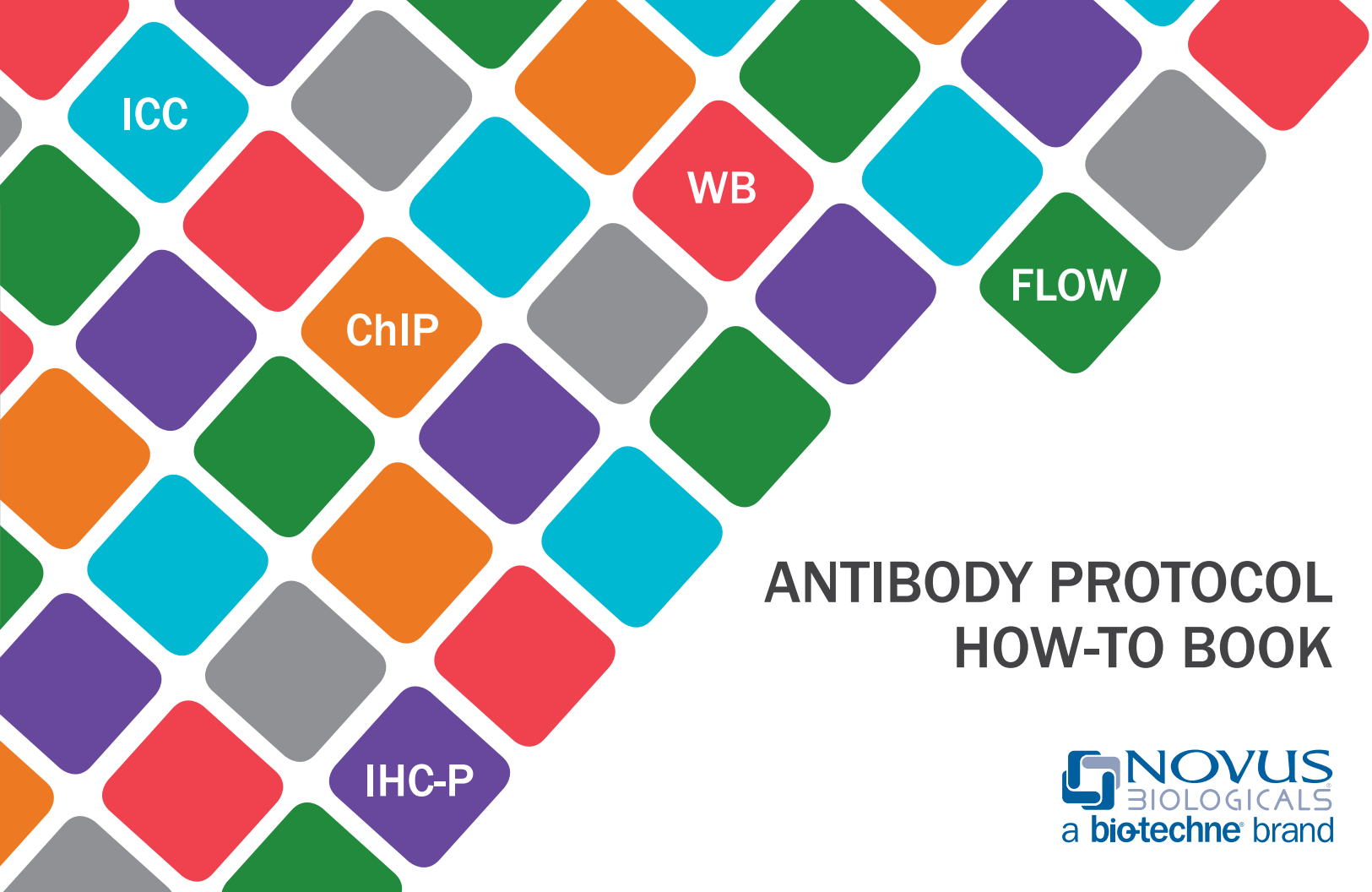

ANTIBODY PROTOCOL HOW-TO BOOK

NOVUS BIOLOGICALS
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PROTOCOL FOR SDS PAGE & WESTERN BLOTTING

SDS-PAGE

1. Prepare or purchase an appropriate percentage polyacrylamide gel to best resolve your protein of interest based on its estimated molecular weight.

> **TIP** *Low molecular weight proteins are best resolved on high percentage gels (15-20%), whereas large proteins 200+ kDa will require 4-6% gels for sufficient resolution. If your protein of interest has multiple isoforms of low to high molecular sizes, gradient gels would be your best option for achieving efficient separation of the proteins. Precast gels with different formats and prepared electrophoresis buffer stocks are available from a number of manufacturers to make SDS-PAGE more reproducible and convenient.*

2. In the first well, load a protein molecular weight standard. Novus Scientists typically load a pre-stained standard to monitor the progress of the run. Optional: If you wish to visualize the molecular weight markers on X-ray film or on a digital photograph, we recommend running 1-4 µL of an all blue prestained molecular weight marker and use the mouse monoclonal Anti-Blue Marker Antibody (6F4-F6) (NBP2-33376) in conjunction with your primary antibody. If HRP is the method of detection for your Western blot, a convenient Anti-Blue Marker Antibody (6F4F6) (NBP2-33376H) - HRP conjugate is also available.

3. Load protein lysates (10-50 µg) prepared in 1X Laemmli sample buffer into the remaining wells of the gel.

> **TIP** *For preparing reduced protein samples, a reducing agent such as DTT, βME or an equivalent must be added at an appropriate concentration. Alternatively, non-reduced protein samples can be prepared in the absence of a reducing agent.*

4. Cover the gel with 1X running buffer as instructed by the manufacturer.

5. Run the gel as recommended by the manufacturer. Voltage may vary depending on research needs.

PROTEIN TRANSFER

1. Prepare all membranes, filter papers and buffers before starting the protein transfer procedure.

> **TIP** *For proteins with a molecular weight less than 30 kDa, use 0.2 µm PVDF membrane, otherwise 0.45 µm PVDF is recommended. Wet the PVDF membrane in methanol and then soak it in distilled water followed by transfer buffer. Handle the PVDF membrane carefully to avoid marring or scratching the surface, as this can increase background during immunoblotting.*

2. After electrophoresis, remove the gels from the plastic casings and equilibrate in transfer buffer for 10 minutes.

3. Carefully assemble the transfer cassette according to the illustration below. Sequentially assemble the layers of the transfer sandwich, gently removing any air bubbles with a roller or pipette.

TIP

Make sure no air bubbles are trapped between the gel and the membrane as they will impede the transfer of protein to the membrane.

4. Perform the transfer of proteins from the gel to the membrane according to the manufacturer's recommendations.

TIP

Low molecular weight proteins <30 kDa require a short transfer time to avoid pulling the protein through the membrane.

5. Once the transfer is complete, gently disassemble the transfer cassette and move the membrane to a clean surface to mark the molecular weight bands with a China marker or pencil. If all blue molecular weight markers have been used, there is no need to mark the bands, as they will be visible on your X-ray film or digital image when used in conjugation with the Anti-Blue Marker antibody.

TIP

At this point the membrane can be stained with a reversible protein stain like 0.1% Ponceau S for 1 or 2 minutes to confirm a successful protein transfer. Rinsing the membrane in distilled water will remove the excess stain and reveal the protein in each lane. Additional marking of the lanes is possible at this time.

6. Remove the excess stain by soaking the membrane in 1X TBST and then transfer to a blocking solution of choice. Novus Scientists typically block their membranes in 5% non-fat milk in TBST or in 5% BSA in TBST for 1 hour with gentle shaking.

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IMMUNOBLOTTING

1. Prepare a working dilution of the primary antibody in 1% non-fat milk -TBST or 1% BSA -TBST. Typical starting points for dilutions are 1-2 µg/mL (check the product datasheet). Each antibody should be optimized as required. Incubate the membrane in the diluted primary antibody for 1 hour at room temperature or overnight at 4 °C with gentle agitation.

   Optional: To image the molecular weight markers along with your protein of interest, you can add 1 µg/mL of the Anti-Blue Marker antibody into your primary antibody solution. This antibody will only bind to the blue dye on each molecular weight marker and will not cross react with your protein lysates.

TIP *Make sure the membrane is completely submerged in the antibody solution to prevent it from drying out.*

2. Wash the membrane in 1X TBST three times for 10 minutes each with agitation.

3. Incubate the membrane in an appropriately diluted secondary antibody solution prepared in the same blocking buffer as the primary antibody. Incubate the membrane for at least 1 hour at room temperature.

   Optional: To detect the molecular weight markers at the same time as your protein of interest, add an anti-mouse HRP conjugated antibody into the secondary antibody solution (if not already anti-mouse), or add *1 µg/mL of* the HRP conjugated Anti-Blue Marker antibody to the secondary antibody solution.

4. Wash the membrane in 1X TBST three times for 10 minutes each with agitation.

5. Prepare the chemiluminescent substrate just before use according to the manufacturer's instructions.

6. Incubate the membrane in the substrate as instructed by the manufacturer.

7. Sandwich the membrane between layers of plastic (ex. sheet protectors) and expose to X-ray film or digitally capture images.

PROTOCOL FOR IMMUNOCYTOCHEMISTRY (ICC)

CELL CULTURE

TIP *Keep all mechanical manipulations to a minimum to avoid compromising the quality of the final cell images. Always treat the cells and coverslips gently, never let them dry out and avoid dropping solutions directly on the cells.*

1. Seed the cells on sterile glass coverslips. Some cell types may require growth on poly-lysine or other treated coverslips for proper adhesion.

TIP *Coverslips can be sterilized by dipping in ethanol and flaming them or by placing them into tissue culture dishes and exposing to UV radiation (present in most tissue culture hoods). Several small circular coverslips can be seeded in a single dish to reduce the number of dishes in your incubator.*

2. Grow the cells to semi-confluency.

FIXATION

1. Aspirate the culture medium from the dish or remove each coverslip as required with tweezers, and gently wash with PBS at room temperature.
2. Incubate the coverslips in freshly prepared 4% paraformaldehyde - PBS at room temperature for 10 minutes. Alternatively, the cells can be fixed in -20°C methanol for 10 minutes.

TIP *Alternative fixations should be tested and compared to determine which is best at preserving the structure and epitope of the protein of interest within the cell.*

3. Wash the coverslips of fixative in PBS for 2 minutes.

PERMEABILIZATION

1. Incubate the coverslips in 0.5% Triton X-100 in PBS at room temperature for 5 minutes. The final working percentage of different detergents (ex. digitonin, Tween-20) should be explored to find the optimal conditions to best preserve the cell structure and protein of interest.
2. Wash the coverslips of the permeablization buffer by incubating in PBS for 5 minutes.

BLOCKING

1. To reduce the background fluorescence, block the coverslips in 1-5% normal serum or BSA prepared in PBS for 1 hour at room temperature. The normal serum block should be of the same species in which the secondary antibody has been raised.

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ANTIBODY INCUBATION

1. Prepare primary antibody dilutions for ICC in 1% normal serum or BSA. Typical working concentrations of antibodies may range from 5-20 µg/mL, however each antibody may require optimization to obtain an appropriate signal.

> **TIP** *Directly conjugated antibodies eliminate the need for secondary antibodies (and hence reduce background) and shorten the time required to complete the ICC procedure.*

2. Incubate the coverslips with the primary antibody dilution for 1 hour at room temperature (37 °C is optional), or overnight at 4 °C.

> **TIP** *Make sure the coverslips do not dry out during the antibody incubations.*

3. Wash the coverslips gently in PBS three times for 5 minutes each. Additional or longer washes maybe required if excessive background remains.

4. Prepare an appropriate dilution of fluorochrome-conjugated secondary antibody in 1% normal serum or BSA. Typical starting dilutions range from 1-2 µg/µL, however optimization may be required for the best image.

5. Incubate the coverslips in the secondary antibody dilution for 1 hour at room temperature in a dark environment.

6. Wash the coverslips gently in PBS for three times 5 minutes each. Additional or longer washes may be required if excessive background remains.

> **TIP** *Double labeling experiments (2 different antibodies from 2 different species) are best carried out by sequentially incubating with the primary and secondary antibodies. Make sure the primary antibodies are from differing host species, and if this option is not available, directly conjugated primary antibodies may be employed. The use of fluorescently labeled phallodin is a good alternative to immunostaining actin as a reference marker in ICC.*

MOUNTING AND IMAGING

1. When all the necessary washing steps have been completed, the coverslips can be counter stained with DAPI or Hoechst (1-10 µg/mL) to stain the nuclei.

2. Invert the coverslip onto a glass slip with a drop of mounting media containing a fluorescence antifade agent.

3. Carefully remove the excess mounting media if necessary and seal as required with nail polish.

> **TIP** *Some mounting media solutions have DAPI already added and will harden after exposure to air, eliminating the need to seal the edges of the coverslip.*

4. Examine the cells under a fluorescence microscope and image as required.

> **TIP** *Avoid long exposure to the excitation wavelength of the fluorochrome to prevent photobleaching.*

ADDITIONAL TIPS

Excessive background

If too much background fluorescence is present in your samples try the following suggestions:

1. Increase the percentage of blocking agents used in the blocking step and in the primary and secondary antibody dilution buffers. Increase the time of blocking and/or reduce the time of antibody incubations.

2. Titrate the amount of primary and secondary antibody used in all the incubations.

3. Some cells may contain high amounts of your protein of interest in the cytoplasm (ex. tubulin and actin). If antibodies to these proteins are used in your ICC procedure, it can result in excesive staining. The level of cytoplasmic staining can be reduced by a brief extraction of the cytoplasm in 0.1% Triton X-100 prior to fixation.

4. Try quenching the fixation agent to eliminate any free aldehyde groups which could non-specifically bind antibody. Quench formaldehyde with 0.1M Tris or Glycine buffer and quench glutaraldehyde with 0.1% sodium borohydride.

DROPPED A COVERSLIP

If a coverslip of cells is accidently dropped and “cell side up” orientation is lost, you can still salvage the experiment by carefully picking up the coverslip with tweezers and gently scraping one surface with a pipette tip to see if any cells are visibly removed.

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PROTOCOL FOR CHROMATIN IMMUNOPRECIPITATION (ChIP)

This is an abbreviated protocol to highlight the main points of ChIP using the ChromataChIP kit (NBP1-71709). A more detailed protocol can be found on our website www.novusbio.com or in online datasheets of ChromataChIP kits.

CHROMATIN IMMUNOPRECIPITATION

1. Dilute each sheared chromatin sample 1:10 with IP dilution buffer containing protease inhibitors. Save an undiluted input sample at 4 °C for the reverse crosslinking step.

2. Add 2 µg of the antibody of interest to each of the diluted samples. We also recommend running the following controls:

   - Positive Control Antibody: An antibody known to work well in ChIP with your primer set.
   - Negative Control Antibody: A non-specific or isotype control antibody.

3. Rotate the tubes overnight at 4 °C.

4. Add 25 µL of washed and suspended Protein A/G magnetic beads to each IP sample.

5. Rotate the tube for 1 hour at 4 °C.

   TIP *Pulse centrifuge the tubes to remove any material from the caps.*

6. Pellet the magnetic beads with a magnetic separator and remove the supernatant. Add 500 µL of cold wash buffer 1 and rotate for 5 minutes at 4 °C. Pellet the magnetic beads with a magnetic separator and discard supernatant.

7. Add 500 µL cold wash buffer 2 and rotate for 5 minutes at 4 °C. Pellet the magnetic beads with a magnetic separator and discard supernatant.

8. Add 500 µL cold wash buffer 3 and rotate for 5 minutes at 4 °C. Pellet the magnetic beads with a magnetic separator and discard supernatant.

9. Add 500 µL cold wash buffer 4 and rotate for 5 minutes at 4 °C. Pellet the magnetic beads with a magnetic separator and discard supernatant.

10. Elute the antibody-protein complex by adding 200 µL of IP elution buffer and rotate at room temperature for 15 minutes. Pellet the magnetic beads with a magnetic separator and keep the supernatant.

REVERSE CROSS-LINKING AND DNA PURIFICATION

1. Add 160 µL of IP elution buffer and 8 µL of 5M Nacl to the in put control sample that did not go through the IP and wash steps.

2. Incubate the sample at 95 °C for 15 minutes.

> **TIP** *Make use of a thermocycler to conveniently hold to the sample at this temperature.*

Optional: After the completion of Step 1, add 2 µL of Proteinase K to the samples and incubate at 62 °C for 2 hours to overnight.

3. Incubate at 95 °C for 10 minutes to deactivate Proteinase K.

4. The DNA samples can now be purified with spin columns or by phenol/chloroform extraction.

DNA PCR AMPLIFICATION

1. Purified DNA can now be measured by PCR. Quantitative real-time PCR is the preferred method of amplification due to its sensitivity. The method described below uses a 2X SYBR green reaction mix containing all necessary components (dNTPs, DNA polymerase, buffers). Run each PCR reaction in triplicate for each sample. Samples to be assayed include: immunoprecipitated sample from the antibody of interest, the positive control sample, the negative control sample, and the purified input control. Forward and reverse primers are also needed for each region that will be amplified. Each sample will use 2 µl of purified DNA as template.

2. For the input control fraction only, dilute the template to 1% of the original concentration (1:100 dilution). All other samples are left undiluted.

3. Start by creating a PCR master mix for each primer set and dispense the mix into each reaction well, then add the template. Your positive control primer set master mix should contain the following:

   - 7 µL of DNase free water
   - 1 µL of 10 µM primers (final concentration 0.5 µM)
   - 10 µL of 2x SYBR reaction mix
   - 2 µL of purified DNA template

> **TIP** *Use PCR plates when you have a large number of reactions.*

4. Perform real time PCR according to manufacturer's recommendations for the SYBR reaction mix.

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PROTOCOL FOR IMMUNOHISTOCHEMISTRY (IHC), PARAFFIN

PREPARATION - PERFUSION AND PARAFFIN EMBEDDING

1. Fix the tissue of interest by immersing it in 10% neutral buffered formalin (4% PFA-PBS) for 4-24 hours at room temperature. Fixation time and temperature depends on tissue type/size. After fixation, wash the tissues thoroughly in PBS.

> **TIP** *Tissues at this stage may be stored in cold PBS until further processing (maximum of 2-3 days). For longer storage, the tissues must be kept in 70% ethanol.*

2. Dehydrate by moving tissue through the following solutions twice for 30 minutes each:

    a. 70% Ethanol
    b. 95% Ethanol
    c. 100% Ethanol
    d. Xylene

3. Embed the tissue in molten paraffin. After the paraffin solidifies keep the blocks at 4°C until sectioning.

4. Use a microtome to cut the embedded tissue into 4-6 µm thick sections and float them in a 50°C water bath containing distilled water.

5. Mount sections onto gelatin or poly-L-lysine coated slides and allow them to dry overnight. Slides can be safely stored at room temperature until ready for staining.

IMMUNOFLUORESCENT STAINING

1. Deparaffinize and rehydrate by immersing the slides through the following solutions:

    a. Xylene: three times for 5 minutes each
    b. 100% Ethanol: twice for 5 minutes each
    c. 95% Ethanol: 5 minutes
    d. 70% Ethanol: 5 minutes
    e. 50% Ethanol: 5 minutes
    f. Distilled water: 5 minutes. Do not let the tissue dry from this point on.

> **TIP** *Before moving to alcohol grades step, make sure to completely deparaffinize the sections. If the sections still have traces of wax, an additional immersion of 5 minutes in Xylene may be employed.*

2. Draw a circle on the slide around the tissue with a hydrophobic barrier pen or use rubber cement.

TIP *Wipe the area around the section with tissue paper before drawing circle. This will help eliminate the chances of hydrophobic ink's dispersal over the sections.*

3. For antigen retrieval using microwave, bring the slides to a boil in 10 mM sodium citrate buffer (pH 6.0) and then maintain at a sub-boiling temperature for 10 minutes. Then, let it cool on bench-top for about 30 minutes and wash the sections by immersing them in distilled water for 5 minutes.

4. To permeabilize the tissue/cells, wash the sections twice for 10 minutes with 1% animal serum in PBS with 0.4% Triton X-100 (PBS-T). The species of the animal serum is dependent on the host of your secondary antibody (e.g. when using a goat anti-mouse secondary, use goat serum).

TIP *Hydrogen peroxide is a light sensitive chemical, therefore, this step should be (preferably) performed away from direct exposure to light.*

5. Block any non-specific binding by incubating the tissue sections with 5% animal serum in PBS-T for 30 minutes at room temperature.

TIP *Novus offers NBP1-27946 and NB100-63473 to block the mouse Fc gamma III/II Receptors (FcRs), to block rat FcRs, we have NBP2-42229.*

6. Add primary antibody diluted in 1% animal serum PBS-T and incubate at room temperature for 1-2 hours followed by overnight at 4 °C in humidified chamber. Use the recommended dilution of the antibody specified on the datasheet. If not specified, the typical starting dilution can be 2-5 µg/ml.

TIP *In addition to positive controls, make sure to include the following negative controls in your assay – secondary antibody only (no primary), no primary or secondary antibody.*

7. Wash sections twice with 1% serum PBS-T for 10 minutes each.

8. Dilute secondary antibody in 1% serum PBS-T and incubate with sections at room temperature for 1-2 hours. Use the recommended dilution of the antibody as specified on the data sheet.

9. Wash sections twice with 1% serum PBS-T for 10 minutes each.

Optional: Double/Nuclear labeling

a. Double labeling: If using a second primary antibody and appropriately matched secondary, repeat steps 5-8.

b. Nuclear labeling: After application of all primary antibodies, DNA binding dyes such as DAPI can be applied, After incubation, wash once for 5 minutes with PBS.

10. Tap off excess wash and apply one drop of anti-fade mounting medium to the slide. Place a coverslip on the tissue sections. Circle the edges of the coverslip with clear fingernail polish to prevent the cells from drying. Allow nail polish to air dry.

11. Slides may now be examined under a microscope with the appropriate fluorescent filter sets. Limit the amount of time each slide is exposed to the microscopes light will prolong the signal and prevent photobleading

12. Slides can be stored between -20°C and 4°C in a dark slide box or slide book.

IMMUNOCHROMOGENIC STAINING (ABC METHOD WITH DAB)

1. Deparaffinize and rehydrate by immersing the slides through the following wells:
   a. Xylene: three times for 5 minutes each
   b. 100% Ethanol: twice for 5 minutes each
   c. 95% Ethanol: 5 minutes
   d. 70% Ethanol: 5 minutes
   e. 50% Ethanol: 5 minutes
   f. Distilled water: 5 minutes. Do not let the tissue dry from this point on.

TIP

Before moving to alcohol grades step, make sure to completely deparaffinize the sections. If the sections still have traces of wax, an additional immersion of 5 minutes in Xylene may be employed.

2. Draw a circle on the slide around the tissue with a hydrophobic barrier pen or with rubber cement.

3. For antigen retrieval using microwave, bring the slides to a boil in 10 mM sodium citrate buffer (pH 6.0) and then maintain at a sub-boiling temperature for 10 minutes. Then, let it cool on bench-top for about 30 minutes and wash the sections by immersing them in distilled water for 5 minutes.

4. To block endogenous peroxidase activity, quench the tissue sections with 3.0% hydrogen peroxide in methanol for at least 15 minutes. Afterwards, wash the sections by immersing them in distilled water for 5 minutes.

5. To permeablize the tissue/cells, wash the sections twice for 10 minutes with 1% animal serum in PBS with 0.4% Triton X-100 (PBS-T). The species of the animal serum is dependent on the host of your secondary antibody. (e.g. when using a goat anti-mouse secondary, use goat serum).

6. Block any non-specific binding by incubating the tissue sections with 5% animal serum in PBS-T for 30 minutes at room temperature.

7. Add primary antibody diluted in 1% animal serum PBS-T and incubate at room temperature for 1-2 hours. Then store overnight at 4°C in humidified chamber. Use the recommended dilution of the antibody specified on the datasheet. If not specified, the typical starting dilution can be 2-5 µg/ml.

8. Wash sections twice with 1% serum PBS-T for 10 minutes each.

9. Add a biotinylated secondary antibody and incubate at room temperature for 1 hour. Use the recommended dilution of the antibody specified on the datasheet.

10. Wash sections twice with 1% serum PBS-T for 10 minutes each.

11. Add ABC-HRP reagent and incubate at room temperature for 1 hour. Follow manufacturer's guidelines for reagent preparation.

12. Wash sections twice in PBS for 10 minutes each.

13. Important: DAB is a carcinogen! Always wear gloves and work in a fume hood when working with DAB. Deactivate and clean work area after use according to manufacturer's instructions.

    Prepare a working solution of DAB and apply to tissue sections. Monitor the reaction as the chromogenic reaction turns the epitope sites brown (time of color development may vary from few seconds to 10 minutes). Proceed to the next step when the intensity of the signal is appropriate for imaging.

14. Wash the sections twice in distilled water for 2 minutes each.

15. To counterstain nuclei, use Hematoxylin according to the manufacturer's instructions. Note: If you are using an aqueous chromogen instead of DAB (i.e. AEC, Fast Red, etc.), skip the following dehydration step and mount in aqueous media instead of organic mounting media.

16. Dehydrate tissue sections by moving slides through the following solutions twice for 2 minutes each:

    a. 95% Ethanol
    b. 100% Ethanol
    c. Xylene

17. Add mounting media to slides and top with coverslips. The DAB reaction is permanent and stable and can be analyzed under a brightfield microscope at any time.

PROTOCOL FOR FLOW CYTOMETRY

SAMPLE PREPARATION

1. Grow cells to 50-75% confluency. Flow cytometry requires between 2 x 10^5 and 1 x 10^6 cells for optimal performance.

2. If cells are adherent, harvest gently by washing once with staining buffer and then scraping. Avoid using trypsin as this can disrupt certain epitopes of interest. If enzymatic harvest is required, use Accutase or Collagenase for a less damaging option.

3. Reserve 100 µL for counting, then transfer cell volume into a 50 mL conical tube and centrifuge for 8 minutes at 400 RCF.

   a. Count cells using a hemocytometer and a 1:1 trypan blue exclusion stain to determine cell viability before starting the flow protocol. If cells appear blue, do not proceed.

4. Re-suspend cells to a concentration of 1 x 10^6 cells/mL in staining buffer (NBP2-26247).

5. Aliquot out 1 mL samples in accordance with your experimental samples.

TIP *When cell surface and intracellular staining are required in the same sample, the cell surface staining should be performed first since the fixation and permeablization steps might reduce the availability of surface antigens.*

CELL SURFACE STAINING (IF PROTEINS OR ANTIGENS ARE INTRA-CELLULAR, PROCEED TO NEXT SECTION)

1. Recommended: Block non-specific interactions using 0.5-1 µg of a species specific Fc-blocking reagent such as an anti-mouse CD16/CD32 antibody (NBP1-27946).

2. Add appropriate amount of each directly conjugated antibody (e.g. 1 test or 1 µg per sample, as experimentally determined) to 100 µL of staining buffer (NBP2-26247) per sample (eg. use 1 mL of staining buffer for 10 samples).

   a. To determine which directly conjugated antibodies work with your instrument, use our panel builder at www.novusbio.com/novusknowsflow.html.

   b. For additional help on selection of conjugated targets, contact technical support at technical@novusbio.com

3. Add 100 µL of diluted antibody to each sample.

4. Incubate on ice (2-8°C) in dark for 1 hour.

5. Add 1-2 mL of staining buffer and centrifuge at 400 RCF for 5 minutes and discard supernatant.

6. Wash twice by re-suspending cells in staining buffer (2 mL for tubes or 200 µL for wells) and centrifuging at 400 RCF for 5 minutes. Discard supernatant.

7. Resuspend in an appropriate volume of staining buffer (usually 500 µL per sample) and proceed with analysis on your flow cytometer.

INTRACELLULAR STAINING

TIP *When performing intracellular staining, it is important to use appropriate fixation and permeabilization reagents based upon the target and its subcellular location. Generally, our Intracellular Flow Assay Kit (NBP2-29450) is a good place to start as it contains an optimized combination of reagents for intracellular staining as well as an inhibitor of intracellular protein transport (necessary if staining secreted proteins). Certain targets may require more gentle or transient permeabilization protocols such as the commonly employed methanol or saponin-based methods.*

PROTOCOL FOR CYTOPLASMIC TARGETS

Optional: Perform cell surface staining as described in the previous section if needed.

1. Fix the cells by adding 100 µL of fixation solution (such as 4% PFA) to each sample for 10-15 minutes.

2. Permeabilize cells by adding 100 µL of a permeabilization buffer to every 1 x 10^6 cells present in the sample. Mix well and incubate at room temperature for 15 minutes.

   a. For cytoplasmic targets, use a gentle permeabilization solution such as 1X PBS + 0.5% Saponin or 0.5% Tween-20.

   b. To maintain the permeabilized state throughout your experiment, use staining buffer + 0.1% of the permeabilization reagent (i.e. 0.1% Tween-20 or 0.1% Saponin).

3. Following the 15 minute incubation, add 2 mL of the staining buffer + 0.1% permeabilizer solution to each sample.

4. Centrifuge for 5 minutes at 400 RCF.

5. Discard supernatant and re-suspend in 1 mL of staining buffer + 0.1% permeabilizer.

6. Stain each sample at 1 µL/ 1 x 10^6 cells of primary antibody or 1-3 µL/ 1 x 10^6 cells for directly conjugated antibodies. Mix well

and incubate on ice for 30 minutes- 1 hour. Gently mix samples every 10-15 minutes.

7. Following the primary/conjugate incubation, add 2 mL/sample of staining buffer +0.1% permeabilizer and centrifuge for 5 minutes at 400 RCF.

8. Remove supernatant and re-suspend each sample in 2 mL staining buffer +0.1% permeabilizer. Repeat wash for 5 minutes at 400 RCF.

9. If using a directly conjugated antibody, after the second wash, re-suspend cell pellet to a final volume of 500 µL per sample and proceed with flow analysis.

PROTOCOL FOR NUCLEAR TARGET (NUCLEAR ENVELOPE AND NUCLEAR MATRIX)

Optional: Perform cell surface staining as described in the previous section and/or the cytoplasmic target staining described above.

1. Fix the cells by adding 100 µL fixation solution (such as 4% PFA) to each sample for 10-15 minutes.

2. Permeabilize cells by adding 100 µL of a permeabiliation buffer to every 1 x 10^6 cells present in the sample. Mix well and incubate at room temperature for 15 minutes.

   a. For nuclear targets, use a gentle permeabilization solution such as 1X PBS (0.1 -1.0%) Triton X-100 or NP-40.

   b. To maintain the permeabilized state throughout your experiment, use staining buffer + 0.1% of the permeabilization reagent (Triton or NP40).

3. Following the 15 minute incubation, add 2 mL of the staining buffer + 0.1% permeabilizer solution to each sample.

4. Centrifuge for 5 minutes at 400 RCF.

5. Discard supernatant and re-suspend in 1 mL of staining buffer + 0.1% permeabilizer.

6. Stain each sample at 1 µL/ 1 x 10^6 cells of primary antibody or 1-3 µL/ 1 x 10^6 cells for directly conjugated antibodies. Mix well and incubate on ice for 30 minutes- 1 hour. Gently mix samples every 10-15 minutes.

7. Following the primary/conjugate incubation, add 2 mL/sample of staining buffer +0.1% permeabilizer and centrifuge for 5 minutes at 400 RCF.

8. Remove supernatant and re-suspend each sample in 2 mL staining buffer +0.1% permeabilizer. Repeat wash for 5 minutes at 400 RCF.

9. If using a directly conjugated antibody, after the second wash, re-suspend cell pellet to a final volume of 500 µL per sample and proceed with flow analysis.

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RUNNING BUFFER

12.5 mM Tris-base	1.51 g
100 mM Glycine	7.5 g
0.05% SDS	0.5 g
Water	To 1000 mL

1X TBST

20 mM Tris-base	2.4 g
150 mM NaCl	8.7 g
0.1% Tween-20	1 mL
Water	To 1000 mL Adjust pH to 7.5

0.1% PONCEAU S PROTEIN STAIN

Ponceau S	0.1 g
Acetic Acid	1 mL
Water	To 100 mL

1X TRANSFER BUFFER

25 mM Tris-base	3.03 g
192 mM Glycine	14.4 g
20% Methanol	200 mL
Water	To 1000 mL

1X PBS

10 mM Na2HPO4	2.17 g
1.8 mM KH2PO4	0.24 g
140 mM NaCl	8 g
2.7 mM KCl	0.2 g
Water	To 1000 mL Adjust to pH 7.4

WESTERN BLOT BLOCK SOLUTIONS

Non-fat Milk or BSA	1 or 5 g
1X TBST	To 100 mL

4% PARAFORMALDEHYDE (PFA)

Paraformaldehyde	4 g
1X PBS	To 100 mL

Gently heat in a fume hood while stirring. Do not boil. Cool to room temperature before use.

1X LAEMMLI SAMPLE BUFFER

62.5 mM Tris-HCl pH 6.8

10% Glycerol

2% SDS

0.005% Bromophenol blue

Add reducing agent to 2.5% as required.

ICC PERMEABILIZATION BUFFER

Triton X-100	0.5 mL
PBS	99.5 mL

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