A Dissertation

entitled

A Multiplex Two-Color Real-Time PCR Method for Quality-Controlled Molecular Diagnostic Testing of FFPE Samples

by

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Measurement of messenger RNA is the most basic component of gene expression profiling methods that include polymerase chain reaction (PCR), microarray or next-generation sequencing. Gene expression measurement by reverse transcription quantitative PCR (RT-qPCR) supports personalized cancer treatment. However, most clinical samples obtained through standard clinical pathology workflow are formalin-fixed, paraffin-embedded (FFPE), and these yield small amounts of low integrity RNA containing PCR interfering substances. Therefore, there is a need for RT-qPCR tests capable of reliable transcript abundance analysis in such samples.

The goal of the dissertation was to develop a new RT-qPCR method to overcome such limitations in a real-time PCR platform. During my studies, I focused on 1) the test development of two-color fluorometric real-time PCR with competitive internal standards, 2) the test validation of two-color fluorometric real-time PCR assay for accurate quantification of highly degraded FFPE samples, and 3) the test application of two-color fluorometric real-time PCR for an accurate detection and quantification of Viral
Hemorrhagic Septicemia virus (VHSv) for fish samples. In addition to the development of the two-color fluorometric assay, I also helped develop a multiplex competitive PCR amplicon library preparation method for measuring transcript abundance by next generation sequencing (RNAseq).

Novel aspects of the two-color fluorometric real-time RT-qPCR method are 1) measurement of each gene relative to a known number of internal standard molecules within an internal standards mixture to control for interfering substances and enable numerical quantification, 2) two-color fluorometric probes enabling the discrimination of native templates and internal standards in real-time PCR platform, 3) external standards mixture for control for inter-experimental variation in fluorescence intensity of two probes and selection of quantification cycles, 4) gene-specific priming in reverse transcription and the pre-amplification method for enhancement of signal. Fitness of this method and reagents was assessed in twenty surgically removed FFPE human samples, and in a variety of fish samples from wild-caught and laboratory raised for VHSv quantification.

Internal standards controlled for experimentally introduced interference, prevented false-negatives and enabled pre-amplification to increase signal without altering the measured absolute initial copies of native templates. Internal standards also enabled multiplexing with many genes (e.g., three gene test with a reference gene) while controlling for inter-gene variation in PCR amplification efficiency. External standards reduced inter-experimental variation (i.e., low coefficient variation in 5 days, 7 PCR reactions). When we tested with 20 surgical FFPE samples for lung cancer diagnostic test, the result showed 93% diagnostic accuracy which was similar to that previously
reported for analysis of fresh samples. Further, the two-color fluorometric RT-qPCR method showed excellent performance for quantification of VHSv without false negative and false positive results compared with other methods.

This quality-controlled two-color fluorometric RT-qPCR approach will facilitate the development of reliable, robust RT-qPCR-based molecular diagnostic tests in FFPE clinical samples and other challenged samples.
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List of Abbreviations

Cq............................Quantification Cycle
CV............................Coefficient (of) Variation

ERCC.........................External RNA Control Consortium
ESM..........................External Standards Mixture

FFPE.........................Formalin-Fixed, Paraffin-Embedded
FNA.........................Fine Needle Aspiration

IS............................Internal Standard
ISM..........................Internal Standards Mixture

LCDT.........................Lung Cancer Diagnostic Test

NGS..........................Next-Generation Sequencing
NT............................Native Template

PCR..........................Polymerase Chain Reaction

RT............................Reverse Transcription
RT-qPCR.....................Reverse Transcription Quantitative-PCR

VHSv........................Viral Hemorrhagic Septicemia Virus
Chapter 1

Introduction

Morphologic analysis of cytology samples obtained by fine needle aspiration (FNA) is the primary method for diagnosing bronchogenic carcinoma because most lung cancers are advanced at the time of diagnosis and therefore are diagnosed by the least invasive possible method; typically a trans-thoracic, -bronchial, or -esophageal FNA. However, in multiple studies that use only the firm diagnosis of malignant (i.e., excluding suspicious or atypical), the sensitivity for diagnosing bronchogenic carcinomas ranged from 65-90%. Because cytologic samples typically have no tissue architecture, diagnosis is not as reliable as histomorphologic analysis of surgical, core needle, or forceps biopsy specimens. Thus, there is a need for strategies to improve accuracy of diagnosis by cytomorphologic criteria alone.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) tests that measure gene expression (transcript abundance) promise to improve cancer diagnostic accuracy for each cancer. However, few assays have met the standards needed for successful clinical implementation. Challenges include lack of methods and reagents for 1) standardization across laboratories, and 2) lack of appropriate bio-specimens. Use of
synthetic internal controls in molecular diagnostic tests is recommended by regulatory agencies, including the International Organization for Standardization (ISO), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). One of the best known FDA approved RT-qPCR based tests are COBAS TaqMan HIV-1 test (Roche Molecular Systems, Inc., Branchburg, NJ). In this assay, the internal control is key to meet FDA requirement to prevent false negative results. However, this diagnostic test measures a single infectious virus. An additional requirement when conducting RT-qPCR tests of human tissues is that both transcripts for a target gene and a loading control gene (e.g., ACTB) must be measured. Thus, for quality-controlled analysis, it is necessary to measure at least four analytes; each target gene native template (NT) relative to a known number of its respective internal standard (IS) molecules, and the loading control gene NT relative to a known number of its IS molecules.

We previously developed a method to implement quality-control and standardization in RT-qPCR by measuring target genes and a loading control gene relative to a known number of its respective synthetic IS molecules in each PCR reaction (Standardized RT-PCR; StaRT-PCR). This approach controls for inter-sample variation in interfering substances (e.g., heme, EDTA, unknown inhibitors) as well as inter-experimental variation (e.g., day to day, operator to operator) in quality and quantity of reagents in PCR reactions or thermal cycling conditions. According to our established method, the IS is designed to be 10% shorter than NT so that they can be separated and quantified by electrophoresis size separation.

The StaRT-PCR assay worked well for clinical samples that yield relatively high quality RNA, such as fresh frozen surgical samples or blood samples. On the other hand,
the same assay may work sub-optimally when using clinical samples yielding low-quality RNA, such as FNA samples used for standard of care cytomorphological diagnosis that are subsequently formalin fixed and paraffin embedded (FFPE). These FFPE FNA cell blocks are typically archived for potential additional analysis, such as immunocytochemistry. Thus, they are a potential resource that could be used to develop tests for prognosis and/or personalized medicine. Despite the importance of these biospecimens, these samples often contain highly-degraded transcripts and demonstrate a poor cellularity. For the reliable analysis of highly degraded RNA samples, including FNA cell block FFPE samples, the optimal PCR amplicon length should be < 100 bp. This is too short for routine quantification with StaRT-PCR in an electrophoresis platform because of size similarity among NT, IS and primer dimers or trimers. For this reason, we developed a new assay that is suitable for analysis of a short amplicon. To meet this challenge we chose to develop two-color fluorometric standardized RT-PCR in a real-time PCR platform.

The first goal of this study was to develop a robust RT-qPCR method for reliable molecular diagnostic testing of FFPE samples because the standard clinical pathology work-flow is to use FFPE processing for both surgical tissues and FNA biopsy cell block samples. Development of such an RT-qPCR method for reliable transcript abundance measurement of highly degraded RNA included 1) designing shorter PCR products (60 to 80 base pairs for each gene), 2) choosing NT and IS-specific fluor-labeled probes that differentiate expression of NT and IS in PCR amplification, and 3) ensuring the specificity of the NT and IS fluor-labeled probes. After the designing of appropriate probes and primers, analytical performance tests (primer efficiency, linearity and
precision) were conducted using synthesized NT or IS sequences and endogenous cDNA made from non-degraded cell line transcripts.

A key part of the rationale to develop this two-color fluorometric method was to enable analysis of small highly degraded samples available from FNA cell block FFPE samples. To achieve this goal, we used gene-specific primers in RT and multiplex pre-amplification to increase cDNA yield, as previously described [1]. Mixture of each sample with the same internal standards mixture prior to the pre-amplification step was key for reliable quantification of transcript abundance. The NT: IS ratio remained constant throughout both the multiplex pre-amplification and the following PCR. One difference from capillary electrophoresis StaRT-PCR was using probe chemistry, and there are at least two possible sources of analytical variation associated with this method. For example, there may be inter-probe variation in fluorescence intensity, including intensity difference among different batch of probes and/or probe intensity may change over time. In addition, inter-experimental variation in instrument selection of a threshold for quantification of each probe may also be significant. To control for these sources of variation, two concentrations of external standards mixture that comprised 1:1 of synthetic NT: IS copies for all targets and a reference gene were measured in each experiment.

After successful development of the two-color fluorometric method, and demonstration of necessary analytical performance, the next goal was the successful demonstration of fitness of purpose for this method in the analysis of surgical FFPE samples. For this purpose, we used the previously reported RT-qPCR lung cancer diagnostic test (LCDT), comprising MYC, E2F1 and CDKN1A measured relative to the
ACTB loading control gene [2]. The purpose of the LCDT is to augment morphologic diagnosis of lung tissue samples. A new real-time two-color fluorometric RT-qPCR method for measuring LCDT was tested for correct classification of malignant from benign diagnosis among twenty surgically removed FFPE samples.

Following successful development of two-color fluorometric real-time PCR, the method will enable the completion of a prospective clinical trial of the LCDT as well as develop and apply other promising tests to facilitate personalized medicine through more clinically meaningful diagnosis and sub-classification of lung cancer than the existing methods.
Chapter 2

Literature Review

2.1 Challenges of Formalin Fixed Paraffin Embedded Sample

2.1.1 RNA Degradation and Isolation Methods

Many thousands of formalin fixed paraffin embedded (FFPE) tissue blocks exist in hospital pathology archives. These can be used for tumor gene expression profiling and biomarker discovery [3-5]. When tissues were prepared to be archival samples, RNA was degraded in the tissue in the various ways. RNA started to be degraded physically and chemically before, during and after fixation [6], as well as paraffin embedding process which caused increased fragmentation at the elevated temperatures. A direct consequence of the fixation process by formaldehyde is chemical modification. Formalin causes addition of mono-methylol (CH2OH) groups to bases at different rates, from 40% for adenine to 4% for uracil. In addition, some adenines undergo dimerization by methylene bridging [7,8]. The crosslinking between nucleic acid themselves or nucleic acid and protein by formalin is not reversed during extraction [3,4,9]. However, the majority of the methylol groups can be removed by elevating the temperature [10,11], and proteinase K digestion solubilizes the fixed tissue and enables the extraction of RNA[12].
Yield of RNA from FFPE tissues is significantly lower than from fresh frozen tissues. RNA fragments extracted from FFPE are < 200 base pairs in length [13-17]. Thus, reverse transcription quantitative polymerase chain reaction (RT-qPCR) with primers coding for short amplicons are more efficient than primers coding for long amplicons [18]. Godfrey suggests that an efficient detection of targets can be achieved with the use of PCR amplicons < 130 base pairs, and optimal RT conditions [15]. Because of RNA modifications in FFPE, results are not directly comparable to fresh tissue RNA. Thus, it is important to develop biomarkers in FFPE, not attempt to discover in fresh sample RNA, then validate in FFPE. The genes may be the same, but the cut-off values likely will be different.

2.1.2 Reverse Transcription Efficiency, Priming, Inhibitors in FFPE Samples

RNA must be reverse transcribed to complementary DNA (cDNA) for RT-qPCR assays. The different priming methods include oligo d(T), random, gene-specific or combined RT priming. The optimal priming method is dependent on the type of sample, quality of the extracted RNA and the purpose of molecular diagnostic test.

Oligo d(T) primers anneal to the poly-A tail of each mRNA molecule while random primers can prime multiple locations throughout each molecule. If mRNA is not degraded, oligo d(T) primers generally yield more cDNA from mRNA because they bind only to mRNA, while random primers also bind to ribosomal RNA which typically comprises 95–98% of total RNA [19]. However, if RNA samples are degraded and/or fixed with formaldehyde, the poly-A tail may be absent or modified and it is recommended that random primers be used [12]. Gene-specific RT priming is the most specific and sensitive option for quantification [19-21]. It is linear over a wider range of
RNA concentration for cDNA yield than random primers. However, gene-specific primers yield cDNA corresponding only to the primed mRNA. Thus, in contrast to cDNA from oligo d(T) or random primers, the sample may not be used to assess every gene in the genome.

Inhibitors can affect the RT and PCR [22-26]. Inhibitors may be co-extracted with RNA or introduced during the nucleic acid extraction from the biological samples such as heme, immunoglobulin G, leukocyte genomic DNA, EDTA and heparin for collected whole blood [19,27-32], bile, salts, urea, lipids and muscle in solid tissues[33], and formalin and a residual paraffin from FFPE samples [34,35]. Inhibitors can generate inaccurate quantitative results, creating false-negative results at worst. In some cases, cDNA may be diluted to remove inhibitors prior to PCR amplification [36], but this is impossible when using very small amounts of RNA extracted (i.e., there is a high ratio of inhibitors to RNA). Several methods have been used to control for the effect of inhibitors, including the use of internal or external controls during RT-qPCR [32,37]. An, et al conclude that the absolute need for inclusion of an endogenous positive control when performing PCR amplification, especially on FFPE tissues [38].

RT efficiency can be measured by Reverse Transcription Standards Mixture (RTSM). An RTSM containing known copy numbers of External RNA Control Consortium (ERCC) 171 RNA and ERCC 113 cDNA was added to each RT reaction. The yield of PCR product from ERCC 171 RNA relative to ERCC 113 cDNA was measured for RT efficiency as a result of RT inhibition. Stanoszek and colleagues showed that increasing RNA amounts in RT inhibited random hexamer-primed RT efficiency [39]. A good example for controlling RT inhibition is inclusion of an RNA
competitor. Kleiboeker and colleagues used synthesized, heterologous competitor RNA for detection of West Nile virus. They demonstrated that 20% of the samples showed either partial or complete inhibition of competitor RNA amplification when the RNA was included at levels that were 10-fold greater than the detectable limits of the assay [40].

2.2 RNA-Based Molecular Diagnostic Test

2.2.1 Reverse Transcription-Polymerase Chain Reaction

RT-PCR is a powerful tool for the detection and quantification of mRNA. It has high sensitivity, good reproducibility, and wide dynamic range of quantification. A cDNA reverse transcribed from mRNA is amplified by PCR. The two broad classes of amplification product detection techniques are the “end-point” measurements of product and “real-time” monitoring of product formation. End-point determinations analyze the reaction after it is completed, and real-time determinations monitor the reaction in the thermal cycler as it progresses [41,42].

Amplified RT-PCR end product is detected by various post-amplification detection methods including ethidium bromide gel staining, radioactivity labeling, high performance liquid chromatography, southern blotting, fluorescence labeling, or densitometric analysis [43,44]. Without appropriate controls, this post-PCR step leads to high intra-, inter-assay variability and lower dynamic range than real time PCR. This property is a drawback for quantitative measurements because small differences in the multiplication factor lead to large differences in the amount of product [42,45].

Heid at al developed real time PCR measuring PCR product accumulation through dual-labeled fluorogenic probes in which one fluorescent dye serves as a reporter, FAM (6-carboxyfluorescein), and its emission is quenched by the second fluorescent dye,
TAMRA (6-carboxy-tetramethylrhodamine). Nuclease degradation of the hybridization probe during each PCR cycle releases the quenching of the FAM fluorescent emission [46,47]. Real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays. This method has an accurate dynamic range of 7 to 8 log orders of magnitude of starting target molecule determination [46,48]. The quantification cycle (Cq) is defined by software as the cycle when sample fluorescence exceeds a chosen threshold above calculated background fluorescence. The Cq is dependent on the starting template copy number, the efficiency of PCR amplification, efficiency of cleavage or hybridization of the fluorogenic probe, and the sensitivity of fluorescence detection. A Cq value is reported for each sample and can be translated into a quantitative result by constructing a standard curve or comparing reference Cq values.

2.2.1.1 Probe Chemistry in Real-Time PCR

In Real time PCR, the linear correlation between PCR product and fluorescence output is used to calculate the amount of template present at the beginning of the reaction [49]. Generally, two methods have been established for quantitative detection of the PCR amplicon: non-specific, double-strand DNA intercalating dye (e.g., SYBR Green I [50]), and sequence-specific fluorescent probes based on hybridization of fluorescence-labeled probes to the correct amplicon (e.g., TaqManTM (Applied BioSystems) [47], molecular beacons (Stratagene) [51], EclipseTM [52]). Initially, ethidium bromide (EtBr) was used as a DNA intercalating dye for nonspecific detection [53]. SYBR TM Green I is the most common intercalating dye used to bind to any double stranded DNA [50,54]. TaqManTM is the best known sequence specific probe which uses the 5’-3’ exonuclease
activity of Taq polymerase. Hydrolysis activity of the Taq polymerase separates probe fluorophore dye from a quencher dye, and results in increased fluorescence signal. When these probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to fluorescent resonance energy transfer (FRET) effects [55]. During PCR, the probe hybridizes to a target sequence and is cleaved by Taq polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye is increased.

2.2.1.2 Quantification Method

Generally, two quantification strategies are used in real-time RT-PCR, relative or absolute quantification. Both methods need normalization to correct for sample-to-sample variations in loading.

2.2.1.2.1 Normalization to Control for Loading

A major potential source of analytical variation is inter-sample variation in the amount loaded into an RT-PCR reaction which can cause RT-PCR specific errors in the quantification. One way to control for this source of variation is to amplify simultaneously a target RNA along with a reference RNA that serves as a loading control. The target RNA is normalized by a reference RNA. This reference gene can be co-amplified in the same tube (in the case of multiplex assay) or can be amplified in a separate tube [19]. Well known reference genes include housekeeping genes such as β-actin, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), glucuronidase, beta (GUSB), and ribosomal RNAs (rRNA). The ideal reference gene should be expressed at a constant level among different tissues, at all stages of development, and should be unaffected by the experimental treatment [56]. However, housekeeping genes may be
affected by different treatments, biological processes, and even different tissues or cell types [56-58]. rRNAs are not proper references because of inter-sample variation in the balance between rRNA and mRNA fractions. rRNAs are transcribed with a different polymerase than mRNAs (i.e., rRNA by the RNA polymerases I, mRNA by RNA polymerases II), so changes in polymerase activity may not affect both types of RNA expression equally [48,59,60]. Vandesompele et al quantified the errors associated with the use of a single (non-validated) reference gene [61]. They recommended the use of multiple reference genes depending on the observed inherent expression variation of candidate reference genes and the tissue heterogeneity of the samples under investigation.

2.2.1.2.2 Relative Quantification

In relative quantification, the relative mRNA levels of two or more genes are compared across samples. For example, fold difference of a target gene versus a reference gene (housekeeping or endogenous control gene) in one sample to another sample is compared. A reference gene is considered to have a constant difference in mRNA levels for the gene of interest. Therefore, fold difference of target mRNA to one or more reference mRNA could be reported. The original RNA concentration is not reported because the result is a ratio of expression level of target vs reference gene. This quantification method is adequate to investigate physiological changes comparing control vs treated samples [42].

Basically, there are two calculation methods of the relative quantification, with efficiency correction [62] and without [63]. For efficiency correction, the slope of the standard curve is required. A significant statistical bias can be introduced that results in misleading biological interpretation when the expression levels of target and normalizer
is too different or when the target gene is expressed at very low levels, as the relationship between the two may not be linear at these case [64].

Even if there are issues about reference gene, relative quantification is more convenient than absolute quantification, because it requires less stringent controls. However, when performing relative quantitation, the data (Cq) used for comparison are arbitrary values and only applicable to the samples run within the same PCR. For comparison of PCR results from two different experiments, it is necessary to include a standard control in every plate or run [48].

2.2.1.2.3 Absolute Quantification

Absolute quantification reports the final result in copy numbers per total RNA concentration, per genome, per cell, per gram of tissue, ml blood, etc. Generally, there are two approaches for absolute quantification: “standard curve method” and “competitive PCR”. The standard curve method measures expression of a particular gene using serial dilutions of known copy number or concentration of a selected sequence in a separate PCR assay. Competitive PCR measures endogenous gene expression relative to known numbers of synthetic RNA or DNA sequences placed in each PCR assay. In cases where data compared are assayed on different days or in different laboratories, absolute quantitation may be preferred because results are based on constant reference agent [48,65].

2.2.1.2.3.1 External Standards for Standard Curve

In real time RT-PCR, the threshold method assumes that all samples that have the same concentration of DNA produce the same Cqs. Therefore, in standard curve method, an unknown sample Cq is compared to Cqs generated from a series of five to six serial
dilution of known concentration or copy number [66]. Standard curve quantification method is highly reproducible with a relatively low intra-, inter-assay variation under optimal conditions [42,62]. However, the accuracy of this method depends on the accuracy of the standards. Standard production, determination of the exact concentration, and storage stability can be problematic. Known concentrations of DNA molecules (e.g., recombinant plasmid DNA, genomic DNA, RT-PCR product, and commercially synthesized oligonucleotide) can be used as standard molecules [43,62].

As the PCR efficiency can be affected by the variations of reagents, primers (sequence alterations), a variation of day-to-day or sample-to-sample or operator-to-operator, this approach can make errors into the quantification [50]. The main disadvantage of using an external standard is that it cannot provide a control for detecting inhibitors of the PCR reaction. Some of the unknown samples may contain substances that significantly reduce the efficiency of the PCR. This requires an addition of internal control templates that generate more confidence in negative results where no template is detectable [66].

**2.2.1.2.3.2 Competitive PCR**

A competitive approach for quantitative PCR was introduced by Gilliland et at [67]. The general concept of competitive PCR consists of co-amplification of two different templates bearing the same primer recognition sequences in the same tube. This approach ensures identical thermodynamics and amplification efficiency for both template species. The quantity of a synthetic competitive template should be known to quantify an unknown sample, and the ratio of their initial copy numbers remains constant throughout the amplification. Their amplicons can be distinguished by the addition of a
restriction enzyme site to the standard [67,68], or by varying its size or sequence [56,69-72].

For competitive templates, synthetic internal standard (IS) RNA molecules could be spiked into the RNA samples before RT [73,74] or synthetic IS DNA molecules that differ from the cDNA of interest could be added to the endogenous cDNA following RT [75]. By including a known number of IS RNA molecules in RNA sample prior to RT, variable effects due to differences in conditions of the RT and the PCR amplification could be internally controlled [76]. However, pipetting errors may occur at two points: placing the same amount of RNA from each sample into its respective RT reaction and pipetting cDNA from each RT reaction into each PCR assay. Further, competitive RNA may degrade during long-term storage, and hetero-duplex formation between the nearly identical standard and target can result in variable sensitivity and accuracy [45,56]. When Nolan and colleagues used a serial dilution of RNA for RT and then quantified the specific cDNA targets with real-time PCR, non-linear relationship between the initial concentration of RNA and the level of the specific cDNA yield was found [21]. Additionally, the RT efficiency of a synthetic RNA IS and an endogenous transcript may be variable, likely due to variation in hetero-duplex formation, and IS for one gene may not effectively control for RT efficiency of all other transcripts [77].

When fluorescence is used to detect the endogenous and standards amounts (e.g., TaqMan probes, DNA intercalating dye), it is recommended to add an amount of DNA standard that does not differ too much from the amount of target (within a 10-fold ratio), because there are upper and lower limits of detection of fluorescence [43-45]. If RNA competitor is used during RT and will be detected using fluorescence method, it is
difficult to know beforehand the proper amount of IS for each gene to be placed into the RNA sample before RT. Therefore, several RT reactions per sample (i.e., a constant amount of the target of interest and varying amounts of competitor) will be needed. This is a very inconvenient process that depletes RNA stores and limits analysis of the cDNA to the genes for which an RNA IS was included in RT. In contrast, when synthetic DNA is used as competitors in PCR only, several reactions with different competitor : target ratios for each unknown target can be completed with more abundant supply of cDNA rather than the limited source of RNA. Thus, when fluorescence emission is used to detect the cDNA signal, Willey and colleagues state that measurement of cDNA with synthetic DNA competitor is most practical [78].

However, when non-fluorescent methods of detection (e.g., next generation sequencing) are used it is not necessary to keep the NT and IS within close ratio [72] and RNA or cDNA standards may be employed.

Ultimately, the main advantage of competitive PCR is that the results are not affected by tube to tube variations in amplification efficiency controlling for inter-sample variation in interfering substances [56].

2.2.1.3 Two-Color fluorometric Real-Time PCR

The COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche Molecular Systems, Inc., Branchburg, NJ) (COBAS) [79-82] and Abbott Real time HIV-1 assay (Abbott Molecular Inc., Des Plaines, IL) (Abbott) [83,84] are absolute quantification method for one gene test in real-time PCR platform using competitive method and standard curve method, respectively. COBAS and Abbott are each designed to detect and precisely measure levels of the human immunodeficiency virus (HIV)-1 circulating in a patient’s
blood. It was approved by the U.S. Food and Drug Administration (FDA) for in vitro diagnostic use [81,84]. Each of these assays is commercially available for quantification of HIV-1 RNA in plasma, and each is intended for use as a marker of disease prognosis and as an aid in assessing viral response to antiretroviral treatment. Each kit includes materials for specimen preparation to isolate HIV-1 RNA, RT of the target RNA to generate complementary DNA (cDNA), and simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target. Two probes labeled with different fluorescent reporter dyes were used for detection, one to detect the synthetic internal control and one to detect the target sequence at PCR step. The competitive template quantitation standard (QS) RNA (COBAS) or internal control (IC) RNA (Abbott) controls for effects of inhibition and controls the preparation and amplification processes. It allows more accurate quantitation of HIV-1 RNA in each specimen than without synthetic IS controls. Copies/mL unit is reported in both assays. To verify run validity, an HIV-1 negative control, a low positive control, and a high positive control are included in each run at both assays.

2.2.1.3.1 The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test

The quantitation of HIV-1 viral RNA is performed using the HIV-1 QS Armored RNA. The HIV-1 QS is a non-infectious Armored RNA construct that contains HIV sequences with identical primer binding sites as the HIV-1 target RNA. The probe binding region of the HIV-1 QS has been modified to differentiate HIV-1 QS amplicon from HIV-1 target amplicon. The HIV-1 probe and HIV-1 QS probe are labeled with different fluorescent reporter dyes. The HIV-1 QS is added to each specimen at a known
copy number and is carried through the specimen preparation, RT, PCR amplification and
detection steps [79-81] .

2.2.1.3.2 Abbott Test

A non-competitive Armored RNA® IC comprising an RNA sequence unrelated to
the HIV-1 target sequence derived from the hydroxypyruvate reductase gene of the
pumpkin plant, Cucurbita pepo. It is added into the sample lysis buffer during extraction
and processed simultaneously with each sample. In the presence of the HIV-1 target
sequence or IC sequence, the HIV-1 probe preferentially hybridizes to the target
sequence and the IC probe hybridizes to the IC sequence, allowing two different
fluorescent detections. A calibration curve is required to quantitate the HIV-1 RNA
concentration of specimens and controls. Two calibrators are run in triplicate to generate
a calibration curve. As consistent quantity of IC is introduced into each specimen,
calibrator, and control at the beginning of sample preparation, it demonstrates proper
specimen processing and assay validity [83,84].

When Sloma and colleagues compared between COBAS and Abbott, they found
good correlation of quantitative results (r = 0.95) among clinical specimens, with a mean
difference of -0.34 log10 copies/mL, and direct reagent and consumable costs for each
assay were comparable [85]

2.2.1.4 Our Method (Competitive Multiplexing PCR)

Our developed two-color fluorometric PCR method is a competitive PCR for
absolute quantification in real-time PCR format for tests comprising multiple gene
analytes. It is designed for quantification of challenged mRNA, such as FFPE samples
having chemical modification and physical fragmentation and crosslinking between
nucleic acid themselves or nucleic acid and protein by formalin. In contrast to the COBAS and Abbott methods for the HIV-1 test, two main differences with our method are the application of internal standards mixture (ISM) and external standards mixture (ESM) for the quality control. Both COBAS and Abbott assays are developed for only one target gene (HIV-1), not for multiple genes (i.e., targets and references). As at least one reference gene is needed for the loading control, a method for measuring at least two genes (i.e., a target and a reference) is necessary for accurate transcript abundance measurements.

ISM is a mixture of synthetic competitive IS with a known concentration of IS for each of multiple represented genes (e.g., one or more reference genes and one or more target genes). In each PCR reaction, the ISM controls for inter-sample variation due to interfering substances and prevents false negative results. If the same ISM is used across PCR experiments and laboratories, it controls for analytical variation across multiple runs in different laboratories [1,32,75,86].

ESM is a mixture of synthetic native templates (NT) and IS templates of genes of interest keeping 1:1 ratio of NT: IS for each gene and a constant inter-gene relative concentration. It controls the fluorescence intensity difference between two probes labeled with different dyes due to the variation of degradation of probes or software selection of Cq values in each plate of PCR. In each plate, two external standards PCR reactions per each gene are run, and the mean of two ESM’s Cq difference (NT Cq- Is Cq) was used for normalization of an unknown sample’s Cq difference for accurate quantification of initial copy numbers. The final report is NT copies of a target gene per million of a reference gene (ACTB).
2.2.2 Sequencing

Automated Sanger sequencing dominated the sequencing industry for almost two decades. Completion of the human genome sequence [87] was by Sanger sequencing. But, this approach is relatively low throughput, expensive and generally not quantitative (i.e., for measuring the number of copies). It is considered ‘first-generation’ technology, and newer methods are referred to as next-generation sequencing (NGS) [88].

NGS provides massively parallel throughput and enables extensive genome analyses of groups of individuals. It includes analyses of sequence differences, polymorphisms, mutations, copy number variations, epigenetic variations and transcript abundance [88-90]. The transcriptome, the complete set of transcripts in an RNA sample, is characterized through cDNA sequencing following RT. Transcriptomics includes the study of all species of RNA, such as mRNAs, non-coding RNAs and small RNAs [88]. Applications of seq-based method are described for measuring genome-wide profiles of immunoprecipitated DNA-protein complexes (ChIP-seq) [91], methylation sites (Methyl-seq) [92] and DNase I hypersensitivity sites (DNase-seq) [93].

RNA-Seq is a recently developed approach using NGS technologies (i.e., sequencing the same region multiple times) for simultaneous transcriptome profiling, mapping and quantifying transcriptomes of targeted regions of interest or whole transcriptomes. The relative abundances of individual transcripts in a transcriptome can differ by several orders of magnitude. For the detection and quantification of low abundance transcripts with RNA-seq, the total number of reads per library can be increased [94].
A sequencing approach offers many advantages over qPCR and microarray technologies for transcriptome analysis. First, lower input amounts and samples with relatively poor integrity, such as archival FFPE tissues can be used. Second, RNA-seq does not have an upper limit for quantification with a large dynamic range of expression levels. Third, without prior knowledge of a particular gene sequence, RNA-seq can identify and quantify rare transcripts and provide information regarding sequence variation. It allows analysis of the entire transcriptome including determining exon/intron boundaries. Additionally, single-base resolution (i.e., sequence information of each nucleotide), possibly could affect gene expression measurements in other platforms due to probe specificity, is obtained [88,90,91,95,96]. The major advantage of NGS is the relatively low cost of obtaining a large volume of data. But, for smaller-scale projects and longer DNA sequence read (>500 bp), the Sanger method is still widely used. Direct RNA sequencing technology for single molecule is currently under development by Helicos [97,98]. However, for most NGS, the requirement of RNA or cDNA fragmentation in the library construction step creates a bias in the outcome [99]. Further, the cost of handling large amounts of data and the greater sequencing depth required for a rare transcript or variants is often a limiting factor [100].

Major steps of NGS are template preparation, sequencing, genome alignment and assembly [89]. In general, the total or fraction of RNA is converted to a library of cDNA fragments with platform specific adapters attached to one or both ends. Then, the short product of each molecule is sequenced with or without amplification from one end (single-end sequencing) or both ends (pair-end sequencing). The read lengths are usually 30-400bp, depending on the sequencer. Following sequencing, the resulting reads are
either aligned to a reference genome or reference transcripts, or assembled de novo without the genomic sequence to produce a genome-scale transcription map [88]. Metzler et al. described the pros and cons for the choice of sequencing instruments. Several NGS platforms, Illumina/Solexa, Roche/454, Life Technologies/APC and Helicos BioSciences, used for RNA-seq are commercially available, and new technologies are in development [89]. Sequencing capabilities, such as throughput, read length, error rate and ability to perform paired reads vary among platforms.

2.2.2.1 Whole Transcriptome RNA-seq

Whole transcriptome RNA-sequencing (total RNA sequencing) captures a broad range of gene expression levels (more than five orders of magnitude) and enables the detection of novel transcripts in both coding and non-coding RNA species. It provides insight into complex diseases by understanding of altered expression of genetic variants. As well as transcript abundance quantification, detailed transcript structure information, including representation of alternative transcripts (splicing pattern and transcript isoforms) [101], polymorphisms, and mutations, including translocation and fusion transcripts are available [102].

Microgram quantities of total RNA are required for whole transcriptome sequencing. Removal of rRNA prior to analysis optimizes the percentage of reads covering RNA species of interest because rRNAs constitute 95-98% of total cellular RNA. Enrichment of mRNA is conducted by either poly-A selection (mRNA selection) or depletion of rRNA [103,104]. About 1µg of total enriched mRNA is subjected to cDNA synthesis by RT. Fragmentation methods are used for either RNA (i.e., RNA hydrolysis or nebulization) or cDNA (i.e. DNase I treatment or sonication). The
fragmented cDNA is size-selected and ligated with platform-specific adaptors with or without a barcode. Then the library preparation is purified before sequencing [105]. Basically, two different assembly methods are used for producing a transcriptome from raw sequence reads: de novo and genome-guided. The de novo approach does not rely on the presence of a reference genome to reconstruct the sequence [106,107]. The reference genome approach is cheaper for mapping using tools, such as Bowtie [108], TopHat splice junction mapper [109], filtering only unique hits to the reference sequence [102,105].

The interest in single cell whole transcriptome analysis is growing rapidly, especially for profiling rare or heterogeneous populations of cells [110,111]. However, the reproducibility of such low-quantity RNA-seq approaches is negatively affected by stochastic amplification bias that results in the drop-out of some RNA species and preferential amplification of others [90,112].

For routine molecular diagnostic testing, the cost (due to need to sequence high abundance targets many times in order to quantify low abundance targets) and complexity (i.e., difficulty in sequence alignment) of whole transcriptome RNA-sequencing data sets are barriers to use of this method [72].

2.2.2.2 Targeted RNA-seq

Traditionally, RNA sequencing experiments have started with whole transcriptome library preparation. However, in many cases, whole transcriptome sequencing is not required and is less suitable than targeted RNA sequencing. Targeted RNA sequencing is a method for measuring of transcripts of interest with quantitative or qualitative information [113-115]. It would be better for a diagnostic test than whole transcriptome
RNA-seq. Targeted RNA sequencing allows for allele-specific expression measurement \[116\] and differential expression analysis \[117\] as well as detection of the fusion transcripts from chromosomal rearrangements \[118\]. Further, data analysis of targeted RNA-seq is significantly faster than whole transcriptome RNA-sequencing because it takes less time for alignment. However, these studies also underscored some challenges, for example, the high sequencing depth required to cover sufficiently low-abundance transcripts \[90,100\].

\[2.2.2.2.1\] Ion AmpliSeqTM Approach

Ion AmpliSeqTM \[119\] is a type of targeted RNA sequencing using semi-conduct chip. Extracted RNA is reverse transcribed into cDNA. The cDNA is amplified with a pool of specific gene targets. Using RNA custom panels, a pool of gene specific assays (12 up to 1200 in a pool, up to 96 barcodes) can be designed to measure the abundance of multiple genes at one time. Current design is one amplicon per gene at exon-exon boundary. As the next step, primers are partially digested, and Ion Torrent specific adaptors or barcode adaptors ligated onto the amplicons. Then, the library is amplified with PCR and purified with magnetic bead for enrichment. The purified library is sequenced on the sequencer. After sequencing with Ion Torrent ProtonTM or Personal Genome Machine® (PGMTM), data was analyzed to give the number of counts per amplicon, and \(<10\) counts were not used. Counts corresponds the abundance of gene target. Therefore, gene expression is calculated relatively among genes showing fold changes using a total number of reads. The company compared Ion AmpliSeqTM with TaqMan PCR assay, showing a good correlation.
For many RNA-Seq-based projects, the budget for sequencing costs, and thus the total number of reads that can be obtained, is constrained. Thus, researchers designing RNA-Seq experiments must often determine the correct balance between sequencing depth (i.e., the number of reads per sample) and the number of samples sequenced [94]. For the low transcript abundance detection, read counts should be increased, because highly abundant genes occupy the most space of on a sequencing chip that causes fewer low expressed genes to be represented on a chip. To determine how many samples can be measured on a single Ion Chip, a researcher must take into account: the number of genes in their panel, the dynamic range of the panel, the size of the Ion Chip. Therefore, the company recommends a pilot run [120].

As Ion AmpliSeq™ is a PCR-based target selection, the restriction of PCR cycles to exponential phase is required for library preparation. This is not required for real-time PCR because of use of probe chemistry, detecting and reporting early stage of amplification [119]. If the amplification is extended beyond the exponential phase into the saturation phase, tubes containing more starting material (e.g., ACTB, rRNA) will reach the saturation phase sooner than tubes containing a smaller amount. Therefore, inter-sample and inter-experimental variation in plateau phase may not be controlled.

**2.2.2.2 Our Method (Competitive PCR Library Preparation)**

We developed a competitive multiplex PCR-based amplicon sequencing library preparation method for RNA-sequencing (Standardized sequencing: Star-Seq). This method not only targets the sequences of interest, but also controls for inter-target variation in PCR amplification during library preparation by measuring each transcript NT relative to a known number of synthetic competitive template IS controls. Briefly,
mixed cDNA (NT) and ISM were PCR amplified first with tailed target-specific primers, and then with primers that added barcoding sequences, then with primers that added platform-specific adapter sequences. Individual library for each sample was mixed, gel purified, and then sent for Ion Torrent PGMTM sequencing or Illumina HiSeq or MiSeq. The competitive multiplex-PCR amplicon library preparation method provides the quality control, reproducibility, and reduced sequencing read depth for low cost, covering low-abundance transcripts sufficiently [72]. A key advantage of competitive PCR is its insensitivity to the effect of saturation of the PCR [43]. This overcomes the limitation of Ampliseq, described above.

2.3 Rationale for Competitive RT-PCR

The importance and need for standardization and extended quality control studies in RT-PCR were emphasized by researchers [73,121]. The significant difference in our assays (i.e., both, two-color fluorometric real-time PCR and Star-Seq) from commonly used real time PCR and NGS is the implementation of the known number of synthetic internal competitive template molecules for each gene in one mixture.

The competitive IS molecule was designed with 4-6bp (two-color fluorometric real-time PCR) and 6bp (Star-Seq) difference from each native target gene template (NT). This alteration from NT could be distinguished by probe (two-color fluorometric real-time PCR) or sequencing program (Star-Seq). After synthesis of IS, each IS was mixed in an ISM with a constant ratio, and it was used for co-amplification of an unknown number of NT, in a single PCR reaction, for direct quantification (two-color fluorometric real-time PCR) and library preparation for sequencing (Star-Seq).

2.3.1 Quality Control
2.3.1.1 Multiplex PCR with Quality Control

Multiplex PCR was first described in 1988 by Chamberlain, et al for screening of gene deletions [122]. It used for analysis of microsatellites [123] and SNPs [124]. Multiplex PCR consists of multiple primer sets within a single PCR for targeting multiple genes at once. Amplicon sizes were different to be visualized by gel electrophoresis. In the real-time PCR platform, the sequence specific probes can be labeled with different reporter dyes that allow the detection of products from several sequences [19]. Generally, a single test with multiplex PCR requires less time to perform PCR, less consumption of reagents and variations of loading than separate PCR. However, with competition of primers in one reaction, PCR efficiency of each gene could be different affecting quantification of transcript abundance. As we co-amplify ISM with unknown target NT in a single PCR, any source of variations, such as PCR efficiency, has the same effect on both IS and NT. Therefore, the quantification of unknown NT will not be influenced by source of variations controlled by ISM.

2.3.1.2 Multiple PCR Rounds with Quality Control

The quantification of very low copy number targets, for example, small biopsies such as fine needle aspiration (FNA), colonoscopies, are challenged because the Cq values of targets are close to the Cq of no template control. Because of the background variation of fluorescence at each PCR and the low signal to background, the obtained Cqs are not comparable among the different PCR [19]. Lau, et al, demonstrated the enhancement of the limit of detection with pre-amplification in real-time PCR to detect the coronavirus associated with severe acute respiratory syndrome (SARS-CoV) [125]. The TaqMan® PreAmp Master Mix kit is available commercially recommending 10 or
14 cycles of pre-amplification and then 1:5 or 1:20 dilution of pre-amplified product for the second amplification [126,127]. Crawford, et al, successfully demonstrated the two-step PCR with implementation of ISM for fresh FNA samples in the capillary electrophoresis platform [1]. Based on previous work [1], pre-amplification method (two-step PCR) was applied in the standardized two-color fluorometric real-time PCR for FFPE samples to increase signal from background and amount of cDNA reverse transcribed from the limited amount of RNA.

Star-Seq uses multiple steps of PCR for library preparation for tagging, barcoding, adapting with different sets of primers. Each PCR step can cause a bias, especially in dilution of PCR products and separate PCR runs for each target gene affecting the quantification of transcript abundance. However, the initial ratio between NT: IS is not changed through multiple PCRs enabling multiple PCR rounds and controlling each PCR.

2.3.2 Numerical Quantification

2.3.2.1 Important to Avoid Stochastic Sampling Variation

For the evaluation of the absolute copy number, the limiting dilution PCR can be used in combination with Poisson statistics. The principle of limiting dilution is based on the use of a qualitative all-or-none end point PCR [128]. At serial limiting dilution, where some end points are positive, and some are negative, the proportion of the presence or absence of targets can be observed by replicates of PCR. And the result can be compared with the expected proportion of the presence or absence by using Poisson statistics. As expected, the confidence interval of the estimated initial copy number of the target is larger for a low initial copy number [45,129,130]. At very low copy numbers, under 20 copies, the random variation due to stochastic sampling error (i.e., Poisson sampling error)
becomes significant demonstrated multiple replicates of PCR at serial dilutions of the material [42,129]. For example, Jiang et al observed significantly larger imprecision than expected under Poisson sampling errors in traditional RNA-sequencing method [131]. Numerical initial unknown copy numbers of samples could be quantified with co-amplification of the known number of ISM in a PCR reaction [132]. As the stochastic sampling error could not be controlled when low copies exist in the samples, each PCR step taking out the cDNA or PCR products from the previous step can cause stochastic sampling variation. However, with co-amplification of the absolute known number of ISM, we know whether stochastic error occurred, or just expression level is low.

Competitive PCR strategy can be applied to a variety of studies of gene expression requiring accurate measurement of mRNA species in low abundance or from small numbers of cells [67].

2.4 Contributions

2.4.1 Manuscript I

Manuscript I: is entitled, “A multiplex two-color real-time PCR method for quality-controlled molecular diagnostic testing of FFPE sample.” This manuscript has been accepted and published in PLOS One [133]. The aim of this study was to develop a quality controlled method for highly degraded samples, especially FFPE samples, on the real-time PCR platform. The implementation of development involved four steps. 1) A short PCR target size (60-80bp) was made for fragmented FFPE RNA. 2) Co-amplification of ISM with unknown samples allowed absolute quantification among genes, at least one reference gene and many target genes, without inter-well and/or inter-platform variation in PCR conditions. 3) ISM also enabled multiplex pre-amplification
PCR for lowly expressed degraded and limited amounts of RNA. Without ISM, two-step PCR can cause significant changes in transcription abundance measurement. 4) The novel development of ESM controlled for probe fluorescence intensity and collection of quantification cycles of the two probes, NT Cq and IS Cq. The successful demonstration was conducted by 20 surgically removed FFPE samples with known values of the lung cancer diagnostic test (LCDT) that comprises MYC, E2F1, CDKN1A and ACTB reference gene expression [2].

2.4.2 Manuscript II

Manuscript II: is entitled “Accurate detection and quantification of the fish viral hemorrhagic septicemia virus (VHSV) with a two-color fluorometric real-time PCR assay.” This manuscript has been accepted and published in PLOS One [134]. The aim of this study was to develop a quality controlled molecular diagnostic test using real time platform to detect and measure VHSV. StaRT-PCR capillary electrophoresis platform for VHSV detection and quantification was developed with collaboration of the Lake Erie Center in 2013 [135]. However, we wanted to develop a commonly available assay on a real-time PCR platform, for VHSV detection and quantification. Then new test demonstrated high signal-to-analyte response (slope = 1.00±0.02) and a linear dynamic range that spanned seven orders of magnitude (R² = 0.99), ranging from 6 to 6,000,000 molecules of VHSV quantification. This assay is rapid and inexpensive, and the test has significantly greater accuracy than other published RT-qPCR tests and traditional cell culture diagnostics. My contribution to the work included assay design, data analysis and troubleshooting [134].

2.4.3 Manuscript III
Manuscript III: is entitled “Targeted RNA-sequencing with competitive multiplex-PCR amplicon libraries.” This manuscript has been accepted and published in PLOS One [72]. The aim of this study was the development of targeted quantitative RNA-sequencing method that is reproducible and reduces the number of sequencing reads. We developed a competitive multiplex PCR-based amplicon sequencing library preparation method that a) targets only the sequences of interest and b) controls for inter-target variation in PCR amplification during library preparation by measuring each transcript NT relative to a known number of synthetic competitive template IS copies. My contribution to the work was conduction of a comparison of two platforms, two-color fluorometric real-time PCR and targeted RNA-sequencing. First of all, LCDT genes were pre-amplified with mixture of LCDT primers for three FFPE samples. Then, the pre-amplified product was divided between the two platforms and transcript abundance was measured using each platform. The results showed very good concordance (linearity) between platforms, even without EMS normalization. With ESM normalization, the quantified copies of each gene measured using each of the two platforms were within two-fold ranges (slope: 0.9454, R2:0.9747). We figured out that the variation of transcript abundance measurements between the two different platforms could be controlled by the use of ESM.

2.5 Future study

Through funding in part from RC2 CA148572 and HL108016, we have collected normal bronchial epithelial cell samples from over 500 subjects with lung cancer and/or chronic obstructive pulmonary disease (COPD) or demographically at risk for lung cancer or COPD. We will identify heritable susceptibility factors that contribute to lung cancer and
COPD risk. We will focus on discovery of cis-regulatory SNPs that affect inter-individual variation in the expression of genes with altered regulation in subjects with lung cancer or COPD. To do that, for the multiple putative risk genes, we will assess genotype at putative cis-regulatory SNPs in gDNA from over 500 subjects, and allele-specific expression and total expression in normal bronchial epithelial cell samples from matched over 500 subjects using targeted competitive multiplex NGS method.


110. (!!! INVALID CITATION !!!).


Chapter 3

A Multiplex Two-Color Real-Time PCR Method for Quality-Controlled Molecular Diagnostic Testing of FFPE Samples

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3.1 Abstract

Reverse transcription quantitative real-time PCR (RT-qPCR) tests support personalized cancer treatment through more clinically meaningful diagnosis. However, samples obtained through standard clinical pathology procedures are formalin-fixed, paraffin-embedded (FFPE) and yield small samples with low integrity RNA containing PCR interfering substances. RT-qPCR tests able to assess FFPE samples with quality control and inter-laboratory reproducibility are needed.

We developed an RT-qPCR method by which 1) each gene was measured relative to a known number of its respective competitive internal standard molecules to control for interfering substances, 2) two-color fluorometric hydrolysis probes enabled analysis on a real-time platform, 3) external standards controlled for variation in probe fluorescence intensity, and 4) pre-amplification maximized signal from FFPE RNA samples. Reagents were developed for four genes comprised by a previously reported lung cancer diagnostic test (LCDT) then subjected to analytical validation using synthetic native templates as test articles to assess linearity, signal-to-analyte response, lower detection threshold, imprecision and accuracy. Fitness of this method and these reagents for clinical testing was assessed in FFPE normal (N=10) and malignant (N=10) lung samples.

Reagents for each of four genes, MYC, E2F1, CDKN1A and ACTB comprised by the LCDT had acceptable linearity (R² > 0.99), signal-to-analyte response (slope 1.0 ± 0.05), lower detection threshold (< 10 molecules) and imprecision (CV < 20%). Poisson analysis confirmed accuracy of internal standard concentrations. Internal standards controlled for experimentally introduced interference, prevented false-negatives and
enabled pre-amplification to increase signal without altering measured values. In the fitness for purpose testing of this two-color fluorometric LCDT using surgical FFPE samples, the diagnostic accuracy was 93% which was similar to that previously reported for analysis of fresh samples.

This quality-controlled two-color fluorometric RT-qPCR approach will facilitate the development of reliable, robust RT-qPCR-based molecular diagnostic tests in FFPE clinical samples.
3.2 Introduction

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) tests that measure transcript abundance of selected genes in clinical specimens have been demonstrated to increase cancer diagnostic accuracy and enable “personalized medicine” through selection of the most effective treatment for each cancer [1-3]. However, a key challenge is that current clinical pathology sample collection and processing procedures focus on formalin fixation and paraffin embedding (FFPE) and fresh/fresh-frozen tissues rarely are available for molecular analysis. FFPE samples are difficult to work with because they yield RNA that 1) often contains PCR-interfering substances, and 2) is uniformly highly fragmented and often in low abundance [4-8]. Economic factors prevent changing this workflow to ensure collection of samples in a form more conducive to molecular genetic analysis, such as fresh frozen, therefore, there is a need to develop methods that are sufficiently robust to reliably conduct molecular genetic analysis in FFPE samples.

One way to address the challenge of interfering substances is to incorporate quality control in qPCR through measurement of each analyte relative to a known number of competitive template internal standard (IS) copies. This quality control method is recommended by regulatory agencies, including the EPA [9], ISO [10], and FDA [11], and is implemented in many FDA-approved single analyte RT-qPCR tests [12-14].

Multiple gene RT-qPCR tests present an even more complex quality control challenge that can be addressed by combining the IS for each of the genes into an internal standards mixture (ISM) and using an aliquot of this ISM in each experiment, as previously described [15-17]. Each target gene and loading control gene then is measured relative to a
known number of its respective competitive template IS molecules in each PCR reaction [18,19].

To address the challenge of analyzing clinical samples that yield a low amount of RNA, we previously described a competitive multiplex PCR method, in which all reference and target genes are first co-amplified with ISM in a first round of PCR, followed by amplification of individual gene in the second round [20]. This approach enables reliable measurement of many genes from the amount of RNA that, without pre-amplification, would be sufficient for measurement of only a single gene.

The primary goal of this study was to develop a robust RT-qPCR method for more reliable molecular diagnostic testing of FFPE samples including those stored in existing large archives. To meet this need, a method was designed with four elements: 1) Synthetic competitive IS formulated into an ISM to control for sub-optimal PCR, including interference with PCR caused by substances present in FFPE samples [21-23], sub-optimal quantity or quality of PCR reaction reagents, and inter-well and/or inter-platform variation in PCR conditions; 2) Fluorometric hydrolysis probe real-time PCR to enable quantification of short PCR amplicons (60-80 base pairs) that are optimal for reliable analysis of FFPE samples; 3) An external standards mixture (ESM) in each experiment to control for inter-lot and inter-experimental variation in probe fluorescence intensity; and 4) Reverse transcription with gene specific primers (GSP) followed by competitive multiplex pre-amplification to enable measurement of lowly expressed genes in pauci-cellular samples with degraded and limited amounts of RNA.

To validate this approach, we developed reagents for measurement of previously reported lung cancer diagnostic test (LCDT) comprising v-myec avian myelocytomatosis
viral oncogene homolog (*MYC*), E2F transcription factor 1 (*E2F1*), and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) genes measured relative to actin, beta (*ACTB*) [24]. These reagents were subjected to analytical validation using synthetic templates as test articles and fitness for the purpose of testing using surgical benign and malignant FFPE samples according to recommended practices [25].
3.3 Materials and Methods

3.3.1 Ethics Statement

Twenty archived surgical FFPE lung tissues that had been processed according to the standard University of Toledo Medical Center (UTMC) Department of Pathology practice between 2010 and 2012 were obtained for this study under UTMC IRB # 107790. According to the UTMC IRB # 107790 protocol, each FFPE sample was assigned a non-identifying number by the pathologist and transferred to the research laboratory. The link between the non-identifying number and identifying information was destroyed by the pathologist immediately following sample transfer. Accordingly, on March 27, 2013 the UTMC IRB #107790 protocol was approved by the Chair of the UTMC Biomedical Institutional Review Board as exempt research and the requirement to obtain a signed consent/authorization form for use and disclosure of protected health information was waived as this research was determined to be minimal risk.

3.3.2 FFPE Sample Preparation

Microtome sections (10 micrometre thickness) were obtained from each sample. Six strips per sample (1 strip = 4 sections) were obtained, and each strip was put in one 1.5 ml micro-centrifuge tube for RNA extraction. Therefore, 24 sections (240 micrometres) of each sample block were collected for RNA extraction.

3.3.3 RNA Extraction and Reverse Transcription

RNA was extracted from the surgical FFPE samples using the RNeasy® FFPE Kit (Qiagen, Valencia, CA). RNA was treated with DNase in the RNeasy® FFPE Kit RNA extraction protocol in order to minimize the effect of contaminating genomic DNA. RNA purity and integrity were assessed using absorbance at 260/280 nm ratios and RNA
integrity number (RIN) scores as detected on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The effect of different conditions on reverse transcription (RT) efficiency, including priming with random hexamer primers (RHP) or GSP and use of 1 or 5 µg of RNA in the 30µl RT reaction, was assessed with three FFPE samples. A previously described test using the External RNA Control Consortium (ERCC) standards was used to measure RT efficiency [26]. After completion of these studies, optimal RT conditions were selected consisting of a 30µl RT reaction with 1 µg of RNA, gene-specific RT primer (the PCR reverse primer) (3 µM), and SuperScript III First Strand Synthesis System (Life Technologies, Grand Island, NY).

3.3.4 Primer Design and Testing of Efficiency and Specificity

For each gene (MYC, E2F1, CDKN1A and ACTB) primers were designed to 1) amplify the shortest possible PCR product size (60-80 base pairs) and 2) span intron/exon splice junctions to minimize the effect of residual genomic DNA contamination (Table 1).

Each candidate primer pair was assessed for efficiency in a serially diluted mixture of H23 cell line cDNA and ISM using endpoint PCR. After 35 PCR cycles, products were electrophoretically separated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), the electropherogram was inspected for presence or absence of non-specific products, and appropriately sized peaks were quantified by densitometry.

3.3.5 Design of Probes and IS Templates

For each gene target, after native template (NT) primers with sufficient efficiency and specificity were identified, we developed real-time PCR assays using fluorometric hydrolysis probes [27]. First, a probe for the NT was designed followed by the design of an IS probe for the same DNA region but with 4-6 base pair alterations from the NT probe.
sequence. An IS template with corresponding alterations was synthesized as described in the synthesis and purification of standards section below. Use of multiple base changes in the IS probe ensured specificity of NT probe (FAM labeled) for the NT and IS probe (Quasar 670 labeled) for the IS. Probes with a fluorescent label at the 5’ end and a Black Hole Quencher at the 3’ end (BHQplus, Biosearch Technologies, Novato, CA) were designed using real-time design software from the Biosearch Technologies web site (Figure 1A, Table 1).

3.3.6 Synthesis and Purification of Standards

For each gene, we synthesized an NT (to be used in the ESM) and an IS via commercial vendor (Life Technologies, Grand Island, NY). The products of such syntheses are single-stranded and contain a significant fraction of incompletely synthesized (less than full-length) molecules. Thus, we PCR-amplified each synthesized NT or IS with GSP to generate completely synthesized, double-stranded nucleic acid templates. This was followed by electrophoretic separation of the PCR products on agarose gel, selection of the correct size band, and purification from agarose using QIAx II gel extraction kit (Qiagen, Valencia, CA) (Table 1).

3.3.7 Probe Specificity Test

Specificity of each probe was tested by including it in PCR assays containing the synthetic NT or IS serially diluted from $10^{-11} \text{ M}$ to $10^{-15} \text{ M}$. For each probe, at each NT or IS dilution, the signal (Cq value: quantification cycle) [28] observed with amplification in the presence of the non-homologous template was compared to Cq value observed with amplification in the presence of the homologous template. The non-specific binding rate was calculated using $2^{-\text{delta Cq}}$ (delta Cq = non-homologous template Cq – homologous template Cq) at
each dilution. If, at any concentration, the number of input non-homologous molecules detected by the probe was more than 10% of the number of homologous molecules detected, then the probe was re-designed.

3.3.8 Preparation of Internal Standards Mixture (ISM)

Known quantities of the IS for each gene were combined into an ISM. Use of the ISM rather than individual IS in each experiment minimized inter-experimental variation [16] as described in Table S1A in File Supporting information. Six different ISM were prepared (ISM A-F) containing different concentrations of target gene IS mixture (MYC, E2F1, CDKN1A) relative to the reference gene (ACTB) IS.

3.3.9 External Standards Mixture (ESM)

Known quantities of purified synthetic NT and IS for each gene were combined into an ESM. The ESM was used to control for inter-experimental variation resulting from 1) instability or intensity differences of one fluor relative to the other and 2) software selection of Cq. We prepared a stock ESM with $10^{-11}$ M NT/$10^{-11}$ M IS, and then diluted it to two working concentrations of $10^{-13}$ M NT/$10^{-13}$ M IS and $10^{-14}$ M NT/$10^{-14}$ M IS. Each of these two ESM concentrations was measured in each experiment and for each gene the average measured Cq difference [NT Cq - IS Cq] from the two ESM was used to correct the [NT Cq - IS Cq] value measured in each unknown sample (Table S1B in File Supporting information, Figure S1 in File Supporting information).

3.3.10 Pre-Amplification (1st round PCR)

The pre-amplification reaction for each sample was prepared in a 20 µl volume and included 1) 2 µl primer mixture (ACTB, MYC, E2F1, CDKN1A) with concentration of each primer at 800 nM (final concentration of each primer in PCR of 80 nM), 2) 1 µl cDNA
sample, 3) 1 µl ISM, and 4) 10 µl TaqMan Universal Master Mix II (No UNG: Uracil N-Glycosylase, Life Technologies, Grand Island, NY) with 6 µl RNase-free water. Probes were not used in the pre-amplification. Cycle parameters were 95 °C for 10 min then 18 cycles at 95 °C for 15 s and 60 °C for 1 min. The ABI 7500 Fast real-time PCR system was used with standard mode (software v2.0.6, Life Technologies).

3.3.11 Second-Round PCR

Pre-amplified PCR products were diluted 1000-fold with TE buffer. A 20 µl reaction was prepared for each gene with 1) 1 µl of diluted pre-amplified product, 2) 2 µl of a primer mixture containing each primer for each gene at 8 µM (final concentration of each primer, 800 nM), 3) 2 µl of 2 µM NT probe and 2 µl of 2 µM IS probe (final concentration of each probe, 200 nM), 4) 10 µl TaqMan Universal Master Mix II (No UNG) with 3 µl RNase-free water, and subjected to 40 cycles of PCR using the same cycle parameters as in the pre-amplification. Automatic threshold was used to determine Cq values (Figure 1B, Figure S1 in File Supporting information).

3.3.12 Calculation of Gene Expression

To quantify the copy number for each gene NT in a cDNA sample, 1) the \([\text{NT Cq - IS Cq}]_{\text{Sample}}\) for the unknown sample and the average \([\text{NT Cq - IS Cq}]_{\text{ESM}}\) of two concentrations of ESM \((\text{[NT Cq - IS Cq]}_{\text{ESM}})\) were calculated, 2) The corrected delta Cq was calculated as: \([\text{NT Cq - IS Cq}]_{\text{Sample}} - [\text{NT Cq - IS Cq}]_{\text{ESM}}\), 3) \(2^{(\text{corrected delta Cq})}\) was multiplied times the known number of input IS copies in the reaction to obtain the gene NT copy number, and 4) each target gene NT value was normalized to the \(ACTB\) loading control gene NT value, and presented as target gene NT molecules /10^6\(\text{ACTB}\) molecules (Table 2, Table S1B in File Supporting information).
3.3.13 Accuracy

The concentration of each stock (purified) IS was determined using densitometric quantification of the appropriately sized peak after electrophoretic separation on the Agilent Bioanalyzer 2100. Then the appropriate volume of each IS was combined to make an ISM. After preparing the ISM, limiting dilution PCR and Poisson analysis were used to determine the true concentration of each IS in the ISM. Specifically, the stock ISM solution was serially diluted to a concentration expected to contain 40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules of each IS in each microliter. The expected frequency of reactions with detectable PCR product at each dilution was tested with real time PCR using the pre-amplification method to increase the signal to background ratio (See the above section: Pre-amplification (1st round PCR) and second-round PCR. As an example, when 1 µl of the dilution expected to contain 0.7 molecules per microliter solution was included in the PCR, the expected frequency of positive reactions was 50.3% by Poisson analysis. The nine replicate samples of each dilution for each gene (ACTB, MYC, E2F1, CDKN1A) were measured. For each dilution the observed frequency of positive values (true concentration value) was plotted versus the frequency expected if the concentration determined by Agilent Bioanalyzer 2100 was correct [29].

3.3.14 Linearity

For each gene, the linearity of the assay was assessed through serial 10-fold dilution of the external standard stock solution (10^{-11}M NT/10^{-11}M IS to 10^{-17}M NT/10^{-17}M IS) or serial dilution keeping IS constant and diluting NT up to 1/80-fold relative to IS and vice versa. For each dilution series, correlation coefficient (r^2) and slope (linearity) were calculated.
3.3.15 Imprecision

For each gene, the imprecision was measured as the coefficient of variation (CV) of the copy number measured at each dilution used in the linearity test. The CV was calculated as the standard deviation divided by the mean derived from multiple replicate measurements (at least three). The average CV across all dilutions for each gene, and the average CV across all four genes were calculated.

3.3.16 Robustness and Interference Tests

The effect of intentionally perturbing PCR conditions was assessed. Conditions altered included PCR volume and concentration of primer, probe, or EDTA [30]. Samples used for this analysis were cDNA reverse transcribed from non-FFPE treated benign lung tissue RNA (Life Technologies, Grand Island, NY) or FFPE-processed, surgically-removed, malignant lung tissue sample 1 or 8 RNA (SM1, SM8).

3.3.16.1 EDTA Concentration Variation

The effect of variation in PCR EDTA concentration on MYC and ACTB measurement was assessed in triplicate 20 µl PCR assays containing non-FFPE, benign lung cDNA pre-amplified with ISM. EDTA concentrations tested were 0, 0.5, 1, 1.4, 1.8, 2.2, 2.6, 2.8, 3.0, 3.2, 3.4, 3.6 and 4.0 mM.

3.3.17 Effect of ESM on Quality-Control

The effect of variation in fluorescence intensity on reliability of measurement was tested by varying ratio of [labeled probe]/ [unlabeled probe] keeping [total probe] in the PCR constant at 200 nM. PCR assays were conducted for MYC and ACTB measurement in non-FFPE, benign lung cDNA pre-amplified with ISM. The IS labeled probe concentration in the PCR was kept constant while NT labeled probe was diluted with
unlabeled probe to 200, 150, 100, 80, 40, 20, 10, 5, 0 nM or vice versa. Unlabeled probe was obtained from Life Technologies, Grand Island, NY.

Inter-day experimental variation without or with ESM was tested in FFPE sample SM8 cDNA in seven PCR reactions on five different days. The automatically selected Cq values were used to measure $MYC/10^6 \ ACTB$.

3.3.18 Statistical Analysis

The transcript abundance value (target gene molecules/$10^6 \ ACTB$ molecules) for each LCDT gene was measured in triplicate and variation was measured as the CV. We used the Student’s t-test to determine a significant ($P < 0.05$) difference in mean LCDT value of the malignant group compared to the benign group. Levene’s test was used to assess the equality of variances in different samples for Student’s t-test using R program (v 2.15.2). The receiver operator characteristic (ROC) plot was generated by GraphPad Prism 6.
3.4 Results

3.4.1 Analytical Validation of Primers, Probes, and Internal Standard Mixtures

3.4.1.1 Primer Efficiency and Specificity

Primer efficiency was determined by PCR analysis of serially diluted IS. For each gene, at the dilution predicted to contain a single molecule of IS based on Agilent 2100 concentration analysis, the fraction of measured replicates that had detectable PCR product was consistent with the frequency predicted by Poisson analysis (see Accuracy section below). The Poisson analysis results support the conclusion that the IS concentration was accurate and that the primers had efficiency necessary to generate a detectable signal from a single molecule after 40 cycles [29].

3.4.1.2 Probe Specificity

For the first E2F1 assay design, the NT probe had >10% non-specific binding, so it was re-designed to increase the number of changes in IS compared to NT from four to six base pairs. After the re-design of the E2F1 IS and its respective probe, non-homologous (non-specific) binding was <1% for both NT and IS probes for all genes, more than meeting our threshold acceptance criteria.

3.4.1.3 Internal Standards Mixture Accuracy

After the IS were combined into the ISM, the ISM was serially diluted beyond the level expected to contain a single molecule in a PCR assay, and the IS for each of the four genes was PCR-amplified in the PCR assays containing each ISM concentration. The observed frequency of the positive result was highly correlated ($R^2 = 0.94$) with the expected positive frequency predicted by Poisson analysis (Figure 2, Figure S2 in File...
Supporting information), indicating that the intended concentration for each IS in the ISM was accurate.

3.4.2 Analytical Validation of the Competitive Multiplex Two-Color Real-Time Method

3.4.2.1 Linearity

The linearity of the two-color fluorometric assay was determined by analysis of serial dilutions of synthetic NT and IS for each gene. In a serial dilution of the stock ESM (a 1/1 mixture of NT/IS) over seven orders of magnitude (from $10^{-11} \text{ M}$ through $10^{-17} \text{ M}$), the correlation coefficient for the measurement of each gene relative to its respective IS was $> 0.99$, and the average slope for the signal-to-analyte response was $1.0 \pm 0.05$ (Figure 3A, B, Figure S3 in File Supporting information).

To more stringently assess linearity, the NT was serially diluted relative to a constant IS concentration of $10^{-12} \text{ M}$ and the IS was diluted relative to a constant NT concentration of $10^{-13} \text{ M}$. In the dilutions with NT/IS or IS/NT ratio of $\leq 10$, the average slope for the four genes ($ACTB$, $E2F1$, $MYC$, $CDKN1A$) was $1.0 \pm 0.10$ in each set of dilution series. At dilutions with NT/IS or IS/NT $\geq 10$, there was a slight deviation of the slope from 1.0 (Figure 3C-E, Figure S4 in File Supporting information, S5 in File Supporting information).

3.4.2.2 Imprecision

The imprecision for measurement of the LCDT genes was measured among both the ESM dilution samples and the NT/IS dilution samples.

At each serial 10-fold dilution of ESM ($10^{-11} \text{ M} \ NT/10^{-11} \text{ M} \ IS$ to $10^{-17} \text{ M} \ NT/10^{-17} \text{ M} \ IS$), the average coefficient of variation (CV) for measurement of each of the four genes
was < 10% for > 60 molecules input (10^{-11} \text{ M NT}/10^{-11} \text{ M IS} \text{ to } 10^{-16} \text{ M NT}/10^{-16} \text{ M IS}) and < 30% for > 6 molecules input (10^{-11} \text{ M NT}/10^{-11} \text{ M IS} \text{ to } 10^{-17} \text{ M NT}/10^{-17} \text{ M IS}) with little inter-gene variation (Table S2 in File Supporting information).

Among the NT/IS dilution samples, the average CV among the four LCDT genes was calculated for different ranges of dilution. For an NT dilution from 1/1 to 1/10-fold relative to a constant IS, the average CV among the four genes was 12%. At dilutions beyond 1/10, the CV increased, but from 1/1 to 1/80 NT dilution the average CV was only 20% (Table S3 in File Supporting information). Similar results were observed for an average CV for each of the four genes in the IS dilution relative to a constant NT.

3.4.2.3 Robustness and Interference Testing

Changing the volumes and/or the concentrations of primers or probes did not lead to significant differences in expression measurement of \textit{MYC} or \textit{ACTB} in FFPE SM1 cDNA with or without pre-amplification (Figure S6 in File Supporting information).

As EDTA concentration was increased, Cq value of each of the four analytes tested in non-FFPE, pre-amplified, benign lung cDNA (\textit{MYC} IS and NT, \textit{ACTB} IS and NT) increased, ultimately resulting in no signal (Figure 4). However, the \textit{MYC} NT and \textit{ACTB} NT values calculated relative to their respective IS were constant, and due to the loss of signal for IS at highest EDTA concentration, no false negative values were reported.

3.4.2.4 Use of ESM to Control for Variation in Fluorescent Labeling of Probe and Selection of Threshold

The specific activity of probe labeling with fluor (i.e., [labeled probe]/[total probe]) may vary between experiments due to freezing and thawing of probes or due to lot differences, the effect of variation in fluorescence specific activity on measurement of \textit{MYC}
in benign, non-FFPE lung cDNA was tested. As the labeled probe concentration decreased in the reaction, the Cq increased (Figure 5A, C). However, this potential source of analytical variation was controlled by correcting the measured lung sample [NT Cq - IS Cq] values relative to the ESM [NT Cq - IS Cq] values (Figure 5B, D). The ESM contained a known 1:1 concentration of each NT and IS that was constant among experiments, any variation in the observed ESM [NT Cq - IS Cq] relative to the expected value of 0 was attributable to variation in experimental conditions, including fluorescence intensity.

Another potential source of inter-experimental variation is inter-experimental variation in selection of Cq threshold. Even when the Auto Cq mode is used to select automatically the optimal Cq threshold, there was large inter-experimental variation in NT/IS Cq difference based on amplification plot and amount of cDNA loaded (Figure 5E). Thus, whether the threshold was selected through the automatic method or the manual method, there was day-to-day variation in the selected Cq threshold setting. However, because the inter-experimental variation in the Cq threshold had the same effect on sample Cq and ESM Cq, inter-experimental variation in sample Cq was controlled by ESM Cq as described in the previous paragraph. For example, MYC/10^6 ACTB was measured in FFPE sample SM8 cDNA in seven PCR replicates on five different days and the Cq threshold value automatically selected in each PCR was different (Figure 5E) resulting in high CV of 0.99. However, with the ESM correction, the CV of measured MYC/ 10^6 ACTB was reduced to 0.32 (Table 2).

3.4.3 Fitness for Purpose Testing in FFPE Samples
The histomorphologic diagnosis of benign or malignant FFPE samples used in this study is presented in Table S4 in File Supporting information. The RNA yield and purity are presented in Table S5 in File Supporting information.

3.4.3.1 Optimization of FFPE Reverse Transcription

Efficiency of RT with GSP or RHP was assessed in three (two malignant and one benign) surgical FFPE samples (SM1, SM2, SB1). The average yield of cDNA from 1 µg RNA was more than 50-fold higher with GSP. Based on this, analysis of FFPE samples was conducted with GSP in RT. The RT yield was increased another 4.6-fold by increasing RNA in RT to 5 µg.

3.4.3.2 Effect of Pre-amplification

Results for analysis of LCDT genes in sample SM1 with or without pre-amplification were compared to quantify the increase in signal relative to background resulting from pre-amplification and to confirm that it did not significantly alter the result. Importantly, for each gene the signal was increased (Cq decreased) with pre-amplification. Specifically, Cq value decreased for ACTB, MYC, E2F1, and CDKN1A by 9, 10, 9 and 10, respectively, following pre-amplification and 1000-fold dilution of the pre-amplification product prior to second round amplification. Yet, because each target was measured relative to a known number of its respective IS molecules, the value measured with the pre-amplification method was not significantly different from that measured with the no pre-amplification (Figure S6 in File Supporting information).

3.4.3.3 Analysis of MYC, E2F1, CDKN1A and ACTB in FFPE Samples

The comparison of the LCDT index in benign and malignant surgical FFPE samples is presented in Figure 6A, and the ROC curve analysis is presented in Figure 6B.
Based on the linearity and imprecision results, for analysis of clinical samples, we chose to restrict the conditions for calculation of results to 1/10 to 10/1-fold difference between the NT and the IS.

The LCDT optimal cut-off value had 90% specificity and 90% sensitivity to classify samples as cancer or non-cancer, similar to previous reports with non-FFPE fine needle aspirate (FNA) samples [31,32]. The ROC area under the curve was 0.93 with a 95% confidence interval of 0.82 to 1.04 and the $P$-value of Student’s t-test for stratification of malignant from non-malignant was 0.0061. The average CV among surgical FFPE samples for measurement of $MYC$, $E2F1$, and $CDKN1A$ relative to $ACTB$ was 0.27, 0.41, and 0.26, respectively (Table 3). These data confirm fitness for purpose of this optimized LCDT test in FFPE samples.
3.5 Discussion

Here we report the analytical validation and fitness for purpose testing of an RT-qPCR method suitable for reliable analysis of FFPE samples. Key features of this optimized method are highlighted here.

3.5.1 Internal Standards Provide Quality Control

The endogenous amount (NT) of each of multiple genes was measured relative to a known number of respective IS molecules. Each IS amplified with the same efficiency as the NT, and this controlled for inter-sample variation in PCR interfering substances and inter-experimental variation in PCR reagent quality or quantity or thermal cycling conditions as previously described [30,33,34]. Key to the elimination of inter-experimental variation when measuring multiple genes was use of the same ISM comprising a known concentration of IS for each of the genes to be measured [16]. The ISM was both stable and simple and inexpensive to prepare.

3.5.2 Two-Color Fluorometric Real-Time PCR

For reliable analysis of FFPE samples, it is important to use primers that yield short PCR products (e.g. 60-80 base pairs). Such products are readily quantified using real-time PCR. Competitive PCR analysis involves simultaneous quantification of each target gene NT and its respective IS. To do this by real-time PCR requires inclusion of two different sequence-specific probes in the same PCR reaction, each with a different fluor [12,13,35]. Calculating each NT analyte relative to its respective IS requires an additional measurement, and, in some studies, this may be associated with a tendency to increased imprecision [36]. However, as previously reported, the imprecision observed in this study was less than 10% except for the measurement of very low copy numbers (<60 copies), at
which point imprecision is determined largely by the natural law governing stochastic sampling variation rather than method-specific characteristics [16].

3.5.3 ESM Controlled for Inter-Run Variation in Probe Fluorescence

Multiple different factors may cause inter-experimental variation in fluor signal detection including variation in fluor concentration (Figure 5A-D), variation in cycle threshold setting (Figure 5E), and as yet unknown sources. The use of the ESM significantly reduced these sources of inter-experimental variation (Table 2). In addition to use in multiple analyte assays, such as the one presented here, this approach is applicable to single analyte two-color fluorometric assays [12,13] and may demonstrate similar utility if so employed.

3.5.4 Multiplex Pre-amplification was Enabled by Use of Internal Standards

Using IS in conjunction with multiplex pre-amplification enabled reliable analysis of even lowly expressed genes in very small amounts of cDNA. Specifically, it was possible to determine the starting number of NT molecules, even after two rounds of PCR, by measuring the NT signal relative to the IS signal (Figure S6 in File Supporting information). This is because a known number of its respective IS molecules was included in the pre-amplification reaction for each gene, and because the NT and IS amplified with the same efficiency [20].

Competitive multiplex pre-amplification improved measurement of FFPE samples in the following ways. First, cDNA consumption was reduced. In the multiplex pre-amplification, reduction in cDNA consumption depends on the number of targets and reference genes. Thus, in this study involving only three target genes and a single reference gene, cDNA consumption was reduced four-fold. We have previously conducted
competitive multiplex pre-amplification with 96 genes, and this enabled a marked reduction in cDNA required for measurement of each gene [20,37].

Second, pre-amplification markedly increased signal above the background signal typically observed in the no template control at 35 Cq. Specifically, with one round of PCR (no pre-amplification) the Cq for each NT and IS ranged from 20-35. In contrast, using pre-amplified and 1000-fold diluted samples, the Cq for each NT and IS after a second round of PCR ranged from 11-26. The amount of dilution of first round amplification product can be reduced if necessary to ensure sufficient signal in the second round for very low input of sample cDNA. Further, for analysis of FNA FFPE samples with very low input cDNA, the higher signal following pre-amplification is associated with better precision than no pre-amplification (data not shown).

3.5.5 Gene Specific Reverse Transcription.

We recently reported that use of gene specific priming in RT increases yield of cDNA by 10-100 fold compared to oligo dT or random hexamer priming when applied to RNA from human peripheral blood leukocytes [26]. Because FFPE treatment typically reduces yield of cDNA from RNA by 100-fold, we evaluated the utility of gene specific priming relative to random hexamer priming to increase signal. The more than 50-fold increase in cDNA yield with gene specific priming RT compared to random hexamer priming RT observed in this study is consistent with our results from the prior study with leukocytes.

3.5.6 Fitness of Method for FFPE Sample Analysis

Fitness of this two color fluorometric method for analysis of FFPE samples was evaluated by measuring a previously described test for lung cancer diagnosis for non-FFPE
FNA samples [31,32] in a small number of surgical FFPE benign and malignant lung samples. The results support the utility of this optimized method for analysis of FFPE samples. Specifically, imprecision was acceptable, and the optimal cut-off for the LCDT had similar accuracy in separating benign from malignant compared to what was reported previously for fresh FNA samples [31,32]. These results support the conclusion that the method presented here is suitable for use in a planned clinical validation trial in which the LCDT will be evaluated for utility to augment cytomorphology in analysis of FNA cell block FFPE samples.

3.5.7 Summary

Successful analytical validation described here of the quality-controlled two-color fluorometric real-time PCR method for analysis of the LCDT in FFPE samples supports use of this approach in development and implementation of promising RT-qPCR based diagnostic tests that require analysis of RNA extracted from FFPE samples.
3.6 Table and Figure Legends

Table 3.1 Sequences of primers, probes, and standard templates for two-color fluorometric real-time measurement.

<table>
<thead>
<tr>
<th>Primer/Probe/Template</th>
<th>NT: native template</th>
<th>IS: internal standard</th>
<th>BHQ: black hole quencher</th>
<th>Bold: modified nucleotides</th>
<th>Underline: probe binding site</th>
</tr>
</thead>
</table>

Table 3.2 Effect of external standards mixture (ESM) on quality control. Inter-day experimental variation without and with ESM.

Note: SM8: surgically removed, formalin-fixed, paraffin-embedded (FFPE) sample

8. ISM D (-12/-14) contains $ACTB$ IS $10^{-12}$M /target gene IS $10^{-14}$M that corresponds to $ACTB$ IS 600000 /target gene IS 6000 molecules. ISM E (-12/-15) contains $ACTB$ IS 600000 /target gene IS 600 molecules. Cq: quantification cycle. ΔCq: quantification cycle difference of NT and IS: NT Cq - IS Cq. CV: coefficient of variation. NT: native template. IS: internal standard. ISM: internal standards mixture.

Figure 3.1 Schematic illustration of the probe design (A) and pre-amplification PCR (B).

Native template (NT) binding hydrolysis probes were labeled with FAM. Internal standard (IS) binding hydrolysis probes were labeled with Quasar 670. (A) For each gene, NT and IS had the same primer binding sites but there was a 4-6 bp difference in probe binding sites. (B) Varying concentrations of internal standards mixture (ISM) relative to cDNA were used to ensure that NT: IS ratio was $>$1:10 and $<$10:1.
Figure 3.2  Observed compared to expected positive PCR with limiting dilution.

Frequency of observed relative to expected positive PCR signal was measured. Poisson analysis was used to calculate expected positive frequency. Results from the average of nine replicates at each of 10 internal standard mixture dilution points (40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules/µl) averaged across the four genes (ACTB, MYC, E2F1, CDKN1A) were compiled and plotted. Each gene plot is presented in Figure S2 in File Supporting information.

Figure 3.3  Observed compared to expected E2F1 NT molecule values measured by two-color fluorometric assay in dilution series samples.

Linearity graphs (A, C, E) and amplification plots of E2F1 (B, D, F). (A, B) Serial dilution of external standards mixture (ESM, 1/1 mixture of NT/IS) from $10^{-11}M$ through $10^{-17}M$ (triplicate measurements, with error bars). (C, D) NT dilution relative to constant IS from 1/1 NT/IS ($10^{-12}M$) down to 1/80 (NT/IS) (triplicate measurements with error bars). (E, F) IS dilution relative to constant NT from 1/1 NT/IS ($10^{-13}M$) down to 1/80-fold (one replicate). NT: native template. IS: internal standard.

Figure 3.4  Internal standards control for PCR inhibition by EDTA. MYC and ACTB were measured in the presence of varying EDTA concentration.

(A) Quantification cycle (Cq) values of MYC IS, MYC NT, ACTB IS, ACTB NT. (B) Molecules of each gene and normalized value of MYC/ $10^6$ACTB molecules (triplicate measurements) analyzed in benign, non-FFPE lung cDNA reverse transcribed with gene
specific primers. NT: native template. IS: internal standard. FFPE: formalin-fixed, paraffin-embedded. The asterisk (*) indicates that Cq values were undetermined by software.

**Figure 3.5** External standards mixture controls for inter-experimental variation in fluor signal or quantification cycle (Cq) selection.

(A-D) Effect of diluting labeled probe with unlabeled probe on measurement of MYC in benign, non-FFPE lung cDNA reverse transcribed with gene specific primers (triplicate measurements, with error bars). (A, B) NT labeled probe diluted with NT unlabeled probe. (C, D) IS labeled probe diluted with IS unlabeled probe. (E) Effect of inter-day variation in threshold selection on measurement of MYC and ACTB in surgically removed, FFPE sample 8 (SM8). NT: native template. IS: internal standard. FFPE: formalin-fixed, paraffin-embedded. The asterisk (*) indicates that Cq values were undetermined by software.

**Figure 3.6** Validation of two-color fluorometric assay in 20 surgically removed, FFPE lung samples.

(A) lung cancer diagnostic test (LCDT) index values by diagnostic class. (B) receiver operator characteristic curve (ROC) of LCDT index.
### 3.7 Tables and Figures

**Table 3.1**

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<td>Reverse</td>
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**Probe sequences**

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<td>BHQ plus-2</td>
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**NT and IS sequences**

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Table 3.2

Without ESM correction

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<th>ACTB</th>
<th>MYC</th>
<th>Molecules/Assay</th>
<th>MYC/10⁶ ACTB</th>
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<td>IS Cq</td>
<td>Raw ΔCq</td>
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With ESM correction

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<th>ACTB</th>
<th>MYC</th>
<th>Molecules/Assay</th>
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<td>IS Cq</td>
<td>Raw ΔCq</td>
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Figure 3.1

A  
Example: ACTB (60bp)

![Diagram showing primer and probe binding sites](image1)

B

1st round PCR
- Sample cDNA
- 6x10^6 molec.IS (Gene A, B, C, D, n,..)
- Primer mix (Gene A, B, C, D, n,..)
- No probes
- 18 cycles

![Diagram showing PCR process](image2)

2nd round PCR
- Diluted 1st round product
- Primers for one gene
- Probes (NT and IS) for one gene
- 40 cycles

![Diagram showing 2nd round PCR process](image3)
Figure 3.2

Frequency of Positive PCR

\[ y = 0.920x + 0.02 \]
\[ R^2 = 0.942 \]
Figure 3.3

A. NT/IS (1/1) Serial Dilution

\[ y = 1.040x - 0.183 \]
\[ R^2 = 0.998 \]

B. Cycle

C. NT Dilution (1/80-fold)

\[ y = 1.083x - 0.414 \]
\[ R^2 = 0.997 \]

D. Cycle

E. IS Dilution (1/80-fold)

\[ y = 0.972x + 0.128 \]
\[ R^2 = 0.983 \]

F. Cycle
Figure 3.4
Figure 3.5

A. Cq Variation by NT Probe Con.

B. Measured MYC Molecules

C. Cq Variation by IS Probe Con.

D. Measured MYC Molecules

E. Inter-experimental variation in Cq threshold

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<tr>
<th>Day</th>
<th>ACTB-NT</th>
<th>ACTB-IS</th>
<th>MYC-NT</th>
<th>MYC-IS</th>
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</tr>
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<td>0.03</td>
<td>0.31</td>
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<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
<td>0.04</td>
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</table>
Figure 3.6
3.8 Supplemental Table and Figure Legends

Table S3.1 (A) Composition of Internal Standards Mixture (ISM) A-F. (B) Steps to calculate $MYC/10^6\ ACTB$ value for surgically removed, malignant sample 1 (SM1).

To quantify the copy number for each target gene native template (NT) in a cDNA sample, 1) the $\Delta Cq$: $[NT\ Cq - IS\ Cq]_{Sample}$ for unknown sample and the average of two concentrations of ESM $\Delta Cq$: $[NT\ Cq - IS\ Cq]_{ESM}$ were calculated, 2) The corrected delta Cq was calculated as: $[NT\ Cq - IS\ Cq]_{Sample} - [NT\ Cq - IS\ Cq]_{ESM}$, 3) $2^{(-\text{corrected}\ delta\ Cq)}$ was multiplied times the known number of input IS copies in the reaction to obtain the gene NT copy number, and 4) each target gene NT value was normalized to the $ACTB$ loading control gene NT value, and presented as target gene NT molecules $/10^6\ ACTB$ molecules.

ISM D (-13/-15) contains $ACTB$ IS $10^{13}M$/each target gene IS $10^{15}M$ that corresponds to $ACTB$ IS 60000 /each target gene IS 600 molecules.

Table S3.2 Effect of external standards mixture (ESM) dilution on precision in measurement of each lung cancer diagnostic test (LCDT) genes by two-color fluorometric real-time assay.

A serially diluted 1:1 ratio of native template (NT): internal standard (IS) from $10^{-11}M$ through $10^{-17}M$ was analyzed in triplicate.

Table S3.3 Effect of native template (NT) dilution relative to internal standard (IS) on precision in measurement of the lung cancer diagnostic test (LCDT) genes by two-color fluorometric real-time assay.
For each gene serial dilution of synthetic NT up to 1/80-fold relative to constant IS was measured at each dilution. At each NT dilution points the compiled data across the four genes (*ACTB, MYC, E2F1, CDKN1A*) in triplicate measurements were presented.

**Table S3.4** Histomorphological diagnosis of surgically removed, formalin-fixed, paraffin-embedded samples.

**Table S3.5** Total RNA sample quantity and purity assessment (A) (n=20).

**Figure S3.1** Schematic plot of experiment set up for 96 well plate.

After dilution of pre-amplified PCR product containing cDNA and internal standards mixture (ISM), an aliquot of each diluted products was distributed into individual wells for 2\textsuperscript{nd} round amplification for each individual gene native template (NT) and respective internal standard (IS) using gene-specific primers and probes. ISM C(-13/-15) was presented in the figure as an example, containing *ACTB* IS $10^{-13}$M / each target gene IS $10^{-15}$M corresponding to *ACTB* IS 60000 molecules/ each target gene IS 600 molecules. The PCR amplification plots for *ACTB* from the two external standard mixtures (ESM), NT and IS each at $10^{-13}$M or NT and IS each at $10^{-14}$M, are presented in one plot in the middle. Green is NT and red is IS in the plot. SM: surgically removed, malignant sample. NTC: no template control.

**Figure S3.2** Observed compared to expected positive PCR with limiting dilution PCR for each gene.
Pre-amplification method was used for testing 9 replicates. Each of 10 dilution points of internal standards mixture (ISM) (40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules) was multiplex pre-amplified nine times then observed PCR positivity for each gene at 2\textsuperscript{nd} amplification.

**Figure S3.3** Observed compared to expected native template (NT) molecule values measured by two-color fluorometric assay in external standards mixture (ESM) dilution series samples.

ESM (1/1 mixture of NT and internal standard (IS)) was serial 10-fold diluted from NT $10^{-11}\text{M}/\text{IS }10^{-11}\text{M}$ to NT $10^{-17}\text{M}/\text{IS }10^{-17}\text{M}$ and each dilution sample analyzed in triplicate.

**Figure S3.4** Observed compared to expected native template (NT) molecule values measured by two-color fluorometric assay in serially diluted synthetic NT relative to constant synthetic internal standard (IS) dilution series samples.

*ACTB, MYC, E2F1, or CDKN1A* synthetic NT was serially diluted relative to constant synthetic IS, starting with 1/1 NT/IS mixture at $10^{-12}\text{M}$. A, C, E, G: Linearity from 1/1 to 1/10-fold NT dilution. B, D, F, H: Linearity from 1/1 to 1/80-fold NT dilution. We assessed 1/1, 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, 1/9, 1/10, 1/12, 1/14, 1/16, 1/18, 1/20, 1/24, 1/28, 1/32, 1/36, 1/40, 1/48, 1/56, 1/64, 1/72, 1/80-fold dilutions of NT relative to constant IS. Data were analyzed with triplicate measurement.
Figure S3.5 Observed compared to expected native template (NT) molecule values measured by two-color fluorometric assay in serially diluted synthetic internal standard (IS) relative to constant synthetic NT dilution series samples.  

*ACTB, MYC, E2F1, or CDKN1A* synthetic IS was serially diluted relative to constant synthetic NT starting with 1/1 of NT/IS mixture at \(10^{-13}\) M. A, C, E, G: Linearity from 1/1 to 1/10-fold IS dilution. B, D, F, H: Linearity from 1/1 to 1/80-fold IS dilution. We assessed 1/1, 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, 1/9, 1/10, 1/12, 1/14, 1/16, 1/18, 1/20, 1/24, 1/28, 1/32, 1/36, 1/40, 1/48, 1/56, 1/64, 1/72, 1/80-fold dilution of IS relative to constant NT (one replicate measurement). Auto Cq could not be generated for the 1/56, 1/64, 1/72, 1/80-fold IS dilutions of *ACTB*.

Figure S3.6 Effect of PCR reaction conditions on lung cancer diagnostic test (LCDT) gene expression values measured in cDNA with or without pre-amplification. 

A: pre-amplification. B: no pre-amplification. Surgically removed, formalin-fixed, paraffin-embedded, malignant sample 1(SM1) reverse transcribed with gene specific primers was used. The reference optimal PCR condition was 20 µl reaction volume (2V), 800 nM of primers (1Pm) and 200 nM of probes (1Pb). To test robustness, we reduced volume by half, and/or doubled primer or probe concentration in each of the two conditions (pre-amp or no pre-amp).
3.9 Supplemental Tables and Figures

Table S3.1

(A) ISM composition

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<th>ISM (M)</th>
<th>ACTB IS mol./µl</th>
<th>Target Gene IS mol./µl</th>
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<td>6000000</td>
</tr>
<tr>
<td>B(-12/-12)</td>
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<td>600000</td>
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<tr>
<td>C(-12/-13)</td>
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<tr>
<td>F(-12/-16)</td>
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<td>60</td>
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(B) Example of MYC/10^6 ACTB calculation in Sample SM1

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<th>IS Cq</th>
<th>ΔCq</th>
<th>Ave. of ESM ΔCq</th>
<th>Corrected ΔCq</th>
<th>2^(-corrected ΔCq)</th>
<th>IS mol.</th>
<th>Target mol.</th>
<th>MYC/10^6 ACTB</th>
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</table>
ISM: internal standards mixture. ESM: external standards mixture. NT Cq: native template quantification cycle. IS Cq: internal standard quantification cycle. ΔCq: quantification cycle difference of NT and IS, NT Cq - IS Cq. mol.: molecules. SM1: surgically removed, formalin-fixed, paraffin-embedded sample 1.
Table S3.2

(A) **ACTB**

<table>
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<tr>
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Average of CV from $10^{11}$M to $10^{16}$M 0.11
Average of CV from $10^{11}$M to $10^{17}$M 0.25

(B) **MYC**

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(C) **E2F1**

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(D) **CDKN1A**

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Table S3.3

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NT: native template. SD: standard deviation. CV: coefficient of variation.
Table S3.4

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<td>SB6 UIP</td>
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### Table S3.5

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SB: surgically removed, benign sample. SM: surgically removed, malignant sample.
Figure S3.1

Experiment Setup in 96 Well Plate

- **1st round PCR**
  - 20 Samples: Each sample cDNA was mixed with aliquot of ISM and pre-amplified.

  - **Sample 1**
    - Mixed internal standards
    - 60,000 molecules ACTB IS
    - 600 molecules MYC IS
    - 600 molecules E2F1 IS
    - 600 molecules CDKN1A IS
    - Primer mix
    - No probes
    - Universal Master Mix

- **2nd round PCR**
  - Measure each gene relative to known number of IS molecules within ISM
  - Measure four genes/Sample
  - Measure each gene in ESM at $10^{-13}$ M and $10^{-14}$ M
  - No template control (control for false positives)

  - **Sample 1**
    - **Primer**
      - ACTB
      - MYC
      - E2F1
      - CDKN1A
    - **Probe**
      - ACTB-FAM
      - ACTB-Quasar
      - MYC-FAM
      - MYC-Quasar
      - E2F1-FAM
      - E2F1-Quasar
      - CDKN1A-FAM
      - CDKN1A-Quasar
    - FAM probe for native template
    - Quasar 870 probe for IS
    - Universal Master Mix

- **Two External Standards Plot**
  - 10-13M, 10-14M
  - e.g.) ACTB
Figure S3.2

A. ACTB

\[ y = 0.845x + 0.136 \]

\[ R^2 = 0.805 \]

B. MYC

\[ y = 0.803x - 0.008 \]

\[ R^2 = 0.873 \]

C. E2F1

\[ y = 0.988x + 0.087 \]

\[ R^2 = 0.831 \]

D. CDKN1A

\[ y = 1.044x - 0.015 \]

\[ R^2 = 0.862 \]
Figure S3.3

A. ACTB

\[ y = 1.010x - 0.028 \]
\[ R^2 = 0.999 \]

B. MYC

\[ y = 1.049x - 0.225 \]
\[ R^2 = 0.999 \]

C. E2F1

\[ y = 1.040x - 0.183 \]
\[ R^2 = 0.998 \]

D. CDKN1A

\[ y = 1.028x - 0.140 \]
\[ R^2 = 0.998 \]
Figure S3.4
Figure S3.5

A. ACTB (1/10-fold dilution)
   \[ y = 1.031x - 0.156 \]
   \[ R^2 = 0.992 \]

B. ACTB (1/48-fold dilution)
   \[ y = 1.136x - 0.588 \]
   \[ R^2 = 0.986 \]

C. MYC (1/10-fold dilution)
   \[ y = 1.037x - 0.199 \]
   \[ R^2 = 0.993 \]

D. MYC (1/80-fold dilution)
   \[ y = 1.037x - 0.189 \]
   \[ R^2 = 0.995 \]

E. E2F1 (1/10-fold dilution)
   \[ y = 0.922x + 0.324 \]
   \[ R^2 = 0.987 \]

F. E2F1 (1/80-fold dilution)
   \[ y = 0.972x + 0.126 \]
   \[ R^2 = 0.983 \]

G. CDKN1A (1/10-fold dilution)
   \[ y = 1.080x - 0.386 \]
   \[ R^2 = 0.993 \]

H. CDKN1A (1/80-fold dilution)
   \[ y = 1.227x - 0.984 \]
   \[ R^2 = 0.988 \]
Figure S3.6

A  Pre-amplification

B  No Pre-amplification
3.10 References


Chapter 4

Accurate Detection and Quantification of the Fish Viral Hemorrhagic Septicemia virus (VHSv) with a Two-Color Fluorometric Real-Time PCR Assay

Lindsey R. Pierce\textsuperscript{1,a}, James C. Willey\textsuperscript{2,a}, Vrushalee V. Palsule\textsuperscript{1}, Jiyoun Yeo\textsuperscript{2}, Brian S. Shepherd\textsuperscript{3}, Erin L. Crawford\textsuperscript{2}, and Carol A. Stepien\textsuperscript{1,*}

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\textsuperscript{a} = shared first author

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4.1 Abstract

Viral Hemorrhagic Septicemia virus (VHSv) is one of the world's most serious fish pathogens, infecting > 80 marine, freshwater, and estuarine fish species from Eurasia and North America. A novel and especially virulent strain – IVb – appeared in the Great Lakes in 2003, has killed many game fish species in a series of outbreaks in subsequent years, and shut down interstate transport of baitfish. Cell culture is the diagnostic method approved by the USDA-APHIS, which takes a month or longer, lacks sensitivity, and does not quantify the amount of virus. We thus present a novel, easy, rapid, and highly sensitive reverse transcription real-time quantitative PCR (qRT-PCR) assay that incorporates synthetic competitive template internal standards for quality control to circumvent false negative results. Results demonstrate high signal-to-analyte response (slope = 1.00 ± 0.02) and a linear dynamic range that spans seven orders of magnitude ($R^2 = 0.99$), ranging from 6 to 6,000,000 molecules. Infected fishes are found to harbor levels of virus that range to 1,200,000 VHSv molecules/$10^6$ $actb1$ molecules with 1,000 being a rough cut-off for clinical signs of disease. This new assay is rapid, inexpensive, and has significantly greater accuracy than other published real-time PCR tests and traditional cell culture diagnostics.
4.2 Introduction

Molecular diagnostic tools have facilitated the early detection, prevention, and spread of many important pathogens [1], led by the speed, sensitivity, and accuracy of Polymerase Chain Reaction (PCR)-based assays [2]. Their ability to diagnose targeted genetic sequences and quantify levels of infectious agents with hybridization probes has advanced screening technology for multiple human diseases, including influenza, hepatitis, and HIV [3,4]. Use of these approaches to elucidate and characterize plant and animal pathogens likewise is growing at a rapid pace [5,6].

Viral Hemorrhagic Septicemia virus (VHSv) causes one of the world's most serious finfish diseases, infecting > 80 species across the Northern Hemisphere [7], yet there remains a need for a fast, sensitive, accurate, and inexpensive diagnostic test. VHSv is a negative-sense, single stranded RNA Novirhabdovirus of ~ 12,000 nucleotides, with six open reading frames of 3'N-P-M-G-Nv-L'5 [8]. Infected fishes often swim erratically and have bulging eyes, distended abdomens, and extensive external/internal hemorrhaging [9]. The virus can survive for up to 13 days in the water [10], and can be spread via ballast water, boating, equipment, and aquatic animals (e.g. birds, turtles, leeches, and amphipod crustaceans) [11–14]. It is transmitted most readily during the spring spawning season through fish waste, reproductive fluids, and skin secretions [11].

VHSv first was described in the late 1930s as “Nierenschwellung” in aquacultured rainbow trout (Oncorhynchus mykiss) in Europe [15]. It now occurs across the Northern Hemisphere as four genetically and geographically distinct strains (I-IV) and substrains, whose evolutionary and biogeographic patterns recently were analyzed by Pierce and Stepien [16]. Strains I–III primarily occur in Europe, where they infect a wide variety of...
marine, estuarine, and freshwater fishes. Strain IV (now classified as IVa; [17]) first was discovered in 1988 from North American Pacific coastal fishes, including salmonids [18, 19], and now also occurs in Japan [20]. In 2000, another IV substrain (now designated as IVc per [16]) was discovered off the coast of New Brunswick, Canada, infecting the estuarine mummichog (*Fundulus heteroclitus*) and three-spined stickleback (*Gasterosteus aculeatus*) [21]. In 2003, the novel and especially virulent substrain, IVb, was described from a moribund muskellunge (*Esox masquinongy*) in Lake St. Clair of the freshwater Laurentian Great Lakes system [17]. Substrain IVb then spread throughout all five of the Great Lakes, infecting > 30 species, including many commercially and ecologically important fishes, such as muskellunge, drum (*Aplodinotus grunniens*), walleye (*Sander vitreus*), yellow perch (*Perca flavescens*), and the round goby (*Neogobius melanostomus*). Substrain IVb now contains at least 16 glycoprotein (*G*)-gene sequence variants [22], whose rapid spread and diversification in a quasispecies mode suggest that this strain mutates rapidly and may be highly adaptable (see [16]).

To avoid outbreaks of the virus, the Aquatic Invasive Species Action Plan [23] requires that aquaculture and baitfish vendors from U.S. states (Illinois, Indiana, Michigan, Minnesota, New York, Ohio, Pennsylvania, and Wisconsin) and Canadian provinces (Ontario and Quebec) have their fish products certified as VHSv-free prior to interstate or international transport. Cell culture is the VHSv diagnostic that is approved by the World Organization for Animal Health [24], along with the Fish Health Section of the U.S. Fish and Wildlife Service and the American Fisheries Society [25]. The cell culture process takes a month or longer for cell growth, cell confluency, viral growth, and confirmation PCR. It moreover lacks the sensitivity to detect low viral concentrations in
carrier fish, and results in false negative levels reported as 43–95% [26–28].

Real-time quantitative reverse transcription (qRT)-PCR assays for detecting VHSV [26–34] likewise have substantially high false negative rates that ranged from 15–92% [26–28]. The high false negative rates in those assays may have resulted from unknown and/or unmonitored effects from interfering substances in the PCR or reverse transcription reactions (rxn), which prevented detection of the target gene [35].

To avoid those issues, the present research describes and evaluates a new, accurate, fast, and highly reliable assay that diagnoses and quantifies VHSV. This assay uses Standardized Reverse-Transcriptase Polymerase Chain Reaction, i.e. StaRT-PCR, which is a form of competitive template RT-PCR that allows rapid, reproducible, standardized, and quantitative measurement of data for many genes simultaneously [36]. StaRT-PCR uniquely incorporates internal standards (IS) in the rxn mixture to improve accuracy and circumvent false negative results. Our new assay is based on real-time PCR equipment that is readily available in most diagnostic laboratories, markedly improving on a previous version of our VHSV test [37], which also used StaRT-PCR, but relied on less common Agilent capillary electrophoresis equipment. Results from both assays are evaluated by us here to determine the presence or absence of VHSV and measure concentration of the virus from fish samples in targeted field and laboratory studies. We assay the VHSV nucleoprotein (N)-gene and the fish reference beta-actin 1 (actb1) gene, assessing amplification relative to known numbers of their respective competitive IS molecules. Our new approach uses sequence specific fluor-labeled hydrolysis probes that can be used on a variety of real-time PCR thermal cyclers, for which positive VHSV results are visible as two colors on the real-time PCR plot (see Figure 1a; green = native
template (NT), red = IS).

Results of our 2-color fluorometric assay are compared to those from our previously reported Agilent capillary electrophoresis test [37], SYBR® green qRT-PCR, and cell culture [24], using the same biological samples. The numbers of VHSv molecules are quantified from field-caught and laboratory-challenged VHSv-infected fish samples using the new 2-color fluorometric assay in comparison to the Agilent capillary electrophoresis test [37].
4.3 Materials and Methods

4.3.1 VHSv Assay Development

All primers and NT probes were matched to homologous sequences of the VHSv N-gene, including all VHSv strains and substrain variants from NIH GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the literature, using Biosearch Technologies Real Time Design software (Novato, CA; http://www.biosearchtech.com/). The original muskellunge isolate MI03GL from the Great Lakes (GenBank Accession no. DQ427105) served as the reference for VHSv, and actb1 mRNA from the yellow perch Perca flavescens (AY332493) was used as the fish reference gene sequence. Selection criteria included: product lengths that were < 100 bp, with optimal melting temperatures of 65–68°C for primers and 68–72°C for probes. NT probes for the target and reference genes were labeled with FAM (fluorescein amidite dye).

The competitive template IS probes for the VHSv N-gene and the fish reference actb1 gene each were constructed by altering 5–6 bp of the NT probe sequences, and were labeled with Quasar dye having 670 nm maximum absorbance (Biosearch Technologies). The IS probes were designed to: minimize cytosine and thymine (30%), maximize adenine (50%), avoid guanine at the 5’ end, have lengths < 24 bp, and have predicted melting temperatures ± 0.02°C of the NT probe. Synthetic NT and IS templates for VHSv and actb1 were assembled by combining the forward primer, probe, and connecting sequence through the reverse primer (Table 1), and were synthesized by Life Technologies (Grant Island, NY; http://www.lifetechnologies.com/us/en/home.html). The BLAST procedure (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was employed to verify that all primers, probes, and IS did not recognize other viral or fish DNA sequences.
To ensure that the probes did not bind to non-homologous template, their specificities were tested using synthetic templates for the VHSv N- and actb1 genes. Both synthetic templates (NT and IS) were serially diluted 10-fold from $10^{-11}$ M to $10^{-15}$ M and tested with all probes in PCR amplification experiments, following the directions for “Performing the VHSv Assay”, as detailed below. For example, the VHS N-gene IS synthetic template was tested using the VHS N-gene IS probe, as well as the VHS N-gene NT probe, and vice versa. The same was done for actb1. Cycle thresholds ($C_t$) from the homologous and non-homologous templates were compared at each dilution, and the non-homologous amplifications were calculated using formula $2^{(-\Delta C_t)}$ and multiplied by the known number of input copies. If the resulting numbers of molecules were > 10% of the known input copy number, then the probe was re-designed, and the process was repeated.

After synthesis, the NT and IS for each gene were PCR amplified (Table 1) in 10 individual 10 µl rxns, containing 1 µl 750 nM of each primer, 0.5 U Go-TAQ polymerase (Promega, Madison, WI), 1 µl 10X MgCl$_2$ PCR buffer, 0.2 mM dNTPs, and RNase-free water on a Rapid Cycler 2 (Idaho Technology, Inc., Salt Lake City, UT; www.biofiredx.com/). Rxns were run for 35 cycles of 5 sec at 94°C, 10 sec at 58°C, and 15 sec at 72°C, with a slope of 9.9. To purify the NT and IS, all 10 replicate PCR products per template were combined into a single tube, loaded onto individual 2% low melting pre-cast agarose gels from E-Gel iBase (Invitrogen, Grand Island, NY; www.invitrogen.com/), separated by electrophoresis, and visualized on a UV transilluminator. The NT and IS bands for the VHSv N- and actb1 genes were harvested from their respective gel collection wells. The mean concentrations (ng/µl) of each were
calculated from 1 µl of their purified products as measured with an Agilent 2100 Bioanalyzer in triplicate, and converted into molarities according to the formula (1):

\[ \text{Molarity} = \frac{\text{concentration (µg/µl)}}{\text{molecular wt. (µg/µmole)}} \]

To control for inter-sample and inter-experimental pipetting variation, a synthetic internal standard mixture (ISM) was created using the purified IS described above. To prepare the original stock ISM “A”, we estimated the relative concentrations of the VHSv N-gene and actb1 IS needed to achieve a 1:1 cDNA NT:IS relationship in a variety of samples (Table 2). Briefly, we mixed \(10^{-10}\) M of the VHSv N-gene IS and \(10^{-11}\) M of the actb1 IS in an initial stock as ISM “A”. To measure various levels of gene expression, additional ISM mixtures (ISM B–H) were constructed using 10-fold serial dilutions of the VHSv N-gene relative to a constant concentration of the actb1 gene IS at \(10^{-11}\) M (Table 2). Additional 10-fold dilutions of each ISM (A–H) stock then were prepared using 0.1 ng/µl yeast tRNA carrier (Invitrogen, Carlsbad, CA) to prevent adherence of the negatively charged IS molecules to the tube or pipette tip surfaces (Table 2, rows 2–8).

An External Standardized Mixture - ESM (comprised of the synthesized NT and IS for the VHSv N- and actb1 genes) - was made to control for inter-lot and inter-experimental variation in probe fluorescence intensity, to guard against inter-experimental variation in C\(_t\) selection, and to normalize the probe (see equation (2), “Correction for variation in fluorescence among probes”). Stock ESM containing \(10^{-11}\) M NT/\(10^{-11}\) M IS for the VHSv N- and actb1 genes was diluted to a working concentration of \(10^{-13}\) M NT/\(10^{-13}\) M IS and \(10^{-14}\) M NT/\(10^{-14}\) M IS using yeast tRNA (Invitrogen,
4.3.2 Fish Samples Used to Evaluate the VHSv Assay

Spleen tissues from a variety of fish samples were used to test our assay for the presence and concentration of VHSv (and to compare our results to other assays, using the same samples). Fish were obtained, maintained, anesthetized, and sacrificed following the Institutional Animal Care and Use Committee (IACUC) approved protocols from the University of Toledo (#106419), Michigan State University (MSU; East Lansing, MI) (#AUF 07/07-123-00), and the U.S. Geological Survey's (USGS) Western Fisheries Research Center Challenge Facility (WFCCF; Seattle, WA) (#2008-17). Fish were euthanized with an overdose of 25 mg/ml tricaine methanesulfonate (MS-222; Argent Chemical Lab, Redmond, WA) and decapitated to ensure death. To remove any external viral particles, each fish was washed separately by submerging it 3X in double distilled H$\text{}_2\text{O}$. The surgical site (anus to operculum) was disinfected with 100% ethanol and betadine using sterile equipment. Spleen tissue was removed, placed into individual 1.5 mL eppendorf tubes, flash frozen in liquid nitrogen or stored in RNAlater (Qiagen), and kept at -80°C until further processing. Gloves and all equipment were changed between each fish to ensure sterile conditions. Specimens were disposed of following the respective approved biohazard protocols of the University of Toledo, MSU, and USGS.

Samples tested for VHSv included cDNA from 23 wild-caught Great Lakes fishes, including 10 infected and 13 negatives: two bluegill (*Lepomis macrochirus*), a brown bullhead (*Ameiurus nebulosus*), a freshwater drum, seven largemouth bass (*Micropterus salmoides*), a smallmouth bass (*Micropterus dolomieu*), and 11 lake herring (*Coregonus artedi*). We also tested 40 fish from VHSv laboratory challenge experiments, including
20 muskellunge (15 VHSv infected and 5 negative controls) from the MSU-Aquatic Animal Health Laboratory (AAHL), and 20 yellow perch (14 VHSv-infected and 6 negative controls) from USGS-WFRCCF.

A series of laboratory challenge experiments were conducted by MSU-AAHL on certified VHSv-free juvenile muskellunge (Rathburn National Fish Hatchery, Moravia, Iowa) under MSU IACUC protocols AUF 07/07-123-00. Muskellunge were challenged via water immersion for 90 min with VHSv-IVb (isolate MI03GL) at $4.0 \times 10^3$ pfu/ml, and the negative controls with 1 ml sterile maintenance minimum essential media. Fish then were placed into clean VHSv-free water, and later randomly sacrificed at pre-determined intervals, as previously described.

We also analyzed RNA from a series of juvenile yellow perch laboratory challenge experiments, using six-month-old (VHSv-certified-free) Choptank broodstrain [42] from the University of Wisconsin-Milwaukee’s Great Lakes WATER Institute (Milwaukee, WI), which were conducted at USGS- WFRCCF under their 2008-17 IACUC protocol. Perch were challenged either via intra-peritoneal injection of $1.0 \times 10^5$ pfu/ml VHSv-IVb (strain MI03GL) or via immersion for two hours in the same dosage, while control groups had a dose of minimum essential media. Fish were selected randomly in days 0–6 for euthanization with 240 mg/L MS-222 and 1.2g/L NaHCO$_3$. Dissection followed protocols described above.

4.3.3 Performing the VHSv Assay

Spleen tissue (0.25–0.50 g) was ground using a sterile mortar and pestle under liquid nitrogen, and its RNA was extracted using the TriREAGENT® (Molecular Research Center, Inc., Cincinnati, OH) protocol. The RNA was re-suspended in 30 µl
RNase-free water, quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and adjusted to a 1 μg RNA/μl concentration. DNA-free DNase Treatment and Removal Reagents (Ambion Life Technologies, Grand Island, NY) were used to eliminate any contaminating gDNA. The purified RNA was reverse-transcribed to cDNA, with 1 μg RNA, 5X First Strand buffer, 10 mM dNTPs, 0.05 mM random hexamers, 25 U/μl RNasin, and 200 U/μl M-MLV in a 90 μl rxn volume, using 94°C for 5 min, 37°C for 1 h, and 94°C for 5 min. The cDNA was stored at –20°C.

A set of PCR rxns was run per each cDNA sample to determine the appropriate concentrations of NT and IS for actb1 to achieve a ratio of > 1:10 and < 10:1 of amplified products. Once the NT:IS products were in balance, pre-amplification of the VHSv N- and actb1 target genes were performed simultaneously to increase the signal (i.e. lower C_t) relative to non-specific background.

For each pre-amplification, a 10 μl volume of a master mixture was prepared that contained 5 μl TaqMan® Universal Master Mix II (without uracil N-glycosylase; Applied Biosystems, Grand Island, NY), 1 μl of 10X primer solution (Final concentration: 75 nM) containing the forward and reverse primer pairs of the VHS N and actb1 genes mixed together), and RNAse-free water. Eight μl of this master mixture was dispensed into individual wells of 0.1 mL 96-well TempPlate® (USA Scientific, Inc.; www.usascientific.com/) containing 1 μl cDNA and 1 μl of the appropriate ISM concentration (Table 2). This was done in triplicate to allow calculation of the mean and standard error (S.E.) of the relative VHSv N-gene concentration/10^6 actb1 molecules per fish sample. The plates then were sealed with a TempPlate® RT Optical Film and centrifuged for 2 min at 2000 rpm. PCR rxns were conducted on an ABI 7500 Fast using
standard mode cycling conditions: 10 min at 95°C, followed by 13 cycles of 15 sec at
95°C, and 1 min at 60°C. For the Poisson distribution experiments, 25 pre-amplification
cycles were used due to lower amount of starting template. Three no-template controls
per rxn, located on separated areas on the plate, were used to control for possible
contamination.

A second round of amplification was performed, in which each pre-amplified
sample was diluted 1000-fold with TE buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 7.4); 2
µl of each diluted product was placed into each well of a new 0.1 mL 96-well
TempPlate®, along with 18 µl of a master mixture containing 10 µl TaqMan® Universal
Master Mix II (without uracil N-glycosylase), 2 µl of each 10X primer solution (Final
concentration: 750 nM), 2 µl of each NT and IS probe (Final concentration: 200 nM), and
RNAse-free water. This second amplification was conducted as described above, except
run for 40 cycles. The number of VHSv molecules/10^6 actb1 molecules was calculated
using equations (2) and (3) below.

\[
\text{Measured NT molecules for the VHSv } N \text{ and } actb1 \text{ genes:}
\]
(a) Correction for variation in fluorescence among probes =
the mean of two ESM concentrations \(\left[\text{measured NT cycle threshold (C_t)}\right] - \left[\text{measured IS C_t}\right]\)
(b) Measured NT signal relative to the IS signal for each gene \(\Delta C_t\) =
each gene \(\left[\text{NT C_t} - \left[\text{IS C_t}\right]\right] - \text{normalizing value}
(c) Measured NT molecule copy number = \# input IS molecules from ISM \times \left[2^{\left(-\Delta C_t\right)}\right]

\[
\text{Measured NT molecules for the VHSv } N \text{ and } actb1 \text{ genes:}
\]
(a) Correction for variation in fluorescence among probes =
the mean of two ESM concentrations \(\left[\text{measured NT cycle threshold (C_t)}\right] - \left[\text{measured IS C_t}\right]\)
(b) Measured NT signal relative to the IS signal for each gene \(\Delta C_t\) =
each gene \(\left[\text{NT C_t} - \left[\text{IS C_t}\right]\right] - \text{normalizing value}
(c) Measured NT molecule copy number = \# input IS molecules from ISM \times \left[2^{\left(-\Delta C_t\right)}\right]

\[
\text{Final number of molecules for the target VHSv } N \text{ gene and reference } actb1 \text{ gene:}
\]
VHSv molecules/10^6 actb1 molecules = \[\text{VHSv measured NT}] / [\text{actb1 measured NT}] \times 10^6

4.3.4 Specificity, True Accuracy, and Linearity

Our assay was tested for non-specific amplification using two human viruses
(Encephalomyocarditis virus and Vesicular Stomatitis virus) and five fish viruses related to VHSv (Hirame Rhabdovirus, Infectious Hematopoietic Necrosis virus, Infectious Pancreatic Necrosis virus, Spring Viremia of Carp virus, and Snakehead Rhabdovirus). The Snakehead Rhabdovirus is the nearest relative to VHSv, with 62% sequence similarity [8, 16]. Twenty-five VHSv isolates were tested to evaluate amplification for a range of European, Asian, and North American variants (Table 3), encompassing all four strains. All samples were assayed in triplicate.

To measure true accuracy - the agreement between a measurement and its known value [38] – the relationship between the observed versus expected numbers of VHSv N-gene and actb1 molecules based on Poisson analysis was determined [39]. Ten replicates were amplified for the VHSv N- and actb1 genes over a series of limiting PCR dilutions, which were predicted to contain 40, 20, 10, 7, 6, 5, 4, 2, 1, 0.7, 0.4, and 0.1 molecules. Linear regression analysis was performed in the R statistical suite software v2.15.2 [40]. A $\chi^2$ test (in Microsoft Excel) compared the number of molecules measured with the 2-color fluorometric assay versus those from the Agilent 2100 Bioanalyzer, at the same dilutions.

Linearity was measured over two series of dilution experiments to: 1) determine the maximum and minimum ratio of NT to IS that produces reproducible results, and 2) verify that our test follows a linear trend in calculating the expected number of molecules per dilution. The first dilution set was prepared by mixing a constant amount of synthetic NT with decreasing amounts of IS to generate dilutions of: 1:1 ($6 \times 10^4$ molecules), 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:12, 1:14, 1:16, 1:18, and 1:20 ($3 \times 10^3$ molecules) for both genes. Identical procedures were performed by holding the IS constant, while
varying the NT. The second dilution series evaluated linearity for the VHSv N- and actb1 genes using 10-fold serial dilutions of the ESM at a 1:1 ratio, with dilutions of $6 \times 10^6$, $6 \times 10^5$, $6 \times 10^4$, $6 \times 10^3$, $6 \times 10^2$, 60, 6, and 0.6 molecules. Regression analyses were conducted to determine correlation ($R^2$), slope (linearity), and relation to a linear trend ($F$) among the various dilutions of NT:IS and IS:NT for each gene. Imprecision was reported as the coefficient of variation (CV), which was calculated as the standard deviation divided by the mean of triplicate measurements at each dilution (reported in %) (in Microsoft Excel) [41]. In addition, S.E. was calculated for each sample. For these linearity experiments, PCR was performed as specified above in “Performing the VHSv Assay”, but substituting the cDNA and ISM with either 2 µl of the ESM (from dilution 1) or a concentration of 1:1 NT/IS (dilution 2). Each dilution was run in triplicate, with a negative/no template control for each run.

4.3.5 VHSv Detection Comparisons of Our Assay to Others

Results from the new 2-color fluorometric test are compared to those from our prior Agilent capillary electrophoresis assay [37], conventional SYBR® green qRT-PCR, and cell culture to evaluate their relative abilities to detect VHSv in 63 fish samples (see “Fish Samples used to Evaluate the VHSv Assay”). All samples were analyzed in triplicate and all runs had positive and negative controls. Each PCR rxn included a known cell culture positive, a negative VHSv cDNA, and a reagent negative control (nuclease-free H$_2$O). PCR products were visualized on 1% agarose gels to confirm positive/negative results. The amount of yellow perch fish tissue available from the USGS laboratory challenge experiments precluded their analysis with cell culture. $\chi^2$ tests [43] were used to compare the results among the approaches.
SYBR® green qRT-PCR experiments used a Mastercycler Realplex Thermocycler (Eppendorf, Inc., Westbury, NY) with 25 μl rxns, containing 0.05 μg of each primer (the same primers used for the Agilent capillary electrophoresis assay [37]), 2 μl cDNA product, 10 μl SsoFast SYBR® green mix, and RNase-free water. Amplifications were run on a Mastercycler Realplex Thermocycler (Eppendorf, Inc., Westbury, NY), with an initial denaturation of 5 min at 95°C, followed by 40 cycles of 30 sec at 95°C, and 1 min at 60°C.

Cell culture was performed at MSU’s AAHL by M. Faisal and R. Kim, following standard OIE [24] procedures. If results were positive, RNA was extracted from infected cells as described above, reverse transcribed using Affinity Script Multiple Temperature Reverse Transcriptase PCR (Stratagene, La Jolla, CA), and amplified following OIE [24].

4.3.6 VHSV Quantification Using Our Assay

Positive samples were quantified using our new 2-color fluorometric real-time PCR assay and compared to our earlier results from the Agilent capillary electrophoresis procedure [36] from the 63 test fish, using linear regression in R and an $F$-test [43]. Numbers of VHSV/10$^6$ actb1 molecules were measured in triplicate, from which means and S.E. were calculated. Relative numbers of VHSV molecules were compared between laboratory challenged muskellunge showing clinical signs of infection (e.g. external hemorrhages; $N = 9$) versus those without signs ($N = 9$). A χ$^2$ test (Microsoft Excel) was used to determine if a threshold number of VHSV molecules characterized the appearance of the clinical signs. Due to low sample size, a power analysis (G*Power2; [44]) was used to estimate the number of fish needed to achieve 95% confidence, with an effect size of 0.50 [45].

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4.4 Results

4.4.1 Performance of our 2-Color Fluorometric Assay for VHSv

Our test results are negative for all other viruses (i.e. did not result in amplification; Table 3), including human viruses (Encephalomyocarditis virus and Vesicular Stomatitis virus) and fish viruses that are related to VHSv (i.e. Hirame Rhabdovirus, Infectious Hematopoietic Necrosis virus, Infectious Pancreatic Necrosis virus, Spring Viremia of Carp virus, and Snakehead Rhabdovirus). All four VHSv strains (I–IV) and all substrains evaluated (e.g. I, Ia, II, III, IVa, IVb, and IVc) show positive amplification results with our test (Figure 1a; Table 3). Thus this new assay is specific for VHSv.

Amplification results for the VHSv N-gene (Figure 2a) are 100% (10/10 times) for dilutions of 5–40 VHSv molecules, 90% (9/10 times) for 4 molecules, 80% (8/10) for 2 molecules, 60% (6/10) for a single molecule, 30% (3/10) for 0.7 molecules, 20% (2/10) for 0.4 molecules, and 10% (1/10) for 0.1 molecules ($R^2 = 0.98$, $F = 541.50$, df = 1, 10, $p < 0.001$). Values for amplification of the fish actb1 gene are similar (Figure 2b), yielding 100% positives for 4–40 molecules (10/10), 70% at 2 molecules (7/10), 40% for a single molecule and for 0.7 molecules (4/10), 20% for 0.4 molecules (2/10), and 10% at 0.2 molecules (1/10) ($R^2 = 0.97$, $F = 283.60$, df = 1, 10, $p < 0.001$). Results show that the numbers of ISM molecules measured by our assay match those from the bioanalyzer for the VHSv N-gene ($\chi^2 = 0.18$, df = 11, NS) and the actb1 gene ($\chi^2 = 0.23$, df = 11, NS).

The relationship between the amount of PCR product remains linear when the concentration of NT is held constant and the IS is varied for both the VHSv N-gene (Figure 3a: $R^2 = 0.99$, $F = 1363.00$, df = 1, 13, $p < 0.001$) and the actb1 gene (Figure 3b: $R^2 = 0.98$, $F = 541.50$, df = 1, 10, $p < 0.001$).

$R^2 = 0.99, F = 1283.00, \text{df} = 1, 13, p < 0.001$). Figure 1b depicts the results that illustrate this relationship. The mean calculated CV is 5% for the VHSV N-gene over an NT:IS dilution range of 1:1–1:10 (yielding $6.0 \times 10^4 \pm 0.0 \times 10^0$ to $4.6 \times 10^3 \pm 2.8 \times 10^2$ molecules). The CV likewise is 5% for the actb1 gene (yielding $6.0 \times 10^4 \pm 0.00 \times 10^0$ to $7.5 \times 10^3 \pm 4.3 \times 10^2$ molecules). At dilutions beyond 1:10, the CV increases to 7% for both the VHSV N-gene (yielding up to $1.5 \times 10^3 \pm 1.1 \times 10^2$ molecules) and the actb1 gene (yielding up to $3.0 \times 10^3 \pm 1.2 \times 10^2$ molecules) when the NT is held constant.

Analogous results are obtained when the IS is held constant and the NT is varied for the VHSV N-gene (Figures 1c and 3c: $R^2 = 0.99, F = 5124.00, \text{df} = 1, 13, p < 0.001$) and the actb1 gene (Figure 3d: $R^2 = 0.99, F = 2434.00, \text{df} = 1, 13, p < 0.001$). The mean CV for the IS:NT dilution range of 1:1–1:10 is 5% for the VHSV N-gene (yielding $6.0 \times 10^4 \pm 0.0 \times 10^0$ to $5.9 \times 10^3 \pm 2.2 \times 10^3$ molecules) and 3% for the actb1 gene (yielding $6.0 \times 10^4 \pm 0.0 \times 10^0$ to $4.0 \times 10^3 \pm 4.3 \times 10^1$ molecules). At dilutions beyond 1:10, the CV increases to 7% for the VHSV N-gene (yielding up to $2.8 \times 10^3 \pm 1.8 \times 10^2$ molecules) and 6% for the actb1 gene (yielding up to $1.4 \times 10^3 \pm 6.2 \times 10^1$ molecules) when the IS is held constant. Based on these findings, our quantifications are conducted in the range of 1:10 to 10:1 NT:IS to maximize accuracy.

The numbers of VHSV molecules detected show a linear relationship over seven orders of magnitude (serial dilutions of $6 \times 10^6$ to $6 \times 10^0$ molecules) when the NT:IS is 1:1 (Figure 4a), with a slope of 1.00 ($R^2 = 0.99, F = 9404.00, \text{df} = 1, 5, p < 0.001$). This relationship output is shown in Figure 1d, in which NT and IS consistently increase by $\leq 3.2 \text{C}_t$ for each 10-fold serial dilution of the ESM. The mean CV for VHSV is estimated at 7% for samples containing $6 \times 10^6$ to $6 \times 10^1$ molecules (measured as $6.5 \times 10^6 \pm 5.2 \times 10^0$.
10^5 to 7.9 x 10^1 \pm 2.0 x 10^0 molecules), and 9% when the range is extended to 6 x 10^0 molecules (measured as 6.0 x 10^0 \pm 1.0 x 10^0 molecules). Results for actb1 show a similar trend (Figure 4b), having a slope of 1.04 (R^2 = 0.99, F = 1347.00, df = 1, 5, p < 0.001), a mean CV of 7% for 6 x 10^6 to 6 x 10^1 molecules (measured as 6.6 x 10^6 \pm 2.1 x 10^5 to 7.8 x 10^1 \pm 8.0 x 10^0 molecules), and 10% when the range was extended to 6 x 10^0 molecules (measured as 3.0 x 10^0 \pm 0.4 x 10^0 molecules). Stochastic sampling likely contributed to the increased CV and S.E. in the measurements for 6 molecules.

4.4.2 VHSv Detection and Quantification Comparison among Methods

Results reveal that our present 2-color fluorometric real-time PCR assay and previous results from the Agilent capillary electrophoresis-based approach [37] both discriminate identical positives and negatives (i.e. they have the same accuracy; Figure 5; \chi^2 = 0.00, df = 1, NS), and are free of false negatives (Figure 5). In contrast, the cell culture results have 56% false negative error (Figure 5a: \chi^2 = 9.36, df = 1, p = 0.002) and SYBR® green yields 33–44% false negative error (Figure 5a,b: \chi^2 = 5.96, df = 1, p = 0.02). All positives detected by SYBR® green qRT-PCR and cell culture also were positive with both of our StaRT-PCR methods (2-color fluorometric real time and capillary electrophoresis). The false negative range for SYBR® green qRT-PCR was 1.0 x 10^0-1.6 x 10^2 VHSv/10^6 actb1 molecules (= 0.6 x 10^0-2.5 x 10^2 VHSv molecules, as quantified by our 2-color fluorometric method) and 1.0 x 10^0-2.2 x 10^3 VHSv/10^6 actb1 molecules (= 0.6 x 10^0-6.1 x 10^3 VHSv molecules, as quantified by our 2-color fluorometric method) for cell culture. True negatives (including experimental controls) are negative with all assays; i.e. we find no false positives and no contamination.

Numbers of VHSv molecules/10^6 actb1 molecules measured from the spleen
tissues of positive fish are higher in the new assay, ranging to 1.21 \times 10^6 \text{ VHSv molecules}/10^6 \text{ actb1} (= 1.90 \times 10^4 \text{ VHSv molecules}) than for the Agilent capillary-based test, which ranged to 8.4 \times 10^5 \text{ VHSv molecules}/10^6 \text{ actb1} (= 2.7 \times 10^3 \text{ VHSv molecules}). However, both sets of values have a direct linear relationship (Figure 6: \( R^2 = 0.91 \), df = 1, 38, \( F = 396.40, p < 0.001, t = 1.42, \text{df} = 78, \text{NS} \)). Muskellunge exhibiting clinical signs of infection contain a greater mean number of viral molecules (1.4 \times 10^5 \pm 6.5 \times 10^3 \text{ VHSv}/10^6 \text{ actb1 molecules} = 6.9 \times 10^4 \pm 6.9 \times 10^3 \text{ VHSv molecules}) than those without (1.2 \times 10^4 \pm 1.7 \times 10^3 \text{ VHSv}/10^6 \text{ actb1 molecules} = 1.5 \times 10^3 \pm 1.6 \times 10^2 \text{ VHSv molecules}). The estimated threshold at which those individuals display clinical signs of infection is \( \sim 1 \times 10^3 \text{ VHSv}/10^6 \text{ actb1 molecules} (= 3.6 \times 10^2 \text{ VHSv molecules}) \) using our assay. Our sample sizes were not sufficient to further evaluate the relationship between this threshold number of molecules and clinical diagnosis (\( \chi^2 = 0.09, \text{df} = 1, \text{NS} \)). Power analysis estimated that 52 fish samples (26 with and 26 without clinical signs) would be needed to verify this finding.

All data and analyses are publically accessible on the University of Toledo Lake Erie Center’s VHSv webpage
(http://www.utoledo.edu/nsm/lec/research/glgl/VHS/VHS_main.html).
### 4.5 Discussion

Disease diagnostic laboratories depend on rapid, sensitive, and accurate detection methods, which are easy to employ and cost-effective. Cell culture is the VHSv diagnostic approved by the World Organization of Animal Health [24], which takes up to a month to perform in clinical settings and often results in substantial false negatives – as revealed here and by other studies [26–28]. Compared with traditional cell culture, all PCR-based assays [32] – including the present one – show enhanced ability to detect VHSv since they amplify both the negative-strand non-replicating genomic RNA and the positive-strand replicating mRNA transcripts. Amplification of both transcripts may be beneficial since positive results may denote new spread of VHSv or latent cases in the geographic region where samples were taken, which can likely aid in the diagnosis of viral infections.

The present assay detects and quantifies VHSv-IVb in fishes from the Great Lakes using primers and probes that are homologous to the N-gene sequence of the widespread IVb isolate MI03GL, and matches conserved sequence regions among all VHSv strains and substrains. Results demonstrate cross-reaction with all other VHSv strains and substrains tested. Other human and fish viruses do not amplify. Thus, our assay is VHSv-specific and detects all of its known variants.

Other PCR tests developed for VHSv by Chico et al. [26], López-Vázquez et al. [27], Liu et al. [29], Matejusova et al. [30], Cutrín et al. [31], Hope et al. [32], Garver et al. [33], and Jonstrup et al. [28], culminated in high numbers of false negatives, analogous to the SYBR® green test evaluated here (33-44% false negatives). Notably, 15–90% false negatives were reported by López-Vázquez et al. [27], 25–92% by Chico et
al. [26], and values to 42% by Jonstrup et al. [28], for their respective approaches. Unlike those other real-time PCR tests for VHSv [26–34], our method incorporates intrinsic quality control standards (IS) to circumvent false negative results.

Specifically, exogenous (IS) and endogenous controls (the commonly used reference gene \textit{actb1}) facilitate optimal detection of true positives and act to normalize the quantification of viral molecules. Use of IS is recommended by the International Organization for Standardization [46], the U.S. Environmental Protection Agency [47], and the U.S. Food and Drug Administration [48]. Tests for Hepatitis C virus [49] and Human Immunodeficiency virus [50, 51] already have implemented IS in their assays.

Our assay is sensitive, follows a linear relationship with increasing viral concentration, and is highly reproducible. It detects down to five VHSv \textit{N}-gene molecules with 100% accuracy, based on a Poisson distribution. Other real-time PCR assays for VHSv showed much higher detection thresholds. Notably, Liu et al.’s [29] test required $\geq$ 140 viral copies of VHSv, and assays by Hope et al. [32] and Garver et al. [33] needed $\geq$ 100 viral copies. Our results are consistent for samples containing six to 6,000,000 molecules of VHSv. Stochastic variation is evident only at extremely low dilutions (< five molecules). Results confirm reliability from concentrations of 1:1 to 1:20 NT:IS, with some slight increase in CV at dilutions $> 1:10$. We thus recommend adjusting the relative concentrations of NT:IS to maximize accuracy, following recommendations in the Materials and Methods section “Performing the VHSv Assay”. All quantification values reported here fall within this 1:10 range, which allows us to distinguish ~ a 1.25-fold C\textsubscript{t} difference. Our assay also should work well with highly degraded samples (e.g. dead fish in the field), as described for human cancer samples.
using this type of approach by some of our team members [52].

This 2-color fluorometric real-time assay is highly accurate, and is free of the size separation steps required for our previously-published Agilent capillary electrophoresis approach [37]. Here we determine higher numbers of VHSv molecules for the same fish samples, due to the re-design of primers and inclusion of fluorescent-labeled probes. Results of both methods have a linear relationship and are readily cross-calibrated.

Laboratory challenged muskellunge showing clinical signs of infection contain a greater mean number of viral molecules than those without. It is estimated that $1 \times 10^3$ VHSv/10$^6$ actb1 molecules ($= 3.6 \times 10^2$ VHSv molecules) appears to mark a clinical threshold for signs of the disease. However, exhibition of clinical signs at this biomarker could be species-specific, and may differ between fish in the laboratory and those in the field. Further experiments are warranted to validate this assumption.
4.5.1 Conclusions

Our assay is highly sensitive and accurate, free of false negatives, and reliably quantifies a wide range of VHSv in fish tissue samples. Other PCR-based methods and cell culture techniques had high proportions of false negatives since they lacked intrinsic quality control, which could lead to spread of the virus. This new test will aid rapid, accurate, and low-cost diagnosis of the disease. It has wide applicability across the geographic range of the virus, and should be highly successful in elucidating new occurrences and circumventing spread.
4.6 Table and Figure Legends

Table 4.1 Sequences and PCR parameters for our 2-color fluorometric VHSv assay.

Primers, probes, internal standards (IS), and synthetic templates are specified. F = forward primer, R = reverse primer, NT = native template. Italics = modified bp from NT probe.

Table 4.2 Concentrations used for the 2-color fluorometric VHSv assay.

Dilution mixtures (A–H) used for the Internal Standards Mixture (ISM) actb1/VHSv are given in units of 10x M.

Table 4.3 Specificity of the 2-color fluorometric test.

− = negative result (no amplification), + = positive result.

Figure 4.1 Real-time PCR amplification plots for various experiments.

ABI 7500 real-time PCR results for (a) a true VHSv positive fish sample, (b) the relationship between the VHSv Native Template (NT) and Internal Standard (IS) with the NT held constant and the IS varied, (c) the relationship between VHSv NT and IS with the IS held constant and the NT varied, and (d) the relationship between VHSv NT:IS with concentrations held constant for dilutions of 1:1–1:20. Green = NT, red = IS, y = fluorescence of the reporter dye minus the baseline (∆ fluorescence), x = cycle threshold.

Figure 4.2 True accuracy of the 2-color fluorometric test.
Results are based on % positives from 10 separate runs of 12 dilutions using a known Internal Standard Mixture (ISM). Dilutions were: 40, 20, 10, 7, 6, 5, 4, 2, 1, 0.7, 0.4, and 0.1 molecules. The 2-color fluorometric test yielded 100% positives for (a) \( \geq 5 \) molecules of VHSv and (b) \( \geq 4 \) molecules for actb1.

**Figure 4.3 Relationship between the number of observed and expected molecules for NT:IS concentrations of 1:1–1:20.**

The concentration of Native Template (NT) was held constant and the Internal Standard (IS) was varied for dilutions of: 1:1 (6 x 10^4 molecules), 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:12, 1:14, 1:16, 1:18, and 1:20 (3 x 10^3 molecules). The 2-color fluorometric assay yielded a linear relationship for (a) VHSv (\( R^2 = 0.99, F = 1514.00, \text{df} = 1, 13, p < 0.001 \)) with a mean CV of 5% for dilutions 1:1–1:10 and 7% for concentrations down to 1:20, and for (b) actb1 (\( R^2 = 0.99, F = 1283.00, \text{df} = 1, 13, p < 0.001 \)), CV = 5% and 7%. The same linear pattern was observed when the IS was held constant and NT varied for (c) VHSv (\( R^2 = 0.99, F = 5124.00, \text{df} = 1, 13, p < 0.001 \)), CV = 5% for 1:1–1:10 and 7% for dilutions down to 1:20, and (d) actb1 (\( R^2 = 0.99, F = 2434.00, \text{df} = 1, 13, p < 0.001 \)), CV = 3% and 6%. Error bars = standard error of results for triplicate runs. Dotted line = partition of dilutions from 1:1–1:10 (right) and 1:12–1:20 (left).

**Figure 4.4 Relationship between the numbers of observed versus expected molecules when NT:IS concentrations are 1:1.**
Results are based on dilutions of the Native Template (NT) and Internal Standard (IS) of $6 \times 10^6$, $6 \times 10^5$, $6 \times 10^4$, $6 \times 10^3$, $6 \times 10^2$, 60, 6, and 0.6 molecules for VHSv and actb1. The 2-color fluorometric assay yielded a linear relationship for (a) VHSv over seven orders of magnitude (from $6 \times 10^6$ to $6 \times 10^0$ VHSv molecules) with a slope of 1.00 ($R^2 = 0.99$, $F = 9404.00$, $df = 1, 5$, $p < 0.001$), and mean CV of 9%. A linear trend also was obtained for (b) actb1 ($R^2 = 0.99$, $F = 1347.00$, $df = 1, 5$, $p < 0.001$). Slope = 1.04, mean CV = 10%. Error bars = standard error of triplicate runs.

**Figure 4.5 Relative numbers of VHSv positives and negatives from our 2-color fluorometric and capillary electrophoresis StaRT-PCR assays, which indicated identical numbers of positives and negatives.**

Compared to these tests, for 43 fishes (25 positives, 18 negatives (including 5 laboratory controls)), (a) SYBR® green had 44% false negative error ($\chi^2 = 5.37$, $df = 1$, $p = 0.02$), and cell culture had a 56% error ($\chi^2 = 9.36$, $df = 1$, $p = 0.002$). For 63 fish samples (39 positives, 24 negatives (including 11 laboratory controls)), (b) SYBR® green qRT-PCR had 33% false negative error ($\chi^2 = 5.37$, $df = 1$, $p=0.02$), whereas the 2-color fluorometric and capillary electrophoresis tests showed zero false negatives.

**Figure 4.6 Mean log numbers of VHSv molecules/106 actb1 molecules from our new 2-color fluorometric assay versus the prior Agilent capillary electrophoresis approach.**

Results show a linear relationship between the two tests ($R^2 = 0.91$, $df = 1, 38$, $F = 396.40$, $p < 0.001$) and do not significantly differ ($t = 1.42$, $df = 78$, NS). Arrow =
Estimated threshold concentration of VHSV for fish with clinical signs of infection using our new assay, from a $\chi^2$ test of nine symptomatic fish ($1 \times 10^3$ VHSV molecules/$10^6$ actb1 molecules = $3.6 \times 10^2$ VHSV molecules). Triangle = false negative range for SYBR® green qRT-PCR ($1.0 \times 10^0$-$1.6 \times 10^2$ VHSV/$10^6$ actb1 molecules = $0.6 \times 10^0$-$2.5 \times 10^2$ VHSV molecules). Square = false negative range for cell culture ($1.0 \times 10^0$-$2.2 \times 10^3$ VHSV/$10^6$ actb1 molecules = $0.6 \times 10^0$-$6.1 \times 10^3$ VHSV molecules.
### 4.7 Tables and Figures

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Isolates obtained from:

\(^a\) Western Fisheries Research Center, USGS, Seattle, WA, USA
\(^b\) Cornell University College of Veterinary Medicine, Ithaca, NY, USA
\(^c\) Finnish Food Safety Authority Evira, Finland
\(^d\) Universidad de Santiago de Compostela, Spain
\(^e\) Fisheries and Oceans Canada, Pacific Biological Station, BC, Canada
Figure 4.1
Figure 4.2

(a) VHSv

(b) actb1
Figure 4.3

(a) VHSv Constant NT with varying IS

\[ y = 1.20x - 0.90 \]
\[ R^2 = 0.99 \]

(b) actb1 Constant NT with varying IS

\[ y = 0.98x + 0.12 \]
\[ R^2 = 0.99 \]

(c) VHSv Constant IS with varying NT

\[ y = 1.05x - 0.18 \]
\[ R^2 = 0.99 \]

(d) actb1 Constant IS with varying NT

\[ y = 1.26x - 1.18 \]
\[ R^2 = 0.99 \]
Figure 4.4

(a) VHSv

\[ y = 1.00x + 0.02 \]

\[ R^2 = 0.99 \]

(b) acb1

\[ y = 1.04x - 0.19 \]

\[ R^2 = 0.99 \]
Figure 4.5

(a) Results from various methods

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<td>18</td>
</tr>
<tr>
<td>Capillary Electrophoresis</td>
<td>25</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>SYBR® green qRT-PCR</td>
<td>14</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Cell Culture</td>
<td>11</td>
<td>14</td>
<td>18</td>
</tr>
</tbody>
</table>

(b) PCR-based test results

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<tr>
<th>Method</th>
<th>Positives</th>
<th>False Negatives</th>
<th>True Negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Color Fluorometric</td>
<td>39</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>Capillary Electrophoresis</td>
<td>39</td>
<td></td>
<td>24</td>
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<tr>
<td>SYBR® green qRT-PCR</td>
<td>26</td>
<td>13</td>
<td>24</td>
</tr>
</tbody>
</table>
Figure 4.6

The figure shows a logarithmic graph comparing Log 2-Color Fluorometric VHSV/10^6 actb1 Molecules to Log Capillary Electrophoresis VHSV/10^6 actb1 Molecules. The data points follow a linear trend with the equation $y = 1.07x + 0.31$ and an $R^2$ value of 0.91. The threshold is indicated as approximately $1 \times 10^3$ VHSV/10^6 actb1 molecules (~360 VHSV molecules).
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Chapter 5

Targeted RNA-Sequencing with Competitive Multiplex-PCR Amplicon Libraries

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5.1 Abstract:
Whole transcriptome RNA-sequencing is a powerful tool, but is costly and yields complex data sets that limit its utility in molecular diagnostic testing. A targeted quantitative RNA-sequencing method that is reproducible and reduces the number of sequencing reads required to measure transcripts over the full range of expression would be better suited to diagnostic testing. Toward this goal, we developed a competitive multiplex PCR-based amplicon sequencing library preparation method that a) targets only the sequences of interest and b) controls for inter-target variation in PCR amplification during library preparation by measuring each transcript native template relative to a known number of synthetic competitive template internal standard copies. To determine the utility of this method, we intentionally selected PCR conditions that would cause transcript amplification products (amplicons) to converge toward equimolar concentrations (normalization) during library preparation. We then tested whether this approach would enable accurate and reproducible quantification of each transcript across multiple library preparations, and at the same time reduce (through normalization) total sequencing reads required for quantification of transcript targets across a large range of expression. We demonstrate excellent reproducibility ($R^2=0.997$) with 97% accuracy to detect 2-fold change using External RNA Controls Consortium (ERCC) reference materials; high inter-day, inter-site and inter-library concordance ($R^2=0.97-0.99$) using FDA Sequencing Quality Control (SEQC) reference materials; and cross-platform concordance with both TaqMan qPCR ($R^2=0.96$) and whole transcriptome RNA-sequencing following “traditional” library preparation using Illumina NGS kits ($R^2=0.94$). Using this method, sequencing reads required to quantify accurately more than 100 targeted transcripts expressed over a $10^7$-fold range were reduced more than 10,000-fold, from $2.3\times10^9$ to $1.4\times10^5$ sequencing reads.
These studies demonstrate that the competitive multiplex-PCR amplicon library preparation method presented here provides the quality control, reproducibility, and reduced sequencing reads necessary for development and implementation of targeted quantitative RNA-sequencing biomarkers in molecular diagnostic testing.
5.2 Introduction:

Next-generation sequencing (NGS) of RNA derived templates, or RNA-sequencing, is a powerful method with potential to rapidly advance discovery, development and implementation of transcript-based biomarkers in the clinical setting [1]. Whole transcriptome RNA-sequencing provides transcript abundance quantification along with detailed transcript structure information, including representation of alternative transcripts, polymorphisms, and mutations, including translocations. However, the cost and complexity of whole transcriptome RNA-sequencing data sets are barriers to use of this method in routine molecular diagnostic testing. For example, quantification of expression levels as low as 1 transcript/cell with adequate precision typically requires >10^8 sequencing reads [2]. Recently developed targeted approaches reduce data complexity, and to some extent cost due to their focused nature [3-8]. However, targeted approaches reported thus far have limited clinical utility for at least two reasons:

1. Target enrichment steps, including bait hybridization-, capture and ligation- or polymerase chain reaction (PCR) based strategies, for RNA-sequencing may be associated with inter-library variation in measurement of transcript expression [3-11]. Since these targeted methods for RNA-sequencing were recently developed, the specific causes of inter-library variation in measurement have not yet been reported. However, it is reasonable to extrapolate from observations reported with similar techniques as to how variation may occur. As an example, for each of these approaches there may be inter-target variation in melting temperature of hybridization between native nucleic acid targets and enrichment templates. Further, disparity in annealing efficiency may be accentuated by inter-sample and inter-laboratory variation in
conditions [12]. In particular, for multiplex PCR-based amplicon library preparation, sample overloading or excessive amplification cycles may cause different targets to plateau at different cycles depending on the level of expression, the amount of sample loaded, and the total number of cycles used. Array- or solution-based bait enrichment targeted sequencing libraries are also likely to be susceptible to potential overloading and signal saturation effects as is observed with the limited dynamic range in microarrays expression measurements [2]. This can lead to inter-experimental and inter-target variation in signal compression. One way to avoid these effects is to load routinely less sample and/or use fewer amplification cycles for PCR based targeted sequencing approaches [8]. This concept is similar to the earliest semi-quantitative end-point PCR methods, when PCR was performed on serial dilutions of target templates in an effort to find a concentration that yields detectable signal yet is not at plateau phase for any of the targets being measured [12-14]. Historically, these steps can result in loss of signal for lowly expressed target transcripts and false negative reporting. Further, the relative abundance of the highest expressed transcript targets typically is unknown making it difficult to ensure prevention of early plateau in any given experiment. In addition, inter-sample variation in target-specific amplification inhibitors, commonly observed among clinical samples, may cause non-systematic analytical variation and contribute to false negative reporting [15-19]. These sources of a non-systematic variation, if not adequately controlled, will confound inter-sample comparison of transcript abundance data required for clinical diagnostic testing or collection of data for submission to regulatory agencies obtained using current targeted quantitative RNA-sequencing approaches.
2. Since a goal of many targeted RNA-sequencing methods is to maintain the initial relative quantitative representation of targets, a large number of sequencing reads is still required to reproducibly quantify each of them [20]. Specifically, the large range in expression typically exhibited among targeted transcripts in a given sample imposes a need for over-sequencing of the highest expressed transcript target in order to accurately quantify the lowest [2]. In turn, this reduces the number of samples that can be evaluated during each sequencing run, and therefore increases direct sequencing costs per sample [21]. A targeted method which reduces the over-sequencing of highly expressed transcripts relative to lowly expressed ones, yet maintains information regarding the original quantitative relationship between targets is needed for targeted quantitative RNA-sequencing to be cost-effective in the clinical setting.

We hypothesized that a competitive approach to multiplex PCR-based amplicon library preparation would effectively address the quality-control and cost limitations associated with existing PCR-based targeted RNA-sequencing methods [5,7]. With competitive amplicon library preparation, each native target (NT) in a sample (e.g. cDNA) is multiplex PCR-amplified in the presence of a known number of its respective competitive internal standard (IS) molecules within an IS mixture [13,22] (Figures 1 and 2). Introduction of a competitive IS mixture into PCR-based reactions controls for intersample variation in the presence of interfering substances [16-19]. In addition, use of the same competitive IS mixture across PCR experiments and laboratories controls for multiple sources of analytical variation ensuring concordance among data sets [2,15-17,23]. After sequencing a library prepared with competitive multiplex PCR, the number of sequencing reads for each native target is measured relative to the number of reads observed for its
respective competitive IS. We reasoned that this approach would enable highly reproducible quantitative targeted RNA-sequencing.

Introduction of a competitive IS mixture into PCR-based reactions also controls for inter-target variation in amplification efficiency due to the limitation in reagent quality or quantity [16-19]. Specifically, because each target native template (NT) amplifies with the same efficiency as its respective competitive IS, the pair remains in a fixed proportion (NT:IS ratio) throughout amplicon library preparation (Figures 1,2 and Animation S1). Since the NT:IS ratio remains fixed, the starting number of NT molecules can be determined by multiplying the NT:IS ratio at the end of PCR by the known number of IS molecules placed into the library preparation (Figure 1). Importantly, inter-target convergence (i.e. normalization between amplicons) from the original representation is controlled because each target NT is measured relative to a known starting number of competitive IS copies (Figure 2 and Animation S1). Taking advantage of this characteristic, we designed amplification conditions that promote convergence of inter-transcript native amplicon representation toward equimolar proportions at plateau phase (Figure 2 with details in Methods section and Animation S1). Attaining this goal would be important because convergence of representation would reduce the need to over-sequence high abundance templates, enabling analysis of more samples per sequencing chip and thereby reducing direct sequencing costs. The cost savings potential with this approach would be substantial because inter-gene variation in a transcript representation commonly ranges more than one million-fold [2].

In this study, we assessed the utility of the competitive multiplex PCR amplicon library preparation method to support reproducible and cost-effective transcript abundance
measurement on the Ion Torrent NGS platform. To do this, we used External RNA Controls Consortium (ERCC) and FDA-sponsored Sequencing Quality Control (SEQC) project reference material RNA cross-titration pools (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/) [2,24]. We prepared libraries using primers and competitive IS for 178 endogenous and synthetic cDNA targets expressed over a greater than $10^7$-fold range and subjected them to IonTorrent NGS. We then evaluated the method for a) accuracy and reproducibility of nucleic acid abundance measurement on different days within individual test sites, between test sites, and between different preparations of libraries, b) inter-platform concordance with TaqMan qPCR (MAQC-I study) and traditional RNA-sequencing library preparation using Illumina NGS kits (SEQC study), as well as c) reduced number of sequencing reads required for quantification.
5.3 Methods:

5.3.1 Competitive Amplicon Library Preparation Concept

A schematic of the workflow and data analysis for competitive amplicon library preparation is depicted in Figures 1 and 2, with a detailed description provided in the following Method sections, and animated schematic depicting the core concepts in Animation S1.

5.3.2 Samples

5.3.2.1 Genomic DNA Test and Normalization Material. Genomic DNA (gDNA) was extracted by FlexiGene DNA kit (Qiagen), quantified using commercially available genomic DNA qPCR reagents (Accugenomics, Wilmington, NC), and diluted to a stock concentration of 100,000 genomic copies per µL in dilute TE buffer (10 mM Tris-Cl, pH 7.4, 0.1 mM EDTA). Ethics statement: Peripheral whole blood (20 ml) was obtained by phlebotomy from subject ID 723 at the University of Toledo Medical Center (UTMC) under the University of Toledo Biomedical Institutional Review Board (IRB) approved and confirmed protocol number 105081. Both written and oral consent were obtained.

5.3.2.2 RNA Reference Materials. Ten micrograms each of samples A-D reference RNA materials at a concentration of 1 µg/µL were obtained from the FDA sponsored SEQC project (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm) [2]. Sample A comprised Stratagene Universal Human Reference RNA (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) mixed with Ambion External RNA Controls Consortium (ERCC) Spike-In Control RNA mix 1 (Life Technologies, Grand Island, New York, USA). Sample B comprised Human Brain
Reference RNA obtained from Ambion mixed with ERCC Spike-In Control RNA mix 2. ERCC mixtures 1 and 2 were at a final concentration of 2% in samples A and B, respectively, based on total RNA concentration. Each of the two ERCC RNA spike-in mixes contain the controls spanning a range greater than $10^6$, but in different formulations. Each formulation contains the same four subgroups of controls but for each of the four subgroups there is a fold-difference in concentration between mix 1 and 2; 0.5x, 0.67x, 1.0x and 4.0x-fold respectively ([Dataset S1,2]). After mixing samples A and B with ERCC mixes 1 and 2, these were combined in 3:1 and 1:3 proportional mixtures to create samples C and D, respectively. Thus, samples A-D represent a complex mixture of synthetic (ERCC controls) and endogenous RNA targets in known proportions over a dynamic range greater than $10^6$ and $10^7$, respectively.

### 5.3.3 Assay Target Selection

The MicroArray Quality Control (MAQC) consortium (now known as SEQC) previously selected a list of 1,297 genes to evaluate performance of multiple qPCR and microarray platforms [2]. From this list, 150 endogenous targets were selected to develop assays for competitive amplicon library preparation ([Dataset S1]). These 150 assays were chosen, in part, because the gene targets they represent were previously demonstrated to be expressed over a greater than $10^7$ dynamic range across samples A and B. In addition, assays were developed for 28 of 92 ERCC targets ([Dataset S1]). These 28 targets were chosen because they: a) also are present across a large dynamic range (>10$^6$) within ERCC formulation mixes 1 and 2, and b) were distributed evenly among the 4 subgroups that exhibit known fold differences in abundance between mix 1 and 2; 0.5x, 0.67x, 1.0x and 4.0x-fold differences [24].
5.3.4 Reverse Transcription of RNA Reference Materials

5.3.4.1 Reverse Transcription 1 (RT1). For each of samples A-D, two separate 2 µg aliquots of RNA were reverse transcribed in 90 µL volume reactions each using Superscript III reverse transcriptase (Life Technologies) and oligo(dT) priming according to manufacturer’s protocol. After RT, the two 90 µL cDNA products for each sample were combined into a single 180 µL volume.

5.3.4.2 Reverse Transcription 2 (RT2). For sample A, an additional set of two separate 2 µg aliquots of RNA were reverse transcribed in 90 µL volumes and combined. This separate preparation of sample A was used for comparison of inter-library preparation effects.

5.3.5 Reagent Design

5.3.5.1 Primer Design and Synthesis. Forward and reverse PCR primers were designed corresponding to 101-bp amplicon regions for each of 150 uniquely transcribed genes in the human genome as well as 28 ERCC targets (Dataset S1). Each forward and reverse primer set was designed with a uniform 68°C melting temperature using Primer3 software [25]. Primers with high predicted specificity were selected using GenomeTester 1.3 with human reference genome version 19 to predict off-target amplicons less than 1000 bp in size [26]. Each primer was designed with a universal tail sequence not present in the human genome for multi-template PCR addition of barcode and platform specific sequencing adapters. The forward universal tails were identical to sequence adapters used previously for arrayed primer extension (APEX-2) [27], while the reverse tail sequence was the same as the forward with the exception of the four 3’ bases, enabling directionality during sequencing (Dataset S1). Target specific primers with universal tails for the 150
endogenous targets and 28 ERCC targets were synthesized by Integrated DNA Technologies (IDT) and Life Technologies, respectively. Separate primer pools for all endogenous targets or all ERCC targets were created by combining synthesized primers in equimolar ratio and diluting to a final working concentration of 50 nM of each primer in dilute TE buffer.

5.3.5.2 Competitive Internal Standard Mixture Design and Synthesis. Each 101-bp competitive IS was designed to retain target specific priming sites identical to its respective native nucleic acid template (Figure 1A and Dataset S1). Internal to these identical priming sites were six nucleotide changes that enabled differentiation between a competitive IS and its corresponding NT during post-sequencing data analysis (Figure 1A). Six nucleotide changes were empirically determined to eliminate post-sequencing data alignment errors of NT versus competitive IS, or vice versa. The 28 competitive IS corresponding to ERCC targets were synthesized by Life Technologies and the 150 competitive IS corresponding to the endogenous targets were synthesized by IDT.

For the 28 competitive IS templates corresponding to ERCC targets, each standard was separately amplified with forward and reverse primers (without universal sequences), column purified (QIAquick PCR purification kit), visualized, and quantified for abundance of full-length templates on an Agilent 2100 Bioanalyzer using DNA Chips with DNA 1000 Kit reagents according to manufacturer’s protocol (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Quantified standards then were combined at UTMC in a 1:1 stoichiometric molar ratio, to create a stock concentrated mixture of IS.

The concentration of the competitive IS template for each of the 150 endogenous targets was measured by optical density (OD) at IDT. The IS then were combined into an
IS mixture by IDT in a 1:1 stoichiometric molar ratio based on OD measurements. The concentration of each full-length IS in the mixture was empirically determined at UTMC by cross-titrating the competitive IS mixture relative to a constant 100,000 copies (i.e. genome equivalents) of subject ID723 gDNA (Figure 1B). We reasoned that, in gDNA from a phenotypically healthy individual, the majority of endogenous target loci would be in a 1:1 proportion to each other, providing a practical and the cost-effective reference material to determine the “actual” concentration of full-length amplifiable copies for each endogenous target competitive IS template.

The endogenous target IS mixture and ERCC target IS mixture were each serially diluted to working concentrations and used in all subsequent experiments to quantify copies of each transcript in reverse transcribed samples A-D (Figure 1).

5.3.6 Competitive Amplicon Library Preparation

5.3.6.1 Reaction Components. A targeted PCR amplicon sequencing library was prepared from each sample for analysis on an Ion Torrent PGM sequencer using competitive multiplex-PCR (http://ioncommunity.lifetechnologies.com/docs/DOC-7293). For the each library, a 10 µL reaction volume was prepared containing: 1 µL of ID 723 gDNA or sample A-D cDNA, 1 µL of competitive IS mixture at varying input concentrations, 1 µL of corresponding primer-mix containing universally tailed target-specific primers, 1 µL of 2 mM dNTPs, 1 µL of 10x Idaho Technology reaction buffer with 30 mM MgCl₂, 0.1 µL of Promega GoTaq Hot Start Taq polymerase (5U/µL) and 4.9 µL of RNAse free water (Figure 1). For the quantification of each IS in the 150 endogenous target IS mixture, genomic DNA was spiked into 10 separate competitive multiplex-PCR mixtures containing a) 3-fold serial dilutions, ranging in abundance from 2x10⁷ to 1x10³ copies of IS for each
gene, and b) primers for the 150 genes. For quantification of each endogenous target in samples A-D cDNA, RT1 and RT2 cDNA from sample A and RT1 cDNA from samples B, C and D were each spiked into 12 separate competitive multiplex-PCRs containing a) three-fold serial dilutions of IS mixture, ranging in abundance from $6 \times 10^7 - 3.4 \times 10^2$ copies loaded and b) primers for the corresponding genes. A total of 12 µL of each cDNA sample was consumed for endogenous gene analysis, corresponding to 264 ng of RNA for each sample. For analysis of the 28 ERCC targets, RT1 cDNA from samples A-D were each spiked into five PCR reactions containing a) dilutions of IS mixture representing $10^6$, $10^5$, $10^4$, $10^3$ and 300 copies loaded for each of the 28 ERCC targets and b) corresponding primers. A total of 5 µL of each cDNA sample was consumed for ERCC target analysis, corresponding to 110 ng of RNA.

5.3.6.2 Thermal-Cycling Parameters. As the number of targets in multiplex PCR is increased it is necessary to decrease the concentration of primers used in order to obtain successful first round amplification for all targets [17]. The predominant effect of decreased primer concentration in multiplex PCR is to limit amplicon product formation for the most abundant targets at plateau phase which, in turn, prevents dNTPs from becoming a limiting reagent for less abundant targets [28]. The result is that target templates that vary widely in starting concentration converge toward equimolar concentration at PCR endpoint and this reduces over-sampling or -sequencing of high-abundance targets. Because each target is measured relative to a known number of copies of its respective competitive IS, information regarding the initial proportional representation between native templates is preserved (Figures 2, 3 and Animation S1). However, there is a limit to how much the primer concentration can be diluted and still
result in observable amplification product. Dilution of primers, and therefore convergence of template amplicons, was maximized using touchdown PCR and primers with high primer melting temperature [29]. To compensate for low primer concentration and high primer melting temperature, initially high annealing temperatures were incorporated during the earlier cycles of PCR to increase stringency of primer binding and reduce off-target annealing. In subsequent cycles annealing temperature was gradually lowered resulting in increased yield once specific amplicon product was sufficiently formed during earlier higher stringency cycles. Using this framework we developed the following protocol: Each competitive multiplex reaction mixture was cycled in an air thermal cycler (RapidCycler; Idaho Technology, Inc. Idaho Falls, Idaho) for a total of 45 cycles under modified touchdown PCR conditions with low primer concentration: 95°C/3 min (Taq activation); 5 cycles of 94°C/30 sec (denaturation), 72°C/4 min (annealing), and 72°C/15 sec (extension); repeat 5 cycles with annealing temperature decreased 1°C to 71°C; iterate 1°C decrease and 5 cycles until annealing temperature was 64°C. Use of a hot start protocol was absolutely necessary under these conditions to avoid off-target priming that would otherwise result in formation of only primer-dimer products.

5.3.6.3 Addition of Barcodes and Sequencing Adapters. A sample from each of the competitive multiplex-PCR reactions was labeled using a unique set of barcode primers. A set of fusion primers containing the barcode sequences and Ion Torrent amplicon sequencing adapters were designed with 1) their 3’-end complementary to the universal APEX-2 sequence tails added during competitive multiplex-PCR, 2) 5’ to that a four nucleotide index/barcode sequence, and 3) 5’ to that, a forward or reverse Ion Torrent amplicon sequencing adapter (Dataset S1). Forward and reverse sequencing primers were
tailed with different barcodes to dual index each sample and reduce likelihood of false-indexing/barcoding a sequence read [30]. For each barcoding reaction, a 10 µL reaction volume was prepared containing: 1 µL of competitive multiplex-PCR product, 1 µL of 1 uM forward and reverse barcoding primer, 1 µL of 2 mM dNTPs, 1 µL of 10x Idaho Technology reaction buffer with 30 mM MgCl₂, 0.1 µL of Promega GoTaq Hot Start Taq polymerase (5u/µL) and 4.9 µL of RNAse free water. Each barcoding reaction was cycled in an air thermal cycler (RapidCycler; Idaho Technology, Inc. Idaho Falls, Idaho) under the following conditions: 95°C/3 min (Taq activation); 15 cycles of 94°C/5 sec (denaturation), 58°C/10 sec (annealing), and 72°C/15 sec (extension). Reaction vessels were immediately removed and kept at 4°C during all subsequent steps. The goal during this step was to prevent heterodimerization of barcoded amplification product. Depending on the type of heterodimerization, post-sequencing alignment errors can arise from false base calls during sequencing with resultant decrease in measurement precision and accuracy. These false base calls occur because a single sequencing bead will be populated by two fairly similar heterodimerized templates during emulsion PCR step of Ion Torrent bead preparation (e.g. NT and IS heterodimer for same gene target).

5.3.6.4 Sample Multiplexing. Each uniquely barcoded competitive amplicon sequencing library then was individually quantified on an Agilent 2100 Bioanalyzer using DNA Chips with DNA 1000 Kit reagents according to manufacturer’s protocol (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The sequencing libraries then were mixed in a known stoichiometric ratio to optimize the percentage of sequencing reads that each library would eventually receive; in most cases 1:1 was used.
5.3.6.5 **Product Purification and Sequencing.** It is critical during the purification of barcoded sequencing libraries not to use strong denaturants or chaotropic salts, such as guanidine hydrochloride or thiocyanate. These agents result in downstream template heterodimerization that can result in false base calls and subsequent post-sequencing alignment errors for Ion Torrent sequencing (unpublished studies). For this reason, each mixture of barcoded sequencing libraries was purified using Life Technologies E-Gel SizeSelect 2% Agarose gels, which do not require the use of denaturants or chaotropic salts, and can easily be run in a refrigerated room to prevent heat denaturation during electrophoretic separation. Purified sequencing libraries then were quantified using the Kapa Library Quantification kit for Ion Torrent sequencing platforms (Kapa Biosystems). Based on this quantification, libraries were diluted appropriately and prepared for Ion Torrent PGM Sequencing service according to manufacturer’s recommendations at the University of Toledo Medical Center (UTMC) Department of Pathology, Toledo, OH, and Ohio University (OU) Genomics Facility, Athens, OH.

**5.3.7 Data Processing**

**5.3.7.1 FASTQ File Processing.** UTMC and OU Ion Torrent PGM Sequencing services provided raw sequencing data from Ion Torrent Analysis Suite 3.0 in FASTQ format. Sequencing reads greater than 150 bases in length were extracted and each read was parsed into 3 separate FASTQ files: **1)** forward *(query-barcode.fastq)* and **2)** reverse barcode *(query-revbarcode.fastq)* regions (first and last 14 bases of each sequencing read, respectively), as well as **3)** central portion of the amplicon (60 bases) *(query-subject.fastq)* corresponding to the region internal to target specific priming sites where six nucleotide substitutions exist between NT and matching competitive IS.
5.3.7.2 BFAST of Sequences Against Index Databases. Each of the three FASTQ files was aligned with FASTA databases (Dataset S3) corresponding to whether it was a barcode (barcode.fa) or amplicon region (subject.fa) using the BLAT-like fast accurate search tool (BFAST, version 0.7.0a), with file output in sequence alignment/map (SAM) format [31]. Input parameters are outlined in supplementary methods. BFAST match against the index databases and SAM file output was performed for the trimmed FASTQ files containing 1) forward barcode, 2) reverse barcode and 3) captured amplicon subject sequences.

5.3.7.3 Binning of Sequence Counts. Each of the three SAM files from 1) forward and 2) reverse barcode, and 3) amplicon region then were merged into a practical extraction and reporting language (PERL) hash table using the sequence read ID as a key for matching (http://www.perl.org/). The PERL scripts for this data processing step are available upon request. Based on barcode and amplicon alignment, each sequencing read was binned into an array corresponding to the IS input concentration for a given sample preparation (Figure 1), and whether it was called as a NT or IS by BFAST alignment. If the forward and reverse barcode alignment calls did not match, the sequence read was not binned. The resulting hash table of binned sequencing reads was output in comma delimited format (Dataset S2,S4) and processed as outlined in Statistical Methods section.

5.3.8 Statistical Methods

5.3.8.1 Estimate of Native Target Concentration. For each gene target and technical replicate with input concentration of each IS mixture indexed with the subscript $i$, an estimate of the concentration of the native target ($NC_i$) was calculated based on the observed/binned sequence counts of both the native target ($NT_i$) and internal standard ($IS_i$),
as well as the known starting concentration (in units of template copies per library preparation) of the internal standard (SC\textsubscript{i}) (Dataset S2,S4):

\[
\log_{10} NC_i = \log_{10} \frac{NT_i}{IS_i} + \log_{10} SC_i
\]

Systematic variation in pipetting of NT and/or competitive IS mixture was identified as a systematic increase or decrease in NC\textsubscript{i} for all templates for that dilution point technical replicate compared to expected from surrounding dilution point estimates. The median of the systematic difference from expected measured between input concentrations of IS mixture was subtracted from NC\textsubscript{i} to arrive at the corrected NC\textsubscript{i}.

Summarization of the estimates across the technical replicates (NC\textsubscript{j}) provided the estimate of the NT concentration (Figure 1). Four summarization methods (average, median, least squares, and weighted least squares) were evaluated to estimate the target concentration. The percent variance explained (R\textsuperscript{2}) was used as the objective criterion to compare the four methods across a range of the following quality control (QC) parameters corresponding to each transcript: 1) minimum number of sequence counts for an acceptable NT\textsubscript{i} or IS\textsubscript{i} measurement; and 2) the inter-replicate coefficient of variation (CV) across NC\textsubscript{i}. This search across methods and QC parameter sets was conducted to identify an optimal combination that maximized both the R\textsuperscript{2} measure and the number of transcripts retained. This empirical search determined that an optimal method and QC parameter combination for estimating the summarization quantity was, 1) the median (NC\textsubscript{median}) of NC\textsubscript{j} technical replicate measures that have, 2) at least 15 sequencing counts for both NT\textsubscript{i} as well as IS\textsubscript{i}, and 3) coefficient of variation (CV) across NC\textsubscript{i} of less than 1.00 on a base 10 logarithm scale.
5.3.8.2 Methods to Assess Agreement between Estimates of NC\textsubscript{median}. The summarized NC\textsubscript{median} value for each transcript was compared across different laboratories (e.g. sample A, OU vs. UTMC), days (e.g. sample A, Day 1 vs. Day 2), library preparations (sample A, RT1 vs. RT2), and observed versus expected estimates based on the known mixtures of samples A and B in samples C (75% A and 25% B) and D (25% A and 75% B). Bland-Altman difference plots were used to assess agreement of estimates of NC\textsubscript{median} and those obtained from known values (for the ERCC samples) and alternative RNA transcript measurement methods (TaqMan qPCR and traditional RNA-sequencing library preparation using Illumina NGS kits [Dataset S5]) [32]. Briefly, difference plots display the differences between estimates given by two methods. For this particular application, differences were plotted on the base 10 logarithm scale. Along with the difference plots, scatter plots of the data and corresponding R\textsuperscript{2} values (percent variance explained) from linear models also are displayed. In addition, the area under the receiver operating characteristics (ROC) curve (and corresponding 95% confidence interval) was calculated to assess accuracy in the detection of fold differences (fold changes of 1.10, 1.25, 1.50, 2.00, and 4.00) of ERCC controls known to exist between samples A and B, as well as their derivative mixtures resulting in samples C and D. Results for fold-change ROC curve analysis were binned across differential ratio subpools of pairwise comparisons: 1.1-fold change [1.05 – 1.174] (controls n=100, tests n=96); 1.25-fold change [1.175 – 1.374] (controls n=163, tests n=163); 1.5-fold change [1.375 – 1.74] (controls n=229, tests n=227); 2.0-fold change [1.75 – 2.49] (controls n=229, tests n=223); \geq 4.0-fold change [2.5 – 10.0] (controls n=286, tests n=290).
5.3.8.3 Figures. Pairwise comparison plots, Bland-Altman difference plots and ROC curve plots and associated summary statistics were all generated using GraphPad Prism version 6.0. Pairwise comparison linear regression analysis and was performed using a least squares best fit with GraphPad Prism version 6.0. Spearman’s rank correlation analysis was performed using GraphPad Prism version 6.0.
5.4 Results:

5.4.1 Performance Testing of Competitive Amplicon Library Preparation

5.4.1.1 Performance with gDNA. Among the endogenous gene targets, 82% (123 of 150) of designed assays for competitive amplicon library preparation produced one or more valid native target to internal standard (NT:IS) ratio measurements using ID 723 genomic DNA (gDNA) test material (Figure 1B and Table S1). For those assays with at least triplicate measurements, the ratio of NT:IS sequencing reads decreased in direct proportion to increasing amounts of IS placed into the library preparation (average slope = -1.01x) and at each titration point the inter-gene variation among NT:IS ratios was close to a 1:1 relationship (Figure 1B).

Of the 27 assays with no measurement, 26 had too few sequencing reads consistent with low primer efficiency, and only one (1) assay failed due to unacceptable analytical variation as defined in the Statistics section of Methods.

5.4.1.2 Performance with cDNA. Of the 123 assays that produced valid measurements in gDNA, some did not produce the valid measurement in one or more cDNA samples due to low transcript expression and/or low sequencing counts (Table S3). Among the four cDNA samples (A-D), a valid measurement was obtained relative to at least one IS concentration for an average of 100 (96 – 107; samples A-D) of the 123 working endogenous target assays (Table S1). For the endogenous targets measurable in cDNA, the ratio of NT:IS sequencing reads was decreased in direct proportion to increasing amounts of IS placed into the library preparation (average slope = -0.95x) (Figure 1B), as was observed for measurement of gDNA. However, because endogenous cDNA targets
were expressed over a >10^7-fold range, inter-gene variation among NT:IS ratios at each IS cross-titration point was much greater than that observed with gDNA (Figure 1B).

Among the ERCC targets, 26 of 28 designed assays produced one or more measurements in samples A-D (Table S1). For one assay (ERCC 46), sequencing reads were too few for both NT and IS across samples A-D indicating low PCR efficiency, and only one assay (ERCC 96) failed due to failure to titrate NT:IS. For the 26 successful assay measurements, the average slope of cross-titrating NT with competitive IS was -1.02x (Figure 1B).

5.4.2 Competitive Amplicon Library Preparation Reduced Oversampling in RNA-Sequencing

Dynamic range of a method can be defined as the fold difference from highest to lowest measurable value. The observed dynamic range measured as transcript copies per competitive amplicon library preparation was 2.7\times10^6-fold for the 26 ERCC targets and 6.2\times10^7-fold for the approximately 100 endogenous cDNA targets assessed across samples A-D. The number of sequencing reads required to sequence all targets at least fifteen times was 2.3\times10^4 and 1.4\times10^5, respectively, for the same 26 ERCC and 100 endogenous cDNA targets (Figure 3). We chose fifteen (15) sequencing reads as a benchmark for quantification because it represents sufficient sampling of a given target to enable the detection of a 2-fold change in abundance between targets with a type 1 error rate of less than 0.05, and a type 2 error rate less than 0.20. It is important to note that it is assumed that the precision of quantification in RNA-sequencing, targeted or not, is directly proportional to the number of sequencing reads a given transcript or amplicon has, and that
the sampling of low abundance targets fits a Poisson distribution. This assumption may not be true under some conditions as discussed below [24].

For “traditional” RNA-sequencing library preparation methods, the starting proportionality among native targets must be maintained during library preparation in order to achieve reproducible quantification. Assuming that this proportionality is maintained, the total number of sequencing reads required for traditional RNA-sequencing can be calculated as the sum of sequencing reads of all targets that is required to sequence the least abundant target at least fifteen times. In a simplified example of this calculation, if two transcript targets of interest are expressed over a 1 million fold dynamic range, 15 million plus 15 sequencing reads (15,000,015 sequencing reads) would be required in each traditional RNA-sequencing library preparation technical replicate to have sufficient statistical significance to detect a two-fold change in transcript abundance between targets. This summated number of required sequencing reads is much larger when more than two transcript species are present in a given sample. As written above, clarification is required, as it is somewhat difficult to compare sequencing reads obtained from traditional RNA-sequencing versus PCR-based targeted RNA-sequencing approaches. The distinguishing features are that the former has a component of transcript length and percentage of transcript sequenced by at least a certain number of reads (i.e. sequencing coverage), while the latter targeted PCR approach simply requires counting the number of times a specific targeted region is sequenced (i.e. sequencing reads). As an example of the differences, in traditional RNA-sequencing a single RNA molecule of 5 kilobases in length may be fragmented into 50 separate ~100 base length RNA molecules that when processed through ligation steps and sampled with sequencing may not exhibit a similar sampling distribution
as smaller RNA molecules. In contrast, for targeted RNA-sequencing with PCR-based library preparation, transcript length should not impact sampling distribution phenomena because fragmentation is not typically involved. This difference may account for some of the deviations from Poisson distribution sampling laws previously observed in traditional RNA-sequencing methods [24] versus observations of a good fit to Poisson sampling in our targeted approach (Figure 4C). Thus, the authors recognize there are a number of limitations in a direct comparison between our method, traditional RNA-sequencing, and some targeted RNA-sequencing approaches that still require native template fragmentation. That being stated, a careful comparison of sequencing counts between methods is still possible.

Based on assumptions outlined above, for traditional RNA-sequencing library preparation methods the calculated number of reads required to sequence all targets at least fifteen times was 1.5x10^8 and 2.3x10^9, for the 26 ERCC and 100 endogenous cDNA targets respectively (Figure 3B). This approximation is based on the sum of measured transcript copies using competitive amplicon library preparation method with the least abundant target sequenced at least 15 times. Notably, these calculations closely approximate the actual sum of sequencing reads (~5.0x10^9) required to sequence the same 100 endogenous targets at least fifteen times each using traditional RNA sequencing methods in preliminary SEQC project study data (Dataset S5). For our comparative uses of the SEQC preliminary data, it was not relevant if the 15 sequencing reads were in the same location of a specific transcript or 15 sequencing reads distributed across the length of a transcript; they only needed to be at least 15 read counts with sequence unique to that transcript. Thus, compared to traditional RNA-sequencing, targeted quantitative RNA-sequencing with
competitive amplicon library preparation decreased required sequencing reads from $1.5 \times 10^8$ and $2.3 \times 10^9$ to $2.3 \times 10^4$ and $1.4 \times 10^5$, respectively, for the 26 ERCC and 100 endogenous cDNA targets; an average decrease in required reads of $1.15 \times 10^4$-fold ($6.9 \times 10^3$ to $1.6 \times 10^4$) for transcripts expressed over a $>10^6$ fold dynamic range (Figure 3B). This observed reduced sequencing read requirement compared to traditional or other targeted quantitative RNA-sequencing methods can be primarily attributed to the intentional convergence (i.e. normalization) of abundance among amplicons during plateau phase of competitive multiplex PCR-driven library preparation.

5.4.3 Performance of Competitive Amplicon Library Preparation with ERCC Reference Materials

There was high correlation ($R^2=0.94$; Figure 4A) between expected ERCC target copy concentration, based on Ambion reported input ERCC target concentrations (Dataset S2), and measured copy numbers per library preparation. The observed slope and intercept $(1.00x + 0.50)_{\log_{10}}$ indicated no signal compression and good agreement between measured and expected cDNA molecules based on an assumed 100% efficiency in conversion of ERCC RNA to cDNA during reverse transcription. The median intra-assay ERCC measurement coefficient of variation (CV) was 20% across each sample’s technical replicates and 19% across samples A-D (Table S2). As noted in the methods, samples C and D represent a known cross-mixture of total RNA from samples A and B. Thus measurements made in A and B were used to calculate expected measurements for samples C and D (e.g. $0.75*A + 0.25*B$ to predict C; and $0.25*A + 0.75*B$ to predict D). Based on these calculations, there was excellent correlation, and thus reproducibility, between expected and measured for Samples C and D ($R^2=0.997$) (Figure 4C).
The *intra-sample intra-assay* CV for technical replicates progressively increased as the copies loaded per library preparation decreased below $\sim 10^3$ (Figure 4D). This measured increase in CV corresponds well to expected increase in CV based on a Poisson distribution and the stochastic process of sampling low abundances of cDNA molecules during library preparation. Thus, the majority of assay variance at low copy number is due to sampling variance dictated by natural law, and not platform-specific technical variance.

To evaluate accuracy to detect a given fold-change in abundance between samples, the defined ratios between sub-pools of ERCC controls in mixes 1 and 2, which were spiked into samples A and B respectively at known concentrations, were used as truth in receiver operating characteristic (ROC) curve analysis (SEQC main study). Moreover, since samples C and D represent known cross-mixtures of A and B, the factorial comparison of possible pair-wise tests to detect fold-change between control ($n=1007$) and test ($n=999$) measurements for each sub-pool allowed for fine resolution discrimination analysis of expected and observed fold-change (e.g. sample A vs. B, A vs. C, A vs. D, B vs. C, B vs. D and C vs. D). The ROC analysis area under the curve (AUC) provided a measure of relative accuracy to detect a fold-difference between control and test groups of measurements. Using this approach competitive amplicon library preparation had a 97% accuracy (AUC) to detect a two-fold change in ERCC target abundance between samples, with commensurate increase or decrease in accuracy with higher or lower expected fold-change, respectively (Figure 4E).

### 5.4.4 Performance of Competitive Amplicon Library Preparation for Measurement Endogenous cDNA Targets
The reproducibility of endogenous transcript abundance measurements using competitive amplicon library preparation was assessed using correlation coefficients from pairwise comparisons of endogenous transcript abundance measurements on separate days with the same library and sequencing site (Figure 5, panel A; $R^2 = 0.99$), separate days and sites with the same library (panel B; $R^2 = 0.98$), separate day and library preparation with same sequencing site (panel C; $R^2 = 0.98$) and separate day, site and library preparation (panel D; $R^2 = 0.97$). Analysis of library preparation effects includes separate reverse transcriptions (RT1 and RT2).

Because samples C and D represent a known cross-mixture of A and B, measurements made in A and B, can be used to calculate expected measurements for C and D (Figure 4E,F). Observed correlation ($R^2$) between expected and measured for samples C and D was 0.98 and 0.97 respectively.

5.4.5 Cross-Platform Comparison with Competitive Amplicon Library Preparation Measurements

For the vast majority (~90%) of endogenous target measurements the difference in results measured in this study compared to results previously reported for TaqMan qPCR and for traditional RNA-sequencing library preparation using Illumina next-generation sequencing (NGS) kits (http://www.fda.gov/ScienceResearch/BioinformaticsTools/MicroarrayQualityControlProject/default.htm) was systematic across all samples A-D (Figures S1,S2) [2]. For a smaller fraction (~10%), bias was highly different in sample A versus B for both TaqMan qPCR and traditional RNA-Sequencing with Illumina kit (e.g. gene IDs - BAG1, ELAVL1, SOX15 and others). Moreover, in this smaller subset of assay targets, an intermediate level
of systematic bias was observed in samples C and D compared to A and B. This intermediate level of systematic bias is likely due to the fact that samples C and D are cross-titrations of samples A and B. This trend was not observed in any of the ERCC reference RNA controls assessed (Figure 4B). This finding indicates that a small number of endogenous target assays, for each platform, measured a unique signal specific to sample A or B; most likely cross-platform differences in targeting transcript isoforms, as was noted for ELAVL1 in MAQC I study [2].

Spearman’s rank correlation analysis for samples A and B is significant between competitive amplicon library preparation method and TaqMan qPCR ($r_s = 0.69; p<0.005$) as well as traditional RNA-Sequencing with Illumina kit ($r_s = 0.75; p<0.005$) (Figure 6A,B). For each assay, in each platform, a systematic difference was observed (e.g. MMP2; Figure 6A,B). These observed systematic differences for A and B (Figures S1,S2) were averaged and subsequently subtracted away from the raw reported measurements for TaqMan qPCR or traditional RNA-sequencing measurements for samples C and D. These TaqMan- and Illumina-corrected measurements of C and D were plotted against measurements of C and D obtained with competitive amplicon library preparation (Figure 6C,D). This approach and comparison was taken for several reasons. Principally, there are large systematic measurement differences between the method reported here and TaqMan qPCR or traditional RNA-sequencing in samples A and B (Figures S1,S2). This systematic difference was largely recapitulated in samples C and D. We reasoned that these differences were due to how each platform interpreted the assay signal for each target. As an example, the measured signal for a given target in traditional RNA-sequencing is filtered through a set of biases such as GC content, transcript length, transcript fragmentation...
efficiency during library preparation, ligation of sequencing adapters and so forth. These systematic effects can be large at times [33]. In this inter-platform comparison, we sought to demonstrate, that once these large differences were corrected for, both our method as well as TaqMan qPCR and traditional RNA-sequencing will be largely concordant for absolute transcript abundance measurements in Samples C and D. Based on these pairwise comparisons, we observed a high degree of cross-platform agreement between competitive amplicon library preparation targeted RNA-sequencing with TaqMan qPCR ($R^2=0.96$), as well as Illumina RNA-Sequencing ($R^2=0.94$) for absolute expression measurements. It is highly likely that additional correction for transcript isoform differences could result in a higher degree of concordance, however this transformation was not attempted in order to avoid over-fitting of the data.

The caveat for interpreting data from this inter-platform comparison is to understand that there is not an accepted gold-standard for nucleic acid measurement in complex mixtures of endogenous targets as compared to the more straightforward analysis of ERCC cDNA performed above. This is further compounded by platform differences in targeting of naturally occurring endogenous transcript isoforms which are more likely the rule than the exception (unpublished SEQC studies). Because of this, a comparison of fold-change detected between platforms using endogenous materials is fraught with difficulties in parsing out the effects of the platform, and the resulting intrinsic differences in how each transcript signal is interpreted in each platform. Thus, the most important message in this inter-platform comparison is that there can be a large degree of agreement between platforms’ absolute expression measurements, when systematic bias is accounted for.
5.5 Discussion:

Based on data presented here, targeted RNA-sequencing of competitive amplicon library preparations provides a quality-controlled method suitable for transcript-based biomarker development and implementation, including collection of data suitable for the clinical setting as well as submission to regulatory agencies. Numerous regulatory agencies, consensus groups and investigators have recommended that nucleic acid based *in-vitro* diagnostic devices, if applicable, should include quality controls for non-systematic analytical variation and false negative reporting [15,16]. Of note, competitive IS have been used for more than two decades to provide necessary quality control for PCR-based *in vitro* nucleic acid diagnostic devices [13,22]. Competitive IS use in PCR enables accurate and precise quantification at amplification plateau phase and use of the same competitive IS mixture ensures inter-laboratory concordance of results [17-19]. In this study, we apply competitive IS mixtures to targeted amplicon sequencing of RNA derived templates (Figure 1). Using ERCC reference materials of known abundances and proportions (Dataset S2; Figure 4) [24], we demonstrate excellent reproducibility ($R^2=0.997$) with 97% accuracy to detect 2-fold change (Figure 4). In addition, results support utility of this method in reducing required sequencing reads by driving amplicon abundances (through normalization) toward equimolar proportions at plateau phase of library preparation, resulting in a 10,000 fold reduction in required sequencing reads (Figures 2-3 and Animation S1), without compressing the linear dynamic space for signal measurement by utilizing competitive internal standard reference templates (Figures 4-6).

Consistent with previous reports [2,24], systematic differences from expected measurements were observed (Figures 4AB, S1 and S2). Possible reasons for observed
systematic differences in measurements of synthetic ERCC targets include, but are not limited to: 1) concentrations different from those reported in product documentation due to the multi-step process of creating the ERCC RNA targets in mixes 1 and 2 to prepare samples A-D, 2) systematic differences in reverse transcription efficiency between ERCC targets, and 3) variation introduced during the multi-step process of preparing the competitive IS mixture for ERCC targets used in this study. Observed inter-platform variation in measurement of endogenous gene targets (i.e. competitive amplicon library preparation targeted RNA-sequencing vs. TaqMan vs. traditional RNA-sequencing on Illumina NGS) was largely systematic for each target (Figures S1, S2). One source of systematic variation between methods may be ligation bias exhibited during more traditional RNA-sequencing methods, which can be as large as 1000-fold [33]. One possible source of non-systematic inter-platform variation in measurement of a select number of endogenous assay targets is that different transcript isoforms were assessed by the different platforms. This is likely the mechanism for targets where the direction of variation is different for sample A than it is for sample B as is evident for BAG1, ELAVL1, SOX15 and others (Figures S1, S2) [2] (also observed in preliminary SEQC studies). Another reason for systematic differences may relate to inter-platform variation in RT methods used. Notably, because the majority of differences are systematic, they do not cause inter-platform variation in absolute expression measurement for those assays that are targeting the same transcript isoforms (Figures S1, S2 and 6). The study here chose to compare accuracy to detect fold-change in ERCC targets, but not for endogenous cDNA targets. The reasoning here is that for endogenous RNA targets, no gold-standard for truth in measuring fold-change has been established (SEQC preliminary results main study).
However, a comparison between the fold-change observed with a method, and that expected based on the known composition and abundance of each ERCC target, is a very good measure of analytical performance (Figure 4).

For routine molecular diagnostic tests that measure nucleic acids, it is important to have well-characterized set of quality-controlled assays that focus on a specific set of clinically relevant questions. The method presented here reduces the data complexity and costs in a number ways, making it easier for the implementation of focused quantitative sequencing panels in the clinical setting. The chief advantage of this method relative to a typical qPCR clinical diagnostic is reduced cost, qualitative sequencing information and simultaneous measurement of a large number of targets per technical replicate with minimal sample usage. The authors do recognize the need, at times, for whole-transcriptome sequencing for discovery of the occasional clinically relevant transcript alteration that is not routinely assayed. However, in this study we chose to address a separate but equally important need for a method, which enables reproducible quantitative sequencing panels that cost-effectively assess routine clinical entities.

The cost and time for synthesizing competitive IS mixtures is coming down rapidly due to array based synthesis methods [34], as is sequencing cost per read. These trends will enable routine, cost-effective development and implementation of large competitive amplicon sequencing panels. Further, the use of competitive IS mixtures may potentially be adapted to even larger scale targeted RNA-sequencing approaches; such as those performed using array- or solution-based bait libraries [3,6], or even molecular inversion capture probes [7].
5.5.1 Conclusions:

We describe competitive multiplex PCR amplicon library preparation for targeted quantitative RNA-sequencing, which 1) provides quantitative transcript abundance data sets for selected gene targets that are concordant across days, library preparations and laboratories, and 2) reduces sequencing reads required for transcript abundance quantification by more than 10,000-fold.
5.6 Figure Legends:

Figure 5.1 Competitive amplicon library preparation workflow and data analysis overview.

a) Competitive amplicon library preparation workflow. Internal standard (IS) is a single-stranded or double-stranded DNA template that a) is homologous to a specific native target (NT) at the primer sequences (green shaded regions) and therefore competes for amplification with the NT but b) contains one or more base substitutions (depicted by asterisks) internal to the primer sites and therefore is distinguishable from the NT during post-sequencing data analysis. The corresponding IS template for each native target is in a fixed relationship relative to the IS for the other genes in an internal standard mixture, and the concentration of each IS in the mixture is known. The native sample and competitive internal standard mixture are combined in varying ratios and processed according to the flow diagram (details available in Methods section).

b) Linearity of titration between competitive IS relative to native targets in samples A-D (samples used are described in detail in Methods section). With competitive methodologies, it is important to demonstrate that as the abundance of competitive internal standard mixture changes relative to native material (x-axis), the ratio of measured native material to competitive internal standards changes in direct proportion (y-axis), and that the slope is near to 1.00. The proportional relationship among native targets in the original sample is preserved during amplification and sequencing because i) the competition between each NT and its respective IS enables calculation of the initial concentration for each NT, and ii) the IS are in a fixed relationship relative to each other (Figure 2 and Animation S1). Native targets for which values could not be measured across at least three dilution points are not shown. Upper panel: Linearity
of cross titrating competitive Internal Standard Mixture with the constant amount of
genomic DNA (gDNA) for 123 targets (10 titration points). Dotted lines represent 95% prediction interval for NT:IS ratio values. Middle panel: Linearity of cross titrating competitive Internal Standard Mixture with constant amount of endogenous cDNA native targets from samples A-D (same targets as assessed in gDNA; upper panel) (12 titration points). Lower panel: Linearity of cross titrating competitive Internal Standard Mixture with constant amount of 26 ERCC native targets from samples A-D (5 titration points). Each titration and assay measurement represents a single technical replicate.

**Figure 5.2  Schematic depiction of how competitive amplicon library preparation reduces oversampling.**

a) Depicted are two native targets (NT) within a hypothetical cDNA sample. One NT is in high abundance, $10^8$ copies (“Abundant” NT), while another is in low abundance, $10^2$ copies (“Rare” NT), representing one million-fold difference in abundance between targets. This hypothetical cDNA sample is combined with a mixture of internal standards (IS) with a fixed relationship of concentrations at $10^5$ copies. b) Depicted is the competitive multiplex-PCR library preparation for panel A. The PCR amplification plots for both the “Abundant” and “Rare” NT are separated for purposes of clarity, but occur in the same reaction. During competitive multiplex-PCR, each NT competes equally with its respective competitive IS for dNTPs, polymerase and a limiting concentration of primers. Because the starting concentration of each target’s primer-pair is the same, each competitive reaction will plateau around the same end-point concentration ($\sim 10^9$ copies). c) The equal competition between each NT and respective IS preserves the proportional relationship
between NT in the original sample, allowing for measurement of native target abundance without signal compression (also see Animation S1). Yet, a $10^6$ fold range of templates is reduced to $10^3$ after competitive multiplex-PCR library preparation resulting in a 1,000-fold reduction in oversampling/sequencing of the high abundance target.

Figure 5.3 Competitive amplicon library preparation reduced required targeted RNA-sequencing reads up to 10,000-fold.

Each sample comprising multiple native targets was mixed in multiple ratios with a competitive internal standard (IS) mixture (depicted in Figure 1). The goal is for each combined ratio of native material and competitive IS mixture (Figure 1A) to have a subset of targets in “balance” or equivalence (i.e. as close to a 1:1 relationship of NT:IS as possible). The closer to equivalence, the fewer sequencing reads required across all library preparations to meet criteria outlined in Statistical Methods section for valid measurement of a given target. Preparation of multiple sequencing libraries with competitive IS mixture spiked-in over the range of expression will result in a greater decrease in required sequencing reads than what is obtainable with only one spike-in ratio (as depicted in Figure 2 and Animation S1). a) Actual sequencing reads data for 26 ERCC (n=104) and 100 endogenous (n=400) cDNA targets across samples A-D. X-axis represents the abundance of each target in a library preparation normalized to the lowest abundance target (set to $10^0$). Y-axis is in units of normalized sequencing reads (coverage) required to sequence the lowest abundance target at least once. b) Tabular summary of panel A where the number of sequencing reads represents the sum of all sequencing reads to observe all targets at least fifteen times. As discussed in the results section, fifteen sequencing reads is
sufficient to achieve a type 1 error rate less than 0.05, and a type 2 error rate of less than 0.20. The required number of traditional RNA-sequencing reads is calculated based on an assumed relationship between target copies present prior to library preparation. Fold-reduction in required sequencing reads by competitive amplicon library preparation is the quotient of calculated traditional RNA-sequencing and measured competitive amplicon library preparation sequencing reads.

**Figure 5.4 Performance of competitive amplicon library preparation with ERCC Reference Materials.**

a) Measured signal abundance of ERCC targets in samples A, B, C and D. X-axis units are derived from Ambion product literature for the known concentration of ERCC spike-in controls (n=104). b) Difference plots of data in panel A ordered numerically by ERCC ID. Each ERCC target depicted was measured at least once in all four samples A-D. For purposes of clarity, ERCC-170 is highlighted orange in panels A and B (n=104). c) Samples C and D represent a 3:1 and 1:3 mixture, respectively, of samples A and B. These ratios were used to calculate expected measurements for samples C and D (x-axis). Actual measurements of samples C and D are plotted on the y-axis (n=52). d) Coefficient of variation (CV) in measurements of ERCC targets in samples A-D, for those assays with at least two IS dilution points. Red line depicts expected CV based on a Poisson sampling (n=95). e) ROC curves to detect fold change with the corresponding area under the curve (AUC) with 95% confidence intervals. ROC curves are derived from the comparison of differential ratio subpools of ERCC targets in samples: A vs. B, A vs. C, A vs. D, B vs. C, B vs. D and C vs. D. Results for 1.1-fold change represent a range of differential ratio
subpools [1.05 – 1.174] (controls n=100, tests n=96); 1.25-fold change [1.175 – 1.374]
(controls n=163, tests n=163); 1.5-fold change [1.375 – 1.74] (controls n=229, tests n=227);
2.0-fold change [1.75 – 2.49] (controls n=229, tests n=223); ≥4.0-fold change [2.5 – 10.0]
(controls n=286, tests n=290).

**Figure 5.5** Performance of competitive amplicon library preparation with
endogenous cDNA targets.

a-d) Absolute signal abundance of cDNA targets in sample A in units of copies per library
preparation measured on separate days, at different sites (OU = Ohio University; UTMC =
University of Toledo Medical Center), and between different reverse transcription
preparations (RT1 and RT2). a) Inter-day effect (n=88). b) Inter-day and Inter-site effect
(n=81). c) Inter-day and Inter-library effect (n=92). d) Inter-day, Inter-site and Inter-
library effect (n=80). e-f) Samples C and D represent a 3:1 and 1:3 mixture, respectively,
of total RNA from samples A and B. These ratios were used to calculate expected
measurements for samples C and D (x-axis) from measurements of A and B. Plotted on the
y-axis are actual measurements of samples C (n=86) and D (n=90).

**Figure 5.6** Cross-platform comparison of competitive amplicon library preparation
with TaqMan qPCR and Illumina RNA-Sequencing.

a) Comparison of TaqMan qPCR with competitive amplicon library preparation (n=146)
for samples A and B without correction for systematic biases. Data is normalized to a
median relative abundance. b) Comparison of Illumina RNA-Sequencing with
competitive amplicon library preparation (n=170) for samples A and B without correction
for systematic biases. Data is normalized to a median relative abundance. For a) and b), Spearman’s rank correlation coefficient is noted ($r_s$). The average of differences for measurements of samples A and B between competitive amplicon library preparation and TaqMan qPCR (Supplementary Figure 1) or Illumina RNA-sequencing (Supplementary Figure 2) was determined for each endogenous target. This difference was subtracted from TaqMan qPCR or Illumina RNA-sequencing measurements for samples C and D and plotted (x-axis). Competitive amplicon library preparation measurements of C and D are plotted on the y-axis. c) Comparison of TaqMan qPCR with competitive amplicon library preparation (n=146) for samples C and D with correction for platform and assay specific bias. d) Comparison of Illumina RNA-sequencing with competitive amplicon library preparation (n=170) for samples C and D with correction for platform and assay specific bias.
5.7 Figures

**Figure 5.1**

A

- Mix Native Targets (NT) and Internal Standards (IS) in Varying Ratios
- Sample of Native Targets (e.g. cDNA or gDNA)
- Mixture of Internal Standards
- Example of NT:IS mix ratios
- Multiplex competitive PCR with tailed target-specific primers
- Addition of barcode and NGS platform-specific adapter sequences
- Combine individual library preparations, gel purify and then sequence

**Data Analysis:** Identify and count sequences for each target as the native or internal standard. Extrapolate equivalence concentration where [NT] = [IS], based on ratio of NT and IS sequencing counts for each target (Panel B).

- See Statistical Methods Section for use of data and equations -

**Legend:**

- Target-specific forward or reverse priming sites (identical between Native Target and Internal Standard)
- Synthetic Alleles allows for discrimination of Native Target from Internal Standard
Figure 5.2

A. Native Targets (NT) (e.g. cDNA)
- "Abundant" NT: $10^8$ copies
- "Rare" NT: $10^2$ copies

B. Internal Standards (IS)
- "Abundant" Target IS: $10^8$ copies
- "Rare" Target IS: $10^5$ copies

C. Multiplex Competitive PCR

<table>
<thead>
<tr>
<th>Before PCR</th>
<th>After PCR</th>
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<tr>
<td>Abundant</td>
<td>Rare</td>
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<tr>
<td>NT:IS Ratio</td>
<td>1000:1</td>
</tr>
</tbody>
</table>

Native Target (NT) Copies
- $10^8$ $10^2$ $10^9$ $10^6$

Internal Standard (IS) Copies
- $10^5$ $10^5$ $10^6$ $10^9$

Range of all templates
- $(10^8/10^2) = 10^6$
- $(10^9/10^6) = 10^3$
Figure 5.3

A

Normalized number of reads to sequence all targets once

Normalized abundance of each target

B

<table>
<thead>
<tr>
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<th>Sequencing Reads</th>
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<tr>
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<tr>
<td>Traditional RNA-Seq.</td>
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<tr>
<td>Competitive Amplicon Library</td>
<td>$2.3 \times 10^4$</td>
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<tr>
<td>Fold-Reduction</td>
<td>$6.9 \times 10^3$</td>
</tr>
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</table>
Figure 5.5

(A) Sample A - RT1 - Day 1 - OU (copies per library preparation)

(B) Sample A - RT1 - All Days - OU (copies per library preparation)

(C) Sample A - RT2 - Day 2 - OU (copies per library preparation)

(D) Sample A - RT1 - All Days - UTMC (copies per library preparation)

(E) Sample A - RT1 - Day 1 - OU (copies per library preparation)

(F) Sample A - RT2 - All Days - OU (copies per library preparation)

Observed Sample C Measurement (median normalized abundance)

Expected Sample C Measurement (median normalized abundance)

Observed Sample D Measurement (median normalized abundance)

Expected Sample D Measurement (median normalized abundance)
Figure 5.6

Identify each assay’s platform specific systematic bias in samples A & B (depicted in supplementary Figures 1 and 2), then assess for inter-platform measurement concordance in samples C & D based on these bias corrections.
5.8 Supplemental Table, Figure, Dataset, Method Legends

Table S5.1  Competitive amplicon library preparation qualitative assay performance.

Table S5.2  Coefficient of variation (CV) of ERCC measurements.

Table S5.3  “True negative” calls: competitive amplicon library preparation versus TaqMan qPCR and Illumina RNA-sequencing.

Figure S5.1  Difference plots between TaqMan qPCR and competitive amplicon library preparation based measurements.

Figure S5.2  Difference plots between Illumina RNA-sequencing and competitive amplicon library preparation based measurements.

Figure S5.3  Comparison of two platform: Two color fluorometric real-time PCR and standardized library preparation targeted RNA sequencing

Dataset S5.1  Reagent design.

Dataset S5.2  ERCC targets sequencing counts.

Dataset S5.3  FASTA database index input.

Dataset S5.4  Endogenous targets sequencing counts.

Dataset S5.5  Cross-platform comparison data.

Methods S5.1  BFAST input parameters.
5.8 Supplemental Table, Figure, Dataset, Method

Table S5.1

Supplementary Table 1. Assay Performance

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<thead>
<tr>
<th>ERCC Target Measurements</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valid</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>True negative</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Failed</td>
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<table>
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<tr>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>gDNA</th>
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<tbody>
<tr>
<td>Valid</td>
<td>99</td>
<td>99</td>
<td>96</td>
<td>107</td>
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</tr>
<tr>
<td>True Negative</td>
<td>4</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Failed</td>
<td>20</td>
<td>12</td>
<td>21</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

Assay measurement performance as assessed in samples A-D for ERCC as well as endogenous cDNA targets. Endogenous targets were also assessed against gDNA control (depicted in Figure 1B).

True negative measurements occur when sufficient number of competitive internal standard was sequenced (sequenced at least 15 times), but insufficient native template was observed across all spike-in concentrations of internal standard. An upper limit of expression for these assays can still be calculated as \( \left[ \frac{1}{\text{IS sequencing counts}} \right] \times \text{concentration} \) IS loaded into the library preparation with the lowest IS concentration present. These measurements represent true negative measurements and the lower limit of accurate quantification can be determined from these data. In this way, competitive IS mixtures can control for false negative reporting.

Failed assays are measurements where "sequencing depth was too low" for both the NT and IS. These represent true assay failures (neither native or internal standard was sequenced at least 15 times). Increasing sequencing coverage will recover these assays and provide valid measurements.
Table S5.2

**Supplementary Table 2.** Coefficient of variation (CV) of ERCC measurements.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay; Intra-sample CV</td>
<td>0.20</td>
</tr>
<tr>
<td>Intra-assay; Inter-sample CV</td>
<td>0.19</td>
</tr>
</tbody>
</table>

CV of differences is calculated from data presented in Figure 4. CV = (Standard Deviation of measurements)/(mean of measurements).

Intra-assay Intra-sample CV is calculated from the median of intra-assay CV within each sample A-D.

Intra-assay Inter-sample CV is calculated from the median of intra-assay CV across samples A-D.
Table S5.3

Supplementary Table 3. “True negative” calls: competitive amplicon library preparation versus Taqman and RNA-sequencing.

<table>
<thead>
<tr>
<th>HUGO and Sample ID</th>
<th>CALP</th>
<th>Taqman</th>
<th>RNA-Seq</th>
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<tbody>
<tr>
<td>RPL3L Sample-D</td>
<td>-2.4879</td>
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<td>-2.90</td>
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<tr>
<td>ANXA13 Sample-C</td>
<td>-2.127</td>
<td>-3.20</td>
<td>-2.25</td>
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<tr>
<td>MMP7 Sample-C</td>
<td>-1.7003</td>
<td>-3.27</td>
<td>-2.23</td>
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<tr>
<td>KRT24 Sample-D</td>
<td>-1.6589</td>
<td>-3.00</td>
<td>-2.31</td>
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<tr>
<td>KIAA0101 Sample-B</td>
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<td>-1.64</td>
</tr>
<tr>
<td>KRT24 Sample-B</td>
<td>-1.2609</td>
<td>-2.83</td>
<td>-1.88</td>
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<td>STMN2 Sample-A</td>
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<td>IL18R1 Sample-B</td>
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<td>-2.28</td>
<td>-0.81</td>
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<tr>
<td>DPP4 Sample-B</td>
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<td>IL18R1 Sample-D</td>
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<td>-2.37</td>
<td>-1.10</td>
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<td>KIT Sample-C</td>
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<td>-0.18</td>
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<td>ND</td>
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<tr>
<td>POU1F1 Sample-C</td>
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<td>-3.95</td>
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<td>POU1F1 Sample-B</td>
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<td>RPL3L Sample-B</td>
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</tr>
<tr>
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<td>ND</td>
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<tr>
<td>POU1F1 Sample-A</td>
<td>-1.5431</td>
<td>ND</td>
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<tr>
<td>CYP2C9 Sample-D</td>
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<td>ND</td>
<td>-3.41</td>
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<tr>
<td>ABCB11 Sample-B</td>
<td>-1.1123</td>
<td>ND</td>
<td>-2.38</td>
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<tr>
<td>SERPINE7 Sample-B</td>
<td>-1.0772</td>
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<td>-3.15</td>
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<td>DLG7 Sample-B</td>
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<td>FOXA1 Sample-B</td>
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<td>ND</td>
<td>-2.42</td>
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<tr>
<td>CYP2C9 Sample-C</td>
<td>0.37</td>
<td>ND</td>
<td>-3.20</td>
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<tr>
<td>CYP2C9 Sample-B</td>
<td>0.6952</td>
<td>ND</td>
<td>-2.92</td>
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<tr>
<td>CYP2C9 Sample-A</td>
<td>0.7906</td>
<td>ND</td>
<td>-3.16</td>
</tr>
</tbody>
</table>

26 competitive amplicon library measurements had sufficient data to report back a less than measurement.

Of the 26 measurements, TaqMan reported not detected (ND) for 14, and RNA-Seq reported ND for 1. Because competitive amplicon library preparations could detect IS, but not accurately quantify NT present, these represent False Negative detections for TaqMan and RNA-Seq. Less than measurements were calculated as $[1/(IS\text{ sequencing counts})] \times \text{concentration IS loaded into the library preparation.}$
Supplementary Figure 1. Difference plots between TaqMan and competitive amplicon library preparation based measurements.

Measurement differences are systematic for each endogenous target between TaqMan and competitive amplification library preparation across all four SEQC samples: A, B, C and D.
Supplementary Figure 2. Difference plots between Illumina RNA-sequencing and competitive amplicon library preparation based measurements.

Measurement differences are systematic for each endogenous Target between RNA-Seq and competitive amplification library preparation across all four SEQC samples: A, B, C, and D.
Figure S5.3

Comparison of two platforms
In FFPE Samples Analysis

- Open circle: benign; filled circle: malignant
- Target gene copy number/10^5 ACTB
- 2CF: two-color fluorometric real-time RT-qPCR
- StaR-SEQ: standardized library preparation targeted RNA next generation sequencing
- FFPE: Formalin fixed, paraffin embedded

Between two developed platforms, the result showed a very good correlation coefficient ($r^2=0.974$) and linearity (slope=0.9454). The reason of very good $y$-intercept (0.35) was the use of external standards mixture (1:1 ratio of synthetic native and internal standards templates) for 1) normalization of two platforms (real time PCR and NGS), and 2) normalization of two color probes in real time PCR method. The values used in this plot were target gene copy number over one million of the reference gene (ACTB). Three surgically removed human FFPE samples were used.
Dataset S5.1

doi:10.1371/journal.pone.0079120.s006 (XLS)

Dataset S5.2

doi:10.1371/journal.pone.0079120.s007 (XLS)

Dataset S5.3

doi:10.1371/journal.pone.0079120.s008 (XLS)

Dataset S5.4

doi:10.1371/journal.pone.0079120.s009 (XLS)

Dataset S5.5

doi:10.1371/journal.pone.0079120.s010 (XLS)

Methods S5.1

doi:10.1371/journal.pone.0079120.s011 (DOCX)

Animation S1

doi:10.1371/journal.pone.0079120.s012 (PPTX)
5.10 References:


In a review article by Bustin et al [1], it becomes known the current lack of proper controls in real-time PCR assays,

“If all unknown samples record Ct values of around, say 18–25, and the NTC records a Ct of 39, then it is legitimate to ignore the very high Ct values recorded for the NTC and use the data. ~ The NTC is such a crucial part of a good experimental setup that the requirement for an absolutely negative result cannot ever be compromised. Therefore, we propose that if the Ct values recorded by unknowns are above 33–35, then the NTC must always be negative for any results to be valid. Finally, if the unknowns record Ct values in the region of 37–39, it is important to run the reaction for 45 cycles, to be certain that the NTC comes up negative.”

In this article, Bustin discussed which Ct (threshold cycle, quantification cycle, Cq) could be considered a true positive result. Bustin suggested that any Ct that differed by more than 5 cycles from the no template control (NTC) be regarded as probably not caused by any contaminant, especially when the replicate wells are also record positive,
similar Ct values. Regardless, the reliability of the quality controlled reverse transcription quantitative PCR (RT-qPCR) method is achieved via co-amplification of each analyte to a known number of competitive internal standard (IS) copies amplified in the same PCR assay [2-4]. The application of IS was recommended by regulatory agencies [5-7]. The existing quality controlled and FDA approved tests used IS in a real-time RT-qPCR platform was for a single-gene test using intact viral RNA [8,9]. Previously developed quality controlled RT-qPCR tests targeting for multiple genes that used an internal standards mixture (ISM) was excellent for intact RNA using a capillary electrophoresis platform [10].

The goal of the studies presented in this dissertation was to develop a robust RT-qPCR method for reliable molecular diagnostic testing of formalin fixed paraffin embedded (FFPE) samples on a real-time PCR platform. Although archival FFPE samples are abundant in most hospitals, RNA extracted from FFPE samples are highly degraded, sized limited, and relatively impure because of the process of formalin fixation and paraffin embedment. Therefore, it was necessary to optimize signal amplification and control for inter-sample, inter-gene variation in PCR interfering substances. The results of the studies presented here have demonstrated: 1) a quality controlled two-color fluorometric real-time RT-qPCR test for highly degraded FFPE samples (Chapter 3), 2) an application of two-color fluorometric RT-qPCR for accurate detection and quantification of Viral Hemorrhagic Septicemia (VHS) virus (Chapter 4), and 3) an application of external standards mixture (ESM) enabling absolute and accurate quantifications comparing two different platforms: real-time PCR and next generation sequencing (NGS) (Figure S5.3).
Development of Multiplex Two–Color Fluorometric RT-qPCR for Testing of FFPE Samples

Our test needed two probes labeled with different dyes in a real-time PCR platform: one detected the native template (NT) and another detected the IS. After choosing black hole quencher, BHQplus™ probes [11] among conventional available probes, such as minor groove binding Eclipse™ [12] and locked nucleic acids LNA™ [13], we used two different emission wavelengths with minimal crosstalk for the discrimination of IS from NT in one PCR reaction. Then 4-6 nucleotide alterations in an IS probe and a synthetic IS from the selected NT probe sequence was made for each gene. This number of nucleotide alterations was necessary to improve probe specificity, that is, to ensure that an NT probe binds only to an NT, and an IS probe binds only to an IS template (Figure 3.1, Table 3.1). In the case of E2F1, the IS probe was redesigned with a higher number of altered bases, from 4 to 6 because only 4 altered bases was not sufficient to ensure specificity. With these designs, all probes (NT and IS probes for E2F1, MYC, CDKN1A, ACTB) demonstrated < 1% binding to the non-homologous strand.

When we developed the two-color fluorometric real-time RT-qPCR assay, we performed the analytical validation with synthetic templates (NT and IS) before analysis with FFPE samples. The key difference with our method in the application of IS was that all our IS templates were placed in one mixture to reduce inter-gene variations [3]. Next, validation was conducted to check that our ISM contained an accurate number of molecules for each IS. For each gene, the limiting dilution PCR was done to test the true
accuracy for the IS concentration in the ISM. The observed frequency of the positive results was highly correlated ($R^2 = 0.94$) with the expected positive frequency predicted by Poisson analysis in nine replicate PCR reactions for each gene (Figure 3.2).

After testing linearity (serial dilution of 1:1 of NT: IS and dilution of IS to constant NT and vice versa) and precision (coefficient variation at least triplicate measurements) of the assay, the effect of intentionally perturbing PCR conditions was assessed for test of robustness. Conditions altered included PCR volume and concentration of primer, probe, or EDTA. Changing the volumes and/or the concentrations of primers or probes did not lead to significant differences in expression measurement of MYC or ACTB in an FFPE cDNA with or without pre-amplification (Figure S3.6). On the other hand, as EDTA concentration was increased, Cq values of each of the four analytes (MYC IS and NT, ACTB IS and NT) tested in non-FFPE, pre-amplified, benign lung cDNA were increased, ultimately resulting in no signal (Figure 3.4). However, the MYC NT and ACTB NT values calculated relative to their respective IS were constant, and due to the loss of signal for IS at highest EDTA concentration, no false negative values were reported.

The two-color fluorometric real-time RT-qPCR is an absolute quantification method. To quantify the NT copy number for each gene, the $[\text{NT Cq} - \text{IS Cq}]$ for the unknown sample was obtained. I used this Cq value, delta Cq, in the formula of the $2^{(\text{Cq} - \text{delta Cq})}$. Then, this fold difference was multiplied by the known number of input IS copies used in the initial PCR reaction. However, I found that the data showed high coefficient variations without a proper control. The possible causes could be instability or intensity differences of the two fluorescence probes varying between experiments due to freezing
and thawing of probes or due to lot differences. The effect of variation in fluorescence specific activity was tested on measurement of MYC in benign, non-FFPE lung cDNA. As the labeled probe concentration decreased in the reaction, the Cq increased (Figure 3.5A, C). However, this potential source of analytical variation was controlled by correcting the measured \([\text{NT Cq} - \text{IS Cq}]\) values relative to the mean of two concentrations of ESM \([\text{NT Cq} - \text{IS Cq}]\) values (Figure 3.5B, D). The ESM contained a known 1:1 concentration of synthetic NT and IS for each gene that was constant among experiments. In each 96 well PCR plate, we applied two concentrations of ESM for each gene. Another source of variation could be the selection of the threshold for the generation of Cq from each probe. Whether the threshold was selected through the automatic method or the manual method, there was day-to-day variation in the selected Cq threshold setting (Figure 3.5E). For example, MYC/10^6 ACTB was measured in an FFPE sample cDNA in seven PCR replicates on five different days, and the Cq threshold value automatically selected in each PCR was different, resulting in a high CV of 0.99. However, with the ESM correction, the CV of measured MYC/10^6 ACTB was reduced to 0.32 (Table 3.2). The use of the ESM significantly reduced these sources of inter-experimental variation.

Because FFPE samples yield RNA with typically 100-fold reduced RT efficiency [14], and fine needle aspirate (FNA) cell block FFPE samples yield a small amount of low quality RNA, we used a pre-amplification method that a) enabled multiplex assay of many genes with limited input cDNA, and b) increased signal to background. We incorporated ISM in the pre-amplification reaction to control for interfering substances, variations in PCR efficiency and dilution of pre-amplified PCR products. For the first-
round PCR, a mixture of gene-specific primers with ten-fold less concentration than the second-round PCR was used with 18 cycles. After 1000-fold dilution of pre-amplified product with TE buffer, it was transferred to each gene PCR reaction with each primer and probes and conducted for 40 cycles. Pre-amplification markedly increased signal above the background signal typically observed in the no template control at Cq of 35. Specifically, with one round of PCR (no pre-amplification) the Cq for each NT and IS ranged from 20-35. In contrast, using pre-amplified and 1000-fold diluted samples, the Cq for each NT and IS after a second round of PCR ranged from 11-26. The dilution of the first round amplification product can be reduced if necessary to ensure sufficient signal in the second round for very low input of cDNA. Moreover, results with or without pre-amplification were compared using a surgically removed lung cancer FFPE sample to ensure that pre-amplification yielded reliable data. The lung cancer diagnostic test (LCDT) index value measured with the pre-amplification method was not significantly different from that measured with the no pre-amplification method (Figure S3.6).

In addition to the validation of a pre-amplification method to increase signal, we wanted to increase cDNA yield itself by testing reverse transcription priming methods for RNA extracted from FFPE samples. In FFPE samples, the loss of transcripts’ poly-(A) tails was caused by formalin fixation and monomethylol group binding on adenines. Therefore, random hexamers (RHP) or gene specific primers (GSP) were recommended for RT of FFPE samples [15]. Furthermore, Stanoszek, et al reported that the use of gene specific priming in RT increased the yield of cDNA compared to oligo dT or random hexamer priming with human peripheral blood leukocytes [16]. When we compared
gene specific priming with random hexamer using three surgical FFPE samples, we obtained more than 50-fold higher cDNA with GSP from 1 µg RNA. The RT yield was also increased another 4.6-fold by increasing RNA input to 5 µg.

Fitness of this two-color fluorometric method for analysis of FFPE samples was evaluated in 20 surgical FFPE benign and malignant lung samples by measuring a previously described test for lung cancer diagnosis (LCDT) developed using non-FFPE FNA samples [17,18]. The results support the utility of this optimized method for analysis of FFPE samples. Specifically, imprecision was acceptable, and the optimal cut-off for the LCDT had similar accuracy (93%) in separating benign from malignant compared to that previously reported for fresh samples [17,18]. These results support the conclusion that the method presented here is suitable for use in a clinical validation trial in which the LCDT will be evaluated for the utility to augment cytomorphology in analysis of FNA cell block FFPE samples.

Development of an Accurate Quantification of the Fish Viral Hemorrhagic Septicemia virus (VHSV) with a Two-Color Fluorometric Real-Time PCR.

Viral Hemorrhagic Septicemia virus (VHSV) infects more than 80 species of finfish across the Northern Hemisphere [19]. Cell culture is the VHSV diagnostic test that is approved by the World Organization for Animal Health [20], along with the Fish Health Section of the U.S. Fish and Wildlife Service and the American Fisheries Society [21]. However, the culture process takes one month to confirm the infection and lacks the sensitivity for low viral concentrations with high false negative rates such as 43-95% [22-24]. Real-time RT-qPCR for viral detection and quantification is a fast, sensitive
method with a large dynamic range. Without appropriate quality control, studies demonstrated high false negative rates that ranged from 15–92% [22-24]. There was a need for the development of a quality controlled method for VHSv detection and quantification. Therefore, with the collaboration of Willey group and Lake Erie Center, an assay was developed to accurately detect and quantify VHSv using the Agilent Bioanalyzer with standardized reverse transcription PCR (StaRT-PCR) [25]. StaRT-PCR assays improved accuracy and prevented false negative results with the use of internal standards. However, this method is based on capillary electrophoresis, a method less popular than a real-time PCR platform. Meanwhile, we developed a two-color fluorometric real-time PCR for highly degraded FFPE samples. We wanted to apply this new method for VHSv detection and quantification. For that, primers, probes, synthetic NT and IS were designed for detection of N-gene in VHSv–IVb and ACTB (reference gene) in fish. After the analytical validation of the assay showed good specificity, accuracy, linearity and precision (Chapter 4), the two-color fluorometric real-time PCR method was compared to SYBR® green RT-qPCR, cell culture, and capillary electrophoresis StaRT-PCR with wild-caught and laboratory infected fish samples.

False negative reports of fish with low levels of infection were prevented by the use of two assay characteristics: 1) pre-amplification and 2) application of IS. The determination of positive PCR reactions depended on NTC PCR reactions. In the case of the late amplification of VHSv (Cq at 33-35) caused by low levels of virus, the result is ambiguous in confirming assay positivity because of background amplification in NTC reactions. Thus, further experiments, such as cell culture, may be need for accurate diagnosis. Pre-amplification was conducted with internal standards for 15 cycles, and
then PCR products were 1000-fold diluted. The second amplification signal was detected at least 10 Cq values earlier than signals obtained with no pre-amplification, thus enhancing the discrimination of target signals from the NTC background. Furthermore, with IS amplification in each PCR reaction, the absence of IS signal demonstrated PCR reaction failure rather than a false negative result. Both two-color fluorometric real-time PCR assay and capillary electrophoresis StaRT-PCR distinguished identical positives and negatives, and were free of false negatives (Figure 4.5). In contrast, the cell culture results showed a 56% false negative error and SYBR® green results demonstrated a 33–44% false negative error.

**Use of ESM in Two Different Platforms: Real-Time RT-qPCR and Next Generation Sequencing**

When Blomquist, et al developed a competitive multiplex-PCR library preparation method for targeted RNA-sequencing, Blomquist completed a cross-platform comparison with TaqMan qPCR and Illumina RNA-sequencing (traditional NGS) (Figures S5.1, S5.2, 5.6). For each assay, in each platform, a systematic difference was observed. Those observed systematic differences were averaged and subtracted away from the raw reported measurements for TaqMan qPCR or traditional RNA-sequencing measurements. Thus, results in Figure 5.6 C and D demonstrated normalized data after bias corrections. Consequently, a high degree of the cross-platform agreement between the competitive amplicon library preparation targeted RNA-sequencing with TaqMan qPCR ($R^2=0.96$), as well as Illumina RNA-Sequencing ($R^2=0.94$) for absolute expression
measurements was observed. The purpose of this comparison was a demonstration of reliability of the new developed method with existing methods. However, for the usual experiment, this normalization process will not be used, and individuals who want to compare platforms prefer a relative quantification method (fold changes) [26]. As each platform has its own biases, if normalization is conducted differently for the two platforms, such as TaqMan and NGS, there will be more chance of variations.

Like the comparison with TaqMan qPCR, we compared the competitive amplicon library preparation NGS method to the two-color fluorometric real-time RT-qPCR method. ESM (1:1 ratio of synthetic NT: IS) was developed for the two-color fluorometric real-time PCR method to control for the fluorescence differences between the two probes or for the variation of Cqs selection for two probes. In this comparison, the same ESM provided a known number of standard molecules for two platforms enabling an absolute quantification comparison. Even without ESM, the correlation between the two platforms was very good. However, the obtained absolute copies of the reference gene (ACTB) were a mildly different between the two platforms, which can produce variable results of target gene expression after reference gene normalization. However, after ESM normalization for each gene, the difference of the target genes (target/10^6 ACTB) between two platforms demonstrated a two-fold range (y-intercept (log) = 0.35). The result showed a very good correlation coefficient (r^2=0.974) and linearity (slope=0.9454) between the two developed platforms. Three surgically removed human FFPE samples were used for the measurement of the LCDT index genes (E2F1, MYC, CDKN1A, ACTB) (Figure S5.3), and the values in this plot were target gene copy numbers over one million copy numbers of the reference gene (ACTB).
New Contributions from Chapters 3, 4 and 5

1. The developed two-color fluorometric real-time PCR demonstrated good analytical performance. Probe specificity was <1% non-homologous binding, and primers detected < 10 molecules. For the 6 orders of magnitude with 1:1 ratio of NT: IS and in the dilutions of NT to constant IS or vice versa, in the ratio of < 10, R^2 value was > 0.99 and slope was 1.0 ± 0.05. The average coefficient of variation (CV) for measurement of each gene was < 10% for > 60 molecules input.

2. When we use the pre-amplification method in the developed two-color fluorometric real-time PCR, the signal was increased (Cq decreased). Moreover, the NT value measured with the pre-amplification method was not significantly different with or without pre-amplification. Possible variations from two-step PCR including dilution of pre-amplified product or the presence of PCR inhibitors were well controlled by IS.

3. ESM controlled for the variation in fluorescent labeling probes and selection of the threshold. The unknown copies of target NT were calculated by comparison of Cq values of NT and IS: [NT Cq - IS Cq] multiplied by input IS copies. Variations that can affect those Cq values, such as probe quality, activity, and the software selection of Cq were controlled by the mean of the two ESM [NT Cq - IS Cq] values.

4. The efficiency of RT with GSP was higher than RHP in FFPE samples. The average yield of cDNA was more than 50-fold higher with GSP with 1 µg RNA input, and the RT yield was increased another 4.6-fold by increasing RNA input in RT to 5µg.

5. The developed two-color fluorometric real-time PCR showed a reliable transcript abundance measurement in surgically removed FFPE samples for the multiple genes,
MYC, E2F1, CDKN1A and ACTB. The LCDT optimal cut-off value had 90% specificity and 90% sensitivity to classify samples as cancer or non-cancer.

6. The developed two-color fluorometric real-time PCR enabled an accurate quantification of VHSV from a variety of fish samples. This method was more reliable than SYBR green PCR (44% (n=43), 33% (n=63) false negative error) and cell culture method (59% false negative error (n=43)) for VHSV detection, whereas the two-color fluorometric real-time PCR method showed zero false negatives in each fish group.

7. Using an ESM, an accurate data comparison was enabled for the comparison of two different platforms: NGS (competitive library preparation) and real-time PCR (two-color fluorometric). Between two developed platforms, the result showed a very good correlation coefficient ($r^2=0.97$), linearity (slope=0.95), and about two-fold range variations in the comparison of absolute copy numbers (y-intercept (log) = 0.35).


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Chapter 2


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Chapter 3


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Chapter 5


Chapter 6


