

Expression Profiling of Fixed and Unfixed Tissue Using TransPlex™ Whole Transcriptome Amplification Kits



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TransPlex™ Flow Diagram

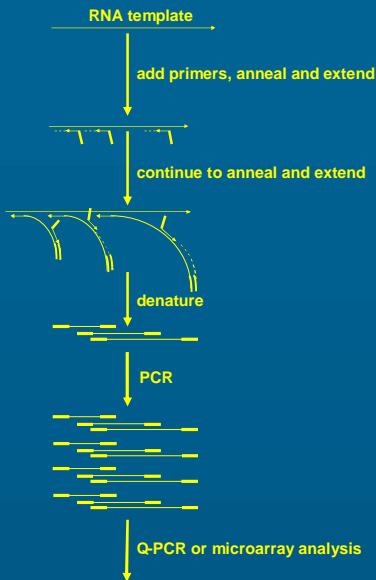
Rubicon TransPlex WTA has two steps, OmniPlex library synthesis followed by library amplification.

To make the library, isolated RNA from fixed or unfixed tissue is first annealed to a nonself-complementary primer comprised of a quasi-random 3' end and a universal 5' end. The primer is extended by polymerase, displacing a single strand that is also copied by primer annealing and elongation, forming an OmniPlex library. The OmniPlex library is comprised of random 100 - 1,000 bases fragments with overlapping sequences.

To achieve whole transcriptome amplification, universal-primer PCR is used to amplify the RNA, without gene- or exon-bias. The amplified OmniPlex library can then be analyzed using homogeneous or array-based methods.

Library synthesis and 1000-fold amplification take place in the same tube in less than four hours. Synthesis and amplification is performed in 96- or 384-well format.

The same steps of library synthesis and amplification are used for GenomePlex whole genome amplification (WGA) and MethyPlex whole methylome amplification (WMA), within 2 to 3 hr. WGA products are used for genotyping, sequencing and CGH. WMA products are used to quantify promoter methylation for research or diagnostics.



Protocol for TransPlex WTA

A. Library Preparation

1. Add 10 – 100 ng RNA in up to 16.5 µL in a microtube/plate
2. Add 5 µL Library Synthesis Buffer and 2.5 µL Library Stabilization Buffer
3. Heat at 70 °C for 5 min
4. Add 1 µL Library Synthesis Enzyme
5. Incubate 24 °C, 15 min; 42 °C, 2 hr; 95 °C, 5 min
6. Cool to 4 °C

B. Library Amplification

1. Add to each 5 - 10 ng library aliquot: 15 µL of 5 X Amplification Mix and 1.5 µL dNTP mix.* Bring to 74 µL with nuclease free water
2. Add 1 µL TITANIUM Taq DNA Polymerase and perform 17 cycles of PCR
 Denature 94 °C for 20 sec
 Anneal/Extend 65 °C for 5 min
3. Amplified cDNA should be purified to remove residual primers and nucleotides, e.g., Qiagen Qiaquick®, or Millipore Montage® filter plates

* Note: For incorporation of label during amplification substitute labeled dNTP mix
 Kit contains sufficient amplification mix to perform 125 amplifications yielding 4 – 6 µg each

ABSTRACT

Rubicon's proprietary TransPlex™ Whole Transcriptome Amplification (WTA) Kit contains reagents and protocol for producing a highly representative library from total or pre-selected messenger RNA. Sample RNA including those isolated from human blood, cell culture, biopsy, or from non-human samples such as bacteria, plants and animals may serve as template. Accurate expression profiling can be done from nanogram amounts of formalin fixed or degraded RNA while retaining native gene- and exon-representation. Within 4 hours, microgram quantities of DNA are generated from nanograms of starting materials. Amplified cDNA, after routine purification procedure, can be used for a wide variety of downstream genetic and expression analyses or stored in this stable format for future analysis. The TransPlex Kit offers a simple, robust, and accurate method for amplification when RNA sample amounts are limiting. Rubicon provides rapid results in a flexible platform for expression analysis.

Rubicon customers have used TransPlex WTA kits in real-time PCR and microarray assays. Customer results were obtained from WTA reagents and protocols identical to the commercial TransPlex WTA kits currently sold by Rubicon Genomics since September, 2005.

Oligonucleotide and cDNA microarrays give excellent results with TransPlex WTA products using standard protocols for labeling, hybridization, and computer analysis. Results from six customers are summarized below.

Laboratory 1—Cancer Tissue Repository

Expression analysis of 1 – 10 y-old formalin-fixed paraffin-embedded breast cancer biopsies using Affymetrix X3P and U133 microarrays to study estrogen receptor genes. In this project 25 - 100 ng of total RNA was amplified to 10 micrograms cDNA product that was biotin-labeled using standard DNaseI-TdT protocol. The gold standard reference was IHC of a different section from the same block.

RESULTS:

1. TransPlex gave 17-24% gene present rate with 10 y-old biopsies
2. TransPlex gave >30% present rate with new FFPE sections (fresh tissue gave 50% present rate)
3. TransPlex gave excellent 83% correlation with estrogen receptor or IHC results.
4. Same results on X3P and U133 chips for every sample.

5. PAM analysis of WTA products

Automated classifier analysis from all tissue samples was able to correctly classify the estrogen receptor status from IHC.

Performance of PAM classifier during cross-validation:
 Let, for some class A

n11 = number of class A samples predicted as A
 n12 = number of class A samples predicted as non-A
 n21 = number of non-A samples predicted as A
 n22 = number of non-A samples predicted as non-A
 Then the following parameters can characterize performance of PAM classifier:
 Sensitivity = n11/(n11+n12)
 Specificity = n22/(n21+n22)
 Positive Predictive Value (PPV) = n11/(n11+n21)
 Negative Predictive Value (NPV) = n22/(n12+n22)

These parameters are listed in the table below

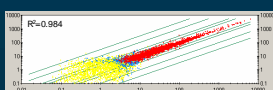
Class	Sensitivity	Specificity	PPV	NPV
N	0.857	1	1	0.875
P	1	0.857	0.875	1

Laboratory 2—Microarray Development Laboratory

Expression analysis using small amounts of total RNA, using Q-PCR, Affymetrix U133 arrays, and custom plant arrays. Input 5 ng to 100 ng total RNA was amplified to produce 12 micrograms of cDNA labeled with biotin or cy3 or cy5.

RESULTS:

1. Excellent results on U133 arrays, except for 5 ng, when rare transcripts are not well represented.
2. 38% gene present rates.
3. Excellent Q-PCR concordance between TransPlex products from 5 ng total RNA and unamplified cDNA made with the ABI kit, except for beta-actin. Representation was the same between 5 and 100 ng starting total RNA
4. TransPlex gave better array and Q-PCR results with much less input and with much less effort and time than using standard IVT.
5. TransPlex performed very well for degraded RNA.
6. Significantly better plant array results after WTA than using unamplified plant RNA, because WTA had diluted plant inhibitors.
7. Three serial amplifications (100 million-fold amplification) without loss of any information about gene expression.
8. Excellent correlation (98%) between independent WTA reactions and hybridizations from 50 ng of template.



Laboratory 3—Microarray Development Laboratory

Affymetrix and custom oligonucleotide expression array hybridization of WTA cDNA amplified from 50 ng total RNA. Ten micrograms of cDNA products end-labeled with biotin and directly compared with ten micrograms of unamplified cDNA.

RESULTS:

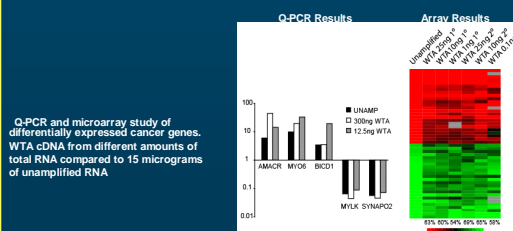
1. WTA cDNA and unamplified cDNA gave equally reproducible results
2. WTA cDNA gave equal or higher microarray hybridization signal.
3. WTA cDNA gave 58% gene present rates vs. 60% for unamplified cDNA
4. WTA cDNA gave equal or higher microarray Discrimination Scores.
5. WTA cDNA gave equal average representation at 5' and 3' ends of transcripts (no 3' bias) for transcripts >5 kb long.
6. WTA and unamplified cDNA gave same differential expression results measured by real-time PCR.

Laboratory 4—University Department of Pathology Laboratory

Affymetrix and cDNA expression arrays used to discover gene differentially regulated in prostate cancer. Studies involve 10 – 100 ng of total RNA amplified 100 – 1,000X using TransPlex kits. For array hybridizations, 10 – 20 micrograms of WTA products were labeled by incorporation of amino-allyl dUTP and CY3/CY5 labeling. Fixed and frozen tissue was compared, and LCM used to harvest RNA from 5,000 cells.

RESULTS:

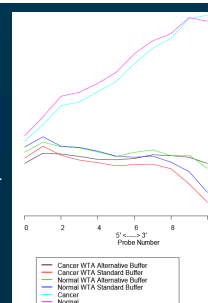
1. WTA gave Q-PCR results that agreed with unamplified.
2. WTA gave 90%+ correlation for top 100 up- and down-regulated prostate cancer genes.
3. WTA gave excellent differential expression results from LCM.
4. WTA gave excellent results from formalin-fixed paraffin-embedded sections.
5. TransPlex concluded to be faster and simpler than other RNA amplification methods.



Q-PCR and microarray study of differentially expressed cancer genes. WTA cDNA from different amounts of total RNA compared to 15 micrograms of unamplified RNA

Q-PCR study of differentially expressed cancer genes—WTA cDNA 25 ng of RNA from FFPE tissue compared to 15 micrograms of unamplified RNA from unfixed tissue

Lack of 3' bias of WTA cDNA, measured by Affymetrix array hybridization of probe sets located different distances from the 5' end of specific transcripts. Experiment performed on cancer and normal samples. Top two lines are signals from unamplified cDNA, labeled by incorporation of amino allyl dUTP from a polydT primer. Lower lines are hybridization signals from WTA cDNA made from TransPlex WTA cDNA labeled by PCR incorporation of amino-allyl dUTP.

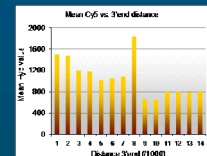


Laboratory 5—Microarray Development Laboratory

TransPlex WTA was evaluated for purposes of determining exon splice variations in fresh and fixed tissue using oligonucleotide microarrays and Q-PCR. cDNA labeled with CY3/CY5

RESULTS:

1. Self-self hybridizations were performed on two 30 ng aliquots of unfixed RNA that were amplified to produce 10 micrograms WTA cDNA. Only 0.4% of over 8,000 genes gave false positive results for apparent deregulation (>2X change in apparent expression). This is better than reproducibility of hybridization from 10 micrograms of unamplified cDNA, which gave 1 – 2% false positive results.
2. Very little 3' bias of amplified products over exons as far as 14,000 bases from 3' end, starting from 10 ng total RNA template.



Laboratory 6—Diagnostics Company

TransPlex WTA was evaluated for purposes of discovery of diagnostic RNA markers for non-invasive cancer diagnostics from blood. RNA was isolated from blood, amplified using a TransPlex kit and tested by Q-PCR and Affymetrix U133 hybridization.

RESULTS:

1. TransPlex WTA cDNA gave excellent results using Q-PCR and U133 on 21 gene expression markers.

SUMMARY OF EXPERIENCE USING TRANSPLEX WTA cDNA FOR EXPRESSION STUDIES

1. TransPlex WTA is more rapid and less labor-intensive than IVT or other methods to amplify total RNA. WTA is rapid and inexpensive enough for HTS.
2. TransPlex WTA has dual advantage of exponential amplification kinetics, and linear transcript and exon representation.
3. TransPlex WTA gives reliable amplification on all sample types, with extremely high correlation coefficients among technical replicates.
4. TransPlex WTA gives excellent array or Q-PCR results with as little as 5 – 10 ng of total RNA from fresh tissue, 50 ng of total RNA from FFPE biopsies, or 5,000 LCM cells.
5. TransPlex WTA of FFPE biopsies gives excellent correlation with IHC of same blocks.
6. TransPlex WTA does not show exon bias as far as 15,000 b from 3' ends, for study splice variants in fresh and fixed tissue.
7. TransPlex WTA facilitates expression studies from plants and other organisms with RT-PCR or hybridization inhibitors.
8. TransPlex WTA can be used to simultaneously amplify RNA and DNA from a mixture of viruses for diagnostics.
9. TransPlex kits are available from Rubicon Genomics (kits@rubicongenomics.com, www.rubicongenomics.com, or (734) 677-6210).