CNV Hybridization Protocol

Contents

Overview

The nCounter® Custom CNV Assay measures copy number variation for up to 800 loci in a single reaction via digital detection of color-coded barcodes. Probes are designed to target regions in the genome of interest; custom designs are available.

Aberrations in copy number are implicated in many diseases, from genetic disorders to cancer. FISH has traditionally been used to detect CNVs, but the growing number and importance of CNVs has made higher-plex technologies such as microarrays and NGS more attractive. However, these approaches require cumbersome and time-consuming workflows and a significant amount of expertise. Additionally, most microarrays are not able to resolve CNVs from FFPE samples.

NanoString's nCounter technology makes it easy to directly quantify CNVs from up to 800 loci with:

- Robust performance on FFPE
- A simple assay not requiring expertise
- Minimal hands-on time and fast results
- Lower cost than FFPE microarray or NGS

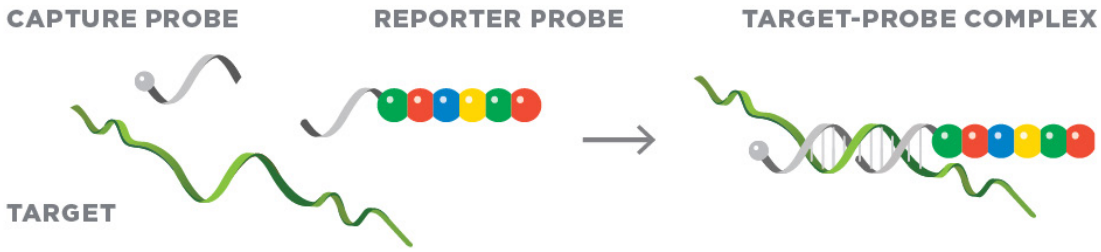


Figure 1. CodeSet chemistry: Capture and Reporter probes bind to the target.

Materials and Reagents

Materials Supplied by NanoString

Table 1. Materials supplied in the nCounter CNV DNA Prep Kit (store at -20°C)

Reagent	Description	Storage
CNV CodeSet	192 rxns of barcoded Reporter and Capture probes	At or below -80°C
Hybridization Buffer	Supplied with nCounter Master Kits and SPRINT Reagent Packs	RT (15–25°C)
10X AluI Fragmentation Buffer	192 rxns of DNA fragmentation buffer	-20°C
10X CNV DNA Prep Control	192 rxns	-20°C
AluI Fragmentation Enzyme (5 U/µL, supplied with kit)	192 rxns of enzyme for DNA fragmentation	-20°C

Additional Materials Required

The additional materials listed in Table 2 are required to complete the hybridization setup.

Table 2. Additional materials to run the CNV hybridization

Item	Manufacturer	Part #
NanoDrop ND-2000 **OR** Qubit 4 Fluorometer*	Thermo Fisher	ND-2000 **OR** Q33228
Bioanalyzer 2100*	Agilent	G2940CA
Thermal Cycler	Various	Various
Microfuge or picofuge	Various	Various
Multi-channel pipetter	Various	Various
0.2–0.5 mL PCR tube	Various	Various
12-tube PCR hybridization strip	Various	Various
Pipettes for 0.5–10 µL	Rainin	L-10XLS+
Pipettes for 2–20 µL	Rainin	L-20XLS+
Pipettes for 20–200 µL	Rainin	L-200XLS+
Disposable gloves	Various	Various

** Equivalent products from another manufacturer are acceptable*

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 1,000x*g* 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.

General Considerations for CNV Assays

Much like NanoString's RNA-based assays, the nCounter CNV assay is compatible with a wide variety of DNA sample types. However, additional handling steps are generally required for processing DNA on an nCounter instrument. Below are some important considerations before using an nCounter assay with DNA samples. If you are considering using samples that are not addressed in this manual, contact NanoString Support.

Fragmentation

Purified genomic DNA is typically longer in length (> 20 kb) than the average RNA (~1.5 kb) and too long for accessing NanoString's fluorescent probes without spatial hindrances imposed by DNA's bulkiness. In addition, DNA is stable under the hybridization conditions used. Thus, regardless of sample type, the DNA must be fragmented prior to analysis on nCounter.

A DNA prep kit is supplied with all nCounter CNV assays and contains the reagents necessary for fragmentation of genomic DNA via the restriction endonuclease AluI. Digestion of human genomic DNA with AluI results in an average fragment size of 500 base pairs. Optimal target fragments for hybridization (~100–500 base pairs) that do not contain the AluI sequence, are selected in the Covarprobe design process.

Other fragmentation methods may be compatible with the CNV assay. For the most accurate results when using FFPE samples, NanoString recommends fragmentation via Covaris AFA technology, with an optimal target size between 200–300 bases. For best results, all samples (including reference samples) should have similar fragmentation profiles. Contact NanoString Support to review guidelines for each approach.

Denaturation

DNA is typically double-stranded in its biological conformation , and nCounter probes are designed to hybridize with a single-stranded target. Therefore, DNA must be denatured prior to hybridization, to induce a single stranded conformation accessible by NanoString fluorescent probes.

> **IMPORTANT:** The sample denaturation temperature must be 95°C. Incomplete denaturation may result in decreased counting efficiency. Denaturation must be done just prior to hybridization; denatured samples will *not* stay denatured for even short storage times.

DNA Sample Input Guidelines

NanoString recommends using 150 ng (for SPRINT) to 300 ng (for MAX/FLEX) of input genomic DNA for all nCounter DNA assays. This amount is three times greater than the mass of RNA input required for the nCounter gene expression assays. A larger sample mass is required because most non-repetitive genomic DNA sequences have, on average, only two copies per cell in normal diploid samples. In contrast, the amount of a given mRNA sequence can range from one to many thousands of copies per cell depending on its level of expression.

The low copy number in individual cells provides some flexibility to optimize assay results by adjusting the sample amount. If the sample amount is not limiting, increasing input DNA amount to 300–600 ng may provide better resolution for single copy changes (see Tech Note: Analyzing FFPE Specimens with the nCounter CNV Assay for more details). For DNA that has been enriched (via immunoprecipitation, NGS exome capture, target enrichment, etc.), the input requirement is typically lower than for standard genomic DNA. For these sample types, the recommended starting input ranges from 2.5–50 ng depending on the level of enrichment and the nCounter instrument model.

The maximum sample input volume for hybridization with DNA is 10 µL. Thus, sample concentration must be high enough to provide adequate sample mass (as discussed above).

DNA Sample Quality Guidelines

DNA used in the nCounter assay MUST be free of contaminating RNA for accurate copy number analysis. DNA used in the assay should be free of contaminating RNA, which can negatively impact the quality of the data in two ways:

- Although probes are designed to be non-exonic, non-coding strand sequences, contaminating RNA may result in reduced counting efficiency.
- RNA may also result in over estimation of DNA concentration when measured by UV absorbance, leading to lower effective DNA input amounts than recommended and lower counts.

For pure genomic DNA preparations, NanoString recommends that the A260/A280 ratio should be between 1.7 and 1.9, and that the A260/A230 ratio should be between 1.3 and 2.0. Fluorescence-based assays (e.g., QuBit from Life Technologies) that use dyes specific for DNA may provide the most accurate concentration measurements if RNA contamination is suspected.

DNA extracted from FFPE is typically degraded due to the fixation and storage process. The nCounter FFPE protocol and analysis guidelines have been optimized to produce accurate results and recommend that the average size of DNA extracted from FFPE tissues be greater than 1 kb (prior to fragmentation). See the section on DNA Fragmentation Quality Control for an example of size variability of FFPE samples. (see Tech Note: Analyzing FFPE Specimens with the nCounter CNV Assay.

DNA Fragmentation Guidelines

Genomic DNA *must* be fragmented prior to hybridization. Two methods of fragmentation are acceptable: AluI restriction enzyme digestion or Covaris AFA-based fragmentation. When using non degraded DNA such as (DNA extracted from cell lines, blood, or fresh or frozen tissue, NanoString recommends using AluI-based fragmentation. For degraded genomic DNA, either from FFPE samples or other sources, NanoString recommends the Covaris-based fragmentation method, although AluI can also be used. If samples were previously fragmented by other methods or do not meet the optimal fragmentation profiles described below, please contact NanoString Support or a Field Application Scientist for guidance.

AluI Restriction Digest Fragmentation

IMPORTANT: It is critical that all samples have similar fragmentation profiles for accurate copy number results (see DNA Fragmentation Quality Control). Contact NanoString Support for additional information.

IMPORTANT: If setting up a master mix, add reagents for 13 reactions to account for dead volume. When using a master mix, a minimum digest volume of 10 µL (300 ng DNA) is recommended. If your DNA concentration is less than 29 ng/µL, NanoString recommends ethanol precipitation with a carrier such as linear acrylamide (see the Covaris AFA Fragmentation protocol for an example). Vacuum drying is not recommended, as this method concentrates salts and other components that may inhibit the restriction digest.

NOTE: If desired, the DNA Prep Kit reagent can be separated into 14 µL aliquots for storage (14 µL is enough to prepare one master mix for 12 restriction digests).

1. Set up the restriction digest in a 0.2–0.5 mL PCR tube. Recommended DNA input is 300 ng in 7 µL of sample (minimum of 200 ng). The minimum recommended concentration of genomic DNA should be 29 ng/µL prior to its addition to the restriction digest.

   The non-sample components can be set up in a master mix for multiple samples if necessary (see Note above). Use the following volumes for each component for a total volume of 10 µL per digest:

   CAUTION: RNA is a component of the Reporter CodeSet. To reduce the risk of RNase contamination during set-up, use RNase-free water, tips, and gloves.

Caution: make sure to change pipette tips to avoid sample cross contamination

- 1 µL 10X AluI Fragmentation Buffer (supplied with kit)
- 1 µL 10X CNV DNA Prep Control (supplied with kit)
- 1 µL AluI fragmentation enzyme (5 U/µL, supplied with kit)
- 7 µL containing 300 ng DNA in RNase-free water, Tris pH 8.0, or similar

2. Mix and spin briefly to bring contents to the bottom of each tube.
3. Incubate the AluI restriction digest at 37°C for 1–2 hours in a heat block or a thermal cycler with the heated lid turned on.

Covaris AFA Fragmentation

1. Begin with approximately three 10 µm slices of FFPE tissue.

   NOTE: The yield of DNA from a given FFPE sample is highly variable. In general, NanoString has found that three 10 µm slices (100 ng of DNA per slice, or 300 ng total) will yield a sufficient amount of input material for most assays. Accuracy of results may increase with greater DNA input.

2. Isolate DNA from FFPE tissue using an accepted protocol (e.g., the QIAamp DNA FFPE Tissue Kit from QIAGEN). Ensure that the genomic DNA is free of contaminating RNA.
3. Confirm DNA yield by checking concentration with a NanoDrop instrument or a fluorescent-based dye detection method.
4. Dilute between 500 ng and 1 µg of DNA in 130 µL of 10 mM Tris.
5. Fragment the diluted DNA with a Covaris AFA instrument. Use the settings defined by the manufacturer to produce 200 bp fragments (actual settings may vary depending on the instrument model).
6. After fragmentation is complete, assay 1 µL of sample (approximately 4 ng) on an Agilent 2100 Bioanalyzer using a High Sensitivity DNA Kit to confirm the desired degree of fragmentation. Successful sonication should produce a single peak centered between 200–300 bp with an average mass between 250–450 bp. The presence of multiple peaks or at greater than 300 bp will indicate less-than-optimal sonication. Presence of peaks at lower than XX? Bp will indicate too much fragmentation and sample degradation.
7. Isolate the fragmented DNA via ethanol precipitation using linear acrylamide as the carrier† (other non-nucleic acid-based carriers such as glycogen can also be used).
   a. Add the following reagents to 130 µL of the sonicated sample:
      - 2 µL linear acrylamide (Ambion; 5 mg/ml)
      - 14.7 µL sodium acetate (3M; pH 5.5)
      - 367 µL ethanol
   b. Cool at -20°C for at least 2 hours.
   c. Spin down at 4°C at max speed (16,000 RCF) for 30 minutes using a microcentrifuge.
   d. Carefully remove the supernatant.
   e. Add 250 µL 70% ethanol.
   f. Spin down at 4°C at max speed for 5 minutes.
   g. Carefully remove the ethanol, being careful not to disturb the pellet.
8. Resuspend the precipitated DNA in 11.5 µL of 10 mM Tris. Repeat Step 3 to confirm DNA concentration.
9. When fragmentation is complete, denature samples at 95°C for 5 minutes. Immediately cool samples on ice for 2 minutes. Keep on ice until ready to set up the hybridization reaction.

10. Proceed to the hybridization protocol. Any remainder of the fragmented DNA sample can be stored at -20°C for future use. Remember to denature the sample (Step 9) few minutes prior to use.

†Gaillard C and Strauss F. (1990) Ethanol precipitation of DNA with linear polyacrylamide as carrier. Nucleic Acids Res (18)2:378.

DNA Fragmentation Quality Control

After fragmentation, check the concentration and fragmentation profile to verify successful fragmentation. For FFPE, adjust the number of slices if needed to reach the sample concentration/input amount necessary for detection of targets.

Ideal Fragmentation Profile of AluI-digested DNA

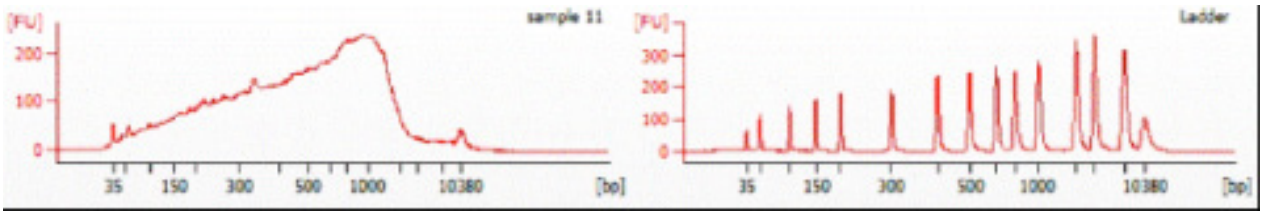


Figure 2. Agilent Bioanalyzer High-Sensitivity DNA assay profile of 20 ng human genomic DNA digested with AluI enzyme for 2 hours at 37°C.

Ideal Fragmentation Profile of Sonicated Genomic DNA

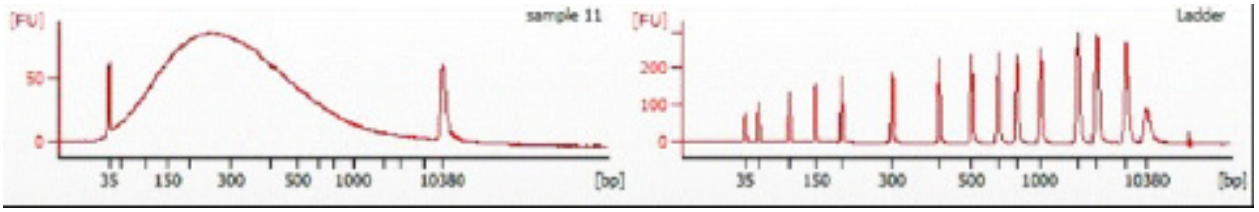


Figure 3. Agilent Bioanalyzer High-Sensitivity DNA assay profile of 20 ng human genomic DNA fragmented with Covaris-AFA technology using manufacturer's recommended settings for 200 bp peak.

Incomplete Sonication

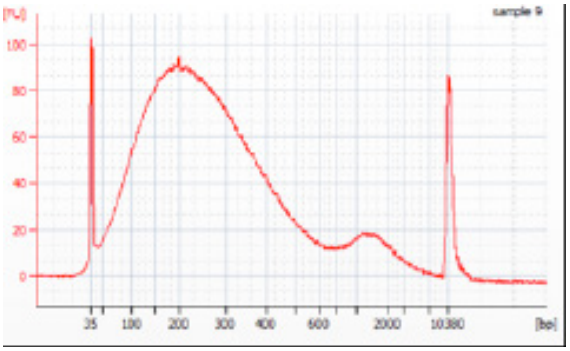


Figure 4. A Bioanalyzer High-Sensitivity DNA assay profile of 4 ng human genomic DNA fragmented with Covaris AFA technology.

While the majority of the DNA is the correct size (~200 bp) the presence of a high molecular weight peak (1,000-1,500 bp) indicates that the fragmentation was not complete.

FFPE Gel Examples

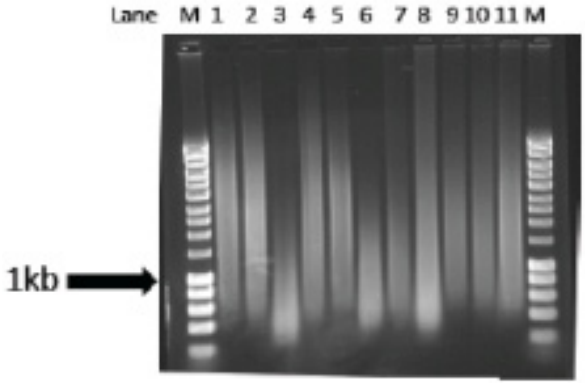


Figure 5. Analysis of genomic DNA size from FFPE tissues.

DNA was extracted from three 10 µm slices of 11 different FFPE tissues using the DNeasy FFPE Kit (QIAGEN). To assess DNA size, 150 ng of extracted DNA was run on a 0.8% agarose gel. HyperLadder 1 kb (Bioline) was used as a marker. Samples 3, 6, and 7 have average sizes below 1 kb and should be considered "lower quality."

Summary

Table 3. Methods for performing quality control on fragmented DNA

Sample Type	Recommended Assay Input	Recommended Concentration	QC Method
Genomic DNA (un-enriched)	150–600 ng	> 30 ng/µL	Agarose gel; confirm complete digestion with nCounter restriction digest controls

CNV Hybridization Protocol

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 5 µL of hybridization buffer, up to 10 µL of denatured sample DNA, and 2 µL of Capture ProbeSet.

1. Remove aliquots of both Reporter CodeSet and Capture ProbeSet reagent from the freezer to thaw at room temperature. Invert several times to mix well and spin down reagent.

   IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

2. Create a master mix by adding 70 µL of hybridization buffer to the tube of Reporter Probes. Do not remove the Reporter Probes from the tube. RNase-free water may also be added to this mix if the volume of the individual DNA samples is less than 5 µL and is constant. (Add enough water for 14 assays to allow two assays' worth of dead volume.) Do not add the Capture ProbeSet to the master mix. Invert repeatedly to mix and spin down master mix.

3. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.

4. Add 8 µL of master mix to each of the 12 tubes. NanoString recommends using a fresh tip for each pipetting step. The CodeSet has components that can wick up into the tip, and you may not dispense the correct amount if you use the same tip.

   IMPORTANT: If not already done, denature DNA samples at 95°C for 5 minutes. Immediately place DNA samples on ice for 2 minutes to minimize re-annealing. Denaturation of DNA samples is critical for optimal assay performance.

5. Briefly spin down denatured DNA samples in a picofuge.

6. Add up to 10 µL of sample to each tube.

7. If necessary, add RNase-free water to bring the volume of each assay to 18 µL.

8. Invert the Capture ProbeSet tube to mix and spin down the contents. Add 2 µL of Capture ProbeSet to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down and immediately place the tubes in the pre-set 65°C thermal cycler.

   IMPORTANT: If not already done, denature DNA samples at 95°C for 5 minutes. Immediately place DNA samples on ice for 2 minutes to minimize re-annealing. Denaturation of DNA samples is critical for optimal assay performance.

9. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

   NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to 4°C is to ensure equivalent hybridization times of all assays being directly compared in

the same series of experiments. Counts continue to accumulate with time, with total counts typically increasing 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will increase sensitivity by increasing counts without significantly increasing background.

Quick Reference

CNV Hybridization Protocol

Please read through the full protocol before beginning. This illustrated workflow is intended for quick reference at the bench.

1 Fragment DNA

- ☐ Follow guidelines to fragment DNA by using restriction digest with AluI or using sonication. Confirm fragmentation profile (~50% of sample > 300 bases).

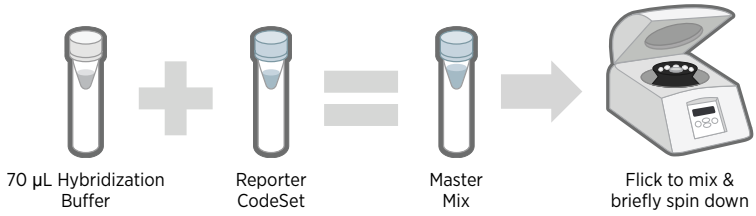
2 Prepare for hybridization

- ☐ Preheat thermocycler to **65° C**.
- ☐ **Thaw** codeset & samples.

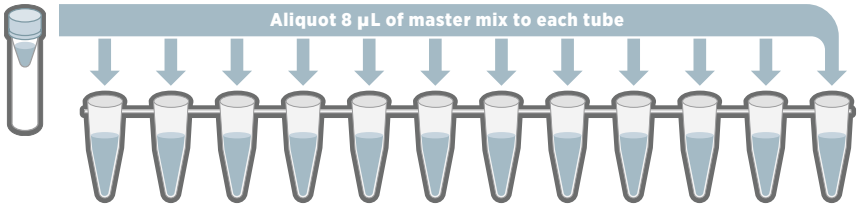
Preheat to 65°C

3 Create & aliquot Master Mix

- ☐ Add **70 µL of Hybridization Buffer to the Reporter CodeSet tube** to create Master Mix.
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **8 µL of Master Mix** into each tube of a labeled 12-tube strip.



70 µL Hybridization Buffer | Reporter CodeSet | Master Mix | Flick to mix & briefly spin down

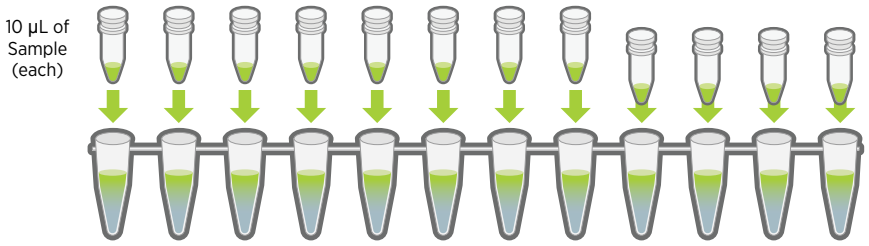


4 Denature DNA

- ☐ Denature DNA samples at **95°C** for **5 minutes**.
- ☐ Immediately place samples on ice for **2 minutes**.

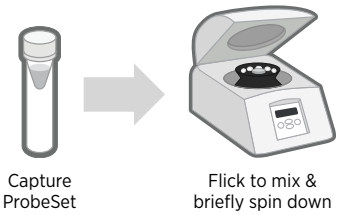
5 Add sample

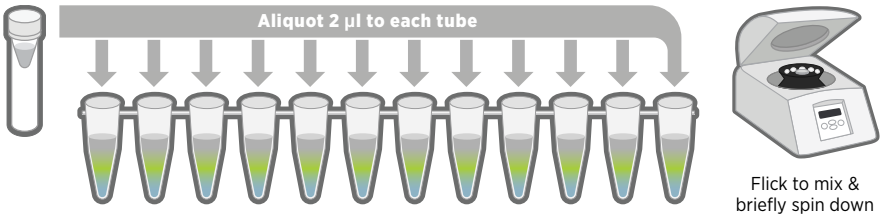
- ☐ Add **10 µl of sample** to each tube.



6 Add Capture ProbeSet

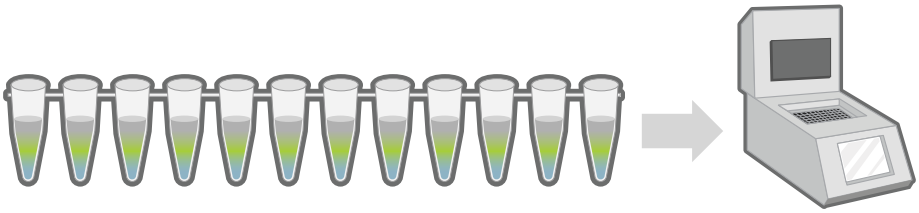
- ☐ **Flick-mix** Capture ProbeSet and **spin down briefly**.
- ☐ Add **2 µL of Capture ProbeSet** to each tube, for a total volume of 20 uL.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.





7 Hybridize

- ☐ **Immediately** place tubes in the thermocycler at **65° C** for **16-24 hours.** Maximum hybridization time should not exceed 48 hours.

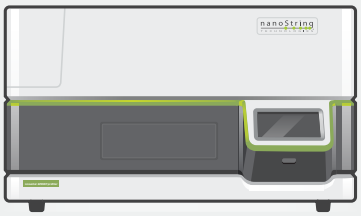


OPTION A: Use nCounter® MAX/FLEX

7a Load nCounter MAX/FLEX

See **MAN-C0035, nCounter Analysis System User Manual** for details.

OPTION B: Use nCounter® SPRINT



7b Load nCounter SPRINT

See **MAN-10017, nCounter SPRINT Profiler User Manual** for details.

CNV Hybridization Protocol with Panel Plus Reagents

Set up a hybridization reaction at room temperature for each sample with the following components: 3 µL of Reporter CodeSet, 2 µL of Reporter Plus,5 µL of hybridization buffer, up to 10 µL of denatured sample DNA, 2 µL of Capture ProbeSet, and 1 µL of Capture Plus.

1. Remove aliquots of Reporter CodeSet, Capture ProbeSet, and Plus reagents from the freezer to thaw at room temperature. Invert several times to mix well and spin down reagent.

   IMPORTANT: After it has thawed, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for 10 minutes and cool at room temperature before using.

2. Add 28 µL of Reporter Plus reagent to the thawed Reporter CodeSet. Invert several times to mix well, and spin down reagents.

3. Create a master mix by adding 70 µL of hybridization buffer to the tube containing the Reporter CodeSet and the Reporter Plus reagents. Do not remove the Reporter CodeSet from the tube. RNase-free water may also be added to this mix if the volume of the individual DNA samples is less than 10 µL and is constant. (Add enough water for 14 assays to allow two assays' worth of dead volume.) **Do not add the Capture ProbeSet or Capture Plus reagent to the master mix.** Invert repeatedly to mix and spin down master mix.

4. Label the hybridization tubes. If using strip tubes, ensure they fit in a microfuge or picofuge and cut the strip in half if necessary.

5. Add 10 µL of master mix to each of the 12 tubes. NanoString recommends using a fresh tip for each pipetting step. The CodeSet has components that can wick up into the tip, and you may not dispense the correct amount if you use the same tip.

   IMPORTANT: If not already done, denature DNA samples at 95°C for 5 minutes. Immediately place DNA samples on ice for 2 minutes to minimize re-annealing. Denaturation of DNA samples is critical for optimal assay performance.

6. Briefly spin down denatured DNA samples in a picofuge.

7. Add up to 10 µL of sample to each tube.

8. If necessary, add RNase-free water to bring the volume of each assay to 20 µL.

9. Add 14 µL of Capture Plus reagent to the thawed Capture ProbeSet. Invert several times to mix well, and spin down reagents.

10. Add 3 µL of Capture ProbeSet and Capture Plus reagent mix to each tube immediately before placing at 65°C. Cap tubes and mix the reagents by inverting the tubes several times and flicking with a finger to ensure complete mixing. Briefly spin down.

11. Incubate reactions for at least 16 hours. Maximum hybridization time should not exceed 48 hours. Ramp reactions down to 4°C and process the following day. Do not leave the reactions at 4°C for more than 24 hours or increased background may result.

    NOTE: The purpose of selecting a fixed hybridization time followed by a ramp down to

4°C is to ensure equivalent hybridization times of all assays being directly compared in the same series of experiments. Counts continue to accumulate with time, with total counts typically increasing 5% per hour between 16 and 24 hours. Although a 16-hour incubation is adequate for most purposes, a longer incubation will increase sensitivity by increasing counts without significantly increasing background.

Quick Reference

CNV Plus Hybridization Protocol

Please read through the full protocol before beginning. This illustrated workflow is intended for quick reference at the bench.

1 Fragment DNA

- ☐ Follow guidelines to fragment DNA by using restriction digest with AluI or using sonication. Confirm fragmentation profile (~50% of sample > 300 bases).

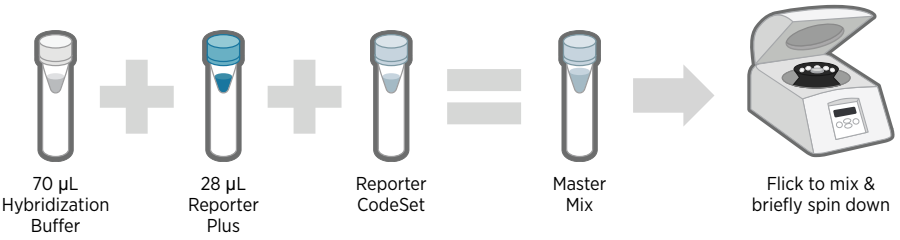
2 Prepare for hybridization

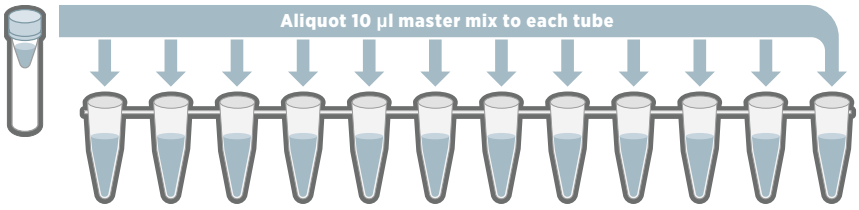
- ☐ Preheat thermocycler to **65° C**.
- ☐ **Thaw samples** and Codeset and CodeSet Plus tubes

Preheat to 65°C

3 Create & aliquot Master Mix

- ☐ Add **70 µL of Hybridization Buffer** and **28 uL of Reporter Plus** to the **Reporter CodeSet** tube to create **Master Mix.**
- ☐ Flick to mix, then briefly spin down contents.
- ☐ Aliquot **10 uL of Master Mix** into each tube of a labeled 12-tube strip.





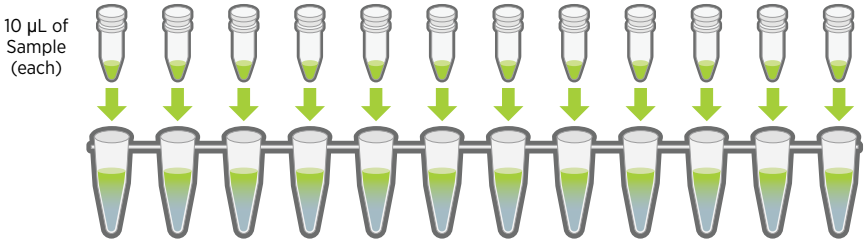
4 Denature DNA

- ☐ Denature DNA samples at **95°C** for **5 minutes**.
- ☐ Immediately place samples on ice for **2 minutes**.

5 Add sample

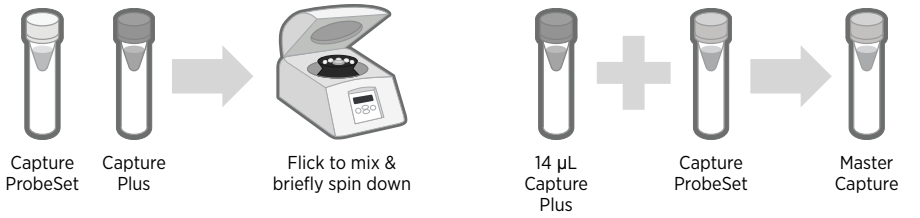
- ☐ Add **10 µl of sample** to each tube.

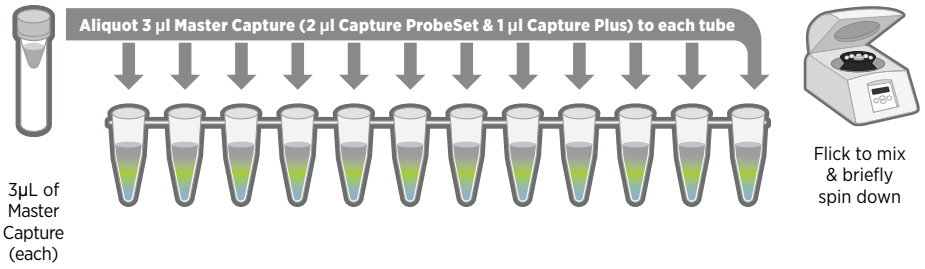
Note: If using less than 5 µL of sample, add RNAse-free water to each tube to bring the volume to 15 µL



6 Add Master Capture

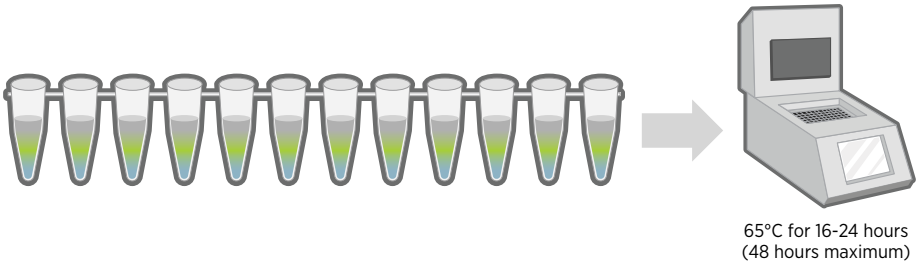
- ☐ **Flick-mix** Capture ProbeSet and Capture Plus tubes and **spin down briefly**.
- ☐ Add **14 µL of Capture Plus** to the **Capture ProbeSet** to create the **Master Capture.** Flick to mix, then **briefly spin down** contents.
- ☐ Add **3 µL** of **Master Capture** to each tube, for a total of 21 uL.
- ☐ Cap tightly, **flick-mix**, and **spin down briefly**.





7 Hybridize

- ☐ **Immediately** place tubes in the thermocycler at **65° C** for **16-24 hours.** Maximum hybridization time should not exceed 48 hours.

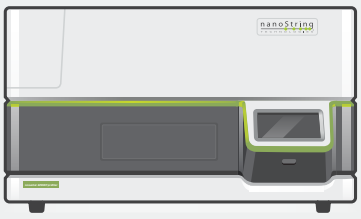


OPTION A: Use nCounter® MAX/FLEX

7a Load nCounter MAX/FLEX

See **MAN-C0035, nCounter Analysis System User Manual** for details.

OPTION B: Use nCounter® SPRINT



7b Load nCounter SPRINT

See **MAN-10017, nCounter SPRINT Profiler User Manual** for details.

Trademarks

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