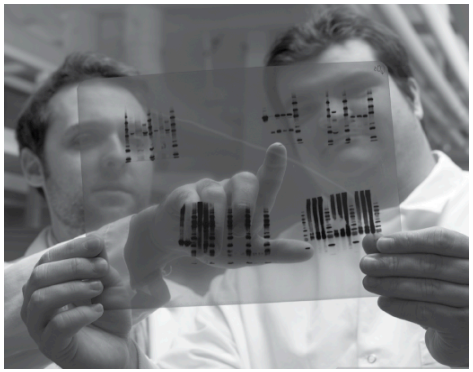
SOLUTIONS FOR WESTERN BLOTTING

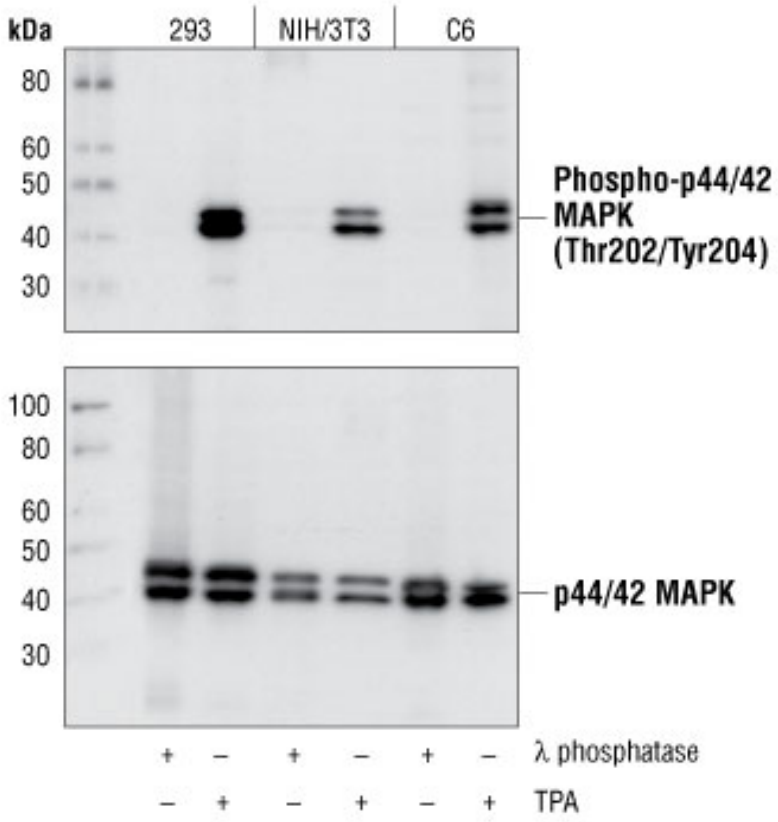
Edward Verwayen



Cell Signaling TECHNOLOGY®

What is the aim of today's seminar?

To get you generating blots that look like this…



Agenda

Part 1: Western blot tools for success

Basic concepts and methods

The importance of antibody validation

Primary antibodies – XP® antibodies

Antibody kits

Secondary antibodies and detection platforms

Part 2: Western blot protocol for good results

Western blot step by step

Troubleshooting

How can CST support you

Cell Signaling TECHNOLOGY®

Cell Signaling Technology Mission®

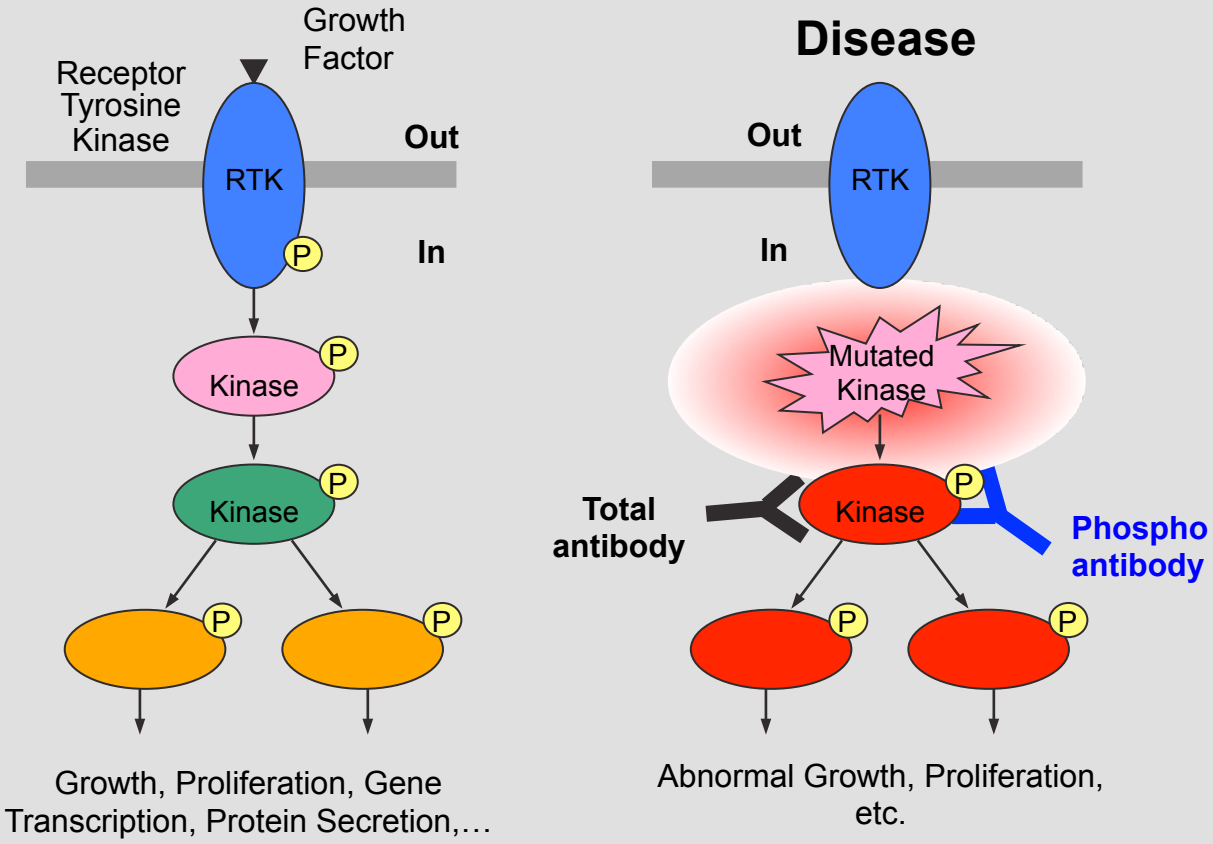
"To deliver the world's highest quality research products that accelerate biological understanding and enable personalized medicine"

UNPARALLELED PRODUCT QUALITY, VALIDATION, AND TECHNICAL SUPPORT

Dedicated to making high quality products	Rigorous validation of products in several applications	Technical support provided by CST's scientists

Cell Signaling TECHNOLOGY®

Cell Signaling Technology® product expertise



Western Blotting can be used to successfully analyze expression and/or modification status of a protein

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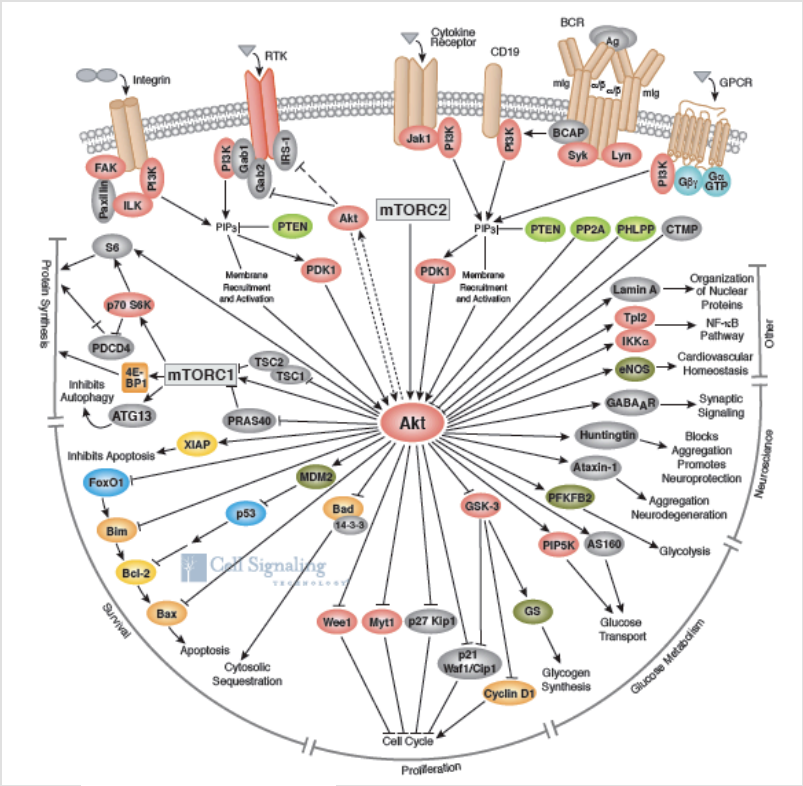
Cell Signaling Technology® Products



CST's antibodies cover most signaling pathways and cellular processes

- Total and activated state polyclonal and monoclonal antibodies, secondary, conjugated and motif antibodies
- Antibody kits for WB, ELISA, ChIP…
- Companion reagents
- Services: carrier-free, custom-formulated antibodies, bulk and proteomics services

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Western Blot Tools for success

Solutions for consistently better blotting

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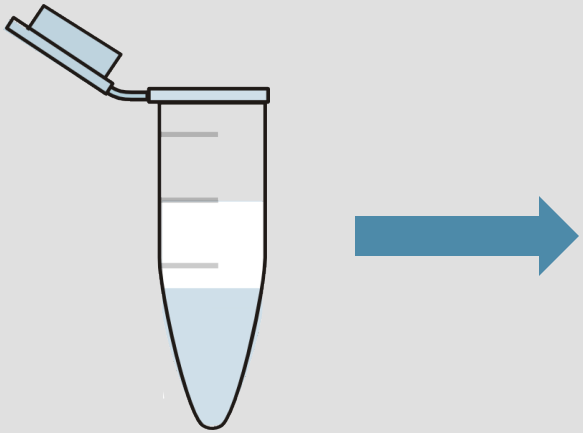
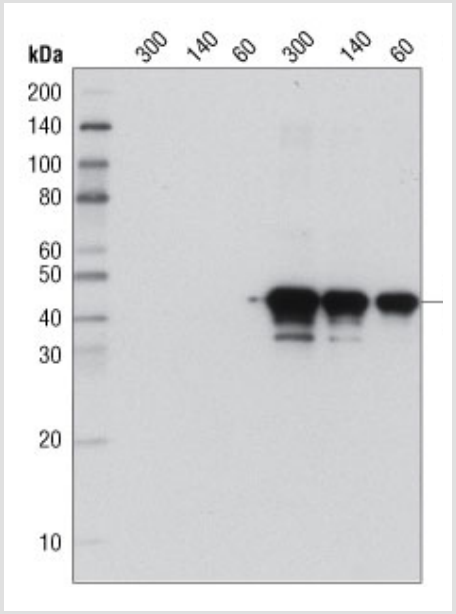
What is Western Blot?

WB is the most common technique used to detect proteins in a sample



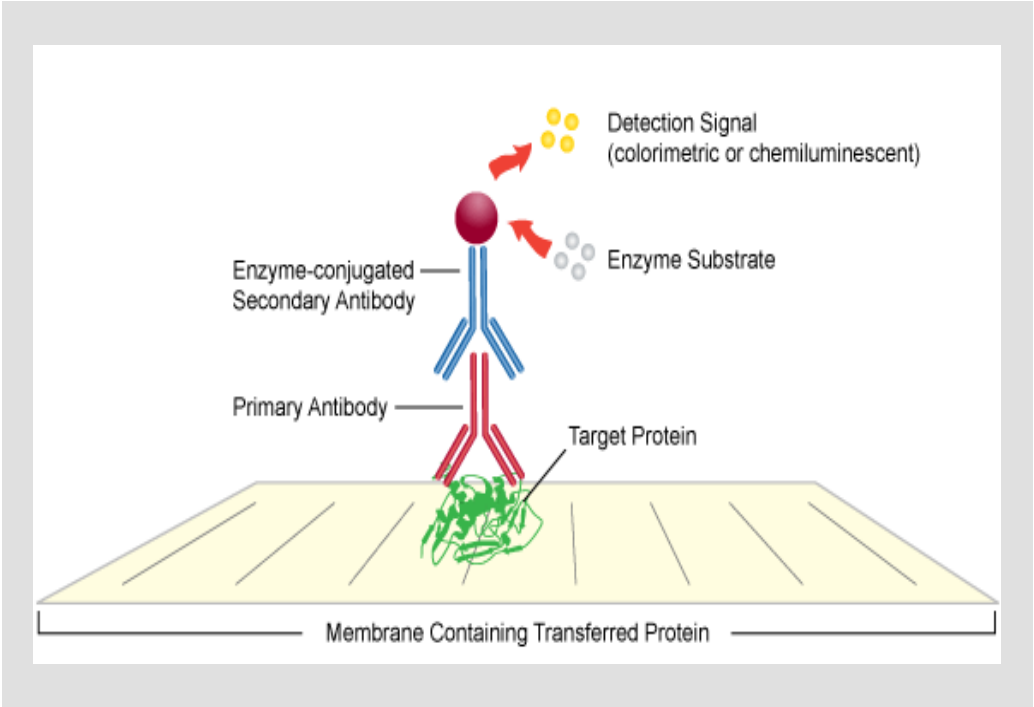


WB based on:

1. Molecular weight
2. Antibody binding specificity

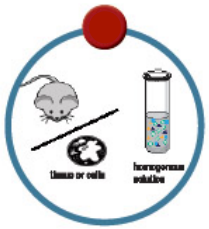
Applications of Western Blot

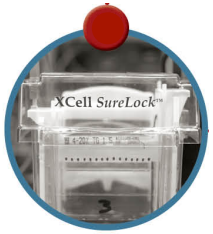
- Is the protein of interest expressed in my cells/tissues?
- Does expression of my protein changes after treatment?
- How does protein expression compare between different cells/ tissues?
- Medical diagnostics

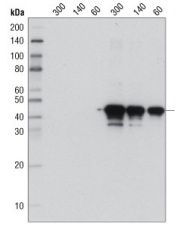


The Steps of Western Blot

Sample preparation → Electrophoresis → Transfer (blotting) → Antibody probing → Detection → Imaging and analysis







http://www.cellsignal.com/WB

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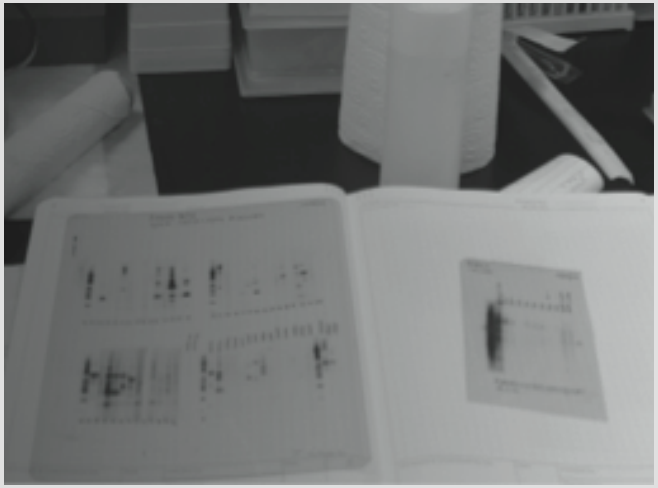
Important factors for good western blotting

- Quality reagents
- Western Blot validated antibodies
- Suitable controls
- Optimized protocol
- Appropriate detection system

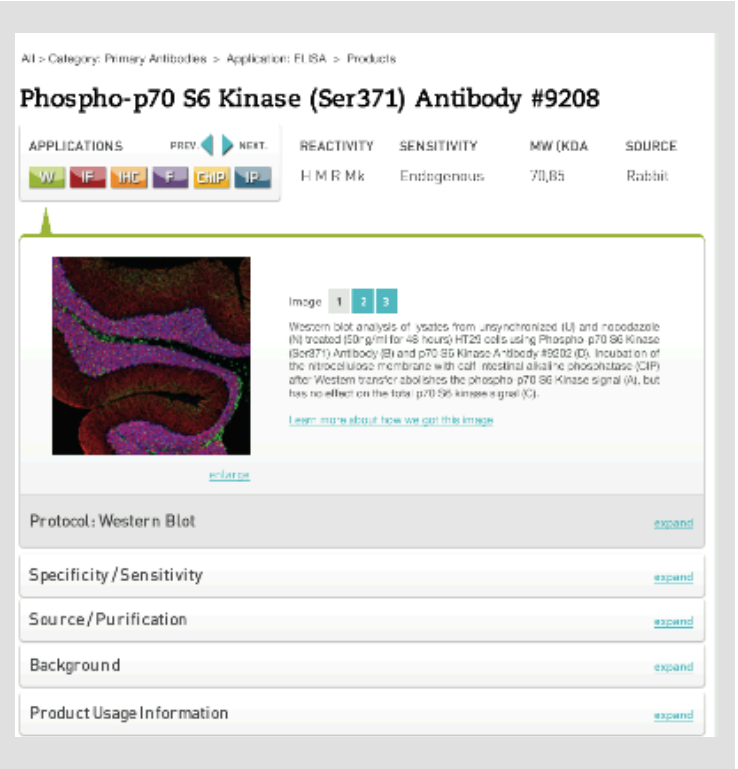


All CST antibodies are validated for western blot to help you achieve the best possible results

CST validation experiments

Combination of tests to provide the highest quality and most thoroughly tested antibodies

- Verification of specificity using multiple approaches:
  - True positive and negative expressing cell lines
  - Use of chemical inhibitors and activators
  - Phosphatase treatment
  - siRNA knockdown models
- Optimization of dilution, buffers and protocols
- Lot-to-lot testing in each application to ensure reproducibility



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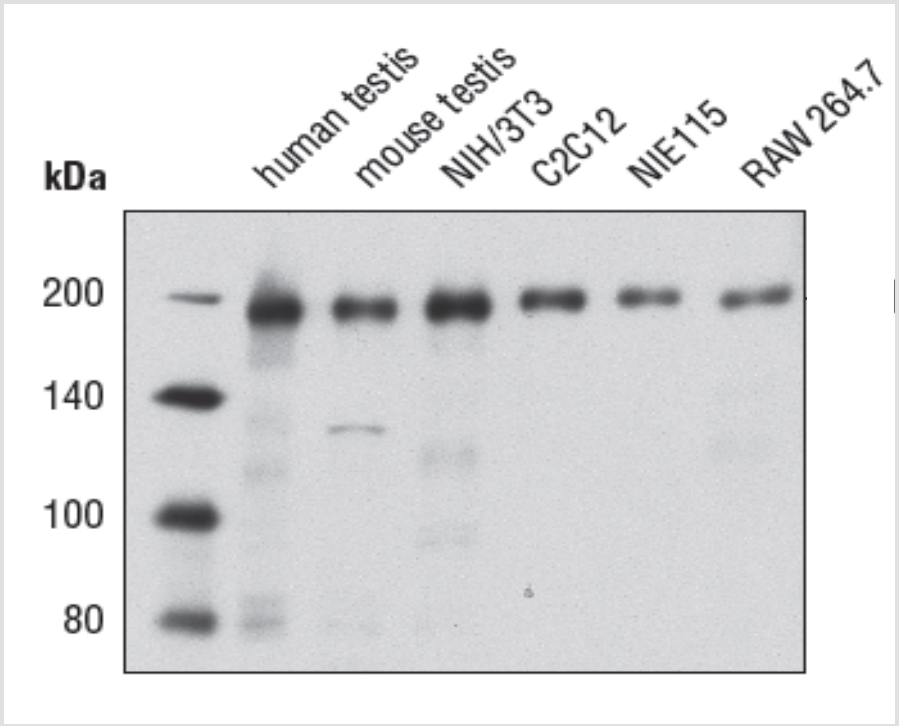
Validation of Antibody Specificity by Western Blot

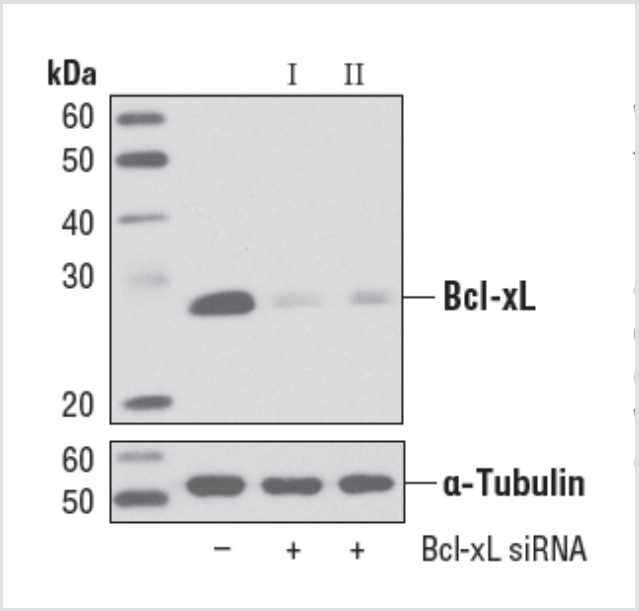
- Analysis of multiple cell lines and tissues



JIP4/SPAG9 (D72F4) XP® rabbit mAb

Validation of Antibody Specificity by Western Blot

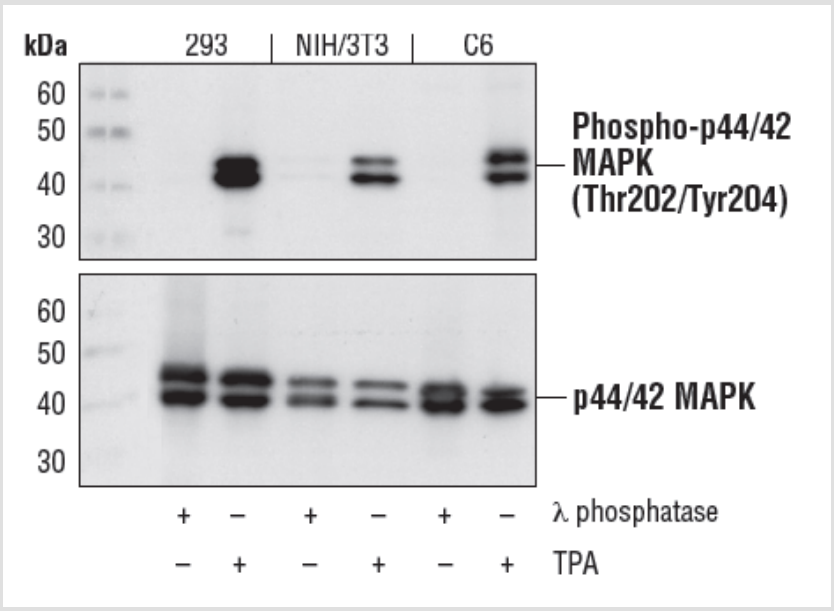
- siRNA Knock-down models



Bcl-xL (54H6) Rabbit mAb #2764

α-tubulin (11H10) rabbit mAb #2125

- Phosphatase and activation treatment

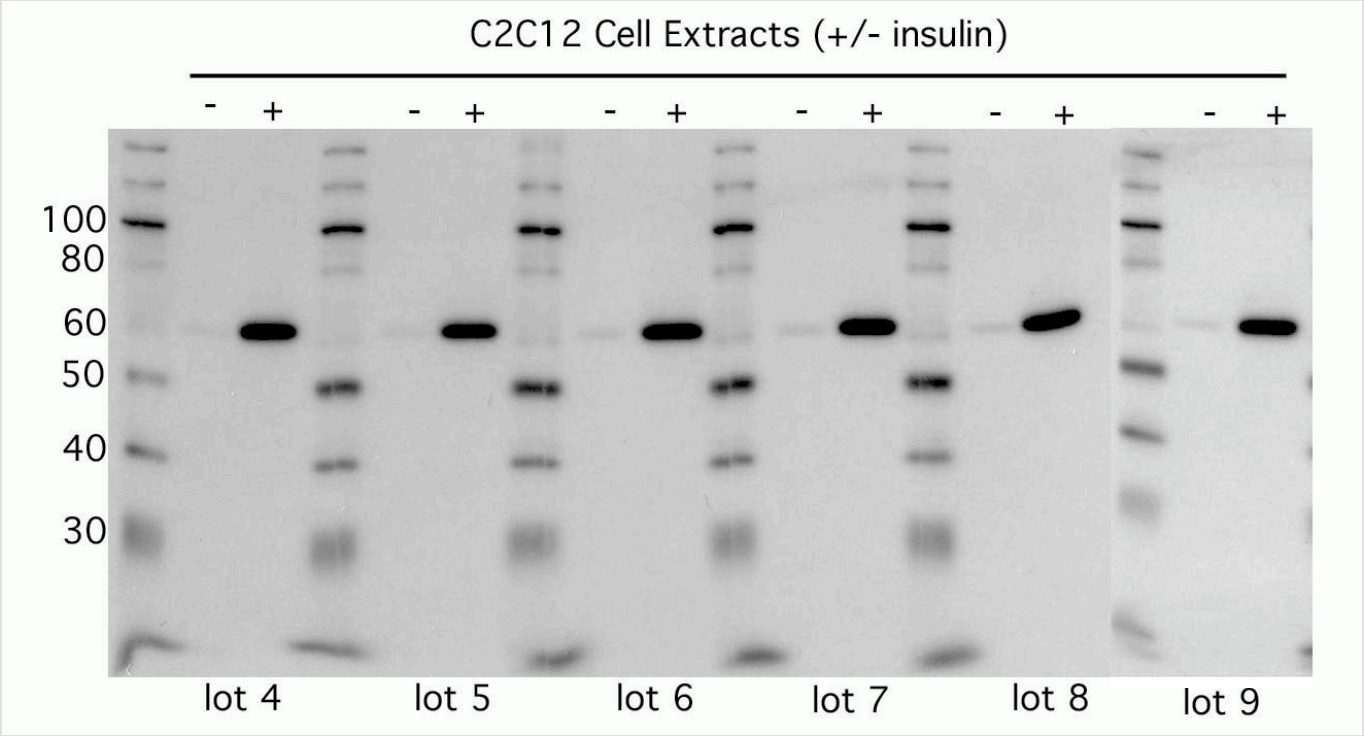


Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® mAb#4370

p44/42 MAPK (Erk1/2) (137F5) mAb #4695

Testing Antibody Reproducibility by Western Blot

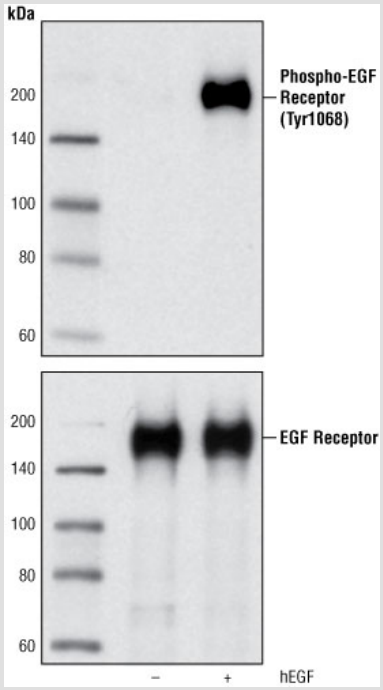
- Side by side comparison of new lots with previous lots



Phospho-Akt (Ser473) Antibody #9271

Optimization of Antibody Performance by Western Blot

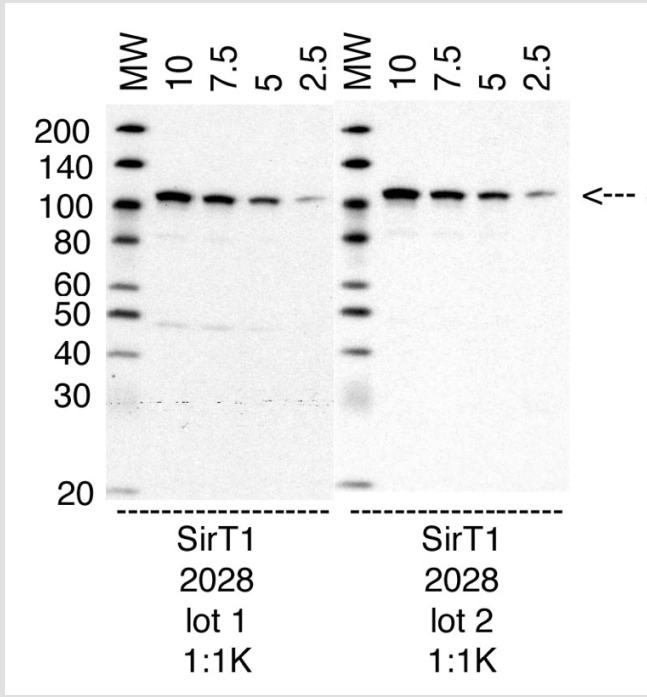
- Identification of positive and negative controls



Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777

EGF Receptor (D38B1) XP® Rabbit mAb #4267

- Titration analysis to determine optimal antibody dilution



SirT1 Antibody (Mouse Specific) #2028

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What does Antibody Validation mean for you?

The accuracy of western blot results is dependent on the quality of the primary antibody used

- Publication-quality results that you can trust
- Ready-to-go protocol for each antibody
- Guaranteed antibody performance
- No more wasting of time and samples

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Antibody Products for Western Blot

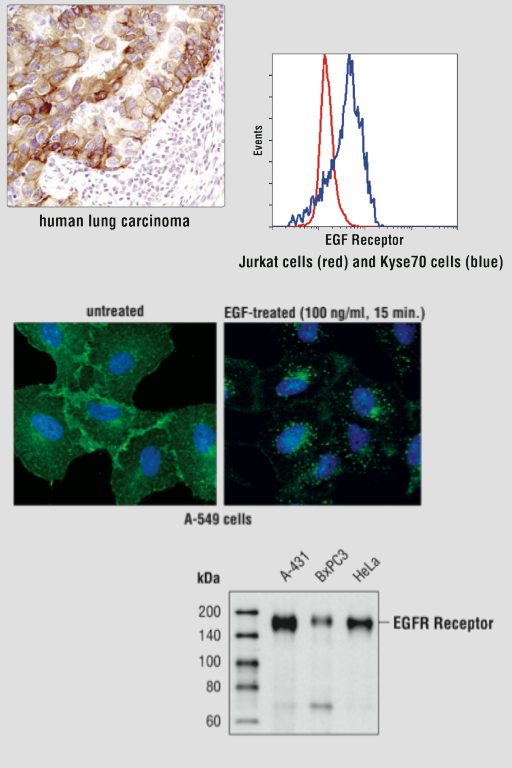
XP® Antibodies

Cell Signaling TECHNOLOGY®

What are XP® Antibodies?

XP® antibodies show superior performance in key applications: one antibody for all your research needs

- High quality rabbit monoclonal antibodies
- Selected based on superior performance in at least 2 applications
- Generated using XMT® technology
- Extensive validation and stringent quality control
- Exceptional specificity, sensitivity, stability and reproducibility



EGF Receptor (D38B1) XP® Rabbit mAb #4267

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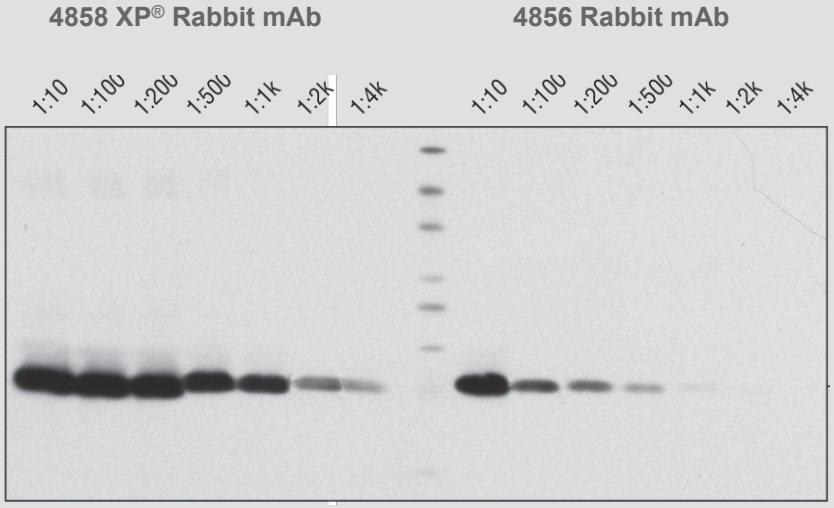
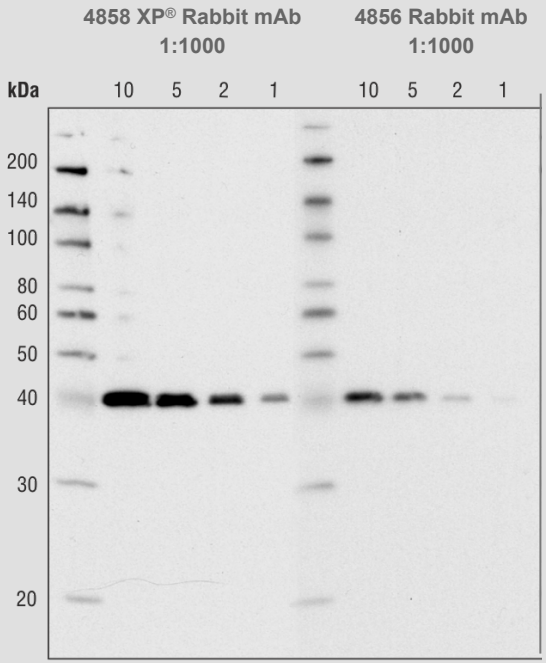
XP® Antibodies Display Stronger Sensitivity

Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858 compared to Phospho-S6 Ribosomal Protein (Ser235/236) (91B2) Rabbit mAb #4856



Antibody starting concentration: 1 µg/µl
5 second exposure

Serial antibody titration



5 second exposure

Serial dilution of extracts

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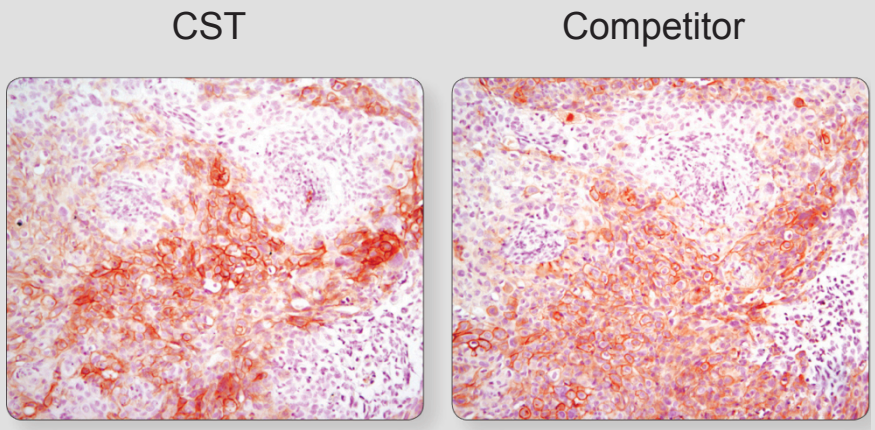
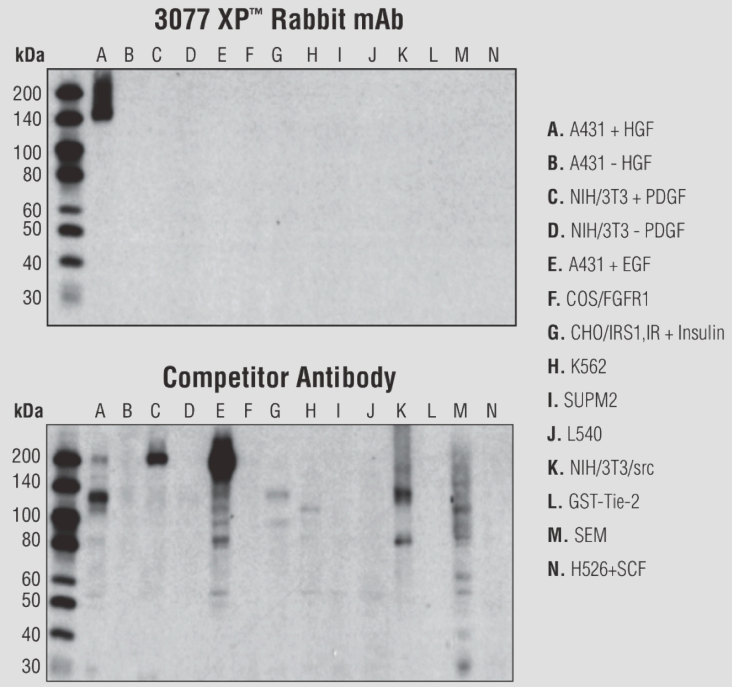
XP® Antibodies Display Stronger Specificity

Phospho-Met (Tyr1234/1235) (D26) XP® Rabbit mAb #3077 compared to a competitor's product



Comparison of CST #3077 and a competitor's product on HCC827 xenograft



Western analysis of RTK overexpressing cell lines

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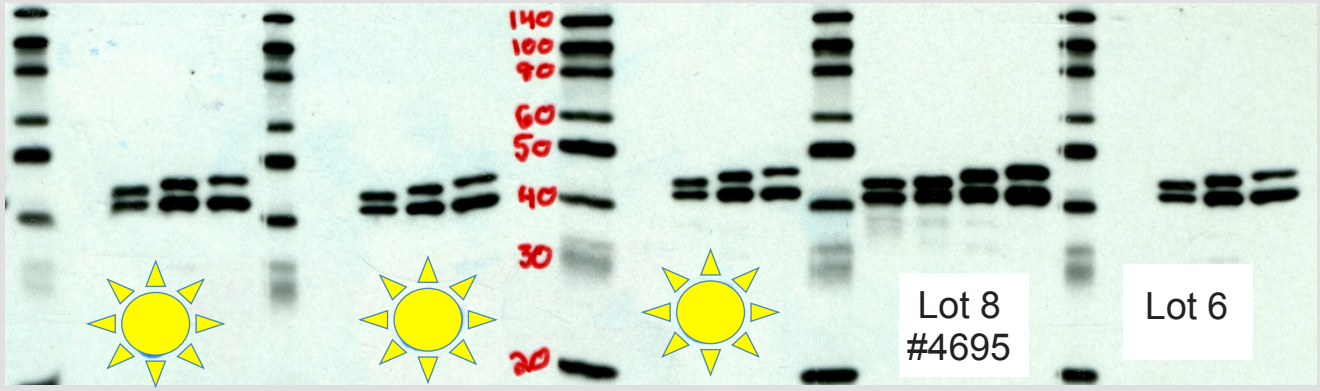
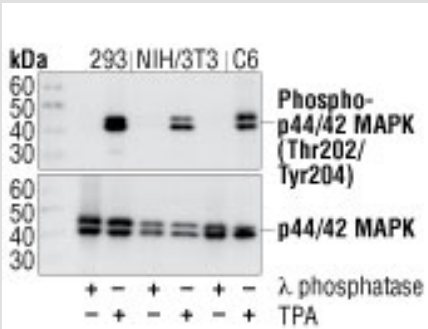
Testing Antibody Stability by Western Blot

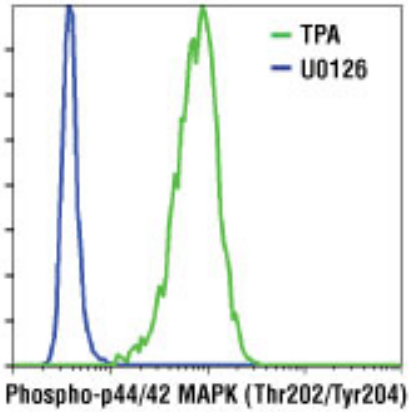
- Side-by-side analysis using the antibody kept at different conditions

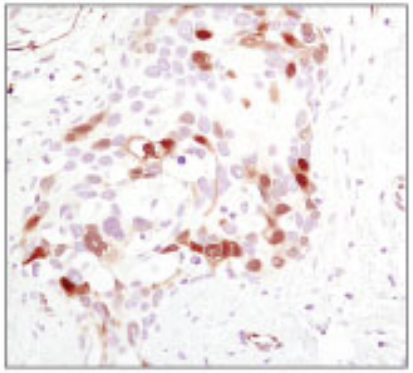


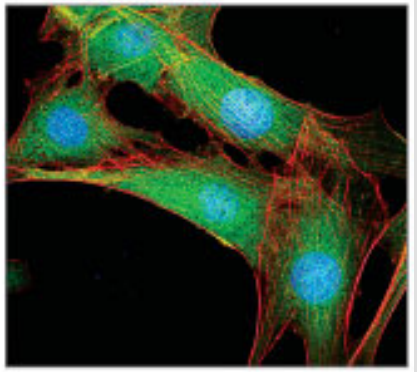
Phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370

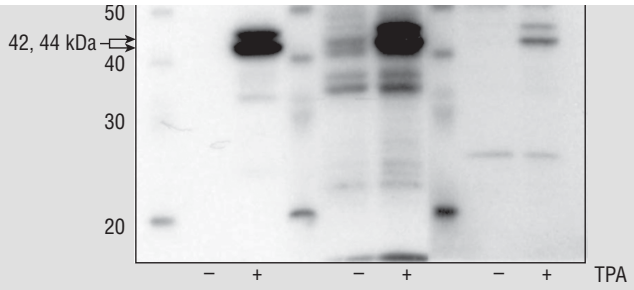
XP® antibodies performance in multiple applications

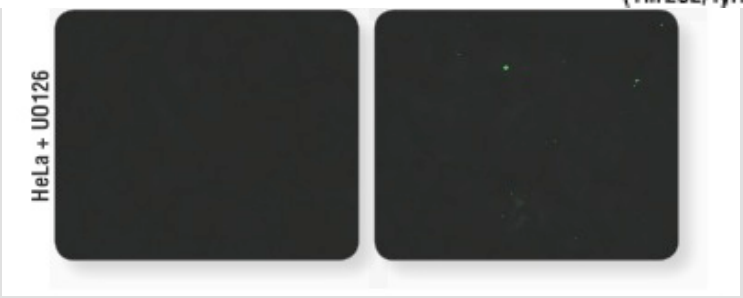








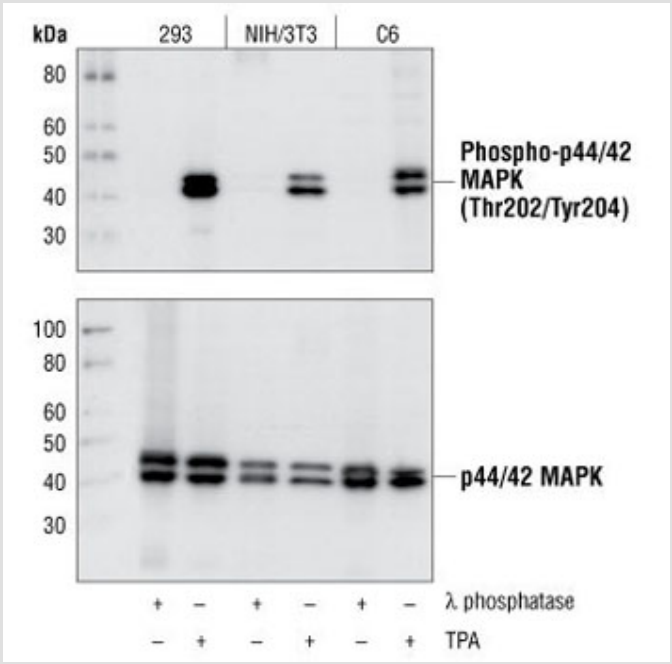




Phospho-p44/42 MAPK (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370

What does XP® bring to you?

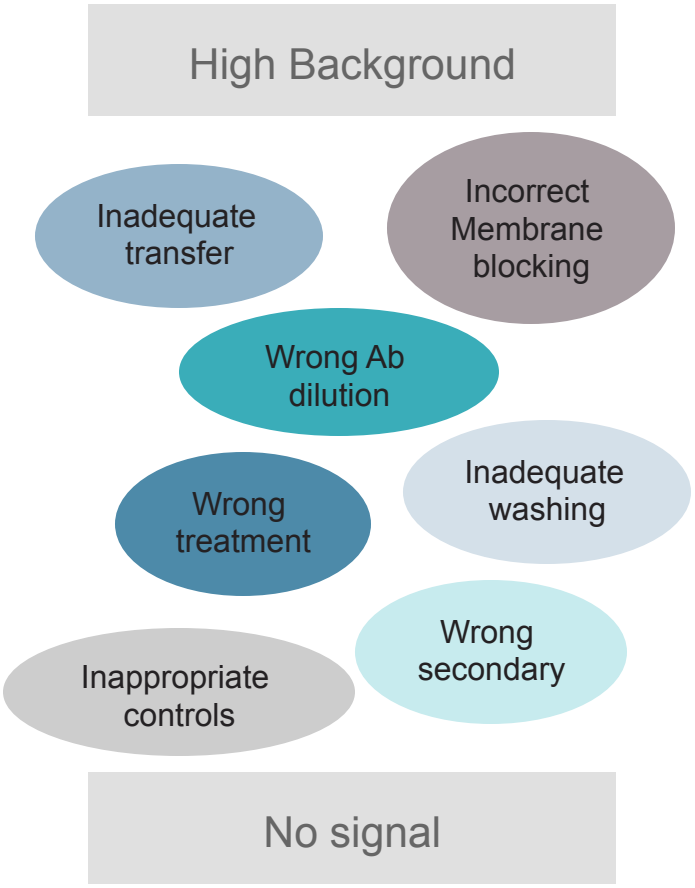
- Validated for all relevant applications
- Best specificity and sensitivity
- High quality antibodies for challenging and clinically relevant targets
- www.cellsignal.com/technologies/xmt/index



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370

Summary

- The accuracy of western blot results is dependent on the quality of the primary antibody used
- CST validates all antibody products by testing in biologically relevant systems
- WB is a time consuming technique, and following the right protocol is key to get good results



How to perform successful western blotting

Solutions for consistently better blotting

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Agenda

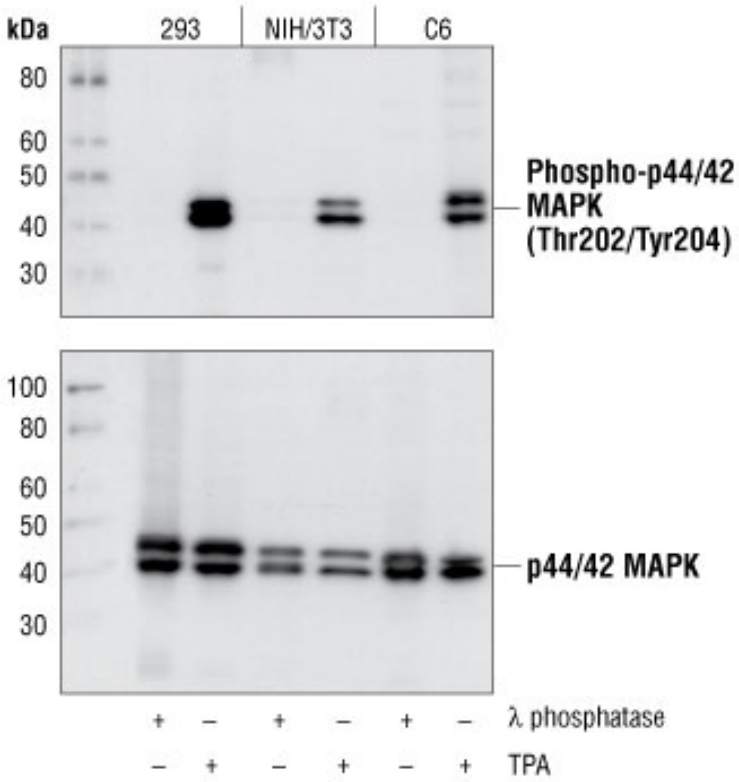
Part 2: Western blot protocol for good results

Western blot step by step

Troubleshooting

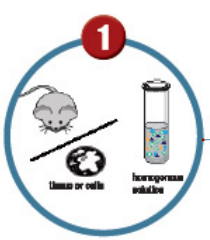
How can CST support you

Why western blot?

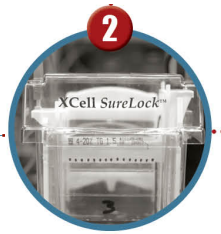


- Western blot is a widely accepted analytical technique used to detect specific proteins in a sample of tissue homogenate or cell extract
- To produce high quality western blot you need:
  - Good quality antibodies
  - Good quality reagents
  - Optimized protocol

The 10 steps of western blot



Sample Preparation
Lysis buffer & sonication = better release of proteins



SDS-PAGE
Run with appropriate controls

Wet Transfer
To nitrocellulose membranes

Blocking
5% milk in TBST

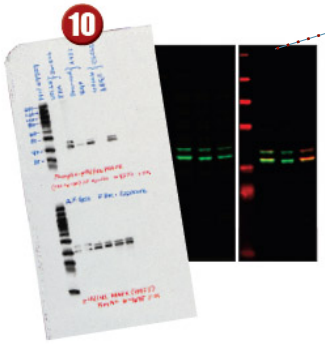
Primary Antibody Incubation
At 4°C overnight in 5% BSA or nonfat milk* in TBST
*as recommended on product

Washing
3 x 5 min in TBST

Secondary Antibody Incubation
For 1 hr at room temperature

Washing
3 x 5 min in TBST

Detection
Mix reagents immediately before



Imagir
Chemilu imaging offers greater flexibility and ease of use

How critical is each protocol step to obtain informative results?

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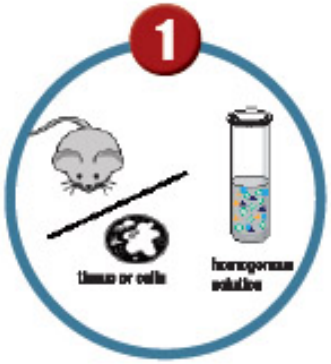
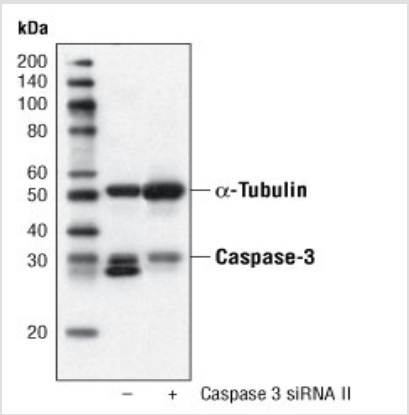
1. Sample preparation



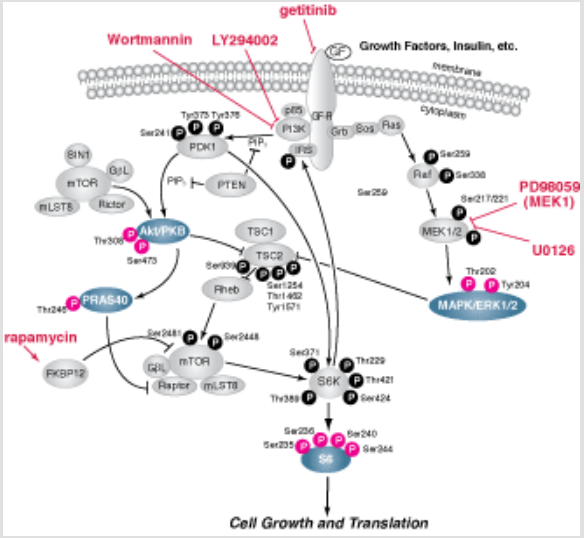
1. Treat samples – *Ensure appropriate treatment:*
   - Cytokines, growth factors
   - Chemical activators or inhibitors
2. Lysis of sample using lysis buffer- *Phosphatase/protease inhibitors needed*
3. Sonicate to complete cell lysis: 10-15 sec.
4. Denature by heating: 95-100°C, 5 min.
5. Load samples onto SDS-PAGE gel

The importance of controls

- Including controls helps you understand and interpret your results
- Shows that your assay works
- Positive and negative cell extracts and proteins made and used in-house



SignalSilence® siRNA: Caspase 3 siRNA II #6520



Chemical inhibitors and activators

www.cellsignal.com/support/controls.html

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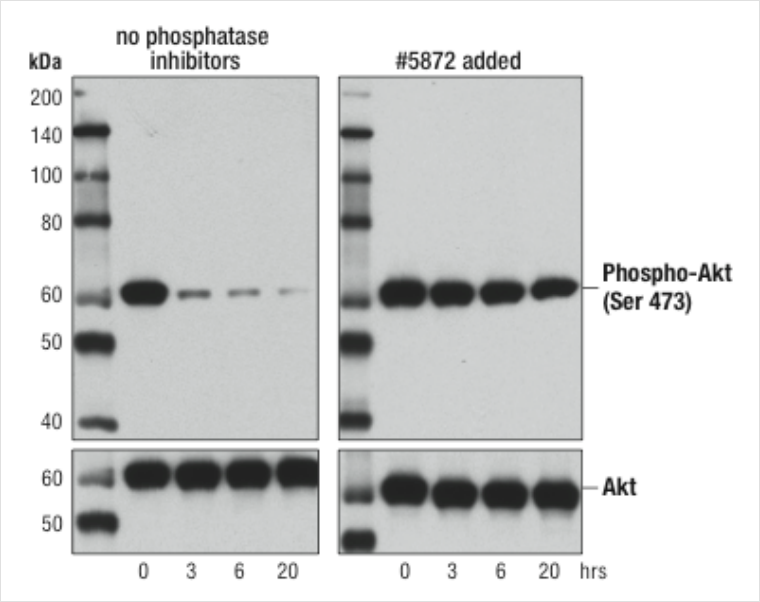
What lysis buffer do I use?

Lysis Buffers

- Cell Lysis Buffer (10X) #9803 – Whole cell/ non denaturing conditions
- Chaps Cell Extract Buffer (10X) #9852 – cytoplasmic lysates
- RIPA Buffer (10X) #9806 – Whole cells or tissues

Protease & Phosphatase inhibitors

- Phosphatase Inhibitor Cocktail (100X) #5870
- Protease Inhibitor Cocktail (100X) #5871
- Protease/Phosphatase Inhibitor Cocktail (100X) #5872



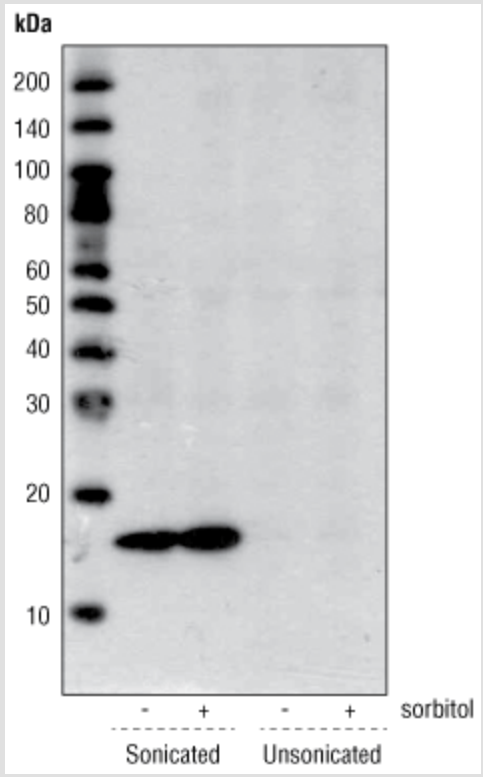
Protect your samples by always including protease and phosphatase inhibitors in your lysis buffer

Other tips for preparing your samples

We recommend sonication of all cell and tissue extracts and, in particular, for nuclear and chromatin associated proteins

The lysis buffer used is dependent on the location on the protein of interest

- Cytoplasmic proteins
  - 0.5-1% Triton or NP40 buffer
  - Spin down nuclei
- Membrane proteins associated to lipid rafts
  - Add additional detergent or use CHAPS buffer
- Nuclear proteins
  - RIPA buffer + sonication



Phospho-Histone H3 (Ser10) Antibody #9701

2. Gel electrophoresis

1. Prepare your gel if necessary
2. Load your samples
   - **Loading buffers:** Blue Loading Buffer Pack #7722 and Red Loading Buffer Pack #7723 – Contains 3x SDS loading buffer and 30x DTT reducing agent
3. Run gel: 1.5 – 2 hrs, 70V, 4°C
   - **Running buffer:** Tris-Glycine SDS Running Buffer (10X) #4050
4. Denature by heating: 95-100°C, 5 min.
5. Load samples onto SDS-PAGE gel

What to take into account when preparing your gel

CST recommends

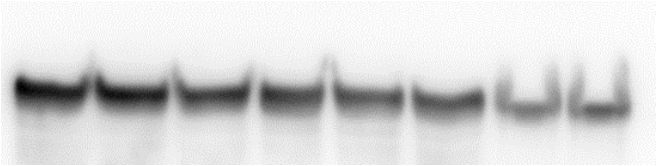
- Tris-glycine pre-cast mini gels
- Tris acetate gels for high molecular weight markers

When making the gel, remember

- Gel percentage depends on the target protein's size
- The size of the gel depends on the protein quantity to be loaded

Protein Size (KDa)	Gel percentage (%)
4-40 kDa	20%
12-45 kDa	15%
10-70 kDa	12.5%
15-100 kDa	10%
25-200 kDa	8%
Gradient gels	varies

µg protein	No. Wells
20-30	12
15-20	18
8-10	26



Overloaded gel, U-shaped bands

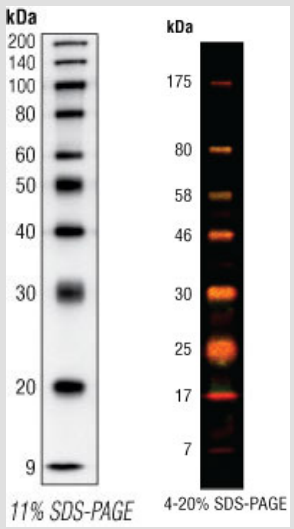
Electrophoresis controls

Molecular weight markers

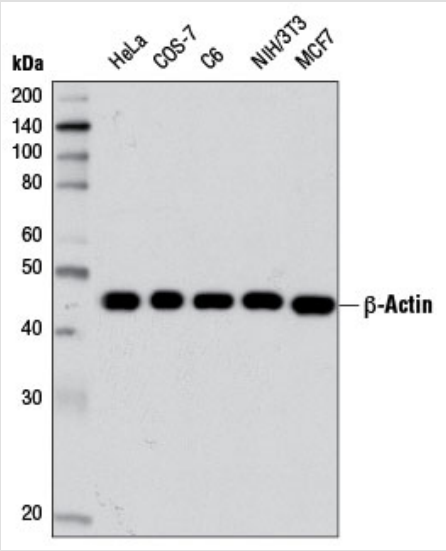
- Biotinylated Protein Ladder Detection Pack #7727
- Prestained Protein Marker, Broad Range (Premixed Format) #7720

Loading controls

- Used to ensure equal loading of the gel
- Control depends on location of nature of protein of interest



Protein ladders #7727 and #7720

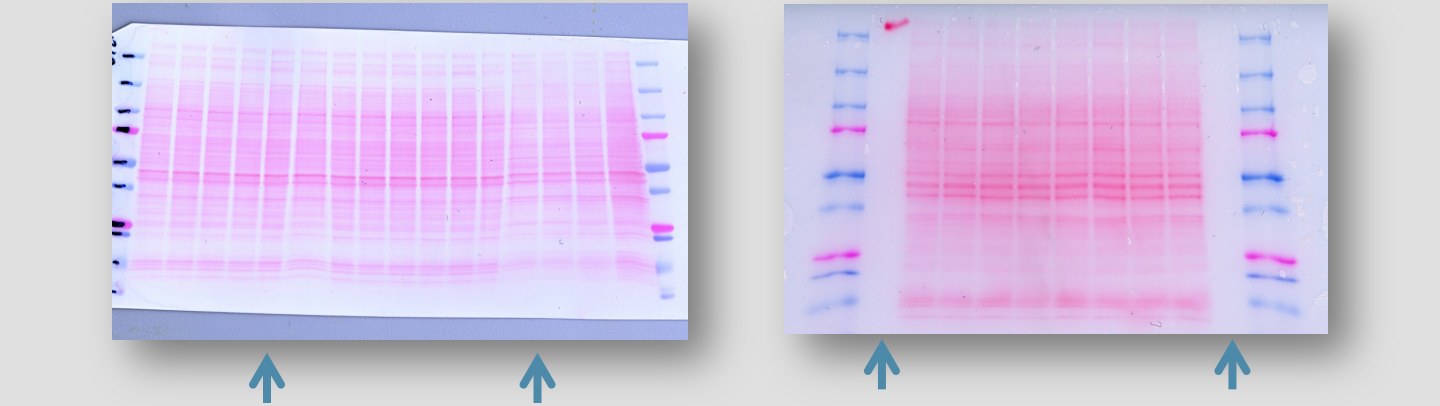


β-Actin (D6A8) Rabbit mAb #8457

www.cellsignal.com/catalog/loading-controls.html

Other tips when preparing your gel

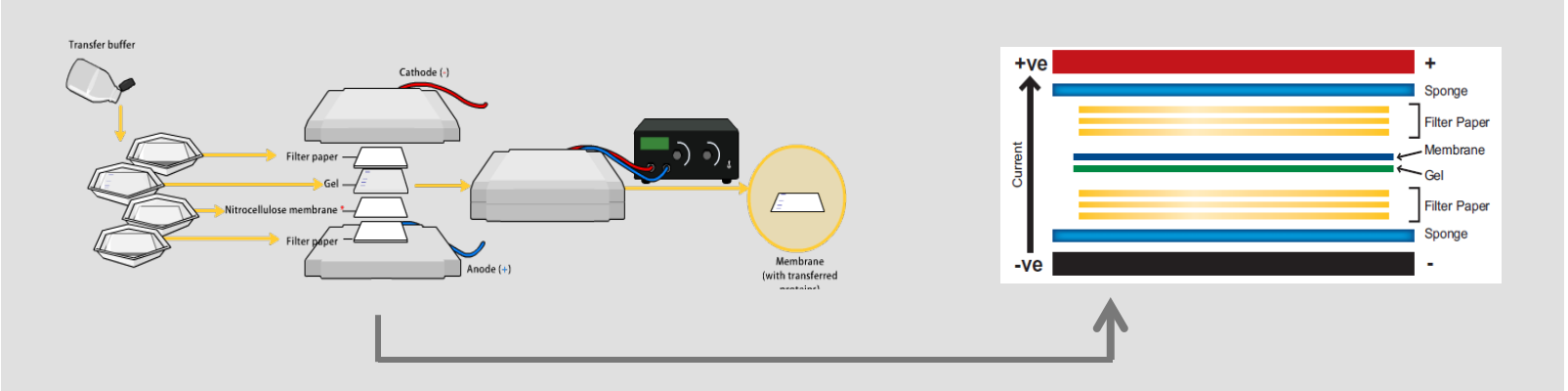
- Measure protein concentration
- Add lysis buffer to ensure all wells have the same volume
- Make enough sample to run several gels, freeze



Load 1 x sample buffer in lysis buffer (important in gradient gels)

3. Protein transfer

1. CST recommends wet transfer, 70V, 1.5 hrs
   - Tris-Glycine Running Buffer + 0,2 M glycine + 20% methanol +/- 0,1% SDS
2. Use nitrocellulose (#12369) or PVDF membranes
3. Block in 5% milk/ TBST, 1hr



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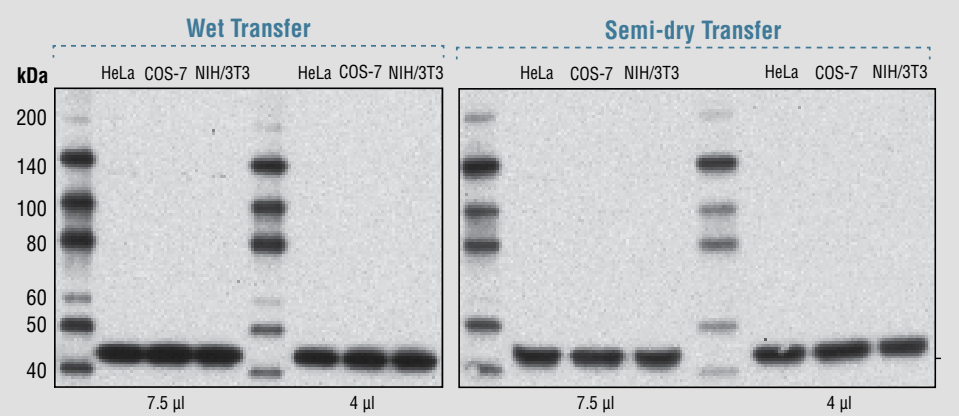
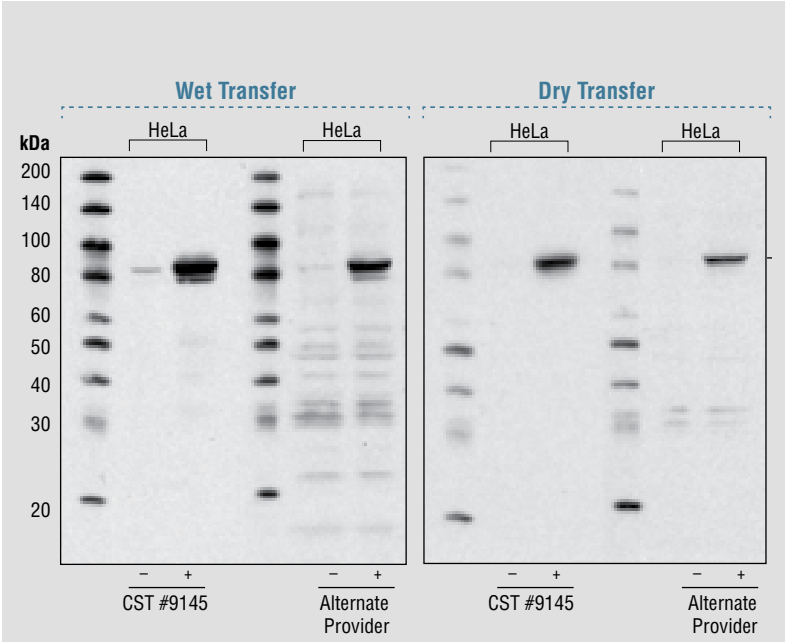
Important points for protein transfer

Wet, semi-dry or dry transfer?

- Semi-dry transfer works well with low to mid-weight proteins, but it can yield higher background
- Dry transfer yields lower signal levels



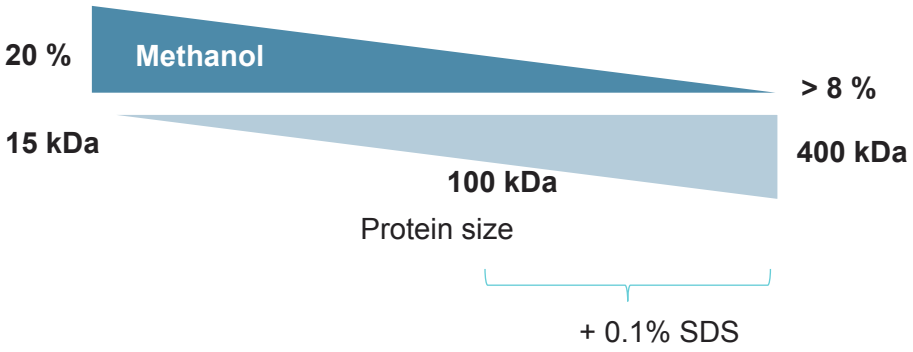
Pan-Actin (D18C11) Rabbit mAb #8456



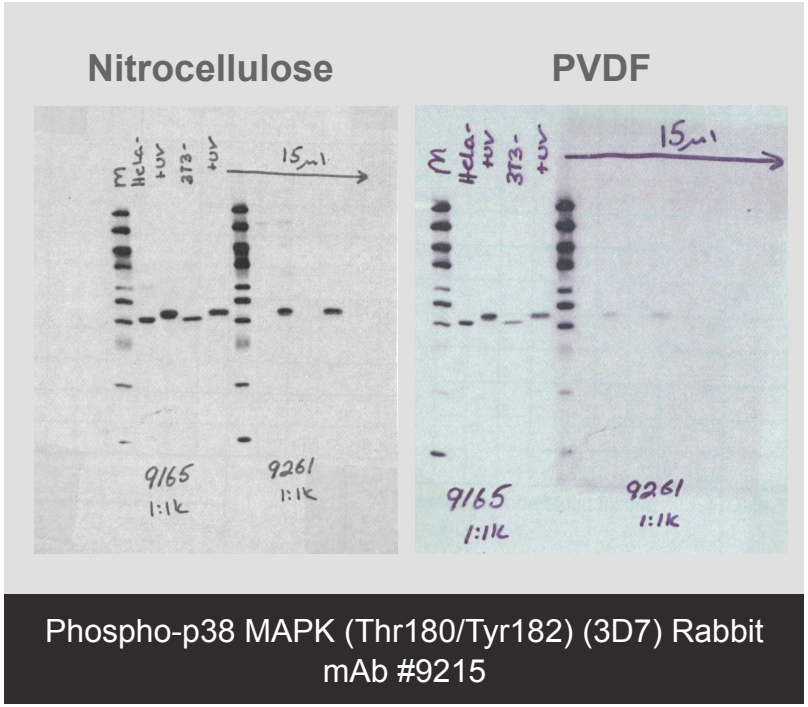
Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145

Important points for protein transfer

Membrane: Nitrocellulose sandwiches #12369



Protein size	Methanol	SDS	Membrane	Size
Large (>100 KDa)	Less than 10%	Final step	Nitrocellulose	0,4 µm
Small (< 100 KDa)	20%	No	Nitrocellulose or PVDF	0,2 µm



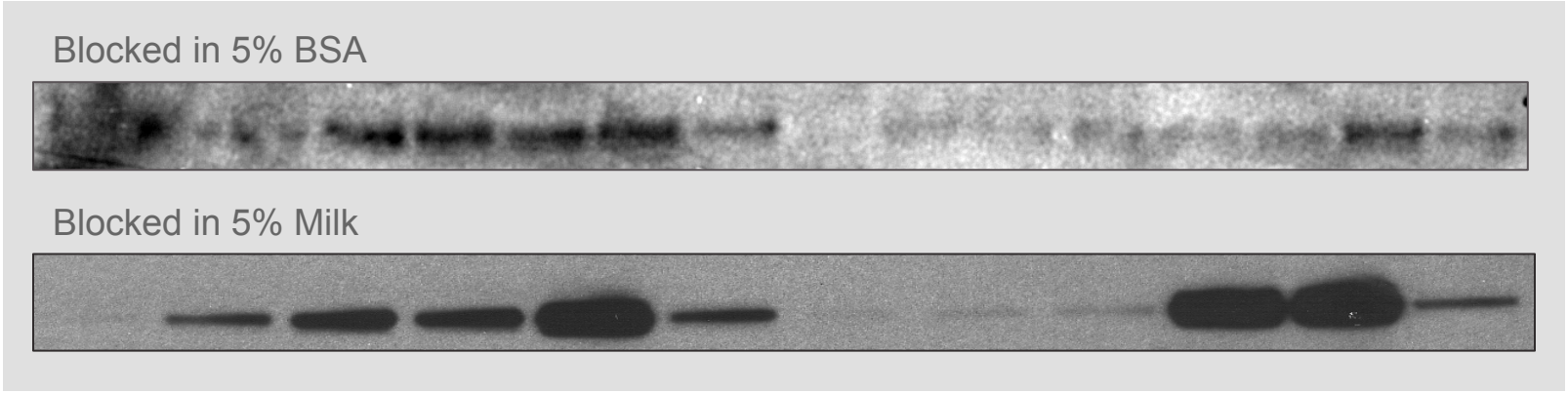
Phospho-p38 MAPK (Thr180/Tyr182) (3D7) Rabbit mAb #9215

4. Blocking

- CST recommends blocking in 5% milk/TBST, 1hr, R.T.

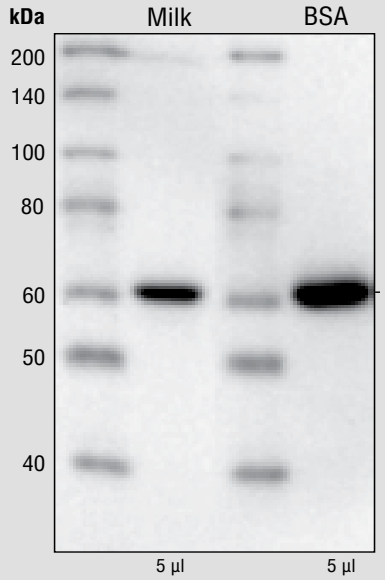
Nonfat dry Milk, #9999

Tris Buffered Saline with Tween 20 (TBST 10X), #9997



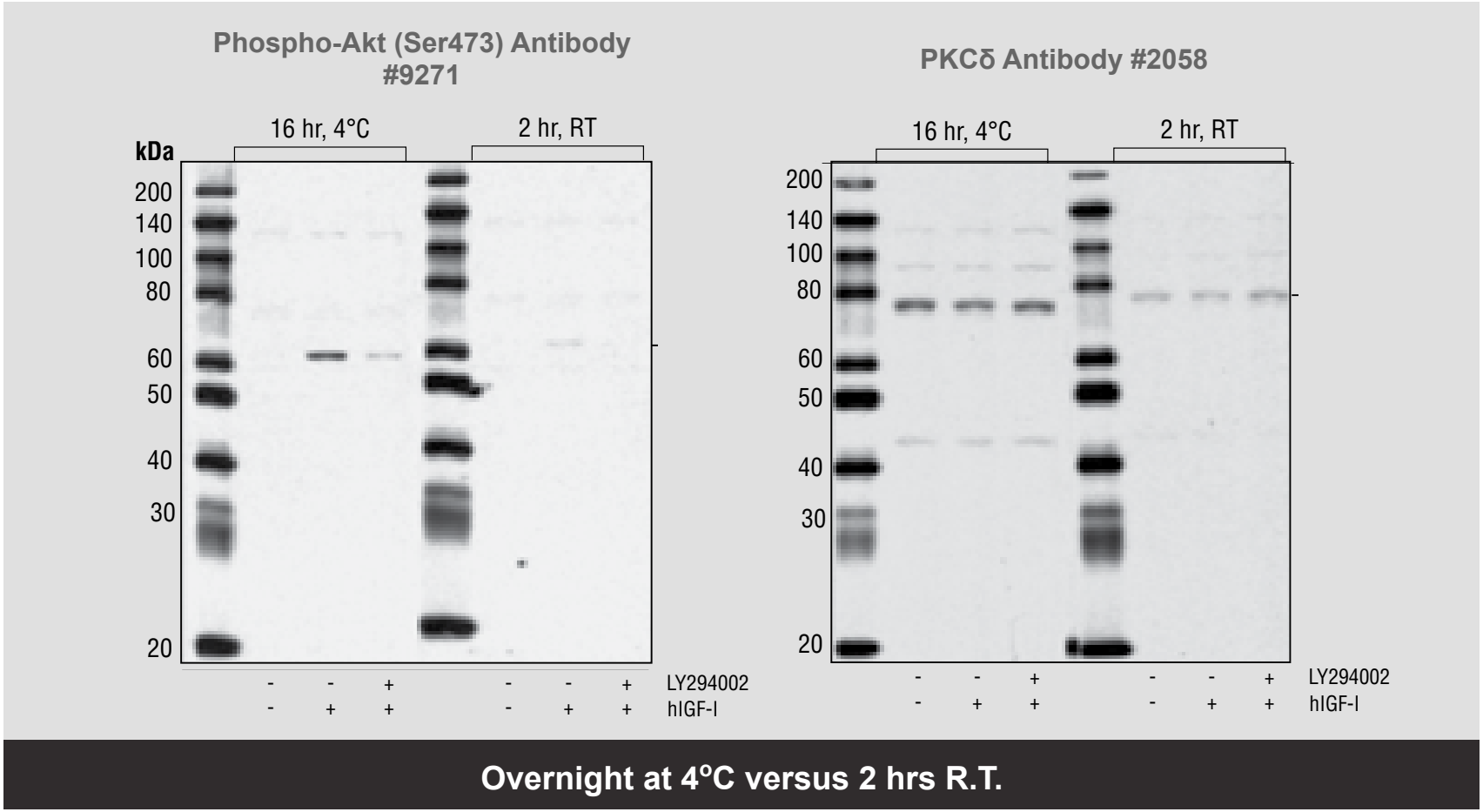
5. Primary antibody incubation

1. Dilute primary antibody in TBST + 5% BSA or 5% milk
   - 1x TBS, 0,1% Tween-20 + 5% milk – ***Mouse monoclonal antibodies***
   - 1x TBS, 0,1% Tween-20 + 5% BSA - ***Polyclonal & rabbit monoclonal antibodies***
2. Incubate at 4°C overnight
3. Wash using TBST 3x 5 min



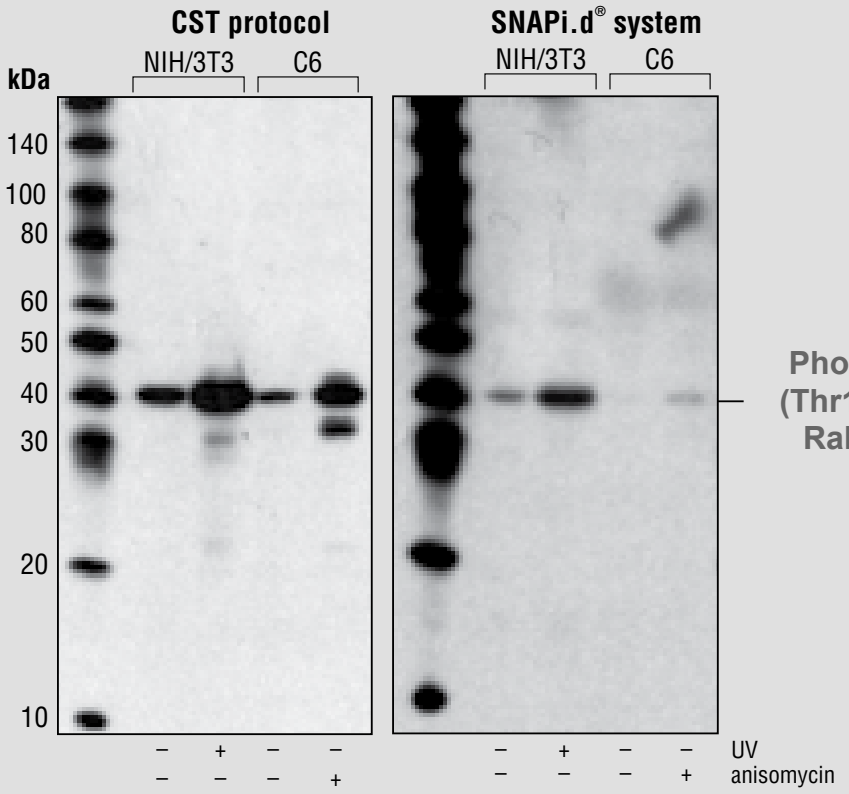
Phospho-Akt (Ser473) #9271

The importance of timing for primary antibody incubation



Overnight at 4°C versus 2 hrs R.T.

The importance of timing for primary antibody incubation



Overnight at 4°C versus Abbreviated Incubation Systems

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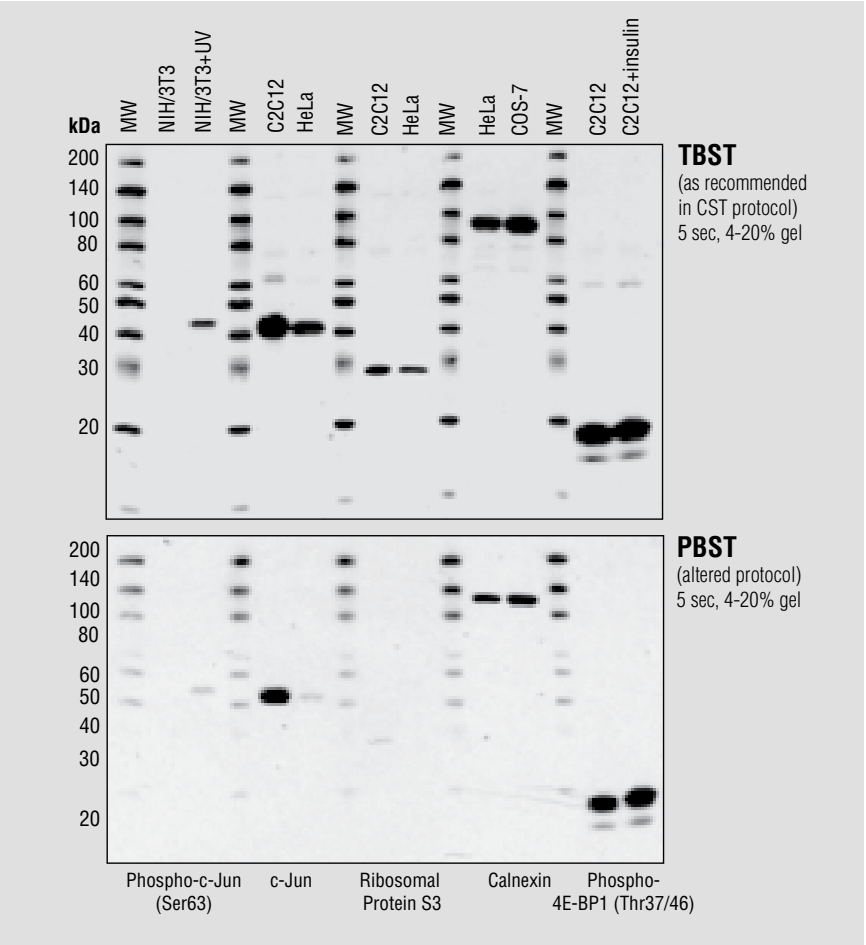
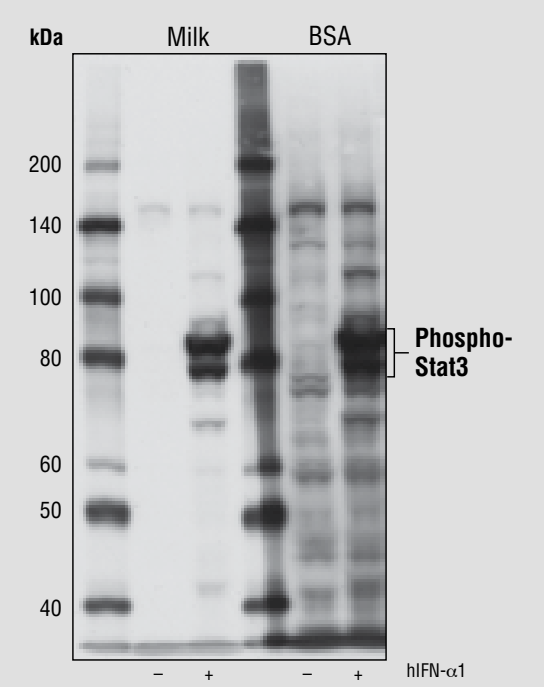
6. Washing steps are also important

- Wash 3x5m, always in TBST



7. Secondary antibody incubation

1. Incubate with the appropriate species conjugated antibody
2. Keep concentration low to avoid background - 1:5000 or 1:2000
3. In 5% milk TBST
4. Incubate 1hr, R.T.

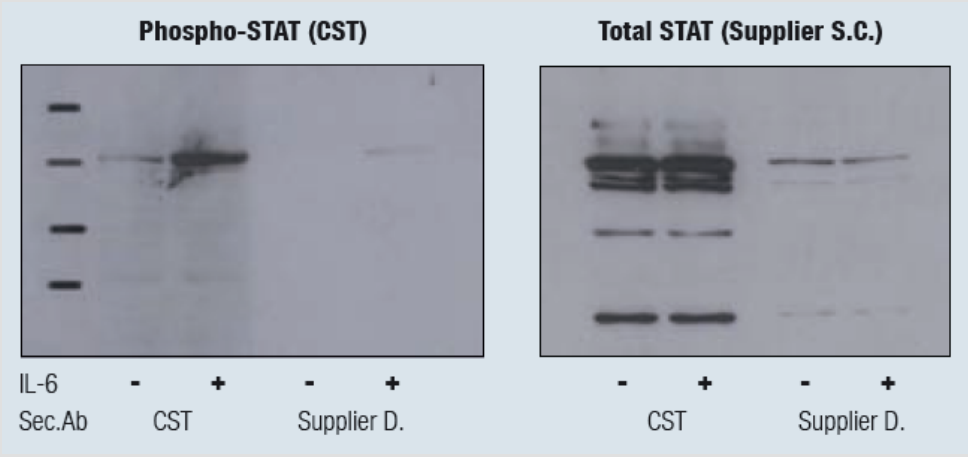


Diluting secondary antibody in milk yields lower background

Secondary antibodies for chemiluminescence detection

Anti-rabbit IgG, HRP-linked antibody #7074

- High sensitivity with short reaction times
- For use with rabbit monoclonal and polyclonal antibodies
- Used in-house to validate CST's primary antibodies
- Also validated for specificity



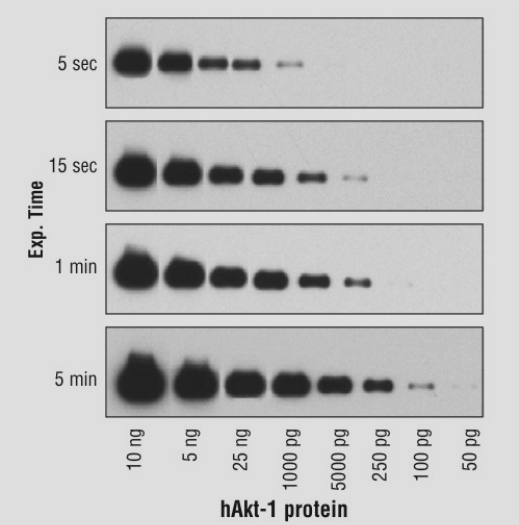
Anti-rabbit IgG, HRP-linked antibody #7074 compared to an equivalent competitor product

9. Detection (chemiluminescent WB)

- Add appropriate detection reagent
  - Amount of protein present on the blot
  - Duration of the signal

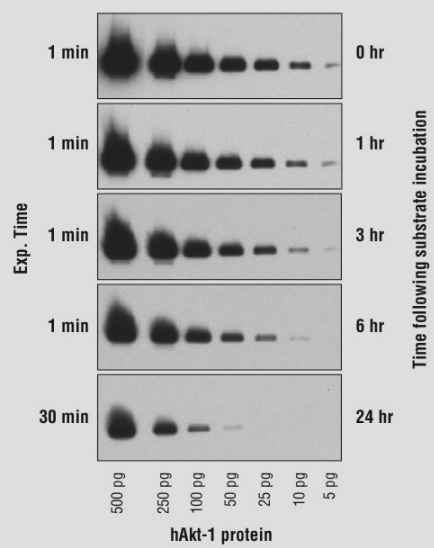
Detection reagents for chemiluminescent WB

SignalFire® ECL reagent #6883



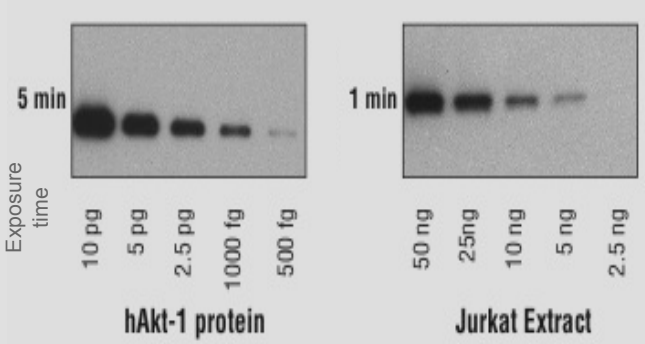
- Detects picogram amounts of protein
- Cost effective

SignalFire® Plus ECL reagent #6883



- Longest signal duration
- Detects low picogram amounts of protein

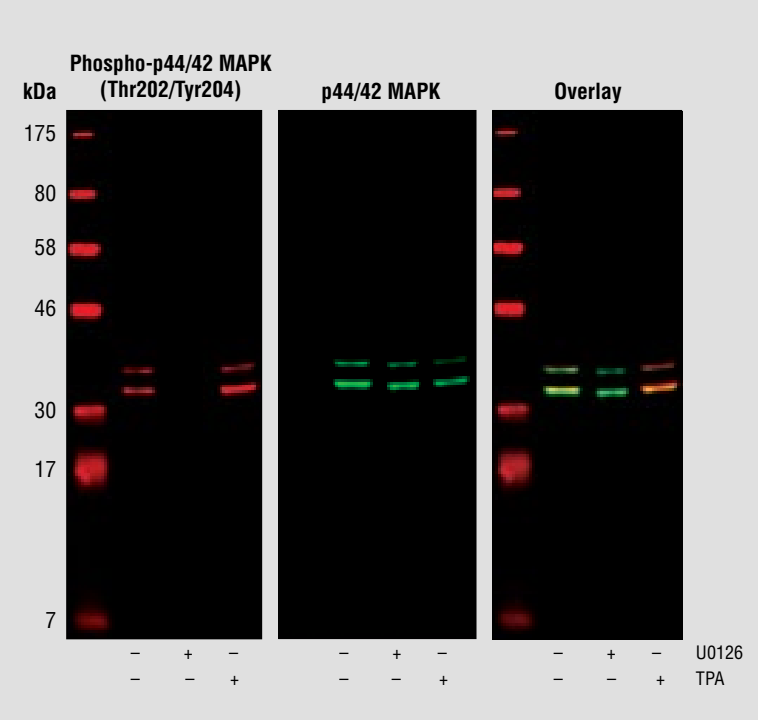
SignalFire® Elite ECL reagent #6883



- Greatest sensitivity: Detects femtogram amounts of protein

Secondary antibodies for fluorescent detection

- Fluorescent detection provides faster visualization and semi-quantification of results
- Allows 2-color western blot for:
  - Proteins that differ significantly in size
  - Phospho- and total protein - if no hindrance of the epitopes (optimization needed)
- 2-color western blots require primary antibodies from different species and secondary antibodies labeled with different dyes:
  - Anti-mouse IgG #5470 and anti-rabbit #5366 DyLight® 680 conjugates
  - Anti-mouse IgG #5257 and anti-rabbit #5151 DyLight® 800 conjugates



"multiplex'" Fluorescent WB

Chemiluminescent or fluorescent detection?

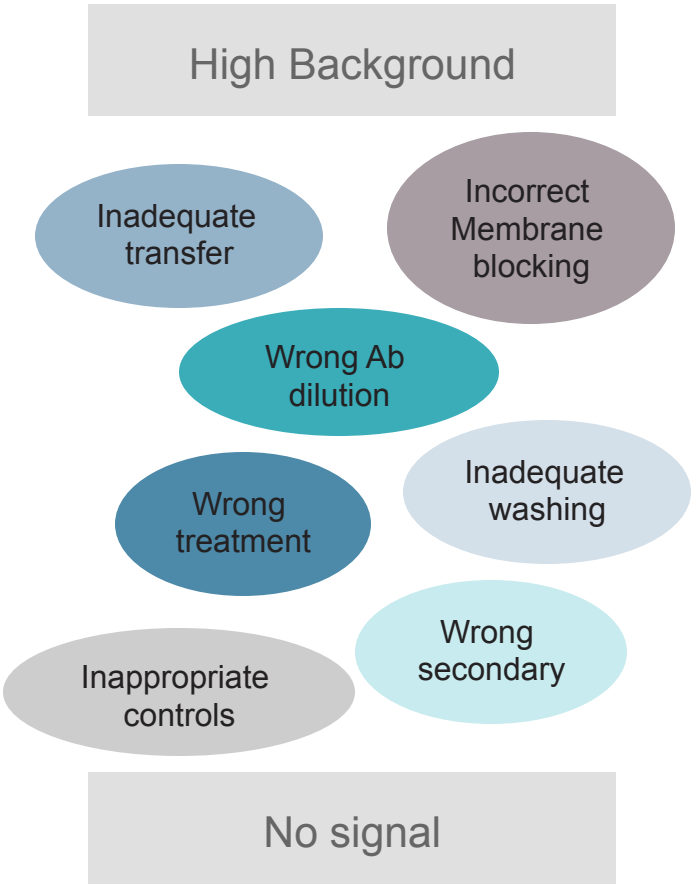
	Chemiluminescent WB	Fluorescent WB
Signal generation	Enzymatic reaction: 2-step HRP-conjugated antibody + detection substrate	Secondary antibody labeled with a fluorescent dye (direct detection)
Sensitivity	+++	++/ +++
Multiple detection	Yes, if enough difference in weight	Yes, if no hindrance of epitopes
Signal stability	Hours (days if correct detection reagent is used)	Months/ years
Detection/ Instrumentation	• Film exposure/ digital imaging • No need of special instrumentation	• Digital imager • A fluorescent imager is required
Other	Cheaper/ more established	Reduced chemical waste

Troubleshooting

And frequently asked questions

Critical points to get your western blot results

- Ensure correct treatment of your sample
- Load an appropriate amount of cell lysate into the gel
- Check species reactivity of your primary antibody
- Do not over-wash the membrane
- Do not over block
- Use the right secondary antibody



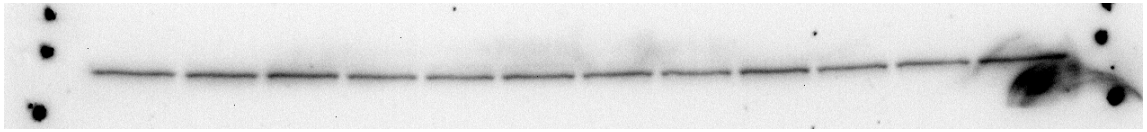
Problem: High background

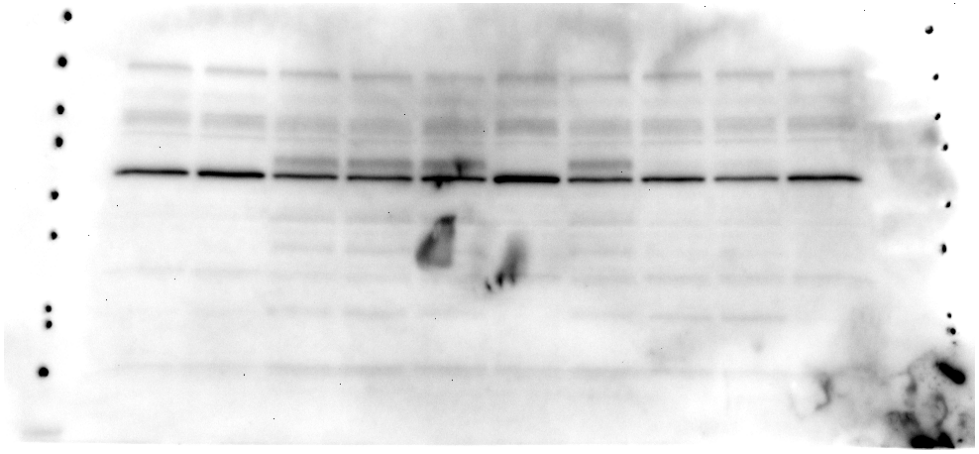
Cause	Solution
Using tissue extract	• Use fresh, sonicated and clarified cell extracts • Use RIPA buffer for lysis
Primary antibody incubation	• Follow recommended dilution and buffer
Membrane blocking	• Do not block less than 1hr • Block in 5% milk
Washing	• Wash 3 x 5 mins • Add a mild detergent
Membrane	• Ensure pore size is adequate for the test protein • Use only nitrocellulose or PVDF membranes • Pretreat PVDF membranes
Secondary antibody incubation	• Ensure secondary is specific for primary antibody • Always incubate in 5% milk
Protein level	• Ensure correct protein amount is loaded in the gel • Use appropriate controls

Problem: Low or no signal

Cause	Solution
Appropriate treatment	• Use treatment to induce expression or protein activation
Primary antibody incubation	• Follow recommended dilution and buffer
Washing	• Wash 3 x 5 mins
Protein transfer	• Use adequate transfer time • Ensure pore size is adequate for the test protein • Use of methanol versus protein size
Membrane blocking	• No longer than 1hr in 5% milk
Secondary antibody incubation	• Ensure secondary is specific for primary antibody • Use low antibody concentration
Protein level	• Ensure your cell line is expressing adequate protein levels • Use a detection reagent that detects lower protein levels • IP for low concentration proteins

And one last tip…





Always treat the membrane carefully!

Technical support

CST's help to you

Cell Signaling TECHNOLOGY®

Technical support

The same scientists who develop and validate CST antibodies are available as technical resources to help you in your research

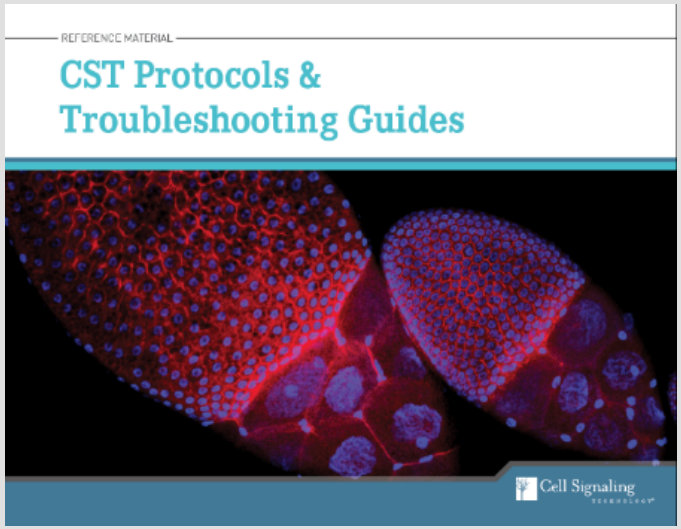
- Protocol- sample preparation/staining/detection
- Antibody concentrations
- Has antibody been tested/validated for my test species?
- Recommendations for best choice when multiple antibodies available

Contact us at:
eusupport@cellsignal.eu

Optimized protocols available

The most common troubleshooting cases can be solved using CST's optimized protocols

- We test our products to obtain the best possible results
- We strongly recommend using our optimized application-specific protocols for each product
- These guarantee accurate and reproducible results



Summary

Western blot is a complex technique with a lot of variables

- Follow the recommended protocol and dilutions
- Ensure protein expression in your extracts – Use controls
- A good informative western blot needs quality reagents
- If in doubt, contact us at eusupport@cellsignal.eu

Thanks for listening!

Questions?

About Us

Founded by research scientists in 1999, Cell Signaling Technology (CST) is a private, family-owned company with over 400 employees worldwide. Active in the field of applied systems biology research, particularly as it relates to cancer, CST understands the importance of using antibodies with high levels of specificity and lot-to-lot consistency. It's why we produce all of our antibodies in house, and perform painstaking validations for multiple applications. And the same CST scientists who produce our antibodies also provide technical support for customers, helping them design experiments, troubleshoot, and achieve reliable results. We do this because that's what we'd want if we were in the lab. Because, actually, we are.