Protein Processing for FFPE Samples

In this workflow, proteins will be immunostained on an FFPE-mounted tissue section. The slide will be deparaffinized followed by antigen retrieval and incubation with the antibody mix. Slides are then washed and subjected to UV light to cleave the nCounter® oligonucleotide tags from the bound antibodies. These oligo tags are then heat denatured for hybridization and nCounter analysis.

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

- Protein TagSet (D) **contains ERCC controls** and is used as 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.

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Recommended Materials

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

Table 1. Reagents provided by NanoString

<table>
<tr><th colspan="2">Reagents</th><th>Description</th><th>Storage</th></tr>
<tr><td rowspan="4">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.</td><td>Protein TagSet (D) or (R)</td><td>Panel</td><td>At or below -80°C</td></tr>
<tr><td>Antibody Mix</td><td>Oligonucleotide tags bound to antibodies</td><td>At or below -80°C</td></tr>
<tr><td>Buffer W</td><td>Wash buffer</td><td>2–8°C</td></tr>
<tr><td>Buffer T</td><td>Elution buffer</td><td>RT</td></tr>
</table>

Table 2. Required materials and reagents

Materials	Manufacturer	Part Number(s)
Pipettes for 5–1,000 µL	Various	Various
Filter tips (RNase/DNase free)	Various	Various
Benchtop centrifuge	Various	Various
Tissue-Tek® Manual Slide Set (plastic Coplin jars) or equivalent	Sakura	4451
Thermal cycler	Various	Various
TintoRetriever Pressure cooker*	BioSB	BSB 7008
D- or R-Limonene	Various	Various
100% Ethanol (EtOH), ACS grade or better	Various	Various
Hydrophobic barrier pen	Various	Various
UV gel box	Various	Various
Humidity chamber	Various	Various
10X Citrate buffer pH6	Sigma	C9999
Tris Buffered Saline with Tween® 20 (TBST-10X)	Cell Signaling Technologies	9997S
Double-distilled water (ddH2O)	Various	Various

**or similar*

Protocol Summary

Table 2. Estimated timeline for FFPE sample protein preparation. Hands-on time may be less than the processing times listed.

Step	Minimum Time Required
Advance Preparation	10 mins
Deparaffinization of Slide-mounted FFPE Tissues	65 mins
Antigen Retrieval	45 mins (15 and 25 min incubations included)
Primary Antibody Incubation	2 hours and then overnight incubation
Slide Wash and UV Cleavage of nCounter Tags	2 hours and 15 mins

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 500 mL of 95% ethanol by adding 25 mL of ddH20 to 475 mL of 100% ethanol.
3. Prepare 5 L of 1X TBST by adding 500 mL of 10X TBST to 4500 mL of ddH20. Volume is dependent on the wash container size. Additional TBST may be required.

FFPE 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 pipettes
- Change gloves often to avoid contamination
- Be careful to not touch tissue sections while handling slides
- **DO NOT** allow the tissue section to dry out

Deparaffinization of Slide-mounted FFPE Tissues

1. Deparaffinize and rehydrate FFPE tissue sections by placing the slides in a rack, and gently perform the following washes using Coplin jars.

   NOTE: These are recommended wash times. Shorter wash times may be sufficient but are not verified.

   - D- or R-Limonene: 3 washes for 5 minutes each
   - 100% ethanol: 2 washes for 10 minutes each
   - 95% ethanol: 2 washes for 10 minutes each
   - ddH20: 2 washes for 5 minutes each

Antigen Retrieval

NOTE: This protocol recommends a TintoRetriever Pressure Cooker (BioSB), although other brands should work. Other heat-induced methods have not been tested.

1. Make sure that the water in the pressure cooker is at the correct level per the manufacturer's instructions (for the TintoRetriever, above 4 cups/1 liter).
2. Place FFPE slides in a Coplin jar containing 1X Citrate Buffer pH 6. Place a lid on the Coplin jar to prevent evaporation; to prevent pressurization, do not seal the jar.
3. Place the Coplin jar containing the slides and lid into the pressure cooker.
4. Attach the pressure cooker lid and run on high pressure for 15 minutes.
5. When the timer reaches zero, release the pressure and carefully transfer the Coplin jar with slides to room temperature, remove the Coplin jar lid, and let stand for 25 minutes.

Primary Antibody Incubation

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

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.

1. Wash the slides with 5 changes of 1X TBST using Coplin jars for 2 minutes each.
2. Carefully blot the excess buffer from the edge of the slide with an absorbent wipe to remove excess TBST after the final wash.
3. Make a closed square or circular 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.
4. Place the slide in a horizontal position and add enough Buffer W to completely cover the tissue (Approximately 50–200 µl, depending on the size of the tissue).
5. Incubate with Buffer W for 1 hour at room temperature in a closed humidity chamber.
6. Thaw antibody mix on ice.
7. After the 1-hour incubation, place the slides in a rack, and gently wash with 3 changes of 1X TBST using Coplin jars for 2 minutes each.
8. Make a working antibody solution by adding 64 µl of the antibody mix to 2.4 ml of Buffer W in a 5 mL conical tube.
9. Blot the excess buffer from the slide with an absorbent wipe.
10. Cover the tissue with the diluted antibody cocktail. **Make sure the entire tissue is covered.** (Approximately 50–150 µl, depending on the size of the tissue).
11. Incubate the slides in a closed humidity chamber overnight at 4°C. Minimize exposure to light.

Overnight Stopping Point

Slide Wash and UV Cleavage of nCounter Tags

IMPORTANT: Do not touch the tissue or allow it to dry throughout this process.

1. Carefully aspirate the primary antibody cocktail from the slide.
2. Place the tissues in a rack, and gently wash with 3 changes of 1X TBST using Coplin jars for 10 minutes each. For larger tissues, add an additional wash for a total of 4 washes.
3. Blot the excess buffer from the slide with an absorbent wipe.
4. Place the slide in a horizontal position and cover the tissue with enough Buffer T to completely cover the tissue (Approximately 50–200 µl, depending on the size of the tissue).
5. Place slides directly, or on a clear tray, onto a UV transilluminator and expose to 302/312 nm UV light for 3 minutes to cleave tags from antibodies.
6. Carefully pipet the buffer on the tissue up and down a few times without creating excess bubbles and then transfer to a micro-centrifuge tube.

NOTE: To measure the areas of the tissues analyzed, allow them to dry and then measure.

Proceed to Protein-only Hybridization Setup (MAN-10059) or store at -20°C or -80°C.