Preparing Nucleic Acid from Fresh/Frozen Samples for Use with nCounter Assays

Introduction

Many nCounter assays are compatible with both extracted nucleic acid or cell lysates. This document outlines important information related to the use of fresh/frozen samples in nCounter assays, including:

- extraction recommendations
- nucleic acid QC information
- lysis buffer recommendations
- sample input recommendations

Quantifying Purified RNA Concentration and Assessing Quality

Quantify RNA using fluorescence (e.g., Bioanalyzer) 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 close to the ideal (see below).

- 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 that 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 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 50-300 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 300 nt and samples with extremely low concentration (<10 ng/uL).

2. For most nCounter applications, sample input volumes are 5 µL and a minimum of 100–300 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 1 ng (2 ng/µL) of RNA from fresh/frozen 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 provide 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 total sample input of 5 ng will be used in a maximum 5 µL total volume. 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**.

Whole Cell Lysates

NanoString recommends a minimum of 5,000 to 10,000 cells (or cell equivalents) per hybridization reaction for measuring gene expression, depending on cell type. Some cell types, such as freshly isolated immune cells, may require up to 20,000 to 30,000 cells for adequate input. The required number of cells for any given application will ultimately be dependent on the abundance of the mRNA targets of interest in the sample to be assayed and should be determined empirically by the end user for their biological system. Furthermore, the maximum sample input when using cell lysates depends on the type of lysis buffer used.

Detergent-based lysis buffers, such as Buffer LH that do not contain chaotropic salts are fully compatible with nCounter hybridization reagents; as much as 5 µl may be added to each hybridization reaction. Other lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics and are compatible with nCounter reagents with some modifications to protocol. These include Buffer RLT and other buffers with a high concentration of guanidine isothiocyanate. NanoString recommends using no

more than 1.5 µL of these lysis buffers per nCounter hybridization reaction. For this reason, NanoString recommends the use of Buffer RLT only for applications in which cells can be pelleted to achieve a minimum cell concentration of 3,500–6,500 cells per µL.

> **NOTE:** See MAN-10054 for additional information on measuring RNA directly from concentrated detergent-free protein lysates for 3D Biology™ Applications.

Table 1. Recommended sample lysis concentrations

Initial Number of Cells	Recommended Lysis Buffer	Recommended Lysis/Sample Concentration	
		MAX/FLEX	SPRINT
50,000 cells or more	RLT or other buffer with a high concentration of guanidine isothiocyanate	~6,500 cells/µL	~3,500 cells/µL
50,000 cells or less	iScript™ RT-qPCR Sample Preparation Reagent Buffer LH or other detergent/chemical lysis buffer	~2,000 cells/µL	~1,000 cells/µL

To prepare cell lysates, NanoString recommends following guidance provided by the lysis buffer supplier. Table 4 contains a list of suggested lysis buffers and associated catalog numbers.

Table 2. Suggested lysis buffers

Lysis Buffer	Supplier	Catalog Number
Buffer LH	NanoString	122000001
iScript RT-qPCR Sample Preparation Reagent	BioRad	170-8899
Cells-to-CT	Life Technologies	4391851C
Buffer RLT	QIAGEN	79216

Preparing Cell Lysates with Non-chaotropic Buffers

For applications involving small numbers of initial cells, NanoString recommends sorting directly into a chemical- or detergent-based buffer (such as Cells-to-Ct) to maximize the concentration of cells in the lysate (up to ~2,000 cells/µL). NanoString does not recommend using a chemical- or detergent-based buffer at concentrations >2,000 cells/µL because this may result in incomplete cell lysis. Additionally, it is important to remove growth medium from cells as it may inhibit lysis and result in reduced assay performance.

Preparing Cell Lysates with Chaotropic Buffers

To prepare cell lysates with Buffer RLT (QIAGEN) or other buffers containing guanidine isothiocyanate, NanoString recommends following the guidance provided in the QIAGEN RNeasy® protocol (see Important Notes, pp 16–27, of the RNeasy Mini Handbook v.06/2012). For most mammalian cell lines grown in tissue culture, the basic steps are:

1. Harvest an appropriate number of cells, and pellet by centrifugation for 5 minutes at 300 RCF in a microcentrifuge tube. Carefully remove all supernatant by aspiration. Failure to remove all supernatant may dilute lysis buffer and result in incomplete cell lysis.

2. Disrupt cells by adding QIAGEN Buffer RLT. Addition of ß-mercaptoethanol to RLT is optional but may improve RNase inactivation in cell lines expressing high levels of RNase. Use 10 µL ß-mercaptoethanol per 1 mL RLT. NanoString does not recommend lysis of highly concentrated material (i.e., > 20,000 cells/µL) as this may result in incomplete lysis and reduced assay performance.
3. Homogenize cells by vortexing for 1 minute. Centrifuge briefly to recover all material to bottom of tube. (It is not necessary to centrifuge cellular debris and remove the supernatant. Hybridization can be performed using the complete lysate.)
4. Proceed immediately to hybridization (using no more than 1.5 µL lysate in each hybridization reaction) or freeze lysate at -80°C.