

# Amplification of mRNA without 3' bias from formalin-fixed paraffin-embedded (FFPE) breast cancer tissues and multiplex expression analysis on flow-through microarrays

Englert, D, Wilson, DJ, Laken, S.  
Xceed Molecular, Toronto, Ontario, Canada



## Abstract

Total RNA extracted from FFPE samples was subjected to two rounds of amplification with the ExpressArt *TRinucleotide* reagents (AmpTec) and hybridized on flow-through TipChips on the Zplex<sup>®</sup> Automated Workstation. Performance was assessed by the analysis of titration mixtures prepared from mRNA from breast cancer and colon cancer FFPE tissue blocks, and from control breast cancer samples comprising two pools of RNA from tumors with expression profiles characteristic of high or low risk of recurrence.

The results demonstrate the feasibility of global mRNA amplification and quantification of transcript abundance in degraded mRNA from FFPE samples. Expression differences of two-fold or less may be analyzed with tens or hundreds of probes for translational research and clinical assay development on the Zplex Automated Workstation. Many probes may be tested in parallel to optimize probe sets for specific tests.

## Background

FFPE samples are an abundant sample resource for validation of expression-based assays and are well suited for routine clinical testing. Analysis on microarrays enables the analysis of many transcripts from samples with small amounts of RNA. However, mRNA in FFPE sections is degraded and cross-linked, with RNA fragment sizes of a few hundred bases or less, and consequently standard methods of microarray sample preparation are not reliable unless probes are very strongly biased to the 3' end. It is not always possible to design high quality probes within this restricted region of mRNAs. Amplification of target sequences any place within transcripts would permit greater flexibility in probe design and optimization of probe sets for reliable multiplex analysis.

## Objective

The objective of this study was to determine whether global amplification of mRNA with *TRinucleotide* cDNA priming, *in vitro* transcription and hybridization on flow-through microarrays is capable of quantifying differential gene expression of two-fold or less from archived FFPE sections.

## Materials and Methods

Total RNA extracted from FFPE samples was subjected to two rounds of amplification with the ExpressArt *TRinucleotide* Nano C&E kit (AmpTec, GmbH). Biotin-labeled anti-sense RNA (aRNA) was prepared from the second round cDNA with the Illumina<sup>®</sup> TotalPrep<sup>™</sup> RNA kit (Applied Biosystems). The labeled aRNA was hybridized to about 500 or 250 probes immobilized in singlet or duplicate on flow-through microarrays (TipChips) on the Zplex Automated Workstation (Xceed Molecular).

## mRNA Amplification and Labeling

*TRinucleotide* primers (AmpTec GmbH, [www.amp-tec.com](http://www.amp-tec.com)) amplify RNA fragments preferentially from the ends. There is no bias for 3' ends of intact transcripts and a strong bias against ribosomal RNA amplification.

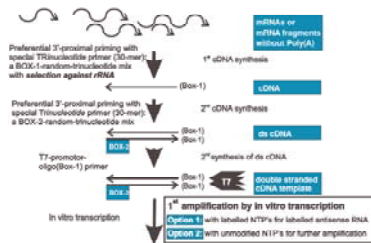


Figure 1 – mRNA amplification from total RNA extracted from FFPE sections with AmpTec ExpressArt reagents.

## Sample Processing/Data Analysis

During processing on the Zplex Automated Workstation samples and reagents are passed through the probe arrays in microplate wells for efficient mixing and target transport, and numerical results are available within three hours of the start of hybridization. Quality control metrics are automatically assessed to ensure the output of only high quality data. Some samples are too degraded to amplify well, resulting in low signal intensities and high background. These samples are flagged by Zplex quality control metrics.



Figure 2 – Zplex Automated Workstation with TipChips and reagent consumables.

## “MAQC”- like Sample Titration

In an experiment analogous to the MAQC study (Nature Biotechnology, September, 2006) total RNA extracted from FFPE sections from breast cancer (sample A) and colorectal cancer (sample B) were mixed in the proportions shown to create the C and D titration mixtures. Aliquots of the four samples were amplified and then hybridized on the Zplex Workstation. There was good agreement ( $R^2 > 0.95$ ) between the observed results for the C and D samples and the predicted results of the C and D mixtures calculated from the A and B expression results (Figure 3). Median CVs of the A, B, C and D samples were 19.9, 18.7, 24.3 and 19.6 %, respectively.

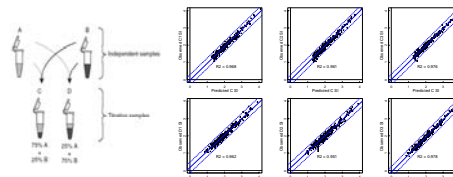


Figure 3 - Observed normalized signal intensities for C and D samples vs. predicted intensities calculated from A and B samples.

## Probes for Hormone Receptor Genes

More than one probe design for a given gene can be compared for a set of pilot samples to screen probes for the best performance.

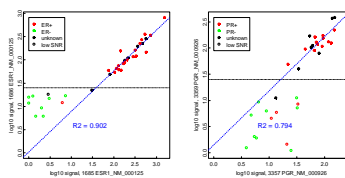


Figure 4 - Comparison of signal intensities of two Zplex probes for each of the ESR1 and PGR genes for 38 breast cancer samples. The ER or PR status of the patients based on immunohistochemistry, where known, are indicated by red or green symbols. Samples with very weak signals are indicated with open circles.

## Breast Cancer Control Samples

Total RNA was extracted from two sets of FFPE sections characterized by expression profiles with high or low risk of recurrence. Replicates of these samples were independently prepared and hybridized in separate batches to test repeatability of the process.

All correlations between four replicates of each of the control samples are shown in Figure 5. All  $R^2$  values were greater than 0.9, and there were few residuals from the regression greater than two-fold (outside the dotted lines).

Differential expression between the two control samples is shown in Figure 5. Expression differences greater than two-fold (to the right or left of the vertical dotted lines) were detected with small P values. The genes expressed at relatively high levels in the low risk sample pool were mainly associated with the estrogen response (luminal subtypes). The genes expressed at relatively high levels in the high risk sample pool are associated with subtypes with relatively poor prognosis.

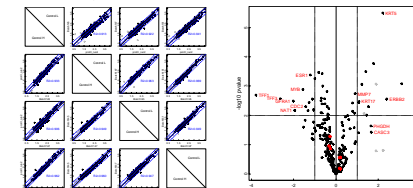


Figure 5, left - All correlations between  $\log_{10}$  signal intensities of four replicates of two control samples. Lower left: sample pool representative of relatively low risk of recurrence. Upper right: sample pool representative of relatively high risk of recurrence.

Figure 5, right - The negative  $\log_{10}$  of P values from a t-test vs.  $\log_2$  of differential expression for the two control samples. Certain genes known to be differentially expressed between subtypes are labeled. Probes plotted in red were used to normalize the data.

## Conclusions

- The results demonstrate the feasibility of amplifying and quantifying sequences at any position within transcripts in degraded mRNA from FFPE samples. Results accurately reflect transcript abundance in total RNA samples.
- Expression differences of two-fold or less may be analyzed with tens or hundreds of probes for translational research and clinical assay development on the Zplex Automated Workstation.
- Many probes may be tested in parallel during assay development to optimize probe sets.