Western Blot Protocol

Standard Operating Procedure — SOP-NB-WB-004

Nexa Biologics • Protein Analysis Core Facility • Rev. 2.1 • 14 January 2025

1 Protocol Overview & Scope

Purpose

This Standard Operating Procedure (SOP) describes the complete workflow for performing western blot (immunoblot) analysis at Nexa Biologics Research Division. It covers sample preparation through chemiluminescent detection of target proteins, with special emphasis on reproducibility, signal quantification, and troubleshooting.

Scope

Applicable to all research scientists and laboratory technicians in the Protein Analysis Core Facility. Covers SDS-PAGE under denaturing conditions (reducing and non-reducing), wet and semi-dry transfer, and HRP-based ECL detection.

Responsible Personnel

- **Protocol Owner:** Dr. Elena Voss, Principal Scientist (Proteomics)
- **Laboratory Supervisor:** Dr. Marcus Hale, Core Facility Director
- **Review Cycle:** Annual (next review: March 2026)

Document Info

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Required Equipment & Reagents

Equipment

- SDS-PAGE electrophoresis system (NexaGel™ Pro)
- Trans-Blot wet transfer cell or semi-dry transfer unit
- PVDF or nitrocellulose membrane (0.2 μm or 0.45 μm pore)
- Chemiluminescence imaging system (VertexImager™ 5000)
- Rocking/orbital shaker (variable speed)
- Microcentrifuge (16 000 $\times$ g capable)
- Heating block or water bath (95–100 °C)
- Protein quantification spectrophotometer

Key Reagents

- NexaLysis™ RIPA Buffer (Cat. NB-LYS-001)
- Protease inhibitor cocktail (Cat. NB-INH-003)
- Laemmli sample buffer (4 $\times$, reducing)
- BrightMark™ Pre-stained Protein Ladder (10–250 kDa)
- Primary antibodies (see Section 5)
- HRP-conjugated secondary antibodies
- NexaLuminol™ ECL substrate (Cat. NB-ECL-010)
- TBS-T (Tris-buffered saline + 0.1% Tween-20)
- Blocking reagent: 5% non-fat dry milk or 5% BSA in TBS-T

⚠ Hazard Notice

SDS is a hazardous irritant. Acrylamide monomer (in gel preparation) is a suspected neurotoxin and carcinogen. Always wear appropriate PPE (nitrile gloves, lab coat, safety glasses). Dispose of acrylamide waste per Nexa Biologics Waste Disposal SOP-NB-ENV-001.

2 Sample Preparation

2.1. Cell or Tissue Lysis

1. **Cell culture:** Aspirate medium. Wash cells twice with ice-cold PBS. Place plate on ice. Add 200–500 µL NexaLysis™ RIPA Buffer per 60 mm dish (scale proportionally).
2. Add 1× protease inhibitor cocktail immediately before use. Optional: add phosphatase inhibitors (Cat. NB-INH-007) if phospho-proteins are targets.
3. Scrape cells into a 1.5 mL microcentrifuge tube. Incubate on ice for 30 min with occasional vortexing every 5 min.
4. **Tissue:** Homogenise 10–30 mg tissue in 300 µL lysis buffer using a pre-chilled pestle. Proceed identically to step 3.
5. Centrifuge at 16 000 × *g*, 4 °C, 15 min. Carefully transfer the supernatant (whole-cell lysate) to a fresh tube. Discard pellet.
6. Snap-freeze aliquots in liquid nitrogen or dry ice/ethanol slurry. Store at -80 °C. Avoid more than three freeze-thaw cycles.

✓ Best Practice — Nuclear Fractions

If nuclear or membrane proteins are targets, use a fractionation kit (NB-FRAC-002) rather than bulk RIPA lysis, which may under-extract these compartments.

2.2. Protein Quantification

Use the BCA or Bradford assay according to kit instructions. Recommended range: 0.5–5 µg/µL. Measure absorbance at 562 nm (BCA) or 595 nm (Bradford). Ensure standards are prepared in the same lysis buffer as samples to minimise matrix effects.

Target Protein	Load Amount	Notes
Abundant (e.g., GAPDH, Actin)	10–20 µg	Reduce if signal saturates
Moderately expressed	20–30 µg	Standard starting point
Low-abundance / secreted	40–60 µg	Concentrate if needed
Phospho-proteins	30–50 µg	Use phosphatase inhibitors

2.3. Sample Denaturation

1. Dilute samples to a uniform protein concentration in lysis buffer. Equalise volumes across all lanes.
2. Add Laemmli sample buffer (4×) at a ratio of 1 : 3 (buffer : sample) to reach 1× final concentration.

3. Heat at 95 °C for 5 min (or 70 °C for 10 min for membrane proteins such as multi-pass transmembrane receptors).
4. Briefly centrifuge at 800 × *g* for 1 min to collect condensate.
5. Samples are ready for immediate loading or may be stored at -20 °C for up to two weeks.

DTT vs. β-Mercaptoethanol

Reducing agent choice affects downstream assays. DTT (100 mM final) is odourless and preferred for ECL. β-Mercaptoethanol (5 %) provides stronger reduction for stubborn disulfide bridges but must be handled in a fume hood.

3 SDS-PAGE Electrophoresis

3.1. Gel Selection & Preparation

Select gel percentage based on target molecular weight:

Acrylamide %	Optimal Range (kDa)	Example Targets
8%	80–200	p53, HSP90, EGFR
10%	40–130	ERK1/2, Akt, STAT3
12%	20–70	GAPDH, Actin, Cyclins
15%	10–40	Histones, Small GTPases
4–20% gradient	10–250	Multiplex / unknown MW

Pre-cast NexaGel™ Pro gels are recommended for consistency. If pouring gels, de-gas the acrylamide solution under vacuum for 10 min before adding APS and TEMED.

3.2. Electrophoresis

1. Assemble gel in the electrophoresis tank. Fill inner chamber with 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3).
2. Load 5 μ L BrightMark™ ladder into lane 1. Load prepared samples into remaining lanes. Include a positive control and negative control in every run.
3. Run at **80 V** through the stacking gel (~20 min), then increase to **120 V** through the resolving gel (~60–90 min).
4. Stop electrophoresis when the dye front reaches 0.5 cm from the gel base.

4 Protein Transfer

4.1. Membrane Preparation

1. **PVDF membrane:** Activate in 100% methanol for 30 seconds min, then equilibrate in transfer buffer for 5 min. *PVDF is preferred for low-abundance proteins due to lower background.*
2. **Nitrocellulose membrane:** Equilibrate directly in transfer buffer for 10 min. Suitable for most standard targets.
3. Cut membrane to gel dimensions. Mark orientation with pencil.

4.2. Transfer Settings

Wet Transfer — NexaGel™ System

Parameter	Standard	High-MW
Buffer	Transfer	Transfer + 20% MeOH
Voltage	100 V	30 V
Current	350 mA	100 mA
Duration	1 h	16 h (4°C)
Temperature	RT	4°C

Semi-Dry Transfer — VertexBlot™

Parameter	Setting
Current (constant)	80 mA per gel
Voltage limit	25 V
Duration	30 min–40 min
Membrane	PVDF (0.2 μ m)
Buffer stacks	3 Whatman sheets each

4. After transfer, stain membrane with **Ponceau S** (0.1% in 5% acetic acid) for 5 min to confirm transfer efficiency. Photograph before washing.
5. Wash with TBS-T (5 min, 3×) to remove Ponceau S completely.

Transfer Efficiency Check

Poor Ponceau S staining indicates failed transfer. Common causes: incorrect buffer composition, air bubbles in the sandwich, or reversed membrane/gel orientation. Re-transfer from the gel if it still stains with Coomassie.

5 Blocking & Antibody Incubation

5.1. Blocking

1. Incubate membrane in **blocking buffer** at room temperature for 1 h with gentle rocking.
2. **Blocking buffer choice:**
   - 5% non-fat dry milk in TBS-T — for non-phospho targets
   - 5% BSA in TBS-T — for phospho-protein detection (milk contains casein, which is phosphorylated and causes background)
   - NexaBlock™ (Cat. NB-BLK-002) — universal, for total and phospho targets on the same membrane
3. Discard blocking buffer. Do *not* wash between blocking and primary antibody incubation.

5.2. Primary Antibody Incubation

1. Dilute primary antibody in the appropriate blocking buffer (see dilution table below).
2. Incubate membrane with primary antibody solution at 4 °C overnight (16–18 h) with gentle rocking, or at room temperature for 2 h if validated.
3. Wash with TBS-T: 3×10 *min* at room temperature.

5.3. Secondary Antibody Incubation

1. Dilute HRP-conjugated secondary antibody in blocking buffer (typically 1:5 000–1:10 000, see table).
2. Incubate for 1 h at room temperature with gentle rocking. Protect from light if using fluorescent secondary.
3. Wash with TBS-T: 3×10 *min*.
4. Final wash with TBS (without Tween-20) for 5 min to remove residual detergent before ECL.

5.4. Antibody Dilution Reference Table

Target	MW (kDa)	Host	1° Dilution	2° Dilution	Block / Buffer
GAPDH	37	Rabbit	1:10 000	1:10 000	5% Milk / TBS-T
β-Actin	42	Mouse	1:5 000	1:5 000	5% Milk / TBS-T
Akt (pan)	60	Rabbit	1:1 000	1:5 000	5% BSA / TBS-T
p-Akt (Ser473)	60	Rabbit	1:1 000	1:5 000	5% BSA / TBS-T
ERK1/2	42/44	Rabbit	1:1 000	1:5 000	5% Milk / TBS-T
p-ERK1/2 (Thr202/Tyr204)	42/44	Mouse	1:2 000	1:5 000	5% BSA / TBS-T
STAT3	92	Rabbit	1:1 000	1:5 000	5% Milk / TBS-T
p-STAT3 (Tyr705)	92	Rabbit	1:500	1:5 000	5% BSA / TBS-T
p53 (DO-7)	53	Mouse	1:500	1:5 000	5% Milk / TBS-T
Caspase-3	35/17	Rabbit	1:1 000	1:5 000	5% Milk / TBS-T
Bcl-2	26	Mouse	1:500	1:5 000	5% Milk / TBS-T
EGFR	134	Rabbit	1:1 000	1:5 000	5% BSA / TBS-T
p-EGFR (Tyr1068)	134	Rabbit	1:1 000	1:5 000	5% BSA / TBS-T
HSP90	90	Rabbit	1:2 000	1:5 000	5% Milk / TBS-T
Histone H3	17	Rabbit	1:5 000	1:10 000	5% Milk / TBS-T

All primary antibodies validated in-house at Nexa Biologics. Catalogue numbers available from Dr. Elena Voss (e.voss@nexabio.internal). Dilutions may require optimisation for new cell/tissue types.

Antibody Stripping & Re-probing

To re-probe for another target on the same membrane, use NexaStrip™ Harsh Stripping Buffer (Cat. NB-STR-004): incubate at 50 °C for 20 min with rocking. Confirm complete stripping with ECL before re-blocking and re-probing. Limit stripping cycles to two to preserve membrane integrity.

6 Detection & Signal Quantification

6.1. ECL Chemiluminescent Detection

1. Prepare NexaLuminol™ ECL substrate by mixing equal volumes of Reagent A and Reagent B immediately before use. Use 1000 µL per 8 × 10 cm membrane.
2. Drain excess wash buffer from the membrane. Place protein-side up on a clean sheet of plastic wrap.
3. Apply ECL substrate evenly across the membrane. Incubate for exactly 1 min at room temperature. Do not agitate.
4. Drain excess substrate. Wrap membrane in plastic wrap; avoid bubbles.

5. Transfer membrane to the VertexImager™ 5000 imaging cassette.
6. Acquire images with exposure times ranging from **10 seconds** to **10 minutes** to determine optimal exposure. Use automatic exposure mode as a starting point.
7. Save raw images in 16-bit TIFF format for quantification. Never adjust contrast on raw images prior to densitometric analysis.

6.2. Image Quantification

1. Open raw 16-bit TIFF in NexaQuant™ or ImageJ/Fiji.
2. Draw rectangular selection boxes of equal area over each band of interest and corresponding loading control bands.
3. Measure integrated density (or mean grey value × area). Subtract local background from each measurement.
4. Normalise target band densitometry to the loading control (e.g., GAPDH or β-Actin) for each lane.
5. Express results as fold-change relative to the untreated / control condition.
6. Statistical analysis: perform at minimum three independent biological replicates. Report mean ± SEM. Use two-tailed Student's t-test or one-way ANOVA with post hoc correction (Tukey or Dunnett) as appropriate.

Linear Dynamic Range

Ensure band signals fall within the instrument's linear range. Saturated bands (clipped white pixels in 16-bit mode) must be re-imaged at lower exposure. Quantification of saturated bands is **not acceptable** for publication.

Data Recording Requirements

- Record all experimental conditions, antibody lots, and exposure times in the Nexa ELN (Electronic Lab Notebook) system.
- Retain all raw image files and original uncropped blot images in the `/projects/NB-WB/raw_data/` shared drive.
- Minimum retention period: 10 years per Nexa Data Integrity Policy (DIP-NB-003).
- Cropped bands in figures must be clearly delineated with a visible border; lane splicing must be disclosed in figure legends.

Publication Checklist

- ✔ ≥ 3 biological replicates
- ✔ Uncropped blot in supplement
- ✔ MW ladder visible
- ✔ Loading control shown
- ✔ Statistics reported
- ✔ Raw data archived

7 Troubleshooting Appendix

The table below covers the most common problems encountered during western blot workflows at Nexa Biologics, based on accumulated facility data (2019–2025).

Problem	Probable Cause(s)	Corrective Action
No signal / very faint band	Protein not expressed; degraded sample; antibody issue; poor transfer	Verify expression by RT-qPCR. Check sample freshness and protease inhibitor use. Increase protein load. Increase primary antibody concentration. Confirm transfer efficiency with Ponceau S.
High background (entire membrane)	Over-blocking; high antibody concentration; insufficient washing; ECL over-exposure	Reduce primary/secondary antibody dilution. Extend wash steps to 5×10 *min*. Reduce ECL incubation time or switch to low-sensitivity substrate.
High background (speckled)	Dry spots during blocking; particulates in antibody solution	Ensure membrane stays wet throughout. Filter antibody solutions through $0.22\,\mu$ m membrane before use. Increase rocking speed during incubation.
Multiple / non-specific bands	Antibody cross-reactivity; protein degradation; antibody concentration too high	Reduce primary antibody concentration. Add 0.1% Tween-20 to primary antibody diluent. Check sample for proteolysis. Use validated positive control lysate.
Band at wrong molecular weight	Post-translational modifications; isoforms; running artefact	Consult antibody datasheet for all reported isoforms. Verify with positive control. Check ladder alignment. Consider dephosphorylation treatment if MW shift is $\sim$2–10 kDa.
Smearing / streaking of bands	Overloaded lane; salt in sample; partially denatured protein	Reduce protein load. Dialyse or dilute sample to remove excess salt. Increase denaturation time at 95 °C.
Wavy or distorted bands	Uneven gel polymerisation; air bubbles; uneven current distribution	Use pre-cast gels. Ensure level gel tank. Check electrode contact. Run at lower voltage for more uniform migration.
White (reversed) bands	Hook effect from antigen excess overwhelming antibody	Reduce protein load. Serially dilute samples to confirm dose-dependence.
Inconsistent signal between lanes	Unequal loading; pipetting error; edge effects	Re-quantify all samples. Verify loading with colloidal Coomassie staining or total protein normalisation stain. Avoid outermost lanes for critical comparisons.
Membrane tears or holes	Excessive stripping; over-drying PVDF; mechanical damage	Limit harsh stripping cycles to two per membrane. Never allow PVDF to dry completely. Handle membrane only by the edges using blunt-nosed forceps.
ECL substrate crystallisation	Reagents mixed too far in advance; temperature too low	Mix substrates immediately before use. Warm to room temperature before mixing. Use within 15 minutes of preparation.

Escalation Procedure

If a problem cannot be resolved after two attempts using corrective actions above, contact the Core Facility Director (Dr. Marcus Hale) and log a trouble ticket in the NexaLIMS portal (lims.nexabio.internal). Attach blot images and the completed Experiment Record Form (ERF-NB-WB-004).

Document Control: This SOP was reviewed and approved by the Nexa Biologics Quality Assurance Committee on 14 January 2025. Printed or downloaded copies are *uncontrolled* — always verify the version at docs.nexabio.internal before use in a regulated context.

Revision History: Rev. 1.0 (Jan 2022): Initial release. Rev. 2.0 (Jun 2023): Added semi-dry transfer settings, expanded antibody table. Rev. 2.1 (Jan 2025): Updated ECL substrate instructions; added publication checklist.

Key References

7 References