Intracellular Protein Processing from Cell Suspensions
with Intracellular-Compatible Universal Cell Capture Kit

In this workflow, cells are collected and then bound to the Universal Cell Capture Beads; then the RNA and Protein samples are prepared for hybridization.

The Intracellular compatible Universal Cell Capture Kit can be used with the nCounter® Vantage 3D™ RNA:Protein Immune Cell Signaling Assay for cell suspensions, and the Vantage 3D Protein Immune Cell Signaling Panel for cell suspensions.

This protocol is compatible with intact cell suspensions from cell lines, PBMCs, and other primary human cells. FFPE and fresh frozen tissue are not compatible with this protocol. Following sample preparation, the RNA and protein components are combined in a single hybridization reaction. Contact NanoString Support (support@nanostring.com) to receive additional assistance with this assay.

Table 1. Materials provided by NanoString

Product	Material	Description	Storage
nCounter Vantage 3D™ Protein (D) (ERCC controls)	Protein TagSet	Panel	At or below -80°C
	Antibody Mix	Oligonucleotide tags bound to antibodies	At or below -80°C
or			
nCounter Vantage 3D Protein (R) (no ERCC controls)	Protein Plus	Panel	At or below -80°C
	Antibody Mix	Oligonucleotide tags bound to antibodies	At or below -80°C
or			
nCounter Vantage 3D RNA:Protein (no ERCC controls)	Protein Plus	Panel	At or below -80°C
	Antibody Mix	Oligonucleotide tags bound to antibodies	At or below -80°C

Product	Material	Description	Storage
Intracellular compatible Universal Cell Capture Kit	Universal Cell Capture Beads	Magnetic bead solution	4°C (Do not freeze)
	Buffer FD	Fixation diluent	4°C (2–8)°C
	Buffer FC	Fixation concentrate	4°C (2–8)°C
	Buffer PW	Permeabilization and wash buffer	4°C (2–8)°C
	Buffer W	Blocking and wash buffer	4°C
	Buffer LH	Lysis buffer	RT

Table 2. Required materials and reagents

Item	Manufacturer	Part Numbers
96-well plate magnet separator*	Thermo Fisher Scientific	12027
	Stemcell Technologies	18102
96-well clear polystyrene round-bottom plate	Corning	351177
Pipettes for 10–1,000 µL*	Various	Various
Manual multi-channel pipette for 200 µL*	Rainin	L12-200XLS+
12-strip standard tubes*	Bioexpress	T-3034-1
15 mL conical tubes*	FisherBrand	S50712
Hemocytometer*	Various	Various
Trypan Blue*	Various	Various
1X phosphate buffered saline (PBS; pH 7.4)*	Thermo Fisher Scientific	10010-023
Human Trustain FcX™**	Biolegend	422301 or 422302
RNase/DNase-free H_2O	Thermo Fisher Scientific	4387937
Brefeldin A solution 1000X#	Biolegend	420601

**Alternative products can be used if they offer similar function and reliability.*

***Only required for samples containing human Fc receptor (e.g., PBMCs).*

 # Only use Brefeldin A solution for fresh cell suspensions. Use with cryopreserved cells may lead to a significant loss in cell viability.

Table 3. Estimated timeline for intracellular protein detection from cell suspensions. Hands-on time may be less than the processing times listed below.

Step	Minimum Time Required
Advance Preparation	Up to 4.5 hours depending on if Brefeldin A treatment is needed
Sample Collection	Depends on the number of samples. 15 minutes minimum
Bind Sample to Universal Cell Capture Beads	35 minutes
RNA Sample Preparation	30 minutes
Protein Sample Preparation	3 hours

Advance Preparation

The following procedure is used for performing 12 reactions. Scale the number of wells according to the number of reactions in your experiment.

1. Prepare fresh 1X Fix Buffer (on day of sample collection)
   a. Add 700 µl Buffer FC to 2.1 mL of Buffer FD.
2. **Optional:** Treat cells with Brefeldin A
   a. Add 1000X Brefeldin A to a tissue culture flask containing cells at 1 µL per mL of culture media.
   b. Incubate at 37° for 4 hours.

   NOTE: Incubation time may need optimization depending on cell type.

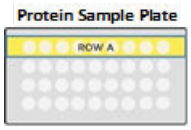
   NOTE: Extended treatment with Brefeldin A may reduce viability and affect cell yield when performing this assay. **Do not treat cryopreserved cells with Brefeldin A**.

Sample Collection

NOTES:

- This section is performed in the Protein Sample Plate.
- 50,000 cells (or 100,000 primary cells such as PBMCs) per sample are recommended for use per reaction.
- The number of cells can be decreased to a minimum of 20,000 cells (or 50,000 primary cells such as PBMC) if cell numbers are limited but note that using less than the recommended cell number above may result in reduced signal.
- Using more than the recommended cell number may require increasing the volume of Buffer LH. See Table 4 (RNA Sample Preparation) and Table 5 (Protein Sample Preparation) for guidelines.
- Perform steps where temperature is not specified at room temperature.

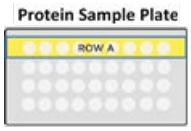
1. Determine the concentration of total viable cells in each sample.
2. For each sample, collect the recommended number of cells in 1X PBS containing 2% FBS, or warmed (37°C) cell culture medium in a 1.7 mL microcentrifuge tube.
3. Transfer cell samples to each well in the **Top** row (A) of the Protein Sample Plate. Add culture media or 1X PBS containing 2% FBS necessary to bring the final volume of each sample well to 200 µL.



Bind the Sample to Universal Cell Capture Beads

NOTE: Keep the plate in contact with the magnet at all times during the step to avoid sample/bead loss. Removing residual liquid after flicking/blotting is not necessary. If buffer is removed by the pipetting method, remove as much of the residual buffer as possible to avoid leaving variable amounts of remaining buffer in the wells. Failure to do so may result in poor quality data. The minimum time for magnet pulldown of beads is at least 3 min but 5 min is recommended. Less than 3 min may result in sample/bead loss and reduced signal in this assay.

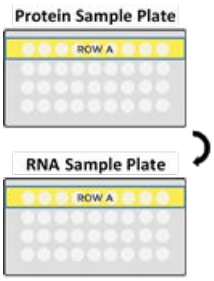
1. Prior to opening the vial of Universal Cell Capture Beads, ensure no beads are on the cap, and then thoroughly re-suspend the beads by pipetting.
2. Add 9 µL of Universal Cell Capture Beads to each sample well (A) in the **Protein Sample Plate**. Use a new pipette tip for each sample well.



3. Mix the samples using a multichannel pipette set to half the sample volume (100 µL).
4. Incubate plate on a flat surface for 30 min at RT.

RNA Sample Preparation (Optional)

1. Mix samples thoroughly by pipetting and transfer 130 µL of each sample from the Protein Sample Plate (Row A) to the corresponding wells in the **RNA Sample Plate** (Row A).



NOTE: RNA and Protein samples may be processed sequentially or in parallel from this point. If sequential sample processing is preferred, keep the Protein Sample Plate on ice until ready to proceed with CodeSet (MAN-10060) or TagSet (MAN-10065) hybridization.

2. Immobilize the bead/cell complexes by placing the RNA Sample Plate on a 96-well plate magnet. Leave plate on the magnet for 5 minutes, undisturbed.
3. Remove and discard the supernatant by either:
   - firmly holding the plate **on the magnet** and inverting and flicking the plate/magnet only once, followed by blotting of the inverted plate on a fresh paper towel or lab wipe; **or**
   - using a single-channel pipette to carefully remove supernatant from each sample well while holding the plate **on the magnet.**

   NOTE: Keep the plate in contact with the magnet at all times during this step to avoid sample/bead loss
4. Resuspend cells in 200 µL of Buffer W.
5. Immobilize the bead/cell complexes by placing the RNA Sample Plate on a 96-well plate magnet. Leave plate on the magnet for 5 minutes, undisturbed.
6. Remove and discard the supernatant by either:
   - firmly holding the plate **on the magnet** and inverting and flicking the plate/magnet only once, followed by blotting of the inverted plate on a fresh paper towel or lab wipe; **or**

- using a single-channel pipette to carefully remove supernatant from each sample well while holding the plate **on the magnet.**

NOTE: **Keep the plate in contact with the magnet at all times during this step** to avoid sample/bead loss.

7. Without disturbing the bead/cell pellets, use a single channel pipette to carefully remove remaining residual buffer from each sample well.
8. Add Buffer LH to each sample well.

   NOTE: The volume of Buffer LH is dependent upon the initial number of cells in each sample. Refer to Table 4 to determine the volume of Buffer LH to add to each sample well. For example, if starting with 20,000 cells, add 6 µL of Buffer LH to the RNA sample plate.

   Table 4. Buffer LH volume based on initial number of cells

Initial **Total** Cells (cell lines)	Initial **Total** Cells (primary cells)	Buffer LH for RNA Lysates
20,000	50,000	6 µL
50,000	100,000	12 µL

9. Pipette thoroughly to lyse cells directly on the beads. Incubate the RNA lysates for 2–3 min at room temperature.

   NOTE: Avoid creating bubbles during lysis step by setting the pipette to half the volume (e.g., 3 µL in this example of Buffer LH). Failure to do so may result in a loss of sample.

10. (Optional) If the lysate is very viscous, add an additional volume equivalent to Step 8 (e.g., 6 µL in this example) of Buffer LH.
11. Place the RNA Sample Plate on a 96-well plate magnet. Leave plate on the magnet for 5 minutes, undisturbed. **Do not discard the supernatant.**
12. Without disturbing the bead/cell pellets, carefully collect each RNA lysate/supernatant sample and transfer to a 12-well strip tube using a single channel pipette.
13. Keep the lysates on ice until you are ready to perform CodeSet (MAN-10060) or TagSet (MAN-10065) hybridization. If not using immediately, samples can be stored at -80°C.

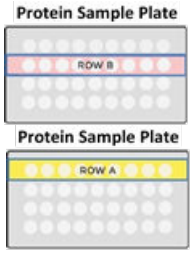
Protein Sample Preparation

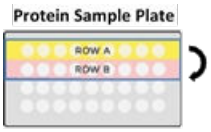
NOTES:

- This section is performed in the Protein Sample Plate.
- Avoid creating bubbles during wash steps by setting pipettes to half the sample volume.

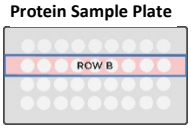
1. Immobilize the bead/cell complexes in the 70 µL of remaining sample by placing the Protein Sample Plate on a 96-well plate magnet. Leave plate on the magnet for 5 min, undisturbed.
2. Remove and discard the supernatant by either:
   - firmly holding the plate **on the magnet** and inverting and flicking the plate/magnet only once, followed by brief blotting of the inverted plate on a fresh paper towel or lab wipe, **or**
   - using a multichannel pipette to carefully remove supernatant from each sample well while holding the plate **on the magnet.**

   NOTE: Keep the plate in contact with the magnet at all times during this step to avoid sample/bead loss.
3. Add 300 µL 1X Buffer PW to Row B of the Protein Sample Plate.
4. Fix the cells by resuspending each of the bead/cell pellets in Row A in 200 µL of 1X Fix Buffer.
5. Incubate at RT for 30 min (there should be buffer and sample in Row A, and buffer only in Row B for pre-blocking).
6. Place the Protein Sample Plate on a 96-well magnet. Leave on the magnet for 5 min, undisturbed.
7. Remove and discard the supernatant from both Row A and Row B.
8. Wash the cells by resuspending each of the bead/cell pellets in Row A with 200 µL Buffer PW and transfer to Row B.
9. Place the Protein Sample Plate on a 96-well magnet. Leave on the magnet for 5 minutes, undisturbed.
10. Remove and discard the supernatant.
11. For primary cells and cell lines expressing human Fc receptors (e.g., cells expressing CD16, CD64, and/or CD32), blocking Fc receptor-mediated antibody binding is necessary to avoid increased background in the assay. **If this situation does NOT apply, proceed to Step 12.**
    a. Prepare 1X Fc Receptor Blocking Solution by diluting 65 µL of BioLegend TruStain FcX in 585 µL of Buffer PW (Extra volume is included to account for variation in pipetting).
    b. Add 50 µL 1X Fc Receptor Blocking Solution to each sample well and thoroughly resuspend beads by pipetting gently.
    c. Incubate for 10 min at room temperature.
    d. Add 150 µL Buffer PW to each sample well and proceed to Step 13.

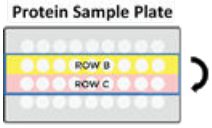




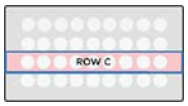
12. Resuspend cells in 200 µL of Buffer PW.
13. Incubate at for 30 min at room temperature.
14. Add 10 µL antibody mix (Ab mix) to each sample. Use a new tip for each sample.
15. Thoroughly mix the samples by pipetting gently with pipette set to half the sample volume (100 µL) and incubate for 60 min at RT.
16. Immobilize the bead/cell complex for 5 minutes on the plate magnet.
17. Remove and discard the supernatant from the Protein Sample Plate.
18. Perform a total of 4 washes as follows:
    a. Remove the plate from the magnet and add 200 µL Buffer PW to each sample well. Mix gently by pipetting.



    b. Immobilize the bead/cell complex for 5 min on the plate magnet, followed by removal and disposal of the supernatant.
    c. Repeat Steps 18a–b for a second wash.
    d. Add 200 µL Buffer PW to each sample. Mix gently by pipetting and **transfer the samples to the empty row (C).**



    NOTE: Decreased assay sensitivity may occur if this step is not performed.
    e. Immobilize the bead/cell complex for 5 min on the plate magnet, followed by removal and disposal of the supernatant.
    f. Repeat Steps 18a–b once more. No additional well transfers are necessary.



19. Without disturbing the bead/cell pellets, use a single channel carefully remove remaining residual buffer from each sample well.
20. Add Buffer LH to each sample well.

    NOTE: The volume of Buffer LH is dependent upon the initial number of cells in each sample. Refer to Table 5 to determine the volume of Buffer LH to add to each sample well. For example, if starting with 20,000 cells for RNA:Protein, add 10 µL of Buffer LH.

    Table 5. Buffer LH volume based on initial number of cells

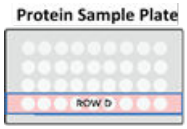
Initial **Total** Cells (cell lines and primary cells)	Buffer LH for Protein Lysates
20,000	10 µL
50,000	25 µL
100,000	50 µL

21. Pipette thoroughly to lyse cells directly on the beads.

    NOTE: Avoid creating bubbles during lysis step (by setting the pipette to half the volume e.g., 5 µL in this example of Buffer LH). Failure to do so may result in a loss of sample.

NOTE: If the lysate is very viscous, add an additional volume equivalent to Step 20 (e.g., 10 µL in this example) of Buffer LH.

22. Transfer cell lysates to a strip tube and incubate for 15 minutes at 95°C in a thermocycler with a heated lid at 100°C, and then immediately ramp down to 4°C or snap cool on ice for a minimum of 2 minutes.
23. Transfer cell lysates back to row (D) of the Protein Sample Plate.



24. Place the Protein Sample Plate on a 96-well plate magnet to immobilize the bead/cell complexes. Leave plate on the magnet for 5 minutes, undisturbed. **Do not discard the supernatant.**
25. Without disturbing the bead/cell pellets, carefully collect each protein lysate/supernatant sample and transfer to a 12-well strip tube using a single channel pipette.
26. Keep the lysates on ice until you are ready to perform CodeSet (MAN-10060) or TagSet (MAN-10065) hybridization. If not using immediately, samples can be stored at -80°C

NOTE: Denaturation of protein lysates is critical for optimal assay performance. It is not necessary to denature the RNA lysates.