TECHNICAL REPORT

# Performance verification of the automated NuGEN® WT-Ovation™ FFPE System V2

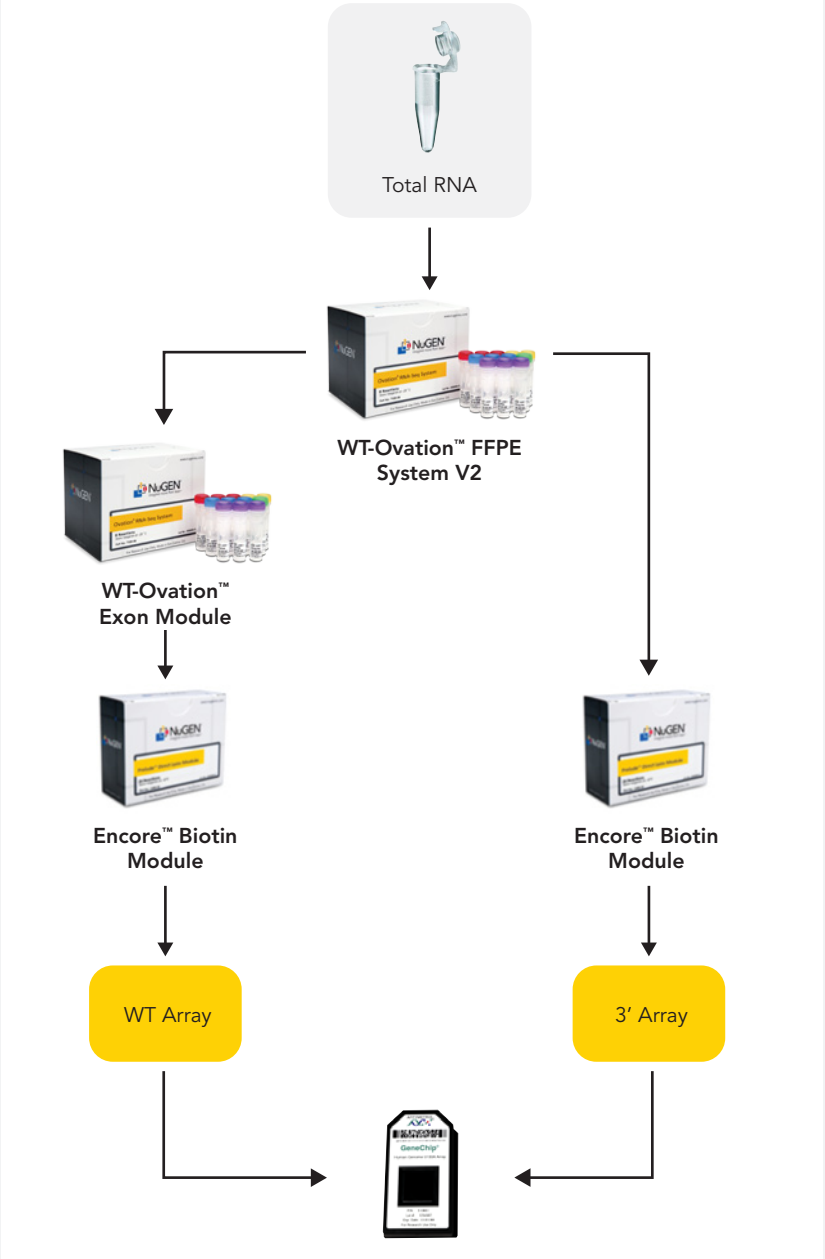
FFPE specimens are routinely collected in clinical environments and are a rich source of well-documented samples, playing an important role in cancer research studies. NuGEN provides solutions to enable transcriptome-wide expression analysis from challenging FFPE-derived samples on the analysis platform of your choice.

FFPE samples yield genomic material, including total RNA, that exhibits a wide range of sample quality, depending on the protocol used to fix the samples, the sample age and RNA extraction procedure. This variance in sample quality is one source of noise that can lead to variability in gene expression studies. As with any experimental design, it is always advised to limit the introduction of noise per step to an absolute minimum.

One way to achieve this is to automate sample processing. NuGEN has automated the WT-Ovation FFPE System V2* on the GeneChip® Array Station (GCAS) as well as on Beckman Biomek FX-based systems. The GCAS is a liquid handling system based on the Caliper Sciclone connected to a Twister II with a thermal cycler integrated on the deck. This technical report describes the performance of the automated NuGEN WT-Ovation FFPE System V2 on the GCAS.

The automated protocol is broken into independent modules which are grouped together in two main

FIGURE 1. **Sample preparation concept for analysis on 3´-based or WT-based array types**




NuGEN® imagine more from less™

categories: 1. Amplification and 2. Fragmentation and Labeling. These protocols are intuitive and easy to operate, offering sample processing from as few as 8 samples up to a full plate of 96 samples. The assay is designed to yield cDNA target (SPIA® product) compatible with 3´-based arrays (i.e., Affymetrix GeneChip® HG-U133 Plus 2.0 or related) as well as WT-based arrays (i.e., GeneChip Gene ST or Exon ST Arrays). The user interface offers the option to incorporate the labeled target into the appropriate hybridization cocktail formulated for either cartridge or peg arrays. The entire process from total RNA to a hybridization cocktail that is ready for use on Affymetrix expression arrays takes between 11.5 and 16 hours and is typically performed in two convenient work days. As an added benefit, there are several stopping points in the assay to enable maximum workflow flexibility.

| Method | Approximate Time | Optional Start/Stop Point |
|---|---|---|
| Primer Anneal | 25 minutes | ✓ |
| First Strand | 80 minutes | ✓ |
| Second Strand | 60 minutes | ✓ |
| Second Strand Purification | 60 minutes | ✓ |
| SPIA Amplification I | 70 minutes | ✓ |
| SPIA Amplification II | 120 minutes | |
| cDNA Purification | 50 minutes | ✓ |
| cDNA Quantitation | 15 minutes | ✓ |
| Normalization | 30 minutes | ✓ |
| Total time | 8.5 hours | |

**Table 1:** Estimated processing times for the individual methods of the automated NuGEN WT-Ovation FFPE System V2 on GCAS (processing times are for 96 samples).

## Protocol

The WT-Ovation FFPE System V2 automated on GCAS consists of 9 methods listed in **Table 1**. After initial deck layout and preparation of the required master mixes, the robot will independently run through to the cDNA quantitation step. This step requires assistance by the user and takes about 15 minutes in total. When processing more than 48 samples, two additional short user interventions are required to retrieve one PCR plate from the instrument and deliver it to an off-deck thermal cycler. The amplification protocol ends with normalized SPIA product that can be safely stored at –20°C for subsequent steps.

If 3´-based Affymetrix arrays are the intended method of data acquisition, fragmentation and labeling are carried out by the NuGEN Encore™ Biotin Module. This module is automated as a standalone protocol consisting of three methods that take approximately three hours to complete with 96 samples.

This technical report describes sample preparation for 3´-based arrays. For WT-based arrays, the amplified SPIA product must be converted by the WT-Ovation Exon Module prior to fragmentation and labeling by the Encore Biotin Module. This Module is also available as a standalone protocol for GCAS and takes just over 4 hours to complete with 96 samples. The modular form of the scripts allows maximum flexibility and easy integration into the laboratory workflow. For an overview on the different workflow options, see **Figure 1**.

The WT-Ovation FFPE System V2 is a very potent system for amplification of degraded RNA. As with every amplification system, extra care has to be taken in order to prevent sample carryover or contamination of the equipment with amplified material which could potentially result in cross contamination of samples. The robotics script was optimized to address these potential issues including reducing the speed of the 96-channel head at selected steps, using special consumables in the cleanup routine of SPIA product and using barrier tips as opposed to nested tips.

All NuGEN assays scripted on the GCAS are optimized for ease of use and **Figure 2** shows an example of the intuitive user interface that helps to prepare the robot for the run and minimizes the risk of operator-introduced errors.

## Assay Setup and Performance

Several sample types were tested to characterize the assay performance with intact and degraded RNA. As a model for good quality RNA, MAQC-A (UHR, Stratagene) and MAQC-B (Ambion) Reference RNAs were used in two input

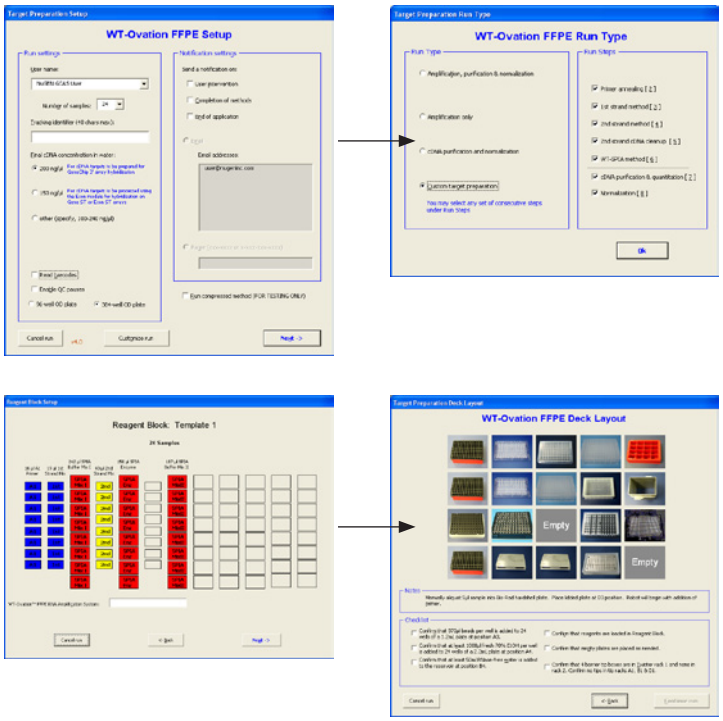
amounts of 50 and 100 ng, respectively. MAQC-A RNA was included in three robotic runs in a total of 11 replicates.

Two clinical FFPE tissue types were used: Sample type 1 is a mixture of lung tumor tissue blocks with 13 technical replicates and was also applied with 50 and 100 ng input RNA. Sample type 2 are sarcoma samples from nine independent blocks with two replicates each where each replicate represents a different RNA extraction from a different slice. Total RNA input amount was 50 ng for each reaction. In addition, three blocks were processed with six replicates in one robotics run and another six replicates in a second run. These 12 replicates allow assessment of run-to-run performance.

**Table 2** contains consolidated information from a total of three robotic runs. It gives an overview of sample yield and coefficient of variation (CV%) across the replicates. It is evident that the good quality RNA gives about three times higher yield of SPIA product but only a modest effect can be observed from the input RNA amount. This is also confirmed by the array data where neither the Pearson correlation matrix nor Principle Component Analysis shows significant differences based on the amount of total RNA input (data not shown). The CV% of cDNA yield is low confirming both the robustness of the WT-Ovation chemistry in general as well as the reliable performance of the robot.

For hybridization of the amplified cDNA to standard Affymetrix expression arrays, the automated Encore Biotin Module prepares the hybridization cocktail in 220 µL total volume. With FFPE samples, it is necessary to aim at a target concentration of 18–23 ng/µL which translates into a minimum yield requirement of 4 µg of SPIA product after the WT-Ovation FFPE System V2. This yield was achieved with every sample processed during the three runs.

FIGURE 2. **User Interface of the automated NuGEN WT-Ovation FFPE System V2 Protocol on GCAS.**



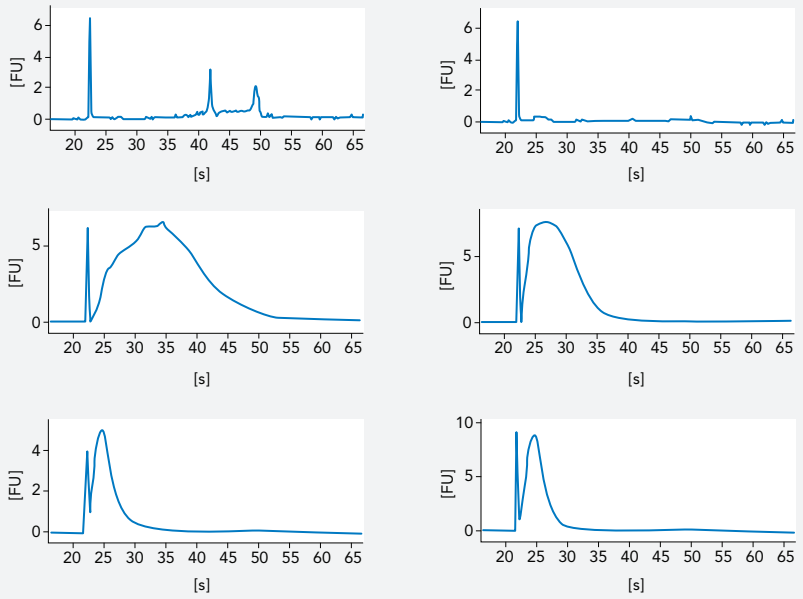

| Sample Type | RNA Input | Replicates | Avg Yield | CV% |
|---|---|---|---|---|
| MAQC-A | 50 ng | 7 | 18.5 µg | 10.5 |
| | 100 ng | 7 | 21.1 µg | 6.2 |
| MAQC B | 50 ng | 7 | 13.7 µg | 21.5 |
| | 100 ng | 7 | 17.9 µg | 7.6 |
| FFPE Sample Type 1 | 50 ng | 13 | 5.4 µg | 14.3 |
| | 100 ng | 13 | 5.6 µg | 6.8 |
| FFPE Sample Type 2 | 50 ng | 12 | 5.9 µg | 10.4 |
| | 50 ng | 11* | 6.4 µg | 6.1 |
| | 50 ng | 12 | 5.4 µg | 9.1 |

**Table 2:** Average yield of different sample types and coefficient of variation across replicates. FFPE sample type 1 is a lung carcinoma tissue in replicates from the same block. FFPE sample type 2 is a sarcoma tissue from three different blocks and processed in two independent robotic runs.

* One sample well in run #2 showed an elevated yield of 11.8 µg which was treated as an outlier and removed from the average yield calculation. This sample was hybridized to arrays and is included in the array data.

**Figure 3** shows traces from the Agilent Bioanalyzer documenting RNA conversion over the course of the assay. MAQC-A model RNA is of good quality with a RNA Integrity Number (RIN) of 8. The amplified SPIA product has a mean fragment size of 500 bases which is fragmented to <100 bases by the fragmentation and labeling reaction. In contrast, a typical FFPE RNA in this experiment has a RIN of 1 which indicates severe degradation and under normal circumstances would make the RNA inaccessible for transcriptome-wide expression analyses. The SPIA product from the FFPE RNA consequently shows a mean fragment size below 200 bases. However, it is also fragmented to <100 bases by the Encore Biotin Module.

FIGURE 3. **Typical Bioanalyzer traces**




**Top row:** total RNA. **Middle row:** SPIA product. **Bottom row:** fragmented and labeled hybridization target.

**Left column:** RNA from MAQC-A with a RIN of 8.0. **Right column:** RNA from an FFPE sample with a RIN of 1.0

The average fragment size of SPIA product is 500 for MAQC-A and below 200 for FFPE reflecting the degraded input RNA. cDNA target is fragmented to <100 bp for both sample types regardless of the SPIA product length.

## Array Performance

A total of 56 samples were hybridized to GeneChip HG-U133 Plus 2.0 Arrays according to the NuGEN Encore Biotin Module User Guide and the Affymetrix GeneChip Expression Analysis protocol. Basic data analysis was done with the Affymetrix Expression Console applying the MAS5 algorithm with default settings. More in-depth analysis was done with Partek Genomics Suite (Partek Inc., USA).

Array QC metrics are shown in **Table 3**. While intact Model RNA exhibits a mean %P value of roughly 66%, the FFPE RNA samples of type 1 average at about 32%, which again reflects the highly compromised RNA quality. Three blocks of FFPE RNA samples of type 2 are listed and each block averages at its own %P value showing the individuality of each block. Furthermore, the %P value correlates with the overall brightness of an array as can be seen from the scaling factors: a higher scaling factor correlates to lower signal intensity from the array and a lower percentage of genes detected. The coefficient of variation of the %P value across FFPE sample replicates is low for all sample

| Sample Type | SF (TGT = 500) | BG | 3/5 Ratio GAPDH | % Present | CV% | Reps |
|---|---|---|---|---|---|---|
| MAQC-A | 4.76 | 29.70 | 1.28 | 68.09 | 3.5 | 11* |
| MAQC B | 7.51 | 28.41 | 1.57 | 65.73 | 2.3 | 3 |
| FFPE Sample Type 1 | 55.17 | 26.00 | 2.70 | 32.06 | 9.6 | 11 |
| FFPE Sample Type 2 | 16.7 | 27.67 | 3.82 | 44.39 | 1.6 | 12 |
| FFPE Sample Type 2 | 14.86 | 28.01 | 3.28 | 46.25 | 1.3 | 12 |
| FFPE Sample Type 2 | 25.67 | 27.11 | 3.55 | 38.47 | 2.6 | 12 |

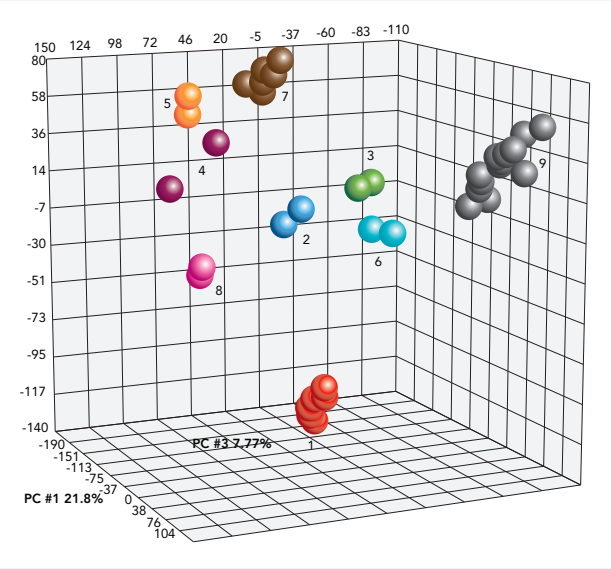
*11 replicates of MAQC-A through 3 different robotic runs

**Table 3:** Average performance metrics of different sample types on GeneChip HG-U133 Plus 2.0 Arrays. FFPE sample type 1 is a lung carcinoma and sample type 2 are sarcoma samples from three different individuals. Type 2 samples were processed in two robotic runs with six replicates each. A strong correlation can be observed between scaling factor, average %P and the associated CV% across the replicates.

types, again confirming the robust overall performance of the assay and the robot.

**Figure 4** shows the Principal Component Analysis (PCA) of the FFPE type 2 samples. Samples cluster according to their origin. Furthermore, correlation coefficients of 0.969 or higher within the sample types are calculated by a Pearson correlation analysis based on signal intensities (data not shown).

FIGURE 4. **PCA plot of 9 different FFPE blocks in replicates**




Samples are color coded and numbered according to their origin and replicates represent different slices from the same block. Samples 1 (red), 7 (brown) and 9 (grey) were processed in triplicates per slice and applied in two different robotic runs yielding a total of 12 replicates for each of these blocks.

## Conclusion

The NuGEN WT-Ovation FFPE System V2, currently automated on the Caliper Sciclone-based GCAS as well as on Biomek FX derived systems (Beckman), enables users to efficiently run large numbers of samples while minimizing the risk of user-introduced errors and greatly reducing labor time. This technical report describes the methods used to process samples on the GCAS.

After initial deck setup and preparation of master mixes, only a single user intervention is required to finish the entire protocol through to formulation of the hybridization cocktail. The intuitive user interface guides the user through the setup process and serves as a reference for correct deck layout and master mix preparation. Both 3´-based and WT-type arrays can be used for sample readout and the entire protocol takes between 11.5 and 16 hours to complete a full plate of 96 samples, depending on the desired array type. The protocol can be stopped at several points throughout the assay, providing maximum flexibility. Subsequent hybridization and handling of the Affymetrix expression arrays utilizes the default Affymetrix protocol.

Highly degraded FFPE RNA from as little as 50 ng can be reliably amplified to yield sufficient SPIA product for subsequent hybridization on expression arrays. Furthermore, it is demonstrated that the Encore Biotin Module robustly fragments the SPIA product to a length of <100 bases regardless of the fragment length of the input material itself.

Analysis of the expression data from hybridization to Affymetrix 3' arrays shows that the system yields elevated and robust %P values. In this study, the overall concordance of FFPE sample replicates is above 0.9691, even between two different robotics runs. This high concordance makes it possible to clearly discriminate between different individual blocks of sarcoma samples by both a correlation matrix as well as a PCA plot.

NuGEN offers enabling technologies that make possible analysis of the most valuable specimens in clinical research — FFPE samples are typically documented with the clinical outcome of a patient. This robust system makes retrospective studies possible and eases detection of biomarkers for classification of disease subtypes and possible drug development for a more targeted, personalized medicine approach.

*In June 2011, NuGEN released a newer version of this product, the Ovation® FFPE WTA System (Part No. 3403).

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