Preparing Nucleic Acid from FFPE Samples for Use with nCounter Assays

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue specimens are highly valuable sources of sample material for biological assays. However, this material can often be challenging to process for downstream analyses. Many parameters of both the FFPE samples (such as storage time and conditions, fixation time, and specimen size) and nucleic acid extraction methodology can impact the quality and quantity of extracted material. This document outlines important information related to the use and extraction of nucleic acid from FFPE samples for use in nCounter assays. For information on working with fresh/frozen samples, see Preparing Nucleic Acid from Fresh Frozen Samples for Use with nCounter Assays (MAN-10051).

Factors Influencing Nucleic Acid Yield

Nucleic acid yield from FFPE samples is impacted by many factors, including time from excision to fixation, tissue type, sample age, surface area of the section, cellularity, section thickness and extraction method. It is important to take such factors into consideration when determining the amount of tissue required for any given assay.

For many sample types, sections as thin as 5 µm may be used, however, yield is generally optimal with sections that are 10–20 µm thick due to the higher percentage of intact cells in such sections.

The tissue type can significantly influence the overall cellularity of a sample, which is directly correlated with yield. It is critical to understand the cellular makeup of your tissues prior to extraction; NanoString recommends serial sections be taken for histological or pathological evaluation before and after the sections are cut to be used for RNA extraction.

A wide variety of extraction methods can be employed to isolate nucleic acid from FFPE samples. Regardless of the extraction method employed, it is important to quantify and QC extracted material prior to hybridization. This protocol uses the Qiagen AllPrep DNA/RNA FFPE Kit for copurification of DNA and RNA from a single section and provides important considerations for assessment of nucleic acid quality and yield.

Deparaffinization and Purification Protocol

NOTE: DNA/RNA Prep from FFPE takes a minimum of 5 hours to complete when using the AllPrep kit with this deparaffinization protocol.

IMPORTANT: Do not deparaffinize a slide for protein preparation using this protocol. A different protocol is outlined for this purpose in Protein Processing for FFPE Samples (MAN-10053).

Table 1. Materials for extracting and quantifying nucleic material from FFPE samples

Material	Manufacturer	Part
AllPrep DNA/RNA FFPE kit	QIAGEN	80234
CitriSolv	Decon Labs, Inc.	1601
Ethyl Alcohol, 200 Proof, Absolute	Various	Various
Glycerol	Various	Various
Agilent Bioanalyzer (or similar system)	Various	Various

1. If the starting material is FFPE **curls**, follow the instructions outlined in the AllPrep manual for purifying RNA and DNA from FFPE samples.

   If the starting material is **slide-mounted** FFPE tissue sections, proceed with Step 2.
2. Place the slides in a rack and gently performing the following washes using Coplin jars:
   a. Incubate the slides in a CitriSolv bath for 2 minutes.
   b. Change the CitriSolv bath and incubate the slides for an additional 2 minutes.
   c. Incubate the slides in a 100% ethanol bath for 2 minutes.
   d. Incubate the slides in nuclease-free water for 1 minute.
3. Air dry the slides.
4. Once the slides are dry, add enough 3% glycerol to cover the tissue in order to prevent pellet loss.
5. Wipe up excess 3% glycerol around the cell sections using an absorbent tissue.
6. Scrape the sections in a single direction on the slide with a clean razor blade to create a cohesive mass.
7. Use a small volume (< 150 µL) of Buffer PKD from the AllPrep DNA/RNA FFPE Kit to transfer the cohesive mass into a 1.5 mL tube.
8. Add Buffer PKD to the sample to bring the final volume to 150 µl total.
9. Add 10 µl proteinase K and mix by vortexing.
10. Perform the remaining post-PKD/ProK-digestion steps outlined in the AllPrep DNA/RNA FFPE Kit instructions to purify RNA and DNA.
11. Extracted nucleic acid should be stored at -80°C.

Optional Stopping Point

Quantifying Purified RNA and Assessing Quality

Quantify RNA using fluorescence (e.g., Bioanalyzer, Qubit, etc.) or spectrophotometry (e.g., Nanodrop) methods. Note that quantification of dilute material via spectrophotometry (below ~20 ng/µL) should be interpreted with caution and absorbance readings should be examined. Quantification tends to be most accurate when the A260/280 and A260/230 ratios are high:

- The A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample as aromatic proteins have a strong UV absorbance at 280 nm. For pure RNA and DNA, A260/280 ratios should be ~2.1 for DNA and ~1.8 for RNA. A lower ratio indicates likely protein contamination, which may artificially inflate RNA quantity measurements.
- The A260/230 ratio indicates the presence of organic contaminants, such as (but not limited to): phenol, TRIzol, chaotropic salts and other aromatic compounds. Samples with 260/230 ratios below 1.8 typically have a significant amount of these contaminants and these may interfere with downstream applications involving enzymes, such as amplification. In a pure sample, the A260/230 should be close to 2.0.

Protocol

1. Evaluate RNA quality using a fragment analysis system to measure nucleic acid fragmentation. NanoString recommends that at least 50% of the sample be greater than 300 nucleotides (nt) in length for optimal performance. RNA samples that exhibit greater levels of fragmentation may still be used, but input levels may need to be increased (see below).

   Appropriate input may be estimated with the following equation: (100/percent of sample >300 nt) x 100 ng. The percent of samples greater than 300 nt can be estimated by having the BioAnalyzer or Tape Station calculate the percent of the sample between 50-300nt and subtracting that quantity from 100%. This calculation is a tool to help estimate ideal input, but not a complete predictor of success; it is less predictive in samples with less than 25% of fragments greater than 300 nt and samples with extremely low concentration (<10 ng/µL).

2. For most nCounter applications, sample input volumes are 5 µL and a range of 25–300 ng, starting with 100 ng, is recommended. As such, purified RNA samples should have a minimum concentration of 20–60 ng/µL. For samples that are more dilute, concentration may be performed by column concentration (such as Amicon Ultra YM-3, 3000 kDa MWCO by Millipore) or ethanol precipitation. For samples that have less total RNA abundance, amplification may be required prior to inclusion in an nCounter hybridization. In such cases, as little as 10 ng (2 ng/µL) of RNA from FFPE may be used.

   NOTE: See MAN-10046 for additional information on the use of the nCounter Low RNA Input Amplification Kit.

3. Store purified RNA at **-80°C**.

Quantifying Purified DNA and Assessing Quality

1. Quantify DNA using a Qubit dsDNA HS Assay Kit and Qubit Fluorometer or a comparable PicoGreen fluorescence-based method. Quantification by spectrophotometry is prone to inaccurate and overestimated measures of DNA concentration.
2. Evaluate DNA quality using a fragment analysis system to measure nucleic acid fragmentation.
    a. The peak of the fragmented DNA profile should be greater than 200 base pairs (bp). DNA Integrity Numbers (DIN) ranging from 1.7–6.9 have all yielded accurate SNV detection from this workflow. However, samples with a DIN of 3 or lower may require additional PCR cycles in amplification; samples of this low quality are less likely to yield reliable results.
    b. Other methods to determine the quality of FFPE-derived gDNA use qPCR methods to measure the relative amplifiability of the DNA. If you choose to evaluate the DNA quality of your samples in this manner, choose a kit that measures whether 200 bp amplicons can be robustly amplified.
3. Create dilute working stocks of DNA. A sample input of 5 ng is required in a maximum volume of 5 µL. For ease of reaction set-up, dilute to 2.5 ng/µL (2 µL per reaction) in nuclease-free water or TE.
4. Store purified DNA at **-80°C**.