nanoString TECHNOLOGIES®

nCounter® Vantage 3D™ Protein Only Solid Tumor Panel for FFPE

Protocol

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nCounter Vantage 3D Protein Only Solid Tumor Panel for FFPE Protocol

Overview

nCounter® technology can be used to detect nucleic acids of increasing variety, including mRNA, miRNA, and DNA. However, other molecules can also be detected using intermediate proxies. NanoString has developed a method for protein analysis using DNA oligonucleotides that are covalently attached to primary antibodies specific to the proteins of interest. For FFPE samples, after an overnight incubation with the primary antibodies, the DNA oligonucleotides are cleaved using a UV gel box and then collected. Each DNA oligonucleotide is then recognized by a unique Reporter probe that contains a fluorescent barcode. Reporter probes are imaged and counted by the nCounter Analysis System to provide a direct, digital readout of protein expression.

The procedures described in this document are compatible with slide-mounted FFPE tissues. **For protein analysis, we recommend a 5 µM mounted section**. Diameter will depend on the tissue type and relative expression but samples down to 1mm^2 have been analyzed using the workflow described here. Contact NanoString Support (support@nanostring.com) to receive additional assistance with this assay.

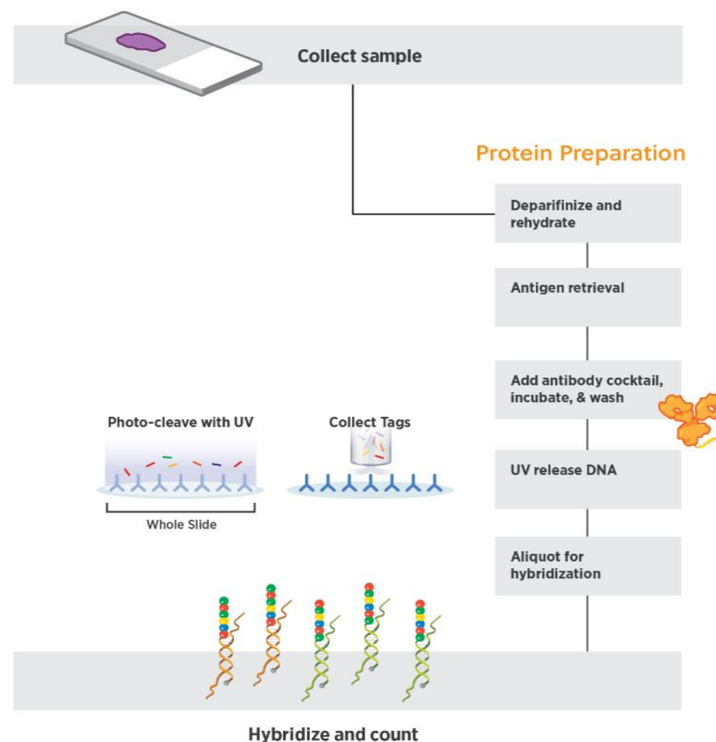

FIGURE 1. Illustration of the nCounter Vantage 3D Protein Only Solid Tumor Panel for FFPE Protocol.

Materials and Reagents

TABLE 1: Reagents provided by NanoString for the protein only assay.

Kit	Reagents
nCounter Vantage 3D Protein (D)	Protein TagSet Antibody Mix
nCounter Vantage 3D Protein (R)	Protein Plus Antibody Mix

TABLE 2: Materials required for the protein only assay.

Materials	Manufacturer	Part Number(s)
Pipettes for 5–1,000 µL	Rainin	L-1000XLS+
Filter Tips (RNase/Dnase free)	Various	Various
Benchtop Centrifuge	Various	Various
Plastic Coplin Jars	Various	Various
Thermal Cycler	Various	Various
Salmon Sperm DNA	Sigma-Aldrich	D7656
Dextran Sulfate (200kDa)	Sigma-Aldrich	67578-5G
TintoRetriever Pressure Cooker	BioSB	BSB 7008
CitriSolv	Decon Labs, Inc.	1601
Ethyl Alcohol, 200 Proof, Absolute	Various	Various
Hydrophobic Barrier Pen	Various	Various
UV gel box	Various	Various
Humidity Chamber	Various	Various
Citrate Buffer pH6	Sigma	C9999
SignalStain Antibody Diluent	Cell Signaling Technologies	8112
Tris Buffered Saline with Tween 20 (TBST-10X)	Cell Signaling Technologies	9997
Phosphate Buffered Saline with Tween 20 (PBST-20X)	Cell Signaling Technologies	9809
Goat Serum	Various	Various

Advance Preparation

1. Prepare the humidity chamber.
    a. Add damp paper towels to a plastic tray with a lid.
    b. Cover the lid with aluminum foil to minimize light exposure.
2. Prepare 10 mL of blocking buffer (1X TBST, 5% Goat Serum, salmon sperm DNA (0.1mg/mL), Dextran Sulfate (200kDa) (10mg/mL)) as follows:
    a. Add 100 mg of dextran sulfate to 9.3 mL of 1X TBST. It may require 10 minutes on a rotator to dissolve.
    b. Denature salmon sperm DNA by heating for 5 minutes at 95°C. Keep on ice when finished. (This step can be performed while waiting for the dextran sulfate to dissolve).
    c. Add 100 µL of denatured salmon sperm DNA.
    d. Add 500 µL of goat serum.
3. Prepare 10 mL of antibody diluent (signal stain antibody diluent, salmon sperm DNA (0.1 mg/mL), dextran sulfate (200kDa) (10 mg/mL)) as follows:
    a. Add 100 mg of dextran sulfate to 9.8 mL of signal stain antibody diluent (supplied as a working solution). It may require 10 minutes on a rotator to dissolve.
    b. Denature salmon sperm DNA by heating for 5 minutes at 95°C. Keep on ice when finished. (This step can be performed while waiting for dextran sulfate to dissolve).
    c. Add 100 µL of denatured salmon sperm DNA.
4. Store the prepared blocking buffer and antibody diluent at 4°C for up to 1 week until ready for use, and keep on ice during sample collection and preparation.
5. Prepare 500 mL of 95% ethanol by adding 25 mL of dH_2O to 475 mL of 100% ethanol.
6. Prepare 50 mL of 1X PBST by adding 5 mL of 10X PBST to 45 mL of dH_2O.
7. Prepare 5 L of 1X TBST by adding 500 mL of 10X TBST to 4500 mL of dH_2O. Additional TBST may be required depending on the wash container size. This formulation is in **excess** of what is required for a 12 reaction assay.

FFPE Protein Sample Preparation

NOTE:

- The antibody mix provided contains high levels of target oligonucleotide. We recommend following the precautions listed below, as failure to do so may result in high background and poor data quality.
    - We recommend the use of filter tips to avoid contamination of pipettes used in this assay.
    - Do not use the same pipet tip twice after addition of the antibody mix.
    - Change gloves often to avoid assay contamination.
- It is important that your sample remain wet throughout processing. Do not let samples dry out.

De-paraffinize and rehydrate slide-mounted FFPE tissue sections by placing the slides in a rack, and gently perform the following washes using plastic Coplin jars:

	Track Washes
1. Incubate sections in 3 washes of CitriSolv for 5 minutes each.	☐☐☐
2. Incubate sections in 2 washes of 100% ethanol for 10 minutes each.	☐☐
3. Incubate sections in 2 washes of 95% ethanol for 10 minutes each.	☐☐
4. Incubate sections in 2 washes of dH_2O for 5 minutes each.	☐☐

Epitope Retrieval:

NOTE: For antigen retrieval we use a TintoRetriever Pressure Cooker (BioSB). Any other commercial pressure cooker is likely to work, but this is the recommended model for use in this assay. We have not tried other heat-induced epitope retrieval methods with this protocol.

5. Make sure that the water in the pressure cooker is at the correct level per the manufacturer's instructions (for the TintoRetriever, above 4).
6. Place FFPE slides in a plastic Coplin jar containing 1X Citrate Buffer pH 6. Place a lid on the Coplin jar to prevent evaporation.
7. Place the Coplin jar containing the slides and covered with the lid, into the pressure cooker.
8. Attach the pressure cooker lid and run on high pressure for 15 minutes.
9. Release the pressure and carefully transfer the Coplin jar containing the slides to room temperature, remove the lid of the Coplin jar, and let stand for 25 minutes.

   NOTE: For consistency, release pressure and remove the Coplin jar from the pressure cooker when the timer reaches zero.

Primary Antibody Incubation:

10. Wash the tissues with 5 changes of 1X TBST using Coplin jars for 2 minutes each. ☐ ☐ ☐ ☐ ☐
11. Carefully blot the excess buffer from the edge of the slide with an absorbent wipe to remove excess TBST after the last wash.

    CAUTION: Do not touch the tissue or allow it to dry.

12. Draw a hydrophobic barrier around each tissue section with a hydrophobic pen. **Ensure that a complete barrier is made**.

    CAUTION: Do not touch the tissue with the hydrophobic pen.

13. Place the slide in a horizontal position and add enough of the prepared blocking buffer (see *Advance Preparation*) to completely cover the tissue.

14. Incubate with the blocking buffer for 1 hour at room temperature in a closed humidity chamber.

15. Carefully aspirate the blocking buffer.

    CAUTION: Do not touch the tissue or allow it to dry.

16. Place the tissues/slides in a rack, and gently wash with 3 changes of 1X TBST using plastic Coplin jars for 2 minutes each. ☐ ☐ ☐

    IMPORTANT: Due to the high sensitivity of this assay, it is highly recommended that you change pipette filter tips for every step, change gloves frequently, and use fresh wipes for liquid removal.

17. Make a working antibody solution by adding 64 µl of the antibody mix to 2.5 ml of the antibody diluent in a 5 mL conical tube.

18. Blot the excess buffer from the slide in Step 16 with an absorbent wipe. Place the slide in a horizontal position and cover the tissue with the diluted antibody cocktail.

    CAUTION: Do not touch the tissue or allow it to dry. Make sure the entire tissue is covered with the antibody cocktail. 100 µl of the antibody cocktail is sufficient to cover roughly 100mm^2 of tissue.

19. Incubate the slides in a closed humidity chamber overnight at 4°C. Minimize exposure to light.

UV cleavage of DNA oligonucleotides using a UV gel box:

20. Carefully aspirate the primary antibody cocktail from the slide.

    CAUTION: Do not touch the tissue or allow it to dry

21. Place the tissues in a rack, and gently wash with 3 changes of 1X TBST using Coplin jars for 10 minutes each. ☐ ☐ ☐

22. Blot the excess buffer from the slide with an absorbent wipe.

    CAUTION: Do not touch the tissue or allow it to dry

23. Place the slide in a horizontal position and cover the tissue with 100 µl of 1X TBST.

    NOTE: Make sure the entire tissue is covered with 1X TBST. Approximately 100 µl of 1X TBST is sufficient to cover roughly 100mm^2 of tissue.

24. Place slides directly, or on a clear tray, onto a UV gel box and expose to UV for 3 minutes to cleave tags from antibodies.

25. Carefully mix 1X TBST covering tissue by gently pipetting up and down a few times and then transfer to a micro-centrifuge tube.

    NOTE: If you would like to measure the areas of the tissues analyzed, save the slides, let them dry, then measure.

Denature cleaved protein:

26. Dilute the samples 1:10 in 1X PBST.

    NOTE: This dilution is necessary to prevent saturation of the nCounter assay cartridge. The optimal dilution may vary depending on tissue type, tissue size, and antibodies used. Save the remaining undiluted sample at -20°C or -80°C in order to re-run the assay if adjustments are to be made.

27. Denature the diluted sample for 3 minutes in a thermal cycler at 95°C with a heated lid at 100°C, and then immediately ramp down to 4°C or quickly cool on ice for 2 minutes. Denaturation is critical for optimal assay performance.

    NOTE: This step will denature the dsDNA oligonucleotide tags so they can hybridize to the reporters.

IMPORTANT: Check your instrument manual before proceeding to the *Vantage Protein Only Hybridization Assay*:

- If using nCounter MAX™ or nCounter FLEX™ system, only 2 µL of the denatured sample in Step 27 is required per hybridization reaction.

- If using nCounter SPRINT™ system, further dilute the denatured sample in Step 27 with an equal amount of nuclease-free water. A 2 µL aliquot of this sample is required per hybridization reaction.

Vantage Protein Only Hybridization Assay

GENERAL PROBE HANDLING INSTRUCTIONS:

- During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Protein TagSet.
- Mixing should be done by flicking or inverting the tubes.
- If using a microfuge to spin down tubes, do not spin any faster than 1,000 RCF for more than 30 seconds.
- Do not "pulse" to spin because that will cause the microfuge to go to maximum speed and may spin the TagSet out of solution.

IMPORTANT: Pre-heat the thermal cycler to 65°C with a heated lid at 70°C.

1. Remove an aliquot of Protein TagSet from the freezer and thaw at room temperature. Invert several times to mix well, then spin down reagents.

   NOTE: Inspect the thawed tube of Protein TagSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

2. Create a master mix by adding the following reagents to the tube containing the Protein TagSet:

   - 70 µL of Hybridization Buffer
   - 84 µL of RNase-free water

   Invert repeatedly to mix, then spin down master mix.
   NOTE: Do not remove the Protein TagSet from this tube. Do not add the Capture ProbeSet to the master mix.

3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge; cut the strip in half if necessary.
4. Add 13 µL of master mix to each of the 12 tubes. Use a fresh tip for each pipetting step.
5. Add 2 µL volume of the cleaved protein sample from Step 27 of *FFPE Protein Sample Preparation* to each tube.
6. Cap tubes and mix the reagents by inverting the tubes several times and flicking to ensure complete mixing.
7. Briefly spin down and immediately place the tubes in the pre-heated 65°C thermal cycler.
8. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours.

9. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

   NOTE: Selecting a fixed hybridization time followed by a ramp down to 4°C ensures equivalent hybridization time for all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, with total counts typically increasing 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation increases sensitivity by increasing counts without significantly increasing background

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FFPE MAN Changes

DC Approval/Release

Name/Signature	Title	Date	Meaning/Reason
Jamie Kuhar (JKUHAR)	Assoc Product Mgmt Intern	16 Nov 2016, 11:13:14 AM	Approved
Anna Berdine (ABERDINE)	VP, Marketing-LS & Clin Market	16 Nov 2016, 05:26:50 PM	Approved
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