Transplex Whole Transcriptome RNA Amplifications

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Abstract

The efficacy of amplification of small quantities of total RNA with the TransPlex® Complete Whole Transcriptome Amplification Kit (WTA2) was examined in this study. Total RNA extracted from decreasing numbers of FACS-isolated bone marrow stem cells (10-, 100-, and 1000-cells samples) was amplified with the TransPlex WTA2 kit. A call rate of 58.8% of unique biological array features was observed for the 10-cell vs. 100-cell microarray analysis, with a similar call rate of 61.46% for 10-cells vs. 1000-cells. Greater than 90% commonality existed between the intersecting data sets for the two analyses.

After a more stringent screening (p = 0.0001), the 10-cell vs. 100-cell comparison revealed 5568 intersecting features. The comparable analysis for 10 cells vs. 1000 cells resulted in 4977 features, with 3862 features common to both comparisons. In addition, the effect of decreasing RNA input on amplification efficiency was examined. Results indicated that an adjustment to the library synthesis primer concentration allowed for maintenance of linear amplification and representative qPCR at low RNA input quantities. This adjustment proved to be critical for downstream qPCR applications, but not for the case where the amplification product was used as microarray target. This study confirms that the TransPlex Complete Whole Transcriptome Amplification Kit can effectively amplify low input quantities of RNA, approaching the single cell level.

Introduction

The TransPlex Complete WTA2 kit was initially developed for enhanced amplification of damaged RNA, described at sigma.com/wta2, “Detection of Prostate Cancer Biomarkers by Expression Microarray Analysis of TransPlex® WTA2-Amplified FFPE Tissue RNA”.

Moreover, efficient amplification of small reaction input quantities of RNA, approaching the single cell level, has been demonstrated using the TransPlex Complete WTA2 kit. RNA isolated from as few as 10 fluorescence-activated sorted cells (FACS) is amplified, maintaining expression patterns observed for 10- and 100-fold larger cell samples. Challenges unique to exponential amplification of small RNA input quantities are discussed and effectively addressed for downstream qPCR and microarray applications.

Materials and Methods

FACS-isolated bone marrow stem cells were provided by Albert Donnenberg, University of Pittsburgh. Cells were sorted directly into GenEluteTM Mammalian RNA Extraction Kit Lysis Buffer (Cat. No. RTN10, component No. L8265), frozen and stored at -70°C until extraction. Total RNA was extracted from 10-, 100-, and 1000-cell samples using the GenElute Mammalian RNA Isolation kit. Sample RNA was eluted at a reduced volume of 40 µl with nuclease-free water, and subjected to a scaled-up (5X) RNasefree DNase digestion (Cat. No. AMP-D1), for a final volume of 97 µl. Each RNA sample was amplified in its entirety using the TransPlex Complete WTA2 kit: the entire heat-attenuated DNase digestion was directly added to the recommended bulk amplification reaction volume of 375 µl, compensating by subtraction of water volume. As per kit instructions, the 375 µl volume was split into five 75 µl reactions for amplification. Each reaction was carried through two cycles beyond stationary amplification (“plateau”). Unincorporated primers and other reaction components were removed from the amplification product using the GenElute PCR Cleanup kit (Cat. No. NA1020). Quantitative PCR was performed using SYBR® Green JumpStart™ Taq ReadyMix™ (Cat. No. S4438), with 250 nM primer concentration. Primer pairs, with the exception of the 18S rRNA set, are specific for cDNA detection.

Amplified RNA (double-stranded cDNA) was labeled with cy3 or cy5 following the Agilent Genomic DNA Labeling Kit Plus procedure (Cat. No. 5188-5309). Two amplified, differentially-labeled cDNA targets were combined, heat-denatured, applied to Agilent Whole Genome™ arrays (G4112F), and incubated at 65°C for 40 hours. Hybridization and subsequent wash procedures for the Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis were followed, except for the omission of COT-1 DNA block during hybridization. Array were scanned (Agilent scanner model G2505B) and features extracted. Intensities representing non-uniform features or associated with local non-uniform background, in addition to “absent” and saturated array feature intensities, were removed using Microsoft Excel™. All intensities less than 100 were removed. Intensities were then subjected to median normalization, Welch’s t-test, Benjamini-Hochberg false discovery rate correction, and a +1.5-fold differential threshold using Genesifter pair-wise comparison, except where only median normalization was performed, as indicated. Normalized-only intensities and intensities subjected to Genesifter™ (Geospiza) pairwise analysis were further analyzed using the Genesifter
Intersector software and Spotfire™ (TIBCO).

For the study of the effect of reduced library synthesis primer concentration on amplification of decreasing quantities of RNA input, mRNA levels for high- and medium-copy transcripts in the amplification products, plus a “rare” transcript, were evaluated by real-time qPCR. Preparation of the lowered concentrations of library synthesis primers was accomplished by dilution of the kit Library Synthesis Solution (component# L9293) with 5 mM dNTPs (a 2-fold dilution of 10 mM dNTPs, Cat. No. D7295).

Results and Discussion

Amplification of Cellular RNA.

Total RNA was extracted from FACS-isolated human bone marrow stem cells as described in the Materials and Methods. Amplification for all samples was allowed to proceed through 2 cycles of “plateau”, or stationary amplification (Figure 1). Amplification product was pre-evaluated by qPCR and agarose gel electrophoresis prior to microarray analysis (Figure 2). An intense low molecular weight band is observed for the “No RNA” control reaction. This banding pattern is occasionally observed for very low input quantities of RNA, though not in the case presented here. This fragment has not been sequencelabeled, though it is likely to be a multimer of the TransPlex WTA2 library synthesis primer sequence. The 3’ region of TransPlex WTA “quasi-random” primers are non-self-complementary, providing a vast improvement in the efficiency of amplification over poly-N priming. However, the primer for the Transplex WTA2 kit has been re-engineered for amplification of fragmented RNA. In so doing, concatemerization of the primer could occur in the absence of template. An alternative explanation will be discussed below.

Figure 1 TransPlex WTA Methodology.

Total RNA is combined with Library Synthesis Solution and heat denatured. This is followed with the addition of Reaction Buffer and the strand-displacing Library Synthesis Enzyme, for “single-tube” reverse-transcription and secondstrand cDNA synthesis (OmniPlex™ Library). The 3’ end of the Library Synthesis Primer is “quasi-random” and substantially non-self-complementary, while the 5’ end is a single, constant non-self-complementary sequence that serves as the annealing site for the universal amplification primer.
Figure 2 RNA Amplification Profiles.

Total RNA was extracted from cell samples as described in Materials and Methods. RNA amplification was allowed to proceed through 2 cycles of stationary amplification (observed by real time PCR using SYBR Green dye). One thousand cell reactions were stopped at 19 cycles; 100- and 10-cell samples at 23 cycles.

Microarray Analysis of Whole Cell Samples

A strong concordance between the three stem cell samples is observed for detection of microarray features, in this case, normalized intensities (> 100) (Figure 3). Greater than 85% of features detected in the 10-cell sample are also detected for the 100-cell sample, while > 90% of 10-cell features are also detected in the 1000-cell sample. Considering the total number of unique biological probes (41,000) on the array, detected features for the 10-cell and 100-cell comparison account for 59% of total features, with 48% of features shared between the data sets. Similarly, the 10-cell and 1000-cell sample comparison, 61% of total array features are detected, with 52% of features shared. It is noteworthy that exclusion of the data less than 100 denotes a conservative measure, since the Agilent feature extraction software removes calculated background.

Figure 3 Amplification Qualitative Test. Prior to microarray analysis, amplification reaction products were evaluated by real-time qPCR using primer pairs for 18S, GAPDH, and β-actin transcripts (A, B). qPCR amplicons were further checked by 1% agarose
Application of statistical tests provides additional confidence to the normalized data (Figure 4). An excess of 55,000 features were common for the 10-cell and 100-cell samples at a p = 0.0001. (This stringency reflects the analysis of an n = 4 for each data point). Moreover, 70% of features common to the two data sets (the 10-cell/100-cell comparison) were also detected in the 10-cell/1000-cell intersection. Similar percent values of commonality are observed in other studies, such as comparing dilutions of intact RNA, matched FFPE and frozen tissue sample RNA, or amplified versus unamplified RNA (not shown). Testing is underway to determine whether amplified transcripts represented in a “unique” data set are present in the unamplified sample. We hypothesize that the TransPlex WTA2 amplification method is sufficiently sensitive to detect basal expression. Such highly stochastic signal should, however, be able to eliminate with sufficient replication and stringent statistical testing.

Amplification of Low RNA Input: Effect of Banding Pattern on qPCR Analyses

The low molecular weight banding pattern, observed for the No RNA Control in Figure 2, has been observed in some 10-cell amplifications, and occasionally in the 100-cell amplification (not shown). It was suggested above that the phenomenon might be reasonably explained by concatemerization of the library synthesis (LS) primer. An alternative hypothesis is that the relatively high concentration of the LS primer results in over-priming, and ultimately, a diminutive amplification product. This is possible since library synthesis (first and second strand cDNA synthesis) occurs in a single tube, driven by an enzyme with strand-displacement capability. To test this idea, decreasing human prostate RNA input quantities were matched with similarly decreasing LS primer concentration during the library synthesis step. Two-fold serial dilutions of each component allowed for evaluation of twelve RNA input values ranging from 25 ng to 12.2 pg, and LS concentration starting at 1X kit concentration, to 0.0078X, on a 96-well plate. A marked loss in linearity of amplification efficiency is observed when using the 1X LS primer concentration, at RNA input quantities less than ~800 pg (Figure 6A). Linearity returns for lower RNA input quantities as the concentration of the 1X LS primer concentration is reduced by 2-fold increments (data not shown), corresponding to the reduction of the low molecular weight banding pattern (Figure 5). Quantitative PCR results using primer sets for high- (18S rRNA), medium- (beta-actin, GAPDH), and low-copy TRCC2 transcripts corroborate these observations (Figures 7A-D). Interestingly, detection of TRCC2, presumably a low copy transcript in prostate, is problematic at the 1X concentration, with only a background signal observed for an intermediate RNA input quantity. TRCC2 amplicon is detected for all RNA input quantities at an 8-fold LS primer dilution, indicating an 8-fold dilution of the WTA2 LS primer optimal for TRCC2 for downstream qPCR.

Figure 4 Microarray analysis revealed a strong concordance between the cell samples. Overlapping regions represent median-normalized intensities (>100). Greater than 85% of detected features represented in the 10-cell sample are detected for the 100-cell sample, while >90% of 10-cell features are also detected in the 1000-cell sample. In addition, the overlapping regions are highly concordant, with greater than 98% of 10-cell/100-cell overlapping intensities detected in the 10-cell/1000-cell intersection.

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Figure 5  A substantial number of statistically significant microarray features (>5500) are detected, with concordance between the two overlapping regions of the cell sample data sets maintained following stringent statistical analysis and corrections of the data presented in Figure 3 (see Materials and Methods). Seventy percent of 10-cell/100-cell overlapping intensities are detected in the 10-cell/1000-cell intersection.

Figure 6  The effect of library synthesis primer concentration on amplification of small quantities of input RNA was examined. Two-fold serial dilutions of human prostate total RNA were added to reactions, with input quantities ranging from 25 ng to 12.2 pg. In addition, decreasing concentrations of library synthesis primer were evaluated: the 1X Transplex WTA2 Kit concentration and
three additional 2-fold serial dilutions. C(t) values measured at early exponential growth (threshold, 0.025) for each amplification reaction are plotted against the input quantity of RNA for each library synthesis primer concentration tested. The results for the kit concentration primer (1X) and a 2-, 4-, and 8-fold dilution of primer are shown. These results suggest that RNA input quantities less than ~800 pg are not efficiently amplified in the presence of 1X library synthesis primer concentration. Intermediate primer concentrations (0.5X and 0.25X) show increasing improvement of amplification efficiency. Overall efficiency appears to be maintained when the 8-fold dilution of library synthesis primer is used.

Figure 7 Amplification products were subjected to qPCR evaluation using primer sets for 18S rRNA (A), β-actin (B), GAPDH (C), and TMCC2 (D). Inserts show the melt curves for amplification product at the decreasing RNA reaction input quantities, and for the 1X and 0.125X primer concentrations. A–C represent high-copy rRNA and medium-copy and house-keeping transcripts; TMCC2, human transmembrane and coiled-coil domain family 2 transcript, was noted to be inconsistently detectable in previous studies, with C(t)s in the mid-30s. Interestingly, a pattern of biphasic inefficient amplification is observed for 18S rRNA, at highest and lowest input quantities for both library synthesis primer concentrations. Generally consistent C(t) values are observed for β-actin and GAPDH at the 0.125X primer concentration, while a gradual decrease in efficiency is observed as input quantities decrease at the 1X primer concentration. At the 1X primer concentration, no amplification product is detected for TMCC2. Only a spurious signal at 75 degrees (primer-dimer, gel data not shown) is observed. However, at the low library synthesis primer concentration, TMCC2 amplicon is detectable at all input quantities.

Amplification of Low RNA Input: Effect of Banding Pattern on Microarray Analyses

To determine whether the use of a reduced LS primer concentration is appropriate for microarray analysis of low RNA input quantities, amplification product from three input quantities was tested (Figure 8): 6.25 ng (similar to the kit-equivalent of 5 ng per 75 ul reaction) (1), 391 pg (just less than the 800 pg demarcation observed for reduced amplification efficiency for 1X LS primer concentration in Figure 6A) (2), and 48.8 pg (approximately the RNA mass equivalent of a ‘single’ cell) (3). Amplification products were labeled and subjected to microarray analyses as described in Materials and Methods. In Figure 8A (non-normalized intensities) reproducibility between same-dye duplicate data
for each original RNA input quantity is shown. Figure 8B (also non-normalized intensities) shows a similar comparison of duplicates for each input quantity (n = 4). Each comparison shows an increase in variance between the two sets of data. However, one would postulate that there is a considerably less loss of quantitative and qualitative correspondence between the duplicates generated in 1X LS primer than those prepared with the 8-fold dilution. This observation is extended to Figure 8C, where the data sets have been statistically tested (t-test, false discovery rate correction, and +1.5 threshold). Unquestionably, the 1X library synthesis primer concentration performs better for microarray analysis.

Figure 8 Amplification product generated from 6.25 ng, 390 pg, or 48 pg input quantities, prepared with 1X or 0.125X library synthesis primer, was labeled for microarray analysis as described in Materials and Methods. Results are shown in Panels A through C (log intensities vs. log2 intensities). Panel A shows reproducibility for duplicate 6.25 ng reactions (1), 390 pg reactions (2), and 50 pg reactions (3). In Panel B, 6.25 ng reaction intensities are plotted versus 390 pg reaction intensities (1), 6.25-ng reaction intensities versus 48-pg reaction intensities (2), and 390 pg reaction intensities are plotted versus 48 pg reaction intensities (3). Statistically analyzed and normalized intensities are shown in Panel C: 6.25 ng reaction intensities versus 390 pg reaction intensities (1), 6.25 ng reaction intensities versus 48-pg reaction intensities (2), and 390 pg reaction intensities versus 48 pg reaction intensities (3). The positive effect of lowered library synthesis primer concentration observed for quantitative PCR is not evident in microarray results.

Conclusions

These observations indicate that reduced library synthesis primer concentration enhances detection of low-copy transcripts when performing downstream qPCR application. At 1X LS primer concentration, medium-copy transcripts can be reliably detected. However, at this concentration, low-copy transcripts may be overprimed, resulting in template too
short for both qPCR primers of a set to find their respective annealing sites. This would not appear be a problem for microarray amplifications, where such size reduction of the amplification product would be essentially equivalent to target fragmentation. However, reduced primer concentration decreases the probability of the LS primer to annealing to all potential templates during library synthesis, thus preventing uniform amplification of the transcriptome. This is not to suggest that the observed effect of higher LS primer concentration on amplification efficiency (Figure 6) is not affecting microarray results. This effect manifests itself in predictable and manageable small increases in expression differentials for low-copy transcripts, and a similar minor compression of those of high-copy transcripts.

Therefore, the low library synthesis primer concentration is optimal for detection of low-copy transcripts by qPCR, and the standard kit concentration for representative amplification for all RNA input quantities when following with microarray analysis.

For the investigator utilizing TransPlex WTA2 amplification product for qPCR, it should be noted that the 8-fold dilution of the LS primer was shown here to be optimal for TRCC2. This could vary with individual amplicons. It is suggested that an investigator test serial dilutions of the LS primer to find the concentration optimal for a given amplicon, as described in this presentation. (Directions for LS primer dilution are provided in Materials and Methods.)

In conclusion, the TransPlex Complete WTA2 kit provides a robust, sensitive method for amplifying RNA quantities approaching the single cell level, while maintaining the representative pattern of expression of higher quantities of RNA input.

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Materials

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References