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entitled

Biospecimen RNA Quality Control in Reverse-Transcription, Quantitative PCR (RT-qPCR) Clinical Tests

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in Biomedical Sciences

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An Abstract of

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Although numerous platforms exist to measure gene expression, RT-qPCR assays remain the most sensitive and accurate. The accuracy and reproducibility of any RT-qPCR-based molecular test is dependent on adherence to stringent quality control criteria involving biospecimen collection and storage, RNA isolation and reverse transcription and PCR amplification of cDNA.

The following work was focused on three parameters of RNA quality control: RNA integrity, reverse transcription efficiency and PCR efficiency in three RT-qPCR-based assays. All studies used a form of competitive, RT-qPCR known as standardized, reverse transcription PCR (StaRT-PCR) that allows measurement of each gene relative to a known number of internal standards in each PCR assay using the same PCR primers, thus controlling for PCR efficiency. Additionally all studies use a novel Reverse Transcription Standards Mixture (RTSM), prepared by combining known amounts of in-vitro transcribed, synthetic External RNA Control Consortium (ERCC) RNA standards to determine the efficiency of each RT reaction including a specific volume of RTSM.
The first study was designed to gather empiric data to determine the impact of room temperature storage time, different RT primers and RNA input in the RT reaction on BCR-ABL1 and β-glucuronidase (GUSB) cDNA yield in both whole blood samples from patients with chronic myelogenous leukemia and anonymized normal whole blood mixed with BCR-ABL1-positive K562 cells. After completing these experiments, we concluded that the hypothesis that BCR-ABL1/GUSB measurement in blood samples can be reliably measured within 48 hours is not supported by the results. Further the hypothesis that the current standard random priming method for reverse transcription in BCR-ABL1 testing is suboptimal was supported by our data. Therefore to achieve accurate and sensitive BCR-ABL1 normalized to GUSB measurement, whole blood should be processed within 24 hours after collection and gene-specific primers should be used in the RT reaction.

The second study tested the hypothesis that increasing RNA input in the RT reaction of RNA extracted from a smallmouth bass infected with Viral Hemorrhagic Septicemia virus (VHSV) strain IVb would significantly change the measurement of VHSV-IVb – N gene and reference genes actb1 and ef1a compared to a baseline RNA input due to reductions in RT efficiency measured by the RTSM. While certain results supported the hypothesis that increasing amounts of fish RNA in RT reactions reduces RT efficiency, thus causing significant changes in VHSV-IVb/actb1 measurement across different RNA inputs, actb1/ef1a values did not statistically change with increasing RNA input. Therefore, a standard RNA input per RT reaction should be employed during VHSV measurement, for which 1µg total RNA/90 µL reaction is recommended.
The third study tested two hypotheses. First, we hypothesized that certain RNA extraction methods specific for formalin-fixed, paraffin-embedded (FFPE) samples would provide RNA eluates from A549 FFPE samples with different levels of RT inhibitors that would reduce the RT efficiency of ERCC 171 RNA within the RTSM. Second, we hypothesized that use of gene-specific primers in RT reactions of RNA extracted from surgical FFPE lung tissue would yield a higher quantity of ACTB cDNA compared to random hexamers. The results supported the hypotheses, demonstrating that the RNeasy® FFPE kit provides less RT inhibitors co-extracted with RNA, and ACTB cDNA yield increased with the use of gene-specific RT priming. The work presented here will demonstrate the significance of RNA quality control criteria to ensure the accuracy and sensitivity of three RT-qPCR clinical tests.
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Chapter 1

Introduction

To develop a molecular diagnostic test, especially those based on transcript abundance of RNA extracted from clinical biospecimens, several parameters must be defined. Important among these are specifying a clinical purpose of the molecular diagnostic test, defining those analytes to be measured, identifying the most optimal assay platform to measure chosen analytes and setting particular quality control parameters that must be met to ensure test accuracy and reproducibility.

First, the clinical purpose of the molecular diagnostic test must be identified in the patient population. Examples include measuring risk, presence, severity, stratification or chemotherapeutic sensitivity for a specific disease. Also, the clinical purpose of a new molecular diagnostic test may be to improve a currently used test by optimizing sensitivity and/or specificity.

Second, the analytes to be measured in the patient population must be specified. With respect to a transcript-based molecular diagnostic test, it must be determined which transcripts classify subjects into the population with the clinically significant disease (cases) from the population without the disease (controls). While specific transcripts may be qualitatively present in both case and control populations, the quantitative level of
particular transcripts may be significantly different between cases and controls. For example, because most cancers are genetically complex, usually transcript abundance differences of a single transcript are not sensitive or specific enough to discriminate between case and control populations. As a result, molecular diagnostic tests intended to stratify subjects on the basis of cancer risk, diagnosis and/or severity of disease are based on the transcript abundance measurement of multiple genes. In addition to choosing appropriate genes that separate cases from controls, any RT-qPCR-based molecular diagnostic test requires the identification of a proper internal reference gene that will control for loading during reverse transcription and PCR amplification steps of the test. A validated internal reference gene must be expressed at levels that are not statistically different between case and control populations. The expression of the chosen internal reference gene must also not be affected by such conditions as patient gender, age, ethnicity and/or treatment status.

Third, the most optimal assay platform must be identified to measure the predetermined set of analytes. Both the clinical purpose and set of specific transcripts to be measured will greatly affect the choice of assay platform for the molecular diagnostic test. If analytes to be measured are RNA transcripts, have a wide range of expression and/or are expressed only at low levels, a highly sensitive assay must be chosen, such as RT-qPCR. The best combination of chosen analytes and measurement platform will distinguish patient from non-patient populations. The chosen assay must provide highly specific and sensitive measurement of specific analytes.

Finally, quality control measures must be identified to ensure that molecular diagnostic test values measured in specific biospecimens are indeed accurate and
reproducible. Each step of the RT-qPCR molecular diagnostic test, from biospecimen collection to PCR amplification, must meet specific quality control criteria to ensure accurate results. Ideally, for all transcripts measured, expression levels should not be affected by variation in biospecimen collection/storage or any downstream laboratory protocol, including RNA extraction, reverse transcription and/or PCR amplification. This is because inter-sample variation in RNA quality, presence of RT- or PCR-specific inhibitors and/or suboptimal test reagents can produce inaccurate test values. Therefore each biospecimen sample used for molecular diagnostic testing must pass stringent quality control criteria ensure that the level of RNA integrity, reverse transcription efficiency and PCR amplification efficiency will yield a reliable test result.
Chapter 2

Literature Review

2.1 RNA degradation

Messenger ribonucleic acid (mRNA) is the macromolecule that serves to transfer information from a cell’s genetic code within deoxyribonucleic acid (DNA) to ribosomes in the cytoplasm to produce necessary proteins. As a whole, however, mRNA only accounts for ~2-5% of total RNA in a cell. Most RNA in a eukaryotic cell is in the form of ribosomal RNA (rRNA), while an additional small percentage of remaining total RNA is transfer RNA (tRNA). In comparison to DNA, RNA contains a 2’ hydroxyl (-OH) group that makes it more susceptible to degradation by ribonucleases and/or spontaneous cleavage at the phosphodiester backbone via acid-base catalysis.\(^1,2\) Deprotonation of the 2’OH group enhances its nucleophilicity and encourages attack on the the adjacent 3’ phosphorus, forming both a cyclic, intermediate 2’,3’phosphate and 5’ oxyanion leaving group.

Stability of eukaryotic mRNAs is maintained with the 5’ 7-methylguanosine cap and the 3’ poly (A) tail, both of which protect the transcript from exonucleases.\(^3\) Messenger RNAs are progressively degraded 3’ to 5’ after deadenylation by a complex of exonucleases known as the exosome; this is one of the primary pathways for degradation
of mRNA in cells. In addition to primary 3'-5' degradation, subsequent 5'-3' decay by the XRN1 exoribonuclease may occur subsequently after decapping. Transcript degradation may also occur by endonucleolytic cleavage, thus producing two RNA fragments susceptible to exonuclease activity. Although cellular ribonucleases (RNases) play key roles in mRNA maturation, structured decay and necessary digestion, their presence can result in very short lifespans for RNA molecules. Cellular mRNAs have highly different rates of degradation, with some lasting only minutes, while others have half-lives of 50 hours in vivo.

Coupled with the presence of ubiquitous RNases in the environment, extracellular and intracellular chemical RNA degradation by enzyme-catalyzed hydrolysis remains a serious threat to the accuracy of transcript-based molecular diagnostic tests using *ex vivo* biospecimens. To provide undegraded, intact RNA for a particular qRT-PCR-based assay, all steps from biospecimen collection to the reverse transcription reaction must ensure that RNA’s physical integrity is protected and downstream molecular analyses remain accurate.

Preanalytical factors affecting the protection of RNA integrity include biospecimen collection, preservation and storage. Levels of interleukin, cytokine, chemokine and other stress-response genes’ transcript abundance may significantly change due to processes such as inflammation, ischemia and/or necrosis in collected *in vivo* or *ex vivo* clinical samples including blood, surgically removed solid tissues and fine-needle aspiration biopsies. Such changes in gene expression due to inflammatory or hypoxic responses in procurement and/or storage of biospecimens must be assessed so optimal biospecimen handling can be standardized. Additionally these
sample removal techniques may introduce exogenous RNAses, and therefore, all instrumentation and collection devices must be free of RNAses before coming into contact with the biospecimen sample. Ideally RNA from the removed sample should be extracted immediately post-collection. Most of the time, however, the site of biospecimen collection and the site of sample processing/analysis are different. Because RNA extraction is postponed, the biospecimen must be adequately preserved and stored so as not to cause any change in gene expression values. Although several organization have addressed best practices for specimen handling,\textsuperscript{29-31} many studies reviewed (Sections 2.1-2.4) demonstrate conflicting results as to the the best quality control criteria per each biospecimen.

2.1.1 Measurement of RNA integrity

2.1.1.1 Ribosomal RNA-based methods

2.1.1.1.1 Ribosomal 28S/18S ratio

Because RNA is vulnerable to degradation, it is imperative that researchers be armed with accurate and reproducible methods to measure the amount of degradation RNA samples have incurred. Although methods exist to assess RNA integrity, the research community has not uniformly accepted any specific method. Traditionally RNA integrity is measured via agarose gel electrophoresis with ethidium bromide staining. RNA integrity is determined by the band intensity ratio between eukaryotic 28S and 18S rRNA subunits; a ratio of 2.0 or higher is considered high quality RNA.\textsuperscript{32} Yet, some studies have demonstrated that the 28S/18S rRNA ratio in peripheral blood decreases with age, especially with Alzheimer’s disease,\textsuperscript{33} in mouse brain and liver blood\textsuperscript{34} and murine hepatitis virus (MHV)-infected murine cell RNA.\textsuperscript{35} Schoor and colleagues
determined that use of the 28S/18S ratio may be misleading; a highly degraded sample from an intentional room-temperature incubation of pulverized normal renal tissue showed a measured 28S/18S ratio close to that from a freshly isolated RNA sample.\textsuperscript{36} Fleige and colleagues determined that there was no predictable relationship between transcript abundance and rRNA 28S/18S ratio for different bovine tissue and cultured cell biospecimens intentionally degraded by skin RNAses or ultraviolet light irradiation.\textsuperscript{15} While Miller and colleagues demonstrate that high rRNA ratios corresponded to poor-quality GAPDH, KYNF, NEFL and β2M transcripts in random-primed RNA extracts from postmortem human brain tissue,\textsuperscript{37} Malik and colleagues found that oligo(d)T-primed porcine retinal RNA maintained in situ for various time points yielded good RT-qPCR signal that did not correlate with increasingly poor 28S to 18S ratios.\textsuperscript{38}

2.1.1.1.2 RNA Integrity Number (RIN)

An improvement to this methodology was developed with the introduction of the Agilent 2100 Bioanalyzer, a device that uses microcapillary electrophoresis and laser-induced fluorescence to detect nucleic acid amounts of a given size.\textsuperscript{39} To standardize measurements of RNA integrity, Agilent developed a user-independent, software algorithm that calculates an RNA Integrity Number (RIN) for each analyzed RNA sample.\textsuperscript{40} The algorithm incorporates information from both the 28S:18S rRNA peak ratio and the size distribution of smaller RNA fragments.\textsuperscript{40, 41} The RIN Score given for a particular RNA sample is a whole integer or decimal number ranging from 1 (completely degraded) to 10 (undegraded). Although RIN Score analysis may be the current gold-standard in measurement of RNA integrity, its value is limited. In some cases, electropherogram signals are of unusual shape, including peaks at unexpected migration
times, spikes or abnormal fluctuation of the baseline; in these cases, a reliable RIN computation is impossible.\textsuperscript{42} If clinical sample RNA is limiting, numerous repeat measurements may not be possible. However, even if an adequate RIN Score is calculated for a particular RNA sample, the measurement is of rRNA structural stability, and the integrity of rRNA may not necessarily be an accurate measure of mRNA integrity due to structural differences between the two types of RNA. Maintained integrity of rRNA measured by the Agilent Bioanalyzer 2100 is no guarantee of the stability of specific transcripts.\textsuperscript{43} While Auer proposes that a RIN score of greater than seven is considered to be intact RNA,\textsuperscript{44} Popova warns that up to 30\% of RNA transcripts that possess RIN scores of greater than 7.8 can be degraded prior to use in hybridization assays.\textsuperscript{45} Additionally, studies by Hagedorn reveal that RNA extracted from a frozen liver biospecimen incubated at room temperature for 0, 5 and 10 minutes provided RIN scores of >7, while RNA-sequencing data showed a >50\% reduction in exonic sequencing reads observed in 2.7\% of mRNA transcripts by 5 minutes, 14\% at 10 minutes and 76\% at 15 minutes (RIN score of 6.7).\textsuperscript{46} Even the report by Shroeder advocating use of RIN Score for measuring RNA integrity showed a suboptimal correlation between RIN Score and reference gene GAPDH, KYNF, $\beta$2M and NEFL transcript abundance in RNA extracts of postmortem human brain tissue.\textsuperscript{47} Skrypina and colleagues demonstrated that for some genes measured in human bladder carcinoma cell line T24P total RNA treated with destabilizing MgCl$_2$, rRNA and mRNA degradation profiles are similar, while for other transcripts like GAPDH, they are not.\textsuperscript{48} Additionally Mayne and colleagues showed that in RNA collected from brains of rats killed by high-
energy focused microwave irradiation, rRNA was highly degraded but GAPDH and TNF-α mRNAs remained intact.\textsuperscript{49}

2.1.1.2 Messenger RNA-based methods

A more sensitive measurement of mRNA integrity may be a transcript-based RNA integrity quality control metric compared to rRNA-based RIN Score methodology.\textsuperscript{50, 51} In 2000, Swift and colleagues described a transcript-based integrity test that compared the fraction of four, short PCR amplicons (each with its own forward and reverse primer) measured from the \textit{Gallus gallus} (chicken) 10-kb fatty acid synthase (FAS) mRNA that extends 2, 6, 8 or 9 kb from the polyA tail, using oligo(d)T RT priming.\textsuperscript{52} The Swift study used the FAS-based integrity test in seven different RNA preparations of pooled embryonic chicken liver, stomach, pancreas and intestine, including one high-quality RNA preparation from an older embryo to determine which RNA preparation could be used with confidence for construction of a cDNA library. Yet, the authors acknowledge that a combination of factors such as nuclease hypersensitive sites and secondary structures could increase the probability of terminating cDNA synthesis at locations on the transcript furthest from the polyA-tail and that other researchers may need to create a similar integrity test with other long, ubiquitous mRNAs.

In 2001 work by Sugita described a one-step, RT-qPCR method for assessment of mRNA stability using multiple primers (2 forward and 1 reverse) for PCR of human ACTB, measuring a density ratio of the two, long (637 bp) and short (178), band segments created in the primer set.\textsuperscript{53} In an autolytic degradation experiment, CAMA-1 cells in EDTA were placed at room temperature at regular time intervals between time 0
and 96 hours; Sugita and colleagues determined a linear \( y = 0.1053 x + 1.98, r^2 = 0.97 \) relationship between incubation time in hours and short:long band density ratio from 0 to 48 hours with ratios given as 1.7 and 7, respectively.~\(^{53}\) While the Sugita study gives recommendations for ACTB duplex density ratio and usability of RNA specimens for downstream applications, it did show evidence that for at least one transcript MUC1, RT-qPCR product was detected undiminished up to 16 hours of room temperature incubation, while the ACTB duplex density ratio demonstrated considerable degradation of ACTB mRNA.~\(^{53}\) The results of the Sugita study demonstrate that like the issue of rRNA degradation being used to determine mRNA degradation, the degradation of one particular transcript may not reflect the degradation of all other transcripts in the biological sample.

In addition to using the ACTB-based integrity test described by Sugita, Malik and colleagues developed a similar one-step, RT-qPCR GAPDH integrity test that compared the measured yield of three PCR amplicons, long (636bp), medium (525bp) and short (195bp), while using the same reverse primer to assess the integrity of RNA extracted from porcine retina and retinal pigment epithelium (RPE) isolated, kept at room temperature for 2 hours, cooled to \(4^\circ\)C and then frozen at \(-80^\circ\)C at various time intervals from 5 to 48 hours.~\(^{38}\) Gel electrophoresis patterns of PCR products from RPE tissue showed two ACTB and three GAPDH amplicons after 5 hours storage at \(4^\circ\)C before freezing; however, at 12 hours and longer the long (636bp) ACTB amplicon and the middle (525 bp) and long (817) GAPDH amplicons were greatly diminished. RPE tissue-specific RPE-65 gene product reflected the same diminishing yield after 5 hours. ACTB, GAPDH and retina tissue-specific RHO gene products from retinal tissue, however,
remained of similar density up to 48 hours, demonstrating that GAPDH, ACTB and RHO mRNA was stable for the same time period.

In a study by Bauer and colleagues to determine if quantification of mRNA degradation in whole blood samples using laser-induced fluorescence capillary electrophoresis (LIF-CE) could be used as an indicator of human postmortem interval (PMI), a FAS transcript-based integrity test was developed that compared yields of four amplification products of different sizes spaced at intervals of approximately 1.5 kb along FAS cDNA. The relative yields of amplicons proximal and distal to the 3’end of the FAS transcript were used as a relative measure of the ratio of intact to fragmented FAS transcript in the sample. Using whole blood collected from living individuals stored at 4°C for up to 4 days, the FAS-based integrity test showed a time-dependent decrease in the fraction of intact mRNA as shown by the quotient of the most distal PCR fragment to the fragment closest to the 5’end of the cDNA ($r^2 = 0.843$, $p<0.01$), half of post-mortem blood samples showed a continuous decrease of the fraction of intact FAS mRNA up to a PMI of 120 hours ($r^2 =0.808$, $p<0.01$), and postmortem parietal cortex brain tissue demonstrated similar results ($r^2 = 0.791$, $p<0.01$). When yields of either of the two internal PCR amplicons were used to calculate the fragmentation ratio of postmortem blood samples, the decrease was less pronounced or absent altogether.

Three years later in 2006, Nolan and colleagues created a 3’:5’ messenger RNA integrity assay that compares the PCR amplification of three amplicons (each with its own set of forward and reverse primers and probes) located along the entire length of the GAPDH sequence after reverse transcription with an oligo d(T) primer. Theoretically, the ratio of measured amplicons demonstrates the ability of the reverse transcriptase to
proceed along the entire length of the transcript. The Nolan study used the GAPDH integrity test to assess RNA integrity in RNA extracted from fresh, frozen and FFPE-treated colonic biopsies, showing that the mean 3’-5’ ratio for fresh tissue was 2, while FFPE-treated tissue provided a value of 90. Although Nolan and colleagues again give recommendations that a 3’-5’ ratio of 1 indicates high integrity RNA while a ratio above 5 suggests degradation, they report that different mRNAs degrade at different rates and GAPDH degradation may not be representative of the integrity of all mRNAs.

In a 2009 study involving murine fresh and frozen lung tissue, Muyal and colleagues developed their own transcript-based integrity test that measures two different GAPDH PCR amplicons that overlap, one short (180bp) and one medium (700bp) with a longer PBGD amplicon (1400bp). The study demonstrated that the short GAPDH product yield was not significantly different between frozen and fresh lung tissue, the medium GAPDH product yield was only significantly higher in fresh versus frozen tissue when a silica-gel column was used for RNA extraction versus a guanidine isothiocyanate extraction technique, and the long PBGD product yield was only significantly higher in fresh versus frozen tissue when a guanidine isothiocyanate extraction technique was used for RNA extraction versus use of a silica-gel column. This study, while presenting another type of mRNA-based integrity test, was most concerned with the ability of different RNA extraction techniques to isolate mRNA in various stages of decay, rather than focusing on the overall differences in mRNA integrity between fresh and frozen tissue.

Also in 2009, Fajardy and colleagues completed a study assessing the integrity of RNA isolated from human placenta either directly placed in RNALater® (Qiagen, Hilden,
Germany), an RNA stabilizing solution, or dissected and stored at 4°C from 0 to 96 hours in 24-hour intervals. RNA integrity was evaluated using both RIN score and quantification of 5’ and 3’ amplicons (each with its own forward and reverse primer) of two large reference genes, FAS and GAPDH. The 5’ and 3’ FAS amplicons were designed according to the Bauer study described above. While RIN Score analysis and the 5’/3’ GAPDH product ratio demonstrated that RNA degradation occurred only for placenta samples not immediately fixed in RNAlater and was significant at 72 hours compared to baseline (0 hrs), the 5’/3’ FAS product ratio for both storage protocols significantly declined at 72 hours, but more pronounced for the storage protocol without RNAlater. According to the RIN score analysis, significant degradation only occurred for placenta samples not immediately fixed in RNAlater, beginning at 72 hours. The Fajardy study showed that the 5’/3’ FAS product ratio for both storage protocols declined at 72 hours from baseline (0 hrs), but was significantly less for placenta samples not immediately stabilized in RNA later. However, the 5’/3’ GAPDH product ratio demonstrated that degradation only occurred for placenta samples not immediately fixed in RNAlater and a significant decrease from baseline (0 hrs) could be detected at 72 hours.

In 2012 Hagedorn and colleagues filed a patent that described the use of yet to be determined “sentinel RNAs,” 5’-methyl guanosine capped transcripts that are abundantly expressed and rapidly degrading, that will be measured in tested biospecimens compared to a degradation reference standard, a known amount of sentinel RNA degradation at one or more timepoints from a control biologic sample. Hagedorn claims that the amount of degradation in an unknown sample will be determined by rates of either or both 3’-5’
processive degradation and 5’-3’ processive degradation, wherein each type of processive degradation is determined from 200 nucleotides from each 5’ and 3’ end of one or more sentinel RNAs.\textsuperscript{46}

The use of any transcript-abundance based mRNA integrity test should only be used with target transcripts that have low inter-individual variation in transcript abundance, similar levels of expression in normal and diseased samples and an absence of pseudogenes. Yet it is important to note that with a transcript-based integrity assay, the reverse transcription and PCR efficiencies of each target transcript must be identical, or differences must be controlled.

2.1.2 Biospecimen-specific effects on RNA integrity

RNA integrity is often biospecimen-specific and dependent on the transport, storage and processing of the sample. Some biospecimens, by nature of the tissue are highly susceptible to degradation of RNA; RNA from dense connective tissue,\textsuperscript{59} RNAs-rich tissue like, pancreas, stomach, spleen and liver\textsuperscript{60,61} and some tumors\textsuperscript{48} is often more degraded than other biospecimens. Additionally, RNA from other tissues, such as brain, is reported to be highly stable.\textsuperscript{62-66} Additional evidence of tissue-dependent transcript degradation is shown by forensic studies demonstrating that RNA integrity among different postmortem tissues or organs is known to behave differently.\textsuperscript{67,68} It is important to note that the stability of various transcripts is not only significantly different in different tissues, but different within the same tissue type depending on the degradation status.\textsuperscript{69}

2.1.2.1 Whole blood

2.1.2.1.1 Blood collection tubes with anticoagulant
Whole blood from patients is immediately placed into collection tubes with a variety of anti-coagulants, including citrate salts, heparin or EDTA. Studies show that with current methods of blood collection changes in gene expression in whole blood may occur minutes after venipuncture due to induction of specific genes or degradation of already present transcripts. Intracellular RNA may be degraded ex vivo by specific and nonspecific endogenous nucleases. Prolonged storage for 5 days of whole blood in EDTA tubes at room temperature can decrease total RNA yield by greater than 60%. Many specific genes (including interleukins, cytokines, chemokines and proto-oncogenes) show nearly 99% reduction or up to 100-fold induction after 5-day storage in EDTA tubes by either ex vivo RNA down-regulation and/or degradation or gene upregulation when compared to immediately processed blood samples. According to Tanner, p53 was found to be 2- to 10-fold reduced in EDTA-tubed blood after 24 hours of storage at room temperature, while Rainen observed no detection of p53 transcript after 3 days of storage. Such large changes in gene expression are problematic for molecular diagnostic tests based on transcript abundance measurement because collected blood may be shipped between collection sites and those laboratories that conduct RT-qPCR studies.

2.1.2.1.2 Blood collection tubes with RNA stabilizing agent

Delays in sample processing due to transportation of blood samples pose a current threat to the reliability of gene expression measurements taken from whole blood. As a result of such studies, blood collection tubes with RNA stabilizing agents have been developed to prevent RNA induction and/or degradation if blood samples must be stored for long periods of time. Paxgene Blood kit (Qiagen, Hilden, Germany), Tempus Blood
RNA (Life Technologies, Carlsbad, CA) and RNAgard (Biomâtrica, San Diego, CA) collection tubes are believed to prevent changes in the RNA profile after venipuncture. Even with promising studies showing how these RNA stabilizing tubes may help prevent RNA degradation, it becomes apparent that this technology fails at promising stability of a variety of specific transcripts. The results of Kagedal’s study suggest that RNA stabilizing tubes do not prevent all RNA degradation; while some transcripts may be stabilized, others may not. Therefore, research and clinical laboratories must test the stability of target transcripts in both typical anti-coagulant and RNA-stabilizing collecting tubes before using them for RT-qPCR – based molecular diagnostic testing.

2.1.2.2 Surgically-removed tissue

When a patient sample is removed by an invasive surgical procedure, it is either subsequently frozen or fixed in a specific preservative. Factors including surgical technique, transport time and mode from the site of collection, preservation method, storage conditions and freeze-thaw cycling can impact RNA integrity in surgically removed biospecimens. Increased time between sample collection and placement in preservative/freezer causes significant changes in gene expression measurements, due to changes in cellular metabolic activity and RNA degradation. Extent of gene expression changes due to RNA degradation may be tissue- and/or tumor-dependent, thus effects of ischemia (or pre-fixation) time must be empirically tested for each specific biospecimen used for molecular diagnostic testing. Using microarray gene expression analysis with confirmative RT-qPCR, Riis and colleagues demonstrated many differentially expressed genes between matched pre- and post-operative breast cancer tumor samples, two of which (GRB7 and NDRGI) are included in currently marketed
molecular clinical tests, Oncotype DX and Mammaprint, respectively. The authors use these examples to emphasize the importance of determining whether gene expression differences are related to tumor biology or biospecimen handling factors.

2.1.2.2.1 RNeasy®

With regard to RNA preservation, snap freezing of removed samples is considered most optimal. Yet, even with immediate freezing of surgically removed tissue and proper storage, frozen samples must first thaw before RNA extraction. RNA degradation occurs within minutes after initiation of thawing. Botling and Groningen showed that thawing of frozen tonsil and renal tissue, respectively, submerged in RNA stabilization solution RNeasy (Qiagen, Hilden, Germany) prevented such degradation of RNA. Wang and colleagues demonstrated delayed degradation of FOXC1 mRNA in human ocular trabecular meshwork tissue with addition of RNeasy compared with samples stored on ice or frozen at -80°C. A study by Malik and colleagues showed that decay of RPE tissue mRNA occurring at 5 hours could be delayed by at least 24 hours with use of RNeasy. Also, Sherwood demonstrated that use of RNeasy versus snap freezing in the preservation of postmortem brain tissue protects RNA better. While RNA integrity was maintained, some researchers like Florell have shown that RNeasy also preserves the histologic integrity of tissues like lung, liver, heart and skin, while Groningen demonstrates that it does not maintain renal tissue integrity for microscopy or immunohistochemical assessment. Yet, Micke and colleagues found that RNeasy did not improve RNA integrity in larger colon and tonsillar tissue resections compared to storage on ice, in saline or at room temperature for up to 16 hours before storage at -80°C. However, the RNA integrity was measured using ribosomal RNA 28S/18S
ratios, and the published electropherograms show visual evidence of RNA degradation in the absence of RNAlater, as opposed to the 28S/18S ratio. Micke also found that RNAlater produced significant tissue shrinkage and changes in RT-qPCR expression for specific genes.

### 2.1.2.2 Formalin fixation and paraffin embedding

It is important to note that freezing surgically removed tissue samples incurs high cost and increased labor, as well as, providing poor morphological preservation for histopathologic analysis. As a result, the majority of surgically removed tissues are first fixed with a formalin solution with 10% formaldehyde and then paraffin embedded. Unlike fresh frozen tissues, formalin fixed tissue blocks retain tissue and cell architecture for optimized histological and immunohistochemical analyses. Yet, RNA from formalin fixed tissue possesses additions of mono-methylol groups to bases and dimerization of adenine groups by methylene bridging. These interactions can cause significant physical breakage of RNA strands during formalin fixation. Also Williams demonstrated that in RNA extracted from FFPE tissue, as many as 1 in 500 bases are mutated, either C to T or G to A transitions. In RNA extracted from paired fresh frozen and FFPE tissues that was reverse transcribed and PCR amplified, there was variability in the observed differences in specific transcript abundances between the two paired samples. Detection of targets is less efficient from fixed material that the effect on different mRNA species, and even different fragments of the same mRNA, is variable. Additionally, RNA quality within these specimens is greatly dependent on pre-fixation factors such as tissue type and amount and the extent of autolysis before fixation. The pH, temperature and duration of fixation, along with the temperature and duration of storage, all can impact
the extent of nucleic acid degradation.\textsuperscript{92-95} Despite these problems with FFPE tissues, Godfrey suggests that efficient detection of targets can be achieved with the use of PCR amplicons $< 130$ basepairs and optimal RT conditions.\textsuperscript{74} Despite problems with nucleic acid degradation, there remains a wealth of archival tissue FFPE blocks from patients with clinical follow-up information useful for retrospective studies concerned with relating gene expression and clinical outcome. As a result, molecular diagnostic tests should be amenable to using RNA extracted from formalin-fixed and paraffin embedded tissues.

2.1.2.3 Fine needle aspirations

Fine needle aspiration of a patient sample may be performed if a suspect lesion is superficial or surgical removal of a sample is unable to be performed. In this procedure, a pathologist or radiologist uses guidance imagery (computer tomography scan, fluoroscopy or ultrasound) to target the lesion of interest and a suitable point of entry. Then, a specific gauge needle is advanced toward the lesion under imaging guidance. Once site of sampling has been reached, the needle is passed through the tissue of interest, collecting cellular material within the needle lumen. Generally most of the collected material is used for preparation of a cytologic smear on a glass slide for preliminary interpretation. Additional material (either from the residual first sample or from subsequent samples) is processed according to the presumptive diagnostic interpretation and physical characteristics of the collected sample to be triaged for either more smears, cell block, cultures, flow cytometry, and/or molecular studies. If the original needle wash or additional aspiration material is to be used for qRT-PCR-based
experiments, the aspirated material must be immediately preserved so as to protect RNA from degradation.

2.1.2.3.1 Liquid-based cytology collection mediums

Liquid-based cytology (LBC) collection mediums conserve cell morphology, improve diagnostic accuracy, reduce incidence of inadequate smears and allow for proper storage and transportation of collected cellular material for ancillary analysis.\textsuperscript{96-100} Two widely used and FDA-approved liquid preservatives include ethanol-based ThinPrep Preservcyt\textsuperscript{®} Solution (Cytyc Corp., Londonderry, NH) and methanol-based BD Surepath\textsuperscript{TM} Preservative Fluid (BD Diagnostics, Burlington, NC). Although these liquid preservatives are primarily meant to conserve sample cell architecture, their degree of RNA protection is determined by their ability to prevent RNase activity.

2.1.2.3.1.1 Preservcyt\textsuperscript{®}

Tisserand and colleagues looked at the effect of Preservcyt fixation on rRNA degradation and RT-qPCR p53 amplicon yield in both cultured SKBR3 and MCF-7 cells and patent breast carcinoma fine needle biopsies that were first placed in CytoLyt solution and then transferred into PreservCyt.\textsuperscript{101} In Preservcyt fixed cultured cells, rRNA 28S and 18S peaks were intact after 9 months of storage at -20\textdegree C, were intact after 5 months of storage at 4\textdegree C and showed marked degradation at 1 month of storage at room temperature.\textsuperscript{101} Yield of 1100-bp p53 product after RT-qPCR was identical for cultured cells fixed in Preservcyt kept at 4\textdegree C or -20\textdegree C for regardless of storage time.\textsuperscript{101} Although good amplification of the p53 amplicon was observed in fixed cells stored at room temperature for 1-2 months, an approximate 95% reduction of PCR yield was observed after 9 months of storage.\textsuperscript{101} In five breast carcinoma fine needle samples stored at 4\textdegree C
for 2 and 16 months, there was moderate rRNA degradation for the 2-month storage sample and severe rRNA degradation for the 16-month storage sample; for the 2-month storage samples, there was efficient RT-qPCR yield of a 1000bp p53 product in all five samples, while for the 18-month storage samples, only one of five samples provided a similar p53 amplicon yield.\textsuperscript{101}

Dimulescu and colleagues reported that preserved cord blood lymphocyte cells stored in PreservCyt for 24 hours at room temperature and 4°C, as well as preserved CaSki and HeLa cells stored for 24 hours at 4°C, maintained intact 28S and 18S ribosomal RNA bands on electrophoresis.\textsuperscript{102} RT-qPCR of HPV 16 E7 RNA extracted from a dilution series of CaSki cells in HTB-31 cells stored for 24 hours at 4°C either unfixed or fixed in PreservCyt was performed, and Dimulescu reported a 2.5-fold loss of sensitivity with fixated cells compared to unfixed cells.\textsuperscript{102} Cuschieri identified that HPV RNA from patient cervical samples could be detected for at least 14 days after sample placement in PreservCyt.\textsuperscript{98}

2.1.2.3.1.2 Surepath\textsuperscript{TM}

Additional studies report that RNA extracted from cells fixed in PreservCyt remains intact while RNA extracted from cells fixed in SurePath LBC medium is of poor quality for RT-qPCR.\textsuperscript{103, 104} Powell and colleagues spiked known numbers of CaSki cells into PreservCyt and Surepath media and determined RNA recovery from samples stored at room temperature for various timepoints.\textsuperscript{104} RNA extracted from Surepath-fixed cells showed no 28S or 18S ribosomal subunits after electrophoresis for any timepoint, and recovery of RNA after 168 hours in Surepath was reduced at least 104-fold compared to PreservCyt-fixed cell RNA depending on the RNA extraction method.\textsuperscript{104} Horvath and
colleagues showed that Surepath-fixed HeLa cells compared to fresh, nonfixed cells provided less RNA yield and more RNA degradation, as shown by the absence of ribosomal RNA peaks.\textsuperscript{103} Also, RNA extracted from fixed cells showed poor real-time RT-qPCR amplification with an absent correlation between threshold values and log cDNA concentration.\textsuperscript{103}

\textbf{2.1.2.3.2 RINAlater®}

In addition to LBC solutions to preserve bioptic material, RINAlater has been studied in fine needle aspirate samples. Ellis and colleagues looked at core breast needle biopsies, and showed that RINAlater maintained both cellular integrity for microscopy and RNA integrity measured post-extraction.\textsuperscript{105} Lawson and colleagues demonstrated that low-volume diagnostic lung cancer transbronchial needle aspiration biopsies stored in RINAlater improved extracted RNA yield and quality better than matched patient biopsies immediately snap-frozen in liquid nitrogen.\textsuperscript{106} Although a biospecimen may be initially used for histological or cytological examination, it must be preserved for downstream molecular analysis. Therefore biospecimen storage and transport become significant factors in the preservation of RNA used for transcript-based molecular diagnostic tests.

\textbf{2.2 RNA isolation}

\textbf{2.2.1 Canonical techniques}

Before RNA can be used for downstream molecular analysis, it must first be isolated from the cells of the biospecimen. While there are a wide variety of RNA extraction products available, two of the most common methodologies include liquid-phase, guanidine isothiocyanate-phenol-chloroform extractions and extractions using solid-phase, spin column technology. Phenol-chloroform extractions use a detergent such
as guanidinium thiocyanate to lyse cells, followed by a liquid phase separation based on solubility and alcohol precipitation. Spin column extractions use materials, such as silica, to create linkages between RNA to specific surfaces in the presence of chaotropic salts, and then the RNA is washed and eluted. RNA degradation during extraction is prevented by use of chaotropic salts like guanidium thiocyanate and denaturants like β-mercaptoethanol to inactivate ribonucleases, and all reagents and buffers must be ribonuclease-free.

2.2.2 Special considerations for FFPE samples

In biospecimen samples that have been chemically fixed in formalin and paraffin embedded, additional steps are added to the canonical liquid- or solid-phase RNA extraction procedure, including a deparaffinization step involving xylene, ethanol washes and an additional protease K digestion. Gilbert and colleagues show that in a variety of paired FFPE tissues, RNA extracted with and without a deparaffinization step showed statistically similar yields and ability to PCR amplify HIV RNA. Additionally, some studies show an advantage to incubating FFPE samples at 98°C prior to protease digestion. With regard to optimizing incubation time and temperature of digestion, studies show that while Banerjee and colleagues recommend an incubation of no more than 3 hours at 55°C, the Isola and Gilbert studies show that a prolonged (>24 hours) incubation is better at 55°C and 65°C, respectfully. 

Yields of RNA from FFPE tissues compared to fresh frozen tissues are significantly lower and those RNA fragments extracted are approximately 200 basepairs or less. Crosslinks between nucleic acid and protein caused by formaldehyde are considered to be somewhat heat reversible. While either increased incubation
temperature\textsuperscript{126} or increased incubation time\textsuperscript{95} may be employed, heat-induced degradation of nucleic acid may result.\textsuperscript{127} Many studies have been completed to compare the efficiencies of currently available kits used to extract RNA from FFPE-treated biospecimens.\textsuperscript{128-130}

2.2.3 RNA purity

Ultimately an optimal and efficient extraction assay will provide a high yield of pure RNA. Yield of RNA is determined by spectrophotometry; as per the Beer-Lambert law, the amount of light absorbed by RNA at a wavelength of 260 nm is directly proportional to the concentration of the absorbing material, or RNA. While other methods exist to measure RNA yield, such as microfluidic analysis and fluorescent dye detection, these methods can produce significantly different results, thus data should only be compared using the same method.\textsuperscript{90, 131, 132}

2.2.3.1 Measurement of RNA purity

RNA purity is measured by comparing the absorbance measurements taken at the wavelengths of 260 and 280 nm.\textsuperscript{133} RNA with a ratio of $A_{260}/A_{280} = 1.8\text{-}2.0$ is considered pure. Contaminants, such as protein, phenol or salts that are co-extracted with the RNA or the buffer used to elute RNA may cause the $A_{260}/A_{280}$ ratio to deviate from 2.0.\textsuperscript{14} Bustin and Nolan question the accuracy of the $A_{260}/A_{280}$ ratio to determine purity; a value of 1.8 corresponded to only 40% RNA, but accounted for 60% protein.\textsuperscript{134} Additionally, this measure of RNA purity does not detect other contaminants that absorb ultraviolet light at different wavelengths, and cannot determine the amount of genomic DNA contamination in the RNA sample. Also, Nolan and Bustin concluded that the typical $A_{260}/A_{280}$ ratio measurement does not detect the presence of EDTA in high
enough concentrations to inhibit qPCR amplification. Any contaminants present in the extracted RNA sample may inhibit enzymatic reactions in downstream reverse transcription and/or PCR amplification.

2.2.3.2 Genomic DNA contamination

In transcript abundance-based studies, elimination of genomic DNA from extracted RNA is important to prevent inaccurate molecular diagnostic test results. PCR primers can be created to span introns to prevent amplification of gDNA, however this is not a guarantee. Without DNase treatment, up to 80% of nucleic acid recovered after RNA extraction is DNA instead of RNA. Malik and colleagues state that after DNAse treatment of porcine eye RNA, OD260 was reduced 40% to 60%. As a result, it is recommended that either during or after RNA extraction, DNAse treatment be applied to the sample. Yet, a study by Ivarsson demonstrated a negative impact on RNA with the application of DNAse treatment.

2.2.4 Comparative studies

While multiple studies have been completed to identify the most optimal extraction method, many have determined that liquid-based extractions provide significantly higher RNA yields than spin column-based extractions in a variety of solid biospecimens like kidney, cartilage, lung and sputum. However, Miller and colleagues determined that the two extraction techniques provided the same RNA yield from frozen, postmortem frontal cortex tissue. Yet even various kits employing the same canonical extraction method (e.g. liquid phase separation), can produce statistically significant results in RNA extraction from blood. However, studies show that there is no difference in the purities of RNA extracted with the two techniques.
The purity of RNA is important for any downstream applications such as reverse transcription and PCR amplification. In addition to different RNA yields and purities from the same biospecimen with the use of varying extraction techniques, it is possible that downstream RT-qPCR results may be affected as well. If a particular RNA extraction method preferentially extracts certain transcripts, fails to capture low copy transcripts, or introduces enzyme-inhibitory contaminants, results of RT-qPCR assays may be significantly different among various extraction methods. However, very few publications have addressed the issue of whether different RNA extraction techniques, in addition to providing different RNA yields and purities, cause significantly different RT-qPCR results.\textsuperscript{56, 83, 142}

2.3 Reverse transcription efficiency

In RT-qPCR assays, RNA must first be reverse transcribed to cDNA to measure gene expression. Although the reverse transcription step is of major importance to the accuracy of any RT-qPCR assay, it remains susceptible to low efficiencies of RNA conversion to cDNA templates. Earlier studies show that yield of full-length cDNA templates from mRNA rarely exceed 40\%,\textsuperscript{143} and that the efficiency of reverse transcription can be significantly 3-fold lower when target templates are rare.\textsuperscript{144} In addition, Tichopad and colleagues demonstrated that the reverse transcription of a lowly expressed gene in solid tissue samples generated the most noise.\textsuperscript{145} Efficiency measurement of reverse transcription with a certain reverse transcriptase and RT priming method is highly gene-dependent due to specific transcripts’ secondary and tertiary structures.\textsuperscript{146, 147} Reverse transcription efficiency may decrease by the presence of interfering substances within the extracted RNA, or by suboptimal quality/quantity of
reagents used in the reverse transcription reaction, such as the primer and reverse transcriptase chosen.

2.3.1 Reverse transcription priming

Before a reverse transcription can occur, a primer must anneal to the template RNA. The different priming strategies employed include oligo d(T), random, gene-specific or combined RT priming. Each method of reverse transcription priming has its advantages and disadvantages; the choice of RT primer can result in highly different transcript abundance measurements for the same target.\textsuperscript{148, 149} The optimal priming method is dependent upon the type of biospecimen, the quality of the extracted RNA and the overall purpose of the molecular diagnostic test.

2.3.1.1 Oligo d(T) priming

While oligo d(T) primers specifically anneal to an mRNA’s poly-A tail, random primers can theoretically prime multiple locations along a single RNA sequence. Yet Nam and colleagues found that oligo d(T) primers can prime internal poly-A sequences and result in truncated cDNA.\textsuperscript{150} If mRNA is relatively intact and not degraded, oligo d(T) primers have shown to provide better specificity over random primers.\textsuperscript{132} If oligo d(T) primers are used for RT-qPCR-based assays, the subsequent PCR primer binding sites must not be at the extreme 5’-end of the cDNA sequence to prevent loss of template detection during PCR amplification because oligo d(T) primers cause a 3’ bias in cDNAs reverse transcribed.\textsuperscript{48, 132} However, Sieber and colleagues found the most differences of reverse transcription efficiency among different reverse transcriptases with use of oligo d(T) primers in human tissue biospecimens.\textsuperscript{151}

2.3.1.2 Random priming
If RNA samples are degraded, lack a poly-A tail, or have considerable secondary structure, it is recommended that random primers be used. When random priming is chosen as the method of RT priming, it must then be determined what concentration and length of primer to use. A study by Stangegaard showed that use of random pentadecamers yielded approximately twice as much cDNA as random hexamers in HeLa cell poly(A) mRNA and amplified RNA. A study by Ross confirmed an 85% increase in BCR-ABL1 cDNA yield from patient whole blood with use of random pentadecamers versus hexamers in the RT reaction. Both Nardon and Zhang completed studies showing that cDNA yield can be increased with higher concentrations of both random hexamers and pentadecamers. However, in both the Stangegaard and Ross studies, it was determined that the use of random pentadecamers versus random hexamers in the RT reaction does not improve cDNA yield equally across all transcripts. While two studies have shown that there is not a linear correlation between input target amount and cDNA yield when specific targets are measured with random priming of total RNA extracted from fresh human colonic biopsies, and an unidentified biospecimen, Nardon and colleagues demonstrated linearity with increasing amounts of pooled FFPE colon cancer biopsy total RNA (0.125-5µg) in 20µL RT reactions. Additionally, studies show that increased background RNA has an inverse, yet non-linear effect on transcript abundance measurement of spike-in sequences with use of random priming. On the other hand, random hexamer primers have been shown to overestimate transcript abundance measurements by up to 19-fold compared with a gene-specific RT primers. The disadvantages of using either oligo d(T) or random priming alone may be suppressed by using a combination of the two priming methods. Sieber and colleagues
demonstrated an increase of 25% in reverse transcription efficiency when both oligo d(T) and random hexamers were used.\textsuperscript{151}

2.3.1.3 Gene-specific priming

Gene-specific RT priming is the most specific and sensitive option for quantification; it provides better linearity of results over a wider template range than random primers.\textsuperscript{90, 132, 149} Also, because specific priming performs optimally at higher temperatures, a more thermo-stable enzyme like AMV or Superscript reverse transcriptase should be used.\textsuperscript{154, 156} However, while oligo d(T) and random priming allow reverse transcription of different transcripts in the same RNA pool, use of gene-specific RT primers provides reverse transcription of specific transcripts, and requires prior determination of which transcripts need to be reverse transcribed.

2.3.2 Reverse transcription inhibitors

2.3.2.1 Specific RT inhibitors

Many studies have shown that inhibitors present in extracted RNA from specific biospecimens can impact the accuracy of RT-qPCR, and affect the reverse transcriptase and/or amplifying DNA-dependent, DNA polymerase.\textsuperscript{157-161} These inhibitory compounds may be co-extracted with RNA and/or introduced during the nucleic acid extraction. Examples of biospecimen-specific enzymatic inhibitors include heme, immunoglobulin G, leukocyte gDNA and anticoagulants, EDTA and heparin for collected whole blood, lipids and muscle in solid tissues\textsuperscript{145} and formalin and residual paraffin from FFPE samples.\textsuperscript{167, 168} As an example, Gerard and colleagues demonstrated that the presence of heparin in the RT reaction inhibited 50% of the activity of Superscript II reverse transcriptase.\textsuperscript{169} Inhibitory components introduced by the extraction process include
phenol, guanidine and ethanol.\textsuperscript{170} While it has been shown that diluting RT reaction cDNA used in PCR amplification removes some inhibitors,\textsuperscript{171} such dilutions may not be possible for low-volume biospecimen samples with less RNA. The reverse transcriptase must efficiently convert both low- and high-abundance transcripts into cDNA despite the amount of background RNA in the assay.\textsuperscript{171}

2.3.2.2 Methods to assess presence of RT inhibitors

A study by Bustin concluded that only 65\% of researchers test their nucleic acid samples for the presence of inhibitors.\textsuperscript{131} Several methods have been used to identify the presence of inhibitors, including use of internal or external controls during RT-qPCR.\textsuperscript{172, 173} To identify and/or control for the presence of inhibitors in the RT reaction, assays may use non-target, endogenous genes, single or multiple,\textsuperscript{174} or exogenous, spike-in transcripts,\textsuperscript{175-178} such as Armored RNA\textsuperscript{179, 180} or External RNA Control Consortium standards.\textsuperscript{181} These RNA standards may serve as qualitative, positive controls or quantitative controls from which samples are measured after PCR amplification. While endogenous controls may also control for extraction efficiency as well as RT efficiency,\textsuperscript{182} exogenous controls added to extracted RNA have their molar concentrations known. Whether endogenous genes or spike-in controls are used to assess RT efficiency, it must be acknowledged that the target transcript and control standard may reverse transcribe at different efficiencies in the presence of different inhibitors or the chosen reverse transcriptase and/or primer method.\textsuperscript{183} In addition to any measured control, target genes measured numbering more than one must be assessed for varying RT efficiencies that may impact downstream measurement. As a result, any choice of
exogenous or endogenous control must be optimized for each assay and each biospecimen.

2.3.3 Reverse transcriptase

The efficiency of any reverse transcription reaction will be significantly dependent on the type of reverse transcriptase used. Although there are a variety of reverse transcriptases available to clinical and research laboratories, each enzyme is unique in its ability to reverse transcribe RNA of various lengths and conformations under inhibitory conditions.\cite{132,184} Different reverse transcriptases yield up to 100-fold different amounts of cDNA, depending on the gene analyzed.\cite{155} When RNA is limiting and expected to be degraded, the most sensitive reverse transcriptase should be used.

2.3.3.1 Secondary structure of RNA

A major challenge to reverse transcription efficiency is the secondary structures of RNA that may impede the reaction.\cite{185-190} To prevent secondary structure conformations of RNA during reverse transcription, RNA is first denatured at a high temperature before the reverse transcription. However, the combination of present Mg\textsuperscript{2+} ions and high temperatures may provide an optimal environment for certain RNAses, and permit subsequent degradation of the RNA.\cite{191-193} A study by Gerard recommends use of additional dNTPs during reverse transcription to minimize RNA hydrolysis by chelating destabilizing excess Mg\textsuperscript{2+} ions in the reaction.\cite{193}

2.3.3.2 RNAse H activity

A second problem with reverse transcription is RNA degradation by RNAse H activity of reverse transcriptases; RNAse H removes the RNA strand in RNA:cDNA hybrids in the RT reaction, and may degrade the RNA template before a fully-formed
cDNA is reverse transcribed. Many commercial reverse transcriptases available have inherent RNAse H activity, such as (Avian Myelomatosis Virus (AMV), Murine Moloney Leukemia Virus (MMLV), Sensiscript (Qiagen, Hilden, Germany) or Omniscript (Qiagen) reverse transcriptases, or mutated MMLV with reduced or absent RNAse H activity, such as AffinityScript (Agilent, Santa Clara, CA), Superscript II (Life Technologies, Grand Island, NY), Superscript III (Life Technologies), PowerScript (BD Biosciences Clontech, Palo Alto, CA) or AccuScript (Stratagene, La Jolla, CA). \cite{194, 195}

Studies show that cDNA yield increases when reverse transcription and RNAse H activity are separated; removal of RNAse H activity from the reverse transcriptase not only prevents truncation of cDNAs by template RNA degradation, but allows increased thermal stability of the reverse transcriptase in the presence of a template-primer \cite{151, 170, 195, 196}. The primed mRNA is not only a substrate for the reverse transcriptase, but is also a substrate for RNAse H. \cite{197} Arezi and colleagues state that compared to the half-life of wild-type MMLV reverse transcriptase at 55°C (less than 5 minutes) with template and primers, the half-lives of RNase H-deficient reverse transcriptions are up to 20 minutes. \cite{170}

2.3.3.3 Comparative studies

While some comparative studies show that Superscript III reverse transcriptase is most efficient for measurement of low gene expression in bovine spleen, liver and jejunum \cite{147} and Armored RNA Quant HIV (Asuragen, Austin, TX), \cite{198} other studies show no difference between Superscript III and other RNAse H-negative reverse transcriptases in FirstChoice® Human Reference Brain RNA (Ambion, Foster City, CA), \cite{199} or in the presence of specific inhibitors \cite{170} in Universal Reference RNA (Agilent, Santa Clara,
CA). Suslov and colleagues believe that the Superscript III system contains specific PCR inhibitors that lead to overestimation of amplification efficiency, and hence cause some studies to show superior cDNA yield with Superscript III.\textsuperscript{200} Using a low number of spike-in EGFP transcripts in background bovine testis RNA, Levesque-Sergerie and colleagues determined that Superscript II was superior to Superscript III in cDNA yield using hydrolysis probes for quantitative PCR.\textsuperscript{171} On the other hand, a study using SYBR Green I chemistry during PCR showed that cDNA yield of the testis transcript GNPDA was highest with Sensiscript reverse transcriptase, 50\% more than that seen with Superscript II.\textsuperscript{171} A Bustin study showed that SuperScript II was superior to Sensiscript when a medium-to-low abundance growth hormone receptor transcript was assayed from a colorectal cancer cell line.\textsuperscript{201} In a study by Okello and colleagues, cDNA yields by different reverse transcriptases were compared in Armored RNA Quant Quant HIV standard both alone and with spiked nucleic acid extracts from an FFPE visceral tissue block.\textsuperscript{198} AccuScript and Superscript III showed the most analytical sensitivity and reproducibility for lowly abundant transcript numbers in both experiments while Superscript II did not.\textsuperscript{198} Stahlberg and Curry show that RT efficiency of low levels of transcript may be significantly improved with the use of a carrier such as yeast tRNA or salmon sperm DNA.\textsuperscript{144,147} It is important that studies claiming to compare the efficiencies of different reverse transcriptases using cell lines or exogenous spike-in transcripts that are relatively intact and free from enzymatic inhibitors, confirm their results in sample biospecimens like blood or tissue as differences in biospecimen type and sample extraction influence differences measured in enzyme efficiency.\textsuperscript{155} It must
also be noted that any study recommending the use of a particular reverse transcriptase is specific for the RT priming, PCR amplification system, PCR primers and measured gene.

2.4 PCR efficiency

Post-reverse transcription, cDNA sequences are exponentially amplified in a PCR assay. For any molecular diagnostic test that relies on RT-qPCR to obtain accurate results, the efficiency of PCR amplification is dependent on efficiencies of sample collection, RNA extraction and reverse transcription. Like other enzymatic reactions, efficient PCR requires the stringent removal of or control for inhibitors. Such actions will ensure accurate and reproducible results, including prevention of false negative results.

2.4.1 Specific PCR inhibitors

Many inhibitors of the reverse transcription reaction also inhibit the PCR assay. As stated before, inhibitors of PCR can be biospecimen specific and co-extracted with total RNA or introduced during the extraction process. Additionally, PCR can be affected by the presence of residual reverse transcriptase enzyme in the RT reaction cDNA used for PCR amplification. Many studies have shown that reverse transcriptase inhibits PCR.\textsuperscript{157, 200, 202-205} Typically, the reverse transcriptase is inactivated by a short incubation of the RT reaction at a high temperature before use of the cDNA solution for PCR amplification. However, if this step is incomplete, the PCR assay may be affected.

2.4.2 Methods to remove PCR inhibitors

Many techniques exist to remove any potential PCR inhibitors. Levesque-Sergerie and colleagues suggest that the simplest method is to dilute RT reaction samples before PCR amplification, thereby diluting out potential PCR inhibitors.\textsuperscript{171} On the other hand, this technique is not an option for small volume biospecimen samples that have low
levels of target molecules. Additionally, other researchers such as Bustin and Stahlberg do not recommend RT reaction dilution because of inconsistent detection of target molecules after PCR amplification.\textsuperscript{132,155} A second option available to remove PCR inhibitors is to complete an RT reaction cleanup using a silica-based column or an organic extraction. While this method is recommended by Suslov,\textsuperscript{200} other studies show that completion of this technique results in up to 50% loss of sample.\textsuperscript{171} In studies that compare PCR results from cDNA that is purified using different methods, three studies show a significant decrease in target signal when silica-based column purification is used compared to organic phase purification.\textsuperscript{198,201,206}

2.4.3 Methods to assess presence of PCR inhibitors

2.4.3.1 SPUD assay

Whether or not dilution or purification methodology is employed to remove inhibitors, every PCR assay should include mechanisms to control for any inhibition present. Nolan and colleagues suggest use of a quantitative PCR assay, SPUD, which detects inhibitors in nucleic acid preparations.\textsuperscript{177} An artificial amplicon, SPUD-A, is PCR amplified with both sample cDNA and water, and measured using SPUD-specific PCR primers and a single SPUD hydrolysis probe. Measurements of the SPUD amplicon with only water and with sample cDNA are compared to determine the amount of inhibition. In retrospect, the SPUD assay can only detect the presence of inhibitors that impede PCR amplification of the SPUD amplicon.

2.4.3.2 Internal amplification control (IAC)

Just as different target sequences may be reverse transcribed at different efficiencies in the presence of inhibitors, different sequences may be PCR amplified at
different efficiencies as well. As a result, optimal control for variation in PCR efficiency is the use of a true internal amplification control (IAC), a standard that is close in sequence to the target sequence, PCR amplified with the same primers as the target sequence so that the PCR efficiency is the same, and PCR amplified in the same reaction as the target sequence.\textsuperscript{172, 207-209} When target product is measured relative to a known number of IAC standards, use of IAC controls for variation in amplification efficiency caused by inter-sample variation in the presence of interfering substances as well as inter-gene variation in amplification efficiency due to limitation in reagent quality or quantity.\textsuperscript{183} Additionally, the presence of IAC product serves as a positive control that the PCR amplification was successful. If no target product is produced, it is a truly negative sample instead of a false negative result. Use of an IAC in molecular diagnostic tests is highly recommended by regulatory agencies, including the EPA,\textsuperscript{210} ISO\textsuperscript{211} and FDA,\textsuperscript{212} and are currently used in FDA-approved RT-qPCR tests.\textsuperscript{213-215} Because each gene is measured relative to a known copy number of its specific synthetic internal standard, and both the native gene template and internal standard are PCR amplified with the same efficiency, quantification can be done at plateau phase, allowing accurate and reproducible results with endpoint PCR. If the competitive internal standard sequence is designed to be shorter (typically by 10\%) than the native sequence, each sequence can be size separated by electrophoresis.\textsuperscript{216} On a real-time PCR platform, the internal standard and native template are the same size, but have varying sequences internal to the forward and reverse PCR primers.\textsuperscript{217} In this two-color fluorometric assay, the internal standard and native template anneal to hydrolysis probes with different fluorophores. As a result, gene native template product is measured relative to its internal standard instead of being
compared to an external calibration curve for absolute quantification. Biospecimen RNA may contain inhibitors that are not present in the nucleic acid samples used to construct the standard curve and thus, underestimation of transcript abundance may occur in samples tested. \(^{218}\)

2.5 Manuscript contributions

The accuracy and reproducibility of any molecular diagnostic test that employs RT-qPCR to measure transcript abundance is dependent upon many factors including sample acquisition, storage, transport, RNA extraction, reverse transcription and PCR amplification. The purpose of our studies described in this document were completed to assess how RNA degradation and reverse transcription inhibition affect gene expression measurements in developed molecular diagnostic tests of various biospecimens including human whole blood, homogenized fish tissue and FFPE-treated human lung tissue. All studies were completed with the use of internal standards during PCR and hence, controlled for PCR inhibition. As a result, we can be confident that our results are indicative of experimental changes not caused by PCR inhibition. Our results will help to influence quality control criteria required for specific biospecimens in molecular biology research. The work describing these quality control assessments is presented here in three manuscripts.

2.5.1 Manuscript #1

Manuscript I: is entitled “Quality control methods for optimal BCR-ABL1 clinical testing in human whole blood samples.” This manuscript has been accepted and published in the Journal of Molecular Diagnostics.\(^{219}\) The aim of this study was to 1) compare our BCR-ABL1 quantitative test with internal standards to other laboratory tests
and 2) determine the impact of RNA degradation and reverse transcription factors on BCR-ABL1/GUSB measurement in human whole blood. As stated previously, collected whole blood contains a variety of enzymatic inhibitors either specific to blood or specific to the chemical composition of the collection medium including EDTA. Use of internal standards in the PCR assay controls for these inhibitors; hence, any demonstrated changes in gene expression measurements are attributed to pre-PCR variables such as RNA degradation and/or reverse transcription efficiency.

2.5.2 Manuscript #2

Manuscript II: is entitled “A new StaRT-PCR approach to detect and quantify fish Viral Hemorrhagic Septicemia virus (VHSv): Enhanced quality control with internal standards.” This manuscript has been accepted and published in the Journal of Virological Methods. The main aim of this study was to demonstrate the use of novel RT-qPCR molecular test to detect and measure VHSv using internal standards. My contribution to the work presented involved assessment of RT efficiency using the RTSM with increasing fish-derived RNA input in RT reactions using random hexamers and MMLV reverse transcriptase. Additionally I assisted with analysis of the effect of increasing fish-derived RNA input on specific gene expression measurements, and how this affected VHSv molecular diagnostic test results. In the case of the BCR-ABL1/GUSB molecular diagnostic test with random hexamers, BCR-ABL1 and GUSB transcripts appeared to be reverse transcribed at the same efficiency as whole blood total RNA input increased. We wanted to determine the effect of increasing homogenized fish tissue total RNA in RT reactions and its effect on RT efficiency and the measurement of VHSv N-gene, actb and ef1a product yield.
2.5.3 Manuscript #3

Manuscript III: is entitled “A multiplex two-color real-time PCR method that enables quality-controlled molecular diagnostic testing in FFPE samples.” This manuscript has been submitted to the journal Clinical Chemistry. The aim of this study was to develop a lung cancer RT-qPCR-based diagnostic test with internal standards for use on a real-time PCR platform. This particular test is amenable to use of formalin, fixed and paraffin embedded lung tissue samples because short PCR products can be amplified, an experimental necessity for highly degraded RNA samples. The molecular diagnostic test uses the gene expression of three genes, CMYC, E2F1 and p21, normalized to ACTB to diagnose lung cancer in lung FFPE-treated biospecimen. My contribution to the work presented included identifying the most optimal FFPE RNA extraction method to be used in this study, and assessing the most optimal RT priming method for these FFPE biospecimens.
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Chapter 3

Quality control methods for optimal BCR-ABL1 clinical testing in human whole blood samples

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3.1 Abstract

Reliable BCR-ABL1 measurement is essential for optimal management of chronic myelogenous leukemia. There is a need to optimize quality control, sensitivity and reliability of methods used to measure a Major Molecular Response (MMR) and/or treatment failure. Effects of room temperature storage time, different primers and RNA input in reverse transcription (RT) reaction on BCR-ABL1 and β-glucuronidase (GUSB) cDNA yield were assessed in whole blood samples mixed with K562 cells. BCR-ABL1 was measured relative to GUSB to control for sample loading and each gene was measured relative to known numbers of respective internal standard molecules to control for variation in quality and quantity of reagents, thermal cycler conditions and presence of PCR inhibitors. Clinical sample and reference material measurements with this test were concordant with results reported by other laboratories. BCR-ABL1/10^3 GUSB values were significantly reduced (p = 0.004) after 48-hour storage. Gene specific primers yielded more BCR-ABL1 cDNA than random hexamers at each RNA input. Additionally, increasing RNA inhibited the RT reaction with random hexamers but not with gene-specific primers. Consequently, yield of BCR-ABL1 was higher with gene-specific RT primers at all RNA inputs tested, increasing to as much as 158-fold. We conclude that optimal measurement of BCR-ABL1/10^3 GUSB in whole blood is obtained when gene-specific primers are used in RT and samples are analyzed within 24 hours following blood collection.
3.2 Introduction

Chronic myelogenous leukemia (CML) represents 15% of all adult leukemias in Western populations.\(^1\) This malignancy is caused by a chromosomal translocation t(9:22)(q34;q11); a segment of the Breakpoint Cluster Region (BCR) gene from chromosome 22 (region q11) is fused to a site within the Abelson (ABL1) gene from chromosome 9 (region q34).\(^2,3\) Most BCR breakpoints occur within exons e12-e16 (b1-b5), an area known as the major breakpoint cluster region (M-bcr), and fuse to ABL1 exon 2 breakpoints resulting in e13a2 (b2a2) or e14a2 (b3a2) fusion transcripts that encode 210 kDa proteins.\(^4\) The BCR-ABL1 fusion protein is a constitutively active tyrosine kinase responsible for the uncontrolled proliferation observed in CML.\(^5-7\) For the last decade, first-line therapy for chronic phase CML has been imatinib mesylate (Gleevec) which inhibits the tyrosine kinase activity of the BCR-ABL1 protein.\(^8,9\) Imatinib mesylate binds to the ATP-binding pocket of the BCR-ABL1 fusion protein, stabilizing it in its inactive form.\(^10\) While second-generation tyrosine kinase inhibitors, like dasatinib and nilotinib, have been used in imatinib-resistant or intolerant CML patients, recent studies show promise in these agents as more potent first-line therapies for those newly diagnosed with chronic-phase CML.\(^11-16\)

Following treatment, a major molecular response (MMR) is defined, based on results from the International Randomized Interferon versus STI-571 (IRIS) study,\(^17\) as a three-log\(_{10}\) reduction from a standardized median baseline value in the level of measured BCR-ABL1 transcript. In the IRIS study patients with a BCR-ABL1 value at or below MMR within 12-18 months of beginning imatinib treatment were 100% free from accelerated phase or blast crisis at five years.\(^18\) A subsequent 0.5-log\(_{10}\)
increase in BCR-ABL1 transcript abundance from treated baseline indicates treatment resistance and the need to begin a second-generation tyrosine kinase inhibitor or plan for allogeneic stem cell transplantation.\textsuperscript{19-21} National Comprehensive Cancer Network (NCCN) Guidelines recommend BCR-ABL1 monitoring every three months to provide fusion transcript level trend data.\textsuperscript{19, 20, 22} Timely BCR-ABL1 monitoring is essential to identify patients who are unresponsive to therapy in the early phase of treatment. Therefore, an accurate baseline or normalized value of BCR-ABL1 fusion transcript abundance must be obtained at time of diagnosis and subsequent BCR-ABL1 levels must be accurately measured to monitor therapeutic efficacy over time.\textsuperscript{18}

Several quantitative, reverse-transcriptase PCR-based BCR-ABL1 diagnostic tests are commercially available. In addition, standardized methods have been developed for clinical laboratory measurement of BCR-ABL1 in whole blood,\textsuperscript{4, 22, 23} including use of the International Scale for inter-laboratory harmonization.\textsuperscript{24-29} The IRIS standardized diagnostic baseline is defined as 100\% International Scale value and MMR is defined as 0.1\% International Scale value.\textsuperscript{30} BCR-ABL1 measurement results are converted to the International Scale via use of the World Health Organization panel of reference reagents with designated BCR-ABL1 International Scale values.\textsuperscript{31, 32}

An element missing from existing BCR-ABL1 tests is adequate control for inter-sample variation in PCR interfering substances, and inter-reaction variation in quality and quantity of PCR reagents or thermal cycling efficiency. These problems could cause inaccurate and possibly false negative results. To address this, in the
method described here BCR-ABL1 and GUSB were each measured relative to a
known number of respective synthetic internal standard (IS) molecules.

Another potential source of inconsistency in BCR-ABL1 measurement is
variation in the efficiency of mRNA-to-cDNA conversion during reverse transcription
(RT). Blood-specific inhibitors of RT may be present within RNA extracted from whole
blood, including heme, immunoglobulin G, leukocyte genomic DNA and the
anticoagulants, EDTA and heparin.\textsuperscript{33-37} RNA extracted from whole blood is mostly from
leukocytes; however, a fraction of the RNA comes from reticulocytes. Reticulocytes
contribute an abundance of interfering alpha- and beta-globin mRNA that may compete
with lowly expressed transcripts such as BCR-ABL1 for reagents within an RT
reaction.\textsuperscript{38, 39} Reverse transcription efficiency is also dependent on the priming method
used.

Clinical laboratories that conduct BCR-ABL1 molecular monitoring generally
analyze whole blood samples collected in tubes containing EDTA to prevent coagulation-
associated cytolysis. However, even in such tubes relative representation of certain genes
may change as early as minutes after venipuncture due to altered regulation and/or
degradation rate.\textsuperscript{40, 41} Thus, there is a need to identify the optimal RT priming method for
both BCR-ABL1 and the reference gene, GUSB under the most optimal storage time.
The optimal amount of whole blood RNA to include in the RT reaction may be affected
by each of the above factors.

In an effort to establish optimal conditions for measurement of BCR-ABL1, we a)
developed a quality-controlled, quantitative PCR method, b) measured effect of total
RNA input or type of RT primers on RT efficiency, and c) assessed effect of storage time on BCR-ABL1 measurement.
3.3 Materials and Methods

3.3.1 Samples from College of American Pathologists Minimal Residual Disease (MRD-B) – BCR-ABL1 p210 2011 Proficiency Survey

Three proficiency samples were obtained from the College of American Pathologists (CAP, Northfield, IL). Sample MRD-04 was RNA extracted from the K562 cell line that expresses the b3a2 BCR-ABL1 transcript. Sample MRD-05 was RNA extracted from a BCR-ABL1 – negative cell line. Sample MRD-06 was RNA from MRD-04 diluted 1:10,000 in BCR-ABL1 – negative RNA (MRD-05). Each RNA sample was DNase treated with DNA-free™ (Life Technologies, Grand Island, NY).

3.3.2 Blood samples

All blood samples were collected under protocols approved by the University of Toledo Medical Center institutional review board. Whole blood samples were collected in 4mL dipotassium EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) at ambient temperature at the University of Toledo Medical Center. Blood used to mix with K562 cells (see below) was obtained through the UTMC Department of Pathology from patients undergoing routine venipuncture for laboratory testing. Leftover blood not needed for standard of care purposes from patients with normal Complete Blood Count (CBC) was anonymized and provided to the research laboratory. Peripheral blood was obtained from 3 CML patients (2 chronic phase (CP)-CML newly diagnosed and 1 CP-CML at MMR under treatment) at the same time blood was drawn for shipment to ARUP Laboratories (Salt Lake City, Utah) for BCR-ABL1 measurement and conversion of results to the International Scale.

3.3.3 Samples for kinetic stability study
The K562 cell line, expressing BCR-ABL1 b3a2 fusion transcript, was purchased from ATCC (Manassas, VA) and incubated in RPMI 1640 medium + 10% FBS at 37°C, in an atmosphere containing 5% CO₂ and 90% humidity. K562 cells were isolated from culture at 80% confluency. In each of two experiments, whole blood samples were collected in EDTA tubes from three anonymized individuals and each sample was spiked with a known number of K562 cells suspended in PBS. In the first experiment (samples A, B, C) the concentration of K562 cells in each whole blood sample was 5.8E5 cells/mL (2.32E6 cells total) and in the second experiment (samples D, E and F) the concentration in each sample was 4.7E6 K562 cells/mL (1.88E7 cells total). Each whole blood/K562 cell sample was incubated in 15mL conical tubes at room temperature for various amounts of time. For each sample the time course was initiated within 6 hours of venipuncture. At each timepoint, individual conical tubes were inverted three times before RNA extraction.

3.3.4 Samples for comparison of three RT priming methods study

Known numbers of K562 cells were spiked into anonymized whole blood samples collected in EDTA tubes from one de-identified individual (K). The concentration of K562 cells in sample K was 5.8E5 cells/mL (2.32E6 cells total).

3.3.5 Samples for RNA input versus RT priming methods study

Known numbers of K562 cells were spiked into anonymized whole blood samples collected in EDTA tubes from each of three individuals (G, H, I) for the random hexamer-primed RT efficiency study. The concentration of K562 cells was 4.4E6 K562 cells/mL (1.76E7 cells total) in samples G and H and 5.0E6 K562 cells/mL (2.00E7 cells total) in sample I. For the gene-specific primed RT efficiency study blood from one
individual (J) was used. The concentration of K562 cells was 4.9E6 K562 cells/mL (1.76E7 cells total).

3.3.6 Preparation of RTSM

In vitro-transcribed synthetic, “alien” RNA standards developed by the External RNA Control Consortium (ERCC), termed ERCC 171 and 113, were donated by Dr. Marc Salit, NIST, USA.43,44,45 RNA stocks from ERCC 113 and 171 standards were first diluted in RNase-free water and RNA concentration was measured using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). ERCC 113 and 171 standards were each secondarily diluted in 100 ng/µL salmon sperm DNA to a final concentration of 1.0(10⁻¹⁰) M (Invitrogen, Carlsbad, CA). The ERCC 113 standard was reverse transcribed into cDNA and the cDNA was quantified using the Agilent 2100 Bioanalyzer. A Reverse Transcription Standards Mixture (RTSM) was prepared by combining known molar amounts of ERCC 171 RNA and ERCC 113 cDNA (previously reverse transcribed). A 1µL aliquot of RTSM was included in each RT reaction to assess RT efficiency. The ERCC 171 RNA was reverse transcribed into cDNA along with other RNA species in the RT reaction while the ERCC 113 cDNA remained unchanged. Yield of ERCC 171 cDNA was a measure of RT efficiency and ERCC 113 cDNA was included as a sample loading control. Each 1µL aliquot of RTSM contained 1.42E4 (+ 6.7E3) ERCC 171 cDNA molecules and 1.68E4 (+ 7.4E3) ERCC 113 cDNA molecules when measured in RT reactions with no background RNA.

3.3.7 Measurement of RT efficiency

After RT, an aliquot of RT product cDNA was subjected to PCR in the presence of a known number of IS molecules for ERCC171 and ERCC113, respectively. ERCC
171 RNA RT efficiency was determined by measuring ERCC 171 cDNA NT/IS PCR product ratio over ERCC 113 NT/IS product ratio. In this way, normalization of ERCC 171 cDNA molecules to ERCC 113 cDNA molecules controlled for variation in RTSM sample loading, enabling reliable measurement of RT efficiency.

3.3.8 Sequencing of background peaks

PCR products associated with the background noise peaks were isolated using an E-Gel® SizeSelect 2%™ gel with the E-Gel® iBase™ Power System (Invitrogen, Carlsbad, CA) and tailed BCR-ABL1 b3a2 sequencing primers corresponding to Ion Torrent Amplicon Sequencing adapters were used to amplify both isolated native and off-target products (Ion Torrent Amplicon Application Note, 4/4/2011: Amplicon Sequencing. South San Francisco, CA). Amplified products with attached Ion Torrent Amplicon Sequencing adapters were gel purified using E-Gel® Size Select 2%™ and sent to Ohio University Genomics Facility for Ion Torrent 314 DNA Chip Sequencing Service (Ohio University, Athens, OH, USA). Sequencing data captured internally to the sequencing reagents were used to build consensus sequences using ClustalX 2.1.46 These consensus sequences were then subjected to BLAST search of the NCBI Human genomic and transcript databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3.9 RNA extraction

RNA was extracted with the QIAamp RNA Blood Mini Kit provided by QIAGEN (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. Each QiaAmp spin-column can prepare up to 1.5mL of human whole blood. Each RNA sample was eluted with RNase-free water. In the kinetic cytolysis and RT primer comparison studies, RNA was extracted from single 1mL anonymized whole blood/K562 samples per
individual. In the RNA input versus RT efficiency study, RNA was extracted from four separate spin columns (1mL each) with the same eluate volume of RNase-free water applied to each of the four columns.

For the low BCR-ABL1 RNA RT priming comparison, patient blood sample RNA with undetectable BCR-ABL1 levels was extracted using the QiaAmp RNA Blood Mini Kit (QIAGEN, Hilden, Germany). Extracted patient blood RNA was mixed with a 0.6µL K562 cell RNA (5.66 ng/µL) that was extracted using TriREAGENT® (Molecular Research Center, Inc., Cincinnati, OH).

For all samples, RNA concentration and purity were each measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was evaluated on an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) for quantification and characterization of 28s and 18s ribosomal RNA bands. Agilent software calculated an RNA integrity number (RIN) for each sample assessed. RNA samples were stored at -20°C.

3.3.10 Reverse transcription

All total RNA samples were reverse transcribed using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). A 30µL RT reaction volume was used and contained 0.5 µg total RNA (kinetic stability study), various RNA inputs including 0.9, 3, 5, 9, and 18 µg (RT input study) or 1.0 µg total RNA (RT priming comparison study). Reverse transcription reaction volumes were increased for experiments including the CAP Proficiency Sample Study (75uL) and CML Patient Study (Subject 806:40uL and Subjects 807 and 808:60uL). In a 30µL RT reaction, sample RNA in RNase-free water (12µL) was denatured by five-minute incubation at
94°C and then placed on ice to cool. An 18-µL RT master mix was then added and the RT reaction mixture was incubated at 50°C for one hour, followed by five-minute incubation at 94°C. RT master mix included: 10X RT buffer, 25mM MgCl₂, 0.1M DTT, 10mM dNTP mix, RnaseOUT™ (40U/µL), SuperScript™ III RT (200U/µL) with either random hexamers (25µM), oligo (d)T primers (50µM), or gene-specific primers (3µM) (Invitrogen, Carlsbad, CA). The PCR reverse primer was used as the gene-specific RT primer for GUSB, while BCR-ABL1 gene-specific RT primers included either the PCR reverse primer (19 bases) or an extended primer (29 bases).

3.3.11 Transcript abundance measurement

Sample loading was controlled by measuring BCR-ABL1 relative to GUSB. According to the standardized reverse transcription–polymerase chain reaction (StaRT–PCR) method, BCR-ABL1 and loading control gene GUSB were each measured relative to a known number of their respective IS molecules within a standardized mixture of internal standards (SMIS). SMIS used for these studies was purchased from Accugenomics, Inc. (Wilmington, NC). Transcript abundance values were then calculated as BCR-ABL1 molecules/10³ GUSB molecules. The competitive template IS for BCR-ABL1 and GUSB were each 10–20% shorter in length than the target gene native template (NT) PCR product but both were amplified with the same efficiency by the same pair of primers. BCR-ABL1 b3a2 and b2a2 fusion transcript cDNA were measured with the same BCR-ABL gene-specific PCR forward and reverse primer and measured relative to the same IS sequence. BCR-ABL1 b3a2 and b2a2 PCR products were different lengths that could be electrophoretically separated. Sequence information for primers and internal standards is provided in Table 1. The presence of an internal
standard controlled for sample loading, inter-sample variation in the presence of PCR inhibitors (which often are gene-specific) and ensured no false negatives (if the PCR failed the internal standard PCR product was not observed and there were no data to report). False positives due to contamination were eliminated through use of a control PCR sample with neither native nor competitive template.

Prior to amplification, cDNA and SMIS were combined into a master mix along with the appropriate volume of RNase-free H₂O, 30 mM 10X buffer containing MgCl₂ (Idaho Technology, Salt Lake City, UT), 2mM dNTPs (Promega, Madison, WI), 50 ng/µL gene-specific primers and a minimum of 0.5 U Taq polymerase (Promega, Madison, WI). For PCR amplification of blood/K562 cDNA samples that had been reverse transcribed with the 29-base BCR-ABL1 RT gene-specific primer, a minimum of 0.5 U of Gotaq® Hotstart polymerase (Promega, Madison, WI) was used. For each experiment, sufficient master mixture was prepared to measure each of the desired genes. This mixture was divided into tubes containing primers for single genes. All PCRs were performed in a Rapidcycler (Idaho Technology, Salt Lake City, UT) for 35 cycles. All reactions were denatured for 5 s at 94°C (2 min at 94°C for Gotaq® Hotstart polymerase), annealed for 10 s at 58°C and elongated for 15 s at 72°C. Following PCR amplification, the IS and NT for each gene were electrophoretically separated and quantified on an Agilent 2100 Bioanalyzer using DNA chips with DNA 1000 kit reagents for visualization according to the manufacturer’s protocol (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Area under the curve (AUC) values for the NT and IS electrophoretic peaks for each gene were used for gene expression measurement.
3.3.12 Statistical analysis

Each measurement was performed in at least triplicate. Results were expressed as a mean + SD. Statistical significance was calculated using Student’s t-test from Data Analysis Tools in Microsoft Office Excel (Microsoft Corp, USA) and figures were created with GraphPad Prism 6 (La Jolla, CA). The differences were considered significant if p < 0.05.
3.4 Results

3.4.1 CAP MRD-B – BCR-ABL1 p210 2011 proficiency survey sample study

BCR-ABL1 b3a2/10^3 GUSB values were measured in the three CAP proficiency samples. As expected, BCR-ABL1 b3a2 was not detected in BCR-ABL1-negative sample MRD-05. Compared to baseline sample MRD-04, BCR-ABL1/10^3 GUSB was 3.55-log_{10} lower in sample MRD-06 (Figure 1A). As indicated in the figure, this result was not significantly different from the mean difference measured by other laboratories in the survey.

3.4.2 Patient sample comparison between BCR-ABL1 per 10^3 GUSB and International Scale values

Change in BCR-ABL1/10^3 GUSB values were closely correlated with change in values measured by ARUP Laboratories for the three CML patient samples. ARUP measured BCR-ABL1 relative to ABL1 and converted values to International Scale. A linear trendline fit BCR-ABL1/10^3 GUSB to International Scale data points with high correlation (R^2 = 0.99) (Figure 1B).

3.4.3 Effect of incubation at room temperature on BCR-ABL1 per 10^3 GUSB values

BCR-ABL1/10^3 GUSB transcript abundance and RIN score were measured at each timepoint and plotted as change relative to baseline (undegraded) values (Figure 2). When data from six anonymized individuals were combined, the BCR-ABL1/10^3 GUSB mean value trended down at 24h but was not significantly decreased until 48h (51% decrease, p=0.004). Relative to baseline, RIN Score remained unchanged at across all timepoints measured.

3.4.4 RT primer effect on yield of BCR-ABL1 b3a2 or GUSB
We compared oligo (d)T, random hexamer, and gene-specific RT primers to determine which gave greatest yield of BCR-ABL1 and GUSB cDNA (Figure 3A). For both BCR-ABL1 b3a2 and GUSB, with RNA input into the RT reaction held constant (1 µg RNA/RT), highest yield of cDNA was obtained with the use of gene-specific primers in the RT reaction. The yield with gene-specific primers was 17-fold (p=0.0001) and 21-fold (p=0.0008) higher for BCR-ABL1 b3a2 and GUSB compared with random hexamers (Figure 3A). Because yield of each gene increased about the same amount, normalized (BCR-ABL1 b3a2/10^3 GUSB) values measured with random hexamer versus gene-specific RT primers were not significantly different. In contrast, the BCR-ABL1 yield with oligo (d)T primers was 4-fold lower (p=0.0007) and GUSB cDNA yield was 17-fold higher (p=0.0001) with oligo (d)T compared to random hexamer primers (Figure 3A). Because the effect was different on BCR-ABL1 and GUSB, BCR-ABL1 b3a2/10^3 GUSB values were significantly different compared to those from random hexamer or gene-specific primers (p=0.0001).

### 3.4.5 Effect of RNA input and RT primer on yield of BCR-ABL1 b3a2 or GUSB cDNA

When random hexamer primers were used in RT, a maximum 3-fold increase (p=0.003) in BCR-ABL1 b3a2 (molecules/µL cDNA) was observed at 18 µg RNA/RT compared to baseline at 0.9 µg RNA/RT. Because the slope for GUSB was not significantly different from that for BCR-ABL1 (p≥ 0.35), there was no significant difference among the BCR-ABL1 b3a2/10^3 GUSB values for each level of RNA input (Figure 3B).
The effect of increasing RNA input into RT reactions using gene-specific RT priming was also tested. Yields of BCR-ABL1 and GUSB molecules/µL cDNA were respectively 17-fold and 20-fold higher at baseline 0.9 µg RNA/RT reaction (30 ng RNA/µL cDNA) compared to samples primed with random hexamers (Figure 3B). Consequently, with gene specific primers, at 18 µg RNA/RT reaction (600 ng RNA/µL cDNA), BCR-ABL1 and GUSB yield were respectively 158-fold and 312-fold higher with gene-specific RT priming compared to random hexamer RT priming. As with the cDNA from random hexamer RT, because the slope for GUSB was not significantly different from that for BCR-ABL1 (p≥0.35), there was no significant difference among the BCR-ABL1 b3a2/10^3 GUSB values for each level of RNA input.

### 3.4.6 RNA input effect on RT efficiency with random hexamers (ERCC 171/113)

The RTSM (see Methods) was used to directly measure whether reduced cDNA yield with random hexamers was due to RT inhibition. Compared to baseline (0 µg background RNA/RT), ERCC 171/113 RT efficiency with random hexamer primers was reduced 52% (p=0.02) at 3 µg RNA/RT (100 ng RNA/µL cDNA) and reduced 80% (p = 0.002) at 18 µg RNA/RT (600 ng RNA/µL cDNA). A semi-log plot trendline fits the data points with high correlation (R^2 = 0.98) (Figure 3C). ERCC 171/113 values were measured in the same random hexamer-primed cDNAs used in Figure 3B.

### 3.4.7 Extended BCR-ABL1 gene-specific RT primer effect on yield of BCR-ABL1 per 10^3 GUSB values

Although use of gene-specific primers in RT markedly increased cDNA yield and therefore has the potential to significantly increase the sensitivity of the BCR-ABL1 test, the 19-base, gene-specific BCR-ABL1 RT primer caused high background noise in the
electropherogram when low level of BCR-ABL1 was loaded (Figure 4Ai). In an effort to identify and eliminate the source of this high background, PCR products associated with the background noise peaks were isolated and sequenced. Greater than 95% of the generated consensus sequences mapped to the RANBP3 gene (Entrez Gene ID: 8498), and its multiple splice isoforms RANBP3-a,-b,-d. Based on this mapping we identified significant homology between the 19-base, gene-specific BCR-ABL1 RT primer used and the RANBP3 transcript isoform Family (Table 2). We hypothesized that these splice variants resulted in non-specific electrophoretic peaks at lower input concentrations of BCR-ABL1 native product. In order to test this hypothesis, we lengthened the 19-base, gene-specific BCR-ABL1 RT primer by ten bases at the 3’ end to minimize homology of the gene-specific BCR-ABL1 RT primer to the RANBP3 transcript isoform family.

In contrast to the 19-base RT primer, when low level BCR-ABL1 was loaded into the RT reaction with 29 bp RT primer, the electrophoretic background noise was low (Figure 4Aii). Compared to yields obtained with random hexamers, use of the extended BCR-ABL1 gene-specific RT primer and the original GUSB gene-specific RT primer increased BCR-ABL1 b3a2 yield 8.5-fold (p=0.03) and GUSB yield 6.6-fold (p=0.007) respectively in RNA samples with low BCR-ABL1 levels. Because yield of each gene was increased about the same amount, normalized (BCR-ABL1 b3a2/10^3 GUSB) values measured with random hexamer versus gene-specific RT primers were not significantly different (p=0.17) (Figure 4B).
3.5 Discussion

Accurate measurement of BCR-ABL1 load in peripheral blood samples is necessary for optimal management of chronic myelogenous leukemia. The test and methods that we have developed should enable reliable inter-laboratory comparison of results. The values obtained with the BCR-ABL1 kits have high correlation with results obtained in the CAP MRD-B – BCR-ABL1 p210 2011 Survey and from analysis of CML patient samples with an established commercial test in a clinical laboratory. The synthetic internal standards used in this kit were prepared in large volume and are stable so that they may be shared with other laboratories. This approach not only enables reliable inter-laboratory comparison but also controls for inter-sample variation in interfering substances and/or inter-experimental variation in quality or quantity of PCR reagents, and/or thermal-cycler performance; all factors that may lead to incorrectly low measured values or false negatives. In addition, use of gene-specific primers in RT increased sensitivity of the test by at least 10-fold to a maximum of 100-fold compared to random hexamer primers, depending on the amount of RNA input in the RT reaction. This will enable reduction in amount of RNA required and/or will enable measurement of MMR at a log-lower level.

In order to ensure reliable BCR-ABL1 measurement in clinical testing, it is clear that in clinical application optimal RT conditions, including primers and RNA input, should be established and adhered to, and that blood storage time prior to measurement should be kept to a minimum and kept relatively constant. The effects of varying these conditions are of sufficient magnitude to affect reliability of measuring a $0.5\log_{10}$ increase in BCR-ABL1 load, an accepted indication of treatment failure, and thereby
could negatively impact patient treatment. We conclude that optimal measurement of BCR-ABL1/10^3 GUSB in whole blood is obtained when gene-specific primers are used in RT, and samples are processed within 24 hours following blood collection.

For monitoring CML, patient blood samples typically are collected at one site and then shipped to a specialized laboratory at a different site for BCR-ABL1 testing. Consequently RNA may not be extracted from blood samples until more than 48 hours following collection. Our results, like those of van der Velden\textsuperscript{52} and Moravcova,\textsuperscript{53} suggest that blood samples kept at ambient temperature for extended amounts of time result in altered BCR-ABL1 measurement. Specifically BCR-ABL1 b3a2/10^3 GUSB values decreased by ~50% in 48 hours (Figure 2). Such a sizable decrease combined with other sources of analytical variation could mask a 0.5-log\(_{10}\) increase. Delay of change in treatment due to inaccurate BCR-ABL1 measurement could lead to decreased survival time for patients. Although RIN Score is commonly used as measurement of RNA integrity, in this study it was an insensitive indicator of RNA degradation. However, RIN score may be useful as a convenient first analysis.

To be effective, the BCR-ABL1 measurement method used for molecular CML monitoring must reliably measure low copy numbers. In quantitative, reverse-transcriptase PCR-based assays that measure BCR-ABL1 load, sensitivity of BCR-ABL1 detection can be heightened by increasing the amount of extracted RNA from patient samples into the RT reaction before PCR amplification. Due to increased analyte loaded, there is an advantage to loading up to 600 ng RNA/uL reaction (18 µg RNA/30 µL reaction) when random hexamers are used for priming in the RT reactions. However, when random hexamers are used, adding more RNA to the RT reaction only marginally
improves sensitivity of BCR-ABL1 detection due to reduced RT cDNA yield (Figure 3B). Analysis with the RTSM confirmed that increasing RNA in the RT reaction inhibits random hexamer-primed RT. Possible causes of decrease in RT efficiency in these conditions include limiting substrates, increased presence of blood-specific RT inhibitors or a combination of both. In studies not presented, attempts to optimize substrate concentrations in RT did not improve RT efficiency with random hexamer priming at higher RNA load. In contrast, when gene-specific RT primers were used, BCR-ABL1 yield was markedly higher and there was no interference with RT as the level of RNA input into the RT reaction increased. When RNA input was increased from 0.9 μg to 18 μg RNA/30 μL RT reaction, there was a 3-fold increase in BCR-ABL1 yield with random hexamers but a 158-fold increase with gene-specific primers, providing a nearly 50-fold higher yield of BCR-ABL1 product with gene specific primers. Thus, if enough blood-derived RNA from a sample is available, increasing the RNA input of the RT reaction to as much a 600 ng RNA/μL reaction will markedly improve sensitivity of BCR-ABL1 detection when gene-specific RT priming is used, but provide only small improvement with random hexamer primers.

Although oligo (d)T primers are commonly used for reverse transcription of eukaryotic mRNAs with a 3’ poly-A tail, they have reduced RT efficiency for BCR-ABL1 fusion transcript (Figure 3A), possibly due to the presence of a large distance (~1000 nucleotides) between the poly-A tail and ABL1 exon 2.24 As a result, BCR-ABL1 molecular monitoring laboratories generally use random hexamers as the RT priming method instead of oligo (d)T primers.30 Although the use of random hexamer primers allows the reverse transcription of most transcripts from the same RNA sample, gene-
specific RT priming is reported to provide more efficient RT of specific transcripts. Previous studies showed that the use of random pentadecamer primers increased BCR-ABL1 yield 86% compared to random hexamers. In our study, gene-specific RT primers produced the optimal yield of BCR-ABL1 b3a2 and GUSB cDNA; generally 1-2 logs more than that obtained with random hexamers depending on the RNA input into the RT reaction.

In our construction of the extended BCR-ABL1 gene-specific RT primer to eliminate off-target RT priming, we determined that the RANBP3 transcript isoform Family has a significant degree of sequence homology to ABL1 in exon 2. This particular finding is important, as it may enable optimization of other nucleic acid – based CML monitoring methods in order to avoid false-positive measurement of RANBP3 transcripts.

We conclude that use of gene-specific RT primers will be optimal for molecular monitoring of BCR-ABL1 load and that this is especially important when BCR-ABL1 copy number is low, for example, when patients achieve a major or complete molecular response. It is recognized that use of random hexamers to prime blood-derived RNA for cDNA synthesis enables the reverse transcription of all possible BCR-ABL1 fusion transcripts and this is optimal for initial CML diagnosis. However, because gene-specific primers have much higher RT efficiency, they are more suitable for monitoring purposes when the specific fusion transcript splice variant is known.
3.6 Table and figure legends

Table 3.1 – Primer and internal standard sequences

<table>
<thead>
<tr>
<th>Primer and Internal Standard Sequences</th>
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<tr>
<td>Primer 1: ACCTGACCAATTAGCCCTAG</td>
</tr>
<tr>
<td>Primer 2: CGTATGCGCCCTAGGATAG</td>
</tr>
<tr>
<td>Internal standard: TACCCGCGGGTGAC</td>
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Table 3.2 – Homology of BCR-ABL1 gene-specific RT priming site in AB1 transcript and RANBP3 transcript isoform family

The “~” symbol represents a small insertion sequence (2-5 bases) seen in a small fraction of captured splice variants (<1%). Complementary bases are in bold.

Figure 3.1 – Interlaboratory comparison of BCR-ABL1 measurement

(A.) Log_{10} reduction of BCR-ABL1/10^3 GUSB measurement in College of American Pathologists (CAP) Minimal Residual Disease (MRD)-B - BCR/ABL1 p210 2011 Survey measured at the University of Toledo Medical Center (UTMC) compared to median log_{10} reduction values of survey participants. (B.) Comparison of BCR-ABL1/10^3 GUSB measurement to International Scale percent values reported by ARUP Laboratories (Salt Lake City, Utah) in three CML patient blood samples (2 CP-CML newly diagnosed without treatment, 1 CP-CML at MMR under treatment). Gene-specific RT primers were used for newly-diagnosed CML patient samples and random hexamer RT primers were used for the MMR CML patient sample. The UTMC data presented are mean values for ≥ 3 replicate measurements. Error bars, SD of the mean results.

Figure 3.2 – Effect of storage time on BCR-ABL1 measurement and RIN score
Effect of incubation at room temperature on BCR-ABL1/10\(^3\) GUSB measurement and RIN Score value relative to baseline time equal to 0 hours. RNA was extracted from whole blood collected in EDTA tubes, mixed with K562 cells, and then incubated at room temperature for 3 days. Random hexamers were used for reverse transcription. Asterisks denote statistical significance (* if p<0.01). The results are mean values for samples from six donors, with $\geq 3$ replicate measurements of each sample.

**Figure 3.3 – Effect of RT primer and RNA input on measured transcript abundance**

(A) Effect of RT priming method on yield of BCR-ABL1 b3a2 and GUSB molecules/PCR assay and BCR-ABL1 b3a2/10\(^3\) GUSB measurement following qRT-PCR of RNA extracted from whole blood/K562 mixture with high level of BCR-ABL1 transcript. RH is random hexamer and GSP-19 is gene-specific (19-base primer) (B) Effect of blood/K562 cell mixture RNA concentration in RT reaction (30µL volume) and RT priming method on yield of BCR-ABL1 b3a2 or GUSB cDNA relative to random hexamer primed - baseline (0.9 µg RNA/RT). GSP-RT is gene-specific primed RT reaction and RH-RT is random hexamer primed RT reaction. (C) Effect of RNA input on random hexamer-primed RT efficiency. RT efficiency is ERCC 171/113 measurement normalized to ERCC 171/113 measurement for 0µg RNA input. The results are mean values for samples with $\geq 3$ replicate measurements of each sample. Error bars, SD of the mean results.

**Figure 3.4 – Effect of extended BCR-ABL1 gene-specific RT primer on target specificity**
(A) Agilent Bioanalyzer electropherograms demonstrating (upper) off-target reverse transcription priming with 19-base BCR-ABL1 gene-specific RT primer with sample having low level BCR-ABL1. NT and IS product peaks are outcompeted in polymerase chain reaction by RANBP3 transcript isoforms, (lower) presence of clean NT and IS peaks with 29-base BCR-ABL1 gene-specific RT primer. (B) Effect of RT priming method on BCR-ABL1 b3a2/10^3 GUSB measurement following reverse transcription of low level of BCR-ABL1 transcript in a blood background. RH is random hexamer and GSP-29 is gene-specific primer (29 bases). The data presented are mean values for ≥ 3 replicate measurements. Error bars, SD of the mean results.
### Table 3.1

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<th>IS length</th>
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Table 3.2

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<tr>
<td>BCR-ABL1 19-nucleotide gene-specific RT primer</td>
<td>5'- TTGGGGTCATTTCCAGTCCAGG -3'</td>
</tr>
<tr>
<td>RANBP3 transcript isoform Family</td>
<td>5'- GAGGAGAAAGGCCCAG~AAAAATGAGTCAG -3'</td>
</tr>
<tr>
<td>BCR-ABL1 29-nucleotide gene-specific RT primer</td>
<td>5'- TTGGGTCATTTCCACTGGGTCAGGAAGGT -3'</td>
</tr>
</tbody>
</table>
Figure 3.1

A. UTMC and CAP Survey Results

B. Correlation of ARUP and International Scale Percent Value

y = 0.85x + 0.68
R^2 = 0.997
Figure 3.2

![Graph showing time (hours) vs. fold change from baseline for RIN Score and BCR-ABL1 (b3a2/10^3 GUSB).]
Figure 3.3

**A.**

- BCR-ABL1 (b3a2)
- GUSB

**B.**

- BCR-ABL1 GSP-RT
- GUSB GSP-RT
- BCR-ABL1 RH-RT
- GUSB RH-RT

**C.**

- ERCC 171/113

Log-log plots showing fold change from baseline with RNA input (µg)/RT. The plots are labeled with their respective equations and correlation coefficients:

- BCR-ABL1 (b3a2): $y = 1.16x$, $R^2 > 0.98$
- GUSB: $y = 0.29x$, $R^2 = 0.68$
- ERCC 171/113: $y = -0.33x$, $R^2 = 0.98$
Figure 3.4

A.  

Relative Fluorescence Units

Off-target Priming
(>95% RANBP3)

Specific RT Priming
of BCR-ABL1

B.

BCR-ABL1 (b3a2/10³ GUSB)

\[ \rho = 0.17; t\text{-test} \]
3.8 Supplemental figure legend

Figure S3.1 – Effect of gene-specific RT priming concentration on measured BCR-ABL1 transcript abundance

Effect of various concentrations of BCR-ABL1 gene-specific RT primers on BCR-ABL1 yield (number of molecules/µL cDNA) compared to yield using baseline primer concentration (3µM). Two K562 cell total RNA inputs were assessed: 5µg (solid line) and 18µg (dotted line) RNA/30µL RT reaction using Superscript III first strand cDNA synthesis reagent kit (Life Technologies).
3.9 Supplemental figure

Figure S3.1

[Graph showing BCR-ABL1 molecules/μL cDNA (change from 3μM baseline) against concentration of gene specific primers (μM)]
3.10 References


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Chapter 4

A new StaRT-PCR approach to detect and quantify fish Viral Hemorrhagic Septicemia virus (VHSv): enhanced quality control with internal standards

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(Technical questions regarding the StaRT-PCR method should be requested to James Willey, james.willey2@utoledo.edu)

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4.1 Abstract

Viral Hemorrhagic Septicemia virus (VHSV) causes one of the world’s most important finfish diseases, killing >80 species across Eurasia and North America. A new and especially virulent strain (IVb) emerged in the North American Great Lakes in 2003, threatening fisheries, baitfish, and aquaculture industries. Weeks-long and costly cell culture is the OIE and USDA-APHIS approved diagnostic. A new Standardized Reverse Transcriptase Polymerase Chain Reaction (StaRT-PCR) assay that uniquely incorporates internal standards to improve accuracy and prevent false negatives was developed and evaluated for its ability to detect and quantify VHSV. Results from StaRT-PCR, SYBR® green real time qRT-PCR, and cell culture were compared, as well as the effects of potential PCR inhibitors (EDTA and high RNA). Findings show that StaRT-PCR is sensitive, detecting a single molecule, with 100% accuracy at six molecules, and had no false negatives. In comparison, false negatives ranged from 14-47% in SYBR® green real time qRT-PCR tests, and 47-70% with cell culture. StaRT-PCR uniquely controlled for EDTA and RNA interference. Range of VHSV quantitation by StaRT-PCR was 1.0x10^0-1.2x10^5 VHSV/10^6 actb1 molecules in wild caught fishes and 1.0x10^0-8.4x10^5 molecules in laboratory challenged specimens. In the latter experiments, muskellunge with skin lesions had significantly more viral molecules (mean = 1.9x10^4) than those without (1.1x10^3) (p < 0.04). VHSV infection was detected earlier in injection than in immersion challenged yellow perch (two versus three days), with molecule numbers in both being comparable and relatively consistent over the remaining course of the experiment. Our results show that the StaRT-PCR test accurately and reliably detects and quantifies VHSV.
4.2 Introduction

4.2.1 VHSv characteristics and spread

Viral Hemorrhagic Septicemia virus (VHSv) is one of the world's most serious fish pathogens, killing over 80 marine and freshwater species (including trout, salmon, and perch) across the Northern Hemisphere (Faisal et al., 2012), yet has lacked a rapid and accurate diagnostic test. The virus 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 (Ammayappan and Vakharia, 2009). A new and especially virulent VHSv substrain (IVb) emerged in the Laurentian Great Lakes of North America in 2003, causing massive fish kills (Elsayed et al., 2006; Faisal et al., 2012) that have threatened the fisheries, aquaculture, baitfish, and tourism industries (Leighton, 2011).

Transmission of VHSv occurs via fish waste, reproductive fluids, and skin secretions. Its viral particles can live up to 13 days in the water (Hawley and Garver, 2008) and are transported via boating, ballast water, fishing tackle, and animals – e.g., amphipod crustaceans, leeches, turtles, and birds (Faisal and Schulz, 2009; Bain et al., 2010; Faisal and Winters, 2011; Goodwin and Merry, 2011). Clinical signs of infection vary, ranging from erratic swimming, exophthalmia (bulging eyes), distended abdomens, to extensive external/internal bleeding (Winton and Einer-Jensen, 2002). Since November 2006, the eight U.S. states (Illinois, Indiana, Ohio, Pennsylvania, Michigan, Minnesota, New York, Wisconsin) and two Canadian provinces (Ontario and Quebec) that surround the Great Lakes have required that fish are certified as VHSv-free prior to interstate transport (Aquatic Invasive Species Action Plan, 2011), for which the Office International des Epizooties (OIE) recommends a month long cell culture process (OIE,
VHSV (originally called “Niwensckwellzing”) first was described from European salmonid aquaculture (Schäperclaus, 1938), and was isolated in 1962 from infected rainbow trout (*Oncorhynchus mykiss*) (Einer-Jensen et al., 2004). Four genetically distinct strains (I-IV) and various substrains have been recognized (Snow et al., 1999; Einer-Jensen et al., 2004), whose phylogenetic and biogeographic relationships recently were analyzed by Pierce and Stepien (2012). Strains I-III are found in Europe, where strain I infects >13 freshwater species including rainbow and brown trout (*Salmo trutta*). Strain II comprises a tight genetic cluster (Pierce and Stepien, 2012), which primarily infects Pacific herring (*Clupea pallasii*) in the Baltic Sea and Finland Archipelago (Gadd et al., 2011). Strain III is distributed peripherally to strain I, infecting Atlantic cod (*Gadus morhua*), European eel (*Anguilla anguilla*), haddock (*Melanogrammus aeglefinus*), Norway pout (*Trisopterus esmarki*), Pacific herring, rainbow and brown trout, turbot (*Scophthalmus maximus*), whiting (*Merlangius merlangus*), and others. Strain IV was described in 1988 from the North American Pacific Northwest (substrain a), where it is found in a wide variety of marine fish species (including chinook salmon (*Oncorhynchus tshawytsha*), coho salmon (*Oncorhynchus kisutch*), Pacific cod (*Gadus macrocephalus*), Pacific sardine (*Sardinops sagax*), and smelt (*Thaleichthys pacificus*)), with a few occurrences in Japan and Korea (in black seabream (*Spondyliosoma cantharus*) and olive flounder (*Paralichthys olivaceus*)) (Kim and Faisal, 2011). Substrain IVb was identified in the Great Lakes (Lake St. Clair) basin from an adult muskellunge (*Esox masquinongy*) collected in 2003 (MI03GL; Elsayed et al., 2006). IVb has spread to infect 31 freshwater fish species across all five of the Great Lakes (Thompson et al., 2011), with some isolates
found in invertebrates, indicating that they might serve as transmission vectors (Faisal and Schulz, 2009; Faisal and Winters, 2011).

4.2.2 Need for a new VHSv diagnostic test

Screening methods for VHSv that are approved by the World Organization for Animal Health (OIE, 2009), and the Fish Health Section of the U.S. Fish and Wildlife Service and the American Fisheries Society (2010) are based on identification via cell culture, followed by confirmation either with reverse transcriptase PCR or serologically via an indirect fluorescent antibody test or an enzyme-linked immunosorbent assay. Although those methods readily detect high concentrations of the virus, they may fail to detect low levels of virus in carrier fish. Notably, fish have been shown to shed virus for up to 15 weeks post infection (Kim and Faisal, 2012).

Cell culture identification and subsequent confirmation is labor intensive, time consuming (up to four weeks), and less sensitive than direct PCR-based detection, with false negative rates reported up to 95% (López-Vázquez et al., 2006; Miller et al., 1998; Winton and Einer-Jensen, 2002). For fish farms, hatcheries, and baitfish operators, a month-long holding period for viral detection leads to economic loss and possible viral spread. Additionally, cell culture facilities typically “pool” samples (i.e., tissues from several fish samples are homogenized together), which dilutes the number of viral particles and circumvents pinpointing exactly which samples are VHS positive. Further, cell culture identification methods, such as plaque assay, may not precisely quantify the number of viral particles or allow determination of the amount leading to infection.

Non-lethal techniques to detect neutralizing glycoprotein (G) gene antibodies in fish blood have been developed, with experiments showing that VHSv remained
detectable for ≥90 days post exposure (Millard and Faisal, 2012). However, that antibody approach relied on confluent cultured cells followed by a 6-7 day incubation and an optimum virus concentration for stimulating antibody production (which remains to be characterized). Thus, that weeks-long test may be insufficient for detecting low amounts of VHSV in carrier fish. An assay using monoclonal antibodies by Ito et al. (2012) was developed to identify VHSV strains and substrains. However, that method also used cultured cells and relied on generation of specific antibodies, which are lengthy and costly procedures.

Quantitative real-time PCR (qRT-PCR) approaches have greater sensitivity and reduced detection time in comparison to cell culture and plaque assays (Bruchhof et al., 1995; Miller et al., 1998; Guillou et al., 1999). Several qRT-PCR assays for VHSV have been developed, including: Chico et al. (2006), López-Vázquez et al. (2006), Liu et al. (2008), Matejusova et al. (2008), Cutrín et al. (2009), Hope et al. (2010), Garver et al. (2011), Jonstrup et al. (2012), and Phelps et al. (2012); however, all of these have accuracy limitations. Just three of those tests quantified VHSV levels: Liu et al. (2008), Hope et al. (2010), and Garver et al. (2011). For example, Liu et al. (2008) distinguished to 140 viral copies of a single VHSV type (Ia; isolate Fil3); however, it remains unknown whether their test method, which was based on the G-gene, would work on other VHSV variants. Other assays used the nucleoprotein (N) gene (Chico et al. 2006; Cutrín et al. 2009; Garver et al. 2011; Jonstrup et al. 2012; López-Vázquez et al. 2006; Matejusova et al. 2008; OIE 2009), including a test by the Hope et al. (2010) that reliably distinguished to 100 viral copies. However, higher amounts of RNA (1 µg) led to a 20-fold reduction in their PCR amplification signal, with further signal decline at RNA concentrations of 4-8
μg (Hope et al., 2010). That decline likely was due to interference and/or reagent carry over and would be prone to generate false negative results. The assay by Garver et al. (2011) detected to 100 viral copies, based on results from several laboratories in blind experiments. Jonstrup et al. (2012) reported increased sensitivity compared to other assays because they observed amplification at a lower cycle threshold ($C_t$; the number of cycles at which the fluorescence exceeded the threshold). Their method was evaluated for all four VHSv strains and 79 isolates, yet did not discern significantly more VHS positives than cell culture ($\chi^2 = 0.10$, df = 1, NS; statistical analyses performed in the present study using their results). Their findings suggested a large proportion of false negatives (Jonstrup et al., 2012).

4.2.3 The StaRT-PCR method and study objectives

To date, qRT-PCR and plaque/immunological assays for VHSv have lacked Internal Standards (IS) to control for interfering substances and false negative results, which may lead to misdiagnosis and potential viral spread. The present study thus developed a new PCR-based test for VHSv, which incorporates a standardized mixture of internal standards (SMIS) in a Standardized Reverse Transcriptase Polymerase Chain Reaction (StaRT-PCR); this method follows the StaRT-PCR approach outlined by Crawford et al. (2002), Willey et al. (2004), and Canales et al. (2006).

StaRT-PCR is a form of competitive PCR (Gilliland et al., 1990; Celi et al., 1993) that measures expression of each gene relative to a known number of copies of a synthetic competitive template IS within a SMIS (Willey, 2004). An IS is constructed for each target gene (i.e., VHSv N-gene) and for one or more reference genes (i.e., genes that are expressed at a relatively consistent level across many tissues and conditions), which
are combined into a SMIS. An IS is constructed for each target gene (i.e., VHSv N-gene) and for one or more reference genes (i.e., genes that are expressed at a relatively consistent level across many tissues and conditions,) which are combined into a SMIS. The SMIS is loaded into each reaction (rxn). This approach also controls for interfering substances, such as PCR inhibitors, and prevents false negative results. For example, if the IS PCR product is not observed, it will be interpreted as a failed test and not as valid assessment for the absence of VHSv. StaRT-PCR is designed to yield rapid, reproducible, standardized, and quantified measures from several genes simultaneously.

The research aim of the present study was to evaluate the performance and accuracy of the newly developed StaRT-PCR VHSv test that incorporates synthetic IS, in comparison to conventional qRT-PCR-based assays (e.g., SYBR® green qRT-PCR) that lack IS, and cell culture. Experiments were conducted to test the ability of StaRT-PCR to discern and quantify VHSv from (A) pellets of cells infected in vitro, (B) wild caught fishes, (C) fish (muskellunge and yellow perch (Perca flavescens)) that were experimentally challenged with VHSv, and (D) laboratory altered samples containing various PCR inhibitors. The latter included both exogenous inhibitors (e.g. Ethylenediaminetetraacetic acid (EDTA)) and endogenous inhibitors from high levels of RNA in reverse transcription. The experiments were designed to discern if StaRT-PCR is (1) VHSv-specific, (2) accurately measures the amount of VHSv in infected fish cells, (3) controls for PCR inhibitors, (4) accurately and reliably diagnoses and quantifies VHSv in field and laboratory fish samples, and (5) has greater sensitivity and reliability than cell culture or other qRT-PCR methods. Laboratory challenge experiments also were conducted to determine whether the number of VHSv molecules significantly differs in
challenged fish (6) with or without clinical signs of infection, (7) over the course of early infection (to day six), and/or (8) between immersion and injected challenged individuals. These evaluations provided examples of the potential applications of the StaRT-PCR VHSv test.
4.3 Materials and Methods

4.3.1 Design of the StaRT-PCR test

Primers and the IS designed for the StaRT-PCR VHSv test targeted the central portion of the VHSv N-gene (Fig. 1). The N-gene expresses RNA transcripts most abundantly in rhabdovirus infections (Chico et al., 2006) and is relatively conserved across species due to its RNA-binding function (Hope et al., 2011). Other studies showed that primers targeting the VHSv N-gene were more efficient and sensitive than those targeting the G-gene (Chico et al., 2006; Cutrín et al., 2009). In the present study, N-gene sequences were aligned from all VHSv variants, related Novirhabdoviruses, and other viruses from NIH GenBank (http://www.ncbi.nlm.nih.gov/genbank/) using the BLAST procedure (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that the designed primers did not recognize other viruses. VHSv-specific inter-variant homologous sequences then were targeted for primer design, and Oligo software (http://www.oligo.net/) was used to select primers based on the absence of stable duplex formation, low likelihood of false priming sites, and an optimal annealing temperature of 58°C. Primer sequences are given in Table 1, and their locations and relationships to those from other assays are shown in Fig. 1.

Primer sets and IS for three fish reference genes - β-actin (actb1), elongation factor 1 alpha subunit (ef1a), and 18S ribosomal RNA (18srRNA) - were developed to ensure accurate diagnosis and quantitation under a variety of PCR and sample conditions (Table 1). Reference gene sequences for primers and IS were selected from conserved regions across 10 VHSv-affected fish species (five sport fish species: yellow perch, smallmouth bass (Micropterus dolomieu), round goby (Neogobius melanostomus),
freshwater drum (*Aplodinotus grunniens*), and walleye (*Sander vitreus*) and five baitfish species: emerald shiner (*Notropis atherinoides*), golden shiner (*Notemigonus crysoleucas*), fathead minnow (*Pimephales promelas*), spottail shiner (*Notropis hudsonius*), and alewife (*Alosa pseudoharengus*). Each reference gene was measured to determine whether it was expressed at a consistent level in VHSv infected and non-infected fish, to validate its use as a control for variation in loading of sample into PCR. In these experiments, the reference genes were measured for five VHS positive and five negative control fish samples (muskellunge and yellow perch).

The IS for the VHSv N-gene and each of the three reference genes were prepared following the method of Celi et al. (1993; see their Fig. 1A). First, primers were designed and synthesized for each of the four genes. Then, a modified reverse primer, called the IS primer, was constructed such that the 3’ end had sequence homology to a region between the forward and reverse primers and the 5’end of the IS primer was identical to the reverse primer (Table 1). PCR extension only occurred from the sequence annealed at the 3’ end. This resulted in a PCR product for the IS that was shorter than the VHSv native target (NT) sequence. This difference in size enabled electrophoretic size separation and quantitation of IS and NT. The IS primer was used only to create the IS and was not used in the StaRT-PCR assays.

To develop the SMIS, each IS was generated by separate PCR amplification of fish cDNA using the forward primer (19-21 bp; Table 1) and the IS primer (38-42 bp; Table 1) in five 10 µl PCR replicates containing 1 µl (0.05 µg) of each primer, 0.5 U/µl Go-TAQ polymerase (Promega, Madison, WI), 1 µl 10X MgCl₂ PCR buffer, 1 µl 0.2 mM dNTPs, 5.5 µl RNase-free water, and 1 µl cDNA resulting from a 90 µl reverse
transcription rxn containing 1 µg of VHSv positive fish RNA. Each of the five replicate samples then were transferred to LightCycler® capillaries (Roche, Indianapolis, IN) and cycled on a Rapid Cycler 2 (Idaho Technology, Inc., Salt Lake City, UT), with each cycle being 5 s at 94°C, 10 s at 58°C, and 15 s at 72°C, and a slope of 9.9 for rapid temperature change between cycles (35 cycles total). PCR products from the five replicates then were combined, separated by electrophoresis on a 2% low melting agarose gel (Fisher Scientific, Fair Lawn, NJ) containing 5 µl ethidium bromide (10 mg/ml) per 100 ml 1X TRISacetate EDTA buffer, and visualized on a UV transilluminator. The band corresponding to each IS was excised from the gel and purified using a Qiaquick Gel Extraction Qiagen Kit (Qiagen, Germantown, MD). Molarities were calculated by analysis of 1 µl of the purified products on a Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA) and concentrations were converted into molecules using the below formula (1):

(1) \[ \text{Moles/Liter} / [1.0 \times 10^6 \text{µl/L}] \times [6.0 \times 10^{23} \text{molecules/Mole}] = \text{molecules/µl} \]

A Standardized Mixture of Internal Standards (SMIS) was created by mixing the individual gene IS together, as shown in Table 2, to enable measurement of expression across a range of possible numbers of target molecules. To obtain SMIS “A”, a stock was created such that the IS for 18srRNA was at $10^{-10}$ M, the IS for actb1 was at $10^{-11}$ M, the IS for ef1a was at $10^{-10}$ M, and the IS for VHSv was at $10^{-10}$ M. A separate mixture of the three reference gene IS, which did not contain VHSv IS, was created with 18srRNA at $10^{-10}$ M, actb1 at $10^{-11}$ M, and ef1a at $10^{-10}$. This reference gene IS mixture was used in a 10-fold serial dilution of the SMIS “A” stock to create stocks for SMIS B-H (row 1 of Table
2). A solution of 0.1 ng/µl yeast tRNA carrier (Invitrogen, Carlsbad, CA) was used in all dilutions to prevent adherence of the negatively charged IS molecules to the plastic tube or pipette tip surfaces/diluents. Stock SMIS then was diluted down serially with carrier solution (rows 2-8 of Table 2) to generate a range of concentrations and enable measurement over several orders of magnitude.

4.3.2. How to perform StaRT-PCR

StaRT-PCR methods were used as described by Willey et al. (1998, 2004). First, 0.25-0.50 g fish tissue (spleen was preferred since it is readily identifiable by collectors unfamiliar with fish anatomy) was ground under liquid nitrogen using a sterile mortar and pestle, and RNA was extracted using TriREAGENT® (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer’s protocol. Second, RNA was re-suspended in 30 µl RNase-free water, quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the concentration was adjusted to 1 μg RNA/µl. A 30 µl RNA volume was treated with DNA-free DNase and DNAse Removal Reagents (Ambion Life Technologies, Grand Island, NY) to remove any contaminating gDNA. Third, reverse transcription of this purified RNA to cDNA was conducted using 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. Reverse transcription rxns were carried out at 94°C for 5 min, 37°C for 1 h, and 94°C for 5 min. All cDNA was stored at –20°C until further use.

For each sample assessed, an initial set of PCR amplifications was conducted to determine how much cDNA and how much reference gene (actb1) IS should be combined to achieve approximately (within a 10-fold range) a 1:1 ratio between the NT
and IS for *actb1*. For subsequent reactions, the *actb1* NT and IS concentrations were held constant for each sample, while the SMIS used (A-H) varied (Table 2) to achieve approximately (within a 10-fold range) a 1:1 ratio of the VHSv NT relative to the VHSv IS. For example, if the SMIS “D” concentration was used to assay an unknown quantity of VHSv from a fish sample, and the NT amount of VHSv was found to be >10-fold more than the VHSv IS, the next step would be to repeat the experiment using the same amount of cDNA and substituting with SMIS “C”, which has a 10-fold higher concentration of VHSv IS (Willey et al., 2004).

StaRT-PCR was conducted in 10 µl rxn volumes as follows:

(A) Primer pairs for each gene were mixed together (each at 0.05 µg/µl) to decrease pipetting error, then 1 µl of the mixture for the VHSv target gene and *actb1* reference gene was placed in separate tubes.

(B) A master mixture was prepared containing both the appropriate concentration of cDNA and the appropriate SMIS (as determined in the initial set of PCR) to ensure equal loading of both into the separate rxn tubes for measurement of VHSv or *actb1*. Also included in the master mix were: Go-TAQ polymerase (final conc. of 0.1 U/µl), 10X PCR buffer containing 30 mM MgCl₂ (final conc. of 1X with 3 mM MgCl₂), dNTPs (final conc. of 0.2 mM), and RNase-free water.

(C) 9 µl of the master mixture was added into each of the tubes from (A), mixed, transferred into LightCycler® capillaries, and then PCR-amplified on a Rapid Cycler 2, as described in Section 2.1.

(D) To check for contamination in the PCR reagents and to confirm that reactions worked, a total of seven additional tubes were prepared for each experiment: (1) SMIS
only (no cDNA) with primers for each gene, (2) a known VHSv positive and negative fish with primers for each gene, and (3) a water/reagent control with primers for each gene (nuclease-free H₂O).

(E) PCR products were visualized on an Agilent 2100 Bioanalyzer, as above.

(F) This process (A-E) was repeated 3X to determine a mean and standard deviation, and calculate the standard error, for the number of VHSv molecules/10⁶ actb1 using the equations below (2 and 3).

(2) Correcting NT product size (this is necessary because quantitation was by optical density of intercalator dye and this, in turn, was related to molecule length as well as copy number).

(a) \[
\frac{\text{Expected IS bp}}{\text{Expected NT bp}} \times \text{[NT area under curve on Agilent graph]} = \text{Corrected NT area under curve}
\]

(b) \[
\frac{\text{Corrected NT area under curve}}{\text{IS area under curve}} \times \text{[number of IS molecules in the rxn]} = \text{number of NT molecules}
\]

(3) Normalizing VHSv to actb1

\[
\frac{\text{Number of NT molecules VHSv}}{\text{Number of NT molecules actb1}} \times [10^6] = \text{VHSv molecules}/10^6 \text{actb1 molecules}
\]

4.3.3 Specificity, linearity, precision and accuracy of the VHSv StaRT-PCR test

The VHSv StaRT-PCR assay was tested for non-specific amplification of several viruses (Table 3) in separate experiments. Viruses assessed included the human 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 latter is sister species of VHSv and has 62% similarity (Ammayappan and Vakharia, 2009; Pierce and Stepies, 2012). Additionally, all four strains of VHSv were tested (I-IV), including 25 VHSv isolates and European, Asian, and North American variants (Table 3). Viruses were obtained either as cell culture supernatant, RNA, or tissue from infected fishes. These samples were processed following the same procedure, except that cell culture supernatants were extracted using TriREAGENT-LS® (Molecular Research Center Inc.). All samples were assayed in triplicate.

Linearity of the StaRT-PCR assay was tested by inoculating the *Epithelioma papulosum cyprini* (EPC) cell line with known amounts of VHSv. The EPC cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were propagated following Kim and Faisal (2010), trypsinized from the plate once deemed confluent, and collected as pellets after centrifugation. Pellets (three per dilution) were spiked with a known dilution of $10^0$-$10^5$ pfu VHSv-IVb (strain MI03GL)/$10^6$ cells. Two cell pellets containing nuclease-free H$_2$O served as negative controls. StaRT-PCR was used to measure VHSv in each sample in triplicate (totaling nine measurements per dilution). A log-log (i.e., power) regression analysis was employed to determine whether the number of molecules detected with StaRT-PCR followed a linear trend with viral dilution. Relative accuracy was quantified as the distribution of the percent difference between the number of molecules measured versus the number expected across all dilutions (Shabir, 2003). Precision, the measure of the degree of repeatability of an analytical method (Shabir, 2003), was assessed by calculating the coefficient of variation for each sample across all three experiments. Values are reported as percentages.
The true accuracy of the StaRT-PCR VHSv assay, defined as the agreement between a measurement and its known value (Shabir, 2003), was evaluated by Poisson distribution analysis (Vogelstein and Kinzler, 1999). According to this method, the laws of chance governing stochastic sampling variation were used to calculate the concentration based on the relationship between the fraction of PCRs observed to be positive relative to the fraction expected. The observations were based on nine separate PCRs each of 16 extreme limiting dilutions of a VHS-IVb sample. VHS-IVb was prepared from a smallmouth bass isolate MI03GL (a homogenate of spleen, kidney, and brain tissue; provided by P. Bowser, Cornell University College of Veterinary Medicine, Ithaca, NY). MI03GL was the original IVb isolate (Elsayed et al., 2006) and is the most widespread in the Great Lakes (Thompson et al., 2011). PCRs were run on dilutions expected to contain 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 25, 50, 1.0x10^2, 2.0x10^2, 6.0x10^2, 6.0x10^3, and 6.0x10^6 VHSv molecules, which were mixed with the appropriate SMIS to achieve an approximate VHSv NT:IS ratio of 1:1. An exponential regression analysis (in SPSS v21; http://www01.ibm.com/software/analytics/spss/; SPSS Inc., Chicago, IL; Norusis, 2008) was used to calculate the concentration of VHSv in the SMIS based on the observed: expected values. The concentration of the SMIS based on StaRT-PCR limiting dilution Poisson distribution analysis and the concentration measured by NanoDrop Spectrophotometer 2000 analysis of the undiluted stock concentration were statistically compared using a \( \chi^2 \) test (in Microsoft Excel).

4.3.4 Effect of interfering substances on StaRT-PCR

To evaluate accuracy and performance of StaRT-PCR when subjected to possible interfering substances at the PCR level, RNA from a VHSv-IVb (MI03GL) positive
smallmouth bass (also used in Section 2.3) was treated with DNA-free™ (Ambion Life Technologies, Carlsbad, CA) to remove any contaminating gDNA, and was reverse transcribed to cDNA using 1 µg RNA/90 µl rxn. The cDNA then was spiked with 0.1, 0.5, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 2.0 mM concentrations of EDTA per PCR and amplified in three separate StaRT-PCR runs, one series with a SMIS and the other lacking a SMIS, to assess each for the occurrence of false negatives. Products of PCR at each EDTA concentration were measured in two ways. First, the mean number of VHSV-IVb molecules/$10^6$ actb1 molecules was calculated for each sample (as detailed in Section 4.3.2., Eqs.(2) and (3)). The number of VHSV molecules measured at 0 mM EDTA concentration was set as the 100% baseline, serving as the control. A one-way ANOVA (Sokal and Rohlf, 1995) in SPSS was used to test for significant difference in the mean number of viral molecules/$10^6$ actb1 measured. Second, the total fluorescent light, measured in fluorescent light units, emitted from the Agilent intercalating dye bound to the two PCR product peaks (NT and IS) was measured on an Agilent 2100 Bioanalyzer and combined. The mean fluorescence values for each sample were calibrated as the percent change from the baseline value (0 mM EDTA control).

Reverse transcription efficiency, which is the fraction of mRNA molecules converted into corresponding cDNA molecules (Bustin and Nolan, 2004; Ståhlberg et al., 2004), was analyzed by comparing results from 1 (control), 5, 10, 20, and 30 µg of VHSV RNA/90 µl rxn. The same gDNA-free VHSV-IVb positive sample of RNA used for the EDTA test (above) was used in these experiments. A Reverse Transcription Standards Mixture (RTSM) was constructed using in vitro-transcribed RNA standards developed by the External RNA Control Consortium (ERCC; http://www.nist.gov/mml/), termed
ERCC 113 and 171, and obtained from M. Salit, National Institutes of Standards and Technology, Gaithersburg, MD (Table 1). The concentration of each RNA standard was measured using a NanoDrop 2000 spectrophotometer. The ERCC 113 standard was reverse transcribed into cDNA, and the cDNA was quantified using the Agilent 2100 BioAnalyzer. The ERCC 171 standard then was diluted to 1.0x10^{-10} M using 100 ng/µl yeast tRNA as a carrier, and combined with the ERCC 113 cDNA to yield a mixture of 1.59x10^3±1.6x10^2 ERCC 171 RNA molecules and 1.7x10^3±1.8x10^2 ERCC 113 cDNA molecules per 2 µl aliquot. A 2 µl aliquot of the RTSM was included in each of the reverse transcription rxns. The ERCC 171 RNA was converted into cDNA along with other RNA species in the reverse transcription rxn while the ERCC 113 cDNA remained unaltered. Thus, by comparing the ratio of ERCC 171 and ERCC 113 cDNAs after reverse transcription, using StaRT-PCR, it was possible to calculate the reverse transcription efficiency. The numbers of VHSv, *actb1*, and *ef1a* molecules also were quantified in all samples using Eqs. (2) and (3) from Section 4.3.2. One-way ANOVAs and *t*-tests (Sokal and Rohlf, 1995) were used to identify possible differences in reverse transcription efficiency and variations in the numbers of measured molecules among the five concentrations.

### 4.3.5 Laboratory challenged fish experiments

VHSv laboratory challenge experiments were conducted at the Fish Health Laboratory Containment Facility at Michigan State University (MSU). Certified VHSv-free juvenile muskellunge were obtained from the Rathburn National Fish Hatchery (Moravia, Iowa) and maintained in covered 1,900 L tanks in UV-sterilized 12±1°C oxygenated water under controlled ambient light for three weeks acclimation.
Muskellunge were fed VHSv-free fathead minnows (Robinson Wholesale, Inc.) that had been determined to be VHS-free using OIE (2009) approved tissue culture isolation assays with confirmation qRT-PCR.

A subset of the 360 muskellunge (20.0±10.9 g; total length 17.1±1.5 cm) were randomly selected and assigned into four test groups (90 fish per group). Each group was challenged via water immersion for 90 min in 37.8 L with either: (1) a low dose of 100 pfu/ml VHSv-IVb (MI03GL), (2) a medium dose of 4.0x10³ pfu/ml, (3) a high dose of 1.0x10⁵ pfu/ml, or (4) 1 ml sterile maintenance minimum essential media (MEM), which served as the negative control. The fish groups then were divided into two tank replicates (45 fish x 2), from which two fish each were selected randomly for analysis (measuring VHSv levels in their spleen tissue) at pre-determined time intervals (0, 6, 12, 24, 36 h; 2, 4, 6, 8, 15, 22, 28, 35, 42 days). Fish were euthanized immediately with an overdose of 25 mg/ml tricaine methanesulfonate (MS-222; Argent Chemical Lab), following MSU Institutional Animal Care and Use Committee approved protocols (AUF 07/07-123-00). Tanks were monitored every 8 h, and any moribund or dead fish were removed. Exterior viral particles were eliminated from the fish by submerging each 3X in double distilled H₂O. Fish were dissected under aseptic conditions with the surgical site (anus to operculum) disinfected using 100% ethanol and betadine. Spleen and head kidney were removed, placed into separate 1.5 ml tubes, flash frozen in liquid nitrogen or placed in RNAlater (Qiagen), and stored at -80°C awaiting StaRT-PCR. Nets were disinfected using a 2% chlorhexidine solution, and sterile equipment and new gloves were used for each fish. Specimen disposal followed MSU biohazard protocols.

A second set of VHSv-IVb laboratory challenge experiments was conducted at
the United States Geological Survey (USGS) Western Fisheries Research Center (WFRC) Challenge Facility in Seattle WA (under the supervision of J. Winton and F. Goetz) using six-month-old yellow perch, which were VHSv-certified-free Choptank broodstrain (Rosauer et al., 2011) from the University of Wisconsin’s Great Lakes WATER Institute (Milwaukee, WI). Laboratory work was conducted under USGS-WFRC Animal Care and Use guidelines. Perch were kept in ~8 m diameter covered 278 L aquaria at 18-20°C under ambient light conditions that mirrored the seasonal photoperiod, and fed 1.2 mm pellet feed (Oregon Biodiet, Longview, WA) every other day until satiation. A total of 210 perch were randomly selected and assigned into six groups: two groups of 38 fish (mean = 15 g) were challenged via intra-peritoneal injection of 1.0x10⁵ pfu/ml VHSv-IVb (MI03GL), a group of 20 fish (mean = 15 g) was immediately euthanized, serving as a negative control, and three groups of 38 fish each (mean = ~2 g) were used for the VHSv immersion challenge, with two groups immersed for two hours in the same VHSv dosage (1.0x10⁵ pfu/ml), and the final group challenged with a control dose of MEM-0 (as described above). Fish were randomly selected at predetermined intervals (10 fish in day 1, eight in day 2, and five each for days 3-6) and euthanized using 240 mg/L MS-222 and 1.2g/L NaHCO₃ following USGS-WFRC Institutional Animal Care and Use Committee protocols (2008-17). Spleen and head kidney were removed from each fish with sterile equipment, labeled, flash frozen in liquid nitrogen, and stored at -80°C. Specimens were disposed of following University of Wisconsin’s Great Lakes WATER Institute and University of Toledo biohazard protocols.

4.3.6 Testing for VHSv infection using StaRT-PCR and other assays
The relative performances of StaRT-PCR, conventional SYBR® green qRT-PCR, and cell culture to detect VHSv infection were compared using a $\chi^2$ test (Sokal and Rohlf, 1995) for 23 wild-caught fishes from the Great Lakes, including: two bluegill (\textit{Lepomis macrochirus}), May 2011, Budd Lake, MI), a brown bullhead (\textit{Ameiurus nebulosus}), May 2012, Maumee Bay, Lake Erie), a freshwater drum (April 2012, Sandusky Bay, Lake Erie), seven largemouth bass (four from May 2011 and two from July 2011, Budd Lake, MI, and one from April 2012, Sandusky Bay, Lake Erie), one smallmouth bass (May 2006, Sodus Bay, Lake Ontario), and 11 lake herring (\textit{Coregonus artedi}), December 2009, Apostle Islands, Lake Superior). Other evaluations with StaRT-PCR included 20 experimentally challenged muskellunge (15 VHSv infected and five negative controls) and 20 of the challenged yellow perch (including seven infected fish and three negative controls from the immersion and injection challenge experiments). Cell culture was not performed on the perch samples due to the low tissue quantities available from the WFRC.

SYBR® green qRT-PCR was conducted in 25 $\mu$l rxns, containing 0.05 $\mu$g of each primer (primers were the same as those for StaRT-PCR; Table 1), 2 $\mu$l cDNA product, 10 $\mu$l SsoFast SYBR® green mix, and RNase-free water. Amplifications were carried out on a Mastercycler Realplex Thermocycler (Eppendorf, Inc., Westbury, NY) using an initial denaturation of 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, and 1 min at 60°C. Each rxn included a known cell culture positive, a negative VHSv cDNA, and a reagent negative control (nuclease-free H$_2$O). Positive versus negative results for VHSv were determined based on $C_t$, with positives resulting in a $C_t \leq 38$. Use of that high of a $C_t$ was facilitated by the high signal to background achieved with these optimized reagents.
samples were analyzed in triplicate, and the products were visualized on 1% agarose gels to confirm positive/negative results.

Cell culture was performed following standard OIE (2009) procedures. Spleen tissue from individual fish samples was homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd., Deans La York, UK) at high speed for 2 min and diluted with Earl’s Salt-Based MEM (Invitrogen, Grand Island, NY) supplemented with 12 mM TRIS buffer (Sigma, St. Louis, MO), penicillin (100 IU/ml), streptomycin (100 µg/ml; Invitrogen), and amphotericin B (250 µg/ml; Invitrogen) to produce a 1:4 dilution of the original tissues. The dilutions were centrifuged at 2000 g and the supernatants were placed into individual wells of a 24 well plate containing confluent EPC cells, MEM, and 5% fetal bovine serum. Plates were incubated at 15°C for seven days and observed for the formation of cytopathic effects. A second and third passage was performed before concluding infectivity.

If results were positive for cell culture (i.e., cytopathic effects were observed), RNA was extracted from infected cells following the method used for StaRT-PCR, reverse transcribed using Affinity Script Multiple Temperature Reverse Transcriptase PCR (Stratagene, La Jolla, CA), and amplified using previously described recommended standard procedures and VHSv N-gene primers (Fig. 1) (OIE, 2009).

4.3.7 Quantitative analyses using StaRT-PCR

Mean VHSv molecules calculated (from independent triplicate StaRT-PCR experiments) were compared for the wild caught and laboratory challenged fish samples. Numbers of VHSv molecules in 18 laboratory challenged muskellunge were compared between nine fish having clinical signs of infection (e.g., gross lesions and hemorrhages)
and nine without signs, which were sampled at identical time points. A non-parametric Mann-Whitney U test (using SPSS) was used to rank the relative numbers of molecules in each fish. A $\chi^2$ test (Microsoft Excel) was used to evaluate whether a threshold number of VHSv molecules characterized the appearance of clinical signs. A power analysis (G*Power2; Erdfelder et al., 1996) estimated the sample size needed for 95% confidence, using an effect size of 0.50 (Cohen, 1992). Differences in the number of VHSv molecules across days 1-6 of infection in the laboratory challenged juvenile yellow perch were tested using a one-way ANOVA. Additionally, numbers of VHSv molecules were compared between the immersion versus injected challenged individuals (60 of each).
4.4 Results

4.4.1 Performance of the VHSv StaRT-PCR test

The StaRT-PCR test was determined to be specific for VHSv diagnosis. Specifically, StaRT-PCR VHSv results were negative (no amplification) for human viruses (Encephalomyocarditis virus and Vesicular Stomatitis virus) and for other fish viruses (i.e., Hirame Rhabdovirus, Infectious Hematopoietic Necrosis virus, Infectious Pancreatic Necrosis virus, Snakehead Rhabdovirus, and Spring Viremia of Carp virus). All tests of VHSv strains/substrains (I, Ia, II, III, IVa, IVb, IVc) were positive (Table 3).

Regression analyses showed that 100% of StaRT-PCR tests at concentrations ≥6 VHSv molecules in the sample had 100% true accuracy (Fig. 2; $R^2 = 0.93, F = 190.10, df = 1, 14, p < 0.0001$). Experiments yielded 100% (9/9 times) amplification for dilutions of ≥6 VHSv molecules, 88% (8/9 times) for 4 and 5 molecules, 44% (4/9) for 2 molecules, 33% (3/9) for 1 molecule, and 22% (2/9) for 0.5 molecules. The number of molecules calculated from StaRT-PCR did not significantly differ from the NanoDrop measurements (Fig. 2; $\chi^2 = 0.47, df = 15, NS$).

StaRT-PCR quantitation of VHSv molecules in the dilution test series using biological samples followed a linear relationship to results from plaque assays, across a range of 0.1x10^0-1.0x10^3 VHSv/10^6 actb1 molecules (Fig. 3; $R^2 = 0.98, F = 1797.86, df = 1, 43, p < 0.001$). The precision of the test was calculated as 9.57% for samples ≥11 expected VHSv/10^6 actb1 molecules and 37.69% for ≤11 molecules.

4.4.2 StaRT-PCR control for interference in PCR

As measured by fluorescent light units, the amount of PCR product from positive VHSv-IVb fish RNA decreased in proportion to the amount of interfering substances in
the StaRT-PCR assay (Fig. 4). For example, as EDTA increased from 0.1-1.3 mM, the fluorescence signal for both VHSv NT and IS PCR product decreased relative to the baseline and then disappeared at 1.4 mM or higher. However, because the number of VHSv NT molecules was measured relative to IS molecules, and because both NT and IS were affected the same way by presence of EDTA, the number of VHSv NT molecules measured was unaffected until EDTA concentration increased to 1.3 mM. Even in this condition, despite reduction of fluorescence signal to 65% of control, measured VHSv molecules/10^6 actb1 declined only slightly (by 6%; \( F = 4.46, \text{df} = 6, 14, p = 0.01 \)) compared to the control. At concentrations ≥ 1.4 mM EDTA, no amplification of VHSv NT or IS occurred. Since the IS was not observed, this result would be recorded as non-functioning assay, and not as a false negative. When testing these samples using the same protocol but without the SMIS, VHSv amplification progressively decreased with increasing EDTA concentration, with no signal at ≥ 1.4 mM EDTA. This comparison indicates that lack of inclusion of the SMIS would lead to a false negative report (indicated by *).

4.4.3 Optimization of reverse transcription conditions

In tests for possible RNA interference, VHSv and two reference genes, actb1 and ef1a, were measured along with the RTSM across RNA concentrations from 1 (the baseline control), 5, 10, 20, and 30 µg RNA/90 µl rxn (Fig. 5). The reverse transcription efficiency as measured by the RTSM (Fig. 5A) was significantly reduced with increasing RNA input (\( F = 9.05, \text{df} = 4, 10, p = 0.002 \)). Differences were measured using pairwise tests between yields at 1 and 10, 20, and 30, and 10 versus 30 µg (\( t =3.10-6.20, \text{df} = 4, p = 0.003-0.04 \)). Overall, reverse transcription efficiency decreased 23-26% from the
baseline at 20 and 30 µg RNA/90 µl rxn, respectively. Measured numbers of both reference genes increased in parallel with RNA concentration (Fig.5B; actb1: $F = 96.45$, df = 4, 10, $p < 0.001$; ef1a: $F = 27.36$, df = 4, 10, $p < 0.001$), yet remained constant when ef1a was normalized to actb1 ($F=1.01$, df = 4, 10, NS). Measured numbers of VHSv molecules, in contrast to what we predicted, increased significantly with increasing RNA input from 1 to 10 µg (Fig. 5C; $t = 2.82$-3.46, df = 4, $p = 0.03$-0.05), but showed no further increase with additional RNA/reverse transcription ($t = 0.32$-1.68, df = 4, NS). The number of VHSv molecules per $10^6$ actb1 molecules significantly decreased with the addition of RNA/reverse transcription ($F = 282.64$, df = 4, 10, $p < 0.001$).

4.4.4 VHSv detection and quantitation in wild caught and laboratory challenged fishes

StaRT-PCR had greater accuracy than conventional SYBR® green qRT-PCR, with the latter having 40% false negative error in experiments with wild caught fishes (Fig. 6A; 10 vs. 6 positives; $\chi^2=1.53$, df = 1, NS), 47% error for immersion challenge muskellunge (Fig. 6B; 15 vs. 8; $\chi^2=5.01$, df = 1, $p=0.03$), and 14% error for challenged yellow perch (Fig. 6C; 14 vs. 12; $\chi^2=0.44$, df = 1, NS). StaRT-PCR also detected significantly more VHSv positives than did cell culture, with the latter having 70% false negative error for wild caught fishes (Fig. 6A; 10 vs. 3; $\chi^2=10.39$, df = 1, $p=0.001$), and 47% error in laboratory challenged muskellunge (Fig. 6B; 15 vs. 8; $\chi^2=5.01$, df = 1, $p=0.03$).

SYBR® green qRT-PCR correctly detected slightly more positives than did cell culture in the wild caught fishes (Fig. 6A; 6 vs. 3, 50% error difference; $\chi^2=1.24$, df = 1, NS); both diagnosed equivalent numbers of positives in the experimentally challenged
muskellunge (Fig. 6B; 8 vs. 8; \( \chi^2 = 0.00, \text{df} = 1, \text{NS} \)). All positives detected by cell culture and SYBR® green qRT-PCR were also positive with StaRT-PCR. The false negative range for SYBR® green qRT-PCR was \( 1.0 \times 10^0 - 6.5 \times 10^1 \) VHSv molecules (as quantified by StaRT-PCR) and \( 1.0 \times 10^0 - 1.0 \times 10^3 \) molecules for cell culture. For all assays, negative controls yielded negative results; i.e., they had no false positives and no contamination.

Numbers of VHSv molecules measured by StaRT-PCR varied widely among wild caught specimens (\( 1.0 \times 10^0 - 1.2 \times 10^5 \) VHSv molecules/\( 10^6 \) actb1 molecules) and laboratory challenged individuals (\( 1.0 \times 10^0 - 8.4 \times 10^5 / 10^6 \) actb1 molecules). Fig. 7 shows a difference in the mean numbers of VHSv molecules between muskellunge exhibiting clinical signs of infection (\( 1.9 \times 10^4 \pm 1.2 \times 10^4 \)) versus those without (\( 1.1 \times 10^3 \pm 4.5 \times 10^2 \); \( Z = -2.10, \text{U(df)} = 1, p = 0.04, \text{days 6-28} \)). At day 35, no VHSv was detected in remaining fish from either group. Numbers of VHSv molecules appeared to differ at days 6, 9, 15, and 28, with those showing clinical signs of infection having higher values (Fig. 7). One hundred VHSv molecules were estimated to distinguish a threshold at which individuals showed clinical signs of infection (\( \chi^2 = 0.09, \text{df} = 1, \text{NS} \)). Power calculations determined that 52 fish samples (26 with and 26 without clinical signs) would be needed to verify this level (95% confidence interval) in further testing.

Quantities of VHSv measured in the yellow perch laboratory challenge experiments ranged from \( 4.0 \times 10^0 - 1.3 \times 10^5 \) VHSv molecules/\( 10^6 \) actb1 molecules in the immersion challenged fish and \( 1.0 \times 10^0 - 1.8 \times 10^5 \) molecules in the injection challenged individuals. Results of the yellow perch laboratory challenge experiments showed that VHSv infection first was detected at day three in immersion challenged fish versus day two in injection challenged individuals (Fig. 8; \( t = 2.15, \text{df} = 19, p = 0.04 \)). Overall numbers
of VHSv molecules appeared relatively consistent across the remaining course of infection through day six (immersion: $F=0.48$, df=3, 29, NS; injection: $F=2.62$, df=4, 42, $p=0.05$), and did not differ between the two experiments ($t=0.03-0.96$, df=13-18, NS).
4.5 Discussion

4.5.1 Specificity and accuracy of the StaRT-PCR VHSv test

The fisheries, aquaculture, and baitfish industries rely on accurate certification of their products as VHSv-free (Aquatic Invasive Species Action Plan, 2011), for which results from cell culture may take up to 28 days (Garver et al., 2011) and frequently yield false negative conclusions (47-70% reported in this study). Thus, the development of a rapid and accurate diagnostic assay is key to preventing VHSv spread to new areas, species, and populations (Cutrín et al., 2009). Similar to other PCR-based assays developed to detect VHSv, the StaRT-PCR test is VHSv specific. StaRT-PCR detected all VHSv strains and substrains, and did not amplify other viruses.

Antibody assays (Millard and Faisal, 2012) and other PCR tests (Chico et al., 2006; López-Vázquez et al., 2006; Liu et al., 2008; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2012; Phelps et al., 2012), lacked internal controls. Those other PCR tests often resulted in false negatives, leading to inaccurate conclusions, as demonstrated by the present investigation’s SYBR® green qRT-PCR results (14-47% false negative error).

4.5.2 Quantitation of VHSv by StaRT-PCR

Results support hypothesis that StaRT-PCR accurately quantifies VHSv and is effective across all levels of the virus tested, from low to high. Other assay methods were unable to discern lower levels of virus, resulting in false negatives. Notably, cell culture was unable to discern levels from $1.0 \times 10^0 - 1.0 \times 10^3$ VHSv/$10^6$ actb1 molecules and SYBR® green qRT-PCR failed to detect from $1.0 \times 10^1 - 6.5 \times 10^1$ molecules. Fish harboring low levels of the virus thus would pass inspection for VHSv-free certification, using
PCR-based methods have been shown to be more sensitive than traditional cell culture (e.g., Chico et al., 2006; López-Vázquez et al., 2006; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2012) since they amplify replicating (i.e., infectious) and non-replicating (i.e., non-active) transcripts alike. Cell culture solely detects actively replicating virus that is capable of infecting cells. Thus, it was anticipated that StaRT-PCR, like other RT-PCR approaches, would yield more positives than cell culture. However, detection of both replicating and non-replicating VHSV RNA could be advantageous since its presence may indicate that there are active infections in the area where the samples were collected. This information may aid management efforts and aquaculture facilities to identify latent infections that could be negative by cell culture, but potentially infective.

StaRT-PCR detected more positives than SYBR® green qRT-PCR, which was not surprising as it was more thoroughly optimized for PCR efficiency and controlled for false negatives. Compared to other PCR tests, StaRT-PCR detected a lower threshold of molecules. For example, Garver et al. (2011) stated that their two-step qRT-PCR assay diagnosed 100 VHSV N-gene copies with 100% accuracy. In contrast, StaRT-PCR showed 100% accuracy for six molecules and detected samples of a single molecule at a frequency limited only by the laws of chance governing stochastic sampling variation.

4.5.3 Performance in presence of PCR inhibitors

StaRT-PCR effectively controlled for inhibition at the PCR level. In contrast, Hope et al.’s (2010) one-step qRT-PCR test showed that increasing the VHSV concentration in reverse transcription rxns from 50 ng to 1 μg RNA/25 μL resulted in
only a 10-fold increase in PCR product, two-fold less than what they expected, which could lead to false negatives. They observed even more dramatic reduction in reverse transcription efficiency with higher RNA input. Based on our results, we conclude that 1 μg/90 μl reverse transcription rxn will provide a test with the best balance between optimal sensitivity and adverse effects of RNA input on reverse transcription efficiency. Further increase in RNA input to 10 μg/90 μl will yield further significant increase in VHSv amplification, but the gain will be marginal relative to the RNA consumed due to decreasing reverse transcription efficiency.

Degraded or environmentally challenged samples can lead to loss of signal and interfere with detection of the virus (see McCord et al., 2011). Such samples often contain substances that are co-extracted with the RNA or carried over into the cDNA via the reverse transcription rxn, which may inhibit or lower PCR efficiency by binding to the polymerase and/or blocking reagents necessary for amplification (Opel et al., 2010). StaRT-PCR is the sole VHSv diagnostic to test, detect, and control for PCR inhibition, preventing false negative results when samples contained inhibitors such as EDTA.

The amount of VHSv PCR product decreased as the reverse transcription efficiency decreased, in contrast to the increase in product yield from the two reference genes, indicating that detection of this virus may be affected by RNA interference differently than the endogenous controls. The difference between them might be due to contaminating VHSv protein features that are tightly bound and not removed during the RNA extraction procedure. Antigenic sites or secondary structures (α-helicase, β-strands and loops), which are common in Novirhabdoviruses (Walker and Kongsuwan, 1999), may contribute to this difference. Other unknown factors may be responsible, which
merit further investigation. To avoid these issues, a standard RNA input/reverse transcription condition should be employed when assaying for VHSv, for which 1 µg RNA/90 µl rxn is recommended.

4.5.4 StaRT-PCR performance versus other assays

StaRT-PCR results diagnosed and quantified the amount of VHSv in fish samples from the field and laboratory experiments. Like most other PCR-based VHSv detection methods (Chico et al., 2006; López-Vázquez et al., 2006; Hope et al., 2010), StaRT-PCR is more sensitive and accurate than the traditional “gold-standard” cell culture approach in detecting qualitative positives. In contrast to the present findings, Jonstrup et al.’s (2012) found that cell culture significantly exceeded the detection ability of their one-step qRT-PCR test at low titer (1.9x10^2-1.9x10^3 TCID_{50}/ml). Thus, their test, unlike StaRT-PCR, did not outperform cell culture.

Besides the present investigation, only two other studies compared detection levels of different PCR assays. López-Vázquez et al. (2006) found that their two-step nested qRT-PCR assay had 15-80% false negatives, their qualitative one-step qRT-PCR assay had 60-90%, and cell culture had 95%. Chico et al. (2006) reported that their two-step TAQman® qRT-PCR test had an error rate of 25% (detected 9 of 12 positives) versus 92% error in two-step nested qRT-PCR assays (found 1 of 12 positives), and 67% error with cell culture (identified 4 of 12 positives). In contrast, StaRT-PCR in the present study yielded 0 false negatives, versus 14-47% false negatives with two-step SYBR® green qRT-PCR, and 47-70% with cell culture. The error rates found with SYBR® green qRT-PCR and cell culture in the present study thus were similar to those determined by Chico et al. (2006).
Most other PCR methods conducted a dilution series with a regression analysis to evaluate sensitivity (e.g., Chico et al., 2006; López-Vázquez et al., 2006; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2012; Phelps et al., 2012). This also was done in the present study, along with an extreme limiting dilution assay that compared the observed frequency of positive StaRT-PCR assays against those predicted by Poisson distribution. Thus, the current investigation evaluated the absolute accuracy of test results, incorporating stochastic sampling variation. The close agreement between the values measured with spectrophotometric quantitation and those expected from the Poisson distribution supports the reliability of StaRT-PCR quantitation capability (Vogelstein and Kinzler, 1999). Based on this analysis, the StaRT-PCR assay measures to a single VHSv molecule with known true accuracy, unlike other PCR methods (Liu et al., 2008; Hope et al., 2010; Garver et al., 2011), whose minimum detection thresholds were much higher (requiring $\geq 100$ copies of VHSv). Thus, StaRT-PCR has greater sensitivity and accuracy than cell culture or other qRT-PCR tests.

### 4.5.5 Biological levels of VHSv detected with StaRT-PCR

The application of employing StaRT-PCR to quantify VHSv infection was demonstrated in a variety of biological experiments. Results show that levels of VHSv molecules in wild caught fishes vary widely ($1.0 \times 10^0$-$1.2 \times 10^5$ VHSv/$10^6$ actb1 molecules), resembling values measured in laboratory challenged individuals ($1.0 \times 10^0$-$8.4 \times 10^5$). Additionally, mean VHSv levels in laboratory challenged individuals showing signs of VHSv infection were higher than without. This suggests that individuals in a population respond variably to VHSv infection, underscoring the importance of an
improved diagnostic test for managing this disease. A threshold level of 100 VHSv molecules was identified as a potential biomarker for clinical signs of infection. This observation supports the conclusion that the greater sensitivity of StaRT-PCR compared to cell culture or SYBR® green qRT-PCR has biological significance. Further experiments are warranted to validate this finding.

To the authors’ knowledge, only a single other investigation quantified VHSv samples from infected fish samples. Similar to StaRT-PCR results, Chico et al. (2006) determined that VHS viral load varied widely among nine rainbow trout individuals in an immersion challenge (measured as VHSv relative to RNA). Chico et al. (2006) was one of four other studies that have used a reference gene (18srRNA) for quantitation. Matejusova et al. (2008), Garver et al. (2011), and Jonstrup et al. (2012) employed a different reference gene (ef1a) to confirm RNA quality for cDNA synthesis and to serve as a positive control, but did not quantify amounts of VHSv. The present study evaluated both of those reference genes (18srRNA and ef1a), as well as actb1, facilitating accurate quantitation of VHSv.

Over recent years, increasing numbers of individuals and species of Great Lakes fishes have tested positive for VHSv-IVb, yet many of those have appeared healthy (Kim and Faisal, 2010). For example, a freshwater drum collected in the Lake Erie Harbor of Sandusky Bay on April 12, 2012 and a largemouth bass on May 10, 2012 that tested positive for VHSv with StaRT-PCR (3.4x10^2 and 5.9x10^2 VHSv/10^6 actb1 molecules, respectively), did not have lesions, but were swimming erratically. Lack of external VHS hemorrhages renders it difficult to determine whether fish have VHS and potentially could transmit the virus. As described above, our results in experimentally challenged
fish suggest that a measured value of greater than 100 using StaRT-PCR may be associated with clinical signs of infection.

No other study to date has monitored levels of VHSv molecules across the early course of infection. The relative number of VHSv molecules was tracked in experimentally challenged yellow perch from days one to six. Onset of VHSv infection occurred a day later in immersion challenged than in injection challenged fish, with their viral concentrations remaining relatively consistent through day six. Quantities of virus did not differ between the immersion versus injection challenged fish after onset of infection. Both experiments showed a linear increase in the number of VHSv molecules from the onset of infection, followed by a plateau phase, and a decrease at day six. These data may indicate that VHSv levels are highest at the beginning of the infectious stage, level off as the host develops its immune response, and then start to decline. However, these results are based solely on early infection, so future studies that include middle and later stages are needed to better understand VHSv infection patterns, the disease course, and host response.

4.5.6 Summary and conclusions

The cell culture diagnostic approved for VHSv-free certification (Aquatic Invasive Species Action Plan, 2011) is lengthy, labor intensive, and lacks sensitivity compared to PCR-based assays. Reliance on cell culture alone could result in spread of VHSv throughout aquaculture systems, baitfish transport, and/or watersheds, leading to significant losses. The other PCR-based techniques to detect VHSv (Chico et al., 2006; López-Vázquez et al., 2006; Liu et al., 2008; Matejusova et al., 2008; Cutrín et al., 2009; Hope et al., 2010; Garver et al., 2011; Jonstrup et al., 2012; Phelps et al., 2012) lacked
the intrinsic quality control necessary for accurate and reliable detection. Moreover, those tests were unable to measure their accuracy and reverse transcription efficiency. The new StaRT-PCR assay incorporates IS that allow for sensitive and accurate qualitative and quantitative measurements of VHSv. These standards guard against false negative results and correct for interfering substances. In addition, the present investigation uses StaRT-PCR to demonstrate reverse transcription efficiency and determine a biological threshold level for clinical signs of infection. Implementation of the StaRT-PCR test will aid aquaculture, baitfish, and fishery industries via faster, more sensitive, and accurate disease detection. Application may lead to improved management and commerce, and lead to cost-savings for stakeholders. This StaRT-PCR diagnostic will enhance natural resource conservation efforts to detect the virus and track its spread.
4.6 Table and figure legends

Table 4.1 – Sequences and PCR parameters of primers and internal standards (IS) used for StaRT-PCR.

F, forward; R, reverse; CT, competitive template.

Table 4.2 – StaRT-PCR SMIS (Standardized Mixture of Internal Standards) dilution mixtures of the four IS (Internal Standards)

18srRNA/actb1/ef1a/VHSv. Values are reported as 10^5 M.

Table 4.3 – Viral isolates screened using StaRT-PCR.

-: negative result (no amplification); +: positive result.

Figure 4.1 - Nucleotide map of the VHSv N-gene


Figure 4.2 – True accuracy of StaRT-PCR VHSv assay

Relationship between the numbers of VHSv positives discerned by StaRT-PCR (based on the % from nine separate runs of a known positive fish, for 16 dilutions of VHSv molecules) versus the number expected, as calculated via a Poisson distribution (R^2=0.93, F=190.10, df=1, 14, p<0.0001). Dilutions were: 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 25,
50, 1.0x10^2, 2.0x10^2, 6.0x10^2, 6.0x10^3, and 6.0x10^6 VHSv molecules. Results showed that all dilutions ≥6, yielded 100% positives using StaRT-PCR (thus their ratio was 1.0 between observed versus expected). Values under the points are the number of VHSv molecules measured by NanoDrop. The number of molecules detected by StaRT-PCR did not statistically differ from the NanoDrop values ($\chi^2=0.47, \text{df}=15, \text{NS}$).

**Figure 4.3 – Relationship between StaRT-PCR VHSv assay and plaque assay**

The numbers of VHSv molecules determined from StaRT-PCR versus those from plaque assay (as log_{10}) across a known dilution series of 10^0-10^5 pfu VHSv-IVb (strain MI03GL)/10^6 EPC cells. ●, mean of three replicate runs and three replicate measures each (nine total); SE, solid line; range, dotted line. The relationship was linear: $R^2=0.98, F=1797.86, \text{df}=1, 43, p<0.001$.

**Figure 4.4 – Effect of EDTA concentration on StaRT-PCR VHSv measurement**

The relationship between the numbers of VHSv molecules determined from StaRT-PCR versus the EDTA concentration (a test for possible inhibition). ●, mean number of VHSv molecules per 10^6 actb1 molecules from three replicates ± SE (these changed by only 6% from 0-1.3 mM EDTA). ○, mean fluorescent light units from three replicates ± SE (fluorescent light units decreased while the number of VHSv molecules remained relatively constant; $F=4.46, \text{df}=6, 14, p=0.01$). *, no StaRT-PCR amplification occurred in either the NT or the IS (thus, the rxn at this point was inhibited).
Figure 4.5 – Effect of RNA input on RT efficiency of target molecules

Mean reverse transcription efficiency and performance of StaRT-PCR under possible RNA inhibition conditions (± SE from three replicates). (A) The relationship between the mean numbers of ERCC 171 molecules/10^6 ERCC 113 molecules from StaRT-PCR versus the concentration of VHSv positive RNA used in the rxn (µg RNA/90 µl). Reverse transcription efficiency significantly changed from 1 (the baseline control), 5, 10, 20, and 30 µg RNA/90 µl rxn (F=9.05, df=4, 10, p=0.002), with significant pairwise tests at 1 and 10, 20, and 30, and 10 versus 30 µg (t=3.10-6.20, df=4, p=0.003-0.04). (B) The relationship between the number of molecules from StaRT-PCR using the reference genes *actb1* and *ef1a* versus the concentration of RNA used in the rxn (µg RNA/90 µL). The numbers of *actb1* and *ef1a* molecules both increased with RNA concentration (*actb1*: F=96.45, df=4, 10, p<0.001; *ef1a*: F=27.36, df=4, 10, p<0.001), but remained relatively constant (dotted line) when *ef1a* was normalized to *actb1* (reported as log_{10}; F=1.01, df=4, NS). (C) The relationship between the mean number of molecules from StaRT-PCR, using the target gene VHSv and the reference gene *actb1*, versus the concentration of RNA used in the rxn (µg RNA/90 µL). In contrast to predicted values, VHS molecules increased significantly from 1-10 µg (t=2.82-3.46, df=4, p=0.03-0.05), but not with any further increase in RNA (t=0.32-1.68, df=4, NS). The number of VHSv molecules per 10^6 *actb1* molecules significantly decreased (dotted line) as more RNA/reverse transcription was added (reported as log_{10}; F=282.64, df=4, 10, p<0.001).

Figure 4.6 – Comparison of three VHSv infection assays

Comparisons among assay test results for VHSv infection from StaRT-PCR,
SYBR® green qRT-PCR, and cell culture for (A) Wild caught fishes, (B) Muskellunge challenge experiments, and (C) Yellow perch challenge experiments (cell culture not available). Gray = negative test result, gray with hash marks = false negative, white = positive test result. Compared with StaRT-PCR, for (A) SYBR® green qRT-PCR had 40% false negative error ($\chi^2=1.53$, df=1, NS) and cell culture had 70% error ($\chi^2=10.39$, df=1, $p=0.001$). For (B), SYBR® green qRT-PCR and cell culture both had 47% false negative error ($\chi^2=5.01$, df=1, $p=0.03$). For (C), SYBR® green qRT-PCR had 14% false negative error ($\chi^2=0.44$, df=1, NS).

Figure 4.7 – StaRT-PCR VHSv assay quantification in fish with/without clinical signs of infections

Comparative numbers of VHSv molecules per $10^6$ actb1 molecules (log$_{10}$ mean ± SE of three replicate runs) in laboratory challenged muskellunge with (●) and without (○) clinical signs of VHSv infection, measured using StaRT-PCR. Values for the two groups statistically differed in a Mann Whitney ranking test ($Z=-2.10$, U(df)=1, $p=0.04$ for days 6-28).

Figure 4.8 – StaRT-PCR VHSv assay quantification in immersion/injection challenged fish

Numbers of VHSv molecules per $10^6$ actb1 molecules measured by StaRT-PCR (mean ± SE from three replicates; reported as log$_{10}$) across the early stages of infection in yellow perch for (A) Injection challenge and (B) Immersion challenge experiments. ●, overall mean. ○, individual measures. The experiments differed in the onset of infection,
with (A) being three days and (B) being two ($t=2.15$, $df=19$, $p=0.04$). Overall numbers of VHSv molecules remained consistent across the infection course (A: days 3-6, $F=0.48$, $df=3$, 29, NS, B: days 2-6, $F=2.62$, $df=4$, 42, $p=0.05$). There was no difference in the number of VHSv molecules between A and B ($t=0.03-0.96$, $df=13-18$, NS).
### 4.7 Tables and figures

#### Table 4.1

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**Table 4.3**

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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

A.

B.
Figure 4.2

[Graph showing observed vs. expected N VHSv positives]
Figure 4.3

![Graph showing a log-log plot of N VHSV molecules from Start-PCR (log_{10}) against N VHSV molecules from plaque assay (log_{10}).](image)
Figure 4.5

A. Reverse Transcription Efficiency

B. RNA Inhibition in Reference Genes

C. RNA Inhibition in VHSv
Figure 4.6

A. Wild Caught

B. Experimentally Challenged Muskeilunge

C. Experimentally Challenged Yellow Perch
Figure 4.7

![Graph showing mean N VHSv molecules (log10) over days for fish with and without clinical signs of VHSv](image)
Figure 4.8

A. Immersion Challenged

B. Injection Challenged

Mean NHSV x 10^6 actb/1 Moles (log10)

Days

1 2 3 4 5 6

0.00

1.0 x 10^1

1.0 x 10^3

1.0 x 10^5
4.8 References


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Chapter 5

A multiplex two-color real-time PCR method that enables quality-controlled molecular diagnostic testing in FFPE samples

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5.1 Abstract

Reverse transcription quantitative real-time PCR (RT-qPCR) tests promise to personalize 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. There is a need for RT-qPCR tests that can assess such samples with quality control and inter-laboratory reproducibility. We developed a multiplex RT-qPCR method that uses a) two-color fluorometric probes enabling analysis of each gene relative to its respective internal standard on a real-time platform, b) measurement of each target gene and reference gene transcript relative to a known number of synthetic competitive internal standard molecules to control for interfering substances, c) pre-amplification to maximize signal, and d) an external standards mixture to control for variation in probe fluorescence intensity. We validated this method in analysis of a lung cancer diagnostic test (LCDT) in FFPE samples. Reagents for each of four genes, MYC, E2F1, CDKN1A and ACTB comprised by the LCDT had acceptable linearity ($R^2 > 0.99$), signal-to-analyte response (slope $1.0 \pm 0.05$), lower detection threshold ($< 10$ molecules) and imprecision ($CV < 20\%$). Pre-amplification increased signal without altering measured values. Internal standards controlled for experimentally introduced interference and prevented false-negatives. With these optimized methods the LCDT had diagnostic accuracy in FFPE samples of 93% (receiver operator characteristic AUC) similar to that in fresh samples. This quality-controlled two color fluorometric RT-qPCR approach facilitated development of reliable, robust RT-qPCR-based molecular diagnostic tests in FFPE clinical samples.
5.2 Introduction

Reverse transcription quantitative real-time PCR (RT-qPCR) tests that measure transcript abundance of selected genes in clinical specimens promise to improve cancer diagnostic accuracy and enable “personalized medicine” through selection of the most effective treatment for each cancer. However, few assays have met the standards needed for successful clinical implementation. Challenges include RT-qPCR methods and reagents with insufficient quality control and inter-laboratory reproducibility and clinical pathology procedures that are sub-optimal for preservation of RNA. A previously developed method, standardized (Sta) RT-PCR implements quality-control and enables inter-laboratory reproducibility by measuring each target gene and loading control gene relative to a known number of its respective competitive template internal standard (IS) molecules in each PCR reaction. Use of IS in molecular diagnostic tests is recommended by regulatory agencies, including the EPA, ISO, and FDA, and they are implemented in many FDA approved RT-qPCR tests. Control for inter-experimental variation is obtained by combining the IS for each gene into an internal standards mixture (ISM) and using an aliquot of this ISM in each experiment. Previously described methods use IS that are 10% shorter than the native template (NT) so that they can be separated and quantified by electrophoresis. This works well for samples that yield high quality RNA. However, for reliable analysis of highly degraded formalin-fixed, paraffin-embedded (FFPE) samples, the optimal PCR amplicon length is < 100 bp, and this is too short for routine quantification with electrophoresis. 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 key elements: 1) Fluorometric hydrolysis probe real-time PCR to enable quantification of short PCR amplicons (60-80 bp) that are optimal for reliable analysis of FFPE samples. 2) Synthetic competitive internal standards to control for sub-optimal PCR, including substances commonly present in FFPE samples that may interfere with RT and PCR steps, sub-optimal quantity or quality of PCR reaction reagents, and inter-well and/or inter-platform variation in PCR conditions. 3) Multiplex pre-amplification and reverse transcription with gene specific primers to enable measurement of lowly expressed genes in pauci-cellular samples with degraded and limited amount of cDNA. 4) An external standards mixture in each experiment to control for inter-lot and inter-experimental variation in probe fluorescence intensity.

Using this approach we developed reagents for measurement of a lung cancer diagnostic test (LCDT), comprising MYC, E2F1, and CDKN1A genes measured relative to ACTB. We assessed performance of this optimized LCDT in analysis of surgical FFPE lung tissues.
5.3 Materials and Methods

5.3.1 Subjects and FFPE sample preparation

Ten malignant and ten benign surgical archival FFPE lung tissue samples were accessed and used in this study according to a protocol approved by the University of Toledo Medical Center institutional review board (Supplemental Materials and Methods, Supplemental Table S1).

5.3.2 RNA extraction and reverse transcription

RNeasy® FFPE Kit (Qiagen, Valencia, CA) was used to extract RNA from surgical FFPE samples. One µg RNA was reverse transcribed to cDNA using gene-specific primers and SuperScript III reverse transcriptase (Life Technologies, Grand Island, NY) (Supplemental Materials and Methods, Supplemental Table S2, S3).

5.3.3 Primer design and detection sensitivity test

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 effect of potential genomic DNA contamination (Supplemental Table S4A).

Each candidate primer pair was assessed for detection sensitivity in serial dilution of H23 cell line cDNA and IS. We compared peak sizes and inspected for absence of non-specific products using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

5.3.4 Design of probes and IS templates

After NT primers with sufficient sensitivity were identified, for each gene target we designed a) an IS-specific probe with 4-6 bp differences from the NT probe and b) an IS containing the same sequence as the NT except for the 4-6 bp, homologous to the IS-
specific probe. Use of multiple base changes in the IS ensured specificity of NT probe (FAM labeled) for NT and IS probe (Quasar 670 labeled) for IS. Probes with fluorescent label at the 5’ end and Black Hole Quencher at the 3’ end (BHQplus, Biosearch Technologies, Novato, CA) were designed using real-time design software from Biosearch Technologies web site (Fig. 1A, Supplemental Table S4B, C).

5.3.5 Synthesis and purification of standards

For each gene we synthesized an NT (to be used as an external standard) and IS via commercial vendor (Life Technologies). We enriched for completely synthesized nucleic acid templates by PCR amplification of each synthesized NT or IS with gene specific primers, 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).

5.3.6 Probe specificity test

Specificity of each probe was tested by including it in amplification of non-homologous or homologous template serially diluted from $10^{-11} \text{M}$ to $10^{-15} \text{M}$. At each dilution the Cq observed with non-homologous template amplification was compared to that observed with homologous template. Non-specific binding rate was calculated using $2^{(\Delta \text{Cq})}$ ($\Delta \text{Cq} = \text{non-homologous Cq} - \text{homologous Cq}$) at each dilution. If the quantified value indicated that more than 10% of input non-homologous molecules were detected, then the probe was re-designed.

5.3.7 Preparation of internal standards mixture (ISM)

Known quantities of each IS were combined into an ISM to minimize inter-experimental variation as described in Supplemental Table S5A.
5.3.8 External standards mixture (ESM)

Known quantities of each purified synthetic NT and IS were combined into an ESM which was used to control for a) variation resulting from instability or intensity differences of one fluor relative to the other and b) inter-experimental variation in software selection of quantification cycle. We prepared a $10^{-11}$M NT/$10^{-11}$M IS stock ESM, 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. In each experiment and for each gene, the average measured quantification cycle difference [NT Cq - IS Cq] from the two ESM was used to correct each unknown sample gene [NT Cq - IS Cq] (Supplemental Table S5B, Supplemental Fig. S1).

5.3.9 Pre-amplification (1st round) and second-round amplification

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

Pre-amplified PCR products were diluted 1000-fold with TE buffer and a 20µl reaction was prepared for each gene with a) 1 µl of diluted pre-amplified product, b) 2 µl each gene primer (final concentration, 800 nM), c) 2 µl each NT and IS BHQ plus hydrolysis probe (final concentration, 200 nM), d) 10 µl TaqMan Universal Master Mix II (No UNG), and subjected to 40 cycles of PCR using the same cycle parameters as in
pre-amplification. Automatic threshold was used for Cq values (Fig. 1B, Supplemental Fig. S1).

5.3.10 Calculation of gene expression

To quantify the copy number for each gene NT in a cDNA sample, 1) the \([\text{NT Cq} - \text{IS Cq}]\) for unknown sample and the average \([\text{NT Cq} - \text{IS Cq}]\) of two concentrations of ESM were calculated, 2) ESM-corrected \([\text{NT Cq} - \text{IS Cq}]\) (delta Cq) was derived subtracting those two \([\text{NT Cq} - \text{IS Cq}]\), 3) \(2^{-(\text{delta Cq})}\) 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 (Supplemental Table S5B).

5.3.11 Accuracy

Limiting dilution PCR was used to quantify the true IS concentration value for each IS in the ISM based on Poisson analysis (Supplemental Materials and Methods) \(^{24}\).

5.3.12 Linearity

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

5.3.13 Imprecision

For each gene, 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 for multiple replicates (at least three) divided by the
mean copy number of the measurements. The average CV for each gene among all
dilutions, and the average CV among all four genes were calculated.

5.3.14 Robustness test

The effect of intentionally perturbing PCR conditions was assessed. Conditions
altered include volume of PCR and concentration of primer, probe, EDTA or Universal
Master Mix (Supplemental Materials and Methods).

5.3.15 Effect of ESM on quality control

The effect of fluorescence intensity was tested by varying [labeled
probe]/[unlabeled probe], keeping [total probe] constant at 200nM. Unlabeled probe was
synthesized via commercial vender (Life Technologies) (Supplemental Materials and
Methods).
5.4 Results

5.4.1 Primer detection sensitivity

Each synthetic IS was assessed for concentration by Agilent 2100. 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, the fraction of replicates measured that had detectable PCR product was at the frequency predicted by Poisson analysis (see Accuracy section below). This is evidence that primers had efficiency necessary to generate detectable signal from a single molecule after 40 cycles and that the IS concentration was accurate. 24

5.4.2 Probe specificity

Probe specificity was measured as non-homologous binding fraction in serial dilution of synthetic NT relative to IS or IS relative to NT from $10^{-11} \text{M}$ to $10^{-15} \text{M}$. Non-homologous binding was evident only for ACTB NT probe and E2F1 NT probe, each of which showed < 1% non-homologous binding rate to the IS.

5.4.3 Accuracy

After the IS were combined into the ISM, ISM was diluted beyond the level expected to contain a single molecule in a PCR reaction, and PCR was conducted for all four genes to test the true concentration accuracy of the IS for each gene in the ISM. The observed frequency of positive result was highly correlated ($R^2 = 0.94$) with expected positive frequency predicted by Poisson analysis (Fig.2, Supplemental Fig. 2), indicating that the intended concentration for each IS in the ISM was accurate.

5.4.4 Linearity
Linearity of the two-color fluorometric assay was determined by analysis of serial dilutions of each gene synthetic NT and IS. In serial dilution of stock ESM (a 1/1 mixture of NT/IS) over seven orders of magnitude (from $10^{-11}$M through $10^{-17}$M), correlation coefficient for measurement of each gene relative to its respective IS was > 0.99, and high signal-to-analyte response was observed with average slope of 1.0 ± 0.05 (Fig.3A, Supplemental Fig. S3).

When NT was serially diluted relative to a constant IS concentration of $10^{-12}$M and vice versa at a concentration of $10^{-13}$M, the average slope for the four genes (ACTB, E2F1, MYC, CDKN1A) was 1.0 ± 0.10 in both sets of dilution series (for 1/1 to 1/10). At NT/IS or IS/NT dilutions higher than 1/10, there was slight deviation of slope from 1.0 (Fig. 3B, C, Supplemental Fig. S4, S5).

**5.4.5 Imprecision**

Average coefficient of variation (CV) for measurement of each of the four genes at each dilution of the serial 10-fold dilution of external standard ($10^{-11}$M NT/$10^{-11}$M IS to $10^{-17}$M NT/$10^{-17}$M IS) was < 10% down to 60 molecules input and < 30% down to 6 molecules with little inter-gene variation (Supplemental Table S6).

The average CV of NT dilution from 1/1 to 1/10-fold relative to constant IS for each of the 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% (Supplemental Table S7). Similar results were observed for average CV for each of the four genes in the IS dilution relative to constant NT. Based on linearity and imprecision, for analysis of clinical samples, we chose to restrict conditions for calculation of results to 1/10 to 10/1-fold difference between NT and IS.
5.4.6 Pre-amplification

Pre-amplification conditions were developed to increase sensitivity of measurement in highly degraded and/or size limited samples. Results with or without pre-amplification were compared using a surgically removed malignant FFPE sample to ensure that pre-amplification yielded reliable data. For each gene, the value measured with the pre-amplification method was not significantly different from that measured with the no pre-amplification method (Supplemental Fig. S6).

5.4.7 Robustness testing

Changing the volumes and/or the concentrations of primers or probes did not lead to significant differences in gene expression measurement with or without pre-amplification (Supplemental Fig. S6). As EDTA concentration increased, Cq value of each of the four analytes (MYC IS and NT, ACTB IS and NT) increased (Fig. 4). However, calculated relative to their respective IS the MYC and ACTB values were constant and no false negative values were reported.

ACTB was measured in reactions containing Universal Master Mix concentration varying from 10% to 130% of recommended. Below 30%, there was no signal for the IS and therefore the reaction was interpreted as no result due to failed reaction. Without use of internal standard, this could be falsely interpreted as negative result. Between 40% and 130% of optimal concentration there was no significant alteration in the value measured relative to IS (average CV, 0.31) (Supplemental Table S8, Supplemental Fig. S7).

5.4.8 Use of ESM to control for variation in fluorescent labeling of probe
Because 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 was tested. As labeled probe concentration decreased in the reaction, Cq increased. However, this potential source of analytical variation was controlled by correcting measured values relative to expected ESM value [NT Cq - IS Cq]. Because the ESM contained a known concentration of each NT and IS that was constant among experiments, any variation in the observed relative to expected [NT Cq - IS Cq] was attributable to variation in fluorescence intensity (Fig. 5A-D). With ESM correction, the CV of measured ACTB was 0.27, but without ESM correction it was 0.90 (Fig. 5E, F).

5.4.9 Reverse transcription optimization

Yield of target gene cDNA was much greater with gene-specific primers (GSP) than with random hexamer primers (RH). For example, among three surgical FFPE samples, there was an average 660-fold higher yield of ACTB cDNA from 1 µg RNA. Based on this, analysis of FFPE samples was conducted with GSP in RT. The RT yield was further optimized by increasing RNA in RT to 5 µg. This increased the yield of ACTB cDNA in GSP RT by 4.6-fold and in RH RT by 1.9-fold (Supplemental Fig. S8).

5.4.10 Analysis of MYC, E2F1, CDKN1A and ACTB in FFPE samples

The comparison of LCDT index in benign and malignant samples is presented in Fig. 6A and the receiver operator characteristic (ROC) curve analysis is presented in Fig 6B. The LCDT optimal cut-off value had 90% specificity and 90% sensitivity to classify samples as cancer or non-cancer. The ROC area under the curve was 0.93 with 95% confidence interval of 0.82 to 1.04 and 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 (Supplemental Table S9).
5.5 Discussion

In order to achieve success with RT-qPCR analysis of highly degraded, size-limited, and relatively impure RNA extracted from FFPE samples, it is necessary to a) amplify short PCR products (60-80 bp), b) control for inter-sample variation in PCR interfering substances, c) increase signal and d) control for inter-lot and inter-experimental variation in probe fluorescence intensity. These requirements were achieved with a quality-controlled two color fluorometric RT-qPCR approach that included reverse transcription with gene-specific primers followed by a multiplex pre-amplification step with ISM. Use of the ESM to correct for inter-lot and inter-experimental variation in probe fluorescence measurement was a useful step.

5.5.1 Internal standards provide quality control

Each gene was measured relative to a known number of molecules of its respective IS. Because each IS amplified with the same efficiency as the NT, this controlled for inter-sample variation in PCR interfering substances and inter-experimental variation in PCR reagent quality or quantity or thermal cycling conditions.

This approach to implement quality-control in real-time RT-qPCR is similar to the method developed for the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test (Roche, NJ). Each of the COBAS® diagnostic tests measures a single infectious disease native cDNA template analyte relative to a known number of copies of its respective IS. As with the method described here, each NT and its respective IS is quantified with sequence-specific probes, with each NT probe labeled with one fluor and each respective IS labeled with a different fluor. In contrast to the COBAS tests, an
additional requirement when conducting RT-qPCR tests of human tissues is that transcripts for both a target gene and a loading control gene (e.g. ACTB) must be measured. Thus, for quality-controlled analysis, it was necessary to measure four analytes; each of the three target gene NTs (MYC, E2F1, an CDKN1A) relative to a known number of its respective IS molecules, and the loading control gene NT (ACTB) relative to a known number of its IS molecules. To eliminate inter-experimental variation, we prepared a stock ISM containing IS for each of the four genes.

5.5.2 ESM controlled for inter-run variation in probe fluorescence

The ESM provided important quality control for a) inter-experimental variation in NT/IS Cq difference resulting from software selection of threshold (Auto Cq mode) which differs based on amplification plot and by cDNA amount, b) inter-sample and inter-experimental variation in PCR efficiency and probe fluorescence intensity.

5.5.3 Multiplex pre-amplification was enabled with internal standards

Using IS in conjunction with multiplex pre-amplification enabled reliable analysis of even lowly expressed genes in very small amounts of cDNA, such as that available from fine needle aspirate (FNA) cell block samples. A key benefit from pre-amplification in the real time format is increase in target signal relative to the background that is often encountered at around 35 cycles in no template control. The use of IS ensured that the starting relative proportion of genes was unaltered during two rounds of PCR and enabled absolute quantification of each gene.

5.5.4 Reliable measurement in FFPE samples

Successful development of this robust method will enable the completion of a prospective clinical trial to test the utility of the LCDT augment cytomorphology in
analysis of FNA cell block FFPE samples as well as other promising tests to facilitate personalized medicine through more clinically meaningful diagnosis and sub-classification of lung cancer. Analysis of these cell block FFPE samples is particularly challenging both because a) the fraction of transcripts from highly degraded RNA that serve as templates for RT-PCR is only 1% compared to that from undegraded RNA, and b) for most such samples RNA yield is below 1 µg (data not shown in here).

Recently the cellularity of FNA cell block material has increased because standard of care is changing to require that aspirated material from a separate full needle be used for cell block so that there is sufficient material for immunohistochemical diagnosis of non-small cell lung cancer subtypes. This change will increase the feasibility of RT-qPCR molecular diagnostic testing in FNA cell block FFPE samples.

5.5.5 Summary

Successful demonstration of the quality-controlled two-color fluorometric real-time PCR method for analysis of the genes comprised by the LCDT supports use of this approach in development and implementation of other promising RT-qPCR based diagnostic tests, especially those that require analysis of RNA extracted from FFPE samples. Use of IS in molecular diagnostic tests is recommended by regulatory agencies, including the EPA, ISO, and FDA, and they are implemented in many FDA approved RT-qPCR tests. For example, for clinically important tests that require analysis of FFPE samples and that presently are available only through a single CLIA certified laboratory (e.g. OncoTypeDx, Genomic Health, Inc, Redwood, CA; Pervenio™ Lung RS test, Life Technologies Lab, West Sacramento, CA), implementation of quality-
control using the method presented here may facilitate regulatory approval as kits that can be implemented in regional clinical pathology laboratories.

The improved methods reported here will be used in multi-site clinical validation using FFPE samples from surgery and FNA cell block to assess the utility of the LCDT as a means to augment standard morphological and immunohistochemical methods for diagnosis in transthoracic, transbronchial, or transesophageal FNA cytologic samples.
5.6 Figure legends

Figure 5.1 – Schematic illustration of probe design (A) and pre-amplification PCR (B).

NT binding probes were labeled with FAM and IS binding probes were labeled with Quasar 670. IS and NT have same primer binding sites and 4-6 bp differences in probe binding sites. Different concentration ISM were used to ensure that NT: IS ratio was >1:10 and <10:1.

Figure 5.2 – Limiting dilution PCR.

Frequency of observed relative to expected positive PCR signal was measured. Poisson analysis was used to calculate expected positive frequency. Results from nine replicates at each of 10 ISM dilution points (40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules/µl) for each gene (ACTB, MYC, E2F1, CDKN1A) were compiled and plotted.

Figure 5.3 – Linearity graph and amplification plot of E2F1.

A, B: Serial dilution of 1/1 mixture of NT/IS from $10^{-11}$ through $10^{-17}$ (triplicate). C, D: NT dilution relative to constant IS from $10^{-12}$ of 1/1 of NT/IS up to 1/80-fold (triplicate). E, F: IS dilution relative to constant NT from $10^{-13}$ of 1/1 of NT/IS up to 1/80-fold (one replicate).

Figure 5.4 – Internal standards control for PCR inhibition by EDTA

MYC and ACTB in the presence of varying EDTA concentrations were measured. A. 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. Note: The asterisk (*) indicates that Cq values were undetermined by software.

**Figure 5.5 – Effect of external standards mixture on quality control**

A, B: Dilution of NT probe. C, D: Dilution of IS probe. Presented molecule numbers (B, D) are MYC/ $10^6$ACTB. E, F: Inter-day experiment variation with ESM or without ESM. The asterisk (*) indicates that Cq values were undetermined by software. Note: SM8: surgically removed FFPE sample 8.

**Figure 5.6 – Validation of two-color fluorometric assay in 20 lung biopsy FFPE samples.**

Validation of two-color fluorometric assay in 20 lung biopsy FFPE samples. A, LCDT index by diagnostic class. B, ROC curve of LCDT index.
5.7 Figures

Figure 5.1

A  ❀ Example: ACTB (60bp)

B  

1st round PCR
- Sample cDNA
- $6 \times 10^3$ molec.IS (Gene A, B, C, D, n,...)
- Primer mix (Gene A, B, C, D, n,...)
- No probes
- 18 cycles

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

Table:

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<th>Primer</th>
<th>Probe</th>
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<td>NT-FAM, IS-Quasar670</td>
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<tr>
<td>B</td>
<td>Gene B</td>
<td>NT-FAM, IS-Quasar670</td>
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<tr>
<td>C</td>
<td>Gene C</td>
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<tr>
<td>D</td>
<td>Gene D</td>
<td>NT-FAM, IS-Quasar670</td>
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</table>

Chart:

- NT: IS
  - 4:2
  - 2:2
  - 1:2
  - 2:2
Figure 5.2

Frequency of Positive PCR

\[ y = 0.920x + 0.02 \]
\[ R^2 = 0.942 \]
Figure 5.3

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

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

(C) NT Dilution (1/80-fold)

\[ y = 1.063x - 0.414 \]
\[ R^2 = 0.997 \]

(E) IS Dilution (1/80-fold)

\[ y = 0.972x + 0.128 \]
\[ R^2 = 0.983 \]
Figure 5.4

A

Quantification Cycle (Cq)

EDTA concentration (mM)

B

Molecule Number (log)

EDTA concentration (mM)
Figure 5.5

A. Cq Variation by NT Probe Con.

B. Measured MYC Molecules

C. Cq Variation by IS Probe Con.

D. Measured MYC Molecules

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<th>Sample</th>
<th>Day</th>
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<th>IS Cq</th>
<th>Raw ΔCq</th>
<th>ESM Corrected ΔCq</th>
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E. CV 0.27 0.90

F. Inter-experiment Variation

---

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Figure 5.6

A

B

LCDDT Index

Sensitivity

100% - Specificity%

Benign  Malignant
5.8 Supplemental Materials and Methods

5.8.1 FFPE samples and preparation

5.8.1.1 Patient samples

Twenty archived surgical FFPE lung tissues that had been processed according to standard University of Toledo Medical Center (UTMC) Department of Pathology practice between 2010 and 2012 were obtained according to a protocol approved by the institutional review board. Ten had been diagnosed as non-malignant tissue and ten had been diagnosed as lung cancer. The individual diagnosis and demographic characteristics are presented in Supplemental Table S1. Microtome sections (10 micron 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, total RNA was extracted from 240 microns of each sample block.

5.8.1.2 A549 cell samples

The A549 cell line was purchased from ATCC (Manassas, VA) and incubated in RPMI 1640 medium + 10% fetal bovine serum at 37°C, in an atmosphere containing 5% CO₂ and 90% humidity. A549 cells were trypsinized and then harvested at 90% confluency. Cell pellet of 1.5x10^7 cells was formed by pouring off supernatant after centrifugation. Nine drops of serum and 3 drops of thrombin were added to the cell pellet, allowing 10 minutes of room temperature incubation. The treated cell pellet was given to technicians in the UTMC Department of Pathology and formalin, fixed and paraffin embedded according to standard processing used for patient samples in this study. Microtome sections (5 micron thickness) were obtained from the A549 cell pellet block.
Technicians obtained two sets of 2, 4, 6 and 8 strips and placed the desired number of strips in 1.5 ml micro-centrifuge tubes for RNA extraction.

5.8.2 RNA extraction

RNeasy® FFPE Kit (Qiagen, Valencia, CA) was used following comparison with another commercially available kit, Agilent Absolutely FFPE RNA Kit (Agilent Technologies, Santa Clara, CA). The kits produced similar yields and purities but RNA extracted with the RNeasy® Kit had a statistically significantly better RT efficiency, and took less time and cost less (Supplemental Table S3). We compared the RNA purity and integrity of all samples using absorbance at 260/280 nm ratios and RIN (RNA integrity number) scores as detected on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was treated with DNase in the RNeasy® FFPE Kit RNA extraction protocol in order to minimize effect of contaminating genomic DNA.

5.8.3 Reverse transcription

5.8.3.1 Patient FFPE samples

RNA extracted from surgical FFPE samples (1 µg) was reverse transcribed to cDNA using gene-specific primers and SuperScript III First Strand Synthesis System according to manufacturer’s protocol in a 30µL RT reaction (Life Technologies, Grand Island, NY). The PCR reverse primer was used as the gene-specific RT primer (3µM).

5.8.3.2 A549 FFPE samples

Reverse transcription efficiency was measured using a Reverse Transcription Standards Mixture (RTSM) described in detail in Stanoszek et al. (JMD, 2013) This study used a 3.33µL aliquot of RTSM in each 50µL RT reaction. Each 2 µL aliquot of RTSM contained $4.85 \times 10^4 \pm 1.27 \times 10^4$ ERCC 171 cDNA molecules and $3.66 \times 10^4$
$10^3(\pm 9.41 \times 10^2)$ ERCC 113 cDNA molecules when measured in 30 µL RT reactions with no background RNA. RT reactions with RTSM used 500 ng total RNA extracted from the FFPE A549 cell block, random hexamers (50µM) and Superscript II First Strand Synthesis System (Life Technologies, Grand Island, NY) according to manufacturer’s protocol in a 50µL RT reaction.

5.8.4 Transcript abundance of RT efficiency study

According to standardized, reverse transcription PCR (StaRT-PCR) described previously (Stanoszek), both ERCC 171 and ERCC 113 were measured relative to a known number of their respective IS molecules within a Standardized Mixture of Internal Standards (SMIS). Before amplification cDNA and SMIS were combined into a master mix, along with the appropriate volume of RNase-free H₂O, 30 mmol/L 10x buffer containing MgCl₂ (Idaho Technology, Salt Lake City, UT), 2 mmol/L dNTPs (Promega, Madison, WI), 50 ng/µL gene-specific primers and a minimum of 0.5 U Taq polymerase (Promega). This mixture was divided into tubes containing primers for each template. All PCRs were performed in a Rapidcycler (Idaho Technology) for 35 cycles. All reactions were denatured for 5 seconds at 94°C, annealed for 10 seconds at 58°C and elongated for 15 seconds at 72°C. After PCR amplification, the internal standard (IS) and native template (NT) for each template were electrophoretically separated and quantified on an Agilent 2100 Bioanalyzer using DNA chips with DNA 1000 kit reagents for visualization, according to the manufacturer’s protocol (Agilent Technologies). Area under the curve values for the NT and IS electrophoretic peaks for each gene was used for gene expression measurement.

5.8.5 Accuracy

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Limiting dilution PCR was used to quantify the true IS concentration value for each IS in the ISM based on Poisson analysis. The concentration of each stock (purified) IS was quantified using the Agilent Bioanalyzer 2100 before combining to make an ISM. Stock ISM solution then was serially diluted down to a concentration expected to contain 40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules in each microliter. Nine replicate samples of each dilution for each gene (ACTB, MYC, E2F1, and CDKN1A) were measured. Assuming that the concentration determined by Agilent Bioanalyzer 2100 was correct, Poisson analysis was used to calculate the expected frequency of reactions with positive PCR product. The observed/expected frequency was plotted for each dilution to determine true concentration for each IS.

5.8.6 Robustness

5.8.6.1 PCR volume, primer concentration, probe concentration variance

Optimal PCR conditions were altered using various combinations of decreased PCR volume and increased primer and probe concentration. Each condition was tested using the pre-amplification and no pre-amplification method.

5.8.6.2 EDTA inhibition

PCR inhibition test was conducted by adding EDTA with increasing concentration from 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 in 20 µl PCR reactions for MYC and ACTB with triplicate measurement.

5.8.6.3 Universal Master Mix concentration variance

Universal Master Mix II (no UNG) (Applied Biosystems, Foster City, CA) concentration was changed from 130% to 10% for ACTB measurement with three different kinds of cDNA: human lung total RNA (Life Technologies, Grand Island, NY)
(reverse transcribed with either random hexamer or gene-specific primer), and surgically removed FFPE malignant tissue (reverse transcribed with gene-specific primer).

5.8.6.4 Effect of ESM on quality control

The effect of fluorescence intensity was tested by varying [labeled probe]/[unlabeled probe] keeping [total probe] constant at 200nM. Serial dilutions keeping IS probe constant and diluting labeled NT probe as 200, 150, 100, 80, 40, 20, 10, 5, 0 nM or vice versa were conducted for MYC and ACTB measurement. Unlabeled probe (Life Technologies) was used to make dilution and control for probe binding efficiency and mimic degraded probes. The obtained Cq values were used for calculation of MYC/10^6ACTB.

5.8.6.5 Statistical analysis

Transcript abundance value (target molecules/10^6 ACTB molecules) for each LCDT gene was measured in triplicate and variation was measured as coefficient of variation (CV). We used the Student’s t-test to determine significant (p < 0.05) difference in mean LCDT value of a malignant group compared to a 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.
5.9 Supplemental table and figure legends

Table S5.1 – Biological characteristics of surgical FFPE sample subjects.

SB: surgically removed, benign sample; SM: surgically removed, malignant sample.

Table S5.2 – Total RNA sample quantity and purity assessment (A) (n=20).

SB: surgically removed, benign sample; SM: surgically removed, malignant sample.

Table S5.3 – Comparison of FFPE RNA extraction kits.

For RT efficiency assessment, RNA extracted from FFPE A549 cell pellet was reverse transcribed in the presence of Reverse Transcription Standard Mixture (RTSM) that comprises known concentration of External RNA Control Consortium (ERCC) standards, non-endogenous alien sequences ERCC 171 (RNA) and ERCC 113 (cDNA).

Table S5.4 - Sequences of primers, probes, and standard templates.

Table S5.5 - ISM composition (A) and an example of MYC/10\(^6\) ACTB calculation (B).

To quantify the copy number for each target gene NT in a cDNA sample, the ESM-corrected \([\text{NT Ct} - \text{IS Ct}]\) (delta Ct) was calculated and \(2^{(-\text{delta Ct})}\) was multiplied by the known number of input IS copies in the reaction. Then each target gene NT value
was normalized to the ACTB endogenous loading control gene NT value, and presented as target gene NT molecules /10^6 ACTB molecules.

**Table S5.6 - Precision of the two-color fluorometric assay (NT, IS held constant).**

A serially diluted 1:1 mixture of NT: IS from 10^{-11}\text{M} through 10^{-17}\text{M} was analyzed in triplicate.

**Table S5.7 - Precision of the two-color fluorometric assay (NT serially diluted, IS held constant).**

For each gene serial dilution of keeping IS constant and diluting NT up to 1/80-fold relative to IS was measured in triplicate at each dilution. The compiled data of ACTB, MYC, E2F1 and CDKN1A are presented.

**Table S5.8 - Assay robustness by Universal Master Mix variance.**

Universal Master Mix II (no UNG) concentration was changed from 130% to 10% and measured ACTB in three different kinds of cDNA: surgically removed normal lung (RT with random hexamer (RH) and gene-specific primer (GSP)) and surgically removed FFPE malignant (RT with GSP).

**Table S5.9 - Each gene measurement and LCDT index in surgical FFPE samples (n=20).**

SB: surgically removed, benign sample; SM: surgically removed, malignant sample.
Figure S5.1 - Schematic plot of experiment set up for 96 well plate.

After dilution of pre-amplified PCR product containing cDNA and ISM, each sample was distributed for 2nd amplification for each gene measurement with each primer and probe in individual wells. ISM C(-13/-15) was presented in the figure as an example containing ACTB 10^{-13}M target genes 10^{-15}M corresponding to ACTB 60000/ target gene 600 molecules. Two external standards (10^{-13}M NT/IS and 10^{-14}M NT/IS) PCR amplification plots were presented in one plot in the middle. Green (NT) and red (IS).

Note: SM, surgically removed sample; ISM, internal standards mixture; ESM, external standards mixture.

Figure S5.2 - Frequency of positive PCR with limiting dilution PCR for each gene.

Pre-amp method was used for testing 9 replicates of 10 dilution points (40, 20, 10, 7, 4, 2, 1, 0.7, 0.4, 0.1 molecules) for each gene.

Figure S5.3 - Linearity of the two-color fluorometric assay (NT, IS held constant).

Serially diluted 1/1 mixture of NT/IS. NT/IS in a 1/1 mixture from 10^{-11}M through 10^{-17}M was analyzed in triplicate.

Figure S5.4 – Linearity of the two-color fluorometric assay (NT serially diluted, IS held constant)

Linearity of ACTB, MYC, E2F1, CDKN1A with serially diluted NT relative to constant IS, starting with 10^{-12}M of 1/1 of NT/IS. 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 dilution of NT relative to constant IS. Data was analyzed with triplicate measurement.

**Figure S5.5 - Linearity of the two-color fluorometric assay (NT held constant, IS serially diluted).**

Serially diluted IS relative to constant NT. Starting with $10^{-13}$M of 1/1 of NT/IS was performed down to 1/10-fold IS dilution (A, C, E, G), and from 1/1 to 1/80-fold IS dilution (B, D, F, H). 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 dilutions of ACTB.

**Figure S5.6 - Comparison of pre-amplification and no pre-amplification with different conditions.**

A, pre-amplification. B, no pre-amplification. The optimal PCR condition was assumed to be 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).

**Figure S5.7 - Assay robustness by Universal Master Mix variance.**

Cq values of ACTB for surgically removed normal lung (RT with random hexamer (RH) and gene-specific primer (GSP)) and surgically removed FFPE malignant...
(RT with GSP) are presented. Universal Master Mix II (no UNG) concentration was changed from 130% to 10% and ACTB was measured in three different kinds of cDNA,

**Figure S5.8 – RT efficiency of RTSM with two FFPE RNA extraction kits.**

For RT efficiency assessment, RNA extracted from FFPE A549 cell pellet was reverse transcribed in the presence of Reverse Transcription Standard Mixture (RTSM) that comprises known concentration of External RNA Control Consortium (ERCC) standards, non-endogenous alien sequences ERCC 171 (RNA) and ERCC 113 (cDNA).

**Figure S5.9 - Comparison of reverse transcription (RT) by priming method and RNA input.**

RT with gene-specific primer (GSP) showed a 660-fold ACTB cDNA increase compared to random hexamer (RH) with 1 µg of surgical FFPE RNA samples, SM1, SM2, and SB1 in 30µl RT reaction. When amount of RNA included in RT was increased to 5 µg from 1 µg, the yield of ACTB cDNA increased 4.6-fold in GSP-primed RT and 1.9-fold in RH-primed RT. SM1: malignant 1, SM2: malignant 2, SB1: benign1
### 5.10 Supplemental tables and figures

**Table S5.1**

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<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB1 TB</td>
<td>SM1 Squamous cell carcinoma</td>
</tr>
<tr>
<td>SB2 Emphysema</td>
<td>SM2 Adenocarcinoma</td>
</tr>
<tr>
<td>SB3 UIP</td>
<td>SM3 Squamous cell carcinoma</td>
</tr>
<tr>
<td>SB4 COPD</td>
<td>SM4 Adenocarcinoma</td>
</tr>
<tr>
<td>SB5 Emphysema</td>
<td>SM5 Squamous cell carcinoma</td>
</tr>
<tr>
<td>SB6 UIP</td>
<td>SM6 Adenocarcinoma</td>
</tr>
<tr>
<td>SB7 Foreign body granulomas</td>
<td>SM7 Adenocarcinoma</td>
</tr>
<tr>
<td>SB8 DAD</td>
<td>SM8 Adenocarcinoma</td>
</tr>
<tr>
<td>SB9 Granulomas</td>
<td>SM9 Squamous cell carcinoma</td>
</tr>
<tr>
<td>SB10 Pneumonia</td>
<td>SM10 Adenocarcinoma</td>
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</tbody>
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Table S5.2

<table>
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<tr>
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<tbody>
<tr>
<td>SB1</td>
<td>21.2</td>
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<td>SM1</td>
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<td>1.92</td>
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<td>2.15</td>
<td>SM3</td>
<td>45.8</td>
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<td>17.9</td>
<td>2.02</td>
<td>SM4</td>
<td>8.4</td>
<td>2.02</td>
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<td>20.2</td>
<td>2.16</td>
<td>SM5</td>
<td>55.4</td>
<td>2.13</td>
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<td>SM6</td>
<td>79.7</td>
<td>2.09</td>
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<td>SB7</td>
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<td>1.79</td>
<td>SM7</td>
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<td>SM8</td>
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<td>2.03</td>
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<td>SB10</td>
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<td>2.04</td>
<td>SM10</td>
<td>24.6</td>
<td>2.00</td>
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<td>Average</td>
<td>17.9</td>
<td></td>
<td>Average</td>
<td>46.8</td>
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</table>
## Table S5.3

<table>
<thead>
<tr>
<th></th>
<th>RNeasy® FFPE Kit (Qiagen)</th>
<th>Absolutely RNA® FFPE Kit (Agilent)</th>
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<tbody>
<tr>
<td>Total thickness (µm)</td>
<td>40 80 120 160</td>
<td>40 80 120 160</td>
</tr>
<tr>
<td>Concentration (ng/µl)</td>
<td>56 39 67 9</td>
<td>16 82 56 31</td>
</tr>
<tr>
<td>Total RNA yield (ng)</td>
<td>1680 1170 2010 260</td>
<td>480 2450 1690 940</td>
</tr>
<tr>
<td>260/280λ</td>
<td>2.05 2.07 2.13 3.65</td>
<td>2.06 2.02 1.98 1.96</td>
</tr>
<tr>
<td>RIN Score</td>
<td>1.9 1.3 2.1 1</td>
<td>1 2.1 1.2 1</td>
</tr>
<tr>
<td>RT efficiency (%)</td>
<td>47.3 45.6 39.9 28.0</td>
<td>20.7 11.2 11.5 8.0</td>
</tr>
</tbody>
</table>
Table S5.4

(A) Primer sequences for two-color fluorometric real-time measurement

<table>
<thead>
<tr>
<th>Gene (GenBank accession no.)</th>
<th>Primer (5’ &gt; 3’)</th>
<th>Product Size</th>
<th>Location</th>
</tr>
</thead>
</table>
| ACTB (NM_001101.3)          | FWD: GCCCTGAGGCCACTCTTCCAG  
REV: TTTCGTGGATGCCACAGGAC | 60 bp | Exon 4, 5 |
| CDKN1A (NM_000389.4)       | FWD: CCTGGAGACTCTCAGGGTGCG  
REV: GCGTTTGGAGTGGTAGAAAT | 66 bp | Exon 5, 6 |
| MYC (NM_002467.4)          | FWD: AGCTGCTTAGACGCTGGATT  
REV: CTAACGTGGAGGGGCTATCGT | 75 bp | Exon 1, 2 |
| E2F1 (NM_005225.2)         | FWD: CTCCTCAGGGCACAGGAA  
REV: CGTGGACTCTTCCGGAGAAGACTTTC | 79 bp | Exon 5, 6 |
### (B) Probe sequences for two-color fluorometric real-time measurement

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
<th>Modification</th>
<th>5'</th>
<th>3'</th>
<th>Probe Sequence (5'-&gt; 3')</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>NT</td>
<td>FAM</td>
<td>BHQ plus-1</td>
<td>CCTTCCTTCTGGGATCAG</td>
<td>18 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IS</td>
<td>Quasar 670</td>
<td>BHQ plus-2</td>
<td>CCAACCTTCCAGGGCA TC</td>
<td>18 bp</td>
<td></td>
</tr>
<tr>
<td>CDKN1A</td>
<td>NT</td>
<td>FAM</td>
<td>BHQ plus-1</td>
<td>AAAAGCGGCAGACAGCA GC</td>
<td>18 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IS</td>
<td>Quasar 670</td>
<td>BHQ plus-2</td>
<td>TTACGGGCGGATGACCA C</td>
<td>17 bp</td>
<td></td>
</tr>
<tr>
<td>MYC</td>
<td>NT</td>
<td>FAM</td>
<td>BHQ plus-1</td>
<td>TAGTGGAAGACGCAGGCTCCT</td>
<td>20 bp</td>
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</tr>
<tr>
<td></td>
<td>IS</td>
<td>Quasar 670</td>
<td>BHQ plus-2</td>
<td>ATGTTGGAATCCGTCGAG CGA</td>
<td>20 bp</td>
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</tr>
<tr>
<td>E2F1</td>
<td>NT</td>
<td>FAM</td>
<td>BHQ plus-1</td>
<td>CATCGATCGGCGCTTCT A</td>
<td>18 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IS</td>
<td>Quasar 670</td>
<td>BHQ plus-2</td>
<td>TTCCGATCGTGCCTTCT A</td>
<td>18 bp</td>
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</tr>
</tbody>
</table>

### (C) Sequence of NT and IS for two-color fluorometric real-time measurement

<table>
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<tr>
<th>Gene</th>
<th>Template</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>NT</td>
<td>GCCCTGAGGCACTCTTCCAGCTTCTTCGCGATGGACTCC TGTGGCATACTCCACGAAAG</td>
</tr>
<tr>
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<td>IS</td>
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</tr>
<tr>
<td>CDKN1A</td>
<td>NT</td>
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<tr>
<td></td>
<td>IS</td>
<td>CCTGGAGACTCTCGGGGCTAGGGTCTGCGATTTCAGGAGCAGCAGCATGA</td>
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<tr>
<td>MYC</td>
<td>NT</td>
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<td>IS</td>
<td>AGCCTCCCGGACAGGTGCCCTCAACGTTAG</td>
</tr>
<tr>
<td>E2F1</td>
<td>NT</td>
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</tr>
<tr>
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<td>IS</td>
<td>CTCCTAGGGCACAGGAAACATCGATCGGGAAGGCTTCTGCTTGAAGATCTCAGGAGGAGGTGCCAG</td>
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Table S5.5

(A) ISM composition

<table>
<thead>
<tr>
<th>ISM (M)</th>
<th>ACTB IS molecules/ reaction</th>
<th>Target Gene IS molecules/ reaction</th>
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<tbody>
<tr>
<td>A(-12/-11)</td>
<td>600000</td>
<td>6000000</td>
</tr>
<tr>
<td>B(-12/-12)</td>
<td>600000</td>
<td>600000</td>
</tr>
<tr>
<td>C(-12/-13)</td>
<td>600000</td>
<td>60000</td>
</tr>
<tr>
<td>D(-12/-14)</td>
<td>600000</td>
<td>6000</td>
</tr>
<tr>
<td>E(-12/-15)</td>
<td>600000</td>
<td>600</td>
</tr>
<tr>
<td>F(-12/-16)</td>
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<td>60</td>
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</table>

(B) Example of MYC/10^6 ACTB calculation in Sample SM1

<table>
<thead>
<tr>
<th>Samples</th>
<th>ISM (M)</th>
<th>NT Cq</th>
<th>IS Cq</th>
<th>ΔCq</th>
<th>Ave. of ESM (NT Cq - IS Cq)</th>
<th>Corrected ΔCq</th>
<th>2^(ΔCq)</th>
<th>IS Molec.</th>
<th>Target Molec.</th>
<th>MYC/10^6 ACTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESM 10^{-13}M</td>
<td>13.9</td>
<td>16.8</td>
<td>2.8</td>
<td>-2.8</td>
<td>-0.9</td>
<td>19000</td>
<td>ACTB</td>
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<td></td>
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<tr>
<td>ESM 10^{-14}M</td>
<td>17.6</td>
<td>20.3</td>
<td>-2.7</td>
<td>(+2.8)</td>
<td>-0.9</td>
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<td>60000</td>
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</tr>
<tr>
<td>SM1</td>
<td>D(-13/-15)</td>
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<td>17.7</td>
<td>-3.7</td>
<td>(+2.8)</td>
<td>-0.9</td>
<td>1.9</td>
<td>60000</td>
<td>114000</td>
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<tr>
<td>ESM 10^{-13}M</td>
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<td>15.4</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>2.5</td>
<td>600</td>
<td>1500</td>
<td>13000</td>
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</tr>
<tr>
<td>ESM 10^{-14}M</td>
<td>20.2</td>
<td>19.0</td>
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<td>(-1.1)</td>
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<td>2.5</td>
<td>600</td>
<td>1500</td>
<td>13000</td>
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</tbody>
</table>

Note: D(-13/-15) contains ACTB 10^{-13}M/ target gene 10^{-15}M that corresponds to ACTB 60000/ target gene 600 molecules. ISM, internal standards mixture; ESM, external standards mixture; SM1, surgically removed malignant sample 1; NT Cq, native template quantification cycle; IS Cq, internal standard quantification cycle.
Table S5.6

(A) ACTB

<table>
<thead>
<tr>
<th>External standard</th>
<th>Expected NT</th>
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<th>SD</th>
<th>CV</th>
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</thead>
<tbody>
<tr>
<td>10⁻¹¹ M</td>
<td>6000000</td>
<td>6780000</td>
<td>600000</td>
<td>0.09</td>
</tr>
<tr>
<td>10⁻¹⁰ M</td>
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<tr>
<td>10⁻⁹ M</td>
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<tr>
<td>10⁻⁸ M</td>
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<td>588000</td>
<td>207</td>
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<tr>
<td>10⁻⁷ M</td>
<td>6000000</td>
<td>577</td>
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<tr>
<td>10⁻⁶ M</td>
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<td>61</td>
<td>26</td>
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<td>10⁻⁵ M</td>
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<td>10</td>
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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

<table>
<thead>
<tr>
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<th>Expected NT</th>
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<th>SD</th>
<th>CV</th>
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<tr>
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<tr>
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<td>42</td>
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<tr>
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Average of CV from 10⁻¹¹ M to 10⁻¹⁶ M 0.08
Average of CV from 10⁻¹¹ M to 10⁻¹⁷ M 0.13

(C) E2F1

<table>
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<tr>
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<th>Expected NT</th>
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<th>SD</th>
<th>CV</th>
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</thead>
<tbody>
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<tr>
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</tr>
<tr>
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<td>6100</td>
<td>114</td>
<td>0.02</td>
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<td>10⁻⁷ M</td>
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<td>0.04</td>
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<tr>
<td>10⁻⁶ M</td>
<td>6000000</td>
<td>60</td>
<td>20</td>
<td>0.34</td>
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<td>10⁻⁵ M</td>
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<td>4</td>
<td>0.95</td>
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Average of CV from 10⁻¹¹ M to 10⁻¹⁶ M 0.08
Average of CV from 10⁻¹¹ M to 10⁻¹⁷ M 0.20

(D) CDKN1A

<table>
<thead>
<tr>
<th>External standard</th>
<th>Expected NT</th>
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<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻¹¹ M</td>
<td>6000000</td>
<td>6140000</td>
<td>260000</td>
<td>0.04</td>
</tr>
<tr>
<td>10⁻¹⁰ M</td>
<td>6000000</td>
<td>5990000</td>
<td>10600</td>
<td>0.02</td>
</tr>
<tr>
<td>10⁻⁹ M</td>
<td>6000000</td>
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<td>6000</td>
<td>138</td>
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<tr>
<td>10⁻⁷ M</td>
<td>6000000</td>
<td>578</td>
<td>23</td>
<td>0.04</td>
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<tr>
<td>10⁻⁶ M</td>
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<td>10⁻⁵ M</td>
<td>6000000</td>
<td>6</td>
<td>9</td>
<td>1.35</td>
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</tbody>
</table>

Average of CV from 10⁻¹¹ M to 10⁻¹⁶ M 0.07
Average of CV from 10⁻¹¹ M to 10⁻¹⁷ M 0.25
<table>
<thead>
<tr>
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<th>Expected NT</th>
<th>Average</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT 1/1</td>
<td>600000</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>300000</td>
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<td>27600</td>
<td>0.09</td>
</tr>
<tr>
<td>NT 1/3</td>
<td>200000</td>
<td>217000</td>
<td>33800</td>
<td>0.16</td>
</tr>
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<td>NT 1/4</td>
<td>150000</td>
<td>156000</td>
<td>16800</td>
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<td>NT 1/5</td>
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<td>NT 1/6</td>
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<td>NT 1/7</td>
<td>85700</td>
<td>87400</td>
<td>13300</td>
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<td>9900</td>
<td>0.14</td>
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<td>67400</td>
<td>9500</td>
<td>0.14</td>
</tr>
<tr>
<td>NT 1/10</td>
<td>60000</td>
<td>61000</td>
<td>8800</td>
<td>0.14</td>
</tr>
</tbody>
</table>

**Average from 1/1 to 1/10 dilution** 0.12
| NT 1/12     | 50000       | 48000   | 9200 | 0.19 |
| NT 1/14     | 42900       | 37300   | 6900 | 0.19 |
| NT 1/16     | 37500       | 31900   | 5000 | 0.16 |
| NT 1/18     | 33300       | 30600   | 4600 | 0.15 |
| NT 1/20     | 30000       | 27400   | 5200 | 0.19 |

**Average from 1/1 to 1/20 dilution** 0.14
| NT 1/24     | 25000       | 22500   | 4600 | 0.20 |
| NT 1/28     | 21400       | 19100   | 4200 | 0.22 |
| NT 1/32     | 18800       | 16600   | 3900 | 0.23 |
| NT 1/36     | 16700       | 14600   | 4600 | 0.31 |
| NT 1/40     | 15000       | 12100   | 3700 | 0.31 |

**Average from 1/1 to 1/40 dilution** 0.17
| NT 1/48     | 12500       | 10200   | 2500 | 0.25 |
| NT 1/56     | 10700       | 8000    | 2700 | 0.34 |
| NT 1/64     | 9380        | 6800    | 2300 | 0.34 |
| NT 1/72     | 8330        | 5700    | 2000 | 0.35 |
| NT 1/80     | 7500        | 3600    | 1600 | 0.45 |

**Average from 1/1 to 1/80 dilution** 0.20
Table S5.8

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Figure S5.1

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
    - 800 molecules MYC IS
    - 800 molecules E2F1 IS
    - 800 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**  **Probe**
    - ACTB  ACTB-FAM  ACTB-Quasar
    - MYC  MYC-FAM  MYC-Quasar
    - E2F1  E2F1-FAM  E2F1-Quasar
    - CDKN1A  CDKN1A-FAM  CDKN1A-Quasar
  - FAM probe for native template
  - Quasar 670 probe for IS
  - Universal Master Mix

- **Two External Standards Plot**
  - 10-13M, 10-14M
  - e.g.) ACTB
Figure S5.2

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 S5.3

(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 S5.4
Figure S5.6

A  Pre-amplication

Gene Expression/
10^6 ACTB

B  No Pre-amplication

Gene Expression/
10^6 ACTB
Figure S5.7

Cq Variation by Universal Master Mix Concentration

Note: The asterisk (*) indicates that Cq values were undetermined by software.
Figure S5.8

The figure compares the RT efficiency (%) of two kits, Qiagen RNeasy Kit and Agilent Absolutely Kit, across different numbers of FFPE block strips/ extractions (2, 4, 6, and 8). The bars show the mean RT efficiency with error bars indicating the standard deviation.
5.11 References


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26. Sidor IF, Dunn JL, Tsongalis GJ, Carlson J, Frasca S, Jr.: A multiplex real-time polymerase chain reaction assay with two internal controls for the detection of
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Chapter 6

Conclusions and Summary

In an effort to individualize the diagnosis and treatment of disease, many biomarker-based clinical assays comprising transcript abundance values for multiple genes have been developed. Yet, the accuracy and reproducibility of RT-qPCR – based clinical tests depends on the quality control parameters of biospecimen collection/storage, RNA extraction, reverse transcription and PCR amplification of cDNA.

Previously described work assessed how RNA degradation and reverse transcription inhibition affect gene expression measurements in developed molecular diagnostic tests of various biospecimens including human whole blood, homogenized fish tissue and FFPE-treated human lung tissue. Use of internal standards in all PCR assays controlled for PCR inhibition and prevented false negative results. The results of the studies presented have demonstrated the significant impact of biospecimen preservation and RT efficiency on downstream transcript abundance measurement.

6.1 Manuscript I

6.1.1 Inter-laboratory comparison of BCR-ABL1 measurement

To verify that our particular BCR-ABL1 test could detect and measure a 4-log change in BCR-ABL1 from a baseline measurement, we employed the use of the College
of American Pathologists’ Minimal Residual Disease BCR-ABL1 Proficiency samples and compared our results to the median fold change determined by all participating laboratories (Figure 3.1). Using untreated and treated CML patient whole blood samples, we compared our test results with ARUP Laboratories’ BCR-ABL1 quantitative test results converted to the WHO International Scale and demonstrated high correlation between the results (Figure 3.1).

6.1.2 Effect of whole blood room temperature incubation on RNA integrity

Next, we wanted to determine the effect of room temperature incubation on BCR-ABL1 measurement. Because BCR-ABL1 testing is often completed at a location different from the place of collection, the time delay to whole blood processing becomes a significant factor that may impact transcript abundance measurements. Typically BCR-ABL1 laboratories will accept whole blood samples within 48 hours of collection. Although some samples may be refrigerated during transport, refrigeration is not a mandate. To model a CML patient sample, we added known amounts of BCR-ABL1-positive K562 cells to anonymized, healthy whole blood and allowed these samples to remain at room temperature for various amounts of time. Our studies using a BCR-ABL1/GUSB quantitative test that controls for PCR inhibition showed that the BCR-ABL1/GUSB metric is not statistically different at 24 hours, but significantly reduced 51% at 48 hours (Figure 3.2). It must be stated that this result is specific to a particular set of methods used in the study including whole blood collected in EDTA collection tubes, RNA isolated using a particular silica-based column technology and random hexamers and Superscript III used in the reverse transcription reaction. To confirm this significant decrease in BCR-ABL1/GUSB1 measurement after 48 hours of room temperature incubation, we further examined the impact of temperature on RNA integrity.
temperature incubation using other collection tubes, RNA isolation methods and/or reverse transcription methods, additional studies will need to be completed. Yet, Moravcova and colleagues demonstrated a 2- to 3-fold decrease in BCR-ABL1/GUSB measurement after 48 hours of room temperature incubation using citrate blood collection tubes, a liquid-phase extraction, AMV reverse transcriptase and a nested PCR assay.² Although the two studies had similar results, the differences in methods used must be addressed. Ultimately, if testing centers measure BCR-ABL1 load normalized to GUSB transcript abundance using the methods described in our manuscript, their protocols must change; whole blood samples must be processed within 24 hours to prevent significant changes in transcript abundance due to RNA degradation. This is of high importance because many studies show that a 0.5-log increase in BCR-ABL1 load from a major molecular response (MMR) is an accurate signal of treatment failure.³⁻⁵ A 51% reduction in BCR-ABL1/GUSB measurement due to RNA degradation could mask a 0.5-log increase in BCR-ABL1 load and prevent physicians from making important decisions about patient care.

**6.1.3 Effect of RNA input and RT primer on yield of BCR-ABL1 b3a2 and GUSB cDNA**

Reliable BCR-ABL1 quantitative tests must be sensitive assays that are able to detect very low levels of BCR-ABL1 fusion transcript in patient blood samples. We began additional experiments to determine if we could improve BCR-ABL1 detection sensitivity by manipulating particular facets of the reverse transcription reaction, including increasing RNA input and using a variety of RT primers. Using a constant total RNA input in the RT reaction with Superscript III reverse transcriptase, we compared the
cDNA yields of BCR-ABL1 and GUSB using oligo d(T), random hexamer and gene-specific primers (the reverse PCR primer). The use of gene-specific RT primers provided the best cDNA yield of BCR-ABL1 and GUSB, nearly 20-fold more than that measured with the use of random hexamers, the current priming method used in BCR-ABL1 testing (Figure 3.3). We then wanted to determine with the use of random hexamers and gene-specific RT primers, if we could improve sensitivity of BCR-ABL1 detection with increasing RNA input. Yet, we realized that by increasing total RNA input into the RT reaction, we would be increasing the amount of potential reverse transcriptase inhibitors in the reaction. Using 0.9, 3, 5, 9 and 18 µg total RNA inputs/30 µL RT reaction volumes, we determined that BCR-ABL1 levels only increased 3-fold at 18 µg RNA input compared to baseline 0.9 µg RNA input (Figure 3.3). Additionally the small increase in BCR-ABL1 with random hexamers was not determined to be linear. On the other hand, use of gene-specific RT primers in the RT reaction provided a linear increase in BCR-ABL1, improving BCR-ABL1 yield 20-fold from baseline 0.9 µg RNA input to 18 µg RNA input (Figure 3.3). Interestingly, for random hexamer- and gene-specific-primed RT reactions, BCR-ABL1 and GUSB cDNA yields increased the same amount. As a result, the BCR-ABL1/GUSB measurement for all RNA input conditions did not statistically change from the baseline measurement at 0.9 µg RNA input (Figure 3.3). We determined that in the presence of potential interfering substances in the RT reaction, BCR-ABL1 and GUSB transcripts were reverse transcribed by Superscript III enzyme at the same efficiency.

6.1.4 Effect of RNA input on RT efficiency (ERCC 171/113)
To elucidate the increased presence of inhibitors in the RT reaction further, we wanted to measure the yield of an External RNA Control Consortium (ERCC) in-vitro transcribed standard in the same random hexamer-primed RT reactions with increasing amounts of whole blood total RNA. Our Reverse Transcription Standards Mixture (RTSM) contained known amounts of ERCC 171 RNA and ERCC 113 cDNA and a 1 µL aliquot was placed in each RT reaction. While the ERCC 171 RNA standard is reverse transcribed into cDNA, ERCC 113 cDNA remains unchanged, and serves as a loading control for the RTSM. As more inhibitors are present in the RT reaction, the reverse transcription efficiency of the ERCC 171 RNA standard should decrease; hence less cDNA product should be measured. We determined that increasing input of whole blood total RNA into the RT reaction caused the linear decrease in ERCC 171/113 measurement (Figure 3.3). This may serve as an explanation as to why BCR-ABL1 and GUSB detection did not increase equally to increase in RNA input in random hexamer-primed RT reaction. Additionally a significant decrease in ERCC 171/113 from baseline 0.9 µg RNA/30 µL RT reaction (30 ng RNA/µL cDNA) occurred at 5 µg RNA/30 µL RT reaction (167 ng RNA/µL cDNA) (Figure 3.3). However, according to the manufacturer’s protocol for Superscript III reverse transcriptase, the upper limit of RNA input is 250 ng RNA/µL cDNA.

6.1.5 Effect of extended BCR-ABL1 gene-specific RT primer on yield of BCR-ABL1 cDNA

All previous studies in this manuscript used RNA samples with high levels of BCR-ABL1 transcript. We wanted to determine whether or not gene-specific RT priming would improve BCR-ABL1 detection in RNA samples with low levels of BCR-ABL1
transcript. It is in whole blood samples from CML patients that have reached either MMR or complete molecular response (CMR) where improved BCR-ABL1 detection sensitivity is needed. After using the same BCR-ABL1 and GUSB gene-specific primer in RT reactions with whole blood total RNA with low levels of BCR-ABL1 transcript, we noticed immense electrophoretic noise after PCR amplification (Figure 3.4). It appeared as if there was additional amplification of products other than BCR-ABL1. Using a method of RNA-sequencing called STARSEQ, we determined that 95% these extra products were sequences from the RANBP3 isoform family. We later determined that our BCR-ABL1 reverse PCR primer (our original BCR-ABL1 gene-specific RT primer) shared 74% homology with the RANBP3 family of sequences. Additionally our BCR-ABL1 forward PCR primers shared 80% homology with these sequences. To circumvent the amplification of RANBP3 cDNA PCR, we chose to extend the BCR-ABL1 gene-specific primer by 10 nucleotides (total of 29 nucleotides in length). Use of the extended BCR-ABL1 gene-specific RT primer eliminated the reverse transcription of RANBP3 transcripts and prevented their PCR amplification (Figure 3.4). Our data shows that in cases where the PCR primers cannot be optimized, you can improve specificity of your assay by modifying and optimizing your gene-specific RT primer. We found that in samples with low levels of BCR-ABL1 transcript, use of gene-specific RT primers compared to random hexamers increases yields of both BCR-ABL1 and GUSB approximately 10-fold with the same RNA input in the RT reaction (Figure 3.4).

6.1.6 Recommend additional studies regarding BCR-ABL1 measurement

In addition to the work presented regarding optimizing quality control of BCR-ABL1 measurement in whole blood samples, there are two additional aspects of BCR-
ABL1 testing that should be acknowledged and further studied. These include 1.) longitudinal, prospective studies of healthy individuals harboring BCR-ABL1 fusion transcripts and 2.) development of methods to assess therapy efficacy of CML patients with the use of gDNA rather than RNA.

6.1.6.1 BCR-ABL1 fusion transcripts in healthy individuals

In 1995, Biernaux and colleagues demonstrated that up to 30% of 74 normal, healthy human adults express low levels (5-20 copies per 5x10^7 – 10^8 white blood cells) of BCR-ABL1 p210 fusion transcript by nested reverse-transcription, polymerase chain reaction (RT-PCR), while only 4.5% of 22 children tested were shown to be positive.\(^6\) Hence, Biernaux found that positive results appeared to correlate with age. Results of the Biernaux study were validated in an independent laboratory in Belgium.

A second study in 1998 by Bose found that 27% of 15 normal, healthy human adults expressed the BCR-ABL1 p210 fusion transcript by a similar nested RT-PCR method including 40 replicates of each individual sample.\(^7\) Yet, the Bose study also showed that 69% of 15 individuals tested expressed the BCR-ABL1 p190 fusion transcript. Of the 40 replicate nested RT-PCR assays, individual samples positive for BCR-ABL1 fusion transcript were positive in 1 to 18 replicates, while the majority of normal individuals showed positivity in less than 5 of 40 replicate assays. Bose and colleagues estimated that the number of circulating BCR-ABL1-positive leukocytes in positive, healthy individuals was probably one to 10 in 10^8 total leukocytes. The Bose study also demonstrated that expression of one or more types of BCR-ABL1 fusion transcripts were detected in 7 non-CML hematopoietic cell lines.
A third study conducted by Song and colleagues in 2011 detected BCR-ABL1 p190 fusion transcripts in 74% of 80 healthy individuals and detected BCR-ABL1 p210 fusion transcripts in 42% of 74 healthy individuals using nested RT-PCR, real-time RT-qPCR and additional direct sequencing of PCR products. Using the results of a K562 cell line dilution study and sample RT-qPCR, it was calculated that the frequency of peripheral blood cells containing BCR-ABL1 p210 transcripts was 1:10,000. For each BCR-ABL1 fusion transcript detected, healthy individuals were divided into six age groups including the neonate, children under 10 years of age, individuals between ages 10 and 25 years, adults between ages 26 and 40 years, adults between ages 41 and 55 years and adults older than 55 years. The authors demonstrated that while no correlation between prevalence and age was observed for BCR-ABL1 p190 fusion transcripts, incidence was highly correlated with age in normal individuals positive for BCR-ABL1 p210 fusion transcripts. 

Despite these three studies, the only risk factors for BCR-ABL1 expression positivity appear to be either age or exposure to ionizing radiation. Also, these studies made no attempt to detect the BCR-ABL1 fusion transcripts in these normal individuals at later timepoints. No longitudinal studies of positive individuals have been conducted to determine if these individuals eventually develop definitive CML. Because the incidence of normal individuals expressing low levels of BCR-AbL1 far exceeds the incidence of CML, most positive individuals will not develop leukemia. Because of this, it is believed that presence of the BCR-ABL1 fusion transcript may not be sufficient for malignant transformation of hematopoietic cells. A BCR-ABL1-positive stem cell may be quiescent for some time before becoming a malignant clone after accumulation of additional
mutations to the genome/epigenome, cellular microenvironment or in immune surveillance of the individual. Experimental findings in mouse models show that the t(9;22) translocation does not immediately initiate a leukemic phenotype, but requires a latency period suggesting that multiple additional mutations may be required for a malignancy.\textsuperscript{11,12,13} Such data would suggest that the pathogenesis of CML is multistep.

There are two case studies in the literature showcasing an asymptomatic BCR-ABL1-positive adult male with spontaneously subsiding leukocytosis,\textsuperscript{14} and an asymptomatic BCR-ABL1-positive adult male following autologous transplantation for multiple myeloma.\textsuperscript{15,16} While the male patient with resolved leukocytosis was not treated with tyrosine kinase inhibitor therapy and only monitored every three months for a year with no cytological progression, the male, multiple myeloma patient was initially monitored for 4 weeks, but placed on imatinib therapy after his BCR-ABL1 levels increased nearly 6-fold from his baseline measurement after transplantation. Prospective, longitudinal studies that follow BCR-ABL1-positive, normal individuals over time could help determine which individuals are more at risk to develop a leukemic phenotype, and provide guidelines for monitoring or treating these individuals in the clinic.

### 6.1.6.2 BCR-ABL1 measurement using gDNA compared to mRNA

Although RT-qPCR is a highly sensitive assay, its use is limited by the quality of RNA used and the efficiency of the reverse transcription step. While CML patients under therapy are routinely monitored with BCR-ABL1 tests using RT-qPCR assays, two studies aimed to determine whether BCR-ABL1 monitoring could be completed with the use of genomic DNA, rather than RNA reverse transcribed from patient RNA. Use of
gDNA in measurement of BCR-ABL1 in CML patients would eliminate any problems associated with RNA quality control.

Zhang and colleagues in 1996 compared the use of gDNA and cDNA in the detection of BCR-ABL1 gDNA and transcripts in CML patients treated with allogeneic bone marrow aspiration. The study compared 24 matched samples collected on the same day from a total of 10 patients analyzed by both RT-PCR (cDNA) with the same PCR primers used for all samples, and nested PCR (gDNA) using patient-specific PCR primers created from individual sequencing data. The authors originally found concordant RT-PCR and nested PCR results in 79% of matched samples tested, but upon repeating RT-PCR analysis for the three RT-PCR – negative, DNA-PCR – positive samples, 88% of samples were concordant. They concluded that patients in remission do not harbor a pool of transcriptionally-silent leukemia cells. The authors also state that it is also unlikely that CML cells express unusually high fusion transcript levels because concordance between RT-PCR and DNA-PCR results was found for both high (> 50 BCR-ABL1/µg leukocyte RNA) and low (<50) levels of expression. Ultimately, however, the authors admit that DNA-PCR could not easily replace RT-PCR as a routine method for monitoring CML patients post-transplantation; in an early study by these authors, fragments containing genomic BCR-ABL1 breakpoints were only able to be amplified in 43% of patients.

Over a decade later, Mattarucchi and colleagues revisited the idea of using gDNA to monitor residual disease in CML patients compared to cDNA analysis. To examine the relationship between a patient’s proportion of leukemic cells and his or her expression of the BCR-ABL1 fusion transcript, the authors developed a real-time DNA-PCR using
patient-specific PCR primers and probes to be compared to conventional real-time, RT-qPCR analysis using the M-bcr FusionQuant kit (Ipsogen, Marseille, France). The study analyzed a total of 57 blood and bone marrow samples for a period of ~25.5 months collected from 10 patients diagnosed in the chronic phase and treated with imatinib mesylate. At diagnosis, researchers found that while levels of BCR-ABL1 fusion transcript were extremely variable among patients, nine out of 10 patients had a leukemic cell proportion close to an average value of 81%. One patient with a 35% proportion of leukemic cells had undetectable leukemic mRNA and gDNA after 12 months. The authors determined that after monitoring patients over time with both methods, BCR-ABL1 expression level is not prognostic at diagnosis, while those patients with a lesser proportion of leukemic cells at onset have a better prognosis. Although the authors admit that the results of BCR-ABL1 gDNA and cDNA measurement are more comparable during follow-up of patients under treatment, they explain that for two patients, mRNA levels increased when measured at 15 months, but was not confirmed with follow-up measurements. Yet, for the same two patients, the unconfirmed increase in BCR-ABL1 transcript load did not correspond to an expansion of the leukemic clone. Additionally, the authors discuss that the absence of BCR-ABL1 gDNA could identify patients that would be good candidates for drug discontinuation rather than relying on absence of BCR-ABL1 mRNA detection. Ultimately the authors admit that use of gDNA for molecular monitoring of CML patients would require more labor-intensive methods to characterize each patient’s breakpoint sequence, but could improve patient care overall.

Both the Zhang and Mattarucchi studies admit that while use of genomic DNA for molecular monitoring of CML patients would eliminate many of the technical issues
affecting the reliability and accuracy of RNA-based tests, the gDNA approach requires additional steps to sequence each patient’s specific breakpoint region so that patient-specific PCR primers can be created for the assay. Additional large, longitudinal studies need to be conducted to ensure that use of gDNA to assess treatment efficacy in CML patients performs as well or even better than use of mRNA.

6.2 Manuscript II

6.2.1 The Hope study

An impetus to our research on RT efficiency of fish-derived tissue transcripts was a publication by Hope and colleagues that, in addition to the Pierce study, suggested that RT-qPCR assays are quicker and more sensitive to VHSv detection than cell culture assays. The Hope study developed a one-step, real-time RT-qPCR test using gene-specific RT primers to detect and quantify VHSv in fish tissue. In addition to development of this test, Hope and colleagues chose to study the impact of increasing RNA input in the RT reaction on VHSv-IVb N-gene molecular yield. The Hope study chose RNA inputs of 50, 100, 500 and 1000 ng total RNA per 25 µL RT reaction (2, 4, 20 and 40 ng RNA/µL cDNA) from two VHSv-IVb infected fish with different viral loads. Total RNA was provided from the supernatant collected from pooled fish tissues (liver, anterior and posterior kidney, spleen and heart) that were lysed, homogenized and centrifuged. Although the extraction method for this particular set of samples is not clearly made, the authors did show no distinguishable difference between phenol-chloroform (RNA Bee) and column-based (Qiagen RNAeasy kit) extraction on measured copies of VHSv-IVb N gene in additional fish samples. In both the high and low viral load fish RNA samples, Hope and colleagues found only a 10-fold increase in VHSv-IVb N molecular yield.
between the baseline and highest RNA input, even though there was a 20-fold increase in RNA input between the two samples. Hope writes that additional experiments using higher RNA inputs (4-8 µg) in 25 µL RT reactions were completed; although this data was not shown, Hope writes that higher VHSv-IVb N-gene expression was measured, but levels were even lower than expected for the additional RNA input.

6.2.2 Reverse transcription efficiency of VHSv-infected fish total RNA using StaRT-PCR

In response to the Hope study, we decided to complete additional RT efficiency studies with our fish samples using a developed two-step, end-point RT-qPCR test with use of internal samples. Compared to the experimental setup of the Hope study, our RT efficiency study used larger RNA inputs including 1, 5, 10, 20 and 30 µg total RNA inputs per 90 µL RT reaction (11.1, 55.6, 111.1, 222.2 and 333.3 ng RNA/µL cDNA), measured three genes (VHSv-IVb N-gene, actb1 and ef1a) and used the RTSM to observe effect of RT inhibitors on the gene expression of ERCC 171 RNA standard. Additionally total RNA for our experiments came from pooled homogenate of smallmouth bass (MI03GL isolate) spleen, kidney and brain extracted via TriReagent® (Molecular Research Center, Cincinnati, OH) phenol-chloroform method. Random hexamers and MMLV reverse transcriptase were used for the RT reactions. Reverse transcription efficiency, as measured by the RTSM, significantly decreased 23-26% from baseline compared to 20 and 30 µg RNA/90 µL RT reaction, respectively (Figure 4.5). When expression of reference genes actb1 and ef1a were measured for each RNA input condition, transcript abundance levels increased in parallel, measuring 28.6- and 24.1-fold increases compared to baseline (1 µg/90 µL RT reaction) (Figure 4.5). As a result,
for all RNA input conditions the measurement values of actb1/ef1α did not statistically change from the baseline measurement. However, when levels of VHSv-IVb N-gene were measured in the varying RT reactions, the level significantly increased 3.7-fold from baseline (1µg/90 µL RT reaction) to 10 µg/90 µL RT reaction, instead of an expected 10-fold increase (Figure 4.5). With increasing RNA inputs above 10 µg, the level of VHSv-IVb N-gene expression remained unchanged. As a result, measurements of VHSv-IVb N-gene normalized to actb1 statistically changed from one RNA input to another. This result is in stark contrast to measurements of actb1 normalized to ef1α where measurements did not statistically change with different RNA inputs in the RT reaction. These results show that RT efficiency is gene-specific within the context of a particular biospecimen. Additionally this data shows how simple adjustments to an experiment, such as increasing RNA input into the RT reaction can have statistically significant effects on gene expression measurement results. These results agree with MIQE guidelines recommending that researchers use a constant amount of RNA in RT reactions.21

6.3 Manuscript III

6.3.1 Comparison of FFPE RNA extraction kits

In our search to identify the most optimal FFPE RNA extraction kit, we decided to compare two commercially available kits: RNeasy® FFPE Kit (Qiagen, Valencia, CA) and Absolutely® RNA FFPE Kit (Agilent Technologies, Santa Clara, CA). As stated before, many enzymatic inhibitors of reverse transcription and PCR amplification are either co-extracted with biospecimen RNA or are introduced into the RNA elution via the elution process itself. We wanted to identify the FFPE RNA extraction kit that provided
the most pure RNA solution from different amounts of FFPE cell blocks with the least presence of inhibitors. Inhibition of reverse transcription was measured using the RTSM; the same aliquot volume of RTSM was placed in each RT reaction and ERCC 171/113 was measured using StaRT-PCR in cDNA from each RT reaction. Because each transcript is measured relative to an internal standard in the PCR assay, control for PCR inhibition allowed differences in ERCC 171/113 measurements to be attributed only by RT efficiency differences.

For each extraction kit, increasing numbers of 5-µm thick, strips (2, 4, 6 and 8 strips) of a formalin-fixed and paraffin embedded A549 cell pellet were used in each RNA extraction kit. Because the amount of FFPE block material used in these experiments surpassed the upper limit of material recommended for each kit, we understood that all downstream RT reactions may be maximally inhibited. Despite both kits providing RNA of similar concentration (ng/µL), purity (A260/A280) and RIN Score, each kit provided statistically different measurements of ERCC 171/113 in cDNA. Overall the Qiagen RNeasy kit provided extracted RNA with less inhibitors and better RT efficiency of ERCC 171 reverse transcription than the RNA provided by the Agilent Absolutely RNA kit for all experimental conditions used. ERCC 171/113 measurements were 2- to 4-fold higher ($p \leq 0.021$) in cDNA reverse transcribed from RNA extracted from the Qiagen RNeasy kit than from the Agilent Absolutely kit (Figure S5.8). ANOVA analysis shows that ERCC 171/113 measurement across all extraction conditions for the Qiagen RNeasy kit were not statistically different ($p = 0.15$), while ERCC 171/113 measurements from RT reactions using RNA extracted using the Agilent Absolutely RNA kit declined significantly as more strips were used in the extractions ($p$
= 0.001) (Figure S5.8). We believe one of the reasons RT efficiency was more suboptimal in the Agilent Absolutely RNA kit because it requires use of less material in the RNA extraction. Although we stressed both the RNA extraction kits with more FFPE block material than recommended, the Agilent Absolutely RNA kit had more stringent limitations in material to be used than the Qiagen RNeasy kit. We understand, as well, that these measurements of RT efficiency are specific to the transcript reverse transcribed, RT priming method and reverse transcriptase used and that use of other reagents may show different RT efficiency measurement results between the two RNA extraction kits.

6.3.2 Effect of RT input and primer on ACTB measurement in FFPE-derived RNA

In addition to identifying the optimal FFPE RNA extraction kit for our experiments, we wanted to assess the most optimal RT priming method for RNA extracted from surgically removed lung tissue FFPE blocks. Using the Qiagen RNeasy FFPE RNA extraction kit, RNA from three clinical FFPE specimens was reverse transcribed using two different RT priming methods: random hexamer priming and gene-specific priming. RNA from FFPE tissue is often highly degraded due to physical breakage of transcripts during formalin fixation. As a result, random priming is used during reverse transcription instead of oligo d(T) priming because many transcripts may have loss of the poly-A tail. Like the Stanoszek study that compared use of random and gene-specific priming in reverse transcription of different input amounts of whole blood total RNA, we wanted to compare different RT priming methods using two different total RNA input amounts of RNA extracted FFPE biospecimens to determine impact on ACTB gene expression measurements. Using the newly described StaRT-PCR two-color
fluorometric real-time assay with use of internal standards, we determined that use of
gene-specific RT primers compared to random hexamers improved ACTB/µL cDNA
yield 658-fold with an input of 1µg RNA/30 µL RT reaction and 1381-fold with an input
of 5 µg RNA/30 µL RT reaction (Figure S5.9). Such an increase in ACTB gene
expression is important for many reasons. These results not only demonstrate that RT-
qPCR gene expression measurements are significantly impacted by RT primer method
used, but also show that less sample RNA may be used to still obtain accurate and
reproducible gene expression measurements. The latter is most important for patient
FFPE samples with less material, such as fine-needle aspirate biopsies that are formalin
fixed and paraffin embedded.

6.4 Contributions from Chapters 3, 4 and 5

1. The developed BCR-ABL1 test could detect and measure approximately a 4-log
change in BCR-ABL1 measurement using CAP MRD-B proficiency survey samples.

2. In CML patient whole blood samples our BCR-ABL1 test measurement showed high
concordance with results from ARUP Laboratories’ BCR-ABL1 quantitative test
converted to the WHO International Scale.

3. Room temperature incubation of 6 samples of normal, whole blood mixed with K562
cells for 48 hours resulted in a significant 51% decline in BCR-ABL1/10^3 GUSB
measurement compared to baseline at 0 hours.

4. With a constant RNA input (1 µg) in the RT reaction (30 µL), gene-specific RT
primers provided a 17-fold and 21-fold higher yield of BCR-ABL1 b3a2 and GUSB
cDNA, respectively, compared with random hexamers.
5. When random hexamers are used in RT reactions, a maximum three-fold increase in BCR-ABL1 b3a2 cDNA yield was observed at 18 µg RNA/RT compared with baseline at 0.9 µg RNA/RT, despite a 20-fold increase in RNA input. Because the slope for GUSB cDNA yield increase was not significantly different from that for BCR-ABL1, there was no significant difference among the BCR-ABL1 b3a2/10^3 GUSB values for each level of RNA input.

6. When a specific volume of RTSM is placed in each random-primed RT reaction with increasing whole blood RNA inputs, ERCC 171/113 measurement was reduced by 52% at 3 µg RNA/RT and reduced by 80% at 18 µg RNA/RT. A semilog plot trendline fit the data points with high correlation (R^2 = 0.98).

7. When gene-specific primers (reverse PCR primers) are used in RT reactions, a maximum 20-fold increase in BCR-ABL1 b3a2 cDNA yield was observed at 18 µg RNA/RT compared with baseline at 0.9 µg RNA/RT. There was no significant difference among the BCR-ABL1 b3a2/10^3 GUSB values for each level of RNA input with gene-specific RT priming.

8. In normal whole blood samples with low levels of BCR-ABL1 transcript, an extended, 29-nucleotide BCR-ABL1 gene-specific RT primer was constructed to prevent reverse transcription of RANBP3 isoform family transcripts. Use of the extended RT primer and the original PCR primers increased the yields of both BCR-ABL1 and GUSB approximately 10-fold compared to random hexamer priming.

9. Increasing inputs up to 30 µg homogenized smallmouth bass total RNA into 90 µL random hexamer – primed RT reactions provided increases of 28.6-, 24-1- and 3.7-fold actb1, ef1a and VhSv-IVb N-gene expression, respectively, from baseline 1 µg RNA.
input. Non-parallel increases in VHSv-IVb N-gene and reference gene transcript abundance result in significantly different normalized VHSv-IV b N gene expression measurements across all tested RNA inputs.

10. Reverse transcription efficiency, measured by the RTSM in RT reactions with increasing inputs of homogenized mallmouth bass total RNA, significantly decreased 23-26\% from baseline 1 µg RNA to 20 and 30 µg RNA/90 µL RT reaction, respectively,

11. The Qiagen RNeasy FFPE RNA extraction kit provided RNA eluates from increasing numbers of A549 FFPE strips with less RT inhibition of the RTSM than RNA eluates from the Agilent Absolutely FFPE RNA extraction kit; ERCC 171/113 values were 2- to 4- fold higher using RNA eluates from the Qiagen RNeasy kit.

12. In surgically removed lung tissue FFPE blocks, use of gene-specific RT primers compared to random hexamers increased ACTB/µL cDNA yield 658- and 1381-fold with 1 and 5 µg RNA/30 µL RT reactions, respectively.
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