nCounter

Preparing RNA from Fresh Frozen Samples

User Manual

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nanoString®

Preparing RNA and Lysates from Fresh/Frozen Samples

Contents

Introduction 2
Materials 3
Sample Input Recommendations for Isolated RNA 4
Guidelines: Quantifying Purified RNA and Assessing Quality 4
Whole Cell Lysates Guidelines 6
Preparing Cell Lysates with Non-chaotropic Buffers 8
Preparing Cell Lysates with Chaotropic Buffers 9
Trademarks 11
Contact Information 11

Introduction

Many nCounter® assays are compatible with both extracted RNA and cell lysates. This document outlines important information related to preparing isolated RNA or lysates from fresh/frozen samples to be used in nCounter assays. For information on working with FFPE samples, see MAN-10050, Preparing Nucleic Acids from FFPE Samples.

Materials

Table 1 lists equipment that is needed to run nCounter® XT RNA Assays. Materials are recommended for RNA purification in Table 2. Information for cell lysates is provided in Whole Cell Lysates Guidelines.

Table 1. Equipment needed for all nCounter assays

Equipment	Manufacturer	Part Number(s)
NanoDrop **OR** Qubit Fluorometer*	Thermo Fisher	Various
Bioanalyzer 2100*	Agilent	G2939BA
Microcentrifuge or picofuge	Various	Various
Thermal cycler with a programmable lid	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*

Table 2. Materials recommended for gene expression assays using total RNA (standard protocol) or crude cell lysates as input

Material	Manufacturer	Part Number(s)
RNeasy Mini Kit*	QIAGEN	74104 or 74106
Proteinase K (20 mg/ml) solution	Various	Various
iScript RT-qPCR Sample Preparation Reagent	BioRad	170-8899
Cells-to-CT	Thermo Fisher	4391851C
Buffer RLT	QIAGEN	79216

** Equivalent products from another manufacturer are acceptable*

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.

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 MAX/FLEX 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 (these recommendations do not apply to the RNA:Protein assay, which is optimized for cell number). With the exception of low input, these recommendations apply to sample mass only; sample volume does not vary based on Instrument. The recommended input is generalized for most cases and is intended to be used as a starting point.

Table 3. Recommended sample input mass for nCounter XT assays

Sample Type	nCounter® Analysis System (MAX/FLEX)	nCounter® SPRINT Profiler
Total RNA	100 ng	50 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

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 ~2.0 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 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 Millipore 3000 kDa MWCO), 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 Amplification Kit.

- Store purified RNA at -80°C.

Whole Cell Lysates Guidelines

For most cell types, NanoString recommends a minimum of 5,000 to 20,000 cells (or cell equivalents) per hybridization reaction for measuring gene expression. Some cell types, such as freshly isolated immune cells, may require 20,000 to 30,000 cells per hybridization reaction 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 volume when using cell lysates depends on the type of lysis buffer used. Cell type and number play an important role when choosing which buffer to use. Use the flow chart below (Figure 1) to determine the appropriate lysis buffer and lysis protocol for your sample preparation.

> **NOTE:** For a successful run, it is important to accurately count cells. NanoString strongly recommends either manually counting your cells with a hemocytometer or determining a scaling factor for your cell counter. Some automated cell counters can falsely inflate the true cell number in a sample resulting in lower-than intended input and poor-quality data.

> **NOTE:** Fixation alters the integrity and quality of nucleic acids. To ensure success of gene expression, lysis of fixed cells will require digestion with proteinase K and incubation at high temperature to reverse the cross linking. contact support@nanostring.com for more information.

The list of suggested lysis buffers found in Figure 1 and their associated catalog numbers are listed here in Table 4.

Table 4. Suggested lysis buffers*

Lysis Buffer (Non- Chaotropic)	Supplier	Catalog Number
iScript RT-qPCR Sample Preparation Reagent	BioRad	170-8899
Cells-to-CT	Thermo Fisher	4391851C
Lysis Buffer (Chaotropic)	**Supplier**	**Catalog Number**
Buffer RLT	QIAGEN	79216

Proteinase K in Table 2 is recommended **during the hybridization step with all lysis buffers in order to degrade any residual protein which has been collected along with the supernatant. Do not add proteinase K to the lysis buffer as this can make it more difficult to pellet the cells after lysis and thus increase the amount of cell debris in the lysate supernatant.*

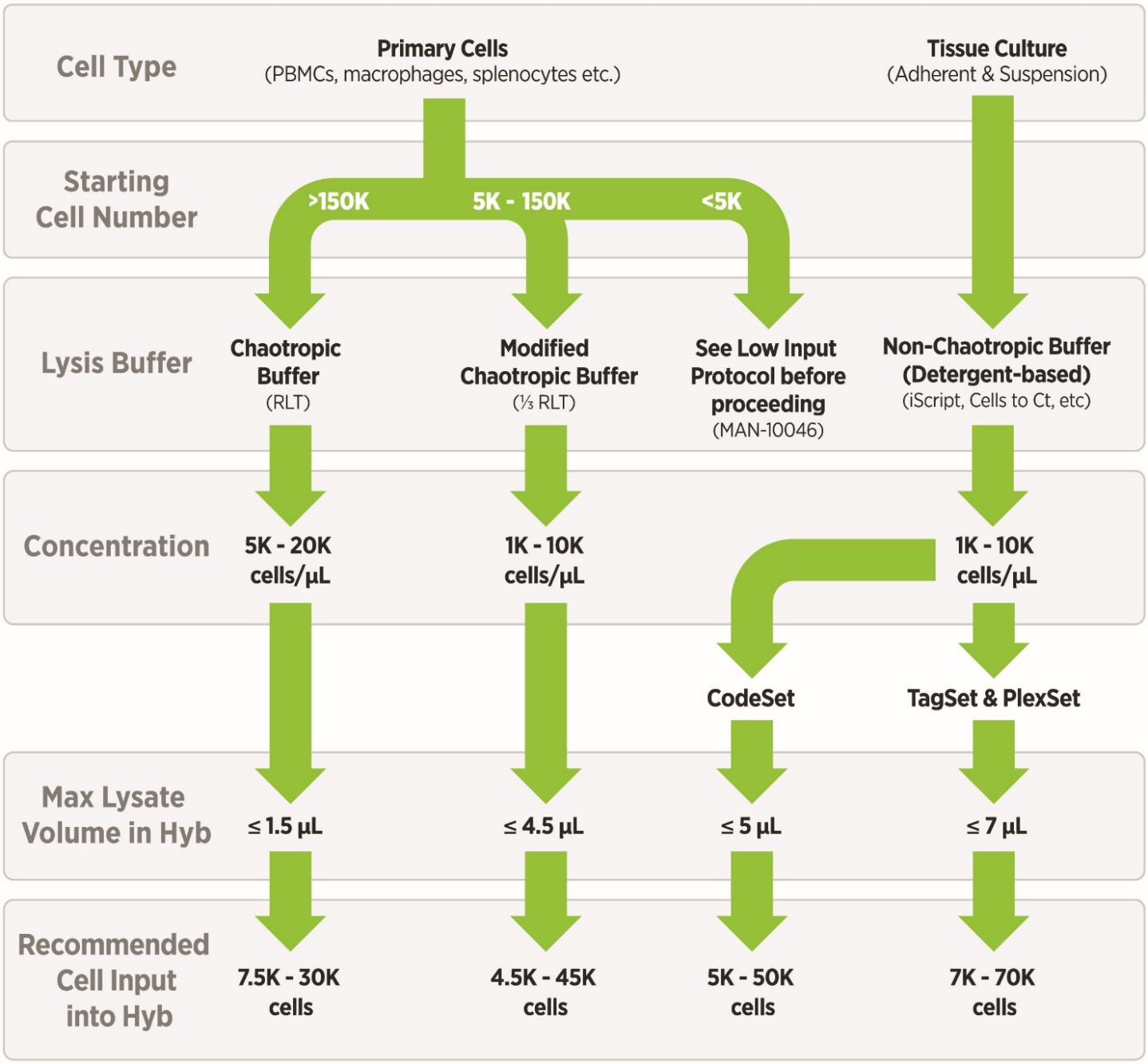

Figure 1. Lysate workflow

Preparing Cell Lysates with Non-chaotropic Buffers

NanoString recommends lysing cells using a chemical- or detergent-based buffer (such as iScript or Cells-to-Ct) for applications involving tissue culture cells (adherent and suspension). These buffers are non-chaotropic, as they do not contain chaotropic salts, making them fully compatible with nCounter hybridization reagents. These buffers should be used such that final cell lysate concentrations are between **1,000–10,000 cells/µL**. Lysate concentrations greater than **10,000 cells/µL** may result in incomplete cell lysis. Up to **5 µL** of lysate may be added to CodeSet hybridization reactions (up to **7 µL** of lysate may be added for TagSet or PlexSet reactions) (Figure 1).

Non-chaotropic buffers are also compatible with the Low RNA Input Kit and can be added to small numbers of primary and tissue culture cells (under **500 cells/µL**) or cells can be sorted directly into lysis buffer. For more information, refer to the Low RNA Input Kit user manual (MAN-10046) or contact support@nanostring.com.

Cell Lysate Protocol for Cultured Cells Using Non-chaotropic Buffers

It is important to start with an optimal number of cells to achieve the minimum cell numbers recommended for cell lysate.

1. Count your cells.
2. For **adherent cells**, skip **Steps 3 and 5**. For **suspension cells**, centrifuge cells in a 96-well round bottom plate or microcentrifuge tube at 500x*g* for 5 minutes @ 4°C.
3. Remove media by tilting plate and carefully aspirating media with a vacuum. Make sure to aspirate as much of the media as possible without disturbing the cell pellet. Residual media can dilute lysis buffer leading to incomplete lysis.
4. Add lysis buffer to each well/tube.

   IMPORTANT: To prevent bubbles, which could lead to incomplete lysis, **DO NOT** go to second pipette stop and **DO NOT** pipette up and down at this step.

5. Set pipette to half the volume of lysis buffer added. Pipette up and down slowly 10 times per sample while avoiding bubble formation. Change pipette tips with each row of cells to prevent cross-contamination.
6. Incubate lysates at room temperature for 5 minutes.
7. Place lysates on ice to use immediately or freeze at -80°C for long-term storage.
8. Use up to the 'Max Hyb Volume' of lysate recommended for your assay in each NanoString hybridization reaction (see Figure 1).

   NOTE: When preparing the hybridization, add Proteinase K to hybridization Master Mix at a final concentration of **200 µg/mL** (For 20 mg/ml Proteinase K solution, add **2.1 µL** into the 14-reaction hybridization Master Mix).

Preparing Cell Lysates with Chaotropic Buffers

As primary cells do not lyse efficiently in non-chaotropic buffers (except at low concentration, see above), complete lysis can be achieved using lysis buffers that contain chaotropic salts. These include Buffer RLT (QIAGEN) and other buffers with high concentrations of guanidine isothiocyanate. These buffers may alter nucleic acid hybridization thermodynamics and therefore some modifications to the protocol are necessary. NanoString recommends adding no more than **1.5 µL** of **cells lysed with chaotropic buffers** to each nCounter hybridization reaction. For this reason, NanoString only recommends the use of Buffer RLT (or other chaotropic lysis buffers) for applications in which cells can be pelleted to achieve a minimum cell concentration of **5,000 cells/µL**. When using lower cell numbers, the RLT protocol can be modified by diluting 1 part RLT buffer with 2 parts water. This allows complete lysis of cells up to **10,000 cells/µL** and up to **4.5 µL** can be loaded to the hybridization reaction (see detailed protocol below).

To prepare cell lysates with Buffer RLT, or other buffers containing guanidine isothiocyanate, NanoString recommends following the guidance provided in the QIAGEN RNeasy® protocol (see Important Notes, page 16–27, of the RNeasy Mini Handbook v.06/2012). For most mammalian cell lines or cells freshly isolated from tissue, follow the basic steps below. For FACS-sorted cells, contact support@nanostring.com for more guidelines.

Cell Lysate Protocol for Primary Cells with Total Starting Cell Number >150,000 per sample)

1. Harvest an appropriate number of cells, and pellet by centrifugation at 400x*g* for 8 minutes @ 4°C in a microcentrifuge tube or a 96-well round bottom plate. Carefully remove all supernatant by aspiration.

   IMPORTANT: 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 cells expressing high levels of RNase. Use 10 µL ß-mercaptoethanol per 1 mL RLT. NanoString recommends lysing cells to achieve a final concentration of **5,000–20,000 cells/µL**. Highly concentrated material (i.e., **>20,000 cells/µL**) may result in incomplete lysis and reduced assay performance.

3. Homogenize cells by vortexing for 1 minute or pipetting up and down 15–20 times (avoid making bubbles by setting your pipette to half the lysis volume). Centrifuge briefly to recover all material to bottom of tube. (It is not necessary to pellet 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.

   NOTE: When preparing the Hybridization, add Proteinase K to hybridization master mix at a final concentration of **200 µg/mL** (For 20 mg/ml Proteinase K solution, add **2.1 µL** into the 14-reaction hybridization master mix).

Cell Lysate Protocol for Primary Cells with Starting Total Cell Number <150,000 per sample

To overcome low sample volume limitations when using chaotropic buffers we have modified the RLT protocol such that cells can be lysed in 1/3 RLT which allows up to **4.5 µL** of the total lysate to be loaded into the hybridization reaction. This protocol is recommended when total number of cells available is less than 150,000. Higher number of cells can be used but lysis volume will need to be adjusted accordingly. For flow cytometry sorted cells, contact support@nanostring.com for additional guidelines.

> **NOTE:** for <5,000 cells, please see the Low RNA Amplification Kit user manual (MAN-10046) before proceeding.

Prior to the start of the lysis experiment, dilute 100% RLT Buffer 1/3 in nuclease-free water. To allow efficient lysis, lysate concentration should not exceed **10,000 cells/µL**. It is important to start with an optimal number of cells so that the final concentration of cells will allow appropriate amount of input to be loaded on to each reaction at maximum volume of **4.5 µL** of cell lysates (see Figure 1).

1/3 Diluted RLT Recipe = 1 part RLT Buffer to 2 parts nuclease-free water

Example: 3 mL RLT Buffer + 6 mL nuclease-free water

1. Spin cells in a microcentrifuge tube or a 96-well round bottom plate at 400x*g* for 8 minutes @ 4°C.
2. Remove media by tilting plate or microcentrifuge tube and carefully aspirating media with a vacuum. Make sure to aspirate as much of the media as possible without disturbing the cell pellet.

   > **IMPORTANT:** Failure to remove all supernatant may dilute lysis buffer and result in incomplete cell lysis.

3. Add 1/3 diluted RLT Buffer to each well or tube to a final concentration of **1,000–10,000 cells/µL**. For a 96-well plate, use a multichannel pipette.

   > **IMPORTANT:** To prevent bubbles, which could lead to incomplete lysis, **DO NOT** go to second pipette stop and **DO NOT** pipette up and down at this step.

4. Set pipette to half the volume of lysis buffer added. Return pipette to the same wells with the lysis buffer and pipette up and down 15 times per sample while avoiding bubble formation. Change pipette tips with each row of cells to prevent cross-contamination.
5. Place lysates on ice to use immediately or freeze at -80°C for long term storage.
6. Use up to **4.5 µL** of lysate in each NanoString hybridization reaction.

   > **NOTE:** When preparing the hybridization, add Proteinase K to hybridization master mix at a final concentration of **200 µg/mL** (For 20 mg/mL Proteinase K solution, add **2.1 µL** into the 14-reaction hybridization master mix).

   > **NOTE:** Use of more than **4.5 µL** of cells lysed with 1/3 Diluted RLT can adversely affect results. Lysis buffers that contain chaotropic salts may alter nucleic acid hybridization thermodynamics.

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

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