nCounter Low RNA Input Amplification Kit

The nCounter Low RNA Input Amplification Kit is designed to produce sufficient target for detection in an nCounter hybridization assay. This multiplexed target enrichment (MTE) is achieved via a two-step process: input RNA is converted to cDNA, which is then amplified using target-specific primers. After MTE enrichment, samples can be directly analyzed with nCounter hybridization reagents that assay target sequences internal to the corresponding MTE primers. No clean-up or dilution of the enriched sample is required prior to analysis, simplifying the workflow and maximizing sensitivity.

Contents

Materials and Equipment

Table 1. Materials provided with the nCounter Low RNA Input Amplification Kit

Kit	Reagents	Description	Storage
nCounter Low RNA Input Amplification Catalog #: LOW-RNA-48, See specific Primer Pool	10X RT Enzyme Mix	Contains reverse transcriptase	-20°C ± 2°C
	10X RT Primer Mix	Contains primers for RT	-20°C ± 2°C
	5X dT Amp Master Mix	Contains amplification polymerase and buffer	-20°C ± 2°C

Table 2. Additional materials required (not provided)

Item	Catalog Number	Manufacturer
Buffer LH	NanoString	PS-GX-BLH
Primers	Various	IDT
Pipettes for 10–1,000 µL*	Various	Various
Manual multi-channel pipette for 200 µL*	L12-200XLS+	Rainin
12-strip standard tubes*	T-3034-1	BioExpress

**Alternative products can be used if they offer similar function and reliability.*

Table 3. Equipment required for use with the nCounter Low RNA Input Amplification Kit

Equipment
NanoString nCounter SPRINT, nCounter MAX, or nCounter FLEX Analysis System
Microfuge or picofuge
Pipettes (p1000, p100, p20, p10)
Calibrated thermal cycler with heated lid

Thermal Cycler Guidelines

Please note that a thermal cycler **with a heated lid** is required for this protocol. 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.

Important Probe Handling Instructions

- During setup, do not vortex or pipette vigorously to mix.
- Mixing should be done by flicking or inverting the tubes.
- If using a microfuge to spin down tubes, do not spin any faster than 1,000xg for more than 30 seconds.
- Do not "pulse" to spin because that will cause the centrifuge to go to the maximum speed and may spin the probes out of solution.

Primers

Primers pools are available for many NanoString Gene Expression panels. Please visit the associated panel product page at www.nanostring.com for additional information.

Upon request, NanoString will provide MTE primer designs flanking the target sequences for custom CodeSets. As part of the CodeSet design process, NanoString can also provide sufficient information to design alternate primers if desired. For additional information or questions about primer design, please contact NanoString Support at support@nanostring.com.

MTE primers should be pooled at a final concentration of 500 nM per oligo in TE Buffer (pH 7.5). NanoString has partnered with Integrated DNA Technologies (IDT) to provide pre-pooled MTE primer sets at the recommended concentration of 500 nM per oligo.

Contact NanoString Support (support@nanostring.com) to receive additional assistance with this assay.

Sample Type Considerations

The nCounter Low RNA Input Amplification Kit is compatible with RNA obtained from a variety of sources, including formalin-fixed, paraffin-embedded (FFPE) samples. To ensure optimal performance with nCounter hybridization reagents in downstream assays, NanoString recommends utilizing the per reaction input amounts shown in Table 4:

Table 4. Sample Input Recommendations

Sample Type	Minimum Recommended Input Amount	
	Mass	Concentration
Purified Fresh/Frozen RNA	1 ng	0.25 ng/µL
Purified FFPE RNA	10 ng	2.5 ng/µL
Cell Lysate	1 ng (or ~100 cells)	200 cells/µL

FFPE

Successful amplification requires RNA fragments of sufficient length. As FFPE-derived RNA is often degraded, NanoString recommends evaluating RNA quality via an Agilent Bioanalyzer (or similar system) prior to amplification. Best results will typically be achieved with RNA samples for which at least 50% of the RNA fragments are 200 nucleotides in length or greater.

Cell Lysates

For applications involving small numbers of initial cells, such as flow-sorting, NanoString recommends sorting directly into a chemical- or detergent-based buffer (such as iScript or Cells-to-Ct) to maximize the concentration of cells in the lysate (up to ~2,000 cells/µL).

- Using a chemical- or detergent-based buffer at concentrations > 2,000 cells/µL is not recommended as this may result in incomplete cell lysis.
- It is important to remove growth medium from cells as it may inhibit lysis and result in reduced assay performance.
- High concentrations of lysis buffer can inhibit the reverse transcription and subsequent amplification steps. **It is important that lysis buffer make up no more than 10% (or 0.5 µL) of the 5 µL RT reaction volume.**

The nCounter Low RNA Input Amplification Kit is incompatible with lysates prepared with chaotropic buffers, e.g. buffers containing guanidine salts, such as RLT.

Low RNA Input Amplification Protocol

cDNA Conversion

IMPORTANT: Keep all components, and set up reactions, on ice during processing unless otherwise specified. Up to 4 µL of sample may be used per reaction. If sample dilution is required, dilution in RNAse free H_20 is recommended.

1. Add up to 4 µL of diluted sample to each well of a strip tube.
2. Make a RT master mix by combining the components as shown below in an RNAse-free 0.5 mL tube. Mix by gently flicking the tubes, followed by brief centrifugation.

Table 5. RT Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
10X RT Enzyme Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL
10X RT Primer Mix	0.5 µL	8 µL	15 µL	22 µL	30 µL

3. Add 1 µL RT master mix to each sample.
4. Cap tubes and gently flick to mix. Briefly spin down at less than 1,000 RPM.
5. Place strip tubes in a thermal cycler with a heated lid and run the following protocol:

Table 6. cDNA Conversion Program

Step	Temperature	Time
Primer anneal	25˚C	10 min
First strand cDNA synthesis	42˚C	60 min
Enzyme inactivation	85˚C	5 min
Hold	4˚C	Forever

6. Proceed to multiplexed target enrichment. Keep reactions at 4˚C if proceeding directly. For long term storage, cDNA should be stored at -80˚C.

NOTE: Up to 1 µL of the RT reaction may be reserved for troubleshooting purposes.

Multiplexed Target Enrichment

1. Make an amplification master mix by combining the components as shown below in an RNAse-free 0.5 mL tube. Mix by gently flicking the tubes, followed by brief centrifugation.

Table 7. Amplification Master Mix

Reagent	Per sample	12 samples	24 samples	36 samples	48 samples
5X dT Amp Master Mix	1.5 µL	21 µL	42 µL	63 µL	84 µL
Gene Specific Primers @ 500 nM per primer	1 µL	14 µL	28 µL	42 µL	56 µL

2. Add 2.5 µL PCR Master Mix directly to each converted cDNA sample.
3. Place new caps on tubes and gently flick to mix, followed by brief centrifugation.
4. Refer to the table below for the suggested number of amplification cycles based on the sample type.

Table 8. Amplification Cycle Recommendations*

Total RNA	Recommended # of Cycles
Purified fresh/frozen RNA	8
Purified FFPE RNA	10
Cell Lysate	8

***** *Optimal cycle number may be sample specific*

5. Place strip tubes in a thermal cycler and run the following protocol:

Table 9. MTE Program

Step		Temperature	Time
Initial Denaturation		95 ˚C	10 minutes
X cycles (see Table 8)	Denature	95 ˚C	15 seconds
	Anneal	60 ˚C	4 minutes
Hold		4 ˚C	Forever

IMPORTANT: After multiplexed target enrichment, incubate the prepared samples for 2 minutes at 95°C and then snap cool on ice for at least two minutes prior to proceeding to the hybridization reaction setup. The entire volume of the MTE reaction should be carried over into the hybridization reaction setup for maximum sensitivity. For long term storage, amplified product should be stored at -80˚C.

NOTE: Successful amplification of purified RNA samples results in production of dsDNA which may be quantified on a Qubit or similar instrument capable of distinguishing dsDNA from ssDNA and RNA. Presence of genomic DNA in a sample will interfere with this type of quantification.

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