

Abstract

eXagenBC™ is a prognostic test for evaluating the risk of distant recurrence in women with early stage, node negative, invasive ductal carcinoma. The test uses fluorescent *in situ* hybridization (FISH) analysis of formalin-fixed paraffin-embedded (FFPE) biopsy specimens. The probe mix contains BAC-derived probes targeting the CYP24, BIRC5, and PDCD6IP genes.

PI calculation: Signal counts are entered into the following equation: PI = [BIRC5 + CYP24] + [PDCD6IP]. Two observers each calculate PI_{50s} from 50 nuclei, and the initial two PI_{50s} are subjected to a concordance test. If necessary a third observer counts a third set of 50 nuclei. The PI is calculated on the basis of the 100 nuclei counted by the two concordant observers. The PI evaluates the risk of distant metastasis, with a threshold of PI ≥ 3.000 defining high risk.

Heterogeneity: Because the test is based on FISH and the signals are observed in individual nuclei, the simultaneous assessment of these prognostic 3 genes in large sets of single nuclei may provide insight into the mechanisms and/or clinical significance of tumor heterogeneity.

Probe Accuracy

Experimental Design
Formalin fixed, paraffin embedded cell lines were tested in replicate on each of 20 days. Counts of individual probes, normalized to the number of nuclei counted, were compared to expected values.

	BIRC5 (green)	CYP24 (orange)	PDCD6IP (red)
mean counts per nucleus	1.91	1.76	1.67
std dev counts per nucleus	0.070	0.077	0.094
%CV counts per nucleus	3.7	4.4	5.6

CYP24: chromosome 20 • 3 copies in GM08123 • 2 copies in GM07216 • expected ratio for GM08123:GM07216 = 1.5

PDCD6IP: chromosome 3 • 3 copies in GM07216 • 2 copies in GM08123 • expected ratio for GM07216:GM08123 = 1.5

BIRC5: chromosome 17 • 2 copies in both cell lines • expected ratio for GM08123:GM07216 probe = 1

Results

%CV of probe counts in a normal diploid cell line range from 3.7 to 5.6% (upper table).

%CV in trisomic cell lines range from 4.4 to 10.1%. Concordance with expected values range from 0.90 to 0.99 (lower table).

probe counts per nucleus	GM08123 (N=40)		GM07216 (N=40)	
	mean	std dev	mean	std dev
BIRC5 green	1.92	0.193	1.89	0.152
	10.1	10.1	8.0	8.0
	concordance 0.99			
CYP24 orange	2.42	0.170	1.66	0.078
	7.0	7.0	4.7	4.7
	concordance 0.97			
PDCD6IP red	1.59	0.069	2.15	0.141
	4.4	4.4	6.5	6.5
	concordance 0.90			

Probe Sensitivity and Specificity

Experimental Design

Probe sensitivity and specificity were evaluated after hybridization of the probe mix to cytogenetically normal metaphase and interphase preparations.

Metaphase	Sensitivity	Specificity	N
PDCD6IP	100%	98.9%	92
BIRC5	98.9%	98.9%	92
CYP24	100%	100%	92
Combined Metaphase	99.6%	99.3%	92
Interphase	Sensitivity	N	
Interphase Sensitivity	95.4%	N/A	283

Metaphase: Chromosomes were identified by Giemsa or DAPI banding patterns. Numbers of probe signals on chromosome bands were tabulated.

Analysis:

% Sensitivity = [# of chromosome bands with correct signals + # of chromosome bands expected to have signal] x 100.

Analysis:

% Specificity = [# of signals on correct chromosome bands + total # of signals] x 100.

Interphase: Signal patterns on consecutive non-overlapping interphase nuclei were tabulated.

Analysis: % Sensitivity is expressed as the [# normal nuclei with the expected normal signal pattern + scored nuclei]. (Definition taken from Wiktor et al, 2006).

Repeatability (Precision) Study

Overall variation of PI by specimen

Sample	S008758	GM08123 (20q13 trisomy)	GM07216 (3pter-p21 trisomy)	Breast Tumor A	Breast Tumor B
run days	20	20	20	20	20
N	40	40	40	40	40
mean PI	2.205	2.716	1.673	2.089	3.771
std dev PI	0.090	0.134	0.172	0.126	0.235
%CV	4.1	4.9	10.3	6.0	6.2

Experimental Design

Samples:

cells lines: 1 normal diploid cell line • two abnormal trisomic cell lines

clinical specimens: one low-risk PI • 1 high-risk PI

All samples were analyzed in replicate each experiment day.

One test run per day for 20 days.

Summary statistics of the PI for each specimen are shown in the table above.

Results: %CV range from 4.1% to 10.3%.

Overall Variability of eXagenBC™

Experimental Design

Six cases of recently diagnosed invasive ductal carcinoma were tested with eXagenBC multiple times at two sites.

Cases "A" (low risk) and "B" (high risk)

60 sections each case were tested at 2 sites:
Each site: 30 sections were tested by 3 operators (10 sections each operator) in 5 runs on 5 days (Replicate testing each day).

Cases "C" and "D" (low risk), "E" and "F" (high risk)

30 sections each case were tested at 2 sites:
Each site: 15 sections were tested by 3 operators (5 sections each operator) in 5 runs on 5 days

Analysis Methods and Results

The grand mean PI (Table 1) used all PI determinations for each case. %CVs ranged from 4.8% to 10.6%.

Analyses are based on a nested ANOVA structure. The within-laboratory variability nested model is Day within Operator within Site, and includes 2 replicates for each day. Within-run %CVs ranged from 1.8% to 11.2% (Table 2).

The overall variability nested model (Table 3) is Operator within Site, with the 5 days representing replicates for each operator. Operator variability ranged from 2.8%CV to 12.1%CV (Tables 2 & 3) and between site variability ranged from 4.8% to 10.6% (Table 3).

Table 1. Overall %CV

Case ID	Grand Mean PI	Overall	
		SD	%CV
A	2.019	0.155	7.7
B	5.249	0.519	9.9
C	2.155	0.147	6.8
D	2.065	0.098	4.8
E	4.151	0.442	10.6
F	3.125	0.320	10.2

Table 1 is based on all cases; table 3 is based on cases C,D, E, and F.

Table 3. Overall Variability

Case ID	Grand Mean PI	Site	Between-operator within-site		Between-site	
			SD	%CV	SD	%CV
C	2.155	1	0.196	9.0	0.147	6.8
		2	0.078	3.7		
D	2.065	1	0.098	4.9	0.098	4.8
		2	0.062	2.9		
E	4.151	1	0.273	6.9	0.442	10.6
		2	0.480	11.0		
F	3.125	1	0.233	7.2	0.320	10.2
		2	0.364	12.1		

Table 2. Cases A & B, Within Laboratory

Case ID	Grand Mean PI	Site	Within-run ¹ SD / %CV			Between-operator within-site		Between-site	
			oper 1	oper 2	oper 3	SD	%CV	SD	%CV
A	2.019	1	0.201 / 10.3	0.130 / 6.8	0.219 / 10.7	0.203	10.3	0.155	7.7
		2	0.060 / 2.9	0.038 / 1.8	0.078 / 3.8				
B	5.249	1	0.557 / 11.2	0.420 / 8.6	0.322 / 6.6	0.398	8.1	0.519	9.9
		2	0.420 / 7.4	0.423 / 7.5	0.396 / 7.3				

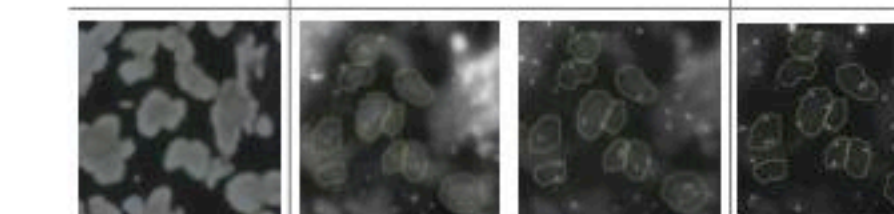
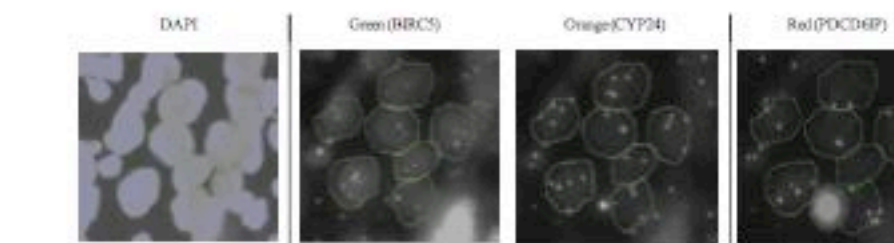
Within-run SDs and %CVs are based on the mean within-run variance of 5 runs for each operator. The mean within-run standard deviation is divided by the mean PI for the operator.

Heterogeneity

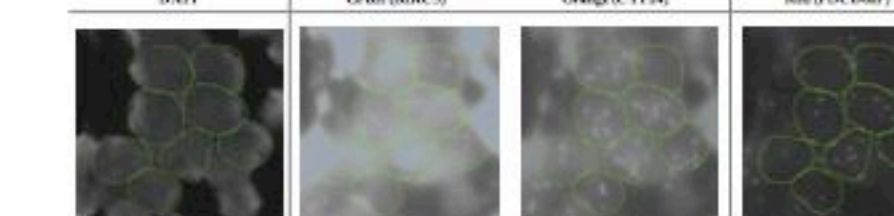
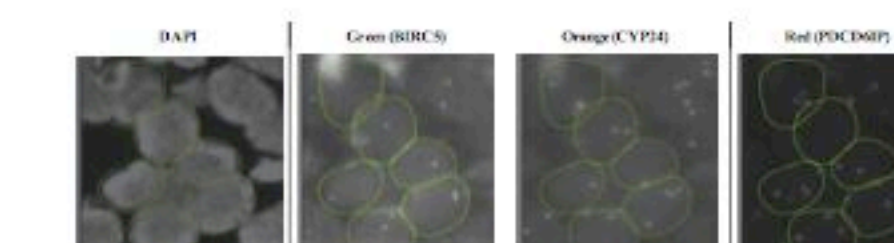
Analysis

For each specimen, two different fields of view from a single section are shown, with the circled nuclei viewed through four filters.

The average number of signals per nucleus, total signal counts, nominal PI based on the circled nuclei, and number of nuclei for each panel are summarized in the table below, right.



Case 1: The orange (Cyp24) signal counts are greater in Field A (high risk) than in Field B (low risk).



Case 2: The signal counts for green (BIRC5), orange (Cyp24), and red (PDCD6IP) are greater in Field B than in Field A; both fields indicate low risk.

Fig.	Field of View	Average signals per nucleus (total)			PI = [G + O] + R	# Nuclei
		Green	Orange	Red		
Case 1	Field A	2.57 (18)	3.86 (27)	1.86 (13)	3.462	7
	Field B	2.10 (21)	2.00 (20)	1.50 (15)		
Case 2	Field A	1.67 (10)	2.17 (13)	2.67 (16)	1.438	6
	Field B	2.25 (18)	6.25 (50)	3.25 (26)		
Case 3	Field A	2.30 (23)	6.50 (65)	2.20 (22)	4.000	10
	Field B	4.13 (33)	8.00 (64)	2.38 (19)		

Case 3: (not shown) The signal counts for green (BIRC5) are greater in Field B than in Field A; both fields indicate high risk.

These three cases demonstrate heterogeneity within single fields of view and between fields of view on single sections. The extent of heterogeneity among tumors is variable. Studies of clinical significance and mechanisms of tumor progression would benefit from analysis of the more highly heterogeneous tumors.

Summary and Discussion

The eXagenBC™ test is repeatable and reproducible. The test probes are accurate, sensitive, and specific. Because the test is FISH based and signals are collected from individual nuclei, tumor heterogeneity can be examined. Heterogeneity analysis could be the subject of clinical significance studies as well as studies addressing mechanisms of tumor progression."

References

Wiktor, AE, Van Dyke, DL, Stupca, PJ, Ketterling, RP, Thorland, EC, Shearer, BM, Fink, SR, Stockero, KJ., Majorowicz, JR, Dewald, GW. (2006). "Preclinical validation of fluorescence in situ hybridization assays for clinical practice". Genetics in Medicine 8:16-23