



*Reprinted from*

# the Journal of Molecular Diagnostics

Official Journal of the Association for Molecular Pathology

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# Performance and Clinical Evaluation of the 92-Gene Real-Time PCR Assay for Tumor Classification

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**Accurate determination of cancer origin is necessary to guide optimal treatment but remains a diagnostic challenge. Gene expression profiling technologies have aided the classification of tumors and, therefore, could be applied in conjunction with clinicopathologic correlates to improve accuracy. We report an expanded version of the previously described 92-gene assay to classify 30 main tumor types and 54 histological subtypes, with coverage of  $\geq 95\%$  of all solid tumors based on incidence. Increased tissue coverage was achieved through expansion of a reference tumor database containing 2206 specimens, with a median of 62 samples per main tumor type. The 92-gene classification algorithm demonstrated sensitivities of 87% and 85% for 30 main types and 54 histological subtypes, respectively, in leave-one-out cross validation, and 83% in a test set of 187 tumors representing 28 of the 30 main cancer types. These findings provide further support that broad and diverse tumor classification can be performed using a relatively compact gene set. An additional 300 consecutive cases submitted for clinical testing were profiled to characterize clinical utility in a real-world setting: the 92-gene assay confirmed 78% of samples having a single suspected primary tumor and provided a single molecular prediction in 74% of cases with two or more differential diagnoses. Further development of the 92-gene RT-PCR assay has resulted in a significant expansion in reportable tumor types and histological features with strong performance characteristics and supports the use of molecular classification as an objective standardized adjunct to current methods. (J Mol Diagn 2011, 13:493-503; DOI: 10.1016/j.jmoldx.2011.04.004)**

Current paradigms of evidence-based patient management require more accurate diagnosis and better classification of tumors. In addition to broad site-directed treatments, newer strategies include agents that selectively target molecular pathways within a specific cellular context or tissue.<sup>1,2</sup> As such, precise determination of the site of tumor origin is an important step toward improving

diagnosis and optimizing treatment selection. In the context of metastatic disease, pathological diagnosis is challenging when the morphological features or clinical presentation of the tumor is overlapping or atypical.<sup>3,4</sup> Immunohistochemical (IHC) analysis is the cornerstone of histopathological evaluation; however, selection of immunostaining protocols and interpretation of equivocal cases remain subjective.<sup>5,6</sup> Integration of standardized molecular assays to aid in identification of the primary tumor site may serve as quantitative complements to current diagnostic procedures.

Numerous studies<sup>7-11</sup> have demonstrated the utility of gene expression profiling technologies in multiclass cancer diagnosis. Several methods have been developed for clinical application and include microarray- and RT-PCR-based platforms that are commercially available.<sup>12-14</sup> Reported predictive accuracies are in the range of 80% to 90% using independent validation sets.<sup>12-15</sup> These cancer classifiers quantify the molecular similarity of the gene expression profile from a sample tissue to a reference database of known tumor types. To obtain sufficient data for accurate classification, the scope of the reference database must include a sufficient number of samples within each class that also represent the inherent cellular heterogeneity of tumors. In addition, tissue coverage, or the number of tumor types and subtypes that are classified, is another important requirement for clinical utility. Limited tissue coverage is a drawback of current molecular tests. Expansion of test panels while maintaining or improving accuracy would potentially increase the clinical impact of these diagnostic assays.

We previously reported the development of a 92-gene real-time RT-PCR assay (CancerTYPE ID; bioTherapeutics, Inc., San Diego, CA), with an accuracy of 84% for the classification of 39 morphological features.<sup>14</sup> The 92 genes, 87 for content and 5 for normalization, were identified by a data-dependent search for gene combinations optimal for multitumor classification from a whole-ge-

The study was funded by and completed at bioTherapeutics, Inc.

Accepted for publication April 15, 2011.

The authors are employees or former employees and stockholders in bioTherapeutics, Inc.

Supplemental material for this article can be found at <http://jmd.amjpathol.org> or at doi: 10.1016/j.jmoldx.2011.04.004.

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nome expression profile of 578 tumor samples.<sup>14</sup> The key advantage of this approach was that the genes evolved as a set, rather than as individual biomarkers, and that their collective expression is used for each tumor type classification. Therefore, the 92-gene panel was developed with inherent scalability to classify a broad spectrum of tumor types.

Herein, we report the use of the previously described 92-gene assay for the generation of a reference tumor database, with a significant increase in tumor coverage and depth. In addition, a novel two-step classification algorithm was developed to enable the classification of 30 main cancer types and 54 histological subtypes. The performance and preliminary clinical utility of this second-generation version of the 92-gene RT-PCR assay are also presented.

## Materials and Methods

### Tumor Samples

Tumor samples for reference database construction ( $N = 2557$ ) and for an independent sample set ( $N = 187$ ) were obtained from multiple tumor banks and commercial sources located in the United States (ABS providers 1–3, Wilmington, DE; Ardais/Cytomyx, Lexington, KY, and Boston, MA; Asterand, Detroit, MI; Bioserve, Beltsville, MD; Exiqon, Tustin, CA; ILSbio, Chestertown, MD; Massachusetts General Hospital, Boston; Proteogenex, San Francisco, CA; Sharp Hospital, San Diego, CA; and Wake Forest University School of Medicine, Winston-Salem, NC) and in Europe (Imperial College, London, UK; and Nice Hospital, Nice, France). Patient consent and Institutional Review Board approval were obtained for all samples, in accordance with the NIH human research study guidelines. Approximately 84% of the tumor samples were from formalin-fixed, paraffin-embedded (FFPE) blocks, and 16% were from snap-frozen tissues. To confirm diagnosis, independent review by a board-certified anatomical pathologist was performed for each case by examining the pathological report and analysis of H&E-stained sections by light microscopy. All cases were reviewed by at least two pathologists. All samples were deidentified, assigned internal accession numbers, and recorded with full pathological diagnosis information (ie, site of biopsy, tumor type, and primary or metastatic biopsy specimen) and basic patient demographics (ie, age, sex, and ethnicity). Samples were analyzed as described later.

### RNA Extraction and Amplification

The H&E slides from all samples were reviewed by a pathologist to mark the areas with the greatest tumor content to reach at least 80% enrichment of tumor cells. Tumor cells were enriched by dissection, and total RNA was isolated from cancer-enriched areas of FFPE specimens and frozen tissue sections (7- $\mu\text{m}$  thick). Total RNA was DNase treated to ensure the complete removal of contaminating genomic DNA. First-strand cDNA was synthesized and then preamplified (PreAmp; Life Technolo-

gies, Carlsbad, CA). The 92-gene assay (real-time RT-PCR) was performed as previously described<sup>14</sup> on the preamplified cDNA product using an ABI 7900HT instrument (Applied Biosystems, Inc., Foster City, CA) with the same primer-probe designs for the 87 tumor-associated genes and 5 reference genes, as previously described.<sup>14</sup>

### Final Database Sample Inclusion-Exclusion

RT-PCR data for 2557 tumor samples were compiled. The mean  $C_T$  values of the five reference genes were calculated for all samples. The samples with a reference mean  $C_T > 28$  were considered as outliers based on box plot analysis and not included in the database. Samples with inadequate tumor content or inconsistent or inconclusive pathological information, based on independent pathological review of the H&E slides and the available pathological information, were also excluded. The final database included 2206 samples.

### Classification Algorithm

The 92-gene expression profiles of the tumor samples in the training set ( $N = 2206$ ) were used as inputs for the modification of a previously described tumor classification algorithm.<sup>14</sup> To correct for input variation, for each sample, cycle threshold ( $C_T$ ) measurements of 87 genes of content were normalized to 5 reference genes. Tumor classification was structured for a two-level labeling scheme: each sample was associated with a main class label (eg, ovary) and a subclass label (eg, ovary-serous). Logistic regression was used to generate a probability for each level of classification generated based on the degree of similarity of the queried sample to the reference tumor database. The predicted main cancer type with the largest probability was considered the main cancer type, and the predicted subtype with the largest probability within that main type was designated the top prediction subtype. In addition to the main predicted type, main types with  $>5\%$  probability and main types with  $<5\%$  probability (or rule-out types) were also calculated and were reported for clinical cases. Leave-one-out cross-validation analyses within the entire 2206-sample reference data set and a separate test set ( $N = 187$ ) were used to estimate the performance of the new algorithm.

### Observational Study of Clinical Case Series

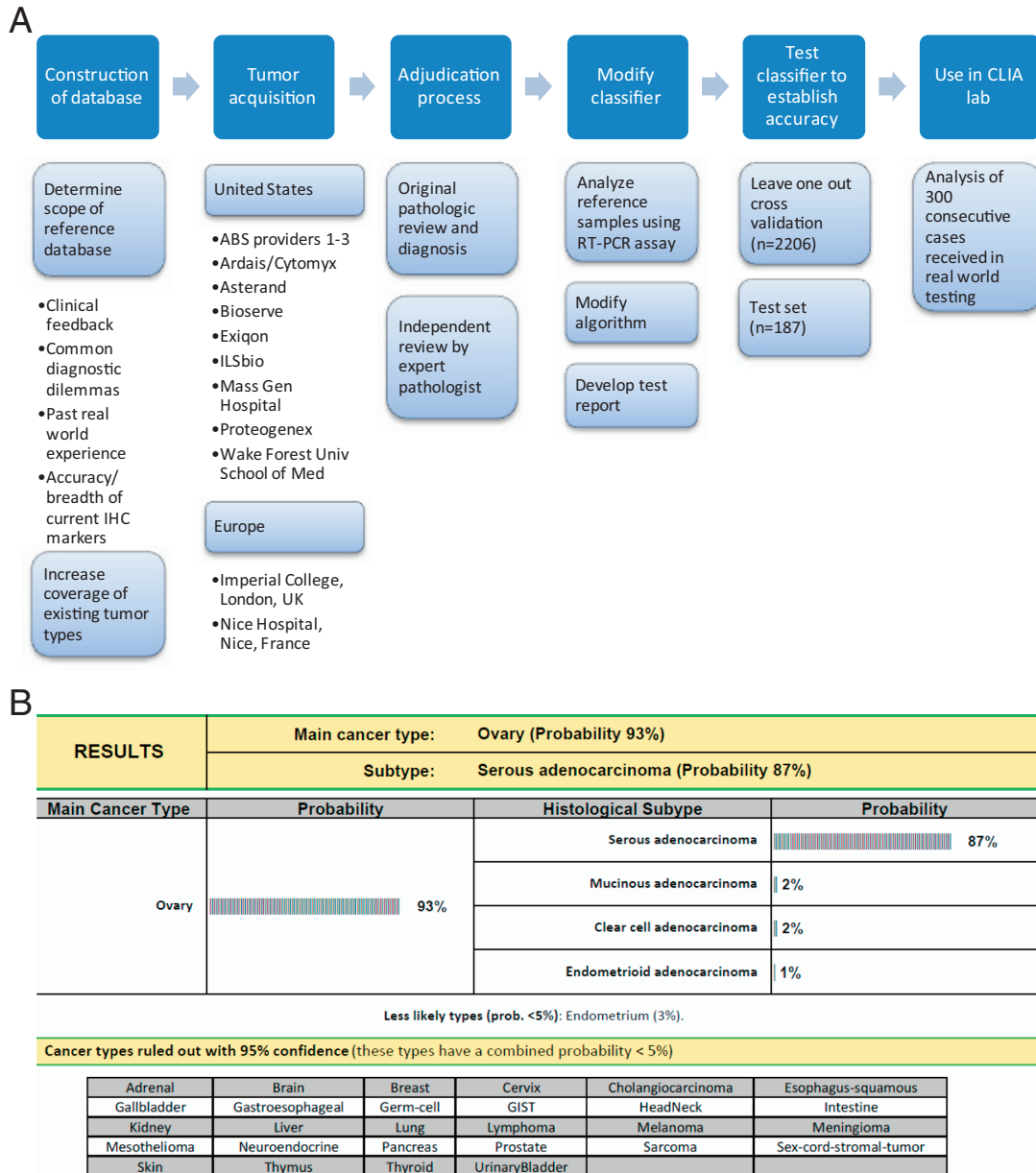
The first 319 consecutive cases submitted for testing that used the expanded version of the 92-gene assay (described herein) were included in a case-series observational study using available data from deidentified delinked patients. During this period, 17 samples did not have sufficient RNA quantity or quality (samples with a reference mean  $C_T > 28$ ) and were not included in this analysis. In addition, two cases that did not have pathological reports available were excluded from the study. A total of 300 cases were subsequently analyzed by the 92-gene assay, and predictions were reported as previously described. Laser microdissection was used in cases from the observational case study, as deemed by

the medical director, a board-certified anatomical pathologist. Per standard operating procedures within the clinical laboratory, the medical director reviewed all cases and completed test sign out on test reports.

*Clinical Case Evaluation*

The biopsy site, suspected primary site(s), morphological characteristics, tumor grading, and IHC analyses for the 300 cases in the observational study were provided by

the physician when requesting the test. In this analysis, descriptions of moderately to poorly differentiated were considered poorly differentiated and of moderately to well differentiated were considered moderately differentiated. In collecting and handling diagnosis data from the provided pathological reports, if only one primary site was listed, the case was considered a suspected primary for confirmation. When two or more suspected primary sites were listed, the case was considered a differential diagnosis. Cases for which the primary site was de-



**Figure 1.** Expansion of the 92-gene assay reference database. **A:** Schematic of the 92-gene assay revisions. The reference database was expanded to include 2206 tumors from multiple tumor banks in the United States and Europe. New types were chosen based on real-world experience from past clinical cases and to encompass difficult-to-diagnose cancer types. Diagnosis of histological tissue types was made by full pathological workup, and all cases were reviewed by at least two independent pathologists. After modification of the database and algorithm, the accuracy of the 92-gene assay was assessed using leave-one-out cross validation of the 2206 samples and an independent test set. CLIA indicates Clinical Laboratory Improvement Amendments. **B:** The 92-gene assay two-tier classification test report. The type and probability of the top prediction is noted at the top of the report in the yellow box, and the probabilities of other tumor types with significant similarity are listed below. The probability for each tumor type is determined based on the similarity of the 87-gene profile of the sample to each tumor type in the reference data set. The bottom table contains tumor types that are not predicted to be the tumor type, with >95% confidence. These main types and their corresponding histological subtypes can be ruled out. ABS, Analytical Biological Services, Inc.; CLIA, Clinical Laboratory Improvement Amendment; GIST, gastrointestinal stromal tumor.

**Table 1.** Scope of Tumor Reference Database

Tumor types	Histological types
Adrenal	
Adrenocortical carcinoma	Adrenal cortical carcinoma
Pheochromocytoma	Pheochromocytoma
Brain	Astrocytoma (anaplastic or fibrillary), oligoastrocytoma, glioblastoma, oligodendroglioma
Breast	Adenocarcinoma of the breast (not otherwise specified, mucinous, medullary, inflammatory, lobular, or ductal)
Cervix	
Adenocarcinoma	Adenocarcinoma of the cervix (endometrioid, clear cell, papillary serous, or mixed), adenosquamous carcinoma of the cervix
Squamous cell carcinoma	Carcinoma of the cervix (squamous cell)
Cholangiocarcinoma	Cholangiocarcinoma
Endometrium	Adenocarcinoma of the endometrium (endometrioid, papillary serous, or clear cell), adenosquamous carcinoma of the uterine body
Esophagus (squamous cell)	Squamous cell carcinoma of the esophagus
Gallbladder	Adenocarcinoma of the gallbladder
Gastroesophageal (adenocarcinoma)	Adenocarcinoma of the stomach, adenocarcinoma of the esophagus
Germ cell	
Nonseminomatous	Tumor, yolk sac tumor of the testis/ovary, carcinoma of the testis embryonal, teratoma of the testis/ovary, choriocarcinoma, mixed germ cell
Seminomatous	Seminoma of the testis; dysgerminoma of the ovary
GIST	Gastrointestinal stromal tumor
Head/neck	
Salivary gland	Adenoid cystic carcinoma; acinar cell carcinoma; adenocarcinoma, not otherwise specified
Squamous cell	Squamous cell carcinoma, basaloid squamous cell carcinoma
Intestine	
Colorectal adenocarcinoma	Colorectal adenocarcinoma
Small intestine Adenocarcinoma	Small-intestine adenocarcinoma
Kidney (renal cell carcinoma)	
Chromophobe	Renal cell carcinoma, chromophobe type
Clear cell	Renal cell carcinoma, clear cell
Papillary	Renal cell carcinoma, papillary
Liver	Hepatocellular carcinoma
Lung	
Adenocarcinoma/large cell	Adenocarcinoma of the lung, carcinoma of the lung (large cell), bronchioloalveolar adenocarcinoma
Squamous cell	Lung carcinoma (squamous cell)
Lymphoma	Lymphoma (large B-cell diffuse), Hodgkin lymphoma, lymphoma (peripheral T cell)
Melanoma	Amelanotic melanoma, nodular melanoma, epithelioid cell melanoma, spindle cell melanoma
Meningioma	Meningioma (atypical, fibroblastic, meningothelial, secretory)
Mesothelioma	Mesothelioma (epithelioid, sarcomatoid, or biphasic)
Neuroendocrine	
Gastrointestinal carcinoid	Small-intestine carcinoid
Lung carcinoid	Lung carcinoid
Small-/large-cell lung cancer	Lung (large-cell carcinoma, neuroendocrine), carcinoma of the lung (small cell)
Pancreatic islet cell carcinoma	Islet cell carcinoma of the pancreas
Merkel cell carcinoma	Carcinoma of the skin (Merkel cell)
Ovary	
Clear cell adenocarcinoma	Adenocarcinoma of the ovary (clear cell)
Endometrioid adenocarcinoma	Adenocarcinoma of the ovary (endometrioid)
Mucinous adenocarcinoma	Adenocarcinoma of the ovary (mucinous)
Serous adenocarcinoma	Adenocarcinoma of the ovary (serous)
Pancreas	Carcinoma of the pancreas (acinar cell), carcinoma of the pancreas (ductal mucinous)
Prostate	Adenocarcinoma of the prostate
Sarcoma	
Leiomyosarcoma	Leiomyosarcoma
Liposarcoma	Liposarcoma
Malignant fibrous histiocytoma	Malignant fibrous histiocytoma, myxofibrosarcoma of the soft tissue
Osteosarcoma	Osteosarcoma, chondrosarcoma
Primitive neuroectodermal tumor	Primitive neuroectodermal tumor, Ewing sarcoma
Synovial sarcoma	Sarcoma (synovial): biphasic or monophasic
Sex cord stromal tumor	Sex cord stromal tumor (granulosa), Sertoli Leydig cell tumor, annular tubules
Skin	
Basal cell carcinoma	Carcinoma of the skin (basal cell)
Squamous cell carcinoma	Carcinoma of the skin (squamous cell)
Thymus	Thymoma, thymic carcinoma
Thyroid	
Follicular/papillary carcinoma	Carcinoma of the thyroid (follicular), carcinoma of the thyroid (papillary), Hürthle cell carcinoma of the thyroid
Medullary carcinoma	Carcinoma of the thyroid (medullary)

(table continues)

**Table 1.** *Continued*

Tumor types	Histological types
Urinary bladder	
Adenocarcinoma	Carcinoma of the bladder (urothelial adenocarcinoma cell)
Squamous cell	Carcinoma of the bladder (squamous cell)
Transitional cell carcinoma	Carcinoma of the bladder (transitional cell)

*n* = 2206.  
 GIST, gastrointestinal stromal tumor.

scribed as unknown or included nonspecific site information were considered unknown or uncertain. Only cases that listed cancer of unknown primary tumor as the pathological diagnosis were considered carcinoma of unknown primary origin.

## Results

### Database Expansion and Algorithm Development

Enhancement of the 92-gene cancer classifier was conducted as schematized in Figure 1A. Clinical inputs provided the basis for further development and included the following: i) clinical experience with the existing 92-gene assay, ii) identification of common diagnostic dilemmas,<sup>16</sup> iii) evaluation of classification gaps with standard IHC markers,<sup>17</sup> and iv) consideration of solid tumor types with indicated targeted therapies.<sup>1,2</sup> Results from these analyses clearly indicated that the reference database should be expanded to include an increase in both the breadth of tumor types and the depth per tumor type in order to include relevant subtypes.

The resulting scope and depth of the reference database encompassed 2206 tumors representing 30 main cancer types and 54 histological subtypes, with a median of 62 tumors per main type, ranging from 26 to 228. Tumor coverage included approximately 95% of all solid tumor types based on annual incidence rates (Table 1). All of the major categories of cancer types (ie, carcinoma, sarcoma, melanoma, and hematolymphoid), 15 types of adenocarcinomas, 6 types of squamous carcinoma, 6 types of sarcoma, 5 subtypes of neuroendocrine tumors, and 4 subtypes of ovarian tumors were included. In addition, the reference database contained tumors to aid in common differential diagnosis of uncertain origin and/or in which IHC markers lacked sensitivity or specificity. Targeted tumor types included gastrointestinal tract tumors (cholangiocarcinoma versus gallbladder versus pancreas versus gastroesophageal versus small intestine versus colorectal), squamous carcinomas (head and neck versus lung), tumors present in the ovary (ovarian mucinous versus gastrointestinal tract), and gynecological tumors (cervical versus endometrial versus ovarian versus breast). Furthermore, the expansion or addition of tumor types with targeted therapies was also incorporated, such as breast cancers, gastroesophageal adenocarcinoma, germ cell, gastrointestinal stromal tumor, colorectal adenocarcinoma, renal cell carcinoma, lung cancers, hep-

atocellular carcinoma, pancreatic islet cell carcinoma, pancreatic adenocarcinoma, and prostate cancer.

The next phase of development was acquisition of tumor samples for the construction of the reference database (Figure 1A). Obtaining the necessary tumor scope and depth per tumor type required an extensive network of commercial and academic sources. Each tumor sample was obtained through an Institutional Review Board–approved process and included an accompanying deidentified delinked pathological report that contained a single diagnosis for the tumor. Only samples with the same diagnosis from the initial pathological report and the adjudication review were included within the reference database; 2257 samples met this criterion.

To develop the algorithm, 92-gene expression profiles for each tumor were generated; 2206 samples that contained sufficient RNA (98% success rate; see *Materials and Methods*) were used to develop the classifier. Tumor classification was structured using a two-level approach (Figure 1B): each sample was associated with a main tumor type (eg, ovary) and a histological subtype (eg, ovary-serous). In some cases, the subclass is the same as the main class (eg, cholangiocarcinoma). The algorithm was designed to compare the gene expression profile from a sample of unknown origin with the gene expression profile from tumors of known origin within the reference database. Next, using logistic regression, direct probability was computed for each classification based on the degree of similarity with tumors within the database. The assay was constructed to generate a test report outlining the top prediction, as determined by the main cancer type and histological subtype with the highest probability (Figure 1B). In addition, rule-out cancer types are listed; these types have a combined probability of <5% for a correct prediction (Figure 1B).

### Analytical Characteristics

#### Classification Performance

Initial characterization of assay performance was conducted with leave-one-out cross validation within the entire 2206-sample reference data set. The sensitivity ( $\pm 95\%$  CI), specificity ( $\pm 95\%$  CI), and positive and negative predictive values are listed in Table 2. The overall sensitivity was 87% at the main type level (30 cancer types) and 85% at the histological subtype level (54 subtypes). In addition, the current expansion of the 92-gene assay included 21 new tumor types

**Table 2.** Performance of Main Type and Subtype Predictions: Internal Validation

Tumor types	n	Internal cross validation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
Adrenal	62	0.95 (0.90–1.00)	1.00 (1.00–1.00)	0.95	1.00
Adrenocortical carcinoma		0.91 (0.82–1.00)	1.00 (1.00–1.00)	0.91	1.00
Pheochromocytoma		1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.00	1.00
Brain	32	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.94	1.00
Breast	56	0.95 (0.89–1.00)	1.00 (1.00–1.00)	0.95	1.00
Cervix	68	0.68 (0.57–0.79)	1.00 (0.99–1.00)	0.82	0.99
Adenocarcinoma		0.63 (0.49–0.77)	1.00 (0.99–1.00)	0.76	0.99
Squamous cell carcinoma		0.68 (0.49–0.88)	1.00 (1.00–1.00)	0.83	1.00
Cholangiocarcinoma	77	0.70 (0.60–0.80)	0.99 (0.99–1.00)	0.74	0.99
Endometrium	50	0.70 (0.57–0.83)	0.99 (0.99–1.00)	0.71	0.99
Esophagus (squamous cell)	26	0.77 (0.61–0.93)	0.99 (0.99–1.00)	0.65	1.00
Gallbladder	54	0.52 (0.39–0.65)	0.99 (0.99–1.00)	0.62	0.99
Gastroesophageal (adenocarcinoma)	89	0.75 (0.66–0.84)	0.99 (0.99–0.99)	0.76	0.99
Germ cell	125	0.90 (0.85–0.96)	1.00 (1.00–1.00)	0.96	0.99
Nonseminomatous		0.88 (0.82–0.95)	1.00 (1.00–1.00)	0.94	0.99
Seminomatous		0.97 (0.91–1.00)	1.00 (1.00–1.00)	1.00	1.00
GIST	33	0.97 (0.91–1.00)	1.00 (1.00–1.00)	0.97	1.00
Head/neck	68	0.76 (0.66–0.87)	0.99 (0.99–1.00)	0.81	0.99
Salivary gland		0.78 (0.62–0.93)	1.00 (1.00–1.00)	0.95	1.00
Squamous cell		0.73 (0.60–0.87)	0.99 (0.99–1.00)	0.71	0.99
Intestine	89	0.82 (0.74–0.90)	0.99 (0.98–0.99)	0.76	0.99
Colorectal carcinoma		0.88 (0.80–0.96)	0.99 (0.99–1.00)	0.79	1.00
Small-intestine carcinoma		0.55 (0.37–0.73)	0.99 (0.99–1.00)	0.55	0.99
Kidney (renal cell carcinoma)	51	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.91	1.00
Chromophobe		1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.88	1.00
Clear		0.93 (0.84–1.00)	1.00 (1.00–1.00)	0.90	1.00
Papillary renal cell carcinoma		1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.83	1.00
Liver	43	0.98 (0.93–1.00)	1.00 (1.00–1.00)	0.95	1.00
Lung	134	0.82 (0.76–0.89)	0.99 (0.99–1.00)	0.86	0.99
Adenocarcinoma/large cell		0.76 (0.67–0.85)	0.99 (0.99–1.00)	0.85	0.99
Squamous cell		0.80 (0.69–0.91)	0.99 (0.99–1.00)	0.76	1.00
Lymphoma	61	0.92 (0.85–0.99)	1.00 (1.00–1.00)	0.92	1.00
Melanoma	54	0.93 (0.86–1.00)	1.00 (1.00–1.00)	0.94	1.00
Meningioma	38	0.97 (0.92–1.00)	1.00 (1.00–1.00)	1.00	1.00
Mesothelioma	62	0.87 (0.79–0.95)	1.00 (1.00–1.00)	0.93	1.00
Neuroendocrine	228	0.97 (0.95–0.99)	0.99 (0.99–1.00)	0.94	1.00
Gastrointestinal carcinoid		0.94 (0.85–1.00)	1.00 (1.00–1.00)	0.97	1.00
Lung carcinoid		0.96 (0.90–1.00)	1.00 (1.00–1.00)	0.93	1.00
Small-/large-cell lung cancer		0.92 (0.87–0.98)	0.99 (0.99–1.00)	0.86	1.00
Pancreatic islet cell carcinoma		0.96 (0.90–1.00)	1.00 (1.00–1.00)	0.94	1.00
Merkel cell carcinoma		0.94 (0.85–1.00)	1.00 (1.00–1.00)	0.94	1.00
Ovary	129	0.88 (0.82–0.93)	0.99 (0.98–0.99)	0.78	0.99
Clear cell		0.91 (0.80–1.00)	1.00 (1.00–1.00)	0.88	1.00
Endometrioid		0.70 (0.42–0.98)	1.00 (1.00–1.00)	0.58	1.00
Mucinous		0.83 (0.72–0.95)	0.99 (0.99–1.00)	0.73	1.00
Serous		0.87 (0.78–0.96)	0.99 (0.99–1.00)	0.78	1.00
Pancreas	80	0.81 (0.73–0.90)	0.99 (0.99–1.00)	0.78	0.99
Prostate	59	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.98	1.00
Sarcoma	121	0.97 (0.94–1.00)	0.99 (0.99–1.00)	0.90	1.00
Leiomyosarcoma		0.89 (0.78–1.00)	1.00 (1.00–1.00)	0.89	1.00
Liposarcoma		0.83 (0.66–1.00)	1.00 (1.00–1.00)	0.94	1.00
MFH		0.95 (0.85–1.00)	1.00 (0.99–1.00)	0.69	1.00
Osteosarcoma		0.92 (0.82–1.00)	1.00 (1.00–1.00)	0.83	1.00
PNET		0.73 (0.46–0.99)	1.00 (1.00–1.00)	0.80	1.00
Synovial sarcoma		0.95 (0.85–1.00)	1.00 (1.00–1.00)	0.86	1.00
Sex cord stromal tumor	28	0.79 (0.63–0.94)	1.00 (1.00–1.00)	0.92	1.00
Skin	69	0.88 (0.81–0.96)	0.99 (0.99–1.00)	0.84	1.00
Basal cell		0.94 (0.85–1.00)	1.00 (1.00–1.00)	1.00	1.00
Squamous cell		0.84 (0.72–0.96)	0.99 (0.99–1.00)	0.72	1.00
Thymus	33	0.91 (0.81–1.00)	1.00 (1.00–1.00)	0.97	1.00
Thyroid	67	0.99 (0.96–1.00)	1.00 (1.00–1.00)	0.96	1.00
Follicular/papillary		0.97 (0.91–1.00)	1.00 (1.00–1.00)	0.91	1.00
Medullary		0.97 (0.92–1.00)	1.00 (1.00–1.00)	0.97	1.00
Urinary bladder	120	0.79 (0.72–0.86)	0.99 (0.99–0.99)	0.83	0.99
Adenocarcinoma		0.44 (0.21–0.67)	1.00 (1.00–1.00)	0.89	1.00
Squamous cell		0.46 (0.26–0.66)	0.99 (0.99–1.00)	0.42	0.99
Transitional cell carcinoma		0.85 (0.77–0.93)	0.99 (0.99–1.00)	0.84	0.99

(table continues)

**Table 2.** *Continued*

Tumor types	<i>n</i>	Internal cross validation			
		Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV
Overall					
Main type accuracy	2206	0.87 (0.85–0.88)	1.00 (0.99–1.00)	0.87	1.00
Subtype accuracy	2206	0.85 (0.83–0.86)	1.00 (1.00–1.00)	0.85	1.00

*n* = 2206.

GIST, gastrointestinal stromal tumor; MFH, malignant fibrous histiocytoma; NPV, negative predictive value; PNET, primitive neuroectodermal tumor; PPV, positive predictive value.

and/or histological subtypes; by leave-one-out cross validation, an overall sensitivity of 89% was achieved for the classification of these 21 new tumor types/subtypes (data not shown). By using this internal validation of the training set, these data provide a preliminary estimate of classification performance.

Furthermore, within a separate test set of 187 FFPE tumor samples, representing 28 of the 30 main cancer types, the 92-gene assay had an overall sensitivity of 83%, with 71% of the main tumor classes demonstrating ≥80% sensitivity (Table 3). Performance within the test set is a preliminary step toward external validation but is definitively addressed in an appropriately powered blinded study. To detect a change in overall sensitivity from 85% to 80%, a minimum sample size of 624 would be required at 95% power.

### Reproducibility

Assay reproducibility was calculated from observed  $C_T$  values (means and SDs) for the 92 genes and 5 normalization genes using positive (universal RNA) and negative (H<sub>2</sub>O) controls. Across 194 independent runs that included four operators, the overall mean percentage CVs for the positive control were 1.69% and 2.19% for the 92 genes and 5 normalization genes, respectively; for the negative controls, the values were 1.25% and 1.66%, respectively. In addition, six tumor types (ie, breast, adrenal, intestine, kidney, thyroid, and prostate) were tested in three independent setups performed by two operators. Across the six tumor samples, the mean percentage CVs were 3.33% and 3.16% for the 92 genes and 5 normalization genes, respectively. The algorithm

**Table 3.** Performance of Main Type Predictions: Test Set

Tumor types	<i>n</i>	Test set			
		Sensitivity*	Specificity <sup>†</sup>	PPV	NPV
Adrenal	2	1.00	1.00	1.00	1.00
Brain	5	1.00	1.00	1.00	1.00
Breast	11	1.00	1.00	1.00	1.00
Cholangiocarcinoma	7	0.71	0.99	0.83	0.99
Endometrium	4	0.75	0.99	0.75	0.99
Gallbladder	6	0.67	0.98	0.50	0.99
Gastroesophageal	14	0.86	0.97	0.67	0.99
Germ cell	6	1.00	0.98	0.67	1.00
GIST	1	1.00	1.00	1.00	1.00
Head/neck	13	0.54	0.99	0.88	0.97
Intestine	16	0.63	1.00	1.00	0.97
Kidney	5	1.00	1.00	1.00	1.00
Liver	7	1.00	1.00	1.00	1.00
Lung	13	0.92	0.98	0.75	0.99
Lymphoma	10	1.00	0.99	0.91	1.00
Melanoma	5	0.80	1.00	1.00	0.99
Meningioma	1	1.00	1.00	1.00	1.00
Mesothelioma	2	1.00	0.99	0.50	1.00
Neuroendocrine	7	1.00	1.00	1.00	1.00
Ovary	6	0.83	0.99	0.83	0.99
Pancreas	8	0.63	0.99	0.83	0.98
Prostate	8	0.88	1.00	1.00	0.99
Sarcoma	6	1.00	0.99	0.75	1.00
Sex cord stromal tumor	1	1.00	1.00	1.00	1.00
Skin	9	0.67	0.99	0.75	0.98
Thymus	2	0.50	1.00	1.00	0.99
Thyroid	5	1.00	1.00	1.00	1.00
Urinary bladder	7	0.86	0.99	0.75	0.99
Overall accuracy	187	0.83	0.99	0.83	0.99

*n* = 187. Overall confidence limits do not apply to individual tumor types (Table 2 provides type-specific data).

\*The 95% CI was 0.78 to 0.88.

†The 95% CI was 0.99 to 0.99.

PPV, positive predictive value; NPV, negative predictive value; GIST, gastrointestinal stromal tumor.

**Table 4.** Characteristics of the Observational Case Study

Characteristics	Value
Male/female (%)	47/53
Age (years)*	62 ± 13
Biopsy type	
Excision/incisional	143 (48)
Core biopsy/biopsy	133 (44)
Cell block/FNA	24 (8)
Biopsy site	
Liver	68 (23)
LN	52 (17)
Lung/pleura	40 (13)
Abdominal/omentum/peritoneum	27 (9)
Soft tissue/mass	25 (8)
Bone/bone marrow	16 (5)
Head/neck	15 (5)
Intestine	12 (4)
Ovary/endometrium/cervix	10 (3)
Breast	9 (3)
Other	26 (9)
Histological grade	
Well differentiated	7 (2)
Moderately differentiated	28 (9)
Poorly differentiated	113 (38)
Undifferentiated	5 (2)
Not otherwise specified	147 (49)
Histopathological diagnosis before cancer type ID	
Carcinoma	274 (91)
Adenocarcinoma	144 (48)
Melanoma	3 (1)
Sarcoma	2 (1)
Neoplasm, NOS	21 (7)

*n* = 300. Data are given as number (percentage) unless otherwise indicated. Percentages may total 100 because of rounding.

\*Data are given as mean ± SD.

FNA, fine-needle aspiration; LN, lymph node; NOS, not otherwise specified.

correctly predicted 17 (94%) of 18 samples (one intestinal sample was incorrectly predicted as gastroesophageal). Within real-world testing, a tumor sample of known origin is assayed every 50 samples. Over 1 year, 32 independent assays, which included three different tumor types, were tested by four clinical laboratory scientists. Means and SDs were calculated from observed  $C_T$  values for the 92 genes and for the 5 normalization genes. The mean percentage CVs were 1.58% (range, 1.41% to 1.69%) for the 92 genes and 1.04% (range, 0.85% to 1.79%) for the 5 normalization genes. The algorithm was 100% concordant for tumor-of-origin predictions across the 32 runs.

### Clinical Case Series

A total of 300 consecutive cases submitted for testing within bioTheranostics' Clinical Laboratory Improvement Amendments–certified College of American Pathologists–accredited high-complexity clinical laboratory were examined. The characteristics of the cases are shown in Table 4. Of the patients, 44% were ≥65 years. The biopsy sites were indicative of metastatic disease (liver, 23%; lymph node, 17%; bone, 5%; and abdomen, 9%). Laser microdissection was performed based on pathological review by the medical director in approximately 85% of

the cases to provide samples with a higher percentage of tumor cells in relation to nontumor cells, necrotic and fibrotic regions, and lymphocyte infiltration. Tumors were predominantly poorly differentiated or undifferentiated where the morphological feature was noted.

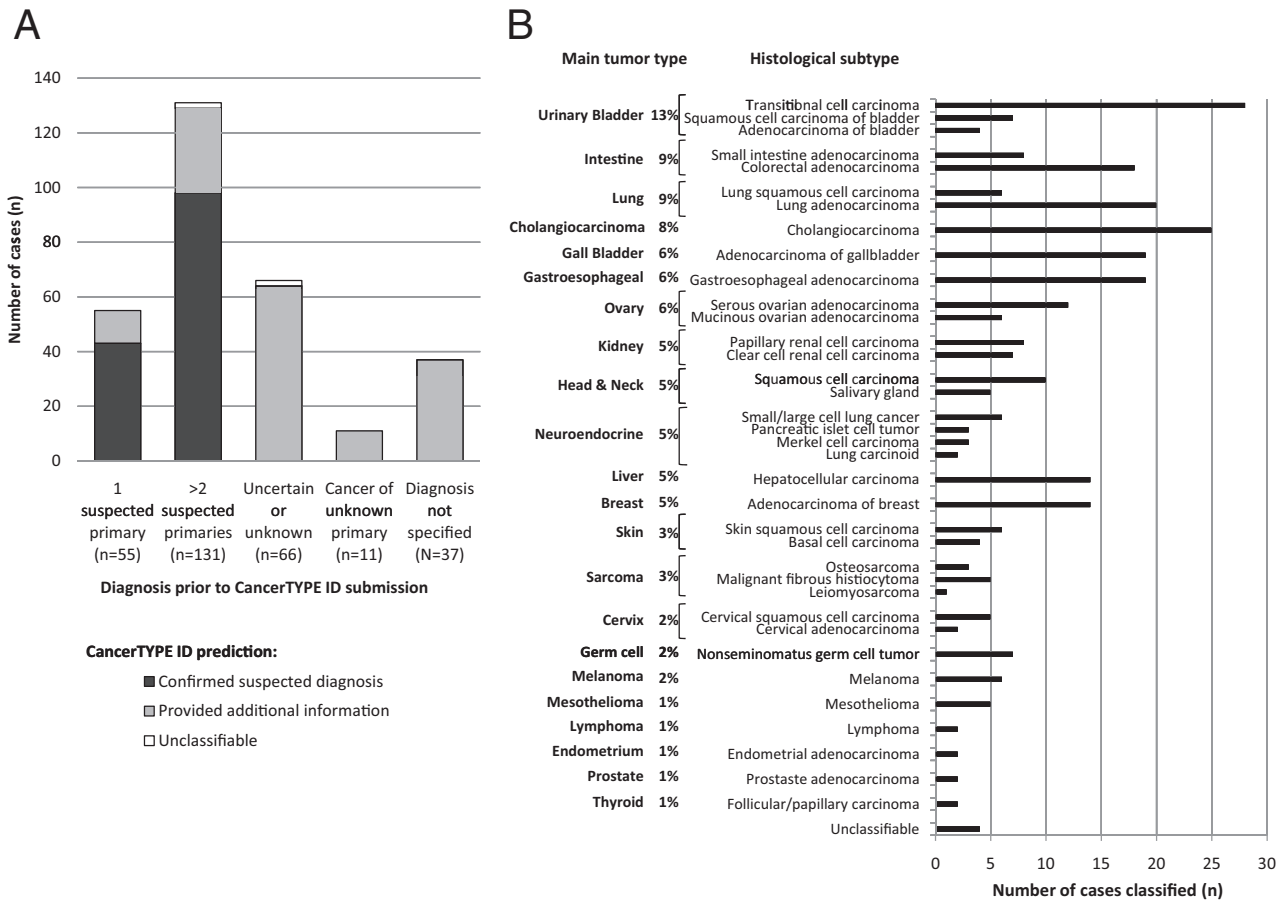
Most of the cases submitted [131 (44%)] were associated with a differential diagnosis with two or more suspected primary tumors. Single suspected primary tumors were present in 18% (*n* = 55) of cases, and a smaller proportion was submitted with a diagnosis of cancer of unknown primary origin (4%, *n* = 11, Figure 2A). In addition, a range of 0 to 28 IHC markers (median, 7) was analyzed before submission to the 92-gene assay. (The frequency of IHC use is provided in Supplemental Figure S1 at <http://jmd.amjpathol.org>). IHC analyses in cases with histopathological diagnoses of carcinoma of unknown primary origin or unknown or uncertain primary origin (*n* = 77) ranged from 0 to 27 markers (median, 7). Cases with a single suspected primary tumor (*n* = 55) showed a similar range in IHC markers of 0 to 26 (median, 6).

Predictions for the 300 cases yielded many diverse tumor types. Results were generated for 22 of the 30 possible main types and 36 of the 54 possible subtype classifications (Figure 2B). Four cases (1.3%) were deemed unclassifiable. All major categories of cancers were predicted (ie, carcinoma, sarcoma, melanoma, and lymphoma); however, the distribution of cancer types predicted differed from respective annual incidences. Predictions for rare tumors were overrepresented and included cholangiocarcinoma, Merkel cell carcinoma, pancreatic islet cell carcinoma, lung carcinoid, small-intestine adenocarcinoma, and mucinous ovarian adenocarcinoma.

The 92-gene assay confirmed the suspected primary tumor in 78% (*n* = 43) of samples submitted with a single diagnosis. Within cases submitted with a differential diagnosis (*n* = 133), the 92-gene assay was consistent with one of the suspected primary tumors in 74% of the cases (*n* = 99), therefore had the potential to add value by reducing the number of possibilities. In a significant proportion of predicted cholangiocarcinomas (75%), ovarian cancers (61%), gallbladder (53%), liver (43%), and mesothelioma (40%), additional molecular data were provided to resolve these differential diagnoses. A limitation of the observational series is that the definitive reference diagnosis associated with each of these cases was not known. However, results from the 92-gene assay were consistent with clinicopathologic impressions (confirmatory for either one or two or more suspected primary tumors) in 47% of the cases and provided additional information in another 37% of the cases with indeterminate, unspecified, or unknown origin.

### Discussion

We report the expanded tumor classification capabilities of the 92-gene assay by producing a reference tumor database and associated algorithm that enables the prediction of 30 main tumor types and 54 histological subtypes. The tumor classifier demonstrated overall analytical sensitivities of 87% and 85% for 30 main cancer types



**Figure 2.** The 92-gene assay test results. **A:** Distribution of primary tumors suspected before the 92-gene assay and concordance of the 92-gene assay prediction with suspected diagnosis. **B:** Distribution of 92-gene assay test subtype predictions. Molecular cancer classification predictions from the clinical experience cases using the expanded algorithm ( $n = 300$ ). The percentage of cases predicted for each main type and the predicted subtype are noted.

and 54 histological subtypes, respectively, by internal validation of the training set, and 83% in a test set of 187 tumors representing 28 of the 30 main cancer types. The 92-gene assay, composed of 87 genes of content and 5 normalization genes, was originally developed to classify 39 tumor types. Herein, our preliminary analyses demonstrate a preservation of overall analytical performance with the addition of new tumor types/histological subtypes that were not included in the original 87-gene selection process. Overall, within the current reference database, 21 new tumor types/histological subtypes were classified, with a mean sensitivity of 85% in the training set and 81% in the test set. The positive predictive values across tumor types/subtypes ranged from 42% to >99%. Tumor subtypes that displayed lower positive predictive values, such as urinary bladder squamous cell carcinoma and small-intestine adenocarcinoma, were indicative of the inherent difficulty in distinguishing primary sites of various squamous cell carcinomas and tumors of the upper gastrointestinal tract, respectively.

The potential capability of the 87 genes to classify such a broad and diverse set of tumor types/histological subtypes is rooted in the discovery method. This involved two essential steps: whole-genome expression profiling of 578 tumors representing 39 types to identify 1001 informative genes for tumor-type discrimination and use

of a genetic algorithm to identify a collective set of genes within the 1001 that had the greatest analytical performance.<sup>14</sup> The key advantage of the genetic algorithm approach is that the discriminatory set of genes evolves as a combination, resulting in the discovery of a non-redundant compact gene set. Within the 87-gene set, there is a high representation of genes that encode either transcription factors involved in the commitment of many cell/tissue lineages or cell surface receptors that activate intracellular transduction pathways.<sup>14</sup> Given this gene content, it is hypothesized that the collective expression of the 87 genes is differentially expressed in other cell lineages not previously profiled and, therefore, discriminatory with respect to classifying new tumor types.

Previous studies<sup>12,18</sup> with microarray-based tumor classification have reported a required range of 40 to 100 genes per tumor type for accurate performance. For example, a recently described classifier includes 1550 genes for the classification of 15 tumor types on a microarray platform.<sup>12</sup> In comparison, gene selection and compaction to 92 genes enabled the development of the real-time RT-PCR assay platform for individual gene expression measurements. Use of real-time quantitative PCR for gene expression measurements is the accepted gold standard because of its robust analytical characteristics, which include high precision, single-copy sensitiv-

ity, and dynamic ranges for individual genes of  $\geq 10^6$ .<sup>19</sup> In contrast, for individual gene expression measurements, microarray platforms have lower sensitivity; are more susceptible to batch effects and, thus, lower precision; and have reported dynamic ranges of approximately  $10^2$ .<sup>20</sup> A decreased sensitivity and dynamic range can affect the informational content that can be obtained for each individual gene expression measurement. After normalization, the median gene expression values for each of the 87 genes across all tumor types within the reference database ( $N = 2206$ ) and clinical case series ( $N = 300$ ) were  $10^8$  (range,  $10^7$  to  $10^9$ ) and  $10^6$  (range,  $10^4$  to  $10^7$ ), respectively. The real-time RT-PCR platform has the capability of capturing this high informational content for each gene; this may be essential for the use of a relatively small gene set (ie, 87 genes) for the classification of new tumor types.

An evaluation of consecutive clinical cases ( $N = 300$ ) revealed several key aspects of potential clinical utility and indications for use for the 92-gene assay. Cases that were submitted for testing originated from  $>10$  biopsy sites and, therefore, had a large degree of heterogeneity of nontumor cells present. As a result, 85% of the tumors were laser microdissected to afford purer histological features. In addition, at least half of the samples were from core biopsy specimens or fine-needle aspirates, having fewer tumor cells available for testing. Furthermore, all samples were FFPE tissue with varying degrees of RNA degradation.<sup>21–23</sup> To overcome these technical issues, the 92-gene assay uses short amplicons (approximately 65 to 85 bp) to enable measurement of degraded RNAs and uses a preamplification step before real-time PCR to increase analytical sensitivity. Therefore, compatibility with standard tissue management and processing and minimal sample requirements for testing were notable characteristics for clinical applicability.

Another interesting feature demonstrated by the clinical cases was that a diverse number of tumor types was reported in real-world testing, which included both prevalent and less common tumors. For example, 19 of the 25 predictions for cholangiocarcinoma, a rare tumor, were from differential diagnoses that listed relatively common gastrointestinal tumor types (eg, colorectal, pancreatic, and gastric). In addition, two different neuroendocrine subtypes were predicted: three Merkel cell carcinomas (960 cases per year) and six small- or large-cell lung cancers (26,000 cases per year) (SEER Cancer Statistics Review, 1975–2007, [http://seer.cancer.gov/csr/1975\\_2007](http://seer.cancer.gov/csr/1975_2007), last accessed July 13, 2011). Overall, 36 different cancer subtypes were predicted by the 92-gene assay in this 300 case series. These findings suggest that the extensive scope and depth of the reference tumor database may be a required specification for a clinically useful molecular cancer classifier.

Interestingly, abstraction of pathological reports provided on submission for testing suggested that the predominant clinical use of the 92-gene assay was to resolve differential diagnoses. Specifically, 10-fold more samples were submitted with a differential diagnosis versus a carcinoma of unknown primary origin diagnosis. This fact

suggests that, in real-world testing, the diagnostic utility of a molecular classifier may extend beyond aiding in the classification of carcinoma of unknown primary origin to those tumor samples in which there are suspected origins with multiple differential diagnoses but no single definitive diagnosis.

Herein, we report the expansion in scope and depth of the tumor reference database and demonstrate the ability of the 92-gene assay to classify 30 cancer types and 54 histological subtypes. To firmly establish the clinical validity of the 92-gene assay, a multi-institutional study is ongoing to determine the analytical performance within many diverse cancer types. In addition, prospective studies are being conducted to assess whether the use of the predictions from the 92-gene assay to select treatment positively affects patient outcome.<sup>24</sup> In summary, this second-generation version of the 92-gene assay might serve as a reliable and valuable molecular complement to current diagnostic modalities.

### Acknowledgments

We thank Jingmei Su, Jose Galindo, Yvette De La Torre, Lavenia Correa, Susan Hicks, Hamid Mobtaker, and Yen Tran for their technical assistance; Hongying Li for statistical support; Amy Marrs for administrative support; and Veena Singh for pathological review.

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CPC B78067