Protein Processing for Lysate Samples

In this workflow, samples will undergo lysis using a detergent-containing buffer followed by detergent removal using spin columns. The lysate will be quantified for total protein concentration, processed for protein (±RNA) detection, and then prepared for hybridization. Lysates are bound to a 96-well protein-binding plate and incubated with the antibody mix from NanoString. Unbound antibodies are then washed away, and bound antibodies are subjected to RLT lysis to collect oligonucleotide tags for hybridization.

There are two versions of the nCounter® Vantage 3D™ Protein panel—with and without ERCC controls.

- Protein TagSet (D) **contains ERCC controls** and is a stand-alone protein assay.
- Protein TagSet (R) **does not contain ERCC controls** and must be **used with Vantage 3D RNA assays**.

IMPORTANT: Do not use Protein TagSet (D) panels with Vantage 3D RNA assays.

Contents

Recommended Materials

Table 1 lists items that are provided by NanoString. Table 2 lists required materials and reagents for Lysate protein processing.

Table 1. Reagents provided by NanoString

Reagents		Description	Storage
See our Protein Panels page for a list of all (D) and (R) Vantage 3D Protein Panels NOTE: (D) contains ERCC controls for stand-alone use, (R) does not. Do not use Protein TagSet (D) panels with Vantage 3D RNA assays.	Protein TagSet (D) or (R)	Panel	At or below -80°C
	Antibody Mix	Oligonucleotide tags bound to antibodies	At or below -80°C
	Buffer WS	Antibody diluent and blocking buffer	4°C

Table 2. Required materials and reagents

Item	Manufacturer	Part Numbers
Pipettes for 10–1,000 µL	Various	Various
Filter tips (RNase/DNase free)	Various	Various
Orbital shaker	Various	Various
Vortexer	Various	Various
Thermal cycler	Various	Various
MaxiSorp™ 96-well U-Bottom plates (60 count)	VWR	62409-046
Buffer RLT (220 ml)	QIAGEN	79216
20X TBS (500 mL)	ThermoFisher Scientific	28358
1X phosphate buffered saline (PBS; pH 7.2 to 7.4)	Various	Various
RNase/DNase Free H_20	Various	Various
Pierce™ Detergent Removal Spin Column	ThermoFisher Scientific	87776 or 87777*
Pierce™ 660 nm Protein Assay Kit	ThermoFisher Scientific	22662
Ionic Detergent Compatibility Reagent (IDCR)**	ThermoFisher Scientific	22663
10% SDS Solution, (4 x 100 mL)	ThermoFisher Scientific	15553027
1.0 M Tris buffer, pH 6.8 (1 L)	VWR	AAJ63831-K2
Anhydrous NaCl	Sigma-Aldrich	S5886
No Weigh DTT	ThermoFisher Scientific	A39255
1.0 M Sodium Azide (NaN_3)	Various	Various
Tween® 20	Various	Various

**See Detergent Removal Columns vs. Plates to determine the correct column size.*

***IDCR ensures residual detergent does not interfere with protein quantification. It is optional for samples lysed in SDS and highly recommended for samples lysed in RIPA.*

Protocol Summary

Table 3. Estimated timeline for lysate sample protein preparation for 12 samples. Hands-on time may be less than the processing times listed

Step	Minimum Time Required
Advance Preparation	30 mins (does not include cell counting if being performed)
Sample Lysis	20 mins
Detergent Removal	30 mins
Protein Lysate Quantification	20 mins
RNA Preparation (optional)	5 mins
Protein Lysate Preparation	Protein Binding: 10 mins then 2 hours or overnight incubation
	Antibody Staining: 3 hours with minimum incubation times

Lysis Buffer Selection

NanoString has validated two lysis buffers that are compatible with downstream lysate sample processing for protein (±RNA):

- SDS lysis buffer: 100 mM Tris, 2% SDS, 50mM DTT, 300 mM NaCl
- Modified RIPA lysis buffer: 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% Sodium Deoxycholate with the addition of 2% SDS, 50mM DTT

IMPORTANT: Once you select a buffer, use it consistently.

Lysis buffer selection is important to ensure consistency of protein counts. We recommend utilizing a consistent lysis buffer for your experiments. Different buffers may solubilize specific proteins with different efficiency or alter binding of proteins to the protein binding plates. When comparing SDS to RIPA + SDS/DTT across multiple cell lines and treatment conditions, protein and RNA expression profiles show a high correlation regardless of buffer used (R^2>0.98). If samples have been stored in RIPA without SDS and DTT, we recommend the addition of SDS and DTT before processing samples for NanoString analysis.

Detergent Removal Columns vs. Plates

NanoString requires that detergent is removed from your sample prior to quantification and processing. While the detergent is necessary for efficient protein solubilization, the presence of detergent in subsequent steps can interfere with protein quantification and inhibit binding of your sample to the protein binding plate. We have validated both the Pierce Detergent Removal Spin Column (87776 or 87777) and the Pierce Detergent Removal Spin Plate (88304). Selection of the columns versus the plates depends on sample volume. The Pierce Detergent Removal Spin Columns are provided in packs of 25 and recommended for processing <24 samples at a time. The Pierce Detergent Removal Spin Plates are provided in packs of 2X96-well plates and are recommended for processing more than 24 samples at a time for a high-throughput workflow. Selection of the columns versus the plates has no impact on assay performance and the option is provided to enable flexibility in sample processing.

For additional questions about the protocol, visit our Knowledge Base and FAQs.

Advance Preparation

Additional Buffer Preparation

1. Prepare 100 mL of TBST (1X TBS with 0.1% Tween). Add 5 mL of 20X TBS and 0.1 mL of Tween-20 to 94.9 mL of RNase/DNase Free H_20. The TBST may be stored at room temperature for up to 1 month. This formulation is in excess of what is required for a 12-reaction assay.
2. Prepare 100 mL of 1X PBS pH 7.2 with 2 mM sodium azide (NaN_3) by adding 0.2 mL of 1M sodium azide to 99.8 mL of 1X PBS. This formulation is in excess of what is required for a 12-reaction assay. This buffer can be stored at room temperature for up to 1 month.

Option 1: SDS Lysis Buffer Preparation

1. Prepare 10 mL of SDS lysis buffer stock (without DTT): Add 1 mL of 1M Tris (pH 6.8), 2 mL of 10% SDS, and 600 µL of 5M NaCl stock solution to 6.4 mL of RNase -free water (This stock solution can be stored at room temp for 6 months).
2. Prepare 1 mL of SDS lysis working buffer (100 mM Tris pH 6.8, 2% SDS, 300 mM NaCl, 50 mM DTT). The volume required will depend on the total number of cells for 10,000 cells per µL and may be more than 1 mL.
    a. Dissolve one vial of No Weigh DTT in 50 µl of lysis buffer stock. (The DTT may take several minutes and some agitation to fully dissolve).
    b. Add 50 µl of the dissolved DTT to 950 µl of the lysis buffer stock. This solution must be used the same day as sample preparation.

Option 2: Modified RIPA Buffer Preparation

1. Prepare 1 mL of modified RIPA lysis buffer
    a. Dissolve one vial of No Weigh DTT in 200 µL of 10% SDS stock solution. Add the 200 µL of dissolved DTT SDS stock solution to 800 µL of RIPA. Prepare enough RIPA lysis solution for 200 µL volume per 1 million cells/pellet sample.

        NOTE: Modified RIPA buffer should be made fresh prior to use.

    b. If samples have previously been lysed in RIPA:
        i. Dissolve 1 vial of no weight DTT in 200 µL of 10% SDS.
        ii. Add the SDS/DTT stock solution 1:5 to the thawed aliquot of RIPA lysate for a final concentration of 2% SDS.

Lysate Processing Recommendations

IMPORTANT: The antibody mix provided contains high levels of target oligonucleotide. Follow these precautions; **failure to do so** may result in **high background** and **poor data quality** due to **oligo contamination**.

- Use filter tips to avoid contamination of pipets.
- Do not use the same pipet tip twice after addition of the antibody mix.
- Change gloves often to avoid assay contamination.
- **DO NOT** allow the tissue section to dry out

Sample Lysis

1. When working with fresh cultured cells, wash cells in PBS to remove any serum from cell culture media prior to lysis. If not proceeding immediately to next step, gradually cool on ice. If lysing in modified RIPA buffer, proceed to Modified RIPA Sample Lysis.

SDS Sample Lysis

1. Add 100 µL of SDS lysis working buffer for every 1 million cells. If starting with fewer than 1 million cells, add lysis buffer to achieve 10,000 cells per µL. This is a recommended lysis buffer-to-cell ratio that typically yields protein concentrations of 500–1500 µg/mL.

   NOTE: We recommend starting the lysis step with 1–2 million cells, depending on the cell size. Smaller cells (e.g., suspension cell lines and primary immune cells) may require higher input. Due to high viscosity, a large-bore pipette tip or transfer pipette may be needed. We recommend a starting protein concentration of ≥500 µg/mL, since sample loss has been observed during detergent removal.

   a. If lysing adherent cells in cell culture plates, make sure that the entire surface is covered and allow 1 minute for lysing. Use a cell scraper to move viscous lysate to one side of the well and then transfer lysate to a tube for boiling.

   b. If lysing cell pellets, flick tube to break pellets if possible, then add lysis buffer. Pipette up and down and/or briefly vortex until cells are fully lysed.

   c. Proceed immediately to the next step.

2. Boil at 95°C for 10 min as soon as possible after lysing.

   NOTE: It is important that the sample reaches 95°C. Do not add water to heat blocks since evaporative cooling may make it difficult for the sample to reach 95°C. Sample will become less viscous when boiled. If the sample is still viscous after 15 minutes, boil an additional 5 minutes.

   a. Cool the SDS lysate to room temperature, spin briefly, vortex, and spin briefly again to ensure a homogenous mixture.

3. Proceed to Detergent Removal

Optional Stopping Point: Lysate is stable at room temperature overnight or indefinitely at -80°C.

Modified RIPA Sample Lysis

1. When starting with fresh or frozen cell pellets, use RIPA with 2% SDS, 50 mM DTT (prepared during advance preparation). Add 200 µL of modified RIPA lysis buffer for every 1 million cells.
2. Boil at 95°C for 10 minutes as soon as possible after lysing.

   NOTE: It is important that the sample reaches 95°C. Do not add water to heat blocks since evaporative cooling may make it difficult for the sample to reach 95°C. Sample will become less viscous when boiled. If the sample is still viscous after 15 minutes, boil an additional 5 minutes.
3. Cool the modified RIPA lysate to room temperature, spin briefly, vortex, and spin briefly again to ensure a homogenous mixture.
4. Proceed to Detergent Removal

Optional Stopping Point: Lysate is stable at room temperature overnight or indefinitely at -80°C.

Sample Previously Lysed in RIPA

1. Dissolve 1 vial of no weight DTT in 200 µl of 10% SDS.
2. Thaw an aliquot of RIPA lysate.
3. Add the SDS DTT stock solution 1:5 to the thawed aliquot of RIPA lysate so the final [SDS] = 2%.
4. Boil the sample 95°C for 15 min, briefly cool to room temperature.
5. Proceed to Detergent Removal.

Optional Stopping Point: Lysate is stable at room temperature overnight or indefinitely at -80°C.

Detergent Removal

1. Remove detergent using the Pierce detergent removal kit of your choice.

   NOTE: For the appropriate size column, refer to Detergent Removal Columns vs. Plates.

   NOTE: Visually inspect column resin volume for consistency. Columns with low resin volumes should not be used.

Detergent Removal Protocol Guidance

1. Place column in a collection tube. Spin at 1500x*g* for 1 minute for 0.5 ml columns or 1000x*g* 1 minute for 0.125 ml columns to remove storage buffer. Place columns in a clean tube and add the sample directly to the resin. Wait 2 minutes and then spin for 2 minutes at either 1500x*g* or 1000x*g*, depending on the column. Proceed to Protein Lysate Quantification.

Optional Stopping Point: Room Temperature or -80°C only. Do not store at 4°C, -20°C, or on ice. Without detergent, hydrophobic proteins will aggregate at low temps.

Protein Lysate Quantification

1. Check the protein concentration of the detergent-free lysate using a Pierce 660 nm protein determination kit with a BSA curve per the manufacturer's instructions (plate-based option available). Scale the ratios (15:1) for the small volumes used in this assay; 60 µL of 660 nm reagent with 4 µL of protein sample.

   NOTE: Protein concentrations can also be quantified from the detergent-free lysate using a traditional Bradford assay or Qubit Protein Assay.

2. Proceed to dilution for RNA and Protein preparation.

RNA Preparation (optional)

RNA can be measured directly from the concentrated detergent-free protein lysates.

1. Using the concentration determined with the Pierce 660nm kit, dilute a small aliquot to 250 µg/mL using nuclease-free water.
   - If using nCounter MAX™ or nCounter FLEX™ system, 4 µl of the diluted detergent-free cell lysate is required for Hybridization.
   - If using nCounter SPRINT™ system, further dilute the lysate with an equal amount of nuclease-free water. A 4 µl aliquot of this diluted cell lysate in required for Hybridization. This will contain approximately 50 ng of RNA.
2. Store at -80°C until ready to proceed with Hybridization.

Protein Lysate Preparation

1. Dilute the detergent-free lysate to 2.5 µg/mL protein concentration using PBS pH 7.2 with 2 mM sodium azide.
2. Pipet 50 µl of the diluted detergent-free lysate into a well of a protein binding plate (MAXISORP plate) and seal with parafilm or a similar material.
3. Incubate at least 2 hours at room temperature or overnight at room temperature.

Optional Overnight Stopping Point

4. Warm Buffer WS to room temperature. Thaw the antibody mix on ice.
5. Add 200 µl of blocking buffer directly to the wells containing the lysate.

   NOTE: DO NOT remove the lysate prior to adding the blocking buffer and DO NOT pipette up and down.

6. Incubate for a minimum of 5 min or up to 1 hour at room temperature.

7. Remove and discard the supernatant by flicking the plate into a sink or basin and then forcefully strike the plate on a fresh paper towel hard enough to remove all the residual liquid.
8. Perform a total of 3 washes as follows:

   IMPORTANT: DO NOT allow the plate to dry completely at any time.

   IMPORTANT: Due to potential for antibody tag contamination:

   - Change pipette filter tips for every step
   - DO NOT pipette up and down
   - Change gloves frequently
   - Use fresh paper towels for blotting the plate after liquid removal

   a. Add 250 µl of room temperature TBST.

   b. Incubate for 1 minute.

   c. Remove and discard the supernatant by flicking the plate into a sink or basin and then forcefully strike the plate on a fresh paper towel hard enough to remove the residual liquid.

   d. Repeat steps a–c 2 more times for a total of 3 washes.
9. Make a working antibody solution by adding 16 µl of the antibody mix to 625 µl of Buffer WS. Mix by inverting and briefly spin down. Do not vortex.
10. Add 50 µl of the working antibody solution to each well.
11. Seal the plate with parafilm or a similar material and incubate at room temperature for 2–3 hours on an orbital shaker at 350 rpm. While shaking is preferable, if a plate shaker is not available, let sit on the benchtop.
12. Carefully use a pipette to remove all the antibody solution. Discard the supernatant.

    IMPORTANT: See Step 8 IMPORTANT note to avoid antibody tag contamination.

    NOTE: Remove all the solution by gently touching the pipette tips to the bottom of the well and without scraping the well surface.
13. Perform a total of 6 washes as follows:

    a. Add 250 µl of room temperature TBST. DO NOT pipette up and down.

    b. Incubate for 5 minutes.

    c. Remove and discard the supernatant by flicking the plate into a sink or basin and then forcefully strike the plate on a fresh paper towel hard enough to remove the residual liquid.

    d. Repeat steps a–c 5 more times for a total of 6 washes.
14. Add 50 µl of RLT per well and seal the plate with parafilm or a similar material.
15. Put the plate on an orbiter shaker for 5 min at 350 rpm.
16. Transfer the RLT lysate to a 12-well PCR strip tube.

17. If not using immediately, store the sample at -80°C until ready to proceed with hybridization.

For additional questions about the protocol, visit our Knowledge Base and FAQs.