GeneSearch™ Breast Lymph Node (BLN) Test Kit REF **2900004**

Σ 30 Reaction Kit

GeneSearch™ RNA Sample Preparation Kit REF **2900005**

Σ 10 Test Kit

Instructions for Use

TABLE OF CONTENTS

INTENDED USE

For *in vitro* diagnostic use only.

The GeneSearch™ Breast Lymph Node (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 to aid in patient staging.

SUMMARY AND EXPLANATION

The presence of metastases in axillary lymph nodes is the most important prognostic indicator in breast cancer.[1-3] Identification of metastases in lymph nodes and the number of positive lymph nodes identified are used in staging to direct treatment of breast cancer patients.

The status of the sentinel lymph nodes (SLNs) has been shown to accurately reflect the presence of metastases in the axillary lymph nodes (ALNs) in patients with breast cancer.[4-9] As summarized by Yared et al.,[10] multiple studies show that SLN examination has a sensitivity of 83.4 % to 100 % for the detection of axillary nodal metastases using paraffin embedded Hematoxylin and Eosin (H&E) histology. When SLN dissection (SLND) is conducted, typically the patient undergoes complete ALN dissection (ALND) only when one or more SLNs test positive for the presence of metastases. Patients with negative SLNs are spared the significant morbidity associated with complete ALND. Patients who undergo ALND have significantly higher rates of increased swelling in the upper arm and forearm (lymphedema), pain, numbness and motion restriction about the shoulder when compared with patients who undergo only SLND.[11] Rapid assessment of the cancer status of SLNs permits the completion of lymphadenectomy of the nodal basin during the same operative procedure, if required, thus avoiding a second surgery.

The recognition of the prognostic importance of the absolute number of involved lymph nodes is in keeping with current clinical practice and is supported by a large body of clinical data.[12-14] One of the changes incorporated into the revised staging system for breast cancer includes mention of molecular techniques, such as Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) used to detect nodal metastases. One limitation to the previously available molecular data was the absence of a large training and test set validating the molecular results as clinically relevant. The GeneSearch™ BLN Assay has been validated against permanent section H&E and immunohistochemistry (IHC) results.

H&E histology has a number of limitations. One is a result of the non-uniform distribution of metastases in the lymph node.[15] The standard for pathological evaluation is to examine one or more H&E-stained section(s) from each lymph node.[16] It has been demonstrated that limited sampling of the lymph node tissue results in misdiagnosis of lymph node-negatives in some breast cancer patients. Further evaluation of H&E-stained sections from deeper levels of the lymph node or by using IHC staining revealed metastases originally not detected by the limited sampling.[10,16-18] In addition, H&E stains may not detect lobular metastases in breast SLNs that are detected by IHC.[19]

The GeneSearch™ BLN Assay provides information similar to that provided by formalin-fixed paraffin-embedded H&E and IHC histology, i.e. the absence or presence of clinically actionable lymph node metastases in patients that have been diagnosed with breast cancer.[1] One advantage of the GeneSearch™ BLN Assay is that the tissue being tested is sampled thoroughly via homogenization. The aliquot being tested is a true representation of the entire tissue being evaluated unlike histology which is a small sampling of the tissue. This increased sampling has resulted in improved sensitivity when compared to Intraoperative Frozen Section (FS) and Intraoperative Touch Imprint Cytology (Intraoperative Touch Prep, ITP). The sensitivity of the

GeneSearch™ BLN Assay was 95.6% whereas the sensitivity for FS was 85.6% and ITP was 45.5%. (Table 13).

The GeneSearch™ BLN Assay has been designed to detect the presence of metastases > 0.2 mm in lymph nodes.[12,13] Analysis of the data collected in the BLN Pivotal Clinical Study showed a high correlation between assay cancer analyte Cycle threshold values (Ct values) and level of metastases reported by permanent section histology (Spearman nonparametric correlation coefficient $\geq$ 0.74). The absolute number of positive SLNs identified using the GeneSearch™ BLN Assay has been shown to correlate with the absolute number of positive SLNs identified with thorough permanent section histology (kappa value of agreement between the two tests was 0.75, 95% confidence interval 0.68 to 0.81). The GeneSearch™ BLN Assay may also be used as an aid in identifying lobular carcinomas, which may be difficult to identify by standard pathology.[19]

PRINCIPLES OF THE PROCEDURE

The GeneSearch™ BLN Assay is a real time RT-PCR assay that detects the presence of breast tumor cell metastasis in lymph nodes through the detection of gene expression markers present in breast tissue, but not in nodal tissue (cell type specific messenger RNA). This assay employs real time RT-PCR utilizing the Cepheid SmartCycler® system to generate quantitative expression data for these genes. The expression results are then applied against predetermined criteria to provide a qualitative (cancer positive/cancer negative) result. Results of the assay have been demonstrated to correlate with detection of metastasis by the current method of paraffin-embedded (permanent section) H&E histology.

Real Time RT-PCR Reaction

The GeneSearch™ BLN Assay qualitatively detects the expression of two genes, Mammaglobin (MG) and Cytokeratin 19 (CK19), which are expressed at a high level in tissue of breast origin but only at background levels in normal lymph node tissue. The specificity of MG for breast tissue and its usefulness in the detection of metastatic disease in lymph nodes, blood and marrow has been well demonstrated.[20,21] CK19 is an epithelial cell marker that has been frequently associated with breast cancer in lymph nodes, bone marrow and blood, and is expressed at levels a million-fold higher in cells associated with cancer compared to normal cells.[22] Using tissue RNA from all available sentinel lymph nodes of 254 subjects, the combination of MG and CK19 was shown to be optimal for the detection of breast cancer metastasis to lymph nodes.[23]

In order to maximize the uniformity of sampling, lymph nodes are divided into sections and alternating sections are combined and processed using the GeneSearch™ BLN Assay. The remaining sections may be used for routine pathology evaluation. Using the GeneSearch™ RNA Sample Preparation Kit, the nodal tissue is homogenized to release RNA molecules. The RNA is purified from the tissue homogenate and RT-PCR is performed on the RNA specimen.

The real time RT-PCR reaction is performed in a homogeneous, one-step, fully contained reaction. Three gene markers (MG, CK19 and an internal control gene [IC; porphobilinogen deaminase, PBGD]) are included in this reaction. A complementary DNA (cDNA) strand is produced from messenger RNA (mRNA) using the reverse transcriptase function of a thermostable DNA polymerase. The reaction mixture (a buffer containing marker-specific DNA primers, marker-specific DNA probes, deoxyribonucleoside triphosphates [dNTPs], and DNA polymerase) is heated to activate the DNA polymerase and then cooled to allow specific annealing of the target-specific reverse (antisense) primers to the target mRNAs. The annealed primers are extended by the DNA polymerase in the presence of excess dNTPs to form cDNA strands.

Following production of cDNAs, the reaction mixture containing the cDNA:RNA hybrid is again heated to denature the strands. The reaction mixture is cooled, allowing the target-specific forward (sense) primers to anneal, and allowing the DNA-dependent DNA polymerase activity to extend the sense strand through to the reverse primer regions. This amplification process results in double-stranded DNA sequences called amplicons. Subsequent cycles of denaturation and annealing/extension exponentially increase the amounts of these amplicons, which are subsequently detected utilizing sequence-specific DNA probes.

Detection of Gene Markers

Production of target amplicon is detected using a probe that contains a DNA sequence specific for part of the target amplicon. This probe is linked to a fluorescent molecule and a molecule that quenches fluorescence. The probe initially anneals to the target sequence and then is cleaved by the exonuclease activity of the DNA polymerase as extension from the primer proceeds past the probe region. As a result of this cleavage, the fluorescent molecule is separated from the quencher, leading to an increase in fluorescence. By measuring fluorescence, the presence of target amplicon can be detected.

Each gene marker is detected using fluorescent molecules with different excitation and emission wavelengths. Fluorescence for each of the gene markers is measured following each temperature cycle. Amplification of the gene markers is detected through increased fluorescence due to release of the fluorophore from the proximity of the quencher. The Ct value is determined when the fluorescent signal exceeds a pre-defined threshold limit. If the external controls are valid, then the Ct value for each gene marker in the patient sample is compared to marker-specific Ct cutoff values. If the Ct value for either or both gene markers is less than the cutoff value then the sample is determined to be positive. All amplification, detection of fluorescence and the interpretation of the signals is done automatically by the SmartCycler® instrument.

MATERIALS PROVIDED

GeneSearch™ Breast Lymph Node (BLN) Test Kit – Part # 2900004

- Instructions for Use
- 0.3 mL Master Mix (White cap): Contains Tris buffer, 0.05% Bovine Serum Albumin, 0.08% Proclin 300, < 0.01% primers and probes
- 0.3 mL Enzyme Mix (Yellow cap): Contains Tris buffer, 0.08% Proclin 300, < 0.02% DNA polymerase (enzyme), and a proprietary stabilizing agent
- 0.05 mL Negative Control (Purple cap): Contains Tris buffer, < 0.001% plasmid DNA, 0.08% Proclin 300
- 0.05 mL Positive Control (Green cap): Contains Tris buffer, < 0.001% plasmid DNA, 0.08% Proclin 300

GeneSearch™ RNA Sample Preparation Kit – Part # 2900005

- 100 mL Homogenization Buffer: Contains ≥ 25% guanidine thiocyanate
- 8 mL Wash Buffer 1: Contains <10% guanidine thiocyanate, 10% ethanol
- 2 mL Wash Buffer 2: Contains proprietary compound mixture, 0.09% sodium azide, pH 7.5
- 1.9 mL RNase-free water
- 10 RNA Spin Columns

GeneSearch™ BLN Protocol Software CD, IVD – Part # 2900006

- GeneSearch™ BLN Protocol Software Release and Install Notes

MATERIALS REQUIRED BUT NOT PROVIDED

Laboratory Reagents

- β-Mercaptoethanol, molecular biology grade, 14.3 M
- 200-proof (absolute) ethanol, molecular biology grade
- Nuclease free water (non DEPC treated)

Laboratory Equipment and Consumables

- Cepheid SmartCycler® Diagnostic System which includes:
  - I-CORE™ blocks
  - Mini-centrifuge
  - Plastic sample racks
  - Computer (including monitor from Cepheid)
  - Cepheid SmartCycler® Diagnostic Operator Manual
  - Cepheid SmartCycler® Dx Software (comes pre-loaded on computer from Cepheid)
    - Tube Puller
    - Cepheid 25 µL reaction tubes for Cepheid SmartCycler®
    - Barcode reader (optional)
- Printer
- 1.5 or 1.7 mL polypropylene microcentrifuge tubes, certified DNase- and RNase-free (Axygen MCT-175-C-S or equivalent)
  - Vacuum System
  - Qiagen® VacConnectors
  - Qiagen® VacValves
  - Vacuum source capable of drawing 800-1000 mbar vacuum
  - Qiagen® Vacuum Manifold
  - Qiagen® Vacuum Regulator
  - Omni Homogenizer
  - Omni Adaptor
  - Omni Tip™ Disposable Probes for Omni Homogenizer
  - 8 mL and 14 mL polypropylene culture tubes for homogenization.
  - Disposable forceps
  - Scale capable of weighing milligram amounts
  - Weighing paper or boats
  - Calibrated pipettors:
    - 1 pipette capable of measuring 5 µL
    - 1 pipette capable of measuring 10 µL
    - 1 pipette capable of measuring 50 µL
    - 1 pipette capable of measuring 400 µL
    - 1 pipette capable of measuring 700 µL
    - 1 additional 1000 uL pipettor capable of measuring 200 µL to 800 µL (used to add ethanol-homogenate mix to column)
  - Pipette-Aid
  - Disposable serological pipettes (10 mL and 25 mL)
  - Mini-centrifuge (optional)
  - Rocker
  - Aerosol resistant, DNase- and RNase-free tips to fit pipettors
  - Microcentrifuge (Eppendorf model 5415D or equivalent)
  - Vortex (Vortex Genie 2, Model A 560 or equivalent)

- Scalpel and blades
- Powder-free disposable gloves

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic use.
2. Before testing samples, read the entire contents of these Instructions for Use and obtain appropriate training.
3. Qualify all equipment prior to use. Refer to the Cepheid SmartCycler® Diagnostic Operator Manual for installation qualification instructions.
4. The GeneSearch™ BLN Test Kit has been designed to detect specific breast tissue mRNA markers in lymph nodes. For accurate test results, avoid contaminating lymph nodes with breast tissue. Testing breast tissue can yield false positive assay results.
5. Do not use expired reagents.
6. **Warning**! All biological specimens and other materials coming into contact with the specimen(s) are considered biohazardous. Handle as if capable of transmitting infection. Treat and dispose of waster using proper precautions and in accordance with local, state and federal regulations. Never pipette by mouth.
7. Results of the assay should be used in conjunction with all clinical information derived from diagnostic tests, physical examination and complete medical history in accordance with appropriate patient management procedures.
8. Use the Run Report, not the Patient Report, to view and report patient results.
9. **Warning**! Homogenization Buffer contains guanidine thiocyanate: Risk and safety phrases * R20/21/22 (Harmful by inhalation, in contact with skin and if swallowed), R32 (Contact with acids liberates very toxic gas), S13 (Keep away from food and drink), S26 (In case of contact with eyes rinse immediately with plenty of water and seek medical advice), S36 (Wear suitable protective clothing), S46 (If swallowed seek medical advice immediately and show label or container). These materials are harmful. Wear appropriate protective clothing when working with these materials. Guanidine thiocyanate can form highly reactive compounds when combined with bleach. Use a suitable laboratory detergent and water to clean spills followed by bleach solution if decontamination is required.
10. **Warning**! Wash Buffer 1 contains 10 % ethanol and is considered flammable (R10, Flammable). Do not use around open flames.
11. **Warning**! Some of the reagents contain Proclin 300 preservative. Symptoms of overexposure to Proclin 300 may include irritation of skin, eyes, mucous membranes and upper respiratory tract.
12. **Warning**! Wash Buffer 2 contains sodium azide**.** Sodium azide may react with lead or copper to form potentially explosive metal azides. When disposing of this reagent, flush plumbing thoroughly with water.
13. The presence of fat on/in the lymph node tissue decreases the sensitivity of the assay and may result in an ‘ invalid’ test result.
14. Lymph nodes from patients diagnosed with lymphoma may give false positive assay results.
15. Fixing tissue in formalin prior to testing will result in an ‘invalid’ test result.
16. Avoid contaminating lymph nodes with primary tumor tissue as it may produce a false-positive test result.

17. Do not pool lymph nodes as it may result in a loss of assay sensitivity and cause an erroneous result.
18. Processing at temperatures above 30ºC may result in a loss of assay sensitivity.
19. Avoid microbial contamination of reagents. Additionally, RNA is susceptible to degradation. It is important to maintain ribonuclease-free conditions.
20. Caution should be exercised in placing the SmartCycler® tubes into the instrument. If tubes are not placed into the instrument in the order specified by the software, the run will be invalid.
21. Environmental contamination of the PCR laboratory by PCR products can cause erroneous results. The sample and control tubes should not be opened under any circumstances in the PCR lab/area to avoid amplicon contamination. If possible, different laboratory areas should be used to prepare the reaction mix and perform the PCR amplification thus minimizing the possibility of amplicon contamination. Ideally, a laminar flow hood should be used during assembly of the PCR reactions to minimize the possibility of contamination. After each run, clean work areas (including applicable equipment) and spills with fresh 10 % bleach/water solution (let sit for 15 minutes) followed by a water rinse.
22. All personnel should follow universal precautions and use laboratory safety equipment (i.e. safety glasses, laboratory coat, gloves). If available, a fume hood should be used for preparing sample preparation kit reagents and a biosafety cabinet for handling biological material. If a hood is not available, it is recommended that a face shield be used during the homogenization step.
23. Avoid contamination of reagents with bleach as it will cause erroneous results.
24. Contamination of reagents may occur if vial caps are interchanged. Only one vial should be open at any given time.
25. In cases where other PCR tests that are open-tube assays are also conducted by the laboratory, separated and segregated working areas should be used for specimen preparation and amplification / detection activities. Supplies and equipment should be dedicated to each area and should not be moved from one area to another. Gloves must always be worn and must be changed before moving from one area to another or before manipulating reagents.
26. Do not use damaged reagent kits.

REAGENT STORAGE AND HANDLING

GeneSearch™ Breast Lymph Node (BLN) Test Kit Storage and Handling

- Store at –15ºC to –25°C, before opening and after opening. Kit is stable for 2 hours at room temperature.
- Follow the instructions for mixing of reagents provided in the "RT-PCR" section of this document. Thaw and mix reagents prior to use. The GeneSearch™ Breast Lymph Node (BLN) Test Kit should be removed from the freezer at least 20 minutes prior to use to allow for complete thawing. Utilize a rocker for thawing and mixing reagents, if available. If a rocker is not available, mix thawed reagents by inverting the vials (cap down) and pulse-vortexing each reagent three times. Spin each reagent briefly in the microcentrifuge or mini-centrifuge to remove any liquid from caps before opening. Inadequate mixing of the PCR reagents prior to use can affect the sensitivity and reproducibility of the assay and cause erroneous assay results.

- Acceptable functional performance for the GeneSearch™ BLN Test Kit was observed following up to 12 freeze-thaw cycles.
- Protect reagents from exposure to direct light.
- When properly stored, reagents are stable until the expiration date printed on the kit label. Do not use expired reagents.
- The GeneSearch™ BLN Test Kit components are manufactured and tested as a master lot. Do not interchange reagents from different lots.
- After completing 30 reactions, any residual reagents must be discarded.
- Once opened, the GeneSearch™ BLN Test Kit, if stored correctly, has been shown to be stable for 6 months, or until kit expiration date, whichever comes first.
- The volume of Master Mix, Enzyme Mix and Sample or control added to the PCR reaction affects the Ct value obtained. Care should be taken to ensure that 10 µL of Master Mix and Enzyme Mix are added to the reaction and 5 µL of sample and/or control are added. The assay can tolerate variations in the volume of any one reactant up to 25%, however, variations in the volumes of multiple reactants (such as those that would result from poor pipetting technique) will cause variations in Ct values that may negatively impact assay results.
- Once PCR reactions are assembled (Master Mix, Enzyme Mix, and sample are added to the Cepheid tube) the thermal cycling should be initiated within 30 minutes. Holding the assembled reactions for longer than 30 minutes before initiation of thermal cycling will result in a loss of assay sensitivity.

GeneSearch™ RNA Sample Preparation Kit Storage and Handling

- Store at 15ºC to 25ºC, i.e. ambient temperature.
- When properly stored, reagents are stable until the expiration date printed on the kit label. Do not use expired reagents.
- Once opened, the GeneSearch™ RNA Sample Preparation Kits can be stored at 15 ºC to 25 ºC (the recommended storage temperature) for a period of 30 days or until kit expiration date, whichever comes first.
- Working reagents prepared for the Sample Preparation Kit (working Homogenization Buffer and working Wash Buffer 2) can be stored for a period of 30 days.
- The RNA Sample Preparation Kit components are manufactured and tested as a master lot. Do not mix and match reagents from different lots.
- After completing 10 purifications, any residual reagents must be discarded.
- Eluted RNA is stable at ambient conditions (15 ºC to 25 ºC) for up to 60 minutes.
- Holding homogenates (homogenized tissue solution) for more than 60 minutes at room temperature may result in a loss of assay sensitivity for some samples. Homogenate solutions should not be held at room temperature for more than 60 minutes. If homogenates will not be tested immediately, solutions should be frozen at –65°C or below until needed.
- The ratio of homogenate to 70% ethanol should be 1:1 (equal parts of homogenate and 70% ethanol solution). Be careful to accurately measure both solutions to ensure that the ratio remains 1:1. Ratios of 1.33, 1.25, 1.0, 0.8 and 0.75 homogenate-to-ethanol were evaluated for the affect on assay performance. A ratio of 0.75 or less (300 µL homogenate:400 µL ethanol) was found to give unacceptable results.
- The 70 % ethanol solution must be mixed prior to use. Failure to mix before use may decrease the sensitivity of the assay.

TISSUE AND ASSAY INTERMEDIATES STORAGE AND HANDLING

- If lymph node tissue will not be tested within 45 minutes after removal from the patient, the tissue should be snap-frozen in liquid nitrogen and stored frozen at –65°C or below until testing occurs. If the sample is frozen, it should not be allowed to thaw prior to homogenization. Keep samples frozen on dry ice during weighing and sectioning until homogenization. Once Homogenization Buffer is added, homogenize immediately.
- Lymph node tissue that is homogenized immediately upon removal from the patient can be stored frozen at –65°C or below for testing within 21 days. Frozen homogenates should be thawed completely at room temperature (until all ice crystals disappear) and mixed prior to use.
- It is not recommended that homogenate-ethanol mixes be stored for use at a later date. These intermediates are stable for up to four hours at ambient temperatures. If they will not be used within 4 hours of being created from the homogenates, they should be discarded and new homogenate-ethanol mixes should be created at the time of RNA purification.
- RNA can be purified from fresh tissue immediately upon removal from the patient and the RNA can be stored frozen for up to nine weeks for testing at a later date. If frozen RNA samples will be used they should be thawed and mixed prior to use.
- If tissue or assay intermediates (homogenates, RNA) will be shipped, they should remain frozen and be shipped on dry ice.

TEST PROCEDURE

Preparation of Working Reagents

Homogenization Buffer

To prepare Working Homogenization Buffer, add 1.0 mL β-mercaptoethanol (β-ME) to one bottle of Homogenization Buffer and mix well. Reagent is stable for 30 days after β-ME addition at ambient temperature. Record expiration date on container. Homogenization Buffer may form a precipitate upon storage. If necessary, redissolve by warming to 30 to 37°C and mix gently by inversion until the precipitate dissolves. Return to ambient temperature before use.

Warning! β-ME is toxic; wear appropriate protective clothing and dispense in a fume hood, if available.

Wash Buffer 2

To prepare Working Wash Buffer 2, add 8 mL absolute 200-proof ethanol to Wash Buffer 2 concentrate in its container and mix well. Reagent is stable when stored at ambient temperature for 30 days after ethanol addition. Record expiration date on container.

70% Ethanol

To prepare 70% ethanol, add 7 mL 200-proof molecular biology grade ethanol to 3 mL of nuclease-free water. Ensure the solution is mixed well prior to use**.** Incomplete mixing of the 70% ethanol solution prior to use may cause erroneous assay results.

Specimen Collection

1. Care must be taken to minimize the contamination of the lymph node with breast or primary tumor tissue, as this may cause a false positive result in the assay. Clean surgical instruments and surgical trays must be used.

2. Lymph node tissue should be placed in a fresh transport container after excision, labeled appropriately and immediately transported to the pathology cut-in area.
3. The lymph node should be prepared as soon as possible to minimize RNA degradation. If tissue must be held for any period of time before processing (e.g., while waiting for additional lymph nodes), keep the tissue on the weighing paper until processing begins. DO NOT place the tissue in the homogenization buffer and allow it to remain for any period of time. Each lymph node should be processed as a separate specimen. Do not use any tissue fixatives on the lymph node prior to preparation. Tissue is stable for 45 minutes at room temperature after removal from the patient. If tissue will not be homogenized within 45 minutes it should be flash frozen in liquid nitrogen and placed in the freezer at -65ºC or below until testing will commence. If frozen tissue will be used, do not allow tissue to thaw before homogenization. Tissue should be placed on dry ice during weighing and sectioning.
4. Before preparation of the lymph node, clean the cutting board and spread a fresh disposable surface on the cutting board. Put a fresh blade on the scalpel and change into a fresh pair of gloves. Change gloves, scalpel blades, forceps and cutting surface between lymph nodes.

   Note: This is essential to minimize sample cross-contamination.
5. Remove the lymph node from its container with gloved hands and clean forceps (do not use forceps that have been in contact with other tissue) and place onto the fresh disposable surface.
6. Clean the lymph node of any fibroadipose tissue (fat) following standard procedures for the laboratory. Fat is a known interfering substance in the assay. Check for and remove any non-lymph node material.
7. Remove tissue sections that are required for routine pathology testing, if required.

Note[1]: During clinical trials approximately 50% of the lymph node was tested with the GeneSearch™ BLN Assay and the remaining tissue was submitted for histology, a portion of which was sampled and tested to obtain the performance results as detailed in the "Clinical Performance" section below.

Note[2]: Lymph nodes from patients diagnosed with lymphoma may cause a false positive result.

Sample Preparation

Homogenization

Homogenization Buffer and Wash Buffer 2 must be prepared per the "Preparation of Working Reagents" section before proceeding further.

1. Determine the sample weight in milligrams (mg) of the tissue to be tested in the GeneSearch™ BLN Assay. Place a fresh piece of weigh paper on the balance, tare and weigh the sample.
2. If a sample is greater than 2 to 3 mm in any dimension, use a fresh scalpel blade to mince the tissue into pieces approximately 2 to 3 mm in diameter. Care should be taken to avoid contamination of the tissue during processing.

   Do not place tissue into homogenization buffer until immediately before homogenization. Once tissue has been introduced to the homogenization buffer, homogenize immediately.

3. Label tubes for homogenization in such a way that source specimens can be identified (with Patient ID and Node ID, if applicable).

4. Add Homogenization Buffer to the homogenization tube (8 mL or 14 mL polypropylene culture tube; for Homogenization Buffer volume below 4 mL use an 8 mL tube, otherwise use a 14 mL tube). Use Table 1 to determine the required volume.

Table 1. Volume of Homogenization Buffer Required

Tissue Weight (mg)	Homogenization Buffer (mL)	Tube Size (mL)
3 - 149	2	8
150 - 199	3	8
200 - 249	4	8
250 - 299	5	14
300 - 349	6	14
350 - 399	7	14
400 - 449	8	14
450 - 499	9	14
500 - 550	10	14
> 550	See note below	

Note: Tissue weighing greater than 550 mg will not be adequately homogenized using the recommended system. The tissue should be divided into equivalent parts prior to homogenization and each part should be homogenized, purified and assayed as an individual specimen.

5. Using clean forceps, transfer the tissue into the Homogenization Buffer.

6. Place a new homogenization probe into the Omni homogenizer.

7. Homogenize each lymph node completely (typically 30 to 60 seconds) using the highest homogenizer setting available.

8. Process the homogenate as described in the "RNA Purification" section.

   Note: Once the homogenates are created they are stable at ambient temperature for up to 60 minutes. If they will not be tested immediately, they should be frozen at –65°C or below until RNA purification will begin. If previously frozen homogenates will be used in the assay, ensure that the homogenates are completely thawed at room temperature (no ice crystals visible) and mixed prior to use.

9. Dispose of the homogenization probe.

RNA Purification

Multiple homogenates can be processed using this procedure. Always ensure that the vacuum is turned to the OFF position prior to the application of a solution to RNA Spin Columns during RNA purification.

1. Label tubes in such a way that source specimens can be identified (with Patient ID and Node ID, if applicable).

2. Add 400 µL of homogenate to 400 µL of 70% ethanol in a 1.5 mL tube and mix by vortexing for 10 seconds.

   Note[1]: The ratio of homogenate to 70% ethanol should be 1:1 (equal parts of homogenate and 70% ethanol solution). Be careful to accurately measure both

solutions to ensure that the ratio remains 1:1. The 70% ethanol solution must be mixed prior to use. Failure to mix before use may decrease the sensitivity of the assay.

Note[2]: The homogenate-ethanol mix solutions can be held for up to 4 hours at ambient temperature. If RNA purification will not be initiated within 4 hours of creation of the homogenate-ethanol mix the homogenate-ethanol mix should be discarded and a new mix should be created from the original homogenate solution.

3. For each sample, attach a VacValve onto a Vacuum Manifold and a disposable VacConnector to each valve.
4. Attach a RNA Spin Column on to the VacConnector, leaving the cap open. Ensure that each VacValve is closed.
5. Aliquot each homogenate/ethanol mix from step 2 onto a separate RNA Spin Column (Column). Label each column with Patient ID and Node ID, if applicable. The volume of homogenate/ethanol mix to be added to the column is based on the original tissue amount and is provided in Table 2.

Table 2. Volume of Homogenate/Ethanol Mix Required

Tissue Weight (mg)	Volume of Homogenate-Ethanol Mix (µL)
3 - 39	700
40 - 49	500
50 - 59	400
60 - 69	350
70 - 79	300
80 - 89	250
90 - 99	225
≥ 100	200

6. Turn VacValves to the ON position and apply vacuum (800-1200 mbars) until sample is filtered (approximately 30 seconds). The vacuum must remain above 800 mbars throughout the filtration.
7. Turn VacValves to OFF position. Add 700 µL of Wash Buffer 1 to each column. Turn VacValves to ON position and allow the solution to filter through the column. Turn VacValves to OFF position.
8. Add 700 µL of Wash Buffer 2 to each column. Turn VacValves to ON position and allow the solution to filter through the column. Turn VacValves to OFF position.
9. Remove each column from the Vacuum Manifold and place into a 2 mL collection tube (supplied with the column).
10. Centrifuge the tube(s) containing the column(s) for 30 seconds at > 10,000 RPM in a microcentrifuge.
11. Discard the collection tube(s). Put each column into a new collection tube (1.5 to 1.7 mL polypropylene microcentrifuge tube).
12. Add 50 µL of RNase-free water directly to the filter membrane in the center of each column.
13. Centrifuge at > 10,000 RPM for 30 seconds in a microcentrifuge.

Before continuing to step 14, ensure that the collection tube has been labeled in such a way that the source specimen can be identified (e.g., Patient ID and Node ID).

14. Discard the column(s). Approximately 50 µL of eluted RNA solution will be contained in each collection tube.
15. Proceed immediately to RT-PCR.

    Note: The eluted RNA solution is stable for up to 60 minutes at ambient temperature. If the RNA solution(s) will not be tested immediately, they should be frozen at –65°C or below until the assay can begin. If previously frozen RNA solutions will be used in the assay, ensure that the RNA solutions are completely thawed (no ice crystals visible) and mixed prior to use.

RT-PCR

It is critical that tissue or other environmental materials do not contaminate the assay reagents and components coming into contact with reagents and specimen. Ensure the work area is clean and free of materials not required to process the GeneSearch™ BLN Assay. Change gloves prior to assembling the PCR tests. Use only PCR-quality disposables and discard if there is a possibility of contamination. A dedicated pipette for the PCR area is recommended.

1. Thaw and mix the reagents before processing.

   Note: Inadequate mixing of the PCR reagents prior to use can affect the sensitivity and reproducibility of the assay.

   Centrifuge the tubes at high speed briefly to collect contents at the bottom of the tube. The GeneSearch™ BLN Assay can be left at room temperature for up to 2 hours.

2. Turn on the computer and SmartCycler® instrument. Log in, input patient ID, node ID, and GeneSearch™ BLN Test Kit lot number.
3. Obtain the required number of Cepheid PCR reaction tubes (one for the Negative Control, one for the Positive Control and one for each patient sample). Label each tube to clearly identify the source specimen and place it in the rack. A maximum of six (6) samples can be tested in each run.

   Note: Accurate pipetting is essential for optimal performance of the BLN Assay. **Care should be taken to ensure that precise volumes are dispensed.**

4. Using a fresh pipette tip for each tube, add 10 µL Master Mix to each tube.
5. Using a fresh pipette tip for each tube, add 10 µL Enzyme Mix to each tube.
6. Using a fresh pipette tip, add 5 µL Negative Control (Purple Cap) to the bottom of the tube labeled “Negative Control.” Close the tube, ensuring the cap has completely snapped in place.
7. Using a fresh pipette tip, add 5 µL Positive Control (Green Cap) to the to the bottom of the tube labeled “Positive Control.” Close the tube, ensuring the cap has completely snapped in place.
8. Using a fresh pipette tip for each specimen, add 5 µL of specimen to the bottom of their respective tubes. Close the tubes, ensuring the caps have completely snapped in place. Proceed immediately to the next step.
9. Centrifuge the tubes for 15 to 30 seconds in the SmartCycler® mini-centrifuge.

10. Set up a run in the SmartCycler® software following the instructions provided in the Cepheid SmartCycler® Diagnostic Operator Manual. Assay run parameters are a part of the protocol selected when the run is set up and are provided on the protocol disk.
11. Place the tubes in the SmartCycler® instrument in the locations selected when the run was set up. When handling SmartCycler® reaction tubes, hold by the ribbed upper portion of the tube. Avoid touching the optical detection windows at the bottom of the tube to ensure an accurate optical reading. Confirm that the appropriate tubes are placed in the appropriate sites of the instrument by checking the site map. Ensure that tubes are firmly seated in SmartCycler® unit.
12. Close the lids on the SmartCycler® instrument and initiate the run. Assay run parameters will be provided with the SmartCycler® software.
13. Ensure that assay setup is done rapidly. Sensitivity of the assay may be reduced if the cycling protocol is not initiated within 30 minutes of assembling the reaction.
14. Following completion of the run, remove the Cepheid PCR reaction tubes using the Tube Puller provided. Discard the tubes **without opening** the tubes.

    Note! It is critical that the tubes NOT be opened following amplification to avoid environmental contamination by the PCR amplicon. If a tube inadvertently opens, immediately decontaminate by wiping the area with a fresh 10% bleach solution and rinsing with water 15 minutes later**.** After decontamination, set up reactions with positive and negative controls to demonstrate a lack of residual environmental contamination.

Retesting

Testing may be repeated based on the mode of failure**.**

External Control Failures: If a run is invalidated due to failure of external controls, the user can repeat the assay using the residual RNA sample from patient lymph node(s) and external controls from the GeneSearch™ BLN Test Kit.

Internal Control Failures: If a sample fails because all markers including the internal control are negative for a given patient (from one or more lymph nodes), RNA can be re-purified from the respective homogenate(s) and the assay can be repeated.

The assay should be repeated as soon as the results are available from the first run**.** The tissue homogenate and purified RNA are stable up to 60 minutes at ambient temperature when proper caution is exercised during their preparation.

QUALITY CONTROL

External Controls

External controls are provided for both cancer markers and for PBGD**.** These controls must be included with each run. External controls consist of linearized plasmids containing sequences capable of being amplified and detected by the primers and probes used in this GeneSearch™ product. The controls monitor reagent quality and instrument performance as they relate to assay performance.

Internal Control

The internal control consists of detection of mRNA from a constitutively expressed gene in lymph node tissue as a control against false negative results. The results of this control are obtained as one of the multiplexed gene markers in the specimen reaction tube. This control

monitors the sample quality, sample preparation and assembly of the RT-PCR reaction in the specimen reaction. It is possible that extremely high expression of the cancer markers will inhibit detection of the internal control. As a result, assays in which one or both cancer markers are positive in at least one lymph node are considered valid, regardless of the result observed for the internal control.

Contamination Controls

The Positive Control and Negative Control also serve as contamination controls. The Positive Control does not contain the target sequence for the internal control and serves as its contamination control. The Negative Control does not contain the cancer marker target sequences and serves as their contamination controls. The contamination control system protects against environmental contamination or nonspecific products that could result in incorrect assay results.

Interpretation of Results

The software will generate results in the form of a report**.** The results presented on the "Run Report" (not the "Patient Report") should be used for reporting patient results. The report provides information in the following sections and corresponding field names as shown in Table 3.

Table 3. Report Sections and Field Names. Refer to the Cepheid SmartCycler® Dx Operators Manual.

Section	Field Names
Run Information	Run Name, User Name, Run Status, Start and Finish Times, Software Version
Assay Information	Assay Name and Version, Kit Lot Number and Expiration date, Assay Status, Assay Type
Patient Results	Number of Instrument Sites used per Patient (referring to the number of samples per patient), Patient ID, Patient Result, Number Positive
Sample Results	Site ID, Patient ID, Sample ID, Assay Result for each site, IC (Internal Control) result by site, any Warning or Error Code, Notes inputted by user, Marker 1 and Marker 2 results
Error(s)	Error(s) or Warnings
Instrument(s)	Instrument and Serial Number
Growth Curve	Plot of fluorescence versus cycle number. The increase in fluorescence is proportional to the amount of amplicon generated and is used to determine the cycle threshold.

The patient result is displayed in the Run Report as determined by utilizing the decision tree depicted in Figure 1 below. The expected qualitative Patient Result will be "Positive", "Negative", or "Invalid" based on the Ct values obtained for each marker in each sample.

Cutoff values

Cutoff values have been determined for each marker. In order to assess patient results, external control values must be valid. Samples with Ct values less than or equal to one or both of the cutoff values for MG or CK19 are considered positive. If the MG and CK19 Ct values are above their cutoffs the Internal Control Ct value must be below its cutoff for MG and CK19 to be considered negative. If the MG and CK19 Ct values are negative and the Ct value for the Internal Control gene is greater than or equal to its cutoff, then the result for that sample will be considered "invalid".

The Cutoff Ct values are as follows: MG ≤ 31, CK19 ≤ 30, Internal Control < 36.

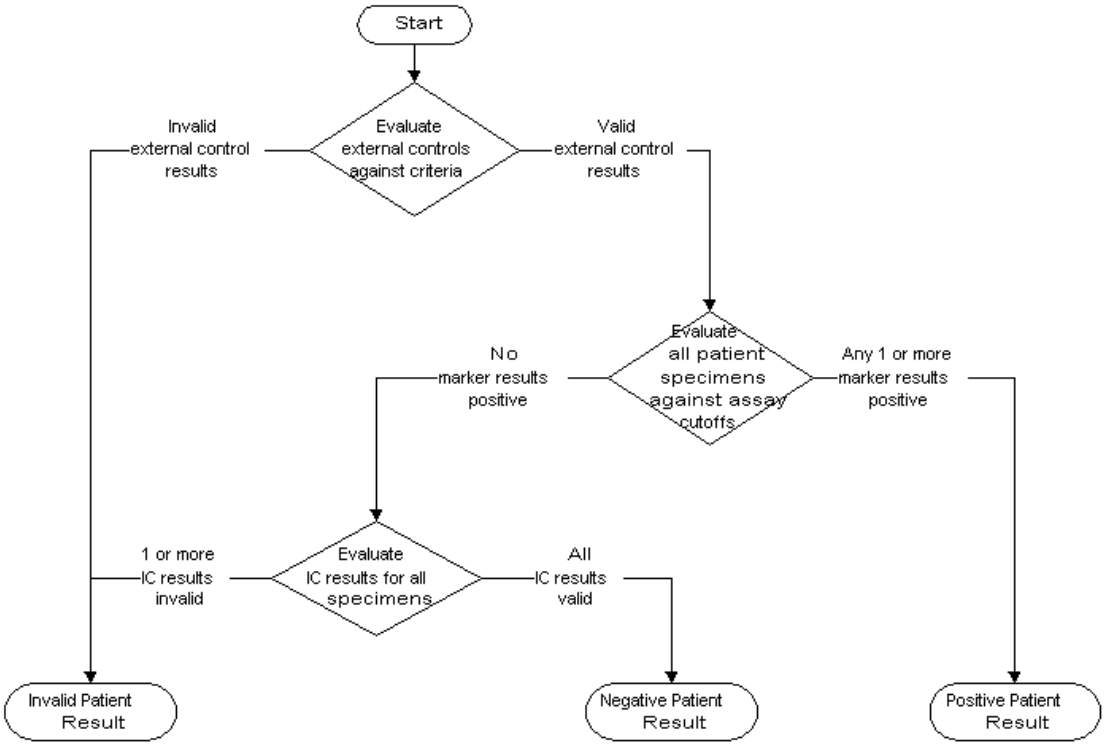


Figure 1. Decision tree to determine patient result.

If the Assay Status field is "Valid", then the Patient Result field in the Patient Results section shows the test result, be it "Positive" or "Negative." This result is per patient and not per lymph node**.** If at least one of the lymph nodes being tested for a patient is positive for either assay marker, then the result reported in the Patient Result field is "Positive", regardless of the results obtained with other samples from that patient**.** However, if the Patient Result is not "Positive" and the result from one or more lymph nodes is "Invalid", then the Patient Result is "Invalid." The Patient Result field in the Patient Results table provides an objective readout of patient status requiring no subjective interpretation by the user.

Note[1]: Ensure the validity of the Positive and Negative Controls before reporting patient results**.** Results from runs with one or more invalid controls must not be reported.

Note[2]: Use the Run Report, not the Patient Report.

If the Assay Status field in Assay Information is "Invalid", then the Patient Result is "Invalid**."** In such cases, assay results obtained in that run are invalid and must not be reported**.** Invalid assay run or instrument error codes or warnings are flagged on-screen and on reports**.** Before reporting GeneSearch™ BLN Assay results, always verify that the assay run is valid**.** Refer to the Cepheid SmartCycler® Diagnostic Operator Manual for printing results.

LIMITATIONS

- The performance of the GeneSearch™ BLN Assay was verified using the procedures provided in these "Instructions For Use" only**.** Modifications to these procedures may alter the performance of the assay.

- Contamination of the specimen with breast tissue (normal, benign or malignant) or breast lymph node tissue from another patient may cause erroneous results**.** Care must be taken in all phases of sample processing to avoid contamination.
- GeneSearch™ BLN Assay results may not be informative if the specimen quality or quantity is inadequate.
- Tissue less than 50 mg in weight may yield a higher Invalid test rate with the GeneSearch™ BLN Assay.
- The GeneSearch™ BLN Assay has been designed to work with SLND procedures yielding no more than 6 lymph nodes per patient per run.
- The GeneSearch™ BLN Assay has been designed for use with fresh tissue and not fixed tissue.
- GeneSearch™ BLN Assay results should be used in conjunction with all clinical information derived from diagnostic tests (i.e. imaging or laboratory tests), physical examination and full medical history in accordance with appropriate patient management procedures.
- The GeneSearch™ BLN Assay has been designed, optimized and tested for use with the GeneSearch™ RNA Sample Preparation Kit and the Cepheid SmartCycler® System.

INTERFERING SUBSTANCES

Potentially interfering substances include, but are not limited to the following:

- A mixture of fat and positive lymph node tissue were processed at different ratios (Fat:Positive Lymph Node 100:0, 75:25, 50:50, 25:75 and 0:100). Fat was found to have an effect on the Assay Result when present in a higher amount than the positive lymph node sample in the mixture (25:75). Fat, if present, should be trimmed from tissue sections being used in the assay to avoid erroneous results.
- Negative lymph nodes were obtained from patients diagnosed with lymphoma and tested using the GeneSearch™ BLN Assay**.** A false positive result was observed with most of these lymph nodes. Patients diagnosed with any other type of cancer such as lymphoma may not be good candidates for the GeneSearch™ BLN Assay, as lymph nodes from these patients may generate a false positive result for the assay.
- GeneSearch™ BLN Assay performance was analyzed in the presence of blood, tissue marking dyes, tracing dyes and technitium^{99} (Tc^{99})**.** Assay performance was not affected by the presence of any of these materials.
- Primary tumor tissue was found to interfere with assay results**.** Contamination with primary tumor may cause false positive or invalid test results.
- Contamination with breast tissue may cause a false positive result.

Analytical Performance

Precision

Precision is a measurement of the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of a homogeneous sample**.** The degree of scatter in multiple measurements of a homogeneous sample is due to several factors such as the operator performing the measurement, the lot number of the material with which the sample is measured, the day on which the measurement is taken and the variability inherent in the measurement system itself**.** The precision of the GeneSearch™ BLN Test Kit was determined using a protocol similar to that recommended in the Clinical Laboratory Standards Institute Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second

Edition (EP05-A2). Two samples and two assay controls were tested on the GeneSearch™ BLN Test Kit using three operators testing both samples and controls on three lots of GeneSearch™ BLN Test Kits each day for eight days with two runs per day with two replicates of each sample and control tested on each run.

The Ct values obtained for each applicable marker in each sample were analyzed to determine the standard deviation (average variation from the mean) of the measurements**.** The standard deviation is divided by the mean Ct value for each marker in each sample and multiplied by 100 to determine the percent Coefficient of Variance (%CV), or degree of scatter in the data.

$$\%CV = (\text{Standard Deviation}/\text{Mean}) \text{ X } 100$$

The Within Run Precision expresses the degree of scatter in measurements taken on a given sample within the same run. The Total Precision expresses the degree of scatter in the measurements on a given sample across all operators, all days, all lots and all runs.

Total assay precision was estimated using the model provided in EP05-A2**.** The formula used to estimate total precision was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2}$$

where S_T = total standard deviation, *dd* = between day, *rr* = between run and *wr* = within run**.** The results are provided in Table 4.

Table 4. Within Run Precision Excluding Lot and Operator

Total Precision (%CV) Excluding Lot and Operator (S_T/Mean Ct)				
	Positive Sample	Negative Sample	Negative Control (NC)	Positive Control (PC)
PBGD	2.9%	4.3%	1.9% (3.1%) [c]	NA [b]
MG	3.1%	NA [a]	NA [b]	1.6 % (6.0%)[c]
CK19	1.5%	NA [a]	NA [b]	1.6% (5.6%) [c]

a. MG and CK19 were not analyzed as these markers are not expressed at appreciable levels in a negative sample. Negative results were obtained as expected in samples not containing these markers.

b. MG and CK19 are not present the Negative Control. PBGD is not present in the Positive Control. Negative results were obtained as expected in samples not containing these markers.

c. Values if results from samples where control was not added (user error) to the reaction are included in the analysis.

Lot-to-lot and operator-to-operator variability were also considered in the reproducibility study design. The formula used to estimate total precision in this case was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2 + S_{op}^2 + S_{lot}^2} \cdot$$

The assay results are reproducible; the values for each sample tested are provided in Table 5.

Table 5. Within Run Precision Including Lot and Operator

Total Precision (%CV) Including Lot and Operator (S_T/Mean Ct)				
	Positive Sample	**Negative Sample**	**Negative Control (NC)**	**Positive Control (PC)**
PBGD	5.6%	5.2%	2.5% (3.4%) c	NA b
MG	5.5%	NA a	NA b	1.8% (6.1%) c
CK19	2.5%	NA a	NA b	1.9% (5.6%) c

a. MG and CK19 were not analyzed as these markers are not expressed at appreciable levels in a negative sample**.** Negative results were obtained as expected in samples not containing these markers.

b. MG and CK19 are not present the Negative Control**.** PBGD is not present in the Positive Control. Negative results were obtained as expected in samples not containing these markers.

c. Values if results from samples where control was not added (user error) to the reaction are included in the analysis.

Linearity

The linearity of an analytical or biological test method is its ability (with a given range) to obtain results that are directly proportional to the concentration of analyte in the sample. The linearity of the GeneSearch™ BLN Test Kit was assessed by preparing samples containing known amounts of *in vitro* transcript (IVT) RNA for each marker, testing these samples on the GeneSearch™ BLN Test Kit and directly comparing the Ct values obtained for each marker in each sample to the concentration of each marker in each sample by regression analysis.

Linearity is expressed by the equation of the line (Y=mx+b) which results from plotting the Ct value of each marker (Y) versus the concentration of target ('x' expressed as the $\log_{10}$(copies/µL)) in each sample (regression analysis). The regression line expresses the best prediction of the Ct value based on the concentration. The closeness of the observed measurements to the resultant line is demonstrated by the R^2 value. An R^2 value of 1 indicates values that lie perfectly on the line.

Samples were prepared by adding IVT RNA in log-fold increments (increases of 10 copies of IVT RNA/µL buffer) and tested on the GeneSearch™ BLN Test Kit. Samples were prepared with IVT RNA for one marker at a time (individual IVT RNA) and in combination with each other (IVT RNA Mix). Regression analysis was performed for each marker for all levels of target that gave detectable signal in the assay. The resultant equations of the lines for each marker and R^2 values are presented in the table below. The assay met the criteria for a linear response (R^2 value > 0.95) between the lowest level detected (shown in Table 6 below) and 10^8 (8 log) copies per µL for all markers tested.

Table 6. Linearity and Detection Limits

Individual IVT RNA

	Equation of the Line Ct = b+(m * ($\log_{10}$(copies/µL))	**R^2 value**	**Lowest Target Level Detected**
IC Ct	PBGD Ct = 44.50 - 3.571 * $\log_{10}$(copies/µL)	0.997	10^3
MG Ct	MG Ct = 41.62 - 3.265 * $\log_{10}$(copies/µL)	0.993	10^2
CK19 Ct	CK19 Ct = 43.62 – 3.559 * $\log_{10}$(copies/µL)	0.999	10^3

IVT RNA Mix

	Equation of the Line $Ct = b+(m * (\log_{10}(copies/\mu L))$	R^2 value	Lowest Target Level Detected
IC Ct	PBGD Ct = 43.58 - 3.419 * $\log_{10}$(copies/µL)	0.995	10^3
MG Ct	MG Ct = 41.7 - 3.269 * $\log_{10}$(copies/µL)	0.994	10^2
CK19 Ct	CK19 Ct = 43.38 – 3.521 * $\log_{10}$(copies/µL)	0.999	10^3

Limits of Detection

A theoretical limit of detection was calculated using the equation generated by regression analysis during linearity testing. The limit of detection is defined as the number of copies of the target sequence detected at 35.9 Ct, the highest value that can be obtained with the GeneSearch™ BLN Assay thermal cycling protocol. Data is shown in Table 7 below.

Table 7. Detection Limits

Individual IVT RNA

	Equation of the Line $Y = b-(m * (\log_{10}(copies/\mu L))$	Analytical Detection Limit
IC Ct	PBGD Ct = 44.5 - 3.571 * $\log_{10}$(copies/µL)	$10^{2.4}$
MG Ct	MG Ct = 41.62 - 3.265 * $\log_{10}$(copies/µL)	$10^{1.8}$
CK19 Ct	CK19 Ct = 43.62 – 3.559 * $\log_{10}$(copies/µL)	$10^{2.2}$

IVT RNA Mix

	Equation of the Line $Y = b-(m * (\log_{10}(copies/\mu L))$	Analytical Detection Limit
IC Ct	PBGD Ct = 43.58 - 3.419 * $\log_{10}$(copies/µL)	$10^{2.3}$
MG Ct	MG Ct = 41.7 - 3.269 * $\log_{10}$(copies/µL)	$10^{1.8}$
CK19 Ct	CK19 Ct = 43.38 – 3.521 * $\log_{10}$(copies/µL)	$10^{2.1}$

CLINICAL PERFORMANCE

Two prospective, multi-site, U.S. clinical trials were conducted to gather the data from which to determine the proper Ct cutoffs for the GeneSearch™ BLN Assay (training set, Cutoff Study, 12 sites, n = 306) and to validate the chosen cutoffs in an independent subject set (test set, Pivotal Study, 11 sites, n = 423). Both studies had identical methods (described below).

Methods

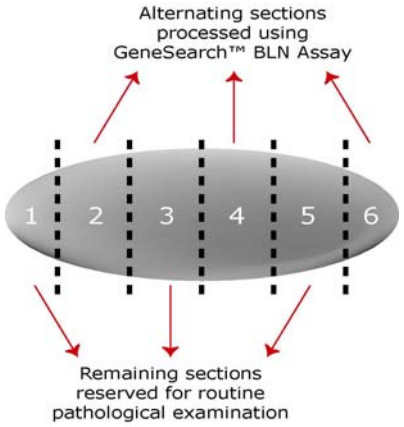
Clinical site personnel performed the GeneSearch™ BLN Assay testing on SLNs freshly removed from female or male patients at least 18 years of age diagnosed with invasive breast cancer. The GeneSearch™ BLN Assay results were compared to rigorous permanent section H&E and IHC sectioning (described below) evaluated by pathologists who were blinded to assay results. Two independent pathologists evaluating a lymph node as having a metastatic focus > 0.2 mm were required for a lymph node (or subject) to be categorized as “positive.”

Sentinel Lymph Node Cut-In and Sharing

All sentinel lymph nodes were bisected along the short axis. Lymph nodes 6.0 mm or less in length were bisected to produce two (2) lymph node tissue portions. Larger lymph nodes were cut along the short axis into an even number of lymph node tissue portions of approximately the same thickness, as indicated in Table 8. This procedure assured that all lymph node portions were between 1.5 to 3.0 mm in thickness, and that there was an equal number of tissue portions for histology and the GeneSearch™ BLN Assay. The figure shows an example of lymph node sharing between the GeneSearch™ BLN Assay and histology for a lymph node that is approximately 12 mm in length.

Table 8. Sentinel Lymph Node Cut-in and Sharing Between the GeneSearch™ BLN Assay and Histology as done in the Clinical Studies. 1.) Lymph node cut-in based on node size. 2.) An example of lymph node sharing between the assay and histology for a lymph node that is approximately 12 mm in length. Central Slides taken for evaluation by study Central Pathologists and Site Slides taken for evaluation by Site Pathologists for patient management.

1. Lymph node cut-in based on node size		2. Example of lymph node sharing
Node size (longest dimension in mm)	**Total number of node tissue portions**	
≤ 6	2	
> 6 and ≤ 10	4	
> 10 and ≤ 15	6	
> 15 and ≤ 20	8	
> 20	10 or more (each portion ≤ 3 mm thick)	



After any desired ITP slides had been taken, alternating lymph node tissue portions from the same lymph node were combined, processed and tested following the Sample Preparation, RNA Purification, and RT-PCR sections of this document.

Remaining tissue portions not used for the GeneSearch™ BLN Assay were processed for permanent section H&E for patient management using standard site procedures (Site Slides). Additional slides were also prepared from the fixed tissue for shipment to the study Central Pathologists for H&E evaluations (Central Slides). Central Slide sections were 4 to 6 µm thick, and three (3) sections were taken from each 1.5 mm to 3.0 mm fixed lymph node portion. The three (3) sections were taken from levels approximately 150 µm apart. Each site determined the number and levels of H&E sections to be evaluated by the site for patient management. IHC evaluations were done when H&E sections were found negative. At the site's discretion, these slides may have been stained (as per standard site procedure) and evaluated by the Site Pathologists (Site Slides) or read by only the Central Pathologists (Central Slides). Thus, for each subject there were two separate sets of H&E slides (Site **and** Central) and for H&E negative subjects, one set of IHC slides (Site **or** Central).

Test Comparator - Histology Interpretation

The combination of permanent section H&E and IHC was used as the comparator test method in these studies to determine the performance of the GeneSearch™ BLN Assay. In an attempt to minimize some of the limitations of current histology, a derived "Overall Histology" result took into account all permanent section H&E and IHC results obtained on the subjects' lymph nodes, as described here. Two Central Pathologists independently read Central Slides. If the results from the two pathologists were discordant, a third Central Pathologist independently read the same slides. The final Central Slide result was determined by majority rule. For Site Slide results, if the site pathologist found the lymph node positive, whereas the Central Slides for the same lymph node were evaluated as negative, the Site Slides were sent to the Central Pathologists for confirmation of positivity. The lymph node (and patient) was considered histology positive if either Site Slides or Central Slides were confirmed positive. For either Site Slide or Central Slide evaluation, if only two pathologists' results were available with one being positive and one negative, the final result was considered undetermined (UND).

For assay performance calculations, histology was divided into two discrete categories of positive or negative, with positive being a metastasis > 0.2 mm, and negative being no detectable metastasis or metastases no larger than 0.2 mm. However, for more in depth analyses, histological results were divided into the following six categories in order of increasing levels of positivity: N – negative, no evidence of tumor cells; N(ITC) - isolated tumor cells only; N(CL) – tumor cell clusters $\leq$ 0.2 mm; P(MI) - micrometastasis > 0.2 to 2 mm; P - metastasis > 0.2 mm but of unknown specific size; and P(MA) - macrometastasis > 2.0 mm. The first three categories were considered negative from a clinical perspective, the last three were considered positive. A lymph node was considered P(MA) only if two Central Pathologists agreed on that level of metastasis.

Central Slide H&E results were a requirement for inclusion of the lymph node in the study. When Site Slide H&E results and/or when any IHC results were not available, the existing Central Slide histology result was the final histology result for the lymph node. When either the final Central Slide and/or final Site Slide histology result was positive, the Overall Histology result for the lymph node was "positive." If one set of slides had a final result of negative for a lymph node, and the other was UND, the final Overall Histology result for that lymph node was considered UND. For P(MA), P(MI), N(CL), N(ITC), or N, the Overall Histology result was always the more positive of the final Central or final Site Slide result.

Overall Histology Results Interpretation for a Subject

It was the subject's Overall Histology result that was used for performance calculations for the GeneSearch™ BLN Assay. The subject's Overall Histology result was negative if the Overall Histology result of all lymph nodes was negative, and positive if at least one lymph node was positive. The subject's Overall Histology result was considered UND if one lymph node was evaluated as UND and there were no other lymph nodes, or all other lymph nodes were either UND or negative. The subject's Overall Histology result was equal to the most positive result seen in any of their lymph nodes, e.g., if one lymph node was P(MA), one P(MI), and one negative, the patient result was P(MA).

Intraoperative Histological Evaluations

The sites' FS or ITP results were collected to compare the performance of these intraoperative methods to the performance of the GeneSearch™ BLN Assay when each was measured against Overall Histology results as described above. There were no Central Pathologist readings of the intraoperative histology slides. The clinical trial sites collected intraoperative results for patient management either on a rare basis on special request from a given surgeon, or as standard

practice for all patients undergoing SLND. For the GeneSearch™ BLN Assay clinical studies, sites were instructed to collect intraoperative histology as per their standard procedures, with the exception that 1) the lymph nodes were to be cut as indicated in Table 8 above, and 2) lymph node portions used for the investigational assay were not frozen. Frozen sections were taken only from the lymph node portions being used for histology. Intraoperative touch preparations could be taken from any lymph node portion, including those to be tested in the GeneSearch™ BLN Assay.

Cutoff Study Results

274 Subjects with valid GeneSearch™ BLN Assay results and defined Overall Histology results were used to determine the cutoffs for the assay IC (PBGD), MG, and CK19. Data from an additional 30 subjects with invalid GeneSearch™ BLN Assay results and two with Overall Histology results of UND were excluded from the cutoff determination, as these subjects would not provide any numerical contribution to a cutoff calculation. Cutoffs for PBGD, MG, and CK19 markers were determined as < 36 Ct, ≤ 31 Ct, and ≤ 30 Ct, respectively. The performance of the GeneSearch™ BLN Assay with these cutoffs in the 274-subject data set was 91.1% sensitivity (95% confidence interval: 82.5% - 96.4%) and 95.9% specificity (95% confidence interval: 92.1% - 98.2%).

Pivotal Study Results

Subject Population

There were 423 subjects (418 females and 5 males) with SLNs removed who met all protocol inclusion criteria. Subject age ranged from 27 to 92 years with a mean age of 60. Nine subjects had chemotherapy and one had radiation therapy. The majority of subjects (80.4%) were diagnosed with invasive ductal cancer either alone or in combination with other breast cancer types. There were 13.9% of subjects with invasive lobular cancer but no invasive ductal cancer, and 5.7% with invasive cancer other than lobular or ductal. The majority of the subjects had either Stage I breast tumors (62.3%), or Stage II (32.0%). There were 5.3% with Stage III and 0.5% with Stage IV. Most subjects were estrogen receptor (ER) positive (79.2%), progesterone receptor (PR) positive (67.8%), and HER-2 negative (74.2%).

SLN Disposition

The mean, median, and maximum numbers of lymph nodes removed were 2.9, 2, and 11, respectively. Two of the 423 subjects had no assay or study histology data available due to their lymph nodes being too small to share tissue for study purposes.

Overall Histology Results

Five of the 421 subjects had Overall Histology results of UND and were not included in assay performance calculations. The prevalence by Overall Histology for the remaining 416 subjects was 29.1% (121 positive subjects of 416), and ranged from 14.3 – 45.5% across all sites. H&E was positive in 120 subjects, and there was one positive subject identified as P(MI) by IHC alone. Positives were most often P(MA) (77.7%), with 19.0% being P(MI), and 3.3% of undetermined size > 0.2 mm (P). Most negatives (93.2%) were completely histologically negative with no evidence of tumor cells (N), 4.7% N(ITC) and 2.0% N(CL).

Central Pathologist Agreement Evaluating the Same Slides

The overall positive/negative agreement on a subject level between the two primary central pathologists evaluating the same slides was 98.3%. There were 92 subjects evaluated with a macrometastasis by one or both central pathologists. Only one of these 92 subjects (1.1%) was

found negative by the other pathologist. In seven of the 92 (7.6%) subjects, the other pathologist evaluated the subject as having micrometastases. There were 19 subjects evaluated by one or both central pathologists as having a micrometastasis (and not evaluated with macrometastases). In 31.6% of these cases (6/19), the other pathologist evaluated the subject as negative. The non-uniform distribution of micrometastases in lymph nodes lowers the probability of correctly evaluating metastases at earlier stages of breast cancer.[15,24] These results illustrate the difficulty of distinguishing between positivity and negativity for clinically relevant metastases.[24,25]

Agreement between Site Pathologist Results and Final Central Pathologist Results from Different H&E Slides

Comparisons were made between the site pathologist H&E results used for patient management *versus* the central pathologist final H&E results on Central Slides. In seven cases one evaluation determined that the subject had macrometastases while the other evaluation found the subject negative. In 12 cases one evaluation determined that the subject had micrometastases while the other found the subject negative. These 19 subjects represent 4.7% of the 408 subjects with site and central pathology results. Site pathology results were unavailable for some subjects. These findings illustrate the inadequacy of current sampling techniques used for histological evaluation of lymph nodes, since significant metastases can be missed in nearby tissue left uncut on the block.[15,26]

Confirmation of Site Slide Positivity

There were 117 subjects reported positive by the site pathologists. Central Slides were also positive in 82.1% of those cases. Of the 21 subjects reported positive by the site and found negative on Central Slides, central pathologist(s) confirmed positivity on the Site Slides in 61.9% (13/21). In 19% (4/21) subjects, both central pathologists found the Site Slides negative. In 4 additional site-positive subjects, missing central pathologist data preclude confirmation of Site Slide positivity - they remain UND. These findings illustrate the subjective nature of histological slide evaluation.[19]

GeneSearch™ BLN Assay Performance Calculations

Of the 421 subjects, the assay resulted in an invalid result for 34 (8.1%), whether due to external control (13) or sample (21) failures. Subject invalid result rates declined to 4.2% with increased operator assay experience (when at least 40 assay runs had been completed). For the purposes of performance calculations, these invalid results were not excluded but were treated as assay "negative," since these results do not provide the clinician with evidence of nodal metastases. Performance calculations were based on the 416 subjects with defined Overall Histology results (the five subjects with Overall Histology results of UND were not included). The GeneSearch™ BLN Assay overall performance (with 95% confidence intervals) is shown below compared to Overall Histology. Also shown is assay performance from the Cutoff Study evaluated by the same methods (assay invalids are treated as "negative" and subjects with incomplete histology are excluded) and the combined performance from both studies.

Table 9. The GeneSearch™ BLN Assay Overall Performance (with 95% Confidence Intervals) Compared to Overall Histology.

STUDY	N	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	PPV	NPV
Pivotal	416	87.6 (80.4-92.9)	94.2 (90.9-96.6)	86.2	94.9

Cutoff	304	82.4 (72.6-89.8)	96.3 (92.9-98.4)	89.7	93.4
Combined	720	85.4 (79.9-90.0)	95.1 (92.9-96.8)	87.6	94.2

PPV = Positive Predictive Value; NPV = Negative Predictive Value

Fourteen of 15 subjects with assay False Negative (FN) results had only one positive lymph node by Overall Histology. Of the 15 FN subjects, two (2) were due to external control failures being interpreted as "negative" for the purposes of assay performance calculations. In these two cases, subject sample Ct values were actually positive for both CK19 and MG. Of the 13 valid assay negative subjects, most had small metastases with nine being P(MI), three P, and only one P(MA) by Overall Histology. Eight of the 13 had positivity found only on Central Slides or Site Slides, but not on both.

There were a total of 17 subjects with assay False Positive (FP) results. Fifteen of the 17 were positive by the assay on only one lymph node.

It is probable that differences in results between the GeneSearch™ BLN Assay and Overall Histology were due predominantly to tissue sampling since the assay evaluated **different** portions of the lymph node than did histology. This conclusion is supported by differences between the evaluations of the two sets of H&E slides collected for the Pivotal Study discussed above. The effect of tissue sampling is evident when comparing site pathologist results on Site H&E Slides to the central pathologist results on Central H&E Slides. This is a comparison of H&E evaluations of **different** sections from the **same** portions of the lymph node. Site pathology had the following "performance" *versus* Central Pathology:

Table 10. Site Pathology vs Central Pathology "Performance"

N	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	PPV	NPV
408	94.1 (87.6-97.8)	95.8 (92.8-97.7)	88.1	98.0

The lack of perfect agreement is due predominantly to different sections (samplings) from the lymph node tissue being taken for Site Pathology evaluation *versus* Central Pathology evaluation.

Sensitivity of the GeneSearch™ BLN Assay for the 94 subjects with P(MA) was 97.9% (95% Confidence Interval: 92.5 - 99.7%). For the 23 subjects with P(MI), assay sensitivity was 56.5% (95% Confidence Interval: 34.5 - 76.8%). The sensitivity for micrometastases for Site Pathology H&E compared to Central Pathology H&E on different but nearby sections of the lymph node is 80.0% (95% Confidence Interval: 51.9-95.7%). These results again show the difficulty of detecting small and infrequently occurring metastases with limited sampling, and the importance of evaluating more than a small proportion of the lymph node.[26]

There were 31 subjects who had breast surgery conducted immediately prior to the SLND. There was 100% agreement between the Overall Histology result and the GeneSearch™ BLN Assay result in these subjects, indicating that conducting breast surgery prior to the SLND did not cause false positive assay results due to contamination of the SLNs with breast tissue.

For the five male subjects, assay results were in 100% agreement with Overall Histology. Three were negative (N) and two were P(MA).

There were 10 subjects who were receiving cancer treatment of chemotherapy (9) or radiation (1). The assay results agreed with Overall Histology (7 True Negative (TN) and 2 True Positive (TP)) in all but one of these subjects. That subject was receiving chemotherapy and was negative by the GeneSearch™ BLN Assay and H&E, and P(MI) only by IHC. The GeneSearch™ BLN Assay, Site Slides H&E and Central Slides H&E were negative for this subject.

Table 11 shows the performance of the GeneSearch™ BLN Assay by tumor histology and tumor size.

Table 11. GeneSearch™ BLN Assay Sensitivity and Specificity Relative to Final Histology Results by Tumor Histology and Tumor Size

	N	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	PPV	NPV
Tumor Histology					
Invasive Ductal	338	88.8 (80.8-94.3)	94.1 (90.3-96.7)	86.1	95.3
Invasive Lobular	59	80.0 (56.3-94.3)	91.9 (78.1-98.3)	84.2	89.5
Invasive Other	24	100 (29.2-100)	100 (83.9-100)	100	100
Tumor Size [a]					
< 1 cm	88	90.9 (58.7-99.8)	100 (95.3-100)	100	98.7
1 - < 2 cm	159	81.4 (66.6-91.6)	92.2 (85.8-96.4)	79.5	93.0
2 – 4.9 cm	127	90.9 (78.3-97.5)	90 (81.2-95.6)	83.3	94.7
≥ 5 cm	22	92.3 (64.0-99.8)	100 (63.1-100)	100	88.9

a. Tumor size was not available for 25 subjects.

The number of axillary lymph nodes that are found with metastases is an important prognostic indicator and is used to make treatment decisions.[1-3] The number of lymph nodes found positive in a subject by the GeneSearch™ BLN Assay compared to the number found positive in the same subject by Overall Histology is shown in Table 12 below. The Kappa value of agreement between the two tests was 0.75 (95% Confidence Interval: 0.68 to 0.81). Kappa values above 0.61 are considered indicative of substantial agreement.[27]

Table 12. Number of lymph nodes found positive in the same subject by Overall Histology

Histology (No. Positive Nodes)	GeneSearch™ BLN Assay (No. Positive Nodes)			
	0 (NEG)	**1**	**2**	**>= 3**
0 (NEG)	278	15	1	1 [a]
1	14	57	8	1
2	1	5	23	1
≥ 3	0	0	1	10

a. The subject with three assay-positive lymph nodes had isolated tumor cells reported by Site IHC in four of the five SLNs.

b. Shaded cells indicate 100% agreement.

Of the 17 cases where the assay was interpreted as FP, 15 (88.2%) subjects were identified as assay positive in only one lymph node. Similarly, of the 15 cases where the assay was interpreted as false negative, 14 (93.3%) subjects were histology positive in only one lymph node. These results are expected when the differences are due to sampling of different lymph node tissue in subjects with less metastatic spread of disease.

Spearman nonparametric correlation coefficient analysis found that there was a high correlation between assay cancer analyte Ct values and level of metastases reported by Overall Histology (n = 383, conclusive histology and valid GeneSearch™ BLN Assay results). The correlation coefficient was 0.77 for MG and 0.74 for CK19 Ct values *versus* the six histology categories of P(MA), P, P(MI), N(CL), N(ITC) and N. The following graph shows that relationship. There are 128 overlapping subjects with negative histology at the 40/40 point in the graph. Those subjects with CK19 Ct values less than 25 or MG Ct values less than 26 were highly likely to have macrometastases (80/88, 90.9%).

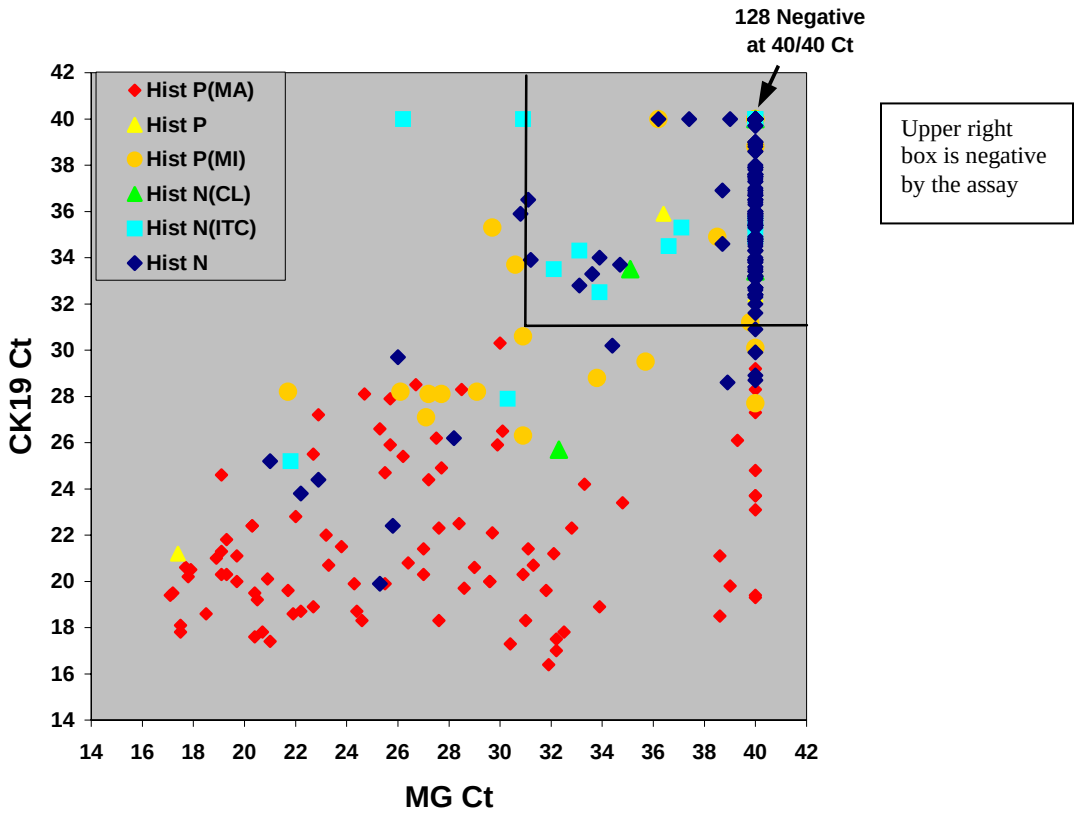


Figure 2. Spearman Nonparametric Correlation Coefficient Analysis. Shows a high correlation between assay cancer analyte Ct values and level of metastases reported by Overall Histology.

Additional Molecular Testing of Assay False Negative and False Positive Samples

Sampling different portions of the lymph node can lead to disagreements between the GeneSearch™ BLN Assay result and the Overall Histology result. Testing the residual assay sample for the expression of other gene markers associated with metastases can provide further information on the lymph node portions tested only by the assay. The Sponsor developed a molecular test (Confirmatory Molecular Test) designed to have very high specificity. This test was a modification of previously published methods.[28] The test had four molecular markers that were not used in the GeneSearch™ BLN Assay.

One lymph node from each of 11 subjects testing negative in both the GeneSearch™ BLN Assay and Overall Histology, TN, were tested in the Confirmatory Molecular Test. All 11 lymph nodes tested negative.

One Overall Histology-positive lymph node was tested from each of 10 GeneSearch™ BLN Assay FN subjects. Residual sample was unavailable in five FN subjects. All 10 lymph nodes tested negative in the Confirmatory Molecular Test, suggesting that the lymph node portions tested in the GeneSearch™ BLN Assay did not contain metastases.

Of the 23 TP lymph nodes from 22 TP subjects tested in the Confirmatory Molecular Assay, 15 lymph nodes (65.2%) from 15 subjects (68.2%) confirmed as positive, 7 were negative, and

one invalid. Lack of 100% agreement was expected since the Confirmatory Molecular Assay was designed to maximize specificity as opposed to sensitivity.

Of the 17 subjects with GeneSearch™ BLN Assay FP results, 13 had residual assay sample available. A total of 15 FP lymph node samples were tested with the Confirmatory Molecular Test. Eleven of the 15 (73.3%) FP lymph nodes and nine of the 13 (69.2%) FP subjects tested positive in the Confirmatory Molecular Test. The proportion of confirmed positive lymph nodes was similar in TP (65.2%) and FP (73.3%) lymph nodes, suggesting that metastases were present in the majority of GeneSearch™ BLN Assay "false" positive samples. In the subject with the three lymph nodes that tested FP in the GeneSearch™ BLN Assay, the Confirmatory Molecular Test confirmed positivity in all three lymph nodes.

Comparison of the GeneSearch™ BLN Assay Performance to Current Intraoperative Tests

Table 13 below compares the performance of the GeneSearch™ BLN Assay to that of current intraoperative methods that were in use at the Pivotal Study sites. For assay performance calculations, the data set was limited to those 324 subjects who had intraoperative frozen section (FS) results. In all cases, the comparator test was Overall Histology.

Sensitivity of the GeneSearch™ BLN Assay was 95.6% compared to 85.6% for FS and 45.5% for ITP. Sensitivity of the GeneSearch™ BLN Assay for subjects with P(MA) was 100% (76/76) compared to 90.8% (69/76) for FS and 57.1% (4/7) for ITP. Sensitivity of the GeneSearch™ BLN Assay for subjects with P(MI) was 81.8% (9/11), and was 54.5% (6/11) for FS and 25.0% (1/4) for ITP.

Specificity for both current intraoperative methods was greater than 97%. Due to the limited sampling involved in current intraoperative techniques, adjacent and more thorough permanent section histology is likely to confirm any metastases seen with FS or ITP.

These data show that the GeneSearch™ BLN Assay detected more metastases than did current intraoperative histological techniques, despite the fact that the comparator test (Overall Histology) was conducted on different portions of the lymph node than those on which the assay was conducted. FS results were, in contrast, generated on the same portions of the lymph node as Overall Histology.

Table 13. Comparison of the GeneSearch™ BLN Assay performance to that of current intraoperative methods used in Pivotal Study sites.

Test	N	Sensitivity (95% Confidence Interval)	Specificity (95% Confidence Interval)	PPV	NPV
BLN Assay	319	95.6 (89.0-98.8)	94.3 (90.5-96.9)	86.9	98.2
FS	319	85.6 (76.6-92.1)	97.8 (95.0-99.3)	93.9	94.5
ITP	29	45.5 (16.7-76.6)	100 (81.5-100)	100	75.0

Summary of Clinical Performance

The agreement between the GeneSearch™ BLN Assay and thorough permanent section histology with review by at least two pathologists is similar to the agreement between Site Pathology review versus Central Pathology review of different H&E sections from the same lymph nodes.

In addition, the absolute number of positive lymph nodes identified by the GeneSearch™ BLN Assay is similar to the number identified by permanent section histological evaluation. In a matched data set, the GeneSearch™ BLN Assay identified more clinically relevant metastases (> 0.2 mm) than did intraoperative histological techniques.

GeneSearch™ BLN Assay Reproducibility

Two operators from each of three sites participated in a Reproducibility Study. All operators tested a Sponsor-provided reproducibility panel composed of human axillary lymph node tissue homogenate supplemented, when needed, with *in vitro* transcript of high or low levels of MG and/or CK19. There were a total of four panel members with one being negative for MG and CK19. Starting from the RNA isolation step, each operator tested panel samples in duplicate in each run using three different lots of the GeneSearch™ BLN Test Kit. Samples were tested with the same lot of reagents on two separate days by each operator. The study design resulted in a total of 72 planned replicate results for each of the four panel members across all lots, sites, days, and operators. The GeneSearch™ BLN Assay results were in 100% agreement with the known presence or absence of target for all individual markers (PBGD, MG and CK19). Percent Coefficient of Variations (CVs) for all marker Ct values were ≤ 6.82 for intra-run, inter-run, inter-site, inter-operator and inter-lot analyses. Standard deviations (SDs) were ≤ 1.88 in all cases (Table 14).

The data show that GeneSearch™ BLN Assay results are highly reproducible on both a quantitative and qualitative level across sites, operators, lots, days and within runs.

Table 14. Variability of Ct Values by Variability Sources. Ct values of invalid MG and CK19 results due to IC failure are not included in the analysis.

Panel		Agreement		Intra-Run		Inter-Run		Inter-Site		Inter-Oper		Inter-Lot	
		%	Mean	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
A (n=74)	**MG: Neg**	100	39.92	0.45	1.12	0 [a]	0	0 [a]	0	0.14	0.36	0	0
	CK19: Neg	100	37.44	1.88	5.03	1.60	4.28	1.11	2.96	0 [a]	0	0.87	2.31
	PBGD: Pos	100	28.47	0.90	3.15	0.41	1.45	0 [a]	0	0.39	1.37	0.94	3.31
B (n=72)	**MG: Low Pos**	100	23.19	0.51	2.18	0.17	0.73	0	0	0.29	1.26	0.71	3.05
	CK19: Neg	100	39.54	1.02	2.57	1.06	2.69	0.48	1.22	0.72	1.82	0.18	0.44
C (n=74)	**MG: Neg**	100	37.98	1.11	2.93	0.30	0.79	0.31	0.82	0 [a]	0	1.18	3.11
	CK19: Low Pos	100	27.96	0.30	1.06	0.25	0.90	0 [a]	0	0.25	0.88	0.74	2.63
D (n=72)	**MG: High Pos**	100	17.66	0.56	3.19	0.16	0.91	0.37	2.11	0.42	2.36	0.61	3.47
	CK19: High Pos	100	20.03	1.37	6.82	0.95	4.76	0.28	1.38	0.83	4.12	0.57	2.85

a. According to NCCLS (CLSI) guideline EP05-A2, variance components less than 0 are recorded as 0.

BIBLIOGRAPHY

1. Albertini JL, Lyman GH, Cox C, et al. Lymphatic mapping and sentinel node biopsy in the patient with breast cancer. *JAMA* 1996; 276:1818-1822.

2. Borgstein PJ, Pijpers R, Comans EF, et al. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J. Am Coll Surg* 1998; 186:275-283.

3. Turner RR, Ollila DW, Krasne DL, et al. Optimal histopathologic examination of the sentinel lymph node for breast carcinoma staging. *Am J Surg Pathol* 1999; 23:263-267.

4. Giuliano AE, Dale PS, Turner RR, et al. Improved axillary staging of breast cancer with sentinel lymph adenectomy. *Ann Surg* 1995; 222:394-401.

5. Morton DL. Intraoperative lymphatic mapping and sentinel lymphandectomy: community standard care or clinical investigation? *Cancer J Sci Am* 1997; 3:328-330.

6. Dauway EL, Giuliano R, Pendas S, et al. Lymphatic mapping: a technique providing accurate staging for breast cancer. *Breast Cancer* 1999; 6:145-154.

7. Veronesi U, Paganelli G, Viale G, et al. Sentinel lymph node biopsy and axillary dissection in breast cancer: results in a large series. *J Natl Cancer Inst* 1999; 91:368-373.

8. Posther KE, Wilke LG, Giuliano AE. Sentinel lymph node dissection and the current status of American trials on breast lymphatic mapping. *Sem Oncol* 2004; 31:426-436.

9.White RL, Wilke LG. Update on the NSABP and ACOSOG breast cancer sentinel node trials. *Am Surg* 2004; 70:420-424.

10. Yared MA, Middleton LP, Smith TL, et al. Recommendations for sentinel lymph node processing in breast cancer. *Am J Surg Pathol* 2002; 26(3):377-382.

11. Schrenk P, Rieger R, Shamiyeh A, et al. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. *Cancer* 2000; 88:608-614.

12. American Joint Committee on Cancer (AJCC) Cancer Staging Handbook, 6th edition, 2002.

13. NCCN Clinical Practice Guidelines in Oncology: Breast Cancer - Version 1, 2004.

14. Whitworth P, McMasters KM, Tafra L, et al. State-of-the-art lymph node staging for breast cancer in the year 2000. *Am J Surg* 2000; 180:262-267.

15. Cserni, G. Metastases in axillary sentinel lymph nodes in breast cancer as detected by intensive histopathological workup. *J Clin Pathol* 1999; 52:922-924.

16. Treseler PA and Tauchi PS. Pathologic Analysis of the Sentinel Lymph Node. *Surg Clin North Am.* 2000; Dec 80(6):1695-1719.

17. Viale G, Bosari S, Maxxarol G, et al. Intraoperative examination of axillary sentinel lymph nodes in breast carcinoma patients. *Cancer* 1999; 85(11):2433-2438.

18. Cserni G. Complete sectioning of axillary sentinel nodes in patients with breast cancer. Analysis of two different step sectioning and immunohistochemistry protocols in 246 patients. *J. Clin Pathol.* 2002; 55:926-931.

19. Van Diest PJ, Torrenga H, Meijer S, et al. Pathologic analysis of sentinel lymph nodes. *Semin Surg Oncol* 2001; 20:238-245.

20. Bernstein JL, Godbold JH, Raptis G, et al. Identification of mammaglobin as a novel serum marker for breast cancer. *Clin Cacncer Res* 2005; 11(18):6528-5635.

21. Zehentner BK, and Carter D. Mammaglobin: a candidate diagnostic marker for breast cancer. *Clin Biochem* 2004; 37:249-257.

22. Schoenfeld A, Kruger KH, Gomm J, et al. The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for Keratin 19. *Eur J Cancer* 1997; 33:854-861.

23. Backus J, Green GA, Xu M, et al. Intraoperative Molecular Analysis of Sentinel Lymph Nodes for the Management of Breast Cancer Surgery. *Clin Cancer Res*; in press.

24. McCready DR, Yong WS, Ng AK, et al. Influence of the new AJCC breast cancer staging system on sentinel lymph node positivity and false-negative rates. *J Natl Cancer Inst* 2004; 96:873-875.

25. Cserni G, Amendoeira I, Apostolikas N, et al. Discrepancies in current practice of pathological evaluation of sentinel lymph nodes in breast cancer. Results of a questionnaire based survey by the European Working Group for Breast Screening Pathology. *J Clin Pathol* 2004; 57:695-701.

26. van Diest PJ, Torrenga H, Borgstein PJ, et al. Reliability of intraoperative frozen section and imprint cytological investigation of sentinel lymph nodes in breast cancer. *Histopath.* 1999; 35(1): 14-18.

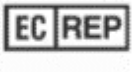
27. Agresti A. Categorical Data Analysis. John Wiley & Sons. 1990: 366-367.

28. Backus J, Laughlin T, Wang Y, et al. Identification and Characterization of Optimal Gene Expression Markers for Detection of Breast Cancer Metastasis. *Journal of Molecular Diagnostics* 2005; 7(3): 327-336.

Technical and Customer Support can be reached 8:00 AM to 8:00 PM Eastern Time.

Veridex, LLC
33 Technology Drive
Warren, NJ 07059
Phone: 1-877-VERIDEX (USA)
00 8000 8374339 (EU)

Key to Symbols: The following symbols may have been used in this instruction for use or in the associated labeling.

	Use by YYYY-MM-DD or YYYY-MM		Catalog number
	Batch code		Authorized representative in the European Community
	Serial number		Temperature limitation
	Caution, consult accompanying documents		Biological risks
	Manufacturer		Consult instructions for use
	Contains sufficient for < n > tests		*In vitro* diagnostic medical device

GeneSearch™ is a trademark of Veridex, LLC. SmartCycler™ is a trademark of Cepheid. Qiagen® is a registered trademark of the QIAGEN Group. Omni Tip™ is a trademark of Omni International.

Mammaglobin markers and assays are licensed under patents owned or controlled by including the following and their non-US counterparts: 5,668,267; 5,922,836; 5,968,754; 6,004,756.

Veridex is licensed under patents owned by Roche Molecular Systems, Inc., and F. Hoffman-La Roche Ltd covering PCR amplification and detection including the following and their non-US counterparts: 5,219,727; 5,310,652; 5,322,770; 5,476,774; 5,641,864; 5,314,809; 5,561,058; 5,693,517; 5,079,352; 5,352,600; 5,407,800; 5,210,015; 5,487,972; 5,804,375.

THE PURCHASE OF THIS PRODUCT ALLOWS THE PURCHASER TO USE IT FOR THE PERFORMANCE OF DIAGNOSTIC SERVICES FOR HUMAN IN VITRO DIAGNOSTICS. NO GENERAL PATENT OR OTHER LICENSE OF ANY KIND OTHER THAN THIS SPECIFIC RIGHT OF USE FROM PURCHASE IS GRANTED HEREBY.

Ortho-Clinical Diagnostics
Johnson & Johnson
50-100 Holmes Farm Way
High Wycombe
Buckinghamshire
HP12 4DP
United Kingdom

Document # PI-1055, Rev. 1, 28 April 2006
Component Code #7800070.00