Multiplex Quantitative Measurement of mRNAs from Fixed Tissue Microarray Sections

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Abstract

The development of prognostic and diagnostic biomarkers, such as those from gene expression studies, requires independent validation in clinical specimens. Immunohistochemistry on tissue microarrays (TMAs) of formalin-fixed paraffin-embedded (FFPE) tissue is often used to increase statistical power, and it is used more often than in situ hybridization (ISH), which can be technically limiting. Here we introduce a method for performing quantitative gene expression analysis across a TMA using an adaptation of 2D-RT-qPCR, a recently developed technology for measuring transcript levels in a histological section while maintaining two-dimensional positional information of the tissue sample. As a demonstration of utility, a TMA with tumor and normal human prostate samples was used to validate expression profiles from previous array-based gene discovery studies of prostate cancer. The results show that 2D-RT-qPCR expands the utility of TMAs to include sensitive and accurate gene expression measurements.

1 Introduction

Gene expression profiling is currently used for analyzing patient samples in the research setting, however the field of molecular diagnostics is now moving toward utilization of expression patterns to predict clinical outcomes in patients [1-3]. Expression microarrays frequently serve as the discovery method and are used to measure a large number of genes in relatively few specimens. Subsequently, the candidate dysregulated genes must be validated by measuring their expression in a larger independent sample set [4]. At present, follow-up validation of profiling data is relatively low throughput. Therefore, it is common to perform immunohistochemistry (IHC) on a large cohort of patient samples of formalin fixed and paraffin embedded tissue (FFPE), including the tissue microarray (TMA) formats, to assess protein levels in a gene by gene manner. However, transcriptome-wide correlation between protein abundance and mRNA expression levels has been reported to be poor [5]. Moreover, there is unique and important biological information contained in the expression status of the transcripts themselves, independent of the protein products, and thus it would be useful to validate mRNAs in addition to their corresponding proteins.

Workflows for semi- or fully-quantitative mRNA expression validation applied to a tissue section include *in situ* hybridization (ISH), *in situ* RT-PCR, microdissection with subsequent RT-qPCR, and macrodissection with RT-qPCR. Each of these methods have significant limitations such as measuring only highly-abundant transcripts and/or the labor-intensiveness of the upfront isolation method [6,7]. For example, ISH can semi-quantitatively detect medium and high abundance mRNAs in a tissue section by direct hybridization of a labeled probe. ISH has

technical challenges when detecting low copy number mRNAs from FFPE tissue, and this is a great limitation because the majority of the transcriptome is found in low-abundance target genes [8], and thus *in-situ* RT-PCR is required to detect and quantify mRNA targets in FFPE tissue sections. Although this method can sometimes provide sensitive and localized detection of low-abundance transcripts, *in-situ* RT-PCR has proven difficult to employ for consistent and reliable measurements [6,7]. Laser-based microdissection allows sensitive and reproducible mRNA quantification by isolating specific cells of interest from a histological tissue section followed by quantitative RT-qPCR, and produces accurate expression data. However, microdissection is both time- and labor-intensive. In contrast, macrodissection, which involves removing a region of a histology slide by scraping with a razor blade or other mechanical tool is faster than microdissection, but the recovered mRNA is derived from a mixture of cells, potentially confounding the results [9-12]. Additionally, for both micro- and macrodissection, the RT-qPCR step itself is laborious to perform simultaneously on a large number of samples due to the extensive number of experimental steps involved [13-15]. These challenges are further compounded when using archival formalin-fixed, paraffin-embedded (FFPE) tissue samples, due to the inherent poor RNA quality [16-18].

To address these limitations, our laboratories recently developed 2D-PCR [6] and 2D-RT-qPCR [7] technologies, which provide a means to mechanically separate a tissue section into individual subregions for qPCR and RT-qPCR measurements, but without the need for laser microdissection or slide scraping. During the procedure, a multi-well array maintains the two-dimensional (2D) layout of the tissue, and the tissue lysis and mRNA purification steps are performed in parallel to minimize the handling time per sample. We have shown successful

DNA and cDNA (mRNA) amplification and measurements across frozen tissue sections [6,7]. In the present report, we describe the adaptation of 2D-RT-qPCR to a TMA constructed of archival formalin fixed, paraffin embedded tissue specimens, and an optimized workflow for its use in the Fluidigm Biomark qPCR assay (Figure 1). 2D-RT-qPCR was applied to a TMA containing human prostate normal and tumor specimens, allowing us to examine the differential expression of 22 mRNA targets across 42 FFPE prostate samples in a single experiment. Our results were then validated *in-silico* by comparing data pooled from 14 independent microarray studies.

2 Materials and Methods

2.1 Tissue Samples & 384-Well Format TMAs

Tissue samples were obtained from the Tissue Array Research Program at the National Cancer Institute (Bethesda, MD) to construct the prostate TMA. Tissue was also obtained for the supplementary studies from the National Cancer Institute, the Cooperative Human Tissue Network, and Pel-Freez Biologicals. Human tissue was obtained either with appropriate bioethics approvals or exemptions from the Office of Human Subjects Protection, NIH. A pathologist (JRC) verified that the cores represented tumor or normal tissue and graded the tumors on the Gleason scale. Tissue microarrays were constructed by standard methods. A 384 well format grid (384-well hard-skirted plates, Bio-Rad, Hercules, CA, USA) was cut to fit into an aluminum macro-embedding mold of 2" x 3" (Super metal base molds, Leica, Wetzlar, Germany). The grid was covered in a thin layer of paraffin (Paraplast X-tra, St. Louis, MO, USA). Individual TMA cores of 3.0 mm were extracted from archival tissue blocks and

centrally embedded vertically into the holes in the grid. The mold was then filled with paraffin and chilled at 4 °C for 15 minutes.

2.2 Tissue Sectioning

TMA blocks were sectioned to 10 µm thickness using a standard microtome (RM2255, Leica, Wetzlar, Germany) onto adhesive film to obtain a uniform thickness for the transfer step (Figure 1C). The film (ARCare 7759, Adhesives Research, Glen Rock, PA, USA), was placed adhesive-side against the TMA and pressed with a roller to create consistent contact. The TMA on the adhesive was deparaffinized in xylene for 60 seconds at room temperature, then dried in a chemical hood for 10 minutes.

2.3 Pretreatment for Recovering mRNA & Transfer to 384-Well Plates

After dewaxing, tissues were exposed to steam in an un-pressurized vessel (Vegetable Steamer, Black&Decker) filled with double-distilled water for 30 minutes. Tissues were then allowed to air dry for 10 minutes. The adhesive-bound, steam-treated tissue was aligned over a clear 384-well flat-top PCR plate (KBioscence/ LGC genomics., Middlesex, UK), where the burr from the flat top of the plate was removed with a blade. This style of plate aids sealing and allows the user to see what is in each well. The film containing tissue was applied such that only a single TMA core was placed in alignment over a single well, as shown in Figure 2. The wells were preloaded with lysis buffer (see below) before the film was applied. The plate was then placed in a PTC-200 thermocycler (MJ Research / Applied Biosystems, Foster City, CA, USA) in a

compression rig [6] to apply 200 pounds of force and 60 °C to the adhesive for 3 minutes, sealing the film to the plate.

2.4 Lysis and Purification

Each well of the plate was preloaded with 14 µL of ChargeSwitch lysis buffer (ChargeSwitch RNA cell kit, Invitrogen/Life Technologies, Grand Island, NY) containing 0.2 mg/mL proteinase K and 5 mM DTT (dithiothreitol). After the plate was sealed, it was inverted, placed back into the compression rig under 200 pounds of force, and heated to 50 °C for 24 hours. After lysis, the plate was centrifuged, and the adhesive film containing the residual tissue core material was peeled off and discarded. A 7 µL solution containing 0.33 µL ChargeSwitch magnetic beads, 1% acetic acid, and 6.6 µL ChargeSwitch binding buffer was added to each well. Total RNA was thereby bound to the surfaces of the beads. The beads were immobilized to the side of each well using a magnet, and the wells were washed twice each with ChargeSwitch wash buffers 13 and 14, removing the magnet between steps. Wash 14 was then removed, with the magnet in place, and the total purified RNA on the beads was assayed either directly by one-step RT-qPCR (SDC – *Supplemental Digital Content*) or indirectly by pre-amplification followed by Fluidigm qPCR (see below).

2.5 Tandem mRNA Pre-Amplification & Fluidigm Assay

After the purification procedure, a pre-amplification mixture was added to the beads, still in the same plate. The pre-amplification procedure is similar to the one used by Stanley [19]. One-Step

AgPath ID reverse-transcriptase PCR mix was prepared according to the manufacturer's instructions except that twice as much enzyme mix was used, and TaqMan primer/probe sets (SDC) were diluted 450-fold (to 40 nM) for each primer set. To each well 10 μL of this reaction mix was added. The plate was heated to 50 °C for 60 minutes, 95 °C for 15 minutes, and thermocycled for 14 cycles of 95 °C for 15 seconds and 60 °C for 4 minutes. The templates were diluted only 4-fold for use in the Fluidigm 48x48 Dynamic Array chip (Fluidigm, South San Francsico, CA, USA). The Fluidigm assay was run in a Biomark HD system according to the manufacturer's protocol, with the exception that a custom PCR mastermix was made as described in the SDC. The experiments were performed by Dr. Kelley Banfield at SAIC-Frederick. Additional information is provided in the SDC.

2.6 Gene Expression Analysis & Statistics

The heat map in Figure 3 was generated using the ArrayMining.org application [20], selecting the option for hierarchical clustering (with *ebayes* supervised feature selection) to place genes and samples into nearest-neighbor groups. Non-detected values were replaced with the average Ct for the gene across all samples with values, and they represented only a small percent of the data

3 Results

3.1 Development of 2D-RT-qPCR On FFPE TMAs

Evolution of the 2D-RT-qPCR assay from application to frozen tissue to FFPE tissue required extensive validation. A series of TMAs were constructed with different core diameters, cut at

different thicknesses to determine optimal tissue sampling required for reliable results. Cores of 3 mm diameter, with 10 um thickness were found to be optimal. Assay sensitivity, linearity and reproducibility were also determined on a panel of control FFPE TMA tissue, and found to be similar to those of a previous Fluidigm study using commercially purified human reference RNA, which found 7% variation for high copy number targets, and 46% and higher variations for low copy number targets [21]. Lastly, we tested the 2D-RT-qPCR method and the RecoverAll kit is provided, which showed improvements in RNA detection efficiency and workflow. Results of these studies and additional controls are described in the SDC.

3.2 Validation of Gene Expression Profiles

A TMA containing tumor and normal human prostate FFPE cores was constructed of archival tissue blocks from the Tissue Array Research Program. The tissue blocks were routine clinical specimens, and not originally procured for research purposes. Each core contained a majority of either tumor or normal cells as determined by a pathologist (JRC). The TMA was sectioned at a 10 μm thickness, with each 3 mm diameter core providing tissue with a volume of approximately 70 nL. In order to meet the sample-size limitation of the Fluidigm 48.48 BioMark assay, 42 cores and 5 controls (commercially purified mRNA or water) were tested. The Fluidigm assay also provides inlets for up to 48 probe sets.

Probe-sets for the Fluidigm assay included eight genes from a previous prostate study as a test set for validation (FOLH, TACSTD, ALCAM, GSTP1, CD10, TIMP3, STAT5B, CD69) [22] and nine commonly dysregulated in cancer (AMACR, SOD2, JAK1, ANG, MUC1, VEGF, H1F1A,

AR, and VHL). Five housekeeping genes provide a reference baseline (HPRT1, B2M, GUSB, CYPA, and GAPDH). The result is the application of 2D-RT-qPCR, to assay 22 transcripts across 18 normal and 24 tumor prostate cores in duplicate.

Of the 2,068 data points collected, 220 were positive and negative controls as described in the SDC. For the remaining 1,848 data points, raw RT-qPCR data are presented in heat map format (Figure 3). The data was collected from a single 10 μm thick section of a TMA constructed of 3.0 mm diameter cores. The data demonstrate two key features of the 2D-RT-qPCR technology. First, consistency is shown by the near-perfect alignment of technical replicates of probe assays to each other. Second, the expression analysis reliably separated the tumor and normal samples as indicated by the clustering of groups. In summary, these data highlight the validity and reproducibility of the 2D-RT-qPCR technology when applied to the molecular analysis of FFPE TMA cores.

In order to accurately compare gene expression values between samples the "delta Ct" was calculated as the expression of a target mRNA minus the average expression of four housekeeping genes (B2M, GUS, GAPDH, and CYPA) to reduce error in normalization for samples prepared by formalin fixation [23]. Delta Cts were subjected to a two-tailed t-test (Welch's assumption, unequal variances) between the tumor and normal sample groups (n=24 and n=18) for each transcript. Based on this metric, we found genes that showed significant differential expression between the two groups (Figure 4).

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Several of the genes showed expression changes that were in agreement with previously published results [22]. Specifically, FOLH, TACSTD, and ALCAM were up-regulated in prostate cancer, while GSTP1, CD10, and TIMP3 were down-regulated (Figure 4). Furthermore, STAT5B and CD69, two genes identified in our previous study [22] as differentiators of high and moderate grade tumors, were also differentially expressed in this TMA study. However, the differential expression of CD69 was not in agreement with previous work [22] as it was expressed lower in tumor cells compared to normal cells, a result that could be due to pathological differences, such as the amount of infiltrating lymphocytes in the sample set. Several tumor-associated genes from the literature were evaluated in the prostate TMA using the 2D-RT-qPCR method and showed similar results to the published data. Specifically, AMACR was over-expressed in tumors, and SOD2, JAK1, ANG, MUC1, and VEGF were under-expressed. In this study, H1F1A was over-expressed in prostate tumors with a fold change of 1.22, which is a marginal yet significant change. As another internal control, we found that HPRT1, another common housekeeping gene, had no differential expression.

The present results were evaluated against *in-silico* microarray datasets from the Oncomine database [24]. To allow a comparison between these data, the Delta Cts in this study were converted to fold-change using the formula $\mathrm{sgn}(\mathit{Delta}\mathrm{Cts}) \cdot 2^{\mathit{abs}(\mathit{Delta}\mathrm{Cts})}$, where sgn($x$) is -1 for $\mathit{Delta}\mathrm{Cts} < 0$ and 1 otherwise, and abs() is the absolute value. The average fold change of differentially expressed genes ($t < 0.05$) from the TMA study was compared with a 14 study microarray dataset, which represented results from 905 prostate samples in total [24]. The average fold changes for the *in-silico* results and for the TMAs are shown in Figure 5, and additional data are provided in the SDC. To quantify the comparison, the Pearson r^2 correlation coefficient was

calculated by comparing the fold change between the overlap in data sets, and was found to be 0.86. It should be noted that H1F1A was not identified as differentially expressed in the *in-silico* data [24]. Interestingly, not one of the microarray datasets identified differential expression in all of the 14 genes that were validated in this study. Overall, the data indicate that the 2D-RT-qPCR technology generates results with strong concordance with previous findings and is capable of accurate and meaningful mRNA measurements from a single tissue section of TMA derived from archival blocks.

4 Discussion

We have described a novel application of 2D-RT-qPCR to a TMA section that allows parallel purification and amplification of mRNA from archival tissue blocks. The Fluidigm assay enabled gene expression measurement of up to 48 mRNAs (or 24 in duplicate) from the samples. Sensitive, specific, and reproducible detection was shown for all transcripts tested and over a wide range of expression levels using archival FFPE patient samples. The technology allowed validation of previously reported gene expression profiles in prostate cancer by utilizing FFPE samples from various sources, highlighting its ability to analyze tissue preserved by the most common preservation method used in archival clinical specimens [25]. The ability to use a small amount of tissue (2.8-70 nL volume depending on TMA core size) for expression analysis opens the possibilities for TMAs to be employed for a wide variety of validation and other studies.

Compared to a widely used commercial kit (RecoverAll, see SDC), whose procedure takes two work days to complete (12 hours) and required a handling time of 6 hours for studying a TMA with 24 tissue core, the 2D-RT-qPCR workflow required only 4 hours with an actual handling time of 1 hour. The time savings came from the lack of macrodissection or manual transfer of tissue lysates. Based on these results, 2D-RT-qPCR is approximately an order of magnitude faster than traditional workflows.

Design and application of the TMA requires special attention. For example, as the TMA is consumed by sectioning, sample heterogeneity may cause divergence from the cell types initially observed (i.e., tumor versus normal), and this may occur unequally as some tissue cores are altered or depleted sooner than others. It is therefore routine practice to stain and visually check a TMA section after every 125-250 μm of sectioning to ensure the histopathology has not changed significantly [26]. Secondly, RNA quality is dependent on the FFPE-derived tissue used in the construction of the TMA [16,17] and depends on the method of tissue preparation, fixation, and age [27]. Although numerous fixatives and fixation protocols have been designed to preserve RNA quality, there is currently no standard protocol [18]. Thus, the cores in a typical TMA may originate from blocks that were prepared in different research centers, using multiple fixation protocols. Therefore, to analyze mRNA in TMAs with tissues originating from multiple institutions, it is useful to compensate for RNA degradation by using the smallest amplicon size feasible for PCR, and to normalize target mRNA values to housekeeping genes in the same samples to correct for sample-to-sample variability in RNA quality [17,28].

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2D-RT-qPCR may be useful in clinical research and diagnostic tests since it can improve sample throughput and use small volumes of tissue. In principle, it is possible to study up to 96 genes in 96 patient core samples by application of a larger TMA and 96x96 Fluidigm qPCR assays, and the system is amenable to automation. 2D-RT-qPCR could also be tailored to clinical diagnostic cancer tests that assay fewer than 96 genes, such as MammaPrint [2,29,30] or Oncotype DX [3], while potentially decreasing the current diagnostic time, from 5 days in the case of the Oncotype DX [3]. Furthermore, pre-amplified cDNA assayed using the Biomark Fluidigm platform has excellent concordance with conventional RT-qPCR [21,31].

Diagnostic tests such as MammaPrint and Oncotype DX depend on the general feature that tumor specimens have more RNA than the surrounding normal tissue [32] and RNA pooled from tumor and normal tissues should already be enriched for tumor RNA. In these particular cases, the contribution of RNA from normal tissue does not affect the diagnostic result, thus allowing the use of gross macrodissection of a few tissue sections to provide sufficient template for a gene expression assay. However, Van De Vjver [2] found that some specimens did not have a high enough proportion of tumor tissue to allow for macrodissection and these specimens could not be included in the study. In fact, many recent studies suggest that isolating cells by microdissection before a molecular test leads to dramatically different genetic profiles [10-12,33] that may in principle change a diagnosis or guide clinicians to different treatment decisions. The use of smaller TMA cores in 2D-RT-qPCR may also help enrich samples for specific cell types prior to analysis.

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5 Acknowledgements

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6 Financial Interests

The authors M.A., M.E-B., M.T., B.S., and E.S. are inventors on patent applications, including issue US patent 8283158 covering the underlying technology and may receive future royalties through the NIH or University of Maryland technology transfer programs.

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Figure Legends

Figure 1. Conceptual schematic of the 2D-RT-qPCR method. A) A TMA block is obtained. B) The block is sectioned to 10 μm thickness on an adhesive film and dewaxed with xylene; steam is also applied to improve mRNA integrity. C) The film with tissue is transferred onto a multi-well plate. D) Total RNA is purified and extracted in each well using solid-phase magnetic purification beads. E) In the same multi-well plate, mRNA is converted to cDNA and pre-amplified by tandem one-step RT-PCR. F) The pre-amplified targets are removed from the plate and quantified using the Fluidigm Biomark RT-qPCR detection system.

Figure 2. Image of a 384-well flat top multi-well plate with a tape containing a TMA section of interest was placed over the plate such that each core (brown circles) is aligned to the center of a well.

Figure 3. Data from the TMA 2D-RT-qPCR experiment shown as heat map representation of genes expression values (rows) indicated by colors and samples (columns). Red indicates over-expression in tumors relative to normal cells, and green indicates under-expression compared to baseline. Tumor = T and normal = N.

Figure 4. Box-and-whisker plot of Delta Cts for mRNA quantified in prostate cancer (colors) compared to normal (white). P-values are displayed above the target gene names. Genes that had significant ($p < 0.05$) overexpression in tumor are colored red while those tumor samples with under expression are colored in green. Samples not showing significant differential expression are shown in grey. Samples were tested in two groups of 24 prostate tumor cases

versus 18 normal prostates. Each box is centered about the mean and extends to the upper and lower quartiles of the data, while the whiskers extend to the full data range.

Figure 5. Fold change of mRNA levels in this TMA study (red circles) compared with the average of 14 Oncomine 3.0 gene expression dataset (black triangles). Values from each gene expression study are also plotted (gray unfilled circles)

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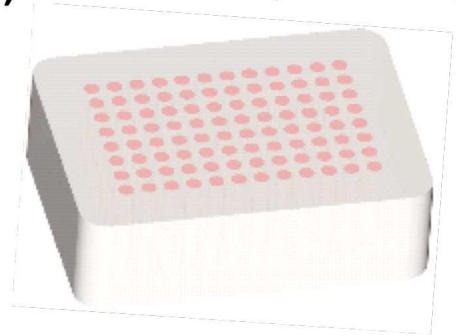
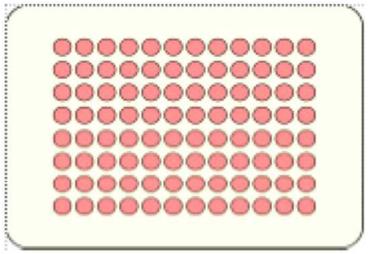
Figure 1

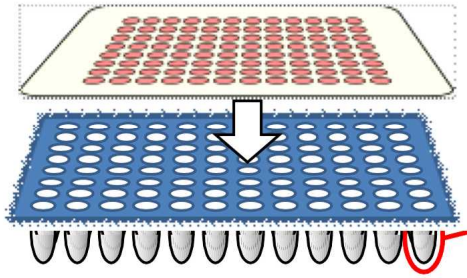
A) TMA with many tissue samples.



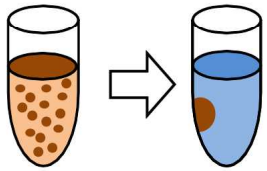
B) 10 µm section on adhesive film. Dewax and treat with steam.



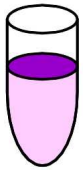
C) Transfer onto multi-well plate.



D) In parallel, extract RNA and then purify using solid-phase purification beads.



E) Pre-amplify targets with gene-specific tandem one-step RT-PCR.



50 °C - 1 hour

<u>18 cycles</u>
95 °C - 15 sec
60 °C - 4 min

F) Assay up to 96 targets vs. 96 samples using Fluidigm qPCR platform.

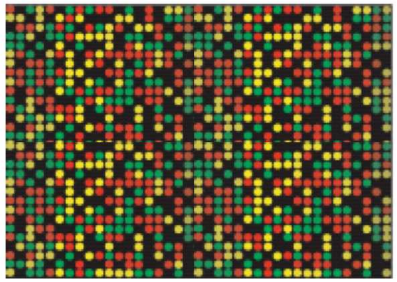


Figure 2

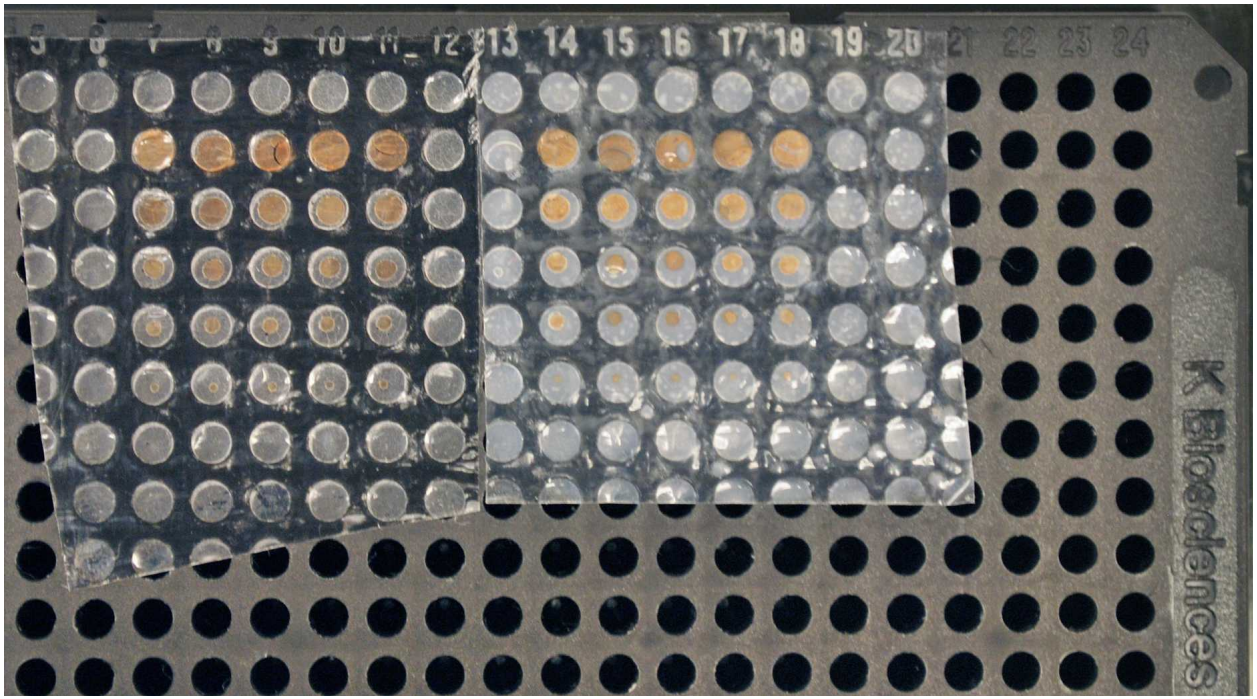
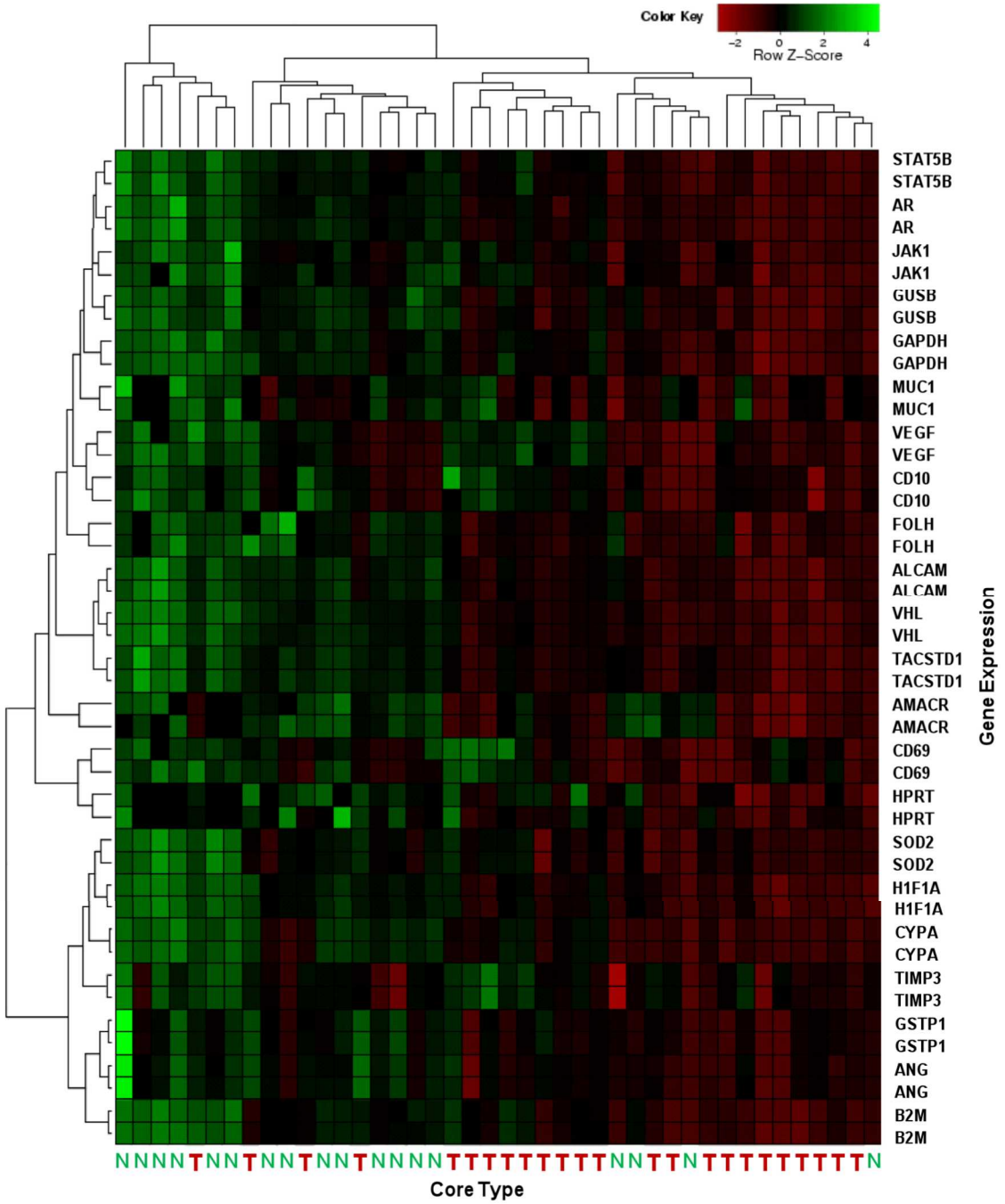


Figure 3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VHL
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TACSTD1
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AMACR
AMACR
CD69
CD69
HPRT
HPRT
SOD2
SOD2
H1F1A
H1F1A
CYPA
CYPA
TIMP3
TIMP3
GSTP1
GSTP1
ANG
ANG
B2M
B2M
Gene Expression
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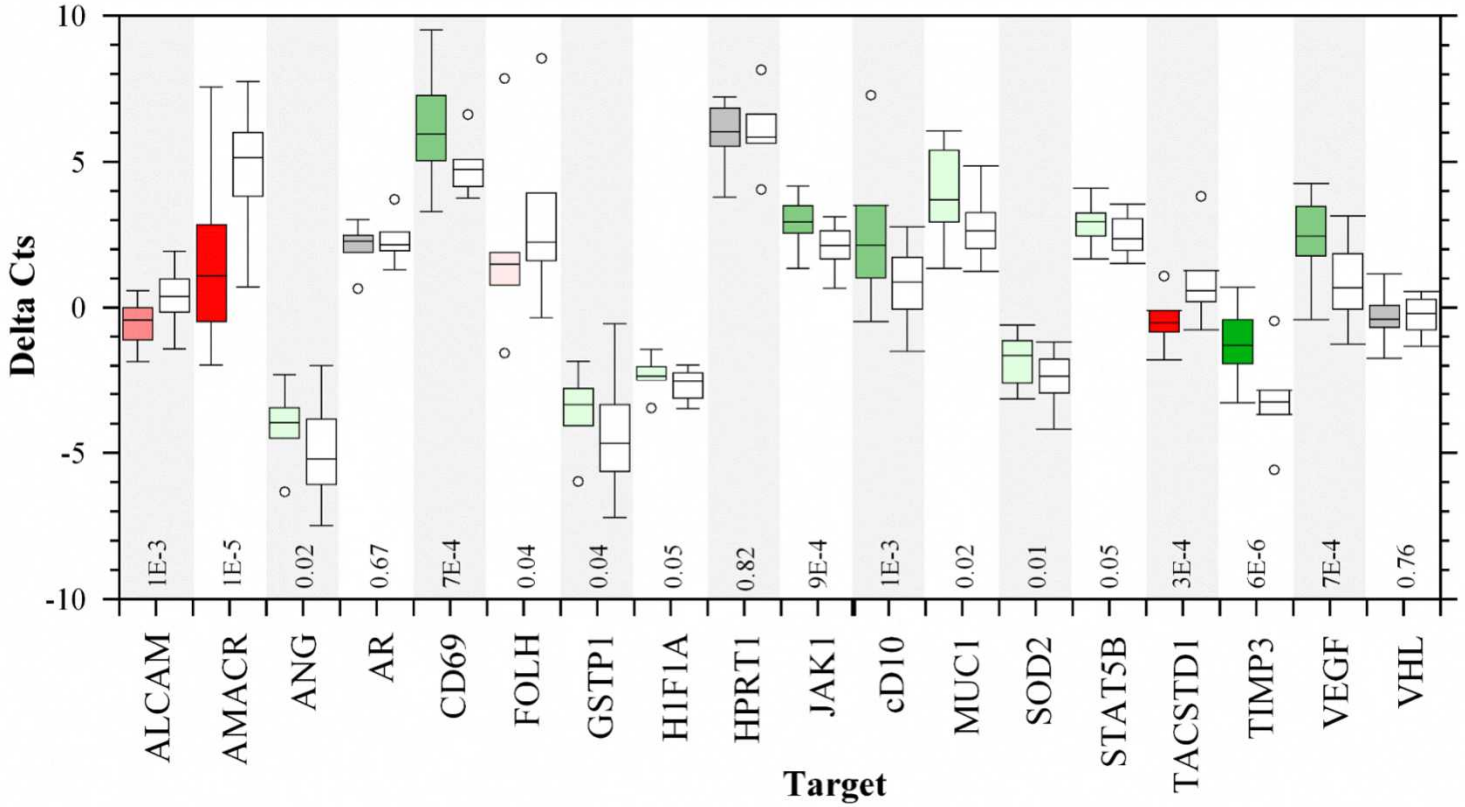
Core Type

Figure 4


Delta Cts
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0
-5
-10
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1E-5
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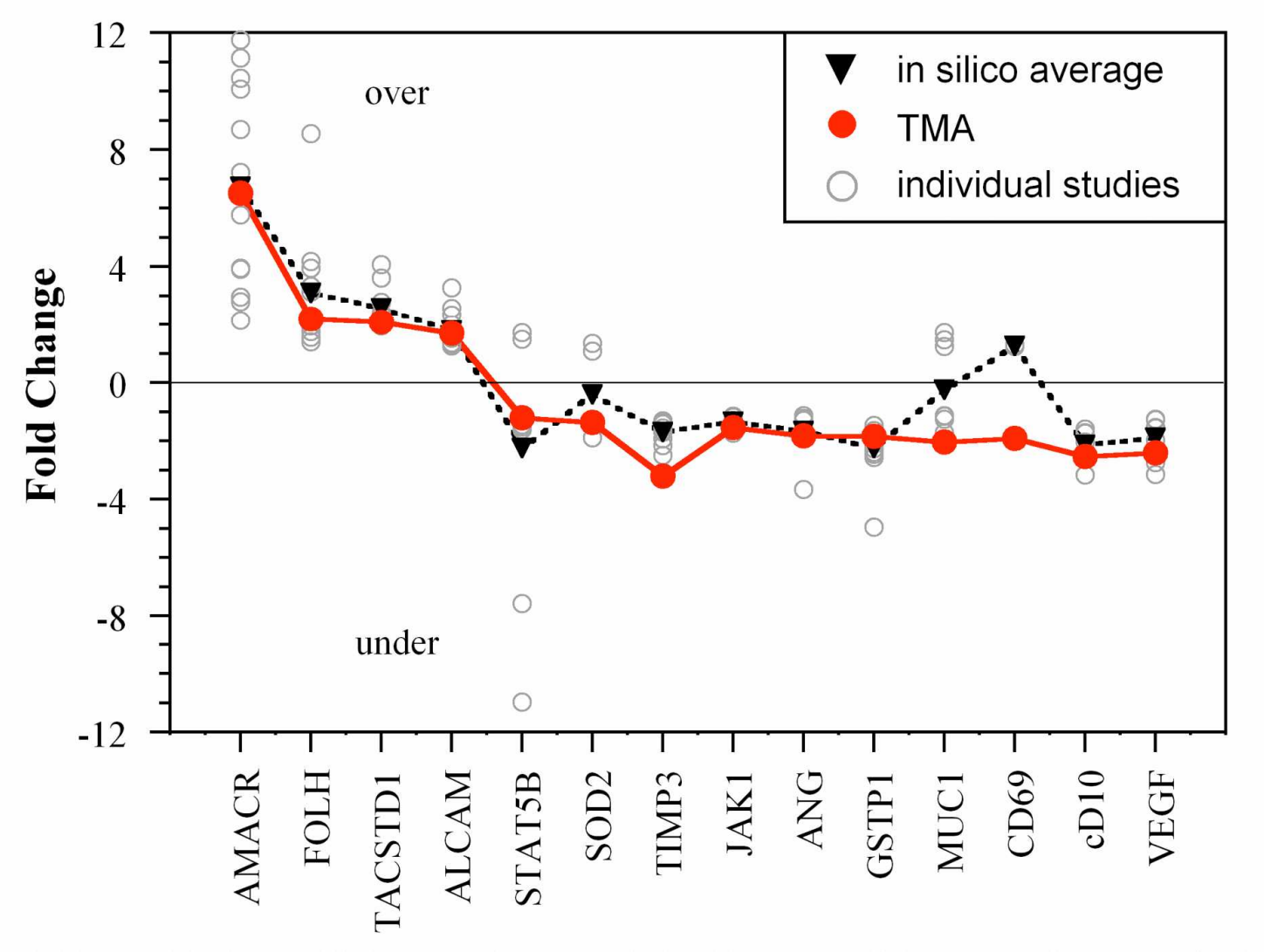
0.67
7E-4
0.04
0.04
0.05
0.82
9E-4
1E-3
0.02
0.01
0.05
3E-4
6E-6
7E-4
0.76
ALCAM
AMACR
ANG
AR
CD69
FOLH
GSTP1
H1F1A
HPRT1
JAK1
cD10
MUC1
SOD2
STAT5B
TACSTD1
TIMP3
VEGF
VHL
Target

Figure 5


Fold Change
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over
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in silico average
TMA
individual studies
AMACR
FOLH
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ALCAM
STAT5B
SOD2
TIMP3
JAK1
ANG
GSTP1
MUC1
CD69
cD10
VEGF

Type of file: table
Label: Supplemental Digital Content
Filename: TMA-2DPCR Supplemental 083013.doc

Electronic Supplemental Information (ESI) for

Multiplex Quantitative Measurement of mRNAs from Fixed Tissue Microarray Sections

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These supplementary materials describe further evaluations of 2D-RT-qPCR.

Section 1. Instructions for performing the one-step RT-qPCR in 384-well plates to detect RNA.

Section 2. Fluidigm assay sensitivity, linearity, and reproducibility on mRNA targets that were pre-amplified using 2D-RT-qPCR.

Section 3. Results from additional control experiments on 2D-RT-qPCR.

Section 4. A comparison of 2D-RT-qPCR with a standard commercially available technique (RecoverAll™), including results for mRNA and a small nuclear RNA.

Section 5. Information for preparing reagents used in the Fluidigm assay and a list of the custom designed primer and probe sets, including the pre-amplification probe mixture used in the experiments.

1 One-Step RNA Assay (RT-qPCR)

For the control experiments on TMAs, mRNA or small RNA was quantified using a one-step RT-qPCR assay. This allowed for the study of up to 3 genes at a time and was performed instead of pre-amplification and subsequent quantification using the Fluidigm assay.

Samples were prepared according to the methods in the main manuscript text. After the lysis and purification steps, a one-step RT-qPCR was prepared according to the manufacturer's instructions (AgPath ID, ABI). Either custom real-time PCR hydrolysis probes or stock TaqMan probe sets from Applied Biosystems were added to the AgPath ID mixture. Both custom and

stock primer/probe sets were mixed to a final concentration of 225 nM (primers) and 62.5 nM (probe). The PCR reagents containing the diluted probe sets were added to the 384-well plates containing purified RNA, which was amplified according to the manufacturer's protocol. RT-qPCR curves were measured using a fluorescent plate reader every 3 to 5 cycles, and Cts were calculated as described previously [1].

2 Assay Sensitivity, Linearity, and Reproducibility

As described in the main text, positive control mRNA and negative controls (water) underwent the same reverse transcription and pre-amplification protocol that was applied to the mRNA isolated from the prostate TMA cores. Specifically, 1 μL of commercially-purified human total RNA at 4 different concentrations in water (1000 ng, 125 ng, 15.6 ng, and 1.95 ng) and a negative control (water) were added to 5 adjacent empty wells and processed as described in the Methods section of the main text.

Using the Cts measured for the control RNA, the assay detection limit was determined to be as low as 1.95 ng for all the targets with the exception of HPRT1, a low-abundance target that had a detection limit of 15.6 ng. Only one false positive (TIMP3) was detected among the negative controls, and it was detected at 26.1 Cts, well outside the range of 15.7 to 21 Cts for the prostate samples that were run in parallel.

The control RNA dilution series showed the Pearson r^2 linearity of the assay (Cts versus ng) that was calculated for each probe set varied from 0.9976 to 0.999, exceeding the performance specifications described by the manufacturers of TaqMan probe sets.

The samples were also assayed for each target in duplicate to allow us to calculate the reproducibility of the Fluidigm system. The reproducibility for each target (n = 2) was first calculated by the standard deviation. However, since the standard deviation from only two samples would provide large bias, we define here the "aggregate variability," which is the average of standard deviations across Cts from all tissue samples (n <= 42) for each target probe set. This measurement is important because it indirectly estimates the combined experimental bias, including the pre-amplification step, randomness in primer-target annealing, error in fluorescent detection using the Fluidigm hardware and software, and variability of PCR across the high dynamic range of samples prepared by 2D-RT-qPCR.

The aggregate variability ranged from 0.06 to 0.49 Cts with a median value of 0.19. The variability of the Fluidigm system had been previously reported to range from 7% for highly abundant targets to 46% for targets with 10 copies [2]. To compare these numbers to our results we converted the measurement from Cts (0.06 to 0.49) to percent change (4.2% to 40.4%) using the formula percent change = $100\% \cdot (1 - 2^{Cts})$. Based on this calculation, our results for the performance of the Fluidigm systems are similar to those previously published. In addition, the lowest aggregate variability came from a high-abundance target (CYPA, mean 18.0 Cts, variability = 0.06 Cts), while the highest variability came from a low-abundance target, (HPRT1, mean 24.4 Cts, variability = 0.49 Cts). Therefore, the performance of 2D-RT-qPCR when applied to TMA tissues in combination with the Fluidigm assay also shows similar performance to that reported for the Fluidigm system alone.

3 Additional Control Experiments

Multiple TMAs were constructed, including mouse and human tissue cores. Evaluation of these TMAs by 2D-RT-qPCR demonstrated that decalcified specimens as well as some archival specimens were inadequate for evaluation due to degradation of biomolecules [3]. Assay specificity was tested with human specific probes against a mouse tissue cores and the converse of mouse specific probes against human tissue. No cross-species transcripts were identified, while all controls performed as specified. To ascertain the specificity and dynamic range of 2D-RT-qPCR in a multiplexed reaction, three mRNA targets (KCNJ1, GYS2, and HPRT1) were co-amplified from mRNA isolated from FFPE mouse tissues, showing target tissue specificity (GYS2 for liver and KCNJ1 for kidney) and a wide dynamic range in qPCR signals (over 14 Cts).

4 Comparison of 2D-RT-qPCR with Commercial Techniques

Initially, a TMA was run through the 2D-RT-qPCR protocol without the use of the Fluidigm system to determine the minimum required tissue core size for sufficient RNA recovery. This was performed on a TMA containing human liver cores with a range of diameters from 0.6 to 2.0 millimeters. The TMA contained 24 cores with 6 replicates over 4 core sizes. To assess different RNA target types, the levels of RNU48, a highly abundant small nucleolar RNA, and SOD2 mRNA were quantified by multiplex RT-qPCR.

The procedure was also repeated and compared against a more common workflow that included the commercially available RecoverAll kit (Invitrogen) to obtain total RNA. As recommended by the manufacturer, RNA was quantified with a two-step procedure of reverse transcription and then real-time qPCR.

The 2D-RT-qPCR workflow followed the steps described in the main text and in Section 1 above. To perform the RecoverAll workflow, a 10 μm section of the TMA was placed on a glass slide and deparaffinized. All 24 cores were macrodissected off of the slide and into a 1.5 mL tube using a razor blade. The RecoverAll procedure was followed as described by the manufacturer, and qPCR assays were performed.

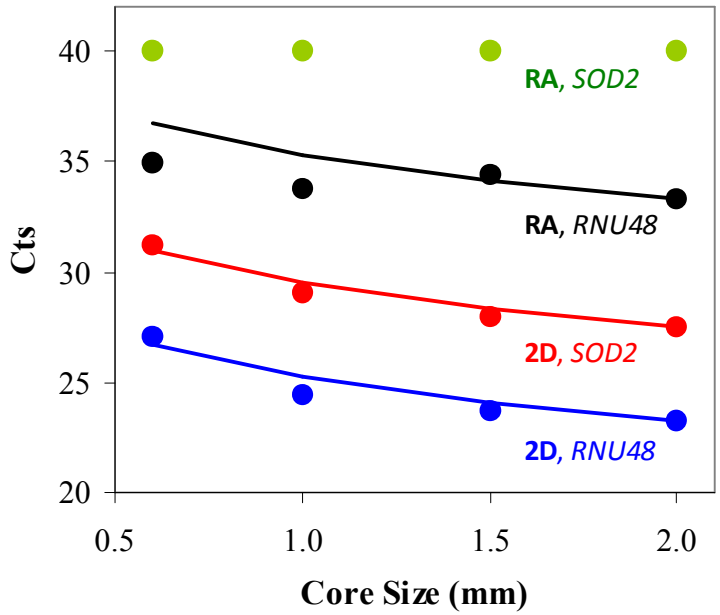

Figure 1. Comparison of RecoverAll (RA, green and black points) and 2D-RT-qPCR (2D, red and blue points) for the recovery and detection of mRNA (SOD2) or small nuclear RNA (RNU48). Points show the experimental data while lines indicate theoretical values assuming 100% PCR efficiency and accounting for the difference in starting material based on core size.

Figure 1 shows the Cts of detection for the two types of RNA versus core diameter. Using 2D-RT-qPCR, both mRNA (red points) and small RNA (blue points) were detected from cores with sizes as small as 0.6 mm. The required Cts were higher for smaller cores, as expected, the number following the theoretical curves for both types of RNA. The theoretical curve is a doubling of template every cycle during the exponential growth phase of PCR.

The RecoverAll procedure allowed for detection of the highly abundant RNU48, but there was a poor correlation with core diameter (black points in Figure 1), and detection came approximately 10 cycles later than with 2D-RT-qPCR. RecoverAll did not result in detection of SOD2 mRNA (green points); the cycling was stopped at 40 cycles. The difference in results obtained with the RecoverAll protocol suggests differential recovery and/or amplification of mRNA damaged during the formalin fixation and storage process [4]. In addition, the concentration of RNA purified with 2D-RT-qPCR was initially higher because it was concentrated on the surface of beads, whereas the RecoverAll system requires dilution of the purified mRNA. In other words, the entirety of the mRNA recovered by 2D-RT-qPCR can be used during PCR, whereas with RecoverAll, it is impractical to use more than a small volume of the recovered sample.

5 Fluidigm Assay

To perform a Fluidigm assay, 24 TaqMan probe sets (in duplicate) were mixed with Fluidigm loading buffer, and a custom made PCR mix was added to the *assay wells* of a 48x48 Fluidigm plate. The PCR mix consisted of 30 mM KCl, 25 mM Tris, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mM dNTPs (New England Biolabs), and 0.04 Units/µL Dynazyme II DNA polymerase.

Then, 48 pre-amplified samples were mixed with Fluidigm loading buffer and added to the *sample wells* of the 48x48 Fluidigm plate, and the plate was run according to the Fluidigm protocol. The samples included 24 prostate tumors, 18 normal prostates, a dilution series of 4 samples, 1 negative control, and one blank well.

24 primers were used in the pre-amplification protocol and are shown in the table below. The pre-amplification mixture contained TaqMan primer/probe sets (those with an Applied Biosystems ID starting with "Hs") that were diluted 450-fold in the final pre-amplification mixture, providing 40 nM of each primer, along with PCR probes that were diluted to below detectable levels (11.1 nM each) and did not affect subsequent qPCR detection. The custom primer/probe sets (B2M and GUS) were also diluted to a final concentration of 40 nM per primer.

Table 1.

Custom and TaqMan probe sets used

Applied Biosystems ID	**Gene**	**Description**	***Included in Analysis?***
Hs00260717_m1	AKT	AKT1 substrate 1 (proline-rich)	*No – primer/probe set failed to show any amplification.*
Hs00233455_m1	ALCAM	activated leukocyte cell adhesion molecule	*Yes*
Hs01091294_m1	AMACR	alpha-methylacyl-CoA racemase	*Yes*
Hs00265741_s1	ANG	angiogenin, ribonuclease, RNase A family, 5	*Yes*
Hs00171172_m1	AR	androgen receptor	*Yes*
Hs00156399_m1	CD69	CD69 molecule	*Yes*
Hs00379515_m1	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	*Yes*
Hs02512067_s1	GSTP1	glutathione S-transferase pi 1	*Yes*
Hs00153153_m1	H1F1A	hypoxia inducible factor 1, alpha subunit	*Yes*
Hs00233820_m1	JAK1	Janus kinase 1	*Yes*
Hs00153510_m1	MME/CD10	membrane metallo-endopeptidase	*Yes*
Hs00410317_m1	MUC1	mucin 1, cell surface associated	*Yes*
Hs00167309_m1	SOD2	superoxide dismutase 2, mitochondrial	*Yes*
Hs00560035_m1	STAT5	signal transducer and activator of transcription 5B	*Yes*
Hs00158980_m1	TACSTD1	EPCAM (epithelial cell adhesion molecule)	*Yes*
Hs00927216_m1	TIMP3	TIMP metallopeptidase inhibitor 3	*Yes - one false positive in the control resulted in us choosing a a cutoff threshold of 26 Cts.*
Hs00173626_m1	VEGF	vascular endothelial growth factor	*Yes*
Hs00184451_m1	VHL	Von Hippel–Lindau tumor suppressor	*Yes*
4326315E	ACTB (endogenous control)	housekeeping gene	*No – "endogenous" primers provided by Applied Biosystems were too dilute to provide efficient amplification.*
4333763	CYPA	housekeeping gene	*Yes*
4333764	GAPDH	housekeeping gene	*Yes*
Hs99999909_m1	HPRT1	housekeeping gene	*Yes*
See B2M forward, reverse, and probe sequences below.	B2M - custom ordered	housekeeping gene	*Yes*

See GUS forward, reverse, and probe sequences below.	GUS - custom ordered	housekeeping gene	*Yes*

mRNA Primer Sets (Biosearch Technologies)

Target	**Forward (5' to 3')**	**Reverse (5' to 3')**
Mouse Liver GYS2	GCCAGACACCTGACACTGA	TCCGTCGTTGGTGGTGATG
Mouse Kidney KCNJ1	GGCGGGAAGACTCTGGTTA	GTGCCAGGAACCAAACCTA
Mouse Control HPRT1	GCAAACTTTGCTTTCCCTGG	ACTTCGAGAGGTCCTTTTCACC
GUSB	GGCCGCTGTGGGAGTCAG	GATGTCATTGAAGCTGGAGGGAAC
B2M	TCTTTCTGGCCTGGAGGCTA	CCATTCTCTGCTGGATGACG

Target	**Probe (5' to 3')**
Mouse Liver GYS2	5'CalFluorOrange560-TTTCCAGACAAATTCCACCTAGAGCCC-BHQ1
Mouse Kidney KCNJ1	5-FAM-AAGCACCGTGGCTGATCTTCCAGA-BHQ1
Mouse Control HPRT1	5'Quasar670-CAGCCCCAAAATGGTTAAGGTTGCAAG-BHQ2
GUS Probe*	5'CalFluorOrange560-CCCCACCGTGGACATGCCA-BHQ1
B2M Probe*	5'CalFluorOrange560-TCCAGCGTACTCCAAAGATTCAGG-BHQ1

*CAL Fluor Orange 560 - detected using VIC reporter on Fluidigm

Small RNA Primer Sets (Biosearch Technologies)

Target	**Forward (5' to 3')**	**Reverse (5' to 3')**
Human RNU48	ATGACCCCAGGTAACTCTGA	GAGCGCTGCGGTGAT

Target	**Probe (5' to 3')**
Human RNU48	5'Quasar670-TGTGTCGCTGATGCC-BHQ2

6 References

1. Armani M, Tangrea M, Shapiro B, et al. Quantifying mRNA levels across tissue sections with 2D-RT-qPCR. *Anal Bioanal Chem.* 2011;400:3383-3393.

2. Devonshire A, Elaswarapu R, Foy C. Applicability of RNA standards for evaluating RT-qPCR assays and platforms. *BMC Genomics.* 2011;12:118.

3. Hewitt S, Lewis F, Cao Y, et al. Tissue handling and specimen preparation in surgical pathology. *Arch Pathol Lab Med.* 2008;132:1929-1935.

4. Masuda N, Ohnishi T, Kawamoto S, et al. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res.* 1999;27:4436-4443.