Application Note: Pairing Covaris truXTRAC™ FFPE RNA pre-analytical extraction with the NanoString Technologies nCounter Analysis System for streamlined analytical assays.

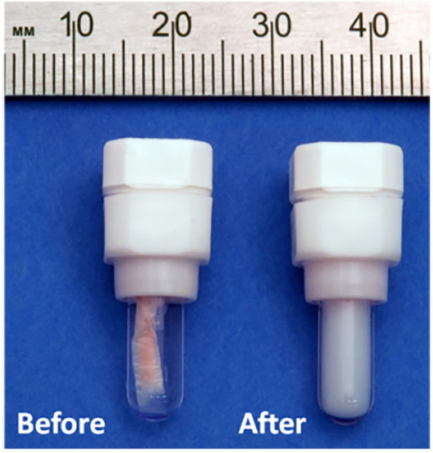
Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissue remains an important source material for clinical and translational research and the increasing number of molecular assays available to analyze these samples makes it critical to provide sensitive and robust methods for nucleic acid extraction and molecular profiling from small amounts of starting material.

Covaris and NanoString Technologies provide two complementary solutions ideal for FFPE stabilized tissues. In this application note, we demonstrate how Covaris truXTRAC can generate high quality sample input material for downstream molecular analysis with the nCounter Gene Expression. Our results show that Covaris AFA (Adaptive Focused Acoustics™) technology is highly reproducible, and yields abundant RNA from as little as one FFPE section, to meet the low input requirement (50- 100 ng) for the nCounter Assay. Furthermore, we demonstrate that a crude FFPE extract from the Covaris AFA treatment can be directly measured in the nCounter hybridization, eliminating the time and effort required for nucleic acid purification, thus enabling an extremely fast and simple workflow.

Covaris technology overview:

Covaris truXTRAC is a robust and efficient method for the extraction of DNA and RNA from FFPE tissues based on Covaris Adaptive Focused Acoustics (AFA) technology. truXTRAC utilizes a highly controlled focused acoustic energy field for the effective removal of paraffin from FFPE tissues. Importantly, the AFA process also promotes simultaneous rapid tissue rehydration, which facilitates subsequent tissue digestion and crosslink reversal. The entire process is fully automated and carried out without the use of any organic solvents or heat. After extraction, nucleic acids can be purified using columns or an automated magnetic bead-based system.

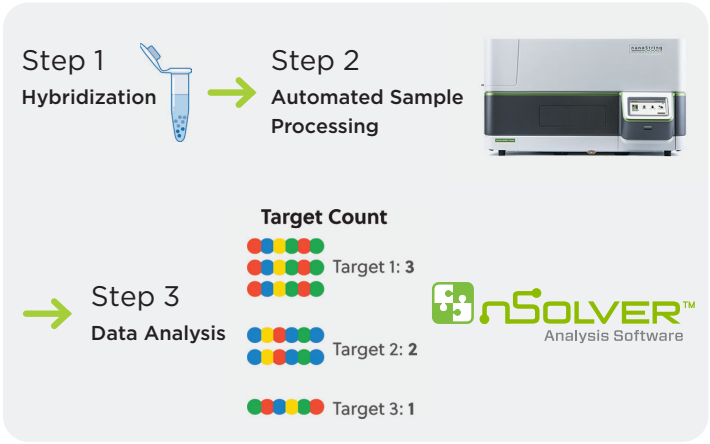


LEGEND: 10 µm FFPE tissue section before (left side) and after (right side) AFA processing

nCounter® Analysis System Overview:

The NanoString nCounter Analysis System provides a direct, sensitive, and digital method for counting relative abundance of RNA, DNA, or protein target molecules in a biological sample. The nCounter Gene Expression and CNV Assay use up to 800 unique, gene-specific probe pairs (Reporter and Capture probe) hybridized in solution with as little as 50-100 ng sample. Each gene target is identified by a unique, fluorescent barcode encoded by the Reporter Probe. NanoString offers two instrumentation options (MAX and SPRINT) for counting individual target molecules, both of which generate equivalent digital data readout for downstream analysis.

Direct Detection and Counting of Fusion Events
No RT-PCR, No Library Prep, No more limitations with FISH assays



(**FIGURE 1:** of Reporter/Capture hybridization, purification, counting)

Materials and Study Design:

All tissue, reagent kits, and instrument accessories were provided by Covaris. The Covaris M220 Focused-ultrasonicator™ (with Holder XTU PN 500414 and Holder XTU Insert microTUBE 130 µl PN 500489) was used for sample processing.

FFPE tissues were provided by CHTN (Cooperative Human Tissue Network, Eastern Division University of Pennsylvania). The FFPE samples used in this protocol were from Prostate, Kidney, and Testis. The excess paraffin was trimmed away from the tissue. For each FFPE tissue types, 20 µm sections were cut in replicates using a microtome. For this study, all sections were adjacent.

Sections were provided as scrolls in Covaris microTUBES.

truXTRAC™ FFPE RNA kit PN 520161

Covaris Heat Block microTUBE Adaptors PN 500456

Detailed Protocols are available at: http://covarisinc.com/products/ffpe-extraction/. Briefly, for the RNA extraction:

1. 110 µl lysis buffer added to each section
2. Covaris AFA deparaffinization/rehydration 5 min
3. Add 10ul Proteinase K
4. Mix Proteinase K with AFA 10 sec
5. Incubate in Pro K 15 min @ 56C
6. Reverse crosslinks 15 min @ 80C
7. Proceed with column purification protocol

Study Design:

Duplicate sections (20 µm) from three different tissue blocks (kidney, testis, prostate) were processed by each method:

1. Covaris truXTRAC FFPE RNA Kit. With the truXTRAC kit, samples were either processed following the full protocol until purified RNA or protocol was stopped after Crosslink reversal but before purification to obtain "crude AFA-extract"
2. Traditional commercial FFPE kit, utilizing chemical deparaffinization, proteinase K digestion, and column-based RNA purification.

***NOTE:** DNase digestion steps were not performed for either Covaris or traditional kits.

Extractions and purifications were performed according to the respective protocols, and nucleic acids were evaluated by Nanodrop, Qubit RNA, or Bioanalyzer. 10 µl of crude AFA-extracts were saved from 2 tissues for direct analysis.

Purified RNA and crude AFA-extract were run with nCounter Gene Expression Assay 236-gene Cancer Reference Codeset.

Results:

The nCounter system is ideally suited for analyzing limited amounts of fragmented RNA, and numerous publications have demonstrated the utility of using the nCounter Gene Expression assay with FFPE samples .

First we evaluated the ability of the Covaris truXTRAC protocol to generate adequate nucleic acid yield and quality for input into the nCounter Assay. From three replicate sections of three independent tissue blocks, we obtained an average yield of 1,458 ng RNA per section which is adequate for more than 10 nCounter multiplex assays per tissue section. The RNA integrity of these samples was evaluated by Agilent Bioanalyzer, using the RNA Nano 6000 kit.

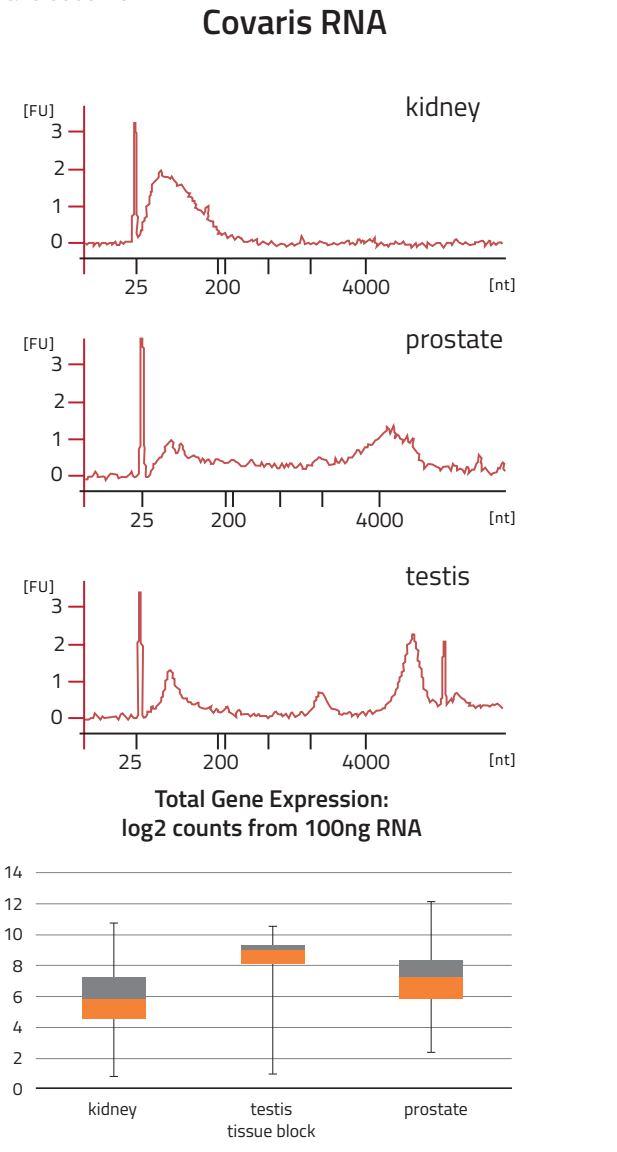


FIGURE 2: Panel A) Bioanalyzer traces of RNA isolated from 3 independent tissue blocks with Covaris truXTRAC kit. Panel B) Box and whisker plot of total Gene Expression counts of RNA samples in Cancer Reference Codeset Assays. Boxes with error bars represent median and maximum/minimum gene expression values, respectively, from the 236 gene panel.

Results (continued):

We assessed the RNA isolated with the truXTRAC method as input for the nCounter Gene Expression. First we analyzed the minimal input recommendation of 100 ng total RNA with the Cancer Reference Gene Expression Codeset (item GXA-CR1.). Figure 2B shows a box and whisker plot of total gene counts for the 236 gene CodeSet from three independent tissue sections. Consistent with previously published data, total gene counts from FFPE samples vary based on RNA degradation, with lowest signal coming from RNA with peak fragment sizes < 200 nucleotides (kidney tissue).

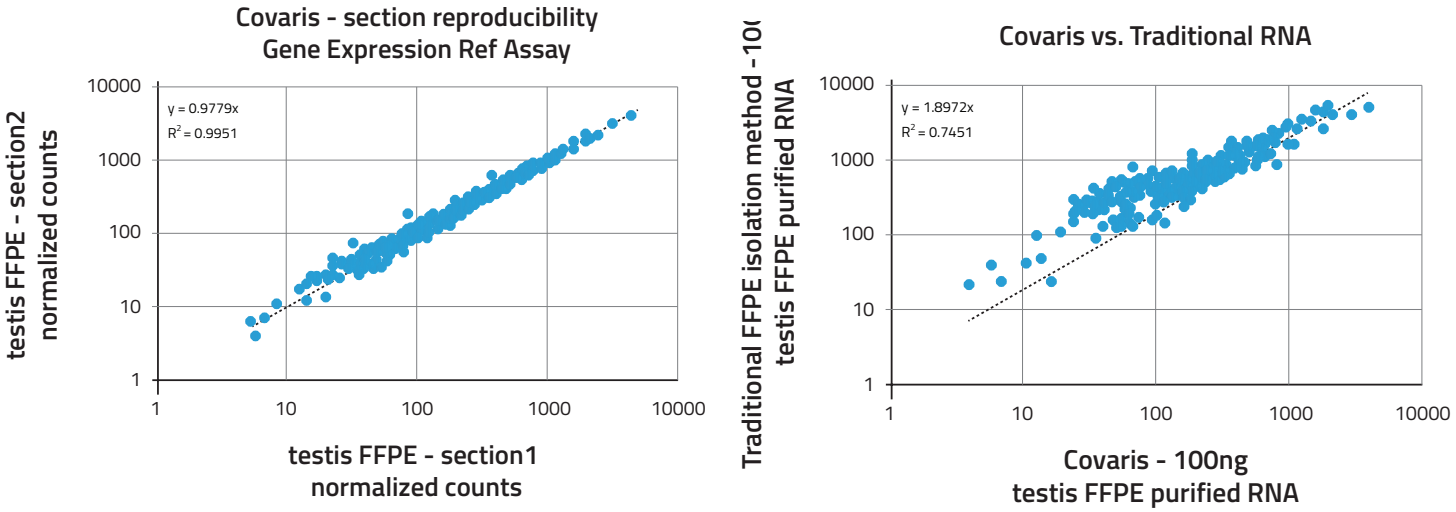


FIGURE 3: Counts from 100 ng RNA using nCounter Cancer Reference Codeset. Panel A) two replicate FFPE sections isolated by truXTRAC kit. Panel B) two replicate sections isolated by truXTRAC and traditional FFPE kits, respectively.

FIGURE 3A: plots the correlation in Reporter counts from RNA isolated from two representative FFPE tissue sections. Replicate RNA samples produce a high correlation (R2>0.99), consistent with published data, demonstrating high technical reproducibility of sample RNA on the nCounter platform.

FIGURE 3B shows positive correlation in gene counts between truXTRAC and traditional FFPE kit RNA isolations. Lower R2 value between kits is expected, due to the different technologies employed in the sample extraction that may introduce minor variation in RNA isolation efficiencies.

An advantage to using the nCounter hybridization system is the ability to use crude, unpurified extracts to directly measure gene expression from various sample types. Covaris truXTRAC RNA extraction is carried out in aqueous buffer, so we were able to test crude AFA treated FFPE extracts in the nCounter Gene Expression assay. Following Covaris AFA treatment, 5 µl of deparaffinized, proteinase K treated and crosslinks reversed FFPE extract was removed, and the remaining sample was carried through the complete truXTRAC column purification process. Figure 4 demonstrates the high correlation in gene expression counts between truXTRAC crude AFA-extract and purified RNA with a technical reproducibility of R2> 0.97. We note that for these replicate tissue sections, the crude AFA-extract produced approximately 2-fold higher counts from an equivalent amount of tissue than purified RNA, suggesting that some RNA may be lost during the column purification procedure.

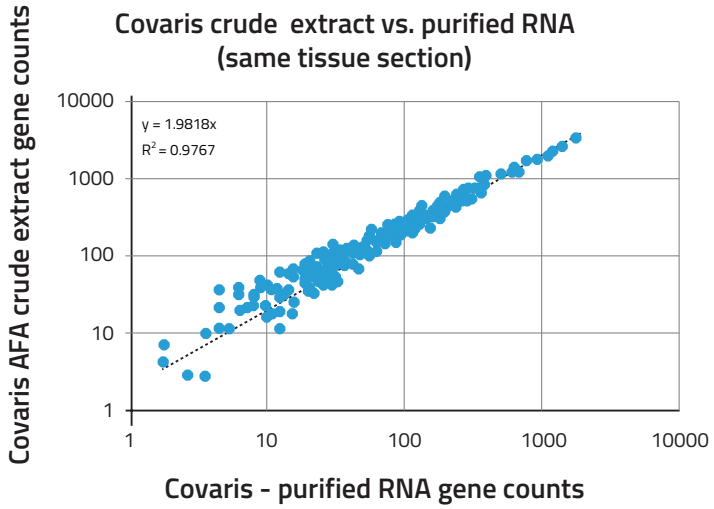


FIGURE 4: correlation between crude FFPE extract and purified RNA from Covaris system. From the same section, an aliquot of crude extract was retained, and the remaining material purified for RNA. Equivalent amounts of tissue by volume were loaded into the nCounter hybridization.

Conclusions

Gene expression and genomic copy number profiling of clinical FFPE derived samples are critical tools in the translational research field. Advances in methodology for easy, reproducible and sensitive preparation and analysis of these FFPE samples will enable more molecular testing on smaller amounts of material.

In this application note we demonstrated the effective pairing of the Covaris truXTRAC FFPE extraction technology with the NanoString's nCounter Analysis platform. RNA extracted using the Covaris system produce robust and reproducible data for the nCounter Gene Expression Assay.

Furthermore, we show that combining the ability of nCounter to work with unpurified material and truXTRAC's organic solvent-free workflow, a crude AFA treated FFPE extract can produce up to two-fold increase in gene expression counts compared to purified RNA. Working directly from the crude extract enables a dramatically simplified single-transfer step workflow requiring less than 40 minutes for the crude extract preparation, saving valuable time and resources during preparation of large numbers of FFPE samples, significantly reducing the probability of handling errors, and precious sample loss.

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LEARN MORE

Visit **www.nanostring.com/nsolver** to learn more about the nSolver 2.5 Analysis Software.

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