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Maximizing RNA yield from archival renal tumors and optimizing gene expression analysis

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Abstract

Formalin-fixed paraffin-embedded tissues are widely available for gene expression analysis using TaqMan PCR. Five methods, including 4 commercial kits, for recovering RNA from paraffin embedded renal tumor tissue were compared. The MasterPure kit from Epicentre produced the highest RNA yield. However, the difference in RNA yield between the kit from Epicenter and Invitrogen's Trizol method was not significant. Using the top 3 RNA isolation methods, the manufacturers' protocols were modified to include an overnight Proteinase K digestion. Overnight protein digestion resulted in significant increase in RNA yield. To improve cDNA production by reverse transcription, random oligonucleotide primers were compared to gene-specific primers targeting the genes of interest. Reverse transcription using gene-specific primers significantly increased the quantity of cDNA detectable by TaqMan PCR. Therefore, expression profiling of formalin-fixed paraffin-embedded tissue using TaqMan qPCR can be optimized by using the Masterpure RNA isolation kit modified to include an overnight proteinase K digestion and gene-specific primers during the reverse transcription.

Keywords

formalin-fixed paraffin-embedded; RNA; TaqMan qPCR

Introduction

Frozen tumor banks of surgical specimens are costly to establish and maintain, and are not widely available. However, formalin-fixed paraffin-embedded (FFPE) tissue samples are routinely archived by nearly every hospital (1;2). Even when frozen tumors are available, the FFPE tumor can often be linked to a greater wealth of clinical information with longer followup. Therefore, FFPE represent a valuable resource for identifying biomarkers that may be useful for diagnosis, determining prognosis, and predicting response to treatment. In oncology, prognostic and predictive markers may allow for patient-specific counseling and treatment (3).

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Competing Interests Statement
The Authors declare no competing interests.

The primary barrier to using FFPE tissue for expression profiling is that the RNA is highly degraded. Strategies have been developed to use FFPE for targeted expression analysis using RT-qPCR and genome-wide expression profiling using microarray platforms(2–7). Optimizing strategies to increase the RNA yield from archival tumors will conserve tissue resources. In this study, five methods for isolating RNA from FFPE tissue were evaluated. The manufacturer's recommended protocols were modified in 3 of the 5 methods to further improve RNA yield. RNA quantification using spectrophotometry (Nanodrop) and flourometric analysis (RediPlate RiboGreen RNA Quantitation) were compared.

Strategies to increase sensitivity of the assay will allow for more accurate quantification of low abundance transcripts. Two strategies for reverse transcription were evaluated, and various platforms for qPCR were compared. Conventional 5ul 384-well TaqMan qPCR reactions, which were set up using a liquid-handling robot, were compared with qPCR reactions using the TaqMan® Custom Array, which is a 384-well microfluidic card that is preloaded for qPCR, thus obviating the need for liquid-handling robots or multichannel pipettes. Finally, qPCR machines from 2 different manufacturers were compared.

Materials and methods

Patient samples and RNA isolation

FFPE renal cell carcinomas procured between 2002 and 2006 were obtained from the Department of Pathology following approval by the Institutional Review Board (I53605). Using five different methods, including 4 commercial kits for RNA isolation, total RNA was recovered from three 10μm sections from 10 patient tumors using each method (50 reactions total). The protocol recommended by the manufacturer was used for the RecoverAll total nucleic acid isolation kit (Ambion, Austin, Tx, USA), High Pure FFPE RNA isolation kit (Roche, Mannheim, Germany), and ArrayGrade FFPE RNA isolation kit (SuperArray, Frederick, MD, USA). Isolation of FFPE samples using the MasterPure RNA Purification Kit (Epicentre, Madison, WI, USA) has been previously described (3).

To isolate RNA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), samples were treated twice with 1.9 ml of xylene for 5 min at 55°C with rotation. Sections were then washed with 100% EtOH and then air dried for 15 min at 55°C. The samples were then digested using 1% SDS and proteinase K (20mg/ml) for 3 hours at 55°C. Trizol was then added and RNA isolation was carried out according to manufacturer's specification. RNA samples were treated with DNAse as recommended by each manufacturer. Fifty nanograms of RNA for each sample were analyzed for residual genomic contamination using TaqMan RT-PCR for β-Actin (ACTB). If ACTB signal was detected below 34 cycles, an additional DNAse treatment was performed. In an attempt to further increase RNA yield, the 3 top performing kits were modified to include an overnight Proteinase K digestion at 55°C.

RNA quantification

Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the Rediplate 96 ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA) were used according to manufacturer's guideline to quantify total RNA. RNA concentrations measured using each of the methods were compared to ACTB Ct determined from qPCR, which served as the gold standard.

Reverse transcription and qPCR analysis

Reverse transcription was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA, USA) according to manufacturer's recommendation. RNA (5 μl) was reverse transcribed in a 50 μl reaction using random primers or specific RT

primers, which consisted of the 3' primer (100 nmol/L) designed for the TaqMan qPCR reaction for each gene being analyzed. TaqMan primers and probes were designed using Beacon Design Software (Bio-Rad, Hercules, CA, USA) using the default parameters for qPCR primers. Amplicon size was limited to less than 100 bp. When possible, amplicons were designed to span intron-exon boundaries. Primer/probe efficiencies were calculated prior to experimental use (8) and amplification efficiencies were greater than 90% for all primer sets.

The qPCR reaction was carried out in 5 or 10 μl volumes using 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 1ul cDNA, 900 nM each primer, and 250 nM probe. The qPCR reactions were performed on an ABI 7900HT (Applied Biosystems) or a CFX384 (Bio-Rad) using the manufacturer's suggested cycling conditions. A Tecan Freedom Evo 200 liquid handling robot was used to set up 384-well TaqMan qPCR reactions. Computer programs specific for the Tecan robot were written to manufacture 23 gene or 63 gene assays in a 384-well format. First, qPCR primers and TaqMan probes were added to the 384-well plates. The plates were then sealed with MicroAmp clear adhesive film (Applied Biosystems) and stored at −20°C. TaqMan Universal PCR Master Mix and cDNA were added to the 384-well plates prior to qPCR.

TaqMan® Custom Array micro fluidic cards

TaqMan® Custom Array (TCA) cards were designed using the Applied Biosystems TCA configurator on their website. The 64 gene format was selected, which allowed 63 genes to be selected from the ABI database and placed on the 384-well card in triplicate. The 63 genes selected were the same genes that were previously designed for use in conventional TaqMan qPCR assays. The cards were loaded with cDNA and TaqMan Master Mix, and run on an ABI 7900HT thermalcycler according to manufacturer's specification.

Results and Discussion

Comparison of commercially available RNA isolation kits

Commercial kits specifically designed for isolating RNA from FFPE tissues are available. To maximize RNA yield from FFPE renal tumors, 5 RNA isolation methods were evaluated, including 4 commercial kits. The Masterpure kit from Epicentre produced the highest RNA yield from archival renal tumors (Figure 1A). However, the difference in RNA yield between the kit from Epicenter and Invitrogen's Trizol method was not significant. Others have reported that RNA yield can be increased by using an overnight Proteinase K digestion rather than the standard 3 hr digestion (2; 5). Using the 3 RNA isolation methods that produced the highest RNA yield, the 3 hr and overnight Proteinase K digestions were compared. For each of the kits analyzed the overnight digestion with proteinase K resulted in greater RNA yield (Figure 1B).

The 10 RNA samples isolated using each of the 5 different methods were analyzed for residual genomic DNA. TaqMan qPCR was performed for ACTB without reverse transcription. The Trizol method had 3 of 10 samples that needed to be retreated with DNAse, while the Ambion, Roche, and SuperArray kits each had 1 of 10 samples that required additional DNAse treatment. The Epicentre kit was the only kit that was free of genomic DNA in all 10 samples. Therefore, the Epicentre kit had the highest RNA yield with no contaminating genomic DNA (Figure 1).

Although others have evaluated commercial kits for isolating RNA (2;9) to the best of our knowledge, no previous study of RNA yield from archival tissue has included the Epicentre kit or the Trizol method. When testing commercially available kits for optimizing RNA extraction for use in microarray experiments, Abramovitz et. al. recovered the most RNA

when using the Ambion and Roche kits in combination with overnight Proteinase K digestion. Doleshal et. al. also evaluated multiple commercial kits and reported that Ambion's RecoverAll gave greatest yield of RNA from FFPE tissue. In addition, the RNA yields were most consistent using the RecoverAll kit when repeat isolations using the same tumor block were compared.

Analysis of RNA quantitation methods

When working with small quantities of highly fragmented RNA, accurate quantitation is important. We evaluated two commonly used methods for measuring RNA concentration. Total RNA from FFPE tumors was measured using the Nanodrop ND-1000 spectrophotometer (Figure 2A) and the Rediplate 96 ribogreen RNA quantitation kit (Figure 2B). Serial dilutions of RNA isolated from FFPE renal tumors were quantified using both methods. The measured RNA concentrations were compared to qPCR Ct for ACTB, which was considered the "gold standard". For both methods there was a linear exponential relationship over a range of concentrations from 1.4 ng/μl to 174.6 ng/μl, which includes typical concentrations of RNA from archival renal tumors. Therefore, both methods accurately reflect differences in RNA concentration. The absolute concentrations reported by the two methods were very similar.

Use of Specific RT Primers to maximize qPCR efficiency

The objective of the remaining studies was to increase the sensitivity for detecting transcripts by qPCR. Primers with poor amplification efficiency may confound attempts to optimize qPCR. Therefore, primer efficiencies were formally calculated for the 23 genes used in this analysis (8). A primer efficiency value of 2 corresponds to 100% efficiency. The last column of Table 1 lists the primer efficiency values for 23 genes that were evaluated. Primer pairs with an efficiency value above 1.8 are considered to be suitable for quantitative PCR. Therefore, all primers evaluated were sufficiently efficient.

To perform expression analysis, cDNA is produced from highly fragmented RNA isolated from FFPE tissue. Conventional reverse transcription (RT) is performed using random oligonucleotide primers; however, others have reported using RT primers that are specific for the genes being assayed (4). The RT reaction is simplified when random primers are used; however, gene specific primers have the potential to increase the sensitivity of the expression analysis.

Table 1 compares the Ct for 23 genes using cDNA produced with random primers and specific primers. Use of specific RT primers increased the abundance of detectable message and decreased the Ct by an average of 2 cycles. To ensure that the use of specific RT primers preserved the relative abundance of each transcript, the Ct were plotted for the 23 genes using random vs. specific RT primers (Figure 3A). The Ct were highly correlated (R^2 0.92), indicating that use of specific primers preserves the ability to quantify gene expression.

Evaluation of TaqMan® Array Micro Fluidic Cards

TCA are micro fluidic cards available from ABI. ABI has developed a large database of TaqMan primer and probe sets; the database contains nearly every gene in the human genome. Investigators select genes they wish to assay, and micro fluidic cards are custom made, preloaded with primer and probe sets. TCA gives researchers the ability to carry out large scale 384-well qPCR projects without the need for an expensive liquid-handling robot or multichannel pipettes. TCA containing 63 genes were compared to conventional 5ul qPCR reactions set up using a liquid handling robot. For the conventional qPCR reactions, the primer and probe sets were designed using commercial software.

Quantitative PCR performed using conventional 384-well plates gave lower Ct values for most of the 63 genes analyzed when compared to qPCR performed using TCA (Figure 3B). This reduction in Ct values shifts the detectable range of the reactions so that less abundant transcripts may be detected. Furthermore, if a liquid handling robot is available and the cost of the robot is not considered, qPCR reactions using the TCA are approximately 5 times more expensive than conventional 5ul reactions using a liquid handling robot. Another advantage to conventional qPCR is that primer/probe sets can be tested and validated prior to initiating a large-scale profiling study. The primer/probe sets on the TCA have not been experimentally validated by the manufacturer.

Comparison of Bio-Rad CFX 384 and ABI 7900HT qPCR systems

Two popular, commercially available systems for 384-well qPCR were compared. The CFX384 (Bio-Rad) system uses an LED based system to excite fluorescence from the probe. The 7900HT (ABI) system uses an Argon laser for excitation. To determine which machine is more sensitive in detecting fluorescence signals, identical qPCR reactions were performed on the 2 systems using four different samples and 23 different genes. For every gene analyzed, the Ct was lower using the ABI system (Figure 3C).

Conclusion

When using FFPE tissue, the Masterpure kit produced the highest RNA yield with no contaminating genomic DNA. RNA yield can be further enhanced by including an overnight Proteinase K digestion. Gene specific primers enhance reverse transcription and increase the sensitivity of qPCR. A liquid handling robotic system and the 7900HT system provide an effective platform for performing 5µl 384-well qPCR assays.

Acknowledgments

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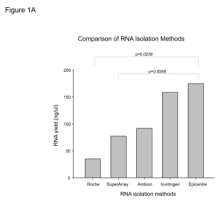

Figure 1B

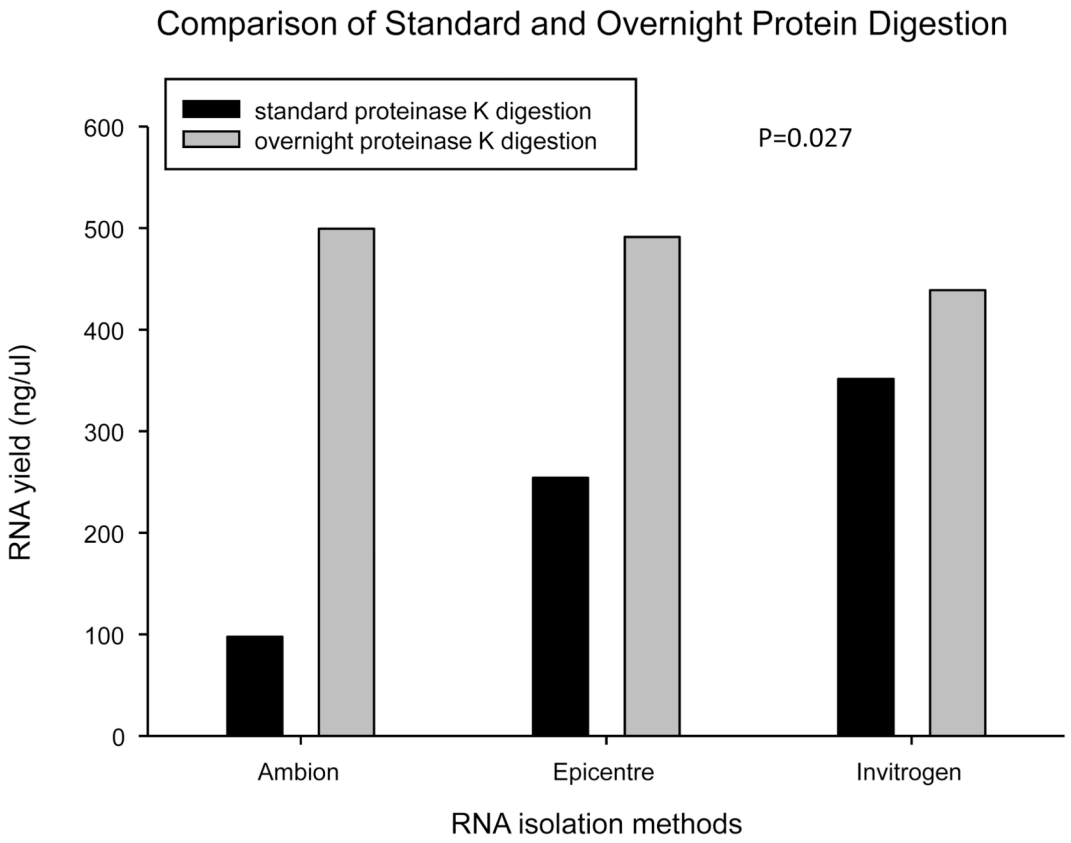

Figure 1. Determination of optimal RNA isolation method for use with archival renal tissue

(A) Comparison of RNA yields from archival renal tumors using 5 different methods for RNA isolation, including 4 commercial kits. Using each method, RNA was isolated from three 10 μm sections from 10 FFPE renal tumors. The total RNA recovered was diluted to a constant volume before measuring RNA concentration using a flourometric method. The Epicentre kit had the highest yield of RNA and the Roche kit had the lowest yield. Paired t-test was performed comparing the indicated groups. (B) Comparison of standard vs. overnight Proteinase K digestion. The 3 top-performing kits were used either with a standard 3 hour Proteinase K digestion or an overnight digestion. The overnight digestion significantly increased RNA yield from the FFPE tissues for all 3 kits. Error bars are not provided since the variability in RNA yield between tumors is greater than the variability in RNA resulting from use of different isolation methods. Paired t-test was performed comparing the 2 Proteinase K digestion groups.

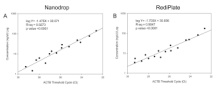

Figure 2. Comparison of methods for RNA quantification

(A) Serial dilutions of total RNA from 3 tumor samples were quantified using the Nanodrop ND-1000. RNA concentrations measured using the Nanodrop were compared to qPCR Ct for ACTB, which was considered the "gold standard". The log linear plot of Ct vs. concentration showed excellent correlation with R^2 of 0.9273 for the log fit. Therefore, the Nanodrop accurately reflects RNA concentration. (B) The same serial dilutions and samples were quantified using the RediPlate 96 RiboGreen RNA quantification kit. The concentrations measured by the RediPlate kit were then compared to the Ct values for ACTB. The log linear plot also showed excellent correlation with R^2 of 0.9047. Therefore, the RediPlate accurately reflects RNA concentrations.

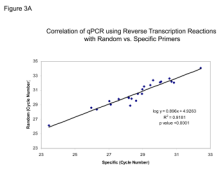

Figure 3B

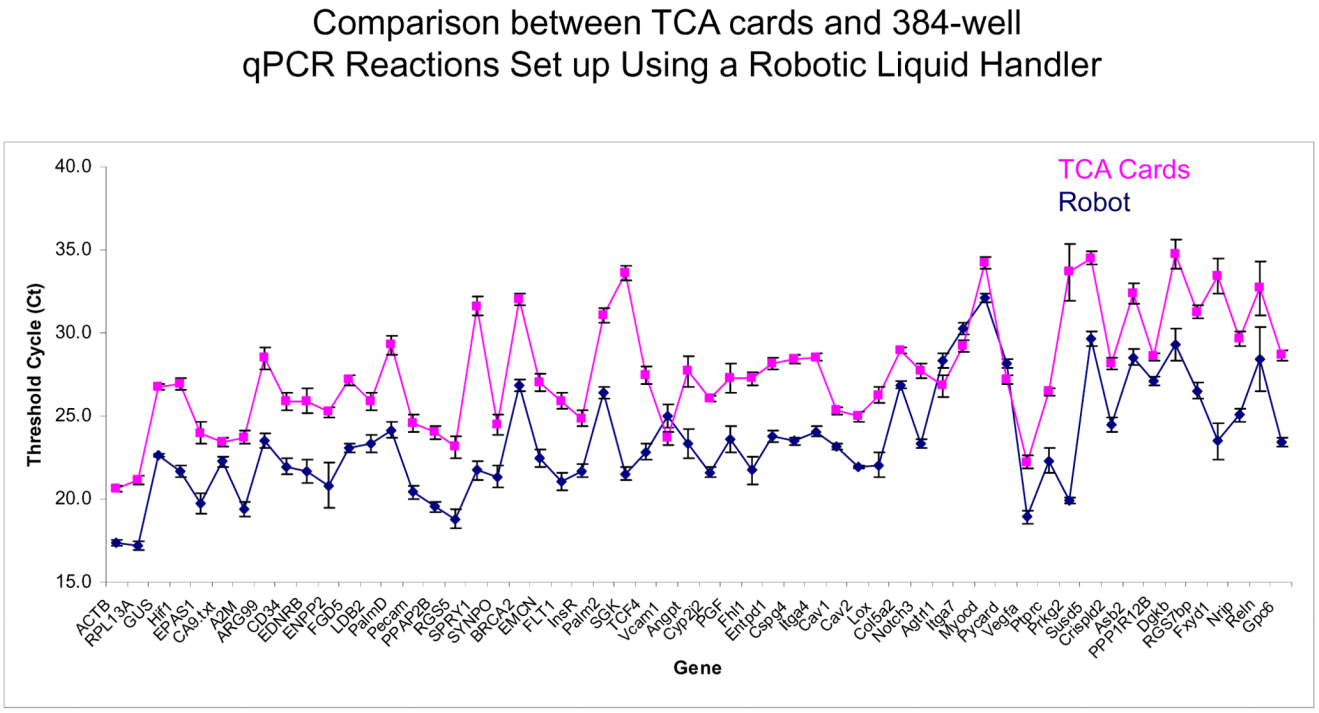

Figure 3C

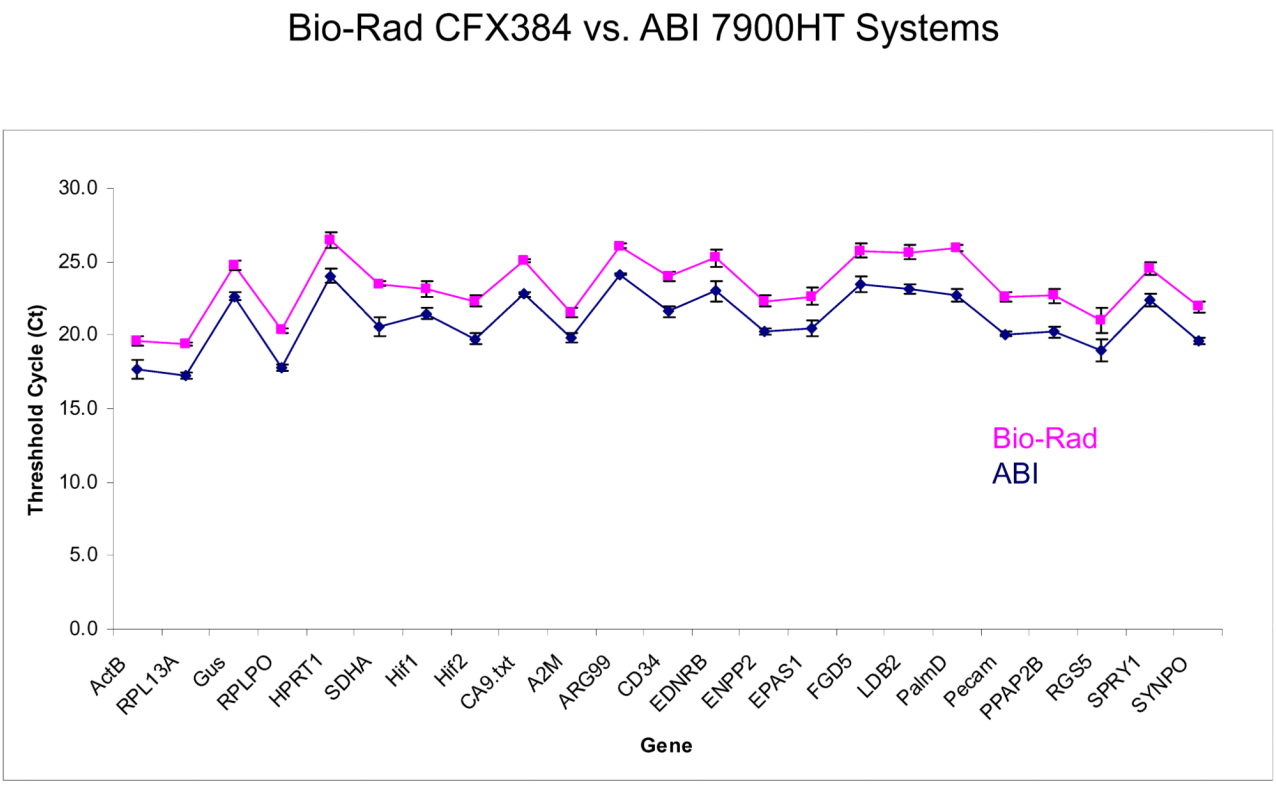

Figure 3. Strategies to increase the sensitivity of qPCR

(A) Scatter plot of qPCR data following reverse transcription reactions using random or specific primers (Table 1). Reverse transcription was carried out with RNA from 6 FFPE renal tumors using either random or gene-specific primers. The average threshold cycle from the qPCR reactions for each of the 23 genes analyzed was plotted to compare the 2 different methods for reverse transcription. Use of specific primers in the RT reaction increased the level of cDNA measured using qPCR. The R^2 was 0.9181, indicating a strong correlation. Therefore the use of specific primers maintains the ability of qPCR to quantify gene expressions. (B) Comparison of TaqMan® Custom Arrays (TCA) and 384-well 5ul qPCR reactions set up using a robotic liquid handler. The Ct for 63 genes were plotted for both the TCA and the standard qPCR reactions. The primers/probes used in the conventional qPCR were designed in-house using Bio-Rads Beacon Designer 3 software. TCA cards were custom-built by Applied Biosystems to assay the same 63 genes. For the majority of genes assessed, conventional qPCR detected transcripts at lower cycles, indicated higher sensitivity. The error bars indicate standard error of the mean for experiments performed in triplicate. (C) Comparison of Bio-Rad CFX384 and ABI 7900HT qPCR systems. Identical 384-well plates were created and qPCR reactions were performed using the 2 qPCR systems. The ABI 7900HT system detected the threshold cycle values earlier, indicating greater sensitivity for detecting transcripts when compared to the Bio-Rad system. The error bars indicate standard error of the mean for experiments performed in triplicate.

Table 1

Analysis of Random and Specific Primers Used in Reverse Transcription Reactions

	Cycle Threshold (Ct) Values RT Primers		Cycle Difference	Fold Enrichment*	Primer Efficiency**
	Random	Specific			
B-Actin	26.2	23.5	2.7	6.5	2.0
RPL13A	28.6	26.0	2.7	6.5	1.9
GUS	32.2	30.7	1.5	2.8	2.0
RPLPO	28.4	26.3	2.1	4.3	1.8
HPRT1	34.1	32.4	1.7	3.2	2.1
SDHA	29.8	27.6	2.2	4.7	2.0
Hif1a	28.9	28.3	0.6	1.6	2.0
Hif2	29.1	27.1	1.9	3.9	2.0
CA9	32.2	30.1	2.1	4.4	1.8
A2M	29.6	28.6	1.0	2.0	1.8
ARG99	32.1	30.8	1.2	2.4	2.0
CD34	30.5	28.9	1.6	3.0	1.9
EDNRB	32.1	30.0	2.1	4.3	1.9
ENPP2	30.5	28.7	1.8	3.5	2.0
EPAS1	31.2	29.0	2.2	4.6	2.0
FGD5	31.5	29.1	2.4	5.4	2.0
LDB2	32.4	29.6	2.8	7.0	2.0
PALMD	32.7	30.6	2.1	4.3	2.0
PECAM1	29.9	28.3	1.5	2.9	2.0
PPAP2B	29.9	28.2	1.8	3.4	2.0
RGS5	29.5	27.0	2.5	5.6	1.9
SPRY1	31.7	29.4	2.3	4.9	1.8
SYNPO	30.1	28.2	1.9	3.7	1.9

* cycle difference of 1 unit corresponds to 2-fold enrichment

** PCR-efficiency = 10 (−1/slope), slope of a plot of Ct vs. serial dilutions of the template