Published in final edited form as:

Appl Immunohistochem Mol Morphol. 2014 ; 22(5): 323–330. doi:10.1097/PDM.0000000000000039.

Multiplex Quantitative Measurement of mRNAs from Fixed Tissue Microarray Sections

Michael Armani, PhD^{1,3}, Michael Tangrea, PhD¹, Brian Yang¹, Alex Rosenberg, BA¹, Kris Ylaya, BSc², Jennifer Morris, BSc², Jaime Rodriguez-Canales, MD¹, Jeffrey Hanson, MSc¹, Benjamin Shapiro, PhD³, Michael R. Emmert-Buck, MD, PhD¹, Elisabeth Smela, PhD⁴, and Stephen M. Hewitt, MD, PhD^{2,*}

¹Pathogenetics Unit, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

²Tissue Array Research Program, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

³Fischell Department of Bioengineering and the Institute for Systems Research, University of Maryland, College Park, MD 20742, USA

⁴Department of Mechanical Engineering, University of Maryland, College Park, MD 20742, USA

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

Corresponding Author: Dr. Stephen M. Hewitt, genejock@helix.nih.gov, Address: 10 Center Drive, Room 2A 33, Rockville, MD 20892, Telephone: 301-496-0040.

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.

relatively few specimens. Subsequently, the candidate dysregulated genes must be validated by measuring their expression in a larger independent sample set⁴. 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 poor⁵. 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 genes⁸, 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 results⁹⁻¹². 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 involved¹³⁻¹⁵. These challenges are further compounded when using archival formalin-fixed, paraffin-embedded (FFPE) tissue samples, due to the inherent poor RNA quality¹⁶⁻¹⁸.

To address these limitations, our laboratories recently developed 2D-PCR⁶ and 2D-RT-qPCR⁷ 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"×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 (KBioscience/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 μ L 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 μ L solution containing 0.33 μ L ChargeSwitch magnetic beads, 1% acetic acid, and 6.6 μ L 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 μ L 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 48 $\times$ 48 Dynamic Array chip (Fluidigm, South San Francisco, 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 μ m 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 targets²¹. 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, HIF1A, 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 fixation²³. 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).

Several of the genes showed expression changes that were in agreement with previously published results²². 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 study²² 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 work²² 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, HIF1A 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 database²⁴. To allow a comparison between these data, the Delta Cts in this study were converted to fold-change using the formula $\text{sgn}(\text{DeltaCts}) \cdot 2^{\text{abs}(\text{DeltaCts})}$, where $\text{sgn}(x)$ is -1 for $\text{DeltaCts} < 0$ and 1 otherwise, and $\text{abs}()$ 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 total²⁴. 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 HIF1A was not identified as differentially expressed in the *in-silico* data²⁴. 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 specimens²⁵. 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}.

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 Vijver 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Kelley Banfield at SAIC-Frederick for running our Fluidigm experiments. We would also like to thank Connie Rogers-Newcome at Adhesives Research for providing samples of ARcare 7759 adhesive films. This work was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

References

1. Ramaswamy S, Ross K, Lander E, et al. A molecular signature of metastasis in primary solid tumors. *Nat Genet.* 2003; 33:49–54. [PubMed: 12469122]
2. van de Vijver M, He Y, van't Veer L, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002; 347:1999–2009. [PubMed: 12490681]
3. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of Tamoxifen-treated, node-negative breast cancer. *N Engl J Med.* 2004; 351:2817–2826. [PubMed: 15591335]
4. Chuaqui R, Bonner R, Best C, et al. Post-analysis follow-up and validation of microarray experiments. *Nature Genet.* 2002; 32:509–514. [PubMed: 12454646]
5. Greenbaum D, Colangelo C, Williams K, et al. Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biology.* 2003; 4:117. [PubMed: 12952525]
6. Armani M, Rodriguez-Canales J, Gillespie J, et al. 2D-PCR: a method of mapping DNA in tissue sections. *Lab Chip.* 2009; 9:3526–3534. [PubMed: 20024032]
7. 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. [PubMed: 21559756]
8. Bioshop J, Morton J, Rosbash M, et al. Three abundance classes in HeLa cell messenger RNA. *Nature.* 1974; 250:199–204. [PubMed: 4855195]
9. Silvestri A, Colombatti A, Calvert V, et al. Protein pathway biomarker analysis of human cancer reveals requirement for upfront cellular-enrichment processing. *Lab Invest.* 2010; 90:787–796. [PubMed: 20195244]
10. Klee E, Erdogan S, Tillmans L, et al. Impact of sample acquisition and linear amplification on gene expression profiling of lung adenocarcinoma: laser capture micro-dissection cell-sampling versus bulk tissue-sampling. *BMC Med Genet.* 2009; 2:13.
11. El-Serag HB, Nurgalieva Z, Mistretta T-A, et al. Gene expression in Barrett's esophagus: laser capture versus whole tissue. *Scand J Gastroentero.* 2009; 44:787–795.
12. Harrell J, Dye W, Harvell D, et al. Contaminating cells alter gene signatures in whole organ versus laser capture microdissected tumors: a comparison of experimental breast cancers and their lymph node metastases. *Clinical & experimental metastasis.* 2008; 25:81–88. [PubMed: 17932773]
13. Bustin S. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol.* 2002; 29:23–39. [PubMed: 12200227]
14. Tian H, Hühmer A, Landers J. Evaluation of silica resins for direct and efficient extraction of DNA from complex biological matrices in a miniaturized format. *Anal Biochem.* 2000; 283:175–191. [PubMed: 10906238]
15. Klebe R, Grant G, Grant A, et al. RT-PCR without RNA isolation. *BioTechniques.* 1996; 21:1094–1100. [PubMed: 8969838]
16. 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. [PubMed: 10536153]
17. Cox M, Eddy S, Stewart Z, et al. Investigating fixative-induced changes in RNA quality and utility by microarray analysis. *Exp Mol Pathol.* 2008; 84:156–172. [PubMed: 18291364]
18. Hewitt S, Lewis F, Cao Y, et al. Tissue handling and specimen preparation in surgical pathology. *Arch Pathol Lab Med.* 2008; 132:1929–1935. [PubMed: 19061293]
19. Stanley K, Szewczuk E. Multiplexed tandem PCR: Gene profiling from small amounts of RNA using SYBR green detection. *Nucleic Acids Res.* 2005; 33:e180. [PubMed: 16314310]

20. Glaab E, J G, Krasnogor J. ArrayMining: a modular web-application for microarray analysis combining ensemble and consensus methods with cross-study normalization. *BMC Bioinformatics*. 2009; 10:358. [PubMed: 19863798]
21. Devonshire A, Elaswarapu R, Foy C. Applicability of RNA standards for evaluating RT-qPCR assays and platforms. *BMC Genomics*. 2011; 12:118. [PubMed: 21332979]
22. Best C, Leiva I, Chuaqui R, et al. Molecular differentiation of high- and moderate-grade human prostate cancer by cDNA microarray analysis. *Diagn Mol Pathol*. 2003; 12:63–70. [PubMed: 12766610]
23. Cronin M, Pho M, Dutta D, et al. Measurement of gene expression in archival paraffin-embedded tissues. *Am J Pathol*. 2004; 164:35–42. [PubMed: 14695316]
24. Rhodes D, Kalyana-Sundaram S, Mahavisno V, et al. Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia*. 2007; 9:166–180. [PubMed: 17356713]
25. Eiseman, E.; Bloom, G.; Brower, J., et al. Case study of existing human tissue repositories: “best practices” for a biospecimen resource for the genomic and proteomic era. Santa Monica, CA: RAND; 2003.
26. Hewitt S. Design, Construction, and Use of Tissue Microarrays. *Meth Mol Biol*. 2004; 264:61–72.
27. Ahlfen S, Missel A, Bendrat K, et al. Determinants of RNA quality from FFPE samples. *PLoS ONE*. 2007; 2:e1261. [PubMed: 18060057]
28. Godfrey T, Kim S, Chavira M, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. *J Mol Diagn*. 2000; 2:84–91. [PubMed: 11272893]
29. Glas A, Floore A, Delahaye L, et al. Converting a breast cancer microarray signature into a high-throughput diagnostic test. *BMC Genomics*. 2006; 7:278. [PubMed: 17074082]
30. van't Veer L, Dai H, van de Vijver M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002; 415:530–536. [PubMed: 11823860]
31. Spurgeon S, Jones R, Ramakrishnan R. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. *PLoS One*. 2008; 3:e1662. [PubMed: 18301740]
32. de Bruin E, van de Pas S, Lips E, et al. Macrodissection versus microdissection of rectal carcinoma: minor influence of stroma cells to tumor cell gene expression profiles. *BMC Genomics*. 2005; 6:142. [PubMed: 16225673]
33. Gerlinger M, Rowan A, Horswell S, et al. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N Engl J Med*. 2012; 366:883–892. [PubMed: 22397650]
34. Tangrea M, Chuaqui R, Gillespie J, et al. Expression microdissection - Operator-independent retrieval of cells for molecular profiling. *Diagn Mol Pathol*. 2004; 13:207–212. [PubMed: 15538110]
35. Hanson J, Tangrea M, Kim S, et al. Expression microdissection adapted to commercial laser dissection instruments. *Nat Protoc*. 2011; 6:457–467. [PubMed: 21412274]
36. Gjerdrum L, Abrahamsen H, Villegas B, et al. The Influence of Immunohistochemistry on mRNA Recovery from Microdissected Frozen and Formalin-Fixed, Paraffin-Embedded Sections. *Diagn Mol Pathol*. 2004; 13:224–233. [PubMed: 15538113]

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)