DNA Amplification for SNV Analysis

The following protocol has been optimized for a fixed DNA sample mass input of 5 ng in a 10 µL amplification reaction. Accurately measure all sample DNA with a fluorescence-based method (e.g., Qubit dsDNA HS Assay Kit).

Materials and Reagents

Table 1. Materials for SNV sample amplification

Reagent	Description	Storage
SNV Primer Pool	Panel-specific primer pool	-20°C
5X dU Amp Master Mix	Amplification master mix	-20°C
Reference DNA	Purified DNA at 2.5 ng/µL	-20°C

Reference Run

For accurate SNV detection, a Reference Sample data set with 12 lanes of data is required prior to analysis of subsequently collected experimental data for each nCounter system that is to be used (here an nCounter system consists of a specific nCounter Prep Station, a specific nCounter Digital Analyzer, and a specific lot of DNA SNV Panel reagents To create a Reference Sample data set, follow the protocol below using the DNA SNV Reference Sample (provided at a concentration of 2.5 ng/µL) as the sample input for all 12 samples to be loaded into an nCounter cartridge. To do this, add 31 uL of the DNA SNV Reference Sample directly to the Reaction Master Mix and distribute 10 uL of this mix to each of the 12 PCR tubes within a strip. It is recommended that the Prep Station and Digital Analyzer serial numbers and the DNA SNV Panel reagent lot be added to the run information by providing it in the Comments of the Cartridge Definition File (CDF). Instructions on how to add information to the Comments field of the CDF can be found in Chapter 5, Section D of the nCounter Analysis System User Manual (MAN-C0035).

The lot-matched Reference Sample data set is necessary to analyze subsequently collected experimental sample data. It is important to maintain a copy of this data set as RCC files for use within nSolver™ Analysis Software. Please also email this RCC file along with the serial numbers for the Prep Station and Digital Analyzer and the lot number of the DNA SNV Panel used to snvqualify@nanostring.com.

DNA Amplification

1. Thaw SNV Primer Pool, 5X dU Amp Master Mix, Reference DNA, and sample DNA on ice. The 5X dU Amp Master Mix and all mixes that contain it should remain on ice until the tubes are put on the thermal cycler. Flick tubes several times to mix well and briefly spin down reagents.

   NOTE: Run the reference sample in one lane as a negative control (recommended, not required).

2. Dilute sample DNA to consistent working concentration between 1 and 2.5 ng/µL.

   NOTE: Sample DNA should be no more dilute than 1 ng/µL to ensure 5 ng of total DNA input. The input amount of **5 ng** is essential, as significantly higher or lower input can negatively impact accuracy of SNV calls.

3. Create a Reaction Master Mix by adding the following components to the 5X dU Amp Master Mix tube containing 31 µL of dNTPs and enzyme.

   Table 2. Reaction Master Mix set up options.

Reagents	PCR Master Mix for DNA at 1 ng/µL	PCR Master Mix for DNA at 2.5 ng/µL
5X dU Amp Master Mix*	31 µL	31 µL
SNV Primer Pool	15.5 µL	15.5 µL
Nuclease-free water	31 µL	78 µL
Total Volume	**77.5 µL**	**124.5 µL**
Master Mix Addition per well	**5 µL**	**8 µL**
DNA Addition per well	**5 µL**	**2 µL**

   **for DNA at lower concentration, adjust water accordingly.*

4. Mix by gentle pipetting, taking care not to produce bubbles. Briefly spin down.
5. Label a 12-well strip PCR tube.
6. Add Reaction Master Mix to each well.
7. Add DNA to each well. Final reaction volume is 10 µL.
8. Mix by gently flicking tubes and briefly spin down.

9. Place the 12-well strip tube in a thermal cycler and run the following thermal cycler protocol:

   IMPORTANT: Ensure tubes are tightly sealed to prevent evaporation.

Table 3. DNA Amplification Thermal Cycler Protocol

Step		Temperature (°C)	Time
UDG digest		37	30 min
UDG inactivation		50	5 min
Initial denature		95	3 min
Denature	18-20 cycles*	95	15 sec
Anneal		56	2 min
Extension		68	30 sec
Final extension		68	5 min
Hold		4	Hold

**For non-FFPE samples and Reference DNA, amplify for 18 cycles. For FFPE-extracted DNA, amplify for 20 cycles. Some particularly degraded samples may require 21 cycles. For accurate variant detection, do not exceed 21 cycles of amplification.*

10. Proceed to SNV Hybridization Setup (MAN-10055) or store amplified DNA at -20°C or -80°C.