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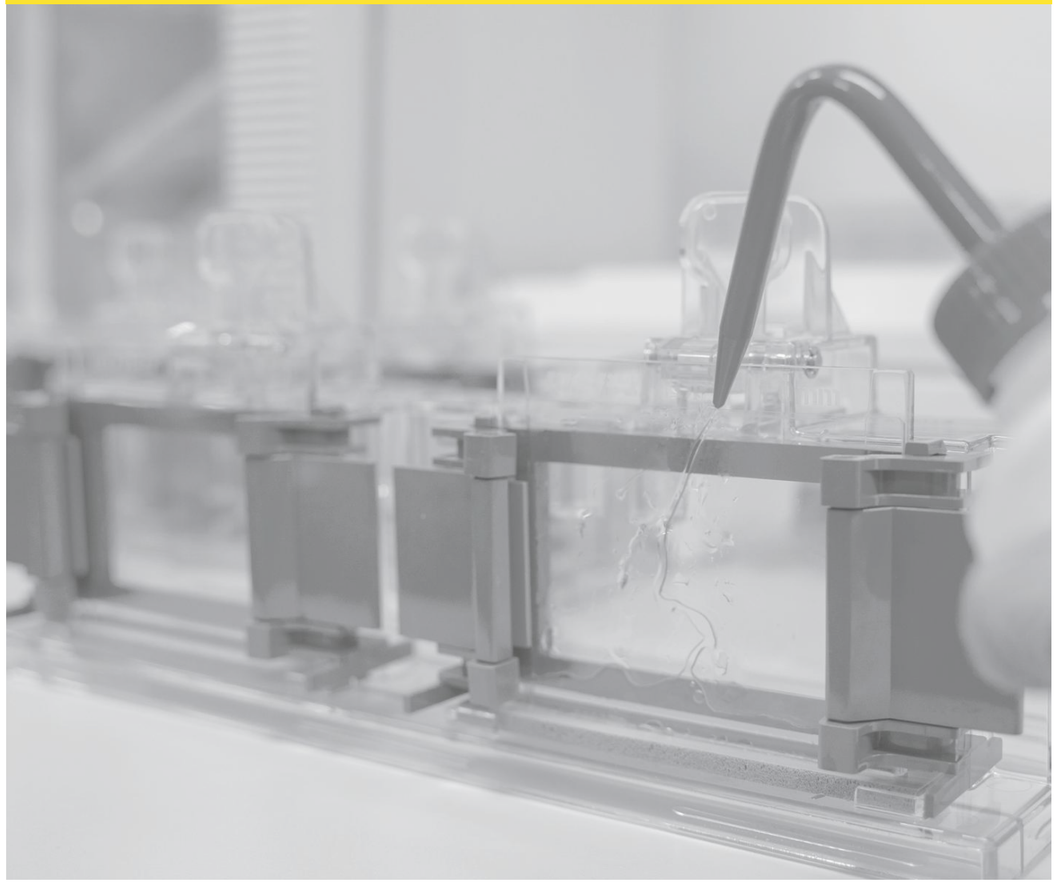
ABclonal Technology®

Western Blot Handbook & Troubleshooting Guide



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COMPANY PROFILE

ABOUT US

ABclonal is an innovative growth company aiming to provide reliable and cost-effective products and services for both basic and translational research in cutting edge biomedical science.

Innovation is in our DNA, with worldwide R&D centers focusing on technical innovation and product development for protein science and molecular biology.

ABclonal always listen carefully to the opinions and feedback from talented scientists across the globe, and quickly translate their needs into a product development pipeline dedicated to developing valuable research tools based on state-of-the-art technologies, to meet the needs of innovation in a timely manner.

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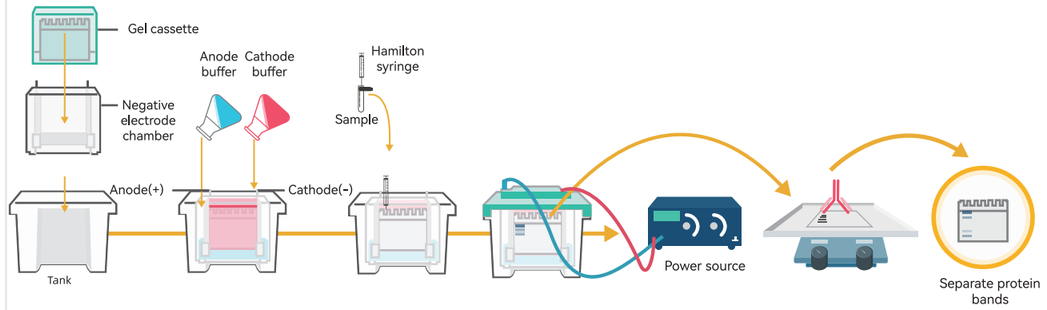
Part One Western Blot Introduction

1.Introduction

Western blotting is an important technique used in cell and molecular biology to separate and identify proteins. In this technique, a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with Labeled antibodies specific to the protein of interest, which allows it to be easily detected and imaged. These steps permit a specific protein to be detected from a mixture of proteins.

2.Technique

Western blotting experiment usually has five steps: sample preparation, loading and running the SDS-PAGE, protein transfer from the gel to the membrane, immunoblotting, and detection.



Part Two Western Blot Protocol

1.Sample Preparation

// 1.1 Background of your target protein and controls

Before the experiment, we recommend you check the database to collect some information about your target protein such as the expression level, protein size, and post-translational modification. Also, using online database to select appropriate controls from the start can save you time and frustration down the road, it will help troubleshoot when your experiment has unexpected sources of error. Here are some commonly used databases:

Uniprot

UniProt (https://www.uniprot.org/) is the world's leading high-quality, comprehensive and freely accessible resource of protein sequence and functional information. It is maintained by the UniProt consortium, which comprises the European Bioinformatics Institute (EBI), the Swiss Institute of Bioinformatics (SIB), and the Protein Information Resource (PIR). Commonly used information that can be queried in the Uniprot database includes:

- **Expression**

Expression

Expressed in prostate cancer and levels increase from the normal to the malignant state (at protein level). Expressed in all human cell types so far analyzed. The Tyr-176 phosphorylated form shows a significant increase in expression in breast cancers during the progressive stages i.e. normal to hyperplasia (ADH), ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and lymph node metastatic (LNMM) stages. 3 Publications

- **PTM/Processing**

PTM / Processing

Molecule processing

Feature key	Position(s)	Description	Actions	Graphical view	Length
Chain (PRO_0000085605)	1 – 480	RAC-alpha serine/threonine-protein kinase	Add BLAST		480

Amino acid modifications

Feature key	Position(s)	Description	Actions	Graphical view	Length
Modified residue	14	N6-acetyllysine 1 Publication			1
Modified residue	20	N6-acetyllysine 1 Publication			1
Disulfide bond	60 ↔ 77	1 Publication			
Modified residue	124	Phosphoserine Combined sources			1
Modified residue	126	Phosphoserine; alternate Combined sources			1
Glycosylation	126	O-linked (GlcNAc) serine; alternate 1 Publication			1
Modified residue	129	Phosphoserine; alternate Combined sources			1
Glycosylation	129	O-linked (GlcNAc) serine; alternate 1 Publication			1
Modified residue	176	Phosphotyrosine; by TNK2 1 Publication			1
Cross-link	284	Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin) 1 Publication			
Disulfide bond	296 ↔ 310	By similarity			
Glycosylation	305	O-linked (GlcNAc) threonine 1 Publication			1
Modified residue	308	Phosphothreonine; by IKKE, PDPK1 and TBK1 7 Publications			1
Glycosylation	312	O-linked (GlcNAc) threonine 1 Publication			1
Modified residue	448	Phosphothreonine Combined sources			1
Modified residue	450	Phosphothreonine Combined sources			1
Modified residue	473	Phosphoserine; by IKKE, MTOR and TBK1; alternate 11 Publications			1
Glycosylation	473	O-linked (GlcNAc) serine; alternate By similarity			1
Modified residue	474	Phosphotyrosine 1 Publication			1

Post-translational modification

O-GlcNAcylation at Thr-305 and Thr-312 inhibits activating phosphorylation at Thr-308 via disrupting the interaction between AKT1 and PDPK1. O-GlcNAcylation at Ser-473 also probably interferes with phosphorylation at this site. 14 Publications

Post-translational modification will lead to a slowdown of electrophoretic mobility, and the corresponding electrophoretic conditions also need to be changed accordingly.

Isoform Sequences

Sequences (2+)

Sequence status: Complete.

This entry describes **2** isoforms produced by **alternative splicing**. Align | Add to basket

This entry has 2 described isoforms and 3 potential isoforms that are computationally mapped. Show all | Align All

Isoform 1 (identifier: **P31749-1**) [UniParc] FASTA | Add to basket

This isoform has been chosen as the canonical sequence. All positional information in this entry refers to it. This is also the sequence that appears in the downloadable versions of the entry.

Show »

Length: 480
Mass (Da): 55,686
Last modified: February 1, 2005 - v2
Checksum: 6EAFF4F8AD436714

BLAST | GO

Isoform 2 (identifier: **P31749-2**) [UniParc] FASTA | Add to basket

The sequence of this isoform differs from the canonical sequence as follows:
1-62: Missing.

Length: 418
Mass (Da): 48,347
Checksum: 671C0EB4BDF8F45F

BLAST | GO

Show »

Computationally mapped potential isoform sequences

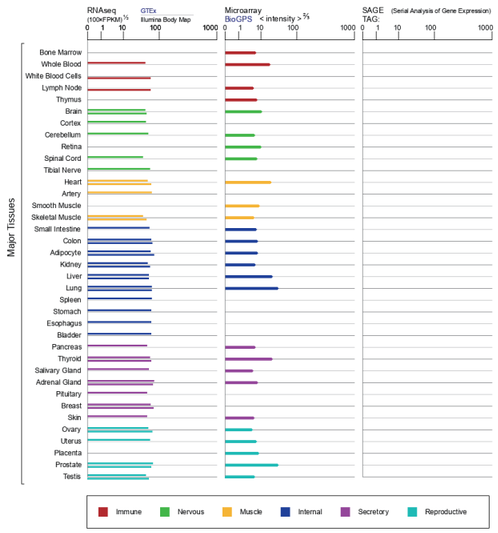
There are 3 potential isoforms mapped to this entry. BLAST | Align | Show all | Add to basket

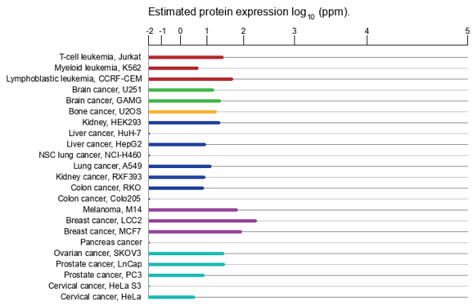
Entry	Entry name		Protein names	Gene names	Length	Annotation
G3V2I6	G3V2I6_HUMAN		RAC-alpha serine/threonine-protein ...	**AKT1**	184	Annotation score:
G3V3X1	G3V3X1_HUMAN		RAC-alpha serine/threonine-protein ...	**AKT1**	170	Annotation score:
A0A087WY56	A0A087WY56_HUMAN		RAC-alpha serine/threonine-protein ...	**AKT1**	146	Annotation score:

It contains the molecular weight size of the target protein, whether there are isoforms, and the sequence differences between isoforms.

GeneCards

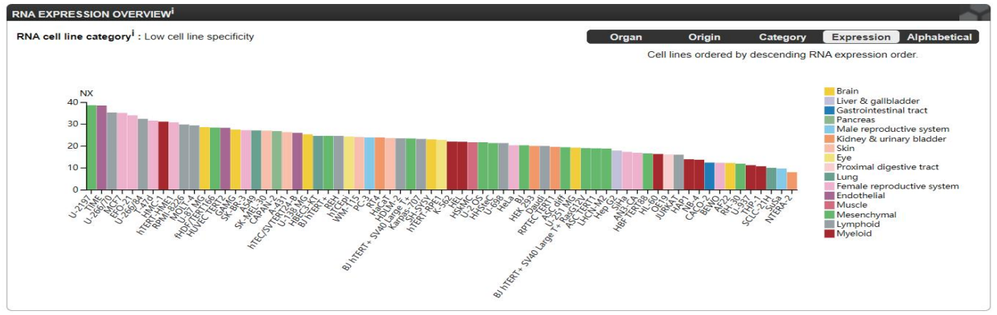
GeneCards (https://www.genecards.org/) is a searchable, integrative database that provides comprehensive, user-friendly information on all annotated and predicted human genes.





The Human Protein Atlas

The Human Protein Atlas (HPA) (https://www.proteinatlas.org/) is an open access resource for human proteins in cells, tissues and organs using integration of various omics technologies, including antibody-based imaging, mass spectrometry-based proteomics, transcriptomics and systems biology.



MGI-Mouse Genome Informatics

MGI (https://www.informatics.jax.org/) is the database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data for the study of human health and disease.

// 1.2.Sample preparation

// Lysis buffer

Refer to the table below for choosing a lysis buffer.

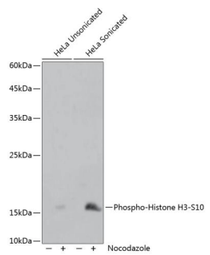
Product Name	Western & IP lysis buffer	RIPA lysis buffer (Strong)	RIPA lysis buffer (Medium)	RIPA lysis buffer (Mild)	NP-40 lysis buffer	SDS lysis buffer
Composition	1% Triton X-100	1% Triton X-100.1% 1% deoxycholate. 0.1% SDS	1% NP-40. 0.5% deoxycholate. 0.1% SDS	1% NP-40.0.25% 0.25% deoxycholate	1% NP-40	1% SDS
Lysis Strength	Mild	Strong	Medium	Mild	Mild	Strong
Membrane-Bound Proteins	Fair	Excellent	Good	Fair	Fair	Excellent
Cytoplasm	Excellent	Excellent	Excellent	Excellent	Excellent	Excellent
Nucleus	Good	Excellent	Good	Good	Good	Excellent
Cytoplasmic Phosphorylated Protein	Excellent	Excellent	Excellent	Excellent	Excellent	Excellent
Whole Cell Lysate	Excellent	Excellent	Excellent	Excellent	Excellent	Excellent

// Sonication Lysis

Cell lysis is the disruption of the cellular membrane. In the laboratory, this is usually done to expose the contents of the cell to purify and study them. Sonication is the physical disruption commonly used to break open cells. The method uses pulsed, high-frequency sound waves to agitate and lyse cells, bacteria, spores, and finely diced tissue. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles that form momentarily and implode, causing shock waves to radiate through a sample. But it produces heat which needs to be mitigated to prevent damage to sensitive proteins, ultrasonic treatment needs to be applied in multiple short bursts to a sample immersed in an ice bath.

ABclonal Recommended Conditions

Sonicate (continuous 39W power) for 3-5 s (no more than 10-20s). It is important to let the sample cool down between sonication steps, the duration of intervals is 3-5s (placed on ice). Repeat the sonication step 3-5 times, and the repeating time mainly depends on the volume of the sample and the strength of added lysis buffer, usually until the sample is uniform and not viscous. Different sonicators' performance may vary and need to be individually optimized according to the manufacturer's recommendations.



Target: Phospho-Histone H3-S10
Molecular weight: 15KD
Sample:
Control group HeLa, HeLa+ Nocodazole (Not sonicated)
Treatment group HeLa, HeLa +Nocodazole (Sonicated)
Result: WB detection of phosphorylation level of Histone 3 Serine 10 in control group and treatment group, the result shows that samples after sonication have stronger signals.

2.Loading & Running SDS-PAGE Gel

// 2.1 SDS-PAGE

SDS-PAGE gel consists of two different gel layers, the upper layer is stacking gel and the lower layer is separating gel. The stacking gel is macroporous gel, the buffer solution is pH 6.7, and the gel percentage is usually 5%; the separating gel is small pore gel, the buffer solution is pH 8.9, and the percentage of the separating gel needs to be determined according to the molecular weight of the protein.

Molecular weight（kDa）	Gel percentage
X ≤ 10	15%
10 < X ≤ 15	13.5%
15< X ≤ 25	12%
25< X ≤ 35	11%
35< X ≤ 40	10%
40< X ≤ 55	9%
55< X ≤ 70	8%
70< X ≤ 100	7%
100< X	6%
Note: X = Protein Size	

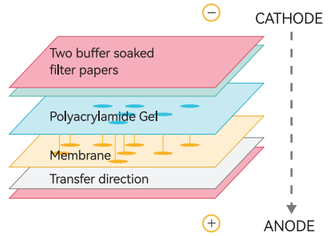
// 2.2 Tips for loading samples and running the gel

In the process of preparing the gel, the speed should not be too fast when mixing the gel components. Be careful and prevent air bubbles when pipetting the liquid. If air bubbles are generated in the gel, do not suck the air bubbles into the pipette tips. If air bubbles are sucked into the pipette tips, wait for them to rise to the surface before pouring the gel, and make sure they stay in the tips. Do not pour it into the chamber too fast, which will cause the generation of air bubbles and cause an uneven gel surface. Make sure to wait until the gel turns solidified.

It is recommended to heat, boil, and then centrifuge before loading the sample. When loading the sample, avoid sticking the pipette tip into the bottom of the tube, otherwise, the pipette tip will be stained with a large amount of sample and will spread when loading the sample. Place the tip of the pipette between the two glass plates, insert it gently (do not insert forcefully or squeeze the glass plate), and slowly pipette out the sample. When all the samples are out, pause for 2 seconds before pulling out the pipette tip.

3.Protein Transfer

The proteins in the SDS-PAGE gel are negatively charged due to binding to SDS, and under an electric field, the negatively charged proteins will be transferred to the membrane. The transfer methods include semi-dry and wet transfer. The principle is exactly the same, except for the mechanism used to fix the gel/film stack and apply the electric field (see figure below). Semi-dry transfer requires less buffer, and the time is shorter, but it is not as stable as wet transfer (semi-dry transfer works better on small proteins); wet transfer requires a large amount of buffer, and the time is relatively long, but it is more stable.



◦Protein transfer illustration

◦Semi-dry transfer cassette

◦Wet transfer cassette

// 3.1 Membrane type

Type	NC membrane	PVDF membrane
Binding capacity	80-110μg/cm²	125-200μg/cm²
Background	Low	High
Features	Brittle	Strong toughness and hydrophobicity
Operation	Wetting with buffer	Wetting in 100% methanol
Price	Low	High

// 3.2 Transfer condition

Molecular weight（kDa)	Transfer condition
X ≤ 10	Constant current 200mA,30min
10 < X ≤ 15	Constant current 200mA,40min
15< X ≤ 20	Constant current 200mA,50min
20< X ≤ 50	Constant current 200mA, 1kDa/min+20min
50< X ≤ 100	Constant current 200mA, 1kDa/min+30min
100< X ≤ 150	Constant current 250mA, 1kDa/min+20min
150< X ≤ 180	Constant current 270mA, 1kDa/min(No more than 3h)
180< X	Constant current 270mA, 1kDa/min(No more than 3h)

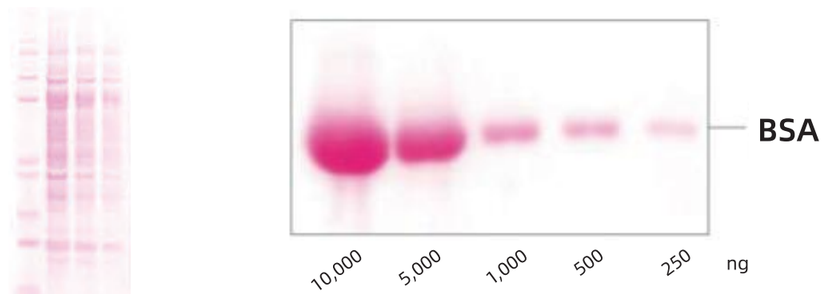
Note: X = Protein Size

// 3.3 Tips for protein transfer

- **Large proteins:** add 0.1% SDS to the transfer buffer; reduce the concentration of methanol to 10% or less, use 4℃ wet transfer instead of semi-dry transfer; increase transfer time, current/voltage.
- **Small proteins:** remove the SDS in the buffer; keep the methanol concentration at 20%; use membrane with small pore size; reduce the transfer time, current/voltage.
- **Membrane orientation:** after the transfer, cut the corner to help distinguish the front and back sides; mark with a pencil.
- **High background signal after transfer:** choose NC membrane.
- **Contamination during transfer:** avoid touching the membrane with fingers, use tweezers to remove the membrane.
- **Size of filter paper:** make sure the filter paper and membrane are the same size as the gel, when using semi-dry transfers, extra part will prevent current flow through the membrane.

// 3.4 Stain the membrane with Ponceau S (Optional)

Negatively charged Ponceau S can reversibly bind to positively charged amino acids on the membrane to confirm that protein transfer was successful. After staining, wash away Ponceau S with several washes in 1x TBST until membrane is clear.



4.Blocking and Antibody Incubation

// 4.1 Membrane blocking

After the protein is transferred to the membrane, there is still unbound protein on the membrane, which can be blocked with 3% or 5% skimmed milk or BSA prepared in TBST to reduce non-specific binding. Skimmed milk usually has stronger blocking effect, but when performing a phosphorylation-related WB experiment, we suggest using 3% or 5% BSA prepared in TBST for blocking when the signal is poor.

// 4.2 Antibody incubation

Primary antibody recognizes the epitope, or the specific amino-acid sequence, of the protein of interest. The secondary antibody recognizes the primary antibody. Secondary antibodies used for western blotting are usually conjugated with an enzyme. We recommend overnight incubation at 4℃ for the primary antibody incubation, which can improve the antibody binding efficiency and help reduce the background signal; the secondary antibody is usually incubated at room temperature.

5.Detection

In western blotting, the method of detection is dependent on which enzyme is conjugated to the secondary antibody. The most commonly used enzyme is HRP, and using ECL as substrate.

Western Blot Protocol

1.Sample preparation

(1) Extract protein from tissue

A. Use sterilized (pre-cooled) tools to separate 200 mg of fresh sample tissue, wash it with normal saline or PBS, and cut the tissue to an appropriate size with clean scissors.
B. Transfer the shredded tissue to a glass grinder, and add an appropriate amount of RIPA-containing protease inhibitors (phosphatase inhibitors if necessary; according to the ratio of 100 μL lysate per 20 mg tissue). Place on ice and grin every 3 minutes, repeat 5 times, and crush the tissue as much as possible until no obvious large cell clumps can be seen. Then place in the ice bath for 30 minutes to fully lyse the tissue cells; a small amount of lysed tissue liquid can be dropped on the glass slide to observe under the microscope to confirm whether the lysis is sufficient.
C. Sonication lyses cells and DNA to reduce sample viscosity and increase protein solubility. The power is 39W, each sonication is about 3-5s, and the interval is 3-5s on ice. Usually 3 to 5 times is enough, mainly depending on the volume of the sample and the lysis capacity of the added lysate.
   Note: To ensure adequate lysis of tissue cells, sonication is recommended. Adjust the ultrasonic instrument to the appropriate frequency and power (ultrasonic power should not be too high, and set ultrasonic intervals to prevent excessive heat generation of the ultrasonic probe), place the ultrasonic probe in the middle of the sample lysate, do not touch the tube wall or bottom, and perform sonication in ice bath.

D. After sonication, continue to incubate the sample on ice for 30 minutes.
E. Centrifuge the above lysed sample at 4°C, 12 000 rpm for 15-20 mins, take the supernatant into a new EP tube, and put it on ice for later use.

(2) Extract protein from cells

A. For adherent cells: aspirate the media out from the cultures, wash the cells with 1X PBS, and add RIPA Lysis Buffer with protease inhibitor (phosphatase inhibitors if necessary; 100 μL each well for 6-well plate, or 500 μL for 10 cm diameter plate) to lyse the cells, then immediately scrape the cells from the plate and transfer the extracts to EP tubes and place on ice.
B. For suspension cells: transfer the suspension cells together with the media to a 15mL centrifuge tube, centrifuge at 1000rpm at room temperature for 5min, collect the cell pellet, and then add RIPA lysate with protease inhibitors (phosphatase inhibitors if necessary; 100 μL each well for 6-well plate, or 500 μL for 10 cm diameter plate), pipette several times, then transfer to a new EP tube and place on ice.
C. Sonication lyses cells and DNA to reduce sample viscosity and increase protein solubility. The power is about 50W, each sonication is about 3-5s, and the interval is 3-5s on ice. Usually 3 to 5 times is enough.
D. After sonication, continue to incubate the sample on ice for 30 minutes.
E. Centrifuge the above lysed sample at 4°C, 12 000 rpm for 15-20 min, take the supernatant into a new EP tube, and put it on ice for later use.

(3) Determine protein concentration (BCA method)

A. Preparation of BCA working solution should accord to the amount of samples, add 50 volumes of BCA reagent A to 1 volume of BCA reagent B (50:1) to prepare an appropriate amount of BCA working solution, and mix well. BCA working solution is stable at room temperature for 24 hours.
B. Take 10μL protein standard (5mg/mL BSA) and dilute to 50μL to make the final concentration 1mg/mL. Diluted protein standards can be stored at -20°C for a long time. Deionized water or 1*PBS can be used as the diluent of this standard solution. Add 0, 1, 2, 4, 8, 12, 16, and 20 μL of the standard into the 96-well plate, and add diluent to make up to 20 μL. Add an appropriate volume of sample to each well of the 96-well plate. If the sample is less than 20 μL, add diluent to make it up to 20 μL. Then add 200μL BCA working solution to each well, and place at 37°C for 20-30min. Use a microplate reader to measure the absorbance of A562 or other wavelengths between 540-595nm. Calculate the protein concentration of the sample from the standard curve and the sample volume used.

(4) Protein denaturation

A. According to the protein concentration of each group obtained from the measurement, contribute 30-50 μg protein per group, and add 1/4 supernatant volume of 5× Loading buffer to the protein extract according to the volume ratio (the final working solution was 1×) and mix well, denatured by heating at 95°C for 10 minutes, and quickly cooled on ice, and stored at -20°C after the liquid was completely cooled.

2.Loading & Running SDS-PAGE Gel

(1) Gel maker installation

Install according to the instruction manual.

(2) Separating gel preparation

According to different protein sizes, choose different concentrations of separating gels (see the table below). Pour the prepared gel solution into the pre-installed gel maker, and add isopropanol to seal the gel. Place horizontally at room temperature for about 30 minutes. Pay attention to prevent the gel from generating bubbles; add TEMED before pouring it into the gel maker to prevent solidification; slowly drag along the side of the glass plate when adding isopropanol.

Separating gel formula	6%	7%	8%	9%	10%	11%	12%	13.5%	15%
Deionized water(mL)	5.3	4.9	4.6	4.3	4.0	3.65	3.3	2.8	2.3
30% Acrylamide(mL)	2	2.4	2.7	3.0	3.3	3.65	4.0	4.5	5.0
1.5M Tris-HCL(pH8.8)(mL)					2.5				
10%SDS(mL)					0.1				
10%AP(mL)					0.1				
TEMED(mL)					0.01				
Total volume(mL)					10				

(3) Stacking gel preparation

After the separating gel has solidified, pour the isopropanol along one side of the glass plate and dry with filter paper. Then prepare stacking gels according to the required volume (see table below). Pour the prepared gel solution into the gel maker, and slowly insert the prepared comb into the gel solution along one end of the glass plate, and place it horizontally at room temperature for 20-30 minutes. Pay attention to avoid air bubbles between the comb and the gel solution.

Stacking gel formula	5% stacking gel	5% stacking gel	5% stacking gel	5% stacking gel	5% stacking gel
Deionized water(mL)	1.1	2.1	2.7	3.4	4.1
30% Acrylamide(mL)	0.33	0.5	0.67	0.83	1.0
1.0M Tris-HCL(pH6.8) (mL)	0.25	0.38	0.5	0.63	0.75
10%SDS(mL)	0.02	0.03	0.04	0.05	0.06
10%AP(mL)	0.02	0.03	0.04	0.05	0.06
TEMED(mL)	0.002	0.003	0.004	0.005	0.006
Total volume(mL)	2	3	4	5	6

(4) Sample loading

After the stacking gel is solidified, pull out the comb vertically with both hands and inject Tris-Glycine SDS running buffer into the inner and outer tanks to form a closed loop. Use a pipette to load the sample vertically, the protein should be 25 μg/well. Note that the Tris-Glycine SDS running buffer in the inner tank needs to be filled, and the Tris-Glycine SDS running buffer in the outer tank only needs to be 3-5cm above the bottom.

(5) Electrophoresis

After the sample is loaded, connect the power supply of the electrophoresis instrument, make sure to connect the positive and negative electrodes correctly, and set the appropriate parameters. The electrophoresis parameter of the stacking gel is a constant voltage of 80V. When the sample enters the separation gel, the voltage can be adjusted to 120V. When the bromophenol blue reaches the bottom of the gel, stop the electrophoresis and turn off the power.

3.Protein transfer

(1) Preparation

A. Put the transfer buffer at least 2 hours in advance (after the start of electrophoresis) in a -20°C refrigerator to pre-cool.
B. According to the size of the gel, cut the filter paper and the membrane to a suitable size.
C. Select 0.45μm NC membrane for the target protein > 20KD; choose 0.2μm NC membrane or 0.22μm PVDF membrane for the target protein < 20KD. Soak the NC membrane in western transfer buffer for later use. If using PVDF membrane, soak in methanol for 5-10 minutes, then soak in western transfer buffer for later use.

(2) Protein transfer

Separate glass plates and retrieve the gel, create a transfer sandwich as follows: three pieces of filter paper, gel, NC membrane, and three pieces of filter paper, on a porous pad in sequence. Relocate the sandwich to the transfer apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the membrane is between the gel and a positive electrode.

4.Blocking

Take the membrane out of the "sandwich" structure, put it into a suitable antibody incubation tank, add 6-7mL western blocking buffer to a 6.3×8.3cm membrane to incubate at room temperature for 1 hour.

5.Primary antibody incubation

A. Dilute the primary antibody with western antibody dilution buffer according to an appropriate ratio.

B. After blocking, pour the diluted primary antibody working solution into the incubation tank, stay at room temperature for 2 hours or overnight at 4℃.

6.Washing

After the primary antibody incubation, wash the membrane with TBST buffer four times for 5 minutes each time.

7.Secondary antibody incubation

A. 10 minutes before the end of primary antibody washing, dilute the secondary antibody with western antibody dilution buffer according to an appropriate ratio.
B. After washing, add the diluted secondary antibody working solution into the incubation tank and stay at room temperature for 1 hour.

8.Washing

After the secondary antibody incubation, wash the membrane with TBST buffer four times for 5 minutes each time.

9.Detection

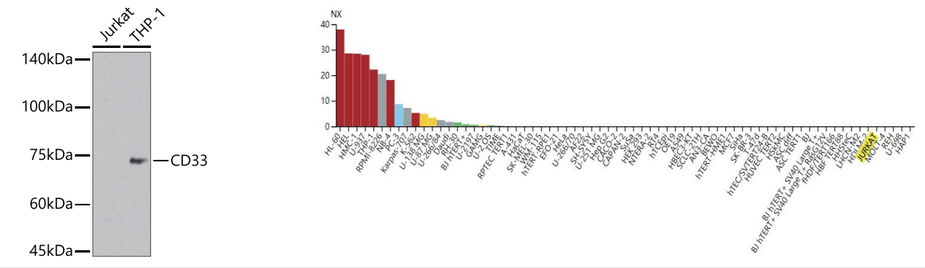
A. According to the size of the membrane, use 1-2mL working solution for every 10cm² membrane, draw equal volumes of Solution I and Solution II in proportion and mix well, and prepare the working solution for detection.
B. Remove the membrane with flat-tip tweezers, and gently touch the lower edge of the membrane to absorbent paper to remove extra fluid on the membrane. Use a pipette to add the working solution to cover the top and the bottom of the membrane, and incubate at room temperature for 1-2min. This step can be completed on a clean plastic wrap or in a plastic box.
C. Detection: Sandwich membrane between layers of plastic (i.e. a sheet protector or plastic wrap) and ensure no bubbles form between membrane and plastic. Expose the membrane to autoradiography film in a dark room or image with a chemiluminescent imaging system. Use multiple exposure lengths to identify the most optimal exposure time.

Part Three Troubleshooting

1.No Bands or Faint Bands

Possible Causes	Solutions
Improper primary or secondary antibody is used	Replace antibody. Use fresh, sterile buffer
The primary antibody and the secondary antibody are not compatible	Make sure you use a secondary antibody raised against the primary antibody species.
Reagents may have lost activity due to improper storage and handling	Check the storage instructions for your products on the datasheet. Use fresh reagents.
The protein of interest isn't present, or not enough protein is present	Run a positive control. Check the scientific literature to see if the protein is expected in your sample. Increase sample load.
Not enough antibody is bound to the protein	Add a higher concentration of primary antibody. Incubate the sample for longer with the antibody (overnight) at 4℃.
HRP inhibitor in solution	Avoid sodium azide in all solutions and utensils.
Overuse of antibodies has reduced their effectiveness	Make sure you use fresh primary and secondary antibodies for each experiment; the effective antibody concentration is lowered after each use.
Too much washing between steps.	Reduce the duration or number of washing steps.
There may not be enough exposure time when imaging the blot	Try imaging the blot again with a longer exposure time.
Over blocking	Reduce the amount of blocking agent or shorten the time and consider changing the blocking agent.
Problems with transfer of proteins to the membrane	Check the transfer was successful using a reversible stain such as Ponceau S before immunostaining. If using a PVDF membrane, make sure you pre-soak the membrane in methanol and then in transfer buffer.

>> Case study 1.1 The sample does not express the protein of interest



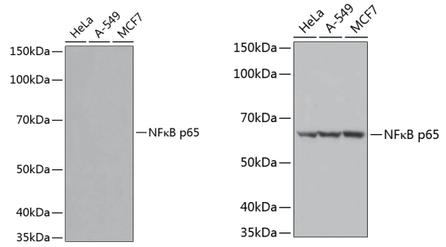
Target: CD33

Molecular weight: 70-80KD

Expression: It has been reported that CD33 is negative in Jurkat cells (PMID: 30519686), and the HPA database shows that THP-1 cells express CD33.

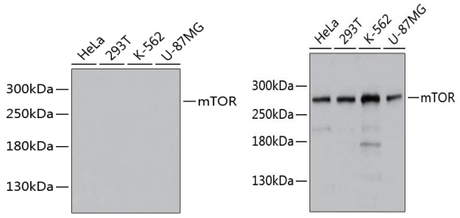
Results: WB detect Jurkat (CD33 negative) and THP-1 cells at the same time, no band was detected in the former sample, but signal could be detected in the latter sample.

>> Case study 1.2 Low antibody concentration



Target: NFκB p65
Molecular weight: 65KD
Antibody dilution ratio: 1:1000 and 1:500
Results: Antibody was diluted at 1:1000 (HeLa cell , A549, MCF-7) and samples were detected by WB, strong exposure for 3 minutes, no band (left); change the antibody dilution ratio to 1:500, increase the antibody concentration, and ordinary exposure for 3 seconds. Obvious target band (right).

>> Case study 1.3 Problems with transfer of proteins to the membrane

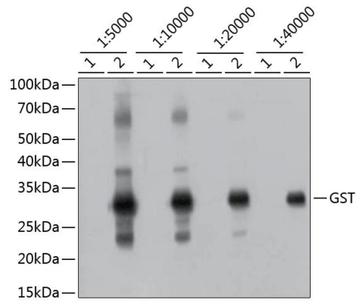


Target: mTOR
Molecular weight: 289KD
Transfer conditions: constant current 270mA, transfer time 1.5h vs. constant current 270mA, transfer time 3.5h
Results: Constant current 270mA, transfer time 1.5h, WB detected HeLa, 293T, K562, U-87 MG samples, no band (left); optimized transfer condition, constant current 270mA, transfer time 3.5h, obvious target band (right).

2.Unexpected or Multiple Bands

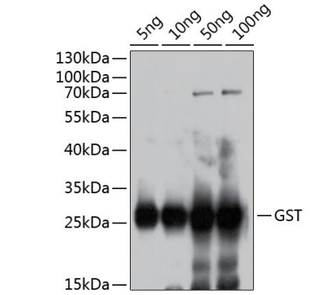
Possible Causes	Solutions
Primary antibody concentration may be too high	Dilute the antibody further to its optimal concentration
The primary antibody is not specific enough	Use monoclonal or affinity-purified antibodies to ensure antibody specificity
Non-specific binding of secondary antibodies	Do not add the primary antibody, and other operating procedures remain unchanged, you can verify whether the background is derived from the secondary antibody; re-select the secondary antibody
Too much protein loaded	Reduce sample load
Protein degradation	Avoid repeated freeze-thaw cycles; use freshly prepared samples and use protease inhibitors
The protein may have multiple isoforms or protein cleavage	Check the literature to see if multiple bands are reported
The cell line may have been passaged too many times.	Go back to the original non-passaged cell line and run these samples in parallel

>>Case study 2.1 Unexpected bands caused by high antibody concentration



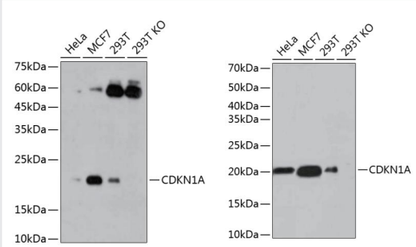
Target: GST protein
Molecular weight: 27KD
Sample: Number "1":
represents the control group, the vector that does not carry the GST tag is transformed into *E. coli* that do not express GST protein after induction.
Number "2":
represents the treatment group, the vector carrying the GST tag is transformed into *E. coli* that express purified GST protein.
Antibody dilution ratio: gradient dilution 1:5000 to 1:40,000
Loading amount: 10ng for each lane
Results: WB detection of four groups of the same samples, with the decrease of antibody concentration, unexpected band gradually decreases.

>>Case study 2.2 Unexpected bands caused by too much sample loads



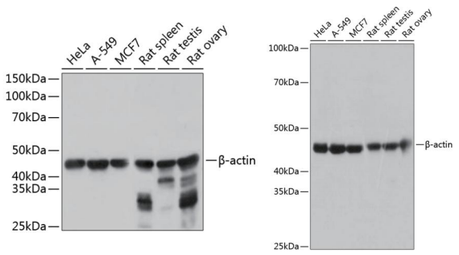
Target: GST protein
Molecular weight: 27KD
Sample: The vector carrying the GST tag is transformed into *E. coli* to express the purified GST protein.
Antibody dilution ratio:1:3000
Results: WB detection of purified GST protein (gradient loading 5ng, 10ng, 50ng, 100ng), as the sample loading increases, the unexpected band gradually increases.

>>Case study 2.3 Unexpected bands caused by poor antibody specificity



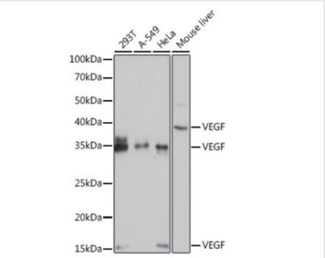
Target: CDKN1A
Molecular weight: 21KD
Antibody dilution ratio: 1:1000
Results: WB detection of HeLa, MCF-7, 293T and 293T knockout samples, the result (left) showed an unexpected band around 60KD, and another group (right) used antibody with good specificity for CDKN1A, and there was no unexpected band.

>> Case study 2.4 Unexpected bands caused by protein degradation



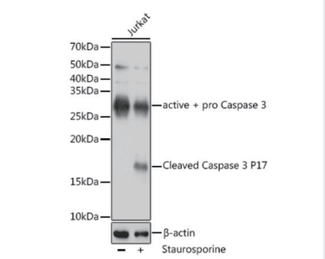
Target: β-actin
Molecular weight: 42KD
Samples: Human cell lines HeLa, A-549, MCF7;
Rat tissue: Spleen,Testis,Ovary
Antibody dilution ratio: 1:10,000
Results: WB detects both human and rat samples at the same time. The human sample has a single band, and the rat sample has multiple bands. The result is caused by sample degradation; a fresh rat sample is re-prepared and tested simultaneously with the human sample. A single band was obtained.

>> Case study 2.5 Unexpected bands caused by isoforms



Target: VEGF
Molecular weight: 15, 20, 25, 36, 40KD and other isoforms (PMID: 8167412)
Results: WB detection of VEGF expression levels in human 293T, A-549, HeLa cell lines and mouse liver tissue,multiple VEGF isoform bands can be detected.

>> Case study 2.6 Unexpected bands casued by protein cleavage

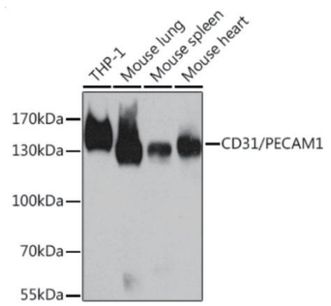


Target: Caspase-3
Molecular weight: precursor 32KD, 17KD cleaved (when apoptosis occurs)
Samples:
Control group: Jurkat cells
Treatment group:Jurkat cells treated with staurosporine (apoptosis induction)
Results: WB detection of Jurkat cells before and after staurosporine treatment, the control group only detected the precursor 32KD, while the treatment group detected both the precursor and the 17KD cleaved protein.

3.Molecular Weight Does Not Match The Theory

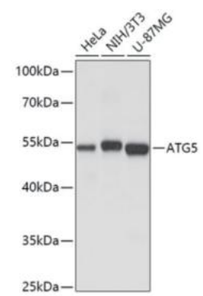
Reason	Type
Protein has post-translational modifications	Glycosylation, acetylation, ubiquitination, etc.
Aggregate form exists	Dimers or polymers
The type of charge carried by the protein	Whether the protein is positively charged or negatively charged affects protein mobility

>> Case study 3.1 Glycosylation causes an increase in molecular weight



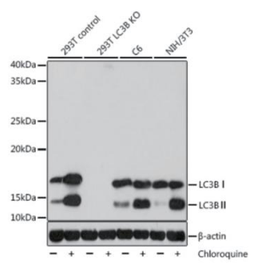
Target: CD31
Molecular weight: theoretical molecular weight 82KD, apparent molecular weight 130KD (PMID: 25971970)
Reason for molecular weight increase: glycosylation modification

>> Case study 3.2 Heterodimer causes apparent molecular weight increase



Target: ATG5
Molecular weight: theoretical 32KD, apparent molecular weight 55KD (PMID: 28928221)
Reason for the increase in molecular weight: ATG5 and ATG12 are covalently combined to form a heterodimer

>> Case study 3.3 The charge on the protein causes the change of molecular weight



Target: LC3B-II

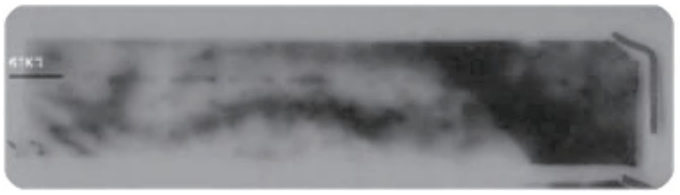
Molecular weight: 14KD

Results: The molecular weight of LC3B-I is about 16KD. Because LC3B-II is bonded with a PE group, theoretically, the molecular weight of LC3B-II is larger than that of LC3B-I. However, since the negatively charged PE changes the properties of LC3B-II, making it more hydrophobic and more mobile in electrophoresis. Therefore, LC3-II stays at a smaller molecular weight (about 14 KD) after electrophoresis.

4.High Background

Possible Causes	Solutions
Blocking of non-specific binding may be insufficient	Increase the blocking incubation period or temperature, and consider changing the blocking agent.
Not enough washing between steps	Wash extensively in buffer between all steps. Add Tween-20 to the wash buffer
Secondary antibody concentration may be too high	Dilute the antibody further to its optimal concentration.
Non-specific background of secondary antibodies	Do not add the primary antibody, and other operating procedures remain unchanged, you can verify whether the background is derived from the secondary antibody; re-select the secondary antibody
The antibody may be binding non-specifically to the blocking agent	Replace the blocking agent; add Tween-20 to the wash buffer to reduce cross-reactivity
Exposure time may be too long	Shorten exposure time
The blot has dried out	Prevent the membrane from drying out during incubation by keeping it covered in buffer.

>> Case study 4.1 Insufficient blocking and washing casues high background

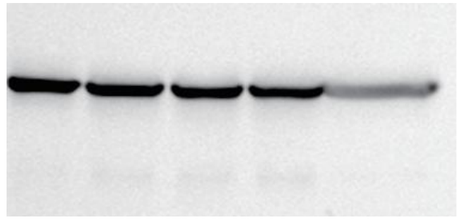


5.Bands are Misshapen or Uneven

Problems	Possible Causes	Solutions
Bands connect in a line	Too much sample loading and high primary antibody concentration	Reduce the loading amount and primary antibody concentration
Smile effect on bands	The gel is not cooled uniformly, the middle is hotter than the ends during electrophoresis; the gel concentration is not suitable; the voltage is too high	Adjust gel concentration; reduce voltage during electrophoresis
Frowning effect on bands	Bubbles at bottom of gel and glass plate; incomplete polymerization on both sides	When adding electrophoretic fluid to the electrophoresis tank, empty the air bubbles under the glass plate; make sure to mix the gel well
Smear	The protein in the sample is not completely dissolved	Boil the sample for 5 minutes before loading and centrifuge for 1 minute
Vertical stripes appear	Sample contains insoluble particles	Boil the sample for 5 minutes before loading and centrifuge for 1 minute
Bands are uneven	The gel has polymerized unevenly. Imbalanced electrodes or skewed loading position	Add an equal amount of sample buffer to the unfilled well, and make sure to mix the gel well
Band scatter	Sample was diluted by electrophoresis buffer during loading; wrong stacking gel preparation or comb base too short	When loading the sample, slowly add the sample from the bottom up; use the newly prepared AP, and prepare the stacking gel according to the formula; about 1.0-1.5cm from the bottom of the comb to the top of the separating gel
Band width unevenness	The protein concentration is different	Add 1*loading buffer to samples with high concentration until the volume of each sample is approximately the same
The background has uneven white spots	Bubbles exist during transfer	Check carefully to avoid the presence of air bubbles
	Uneven distribution of antibodies	Use a shaker when incubating antibodies

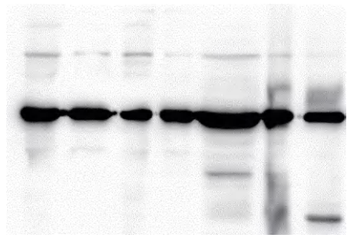
>> Case study 5.1 A large amount of sample is loaded and the bands are connected into a line

>> Case study 5.2 Lane diffusion



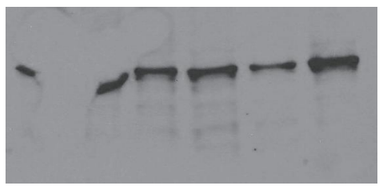
The right lane is empty, and did not use the same volume of 1×loading buffer to load the sample, causing the lane diffusion

>> Case study 5.3 Deformation of bands due to comb bending



>> Case study 5.4 Uneven gel and insufficient solidification

>> Case study 5.5 Band deformation caused by air bubbles



Part Four Case Studies of Representative Target Detection

1.Precautions for Detection of Protein Phosphorylation

Rule 1: Protein phosphorylation tends to come and go quickly

The phosphorylation and dephosphorylation process in cells is usually very fast and may complete within a few minutes to tens of minutes. Therefore, if small molecule compounds are used to treat cells *in vitro* to establish cell models for subsequent related research, it is recommended to use references and databases to explore the drug treatment concentration and treatment period for your cell line. Generally speaking, the phosphorylation modification of many proteins occurs rapidly within a short time (minutes, tens of minutes, 1-2 hours, etc.) after treatment with small molecule compounds and gradually increases over time. However, as the treatment time prolongs, the phosphorylation levels of some proteins will gradually decrease. It will prevent certain proteins (such as kinases) from being activated for too long due to the high level of phosphorylation (as shown in the figure below)

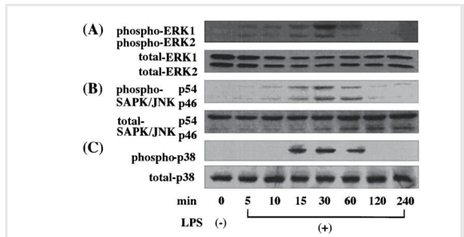


Figure: Time course of J774.1 cells stimulation with LPS (1 mg/mL) to induce phosphorylation of various MAPKs. ERK, SAPK/JNK and the activity of p38 was determined by western blot.

Therefore, it needs to be carefully considered when comparing whether there is a difference in the phosphorylation level of a certain protein between the control group and the treatment group and whether it conforms to the expected pattern. It is strongly recommended that you first use the appropriate drug concentration (based on the literature or preliminary research) to process different time lengths and samples, and only take one best time point treatment group. Samples were subjected to relevant phosphorylation assays and compared to controls to assess changes in results. This suggestion applies to both *in vitro* cultured cell models and animal models.

Rule 2: Certain proteins have background phosphorylation levels when cells are normally cultured

Serum is added to many cells under normal culture conditions, and many nutrients in serum (such as amino acids, lipids, growth factors, etc.) are important factors for maintaining cell growth. However, due to the existence of these components, the phosphorylation of some kinases, including AKT and MAPK, has already undergone phosphorylation modification without adding any small molecule compounds, which is what we usually call background phosphorylation level. Therefore, if some kinase phosphorylation studies are involved in proliferation studies, it is often necessary to pretreat the cells by serum deprivation (culture in a serum-free medium) before the formal experiment.

However, we also need to note that this pretreatment does not apply to any research cell model, and the so-called background phosphorylation level does not occur on all proteins. Serum deprivation can initiate a variety of stress responses, and long treatment may affect the detection of some targets (such as autophagy-related targets). Therefore, to better determine whether the phosphorylation level changes, it is necessary to ensure whether serum deprivation pretreatment is required according to the detected target.

Rule 3: Protein phosphorylation is very sensitive, so be quick and careful when sampling

If we want to detect protein phosphorylation, we often need to extract the protein from the cells under abnormal cell culture conditions (such as taking the cell culture dish out of the incubator). If the operation is improper (such as keeping the cells at a low temperature for a long time, changing the culture conditions or the pH value, exposing the cells to air, heavy metals, and the surface of various materials, etc.), it will cause stress reactions in the cells and then phosphorylation status alert of certain proteins during this time. Therefore, we need to complete the process in the shortest possible time to reduce these perturbations of external factors.

Rule 4: Protein phosphorylation is unstable, fresh samples are the key

After protein extraction, the cell lysate used for protein extraction will contain sufficient protease inhibitors and phosphorylase inhibitors to prevent protein degradation and loss of phosphorylation, but will everything be fine if this is not the case?

Everyone will encounter such a problem sometimes, that is when using fresh samples for the first phosphorylation detection, the results are in line with expectations, but when the protein is stored in the freezer (whether it is -20°C or -80°C) for some time, the experiment could not be repeated by the same batch of extracted samples. This is because the phosphorylation of protein is not stable *in vitro*, especially when the cell lysate used for protein extraction is relatively insufficient (there are too many cells but the lysate added is not enough). Therefore, we recommend:

1) The amount of cell lysate used for protein extraction must be appropriate.
2) Use good quality protease inhibitors and phosphorylase inhibitors.
3) Try to use freshly extracted samples for phosphorylation-related detection, and only use frozen-thawed protein samples when there is no other options. The three experimental repetitions required by the reviewer need to repeat the entire process from cell culture, drug treatment, to sample preparation and testing, instead of making one sample and testing for three times.
4) If it is really necessary to perform technical repetition (extract a sample and perform multiple tests), it is also recommended to divide the protein into different tubes after the protein concentration is determined, add the sample buffer to boil, and then store it at low temperature. Higher concentrations of SDS will also minimize the effect of phosphatases in the sample on phosphorylation. It is advisable to place the sample on ice as soon as possible after taking it out of the freezer and thaw it on the ice, to minimize the influence of the thawing process on phosphorylation.

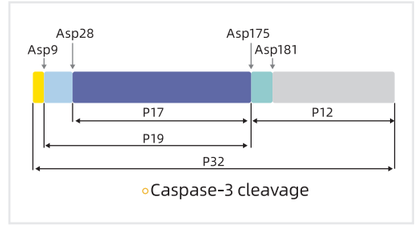
2.Precautions for Detecting Proteins in The Presence of Cleavage

// 2.1 Caspase-3

Caspase-3 belongs to the caspase family and plays an important role in many pathways of apoptotic signal transduction. It usually exists in the cytoplasm in the form of an inactive zymogen (Pro-caspase-3, 32KD). It is activated in the early stage of

apoptosis, and the activated Caspase-3 is composed of large and small subunits, which cleaves the corresponding cytoplasmic and nuclear substrates and finally leads to apoptosis.

The full length of Caspase-3 is 277 amino acids, and there are four enzyme cleavage sites Asp9, Asp28 (Ser29), Asp175 (Ser176), and Asp181 on the zymogen, among which p20 (1-175 amino acids) and p11 (181-277 amino acids) are generated by cutting at Asp175 and Asp181, the large subunit is further cleaved at Asp28 to remove the N-terminal propeptide to generate p17, and cleaved at Asp9 to generate the large subunit of p19, which is related to the activation of the proenzyme of Caspase-3.



One of the key events in the process of apoptosis is the activation of caspase-3. Therefore, detection of cleaved caspase-3 by western blot has become a common method for studying apoptosis, but it is often encountered that cleaved caspase-3 cannot be detected. So where should we start to solve this problem?

Confirm the expression level of Caspase-3 in the tested samples

Caspase-3 is highly expressed in lung, spleen, liver and other tissues, moderately expressed in brain and skeletal muscle, and lowly expressed in testicular tissue; it is also expressed in various cell lines, especially in immune cells. Before performing caspase-3 detection, it is necessary to use literatures or databases as references in advance to understand its background expression level in the tested samples.

Expression[1]

Tissue specificity[1]

Highly expressed in lung, spleen, heart, liver and kidney. Moderate levels in brain and skeletal muscle, and low in testis. Also found in many cell lines, highest expression in cells of the immune system.

Choose the right induction treatment conditions

Cleaved caspase-3 exists only in cells undergoing apoptosis, and it cannot be detected in non-apoptotic or necrotic cells. Therefore, the effectiveness of sample processing is one of the key factors of whether cleaved caspase-3 can be detected. When a precursor of caspase-3 can be detected, we suggest selecting the appropriate inducer and doing a gradient on the inducer concentration and induction time to determine the optimal treatment concentration and sample collection time according to the results. And set up an appropriate positive control for comparative detection at the same time.

Adherent cell lines

Adherent cells may be difficult to adhere after apoptosis. It is recommended to collect all suspended cells by centrifugation, mix with scraped adherent cells and lyse for subsequent detection.

Enough sample loading

In tissue samples, only a small proportion of cells may undergo apoptosis, and it is recommended to load 100 μg of protein per well; for induced cells, it is recommended to load 20–30 μg of protein per well.

Membrane pore size

Cleaved caspase-3 is a small-molecular-weight protein, and the membrane with a pore size of 0.2 μm can minimize the loss during the transfer process. In addition, previous study reports that using glutaraldehyde to fix NC membranes for 30 mins can promote the stable binding of caspase-3 to NC membranes and increase the success rate of caspase-3 detection.

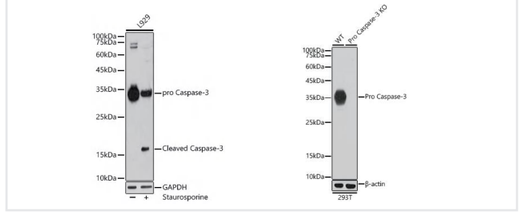
Gel concentration

15% concentration of separating gel (SDS-PAGE) can be used for detection of cleaved caspase-3.

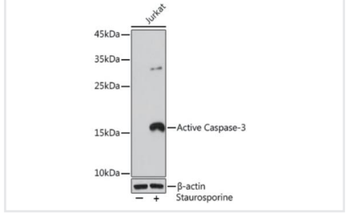
High quality cleaved caspase-3 antibody

When selecting an antibody, you not only need to pay attention to whether the verified species and application meet the requirements, but also need to confirm whether the antibody can recognize cleaved caspase-3.

A19654 [KO Validated] Active + pro Caspase-3 Rabbit mAb



A11021 | Active Caspase-3 Rabbit mAb



// 2.2 LC3

Microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3) is usually abbreviated as LC3. There are four subtypes of mammalian LC3 protein, namely MAP1LC3A (abbreviated as LC3A), MAP1LC3B (abbreviated as LC3B), MAP1LC3B2 (abbreviated as LC3B2), and MAP1LC3C (abbreviated as LC3C), among which LC3A and LC3B are the most studied.

LC3 is ubiquitously present in mammalian tissues and cultured cells and is a key molecule of autophagy. It has a high similarity to the yeast protein ATG8, and its carboxy terminus is rapidly cleaved by ATG4 to produce cytoplasmic localized LC3-I (molecular weight 16KD). During autophagy, LC3-I binds to PE through ubiquitin-like reactions that require ATG7 and ATG3 to produce lipidated LC3-II (molecular weight is 14kD) and localizes to the autophagosome membrane.

LC3-II is an important marker of the autophagosome, which increases with the increase of the autophagosome membrane. Therefore, WB detection of LC3 has also become a commonly used method in autophagy research, but LC3-II often can not be detected.

Confirm the expression level of LC3 in the tested samples

The four isoforms of LC3 have different expression levels in different tissues or cell samples, and should be selected according to the protein expression level in the studied samples.

LC3B is widely expressed in heart, brain, skeletal muscle and testis tissue, but rarely expressed in liver tissue.

Expression

Tissue specificity

Most abundant in heart, brain, skeletal muscle and testis. Little expression observed in liver. 1 Publication

LC3A is widely expressed in heart, brain, liver, skeletal muscle and testis tissue, but not in thymus and peripheral blood leukocytes.

Expression

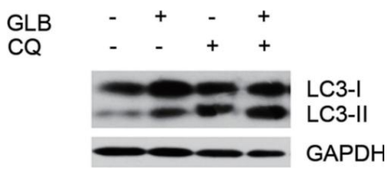
Tissue specificity

Most abundant in heart, brain, liver, skeletal muscle and testis but absent in thymus and peripheral blood leukocytes. 1 Publication

In liver tissue studies, it is more appropriate to detect LC3A. Usually, LC3A/B antibody can also be considered to detect LC3A and LC3B at the same time to improve the success rate of the experiment.

Choose the right sample treatment conditions

There is a process of generation, fusion, and degradation of autophagosomes, that is "autophagic flow". The existence of LC3-I and II is a dynamic process of generation-degradation. Therefore, without autophagy induction, samples only selected for detection at a single time point are likely to coincide with the degradation of autophagic flow, and this makes it hard to detect LC3-II. The expression of LC3-II cannot reflect autophagy, so it needs to use some drugs to block lysosomal degradation (such as chloroquine, bafilomycin A1, etc.). Under the premise of "plugging the leak", we can tell the degree of autophagy according to the LC3-II accumulation over a while.



Sample sonication

As a lipidated and membrane-associated protein, brief sonication on ice after cell lysis can promote protein dissolution and dissociate LC3 from the membrane.

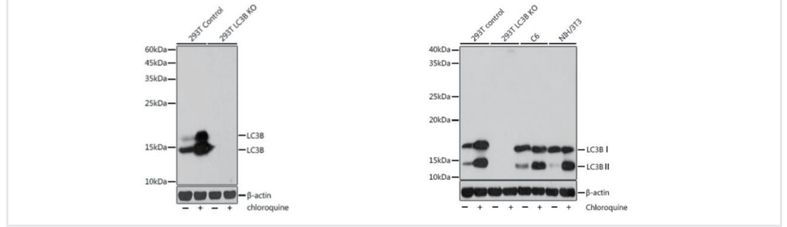
Gel and membrane selection

LC3 is a small molecular weight protein. There is only a 2KD difference between LC3-I (16KD) and LC3-II (14KD). It is recommended to use a high-concentration separating gel such as 15% (SDS-PAGE) for separation, and use a small-pore size 0.2μm membrane for transfer, and set the appropriate transfer conditions.

High quality primary antibody

When selecting an antibody, be sure to select the appropriate antibody according to the species and application being validated.

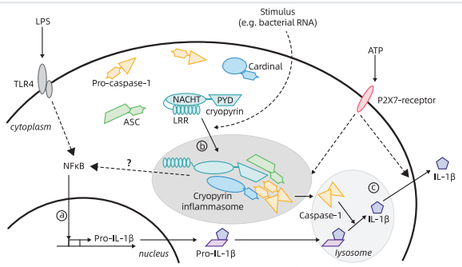
A19665|[KO Validated] LC3B Rabbit mAb



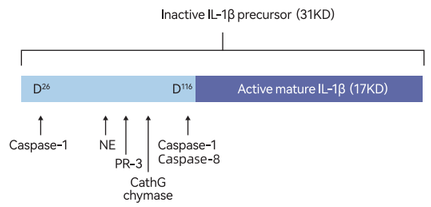
3.Precaution of Secreted Protein Detection

Secreted protein refers to a class of protein that is secreted to function outside the cell after being synthesized. Researchers often need to detect secreted proteins from cultured cell samples, and certain adjustments are required in the sample preparation process. Cell culture supernatants rich in these proteins are usually used as the detected samples. We use IL-1β as an example in following case study.

Interleukin-1β (IL-1β), a member of the IL1 family, is a pro-inflammatory functional cytokine that participates in a variety of autoimmune inflammatory responses and cellular activities, including cell proliferation, differentiation, and apoptosis. IL-1β is produced and secreted by many cells, including immune cells such as monocytes and macrophages. The production and release of IL-1β can be roughly divided into three steps: the production of inactive precursors, the maturation and activation of the precursors, and the secretion of activated proteins to the extracellular space.



Synthesis and Secretion of IL-1β



Splicing of the IL-1β gene

Western blot is an effective way to detect activated IL-1β, in actual detection, there will be cases where the signal cannot be detected.

Choose the right sample treatment conditions

The successful release of IL-1β requires two-step signal stimulation: the first step is the expression of mRNA and subsequent translation that requires the stimulation of PAMPs (pathogen-associated molecular patterns, such as LPS, etc.) from the pathogen. The second step is the rapid and effective processing of the precursor and requires further stimulation of DAMPs (damage-associated molecular patterns, such as ATP) or PAMPs. Studies have shown that without a second stimulus, cells produce only very low amounts of activated IL-1β. Therefore, it is very important to choose and optimize the appropriate sample treatment conditions.

Preparation of cell culture supernatant samples

Since activated IL-1β is secreted extracellularly, but not through the classic protein secretion pathway (ER-Golgi pathway), treatment of cells with traditional inhibitors cannot enrich intracellular IL-1β . Therefore, activated IL-1β can be detected through cell culture supernatant samples. The following sample preparation method (acetone precipitation protein) can be used:

- Medium and cold acetone (volume ratio 1:4) were mixed, stay overnight at -80°C
- Centrifuge at 10,000 g for 10 minutes
- Remove the supernatant, do not touch the protein pellet. Be careful not to over-dry the pellet, otherwise it will not be easy to dissolve later
- Leave the tube open at room temperature for 30 minutes to allow the acetone to evaporate completely
- Use 1X SDS loading buffer to dissolve the precipitate and perform WB detection

Selection of separating gel and membrane

The molecular weight of activated IL-1β is 17KD, which is a small molecular weight protein. It is recommended to use 15% separating gel (SDS-PAGE) for separation, a small pore size 0.2μm membrane for transfer, and set appropriate transfer conditions.

Part Five Appendix:Antibody Selection

1.Primary Antibody Selection

// 1.1 Target protein information

Determine whether the target protein you want to detect matches the information of the antibody recognition protein, and pay attention to the names, aliases and other information.

Some proteins have multiple different isoforms, which requires determining which protein isoform to study before selecting an antibody. There are also some antibodies that can recognize multiple isoforms at the same time because their antigens select peptide regions shared by different isoforms. Some proteins can be converted into mature active forms through cleavage, such as Caspase, Insulin, etc., which requires a specific antibody against the precursor or mature form according to research needs.

// 1.2 Information of antigens used for antibody preparation

Antibodies are prepared by immunizing hosts with various antigens. The types of antigens include full-length proteins, protein fragments, polypeptides, whole organisms (such as bacteria), or cells. Antibody datasheets usually have descriptions of the antigens used to develop antibodies. In some cases, it is necessary to ask the antigen information for antibody selection. For example, when selecting an antibody against a recombinant expressed protein, if it is expressing a partial protein fragment, you need to pay attention to whether the antigenic region of the antibody is within the fragment region. For some special proteins (such as pathogenic proteins) that have not been internally verified for target proteins, you need to ask the manufacturer about the protein homology sequence and combine it with the antigen sequence used to develop the antibody to check the cross-reactivity.

// 1.3 Modification site information

When choosing a suitable antibody for the study of a specific modification site, modifications at different sites may have different mechanisms, and it is not appropriate to generalize. The functions of different modification sites can be determined by references and databases. In addition, some proteins may have different isoforms and highly-homologous sequences, and the sequences around certain phosphorylation modification sites of different isoforms are completely identical, antibodies against these conserved modification sites often recognize different isoforms at the same time.

// 1.4 The species information of the samples

Manufacturers usually carry out in-house validation on common targets of different species, which can be seen as the "Reactivity" of the product manual (such as human, mouse, rat, etc.). After validation, the species that pass the QC will be listed as a reference.

// 1.5 Antibody applications

Manufacturers usually carry out in-house validation on common applicaions, which can be seen as the "application" of the product manual (such as WB, IP, ChIP, IHC, IF, FCM, etc.). After validation, the application that pass the QC will be listed as a reference.

// 1.6 Information of labels

Some experiments, such as multiplex immunofluorescence and flow cytometry, may use directly labeled primary antibodies. When choosing a direct-labeled primary antibody, you need to know the range that can be detected by the instrument, and choose a suitable label for different parameter requirements.

// 1.7 Citations

There may be products with different citations for a certain protein. Many people like to look up in the papers to determine whether the antibody would have good performed.

2.Secondary Antibody Selection

2.1 Host of primary antibody

The selection of secondary antibody is mainly determined by the host species of the primary antibody. If the primary antibody is from mouse, then the secondary antibody can be purchased against mouse.

2.2 Secondary antibody application

For different applications, the secondary antibody is usually conjugated with different labels, such as HRP, Biotin, fluorescein, etc. When doing WB and IHC experiment, HRP and Biotin are often used. But while doing immunofluorescence and flow cytometry, different fluorescein-labeled secondary antibodies can be selected according to the experiment needs, such as FITC, Cy3, PE, etc.

3.Loading Control Selection

The loading control is the internal control generally refers to the proteins expressed by housekeeping genes. Their expression is relatively constant in tissues and cells . When detecting protein expression levels, it is often used as a reference. Here are some commonly used loading controls and notes:

Loading Control	Sample	Molecular weight (kDa)	Note
Beta Actin	Whole Cell / Cytoplasm	43	Not suitable for skeletal muscle samples. Changes in cell growth conditions and interactions with extracellular matrix components can lead to changes in actin protein synthesis
GAPDH	Whole Cell / Cytoplasm	30-40	Under certain physiological conditions, such as hypoxia and diabetes, the expression of GAPDH is increased in some specific cell types
Tubulin	Whole Cell / Cytoplasm	55	Altered expression of tubulin due to differential resistance to antimicrobial and antimitotic drugs
VDAC1/Porin	Mitochondria	31	
COXIV	Mitochondria	16	
Lamin B1	Inside the nucleus	66	Not suitable for samples with nuclear envelope removed
TATA-binding protein, TBP	Inside the nucleus	38	Not suitable for samples with DNA removed

Part Six Featured SCI Citations

PMID	Title	Journal	IF	Type	Cat.No./Species
25083871	MicroRNA directly enhances mitochondrial translation during muscle differentiation	Cell	41.582	Antibody	A4967
28753426	Methyltransferase SETD2-Mediated Methylation of STAT1 Is Critical for Interferon Antiviral Activity	Cell	41.582	Antibody	A3194
28965759	Temporal Control of Mammalian Cortical Neurogenesis by m6A Methylation	Cell	41.582	Antibody	A8530
29290468	Stabilization of Reversed Replication Forks by Telomerase Drives Telomere Catastrophe	Cell	41.582	Antibody	A9555
30146159	Microbial Siderophore Enterobactin Promotes Mitochondrial Iron Uptake and Development of the Host via Interaction with ATP Synthase.	Cell	41.582	Antibody	A5769
31031002	Structure and Degradation of Circular RNAs Regulate PKR Activation in Innate Immunity.	Cell	41.582	Antibody	AP0342
31251911	Pervasive Chromatin-RNA Binding Protein Interactions Enable RNA-Based Regulation of Transcription.	Cell	41.582	Antibody	A5016
31585088	Integrated Proteogenomic Characterization of HBV-Related Hepatocellular Carcinoma	Cell	41.582	Antibody	A11932
32169215	Initiation of Parental Genome Reprogramming in Fertilized Oocyte by Splicing Kinase SRPK1-Catalyzed Protamine Phosphorylation	Cell	41.582	Antibody	A5854
32259487	Distinct Processing of lncRNAs Contributes to Non-conserved Functions in Stem Cells	Cell	41.582	Antibody	A5964
32810437	Formation of NPR1 Condensates Promotes Cell Survival during the Plant Immune Response	Cell	41.582	Antibody	A20262
32931733	Targeting Mitochondria-Located circRNA SCAR Alleviates NASH via Reducing mROS Output	Cell	41.582	Antibody	A9840
33991488	Mouse totipotent stem cells captured and maintained through spliceosomal repression	Cell	41.58	Antibody	A5891, A6709
28297716	RNA m6A methylation regulates the ultraviolet-induced DNA damage response.	Nature	49.962	Antibody	A1848, AP0099
30111841	Modulating plant growth–metabolism coordination for sustainable agriculture	Nature	49.962	Antibody	A16279
30814741	Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma.	Nature	49.962	Antibody	A2091
31485071	Structural basis of nucleosome recognition and modification by MLL methyltransferases.	Nature	49.962	Antibody	A2355, A2357
32376953	Plant 22-nt siRNAs mediate translational repression and stress adaptation	Nature	49.962	Antibody	A2348
32238924	U1 snRNP regulates chromatin retention of noncoding RNAs	Nature	49.962	Antibody	A5761, AP0844
32581380	Reversing a Model of Parkinson's Disease With in Situ Converted Nigral Neurons	Nature	49.962	Antibody	A14150, A6107
33268895	Galactosaminogalactan activates the inflammasome to provide host protection	Nature	49.962	Antibody	A15094, A6724
33361816	Molecular basis of nucleosomal H3K36 methylation by NSD methyltransferases	Nature	49.962	Antibody	A2364
32029600	Enhanced sustainable green revolution yield via nitrogen-responsive chromatin modulation in rice.	Science	47.728	Antibody	A16279

PMID	Title	Journal	IF	Type	Cat.No./Species
33243860	Identification of Integrator-PP2A complex (INTAC), an RNA polymerase II phosphatase	Science	47.728	Antibody	A2355, A7253
33795473	Structural insights into preinitiation complex assembly on core promoters	Science	47.728	Antibody	A2192, A5928
34709882	NIN-like protein transcription factors regulate leghemoglobin genes in legume nodules	Science	47.72	Antibody	A20191
30455424	VCAM-1+ macrophages guide the homing of HSPCs to a vascular niche.	Nature	49.962	Custom Antibody	Zebrafish
30944466	TMK1-mediated auxin signalling regulates differential growth of the apical hook.	Nature	49.962	Custom Antibody	Arabidopsis
31942069	Host-mediated ubiquitination of a mycobacterial protein suppresses immunity	Nature	49.962	Custom Antibody	Mycobacterium tuberculosis
30487604	Stella safeguards the oocyte methylome by preventing de novo methylation mediated by DNMT1.	Nature	49.962	Custom Antibody	Mouse
30356214	Nuclear cGAS suppresses DNA repair and promotes tumorigenesis.	Nature	49.962	Custom Antibody	Human
31243371	UDP-glucose accelerates SNAI1 mRNA decay and impairs lung cancer metastasis.	Nature	49.962	Custom Antibody	Human
32841601	A Defense Pathway Linking Plasma Membrane and Chloroplasts and Co-opted by Pathogens	Cell	41.582	Custom Antibody	Arabidopsis
28552346	Ubiquitination-Deficient Mutations in Human Piwi Cause Male Infertility by Impairing Histone-to-Protamine Exchange during Spermiogenesis	Cell	41.582	Custom Antibody	Mouse
31835033	A Translation-Activating Function of MIWI/piRNA during Mouse Spermiogenesis	Cell	41.582	Custom Antibody	Mouse
30612740	METTL13 Methylation of eEF1A Increases Translational Output to Promote Tumorigenesis.	Cell	41.582	Custom Antibody	Human
27002220	A positive role for polycomb in transcriptional regulation via H4K20me1.	Cell research	25.617	Custom Antibody	Drosophila
32735848	A Bacterial Effector Protein Hijacks Plant Metabolism to Support Pathogen Nutrition	Cell Host Microbe	21.023	Custom Antibody	Tobacco
33176558	A VASt-domain protein regulates autophagy, membrane tension, and sterol homeostasis in rice blast fungus	Autophagy	16.016	Custom Antibody	Magnaporthe grisea
30833710	In rose, transcription factor PTM balances growth and drought survival via PIP2;1 aquaporin	Nature plant	13.256	Custom Antibody	Chinese rose
31075443	Translational Regulation of Plant Response to High Temperature by a Dual-Function tRNA His Guanylyltransferase in Rice	Molecular Plant	13.164	Custom Antibody	Rice
31530735	Intra-Kernel Reallocation of Proteins in Maize Depends on VP1-Mediated Scutellum Development and Nutrient Assimilation	Plant cell	11.277	Custom Antibody	Maize
32155285	miR-210 participates in hepatic ischemia reperfusion injury by forming a negative feedback loop with SMAD4	Hepatology (Baltimore, Md.)	17.425	ELISA	RK00006
33305325	Entropy subspace separation-based clustering for noise reduction (ENCORE) of scRNA-seq data	Nucleic Acids Res	16.971	ELISA	RK00719
34678143	Glycogen accumulation and phase separation drives liver tumor initiation	Cell	41.582	NGS	RK20351, RK20303
33523829	Abnormal neocortex arealization and Sotos-like syndrome–associated behavior in Setd2 mutant mice	Sci Adv	14.136	NGS	RK20202, RK20282
31585088	Integrated Proteogenomic Characterization of HBV-Related Hepatocellular Carcinoma	Cell	41.582	Molecular enzyme	RK20400
32017896	LARP7-Mediated U6 snRNA Modification Ensures Splicing Fidelity and Spermatogenesis in Mice	Molecular Cell	17.97	Molecular enzyme	RK20400