MM16-P
Vol. 25 No. 19

Use of External RNA Controls in Gene Expression Assays; Proposed Guideline

PLEASE

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This proposed document is published for wide and thorough review in the new, accelerated Clinical and Laboratory Standards Institute (CLSI) consensus-review process. The document will undergo concurrent consensus review, Board review, and delegate voting (i.e., candidate for advancement) for 90 days.

Please send your comments on scope, approach, and technical and editorial content to CLSI.

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11 October 2005

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COMMENT

This document provides protocols supporting the use of external RNA controls in microarray and QRT-PCR-based gene expression experiments, including preparation of control transcripts, design of primers and amplicons, quality control, use in final experimental or clinical test application, and analysis and interpretation of data obtained.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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The CLSI voluntary consensus process is a protocol establishing formal criteria for:

- the authorization of a project
- the development and open review of documents
- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

Most documents are subject to two levels of consensus—"proposed" and "approved." Depending on the need for field evaluation or data collection, documents may also be made available for review at an intermediate consensus level.

Proposed A consensus document undergoes the first stage of review by the healthcare community as a proposed standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

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COMMENTS

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VOLUNTEER PARTICIPATION

Healthcare professionals in all specialties are urged to volunteer for participation in CLSI projects. Please contact us at customerservice@clsi.org or +610.688.0100 for additional information on committee participation.

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Use of External RNA Controls in Gene Expression Assays; Proposed Guideline

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Abstract

Clinical and Laboratory Standards Institute (CLSI) document MM16-P—*Use of External RNA Controls in Gene Expression Assays; Proposed Guideline* provides protocols supporting the use of external RNA controls in microarray and QRT-PCR-based gene expression experiments. This guideline addresses important issues associated with the use of external RNA controls as a tool for verification of technical performance, and in support of the evaluation of qualitative results for a specific clinical analyte including preparation of control transcripts, design of primers and amplicons, quality control, use in final experimental or clinical test application, and the analysis and interpretation of data obtained. The guideline will facilitate research and clinical laboratories, regulatory agencies, accrediting agencies, reference laboratories, as well as test, microarray, and reagent manufacturers in measuring the performance of expression assays.

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Contents

Abstract i

Committee Membership iii

Foreword vii

1 Scope 1

2 Introduction 1

3 Standard Precautions 3

4 Terminology 3

4.1 Definitions 3
4.2 Acronyms/Abbreviations 5

5 Overview 5

5.1 Gene Expression Profiling Technology 5
5.2 Gene Expression Profiling in Clinical Applications 6
5.3 A Note on Technical Performance and Clinical Performance 8

6 External RNA Controls and Their Use in Expression Assays 8

6.1 Internal and External RNA Controls in Gene Expression Assays 9
6.2 Characteristics of External RNA Controls 9
6.3 Using Control Materials to Assess Technical and Clinical Performance 10
6.4 Limitations of External Controls 13
6.5 Metrics for Assessing Technical Performance of the Expression Platform 14

7 Recommended Practices/Protocols 16

7.1 Overview 16
7.2 Protocols for Preparation and Assessment of External RNA Controls 17
7.3 External RNA Control Protocols for Microarrays 19
7.4 External RNA Control Protocols for QRT-PCR 25

References 31

The Quality System Approach 36

Related CLSI/NCCLS Publications 37

Foreword

Few inventions in the past twenty years have had as much impact on the research and clinical communities as the polymerase chain reaction (PCR) and microarray technology. These technologies have captured the imaginations of clinical and laboratory researchers and made possible studies heretofore unachievable. The scope and breadth of the studies enabled by these technologies have dramatically changed the practice of laboratory and clinical research, most notably impacting experimental design and data analyses. Whole genome analyses integrating DNA, RNA, and protein information promise deeper insight into biological processes and the discovery of new biomarkers. The volume of data generated by these methods pressures all investigators to design experimental and analytical strategies that will leverage the integration of clinical and molecular information.

This new era of clinical and laboratory research places increased emphasis on collaboration and teamwork, including a significant focus on well-defined clinical database structures, data management, and data access issues. At the very center of this paradigm shift is a requirement for the fundamental tools necessary for measuring technical performance of the platforms used for data collection. Acceptance of microarray and quantitative reverse transcriptase real-time polymerase chain reaction (QRT-PCR) data in the regulatory environment will require standards that assess reliability and quality. The ability to report reliable gene expression results of known quality is key to the successful employment of microarrays and QRT-PCR as tools in toxicogenomics, pharmacogenetics, pharmacogenomics, and as diagnostic devices in clinical medicine.

In this guideline, we present protocols supporting the use of external RNA controls in microarray and QRT-PCR-based gene expression experiments. External RNA controls refer to RNA species that are distinct from the RNAs in the sample that is to be analyzed. The external RNA controls are added prior to enzymatic manipulations such as reverse transcription and labeling. The protocols enable research and clinical laboratories, regulatory agencies, accrediting agencies, reference laboratories, as well as test, microarray, and reagent manufacturers to assess the performance of these expression assays.

Minority Opinion

Please note that during committee voting, the timeliness of this document was raised as a concern. A minority opinion contends that MM16-P is ahead of the technology curve and that clinical applications have not yet been established based upon complex gene-expression technologies on microarray platforms.

"My major reservation is that this document is too closely tied to a single set of controls that are not yet available, and for which there is no experience. Although I agree that there is a need for standardization and controls for this emerging diagnostic technology, the document does not adequately address assay process controls, which ultimately will provide the best quality control for these complex technologies. In my opinion, the quality control concepts and reference materials are not sufficiently well-developed for a CLSI document. An important first step in this process is missing, a publication in the peer-reviewed literature using these concepts and control materials (see "Standardizing global gene expression analysis between laboratories and across platforms." *Nature Methods*. 2005;2:351)."

We invite further comments on this opinion, for committee consideration in advancing the MM16-P guideline to "approved" status in the CLSI consensus process.

Invitation for Participation in the Consensus Process

An important aspect of the development of this and all Clinical and Laboratory Standards Institute (CLSI) documents should be emphasized, and that is the consensus process. Within the context and operation of CLSI, the term "consensus" means more than agreement. In the context of document development, "consensus" is a process by which CLSI, its members, and interested parties (1) have the opportunity to review and to comment on any CLSI publication; and (2) are assured that their comments will be given serious, competent consideration. Any CLSI document will evolve as will technology affecting laboratory or healthcare procedures, methods, and protocols, and therefore, is expected to undergo cycles of evaluation and modification.

The Area Committee on Molecular Methods has attempted to engage the broadest possible worldwide representation in committee deliberations. Consequently, it is reasonable to expect that issues remain unresolved at the time of publication at the proposed level. The review and comment process is the mechanism for resolving such issues.

The CLSI voluntary consensus process is dependent upon the expertise of worldwide reviewers whose comments add value to the effort. At the end of a 90-day comment period, each subcommittee is obligated to review all comments and to respond in writing to all which are substantive. Where appropriate, modifications will be made to the document, and all comments along with the subcommittee's responses will be included as an appendix to the document when it is published at the next consensus level.

A Note on Terminology

Clinical and Laboratory Standards Institute (CLSI), as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all challenges to harmonization. Despite these challenges, CLSI recognizes that harmonization of terms facilitates the global application of standards and is an area that needs immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In keeping with CLSI's commitment to harmonize terminology with that of ISO, the term "sensitivity" will not be used in this document, due to the existence of several alternative common uses of that term. The preferred term "limit of detection" will be used, due to its more precise definition and common use. In many clinical laboratories and diagnostic applications, "sensitivity" or "analytical sensitivity" are used interchangeably with "limit of detection," "lower limit of detection," or "detection limit." However, "sensitivity" is also used in several other ways, some of which are preferred uses in their areas. In different applications, "sensitivity" might be used alone or with modifiers.

Key Words

External RNA controls, gene expression, microarray, molecular methods, QRT-PCR, RNA controls

Use of External RNA Controls in Gene Expression Assays; Proposed Guideline

1 Scope

The recommended external RNA control protocols described in this guideline are for the preparation of individual transcripts, pooling of transcripts, design of primers and amplicons, quality control, use in final experimental or clinical test application, and analysis and interpretation of data obtained using external RNA controls with and without complex background. The protocols will address use on one- and two-color microarray platforms and multiple QRT-PCR systems.

The external RNA controls are a tool for verification of technical performance, but will only support evaluation of qualitative results for a particular clinical sample (see Section 5.3). Technical performance verification does not control for or manage errors arising from human technical error or laboratory error.

This guideline has been developed to provide useful recommendations and protocols for:

- quality requirements for external assay controls;
- statement of control characteristics, performance metrics; and
- explanation of performance specifications, acceptable performance.

The following protocols are not within the scope of this guideline:

- quantitative controls for clinical sample RNA quality;
- controls for BAC, SNP, genotyping, resequencing, and protein arrays;
- preparation of molecular biology reagents;
- fabrication of cloned control materials;
- array manufacture;
- calibration, calibrators for absolute quantification;
- probe design for PCR;
- preanalytic sample handling;
- endogenous controls; and
- prelabeled hybridization controls.

2 Introduction

Microarray and QRT-PCR technologies are emerging as vital components of genomic, evidence-based medicine and are already having an impact.[1] Standard controls, best practice guidelines, and reference materials are expected and required for acceptance of microarray and QRT-PCR results in the regulatory environment, and are necessary for assessing reliability and quality results from these assay platforms. Industry-recognized controls are key to successful use of microarrays and QRT-PCR in toxicogenomics, pharmacogenomics, and as diagnostic devices in clinical medicine.

External RNA controls have been used to validate and interpret data from microarray hybridization experiments since the techniques were first reported in the scientific literature.[2-5] These controls, which are added before labeling the RNA sample, continue to be valuable tools for assessing the limit of detection, linear range, nonspecific background, and reproducibility of microarray platforms and could easily be extended to QRT-PCR assays. Control of these characteristics is essential for reliably implementing gene expression measurements in the clinic.

A set of external RNA controls is being developed in parallel with this guidance by an industry-led consortium, the External RNA Control Consortium (ERCC). The ERCC is working to make these materials readily accessible. They are intended to have broad acceptance within the research and clinical communities. The controls will be made commercially available as individual plasmid clones for the synthesis of polyadenylated transcripts (see Section 6.2). This set of external RNA controls is consistent with recommendations made at the 2003 meeting hosted by National Institute of Standards and Technology (NIST) at Stanford, "Metrology and Standards Needs for Gene Expression Technologies: Universal RNA Standards."[6] This CLSI guidance document is intended to describe a set of protocols recommended for use of the controls to validate gene expression experiments. These controls can be applied in an expression experiment as depicted in Figure 1. This application will control for technical performance, but not sample integrity. Valid clinical results depend on validated platform performance, but clinical results cannot be validated solely with these controls (see Section 5.3).

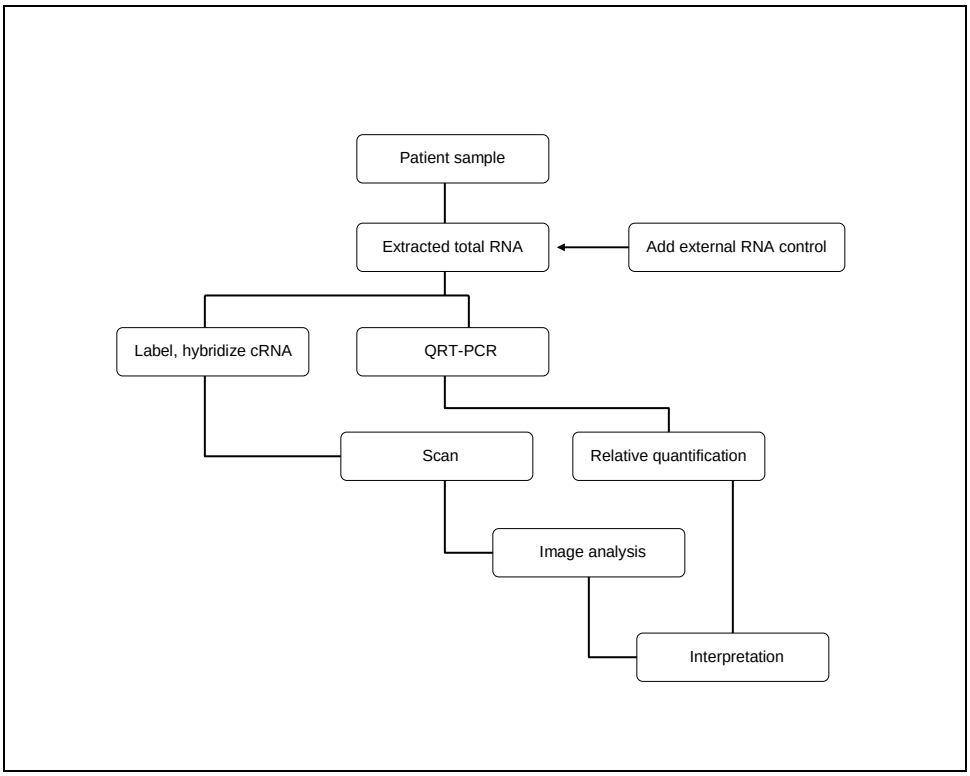

Figure 1. Flowchart Illustrating the Use of External RNA Controls

This document addresses the need among the research and clinical communities to develop standardized external RNA controls for expression analysis by microarray and QRT-PCR assays. It is intended to be useful for assuring assay performance and enabling comparisons of gene expression results. With these goals in mind, stakeholders in regulatory agencies, diagnostics, pharmaceutical, reagent, and microarray manufacturing companies, as well as the research and clinical community have participated in writing and/or reviewing the document, including members of the Association for Molecular Pathology, College of American Pathologists, American College of Medical Genetics, American Society of Human Genetics, Canadian College of Medical Genetics, International Committee on Harmonization, ERCC, Microarray Gene Expression Data Society, American Society of Hematology, American Association for Clinical Chemistry, International Federation of Clinical Chemistry, American Association of Cancer Research, National Institute of Standards and Technology, and the Food and Drug Administration.

MM16 is the second CLSI document presenting guidelines relevant to microarray assays, joining MM12—*Diagnostic Nucleic Acid Microarrays* in that focus. MM16 is the third CLSI document

presenting guidelines related to QRT-PCR with MM5—*Nucleic Acid Amplification Assays for Molecular Hematopathology* and MM6—*Quantitative Molecular Methods for Infectious Diseases*. This document provides an overview of gene expression profiling methods with an emphasis on clinical applications. The main focus of the guidelines in MM16 is the appropriate use of the external RNA controls developed by the External RNA Controls Consortium (available at www.cstl.nist.gov/biotech/workshops/ERCC2003). The chosen protocols and recommendations are not absolute or immutable. The MM16 guidance document addresses the incorporation of the control materials in an assay, including experimental design considerations, and analysis and interpretation of control results.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals.* Infection Control and Hospital Epidemiology. CDC. 1996;17(1):53-80 and *MMWR* 1988;37:377-388). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

4 Terminology

4.1 Definitions

accuracy (of measurement) – closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93).[7]

amplicon – an amplicon is the product of PCR. It is a fragment of DNA that has been synthesized using amplification techniques.

analyte – component represented in the name of a measurable quantity (ISO 17511).[8]

analytical cross-reactivity – evaluation of the level of nonspecific binding of control and/or test probes to nontargeted analytes that may be present in samples.

cycle threshold (Ct) – the Ct value of each RT-PCR reaction depends on the initial template amount (copy number) of the target sequence, and it is inversely proportional to the log of this copy number. In an experiment where all PCR reactions have similar efficiency, the Ct value will be the lowest for reactions, where the initial template copy number was highest.

endogenous control – endogenous controls are used in gene expression studies to normalize gene expression values in the total RNA relative to expression levels of genes whose expression levels are invariant in a given tissue or tissues; **NOTE:** Endogenous controls are not within the scope of this guideline.

external RNA control – the RNA species that are added to an RNA sample prior to enzymatic manipulations.

invariant gene – a gene with an expression level that is unchanging in a given tissue under experimental conditions or in the normal and diseased state.

limit of detection – the lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value (modified from WHO BS/95.1703).[9]

linearity//linear range – the ability (within a given range) to provide results that are directly proportional to the concentration (amount) of the analyte in the test sample (WHO-BS/95.1793)[10]; **NOTE 1:** Linearity typically refers to overall system response (i.e., the final analytical answer rather than the raw instrument output); **NOTE 2:** The linearity of a system is measured by testing levels of an analyte which are known by formulation or known *relative to each other* (not necessarily known absolutely); when the system results are plotted against these values, the degree to which the plotted curve conforms to a straight line is a measure of system linearity.

measurand – particular quantity subject to measurement (VIM93).[7]

normalization – array's normalization is the operation of removing experimental artifacts of non-biological origin such as variations in the sample preparation, variation in the manufacture of arrays, and in the processing of the arrays (labeling efficiency, hybridization, and scanning).

pharmacogenetics – the study of inherited differences (variation) in drug metabolism and response.

pharmacogenomics – the general study of the many different genes that determine drug behavior.

precision (of measurement) – the closeness of agreement between independent test results obtained under stipulated conditions (ISO 3534-1).[11]

primer (QRT-PCR) – a single-stranded nucleic acid that can "prime" replication of a template. More specifically, a single-stranded nucleic acid capable of hybridizing to a template single-stranded nucleic acid in such a way as to leave part of the template to the 3′ end of the primer single-stranded. DNA polymerase can then synthesize a new strand starting from the 3′ end of the primer and adding nucleotides to the growing strand by base complementarity to the template; **NOTE:** Primers used for quantitative reverse transcriptase real-time PCR are generally designed by specialized software taking into account a number of parameters (melting temperature, primers-dimers formation, GC percentages) to optimize the polymerase chain reaction.

probe (microarray) – defined piece of single-stranded nucleic acid used to identify specific DNA or RNA molecules bearing the complementary sequence, which is immobilized on the microarray substrate.

probe (QRT-PCR) – a single-stranded nucleic acid that binds to amplicon sequences between QRT-PCR primers with a fluorescent marker and enhances the specificity of sequence detection and quantification; **NOTE:** One example of this type of probe is in the 5′ nuclease reaction, where fluorescent markers with different excitation/emission profiles can be used on different probes to multiplex QRT-PCR for the simultaneous detection of target sequence and control sequence. The 5′ nuclease reaction signal is generated as free fluorescent molecules are released from the 5′ end of a probe where they had been quenched by a quencher group attached to the 3′ end of the probe.

scaling – the operation of adjusting the total or average intensity of each array to be approximately the same to an arbitrary constant; **NOTE:** Scaling can be done by multiplying each value by a scaling factor (a ratio) which can be determined by comparing the total average intensity of each array to an arbitrary constant.

signal – a quantity that represents the measurand and which is functionally related to it (VIM93)[7]; **NOTE:** In microarray analyses, signal is the fluorescence intensity information captured by the detection system.

target – in expression analysis, the RNA sample or gene of interest that is being assayed; **NOTE:** For microarrays, the target is labeled and hybridized to the array. In QRT-PCR, the target RNA is labeled and hybridized.

trueness (of measurement) – the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value (ISO 3534-1).[11]

4.2 Acronyms/Abbreviations

BAC	Bacterial Artificial Chromosome
BCR/ABL	The translocated ABL gene from chromosome 9 fuses with the remaining portion of the reciprocally translocated BCR gene to form a fusion oncogene BCR/ABL.
cDNA	complementary deoxyribonucleic acid
CLIA	Clinical Laboratory Improvement Amendments
Ct	cycle threshold
DNA	deoxyribonucleic acid
ERCC	External RNA Control Consortium
GLP	good laboratory practice
GMP	good manufacturing practice
ICH	International Conference on Harmonization
ISO	International Organization for Standardization
mRNA	messenger ribonucleic acid
NIST	National Institute of Standards and Technology
$OD_{260/280}$	ratio of the absorbance measured at 260 nm and 280 nm
PCR	polymerase chain reaction
Poly dT	sequence repeat containing several thymine bases
QRT-PCR	quantitative reverse transcriptase real-time PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SNP	single nucleotide polymorphism
VIM	International Vocabulary of Basic and General Terms in Metrology

5 Overview

5.1 Gene Expression Profiling Technology

5.1.1 Gene Expression Overview

Broadly speaking, the term "gene expression profiling" is used to describe experiments that measure changes in mRNA expression levels between different samples or within the same sample over time. Generally, such protocols are carried out under the assumption that measured changes in mRNA expression can be correlated with changes in physiological state or in disease status. The development of high-throughput microarrays and QRT-PCR technologies has provided rapid, accurate methods for measuring such changes. These new tools have become instrumental for basic scientific discovery, for development of new clinical diagnostic tools, and for development and characterization of new drugs and biological therapeutics.

5.1.2 Technology Platforms

5.1.2.1 QRT-PCR

Since the original descriptions of monitoring quantitative PCR in real time, a multitude of instruments and detection approaches have come onto the market place.[12-16] The technology is based on the fact that there is a quantitative relationship between the starting amount of target message and the amount of corresponding PCR product within the linear phase of the reaction for that message. Detection of the target molecules is usually accomplished by measuring changes in fluorescence proportional to the increase in the product of interest. Available instruments range from rapid cycling to high sample density plates and use a variety of detection schemes in order to quantify input target material. Higuchi et al[12] describe the measurement of fluorescence from ethidium bromide. Multiple subsequent detection methods have been developed, applied, and commercialized. The specificity of QRT-PCR, as well as the application of endogenous and exogenous standards for the method, are dramatically improved with the application of fluorescent probes.[17-19]

5.1.2.2 Microarrays

Microarray-based hybridization techniques for peptide and nucleotide applications were first described in the early 1990s.[2,3] With advances in the technology, microarray platforms became more comprehensive in their ability to monitor many genes simultaneously.[4,5] The arrays themselves are solid surfaces onto which single-stranded DNA has been chemically linked either following robotic spotting or via *in situ* synthesis techniques. Each spot of single-stranded DNA molecules represents a single gene and a single array may carry many thousands of spots. Each individual spot acts as a hybridization target for labeled cDNA added in solution to the solid matrix.

Most microarray platforms utilize either one-color or two-color technologies. In two-color approaches, the first sample of interest is labeled with a fluorescent tag of one emission spectrum, while a second sample or reference material is labeled with a distinct and discernable second fluorescent tag. The two samples are mixed in a competitive hybridization reaction, and the final analysis depends on the ratio of one color to the other for each spot on the array. In one-color detection systems, each sample of interest is labeled with a single color and hybridized to its own microarray. Final analysis depends on comparing signal intensities from one sample on one array to corresponding values from a second sample on a second array. This approach relies on manufacturing precision whereby each microarray must reliably compare to each subsequent microarray. In both platforms, changes in the abundance of a particular transcript in the input samples are detected by changes in the fluorescent intensities at its corresponding spot on the microarray(s).

5.2 Gene Expression Profiling in Clinical Applications

The use of sensitive assays that detect changes in gene expression will increase our ability to perform discriminating diagnostic and prognostic molecular tests in the clinical setting. Such technologies are already in use in some contexts, and many more applications are expected soon. For instance, the use of QRT-PCR has become standard of care for some somatic cancers, such as chronic myelogenous leukemia, where monitoring of levels of the aberrant BCR/ABL transcript is used to evaluate response to imatinib therapy and evaluate for presence of residual disease. The presence of this transcript has also been extended to evaluation of some acute leukemias.[20] Other markers, such as the IgH/BCL-2 fusion and NPM-ALK are associated with follicular lymphoma and anaplastic large cell lymphoma respectively, extending the possibility of using QRT-PCR markers for solid tumors.[21,22]

Gene expression measurements by QRT-PCR for genomic markers of immune system function have been used for the assessment of tissue rejection in kidney transplants.[23] Clinical microbiology applications include the detection and characterization of drug resistance in multiple viral strains[24,25] and bacterial,[26]

protozoan parasite,[27] and fungal[28] species. Accurate quantification of gene expression by QRT-PCR has been applied to the detection of microorganisms summarized in Table 1.

Table 1. Clinical Microbiology Applications of QRT-PCR

MICROORGANISM	REFERENCE
Francisella tularensis	McAvin et al 2004[29]
Specific genetic lineages of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks	Polin et al 2004[30]
Periodontopathic bacteria *Tannarella forsythensis* and *Fusobacterium spp.* in periodontal pockets	Suzuki et al 2004[31]
Candida albicans in concentrated oral rinse cultures	White et al 2004[32]
Shigella	Vu et al 2004[33]
Human herpesvirus 8 and Epstein-Barr virus	Friedrichs et al 2004[34]
Parvovirus mutant	Hokynar et al 2004[35]
Severe acute respiratory syndrome (SARS) coronavirus	Hourfar et al 2004[36]; Drosten et al, 2004[37]; Hui et al, 2004[38]

Adoption of QRT-PCR in clinical applications has followed platform developments from a tool for confirmatory assays for hybridization experiments,[39] to a stand-alone technology driven by the universal guidelines for assay development in QRT-PCR,[40] integration of robotic instruments into the assembly of 96- and 384-well qPCR formats,[41] and the development of miniaturized matrices preloaded with primers and probes for simultaneous QRT-PCR measurements of hundreds of gene targets.[42]

For microarrays, the long-term vision includes the identification of well-characterized molecular disease subgroups to which diagnostic and/or prognostic information is attached. Currently, promising results suggest that consistent changes in the expression patterns of a limited number of genes may be sufficient to differentiate important molecular disease subgroups. Microarray-based studies have demonstrated the potential utility of this approach in cancer diagnostics. For example, target genes have been identified that may provide important clinical information about breast cancer outcomes[43-46] and about diagnosis and prognosis in hematologic cancers[47-55] to highlight a few. Although validation and further analysis of these studies is still required, the groundwork has been set for gene expression patterns to become an integral part of patient clinical information in some situations.

In addition to molecular profiling, gene expression-based assays are beginning to play a role in clinical microbiology, as established and novel pathogens are increasingly specifically identified by their gene signatures.[56-60] The long-term vision for such studies would include the generation of microarrays or RNA-based assays that can identify multiple pathogens from a single sample with a rapid turnaround time and high degree of specificity.

RNA-based assays have great potential in clinical tests. However, stringent controls that permit the assessment of limit of detection, specificity, and accuracy of gene expression measurements for any given test are required. Controls are also necessary to compare results between technologies, samples, and laboratories. A number of recent studies examining within-platform performance have demonstrated good reproducibility and repeatability when all aspects of the system are well-controlled.[61-64]

Currently, effective controls for RNA-based assays that span platforms are lacking, limiting the clinical utility of these assays. Without them, comparability of the quality and reproducibility of data produced intralaboratory and interlaboratory, as well as results between platforms, have been misleading or uninformative.[65-67] Sources of variability affecting interpretation of data include operator performance, the characteristics of the sequences being queried, annotation differences, sample integrity, labeling methodology, length of probe and hybridization dynamics (for microarray applications), and identity of reference RNA.[68,69]

The introduction of shared, well-characterized external RNA controls should provide an additional tool for assessing cross-platform comparability.

Because of the diverse nature of the parameters affecting test performance, along with the variation intrinsic in RNA measurements and the potentially subtle changes that may be important clinical parameters, it is critical that methods be established to effectively allow such quality assessments. Without these protocols, interpretation of molecular tests becomes difficult and potentially misleading.[70] Therefore, it is critical that appropriate external RNA controls are implemented to verify technical performance as the first step toward quality management in gene expression profiling for clinical applications.

5.3 A Note on Technical Performance and Clinical Performance

This document refers to two types of performance evaluation: quantitative technical performance and qualitative clinical performance.

Technical performance refers to instrument or system verification and monitoring. It is independent of the RNA tested and does not require the presence of a clinical sample. Technical performance as measured by external RNA controls plays a role in measuring variability introduced by the instrument or system and in establishing acceptable instrument performance criteria. For example, one could verify the equivalence of different lots of reagents or reagents provided by different suppliers by monitoring technical performance with the same external RNA controls. Several *quantitative* measures of technical performance of an assay platform, such as limit of detection, precision, and linearity, can be established by measuring external RNA controls.

Clinical performance measures require the presence of a clinical RNA sample, because they refer to the specific gene or analyte being tested. External RNA enables the detection of systematic errors of different analytes on the same platform; however, external RNA controls do not give specific metrics for the clinical analyte. External RNA controls can provide valuable *qualitative* information about the test, such as the presence of enzyme inhibitors in the clinical sample or catastrophic assay failure. While clinical applications will naturally require additional, more comprehensive strategies for validation, traceability, and quality management, the external RNA controls and protocols described in this document are useful in both clinical and research settings.

6 External RNA Controls and Their Use in Expression Assays

External RNA control materials will play an essential role in developing, validating, and monitoring gene expression assays. Results of an assay where the performance established with external RNA controls is consistent with expectations are likely to be far more reliable than assays run without analytical controls. However, performance assessments made with RNA controls are not intended to extrapolate to performance assessments about particular transcripts in a sample. For this reason, external RNA controls should not be used to predict measures of selectivity/specificity or signal-to-RNA concentration calibration for particular transcripts.

The protocols developed in this document are part of a larger effort to establish standardized methods for gene expression assays. These protocols can be used to validate technical performance when analyzing clinical samples. An example of this use might be establishing performance limits for the signal that arises from a given concentration of a particular external RNA control, and monitoring that metric in patient samples.

6.1 Internal and External RNA Controls in Gene Expression Assays

Two types of external RNA controls are currently available to monitor technical performance in microarray assays.

- Unlabeled, polyadenylated transcripts are combined with RNA samples before cDNA synthesis to monitor the efficiency of target preparation. Often, the controls are a collection of transcripts with different lengths that are mixed in at different concentrations in order to reflect the complex RNA population in a true biological sample.

- Hybridization controls are labeled targets that are prepared separately and then added to the hybridization cocktail for each sample. They are generally used as a set of genes at staggered concentrations, in order to determine the limit of detection, as well as verify the quality of each hybridization.

The external RNA controls discussed in this document refer only to labeling controls. Hybridization controls are not included, as they are not germane or pertinent to QRT-PCR.

Although several sets of labeling controls are commercially available, each set uses different sequences that are microarray specific. For example, one microarray provider uses bacterial sequences for external RNA controls, while another manufacturer includes controls based on Arabidopsis sequences. Therefore, the resources and the results from these external controls are not interchangeable between platforms.

QRT-PCR assays generally rely on an internal RNA control, such as a well-characterized invariant gene. Currently, the choice of invariant gene, as well as the corresponding primers and amplicons, varies between laboratories and assays. A frequent choice of endogenous control for QRT-PCR is 18S RNA, because its quantification is an excellent approximation of the total RNA in the sample. In the specific case of the 5´ nuclease reaction fluorescent primers and probes, reagents are available to multiplex detection of 18S RNA with reproducible and high-PCR efficiency across multiple eukaryotes from yeast to human.[71] The use of external RNA controls will enable standardization by providing a common reference material. It is optimal to use both internal and external RNA controls to assess both clinical and technical parameters. Internal RNA controls aid in the normalization of analyte mass in a clinical sample, whereas external controls provide a standardized reference material for the assessment of technical performance.

6.2 Characteristics of External RNA Controls

The ERCC, an ad-hoc consortium, is developing a set of external RNA controls (available at www.nist.gov). This effort will provide agreement on which external RNA controls can be used on all microarray platforms as well as in QRT-PCR assays. The protocols and applications in this document are based upon a standardized set of external RNA controls, such as those being developed by the ERCC. These control characteristics are intended to be useful across different expression technologies and platforms. The ERCC is working to make these materials readily accessible. They are intended to have broad acceptance within the research and clinical communities.

The external RNA control set being developed by the ERCC will consist of approximately 96 well-characterized polyadenylated transcripts, comprised of random unique and nonmammalian sequences, as would be appropriate to ensure minimal cross-reactivity in mammalian clinical applications. All relevant information regarding the process of sequence selection, verification, quality control, characterization, nucleic acid sequences, recommended handling and storage, stability data, and observed performance characteristics will be published in the open, archival literature and made publicly available by the ERCC. Public availability of this information will facilitate adaptation and implementation by any interested user.

The ERCC is an international organization, and it is expected that the ERCC controls will be internationally recognized.

External RNA Controls

- 96 different RNA transcripts
- 500-2000 nucleotides long
- 20 nucleotide 3´ polyadenylation
- Well-characterized for:
  - sequence
    - integrity
  - concentration
  - source
    - random unique sequences
    - plant
    - bacterial
- Data describing controls will be publicly available in the open literature:
  - sequence
  - unique identifier
  - test and characterization methods
  - test results

Figure 2. Characteristics of ERCC Controls

6.3 Using Control Materials to Assess Technical and Clinical Performance

External RNA controls can be used in both microarray and QRT-PCR experiments. Microarrays allow simultaneous assessment of technical performance of the instrument and quality of the clinical sample. Multiplexed QRT-PCR also can be used to detect external RNA controls with specific primers and probes designed for this purpose. This application is particularly valuable for the identification of factors in the chemical matrix that may affect overall PCR efficiency.

6.3.1 External RNA Controls in Microarray Assays

Microarray applications may use two types of pools containing external RNA controls: calibration curve pools and differential comparison pool pairs. The selection of the pool type is dependent upon the final work product of the assay. If the assay produces values related to the concentrations of mRNA species in a given sample, then the calibration curve approach may be most appropriate. If, alternatively, the assay compares the levels of mRNA species in two different samples, then a differential comparison pool pair may be more appropriate. The formulation and use of these pools are described in Section 7.3.2.

6.3.1.1 Calibration Curve Pools

Calibration curve pools are used to verify the linear range of an assay. For example, a pool of external RNA controls can be added to a clinical RNA sample before target preparation. If the controls were present at different concentrations in the pool, then the expression measure (e.g., signal) for each control could be plotted against its input concentration to generate a standard curve.

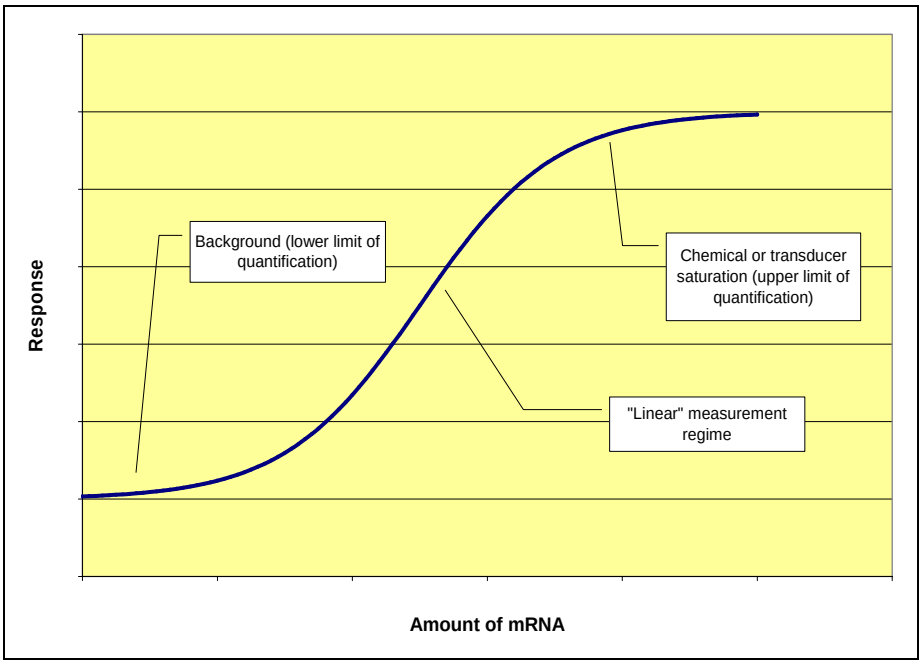

Figure 3. Example of Calibration Curve Based on External RNA Controls

As shown in Figure 3, the expected form of a calibration curve is sigmoid.

- At low concentrations, background signal dominates, so that the constant target signal does not reflect the input mRNA concentration. If background is subtracted aggressively, the signal observed at low concentration mimics the no-signal noise distribution.

- At moderate concentrations, the signal responds linearly to increases in target concentration.

- At high concentrations, saturation phenomena (either of the binding feature or the detection device) cause the signal to flatten at some maximum value.

Thus, a well-constructed calibration series can be used to determine the maximum linearity of a microarray system (see Section 6.5.1.3, and CLSI/NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*). Limits of the assay will be identified as the nonlinear sections. Poor performing assays will be apparent by compression or other changes in the standard curve. Unlike QRT-PCR calibration assays (see Section 6.3.2.1), each concentration in a microarray calibration pool is represented by a different external RNA control. This approach efficiently generates the multiple signals necessary to construct a calibration curve in a single hybridization, but it relies on different probes, with unique sequences and hybridization characteristics, to recognize each external RNA target.

6.3.1.2 Pairs of Differential Comparison Pools

Microarrays are often used to perform differential expression measurements, either by comparing two arrays hybridized to different samples (one-color experiments) or by comparing two samples hybridized to one array (two-color experiments). Differential comparison pools are used to determine the limits of differential detection in either platform. These experiments use two different pools containing the same external RNA controls at different concentrations. For example, one sample is added with a pool that contains a known concentration of an external RNA control. The second sample is added with a different

pool that contains a higher concentration of the same external RNA control. By comparing the results generated with these two samples, one can confirm that the known difference in transcript abundance between the pools is detected.

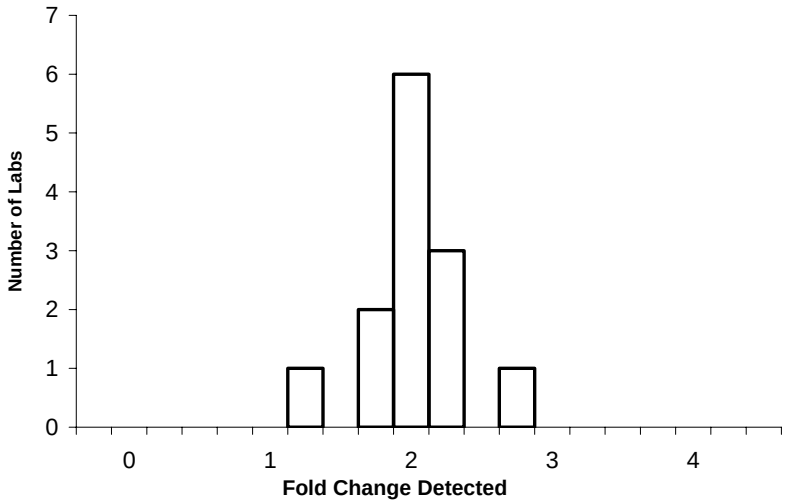

Figure 4. Example of Differential Comparisons Using External RNA Controls

An example of the use of differential comparison pools is presented in Figure 4. In this experiment, three samples with a high concentration, 1:50 000 copies, and three samples with a low concentration, 1:100 000, of the same external RNA control were distributed to multiple laboratories for target generation and hybridization. Signal values for each hybridization were extracted, scaled, and then averaged for the three replicates at each concentration. Figure 4 illustrates the distribution of the change between the high and low concentrations observed in each laboratory. The median value of all laboratories is close to the expected twofold change. It is important to note that in order to compare signals from different arrays or different color channels, comparable linearity should be observed.

6.3.2 External RNA Controls in QRT-PCR Assays

External RNA controls can be used to monitor many facets of technical performance for QRT-PCR assays. Operator competence, general assay precision, accuracy, limit of detection, assay range as well as reagent and PCR platform comparisons can be assessed with standardized RNA controls. These uses of external RNA controls do not require use of a clinical analyte but may also be multiplexed for detection in a clinical analyte.[72] An additional advantage of using a well-defined external RNA control, rather than an internal invariant gene, is that technical performance can be compared across many independent sites and QRT-PCR platforms. External RNA controls in QRT-PCR assays will be used for the evaluation of technical performance in the absence of a clinical analyte, as well as for measuring qualitative clinical performance with an analyte.

6.3.2.1 Standard Curves for Calibration

Individual external RNA controls can be serially diluted in order to provide a standard curve for instrument calibration. This protocol would be carried out using enzyme and buffer reagents compatible with the intended clinical assays, but without including actual clinical samples. As with microarray calibration curves (see Section 6.3.1.1), the expression measure (e.g., Ct) for each sample would be plotted against its input concentration. The calibration curves generated could be used for verifying instrument and reagent performance, as well as competency testing and training.

6.3.2.2 Clinical Performance Evaluation

Individual external RNA controls are selected at a concentration relevant to the concentration of the clinical analyte. This external RNA control is added to each clinical sample and serves to monitor assay performance. The efficiency of amplification of the external RNA control will be similar to that of the clinical analyte. The use of external RNA controls for the assessment of QRT-PCR clinical performance generally utilizes a single control molecule added at a constant concentration to all samples. Diminished amplification efficiency of an external RNA control in a particular sample is a useful indicator of inhibitory effects. In the cases of multiplex instrumentation and multiplex assay design, it is feasible to consider the possible use of at least two different external RNA controls (see Section 7.4.4).

6.4 Limitations of External Controls

Microarrays are complicated by the multiplex nature of the assay, as thousands of transcript levels can be measured simultaneously. Random noise will result in inaccuracies in the individual measurements for some transcripts. The external controls will provide information on the overall quality of the sample labeling and hybridization for that microarray, but not for each individual transcript on the microarray.

In QRT-PCR experiments, external controls added to input RNA do not reflect gene expression levels of that sample. Therefore, quantifying gene levels in clinical samples will still require the use of internal controls specific for the assay design. External controls do not reflect internal gene expression levels of a sample. External controls are used in QRT-PCR to identify matrix effects capable of reducing PCR efficiency and assay limit of detection.

6.4.1 Evaluating Gene Specificity

The ratio of homologous-to-heterologous hybridization for a hybridizing nucleic acid with, for example, 10% mismatches, reveals the level of gene specificity inherent in that probe sequence. Gene specificity is usually tested by hybridizing either a series of gene family members, or a series of oligonucleotides with progressive sequence changes, to an array bearing probe(s) representing that transcript. Gene specificity performance of individual probes is described as the ratio of homologous to heterologous hybridization for a series of nucleic acids with progressively larger percent mismatches. The set of ERCC RNA molecules do not systematically address specificity related to any one gene sequence.

6.4.2 Normalization or Scaling of Microarray Data

Datasets from two or more microarrays, or from two channels in two-color microarray formats are usually adjusted to account for differences in limit of detection between arrays or channels. This normalization or scaling can be done by multiplying expression levels from one array or channel by an adjustment factor such that the mean or median level of hybridization for all arrays or channels is the same. While it is possible to normalize array data by matching the mean or median of a set of RNA controls, this approach is problematic for several reasons. First, the overall mRNA level in eukaryotic tissues is subject to a very robust homeostasis—the level of mRNA per cell does not change perceptibly when expression of subsets of genes are induced and repressed. It is likely to be much less true to assume that the levels of 10 to 100 external RNA controls are identical between samples. Second, the normalization to external RNA controls would not necessarily reflect differences in labeling efficiencies between samples, for example, errors due to inexact pipetting. Third, normalization using hundreds or thousands of genes within the linear range of response of the assay is mathematically more robust than using a small number of external RNA controls. For these reasons, it is recommended that normalization or scaling of microarray data be performed based on many genes intrinsic to the biological samples.

6.4.3 Determining Absolute mRNA Levels

External RNA controls are quite useful to determine linearity of hybridization response, and an approximate limit of detection for hybridization for a given platform. It is not reasonable, however, to extrapolate from a standard curve of 10 to 100 external RNA controls to determine absolute abundances of other intrinsic gene transcripts. This limitation is because probes hybridize to cognate sequences with great variability and unpredictability. While physical chemists have generated a long list of rules to predict which DNA or RNA sequences will not hybridize effectively, our ability to use these predictions at the level of individual oligonucleotide is not well-established. Determination of absolute transcript levels is best done by adding exogenous RNA from the gene in question into hybridizations, or into quantitative QRT-PCR reactions in a standard curve.

6.5 Metrics for Assessing Technical Performance of the Expression Platform

Gene expression technologies were initially used for research purposes, and are now beginning to be used in clinical applications. Because clinical laboratories require a high degree of rigor and validated performance, this transition brings up many questions on the performance of expression assays and whether they are appropriate for clinical use.

Well-designed experiments that include appropriate external controls will help alleviate fears and demonstrate the quality of expression measurements. External RNA controls can be used in a number of ways to verify the technical performance of an assay. In a clean system (buffer), the limit of detection, precision, and linearity of the measurement of the external controls can be determined by replicate assays under defined assay conditions. These results can be compared to similar experiments conducted with external RNA controls added into typical samples. The degree to which the results in the clean system are similar to the results in the typical samples is an indicator of the assay's resistance to sample interference effects or the matrix effects of the test material.

6.5.1 Metrics

Explanation of the following technical performance metrics is helpful in order to best assess the use of external RNA controls in gene expression assays.

6.5.1.1 Precision

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.[11] Intermediate precision refers to the closeness of agreement between interlaboratory results. Note that particular sets of extremes in variation of conditions are termed repeatability (low-varying) and reproducibility (high-varying). The replicate measures can address intralaboratory variability under repeatability conditions, for instance the same RNA sample assayed in the same laboratory on different days, and interlaboratory variability under reproducibility conditions, for instance, the same RNA sample assayed in different laboratories (see the most current edition of CLSI/NCCLS document EP5—*Evaluation of Precision Performance of Quantitative Measurement Methods*).

The external controls can be used as large pools of consistent, known composition that can be repeatedly analyzed in one or many laboratories. The precision of these results can be evaluated as the standard deviation or the coefficient of variation in Ct values.

For microarrays, an intra-array precision metric may also be generated when multiple spots of the same probe are placed at different locations on the same array. Quantitative measures of precision depend critically on the stipulated conditions. In an ideal situation, precision can be assessed by testing replicates of a panel of patient samples. External RNA controls are valuable in evaluating precision in situations

where relevant patient samples are limited in quantity or number and cannot be pooled (i.e., lung biopsy specimens).

The precision of external RNA control amplification and detection may be affected by the QRT-PCR conditions and cycling parameters used for the target RNA. Universal primer and probe design guidelines, amplification (thermocycling) protocols, primer and probe concentrations, and master mix formulations recommended by developers of QRT-PCR platforms are essential to obtain precision values according to the specifications of the platform.

6.5.1.2 Accuracy

Accuracy is the closeness of agreement between the average value obtained from a test result and an accepted value.

NOTE: The term accuracy, when applied to a set of test results, involves a combination of random components and a common systematic error or bias component.[11]

External RNA controls cannot be used to measure accuracy for a particular clinical analyte. Technical performance for a particular assay might be established by comparing the signal for a single control transcript to that established as a reference for that transcript when a method is commissioned and validated in a laboratory.

Demonstration of accuracy is a definitive requirement for quantitative diagnostic tests in which an actual analyte level is determined by the assay. For nonquantitative assays, the term "accuracy" as defined above is inappropriate. For RNA expression assays that are based on pattern information, the positive and negative predictive values of expression patterns will serve as measures of how effectively they detect disease occurrence and susceptibility. The accuracy of the estimated change or Log Ratio expression measures can be evaluated by introducing different differential comparison pools of external RNA controls into samples being compared. Latin square assays have demonstrated that multiple microarray platforms generate accurate results within a defined linear region.

For QRT-PCR, if there is a standard curve or an algorithm for calculating concentration for the assay, then the value obtained for the external RNA control in the QRT-PCR can be compared to the assigned value of the external RNA control to assess accuracy. This result is valid for the control and can also extend to sample RNAs by multiplex detection of external controls spiked throughout multiple steps in the sample isolation, purification, and conversion to cDNA.

6.5.1.3 Linearity

Linearity is the ability within a given range to provide results that are directly proportional to the concentration of the analyte in the test sample; **NOTES:** a) Linearity typically refers to overall system response (i.e., the final analytical answer rather than the raw instrument output); b) The linearity of a system is measured by testing levels of an analyte which are known by formulation or known *relative to each other* (not necessarily known absolutely); when the system results are plotted against these values, the degree to which the plotted curve conforms to a straight line is a measure of system linearity (see the most current edition of CLSI/NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*).

In microarray analysis, linearity refers to the relationship between hybridization signals and known or relative RNA concentrations whereby the log-log plot of the signal generated from a serial dilution of well-characterized and quantified control samples correlates linearly with the known concentrations of those samples.[5] The linear range of an assay is the set of detectable signal intensities that correlate in a linear fashion with known concentrations of the controls and/or analytes. This metric is generally reported

as the slope and intercept of a least squares regression that compares observed amounts to observed signal intensities of known transcripts in the test samples.

The external controls and their corresponding microarray probe or QRT-PCR primer sets will be selected based on their ability to maintain linearity within a specified range of concentrations. An external control RNA pool would be designed to contain multiple controls each at a different concentration within that defined linear range. Although different, the linearity of results for each expression experiment can be confirmed using the collection of relative Ct values for given sets of external controls.

6.5.1.4 Analytical Cross-Reactivity

Measurement of cross-reactivity refers to evaluation of the level of nonspecific binding of control and/or test probes to nontargeted analytes that may be present in samples. Before external RNA controls are chosen for a specific gene expression assay, they should be screened for cross-reactivity to the test probes of the assay (see Section 7.2).

6.5.1.5 Limit of Detection

Limit of detection, is defined as the lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value.[9] **NOTE:** Also called "lower limit of detection," "minimum detectable concentration" (or dose or value), and sometimes used to indicate "sensitivity."

Typically in diagnostic assays, the limit of detection is determined for each analyte being assayed. However, in the case of expression microarrays, it is not realistic to determine the limit of detection of each analyte being probed. In these cases, external RNA controls could be used to determine the limit of detection of a series of representative RNA transcripts on a given platform.

An external RNA control is not useful in defining the limit of detection for any RNA other than itself. Since the RNA control value will be tied to a NIST standard, it can be diluted serially and, for a given set of QRT-PCR conditions, a limit of detection can be determined for the control.

7 Recommended Practices/Protocols

7.1 Overview

The following protocols address the use of external RNA controls in gene expression assays. Diagnostic kit manufacturers may supply external RNA controls that have equivalent performance with the controls described in this document. The use of a set of controls developed by the diagnostic manufacturer could be used in place of the external controls described in this guideline provided the controls are verified and allow technical performance assessment as described here. Section 7.2 includes protocols that apply to both microarrays and QRT-PCR. Sections 7.3 and 7.4 deal with protocols specific to microarrays and QRT-PCR, respectively.

As described in Section 5.3, the external RNA controls can provide quantitative technical performance information and qualitative clinical performance information in gene expression assays. It is important to note that the external RNA controls cannot be used to extrapolate quantitative information about specific clinical analytes.

7.1.1 Scope and Purpose

The full benefits from use of high-quality, well-characterized external RNA controls will be obtained by taking steps to ensure that these controls are used in a well-controlled, well-documented, reproducible

fashion. Several important steps should be taken. Perhaps the most important step is to utilize complete protocols and to record any changes to the protocol during performance of any specific experiment. Written records including protocols and any deviations from the routine protocols are important to ensure that reliability of specific data sets can be assessed. Another important step would be to ensure that any equipment required for making measurements with controls is operating correctly. This can be achieved by performing routine maintenance and calibration of equipment, such as arranged under service contracts. A third important step is to make sure that equipment operators are properly trained and that they routinely keep accurate records regarding instrument service, calibration, and performance, in addition to the experimental data. Finally, algorithms and software used in the analysis and interpretation of data should be controlled and validated. These general principles form the foundation for generating reliable data. More complete discussions of these practices can be found in applicable GLP, GMP, ISO, ICH, and CLIA documents.[73-80]

7.2 Protocols for Preparation and Assessment of External RNA Controls

7.2.1 Synthesis of the External RNA Controls

External RNA controls are generally prepared by *in vitro* transcription of a suitable DNA template, using a phage RNA polymerase, such as T7, T3, or SP6. Optimized kits for performing such transcriptions are available from several manufacturers. The DNA template is produced from either a linearized plasmid construct, or from a template generated by PCR. Most microarray labeling systems take advantage of the presence of a polyadenylated tail on eukaryotic mRNAs. Therefore, external RNA controls are designed to contain artificial polyadenylated tails. In the following discussion of DNA template sources, it is assumed that the template will be produced with a 3′ polyadenylated tail.

Plasmid constructs can be built using any of a number of base plasmids that place a polycloning site between a phage promoter and a poly-A/T region (e.g., pSP64polyA+). Templates can also be built via PCR, by adding 5′ extensions to the primers that result in properly located phage promoter and poly-A/T regions in the final construct. In either case, care must be taken to ensure that the correct sequence direction (sense or antisense) is achieved after *in vitro* transcription. After preparation, external RNA controls should be cleaned according to the kit manufacturer's specifications and assessed for purity and quantity via spectrophotometric methods (see Section 7.2.2).

7.2.2 Quantification and Integrity Confirmation

External RNA controls are best quantified via UV-visible spectroscopy. Mass concentrations of RNA can be estimated using the formula[81]:

Yield of RNA (μg/ml)=(OD_{260})(44)(dilution factor),

and purity can be estimated using:

(OD_{260})/(OD_{280}) ratio (~2.0 for pure RNA).

The ERCC external RNA controls vary in length between 500 and 2000 nucleotides. After quantification, it is important to account for differences in molecular weights of the external RNA controls, because most RNA quantification platforms measure the molar concentration of the mRNA species. The mass concentration produced by the Maniatis formula can be converted into a molar concentration by dividing by the mRNA molecular weight. Alternatively (and more accurately), the molar extinction coefficient for a particular RNA species can be calculated, using standard procedures. Several web-based implementations of these procedures are available, such as that hosted by the Dana-Farber Cancer Institute, found at http://mbcf.dfci.harvard.edu/docs/oligocalc.html.

It is optimal to use full-length external mRNA controls as determined semiquantitatively via gel electrophoresis under RNA-denaturing conditions, or quantitatively via capillary electrophoresis.[82-84]

7.2.3 Range of Concentrations of External RNA Controls

The amount of external RNA controls added to a clinical sample should be guided by the expected range of mRNA concentrations in the sample itself. Generally, the linear range detected by microarray assays is 10^4 and for QRT-PCR assays is ~10^9. For microarray assays, the amount of external RNA control sample added to a clinical sample should be adjusted to span a nominal range from less than 1 mRNA copy per cell to greater than 1000 per cell. This range should be expanded by approximately two orders of magnitude at each end for QRT-PCR methods (i.e., 0.01 mRNA copy per cell to 100 000 mRNAs per cell).

Given generally accepted estimates of the amount of total RNA per cell and the fraction of total RNA that is mRNA, this becomes a calculation of the ratio of external RNA control to total RNA, based on the following assumptions:

The amount of RNA per mammalian cell varies somewhat, but is approximately 26 pg RNA/cell.[85]

- The mRNA/total RNA ratio varies considerably by cell or tissue. Generally reported estimates of mRNA content vary from 1% to 4%. If we rely on an average of 2%, then each cell contains approximately 520 fg mRNA (2% of 26 pg).

- An average mRNA molecule is 1.7 kb (5.6×10^5 g/mol).

Based on these estimates, an average mammalian cell contains 560 000 mRNA molecules. A detailed discussion of these points can be found in the ERCC draft specification available at: www.cstl.nist.gov/biotech/workshops/ERCC2003/031030%20ERCC%20Spec.pdf.

Calculations in this guidance document assume a clinical sample contains 10^5 cells (5.6×10^{10} mRNA molecules). Calculations must be scaled to account for the actual number of cells in a particular clinical sample type. Alternatively (and preferably), the quantity of RNA isolated should be measured prior to addition of external RNA controls, and a constant ratio of added external RNA control to isolated RNA should be maintained.

If the external RNA controls are chosen to have molecular weights near the mammalian average, then this implies a spiking ratio of about 1:500 000 (for polyadenylated RNA) or 1:25 000 000 (for total RNA) to achieve a level equivalent to ~1 copy/cell. The rest of the external RNA control concentration series should then be expanded about this level, to enclose the range of limit of detection of the method used as well as the expected signal range from the clinical sample. Based on these assumptions, the copy number of external RNA controls added to a microarray sample will vary from 10^4 to 10^8, and for QRT-PCR up to 10^9.

In cases where samples consist of cell suspensions, direct counts of cells can be used to determine a spiking ratio.

7.2.4 Testing Controls for Analytical Cross-Reactivity

It is critical to understand the level of nonspecific binding of a control and/or test probes to nontargeted analytes that may be present in samples. Each control can be individually hybridized to the microarray (or equivalent) at 10 times the concentration required in the assay to detect and measure cross-reactivity. Once control probes are selected, they can be tested for cross-reactivity to representative patient samples,

including each tissue type to be assayed. Similarly, external RNA controls should not amplify with the primers and probes used for the sample RNA(s) to be measured in a QRT-PCR assay.

7.2.5 Performance Testing

A solution of external RNA controls may contain contaminants that inhibit efficient amplification and/or labeling. It is therefore important that some assessment of performance be made. This performance testing should be completed before formulation of pools because a labeling inhibitor present in one control RNA can potentially compromise the performance of all of the species present in a mixture. Quality control testing of external RNA controls can be performed via QRT-PCR or on a microarray designed to detect the external controls. One effective method for testing performance is to prepare serial dilutions and evaluate hybridization signal intensity linearity (see Section 6.5.1.3 and CLSI/NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*).

7.2.6 Storage

For optimal preservation, minimize freeze-thaw cycles and keep polyadenylated transcripts of the external RNA controls stored in concentrated aliquots at -80 °C or in liquid nitrogen. Stability of the stored external RNA controls should be evaluated prior to use.

7.3 External RNA Control Protocols for Microarrays

Microarrays allow simultaneous analysis of thousands of transcripts in one hybridization. Pools of external RNA controls, rather than individual control transcripts, can be used in microarray assays. This feature enables monitoring of technical performance and qualitative clinical performance in clinical assays. For optimal benefit, these pooled controls should be included in every assay, in multiple concentrations.

7.3.1 A Comment on Probe Design

Probe design will be performed by the array manufacturer, using their best methods.[86] Access to probe sequence information enables unambiguous interpretation of data. The ERCC will publish information regarding probes used in performance testing of external RNA controls.

7.3.2 Pooling External RNA Controls

After synthesis, quantification, and performance testing of the controls, the individual external RNA controls are combined into two different kinds of pools. Below is a general method for calculating the necessary volume of each external RNA control in a pool or mixture, followed by examples of pool compositions for the two different applications.

7.3.2.1 General Pooling Methods

During the creation of the pools, it is important to remember the following:

- All RNA transcripts must pass integrity and stability QC before mixing.
- Stocks of each RNA transcript should be 1000 to 500 ng/µL.
- A260 measurements should be done on RNA that is relatively similar in stock concentration (i.e., do not compare 1200 ng/µL with 23 ng/µL).

- The total RNA concentrations of the pooled RNA should be ~100 ng/µL.

- At concentrations of 100 ng/µL and below, RNA binding to plastic tubes can be problematic; therefore inclusion of carrier nucleic acid or use of silanized tubes is recommended. Yeast tRNA has been successfully used as a carrier at 10 ng/µL.

- Stocks should be dissolved in one of the storage buffers listed below. Long-term stocks should be stored at -80 °C. In citrate buffer, -20 °C is adequate.

- Stocks should not be freeze/thawed more than ten times. Create stocks useful to avoid multiple freeze/thaws. Aliquot into several tubes.

- To ensure that the external RNA controls are completely dissolved, the solution should be thoroughly vortexed and warmed to 25 to 37 °C.

- Because all external RNA controls are stored as concentrated stocks, dilution will be required. It is best to keep serial dilutions to a minimum of three to four steps. During dilutions, transfers of more than 2 µL should be used. Dilutions should not be stored. Working concentration should be used immediately.

- Good record keeping during pooling is essential for identification and correction of errors. Label all tubes with names, dates, concentrations, and user ID. Make sure notebook or production logs are in agreement with all labels.

- Confirm RNA concentrations after long-term storage. This should not fluctuate more than 10%. Be sure to vortex and heat prior to measurement.

- For quality control, the composition and integrity of each pool can be checked by capillary electrophoresis, or microfluidic methods are recommended for QC of RNA spikes and mixtures. Functional QC should be performed with QRT-PCR, or hybridization to a microarray.

Recommended Storage Buffers

- 1 mM sodium citrate, pH 6.4 +/- 0.2

- 0.1 mM EDTA: in nuclease-free, ultrapure water

- TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.0

7.3.2.2 Calculating Transcript Volumes

Proper laboratory procedure dictates that RNA pools should be constructed via a series of prepools in order to minimize pipetting errors. However, it is also important to place consistency checks on the pre-pooling scheme in order to ensure that the proper amount of each external RNA control is in the final pool. The scheme below outlines a method for directly calculating the volume of each RNA stock present in the final pool, given the initial stock concentration and the desired final concentration in the pool. The actual procedure for pool construction should be cross-checked against this calculation as a quality assurance step.

The final concentration of the i^{th} of N control RNA species in a mixture, $C_i^{(f)}$, can be calculated from the formula:

$$C_i^{(f)} = \frac{c_i V_i}{V_W + \sum_{j=1}^{N} V_j} ,$$

where c_i is the stock concentration of a given mRNA species, V_j is the volume of that stock added to the final formulation, and V_W is the extra volume of water or buffer added to the formulation. Note that this formula works for either mole- or mass-based concentrations.

Generally, the formulator is interested in the mole- or mass-fraction χ_i of the i^{th} mRNA species in the final mixture. This is given by the relationship

$$\chi_i = \frac{c_i V_i}{\sum_{j=1}^{N} c_j V_j} .$$

The above formula can be rearranged into a system of N simultaneous equations for the volumes V_j:

$$0 = \left[\left(\frac{\chi_i}{c_i} \right) \sum_{j=1}^{N} c_j V_j \right] - V_i .$$

This system is generally solved by volumes of the form

$$V_i = \frac{A \chi_i}{c_i} ,$$

where A is an arbitrary constant that is related to the total volume (V_T) of the final mixture by the equation

$$A = \frac{V_T - V_W}{\sum_{j=1}^{N} \frac{\chi_j}{c_j}} .$$

Consider, for example, the set of mole fractions shown in Table 2 (corresponding to the "copies" in Table 3; the mole fractions were calculated by dividing each value of "copies" by the sum of all values of "copies"):

Table 2. Example of Formal Pool Composition

Control ID	Fraction (χ)	Stock Concentration (pM)	Volume (µL)
Control 1	0.00	100	0.00
Control 2	6.84×10^{-5}	100	0.68
Control 3	2.16×10^{-4}	100	2.16
Control 4	6.84×10^{-4}	100	6.84
Control 5	2.16×10^{-3}	100	21.64
Control 6	6.84×10^{-3}	1000	6.84
Control 7	2.16×10^{-2}	1000	21.64
Control 8	6.84×10^{-2}	1000	68.37
Control 9	2.16×10^{-1}	1000	216.35
Control 10	6.84×10^{-1}	1000	683.68

In the above example, the constant A is 1 $pmole^{-1,}$ and V_W is zero. In practice, one might choose to form several subpools, and mix the subpools to achieve the desired final concentrations. However, a subsequent formal calculation of the volume of each stock in the final mixture should correspond to Table 2.

7.3.2.3 Example Composition of a Calibration Pool

Calibration curves are usually presented on a logarithmic scale, so series based on successive doublings or half-log increases (3.16 X) of concentration are most effective. The following table illustrates a series of ten controls that span a detection range for microarray experiments from 10^5 to 10^8 copies per cell (as described in Section 7.2.3).

Table 3. Example of Calibration Pool Composition

External RNA Control	Pool A (# copies)
Control 1	0
Control 2	3.16×10^4
Control 3	10^5
Control 4	3.16×10^5
Control 5	10^6
Control 6	3.16×10^6
Control 7	10^7
Control 8	3.16×10^7
Control 9	10^8
Control 10	3.16×10^8

7.3.2.4 Example Composition of a Differential Comparison Pool

Complete calibration of a differential expression system should evaluate spans of ratios of the same external control between samples, as well as ratios of different external controls within the same sample. This requires a minimum of two samples, with multiple external RNA controls at each concentration, in order to span a range of differential expression ratios. The following table illustrates the composition of two pools that could be used to detect differential expression. Note that the same ratio is evaluated using controls at different amounts. Depending upon application, a larger linear range in ratio space may be required. For example, some sets of differential pools may examine expression ratios representing 1:2, 1:10, 1:50, 1:100, and so on. It is left to the user to determine the most informative range of ratios for any specific intended use.

Table 4. Example of Differential Comparison Pool Composition

External RNA Control	Pool B (# copies)	Pool C (# copies)	Ratio B:C
Control 11	0	0	---
Control 12	3.16×10^4	3.16×10^4	1:1
Control 13	10^5	10^5	1:1
Control 14	3.16×10^5	3.16×10^5	1:1
Control 15	10^6	10^6	1:1
Control 16	3.16×10^6	3.16×10^6	1:1
Control 17	10^7	10^7	1:1
Control 18	3.16×10^7	3.16×10^7	1:1
Control 19	10^8	10^8	1:1
Control 20	3.16×10^8	3.16×10^8	1:1
Control 21	3.16×10^4	1.05×10^4	3:1
Control 22	10^5	3.33×10^4	3:1
Control 23	3.16×10^5	1.05×10^5	3:1
Control 24	10^6	3.33×10^5	3:1
Control 25	3.16×10^6	1.05×10^6	3:1
Control 26	10^7	3.33×10^6	3:1
Control 27	3.16×10^7	1.05×10^7	3:1
Control 28	10^8	3.33×10^7	3:1
Control 29	3.16×10^8	1.05×10^8	3:1
Control 30	1.05×10^4	3.16×10^4	1:3
Control 31	3.33×10^4	10^5	1:3
Control 32	1.05×10^5	3.16×10^5	1:3
Control 33	3.33×10^5	10^6	1:3
Control 34	1.05×10^6	3.16×10^6	1:3
Control 35	3.33×10^6	10^7	1:3
Control 36	1.05×10^7	3.16×10^7	1:3
Control 37	3.33×10^7	10^8	1:3
Control 38	1.05×10^8	3.16×10^8	1:3

7.3.3 Experimental Design

The external RNA controls are added to the samples prior to labeling (see Figure 1). A variety of options exist for optimizing the use of external RNA controls in one- and two-color assays (see Table 5). As shown in Table 5 below, multiple pools can be used in the same sample, provided they contain different sets of external RNA controls.

Table 5. Experimental Design Scenarios

Platform	Sample	External RNA Control	Application
One-color	Tumor 1 Tumor 2 Tumor 3	Pool A Pool A Pool A	Calibration/linear range Technical performance Qualitative clinical performance
One-color	Tumor 1 Normal 1	Pool B Pool C	Differential expression range Technical performance Qualitative clinical performance
One-color	Tumor 1 Tumor 2	Pool A + Pool B Pool A + Pool C	Calibration/linear range Differential expression range Technical performance Qualitative clinical performance
One-color	Tumor 1 Normal 1	Pool A + Pool B Pool A + Pool C	Calibration/linear range Differential expression range Technical performance Qualitative clinical performance
Two-color	Tumor 1-fluor 1 Reference-fluor 2	Pool A Pool A	Calibration/linear range Technical performance Qualitative clinical performance
Two-color	Tumor 1-fluor 1 Reference-fluor 2	Pool B Pool C	Differential expression range Technical performance Qualitative clinical performance
Two-color	Tumor 1-fluor 1 Normal 1-fluor 2	Pool B Pool C	Differential expression range Technical performance Qualitative clinical performance
Two-color	Tumor 1-fluor 1 Tumor 2-fluor 2	Pool A + Pool B Pool A + Pool C	Calibration/linear range Differential expression range Technical performance Qualitative clinical performance
Two-color	Tumor 1-fluor 1 Reference-fluor 2	Pool A + Pool B Pool A + Pool C	Calibration/linear range Differential expression range Technical performance Qualitative clinical performance

7.3.4 Data Preprocessing

7.3.4.1 A Comment on Scaling and Normalization Models

Comparisons between different one-color arrays or between channels of two-color arrays are usually preceded by data processing steps called scaling and normalization. Scaling is the operation of centering each data set to an arbitrary constant. Normalization is the operation of removing experimental artifacts of nonbiological origin such as minor differences in labeling efficiency or scanner settings. A variety of scaling and normalization methods exist.[87-89]

Also, another useful resource for normalization is the Normalization Working Group of the Microarray Gene Expression Data (MGED) organization (http://www.mged.org), which is attempting to define such a standard[90] and have been described in the most current edition of CLSI document MM12—*Diagnostic Nucleic Acid Microarrays*.

An important potential use of external RNA controls is to test the appropriateness of the assumptions underlying scaling and normalization algorithms. If the assumptions are not valid, then the signal intensity differences observed using a pair of differential comparison pools will be skewed. Likewise, the normalized level of signal will vary between samples containing calibration curve pools.

7.4 External RNA Control Protocols for QRT-PCR

QRT-PCR enables quantitative analysis of template concentration. External RNA controls can be used for assessment of technical and clinical assay performance. Generally, technological limitations dictate that one or a few external RNA controls would be used for these purposes. QRT-PCR instrumentation is limited to detection of one or a few (e.g., four) simultaneous targets per reaction tube.

7.4.1 General Use

Two types of QRT-PCR detection technologies exist:

- Single-color DNA-binding dyes (e.g., SYBR Green probe)
  This method permits detection of a single target per assay tube.

- Target-specific, fluorescent hybridization probe(s)
  This method permits detection of several targets per assay tube.

7.4.2 Primer and Amplicon Design and Optimization

As with microarray probes, QRT-PCR primer design and assay optimization are the responsibility of individual investigators. The term amplicon refers to the product sequence that is specifically amplified during PCR.

Primer and probe design are some of the most critical assay components necessary for optimal performance of an assay. Multiple commercial and shareware software are available to assist in primer and probe design. Instrument providers can also provide a source of recommendations for effective primer and probe design. Briefly, several factors to consider for primer, probe, and amplicon design include:

- Amplicon lengths should not exceed 150 bp.

- Dissociation temperatures (T_M) for primers and probes must correspond to specifications associated with annealing temperatures and the chemical matrix of the master mix. In the specific case of the master mix, recommended parameters are:

  Annealing Temperature: 60 ^oC
  T_M for primers: 58 to 60 ^oC
  T_M for 5´ nuclease reaction fluorescent probes: 65 to 67 ^oC

- Primers and probes should be designed to minimize secondary structures.[91]

PCR efficiency can be assessed by analysis of serial dilutions of the various analyte targets. Such dilutions should produce similar slopes for all intended analytes when plotted as semilog plots (cycle threshold (Ct) versus analyte concentration). During assay development, the acceptable variance of slopes should be determined using sound statistical practices and replicate assays.[92-96]

The specific external RNA control(s), primers, and amplicons for the control should be designed following appropriate specifications in order to match PCR efficiencies with intended assay targets.

7.4.3 External RNA Controls to Assess Technical Performance

7.4.3.1 Use of a Single Concentration of an External RNA Control

A large master reaction mix containing all reaction components and an external RNA control at a single concentration can be made. Select a concentration of the external RNA control so that it will be detected within the known linear assay range. If the control concentration is too low, the variance within replicates will be unacceptable. This master mix can be equally distributed to all wells/tubes of an instrument and tested for replicate precision. This type of analysis yields valuable insight into the performance of the instrument and assay. Calculations can be made to determine the precision of replicate measurements and the associated variance. Additionally, the measured variance can help assess realistic limits of discrimination. If, for example, replicate variance of an optimized external RNA control assay approaches twofold within such a replicate assay, twofold discrimination of clinical analytes would not be achievable. Such error could be cause for requesting instrument calibration from the appropriate service organization.

7.4.3.2 Use of a Serial Dilution of External RNA Controls

An optimized QRT-PCR assay designed to detect and measure an external RNA control can be used to train and test assay operators. Replicate serial dilutions can be periodically performed and assessment of replicate slopes and individual control dilutions can be used to determine operator and assay performance levels. Instruments can also be compared using such an experimental design scheme. The minimal molecular amounts represented in dilutions that are successfully discriminated can serve as a general guide to technical capabilities of change discrimination. It is important to avoid over-interpretation of quantitative information obtained from the external controls. External RNA controls serve as a general guide but not an absolute measure of the quantities of clinical target analytes.

7.4.3.3 Use of a Serial Dilution of an External RNA Control for a QRT-PCR Calibration Standard

An appropriate serial dilution of a selected RNA control can be made that reflects the molecular range of intended clinical measurements (see Section 7.3.2 for details on molecular-based formulation). Dilutions should be made fresh from a concentrated stock of external RNA controls. Dilutions should not be used after extended periods of storage. Ideally, concentrations will be selected that range from less than single molecule detection to beyond the upper suspected limit of clinical analyte intended to be measured. The actual dilution factor can be determined by the requirements for assay accuracy, instrument platform (96-well versus 384-well), and of the laboratory. Minimally, at least four dilutions should be made in order to generate adequate data for linear regression analysis.

7.4.4 Use of External RNA Controls to Assess QRT-PCR Clinical Performance

As described above (see Section 6.3), the use of external RNA controls for assessment of QRT-PCR clinical performance will generally utilize a single control molecule added at a constant concentration to all samples. In the case that multiplex instrumentation and multiplex assay design are used, it is feasible to consider the possible use of at least two different external RNA controls.

7.4.4.1 Use of External RNA Controls to Assess Effects of Complex Biological Matrices and Methods of Clinical RNA Extraction

Use of a satisfactory quality control result as an indicator of a valid patient test result requires that the external RNA control be tested in the same mixture as the patient sample. Adding the external RNA control ensures that the control is subjected to at least some of the variables potentially affecting the patient sample during assay analysis. The expected concentration means and variances of the external RNA control can be established by repetitive analysis of this control in buffer (see the most current edition of CLSI/NCCLS document C24—*Statistical Quality Control for Quantitative Measurements:*

Principles and Definitions). The effect of adding the external RNA control to clinical RNA purified from biological matrices (e.g., tissues, cells, body fluids) can be tested next. If significant differences in measurements exist between the buffer and biologically derived RNA experiments, it could suggest reason to examine a different means of RNA purification.

7.4.4.2 Use of External RNA Controls for Assessment of Clinical Assay Results

Protocols supporting scenarios that cover the simplest single color detection systems and multicolor applications are included in this section and referred to as protocols A, B, and C. In a single color system, replicates of a sample are recommended to fully assess the relative level of expression of the target RNA (Protocol A). When multicolor chemistries are used, there are two possible scenarios for external RNA control use. The first scenario uses only a single external RNA control at a constant concentration (Protocol B). If more than three color channels are available, the choice is available to include two different external RNA controls or to use a single constant amount of an external RNA control and increase the number of target genes of interest (Protocol C).

The following protocols assume that basic assay development and assay validation are being performed for each target molecule. Parameters such as assay precision, limit of detection, accuracy, and specificity should be measured for target molecules, including the external RNA controls. External RNA controls will have been selected during assay development and validation, such that they represent a concentration with a reporting cycle threshold of detection (i.e., Ct) at or near the target genes of interest. If two controls are used, they could be selected to be within high and low assay ranges. During assay development, the efficiency of external RNA control amplification should be similar to the amplification efficiency of the targets of interest. Amplification efficiency is measured using serial dilutions of samples and controls.[94-96] Amplification efficiency can be determined from the slope of an amplification curve. The amount of product (N_c) generated in PCR after c cycles is given by $N_c=N_i\ (1+f)^c$ where N_i is the initial number of template molecules and f is the reaction efficiency. In a graph of log N_c versus c, the slope is $\log(1+f)$.[97]

In addition to an external RNA control, an invariant gene is selected and verified for normalization of clinical sample RNA mass loads, during the development and validation of QRT-PCR assays.

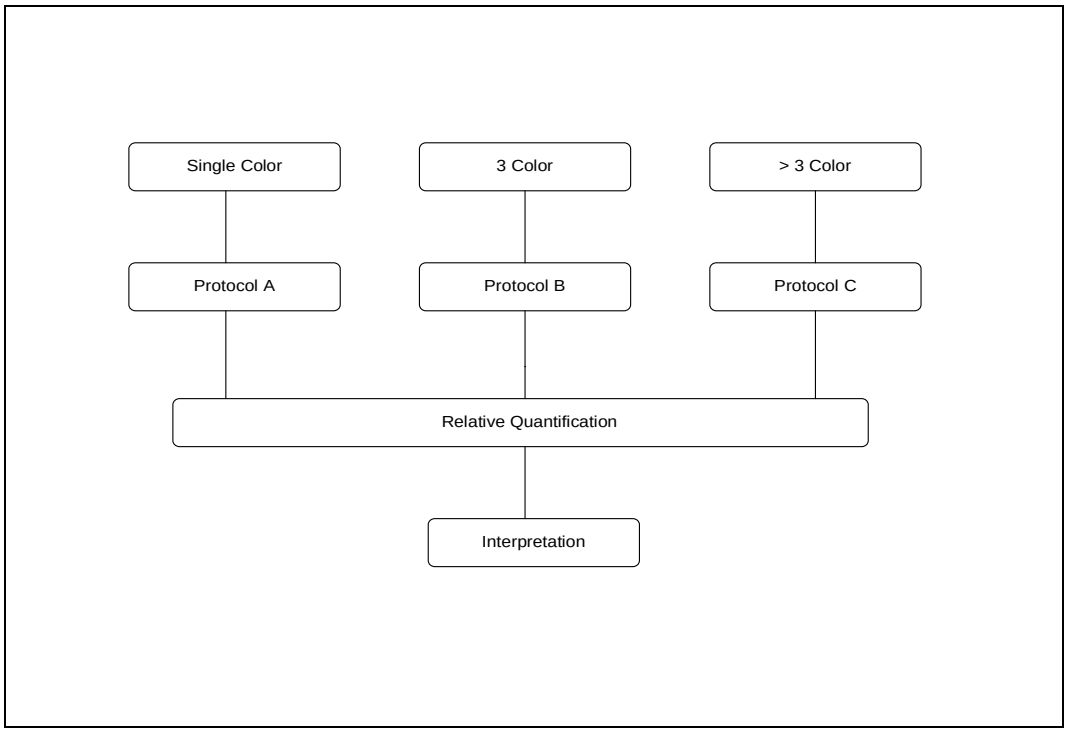

Figure 5. QRT-PCR Protocol Selection for External RNA Controls

CAUTION: Since the polymerase chain reaction yields excessive amounts of final product, it is imperative that good laboratory practice include physical separation of pre-PCR activities, reagents, and equipment from post-PCR materials. Cross-over prevention methods should be employed and negative controls should frequently be used to ensure cleanliness and accurate results. In all cases, note that QRT-PCR conditions, master mix composition, and cycling conditions have been previously developed and verified during assay development.

Protocol A. Single Color System, Replicates

A constant amount of a single external RNA control is chosen based on amplification efficiency match to target genes of interest in the sample. This external RNA control is added to every sample at constant amounts. Replicates of each sample are analyzed in different tubes. Depending on development criteria, multiple replicates may need to be analyzed for each target sequence.

1. The total number of replicates of each of the following reactions will be determined during assay development and governed by availability of clinical sample RNA: Reaction 1, external RNA control; Reaction 2, target gene of interest; Reaction 3, invariant gene endogenous to sample RNA.

   Begin by making appropriate master mixes of primer and probe combinations for each gene to be analyzed. If an external RNA control, one gene of interest, and one internal invariant gene are to be analyzed, this requires three separate mixes of primer and probes, as listed above. Next, make a basic reaction master mix. In a separate tube, pipette the appropriate amount of external RNA control for all assay replicates and add the appropriate aliquot of clinical sample RNA to the external RNA control tube. Add RNA mixture directly to basic reaction master mix. Aliquot appropriate volume of RNA and basic reaction master mix solution to each of the assay reaction replicate tubes. Using separate pipette tips, add the appropriate amount of primer and probe mixture to each assay reaction tube.

2. Perform assay and determine Ct values for each of the three reactions.

3. Compare Ct values for each set of reaction replicates.

4. Comparison of external RNA controls across different clinical samples implies that QRT-PCR reactions are valid and within limits determined during validation.

5. From target gene of interest and endogenous invariant gene, the relative amount of target RNA can be inferred.

Protocol B. Multicolor chemistry, single external RNA control at a constant concentration (three-color QRT-PCR)

1. Add the chosen amount of external RNA control into the extracted clinical sample RNA. Volumes will be chosen based on the total number of replicates to be analyzed.

2. Add the RNA mixture to a basic reaction master mix containing primer and probes for the external RNA control, target gene of interest, invariant gene, and other reaction components. Add appropriate amounts of this mixture to assay reaction tubes.

3. Perform assay and determine Ct values for each of the three above reactions.

4. Compare Ct values for each set of sample replicates.

5. Comparison of external RNA controls implies that QRT-PCR reactions are valid and within limits determined during validation.

6. From target gene of interest and endogenous invariant gene, the relative amount of target RNA can be inferred.

NOTE: This is a multiplex primer and probe assay and adequate attention should be given to optimizing such multiplex assays to prevent inaccurate results.

If the capabilities exist to use more than three colors, Protocol B can be followed and the additional color used for additional target genes of interest. Alternatively, Protocol C can be followed with the additional colors being used to assess additional external RNA controls.

Protocol C. Multicolor chemistry, two different external RNA controls or a single constant amount of external RNA control with multiple target genes (more than three-color QRT-PCR)

1. Add the chosen amount of two or more different external RNA controls into the extracted clinical sample RNA. The concentration of the different external RNA controls should be chosen to lie within the predetermined assay range (based on expected Ct of the clinical analyte), but may be chosen to detect near the lower and higher limits of this range. If a third external RNA control is used, it could be added at a midrange level.

2. Add the RNA mixture into a basic reaction master mix containing primer and probes for external RNA control, target gene of interest, invariant gene, and other reaction components. Add aliquots of this mixture into appropriate reaction replicate tubes.

3. Perform assay and determine Ct values for each of the three above reactions.

4. Compare Ct values for each set of sample replicates.

5. Comparison of external RNA controls implies that QRT-PCR reactions are valid and within limits determined during validation.

   If more than one external RNA control has been used and has been added at high, low, and intermediate predetermined amounts, the analysis of these external RNA controls in each sample can permit one to infer that the test performed within the expected linear range of the assay.

6. From target gene of interest and endogenous invariant gene, the relative amount of target RNA can be inferred.

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The Quality System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality management system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any healthcare service's path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

MM16-P addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X C24 EP5 EP6						M29

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.*

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

MM16-P addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Preexamination				**Examination**			**Postexamination**	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
		MM12	MM12	MM12	MM12	MM12	MM12	MM12

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.*

Related CLSI/NCCLS Publications*

C24-A2 **Statistical Quality Control for Quantitative Measurements: Principles and Definitions; Approved Guideline—Second Edition (1999).** This guideline provides definitions of analytical intervals, planning of quality control procedures, and guidance for quality control applications.

EP5-A2 **Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Second Edition (2004).** This document provides guidance for designing an experiment to evaluate the precision performance of quantitative measurement methods; recommendations on comparing the resulting precision estimates with manufacturers' precision performance claims and determining when such comparisons are valid; as well as manufacturers' guidelines for establishing claims.

EP6-A **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline (2003).** This document provides guidance for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

MM12-P **Diagnostic Nucleic Acid Microarrays; Proposed Guideline (2005).** This guideline provides recommendations for many aspects of the array process including: a method overview; nucleic acid extraction; the preparation, handling, and assessment of genetic material; quality control; analytic validation; and interpretation and reporting of results.

M29-A3 **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on U.S. regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most recent editions.

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Quest Diagnostics Incorporated (CA)
Quintiles Laboratories, Ltd. (GA)
Regional Health Authority Four (NB, Canada)
Regions Hospital
Rex Healthcare (NC)
Rhode Island Department of Health Laboratories
Robert Wood Johnson University Hospital (NJ)
Sahlgrenska Universitetssjukhuset (Sweden)
St. Alexius Medical Center (ND)
St. Agnes Healthcare (MD)
St. Anthony Hospital (CO)
St. Anthony's Hospital (FL)
St. Barnabas Medical Center (NJ)
St. Christopher's Hospital for Children (PA)
St-Eustache Hospital (Quebec, Canada)
St. John Hospital and Medical Center (MI)
St. John Regional Hospital (St. John, NB, Canada)
St. John's Hospital & Health Center (CA)
St. Joseph's Hospital – Marshfield Clinic (WI)
St. Jude Children's Research Hospital (TN)
St. Mary Medical Center (CA)
St. Mary of the Plains Hospital (TX)
St. Michael's Hospital (Toronto, ON, Canada)
St. Vincent's University Hospital (Ireland)
Ste. Justine Hospital (Montreal, PQ, Canada)
San Francisco General Hospital (CA)
Santa Clara Valley Medical Center (CA)
Seoul Nat'l University Hospital (Korea)
Shands at the University of Florida
South Bend Medical Foundation (IN)
South Western Area Pathology Service (Australia)
Southern Maine Medical Center
Specialty Laboratories, Inc. (CA)
State of Connecticut Dept. of Public Health
State of Washington Department of Health
Stony Brook University Hospital (NY)
Stormont-Vail Regional Medical Center (KS)
Sun Health-Boswell Hospital (AZ)
Sunnybrook Health Science Center (ON, Canada)
Sunrise Hospital and Medical Center (NV)
Swedish Medical Center - Providence Campus (WA)
Tenet Odessa Regional Hospital (TX)
The Children's University Hospital (Ireland)
The Permanente Medical Group (CA)
Touro Infirmary (LA)
Tri-Cities Laboratory (WA)
Tripler Army Medical Center (HI)
Truman Medical Center (MO)
Tuen Mun Hospital (Hong Kong)
UCLA Medical Center (CA)
UCSF Medical Center (CA)
UNC Hospitals (NC)
Unidad de Patologia Clinica (Mexico)
Union Clinical Laboratory (Taiwan)
United Laboratories Company (Kuwait)
Universita Campus Bio-Medico (Italy)
University of Chicago Hospitals (IL)
University of Colorado Hospital
University of Debrecen Medical Health and Science Center (Hungary)
University of Maryland Medical System
University of Medicine & Dentistry, NJ University Hospital
University of MN Medical Center - Fairview
University of the Ryukyus (Japan)
The University of the West Indies
University of Virginia Medical Center
University of Washington
US LABS, Inc. (CA)
USA MEDDAC-AK
UZ-KUL Medical Center (Belgium)
VA (Tuskegee) Medical Center (AL)
Virginia Beach General Hospital (VA)
Virginia Department of Health
Washington Adventist Hospital (MD)
Washington State Public Health Laboratory
Washoe Medical Center Laboratory (NV)
Wellstar Health Systems (GA)
West China Second University Hospital, Sichuan University (P.R. China)
West Jefferson Medical Center (LA)
Wilford Hall Medical Center (TX)
William Beaumont Army Medical Center (TX)
William Beaumont Hospital (MI)
Winn Army Community Hospital (GA)
Winnipeg Regional Health Authority (Winnipeg, Canada)
York Hospital (PA)

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