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Analytical Validation of a Novel 6-Gene Signature for Prediction of Distant Recurrence in Estrogen Receptor-Positive, HER2-Negative, Early-Stage Breast Cancer

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BACKGROUND: OncoMasTR is a recently developed multigene prognostic test for early-stage breast cancer. The test has been developed in a kit-based format for decentralized deployment in molecular pathology laboratories. The analytical performance characteristics of the OncoMasTR test are described in this study.

METHODS: Expression levels of 6 genes were measured by 1-step reverse transcription-quantitative PCR on RNA samples prepared from formalin-fixed, paraffin-embedded (FFPE) breast tumor specimens. Assay precision, reproducibility, input range, and interference were determined using FFPE-derived RNA samples representative of low and high prognostic risk scores. A pooled RNA sample derived from 6 FFPE breast tumor specimens was used to establish the linear range, limit of detection, and amplification efficiency of the individual gene expression assays.

RESULTS: The overall precision of the OncoMasTR test was high with an SD of 0.16, which represents less than 2% of the 10-unit risk score range. Test results were reproducible across 4 testing sites, with correlation coefficients of 0.94 to 0.96 for the continuous risk score and concordance of 86% to 96% in low-/high-risk sample classification. Consistent risk scores were obtained across a $>$100-fold RNA input range. Individual gene expression assays were linear up to quantification cycle values of 36.0 to 36.9, with amplification efficiencies of 80% to 102%. Test results were not influenced by agents used during RNA isolation, by low levels of copurified genomic DNA, or by moderate levels of copurified adjacent nontumor tissue.

CONCLUSION: The OncoMasTR prognostic test displays robust analytical performance that is suitable for deployment by local pathology laboratories for decentralized use.

Introduction

Adjuvant endocrine therapy is the standard treatment regime in patients diagnosed with estrogen receptor (ER)-positive, HER2-negative primary breast cancer. However, the cumulative 20-year estimated risk of distant recurrence after 5 years of endocrine therapy is 22% for lymph node-negative patients and 31% for patients with 1 to 3 positive lymph nodes (1). Multigene prognostic signatures can be used to estimate the risk of distant recurrence in patients with ER-positive, HER2-negative breast cancer, and they provide prognostic information independent of traditional clinicopathological factors such as tumor size, tumor grade, and lymph node status (2). Identification of patients with low risk of recurrence when treated exclusively with endocrine therapy can potentially spare unnecessary additional adjunct treatments and the associated side effects in a substantial number of women.

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The OncoMasTR gene panel was derived using a novel bioinformatic approach (ARACNe: Algorithm for the Reconstruction of Accurate Cellular Networks), where common master transcriptional regulators (MTR) were identified that regulate downstream genes present in other existing prognostic breast cancer signatures (3). The MTR gene panel was further refined in a reverse transcription quantitative PCR (RT-qPCR) data set of 225 patients with outcome data, and the most prognostic gene combination was identified (4). The OncoMasTR signature comprises 3 prognostic genes (*FOXM1*, *PTTG1*, and *ZNF367*), which have been demonstrated to have key roles in cell proliferation and in other aspects of cancer biology (5–8). Three reference genes (*GAPDH*, *GUSB*, *TFRC*) are used for normalization of RT-qPCR data. Relative prognostic gene expression measurements are combined with tumor size and nodal status to calculate the OncoMasTR risk score.

Prognostic performance of the OncoMasTR risk score was first demonstrated in the Translational Study of Anastrazole or Tamoxifen Alone or Combined (TransATAC) patient cohort (4). Subsequent blinded studies in a subset of Irish patients enrolled in the TAILORx study (9) and in the Austrian Breast and Colorectal Cancer Study Group (ABCSG) Trial 8 (10) have confirmed that the signature is significantly prognostic for distant recurrence and that it adds significant prognostic information to that provided by standard clinical variables alone.

The OncoMasTR signature has been developed as a RT-qPCR test kit for decentralized use in the local pathology laboratory setting. The analytical performance characteristics of the test are described herein.

Materials and Methods

TEST SAMPLES

Archival formalin-fixed, paraffin-embedded (FFPE) specimens with pathologist-confirmed invasive breast carcinoma were used to determine the performance characteristics of the OncoMasTR test. Resected breast tumor specimens were fixed in neutral 10% buffered formalin and embedded in paraffin using standard protocols. Invasive tumor content was confirmed using 5 μm-hematoxylin and eosin (H&E)-stained tissue sections. Sections adjacent to the H&E section were processed for use in this study. All FFPE specimens were anonymized with an alphanumeric identifier code, and no patient data were available. The studies were conducted in accordance with the Declaration of Helsinki and ethical approval was obtained from the regional ethical review board of Lund University, Sweden.

RNA AND DNA PURIFICATION

Total RNA was purified from FFPE tissue sections (5 μm) manually using the silica membrane spin column RNeasy FFPE kit (Qiagen) in accordance with the manufacturer's instructions incorporating DNase I treatment to yield DNA-free RNA. Genomic DNA was manually purified from FFPE tissue sections (5 μm) using the silica membrane spin column QIAamp DNA FFPE tissue kit (Qiagen) in accordance with the manufacturer's instructions incorporating RNase A treatment to yield RNA-free genomic DNA. Nucleic acid concentrations were determined by spectrophotometry at 260 nm.

GENE EXPRESSION ANALYSIS

The expression of the OncoMasTR gene panel was measured using 1-step RT-qPCR. Hydrolysis probe gene expression assays targeting the 6 genes of interest (*FOXM1*, *PTTG1*, *ZNF367*, *GAPDH*, *GUSB*, *TFRC*) were dried onto MicroAmp™ Fast Optical 96-well reaction plates (Applied Biosystems) with 900 nmol/L forward and reverse primers and 250 nmol/L FAM-MGB–labeled probe per well. Primer sequences are provided in online Supplemental Table 1. PCR reaction mixes were prepared using TaqPath™ 1-Step RT-qPCR Master Mix (Applied Biosystems) in accordance with the manufacturer's instructions. PCR reactions were performed in a final volume of 20 μL per assay well with 10 ng RNA per reaction unless otherwise stated. Thermal cycling was performed on a 7500 Fast Dx real-time PCR instrument (Applied Biosystems) with SDS software v1.4.1 using the fast-cycling mode and the following parameters: 25 °C for 2 min, 50 °C for 15 min, and 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 sec and 60 °C for 30 sec. Data acquisition was performed during the annealing and extension step at 60 °C. The threshold was set at 0.1 and automatic baseline enabled for quantification cycle (Cq) determination. RNA samples were tested in triplicate per expression assay in each run. A positive control human total reference RNA (Agilent Technologies) was tested at 10 ng per well for each expression assay and RT-PCR grade water (Life Technologies) was used as a negative control with 1 reaction per expression assay per PCR plate.

ONCOMASTR RISK SCORE CALCULATION

A model was developed based on a data set of >1500 clinical samples processed at OncoMark during development of the assay (4, 9) to identify outliers in technical replicates; identified outlier Cq measurements were masked in downstream calculations. The arithmetic mean Cq value was calculated for each expression assay; at least 2 Cq values per expression assay were required. For the reference genes (*GAPDH*, *GUSB*, *TFRC*), the geometric mean of the 3 mean Cq values was calculated;

this is referred to as the normalization factor. Test samples where the normalization factor was ≥31 Cq units were considered invalid with respect to sample quality and input. Relative expression levels (ΔCq) of the 3 MTRs (*FOXM1*, *PTTG1*, *ZNF367*) were calculated as the normalization factor Cq minus the mean MTR Cq. For clinical use, relative gene expression levels are combined in a linear model with clinical variables to calculate a continuous OncoMasTR risk score, which ranges from 0 to 10 with a threshold of 5 delineating the high- and low-risk categories. The threshold was established in the RT-qPCR data set used to develop the OncoMasTR multigene signature by dichotomizing the underlying unscaled continuous risk score into low- and high-risk categories, which had sensitivity and specificity for distant recurrence >0.7, and then linearly transforming the risk score so that the threshold value was set at 5 on a 0- to 10-unit scale (4).

The analytical studies described here were conducted without access to clinical variables, and the convention used for risk score calculation across all samples was to assign each sample as lymph node-positive and tumor size of ≤10 mm.

DETECTION LIMITS, LINEAR DYNAMIC RANGE, AND PCR EFFICIENCY

Establishment of expression assay detection limits, linear ranges, and amplification efficiencies was performed using a pooled RNA specimen derived from 6 different FFPE breast tumor specimens. The linear dynamic range was established using a series of 18 × 2-fold dilutions of the RNA pool with concentrations ranging from 40 ng/μL to 0.31 pg/μL. Each RNA dilution was tested across 3 different reagent lots with triplicate measurements per lot resulting in at least 9 Cq measurements per expression assay and RNA concentration. Additional replicates of low RNA concentrations yielding hit-rates of >50% up to the lowest RNA concentration generating a 100% hit-rate were tested to establish the limit of detection (LoD) for each expression assay, with at least 88 replicates per assay used to define the pooled SD of the low concentration samples for LoD calculation. This meets the minimum recommended number of 60 low-concentration replicates for LoD establishment in CLSI guideline EP17-A2 (11). The hit-rate is the proportion of replicates yielding Cq values relative to the total number of replicates tested at a given RNA input. A total of 977 negative control PCR reactions for each expression assay were compiled to establish the limit of blank (LoB).

LoB was calculated as per CLSI EP17-A2 (11) with $\alpha = 5\%$ based on a nonparametric data distribution due to truncation of data at Cq 40, which is the real-time PCR instrument measuring limit. LoD was calculated based on the pooled SD of low-concentration samples for each gene with $\beta = 5\%$ as follows: LoD = LoB − (C_p × SD_L), where SD_L is the pooled SD of the low RNA concentration samples for the gene of interest and C_P is calculated as 1.645 ÷ [1 − (1 ÷ [4 × (number of Cq replicates ÷ number of RNA test levels)])]. As absolute quantification of individual gene transcripts in the pooled RNA sample was not possible due to lack of available recognized calibration standards, LoD was reported as the Cq value for each gene as a surrogate of the transcript level.

The linear dynamic range for each gene was determined as per CLSI EP06-A (12) using all available Cq measurements up to and including the estimated LoD from the serial dilution testing and detection limit establishment experiments, recognizing the inverse relationship between RNA input and Cq. Any outlier Cq values in a dilution step were identified using Grubbs test (13) and removed from analysis. Linear, quadratic, and cubic regression models were fitted to the Cq values and corresponding $\log_2$ RNA input concentrations for each gene. Nonlinear coefficients for the best fit higher order (second or third order) regression models were tested to determine whether they were different from 0 at the 5% significance level. Genes were considered linear where the difference between the linear and best-fit higher order regression model at each RNA input concentration was ±0.5 Cq units. The linear dynamic range for each gene is reported in Cq units corresponding to the mean Cq of the highest and lowest RNA input levels where linearity criteria were met.

Amplification efficiencies for each expression assay were determined from the slope of the linear regression model as follows: % Efficiency = $(2^{-1 \div \text{slope}} - 1) \times 100\%$.

WITHIN-SITE ASSAY PRECISION

A sufficient amount of RNA to conduct the precision study was prepared from multiple RNA extractions performed on different FFPE breast tumor specimens to generate sample pools (>20 μg RNA per pool) representing low, borderline, and high risk scores. The precision study design was balanced (Fig. 1, A) and based on an advanced multifactorial model as per CLSI EP05-A3 (14). RNA samples were tested across multiple potential sources of variation: PCR instruments (n = 3), assay operators (n = 3), reagent lots (n = 3), and loading positions on the PCR plate (n = 2). The study was conducted across a 28-calendar day period. All assay runs included positive and negative controls for each expression assay.

The contribution of each source of variation to the total assay imprecision was calculated by 4-way analysis of variance using R statistical software version 3.4.1 (2017-06-30) "Single Candle." Analysis was performed using OncoMasTR risk scores, normalized ΔCq measurements for the 3 prognostic genes, and absolute Cq values for the 6 prognostic and reference genes.

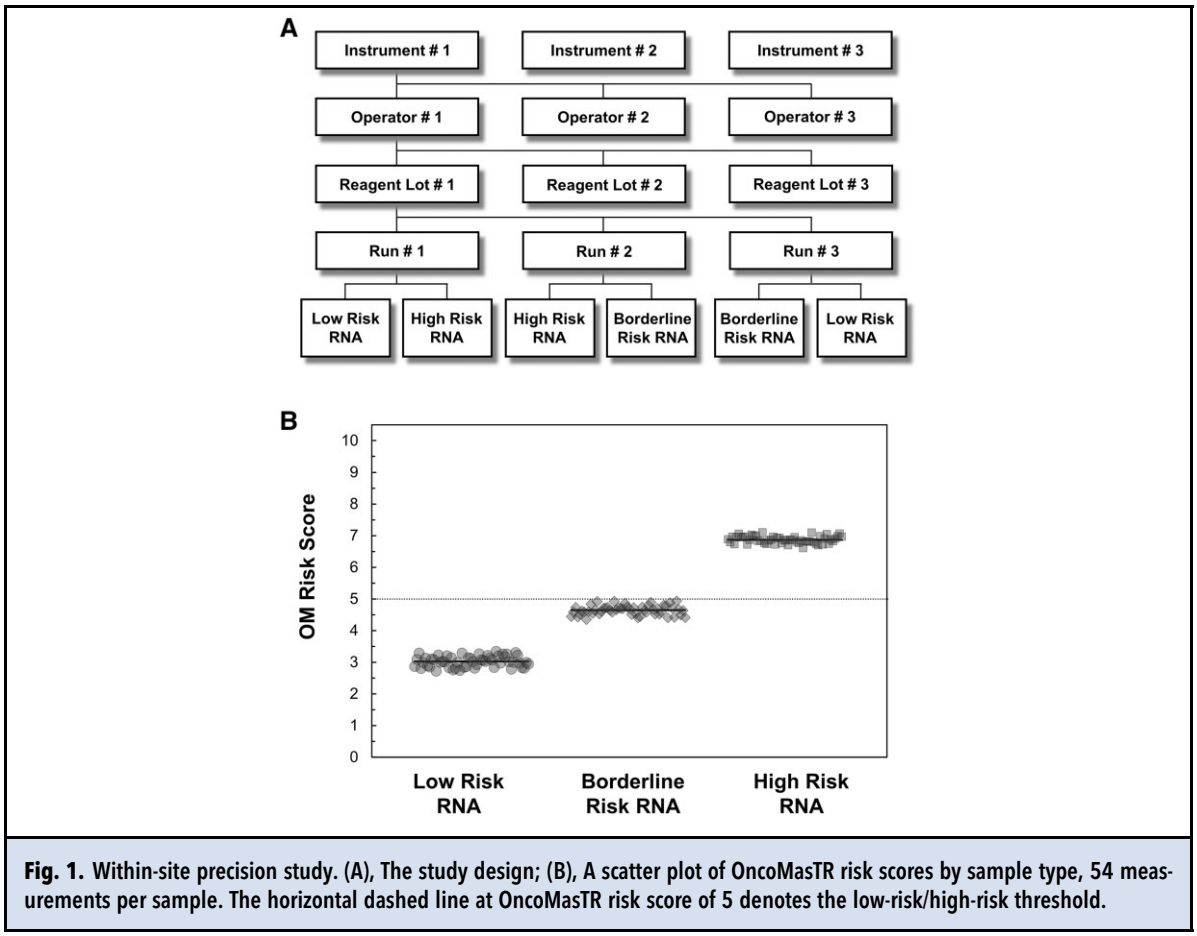

Fig. 1. Within-site precision study. (A), The study design; (B), A scatter plot of OncoMasTR risk scores by sample type, 54 measurements per sample. The horizontal dashed line at OncoMasTR risk score of 5 denotes the low-risk/high-risk threshold.

BETWEEN-SITE TUMOR SAMPLE REPRODUCIBILITY

A panel of 30 FFPE breast tumor blocks was sectioned at 5 μm and mounted on glass slides. Slides were distributed across 4 test sites for pathology review, H&E staining, RNA extraction, and RT-qPCR. Assay results for 2 of the 30 FFPE blocks did not meet prespecified assay quality control criteria from at least 1 test site and were excluded from the analysis. Twenty-eight FFPE specimens yielded passing results at all sites where prespecified quality control criteria were met. The between-site tumor sample reproducibility study was completed within each test site in <28 calendar days, and the total duration of the study from initiation at site 1 to completion at site 4 was approximately 14 months. A single OncoMasTR reagent lot was used within each site, and 2 lots of reagents were used in total.

RNA INPUT RANGE

Five FFPE breast tumor blocks with different OncoMasTR gene expression profiles were selected, and RNA was extracted from multiple sections. Eight different RNA dilutions were prepared for each sample yielding 0.1 to 40 ng RNA input per PCR reaction. Each RNA sample and concentration was tested twice on different reagent lots. The mean and SD in OncoMasTR risk scores at each RNA input level were calculated, and the risk score result at 10 ng RNA per PCR was used as the reference. Results were considered equivalent to the reference value if the SD of the replicate results at the reference and test input levels was <0.46, which is 3× the total SD observed in the within-site assay precision study.

INTERFERENCE STUDIES

Five RNA pools used in the previously described studies were selected for the RNA interference study. RNA samples were spiked with ethanol (up to 1.5% v/v), xylene (up to 0.5% v/v), or a human genomic DNA (gDNA) pool (up to 5 ng/μL) and tested using 1 reagent lot. The gDNA sample was a pool of 10 separate preparations, each extracted from a different FFPE breast tumor sample. Risk score results for each RNA plus interferent test condition were compared to the relevant control RNA sample lacking added interferent and the SD of the paired risk scores calculated. Results were

Table 1. Linear range, detection limits, and amplification efficiencies.

Gene	LoB, Cq	LoD,[a] Cq	Linear dynamic range, log_2 ng RNA per well	Linear dynamic range, Cq	Amplification efficiency,[a] %
FOXM1	40.0	38.4 (37.2-39.5)	−2.7 to +6.3	26.4-36.5	80.2 (77.9-82.7)
PTTG1	40.0	38.8 (38.0-39.6)	−4.7 to +6.3	24.5-36.6	84.6 (82.8-86.6)
ZNF367	40.0	38.6 (37.6-39.6)	−2.7 to +6.3	25.7-36.0	80.3 (78.7-81.9)
GAPDH	40.0	38.0 (36.7-39.4)	−9.7 to +6.3	21.3-36.3	101.5 (99.6-103.6)
GUSB	40.0	38.0 (36.8-39.2)	−8.7 to +6.3	20.0-36.5	87.5 (85.7-89.4)
TFRC	40.0	38.6 (37.6-39.5)	−5.7 to +6.3	24.1-36.9	85.9 (83.4-88.6)

[a]95% CIs are indicated in parenthesis.

considered equivalent, and the test was not subject to interference with the substance and test concentration if the SD of the replicate risk score results was <0.46 for all 5 test samples.

Fifty-four FFPE breast tumor blocks were selected to assess the impact of increasing amounts of adjacent nontumor tissue cells on risk score results. H&E sections were reviewed by 2 board-certified pathologists, and the area of invasive tumor marked on the slides and the invasive tumor content (as a percentage of the total tissue area) was estimated. RNA was extracted from sections adjacent to the H&E section either with macrodissection to enrich for invasive tumor or by sampling the entire tissue section where the resultant sample contained varying amounts of RNA derived from adjacent noninvasive/nontumor cells in addition to the target RNA from the invasive tumor area. Paired RNA samples from each FFPE sample were tested by OncoMasTR, and the equivalence between risk score results due to copurified noninvasive material was determined as previously described for the spiked interferent study.

Results

DETECTION LIMITS, LINEAR DYNAMIC RANGE, AND PCR EFFICIENCY

The linear dynamic range of the individual expression assays was determined as described in CLSI guideline EP06-A (12). All 6 expression assays were linear from the maximum tested concentration of 80 ng RNA per well over at least a 500-fold RNA input range, with lower limits of the linear range falling between 36.0 and 36.9 Cq units (Table 1). Amplification efficiencies ranged from 80% to 102% based on the slope of the gene-specific linear regression models. LoB for all assays was Cq 40, and the LoD ranged from Cq 38.0 to 38.8 (Table 1) for the 6 expression assays.

WITHIN-SITE ONCOMASTR ASSAY PRECISION

Precision of the OncoMasTR test was assessed using pooled breast tumor RNA samples with low, borderline, and high risk scores. Each RNA sample was tested 54 times across different qPCR instruments, reagent lots, loading positions, and assay operators (Fig. 1, A). The overall SD in the risk score across all samples was 0.155, equivalent to approximately 1.5% of the 10-unit risk score range (Table 2). The majority of the imprecision in the risk score was associated with within-run replicate-to-replicate variability in the PCR assays, which was responsible for 66% to 85% of the total observed variance. The test was robust to different operating conditions, which collectively contributed 15% to 35% of the total observed variance. A distribution plot of risk scores is presented in Fig. 1, B. Variance components analysis for Cq and ΔCq measurements is provided in online Supplemental Tables 2 and 3.

BETWEEN-SITE ONCOMASTR TEST REPRODUCIBILITY

Twenty-eight FFPE breast tumor cases were included in the analysis, which yielded valid test results in 4 different laboratories. The study design is depicted in Fig. 2, A. The samples were distributed evenly between risk categories, with a 13 (46%) to 15 (54%) low-risk/high-risk split (Fig. 2, B). OncoMasTR risk scores were highly correlated, with an overall Pearson r of 0.95 for all available paired scores and a concordance of 88.7% in categorization of samples as OncoMasTR low or high risk. Site-to-site continuous risk score correlation ranged from 0.94 to 0.96 (Pearson r), and concordance in sample classification ranged from 85.7% to 96.4%. The overall risk score SD across all 4 sites was 0.237. This larger SD in comparison to that observed in the within-site precision study likely reflects the additional variability introduced in FFPE tissue processing and RNA extraction.

Table 2. Variance components for the OncoMasTR within-site precision study.

RNA sample	Mean OncoMasTR risk score	Variance component[a]					
		Instrument	Operator	Reagent lot	Loading position	Within-run	Total SD
Low risk RNA	3.03	0.0000 (0)	0.0034 (11)	0.0012 (4)	0.0000 (0)	0.0266 (85)	0.176
Borderline risk RNA	4.64	0.0005 (2)	0.0004 (1)	0.0018 (7)	0.0063 (24)	0.0173 (66)	0.162
High risk RNA	6.87	0.0000 (0)	0.0000 (0)	0.0023 (15)	0.0000 (0)	0.0125 (85)	0.122

[a]The percentage of the total variance is shown in parentheses beside the estimated variance.

RNA INPUT RANGE

Consistency in risk score results as a function of RNA input was examined using a panel of 5 FFPE RNA breast tumor samples each tested at 40 ng down to ≤0.25 ng RNA per PCR reaction. These samples were a mix of archival specimens (>25 years old) or contemporary surgical resections (<2 years old). Test results were considered equivalent when the SD of paired risk scores at a variable input level and the nominal input level (10 ng/well) was <0.46 units. Risk scores were stable at all RNA input levels where the normalization factor Cq was <31 (Fig. 3, A). This corresponded to RNA input levels of ≥2.5 ng/well for archival samples and ≥0.25 ng/well for contemporary samples. The normalization factor cutoff has been implemented in all clinical performance evaluations of the OncoMasTR test conducted to date (4, 9, 10).

Absolute RNA input levels were highly correlated with the normalization factor ($r > 0.99$). Reference gene amplification in archival specimens was delayed relative to contemporary samples at equivalent absolute RNA input concentrations (Fig. 3, B), which is consistent with other studies (15) and likely reflects a greater degree of RNA fragmentation in archival samples (16).

ANALYTICAL INTERFERENCE

The sensitivity of the OncoMasTR test to various potential interfering substances was assessed by determining the SD in paired risk score results for control and interferent-spiked test samples. Agents used during RNA purification (xylene and ethanol) did not adversely influence the test results when spiked into a panel of 5 FFPE RNA breast tumor samples at up to 0.5% (w/v) and 1.5% (w/v), respectively (Fig. 4, A). Risk scores were impacted in the presence of 5 ng/μL human gDNA (Fig. 4, A), with underestimation of the risk score by up to 2 units. A similar magnitude of gDNA-mediated risk score bias was replicated by omission of the DNase I treatment step during RNA isolation in a panel of 20 test samples (data not shown). Risk scores in RNA samples containing a lower concentration of human gDNA (0.5 ng/μL) were comparable to control samples (Fig. 4, A) indicating a tolerance of the test to low levels of copurified genomic DNA in RNA test samples. Moderate amounts of nontumor tissue coprocessed with invasive carcinoma tissue did not impact the risk score, and only samples where the invasive tumor content was <30% of the total tissue content yielded results indicative of interference (Fig. 4, B).

Discussion

This study describes the analytical performance characteristics of the OncoMasTR RT-qPCR-based multigene test for estimating the risk of distant recurrence in ER-positive, HER2-negative primary breast cancer. It is 1 of several described multigene signatures for predicting breast cancer outcome, and the analytical performance characteristics of some of these signatures have been described (17–19). First-generation multigene signatures for breast cancer prognosis, such as Oncotype DX recurrence score (20) and MammaPrint (21), were developed as centralized reference laboratory tests due to their high complexity. Further developments in this field resulted in the commercialization of decentralized breast cancer prognostic tests such as EndoPredict (22) and Prosigna (19) for deployment in local pathology laboratories.

Analytical reproducibility is an essential requirement for diagnostic tests, particularly for tests that are intended to be used in a decentralized environment where additional variability may be introduced across laboratory personnel, equipment, and instrumentation. The within-site precision study demonstrated that the OncoMasTR test is robust across different qPCR instruments, reagent lots, and technical operators. The total variation in OncoMasTR results observed in the between-site reproducibility study is consistent with that reported for other decentralized multigene breast cancer tests (19, 22). Although there was good agreement in the continuous risk score ($r = 0.94–0.96$), some discordance in risk classification was observed between test sites in this study for samples with risk scores

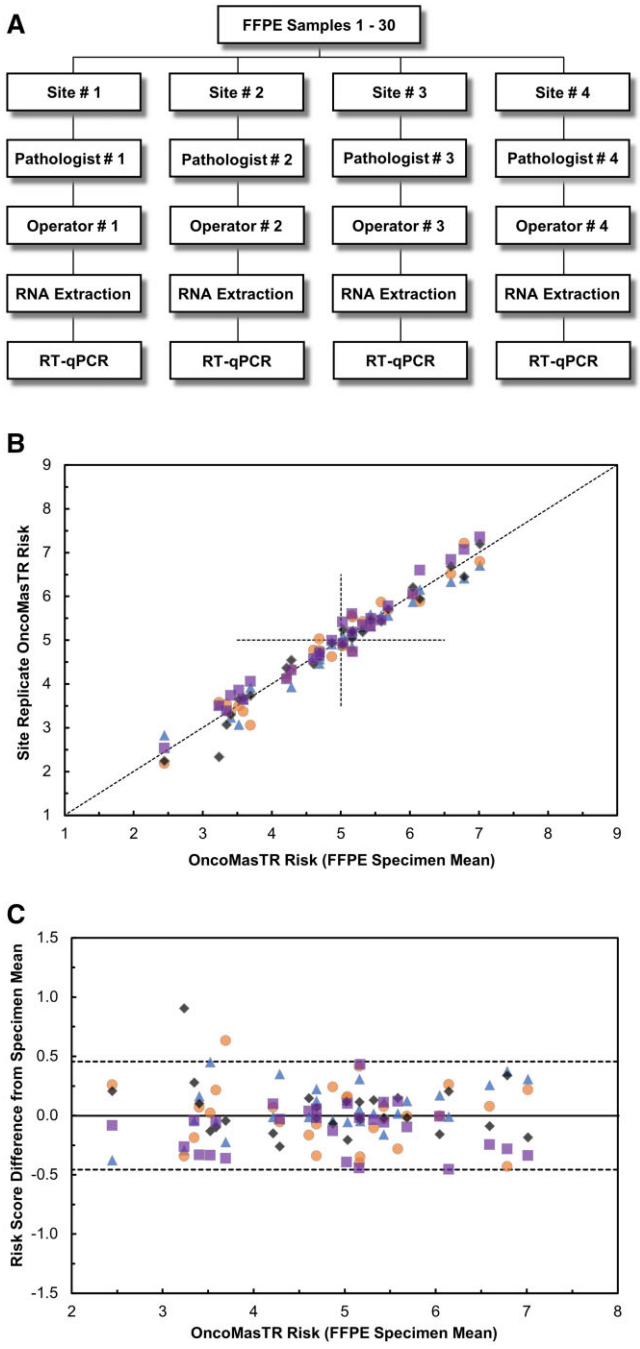

Fig. 2. Between-site tumor sample reproducibility study. (A), The study design; (B), A scatter plot of OncoMasTR risk scores, with the risk score at each test site represented on the y-axis plotted against the mean OncoMasTR risk score across all sites on the x-axis. The vertical and horizontal dashed lines at OncoMasTR risk score of 5 denote the low-risk/high-risk threshold; (C) The risk score difference between each site test result and the overall mean result across the 4 sites for the 28 FFPE specimens analyzed. The upper and lower 95% CIs are denoted by the dashed horizontal lines. Individual sites in (B) and (C) are denoted by the different symbols and colors. Color figure available online at clinchem.org.

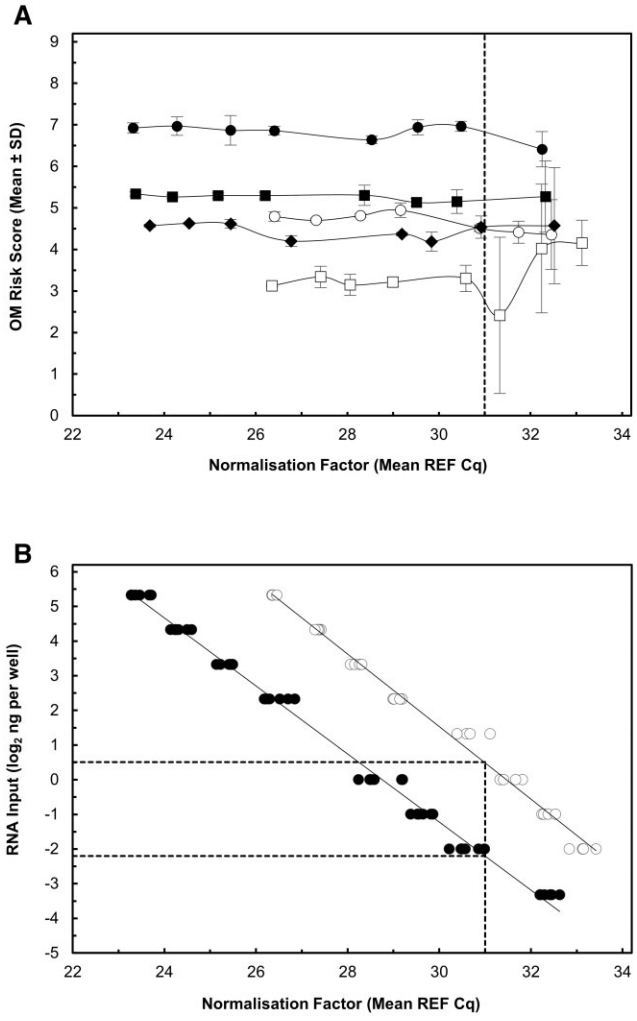

Fig. 3. RNA input study. (A), The relationship between OncoMasTR risk scores (y-axis) and amount of test sample used (x-axis). The normalization factor is a surrogate for the RNA input level and sample quality, with increasing Cq values reflecting decreasing test RNA concentrations in PCR reactions; (B) The relationship between absolute RNA input level and normalization factor and highlights the sample age-related differences shift in absolute Cq measurements. Contemporary <2-year old FFPE specimens are denoted by solid filled symbols, archival approximately 25-year old FFPE specimens are denoted by empty symbols in both graphs.

near the threshold delineating low- and high-risk groups. The OncoMasTR test result is reported not only as a binary classification; the continuous risk score and associated absolute 10-year risk of recurrence are also provided. All information provided by the test, in conjunction with other clinicopathological factors, should be considered in guiding treatment decisions.

The individual OncoMasTR gene expression assays were demonstrated to be linear over a wide range of sample input level with comparable PCR efficiencies in a pooled FFPE breast tumor RNA sample. This ensures robust test results are obtained for individual FFPE specimens that are not critically dependent on absolute RNA input once a minimum threshold for reference gene amplification is met. This analytical validation study was performed using both archival and contemporary FFPE breast tumor samples, and test results were stable for both sample categories in the RNA input range study.

The OncoMasTR multigene signature was relatively robust to admixture of nontumor cells down to

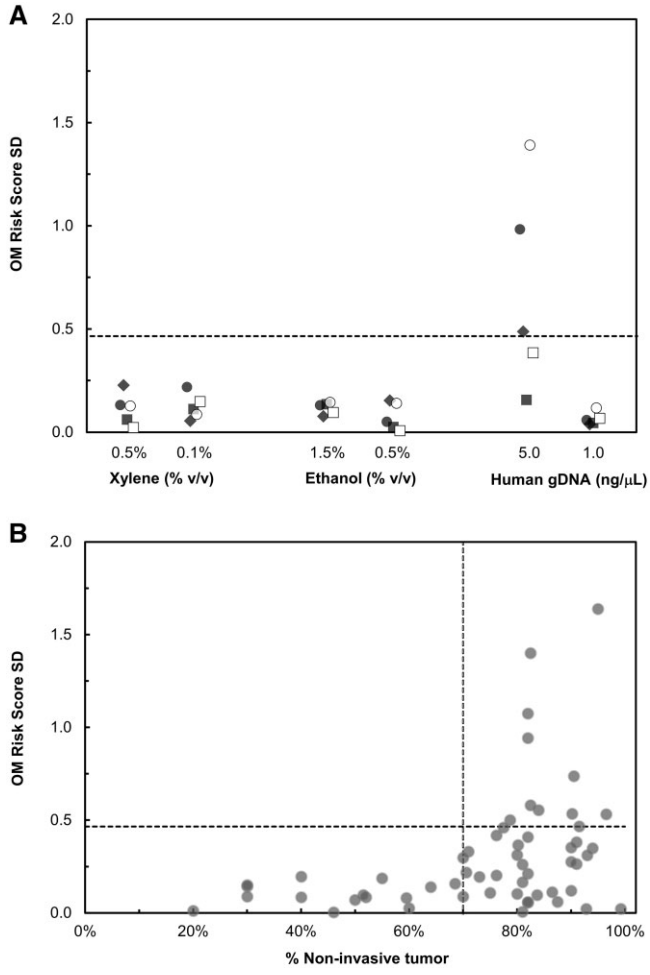

Fig. 4. Interference studies. (A), The impact on risk score in a panel of 5 FFPE RNA specimens spiked with various potential exogenous interfering substances as indicated. The SD in OncoMasTR risk scores for each interferent test condition and the relevant paired control sample was determined; (B), The consistency in OncoMasTR risk score measurement as a function of increasing nontumor content in whole FFPE tissue sections. The SD in OncoMasTR risk scores between macrodissected FFPE tissue and whole FFPE tissue section samples was determined. The dashed horizontal lines in each plot represent the cutoff above which OncoMasTR risk scores were considered adversely impacted by interferents.

a tumor percentage of 30%, which indicates that in clinical practice macrodissection from slides will almost always suffice without the need for laser microdissection.

The OncoMasTR multigene signature has been clinically validated in 2 large cohorts (4, 10) using a prospective-retrospective study design and therefore meets the criteria for level 1B evidence for tumor biomarkers (23). The analytical validity of the test, demonstrated in this study, supports the findings from clinical validation studies and provides a reference point for laboratories adopting the test and verifying the analytical performance characteristics in a decentralized setting.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: ER, estrogen receptor; RT-qPCR, reverse transcription quantitative PCR; FFPE, formalin-fixed, paraffin-embedded; H&E, hematoxylin and eosin; Cq, quantification cycle; LoD, limit of detection; LoB, limit of blank; gDNA, genomic DNA.

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