

NB100-91713 Protocol

Western Blot Protocol for Claudin 5 Antibody (NB100-91713)

Western Blot Protocol:

1. Prepare cell / tissue lysate(s) (see cell / tissue lysis protocol)
2. Make up lysates to a total volume of 10-20 ul with 4x SDS sample buffer (see recipes) containing a reducing agent (either 10% B-ME or 0.3M DTT in the 4x SB).
3. Prepare SDS-PAGE gel (see SDS-PAGE gel pouring).
4. Place gel in running chamber and fill with 1 x SDS Running Buffer. Use 10x stock (see recipes).
5. Heat samples at 95-100C for 5 minutes.
6. Load 5ul of a control pre-stained protein ladder to track sample migration in the first lane of the gel. This is also useful for the transfer step to verify the procedure is successful. These are widely available for most suppliers (Invitrogen, Biorad, Fermentas, etc).
7. Load boiled samples. Electrophorese at constant 100V until the blue bromophenol dye reach the bottom of the gel.

Protein Transfer:

1. Place gel and blotting pads in transfer buffer solution.
2. Transfer gel to filter paper wetted in transfer buffer solution.
3. Assemble transfer sandwich by orientating cathode, filter paper, gel, membrane (nitrocellulose / PVDF), filter paper, anode so protein transfer goes in the direction of cathode to anode. These are typically coloured black (cathode) and red (anode). The choice of membrane (nitrocellulose / PVDF) depends on the protein studied and the antibodies used. Generally you will get the use instructions you need through antibody guidelines. PVDF is particularly useful if you are working with small or highly charged proteins. Remember to make PVDF soaked in methanol for 5 minutes prior to use.
4. Electrophorese for 90 minutes at 100V or overnight at 4 degrees C at 20V. Proceed electrophoresis on ice or in a cold room as the transfer generates a lot of heat!
5. Check membrane for transfer of control protein ladder. If using nitrocellulose, sample transfer and relative concentration can be assessed by staining the membrane with Ponceau S solution (0.1% ponceau, 5% acetic acid). This is a reversible stain which is removed by a simple wash in TBST.

Protein Labeling:

1. Incubate membrane in blocking solution for 1 hour at room temperature or at 4 degrees C overnight with shaking. The blocking solution is normally composed of 5% non-fat milk in TBS-T, and some antibodies require BSA in place of milk. This is normally clear in the manufacturers instructions for the antibody for testing. To make TBS-T, using 10 x TBS, add Tween-20 to a final concentration of 0.05%.
2. Incubate primary antibody overnight or at room temperature for 2 hours prepared in 5% milk/TBST.
3. Remove antibody solution and wash membrane three times for 5-15 minutes in TBST.
4. Incubate membrane with an appropriate secondary antibody (peroxidase conjugated) for 1 hour at room temperature in 5% milk/TBST. For example if your primary antibody was raised in rabbit, a goat anti-rabbit HRP should be used as a secondary antibody.
5. Remove antibody solution and wash membrane three times for 5-15 minutes in TBST.

Protein Detection:

1. There are numerous commercial available chemiluminescence reagents (Amersham, Pierce, Invitrogen) and a range of sensitivities of detection levels is included. These typically take the form of two solutions which are combined and then incubated immediately on the membrane for 1 - 5 minutes.
2. Make membrane exposed to X-ray film for 1 minute to 1 hour, depending on protein signal and chemiluminescence method.