FDA Executive Summary for the GeneSearch BLN assay

Introduction

The presence of cancer in axillary lymph nodes is most often assessed by dissection, either as part of full lymph node dissection and/or in combination with sentinel lymph node dissection, in the routine evaluation of lymphogeneous spread of breast cancer. Both clinical and pathological examination of lymph node tissue contribute as prognostic indicators of metastasis-free and overall survival, contribute to an accurate decision on staging of patients, and provide information for various subsequent treatment decisions. As a result, there are significant short-term and long-term consequences in lymph node evaluation.

The anatomic disruption caused by full axillary lymph node dissection on the functionality and quality of life for early stage breast cancer patients has resulted in frequent use of sentinel lymph node biopsy, the removal and examination of the first and/or second lymph node in the chain of axillary nodes, to evaluate lymphogeneous spread of the primary tumor to other areas of the body. Histopathological evaluation intra-operatively or with subsequent permanent section evaluation of formalin-fixed tissue removed during biopsy has continued to rely on hematoxylin and eosin (H&E) staining. The use of immunohistochemistry and newer molecular-based methods has also been studied as part of the histopathology. But the reliability and clinical consequences from smaller and smaller detected regions containing tumor clusters or cells has caused acceptance of these newer methods to be controversial and delayed.

The staging of breast cancer continues to rely on both clinical and histological evaluation to determine the basic descriptors: tumor size, node involvement, and presence of distant metastasis (or the TNM system). Use of TNM descriptors in the American Joint Committee on Cancer (AJCC) staging system will serve as the standard for this discussion. In the 2002 version of the staging manual, one of the major changes incorporated was a change in the scope of methods used to designate the presence of involved nodes. The staging manual states: “Major classifications of lymph node status are designated according to the number of involved axillary lymph nodes as determined by routine hematoxylin and eosin staining (preferred method) or by immunohistochemical staining.” The 2002 version relies on H&E staining in order to make pathological classification of lymph node status but accepts the use of immunohistochemical evaluations as an additional descriptor. The manual also notes that classification of a lesion identified by RT-PCR alone will be pN0, the classification it would have had using standard histologic staining.

Under current clinical practice in clinically node negative breast cancer subjects, full axillary node dissection takes place intra-operatively only when the sentinel lymph node biopsy indicates at least one positive sentinel node. Patients with negative sentinel nodes usually do not proceed to immediate full axillary node dissection in order to spare patients the associated morbidity. The proposed assay has been described as an additional intra-operative evaluation of lymph node status and provides the same information as subsequent permanent section histological evaluation. The proposed assay has been designed to detect metastases greater than 0.2 mm in size, a size the sponsor believes are clinically relevant and actionable. We accept the characterization of 0.2 mm metastases as clinically relevant and actionable

since the AJCC staging manual has already classified and defined the presence of micrometastases (metastases between 0.2 mm and 2 mm) but recognizes the lack of evidence on 5 year or longer patient survival or progression-free survival outcome.

The GeneSearch BLN assay is a real time reverse transcriptase polymerase chain reaction assay to detect the presence of breast tissue in nodal tissue using 2 tissue specific RNA molecules as biomarkers. The two biomarker RNA's are transcribed from genes expressed at high levels in breast cancer tissue but only at low or background levels in nodal tissue. An additional RNA constitutively expressed from a gene present in normal lymph node tissue is also detected as an internal control gene amplifiable along with the two cancer marker RNA molecules. The presence of appropriate amplification by assay reagents from all three genes would indicate the presence of breast cancer cells metastatic to lymph nodes in the excised sentinel lymph nodes identified with the current visualization techniques. The absence of amplification from these 2 marker genes but successful amplification of the internal control gene would indicate the absence of metastatic breast cancer cells in excised sentinel lymph node tissue.

FDA review of the submission has focused on the intended use population and setting, analytical issues, clinical validity and clinical utility.

Intended Use Population and Setting

As proposed by the sponsor, the GeneSearch BLN assay is a qualitative in vitro test for the rapid detection of clinically relevant (> 0.2 mm) metastases in lymph node tissue removed from breast cancer patients. Results from the assay can be used to guide the decision to excise additional lymph nodes and aid in patient staging.

Though not explicitly noted, the assay is used on fresh lymph node tissue intra-operatively excised during a sentinel lymph node biopsy procedure. The current clinical study has not evaluated use of non-sentinel node tissue, use of tissue other than fresh tissue and use in other lymph node staging procedures such as full axillary node dissection. A specified procedure for cutting node tissue for evaluation in the proposed assay and for histopatho-logical evaluation was performed in the clinical study. Would the node cutting plan be necessary for routine clinical use in order to maximize the detecting ability of the proposed assay and other intra-operative or final permanent section histopathology evaluations?

Since the assay is designed for intra-operative use in approximately 30 minutes, positive assay results suggest immediate follow-up with full axillary node dissection. Negative assay results suggest no further dissection of axillary lymph nodes.

Use of the assay in conjunction with other current intra-operative histological procedures, such as frozen section histology or touch imprint histology, was not explicitly evaluated in the current clinical study, i.e. specific use and performance criteria for these other procedures were not designed into the study. Thus, it is unclear how interpretation of the proposed assay and conflicting results from other intra-operative histology procedures will be arbitrated for a decision to proceed to lymph node dissection of the axillary bed. However, comparative performance of the proposed assay with these 2 intra-operative procedures was performed in the clinical studies on subjects in which one of these other procedures was also performed. Given differences in performance of the proposed assay and other intra-operative procedures, is assay performance sufficiently high to substitute for these intra-operative procedures and accurately guide a decision to proceed with further lymph node removal?

Given the current reliance of the published staging procedures on H&E staining and pathological evaluation and with the resulting TNM designation, how will the proposed assay aid in staging breast cancer patients? Will assay results accurately substitute for other current standard histopathological procedures when that information is not available, or not performed, and accurately provide the equivalent staging information? Will assay results accurately complement current histology procedures? What staging information would be suggested when conflicting assay and histology results are found?

Given the performance characteristics of the assay, a major question for panel consideration arises: Is the balance of false negative results and false positive results sufficiently acceptable to allow approval of the assay for its Intended Use. The immediate outcome of a false negative result would be a failure to get a necessary full axillary node dissection while a false positive result would be an unnecessary surgical dissection with its significant associated morbidity. Longer term outcome data on metastasis-free survival and overall survival from assay positive and negative subjects is currently lacking. Would an equivalent rate of false positive and false negative results be an appropriate balance? Would an unequal balance be acceptable and if so, what would be an acceptable false positive rate given a modest false negative result?

Analytical Issues and Analytical Validation

In the performance of the assay, the fluorescence signal is converted to Cycle threshold values (Ct) using instrument specific-software present in the Cepheid SmartCycler instrument. Ct values of the external positive and negative control are compared with the acceptable range of values for each of the three markers using assay-specific software present in the SmartCycler instrument. Once these controls are within the acceptable ranges for each marker, Ct values are compared with previously determined cutoff values for the 2 cancer markers and the internal control. The strategy of the sponsor's assay is to designate a specimen positive when the Ct value of one or the other of the two cancer markers is below the cutoff Ct value for either respective marker. The Ct value of the internal control gene is not compared with its respective cutoff. A specimen is designated negative when the Ct value of both cancer markers is above the respective cutoff and the Ct value of the internal control is below its respective cutoff implying adequate amplification of the internal control gene from the specimen.

In the clinical study, 34 of 421 subjects (8.1%) had failures of the external controls or internal control gene. Assay results from these subjects were classified as invalid and were considered by the sponsor as assay negative for purposes of performance evaluation. The sponsor has stated that the results were classified as negative since the results do not provide the surgeon with information to support a diagnosis of metastases. The sponsor has stated that such results should be included as part of the "intent to use" population.

The sponsor has not stated that in routine clinical use invalid assay results would be categorized as assay negative. In order to clarify the clinical outcome (rather than analytical outcome) of invalid assay results, the FDA believes that when amplification failures of external controls or amplification failures of the internal control gene occur and in the absence of other intra-operative histology results to guide the surgeon, it would be clinically acceptable to defer a decision to proceed to full axillary node dissection until histological evaluation of permanent sections is completed. When amplification failures of assay external controls or the internal control gene occur, the assay test result indicates an "invalid test" result and a positive/negative assay result would not be expected to be reported to the requestor in most clinical diagnostic laboratories. In the absence of any other intra-operative histology result, significant gross organ observation, or clinical observation, a surgeon would have no information to guide a decision to immediately proceed to full axillary dissection. The surgeon would more likely make no further dissection and complete the sentinel node biopsy operation at that point. The unfortunate result of the assay failure would be a deferred decision for axillary node dissection. The deferral would not necessarily imply a second operation, unless the final histopathology report of the permanent sections indicated a positive result.

Exclusion of invalid assay results from performance calculations for the clinical study indicates that clinical sensitivity and specificity were not statistically different than when invalid results are included. It is unclear to us what the implications of exclusion or inclusion have on the clinical utility of assay results. Therefore, we seek guidance from the panel on the classification of invalid test results (negative or an unreported invalid test result) and a deferral or some other surgical decision when such results occur.

Clinical Validity

The sponsor sought to determine clinical validity through a prospective study of patients with previously diagnosed invasive breast cancer who were aged 18 years or older and who were scheduled to undergo sentinel lymph node biopsy at 11 sites in the United States. Sentinel lymph node tissue identified by standard locating techniques was removed using each site's intra-operative procedure. Each removed node was cut into 2 or more even numbered tissue slabs between 1.5 and 3.0 mm in thickness depending upon the size of the node. The clinical site used alternating tissue slabs for histology and the proposed assay. Patient node tissue destined for the proposed assay was processed intra-operatively. Each site made a determination of the lymph node status (i.e. breast cancer metastases and a subsequent full lymph node dissection procedure) independent of the proposed assay using usual site-specific criteria and pathological methods. Assay results were not used to make subsequent treatment or surgical decisions. Cancer metastasis > 0.2 mm in size was considered as histologically and clinically significant. Permanent section histopathology of nodes was evaluated by site pathologists and by a panel of 3 central pathologists. Site pathologist's evaluation of H&E stained permanent sectioned slides was reviewed by at least 1 of 3 central pathologists

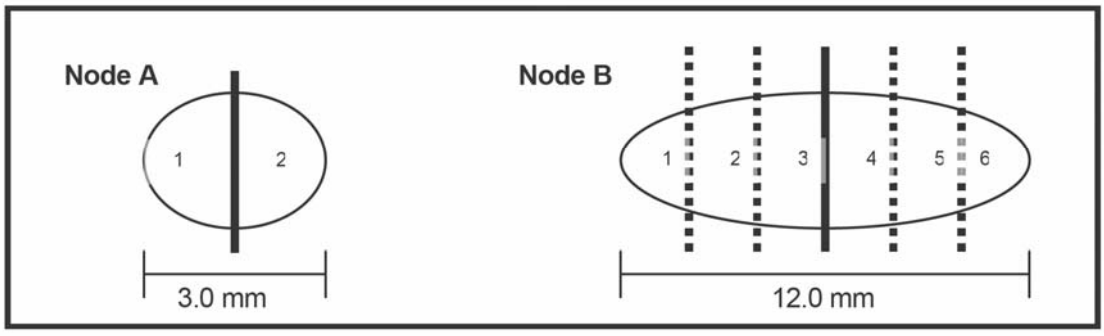
Sections of tissue sent specifically to Central pathology review were cut at 4-6 microns thickness for 3 sections spaced approximately 150 microns apart in each 1.5-3.0 mm thickness tissue slab. Immuno-histochemistry evaluations were performed by site personnel when H&E staining was negative by site pathologists and site evaluations were confirmed by at least 1 central pathologists when in agreement. Performance characteristics of sites' standard intra-operative evaluations were compared with permanent section histopathology in some but not all subjects. A minimum of 200 and maximum of 700 subjects could be enrolled. Sequential analysis of assay results compared with histopathology evaluation was planned and determined the final sample size of 423 subjects. Planned sequential analysis was not undertaken.

For comparison purposes with the proposed assay result only a positive or negative histology determination need be made. A patient was classified as positive if positive by either site pathology, central pathology, or both. If multiple nodes were removed from a patient, a positive histopathology in any tissue slab from any removed node caused a patient to be classified as node positive. A patient was classified as negative if negative by site and central pathology review from all tissue slabs from all removed nodes. The overall histology result for all subjects was the more positive of the final Central or final Site histopathology result. For analysis purposes, size of the metastases was categorized as follows:

- > 0.2 mm but unknown specific size – positive, metastasis
- 2 mm metastases – positive, macrometastases
- 0.2 -2 mm metastases – positive, micrometastases
- < 0.2 mm metastases with clusters of tumor cells – negative, tumor clusters
- < 0.2 mm metastases with isolated tumor cells – negative, isolated tumor cells
- No metastases – negative

All removed nodes were bisected along the short axis. Nodes 6.0 mm or less were bisected only into 2 tissue slabs. Larger nodes were cut into an even number of tissue slabs approximately 1.5 to 3.0 mm in thickness as shown in the following picture taken from the clinical protocol:

Figure 1. Example of node cut-in based on node size



No other node cutting scheme was evaluated by this clinical study since the sponsor and FDA discussed and agreed upon this plan for these initial effectiveness studies. Would the node cutting scheme seriously detract from the clinical validity of this study? In order to be effective and maximize the detecting ability of the proposed assay for future clinical use,

should such a node cutting scheme be recommended for routine surgical and histological evaluation of excised sentinel lymph nodes? Is any node cutting scheme critical to the ability to find cancer metastases in sentinel nodes and, if so, what minimal scheme is acceptable?

The objective of the study was to gather data supporting assay performance initially estimated from previous studies. The assay sensitivity was hypothesized to be 70% or better for the lower 95% confidence limit. The assay specificity was hypothesized to be 90% or better for the lower 95% confidence limit. A secondary objective was to collect long-term clinical outcome data to evaluate the assay and other markers as prognostic markers for long term survival (either overall survival or progression-free survival). The final success or failure of accrual and of the assay centered upon the ability of the assay to meet or exceed the sensitivity and specificity limits specified.

Clinical Utility or Effectiveness

The sponsor combined two study designs (a Beta study and a portion of subjects from the Pivotal study) to create data for evaluation of the cutoffs for the three biomarkers. The inclusion and exclusion criteria for subjects in each study were described as equivalent. Twelve clinical sites and 15 clinical laboratories throughout the U. S. participated. The sponsor notes that 12 study sites provided 274 subjects with valid assay results and defined histology results determined by permanent section hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) evaluation. Both study protocols were amended, with FDA concurrence, to include site H&E and IHC results in the determination of node status, include confirmation of site positives by the central pathologist, and changes to the sensitivity and specificity in the null statistical hypothesis. Testing of node tissue also included additional H&E sections and IHC sections from the opposite block face for tissue samples destined for the proposed assay. This additional sectioning was proposed to provide information on discrepancies between assay positive but histology negative samples due to sampling of different tissue for the assay vs. histology. Site H&E slides and IHC slides evaluated by site pathologists as positive but as negative by one central pathologist were forwarded to another central pathologist for confirmation. Data used in the evaluation have not been provided by the sponsor for confirmation of the appropriateness of the cutoff choice.

In summarizing the findings of the cutoff evaluation, the sponsor notes the following recommendations for the external negative and positive controls:

Control	Internal control gene	Marker 1	Marker 2
External negative control	≥ 25.5 to ≤ 34 Ct	≥ 36 Ct	≥ 36 Ct
External positive control	≥ 36 Ct	≥ 17.0 to ≤ 23.0 Ct	≥ 18.5 to ≤ 23.5 Ct

Marker cutoffs for marker 1 will be ≤ 31 and for marker 2 will be ≤ 30 in test samples. The cutoff for the internal control gene will be < 36.

The submission notes 423 enrolled subjects, 418 females and 5 males. Subjects ranged from 27 to 92 years of age, mean 60 years. Nine subjects were noted to have chemotherapy and one had radiation therapy. The majority of subjects were diagnosed with invasive ductal carcinoma (80%), 14% having invasive lobular carcinoma, and the remaining 6% had invasive cancer other than lobular or ductal type. Of the cancer subjects, 62% had stage I disease, 32% stage II disease, 5% stage III disease, and 0.5% stage IV disease. When information was available, 79% of subjects had estrogen receptor positive tumors, 68% had progesterone positive tumors, and 74% had HER-2 negative tumors. The mean tumor size of all subjects was 1.9 cm. The mean subject age was 60.3 years. The mean and median number of lymph nodes removed was 2.9 and 2 nodes per patient. The overall prevalence of cancer metastases to lymph nodes detected by histology was 29.1% (121 positive subjects in 416 subjects). The prevalence of lymph nodes with metastatic cancer ranged from 14.3% to 45.5% by clinical site. The sponsor notes that of 421 subjects the assay result was invalid for 34 subjects (8.1%). The invalid result was due to external control or subject sample failure. The sponsor states that these invalid results were not excluded from performance calculations but classified as assay negatives since the results do not provide a clinician with evidence of nodal metastases. The sponsor further states that 5 subjects with final histology results of "undetermined" were excluded from the total 421 subjects. This leaves 416 subjects with defined final H&E histology results used for their calculation of device performance. The sensitivity of the proposed assay in this pivotal study was 87.6% (95% confidence interval 80.4 to 92.9%). The specificity of the proposed assay in the pivotal study was 94.2% (95% confidence interval 90.9% to 96.6%).

The role of immunohistochemistry evaluation was examined by comparing the H&E histology categorization with the final histology categorization resulting from H&E and immunohistochemistry evaluations. The observed agreement in the above 6 histological categories plus the undetermined category was 0.952 ± 0.010. The number of subjects who differed between H&E and H&E plus immunohistochemistry was 20, representing 4.8% of 421 subjects. However, only one subject was significantly changed (from negative to positive with micrometastasis), representing less than 1% of 421 subjects. Other changes in categorization were within the 3 negative categories or from negative to undetermined category. Therefore, this supports a conclusion that immunohistochemistry evaluations did not significantly change H&E evaluations. The data also supports a conclusion that H&E evaluations alone are reliable evaluations to determine the final histological status of subjects in this study.

The following shows the categorization of pooled results by site and central H&E histology stratified by GeneSearch assay result without removing subjects with invalid assay results:

GeneSearch Assay positive

	Central H&E histopathology				
Site H&E histopathology	> 2 mm	0.2-2 mm	<0.2 mm	negative	total
>2 mm	78	1	0	3	82
0.2-2 mm	4	5	0	7	16
<0.2 mm	0	1	0	2	3

negative	4	0	0	16	20
Result not available	2	3	0	1	6
Total	88	10	0	29	127

GeneSearch Assay negative

Central H&E histopathology

Site H&E histopathology	> 2 mm	0.2-2 mm	<0.2 mm	negative	total
>2 mm	0	2	0	1	3
0.2-2 mm	0	2	0	2	4
<0.2 mm	0	1	0	4	5
negative	0	3	1	270	274
Result not available	0	0	0	8	8
Total	0	8	1	285	294

All assayed samples

Central H&E histopathology

Site H&E histopathology	> 2 mm	0.2-2 mm	<0.2 mm	negative	total
>2 mm	78	3	0	4	85
0.2-2 mm	4	7	0	9	20
<0.2 mm	0	2	0	6	8
negative	4	3	1	286	294
Result not available	2	3	0	9	14
Total	88	18	1	314	421

Based upon the sponsor's determination of [redacted] subjects, highlighted in the [redacted] section of the first table, were found. [redacted] subjects, highlighted in the [redacted] of the second table, were found. [redacted] subjects, highlighted in the [redacted] of the first table, were found. [redacted] subjects, highlighted in the [redacted] of the second table, were found. The observed overall agreement of site [redacted] was [redacted]. The [redacted] confidence interval of agreement based upon the [redacted] At worst, the greatest disagreement of site and central histopathology categorization was [redacted]. Note further that [redacted] subjects, highlighted in the [redacted] of the third table, were classified with metastases greater than [redacted] by sit istology but were classified as [redacted] by the opposite site or central histology, a percentage of [redacted] of evalua Of 11 false negative subjects, [redacted] were classified with metastases greater than [redacted] by site or central histo assified as completely negative by the opposite site or central histology. Of [redacted] subjects, [redacted] were similarly

When assay and histology results are categorized as positive/negative, the following 2 x 2 table results:

GeneSearch result	Central and site pathology (either or both) positive	negative	total
positive	108	19	127
negative	11	283	294
total	119	302	421

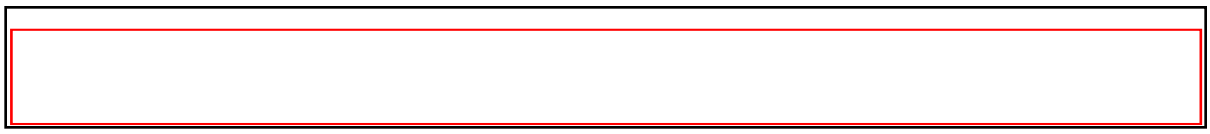
Based upon these histology results, the prevalence of node positive subjects was 28.3% ± 2.2% (exact binomial 95% confidence interval 24.0% to 32.8%). Among histology positive subjects by site or central histology evaluation, the proportion of assay positive subjects was 90.8% ± 2.7% (exact binomial 95% confidence interval 84.1% to 95.3%), a value not statistically different from the sponsor's value of 87.6%. The rate of false negative results in this analysis is 9.2%. Among histology negative subjects, the proportion of assay negative subjects was 93.7% ± 1.4% (exact binomial 95% confidence interval 90.3% to 96.2%), a value not statistically different from the sponsor's value of 94.2%. The rate of false positive results in this analysis was 6.3%. For the hypothesis that the positive predictive value of the GeneSearch assay was greater than the overall prevalence in the above table, the probability was less than 0.001. The positive predictive value of the assay was 85.0% ± 3.2%. The negative predictive value of the assay was 96.3% ± 1.1%.

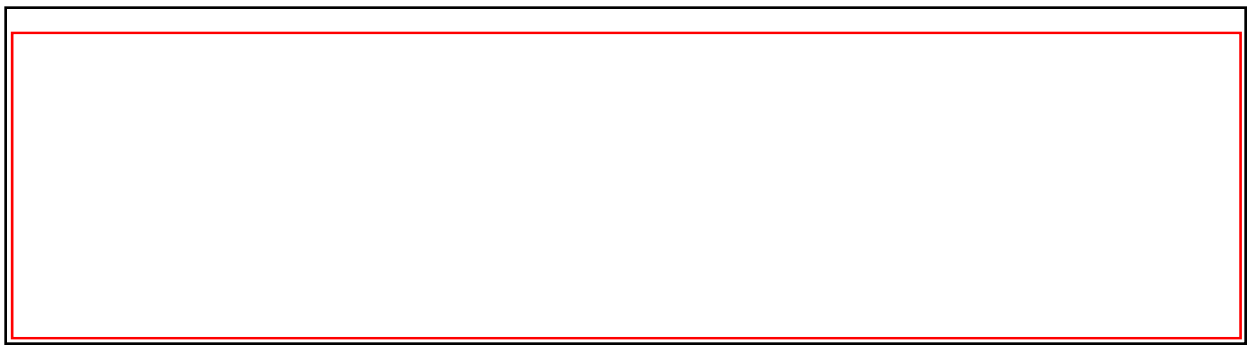
When the assay is used intra-operatively, a positive assay result would indicate an 85% risk of metastatic breast cancer in the subject, no worse than 78% based upon the lower 95% confidence interval of the positive predictive value. When the assay result is negative, the risk of no metastatic breast cancer would be 96%; no worse than 93% based upon the lower 95% confidence interval of the negative predictive value. Therefore when a negative assay result occurs intra-operatively there is 96% confidence (at least 93%) that the subject is absent loco-regional breast cancer metastases and implies little need for complete axillary lymph node dissection to occur during the sentinel lymph node biopsy surgery. This degree of confidence when assay negative could provide a clinical rationale for concluding that the subject does not yet have breast cancer metastatic by lymphogenous spread to the local axillary region. It further supports the conclusion that there is sufficiently little risk of

metastatic cancer (at most 7% risk of cancer though assay negative) to not proceed to level I axillary node dissection and the associated morbidity from that operation. Thus the patient could be spared surgical intervention without serious risk, though at least 2% risk, of metastatic breast cancer from lymphogenous spread of the disease.

The risk of metastatic breast cancer when assay positive is high; at least 78% and as much as 91%. The risk of lymphogenous spread of breast cancer is sufficiently high that the assay could provide a clinical rationale for level I axillary node dissection and histopathological evaluation of other lymph nodes than sentinel lymph nodes. Balancing that risk is the absence of metastatic breast cancer though the assay is positive. In this study the rate of non-metastatic breast cancer though assay positive is estimated to be 15% and as much as 22% (1- lower 95% confidence limit of positive predictive value). This fact suggests that when assay positive that 22% of subjects at most would be subjected to an unnecessary level I axillary node dissection even though absent metastatic spread of breast cancer to the regional lymph nodes. The fact that at most 7% of breast cancer patients with metastatic disease would fail to have a needed surgical intervention because of a negative GeneSearch assay must also be clinically weighed with the fact that at most 22% of subjects would undergo an unnecessary surgical intervention because of a positive GeneSearch assay. It is unclear if surgical oncologists and primary care breast oncologists would consider this assay performance acceptable given these facts.

Of 421 subjects it is possible to exclude from performance calculation the 34 subjects having an invalid assay result. Based upon histology results, the prevalence of node positive subjects was 30.0% ± 2.3% (exact binomial 95% confidence interval 25.4% to 34.3%). Among histology positive subjects by site or central histology evaluation, the proportion of assay positive subjects was 91.4% ± 2.6% (exact binomial 95% confidence interval 84.7% - 95.8%), a value not different from the value, 90.8%, when invalid subjects are included. The proportion of false negative subjects in this analysis was 8.6%. Among histology negative subjects, the proportion of assay negative subjects was 93.4% ± 1.5% (exact binomial 95% confidence interval 89.7% to 96.0%), a value not different from the value, 93.7%, when invalid subjects are included. The proportion of false positive subjects in this analysis was 6.6%. The lower 95% confidence limit of the proportion of assay positive subjects among histology positive subjects was greater than the sponsor target value of 70%. The lower 95% confidence limit of the proportion of assay negative subjects among histology negative subjects was not greater than the sponsor target value of 90% (89.7%). Since this target value was not achieved statistically it would be possible to conclude that the assay did not appropriately categorize histology positive and negative subjects. However, since the difference from the target value is 0.3%, it does not appear clinically appropriate to conclude that the assay did not appropriately categorize subjects with positive or negative histology results when invalid assay results are excluded from calculation. For the hypothesis that the positive predictive value of the GeneSearch assay was greater than the overall prevalence, the probability was less than 0.001. The positive predictive value of the assay was 85.5% ± 3.2%. The negative predictive value of the assay was 96.2% ± 1.2%. The ratio of false positive subjects to false negative subjects in this analysis was 0.770, a value less than 1.0.





A direct comparison of the sensitivities of the proposed assay and frozen section histology results when performed intra-operatively in the same subjects was calculated. The sensitivity of the proposed assay in this comparison was 95.6% while the sensitivity of frozen section histology was 85.2%. The difference in sensitivity (10.1%, 95% confidence interval of the difference 17.8% to 2.5%) was statistically significant ($p = 0.01$). Therefore, in a direct comparison of the proposed assay and frozen section histology the proposed assay has a statistically higher sensitivity than frozen section histology. The ratio of true positive subjects using the proposed assay to the true positive subjects in frozen section histology is 1.12. A direct comparison of the specificities of the proposed assay and frozen section histology results with each other was also calculated. The specificity of the proposed assay in this comparison was 93.9% while the specificity of frozen section histology was 97.8%. The difference in specificity (-3.9%, 95% confidence interval of the difference -7.6% to -0.2%) was statistically significant ($p = 0.037$). Therefore, in a direct comparison of the proposed assay and frozen section histology, the proposed assay has a statistically lower specificity than frozen section histology. Though statistically different, it is not clear if the specificity difference is clinically significant given the high values for each intra-operative procedure. Of note also from this data, the false positive rate of the proposed assay is 2.8-fold higher than the false positive rate of frozen section histology (14 of 229 for the proposed assay compared with 5 of 229 for frozen section histology). Due to the small number of false positive subjects in this comparison, it is not clear if the ratio of false positive rates is clinically meaningful, though statistically different.

The risk of non-metastatic breast cancer (and thus an unnecessary surgery) though GeneSearch assay positive is estimated to be 15% and as much as 22%. The risk of non-metastatic breast cancer though positive by frozen section histology is estimated to be 3% in this direct comparison and as much as 9%. The risk of metastatic disease (and thus a missed necessary surgery) because of a negative GeneSearch assay was at most 5%. The risk of metastatic disease because of a negative frozen section histology was at most 10%. The higher risk profile appears to be when either test is positive. It was previously noted that the overall risk of metastatic breast cancer when GeneSearch assay positive is high; at least 78% and as much as 91%. In this direct comparison of frozen section histology with results of the proposed assay, the risk of metastatic breast cancer when positive by frozen section histology is also high; at least 78% and as much as 93%. The risk of non-metastatic breast cancer though GeneSearch assay positive is estimated to be 15% and as much as 22% (1- lower 95% confidence limit of positive predictive value). The risk of non-metastatic breast cancer though positive by frozen section histology is estimated to be 3% in this direct comparison and as much as 9%. At most 7% of breast cancer patients with metastatic disease would fail to have a needed surgical intervention because of a negative GeneSearch assay and at most

22% of metastasis-free subjects would undergo an unnecessary surgical intervention because of a positive GeneSearch assay. At most 10% of metastatic breast cancer patients would fail to have a needed surgical intervention because of a negative frozen section histology. At most 5% of metastasis-free subjects would undergo an unnecessary surgical intervention because of a positive frozen section histology.

The sponsor provides an analysis of the number of cancer positive nodes in a subject when positive by the proposed assay compared with positive by overall histology in the same subject. The following presents the tabulation by subject:

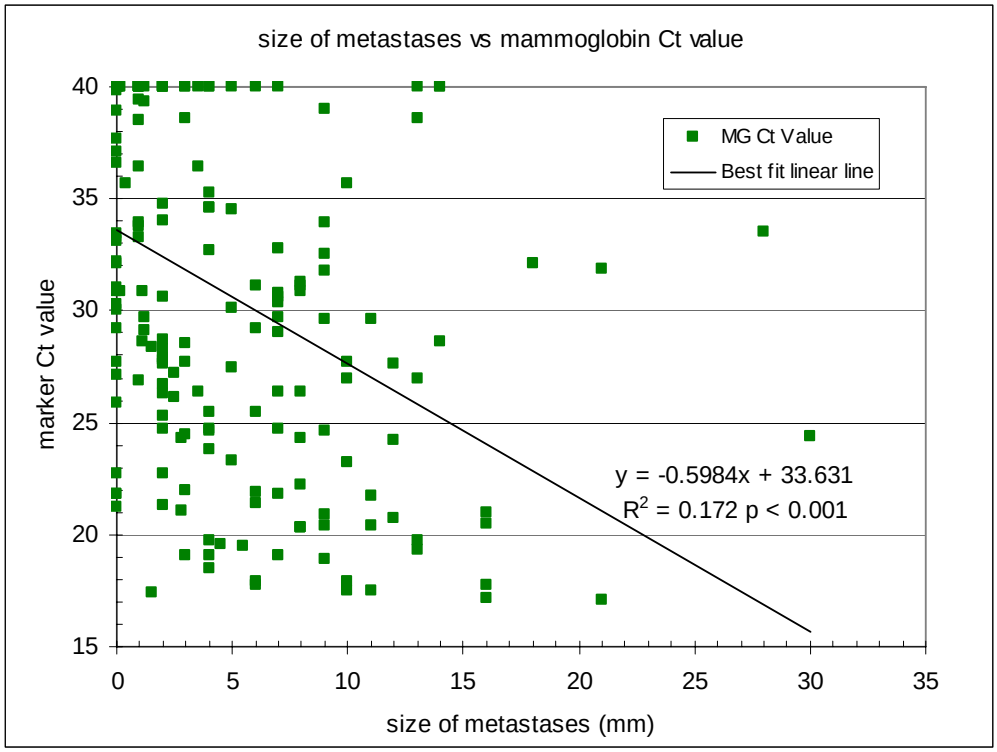
	Proposed assay (number of positive nodes)			
Histology (number of positive nodes)	0	1	2	≥ 3
0	278	15	1	1
1	14	57	8	1
2	1	5	23	1
≥ 3	0	0	1	10

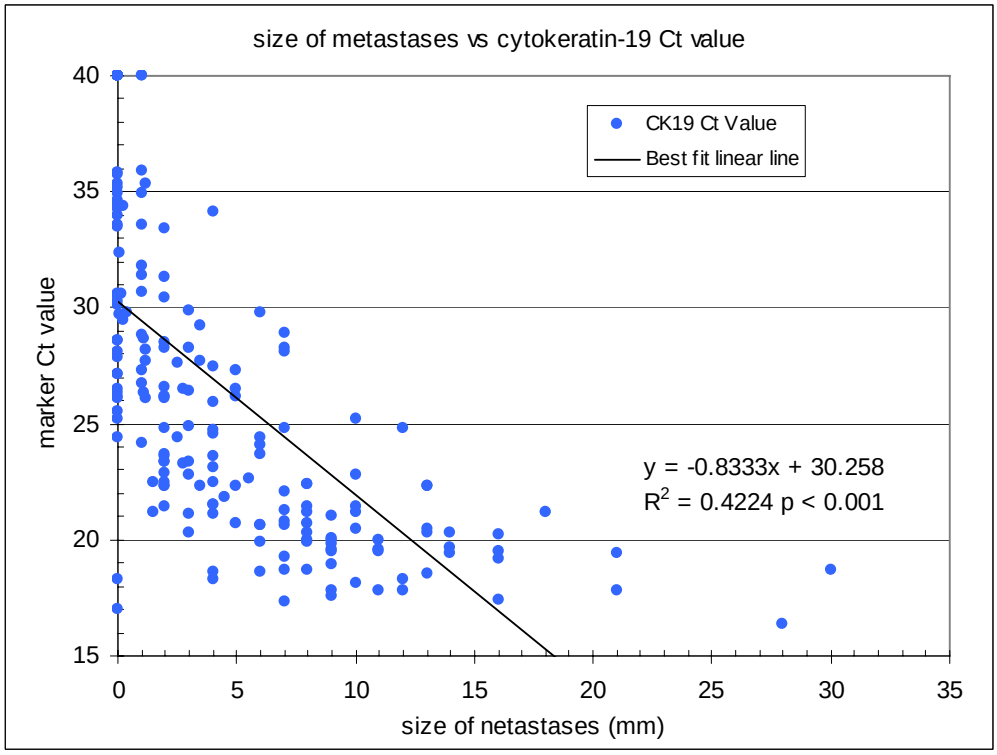
The sponsor notes a kappa value of agreement between the proposed assay and overall histology as 0.75 (95% confidence interval 0.68 – 0.81). The sponsor concludes form this table that kappa values above 0.61 are indicative of substantial agreement. The sponsor does not note that the kappa value is not equivalent with a kappa value of 1.0, indicating perfect agreement of the number of histology positive nodes with the number of assay positive nodes. The upper confidence limit of the kappa value is 0.81 and is not equal to 1.0. The kappa value additionally is not equivalent with a value of 0, indicating agreement equivalent with random chance agreement. The lower confidence interval of the kappa value is 0.68 and is not equal to 0. The main emphasis of sponsor discussion is to note 17 subjects with false positive results, of whom 15 subjects were identified as assay positive in one node only. Of 15 subjects classified as false negative, 14 subjects were identified as histology positive in one node only. The sponsor concludes that these differences are due to tissue sampling differences in subjects with less metastatic spread of disease.

The AJCC staging manual establishes pathologic staging of lymph nodes based upon the number of involved lymph nodes (pN0 – no lymph node metastases histologically; pN1 - metastases to 1-3 axillary lymph nodes; pN2 - metastases to 4-9 axillary lymph nodes; pN3 – metastases to 10 or more axillary lymph nodes). With regard to the correlation of the GeneSearch assay with histology by number of involved lymph nodes, the sponsor does not note that the overall agreement between assay and histology is 89% ± 1.5% (standard error of the mean). In an amendment in response to FDA questioning, the sponsor states that the distribution of patients outside of the diagonal agreement is evenly distributed. The number of subjects above the diagonal is 27 subjects (6.5% of the total) while the number of subjects below the diagonal is 21 subjects (5.0% of the total). Of the 27 subjects above the diagonal, 17 false positive subjects (17 of 295 histology negative = 6% rate of clinical false positive) are present. Of the 21 subjects below the diagonal, 15 false negative subjects (15 of 121 histology positive subjects = 12.4% rate of clinical false negative). The remaining 16 off-

diagonal subjects represent true positive subjects since all are histology and assay positive but are mismatched in the number of positive lymph nodes between the assay and histology. The proportion of mismatched subjects of the total subjects, 3.8%, represents the discrepancy rate between histology and the assay. Of more importance, does this correlation of the assay with number of positive/negative nodes accurately represent the pathologic staging? The assay false positive rate (6%) represents one portion of the inaccuracy since the assay categorized subjects as positive (pN1 or more) when actually the subjects were pN0 by histology. These subjects, in the absence of histology information, could receive an unnecessary axillary node dissection and could be over-staged due to the inaccurate pathologic staging. Of the 121 lymph node positive subjects (29% of total 416 subjects), the assay accurately categorized 116 as pN1 while the remaining 5 (5/121 = 4% or 1.1% of 416 total subjects) were at least pN2. Assay false negative subjects categorized as pN0 by the assay, in the absence of histology information, would be under-staged since histology would categorize subjects as pN1. It is unclear if the under-staged subjects would miss a necessary axillary node dissection but could possibly fail to receive appropriate therapy (chemotherapy, radiation therapy, or some other therapeutic option) based upon the under-staging. Similarly, over-staged subjects could receive an unnecessary axillary node dissection and subsequently receive unnecessary therapy based on the over-staging in the absence of histology. Therefore, 6% of histology negative subjects could be over-staged and 4% of histology positive subjects would be under-staged. The total error rate of 10% (also reflected in the non-agreement rate; 11%) represent staging errors from the assay. It is unclear if this error rate is clinically acceptable. Further it is unclear if accurate staging would occur using only the assay without the complementary histological H&E evaluations.

The sponsor calculated the Spearman rank correlation coefficient of Ct value for each marker gene by final histology results in 6 categories (P(MA), P(MI), P, N(CL), N(ITC), and N). The correlation coefficient for marker 1 was 0.77 and was 0.74 for marker 2. The sponsor notes that the correlation coefficients for each marker are highly correlated with final histology category. The correlation of Ct value for each marker with the 6 histology categories would be even more informative if the correlation were with the measured sizes of metastases. This would indicate a more direct correlation of assay signal, measured as Ct value, with the size of the metastases. The FDA requested of the sponsor the measured size of metastases for each subject where available for comparison with marker Ct value. Metastases size was linearly correlated with Ct value for each marker. The following graphs indicate the relationship.


size of metastases vs mammoglobin Ct value
MG Ct Value
Best fit linear line
y = -0.5984x + 33.631
R2 = 0.172 p < 0.001
marker Ct value
size of metastases (mm)
40
35
30
25
20
15
0
5
10
15
20
25
30
35


size of metastases vs cytokeratin-19 Ct value
CK19 Ct Value
Best fit linear line
y = -0.8333x + 30.258
R2 = 0.4224 p < 0.001
marker Ct value
size of netastases (mm)
40
35
30
25
20
15
0
5
10
15
20
25
30
35