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Meeting Report

Universal RNA Reference Materials for Gene Expression

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A workshop entitled "Metrology and Standards Needs for Gene Expression Technologies: Universal RNA Standards" was held in March 2003 to define the requirements for standardizing RNA-based molecular assays, specifically microarray and quantitative reverse-transcriptase-PCR technologies. NIST sponsored the workshop, and participants represented government, industry, academia, and clinic. Workshop participants concluded that as a first step, two RNA reference materials could be defined that would help in standardization of gene-expression technologies: an Assay Process Reference Material, and a Universal Array Hybridization Reference Material. The specific characteristics of these two standardized materials were broadly outlined. The Assay Process Material was proposed to be a pool of 96 expressed human sequences of defined composition, cloned in a defined vector and pooled in prescribed ways. The Universal Array Hybridization Material was defined as a pool of 12 "alien" synthetic sequences not expressed in any known genome to be used to control for variability in array hybridization methods. Work is underway at NIST and among members of the gene expression array community to further define these materials and make them available.

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A NIST-sponsored workshop entitled "Metrology and Standards Needs for Gene Expression Technologies: Universal RNA Standards" was held on March 28–29, 2003, to define the needs of users of gene expression technologies for standardization of molecular-based assays, specifically microarray and quantitative reverse-transcriptase (RT)-PCR[6] technologies *(1)*. NIST traditionally supports industry by developing the measurements, models, data, and standards needed to accelerate the commercialization of products and to ensure product quality and integrity *(2)*. The primary goal of the workshop was to address the needs of the gene expression community for standardization in this developing field of investigation.

Microarray technology has successfully penetrated the scientific research community where sensitivity and metrology are not top priorities. RT-PCR is already in clinical use, principally in infectious disease diagnosis, where it is used to quantify the viral load of such pathogenic agents as HIV. As applications of RT-PCR expand to include more complex expression profiling, standardized materials similar to those needed for array-based RNA analyses will be required.

To continue moving forward both scientifically and in market growth, gene expression technology must translate from research to medical diagnostics. A recent meeting sponsored by NIST (Gaithersburg, MD, September 2002) elicited the view that this transition will require metrology of RNA, the universal starting material for both microarray devices and RT-PCR. Until the metrologic issues surrounding RNA are resolved, array technology is destined to remain solely a research tool, and RT-PCR will not reach its full potential in the clinic.

The March 2003 conference was an educational forum in which scientists from government agencies, industry, academia, and clinical laboratories shared data, methods, and technologies used in gene expression measurements.

[6] Nonstandard abbreviations: RT-PCR, reverse-transcriptase-PCR; RM, reference material; FDA, US Food and Drug Administration; QC, quality control; IVD, in vitro diagnostic; QRT-PCR, quantitative reverse-transcriptase-PCR; and IVT, in vitro transcription.

The workshop began by defining the metrics important to making gene expression data comparable among technology platforms as well as assuring that inter- and intralaboratory data obtained from the same platform can be compared. It was anticipated that key metrics requiring standardization could be identified in this way and that conference attendees would reach consensus on how standardized materials could be used to meet the needs for technology development, clinical and research applications, and regulatory requirements. The top priority for the workshop was to define the reference materials (RMs) needed and how national, traceable standardized materials *(3)* would address those requirements. The workshop outcome is summarized in this document, which describes the consensus of workshop participants on standardization requirements for RNA and an action plan to meet these needs.

Purpose and Rationale

Gene expression technologies are being used in two major areas of translational research that affect or involve the commercial development of medical products: (*a*) regulatory phases of nonclinical and clinical investigations to better define the potential safety and efficacy of promising therapeutics; and (*b*) clinical diagnostics.

regulatory phases of nonclinical and clinical investigations

Regulatory phases of nonclinical and clinical investigations are incorporating genome-scale gene expression measurements in studies falling within the domains of pharmacogenomics and toxicogenomics *(4)*. In early nonclinical phases of regulatory investigations involving animal and cellular systems, safety concerns of candidate drugs, devices, and biologicals destined for clinical trials are being explored by use of gene expression endpoints that are expected to provide fuller understanding of the broad range and mechanisms of biological responses. In early clinical phases of product investigations, some sponsors are monitoring gene expression responses in accessible tissues to provide improved insight into product efficacy. As product sponsors move expression data from exploratory applications into more pivotal integrated decision-making functions with greater potential to affect patient outcome, the need for reliable data looms larger. Without universally applied RMs for analysis of RNA expression, regulatory agencies attempting to evaluate gene expression profiling data within and across product submissions have no reference point from which to assess the quality of the submitted data. Furthermore, reliable data are also needed in research settings to allow the US Food and Drug Administration (FDA) to review published peer-reviewed literature to benchmark submitted data. To address these needs, both the FDA and the National Institute of Environmental Health Sciences (NIEHS)-sponsored Toxicogenomics Research Consortium have initiated broad collaborative research efforts using standardized preparations of rat and mouse RNA extracted from carefully selected tissues *(1)*.

The availability of a standardized set of RMs would be expected to reduce concerns associated with nonclinical gene expression data submitted for regulatory review to support product approval by providing a means to (*a*) assess and reduce data variability both within and between laboratories and to compare data derived across different platforms; (*b*) assess and improve laboratory competency and data quality; (*c*) reduce data submission requirements; (*d*) enable development of reliable databases; and (*e*) foster harmonized approaches toward data analyses.

Expression profiling is also used to improve quality control (QC) and consistency of cell banks and other biological materials used in the production of certain biologics, including recombinant proteins, vaccines, and cellular and gene therapy products. Gene expression profiling has potential for enhancing QC of, for example, the production of live attenuated viral vaccines and to simplify detection of adventitious agents in transplantation or stem cell products.

clinical diagnostics

Clinical diagnostic applications are numerous and broad. Expression profiles may be useful for profiling tumors (e.g., to improve selection of treatment regimens), for viral serotyping, or for improved detection of blood-borne pathogens. Standardization of such gene expression data is expected as such data are used to support FDA marketing approval for clinical in vitro diagnostic (IVD) devices *(5)*. The intended use/indications for use, analytical and clinical performance characteristics, and labeling constitute the major components of information required to support marketing applications for IVD devices. Marketing approval of an IVD device generally relies on the device satisfactorily demonstrating built-in controls, quantitative calibrators, and/or RMs used in a matrix that will mimic the complexity of the clinical sample to be measured. Furthermore, for CLIA-licensed diagnostic testing laboratories, periodic laboratory proficiency testing is required. Proficiency testing is typically supported by agencies and professional organizations that distribute standard test sets of samples to testing laboratories and grade the quality of the laboratory results for those sample materials relative to the known standard values. No such standard materials exist for RNA expression profiling.

A RM specifically created for clinical use was described in the workshop. The Netherlands Cancer Institute currently uses a 70-gene microarray-defined expression profile for the prognostic screening of breast cancer patients. In a clinical trial to identify patients with tumors likely to metastasize and therefore require adjuvant therapy after surgery *(6)*, this group used a common reference sample for the normalization of their data. The RM consisted of a pool of RNA samples from all study patients, closely

matching the specific sample set. Measurements across the set were sensitive to very subtle changes in expression with respect to the common reference. Because the reference pool was specific to this study and not renewable, the universality of such a classification approach is questionable. Universal RNA materials could help to address this problem if appropriate reference pools could be easily created. Workshop participants recognized that uses of RNA RMs could be many and that it would be necessary to define the end uses of the RNA RMs to improve the likelihood of successful development.

Validating the performance of RNA expression platforms is a necessary step in the development of analytical systems as well, and will be required for laboratories using an expression analysis platform to monitor the performance of such systems. The availability of standardized RMs would allow efficient benchmarking of a system's performance and provide a basis for monitoring performance over time relative to that baseline. Validation across gene expression platforms is also required of collaborating laboratories sharing gene expression profile data generated by different analytical systems. The validation of interlaboratory performance is required for collaborating laboratories even when they are using the same analytical platform. These goals can more effectively be achieved for CLIA-licensed laboratories and FDA-approved devices with standardized RMs.

Gene Expression Profiling Platforms

Although many technologies are available for determining gene expression in a cell or tissue, there are three gene expression platforms for which substantial published data are available for comparisons. Specifically, the workshop focused on the metrics required for the standardization of quantitative RT-PCR (QRT-PCR), cDNA arrays, and oligonucleotide arrays.

QRT-PCR

QRT-PCR can be performed on a variety of commercial platforms where readouts of assay results are generally based on fluorescence measurements. However, results from these various commercial RNA analysis systems are not directly comparable to each other without reference standardization. Similarly, RT-PCR assay quantification depends on calibrating the assay performance of each primer and probe set with known, characterized standardized materials.

cDNA arrays

cDNA arrays are both commercially available and "homemade" by laboratories; such arrays involve spotting of DNA products generated from commercially available clones or from homemade clone libraries on a variety of substrate surfaces. Aside from annotation errors from source materials (which may be significant), sources of error for these arrays include erroneous placement of sequences on array surfaces and variability in the efficiency of oligonucleotide coupling on the substrate surface. The lengths and sequences of probes also vary markedly among laboratories as well as among arrays. Data from these immobilized probe arrays cannot be compared with other expression profiling data without a reference normalization strategy, which could be achieved by a RNA RM.

oligonucleotide arrays

Oligonucleotide arrays also vary in content and composition and are both commercially available and homemade. Commercially available oligonucleotide arrays include both synthesized (photolithographic) and spotted (ink-jet-printed) arrays, in which presynthesized, purified oligonucleotides are chemically immobilized on an active substrate surface. Commercial arrays provide a wide range of designs and compositions from 25mer to 80mer oligonucleotides with various degrees of probe redundancy for detecting individual target sequences and gauging the specificity of hybridization. Because of the variability in array designs, different sets of labeling, hybridization, washing, and imaging conditions are required to achieve optimum performance from each unique array design. Therefore, oligonucleotide array gene expression profiling data are not directly comparable across array types without use of reference normalization.

Sources of Experimental Data Variability

There are numerous significant challenges in generating and sharing validated data obtained from gene expression analysis systems, including sample preparation and storage and the type and extent of data that are necessary to describe any microarray experiment, as outlined by the MIAME (Minimal Information About Microarray Experiments) Conference *(7)*. Gene annotation issues also prevent meaningful sharing of expression data because the definition of a gene and the splice variants expressed by a gene are often not described in the microarray documentation. These were acknowledged to be beyond the scope of the March conference. Instead, participants focused on the sources of experimental variability in gene expression analysis and the usefulness of standardized RMs for the comparison of data among different laboratories and across different gene expression platforms, as well as from platform upgrades from evolving technologies.

The potential sources of variability in processing a sample, from defining the tissue source to isolating the RNA and performing the gene expression measurement, are summarized in Fig. 1. A review of this progression highlights where and how RNA RMs can effectively control these processes.

tissue sample acquisition, preparation, and rna extraction

Tissue sample acquisition and preparation and RNA extraction will require considerable study to fully under-

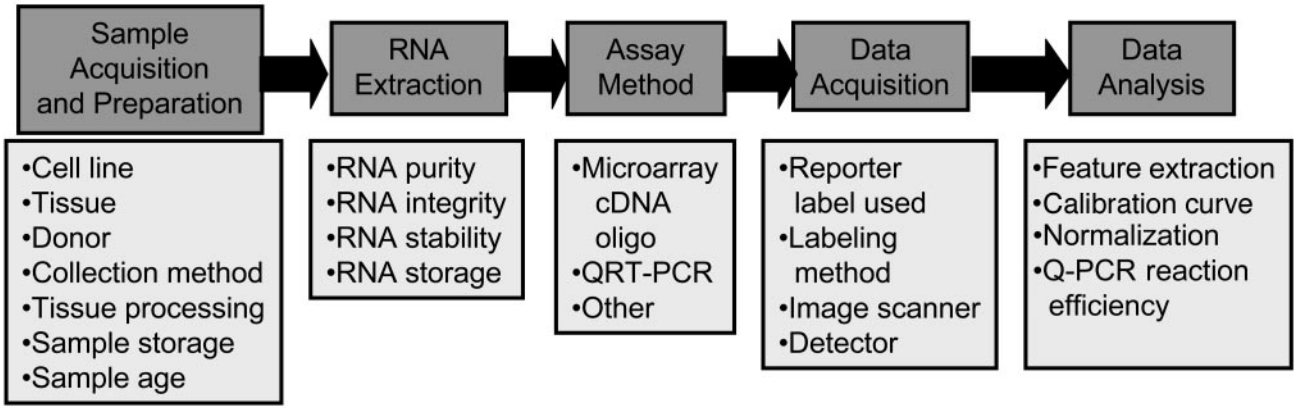

Fig. 1. Potential sources of variability in processing a sample.

Various steps comprising the process elements of RNA expression analysis assays are shown in the *darker boxes* linked by *arrows*. Cumulative sources of process variability are listed in the *lighter gray boxes* below each process step.

stand the sources of variability in the collection and processing of tissues and preparation of tissue extracts. Significant variability may be introduced at these initial sample processing steps. RNA extraction efficiencies vary with tissue source, with blood, pancreas, and spleen being more difficult than other tissues. Best methods to protect RNA within samples from degradation and key control points in tissue procurement, extraction, and storage are still developing. Tissue sample acquisition, tissue preparation, and RNA extraction are difficult to control and lack a clear approach for including an RNA reference standard. It was suggested that a RNA quality index be formulated, taking into account several different physical metrics. Standards that extend to sample preparation issues were not the focus at the March conference, and their design and use are beyond the scope of this document.

RNA preparation for expression analysis

RNA preparation for expression analysis introduces expression data variability even when a single platform is used within one laboratory. This variability derives from the processing steps for performing the assay. For microarrays, this includes target amplification, reporter labeling, array hybridization, and to a lesser extent, scanning of the hybridized array and extraction of image features. For QRT-PCR, sources of variability include the accuracy of RNA quantification, methods and efficiency of reverse transcription, purity of the labeled reporter probe, and accuracy of the assembly of the PCR protocol. Intralaboratory results are confounded by documented and undocumented deviations from standard protocols, which generally result from incomplete documentation, inadequate personnel training, uncontrolled environmental conditions, or uncalibrated or malfunctioning equipment. Additional factors, including changes in reagent lots, vendors, or custom software and inadequately validated revisions to the protocol, can have more subtle effects on data quality. These factors are often not recognized by scientists unaccustomed to the sensitivity of gene expression assays to these perturbations. In clinical settings, adherence to clinical Good Laboratory Practices (cGLPs) minimizes these sources of variability; nevertheless, the need for proficiency testing in the clinical laboratory remains, to verify appropriate control of array-based processes.

Gene expression technologies are susceptible to significant operator-introduced experimental variability for several reasons. Inexperienced users may not realize the effect of subtle changes in cell, lysate, or reaction handling on results and may not have appropriately defined relevant boundary conditions. Given the complexity and expense of array- and RT-PCR-based expression profiling assays, the lack of full characterization of these measurements is easily understood. Despite the complexity of gene expression assays, it is relatively easy to generate interesting and novel results; generating consistent cross-platform verifiable results is another matter. Perturbation of any one part of the assay process, even relatively large perturbations, may not have a significant impact on the final quality of the data, and many small factors may interact to produce systematic data artifacts. Interactions of this type are most effectively discovered and avoided by the rigorous design of experiment approaches not frequently used in research development programs.

Because most gene expression technologies depend on purified RNA (either total RNA or mRNA), it is reasonable to consider the development of standardized RMs with characteristics that mimic RNA extracts. Such a RM would allow correction for processing variability during amplification, target labeling, and signal detection and would allow investigators to evaluate the cumulative assay performance across technology platforms.

Microarrays as test devices

Microarrays as test devices should be tested objectively for performance quality regardless of origin; devices from different vendors and different production lots from a

single vendor should meet the same performance criteria. Because microarrays contribute to the variability of results from gene expression experiments, certification of microarrays by the manufacturer and verification of array performance by the customer is critical in the generation of reliable, consistent, and reproducible data. Microarrays contribute to variability in both overt and subtle aspects of their performance characteristics. Changes in microarray format, e.g., in probe sequence or probe location within a transcript, will impact the nature of the data generated by the array. Subtle changes to fabrication methods, including the efficiency of synthesis chemistry and the characteristics of the substrate (surface), although invisible to the end user, may have profound effects on data quality, e.g., by affecting the specificity of hybridization. The development of a RNA RM for use as an internal assay process control could also serve QC functions during manufacturing. Such an internal "QC template" would permit specific, quantitative measurement of array performance during manufacture as well as end use.

Separate metrics could also be developed for microarrays to measure device performance during the end-use assay. These metrics would measure the relative quality of the overall array synthesis or probe deposition and immobilization processes. This could be accomplished by including a labeled control oligonucleotide or synthetic transcript as a control added to the processed sample before hybridization. The target complementary to the arrayed control probes would be distributed across each microarray according to a defined QC template.

qrt-pcr reactions

QRT-PCR reactions are typically designed by end users applying standard principles built into software programs. Although the performance characteristics of assays resulting from this software design approach may be relatively uniform, assay design validation is still required for the test to be applied to RNA expression analysis and the assay results to be reliably quantitative. The quantitative range for the assay is defined as the concentration range of target where PCR efficiency remains linear with a slope approaching 1. The reproducibility of the assay and specificity of target are also important variables that must be quantitatively evaluated and controlled if the assay is to have clinical utility for monitoring gene expression. The characterization of QRT-PCR assays would benefit from the availability of a validated RNA RM as well as microarrays.

platform performance and data variability

Platform performance and data variability assessment was presented as a function of a standard set of metrics to be used for evaluating gene expression experiments. Although the focus of the workshop was primarily on defining RNA RMs to control the analytical portion of the assays, ultimately such materials need to be set for all steps in the gene expression profiling work flow, and appropriate metrics for their monitoring must be identified.

The consensus among workshop participants was that the following parameters for an RNA RM to be used as an "add-in" to the sample RNA extract should be clearly defined and used to consistently measure performance across different platforms. These parameters include accuracy, dynamic range, sensitivity, specificity, and reproducibility. Standard metrics could then be calculated as an output of any of the gene expression technologies (arrays and QRT-PCR), assuming that the appropriate probes are represented on the array or the sequences have been included as part of an RT-PCR amplicon.

RNA RMs

Workshop participants concluded that two RMs are needed: both a moderately complex pool of highly characterized RNA targets, to serve as an Assay Process RM, and a Universal Hybridization RM. The moderately complex pool would be used as an Assay Process RM across all RNA gene expression profiling platforms, including both RT-PCR- and array-based methods. It would be useful for measuring the accuracy, dynamic range, sensitivity, and specificity of each platform under any given set of conditions. The second recommended standard is specific for arrays. This would be a simple pool of standard sequences adopted by all commercial array manufacturers and available to laboratories making homemade arrays. Its composition would be carefully defined, highly characterized, and compatible with all array formats. Its function would be to provide an internal measure of the quality of any particular array experiment with respect to sensitivity, dynamic range, and specificity, regardless of array design. This RM would provide a basis for comparing results across array platforms and array types as well as make array-to-array comparisons within a single platform. Array manufacturers could also use this material for QC testing of their products during manufacture so that the products could be held to defined performance specifications.

assay process rm

The characteristics of the Assay Process RM are defined as follows:

- Modular RNA components that are individually characterized and pooled in defined ways to permit the pool complexity to be extended over time. Similar RMs can be designed for arrays that profile gene expression in other species.
- Initially, the number of RNA component sequences in the Assay Process RM should be 96 to be consistent with microtiter plate format and automation compatibility.
- Each component sequence should be no longer than 2 kb (2000 bases) beginning from the 3′ end of the transcript or, alternatively, the full-length transcript if it is shorter than 2 kb long.

- Each selected sequence should be conserved, minimally polymorphic, and well characterized for tissue specificity, splice variants, gene family members, pseudogene structure, and typical expression patterns across tissues.
- Each component sequence should be cloned into a standard vector.
- Each clone should be completely sequenced and characterized for polymorphic form.
- Each clone should have at least a 30-base polyadenylation sequence so that the in vitro transcription (IVT) RNA targets generated from the clone are compatible with oligo(dT) priming.
- Each clone should have a promoter sequence for making full-length IVT products with 30mer polyA tails.
- Each component clone should be independently constructed and physically characterized for high purity and high stability, and its IVT and PCR products should be characterized by HPLC and mass spectrometry for purity and exact sequence composition.
- Collectively the RM components should represent a 10^6-fold range of absolute quantitative expression.

A minimum of two forms of the Assay Process RM should be specified. These would each have the identical target sequence composition, but the concentrations of each component species would vary between the two forms. This would ensure that measurement accuracy could be tested over a range of concentration ratios because most RNA expression analysis is focused on measuring relative abundance of specific target sequences and fold changes in expression of specific target sequences. In addition, this type of material would accommodate array platforms that require two differentially labeled samples where one acts as a test sequence and one acts as a reference sequence.

Two additional characteristics for these RMs are desirable:

- The specified concentration ratios for individual sequences between the two forms of the Universal RNA RM should cover a range from 10^{-3} to 10^3.
- The ratios and concentration ranges for both RMs should be independently analytically verified by HPLC or mass spectrometry.

UNIVERSAL HYBRIDIZATION RM

The characteristics of the Universal Hybridization RM are defined as follows:

- A single standard pool composed of 12 RNA target sequences should be made.
- This set of sequences must be represented by complementary, optimized hybridization probes according to a standard template on all array platforms.
- These sequences must be represented with some degree of redundancy on each array (5- to 10-fold rate of repetition) to provide a statistically significant measure of hybridization performance.
- This add-in reference set should be added to every hybridization sample, according to a standard protocol, as an internal positive control of array hybridization performance.
- Component sequences of this material should be "alien" sequences not represented in any naturally occurring test samples.
- The target sequences should be >600 nucleotides long to accommodate the full range of array platform designs.
- These Universal Hybridization RM components should be cloned into the same vector as the Assay Process RM component sequences.
- Clones should have a promoter sequence for generating IVT cRNA products with a 30mer polyA tail for oligo(dT) priming.
- These clones should meet the same purity, sequencing, and stability specifications that are derived for the Assay Process RM.
- Concentrations of the add-in hybridization RM must represent the full dynamic range of the analytical platform.
- Documentation providing explicit instructions on correct use of the add-in internal control material would have to be provided, including data analysis approaches and pass/fail criteria for standard values.

Application of RMs

Critical metrics of an array system are relative accuracy and precision. These are measured by testing for a clearly defined number of copies of a given sequence within a RNA preparation over some linear range. An Assay Process RM provided in two different mixtures of relative abundance could be used to generate a ratio that would allow the laboratory to determine how accurately an array technology reports values relative to an independently verified value. Accuracy and precision for QRT-PCR are conceptually similar in that the assays measure the quantitative accuracy of a defined target copy number over the linear dynamic range of the assay. This again is best accomplished by use of a predefined RM.

A standard set of metrics can be developed for comparing inter- and intralaboratory performance for all gene expression measurement platforms using these RMs. Array and reagent manufacturers could also use this material for QC testing of their products during manufacture so that all inputs into an expression measurement experiment could be held to defined performance specifications across the entire industry.

The following information would be required to successfully implement these metrics industry wide:

- Clear definitions of each of the metrics and how they are correctly applied to each gene expression measurement technology.
- One common QC template for the microarray industry to use for laying out array control probes.

- Industry-wide agreement on the candidate set of target and probe sequences to be used for array QC purposes.
- Common algorithms or an agreed on set of platform-modified algorithms to calculate and apply the industry standard metrics.
- Well-defined protocols for producing and using the synthetic RNA transcripts composing the RMs.

Limitations of Specified Universal RNA RMs

The principle limitation of the Assay Process RM recommended here is its low biological complexity. It is widely recognized that pure, reconstituted, synthetic materials do not typically reflect the behavior of true biological samples. The result is that although the RMs may allow an estimate of relative performance among platforms, the complexity of true samples may include variability in platform performance not accommodated by the RM measurement. Recognizing this problem, CLIA and other regulatory guidelines traditionally recommend using RMs that either are true biological samples or closely mimic actual biological samples.

The workshop membership recognized this limitation of the proposed universal RMs but still recommended that these materials be developed because they will represent major progress in harmonizing data from among expression analysis platforms that currently have no existing standardized RMs of any kind. The meeting attendees further recommended that once experience is gained with these synthetic materials, efforts should be initiated to develop more complex RMs that are manufacturable, renewable, and analytically characterized but more closely approximate a true biological sample.

One proposal to overcome the limitation of the proposed materials and move toward a true universal RM was to identify tissues (from animal, cell culture, and lower-species models) that do not express specific sets of transcripts. Targets based on these nonexpressed transcripts could be developed along the guidelines of the Assay Process RMs and added into extracts of the characterized tissues at defined concentrations. Requirements for a system of this type would have different thresholds for array-based or QRT-PCR RNA analysis platforms but could conceivably be constructed.

Summary

The workshop on Universal RNA Standards successfully completed its mission to define the scope of the need for gene expression measurements. All sectors of the gene expression community were engaged, allowing the discussion to focus on a wide range of applications using the most common analytical platforms. Emerging critical needs that are currently obstacles to the inclusion of gene expression data in the regulatory process were raised. The workshop consensus was that two standardized RMs with defined characteristics would satisfy the most pressing initial needs, although it was acknowledged that these materials have limitations that prevent them from satisfying the full range of RNA expression analysis demands for standardization. RMs representing more biological complexity are an obvious evolution and were discussed at the workshop, but it was agreed that the materials defined here are a critical first step to initiate a more comprehensive program.

The projected breadth of gene expression measurement utility is a powerful motivator driving quantitative gene expression measurement science and the rising demand for demonstrated high-quality expression results. Activities stemming from this workshop are already materializing along the lines projected in this report, to deliver the universal RNA RMs that begin to meet the demand for gene expression metrology. An External RNA Controls Consortium *(8)* has formed to address comparability issues within the gene expression community for experiments addressing human mRNA transcripts. If successful, this consortium will be used as a model for developing universal RMs for expression measurement in other organisms and for further defining the metrics requiring standardization. This nascent technology is rapidly maturing, and an infrastructure to support comparability of results and trustworthiness of conclusions will accelerate its widespread adoption and, ultimately, enable it to meet its promise in discovery research and in the clinic.

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