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Interpreting Gene-Expression Diagnostics: From Molecular Signatures to Clinical Classification

White Paper

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Abstract

Gene-expression diagnostics fundamentally differ from conventional molecular target-detection assays (e.g., pathogen diagnostics) because they characterize patients' biological response patterns rather than the presence or absence of a predefined molecular target. Instead of asking whether a defined DNA or RNA sequence is present, gene-expression assays evaluate coordinated expression patterns of multiple human genes that reflect the current biological state of a tissue. As a consequence, gene-expression classifiers typically generate continuous scores or class probabilities, which may subsequently be translated into clinically interpretable categories, rather than inherently binary results. This white paper explains how multigene-expression classifiers are developed, why probabilistic outputs are expected, and why an equivocal region can represent an appropriate and scientifically justified result category rather than a limitation of the assay.

Gene Expression Diagnostics

Gene-expression diagnostics measure the transcriptional activity of selected human genes, typically by quantifying messenger RNA (mRNA), to characterize the biological state of a tissue. Rather than detecting a single predefined disease-associated molecular target, they assess patterns of cellular activity associated with physiological or pathological processes by evaluating the expression of multiple genes in combination [1,2].

Gene expression represents a dynamic molecular phenotype. Although every nucleated human cell within an individual contains essentially the same genome, only a subset of genes is actively transcribed at any given time. Gene-expression patterns vary with cell type and in response to physiological, environmental, and pathological processes. Consequently, the measured expression profile provides information about the current functional state of a tissue that is not captured by its underlying DNA sequence alone [2,3].

Disease-associated biological processes rarely alter the expression of a single gene in isolation. Instead, they induce coordinated changes across multiple pathways, resulting in characteristic expression patterns – or molecular signatures – that can distinguish healthy tissue from disease or differentiate between clinically similar conditions. These multigene signatures form the basis of modern gene-expression diagnostics [3].

For many complex diseases, a single gene-expression marker may not provide sufficient specificity or robustness for the intended diagnostic application. In such cases, combining the expression of multiple marker genes into a molecular signature can capture complementary biological information and improve diagnostic robustness and performance compared with individual biomarkers [2,4].

A Brief Comparison with Pathogen Diagnostics

Gene-expression diagnostics and pathogen diagnostics answer fundamentally different biological questions.

Nucleic-acid-based pathogen diagnostics directly detect molecules originating from an infectious organism, such as pathogen-specific DNA, RNA, or proteins. The analytical question is therefore whether a predefined molecular target from this organism is present in the sample [5].

Instead of detecting a single disease-specific molecule, gene expression diagnostics evaluate coordinated changes in the expression of multiple human genes. These expression markers are combined into a molecular signature and may be normalized using validated reference genes to provide a robust molecular representation of the underlying biological process [2]. This approach is particularly valuable for inflammatory and other non-infectious diseases, where a disease-specific molecular target may not exist and different conditions may share similar clinical and histopathological features [6]. Consequently, gene-expression diagnostics require the integration and interpretation of multiple expression measurements, rather than solely determining the presence or absence of a predefined molecular target.

This distinction is important because it determines how results are interpreted. Whereas pathogen diagnostics primarily determine whether a predefined molecular target is present, gene-expression diagnostics classify complex biological expression patterns. Because the molecular patterns associated with different biological states may overlap rather than forming completely discrete categories, classifier outputs are naturally continuous and are interpreted using validated decision thresholds rather than representing an inherently binary measurement [7].

	Gene-expression diagnostics	**Pathogen diagnostics**
Primary target	Human gene-expression patterns associated with biological processes or states	Pathogen-derived molecules
Biological question	Which biological state best matches the observed expression pattern?	Is a specific pathogen present?
Measured analyte	Human mRNA expression	Pathogen DNA, RNA or antigen
Typical result	Continuous classifier score / class probability	Target detected / not detected (or quantitative load)
Clinical interpretation	Disease classification using validated decision thresholds	Identification or exclusion of a specific pathogen
Example	Classification of inflammatory skin diseases based on multigene-expression signatures	Detection of HPV DNA, SARS-CoV-2 RNA or fungal DNA

Table 1. ***Comparison of gene-expression diagnostics and pathogen diagnostics***. *The table summarizes the fundamental differences in the biological targets measured, the questions addressed, the methods used, and the resulting clinical information.*

The different biological questions addressed by gene-expression and pathogen diagnostics (Table 1) also determine how their results are interpreted. Whereas pathogen diagnostics primarily identify the presence or absence of a predefined molecular target, gene-expression diagnostics classify complex biological expression patterns that may exist on a continuum. Consequently, their outputs are typically continuous and interpreted using validated decision thresholds rather than representing inherently binary measurements (Figure 1).

From Gene Expression to Diagnostic Classification

Gene-expression diagnostics do not classify individual genes, but the overall expression pattern observed across multiple markers. The development of a diagnostic classifier therefore begins with

a clearly defined clinical question, such as distinguishing between two diseases with overlapping clinical or histopathological features.

During biomarker discovery, well-characterized patient samples are analyzed to identify genes whose combined expression provides diagnostically informative discrimination between the conditions of interest. Rather than relying on a single marker, a carefully selected panel of genes is used to capture complementary information associated with the underlying biological process [2].

To ensure that expression measurements are comparable across samples, marker-gene expression may be normalized using validated reference genes with sufficiently stable expression across the relevant tissues, disease states, and experimental conditions. **Reference genes therefore represent an integral component of the normalization strategy of the diagnostic assay rather than merely serving as internal controls. When appropriately validated for this purpose, reference-gene amplification may additionally serve as an internal process control, providing information on sample adequacy and successful progression of the analytical workflow [2,8,9].**

The normalized expression values form a multidimensional molecular signature that serves as input for a statistical or machine-learning classifier. As part of this process, the normalized marker-gene expression levels may be mathematically transformed or grouped into distinct features. Unlike conventional molecular assays that assess individual targets independently, gene-expression classifiers simultaneously integrate information from all marker and reference genes. Consequently, the diagnostic result is based on the overall molecular expression pattern rather than on the expression level of any individual gene.

Using a training cohort with confirmed reference diagnoses, the classifier is developed to model the mathematical relationship between expression patterns and diagnostic classes. The complete workflow – including biomarker selection, normalization strategy, classifier, and decision thresholds – must subsequently be validated in an independent clinical cohort representative of the intended use population [10].

Although the examples presented in this white paper focus on binary classification for simplicity, the same principles apply to multiclass and multilabel classifiers, which distinguish between more than two diagnostic classes or allow assignment to multiple classes simultaneously.

Interpreting Classifier Outputs

Unlike assays designed solely to detect the presence or absence of a predefined molecular target, gene-expression classifiers can produce a continuous output rather than an inherently binary result. Depending on the classifier and its calibration, this **output may be expressed as a classifier score, confidence measure, or probability of class membership.** Importantly, unless separately demonstrated and validated, **these values should not be interpreted as direct measures of disease severity or progression.** Instead, they reflect the classifier's assessment of the observed gene-expression pattern with respect to the diagnostic classes on which it was trained. Accordingly, the reported value reflects the classifier's interpretation of a multidimensional expression pattern rather than the measurement of an individual analyte. **Higher probabilities or scores indicate stronger agreement with one diagnostic class, whereas intermediate values indicate increasing uncertainty in class assignment [10].**

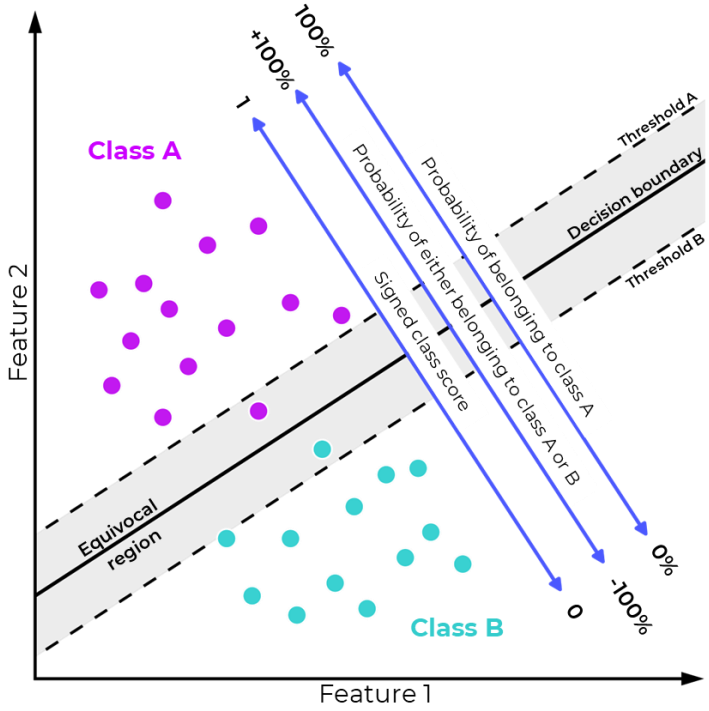
To convert a continuous output into clinically interpretable categories, predefined **decision thresholds** are established and validated during assay development. **Samples with outputs beyond the respective thresholds are assigned to the corresponding diagnostic class in accordance with the validated classification rule. Where an equivocal region is defined, outputs within this region are reported as equivocal rather than being assigned to either diagnostic class. The diagnostic classification is therefore determined by applying the validated decision rule to the classifier output [10].**

Figure 1 illustrates different ways in which a continuous classifier output can be expressed and translated into diagnostic classes using validated decision thresholds. Similar output formats are used in gene-expression assays developed by Dermagnostix: the PsorX-LabDisk reports a probability of assignment to the molecular signature of psoriasis versus eczema, corresponding conceptually to Example 1, whereas the Erysk-TargetDisk uses a signed class score, as illustrated conceptually in Example 3. The numerical values shown in Figure 1 are illustrative and do not represent the decision thresholds of these assays. Samples located close to the decision boundary fall within the equivocal region, where classification into either class does not meet the predefined criteria for a definitive result.

Classifier outputs near a decision boundary may reflect overlapping molecular expression patterns as well as biological and analytical variability, resulting in greater uncertainty in class assignment. Where such uncertainty is relevant to the intended use of the assay, a predefined equivocal region can be used rather than forcing these outputs into one of the diagnostic classes [10].

The equivocal region does not in itself indicate assay failure. Instead, it represents a deliberately defined and validated result category that transparently reflects uncertainty while reducing the risk of overconfident or potentially misleading classifications near the decision boundary. An analytically valid measurement can therefore yield an equivocal classification: analytical validity indicates that the predefined quality requirements for result generation have been met, whereas the equivocal classification describes uncertainty in assigning the measured molecular expression pattern to either diagnostic class.

Samples within the equivocal interval have classifier scores for which the validated assay cannot reliably distinguish between the reference molecular classes. Such results may arise from overlapping molecular phenotypes, biological heterogeneity, intermediate biological features, or analytical and sampling variability. An equivocal result should not be assigned to either class based on the molecular score alone and should be interpreted in conjunction with the clinical, histopathological and other relevant findings.



Example 1: Probability of belonging to class A	
Decision boundary	50%
Equivocal region	40% to 60%
Threshold Class A	> 60%
Threshold Class B	< 40%
Example 2: Probability of either belonging to class A or B	
Decision boundary	0
Equivocal region	-25% to +25%
Threshold Class A	> 25%
Threshold Class B	< -25%
Example 3: Signed class score	
Decision boundary	0.5
Equivocal region	0.4 to 0.6
Threshold Class A	> 0.6
Threshold Class B	< -0.4

Figure 1. ***Continuous classifier outputs and their relationship to a decision boundary.*** *Gene-expression classifiers convert normalized multigene-expression profiles into a continuous classifier output. The schematic*

illustrates three exemplary ways in which the output of a binary classifier may be represented: (1) as the probability of belonging to one class, (2) as the probabilities of belonging to either class A or class B, or (3) as a signed class score. In the two-dimensional feature space shown on the left, the decision boundary separates classes A and B. The classifier output changes with the position of a sample relative to this boundary, with values closer to the boundary generally indicating less distinct class assignment. Depending on the classifier and its calibration, continuous outputs may subsequently be translated into diagnostic categories using predefined and validated decision thresholds. An equivocal region may be defined around the decision boundary for samples that do not meet the predefined criteria for assignment to either class. Samples falling within this region do not meet the predefined criteria for reliable assignment to either diagnostic class and may represent overlapping molecular phenotypes, biological heterogeneity, intermediate biological features, or analytical and sampling variability. The representations and numerical scales shown are illustrative and do not represent universal classifier outputs or diagnostic thresholds.

Conclusion

Gene-expression diagnostics differ fundamentally from molecular target-detection assays because they characterize complex biological expression patterns rather than detecting individual molecular targets. Consequently, diagnostic classification is typically based on continuous molecular information rather than naturally binary measurements. Classifier scores, estimated probabilities, validated decision thresholds, and, where appropriate, an equivocal region are therefore not limitations of gene-expression diagnostics but components of their interpretation and reporting. When developed and clinically validated appropriately, multigene-expression classifiers provide an objective molecular complement to clinical and histopathological assessment, particularly for diseases characterized by overlapping phenotypes.

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