells fixed with 4% PFA.

Recommended fixation step:

10–20 minutes at room temperature (RT) incubation with 2%–4% paraformaldehyde (PFA)

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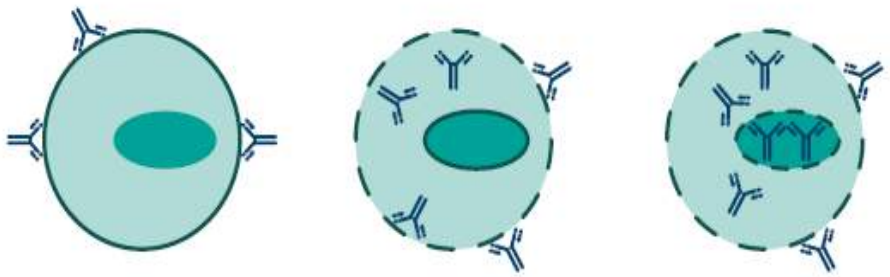
CHOOSE THE RIGHT DETERGENT TO STAIN YOUR TARGET

Aldehyde fixation requires permeabilization step.

Organic solvents does not require permeabilization step.

To access **intracellular targets**, mild reagents (e.g., Digitonin or Saponin) are needed.

To enable staining of **interior membranes** (e.g., nucleus, mitochondria), stronger non-ionic detergents are recommended (e.g., Triton X-100 or NP-40).



Antibody Accessibility with Unpermeabilized Cells and Mild (Digitonin) and Strong non-ionic (Triton X-100) detergent Permeabilized Cells.

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USE APPROPRIATE CONTROLS

Include:

- **The unstained sample** (to determine the autofluorescence background signal).
- Slides stained **only with secondary antibody** (to determine the threshold of background signal).
- **Tissue / cell type control**. Slides with tissue / cells are known to express (or not express) the antigen of interest.
- **Each staining separately** (applies for multistainings to ensure no cross-reactions and appropriate labelling).

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OPTIMIZE DILUTION AND CELL DENSITY

Cells confluence about 50% cell at the time of staining:

- too high: cell architecture is deformed, higher background at low magnifications
- too low: difficult to find the optimal cell pattern

Cut tissue sections at 5 - 10μm thickness:

- too thick: give stronger signals but worse resolution and higher background
- too thin: easy to damage and curl; difficult to embed

ultra-thin sections (nm) - electron microscopy

Special Coating Circumstances:
Cells / tissues difficult to grow on glass surfaces

Cells coat the coverslips with polylysine or extracellular matrices (e.g., collagen or laminin)
Tissues use gelatin-coated or poly-lysine-coated slides

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Primary and secondary antibody optimization

Both antibodies (primary and secondary) should be diluted in the blocking buffer.

Recommendation: 1h at RT with (1%–5%) BSA, milk powder, or serum.

GET PROPER SAMPLE MOUNTING

Mounting media are:

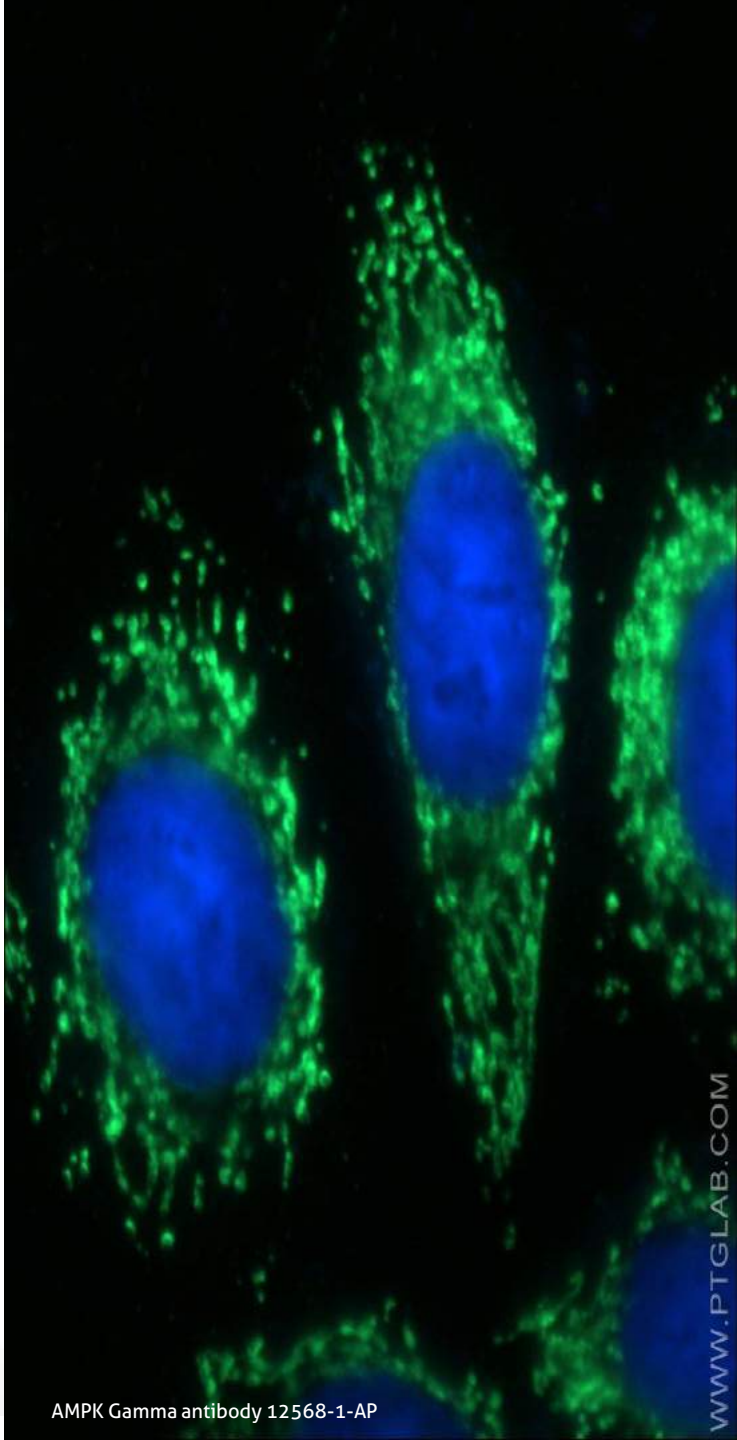
- PBS/Glycerol mixture.

Why mounting media is required:

- Prevent photobleaching/conserve sample.

3 simples steps:

- Mount coverslip/tissue samples
- Seal the samples with nail polish
- Store in the dark at -20°C or +4°C



AMPK Gamma antibody 12568-1-AP

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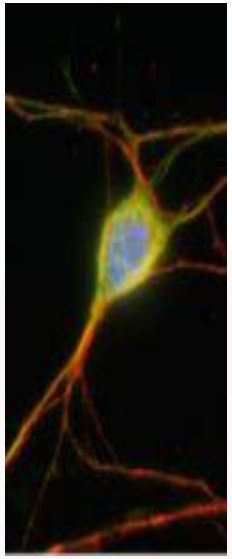
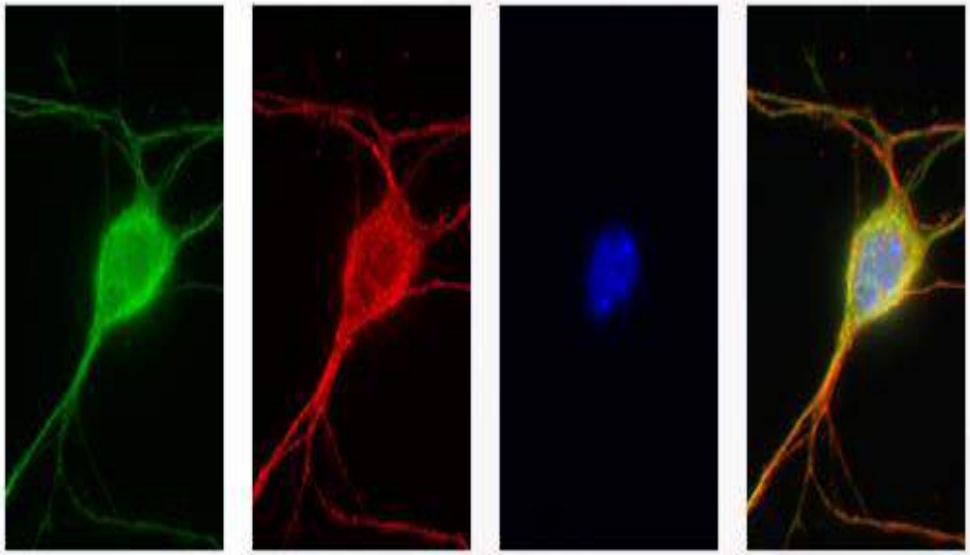


USE COUNTERSTAINS TO HELP IDENTIFY CELLULAR LANDMARKS

- To reduce background fluorescence.
- To identify cellular organelles and provide information regarding signal localization.
- The most common nuclear counterstain dyes are DAPI (diamidino-2-phenylindole), Hoechst 33342, propidium iodide (PI), and Far-Red Draq5.



NF1 ATG5 Nu Merge

10181-2-AP

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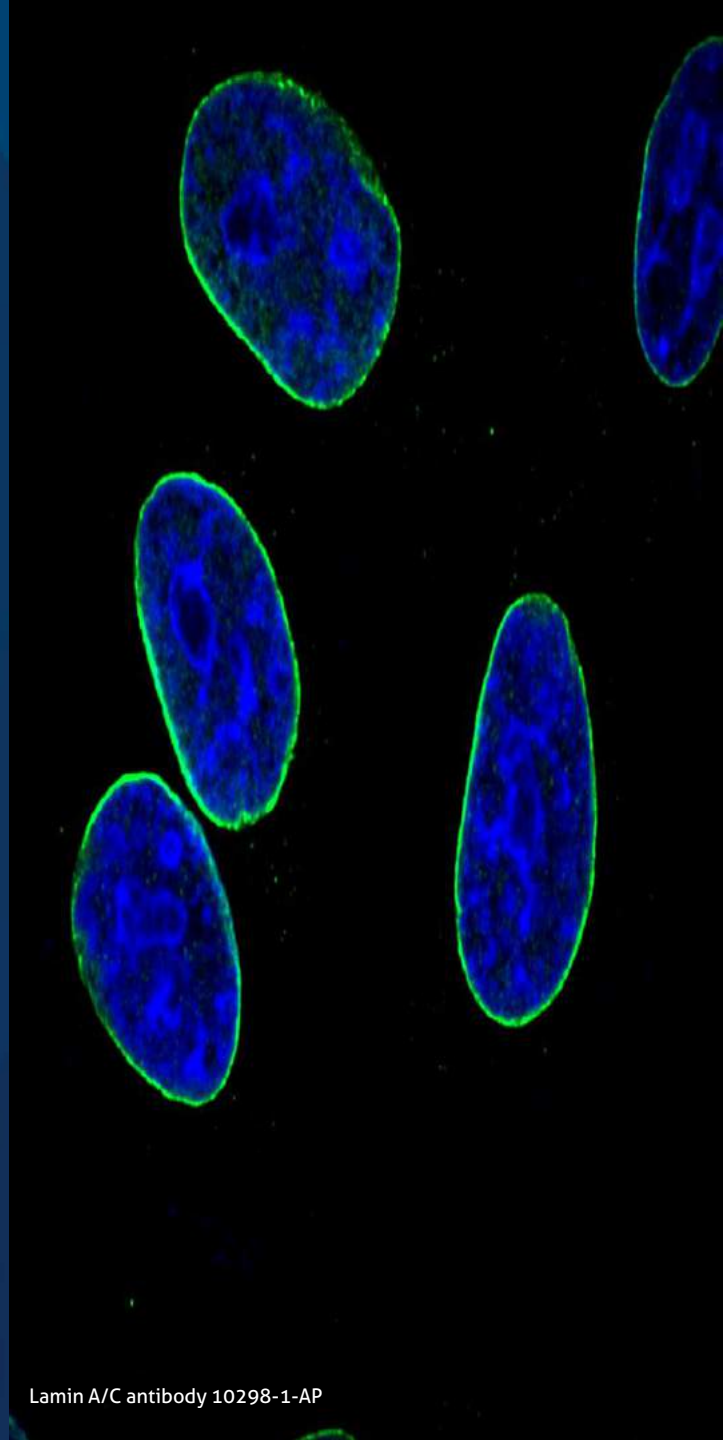
TROUBLESHOOTING



Lamin A/C antibody 10298-1-AP

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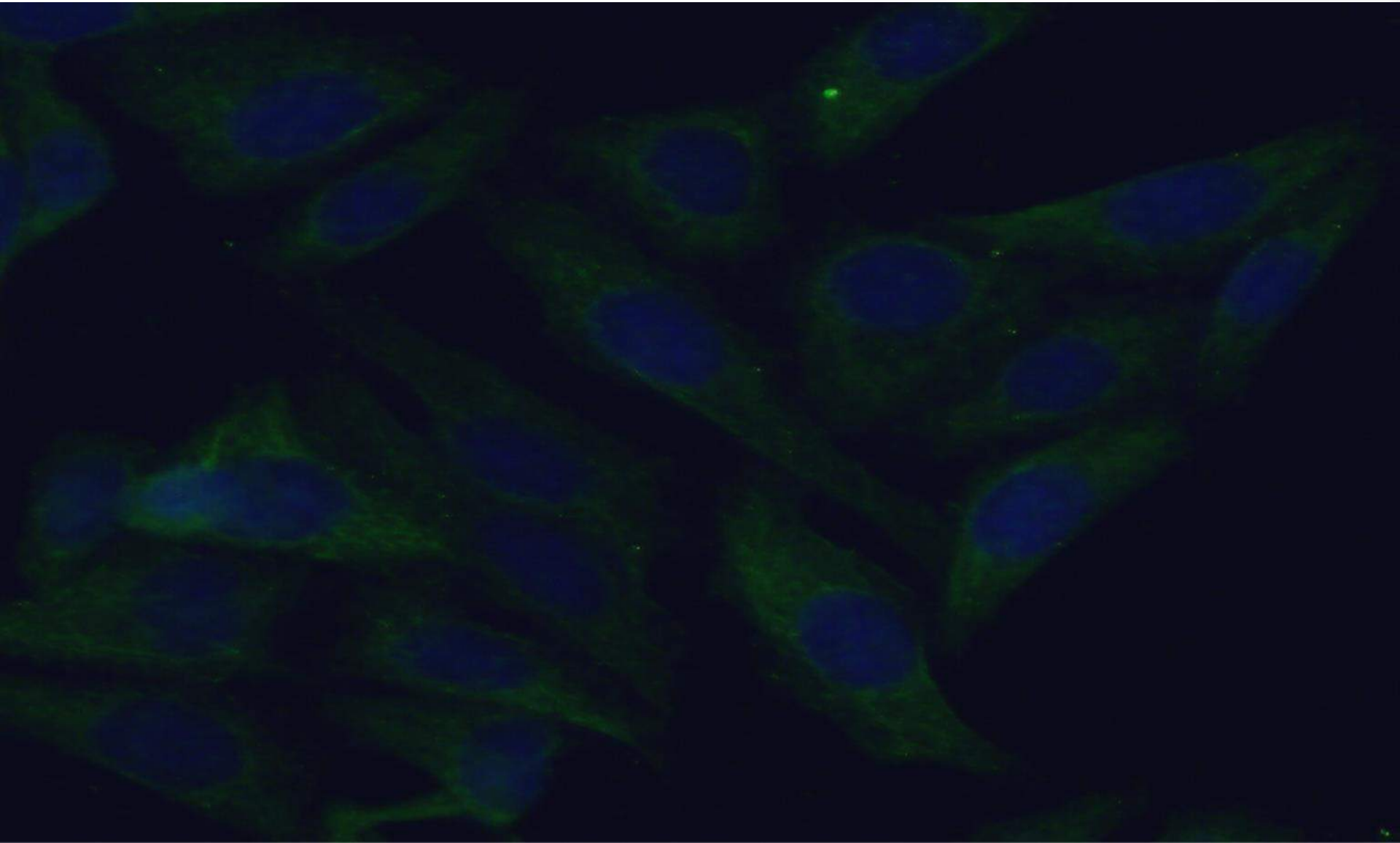

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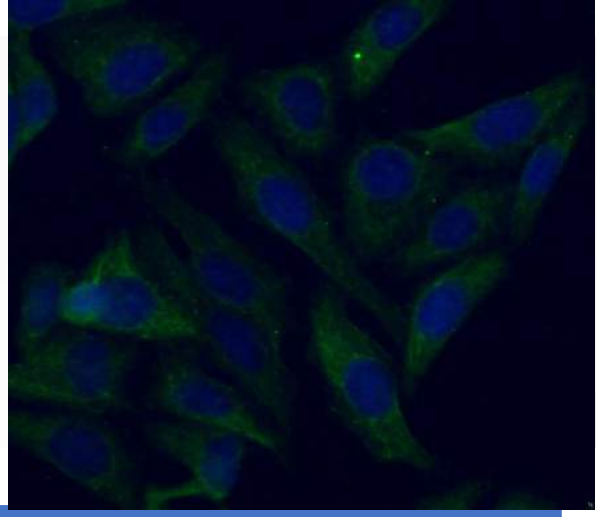

NO/WEAK SIGNAL

Problem	Solution
Microscope	Use positive slide Replace or realign the bulb
Incorrect light source / filter set	Check filters for correct wavelength
Gain / exposure is too low	Increase the exposure time
Fluorescent tag bleached	Avoid over exposure of the slide to light Always store slides in the dark
Antibody is not suitable for detection of protein in its native form in IF	Perform a test on a native western blot (non-denatured)

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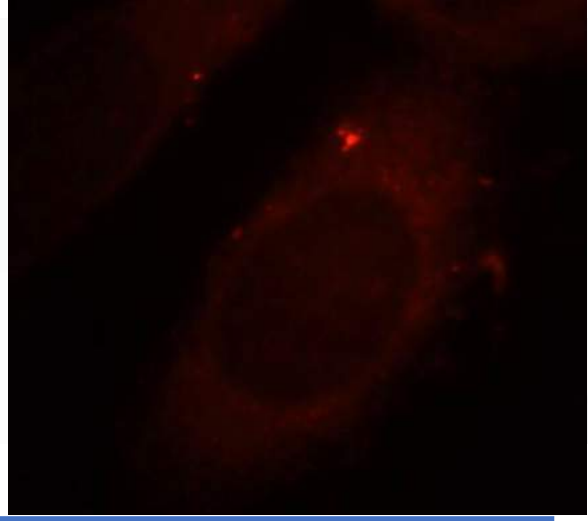

NO/WEAK SIGNAL

Problem	Solution
Protein of interest is low expressed	Use signal amplification when visualizing
Protein of interest is not expressed	Run a positive control
Cells were not permeabilized	Methanol / acetone fixation will permeabilize cells If using formaldehyde, permeabilize cells with Triton X-100
Cell / tissues are over fixed	Reduced the duration of fixation. Perform antigen retrieval to unmask the epitope

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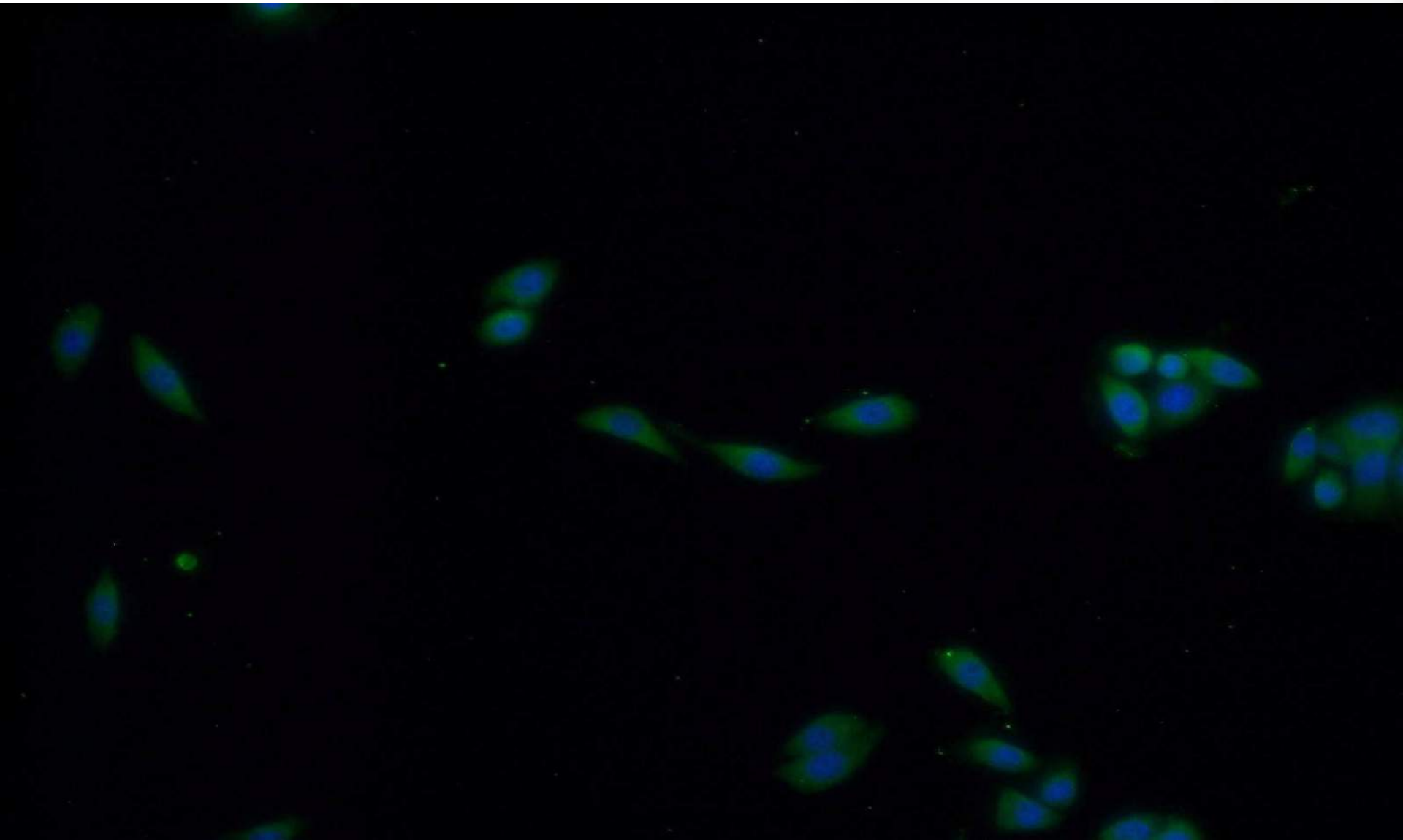

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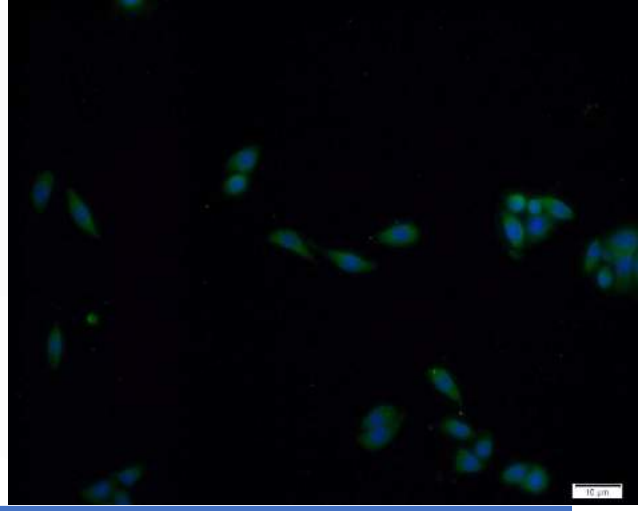

NO/WEAK SIGNAL

Problem	Solution
Cells / tissue dried out; too low/ too high confluency	Samples must be kept covered in liquid Adjust the cell confluency (50-70%); and tissue thickness
Not enough primary antibody	Use a higher concentration of antibody Incubate longer
The primary and secondary antibodies are incompatible	The secondary antibody should be raised against the host of the primary antibody Isotypes should also be compatible
Antibody storage issue	Freeze / thaw cycles can cause degradation Secondaries antibodies should be stored in the dark

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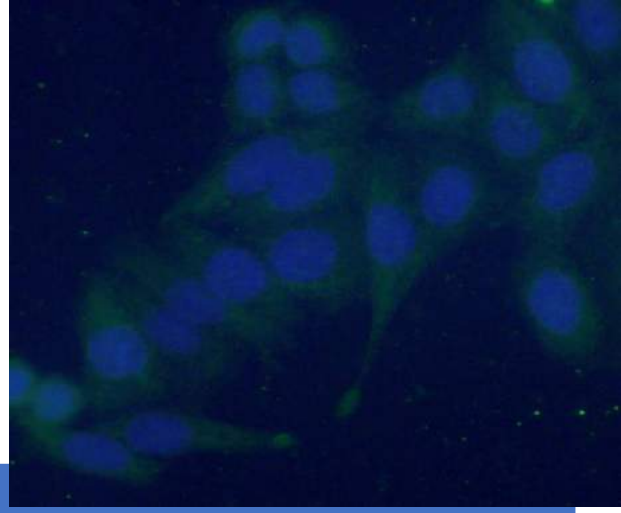
UNSPECIFIC STAINING



Problem	Solution
Target of interest is a nuclear protein	Use a permeabilization step
Antibody concentrations too high	Reduce the concentration, and the incubation time.
The primary is raised again the same species as the tissues stained (e.g. mouse on mouse)	Use a primary that is raised against a different species Block the endogenous IgG with serum from the same species as the secondary Incubate samples with 1% Triton to clean the tissues Use TBS-Tween 20 as a washing buffer, rather than using PBS-Tween 20
Artefacts / Aggregates	Spin down secondary antibodies. Take from the top

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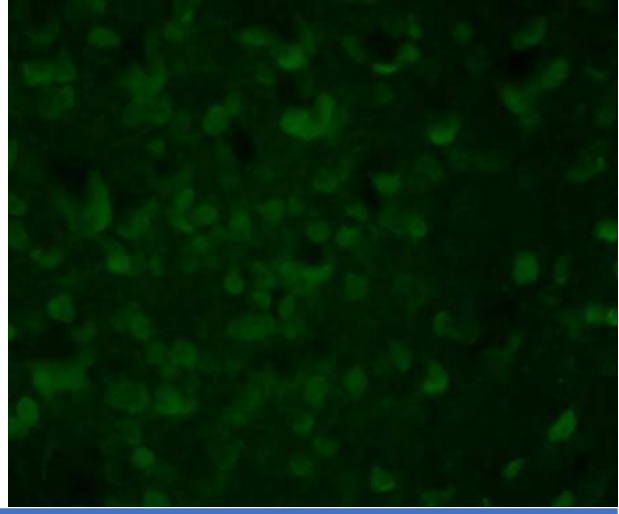
BACKGROUND STAINING



Problem	Solution
Unspecific binding of primary / secondary antibodies	Run control Prolong blocking step
The sample is poorly washed	Repeat or prolong washing step
Inappropriate fixation causes artefacts or damages the antigen	Reduce fixative step Change fixative
Autofluorescence	Check fluorescence in an unstained section Wash with 0.1% sodium borohydride in PBS to remove free aldehyde groups
Too high cell confluency Tissue is too thick	Optimize cell density / confluency Use thinner tissue sections

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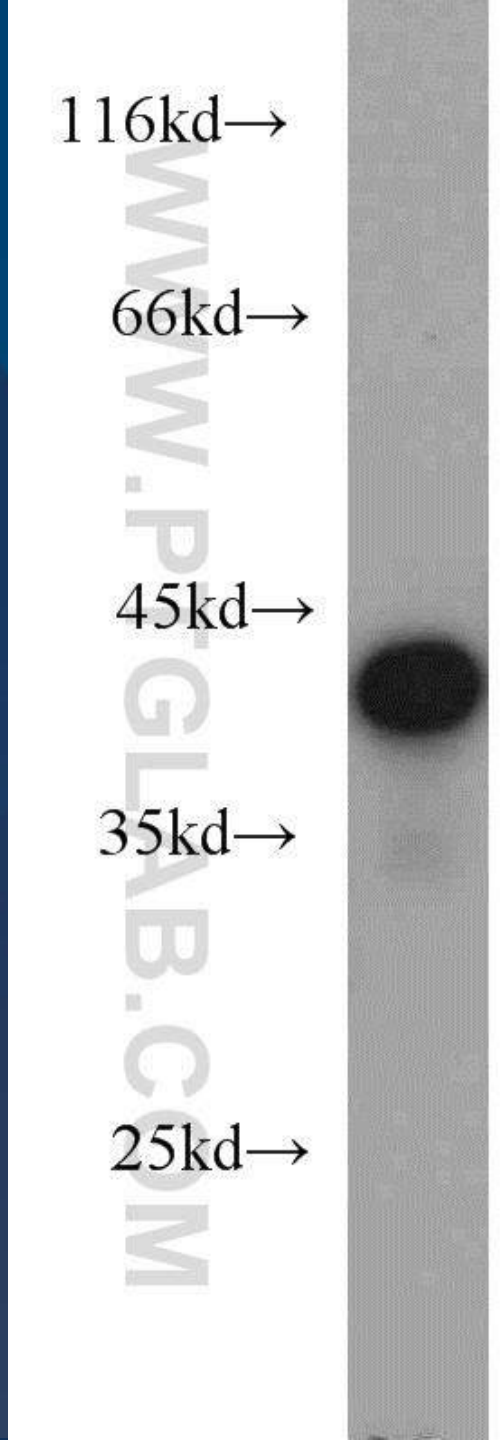
WHY WESTERN BLOTS ARE NOT WORKING



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Western blotting, immunoblotting technique:

- To detect specific proteins in a cell extract or tissue homogenate

The main steps are:

- Sample preparation
- Gel electrophoresis
- Transfer
- Blocking
- Detection
- Analysis

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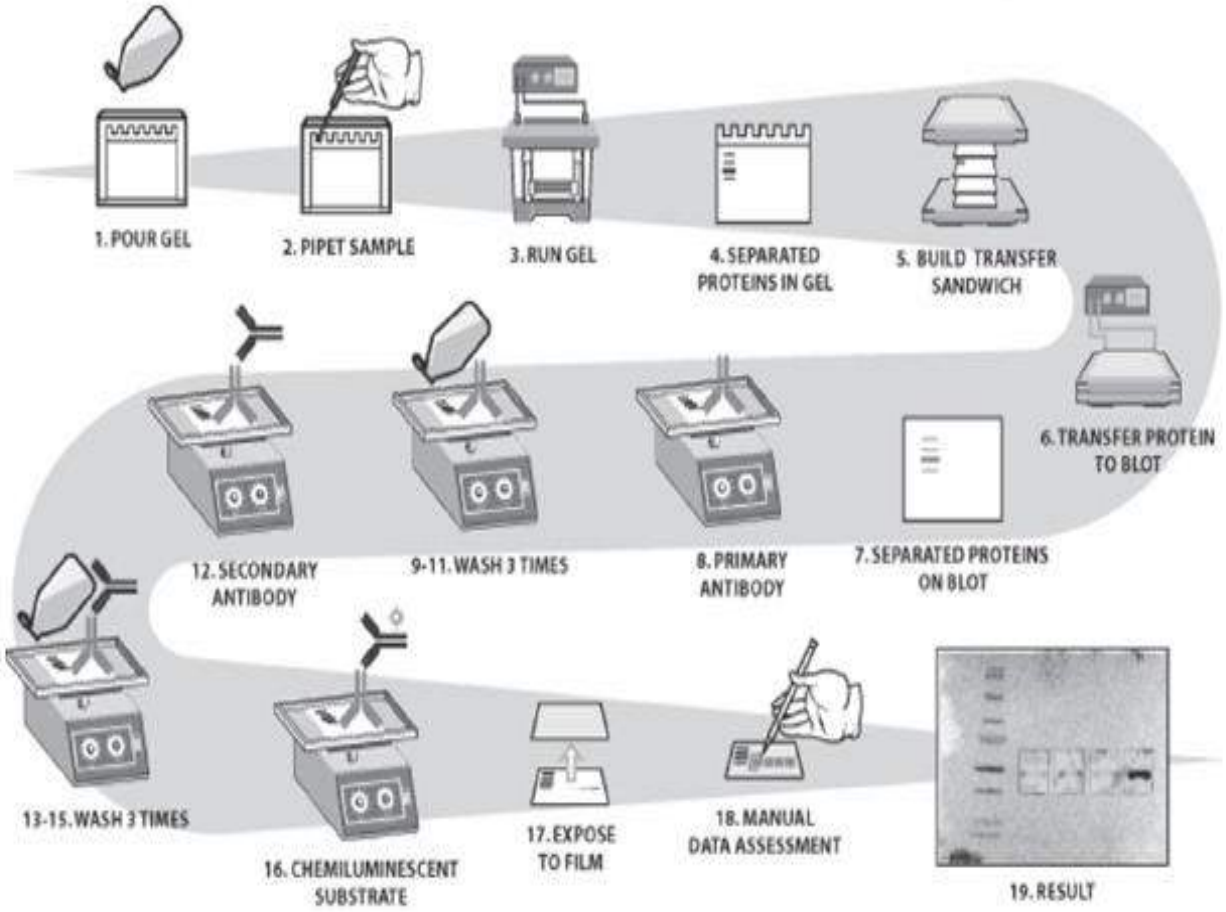
OVERVIEW OF WESTERN BLOTTING



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FACTORS THAT AFFECT WESTERN BLOTTING

Factors	Characteristics
Target	Conformation, epitope.
Polyacrylamide gel	Self-made, manufacturer, percentage, age, lot.
Membrane	Type, manufacturer, lot.
Primary antibody	Type, specificity, epitope, conc., incubation time/ temperature.
Washing	Volume, frequency.
Blocking	Type, conc., cross-reactivity.
Secondary antibody	Type, manufacturer, lot.
Substrate	Sensitivity, manufacturer, lot, age, conc.

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WESTERN BLOTTING

CONSIDERATIONS BEFORE STARTING WB

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CONSIDERATIONS BEFORE STARTING WB FOR POLYACRYLAMIDE GEL

Standard gel:

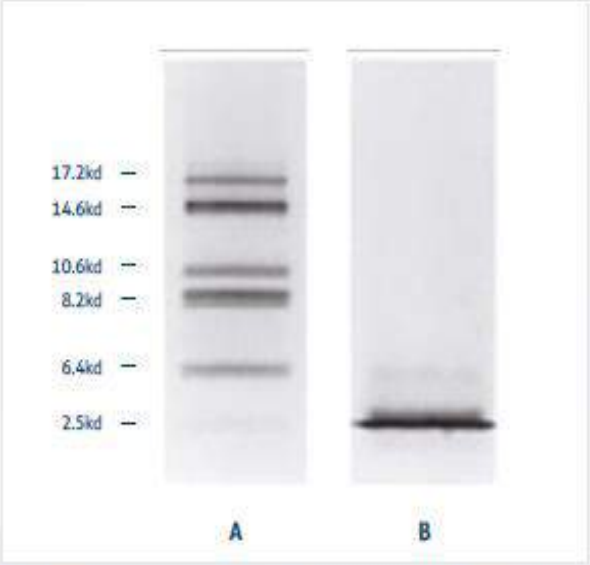
- Tris-glycine buffer system
- Detection of 30–250 kDa proteins

Gel for low molecular weight (MW) proteins:

- Tris-tricine buffer
- Detection of < 30 kDa proteins

Protein Size	Gel Acrylamide Percentage
4–40 kDa	20%
12–45 kDa	15%
10–70 kDa	12.5%
15–100 kDa	10%
25–200 kDa	8%

Tip:
Choose the right gel acrylamide percentage.



H Schägger and G von Jagow. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem. 1987;166(2):368–79.

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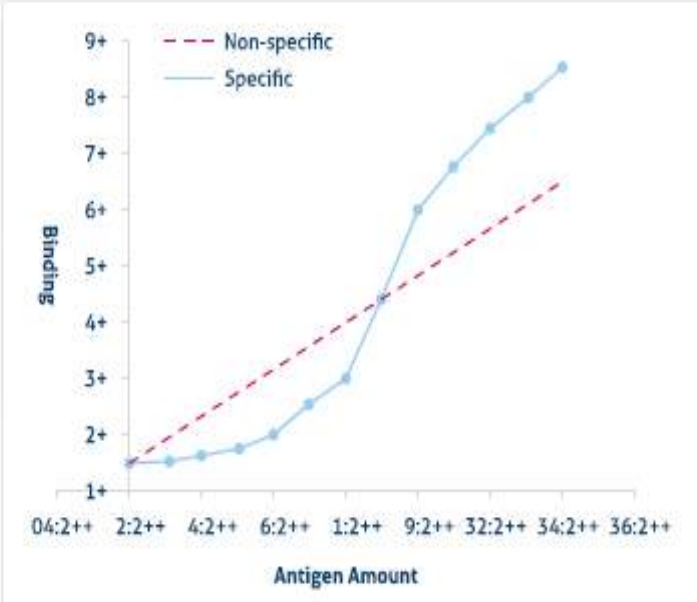
Tip **sonication step**: small aliquots and sonicate 1–2 minutes (high power; 30 seconds sonication, 30 seconds rest)

SAMPLE PREPARATION

- Non-specific binding is favored when a low amount of antigen is present
- For low abundance proteins the following applies:
  - Insufficient amount of antigen might lead to non-specific binding
  - Load more protein



Tip:
Load appropriate amount of the protein lysate.

30ug or more

Protein Abundance Database: **PaxDb**
http://pax-db.org

paxdb

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SAMPLE PREPARATION

- Cell lysis is the breaking down of the cell membrane and the separation of proteins from the non-soluble parts of the cell.
- Dependent on the location of the protein of interest, a different lysis buffer is needed.

Tip:
Choose the right lysis buffer.

Location	Lysis Buffer
Nuclear, Mitochondria	RIPA, Fraction protocol
Membrane-bound proteins, Whole cell lysate	RIPA or NP-40
Cytoplasm	RIPA, Tris, HCL/Triton

Why is RIPA the best lysis buffer?

- Will disrupt protein-protein interactions.
- Is useful for whole cell extracts and membrane-bound proteins.
- Preferable for extracting nuclear proteins.
- Gives the lowest background.

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TRANSFER

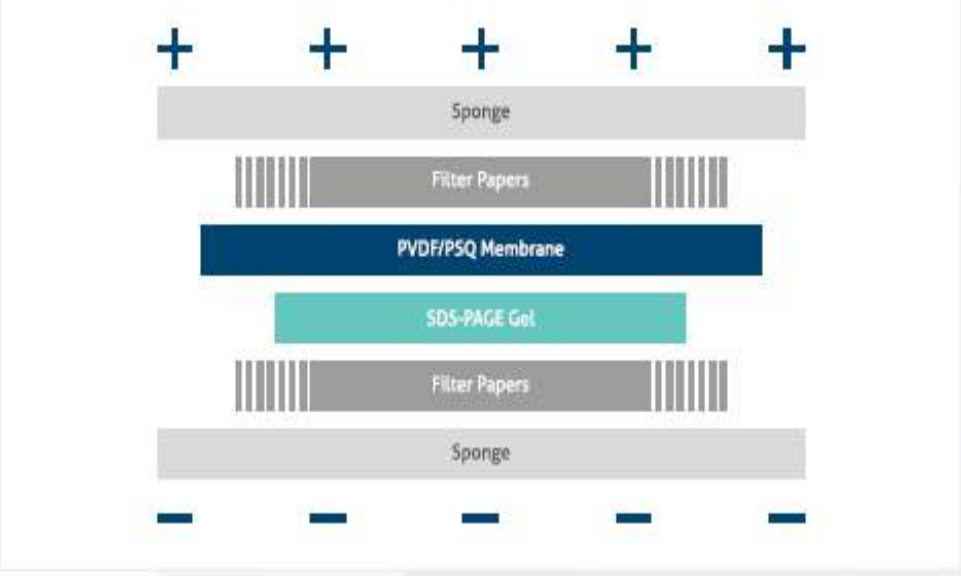
- **Proteins are transferred from the gel to the membrane to make them accessible for antibody detection.**

- **Factors that influence the quality of the protein transfer:**
  - Transfer
  - Temperature
  - Membrane
  - Buffer composition

- **Membrane choice and pore size:**
  - Pore Size
    Dependent on molecular weight (MW) of the target protein
  - Membrane Type
    Dependent on the hydrophilic/hydrophobic character of the target protein

- **Low molecular weight proteins tend to "over transfer":**
  - Transfer slowly and reduce voltage time.

Tip:
Choose the right voltage time for your transfer.



A representation of the components of a transfer "sandwich." Note the orientation of the gel and membrane: the PVDF membrane is situated nearest to the positive electrode. The binding of SDS to proteins results in the complex having an overall negative charge. Therefore, the SDS-bound proteins travel toward the positive electrode.

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Tip:
Choose the right membrane based on the hydrophilic or hydrophobic character of the protein.

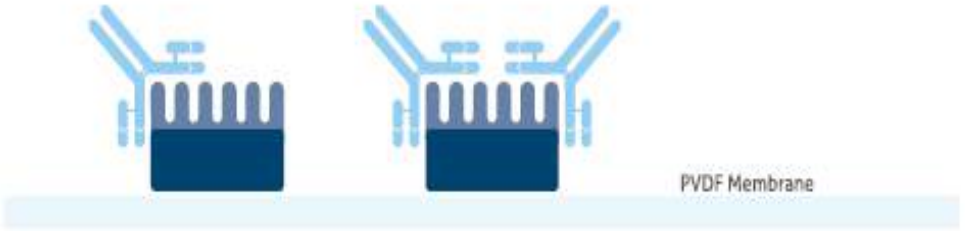
MEMBRANE TYPE

- Protein binding is based on **hydrophobic interactions and charged interactions.**
- **PVDF** and **Nitrocellulose membrane** are the most common used membranes.

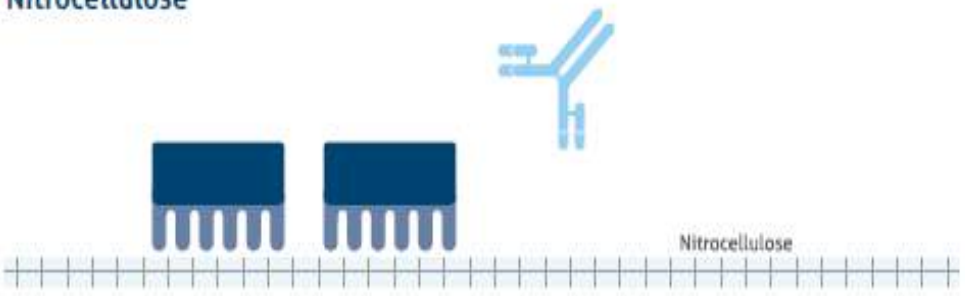
PVDF (Polyviniledene Difluoride)	Nitrocellulose
Less fragile.	Fragile.
Soak in ethanol, isopropanal, or methanol before use.	Soak in transfer buffer before use.
More expensive.	Less expensive.
Can be stripped and reused many times.	Can't be reused as much.
Preferable for proteins with a hydrophilic character.	Preferable for proteins with a hydrophobic character.

Tip:
Choose the right membrane.

PVDF
Immobilon-P (MW>20kDa)
Immobilon-PSQ (MW<20kDa)



Nitrocellulose



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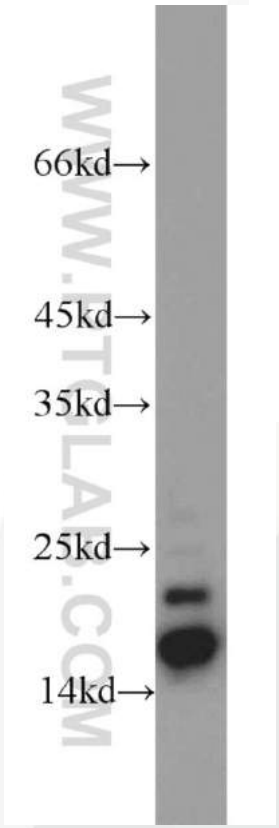
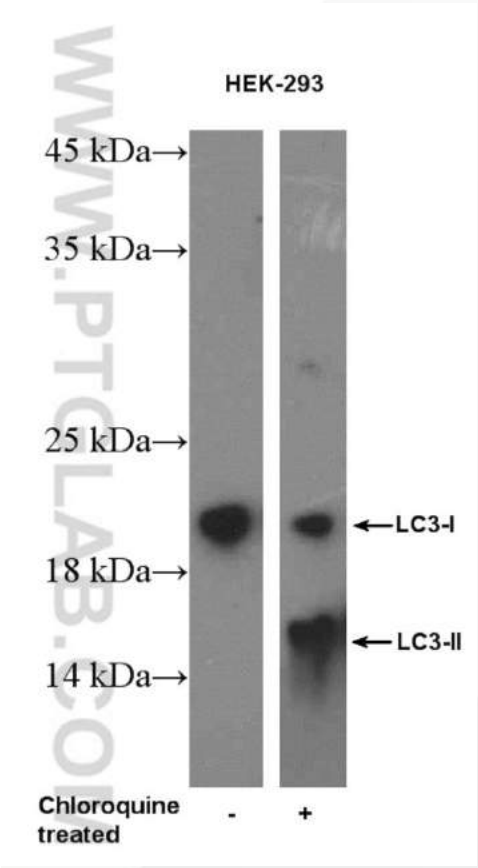
Western blot assay of LC3

- better to use PVDF membrane
- 0.2 um is recommended- small protein,

pore size of 0.45 um - LC3 may cross through the membrane





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LOADING CONTROL

- Loading controls are used for semi-quantification of protein levels.
- They ensure that the observed alteration in target protein is due to experimental manipulations.
- Characteristics of a loading control:
  - High expression
  - Constitutive
  - Unchanged expression during experiment

Tip:
To confirm your results, remember to add a loading control.

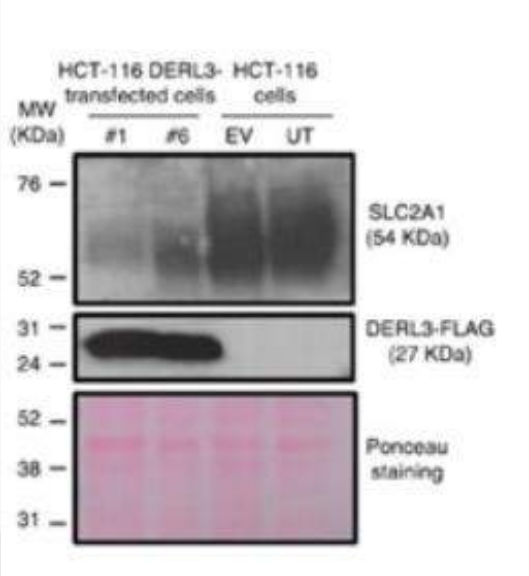
Selected loading controls for different cell locations		
Whole cell/ cytoplasmic	**Nuclear**	**Mitochondrial**
Actin 42 kDa	Lamin B1, 66 kDa	COX-4, 17 kDa
GAPDH, 36 kDa	PCNA, 36 kDa	VDCA1/Porin, 31 kDa
Tubulin, 50–55 kDa	TBP, 38 kDa	–

Loading control for secreted proteins

a ponceau staining of the membrane:

- stain the membrane, check that all wells contain the same amount of protein
- perform the WB using same membrane after washing and blocking
- please remember to block the membrane AFTER ponceau staining!

Lopez-Serra P et al., Nat Commun. 2014



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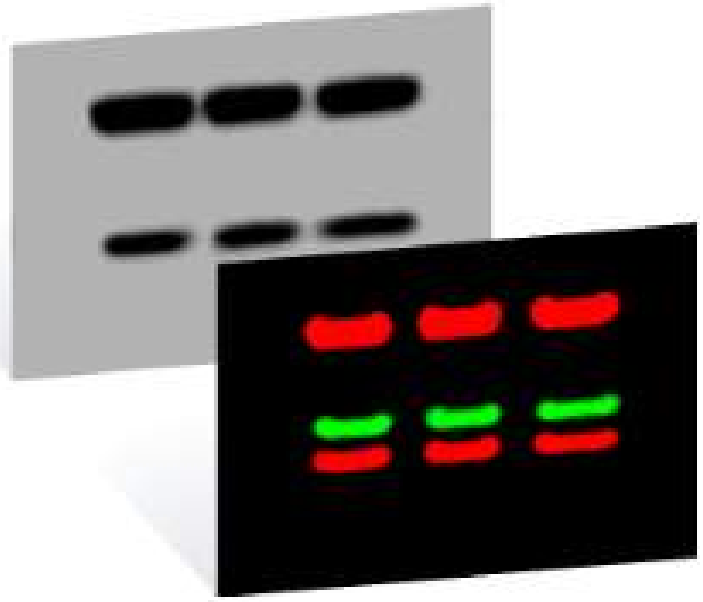
Signal detection for Western Blot

IR Fluorescence

Chemiluminescence



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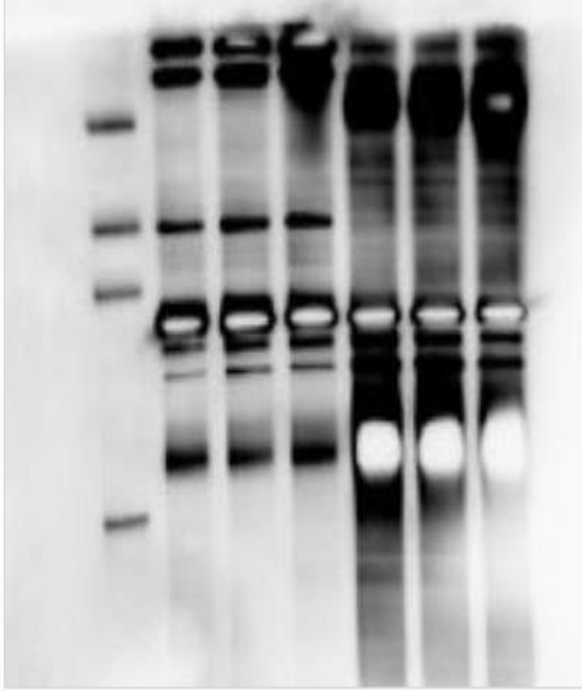
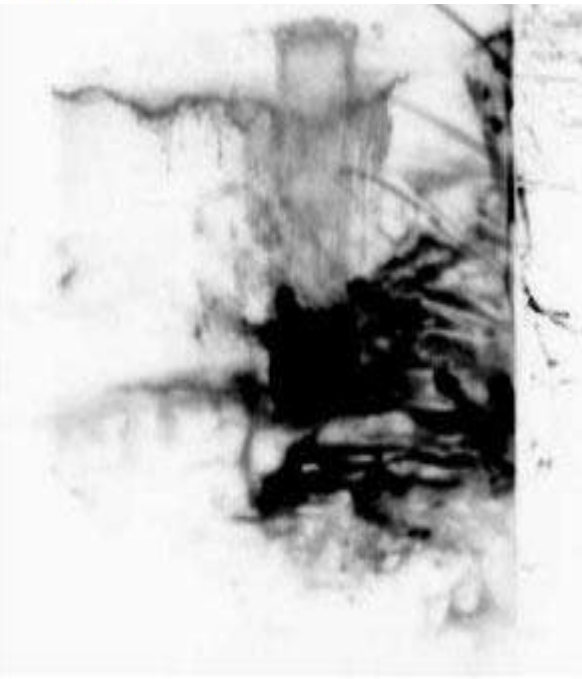
WESTERN BLOTTING

TROUBLESHOOTING



Ghost Bands



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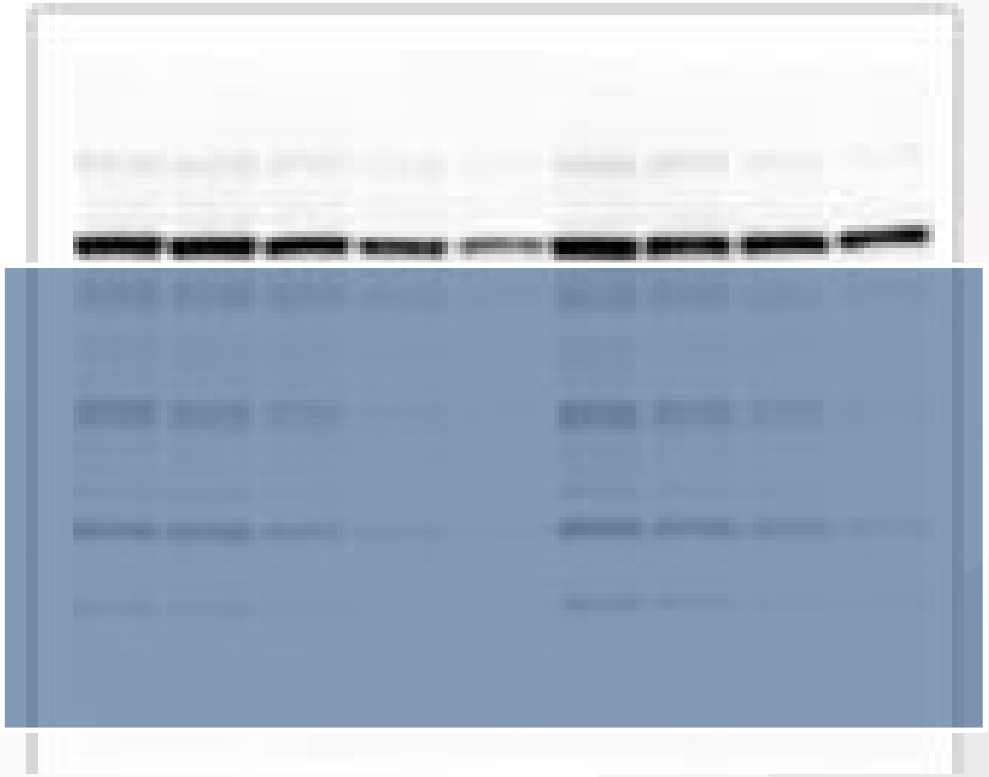
Why Are There So Many Bands?!

UniProt
Uniprot.org



Proteolysis
PTM
Splicing Isoforms

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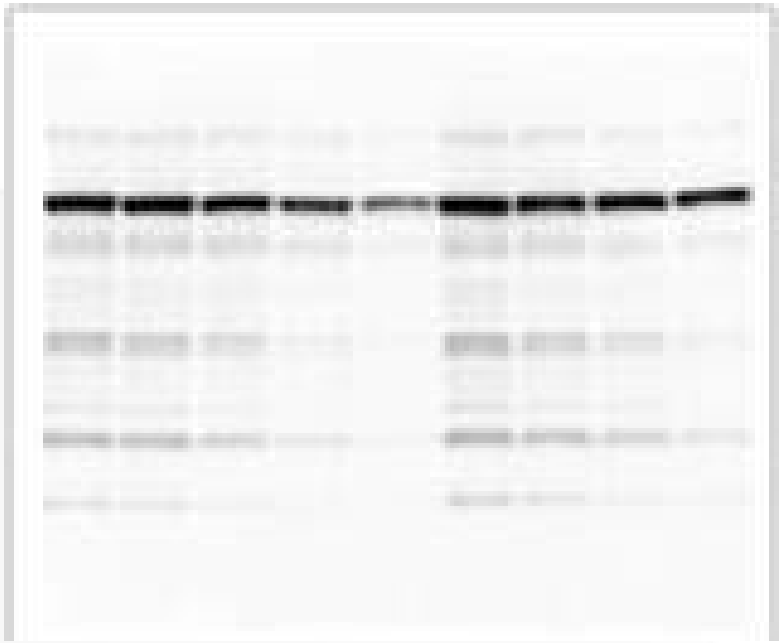
Some Extra Bands Are Result of Proteolysis



Solutions:

1. When possible, use fresh samples
2. Use protease inhibitors in lysis buffer
3. Sonicate samples

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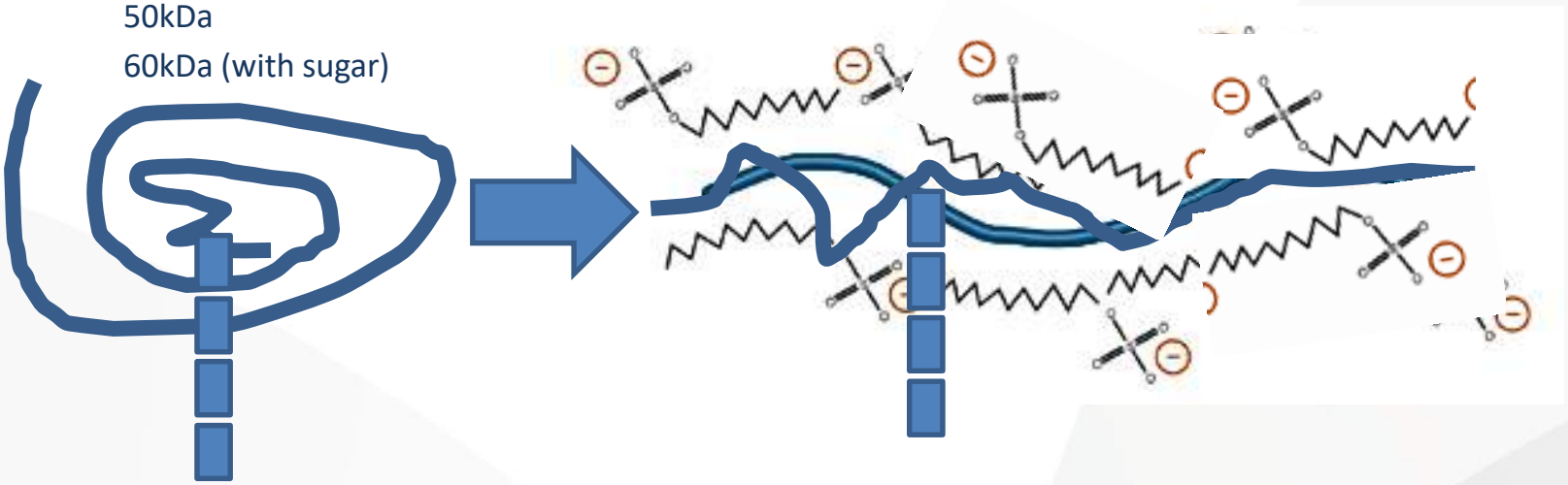
Glycosylation Changes How Proteins Travel through the Gel

50kDa
60kDa (with sugar)



10kDa glycosylation may result in more than 10kDa up the gel

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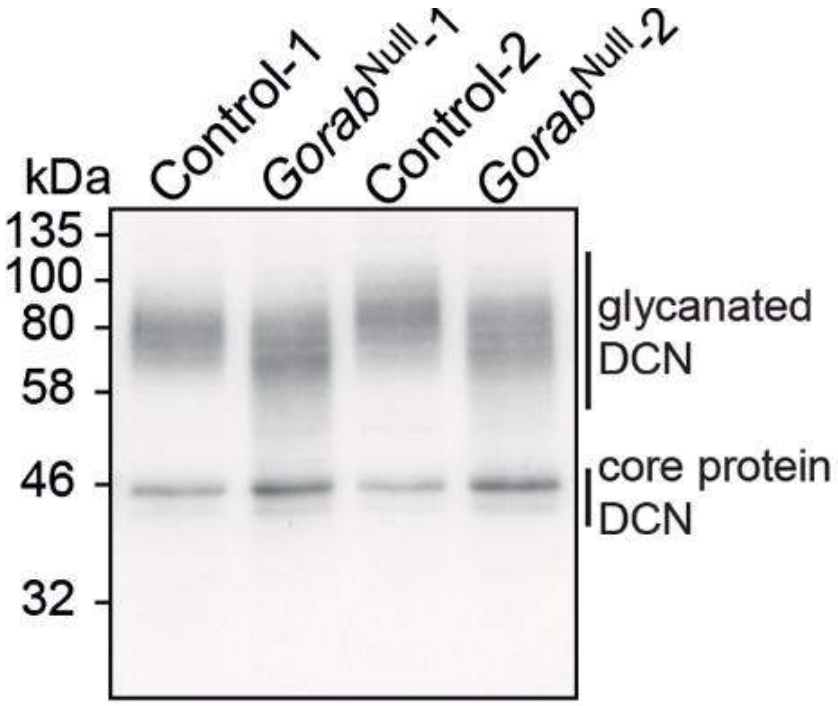
Glycosylation Changes How Proteins Travel through the Gel



Chan WL et al., 2018 14667-1-AP
Impaired proteoglycan glycosylation, elevated TGF-β signaling,
and abnormal osteoblast differentiation as the basis for bone fragility in a mouse model for gerodermia osteodysplastica.

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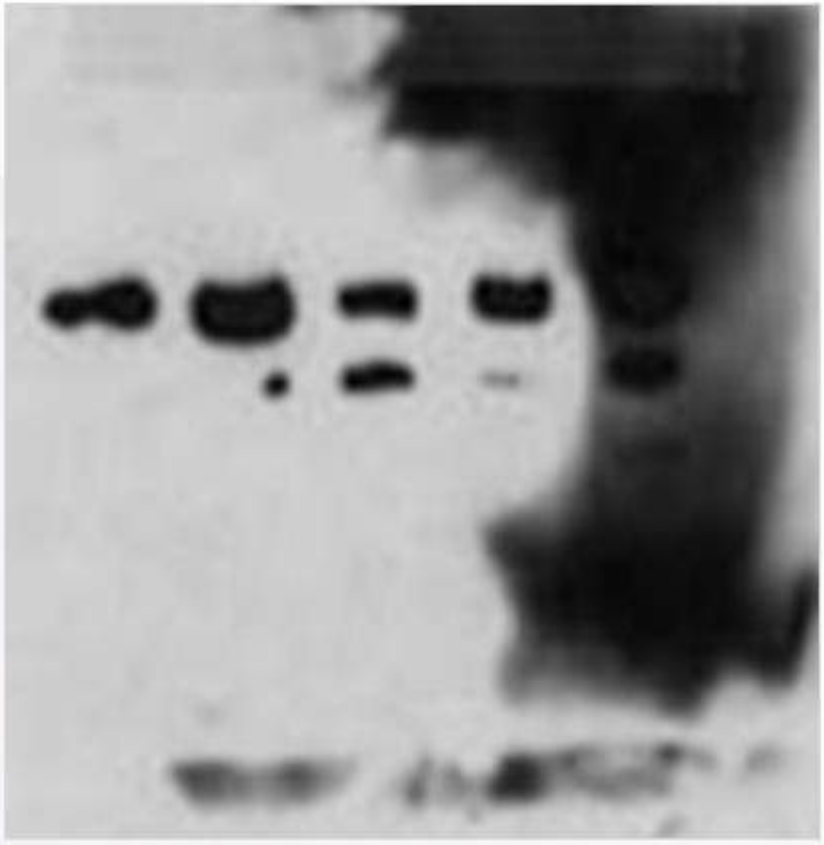
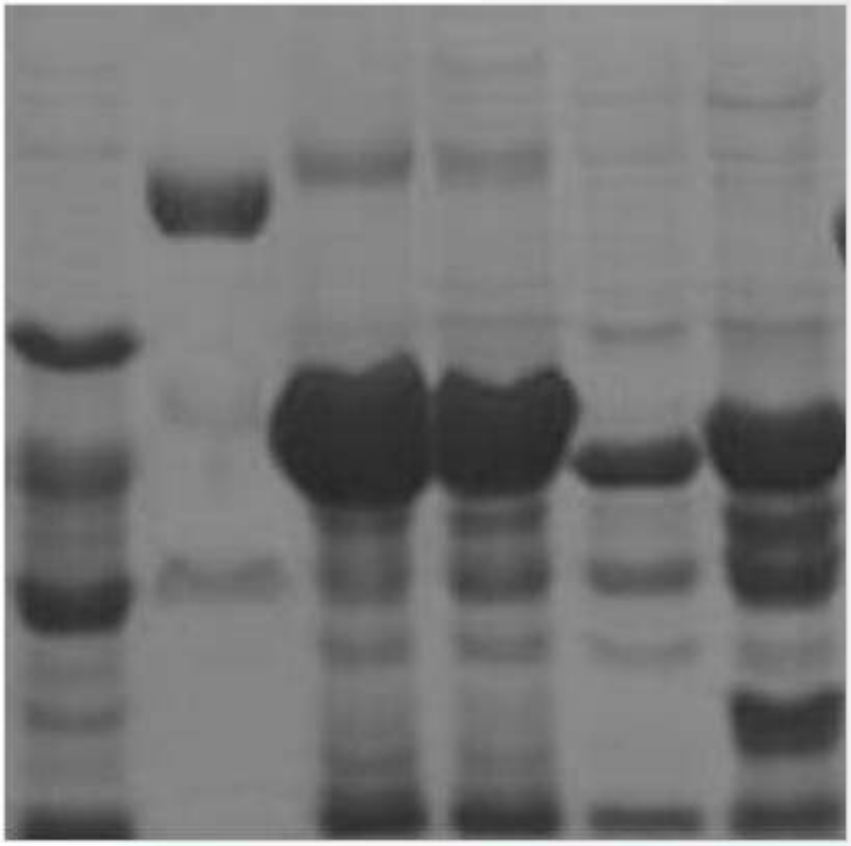
BLOCKING/WASHING BUFFER OPTIMIZATION



Ensure excess ECL is washed away.

SIGNAL OPTIMIZATION



"Smiling Bands"
Electrophoresis too fast/too hot

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NON-SPECIFIC BINDING

Potential Cause	Procedure
Sample degradation	Prepare fresh lysates Include protease inhibitors
Interference from other isoforms	Check literature for known isoforms Use isoform-specific antibody
Target protein abundance is lower than threshold of non-specific binding	Load more protein Enrich low abundance proteins by IP
Inefficient SDS-PAGE separation	Change the gel percentage to suit the target protein's MW.

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WEAK/NO SIGNAL

Potential Cause	Suggested Solution
Issues with the primary and/or secondary antibody	Titrate the antibody Change incubation time and temperature Antibody may have lost activity
Membrane choice	Select PVDF or NC membranes based on hydrophobicity Hydrophilicity of the target protein
Sodium azide contamination	The presence of sodium azide inhibits the activity of HRP Ensure sufficient washing
Detection reagent not sensitive enough	Dilute chemiluminescent reagents in high-purity water Check several exposure times to achieve optimum detection

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HIGH BACKGROUND

Potential Cause	Suggested Solution
Inadequate washing	Increase washing time and volume
Dry membrane	Ensure membrane does not dry out
Film exposure too long/detection reagent too sensitive	Check different types and dilution of the detection reagent
Insufficient blocking	Increase the concentration of blocking reagent Increase blocking time Add Tween 20 to the blocking buffer
Antibody concentration too high	Use higher antibody dilution

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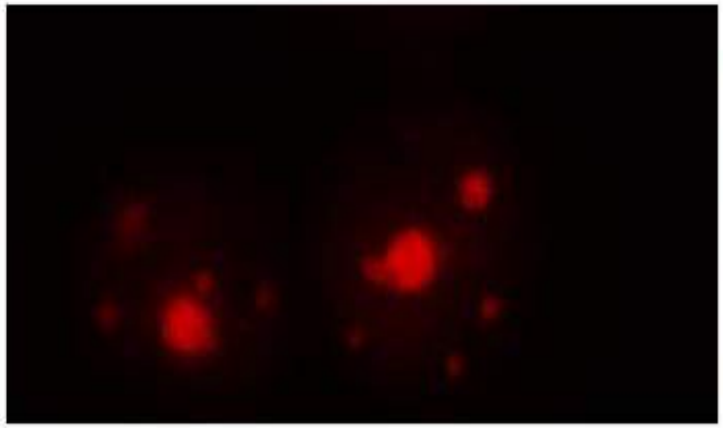
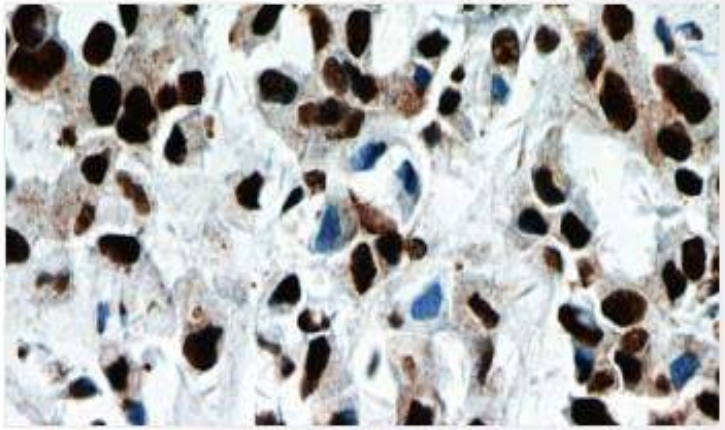
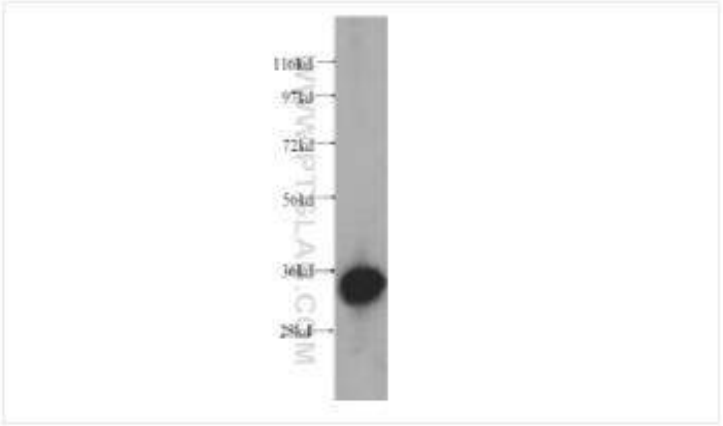
MONOCLONAL OR POLYCLONAL

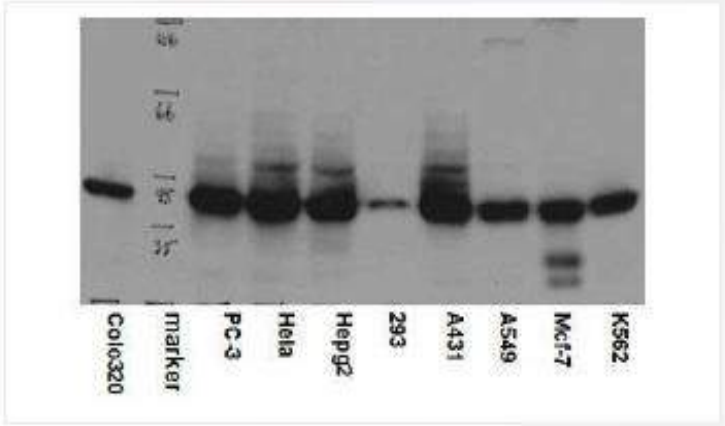
Monoclonal Production:
+/- 6 months





Polyclonal Production:
+/- 3–4 months





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HELPFUL RESOURCES

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USEFUL LINKS

PaxDb Protein Abundance Database:
http://pax-db.org

STRING database Assessment and integration of protein–protein interactions:
https://string-db.org/cgi/input.pl

UniProt Universal Protein resource:
http://www.uniprot.org/uniprot/

NCBI-BLAST Online sequence alignment:
https://blast.ncbi.nlm.nih.gov/Blast.cgi

GeneCards: genomic, proteomic, transcriptomic, genetic and functional information database:
http://www.genecards.org

PubMed: most up-to-date information regarding target research:
https://www.ncbi.nlm.nih.gov/pubmed

Proteintech free protocol guides:
https://ptglab.com/promotions/free-protocol-guides/

Proteintech specific protocols:
https://www.ptglab.com/news/blog/product-specific-protocols/

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- Antibodies against 12,000 targets, all manufactured in-house
- Open access validation data on the website
- First antibody company to use KO/KD to demonstrate specificity
  (The CiteAb awards for the very best suppliers and individuals in the antibody industry)

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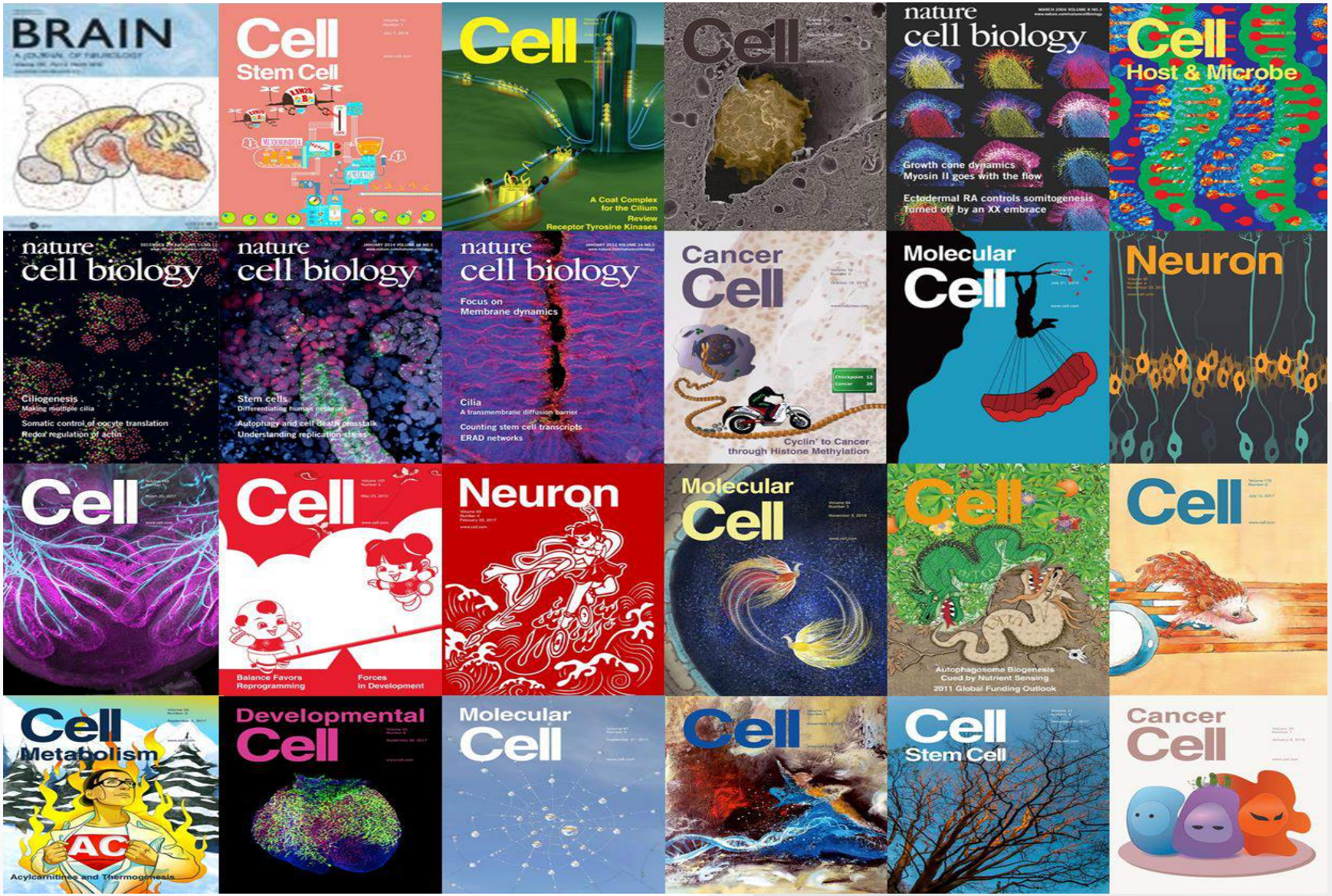
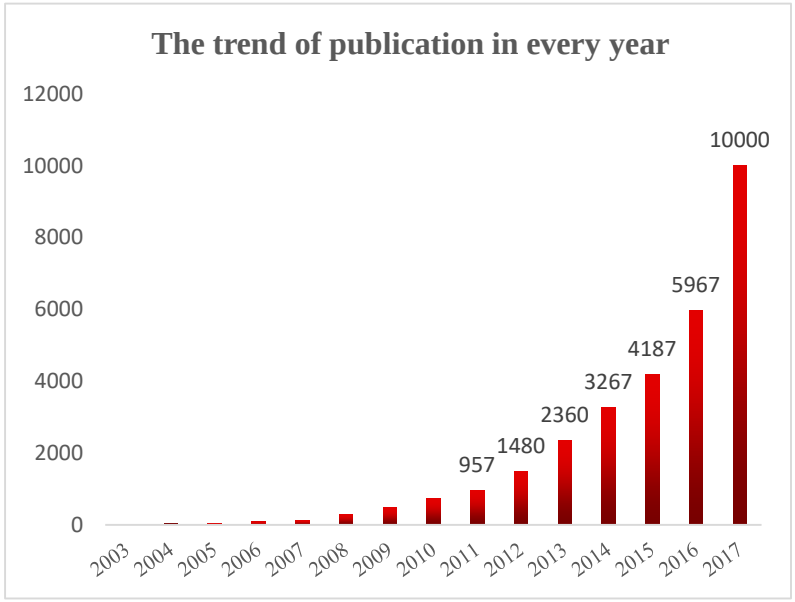
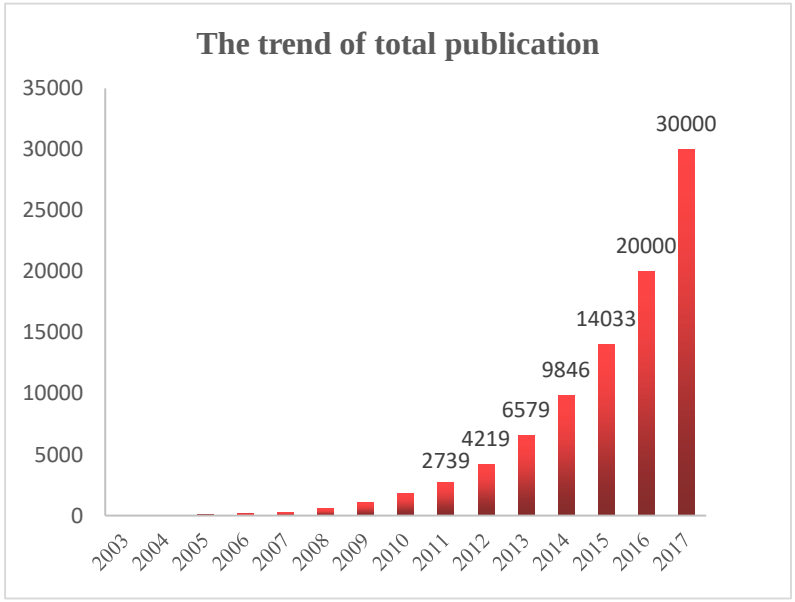


Proteintech in PubMed





CiteAb 2017:

"Highly commended antibody supplier' in CiteAb 2018 awards"

recognizing the company with the largest percentage increase in citations based on data gathered between January 2017 and December 2017

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CONTACT US

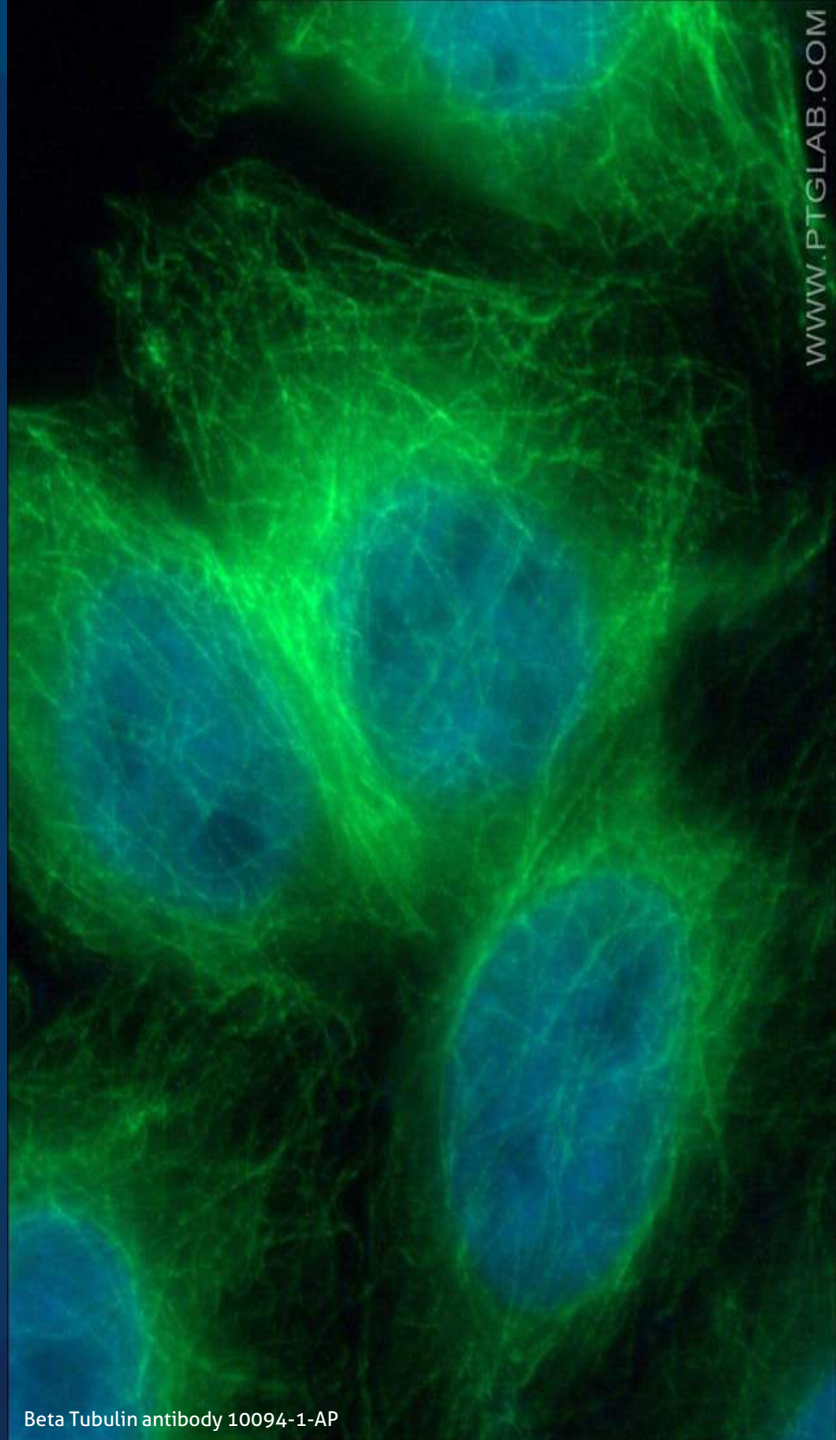
Proteintech Group
US Head Office
proteintech@ptglab.com

Proteintech Europe
UK Office
europe@ptglab.com

Proteintech
China Office
Proteintech-CN@ptglab.com

Support
Available 24 hours via Live Chat and 9–5 (CDT) via phone.

Please visit us at www.ptglab.com for more information about our antibodies and technical tips.



Beta Tubulin antibody 10094-1-AP

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Thank you for your attention!

Dr. Karolina Szczesna
Product Manager and Technical Support
karolina@ptglab.com