

Western Blot Loading Control Handbook



广州奥科生物医学科技有限公司

www.aokemedical.com

Common Loading Control Proteins for Western Blot

kDa	Whole Cell	Cytoskeletal	Mitochondrial	Nuclear	Serum
120	Vinculin				
110					
75					Transferrin
65				Lamin B1	
60			HSP60		
	α -tubulin	α -tubulin		HDAC1	
50	β -tubulin	β -tubulin			
	Actin	Actin			
40	β -actin	β -actin			
35	GAPDH				
			VDCA1/porin	PCNA	
25					
20	Cyclophilin B	Cofilin			
15			COX IV	Histone H3	

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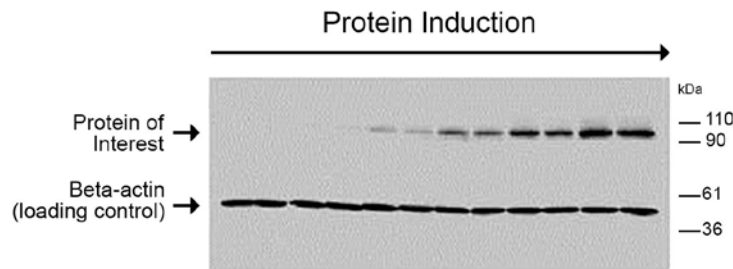
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Introduction to Loading Controls

A Loading Control is Important for Data Normalization

Western blotting is a common laboratory technique used to detect and quantify target protein expression in a complex cell or tissue lysate. This technique provides a semi-quantitative method for the comparison of relative target protein levels across multiple samples. To generate accurate expression data by Western blot analysis, a loading control protein is required. Loading controls provide a means to ensure equal protein loading across wells, as well as a reference point for data normalization.



Prior to western blot analysis, the total amount of sample protein is measured using a biochemical assay (e.g. Bradford, BCA, etc.). Total protein measurements are then used to load equivalent amounts of protein across all wells of the gel. Equal protein loading is required to make accurate comparisons of target protein expression data. Despite experimental measures to prevent unequal loading, subsequent protocol steps, including uneven transfer from gel to membrane, can affect total sample protein. To verify equal loading of the gel and even transfer from gel to membrane, a second control is often required.

A LOADING CONTROL PROTEIN ALLOWS COMPARISON OF TARGET PROTEIN LEVELS ACROSS MULTIPLE SAMPLES

A loading control protein confirms equal sample loading and gel-to-membrane transfer. Following target protein detection, a loading control protein is detected and its expression is used to mathematically compensate for sample-to-sample variation. Normalized expression is then compared across samples to confirm that changes in protein of interest expression represent real differences and are not the result of differences in total protein abundance from sample-to-sample.

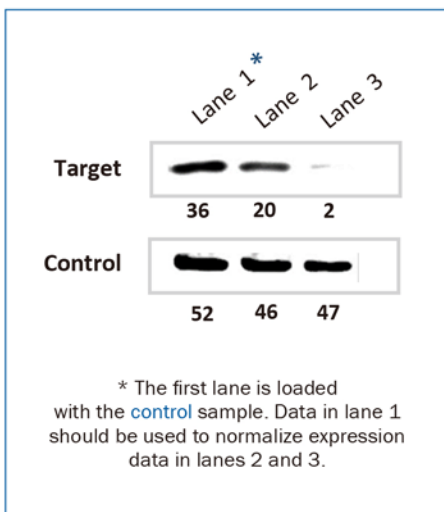
How to Normalize Your Western Blot Data

Normalizing Western Blot Data

Data normalization is required to accurately compare target protein expression across multiple samples in Western blot analysis. To normalize target protein expression, the band intensity of each sample is determined by densitometry. Next, the intensity of the target protein is divided by the intensity of the loading control protein. This calculation adjusts the expression of the protein of interest to a common scale and reduces the impact of sample-to-sample variation. Relative target protein expression can then be compared across all lanes to assess changes in target protein expression across samples.

Step 1

Determine the intensity of each band using densitometry software



Step 2

Normalize the target protein levels to the loading control protein

NORMALIZED DENSITY =

$$\left(\text{Target Protein} \right) \times \frac{\text{Loading Control}^* (\text{lane 1})}{\text{Loading Control} (\text{each lane})}$$

LANE 1 = $36 \times \frac{52}{52} = 36^*$

LANE 2 = $20 \times \frac{52}{46} = 22.6$

LANE 3 = $2 \times \frac{52}{47} = 2.2$

Step 3

Compare relative target protein levels across samples

FOLD DIFFERENCE =

$$\frac{\text{Normalized Density (each lane)}}{\text{Normalized Density}^* (\text{lane 1})}$$

LANE 1 = $\frac{36}{36} = 1.0^*$

LANE 2 = $\frac{22.6}{36} = 0.63$

LANE 3 = $\frac{2.2}{36} = 0.06$

Normalizing Western Blot Data: After band intensity is determined by densitometry software, the loading control protein is used to normalize target protein expression. To normalize expression, multiply the density of the target protein in each lane by the ratio of loading control density from the control sample (lane 1) to the loading control density of other lanes. The fold change can then be calculated by dividing the normalized expression from each lane by the normalized expression of the control sample in lane 1.

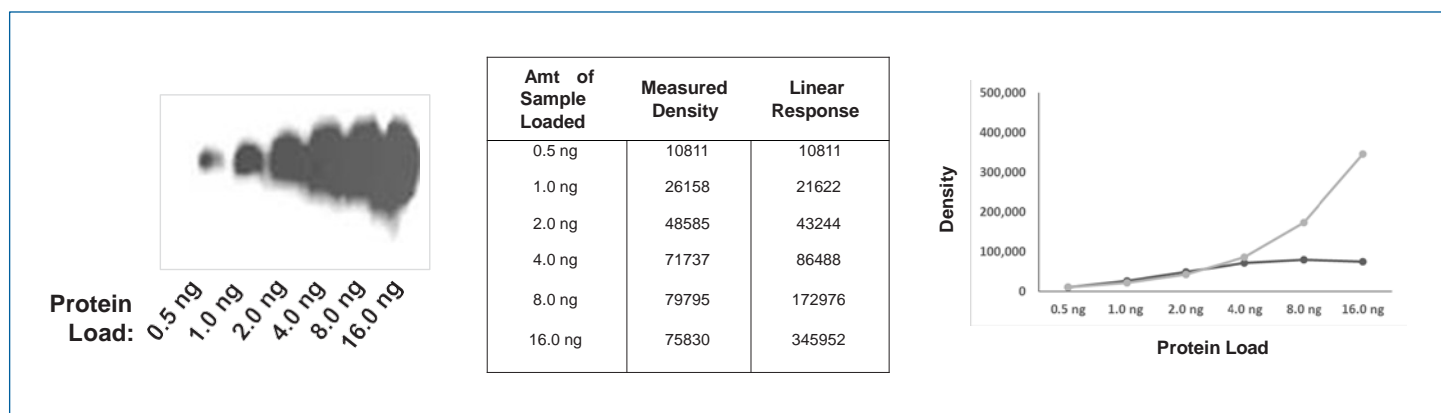
Note: Expression data determined by Western blot analysis is semi-quantitative. Protein abundance is expressed relative to the loading control protein and does not indicate the absolute amount of target protein in your sample.

What to Consider When Using a Loading Control Antibody

Detection Range and Gel Overloading

An ideal loading control demonstrates a linear response over a wide range of sample concentrations. This means the intensity of the loading control protein should increase in equal proportion with sample concentration. For accurate normalization of Western blot data, both the protein of interest and loading control protein should fall within the linear range of detection. Because many target proteins are expressed at low abundance relative to loading control proteins, large amounts of total protein are often loaded on the gel to accurately detect the target protein. However, loading high amounts of total protein often places the loading control outside of the linear range of detection. When this happens, the expression of the loading control protein is no longer proportional to the amount of total protein present in the sample. Deceptively, overloaded gels often give the appearance of uniform loading control band densities across all lanes. However, this uniformity is not due to equal protein loading, but rather samples exceeding the transfer capacity of the membrane or falling outside the linear range of detection.

The optimal amount of total protein to load on the gel should be determined to ensure the accurate quantification of both the target and loading control proteins. To determine the linear range of your samples, create a standard curve using serial dilutions of your sample. The standard curve can then be used to identify the saturation point of the assay and the total amount of starting protein required.



Determining Linear Range: Comparison of the densitometry readings of each sample to the linear response indicates the signal becomes saturated above 4.0 ng. Above 4.0 ng, the band intensity is no longer proportional to total protein abundance: the signal falls outside the linear range of detection. These data determine the optimal total protein load to be 4.0 ng or less for accurate quantification of protein expression.

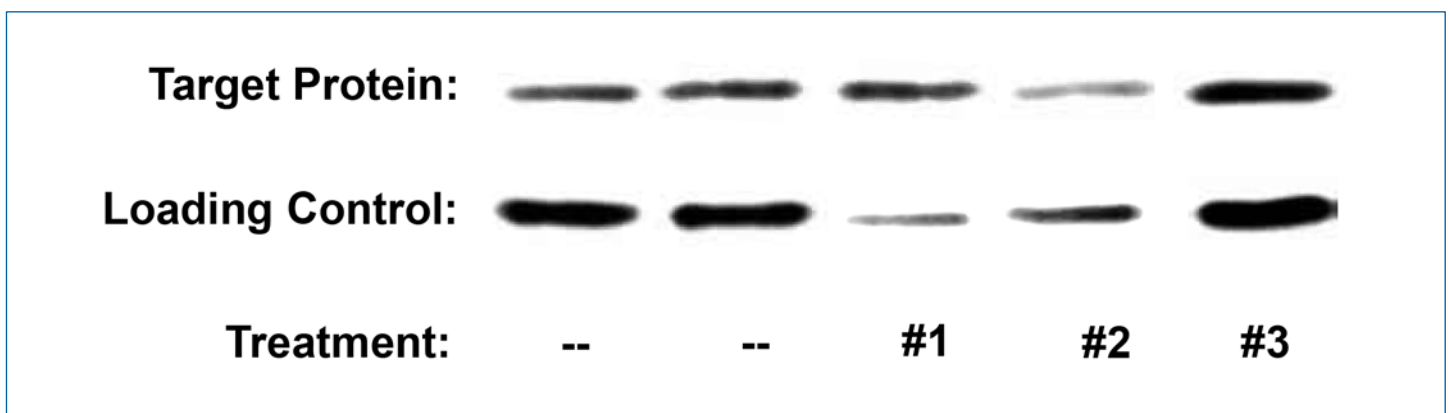
Note: The linear range of detection should be determined for both the target protein and loading control protein. This is to determine the optimal amount of total protein to load to ensure accurate detection of both proteins. This can be performed by running duplicate samples (e.g. ABCD, ABCD) and cutting the blot vertically to separate the samples. One blot is then used to determine the linear range of the loading control, while the other is used for the target protein.

What to Consider When Using a Loading Control Antibody

Concentration Dependence and Expression Consistency

Accurate normalization requires that the protein of interest and loading control protein to vary equally with sample concentration. If unequal variation of one measurement occurs independent of concentration, then normalization fails to bring target protein expression into accurate proportion with the loading control protein. In this case, expression differences between samples can be the result of differences in total protein or loading control abundance and not the result of real differences in target protein expression. Thus, it is crucial to choose a loading control protein with ubiquitous and unchanging expression.

For example, hypoxic conditions have been shown to upregulate the expression of GAPDH, a common whole cell loading control protein. For this reason, GAPDH is not recommended as a loading control protein in oxygen related studies since its expression is subject to change as a result of experimental conditions. In addition to normalizing samples via loading controls, minimizing sources of experimental variability will improve reproducibility, resulting in more accurate quantification of target protein expression by Western blot.

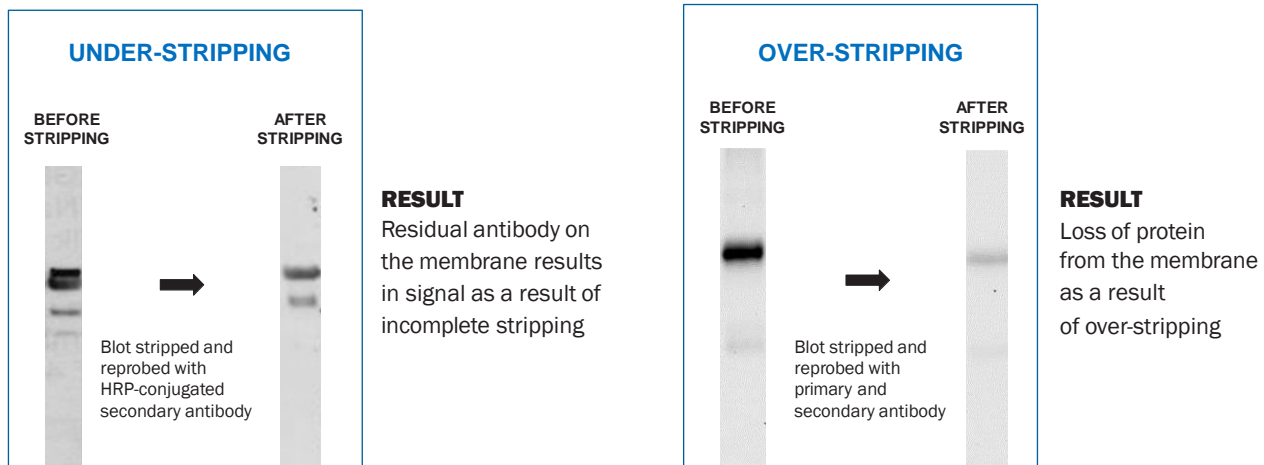


The Expression Level of the Loading Control Protein Changes in Response to Experimental Treatment. If large changes in loading control expression are observed despite loading equal amounts of sample on the gel, then a different loading control protein should be considered. This is exemplified in the image shown above where treatments 1, 2, and 3 result in varying levels of expression of the loading control. Changes in loading control expression due to experimental conditions will affect normalization and result in inaccurate data analysis. Choose a loading control protein whose expression remains constant across all experimental conditions.

What to Consider When Using a Loading Control Antibody

Stripping and Reprobing

A common method to determine expression of a loading control protein is stripping and reprobing. Following detection of the protein of interest, the membrane is stripped of antibodies, then reprobed with a loading control specific antibody. In this method, it is important that all antibody is stripped from the membrane to ensure generation of accurate loading control expression data. Residual antibody on the blot may result in artifacts which may impact loading control expression data. Moreover, blot stripping can result in significant loss of sample protein from the membrane, which could impact expression data and normalization calculations. After stripping, but before reprobing, the membrane should be tested to verify the first set of antibodies was completely removed. To confirm complete antibody removal, the membrane should be washed, blocked, and then stained with secondary antibody. If stripping was complete, then the secondary antibody will remain unbound and produce no detection signal. If signal is detected, then the stripping conditions must be optimized. Often small changes in incubation time or temperature are sufficient to remove the remaining antibody. However, a stronger stripping buffer may be necessary in certain cases for antibodies which are difficult to remove.



Insufficient Stripping: A primary antibody was used to probe expression of a target protein.

Following target protein detection, the blot was stripped to remove antibodies, and then reprobed with HRP-conjugated secondary antibody to confirm complete stripping. The signal generated post-stripping demonstrates the stripping process was incomplete. Residual antibody left on the membrane bound the HRP conjugated secondary antibody, producing chemiluminescent signal.

Excessive Stripping: A primary antibody was used to probe expression of a target protein. Following target protein detection, the blot was stripped to remove antibodies, and then reprobed with the same target protein-specific antibody. The lower signal generated post-stripping demonstrates protein sample was stripped from the membrane. Unequal stripping of protein sample across lanes can result in inaccurate normalization. Special care should be taken when stripping blots consisting of samples from multiple sample types.

Note: See page 7 for a general stripping and reprobing protocol.

Summary: Attributes of an Effective Loading Control Protein

Tips for Choosing the Best Loading Control:

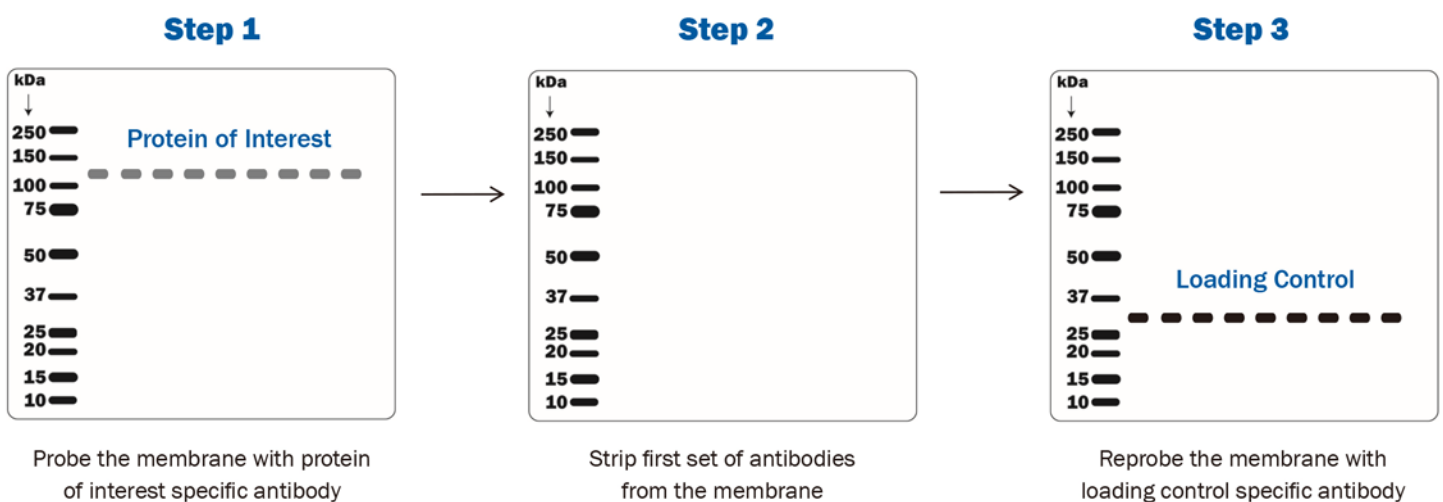
Detection Size	Choose a loading control that can be distinguished in MW from your protein of interest.	e.g. If probing for the expression of the autophagosome marker, LC3B (~14-17 kDa), avoid using loading controls such as Cofilin or Cyclophilin B (~20 kDa), since their molecular weight is similar to the protein of interest.
Expression Consistency	Choose a loading control that is constitutively and ubiquitously expressed. The expression should not change with experimental treatment, cell type, or tissue type.	e.g. Cellular stress has been demonstrated to upregulate HSP60 expression. Thus, HSP60 should be avoided as a control in cellular stress studies, as its expression may differ in stressed versus control samples.
Expression Level	Choose a loading control that is highly expressed in your sample. Common loading controls are highly expressed genes required for basic cellular processes and vitality, also known as housekeeping genes.	e.g. The nuclear loading control, PCNA, is expressed during DNA S phase. Thus, it is not recommended as a control for non-proliferating cells as its expression is down-regulated in non-proliferating cells.

Probing Loading Control Expression

Method 1: Stripping and Reprobing

PROTOCOL

Stripping refers to the method of removing primary and secondary antibodies from a western blot membrane. A single blot can be analyzed sequentially with multiple antibodies by stripping one antibody from the blot and subsequently incubating with another antibody. Membrane stripping is often performed as a means to assess the expression of loading control proteins. Following detection of the protein of interest, a membrane is stripped and reprobed with a loading control antibody to ensure equal expression of the loading control. This practice is also useful when sample is limited.



Protocol

Stripping with heat and detergent protocol

1. In a fume hood, agitate the blot in **stripping buffer #1** for 30 minutes at 50 °C
2. Agitate the blot in **1X PBS** for 10 minutes at room temperature. Repeat with fresh buffer.
3. Proceed to the blocking step of the immunoblotting protocol to reprobe the blot with another antibody.

Stripping Buffer #1

Components:

- 100 mM 2-mercaptoethanol
- 2% SDS
- 62.5 mM Tris-HCl
- Adjust pH to 6.7

Protocol

Stripping with acidic pH protocol

1. Agitate the blot in **stripping buffer #2** for 30 minutes at 50 °C
2. Agitate the blot in **1X PBS** for 10 minutes at room temperature. Repeat with fresh buffer.
3. Proceed to the blocking step of the immunoblotting protocol to reprobe the blot with another antibody.

Stripping Buffer #2

Components:

- 25 mM glycine HCl
- 1% SDS
- Adjust pH to 2

Probing Loading Control Expression

Method 2: Cutting the Membrane

Another common method to probe loading control expression is to cut the membrane horizontally following transfer. Using this method, protein of interest and loading control proteins can be separated and probed independently with different antibodies. The method requires a significant difference in size (kDa) between the protein of interest and loading control. Prestained markers should be used to determine molecular weight and the right location to cut the membrane.

Protocol

Cutting the membrane to probe the expression of two proteins at the same time

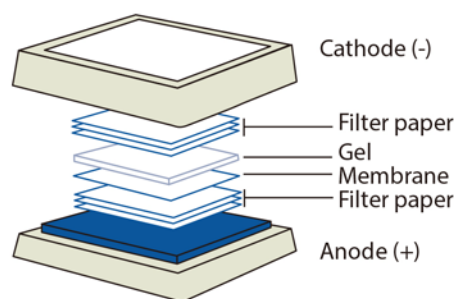
1. After transfer from gel to membrane, carefully disassemble the transfer stack and rinse the membrane briefly in distilled water or 1X TBST.
2. Gently mark the molecular weight ladder bands for size detection.
3. Following membrane blocking, use scissors or a sharp scalpel to horizontally cut the membrane taking care to avoid cutting too close to the proteins of interest. Use rounded tweezers when handling the membrane.

Considerations

- This method requires a significant difference in size (kDa) between the protein of interest and loading control protein to ensure clean separation.
- A prestained marker or ladder should be used to determine the molecular weight and correct location to cut the membrane.
- Prior to using this method, every antibody should be tested on the whole blot to determine antibody specificity.

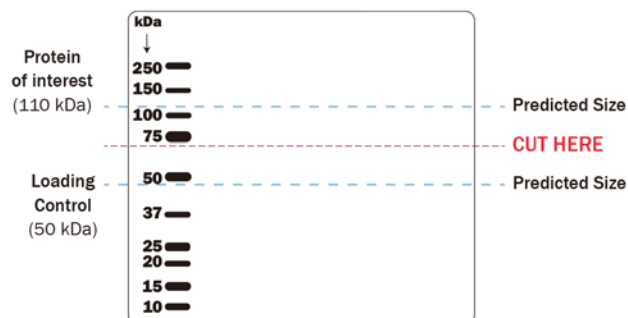
Step 1

Transfer samples from gel to membrane



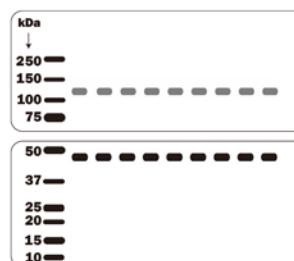
Step 2

Cut the membrane horizontally to separate the loading control and protein of interest



Step 3

Probe one membrane for loading control expression and the other for protein of interest expression



Blot 1

Probe this blot for the protein of interest

Blot 2

Probe this blot for the loading control

Determining the Appropriate Sample Load for Western Blots

Reliable western blot data can be generated only when the proper sample amount of protein is used. Loading too much protein leads to signal saturation in western blots, yet too little produces weak signals. This protocol describes an assay development experiment to determine the appropriate protein load for target and control detection prior to performing the actual western blot experiment.

Protocol:

1. Select a typical sample in which an average amount of target and loading control proteins are present.
2. Load 1, 5, 10, 20, and 50 μg of protein sample to two sets of lanes. Separate the two sets with a prestained protein standard.
3. After transfer, cut the membrane along the protein standards lane and split the membrane into 2 blots.
4. Apply the target protein primary antibody to one blot and the loading control protein antibody to the other.
5. Add substrate to develop the chemiluminescent signal and capture the signals using a CCD camera-based imager.
6. Using the software, read the target protein intensity in each lane and plot the intensity against the protein load. Determine the linear dynamic range under the specific experimental conditions. Select the protein load that gives the quantitative reading in the linear dynamic range for the real experiment.
7. Repeat step 6 to determine the appropriate protein load for quantitative detection of the loading control protein.

Note:

- Procedure details are omitted for steps such as protein sample prep, gel load, gel electrophoresis, transfer, antibody incubation, etc. For details, please refer to the *General Protocol for Western Blotting*, bulletin 6376
- Once the experimental setup and conditions are established for the assay, do not change the sample load, transfer method, transfer time, antibody dilution, antibody incubation time, or temperature in subsequent experiments, as these factors may significantly change the detection signals

