Preparing Nucleic Acid from FFPE Samples

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue specimens are highly valuable sources of sample material for biological assays. However, this material can often be challenging to process for downstream analyses. Many parameters of both the FFPE samples (such as storage time and conditions, fixation time, and specimen size) and nucleic acid extraction methodology can impact the quality and quantity of extracted material. This document outlines important information related to the use and extraction of nucleic acid from FFPE samples for use in nCounter assays. For information on working with fresh/frozen samples, see Preparing RNA and Lysates from Fresh Frozen Samples (MAN-10051).

Contents

Recommended Materials

Table 1 lists materials and instrumentation that are recommended or required to run nCounter XT Assays. Additional materials are recommended for RNA purification in Table 2.

Table 1. Materials recommended for all nCounter assays

Material	Manufacturer	Part Number(s)
Disposable gloves	Various	Various
NanoDrop ND-2000*	Thermo Fisher®	ND-2000
Bioanalyzer® 2100*	Agilent®	G2940CA
Pipette for 0.5–10 µL*	Rainin®	L-10XLS+
Pipette for 2–20 µL*	Rainin	L-20XLS+
Pipette for 20–200 µL*	Rainin	L-200XLS+
Microcentrifuge or picofuge	Various	Various
Thermal cycler†	Various	Various

**nCounter performance data were generated using a Bio-Rad® DNA Engine®.*

†Other instruments can be used but should have a programmable heated lid.

Contact NanoString Support with questions about the compatibility of products not listed here.

Table 2. Additional materials recommended for gene expression assays using total RNA (standard protocol)

Material	Manufacturer	Part Number(s)
RNeasy® Kit (or an equivalent kit from another manufacturer)*	QIAGEN®	74104 74106

**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.

Thermal Cycler Guidelines

Thermal cyclers are produced by a wide variety of manufacturers and possess a wide variety of features. 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.

Sample Type Considerations

Sample input recommendations for nCounter assays were developed using purified total RNA from a variety of tissues, of which mRNA typically composes 5–10% (~5–10 ng in a sample of 100 ng total RNA). Use a NanoDrop™ or other spectrophotometer to measure RNA sample quality. NanoString recommends an A260/A280 ratio of 1.7–2.3 and an A260/A230 ratio of 1.8–2.3.

Many other sample types provide high-quality results with minor adjustments to sample volume or concentration as outlined below. Please consult with your Field Applications Scientist or contact NanoString Support at support@nanostring.com if you have any questions about how to ensure the best results from your experiment.

Blood samples can be assayed using purified total RNA, unpurified blood lysates, or specific blood fractions such as PBMCs isolated from whole blood. NanoString recommends the use of a commercially available kit to collect and purify RNA from blood; kits may also be used for other biological fluids such as sputum or urine. For unpurified RNA, NanoString recommends collecting blood lysate samples in specialized PAXgene® tubes. Additional information on the attenuation of highly expressed genes in some sample types is provided in the Tech Note: Strategies for Successful Gene Expression Assays.

Sample Input Recommendations

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 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 included in this manual. (These recommendations do not apply to the RNA:Protein assay, which is optimized for cell number. For RNA:Protein, refer to RNA:Protein Hybridization Setup) With the exception of single cell assays, these recommendations apply to sample mass only; sample volume does not vary between systems.

Table 3. Recommended sample input mass for nCounter XT assays

Sample Type	nCounter Analysis System (MAX™/FLEX™)	nCounter SPRINT™ Profiler
FFPE-derived RNA	300 ng	150 ng
Total RNA (CodeSets < 400 genes)	100 ng	50 ng
Total RNA (CodeSets > 400 genes)	50 ng	25 ng
Fragmented DNA	300 ng	150 ng
ChIP DNA (unamplified)	10 ng	5 ng
ChIP DNA (whole genome amplification)	100 ng	50 ng
Single Cell or Low Input Material	up to 8 µL of amplified sample	up to 5 µL of amplified sample

Factors Influencing Nucleic Acid Yield

Nucleic acid yield from FFPE samples is impacted by many factors, including time from excision to fixation, tissue type, sample age, surface area of the section, cellularity, section thickness and extraction method. It is important to take such factors into consideration when determining the amount of tissue required for any given assay.

For many sample types, sections as thin as **5 µm** may be used, however, yield is generally optimal with sections that are **10–20 µm** thick due to the higher percentage of intact cells in larger sections.

The tissue type can significantly influence the overall cellularity of a sample, which is directly correlated with yield. It is critical to understand the cellular makeup of your tissues prior to extraction; NanoString recommends serial sections be taken for histological or pathological evaluation before and after the sections are cut to be used for nucleic acid extraction.

A wide variety of extraction methods can be employed to isolate nucleic acid from FFPE samples. Regardless of the extraction method employed, it is important to quantify and QC extracted material prior to hybridization. This protocol uses the Qiagen AllPrep DNA/RNA FFPE Kit for copurification of DNA and RNA from a single section and provides important considerations for assessment of nucleic acid quality and yield.

Protocol: FFPE Sample Deparaffinization and Purification

NOTE: DNA/RNA Prep from FFPE takes a minimum of 5 hours to complete when using the AllPrep kit with this deparaffinization protocol.

IMPORTANT: Do not deparaffinize a slide for protein preparation using this protocol. A different protocol is outlined for this purpose in Protein Processing for FFPE Samples (MAN-10053).

Table 4. Materials for extracting and quantifying nucleic material from FFPE samples

Material	Manufacturer	Part
AllPrep DNA/RNA FFPE kit	QIAGEN	80234
CitriSolv	Decon Labs, Inc.	1601
Ethyl Alcohol, 200 Proof, Absolute	Various	Various
Glycerol	Various	Various
Agilent Bioanalyzer (or similar system)	Various	Various

1. If the starting material is FFPE **curls**, follow the instructions outlined in the AllPrep manual for purifying RNA and DNA from FFPE samples.

   If the starting material is **slide-mounted** FFPE tissue sections, proceed with Step 2.

2. Place the slides in a rack and gently performing the following washes using Coplin jars:
   a. Incubate the slides in a CitriSolv bath for 2 minutes.
   b. Change the CitriSolv bath and incubate the slides for an additional 2 minutes.
   c. Incubate the slides in a 100% ethanol bath for 2 minutes.
   d. Incubate the slides in nuclease-free water for 1 minute.
3. Air dry the slides.
4. Once the slides are dry, add enough 3% glycerol to cover the tissue in order to prevent pellet loss.
5. Wipe up excess 3% glycerol around the cell sections using an absorbent tissue.
6. Scrape the sections in a single direction on the slide with a clean razor blade to create a cohesive mass.
7. Use a small volume (<**150 µL**) of Buffer PKD from the AllPrep DNA/RNA FFPE Kit to transfer the cohesive mass into a 1.5 mL tube.
8. Add Buffer PKD to the sample to bring the final volume to **150 µl** total.
9. Add **10 µl** proteinase K and mix by vortexing.
10. Perform the remaining post-PKD/ProK-digestion steps outlined in the AllPrep DNA/RNA FFPE Kit instructions to purify RNA and DNA.
11. Extracted nucleic acid should be stored at -80˚C.

Guidelines: Quantifying Purified RNA and Assessing Quality

Quantify RNA using fluorescence (e.g., Bioanalyzer, Qubit, etc.) 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 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 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 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 300 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 >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 50–300nt 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/µL**).
- For most nCounter applications, sample input volumes are **5 µL** and a range of **25–300 ng**, starting with **100 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),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 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**.

Working with DNA

SNV Guidelines: Quantifying Purified SNV DNA and Assessing Quality

- 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 inaccurate and overestimated measures of DNA concentration.
- Evaluate DNA quality using a fragment analysis system to measure nucleic acid fragmentation.
    - 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.
    - 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.
- Create dilute working stocks of DNA. A sample input of **5 ng** is required in a maximum volume of **5 µL**. For ease of reaction set-up, dilute to **2.5 ng/µL** (2 µL per reaction) in nuclease-free water or TE.
- Store purified DNA at **-80°C**.

CNV Guidelines: DNA Sample Input for CNV

NanoString recommends using **150–300 ng** of input genomic DNA for CNV assays. 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 and may increase accuracy for highly degraded FFPE samples (see the Tech Note: Analyzing FFPE Specimens with the nCounter CNV Assay for more details).

CNV Guidelines: DNA Fragmentation for CNV

All CNV nCounter DNA assays require that the genomic DNA be fragmented prior to hybridization. Starting material for ChIP-String is typically fragmented during the immunoprecipitation process and may not require additional fragmentation (however, read the section on Guidelines: Special Considerations for ChIP-String Assays).

Two methods of fragmentation are acceptable: Alu1 restriction enzyme digestion or Covaris AFA-based fragmentation. When using intact (i.e., non-degraded) genomic DNA from cell lines, blood, or fresh or frozen tissue, NanoString recommends using Alu1-based fragmentation. For degraded genomic DNA, either from FFPE samples or other sources, NanoString recommends the Covaris-based fragmentation method, although Alu1 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 (support@nanostring.com) or a Field Application Scientist for guidance.

DNA Protocol: Alu1 Restriction Digest Fragmentation

IMPORTANT: If samples were fragmented as part of experimental set-up (e.g., ChIP or NGS library prep) and have an average length under 500 base pairs, this step may be omitted. However, it is critical that all samples have similar fragmentation profiles for accurate copy number results (see the section on DNA Fragmentation Quality Control). Contact NanoString Support at support@nanostring.com 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.

1. Set up the restriction digest in a 0.2–0.5 mL PCR tube. Minimum recommended DNA input is **300 ng** in **7 µL** of sample. The minimum concentration of genomic DNA should be **41 ng/µL** prior to its addition to 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).

   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:

   - **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 **430 ng** DNA in RNase-free water (**750 ng** if using core TagSet plus extension TagSet), Tris pH 8.0, or similar

     IMPORTANT: 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.

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.

   OPTIONAL: You may wish to check the quality of the digest on 1% agarose gel or Bioanalyzer DNA chip. See the section on DNA Fragmentation Quality Control for an example of a typical size distribution for human genomic DNA digested with AluI.

4. When the digest 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.
5. Proceed to the hybridization protocol. Any remainder of the digested DNA sample can be stored at -20°C for future use. Remember to denature the sample (Step 4) prior to use.

DNA Protocol: 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 (an example of a commonly used kit is 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 pH 8.0 or similar.

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 peak heights greater than **300 bp** will indicate less-than-optimal sonication.

7. Isolate the fragmented DNA via ethanol precipitation using linear acrylamide as the carrier[1] (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.

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

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) prior to use.

Guidelines: Special Considerations for ChIP-String Assays

Additional fragmentation beyond that performed during the chromatin immunoprecipitation (ChIP) protocol is not required prior to performing the nCounter ChIP-String DNA assay. However, there are other factors that should be considered prior to beginning the nCounter assay set-up:

- A minimum of **5–10 ng** (**1–2 ng/µL**) of unamplified ChIP'd DNA input is recommended. The input amount is highly dependent on the level of enrichment of the target molecules (see below).
- Direct detection (without amplification) of target molecules requires that the ChIP'd DNA has been significantly enriched. For example, **1 ng** of human genomic DNA has approximately 330 copies of any non-repetitive sequence (i.e., 2 copies per genome). Thus, **5–10 ng** of un-enriched genomic DNA sample would contain approximately 1,600 to 3,300 molecules, roughly equal to the limit of detection for the nCounter system. In order to confidently measure the sequences associated with any given ChIP assay, NanoString recommends an enrichment of at least 10-fold over un-enriched genomic DNA (between 16,000 and 33,000 molecules, and with the same sample mass of **5–10 ng**). Reporter Probe counts increase linearly with an increasing amount of sample input. If the DNA sample is not limiting, increasing the input amount will result in higher counts and higher confidence in the measurements.
- The amount of enrichment is highly dependent on the success of the ChIP protocol, the affinity of the antibody used, and whether the protein of interest is directly bound to genomic DNA or associated with other proteins that are bound to DNA. If a new procedure or ChIP reagents are used, a positive control sample is highly recommended. If ChIP'd DNA is amplified via whole-genome amplification, then a sample input of **50–100 ng** is recommended.

DNA Fragmentation Quality Control (QC)

Ideal Fragmentation Profile of Alu1-Digested DNA

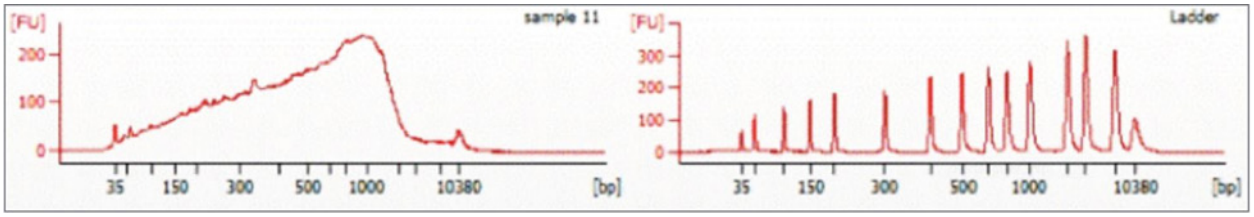

Figure 1. Agilent Bioanalyzer High-Sensitivity DNA assay profile of **20 ng** human genomic DNA digested with Alu1 enzyme for 2 hours at 37°C.

Ideal Fragmentation Profile of Sonicated Genomic DNA

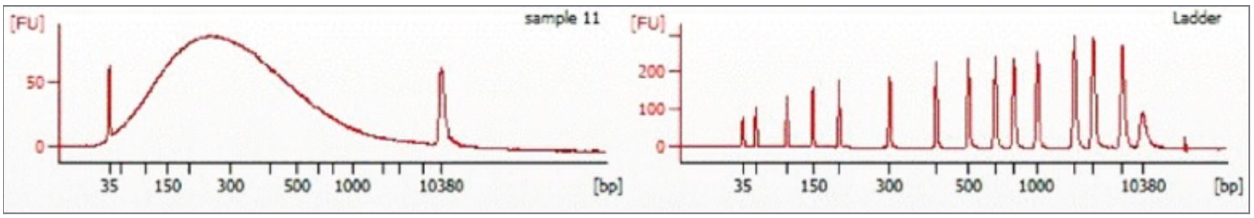

Figure 2. Agilent Bioanalyzer High-Sensitivity DNA assay profile of **20 ng** human genomic DNA fragmented with Covaris-AFA technology using manufacturer's recommended settings for 200 bp peak.

Incomplete Sonication

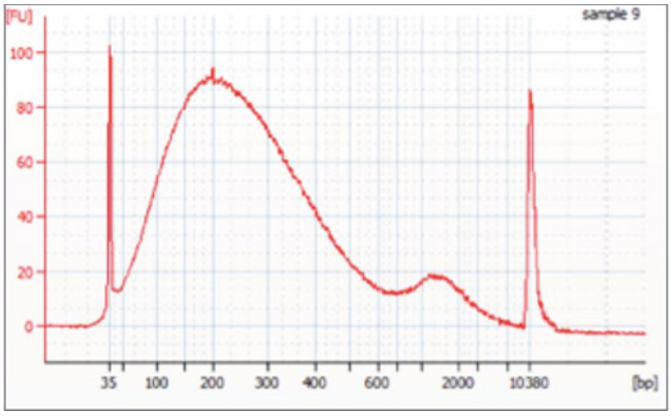

Figure 3. A Bioanalyzer High-Sensitivity DNA assay profile of **4 ng** human genomic DNA fragmented with Covaris AFA technology. While the majority of the DNA is the correct size (~200 bp) the presence of a high molecular weight peak (1,000-1,500 bp) indicates that the fragmentation was not complete.

FFPE Gel Examples

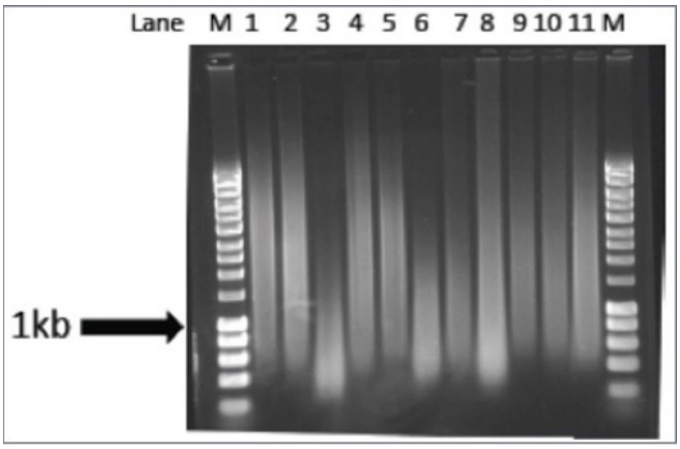

Figure 4. Analysis of genomic DNA size from FFPE tissues. DNA was extracted from **three 10 µm** slices of 11 different FFPE tissues using the DNeasy FFPE Kit (QIAGEN).

QC Methods

Table 5. 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
Target-enriched NGS library or DNA prepared by ChIP	5–50 ng	2-10 ng/µL	Agilent® BioAnalyzer