nCounter

Preparing RNA from FFPE Samples

User Manual

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MAN-10050-05 FEB 2021

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Contents

Introduction 2
Recommended Materials 3
Thermal Cycler Guidelines 4
Sample Input Recommendations for Isolated RNA 5
Factors Influencing RNA Yield 5
Guidelines: Quantifying Purified RNA and Assessing Quality 6
Protocol: FFPE Sample Deparaffinization and Purification 8
Trademarks 9
Contact Information 9

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 RNA and Lysates from Fresh Frozen Samples (MAN-10051).

Recommended Materials

Table 1 lists materials and equipment necessary to run nCounter® XT Assays. Table 2 lists recommended materials for RNA purification.

Table 1. Materials recommended for all nCounter assays

Materials/Equipment	Manufacturer	Part Number(s)
NanoDrop **OR** Qubit Fluorometer*	Thermo Fisher	Various
Bioanalyzer® 2100*	Agilent	G2939BA
Thermal cycler with a programmable lid	Various	Various
Microcentrifuge or picofuge	Various	Various
Pipettes for 0.5–10, 2–20, 20–200 µL*	Rainin	Various
Disposable gloves	Various	Various

** Equivalent products from another manufacturer are acceptable*

Contact NanoString Support with questions about the compatibility of products not listed here.

Table 2. Additional materials recommended for gene expression assays using total RNA (standard protocol)

Material	Manufacturer	Part Number(s)
RNeasy Mini Kit (or an equivalent kit from another manufacturer)	QIAGEN®	74104 or 74106

IMPORTANT: NanoString highly recommends verifying the integrity of total RNA samples via denaturing PAGE or Bioanalyzer before proceeding with hybridization.

IMPORTANT: All assays require PCR tubes to perform the sample hybridization reaction. Ensure that these tubes meet the guidelines provided by the thermal cycler manufacturer. Strip tubes may be helpful, but individual tubes may also be used.

While any thermal cycler-compatible tube will work for hybridization, those tubes will **not** work for the Prep Station. Any hybridizations done in non-NanoString-supplied strip tubes **must** be transferred to the strip tubes supplied in the Master Kit.

Thermal Cycler Guidelines

Thermal cyclers are produced by a wide variety of manufacturers and possess a wide variety of features. NanoString recommends using a model with a programmable heated lid. Models without programmable lids may reach a very high temperature that causes tubes to melt or deform. However, programmable lids may offer different levels of control.

- Ideally, NanoString recommends a thermal cycler with a heated lid that can adjust throughout the protocol. The heated lid should be set to 5°C greater than the current incubation temperature at any moment.
- Some heated lids cannot be assigned a floating temperature. In this situation, program the heated lid to be 5°C greater than the maximum temperature during the protocol. The heated lid should not exceed 110°C.

Sample Input Recommendations for Isolated RNA

The nCounter Analysis System and nCounter SPRINT Profiler utilize different methods for sample processing and digital imaging, although the underlying nCounter chemistry is unchanged. NanoString recommends using 50% less sample for assays performed on the nCounter SPRINT Profiler compared to the nCounter Analysis System to avoid saturation of the imaging surface, which can reduce data quality.

Use Table 3 to determine the recommended sample input for most assays included in this manual. (These recommendations do not apply to the RNA:Protein assay, which is optimized for cell number. For RNA:Protein, refer to RNA:Protein Hybridization Setup). These recommendations apply to sample mass only; sample volume does not vary between systems.

Table 3. Recommended sample input mass for nCounter XT assays

Sample Type	nCounter® Analysis System (MAX/FLEX)	nCounter® SPRINT Profiler
FFPE-derived RNA	300 ng	150 ng
Low Input Material (see MAN-10046 for sample prep guidance)	up to 8 µL of amplified sample	up to 5 µL of amplified sample

Factors Influencing RNA Yield

RNA yield from FFPE samples is impacted by many factors, including time from excision to fixation, fixation time, 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 larger 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 nucleic acid extraction.

A wide variety of extraction methods can be employed to isolate RNA from FFPE samples. Regardless of the extraction method employed, it is important to quantify *and* assess the quality of extracted material prior to hybridization. This protocol uses the Qiagen RNeasy Mini Kit and provides important considerations for assessment of RNA quality and yield.

Guidelines: Quantifying Purified RNA and Assessing Quality

Assess RNA quality using a Fragment Analyzer (e.g., Bioanalyzer) and RNA quantity using fluorescence (e.g., Qubit Fluorometer) or spectrophotometry (e.g., Nanodrop) methods. While fluorometric assays usually provide more accurate results, NanoString input recommendations refer to spectrophotometric (Nanodrop) readings. We recommend determining the 260/280 and 260/230 OD ratios from the spectrophotometer results to assess the purity of the isolated RNA (see below).

> **NOTE:** Quantification of dilute material (below **~20 ng/µL**) via spectrophotometry should be interpreted with caution. Fluorescent-based quantification methods yield more accurate results in these situations.

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, A260/280 ratios should be ~2.1. 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 organic 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.
- 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 200 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 > 200 nt) x 100 ng (or 50 ng for SPRINT)

  The percent of samples greater than 200 nt can be estimated by having the BioAnalyzer or Tape Station calculate the percent of the sample between 50–200 nt 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 200 nt and samples with extremely low concentration (**<10 ng/µL**). Based on the estimations, we also recommend grouping the input amounts in reasonable buckets (e.g., 100 ng/125 ng/150 ng/200 ng), instead of trying to use precise ng values for each sample.

- For most nCounter applications, sample input volumes are **5 µL** and a range of **50–300 ng** for MAX/FLEX **or 25–150 ng** for SPRINT. Starting with **100 ng** for MAX/FLEX or **50 ng** for SPRINT 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 the Amicon Ultracel-3 3000 kDa MWCO or the 3000 kDa MWCO by Millipore), ethanol precipitation, or SpeedVac if no downstream enzymatic steps are required. 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** (**2.5 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 Kit.

- Store purified RNA at -80°C.

Protocol: FFPE Sample Deparaffinization and Purification

NOTE: RNA Prep from FFPE takes a minimum of 5 hours to complete when using the Qiagen RNeasy Mini 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 4. Materials for extracting and quantifying nucleic material from FFPE samples

Material	Manufacturer	Part
Qiagen RNeasy Mini Kit	QIAGEN	73504
D- or R-Limonene	Various	Various
100% Ethyl Alcohol (EtOH), ACS grade or better	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 Qiagen RNeasy Mini Kit manual for purifying RNA 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:

   - ☐ D- or R-Limonene: 2 minutes
   - ☐ New bath of D- or R-Limonene: 2 minutes
   - ☐ 100% ethanol: 2 minutes
   - ☐ Nuclease-free water: 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 Qiagen RNeasy Mini 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 Qiagen RNeasy Mini Kit instructions to purify RNA
11. Extracted nucleic acid should be stored at -80°C.

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