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Review

Quality Assurance of RNA Expression Profiling in Clinical Laboratories

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RNA expression profiles are increasingly used to diagnose and classify disease, based on expression patterns of as many as several thousand RNAs. To ensure quality of expression profiling services in clinical settings, a standard operating procedure incorporates multiple quality indicators and controls, beginning with preanalytic specimen preparation and proceeding thorough analysis, interpretation, and reporting. Before testing, histopathological examination of each cellular specimen, along with optional cell enrichment procedures, ensures adequacy of the input tissue. Other tactics include endogenous controls to evaluate adequacy of RNA and exogenous or spiked controls to evaluate run- and patient-specific performance of the test system, respectively. Unique aspects of quality assurance for array-based tests include controls for the pertinent outcome signatures that often supersede controls for each individual analyte, built-in redundancy for critical analytes or biochemical pathways, and software-supported scrutiny of abundant data by a laboratory physician who interprets the findings in a manner facilitating appropriate medical intervention. Access to high-quality reagents, instruments, and software from commercial sources promotes standardization and adoption in clinical settings, once an assay is vetted in validation studies as being analytically sound and clinically useful. Careful attention to the well-honed principles of laboratory medicine, along with guidance from government and professional groups on strategies to preserve RNA and manage large data sets, promotes clinical-grade assay performance. ***(J Mol Diagn 2012, 14:1–11; DOI: 10.1016/j.jmoldx.2011.09.003)***

Microarray technology and RNA signatures have a long and somewhat rocky track record in basic science laboratories. In the past few years, advances in technology and quality assurance suggest that genomic profiling is reliable enough for medical decision making in clinical trials and ultimately in routine patient care. Factors contributing to adoption in clinical settings include the following: i) good manufacturing practices for reagents, supplies, control instruments, and software; ii) progress in biospecimen science that promotes RNA integrity; iii) advances in bioinformatics, facilitating interpretation of complex data; and iv) novel strategies for quality control, ensuring that each patient test performs as expected.

Microarrays permit measurement of hundreds or even tens of thousands of RNAs simultaneously, including coding and noncoding RNAs of human and microbial sources.[1] RNA profiles that are unique to clinical status can assist with diagnosis, prognosis, monitoring, and predicting efficacy of therapy. To warrant adoption in clinical settings, the assay must provide added value beyond what is already available to patients or their health care system.[2]

RNA profiling is guided by high standards and well-honed principles of laboratory medicine that ensure quality while allowing innovation and progress. The quality systems that are being adapted to expression profiling are emblematic of the novel strategies being developed to manage a wide range of multianalyte technologies, such as full-genome sequencing, proteomics, and single-nucleotide polymorphism chips. The two most impor-

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tant contributors to good outcomes are competent personnel to perform each assay and controls and quality indicators that help these personnel identify and correct problems.[3] Meticulous attention to detail, expert technical knowledge, and medical judgment are required to validate and maintain each assay. During validation work, a standard operating procedure is refined and tested, and evidence is collected to substantiate choices regarding specimen requirements, indications for testing, recommended clinical use of test results, step-by-step analysis, and quality control processes.[4–6] Each new assay or modification to an assay must be vetted by the laboratory director as being analytically sound and clinically useful enough to justify implementation as a medical service. This article reviews the quality assurance measures that are established during assay validation and that are used on a daily basis to ensure good outcomes.

Governmental Oversight of Laboratory Services in the United States

Scrupulous attention to detail and high standards ensure the quality of laboratory work performed in clinical settings. Governmental oversight of testing laboratories, and of the manufacturers providing reagents or test systems to such laboratories, is codified in federal regulations of the Clinical Laboratory Improvement Amendments and Title 21, directing the Food and Drug Administration (FDA). Many RNA-based assays have been FDA cleared or approved, such as tests to detect HIV, hepatitis C virus, mycobacterium tuberculosis, and influenza virus. Quantitative RT-PCR (RT-qPCR) is the technology most frequently used in clinical molecular laboratories, and panels of RT-qPCRs can reasonably be performed on dozens to thousands of different analytes at once.[7,8] For human gene discovery research, even denser arrays provide expression data on virtually all (approximately 22,000) human protein-coding genes and >1000 microRNAs.[1,9]

To date, 10 multianalyte RNA assays have been FDA cleared on Agilent (Santa Clara, CA), Affymetrix (Santa Clara, CA), RT-qPCR, and bead-type platforms. These include Mammaprint (Agendia, Irvine, CA) to help manage selected breast cancer patients,[10,11] Tissue of Origin Test (Pathwork Diagnostics, Redwood City, CA) to help pathologists refine the type of malignancy,[12] AlloMap blood test for acute cellular rejection of a transplanted heart (XDx, Brisbane, CA), BLN assay for breast tumor metastasis to lymph nodes (Veridex, Raritan, NJ; this assay was voluntarily withdrawn from the US market), and six respiratory virus panels from Idaho Technologies (Salt Lake City, UT), Nanosphere (Northbrook, IL), Applied Biosystems (Carlsbad, CA), Focus Diagnostics (Cypress, CA), Gen-Probe (San Diego, CA), and Luminex (Toronto, ON, Canada). After approval of a predicate *in vitro* diagnostic device, the FDA often issues guidance for manufacturers validating similar assays. Many laboratory-developed tests have been implemented as medical services under Clinical Laboratory Improvement Amendment regulations, and examples of such laboratory-developed tests characterizing RNA are *BCR-ABL1* in leukemia[13] and Genomic Health's (Redwood City, CA) Oncotype Dx assay in breast cancer.[14]

The MicroArray Quality Control project began as an initiative to assess the quality of RNA-based microarray expression profiles and to recommend improvements. The project was launched by the FDA and involves nearly 200 academic institutions, commercial manufacturers, and governmental divisions. In work to date, sample exchanges and data set analyses showed that expression profiling is technically robust, is biologically informative, and generates similar profiles across multiple platforms when viewed by functional means.[15–18] Several approaches to prediction modeling were deemed effective.[15,19] The European Union's EMERALD project (Empowering the Microarray-Based European Research Area to Take a Lead in Development and Exploitation) developed software tools for quality metrics of array data.[20]

Guidance from Professional Groups

Several laboratory professional groups offer guidance on clinical-grade procedures and standards. In particular, checklists marketed by the College of American Pathologists (CAP) as part of their worldwide laboratory accreditation program are a blueprint for achieving good outcomes (*http://www.cap.org*, see Reference Resources and Publications section of website; last accessed June 30, 2011). The Clinical and Laboratory Standards Institute produces documents thoroughly describing principles and best practices, such as Diagnostic Nucleic Acid Microarrays,[21] Verification and Validation of Multiplex Nucleic Acid Assays,[22] and Use of External RNA Controls in Gene Expression Assays.[23] Three European organizations developed joint guidelines for microarray profiling of leukemia,[24] and most of the recommended principles and procedures extend to other diseases.

Some multiplexed tests target thousands of RNAs at once, although the minimum number of analytes composing an array is only 11, according to the American Medical Association Current Procedural Terminology Editorial Panel. In the United States, reimbursement by payers for microarray services is accomplished using the physician fee schedule, emphasizing that a pathologist or other laboratory physician is responsible for technical work and uses medical judgment in expert interpretation of the findings. Among the many requirements of Clinical Laboratory Improvement Amendment certification is that a physician consultant be available to discuss indications for testing and patient-specific result interpretation for all tests offered by high-complexity clinical laboratories.

Preanalytic Specimen Preparation

Preanalytic collection, stabilization, transport, and storage conditions are critical for obtaining accurate analytic test results.[25–29] Interestingly, procedures that may seem irrelevant can affect outcome, such as first versus second pass of a needle-core biopsy[30] or freezing of blood or

tissue.[31,32] Guidance from professional groups addresses these preanalytic concerns.[33,34] The testing laboratory takes a trust-and-verify approach by educating health care workers in proper collection and handling procedures, while using quality checks, such as housekeeping transcript levels, to evaluate specimen quality.

RNA is notorious for rapidly degrading if special precautions are not taken to preserve it.[33,35] In stored blood and marrow, Ma et al[36] showed similar RNA profiles for frozen versus TRIzol-preserved cells. Stabilization of RNA at the bedside is feasible using commercial blood collection systems that must be validated for their intended use.[37–42] The advantages of RNA stability must be weighed against the cost of stocking special collection vials at every pertinent blood collection site and the incapacity to interpret smears or apply cell enrichment procedures once the preservative is added.

For solid tissue, 10% neutral-buffered formalin is the fixative favored by nearly all histopathology laboratories. Formalin functions by aldehyde cross-linking to generate a scaffold preventing tissue degradation and diminishing unwanted enzymatic activity (eg, RNases). Diffusion of formalin into tissue is a function of distance and density; thus, slicing tissue into thin pieces promotes rapid fixation. The duration of fixation is critical, with sufficient time required to ensure adequate preservation of analytes, while avoiding overfixation that hardens tissue and increases cross-links with macromolecules, rendering intact RNA difficult to recover.[43–45] A study by Chung et al[46] showed that fixation between 4 and 48 hours was reasonable, although 12 to 24 hours was ideal with respect to downstream RNA quality. Incubating RNA in warm Tris buffer is predicted to reverse, at least in part, the adverse effects of formalin.[45] In validation work, consider splitting fresh specimens and testing pertinent preanalytic variables to gather the evidence required to set limits on acceptable specimen preparation for the particular assay.[47,48]

Fresh or frozen tissue tends to have much better-quality RNA than does formalin-fixed tissue, but formalin-fixed tissue is readily available in clinical settings and, thus, is the specimen of choice. Acid decalcification is not recommended because depurination fragments nucleic acid. Alternative fixatives, particularly alcohol-based solutions, often yield high-quality RNA,[49–51] but these fixatives may suffer when rated by pathologists for histological detail, not to mention the adverse impact on immunostains and other histochemical procedures that are frequently applied in clinical settings.[52] It appears that a molecular test is most likely to be adopted if the test is robust enough to work on customary specimens, recognizing that the term customary encompasses a wide range of nonstandardized formalin fixation protocols.[47] Fortunately, multiple groups[53–55] have succeeded in profiling RNA from archival formalin-fixed tissues. Because some, but not all, target RNA levels are equivalent in paraffin blocks versus matched frozen tissue,[56–61] it is clear that each RNA-based test must be validated for its relevant specimen preparation.

Enrichment of Lesional Cells

To generate an accurate RNA signature, the input tissue must be appropriately representative of the organ or lesion being evaluated.[62] To accomplish this, a pathologist examines a stained slide to confirm histopathological findings and to assess tissue adequacy for the particular test.[63] If indicated, lesional cell enrichment is done by macrodissection or microdissection.[64–66] For blood, marrow, or other cytologic fluids, cell enrichment is achieved using flow cytometry or magnetic bead separation.[67] For plasma or serum, variations in the protocol for cell separation can affect downstream RNA measurement.[68,69]

Criteria for tissue selection are defined during validation work. In tumor profiling, the amount and nature of the malignant and stromal cell components can influence the profile, depending on which RNAs compose the test panel, the anatomical site (eg, primary versus metastatic), and whether the stroma is normal, fibrotic, desmoplastic, or inflammatory.[62,70] In preanalytic work, a pathologist typically selects a portion of tissue in which malignant cell nuclei exceed a given proportion of all nuclei present, which is different from circling malignant areas because those areas might contain only scattered malignant cells in a sea of reactive stromal cells. Expert interpretation of downstream molecular results is done in the context of the input tissue and other correlative data.

RNA Extraction and Quantification

In busy clinical laboratories, automated RNA extraction instruments help control costs and improve reproducibility. Staal et al[24] reported that the extraction method (TRIzol versus RNeasy) affects RNA expression patterns in blood, providing cautionary evidence that substituting extraction protocols can adversely affect results. Skipping extraction altogether is feasible for some robust technologies.[71,72]

It is often worth evaluating RNA quantity and quality before subjecting a specimen to expensive microarray analysis. RNA concentration is measurable by UV spectrophotometry or fluorimetry, and RNA size distribution may be visualized on an electropherogram and scored using a software algorithm (eg, RNA integrity score).[73] The spectrum of RNA size is dramatically larger in fresh or frozen tissue compared with formalin-fixed, paraffin-embedded tissue (Figure 1). A 1-hour delay in fresh specimen processing does not adversely affect the RNA integrity score, although it may disturb individual analytes.[52,74,75] By RT-qPCR, amplicons >500 bp are infrequently achieved from formalin-fixed tissues compared with frozen tissue, and RNA integrity scores do not predict amplifiability from paraffin tissue blocks; instead, levels of housekeeping transcripts, and separate hybridizations of 5′ and 3′ ends of such transcripts, are helpful for assessing specimen quality.[58] The usual precautions to protect RNA are in order, such as use of RNase-free reagents and plasticware, wearing and frequently changing gloves, and keeping a clean work environment by wiping surfaces with 10% bleach or RNase ZAP (Ambion) and using disposable bench covers.

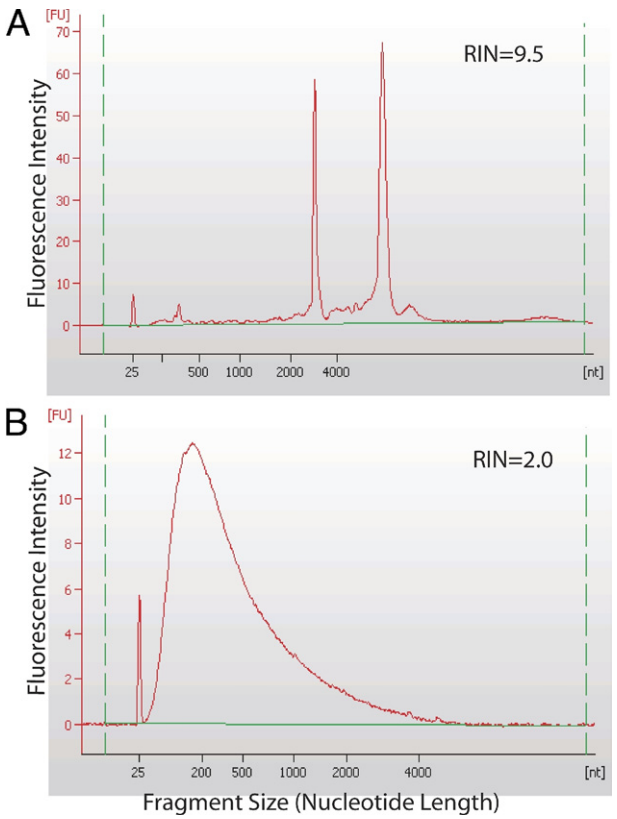

Figure 1. Agilent Bioanalyzer electropherograms reflect RNA size spectrum. **A:** Intact RNA from frozen tissue has prominent 18S and 28S rRNA peaks surrounded by other relatively large RNA molecules. **B:** Degraded RNA from matched paraffin-embedded tissue has much smaller RNA fragments and a lower RNA integrity (RIN) score.

Controls and Limits on Their Acceptability

Quality control is among the most important of quality assurance measures.[3] Although traditional single-analyte assays require inclusion of a positive and a negative control in every run, it is clear that microarray runs cannot possibly include controls for each of the dozens to thousands of target analytes, not to mention the impossibility of representing each of the permutations and combinations of expressed genes that might be found in a given specimen. Thus, a new paradigm of quality control has emerged to accommodate multianalyte arrays.[76] In short, the approach engenders confidence that the pattern of expression is accurate and reproducible.

Controls are used to check assay performance, with special focus on the least robust components. Multiple types of controls are used in RNA profiling. A "no template" control can evaluate background signal and contamination by stray nucleic acid. An exogenous control is run alongside patient specimens to evaluate assay performance in a general manner. A separate exogenous control, representing each of the main outcome groups, could be included for every X patients who are run, with X chosen based on the medical impact of an erroneous classification and the timeliness required to correct any error, recognizing that failure of a control will launch an investigation that questions the results for those patients tested since the last time that the control performed as expected.

An endogenous control checks an inherent feature of patient sample, such as a housekeeping transcript, which is particularly valuable for assessing preanalytic factors (eg, viable cellularity, collection, preservative, shipping, and storage). Because of inherent biological variability in levels of any given gene product, it is recommended to identify several housekeepers that are consistently expressed at low to high levels in the relevant tissue or fluid. Adequate expression of these housekeepers reflects suitable hybridizable RNA, thus permitting rejection of poor-quality specimens or those with faulty technical analysis. In addition, housekeepers can serve as a normalizer by which to quantify target RNAs.[73,77–79] Caution is warranted because *ex vivo* degradation proceeds at different rates for different analytes, reinforcing the need to validate the control and normalization strategy to promote comparability of results across specimens.

Spiked controls are yet another way of checking assay performance. Thanks to the work of the External RNA Controls Consortium, commercial RNA standards are newly available to assess technical accuracy.[80,81] These nonhuman, nonpathogen, spiked RNA products are marketed [by Invitrogen (Carlsbad, CA) and VWR (Radnor, PA)] as mixes of multiple synthetic RNAs at a known concentration and a known sequence. They are spiked into each patient specimen at the earliest informative time point (eg, with lysis buffer) to permit downstream evaluation of assay performance within the patient specimen. Results can detect technical failure and iatrogenic inhibition (eg, residual phenol or heparin anticoagulant) or endogenous interfering substances (eg, hemoglobin or background autofluorescence). Finally, combinations of spiked molecules are proposed as a way of tracking specimen identity through the many steps of specimen preparation and analysis.[82]

Limits on acceptable performance of controls are empirically set by replicate analysis. Consider running a control many times (across different days, technologists, instruments, and lot numbers), and then calculate the mean ± 2 SDs as the limit on its performance. When multiple controls are used, the expected failure rate increases accordingly. For example, a failure rate of 5% for any one control implies that a combination of four controls will fail 18% of the time. This high failure rate emphasizes the benefits of a quality control strategy that includes multiple controls for the many critical aspects of the assay and synthesizes multiple data points to interpret overall success or failure of an assay.

Control results falling outside acceptable limits are documented and investigated for root cause and, when feasible, to take corrective action. When combined with quality indicators (eg, spectrophotometry or histopathological characteristics), results of controls can help pinpoint the source of the problem. Affected patient tests are usually repeated, when sufficient specimen remains. Records of problems, investigations, and potential solutions are fodder for the laboratory's quality improvement program.

Sources of Control Materials

Exogenous controls should resemble patient specimens. Human tissue remaining after clinical analysis can be fractionated and stored for use as a control, although some heterogeneity is expected across aliquots. Xenografts are an alternative source of abundant human cells, although contamination with cells of the host species merits study to show that the xenograft is sufficiently representative of the RNA profile of interest. Fresh blood controls are particularly hard to find, so it is reasonable to use stored white cell pellets or residual RNA or cDNA from previously tested specimens.

A mock specimen may be prepared by mixing a cell line or RNA derived from that cell line with appropriate matrix, and serial dilutions can be used to challenge analytic sensitivity or linearity. Some lot-to-lot variation is expected even when precise criteria are defined for cell culture and harvest. Some scientists favor a mixture of several cell lines to fill in gaps that an individual cell line might have (eg, nonexpressed genes). This approach is exemplified by the Agilent/Stratagene reference RNA manufactured from a mixture of 10 cell lines and well characterized by virtue of its analysis in multiple quality-assurance studies.[16,83,84]

Single-use aliquots prevent multiple freeze-thaw cycles. When the same control material is used in multiple runs, selected numeric results can be tracked over time using Levey-Jennings charts to visualize drift or shift.[85]

Linear Amplification, Labeling, and Two-Color Arrays

When RNA quantity is limited, linear preamplification can boost the signal,[58,64,86–89] and a label can be incorporated to permit downstream detection after hybridization.[84] Labeling efficiency can be checked before hybridization.

In some hybridization strategies, a competitor RNA is cohybridized with patient RNA in a two-color approach.[90] Compared with one-color assays, two-color assays are more expensive and arguably avoidably complex given that MicroArray Quality Control project II studies[91,92] confirm that both one- and two-color strategies perform well. Although the merits and risks of various assay designs can be debated, it is imperative to use the same assay protocol in patient care that was vetted in validation studies.

cDNA Preparation

Before cDNA preparation, DNA is usually removed using DNase. The cDNA production is driven off random primers, oligo dT primers targeting poly A tails of mRNA, or specific primers targeting each RNA. The preparation of cDNA is among the most inconsistent features of RT-qPCRs. Whether replicates are required for this or any other step of analysis depends on the variability observed during reproducibility experiments performed in the validation study. In general, an assay that is so finicky as to require replicate testing may not be reliable enough for clinical use.[93] As a rule, significant differences in expression pattern between two patients must largely represent true biological differences rather than technical error.

Equipment, Reagents, and Test Systems

Lessons learned in the early days of expression profiling led to many improvements in the manufacturing of hardware, reagents, supplies, and software-supporting expression profiling. Manufacturers are vital for supplying testing laboratories with reliable products.[94] Purchasing decisions favor suppliers who comply with the FDA's medical device current good manufacturing practices (*http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/PostmarketRequirements/QualitySystemsRegulations*, last accessed June 30, 2011) or comparable International Standardization Organization systems (*http://www.iso.org/iso/iso_9000_essentials*, last accessed June 30, 2011), promoting quality and consistency.[95,96] Ideally, users and manufacturers communicate so that substantial changes to the product are conveyed to users, and performance of the product in users' hands drives improvement by the manufacturer. Even after extensive vetting by a manufacturer, it remains incumbent on the testing laboratory to ensure that each product performs adequately for its intended use.

Hybridization

Although assay design-related error should have been addressed during assay validation (eg, cross-reactivity, secondary structure diminishing intended base pairing, and competition), other systematic errors may crop up after implementation (eg, defective lot number or instrument). In addition to the error detection strategies previously addressed, consider designing redundancy into the test system by targeting critical analytes numerous times (eg, in different physical quadrants of the array or by probing both ends of a given transcript). Likewise, one could test multiple components of a critical biochemical pathway, multiple markers of a critical phenotype, or multiple conserved segments of an RNA viral genome. These scenarios capitalize on the array's strength in multiplexed testing.

Analytic Interpretation of Results

Analytic interpretation involves generating a reportable result after first evaluating raw data or data generated using decision-support tools, for each control and for the patient at hand. As an example, acceptable input RNA for Affymetrix profiling of paraffin-embedded tissue was defined by Roberts et al[56] as RNA that, by RT-qPCR, had an endogenous *ACTB* 3′/5′ ratio of <20, a C_T <7 between *ACTB* and the exogenous Agilent/Stratagene Universal Reference RNA, and an endogenous 28s ribosomal RNA C_T <15. In our laboratory, quality indicators before Agilent profiling are listed in Table 1. After hybridization, an example of quality metrics used to vet Agilent array data on frozen tissue is shown in Figure 2. For Affymetrix

Table 1. Example Quality Indicators on RNA Processed from Frozen Tissue in Preparation for Expression Profiling of Cancer

Quality indicator	Description
1	The tissue specimen is rejected if there are not at least 30% malignant cells, as determined by a pathologist using microscopy
2	The RNA specimen is rejected if either of the following is true: A260/A280 is <1.8, as determined using Nanodrop (Wilmington, DE) spectrophotometry; or the RNA integrity no. is <6.5, as determined using an Agilent Bioanalyzer 2100
3	The labeled cRNA is rejected if either of the following is true based on results of Nanodrop fluorometry: there is <10 μg of amplified labeled cRNA or specific activity is <8 pmol Cy5/μg labeled cRNA

Quality indicators and limits of acceptability must be established using evidence gathered during validation studies for the particular test being developed.

arrays, Staal et al[24] checked the 3′/5′ ratio for selected housekeeping genes (ideal, 1; good, <3); noise; scaling factor (should be lower than three-fold); visual check of image for bubbles, scratches, and grid alignment; homogeneity of hybridization and uniformity of background; and percentage of genes detectable (>25%).

Individual markers of critical importance may be chosen as a quality check, such as *ESR1* (estrogen receptor) RNA expression in breast cancer compared with ESR1 protein that has been tested by immunohistochemistry (IHC) on the same surgical specimen.[97] Although it is recognized that RNA and protein-based tests may be discrepant for biological rather than technical reasons, the frequency of such discrepancies can be determined during validation studies to identify quality indicators that raise a red flag to be weighed in concert with results of other controls and quality checks.

When redundant assays are present on the array, replicates are examined for consistency or to find problematic variations.[98] As an example, a highly proliferative tumor (identified by visualizing mitotic figures during the tissue selection phase) is expected to express proliferation markers on the array, thus confirming coordinated expression of those phenotypic markers that instill confidence in the array-based results.[99]

Data analysis is done in the context of a thorough understanding of the technical strengths and weaknesses of the test system, based on prior experience gathered during validation work and subsequent clinical practice. Software is relied on to present data in a manner that facilitates its interpretation.[100] A typical standard operating procedure for data analysis involves normalization for background or housekeeping transcript abundance, log transformation to aid in comparing numeric results with those in the validation set, and graphic display of pertinent findings.[77,78,101–104] Overmanipulation of the data should be avoided.[105]

To categorize results in a given patient, a predictive model may be applied that finds patterns across many analytes, as facilitated by a single-sample predictor algorithm.[21,59,106–110] Grouping global patterns of gene expression is a unique strength of RNA expression profiling. A categorical assignment is typically accompanied by a statistic (eg, Spearman's correlation coefficient) representing degree of confidence[12] (Figure 3).

Computer-generated scores or predictors are checked to ensure that they make sense after evaluating pertinent raw data. Unsupervised clustering with a heat map dendrogram visually displays results of a given patient alongside results of a panel of known specimens, to help confirm categorization of the patient into the pre-established group that most closely shares its expression profile.[110] It is important to recognize the limitations of hierarchical clustering, especially because the patient may not belong to any of the pre-established groups. Exogenous controls should be chosen to check categorization at the most critical medical decision points, rather than using only canonical examples that minimally challenge the test system.

Clinical Interpretation and Reporting

Clinical interpretation is the process by which the medical significance of the result is judged in light of the clinical indication for which the test was ordered. Even if the end

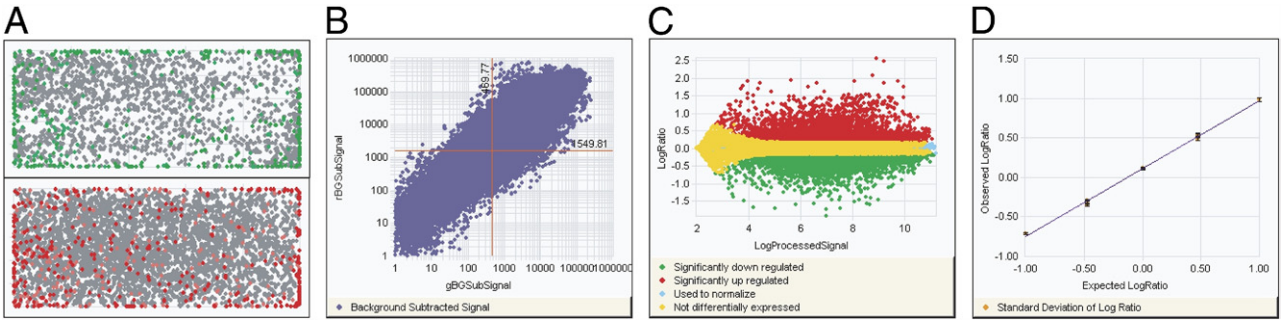

Figure 2. Quality metrics are displayed by Agilent feature extraction software (version 10.5.1.1) from a two-color gene expression experiment on an Agilent microarray system. Acceptability limits might include uniform spatial distribution, with local background of red and green signal <2% and numbers of features nonuniform <5% (**A**); the dynamic range of expression exceeding five orders of magnitude (**B**); even distribution of significantly up- or down-regulated genes across signal intensities (**C**); and spike-in RNA measurements being linear, with a slope >0.9, $R^2 > 0.85$, and replicate reproducibility signified by BGSubSignal <13 and processed signal <6 (**D**).

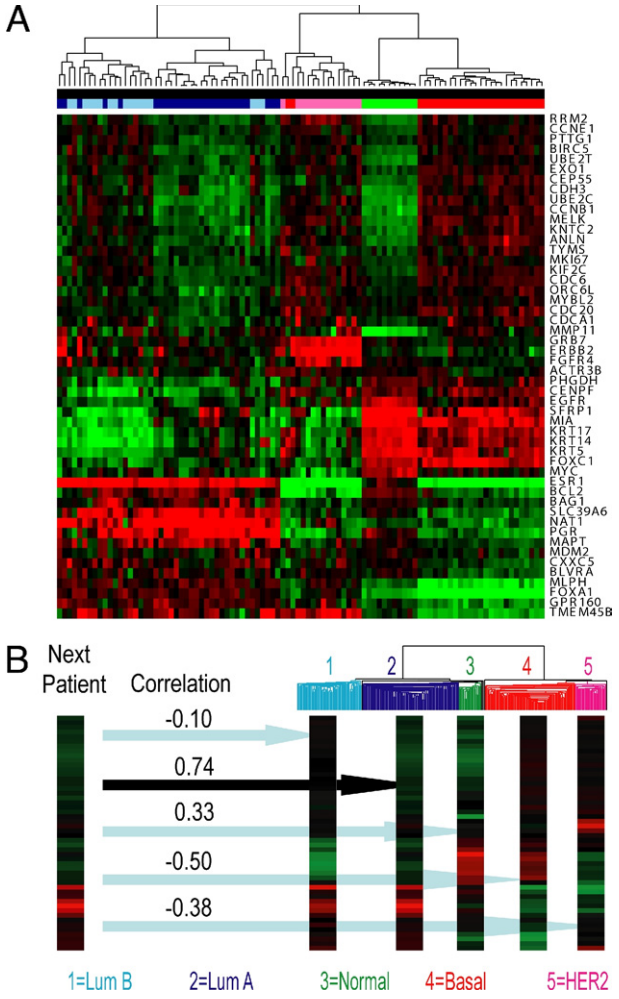

Figure 3. Raw data are interpreted after evaluation of virtually all approximately 22,000 human protein-coding genes using Agilent microarrays. **A:** A heat map shows gene expression profiles of 96 specimens tested for 50 listed genes, and unsupervised hierarchical clustering reveals distinct patterns of expression. **B:** To classify intrinsic subtypes of breast cancer, a single-sample predictor algorithm compares each patient's expression pattern with the consensus pattern for each of the five intrinsic subtypes, and Spearman's correlation coefficients help estimate the certainty of the classification. In the example shown, the patient's profile matches most closely with luminal (Lum) A, although Lum B subtype cannot be excluded, whereas three other subtypes (normal, basal, and HER2) are excluded based on low correlation coefficients.

result of analytic interpretation is an objective numeric score or a discrete disease classification, it is useful for that result to be further interpreted in light of input tissue characteristics; pertinent limitations of the assay, as revealed by controls and quality checks (eg, specimen thawed during transport and marginal housekeeping RNAs recovered); and the level of confidence in the result. Furthermore, correlative analysis with other patient information (eg, age, sex, tumor stage, IHC results, or flow cytometric findings) paints a more complete picture beyond the snapshot of expressed genes. More important, the clinician deserves a clear message about implications for clinical decision making and recommended follow-up. A report describing the *BCR-ABL1* profile in a newly diagnosed lymphoblastic leukemia could expound on the implications for response to tyrosine kinase inhibitor therapy.[111,112] Furthermore, follow-up testing by RT-qPCR might be recommended to identify an amplifiable translocation of *BCR-ABL1* p210 or p190 that could be serially measured as a biomarker of response during therapy.[112]

To maximize the benefit of array-based testing, interpretation by a physician who is expert in disease pathobiological features and molecular technology is required. This laboratory physician takes responsibility for the analysis and for the quality control processes that were used in generating their interpretation. An experienced pathologist is capable of recognizing unusual or outlier results, analogous to unforeseen results generated from histopathological characteristics. For example, careful analysis of RNA expression data may reveal erroneous software-generated calls at variance with clinicopathological findings. A melanoma tissue that was categorized into good versus bad prognosis groups by a software algorithm might, on closer inspection of raw data, lack the typical melanoma markers, thus triggering investigation of whether the patient has a look-alike tumor. Known melanocyte markers that could be used in this evaluation include *MAGEA1*, *MITF*, *MART1* (*melan-A*), *CMM* (*HMB45*), *S100*, and *TYR* (*tyrosinase*), each of which is confirmable using IHC for the corresponding protein.

It is clear that medical judgment is required to identify misleading data and to interpret the significance of findings in light of evidence from patient records, published literature, validation work, databases, and other reliable sources.[113] Pertinent results and interpretation are incorporated into a concise, yet informative, report that is entered into the patient's medical record. As described in CAP guidelines for molecular test reporting,[114] the document should include a written summary of results and an interpretation facilitating medical decision making. Review of draft reports can help catch clerical errors. Related quality assurance tactics include periodic independent appraisals, checking report format after electronic transmission, and surveying client satisfaction.[115]

Data Set Retention

CAP suggests that patient test records be retained for at least 2 years. The US federal regulations protect the privacy of those records and require that the corresponding procedure manual be retained, which serves to annotate each patient data set with the methods that were used to generate it. When the full human transcriptome is profiled, but only selected results are required for data analysis and interpretation, software can then be programmed to mask irrelevant data. Later, one could mine the same data set for the patient's benefit, analogous to re-examination of histopathological slides and blocks in light of the following: i) refined criteria for microscopic diagnosis, ii) availability of novel immunostains, iii) concerns about accuracy, or iv) changes in clinical status. Although RNA profiling might initially be used to help make a diagnosis, later, the same data set could be mined sequentially for prognosis and prediction of response to first- and second-line therapies.

Design Strategies to Minimize Error

Clinical laboratorians tend to choose methods that are tried and true. It is, therefore, not surprising that clinical laboratories are just now adopting expression profiling after years of use and refinement in research settings.[116–118] RNA profiling is a complex process, and each unique reagent, piece of equipment, or manual action could be faulty. A principle of good assay design is to restrict assay components to those that are safe (for patients and health care workers) and simple, while still meeting the clinical objective. Unlike in research settings, turnaround time is critical, so the reliability of each assay component is a key factor in the success of the medical service. Translational research teams should include clinicians who will order the test and act on test results, technology experts who advise on platforms and reagents, and clinical laboratorians who will vet, perform, and interpret test results.

The transfer of a specimen or its derivative to a new vessel requires meticulous care to maintain specimen identity and integrity.[119,120] Barcodes facilitate labeling and tracking throughout the many stages of testing.[120,121] Robotic instruments should be programmed to minimize risk of carryover or aerosolization, and each instrument undergoes regular maintenance and is checked regularly for its performance. Data transfer requires a similarly scrupulous process for maintaining its integrity.

Proficiency Testing

Formal proficiency surveys are offered for many individual RNA-based tests, such as hepatitis C viral load and *BCR-ABL1* quantification. The CAP's Cytogenomic Microarray Survey is a model for interlaboratory comparison of array-based data, although this particular survey challenges DNA-based, rather than RNA-based, microarray services.[122] Proficiency surveys are meant to do the following: i) periodically check assay performance, ii) serve an educational function for laboratory members, and iii) promote quality improvement by sharing information on current methods.[123–125] The CAP and the Association for Molecular Pathology have many resources supporting quality assurance and proficiency, including help in identifying another provider with whom to exchange specimens. In addition to interlaboratory exchange programs to check for comparable signatures or individual analytic test results, an alternative assessment method is to reanalyze internal samples as if they were unknowns.[126]

On the Horizon

This article describes the many principles of laboratory medicine that guide us in validating, implementing, and maintaining RNA-based tests. Strategies are described to check patient-specific and generic assay performance at critical junctures during each multistep protocol. With proper attention to assay design and quality assurance, it is clear that RNA profiling provides robust, accurate, and reproducible results that are powerful by virtue of the number of analytes that are evaluated, the informative signatures reflecting disease status in meaningful new ways, and the redundancy that boosts confidence in the findings. On the horizon are full transcriptome sequencing technologies that are likely to bring greater precision for quantifying RNA and characterizing splice variants.[118]

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