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Intraoperative lymph node assay

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Abstract of the Disclosure

INTRAOPERATIVE LYMPH NODE ASSAY

In an intraoperative lymph node assay for micrometastases, a surgeon identifies a sentinel
5 lymph node during surgery according to known methods. Sentinel nodes are removed and
prepared for the extraction of nucleic acid. Nucleic acid (e.g., RNA) is then rapidly
extracted from the sentinel nodes. Markers indicative of micrometastases, if present, are
then amplified and detected.

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COMPLETE SPECIFICATION

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Invention Title: INTRAOPERATIVE LYMPH NODE ASSAY

The following statement is a full description of this invention, including the best method of performing it known to us:-

INTRAOPERATIVE LYMPH NODE ASSAY

BACKGROUND

5 The invention relates to the field of molecular diagnostics.

Lymph node involvement is the strongest prognostic factor in many solid tumors, and detection of lymph node micrometastases is of great interest to pathologists and surgeons. Current lymph node evaluation involves microscopic examination of H&E-stained tissue sections and suffers from three major limitations: (a) single tumor cells, or small foci of
10 cells, are easily missed; (b) the result is not rapidly available, meaning that any positive result in a sentinel lymph node procedure requires a second surgery for removal of axillary lymph nodes and (c) only one or two tissue sections are studied, and thus the vast majority of each node is left unexamined. Serial sectioning can help overcome the issue of sampling error, and immunohistochemistry (IHC) can help identify individual tumor cells.

15 The combination of these methods, however, is too costly and time consuming for routine analysis and is limited to special cases such as sentinel lymph node examination.

Surgical decisions are often based on intra-operative frozen section analysis of lymph nodes; however, the sensitivity of these methods is relatively poor, ranging from 50-70% relative to standard H&F pathology, leading to an unacceptably high rate of second
20 surgeries. The five-year survival of Stage 0 and I breast cancer patients who do not have lymph node involvement are 92% and 87%, respectively. On the other hand, the five-year survival of later stage breast cancer patients who do have lymph node involvement decrease significantly. For example, the survival of Stage II breast cancer is only 75%, Stage III 46%, and Stage IV 13%. Although node negative breast cancer patients have
25 improved survival, 20-30% of histologically node negative patients suffer disease recurrence. This is most likely due to the limitations of current techniques in the detection of micrometastases including issues related to node sampling and poor sensitivity for detecting individual tumor cells or small tumor foci.

In addition to a need for more accurate and sensitive detection of metastases in breast
30 cancer nodes, there is a similar need for a rapid and sensitive test in melanoma nodes as

well as in the more accurate detection of surgical margins, particularly in the intraoperative setting.

The polymerase chain reaction (PCR) is a powerful tool in the field of molecular biology that could be useful in this setting. This technique allows for replicating/amplifying small amounts of nucleic acid fragments into quantities that can be analyzed in a meaningful way. Furthermore with the development of real-time quantitative RT-PCR (Q-RT-PCR), this technology has become more reliable as well as amenable to automation. Q-RT-PCR is less subject to contamination and provides quantitation of gene expression. Such quantitation could be applied for the detection of micrometastases in intraoperative lymph node assays. PCR in molecular diagnostics, despite its advantages, has several limitations that make it difficult to apply in typical clinical diagnostic setting, particularly in the intraoperative setting.

One such limitation is the time it typically takes to perform PCR diagnoses. Typical PCR reactions take hours, not minutes. Decreasing the time it takes to carry out a PCR reaction is necessary if the technique is to be useful intraoperatively. Further, although Q-RT-PCR can provide quantitative results, to date there have been no known cutoff values for distinguishing positive from negative results based on such technology nor has it been clear which nucleic acid fragments are best detected and correlated to the presence of a micrometastasis. Other methods for the amplification and detection of nucleic acid fragments exist as well and each suffers from similar problems.

An intraoperative molecular lymph node assay that overcomes the existing difficulties would be well accepted by the medical community.

SUMMARY OF THE INVENTION

The invention is an intraoperative lymph node assay for micrometastases. A surgeon identifies a sentinel lymph node during surgery according to known methods. Sentinel nodes are removed and prepared as described below. Nucleic acid (e.g., RNA) is then rapidly extracted from the sentinel nodes. Markers indicative of micrometastases, if present, are then amplified and detected. The surgeon then takes action based upon the outcome of the detection of such Markers.

The assay is most preferably based on a rapid PCR and RT-PCR method that permits execution of a complete PCR reaction in minutes thereby permitting use of PCR in intraoperative diagnoses for detecting micrometastases in sentinel lymph nodes.

5 In one aspect of the invention, an intraoperative molecular diagnostic method includes the following steps conducted during the course of a surgical procedure: obtaining a tissue sample from a patient; extracting RNA from the sample, analyzing the sample by nucleic acid amplification and detection; and determining if the presence of one or more gene expression Markers exceeds a cut-off value. The cut-off value can be an absolute value or a value relative to the expression of a control gene.

10 In another aspect of the invention, the gene expression Markers are nucleic acid fragments specific for particular tissue such as breast tissue.

In yet another aspect of the invention, the gene expression Markers are nucleic acid fragments indicative of malignancy.

15 In yet another aspect of the invention lymph nodes samples are homogenized and then diluted to assure that sufficient RNA is extracted despite an expedited extraction method.

In yet another aspect of the invention the Markers are those of mammaglobin (Seq. ID No. 17) and either PIP (Seq. ID No. 18), B305D (particularly, isoform C, Seq. ID No. 14), B726 (Seq. ID No. 15), GABA (Seq. ID No. 16) or O8E (Seq. ID No. ___) in the case of breast cancer diagnostics and tyrosinase (Seq. ID No. 22) and MART 1 (Seq. ID No. 21)
20 in the case of melanoma.

In yet another aspect of the invention, micrometastases are detected intraoperatively by a method that includes the steps of: obtaining RNA from a sentinel lymph node; performing a quantitative RT-PCR method specific to one or more genes of interest and determining if the presence of the Marker exceeds a predetermined cut-off. The cut-off value can be an
25 absolute value or a value relative to the expression of a control gene.

In a yet further embodiment of the invention, kits contain reagents for conducting intraoperative lymph node assay for micrometastases.

DETAILED DESCRIPTION

Intraoperative methods for cancer diagnostics are presented. These methods employ a rapid method of extracting nucleic acids from a lymph node and a method of amplifying and detecting nucleic acid fragments indicative of metastasis (such fragments are referred to herein as "Markers").

An important aspect of the inventive methods is the rapid amplification and detection of Markers indicative of the expression of certain genes. Provided that such methods can be conducted within a period acceptable for an intraoperative assay (i.e., no more than about 35 minutes), any reliable, sensitive, and specific method can be used. This includes PCR methods, Rolling Circle Amplification methods (RCA), Ligase Chain Reaction methods (LCR), Strand Displacement Amplification methods (SDA), Nucleic Acid Sequence Based Amplification methods (NASBA), and others. The rapid molecular diagnostics involved are most preferably quantitative PCR methods, including QRT-PCR.

Irrespective of the amplification method employed, it is important to adequately sample the tissue used to conduct the assay. This includes proper excision and processing of the sentinel lymph node as well as extraction of RNA from it. Once obtained, it is important to process the nodes properly so that any cancerous cells present are detected.

A variety of techniques are available for extracting nucleic acids from tissue samples. Standard practice in each case is time consuming and can be difficult even when using a commercially available kit designed for this purpose. Typical commercially available nucleic acid extraction kits take at least 15 minutes to extract the nucleic acid. In the methods of the instant invention, nucleic acid is extracted in less than 8 minutes and preferably less than 6 minutes. These rapid extraction methods are the subject of a patent application by R. Belly, J. Toner, and J. Backus of equal date and entitled "Rapid Extraction Of RNA From Cells And Tissues" which is incorporated herein by reference.

The successful isolation of intact RNA generally involves four steps: effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNAase) and removal of contaminating DNA and protein. The disruptive and protective properties of guanidinium thiocyanate (GTC) and B-mercaptoethanol to inactivate the ribonucleases present in cell extracts make them preferred reagents for the first step. When used in conjunction with a surfactant such as sodium dodecylsulfate

(SDS), disruption of nucleoprotein complexes is achieved allowing the RNA to be released into solution and isolated free of protein. Dilution of cell extracts in the presence of high concentrations of GTC causes selective precipitation of cellular proteins to occur while RNA remains in solution. Centrifugation can clear the lysate of precipitated proteins and cellular DNA and is preferably performed through a column. Such columns also shear DNA and reduce the viscosity of the sample. RNA purification is preferably conducted on a spin column containing silica or other material. Manual cell and tissue disruption can be by means of a disposable tissue grinder as described in US Patent 4,715,545.

Homogenization time is within 1 to 2 minute and is more preferably 30-45sec. The sample can then processed with a shredding column (eg., QIAshredder, QIAGEN Inc., Valencia, CA, or suitable substitute) or with an RNA processing device such as the PCR Tissue Homogenization Kit commercially available from Omni International (Warrenton, VA) to reduce its viscosity. RNA is precipitated out via the spin column as described above and centrifugation times are no greater than 30 sec. When using commercial RNA extraction kits such as those available from Qiagen, Inc., filtration is used instead of centrifugation for all steps except for the column drying step. Typically, the sample is diluted with an equal volume of 70% ethanol prior to application on the column. After washes by filtration, the column is dried by centrifugation, and RNA is eluted in RNAase free water. The RNA is selectively precipitated out of solution with ethanol and bound to a substrate (preferably, a silica-containing membrane or filter). The binding of RNA to the substrate occurs rapidly due to the disruption of the water molecules by the chaotropic salts, thus favoring absorption of nucleic acids to the silica. The bound total RNA is further purified from contaminating salts, proteins and cellular impurities by simple washing steps. Finally, the total RNA is eluted from the membrane by the addition of nuclease-free water. The total time of this rapid protocol is less than 8 minutes and preferably less than 6 min.

In summary the rapid RNA extraction method involves the following steps:

- (a) obtaining a sample containing cells from the biological system,
- (b) optionally, removing from the sample, cells without RNA of interest to produce a working sample,
- (c) lysing the cells containing RNA that is of interest and producing a homogenate of them,
- (d) optionally, diluting the homogenate,

- (e) contacting the wetted, homogenized working sample with a substrate containing, or to which is affixed, a material to which RNA binds,
 - (f) allowing the sample to bind to the substrate,
 - (g) removing contaminants and interferents,
 - 5 (h) drying the substrate, and
 - (i) eluting RNA from the substrate;
- in instances in which centrifugation is used, it may occur after steps g, h, or i and vacuum/filtration is preferably applied in extraction steps.

10 The reagents involved in this rapid extraction process are:

- Lysis/Binding buffer (preferably, 4.5M guanidinium-HCl, 100mM sodium phosphate),
- Wash buffer I (preferably, 37% ethanol in 5M guanidine-HCL, 20mM Tris-HCL),
- Wash buffer II (preferably, 80% ethanol in 20mM NaCl, 2mM Tris-HCL),
- 15 Elution buffer, and
- Nuclease-free sterile double distilled water.

Since the distribution of cancer cells in nodes is non-uniform, it is preferable that multiple sections of the node be sampled. Optionally, one or more nodes may also be examined
20 based on pathology. One method for accomplishing both a molecular based test and an examination of the same node sample by pathology is to section the node into at least four sections with one outer and inner section used for pathology, and one outer and inner section for used for molecular testing. As the distribution of metastases and micrometastases in tissues is not uniform in nodes or other tissues, a sufficiently large
25 sample should be obtained so that metastases will not be missed. One approach to this sampling issue in the present method is to homogenize a large tissue sample, and subsequently perform a dilution of the well-mixed homogenized sample to be used in subsequent molecular testing.

30 A typical PCR reaction includes multiple amplification steps, or cycles that selectively amplify target nucleic acid species. A typical PCR reaction includes three steps: a denaturing step in which a target nucleic acid is denatured; an annealing step in which a set of PCR primers (forward and backward primers) anneal to complementary DNA strands;

and an elongation step in which a thermostable DNA polymerase elongates the primers. By repeating this step multiple times, a DNA fragment is amplified to produce an amplicon, corresponding to the target DNA sequence. Typical PCR reactions include 20 or more cycles of denaturation, annealing and elongation. In many cases, the annealing and
5 elongation steps can be performed concurrently, in which case the cycle contains only two steps.

In the inventive method, employing RT-PCR, the RT-PCR amplification reaction is conducted in a time suitable for intraoperative diagnosis, the lengths of each of these steps can be in the seconds range, rather than minutes. Specifically, with certain new thermal
10 cyclers being capable of generating a thermal ramp rate of at least about 5°C per second, RT-PCR amplifications in 30 minutes or less are used. More preferably, amplifications are conducted in less than 25 minutes. With this in mind, the following times provided for each step of the PCR cycle does not include ramp times. The denaturation step may be
15 conducted for times of 10 seconds or less. In fact, some thermal cyclers have settings for "0 seconds" which may be the optimal duration of the denaturation step. That is, it is enough that the thermal cycler reaches the denaturation temperature. The annealing and elongation steps are most preferably less than 10 seconds each, and when conducted at the same temperature, the combination annealing/elongation step may be less than 10 seconds. Some
20 homogeneous probe detection methods, however, may require a separate step for elongation to maximize rapid assay performance. In order to minimize both the total amplification time and the formation of non-specific side reactions, annealing temperatures are typically above 50 C. More preferably annealing temperatures are above 55 C.

A single combined reaction for RT-PCR, with no experimenter intervention, is desirable for several reasons: (1) decreased risk of experimenter error, (2) decreased risk of target or
25 product contamination and (3) increased assay speed. The reaction can consist of either one or two polymerases. In the case of two polymerases, one of these enzymes is typically an RNA-based DNA polymerase (reverse transcriptase) and one is a thermostable DNA-based DNA polymerase. To maximize assay performance, it is preferable to employ a form of
"hot start" technology for both of these enzymatic functions. US Patents 5,411,876 and
30 5,985,619 provide examples of different "hot start" approaches and are incorporated herein by reference. Preferred methods include the use of one or more thermoactivation methods that sequester one or more of the components required for efficient DNA polymerization. US Patents 5,550,044 and 5,413,924 describe methods for preparing reagents for use in

such methods. US Patent 6,403,341 describes a sequestering approach that involves chemical alteration of one of the PCR reagent components. All are incorporated herein by reference. In the most preferred embodiment, both RNA- and DNA-dependent polymerase activities reside in a single enzyme. Other components that are required for efficient
5 amplification include nucleoside triphosphates, divalent salts and buffer components. In some instance, non-specific nucleic acid and enzyme stabilizers may be beneficial.

The specificity of any given amplification-based molecular diagnostic relies heavily, but not exclusively, on the identity of the primer sets. The primer sets are pairs of forward and reverse oligonucleotide primers that anneal to a target DNA sequence to permit
10 amplification of the target sequence, thereby producing a target sequence-specific amplicon. The primers must be capable of amplifying Markers of the disease state of interest. In the case of the instant invention, these Markers are directed to breast cancer and melanoma.

In the case of breast cancer, the inventive method involves the amplification of a tissue
15 marker specific for either breast tissue or breast cancer tissue. A combination of at least two Markers are used such that clinically significant and reliable detection of breast/and/or cancer cells in lymph nodes is detected when present. Preferably, the Markers are amplified and detected in a single reaction vessel at the same time (i.e., they are multiplexed). Most preferably, the primer sets are complimentary to nucleic acid
20 fragments specific to those Markers. The Markers include mammaglobin (Seq. ID No. 17) and one or more (preferably one) of the following: B305D (Seq. ID No. 14), PIP (Seq. ID No. 18), B726 (Seq ID No. 15), GABA (Seq ID No. 16) or O8E (Seq ID No. __). The combination of a tissue specific marker and a cancer specific marker provide sensitivity and specificity that exceeds 90 % and 95 % respectively. Some Markers exist in various
25 isoforms with certain of the isoforms being more specific for one tissue or cancer than others. In the case of B305D, the most preferred isoform is B305D isoform C . It is also the most preferred Marker in combination with the mammaglobin Marker.

In the case of melanoma, the inventive method involves the amplification of markers specific to melanoma cancer. Thus, the primer sets are complimentary to nucleic acid
30 fragments specific to those markers. The Markers include one or more (preferably two or more) of the following: Tyrosinase (Seq. ID No. 22) and MART I (Seq. ID No. 21). These Markers provide sensitivity and specificity that exceeds 90 %.

The reaction must also contain some means of detection of a specific signal. This is preferably accomplished through the use of a reagent that detects a region of DNA sequence derived from polymerization of the target sequence of interest. Preferred reagents for detection give a measurable signal differential when bound to a specific nucleic acid sequence of interest. Often, these methods involve nucleic acid probes that give increased fluorescence when bound to the sequence of interest. The progress of the PCR reactions of the inventive method are typically monitored by analyzing the relative rates of amplicon production for each PCR primer set. Monitoring amplicons production may be achieved by a number of detection reagents and methods, including without limitation, fluorescent primers, fluorogenic probes and fluorescent dyes that bind double-stranded DNA, molecular beacons, Scorpions, and others. A common method of monitoring a PCR reaction employs a fluorescent 5'nuclease assay. This method exploits the 5'nuclease activity of certain thermostable DNA polymerases (such as Taq or Tfl DNA polymerases) to cleave an oligomeric probe during the PCR process. The oligomer is selected to anneal to the amplified target sequence under elongation conditions. The probe typically has a fluorescent reporter on its 5'end and a fluorescent quencher of the reporter at the 3' end. So long as the oligomer is intact, the fluorescent signal from the reporter is quenched. However, when the oligomer is digested during the elongation process, the fluorescent reporter is no longer in proximity to the quencher. The relative accumulation of free fluorescent reporter for a given amplicon may be compared to the accumulation of the same amplicons for a control sample and/or to that of a control gene, such as, without limitation, β -Actin and PBDG (porphobilinogen deaminase) to determine the relative abundance of a given cDNA product of a given RNA in a RNA population. Products and reagents for the fluorescent 5'nuclease assay are readily available commercially, for instance from Applied Biosystems.

The preferred detection reagents are commonly referred to as "Scorpions" and are described in US Patents 6,326,145 and 5,525,494 and incorporated herein by reference. These reagents include one or more molecules comprising a tailed primer and an integrated signaling system. The primer has a template binding region and a tail comprising a linker and a target binding region. The target binding region in the tail hybridizes to complementary sequence in an extension product of the primer. This target specific hybridization event is coupled to a signaling system wherein hybridization leads to a detectable change. In PCR reactions the target binding region and the tail region are

advantageously arranged such that the tail region remains single stranded, ie. uncopied. Thus the tail region is non-amplifiable in the PCR amplification products. The linker comprises a blocking moiety which prevents polymerase mediated chain extension on the primer template.

- 5 Equipment and software also are readily available for controlling and monitoring amplicon accumulation in PCR and QRT-PCR including the Smart Cycler thermocycler commercially available from Cepheid of Sunnyvale, California, and the ABI Prism 7700 Sequence Detection System, commercially available from Applied Biosystems.

In the preferred RT-PCR reactions, the amounts of certain reverse transcriptase and the
10 PCR reaction components are atypical in order to take advantage of the faster ramp times of some thermal cyclers. Specifically, the primer concentrations are very high.

Typical gene-specific primer concentrations for reverse transcriptase reactions are less than about 20 nM. To achieve a rapid reverse transcriptase reaction on the order of one to two minutes, the reverse transcriptase primer concentration was raised to greater than 20 nM,
15 preferably at least about 50 nM, and typically about 100 nM. Standard PCR primer concentrations range from 100 nM to 300 nM. Higher concentrations may be used in standard PCR reactions to compensate for T_m variations. However, for purposes herein, the referenced primer concentrations are for circumstances where no T_m compensation is needed. Proportionately higher concentrations of primers may be empirically determined
20 and used if T_m compensation is necessary or desired. To achieve rapid PCR reactions, the PCR primer concentrations typically are greater than 250 nM, preferably greater than about 300 nM and typically about 500 nM.

Commercially used diagnostics also preferably employ the use of one or more internal positive controls that confirms the operation of a particular amplification reaction for a
25 negative result. Potential causes of false negative results that must be controlled for in an RT-PCR reaction include: inadequate RNA quantity, degradation of RNA, inhibition of RT and/or PCR and experimenter error. In the case of gene expression assays, it is preferable to utilize a gene that is constitutively expressed in the tissue of interest. PBGD (Seq. ID No. 20) is a gene that is commonly used as an internal control due to several factors: it
30 contains no known pseudogenes in humans, it is constitutively expressed in human tissues and it is expressed at a relatively low level and therefore is less likely to cause inhibition of the amplification of target sequences of interest. Use of PBGD as a control minimizes or

eliminates reporting erroneous results arising from all potential sources of false negative results.

In the commercialization of the described methods for QRT-PCR certain kits for detection of specific nucleic acids are particularly useful. In one embodiment, the kit includes reagents for amplifying and detecting Markers. Optionally, the kit includes sample preparation reagents and or articles (e.g., tubes) to extract nucleic acids from lymph node tissue. The kits may also include articles to minimize the risk of sample contamination (e.g., disposable scalpel and surface for lymph node dissection and preparation).

In a preferred kit, reagents necessary for the one-tube QRT-PCR process described above are included such as reverse transcriptase, a reverse transcriptase primer, a corresponding PCR primer set (preferably for Markers and controls), a thermostable DNA polymerase, such as Taq polymerase, and a suitable detection reagent(s), such as, without limitation, a scorpion probe, a probe for a fluorescent 5'nuclease assay, a molecular beacon probe, a single dye primer or a fluorescent dye specific to double-stranded DNA, such as ethidium bromide. The primers are preferably in quantities that yield the high concentrations described above. Thermostable DNA polymerases are commonly and commercially available from a variety of manufacturers. Additional materials in the kit may include: suitable reaction tubes or vials, a barrier composition, typically a wax bead, optionally including magnesium; reaction mixtures (typically 10X) for the reverse transcriptase and the PCR stages, including necessary buffers and reagents such as dNTPs; nuclease-or RNase-free water; RNase inhibitor; control nucleic acid (s) and/or any additional buffers, compounds, co-factors, ionic constituents, proteins and enzymes, polymers, and the like that may be used in reverse transcriptase and/or PCR stages of QRT-PCR reactions. Optionally, the kits include nucleic acid extraction reagents and materials.

The following non-limiting examples help to further describe the invention.

Examples

Real-time PCR

Examples in the present invention are based on the use of real-time PCR. In real-time PCR the products of the polymerase chain reaction are monitored in real-time during the exponential phase of PCR rather than by an end-point measurement. Hence, the quantification of DNA and RNA is much more precise and reproducible. Fluorescence

values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more templates present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background, which is the definition of the (Ct) values. The concept of the threshold cycle (Ct) allows for accurate and reproducible quantification using fluorescence based RT-PCR. Homogeneous detection of PCR products are preferably performed based on: (a) double- stranded DNA binding dyes (e.g., SYBR Green), (b) fluorogenic probes (e.g., Taq Man probes, Molecular Beacons), and (c) direct labeled primers (e.g., Amplifluor primers).

5

Example 1. A comparison of RNA extracted by the Rapid Method and by prior art Method based on real-time PCR with Taqman assays.

A total of 16 H&E positive nodes and 15 H&E negative breast node samples were purchased from Genomics Collaborative. Two 30 mg pieces of tissue were cut from each node.

15

Part I (Prior Art). One 30mg piece of tissue was processed as follows: 600ul of Buffer RLT was added and the tissue sample was homogenized manually for 20-40 sec by means of a disposable tissue grinder (cat 15704-126, VWR Scientific, West Chester, PA). The tube was centrifuged for 3min at maximum speed in Eppendorf model 5415C microcentrifuge. The supernatant fluid was transferred to a new tube, and 1 volume of 70% ethanol was added.

20

Sample (700µL) was applied to an RNeasy mini column placed on the QIAvac 24 vacuum manifold and the vacuum was applied. Buffer RWI (700 µL) and allowed to filter through the column. Buffer RPE (500 µL) was pipetted onto the column and allowed to filter through. Another 500 µL of Buffer RPE was added to the column, and allowed to filter through. The RNeasy column was placed in a 2 ml collection tube and centrifuged in a microfuge at maximum speed for 1min. The RNA was then eluted from the column, by transferring the RNeasy column to a new 1.5 ml collection tube, adding 50ul of RNAase-free water, and centrifuging for 1min at 8000 X g.

30

Part II (Rapid Extraction). The second 30 mg piece of breast node tissue was processed by a rapid protocol as follows: (1) The tissue piece was added to 600 μ L of Buffer RLT and homogenized manually for 20-40 sec by means of a disposable tissue grinder. (2) The homogenate was centrifuged through a QIAshredder column for 30sec at maximum speed.
5 (3) 1 volume of 70% ethanol was added to the lysate and mixed by pipetting. (4) The sample was then applied to an RNeasy mini column placed on the QIAvac 24 vacuum manifold and a vacuum was applied. (5) 700ul of Buffer RW1 was added to the column, and allowed to filter through the column. (6) 500ul of Buffer RPE was added onto the column and allowed to filter through. (7) The column was transferred to a 2ml collection
10 tube, and centrifuged for 30 sec at 10,000 rpm. (8) 25 μ L of RNAase-free water was added to the membrane and the column was centrifuged for 30sec at 10,000rpm to elute the RNA. All centrifugation steps in the rapid protocol were performed on a model 10MVSS VWR centrifuge.
15 Extracted RNA was reverse transcribed, and RNA and cDNA were quantified as described previously.

For Taqman assays, Taqman Core Reagent Kit, Gene Amp 10X PCR Buffer 1, Amp Erase Uracil N-glycosidase, and AmpliTaq Gold DNA Polymerase were purchased from Applied
20 Biosystems, Foster City, CA. All other reagents were from commercial sources described in example 1. Glycerol was purchased from Sigma Chemical Co (St. Louis, Mo.) and Tween 20 from Eastman Organic Chemicals (Rochester, NY)

Mammaglobin primers (SEQ ID NO. 3 and SEQ ID NO. 4) were synthesized by Invitrogen
25 Corp.(Carlsbad, CA) and the mammaglobin Taqman probe (SEQ ID NO. 7) from Epoch Biosciences (San Diego, CA). PBGD primers (SEQ ID NO 8 and SEQ ID NO. 9) were synthesized by QIAGEN Operon (Alameda, CA), and the probe (SEQ ID NO. 23) by SYNTHAGEN, LLC (Houston, TX). B305D primers (SEQ ID NO. 11 and SEQ ID NO.
30 12) were synthesized at Invitrogen Corp and the probe (SEQ ID NO. 13) by Applied Biosystems, Inc. For all Taqman probes, carboxyfluorescein (FAM) and carboxytetramethylrhodamine TAMRA) were used as the dye and quencher pair.

SEQ ID NO. 3 CAAACGGATG AA ACTCTGAG CAATGTTGA

SEQ ID NO. 4 TCTGTGAGCC AAAGG TCTTG CAGA

SEQ ID NO. 7 6-FAM – TGTTTATGCA ATTAATATAT GACAGCAGTC TTTGTG-TAMRA

5 SEQ ID NO. 8 CTGAGGCACC TGGAAGGAGG

SEQ ID NO. 9 CATCTTCATG CTGGGCAGGG

SEQ ID NO. 23 6-FAM-CCTGAGGCAC CTGGAAGGAG GCTGCAG TGT-TAMRA

SEQ ID NO. 11 TCTGATAAAG GCCGTACAAT G

10 SEQ ID NO. 12 TCACGACTTG CTGTTTTTGC TC

SEQ ID NO. 13 6-FAM-ATCAAAAAACA AGCATGGCCTA CACC- TAMRA

For the Taqman assays, a master mix was prepared by adding 10 μ L of 10X PCR Buffer#1, 14 μ L of 25mM MgCl₂, 8 μ L of 10mM dNTP's, 1 μ L of AmpErase UNG (1U/ μ L), 16 μ L of
15 glycerol, 1 μ L of 1%w/v Tween 20, 0.75 μ L of AmpliTaq Gold (5U/ μ L), 6 μ L of each primer from a 5 μ M stock, 0.4 μ L of a 10 μ M stock of probe, and water to a final volume of 96 μ L. Master mix (48 μ L) and 2 μ L of cDNA from each node sample were added to each optical reaction microtiter plate well. After capping with optical caps, the plates were processed in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Inc., Foster
20 City, CA). Commercial reagents including Taqman PCR Core Reagent Kit, Taqman DNA Template Reagents, and Taqman B-actin Detection Reagents were purchased from Applied Biosystems (Foster City, CA) and the assay was performed according to the protocol recommended by the manufacturer. A threshold value of 0.02 was used for analysis with the mammaglobin assay, 0.03 for the B305D assay, 0.08 for the B-actin
25 assay, and 0.1 for the PBDG assay. The average of triplicate determination by Taqman assays are shown in Tables 3 and 4.

A comparison of the real-time measurement of the housekeeping genes B-actin and PBDG are shown in Table 1 in 15 H&E negative node samples as well as 16 H&E positive nodes
30 and two control cDNA samples are shown in Table 1. Results of these experiments indicate good agreement between Ct values obtained with the rapid method of this invention and standard commercial methods (QIAGEN) confirming that RNA extracted by the rapid RNA extraction protocol is capable of being reverse transcribed and PCR

amplified to a similar extent as RNA extracted based on a standard commercial protocol.

Table 2 compares gene expression results based on Ct value for the breast cancer markers
mammaglobin and B305D with the same breast node samples evaluated in Table 1. To
5 determine whether a correlation can be made between H&E positive and H&E negative
breast node samples based on real-time PCR results, the lowest Ct value for each marker in
the H&E negative node samples was identified for both mammaglobin and B305D. An
arbitrary, but conservative, cut-off of 2.5Ct values less than this lowest value among H&E
negative samples was applied to the H&E positive samples. Samples that are shaded in the
10 Table 2 have higher expression for these two breast markers, in that Ct value is at least
2.5Ct values lower than a Ct value observed among H&E negative samples. There was a
good correlation in expression of these markers using both the rapid method of this
invention and the commercial manufacturer recommended protocol (QIAGEN).

15 Using mammaglobin as a marker, Ct values in two samples among the 15 H&E nodes
samples did not strictly correlate between the rapid method of this invention and QIAGEN
RNA extraction protocols. In one of these samples, GCLNC-24, the difference in Ct value
was 0.4 which is within experimental error. The second sample showed a larger difference
in Ct. larger differences in Ct value with both mammaglobin and B305D may be due to
20 difficulties in spectrally quantifying RNA in these samples as well as to possible sampling
problems due to the well established non-uniform distribution of metastases in nodes.
(Cserni. 1999. Metastases in axillary sentinel lymph nodes in breast cancer as detected by
intensive histopathological work up. J. Clin Pathol, 52:0922-924.)

25

Table 1: Comparison of RNA extracted by the rapid method and Prior Art Method as measured by Taqman Assays for the Housekeeping Genes B-actin and PBDG

	Sample	Template	B-Actin Ct Values		PBDG Ct Values	
			Qiagen	Rapid	Qiagen	Rapid
H&E Negative	1	GCLNN-10	20.7	21.9	32.6	34.1
	2	GCLNN-11	20.5	21.0	33.7	32.7
	3	GCLNN-12	21.7	22.5	32.8	32.8
	4	GCLNN-14	21.2	19.1	32.1	31.0
	5	GCLNN-15	19.8	21.3	32.5	39.9
	6	GCLNN-19	22.4	23.0	29.9	39.1
	7	GCLNN-20	17.8	18.6	31.7	30.9
	8	GCLNN-23	21.4	19.2	33.1	31.4
	9	GCLNN-24	21.1	26.1	36.9	37.0
	10	GCLNN-25	19.1	21.4	32.7	33.1
	11	GCLNN-26	23.8	26.7	40.0	39.7
	12	CBLNN-274	20.1	19.8	31.5	30.7
	13	CBLNN-257	20.4	22.6	32.8	34.0
	14	CBLNN-258	23.4	27.6	33.1	35.4
	15	CBLNN-262	23.0	23.6	33.0	32.1
H&E Positive	16	GCLNC-1	18.7	18.5	28.8	29.1
	17	GCLNC-5	20.4	21.6	31.3	31.0
	18	GCLNC-6	20.0	19.9	31.4	30.2
	19	GCLNC-7	20.6	28.2	29.3	32.1
	20	GCLNC-11	18.9	19.5	31.2	30.9
	21	GCLNC-12	26.1	23.8	32.6	31.2
	22	GCLNC-13	20.5	18.9	30.8	27.7
	23	GCLNC-16	20.5	22.7	31.1	30.5
	24	GCLNC-17	22.1	19.4	40.0	29.1
	25	GCLNC-20	20.0	19.5	31.9	29.6
	26	GCLNC-21	21.1	21.3	33.5	32.2
	27	GCLNC-22	24.2	22.9	29.3	32.1
	28	GCLNC-23	18.4	20.7	29.6	35.5
	29	GCLNC-24	19.1	19.5	29.4	30.1
	31	ALNC-1	21.1	19.4	30.2	30.8
	32	ALNN-2	18.9	19.5	31.4	30.0
	33	CBLNN-247	18.2	20.7	31.5	31.1
	Cut Off Values			33.7	37.5	36.1

Table 2: Comparison of RNA extracted by the rapid method and by the Prior Art Method as measured by by Taqman Assays for the Cancer Genes Mammaglobin and B305D

	Sample	Template	MG Ct Values		B305D-A/C Ct Values	
			Qiagen	Rapid	Qiagen	Rapid
H&E Negative	1	GCLNN-10	40.0	40.0	40.0	40.0
	2	GCLNN-11	40.0	40.0	40.0	40.0
	3	GCLNN-12	40.0	40.0	39.7	39.9
	4	GCLNN-14	40.0	40.0	39.6	39.9
	5	GCLNN-15	40.0	40.0	39.6	40.0
	6	GCLNN-19	40.0	40.0	40.0	40.0
	7	GCLNN-20	40.0	40.0	38.6	38.6
	8	GCLNN-23	40.0	40.0	39.0	40.0
	9	GCLNN-24	40.0	40.0	40.0	40.0
	10	GCLNN-25	40.0	40.0	40.0	40.0
	11	GCLNN-26	36.2	40.0	40.0	40.0
	12	CBLNN-274	40.0	40.0	39.3	39.5
	13	CBLNN-257	40.0	40.0	40.0	39.5
	14	CBLNN-258	40.0	40.0	40.0	40.0
	15	CBLNN-262	40.0	40.0	39.6	39.5

H&E Positive	16	GCLNC-1	20.8	22.8	27.1	27.9
	17	GCLNC-5	39.7	40.0	29.2	36.6
	18	GCLNC-6	23.7	22.7	26.8	26.3
	19	GCLNC-7	27.9	28.3	30.0	31.0
	20	GCLNC-11	32.5	32.8	28.2	28.4
	21	GCLNC-12	32.4	30.7	38.6	36.1
	22	GCLNC-13	40.0	39.7	38.9	38.3
	23	GCLNC-16	20.8	21.7	36.5	35.9
	24	GCLNC-17	30.7	28.7	32.3	24.9
	25	GCLNC-20	30.6	30.1	26.8	27.8
	26	GCLNC-21	40.0	38.9	34.0	35.2
	27	GCLNC-22	39.4	36.3	36.4	31.4
	28	GCLNC-23	33.0	33.8	30.1	30.8
	29	GCLNC-24	37.6	37.2	28.0	29.7
	31	ALNC-1	23.8	24.8	25.6	28.6
	32	ALNN-2	27.7	27.6	23.4	23.9
	33	CBLNN-247	35.9	38.2	29.8	30.1

Cut Off Values = 33.7 37.5 36.1 36.1

5

Example 2. Evaluation of a QIAshredder Column

It is the purpose of this example to demonstrate that the QIAshredder column can be used after homogenization of lymph node tissue, and that centrifugation of the homogenate for only 30 seconds yields acceptable RNA extraction.

10

A breast axillary node that was H&E –negative was obtained from Genomics Collaborative (Cambridge, MA). A 490mg slice of tissue was mixed with 5.5 ml of Buffer RLT, and the node was homogenized. Six hundred microliters of homogenate was removed and RNA was extracted using the rapid extraction described in Example 1. As a control, another 600 μ L of homogenate was treated using the same rapid RNA extraction method except that a 3min centrifugation was used instead of the QIAshredder column. The RNA from each reaction was reverse transcribed and quantified as described in Example 1. Real-time PCR was performed using Taqman probes.

Results of these studies indicated similar RNA yields of 0.31 μ g/ μ L in the protocol involving the QIAshredder protocol as compared to 0.34 μ g/ μ L in the centrifugation protocol. Identical 260/280nm ratio's of 2.1 were obtained in both samples indicating high quality RNA. Real-time PCR assays also indicated similar Ct values for Mammaglobin with RNA extracted by the QIAshredder column as compared to the protocol involving (22.5 versus 22.0), as well as with B305D (26.7 versus 26.6) as well as with the housekeeping genes PBDG (29.1 versus 29.0), and B-actin (SEQ ID NO 19, 18.5 versus 18.2).

In summary, this experiment indicates similar RNA yield, quality and real-time PCR amplification results with the protocol involving a QIAshredder instead of a 3min centrifugation step after homogenization. This reduces the time required to perform RNA extraction by 2.5 minutes, which is important in developing protocols suitable for intraoperative and other applications in which fast results are required to impact patient care.

25

Example 3. Effect of Diluting of Lysate on RNA yield and precision

The following example illustrates the effect of dilution of the lysate on RNA yield and precision of RNA recovery. In this example, 20mg, 10mg or 5 mg of frozen pig node tissue was cut. Tissue was ground using a 50ml Disposable Tissue Grinder for 30 sec, and the samples were vortexed. Three replicates were performed for each node weight. One ml of each lysate was transferred to a 1.7 ml microcentrifuge tube which was then centrifuged in an Eppendorf microfuge at maximum speed (14,000RPM) for 30 sec. Seven hundred microliters was centrifuged through a QIAshredder column and the samples were

centrifuged at maximum speed for 30 sec. The column was then washed, the column dried, and RNA eluted as described previously in Example 2, Part I steps 4-8. RNA was quantified by means of a Gene Spec II. Results of these studies are shown in Tables 3 and 4, and illustrates excellent precision at an initial node tissue weight of 2.5mg homogenized in 600ml of homogenization buffer, as compared to samples with higher weights including 10mg and 20mg node tissue. These results indicate that improved precision of RNA recovery can be obtained at lower node weight. Also, it would be expected that more dilute homogenates would be filtered faster through the column, and that there would be even fewer potential problems with filter clogging.

Table 3. Effect of Dilution of Lysate on RNA recovery

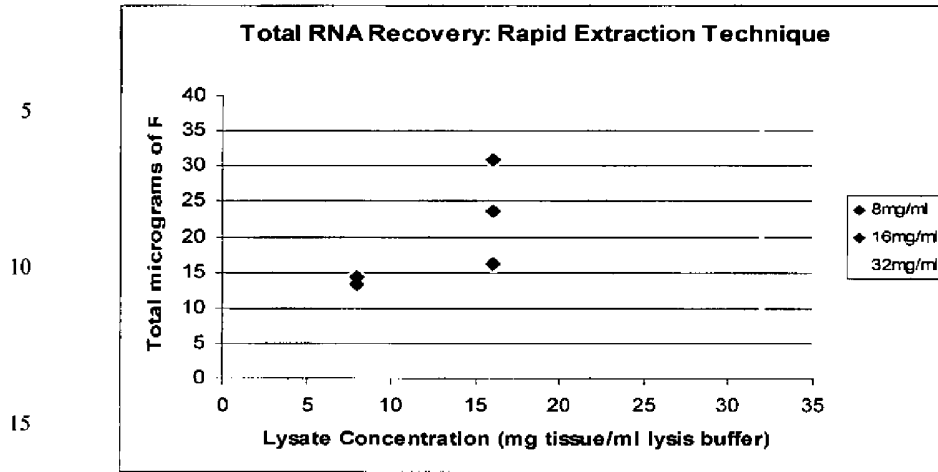
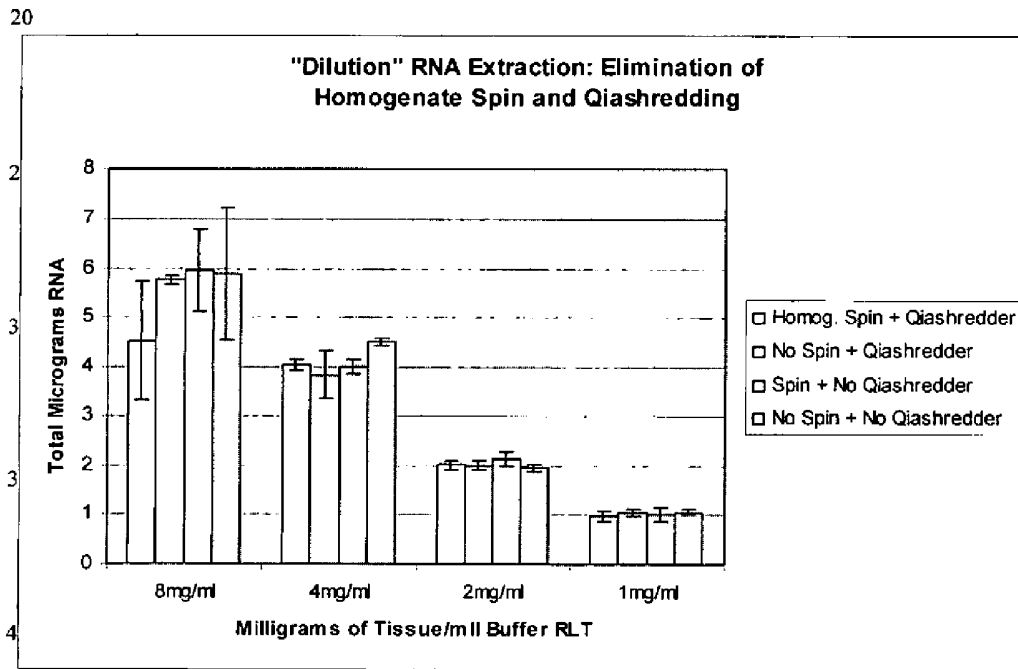


Table 4. Effect of Dilution.



Example 4: TaqMan testing of RNA from lymph node samples

45

Reverse transcription

cDNA was synthesized from 20 µg total RNA at 42 C for 60 min in a 250µl reaction containing 50 mM Tris-Cl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 2000U

Superscript, 400U Rnasin, 2ug oligo dT primer, 0.08 mg/ml BSA and 0.2 mM each dACT, dCTP, dGTP and TTP. The resulting cDNA was diluted 1:8 in water.

PCR amplification

5 2ul of each diluted cDNA was amplified on the Applied Biosystems 7900 with a total volume per well of 50 ml. The amplification was carried out with the following protocol: 50°C for 2 min and 95°C for 10 min, then 40 cycles of 95°C for 15 seconds (sec), 60°C for 1 min (58°C for B726) and 68°C for 1 min. The reaction mix components and final concentrations are as follows: 1X TaqMan buffer A (ABI), 3.5 mM MgCl₂, 0.2 mM of
10 dGTP, dCTP and cATP, 0.4 mM dUTP (ABI), 0.01 u/ul AmpErase UNG (ABI), 0.0375U/ul AmpliTaq Gold (ABI), 8% Glycerol (Sigma), 0.01% Tween 20 (Kodak), 0.3 uM each primer (Invitrogen), RNase/DNase free water (Sigma) and 40 nM 5'-FAM, 3' TAMRA oligonucleotide probe (Synthegen). A threshold of signal relative to an internal reference dye (ROX) was assigned to each primer/probe set in the logarithmic linear range
15 of the increase in fluorescent intensity caused by the increase in PCR product. The resulting Ct values were utilized to determine relative expression of the genes across all samples tested.

Primers utilized:

20

Mammaglobin

Primer 1, SEQ ID NO 3 - CAAACGGATGAAACTCTGAGCAATGTTGA

Primer 2, SEQ ID NO 4 - TCTGTGAGCCAAAGGTCTTGCAGA

Probe, SEQ ID NO 7 - FAM-TGTTTATGCAATTAATATATGACAGCAGTCTTTGTG-

25 TAMRA

B305D

Primer 1, SEQ ID NO 11 - TCTGATAAAGGCCGTACAATG

Primer 2, SEQ ID NO 12 - TCACGACTTGCTGTTTTTGCTC

30 Probe, SEQ ID NO 24 - FAM ATCAAAAACAAGCATGGCCTCACACC

PBGD

Primer 1, SEQ ID NO 25 - CTGCTTCGCTGCATCGCTGAAA

Primer 2, SEQ ID NO 26 - CAGACTCCTCCAGTCAGGTACA

Probe, SEQ ID NO 23 - FAM-CCTGAGGCACCTGGAAGGAGGCTGCAGTGT-TAMRA

PIP

- 5 Primer 1, SEQ ID NO 27 - GCTTGGTGGTTAAACTTACC
Primer 2, SEQ ID NO 28, - TGAACAGTTCTGTTGGTGTA
Probe, SEQ ID NO 29 - FAM-CTGCCTGCCTATGTGACGACAATCCGG-TAMRA

GABA

- 10 Primer 1, SEQ ID NO 30 - CAATTTTGGTGGAGAACCCG
Primer 2, SEQ ID NO 31 - GCTGTCCGAGGTATATGGTG
Probe, SEQ ID NO 32 - FAM CATTTCAGAGAGTAACATGGACTACACA TAMRA

B726

- 15 Primer 1, SEQ ID NO 33 - GCAAGTGCCAATGATCAGAGG
Primer 2, SEQ ID NO 34- ATATAGACTCAGGTATACACACT
Probe, SEQ ID NO 35 - FAM TCCCATCAGAATCCAAACAAGAGGAAG

Results:

20

The attached table contains the Ct values obtained for 11 histology-negative and 24 histology-positive lymph nodes. A cut-off for positivity for each marker was determined by subtracting 2.5 cycles from the lowest Ct observed with the histology-negative samples. Positive samples are denoted shading. Based on these criteria, detection rates for the individual markers were determined. Optimal complementation is seen with mammaglobin combined with B305D A/C, a combination that detects 23/24 histology-positive samples. A combination of mammaglobin, B305D and GABA detected all 24 samples. See Table 5.

Sample	MG	PIP	B305D	GABA	B726	PBGD	Actin
N1	50.0	36.2	35.8	36.5	50.0	30.6	21.6
N2	50.0	38.7	39.0	37.0	50.0	30.4	21.1
N3	50.0	37.8	39.6	39.1	50.0	28.6	23.1
N4	36.3	36.4	39.8	40.0	50.0	32.4	19.8
N5	48.4	40.0	40.0	40.0	50.0	35.8	21.1
N6	46.9	34.8	38.9	36.1	50.0	28.5	20.3
N7	50.0	35.2	40.0	38.4	50.0	27.8	20.3
N8	38.8	38.5	36.9	37.5	50.0	29.4	21.7
N9	50.0	37.0	37.9	36.4	50.0	30.6	20.1
N10	50.0	40.0	38.1	40.0	50.0	31.0	22.3
N11	50.0	39.4	35.8	39.9	50.0	29.4	19.7
C1	25.7	25.1	25.9	36.7	25.8	28.8	21.7
C2	36.0	32.5	35.6	27.2	41.5	29.0	23.2
C3	26.2	30.7	26.8	37.2	35.2	29.9	24.1
C4	25.7	22.9	29.7	30.4	37.9	27.3	20.5
C5	25.9	23.3	34.8	38.0	39.1	27.8	21.1
C6	29.7	36.6	28.8	29.9	40.4	26.2	20.4
C7	35.0	33.9	25.4	23.4	42.7	27.1	22.5
C8	22.7	25.3	29.3	38.0	37.8	27.8	18.9
C9	19.2	26.6	27.7	36.3	36.0	26.2	21.1
C10	20.2	30.0	27.4	33.8	39.7	29.4	23.2
C11	30.4	33.2	29.0	26.2	40.7	27.9	21.4
C12	18.6	27.7	24.3	36.0	33.6	28.8	19.4
C13	23.4	17.0	23.1	38.2	24.3	27.4	21.8
C14	35.9	30.7	28.0	39.8	42.3	31.3	21.7
C15	27.0	28.1	25.6	34.6	25.5	29.7	21.1
C16	32.5	34.5	36.9	23.5	46.1	27.7	27.6
C17	38.8	35.7	27.5	26.3	37.4	28.8	29.7
C18	36.9	34.8	29.2	24.7	50.0	26.4	22.0
C19	31.6	22.9	24.5	38.0	39.1	28.9	24.7
C20	22.3	27.6	21.4	32.6	32.9	27.0	21.6
C21	19.3	24.8	25.1	36.2	28.9	28.4	21.3
C22	32.2	28.4	25.8	37.9	37.3	30.6	23.5
C23	20.7	24.2	27.2	37.4	27.4	28.9	22.6
C24	33.0	27.1	32.6	24.3	40.4	26.4	20.0
cutoff	33.8	32.3	33.3	33.6	37.5		

Positive samples detected 19/24 17/24 21/24 10/24 11/24

Table 5

5

10

Example 5 Multiple Markers

15

Reverse transcription

cDNA was synthesized from 20 ug total RNA at 42 C for 60 min in a 100ul reaction utilizing anchored oligo dT primers. 20 ng of each diluted cDNA was amplified on the Applied Biosystems 7900 with a total volume per well of 50 ml. The amplification was carried out with the following protocol: 94 C for 2 min then 50 cycles of: 94 C for 15 sec,
5 62 C for 15 sec and 72 C for 45 sec. The reaction mix components and final concentrations are as follows: 1X PCR Buffer (ABI), 2.5 mM final MgCl₂, 0.05 mM of dGTP, dCTP and cATP and TTP, 0.05U/ul Taq polymerase containing a 5-20X excess of 2 anti-Taq antibodies (TP4-9 and TP1-12), 0.2 uM each primer (Invitrogen), RNase/DNase free water (Sigma) and a 1:20000 dilution of a SYBR Green stock (Sigma). Amplification primer
10 sequences were identical to those employed in TaqMan testing of lymph nodes. A common threshold of 275 fluorescence units was utilized to determine Ct values. Ct values were then converted into quantitative gene expression and plotted relative to a cut-off that was assumed to approximate the minimum level of gene expression required to differentiate metastatic lymph nodes from background expression .

15

]Primers utilized:

Mammaglobin

Primer 1, SEQ ID NO 3 - CAAACGGATGAAACTCTGAGCAATGTTGA

20 Primer 2, SEQ ID NO 4 - TCTGTGAGCCAAAGGTCTTGCAGA

B305D

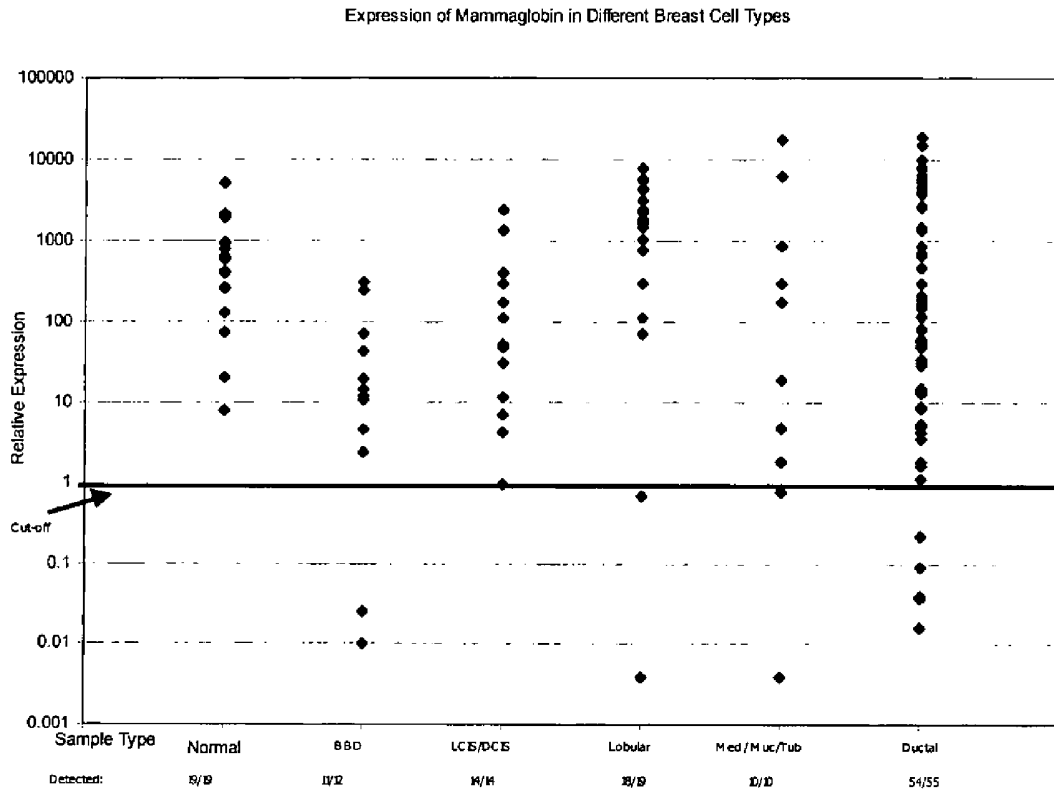
Primer 1, SEQ ID NO 11 - TCTGATAAAGGCCGTACAATG

Primer 2, SEQ ID NO 12 - TCACGACTTGCTGTTTTTGCTC

25

This data displayed in Table 6-8 shows the power of combining genes mammaglobin and B305D signatures for obtaining coverage of the vast majority of breast cancer samples.

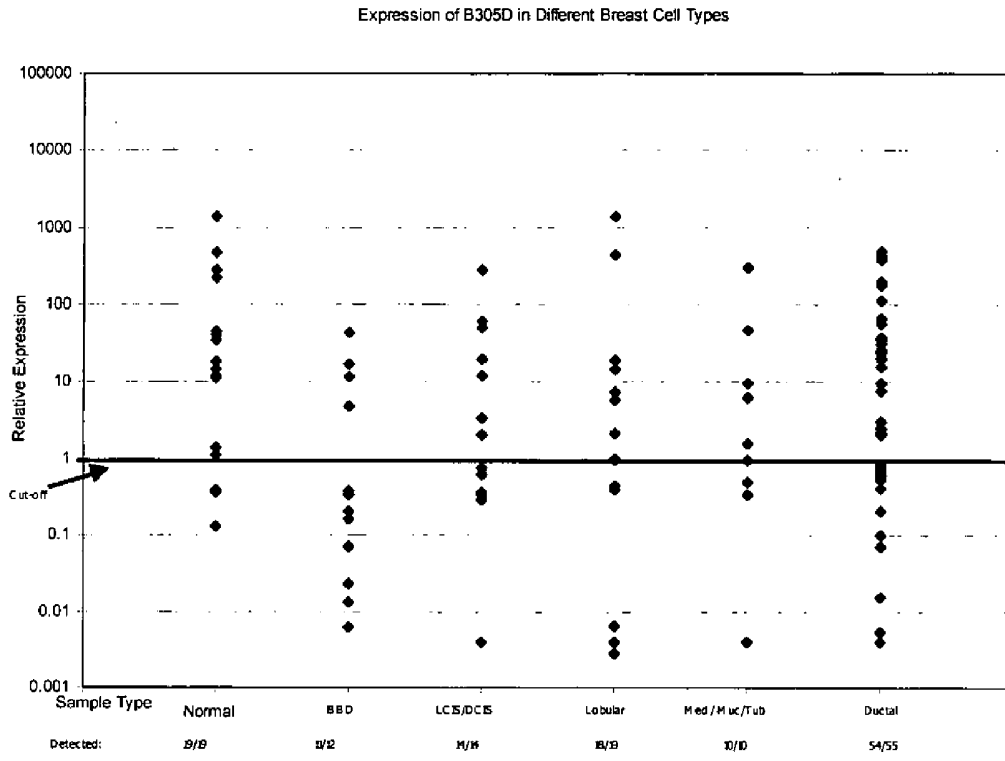
Table 6.



5

10

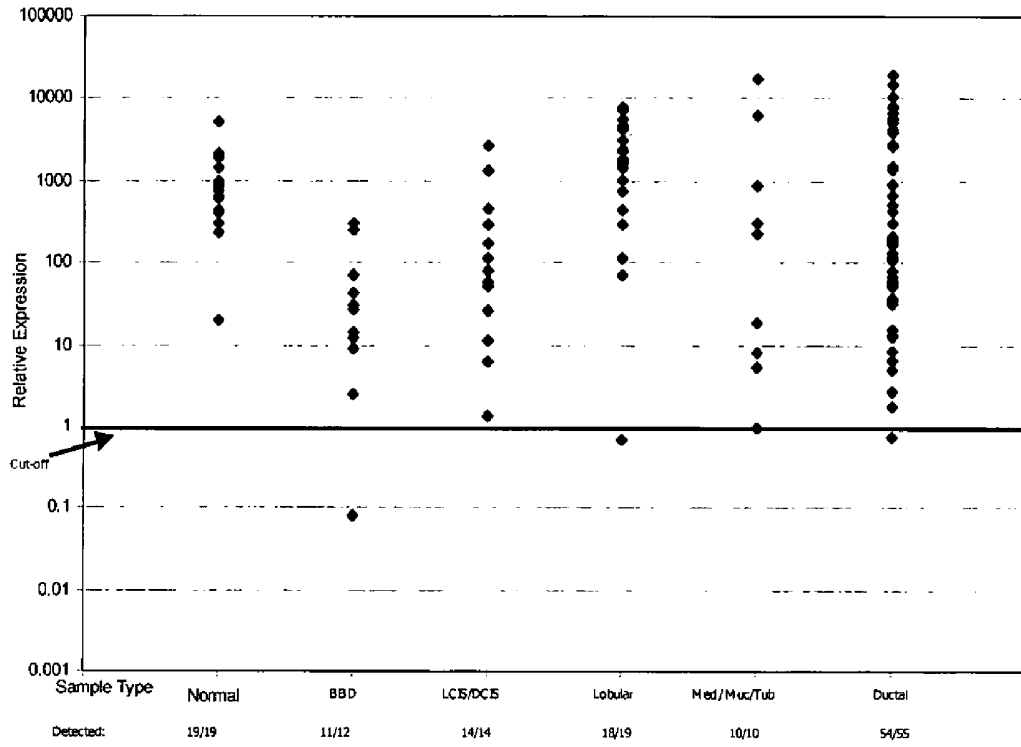
Table 7



5

Table 8

Combined Expression of Mammaglobin & B305D in Different Breast Cell Types



5

Example 5: Rapid RT-PCR from purified RNA

5 Mammaglobin in vitro transcript RNA (100,000 copies) was diluted into 20 ng white blood cell RNA and subjected to one-step RT-PCR amplification in a Cepheid Smart Cycler II in a reaction containing the following: 50 mM Bicine / KOH, pH 8.2, 115 mM Potassium Acetate, 8% v/v Glycerol, 0.2 mg/ml BSA, 150 mM Trehalose, 0.2% v/v Tween 20, 0.2 mM Tris-Cl pH 8, 3.5 mM MnSO₄, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM TTP, 100 U/ml Tth, 20 ug/ml Antibody TP6-25.3, 0.45 uM Mammaglobin Scorpion and 0.5 uM Mammaglobin Primer.

The PCR conditions were as follows (the asterisk denoted the portion of cycling in which the fluorescence was measured:

15

Condition A - 95 C for 3 sec, 60 C for 7 min, 70 C for 2 min, then 40 cycles of: 95 C for 3 sec, 54 C for 6 sec* and 68 C for 6 sec – total time = 34 min

20 Condition B - 95 C for 3 sec, 60 C for 3 min, then 40 cycles of: 95 C for 3 sec, 54 C for 6 sec* and 68 C for 6 sec – total time = 29 min

Condition C - 95 C for 3 sec, 60 C for 3 min, then 40 cycles of: 95 C for 3 sec, 58 C for 6 sec* and 68 C for 6 sec – total time = 26 min

25 The results of this assay are shown in the following table:

Cond	template	mean Ct	Total Assay Time (min)
A	IVT-RNA	30.47	34.00
A	NT	50.00	"
B	IVT-RNA	30.26	29.00
B	NT	50.00	"
C	IVT-RNA	31.15	26.00
C	NT	50.00	"

As can be seen from the data, reducing the length of the reverse transcription step from 9 min to 3 min has no discernable impact on assay performance. Increasing the annealing temperature to 58 C has only a minimal impact of amplification and reduces the total RT-PCR time to 26 minutes. Further reductions in cycling time can be accomplished by primer and probe construction with higher optimal assay temperatures.

Example 6: Rapid multiplex RT-PCR assay for detection of lymph node metastasis (Prophetic)

In this example, purified lymph node RNA (≥ 100 ng total RNA per 100ul reaction utilized to reduce the number of cycles required to generate a positive result) is subjected to one-step RT-PCR amplification in a Cepheid Smart Cycler II. The reaction contains the following components: 50 mM Bicine / KOH, pH 8.2, 115 mM Potassium Acetate, 8% v/v Glycerol, 0.2 mg/ml BSA, 150 mM Trehalose, 0.2% v/v Tween 20, 0.2 mM Tris-Cl pH 8, 3.5 mM MnSO₄ or Mn(acetate), 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM TTP, 100 U/ml Tth, 20 ug/ml Antibody TP6-25.3, 0.4-0.8 uM each Scorpion and primer. Scorpion and primer sequences are designed such that non-specific priming events are minimized and such that they are compatible with multiplexed amplification / detection with the following rapid RT-PCR profile:
95 C for 3 sec, 60 C for 3 min, then 32 cycles of: 95 C for 1 sec, 65 C for 6 sec* and 72 C for 3 sec – total amplification time = 16 min.

The rapid sample preparation method described is utilized that takes ≤ 4 min to complete (refer to sample prep example in application), leading to a total assay time of ≤ 20 min.

For detection of metastasis of breast cancer to lymph nodes, the following genes are amplified: mammaglobin, B305D and PBGD. Mammaglobin and/or B305D signal is indicative of metastasis, while PBGD is utilized as a housekeeping gene. In this example, Ct values for cancer-positive lymph nodes range from 12-30 for mammaglobin and 12-28 for B305D – detection of either gene in this Ct range is indicative of a cancer-positive lymph node. In this example, Ct values for the control gene PBGD must fall in the range of 24-30 cycles in order for a cancer-negative result to be considered valid.

For detection of metastasis of melanoma cancer to lymph nodes, the same conditions are utilized, with the following exceptions: (1) lymph nodes suspected of containing melanoma are processed and (2) the following genes are amplified: MART1, tyrosinase and PBGD.

- 5 MART1 and/or tyrosinase signal is indicative of metastasis of melanoma to the lymph nodes, while PBGD is utilized as a housekeeping gene. In this example, Ct values for cancer-positive lymph nodes range from 18-30 for tyrosinase and 12-27 for MART1 – detection of either gene in this range is indicative of a cancer-positive lymph node. In this example, Ct values for the control gene PBGD must fall in the range of 24-30 cycles in
- 10 order for a cancer-negative result to be considered valid.

The claims defining the invention are as follows:

1. A method of conducting an intraoperative molecular diagnostic assay comprising the steps of: obtaining a lymph node tissue sample from a patient; analyzing the sample by nucleic acid amplification and detection; and determining if the presence of more than one Marker exceeds a cut-off value.
2. The method of claim 1 used for detecting micrometastasis.
3. The method of claim 1 used for detecting breast cancer metastasis.
4. The method of claim 1 used for detecting melanoma metastasis.
5. The method of claim 1 wherein the Markers detect the expression of a gene corresponding to Seq. ID 17 and one or more of the group consisting of Seq ID Nos 14-16 Seq ID No 18.
6. The method of claim 1 wherein all of the steps are conducted during the course of a surgical procedure.
7. The method of claim 1 wherein nucleic acid amplification and detection is conducted by PCR.
8. The method of claim 7 used for detecting micrometastasis.
9. The method of claim 7 used for detecting breast cancer metastasis.
10. The method of claim 7 used for detecting melanoma metastasis.
11. The method of claim 10 wherein the Markers are Seq ID No 21 and Seq ID No 22.
12. The method of claim 7 wherein said PCR is RT-PCR.
13. The method of claim 12 wherein the reverse transcription reaction is conducted for less than 9 minutes.
14. The method of claim 13 wherein the reverse transcription reaction is conducted for about 3 minutes.
15. The method of claim 12, wherein one or more internal control reagents is added to the reverse transcription reaction.

16. The method of claim 1 wherein RNA is extracted from the lymph node by:
- (a) lysing the cells containing RNA that is of interest and producing a homogenate of them,
 - (b) optionally, diluting the homogenate,
 - 5 (c) contacting the wetted, homogenized working sample with a substrate containing, or to which is affixed, a material to which RNA binds,
 - (d) allowing the sample to bind to the substrate,
 - (e) removing contaminants and interferents,
 - (f) drying the substrate, and
 - 10 (g) eluting RNA from the substrate;
- and employing filtration in extraction steps.

17. A kit for conducting an intraoperative lymph node assay comprising: nucleic acid amplification and detection reagents.

- 15 18. The kit of claim 17 wherein said reagents comprise primers having sequences for detecting the presence of a group of Markers selected from the group consisting of Seq. ID No. 14-18 and Seq ID No 21-22.

19 The kit of claim 18 wherein the primers are selected from the group consisting of Seq ID Nos 1-6, 8-12, 25-28, 30, and 33-35.

- 20 20. The of claim 17 comprising RT-PCR reagents.

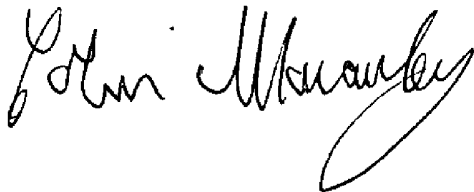
21. A method comprising conducting an intraoperative molecular diagnostic assay and treating a patient in accordance with the outcome of such assay wherein the assay comprises the steps of: obtaining a lymph node tissue sample from the patient; analyzing the sample by nucleic acid amplification and detection; and determining if the presence of
25 more than one Marker exceeds a cut-off value.

Dated this 30th day of April, 2004

VERIDEX, LLC

By Their Patent Attorneys

- 30 CALLINAN LAWRIE



SEQUENCE LISTING

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Belly, Robert
Rosen, Steven
White, Robert

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