

# TransPlex<sup>®</sup> RNA Amplification Kit

## TransPlex Complete Whole Transcriptome Amplification Kit: Whole Transcriptome Amplification of Highly-Degraded RNA from FFPE Tissues

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### Abstract

The TransPlex Complete Whole Transcriptome Amplification (**WTA2**) Kit effectively amplifies intact and highly degraded RNA. To benchmark maintenance of representative RNA levels during amplification, differential gene expression of human liver and brain tissues was examined by microarray analysis. Expression profiles of high-quality RNA amplified with the TransPlex Complete WTA2 Kit or Eberwine linear amplification were compared with that of unamplified cDNA. Results indicate that the TransPlex Complete WTA2 Kit and unamplified profiles correspond closely. Effective amplification of RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue was demonstrated by comparing the array results using target prepared from frozen or FFPE malignant prostate samples, versus matched frozen normal tissue. KEGG pathway profiles and global transcriptome analysis reveal that amplified FFPE target performed comparably to that of frozen malignant tissue. The TransPlex Complete WTA2 Kit is able to amplify nanogram quantities of intact total RNA or highly degraded RNA from FFPE tissue samples while maintaining transcript levels representative of that of the unamplified input RNA.

### Introduction

The sequencing of the human genome has transformed etiological studies of human pathology and the pursuit of novel therapies. Until recently, validated drug targets, demonstrated to be associated with a particular disease or condition, were screened for their response to potential drug candidates. Target-based drug discovery has been increasingly replaced by phenotype-based approaches.<sup>1</sup> Phenotype-based discovery relies on high-throughput and high-content detection and screening processes to identify potential drug targets, which are de-convoluted by further experimentation and analysis. The gene expression microarray, for example, provides a high-content snapshot of sample-specific expression and is able to detect subtle, transcriptome-wide changes in mRNA levels resulting from disease, physiological development, or therapeutic treatment. These platforms have been successfully used to define disease markers.<sup>2,3,4,5</sup> But whereas a relatively small number of RNA source samples may identify potential disease markers, validation requires a substantially larger sample pool. Inaccessibility to a sufficient number of fresh (or fresh frozen) clinical samples impedes the validation process, and consequently, progress towards development of novel treatments for disease.

Over the years, histological studies have amassed large repositories of well-characterized formalin-fixed paraffin-embedded (FFPE) tissue samples. Amplified genomic DNA from fixed tissues has yielded a significant body of relevant information.<sup>6</sup> However, RNA lability and the effects of formaldehyde on RNA<sup>7</sup> give rise to degradation and chemical modification. To date, the scope of application for FFPE RNA has been restricted to rt-qPCR,<sup>8,9,10</sup> and a focused, diagnostic array platform.<sup>11,12</sup> Efforts to utilize amplified FFPE RNA for whole transcriptome microarray applications have met with some success, however only when using mildly degraded RNA, still maintaining a distinct 28S/18S total RNA banding pattern.<sup>13</sup>

In this study, maintenance of relative transcript abundance during TransPlex Complete WTA2 Kit amplification is first validated with commercially prepared high-quality RNAs. Subsequently, microarray expression profiles generated with target prepared from highly degraded RNA extracted from FFPE tissue are shown to be comparable to those prepared from the matched frozen counterpart.

### Materials

Matched normal and malignant human prostate samples, frozen and FFPE, were acquired from the Human Tissue Resource Network, College of Medicine, at Ohio State University. Total human liver and brain RNAs (RIN = 8.5–9.0, defined in *Methods*) were purchased from Ambion (an Applied Biosystems company, Foster City, CA). Agilent 4 × 44 K Whole Genome Microarrays (G4112F, Agilent, Santa Clara, CA) and ancillary consumables were used for microarray analysis (see *Methods*). All other materials and reagents were provided by Sigma-Aldrich Corporation or its affiliates unless otherwise specified.

### Methods

Total RNA was extracted from FFPE tissue samples using the Qiagen RNeasy<sup>®</sup> FFPE Kit; total RNA was extracted from frozen tissue using the GenElute<sup>™</sup> Mammalian Total RNA Extraction Kit (**RTN10**). Contaminating DNA was removed using RNase-free DNase I (**AMPD1**). Nucleic acid was quantified with Nanodrop<sup>™</sup> spectroscopy (a Thermo Fisher Scientific company, Wilmington, Delaware). RNA was assayed following the Agilent RNA 6000 Nano Bioanalyzer Kit protocol and assigned an “RIN” (RNA integrity number) based on a predictive algorithm for RNA quality.<sup>14</sup>

RNA was amplified using the TransPlex Complete WTA2 Kit (5 ng input quantity for intact RNA, 50 ng for FFPE RNA) or Agilent Low RNA Input Linear Amp Kit PLUS, Two-Color (500 ng), per directions. The Agilent RNA Spike-In Kit was used with the linear amplification method to monitor operator-related variance. Microarray cDNA target was direct-labeled using the Agilent Genomic DNA Labeling Kit PLUS. Hybridization conditions for all arrays were 65 °C, 20 rpm, using Agilent Oligo aCGH/ChIP-on-chip Hybridization Kit reagents. Slides were washed and developed using the Oligo aCGH/ChIP-on-Chip Wash Buffer Kit procedure, then scanned using the Agilent microarray scanner (model G2525B). Raw intensities were interpreted with Agilent 9.5.3.1 Feature Extraction software. All absent, saturated, and non-uniform

features were removed in Microsoft® Excel. Processed data were further analyzed using Spotfire® Decisionsite® 9.0 analysis software (Spotfire, Inc.) and Genesifter® (VizX™ Labs).

Dye-swap technical replicate data were processed in Excel, and subjected to Genesifter pair-wise analysis, including Welch's t-test, Benjamini and Hochberg correction, and fold-threshold setting of 1.5. Significant data (adjusted p = 0.05) were screened, referencing Genesifter ontological patterns and KEGG pathway<sup>15</sup> association. For comparative Genesifter KEGG pathway analyses (**Figure 5**), relative differential gene regulation (y-axis) was defined as the difference between the numbers of down- and up-regulated genes divided by the total number of genes in each pathway. Differential gene regulation was normalized (to 0) by subtracting the mean value of each data set from each individual value.

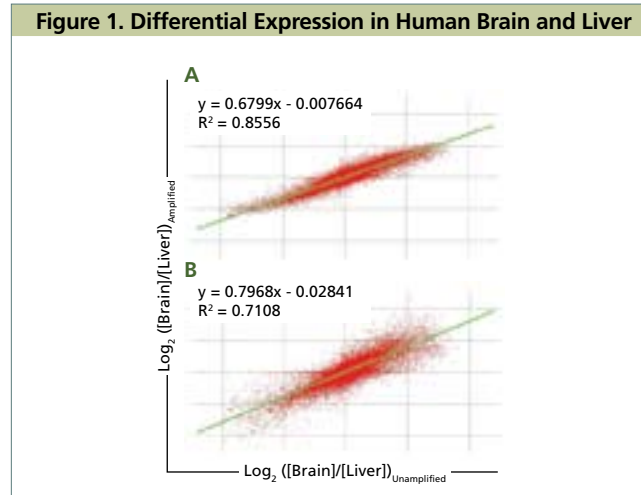
## Results and Discussion

### Amplification of High-Quality RNA

To validate the TransPlex Complete WTA2 Kit, differential expression of human brain and liver was examined by dual-color microarray analysis. cDNA targets (TransPlex Complete WTA2-amplified, linear-amplified,<sup>16</sup> and unamplified control) were prepared from commercially available total RNA, labeled, and hybridized as described in *Methods*. Log ratios of dye-corrected feature intensities for arrays treated with amplified target were plotted against the corresponding log ratios for unamplified control target (**Figure 1**). (The log ratio for each array feature represents the differential expression of a transcript.) Some observations are worthy of note. The slope of the plots for both amplification methods was less than one, indicating a marginal reduction in amplification efficiency with increasing differential expression. It has been proposed that amplification biases due to template length and sequence composition are responsible for this phenomenon.<sup>17,18</sup> The linearity of the TransPlex Complete WTA2 plot, however, indicates uniform amplification with respect to differential expression. Of critical consequence, R<sup>2</sup> was significantly higher for TransPlex Complete WTA2. Increased scatter perpendicular to the trendline at the distal regions of the plot observed for the linear amplification method indicates significant variance for genes showing lower and higher differential expression. The tighter R<sup>2</sup> value for TransPlex Complete WTA2 is interpreted to indicate exceptional maintenance of relative transcript abundance during amplification.

Further comparison of representative amplification for the TransPlex Complete WTA2 and linear methodologies is illustrated in Figure 2. Average log<sub>2</sub> intensities for human liver were plotted versus those of brain, for unamplified and amplified cDNA targets. A defined range of differential expression (log<sub>2</sub>[brain] versus log<sub>2</sub>[liver]) is marked blue in the unamplified control plot (row 1) using Spotfire array analysis software. Axes' coordinates are then transformed to TransPlex Complete WTA2 or linear amplification intensity values (rows 2 and 3, respectively). Less pervasive scatter parallel to the trendline observed for TransPlex Complete WTA2 is interpreted as greater uniformity of amplification efficiency, compared to that seen for the linear method (contrary to published results<sup>17</sup>). Scatter perpendicular to the data trendline (trendline not shown: running from lower left to upper right) is negligible

for TransPlex Complete WTA2 indicating that differential expression (log<sub>2</sub>(intensity<sub>brain</sub>/intensity<sub>liver</sub>)) remains relatively unchanged.



**Figure 1.** Log<sub>2</sub> ratios ([Brain]/[Liver]) for arrays targeted with **(A)** TransPlex Complete WTA2 and **(B)** Eberwine linear amplified-cDNA are plotted against the corresponding log<sub>2</sub> ratios for unamplified cDNA using Spotfire®.

It is essential to understand how these observations relate to a basic principle behind the utility of the TransPlex Complete WTA2 Kit. Variance in amplification efficiency between different transcripts (**Figures 1 and 2**) does not confound assessment of differential expression for a given transcript. Maintenance of the relative abundance of a transcript or “RNA representation” during amplification, however, is absolutely critical. A simple illustration provides explanation. Consider hypothetical unamplified RNA-source samples A and B. For A, the copy number of Transcript 1 is 1000, and Transcript 2, 100 (i.e., a 10-fold copy number ratio for the two different transcripts in sample A). Sample B contains 4000 copies of Transcript 1 and 25 copies of Transcript 2 (an intra-sample ratio of 160). The differential expression of transcripts 1 and 2, between samples A and B, is, however, 4 and 0.25, respectively. Due to biases intrinsic to exponential amplification, 1000-fold amplification is observed for Transcript 1 and 5000-fold amplification for Transcripts 2 (biases exaggerated for sake of illustration). Differential expression is maintained:

**Differential expression** of Transcripts 1 and 2, between Sample A and Sample B (Sample B/Sample A) is constant,

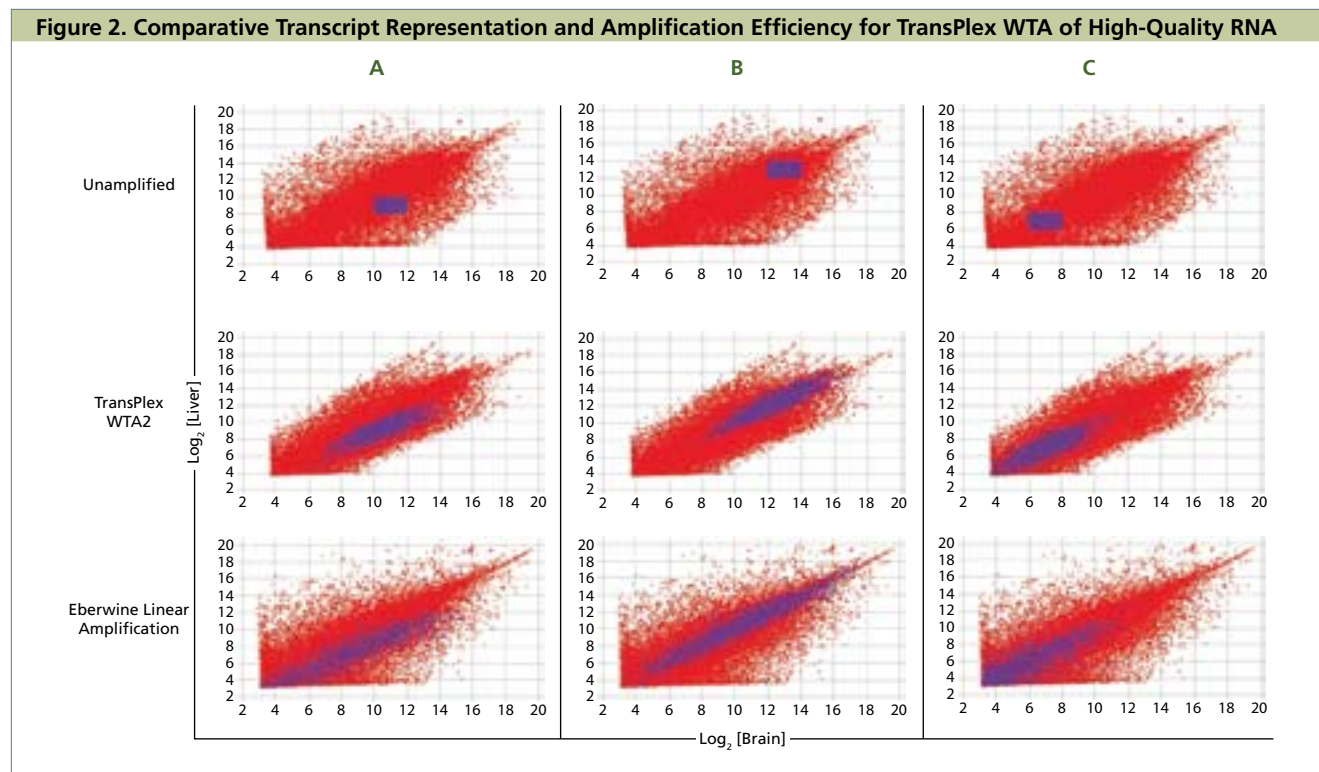
$$\begin{array}{ll} \text{Transcript 1 unamplified} & B/A = 4000/1000 = 4 \\ \text{Transcript 1 amplified} & B/A = (1000 \times 4000)/(1000 \times 1000) = 4 \\ \text{Transcript 2 unamplified} & B/A = 25/100 = 0.25 \\ \text{Transcript 2 amplified} & B/A = (5000 \times 25)/(5000 \times 100) = 0.25 \end{array}$$

whereas, the intra-sample ratio of the two transcript levels (Transcript 1 versus Transcript 2, T1/T2) changes.

$$\begin{array}{ll} \text{Sample A unamplified} & T1/T2 = 1000/100 = 10 \\ \text{Sample A amplified} & T1/T2 = (1000 \times 1000)/(5000 \times 100) = 2 \\ \text{Sample B unamplified} & T1/T2 = 4000/25 = 160 \\ \text{Sample B amplified} & T1/T2 = (1000 \times 4000)/(5000 \times 25) = 32 \end{array}$$

In summary, though the two transcripts are amplified with differing efficiencies, sample-to-sample differential expression of each

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**Figure 2.** For  $\log_2[\text{Liver}]$  vs  $\log_2[\text{Brain}]$ , selected array features are marked (blue) in columns A, B, C for unamplified cDNA (row 1). The corresponding features for TransPlex Complete WTA2 and Eberwine Linear Amplification are shown in rows 2 and 3, respectively.

transcript is maintained. (In cases where comparative expression analysis does not appear to lend itself to a differential comparison, introduction of a reference RNA to establish a differential resolves this dilemma.) Hence, the expression profiles for the amplified samples are facsimiles of the unamplified samples.

The results of this benchmark experiment therefore confirm that TransPlex WTA2 is able to maintain relative differential expression between samples.

## Amplification of Highly Degraded RNA from FFPE Tissue Samples

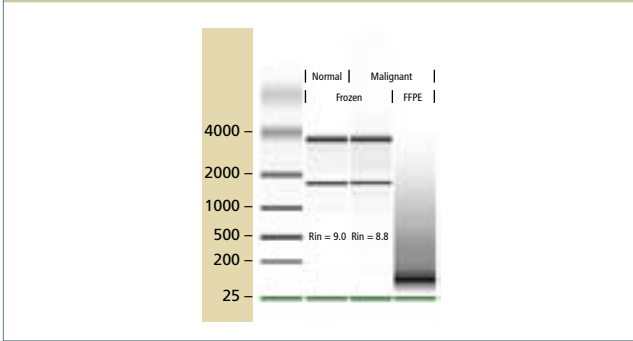
The utility of TransPlex Complete WTA2 for amplifying highly degraded RNA was demonstrated by microarray analysis of differentially labeled target prepared from matched malignant and normal prostate tissues. Amplified RNA from malignant prostate was first extracted from either frozen or FFPE tissue samples, and in both instances, compared to amplified RNA from normal (non-malignant) frozen prostate tissue. RNA extracted from frozen tissue was determined to be of high quality (RIN 9), whereas degraded FFPE sample RNA averaged 100 bases in size, showing no rRNA banding pattern (Figure 5).

Representative and efficient amplification of FFPE RNA was detected with Spotfire analysis (Figure 4). The slope of frozen malignant feature intensities versus frozen normal is 0.9639, and for FFPE malignant versus frozen normal, 0.8905, inferring an overall higher amplification efficiency for frozen RNA, likely due to the

degraded condition of the FFPE RNA. Based on scatter parallel to the slope, amplification efficiency for FFPE RNA appears to be more uniform than for high-quality RNA (Figure 2). Uniform FFPE target size, as indicated by the highly degraded condition of the FFPE RNA (Figure 3) may explain this observation, as opposed to the broader range of target size for intact RNA target (200–400 bp, data not shown). Though representation remains to be generally well maintained, there appears to be more scatter perpendicular to the trendline in Figure 4 for FFPE RNA than was found for intact RNA (Figure 2). This also may be due to the degraded condition and coincident reduction of target complexity of the FFPE RNA, contrary to the case of the frozen tissue target. Also, with the decreased size distribution of the degraded RNA template, semi-degenerate primer-driven amplification may become increasingly difficult.<sup>19</sup>

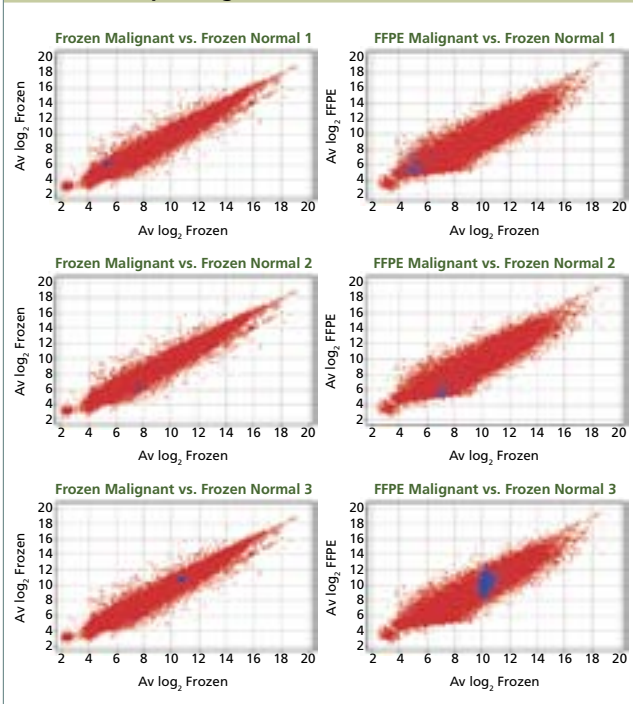
Surprisingly, despite suggestion of a potential loss of relative transcript levels, discussed above in Figure 4, KEGG pathway profiles generated from microarray analysis of FFPE malignant prostate tissue are comparable to those for the frozen malignant tissue (Figure 5). FFPE malignant vs. frozen normal very closely tracks frozen malignant vs. frozen normal for up- and down-regulated expression (panel A) and highly populated KEGG pathways (panel B).

**Figure 3. Bioanalyzer Virtual Gel Image of RNAs Isolated from Frozen and FFPE Tissues**



**Figure 3.** Lanes (l-r): marker, frozen normal prostate, frozen malignant prostate, FFPE malignant prostate. Two hundred fifty nanograms of RNA were applied to each non-marker lane.

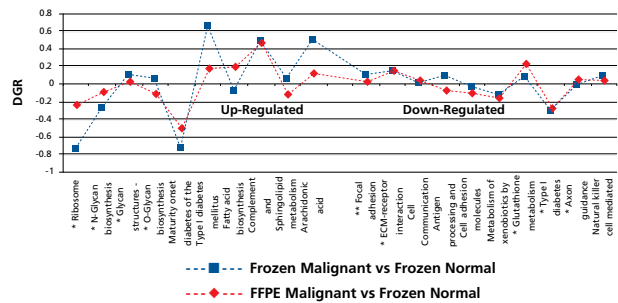
**Figure 4. Comparative Transcript Representation and Amplification Efficiency for FFPE vs. Frozen Sample Target**



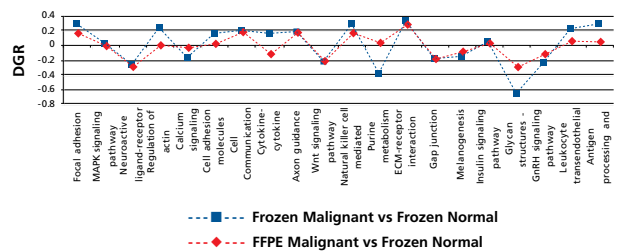
**Figure 4.** Marked array features as described in Figure 2.

**Figure 5. Array Target Prepared from Amplified RNA FFPE vs Frozen Tissue**

**A. Statistically Significant KEGG Pathways**



**B. Highly Populated KEGG Pathways**



**Figure 5.** Maintenance of representation during amplification for FFPE vs. frozen target. **A.** The x-axis corresponds to KEGG pathways having the numerically largest number of functionally related genes. **B.** The x-axis corresponds to the top 10 up-regulated and down-regulated KEGG pathways, based upon the Z-test score for up- or down-regulated genes subsets within the pathway. (A single asterisk indicates a significant Z-score ( $z < 0.5$ , or  $z > 2$ ) for a respective up- or down-regulated gene subset for *TransPlex*<sup>®</sup> WTA2-amplified target; a double-asterisk indicates significance for both up- and down-regulated gene subsets.)

## Conclusions

TransPlex whole transcriptome amplification has previously been reported to provide key advantages over other RNA amplification methods including 1000-fold amplification in less than 4 hours, without 3'- bias.<sup>19</sup> Results presented here demonstrate the ability of the next generation TransPlex WTA2 Kit to effectively maintain relative transcript abundance during amplification of intact and highly degraded RNA.

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