veracyte

Prosigna®
Breast Cancer Assay

Package Insert
Prosigna® Breast Cancer Prognostic Gene Signature Assay

IVD CE Version 02, created 2023-07

1–10 tests

Storage conditions

-20 ° C Store at -20° C or below	Prosigna Breast Cancer Prognostic Gene Signature Assay Cartridges
-80 ° C Store at -80° C or below	Prosigna Breast Cancer Prognostic Gene Signature Assay CodeSet
+15° C +25° C Store at room temperature	Prosigna Breast Cancer Prognostic Gene Signature Assay Prep Pack
+2° C +8° C Store at +4° C	Prosigna Breast Cancer Prognostic Gene Signature Assay Prep Plates

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1 INTENDED USE / PURPOSE

The Prosigna® Breast Cancer Prognostic Gene Signature Assay is an *in vitro* diagnostic assay which uses the gene expression profile of cells found in breast cancer tissue to assess a patient's risk of distant recurrence. The assay measures the gene expression profile using RNA extracted from Formalin-Fixed, Paraffin-Embedded (FFPE) breast tumor tissue. The gene expression data are weighted together with clinical variables to generate both a subtype (luminal A, luminal B, HER2-enriched, or basal-like) and a score indicative of the probability of distant recurrence of disease. The assay is performed on the nCounter® Analysis System using FFPE breast tumor tissue previously diagnosed as invasive breast carcinoma.

The Prosigna Breast Cancer Prognostic Gene Signature Assay is indicated in female breast cancer patients who have undergone either mastectomy or breast-conserving therapy in conjunction with locoregional treatment consistent with standard of care, either as:

a. A prognostic indicator for distant recurrence-free survival at 10 years in post-menopausal women with Hormone Receptor-Positive (HR+), lymph node-negative, Stage I or II breast cancer to be treated with adjuvant endocrine therapy alone, when used in conjunction with other clinicopathological factors.
b. A prognostic indicator for distant recurrence-free survival at 10 years in post-menopausal women with Hormone Receptor-Positive (HR+), lymph node-positive (1–3 positive nodes, or 4 or more positive nodes), Stage II or IIIA breast cancer to be treated with adjuvant endocrine therapy alone, when used in conjunction with other clinicopathological factors.

2 SUMMARY OF THE TEST SYSTEM

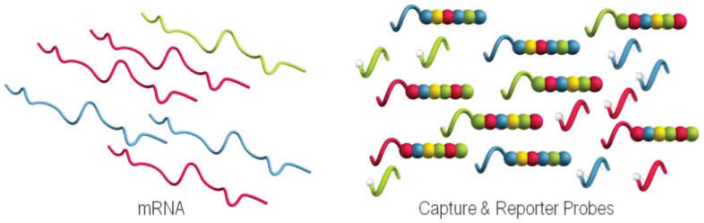
The nCounter Analysis System delivers direct, multiplexed measurements of gene expression through digital readouts of the relative abundance of mRNA transcripts using the following steps: 1) hybridization of the RNA to fluorescent Reporter Probes and Capture Probes, 2) purification of the target/probe complexes using nCounter Prep Plates containing reagents necessary for post-hybridization processing and immobilization onto the nCounter Cartridge on the nCounter Prep Station, and 3) analysis of the nCounter Cartridge on the nCounter Digital Analyzer to provide a test result[1]. Both the Capture and Reporter Probes contain unique DNA probe sequences for target hybridization and purification. The Capture and Reporter Probes are combined with the positive and negative controls to form the CodeSet. Prosigna simultaneously measures the expression levels of 50 genes used for the intrinsic subtype classification algorithm[2], 8 housekeeping genes used for signal normalization, 6 positive controls, and 8 negative controls in a single hybridization reaction using nucleic acid probes designed specifically to those genes. Also included in the Prosigna kit is a Reference Sample consisting of *in vitro* transcribed RNA targets for each of the 58 genes. The Reference Sample is tested with each batch of patient RNA samples to qualify the run and normalize the signal from each gene.

The Prosigna Assay is performed on RNA isolated from FFPE breast tumor tissue. A pathologist examines a hematoxylin and eosin (H&E) stained slide and identifies (and marks) the area of invasive breast carcinoma suitable for the test. The pathologist also measures the tumor surface area, which determines the number of unstained slides required for the test, and the tumor cellularity to ensure the presence of sufficient tumor tissue for the test. A trained technologist macrodissects the area on the unstained slides corresponding to the marked tumor area on the H&E stained slide and isolates RNA from the tissue. The isolated RNA is then tested on the nCounter Analysis System to provide test results, including the intrinsic subtype, Risk of Recurrence (ROR) score, and risk category.

2.1 Principles of the nCounter Analysis System

The nCounter Analysis System uses gene-specific probe pairs (Figure 1) that hybridize directly to the mRNA sample in solution, eliminating enzymatic reactions that might introduce bias in the results. In the first step of the assay the DNA probes are hybridized directly to a 70–100 base pair region of the RNA sample in solution. The fluorescent Reporter Probe consists of a 35–50 base probe sequence that is complementary to the mRNA target and a unique backbone DNA sequence that hybridizes to six RNA segments labeled with one of four fluorescent dyes; red (R), yellow (Y), blue (B), or green (G). The fluorescent segments create a six-position/four-color fluorescent "color code" that is unique to each target. A separate Capture Probe consists of a 35–50 base probe sequence that is complementary to the mRNA target and biotin, which is used for immobilization onto a streptavidin-coated slide.

Figure 1: Hybridize CodeSet to mRNA



After hybridization, all of the sample purification steps are automated on the nCounter Prep Station. First, excess Capture and Reporter Probes are removed (Figure 2) using successive magnetic bead capture steps followed by binding of the probe-target complexes to random locations on the surface of the nCounter Cartridge via a streptavidin-biotin linkage (Figure 3). Finally, probe/target complexes are aligned and immobilized (Figure 4) in the nCounter Cartridge.

Figure 2: Remove Excess Reporters

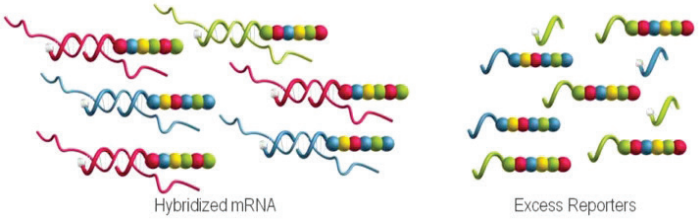


Figure 3: Bind Hybridized Reporters to Surface of Cartridge

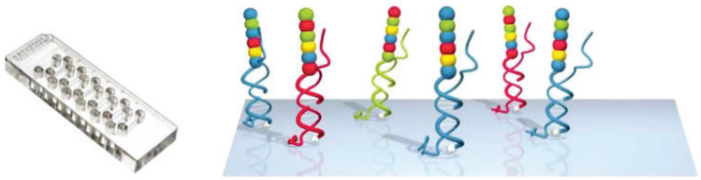
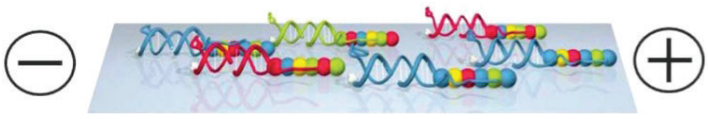


Figure 4: Align and Immobilize Hybridized Reporters



After sample processing has completed, the Cartridge is placed in the nCounter Digital Analyzer for data collection. Each target molecule of interest is identified by the "color code" generated by six ordered fluorescent spots present on its associated Reporter Probe. The Reporter Probes on the surface of the Cartridge are then counted and tabulated for each target molecule and processed with the algorithm (Figure 5).

Figure 5: Data Collection

Code	Gene	Count
	x	3
	y	1
	z	2

2.2 Principles of the Prosigna algorithm for output calculation

The test is based on the reported 50-gene classifier algorithm originally named PAM50[2] and is performed on the nCounter Analysis system using RNA extracted from formalin fixed, paraffin embedded (FFPE) breast tumor tissue samples. The algorithm uses a 50-gene expression profile to assign breast cancer to one of four molecular classes, or intrinsic subtypes: Luminal A, Luminal B, HER2-enriched, or Basal-like[2]. The prototypical gene expression profiles (e.g., centroid) of the four intrinsic subtypes were retrained on the nCounter Analysis System using FFPE breast tumor samples collected from multiple clinical sites in North America. After performing the assay on a patient test sample, a computational algorithm based on a Pearson's correlation compares the normalized 50-gene expression profile of the patient test sample to the prototypical expression profiles of the four breast cancer intrinsic subtypes. The patient test sample is assigned the subtype with the highest Pearson's correlation.

The algorithm further reports a Risk of Recurrence (ROR) score on a 0–100 scale[3], which is correlated with the probability of distant recurrence at ten years for post-menopausal women with hormone receptor-positive, early stage breast cancer[4]. The report also provides a risk category (low, intermediate, or high). The ROR score is calculated using coefficients from a Cox model that includes the Pearson correlation of a 46-gene subset of the 50 genes to each intrinsic subtype centroid, a proliferation score, and gross tumor size. The test variables are multiplied by the corresponding coefficients from the Cox model to generate the score, which is then adjusted to a 0–100 scale based on coefficients generated from the training set of FFPE breast tumor samples. Risk categories are also reported based on cut-offs for ROR determined in a clinical validation study.

3 REAGENTS AND EQUIPMENT PROVIDED

3.1 Prosigna Kit Overview

The Prosigna kit contains reagents sufficient to process 1, 2, 3, 4, or 10 patient samples depending on the product ordered. See below for ordering information. The Prosigna kit contains a CodeSet, one Reference Sample tube for every set of one to ten tests, and consumable components, which are tested together for performance prior to release.

Catalogue Number	Number of tests in Kit	Reference Sample Tubes included
PROSIGNA-001	1	2
PROSIGNA-002	2	2
PROSIGNA-003	3	2
PROSIGNA-004	4	2
PROSIGNA-010	10	2

Recommended for use in conjunction with the Roche FFPET RNA Isolation Kit (Roche-FFPET-025) or the Veracyte FFPE RNA extraction kit (550100). Both RNA extraction kits are available only through Veracyte.

3.2 Prosigna Kit Contents for a 1, 2, 3, 4, or 10 test Prosigna Kit

Number of Tests	1	2	3	4	10
Prosigna CodeSet Box					
Prosigna Reporter CodeSet	1 x 65 µL	1 x 65 µL	1 x 65 µL	1 x 65 µL	1 x 65 µL
Prosigna Capture ProbeSet	1 x 70 µL	1 x 70 µL	1 x 70 µL	1 x 70 µL	1 x 70 µL
Prosigna RNA Reference Sample	1 x 30 µL	1 x 30 µL	1 x 30 µL	1 x 30 µL	1 x 30 µL
CodeSet Barcode Sticker	1	1	1	1	1
Test Configuration Code	1	1	1	1	1
Prosigna Prep Plate Box					
Prep Plates	1	1	1	1	2
Prosigna Cartridge Box					
nCounter Cartridges	1	1	1	1	1
Prosigna Prep Pack Box					
nCounter Prep Station Tips	1	1	1	1	1
nCounter Cartridge Adhesive Cover	2	2	2	2	2
nCounter Tip Sheaths	2	2	2	2	2
nCounter Hybridization Buffer	1 x 580 µL	1 x 580 µL	1 x 580 µL	1 x 580 µL	1 x 580 µL
12-Well Notched Strip Tubes	4	4	4	4	4
12-Well Notched Strip Tube Lids	4	4	4	4	4

Contents Description

Prosigna CodeSet

Prosigna Reporter CodeSet	*buffer, nucleic acids with fluorescent dyes*
Prosigna Capture ProbeSet	*buffer, nucleic acids*
Prosigna RNA Reference Sample	*buffer, nucleic acids*
CodeSet Barcode Sticker	*sticker sheet*
Test Configuration Code	*card with sticker*

Prosigna Prep Plates

Prep Plates	*superparamagnetic beads, buffer, salts, oligonucleotides, polystyrene beads containing fluorescent dyes*

Prosigna Cartridges

nCounter Cartridge(s)	*sample cartridge(s)*

Prosigna Prep Pack

nCounter Hybridization Buffer	*buffer, salts*
12-Well Notched Strip Tubes	*plastic strips*
12-Well Notched Strip Tube Lids	*plastic lids*
nCounter Prep Station Tips	*2 racks of 90 tips + 6 piercers nCounter*
nCounter Cartridge Adhesive Cover	*adhesive films*
nCounter Tip Sheaths	*6-well tip holders*

4 WARNINGS AND PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. This assay is intended to be run by operators trained in highly complex molecular biology techniques, based on local regulations.
3. Do not mix components of kits across Prosigna lots. Functionality can only be assured for Prosigna kit lots as provided, as they are qualified in this manner during manufacture.
4. Any remnant reagents should not be reused in the Prosigna Assay.
5. Discard any reactions with compromised hybridization times or temperatures.
6. It is important to maintain the integrity of the sample chain of custody (tissue to RNA and RNA to assay) to ensure the patient sample ID is associated with the correct test result.
7. Failure to store reagents under the conditions stated on the label could adversely affect assay performance.
8. Always wear gloves while handling reagents and samples.
9. Avoid RNase contamination, which may negatively affect the quality of the results.
10. All biological specimens and materials should be handled as if the potential exists for transmitting infectious agents and should be disposed of with proper precautions in accordance with federal, state, and local regulations.
11. Never pipet by mouth.
12. Avoid reagent contact with eyes, skin and mucous membranes.
13. Use molecular laboratory best practices to prevent cross-contamination between test samples or with high concentration nucleic acid targets (synthetic or PCR-amplified) which may negatively affect results quality.
14. Very low levels of sodium azide (< 0.1%) are contained post-process within the Prosigna Prep Plates and nCounter Cartridges, therefore it is recommended that plastic (not metal) waste receptacles are used for disposal. While extremely unlikely for Prosigna, sodium azide accumulation on metal is known to create an explosive hazard.
15. Additional instrument-specific disposal information can be found in the nCounter Analysis System User Manual and Service Manuals for the Prep Station and Digital Analyzer.
16. Material Safety Data Sheet information for Reporter CodeSet, Capture ProbeSet, Hybridization Buffer, and Prep Plates can be found at www.prosigna.com.
17. All hazardous materials should be disposed of according to your institution's guidelines for hazardous disposal.
18. Any unused CodeSet should be discarded.
19. If a patient's tumor size category is entered into the software incorrectly, the ROR score and risk classification may be adversely affected (e.g., shifted ROR Score and/or mis-classification).
20. If a patient's nodal status is entered into the software incorrectly, the patient's test results may be reported incorrectly (e.g., incorrect risk classification).
21. Do not use RNA of insufficient quality or quantity or tumor samples with insufficient tumor surface area or cellularity in the Prosigna Assay. The Prosigna Assay may be unable to give a valid result and instead will report as assay failure.

5 GENERAL ASSAY CONSIDERATIONS

1. The assay is intended for use only on formalin-fixed, paraffin embedded (FFPE) breast cancer tissue specimens from surgical resection; it is not intended for use on fresh, frozen or non-breast cancer tissue.
2. The gross size of a patient's primary tumor and nodal status are required to perform the assay.
3. Use sterile disposable micropipette tips to avoid microbial and nuclease contamination of reagents or samples during processing.
4. Maintain the isolated RNA samples on wet ice when not actively being manipulated.
5. Calibrated thermometers are required for heat blocks.
6. Do not use kit components in the event that they arrive damaged.
7. It is recommended that clinical controls (e.g., for risk category) are developed and utilized by labs running Prosigna in order to ensure accuracy of results over time as part of standard laboratory quality control procedures.

5.1 Tissue Processing

1. Failure to properly remove surrounding non-tumor/normal tissue by macrodissection during tissue processing could result in an underestimate of risk due to a lower ROR score reported to the physician.
2. Failure to properly remove human genomic DNA during RNA isolation could result in a higher failure rate due to lower assay signal, or an overestimate of risk due to a higher ROR score reported to the physician.
3. All unstained tissue sections should be mounted onto positively-charged microscope slides to avoid detachment during tissue processing.
4. For specimens that require multiple slides, all slides must be processed together.
5. Slide mounted tissue sections may degrade if stored for longer than 9 months in a desiccated environment.
6. Replace 3% glycerol working solution each week, or if solution becomes turbid, to avoid contamination.
7. Change the first D-Limonene wash contents after processing 4 slide sets, and the Ethanol (EtOH) and second D-Limonene staining dish contents after processing 8 slide sets to avoid compromising tissue quality.
8. Use caution when outlining the tumor area on the unstained slide and removing non-tumor tissue to ensure the tumor tissue is not disturbed.

9. Handle sharps with care during macrodissection.
10. Use a fresh razor blade for each tissue sample processed.
11. 10% SDS frequently precipitates at room temperature and should be warmed at 37°C until precipitates have dissolved.*
12. New lots/batches of RNA isolation kits should be tested against the isolation kit specifications to qualify the new kit lots for patient testing (see section 11.5 for details).

*10% SDS required but not provided for the Roche FFPET RNA Isolation kit.

5.2 Performing the Prosigna Assay

1. Ensure the patient's categorical primary gross tumor size is entered correctly into the software.
2. Ensure the patient's categorical nodal status is entered correctly into the software.
3. Verify that the heat block with heated lid required for hybridization meets the specifications and is routinely calibrated.
4. Use only those consumables that were provided with the Prosigna kit. They are designed specifically to work with the nCounter Prep Station and nCounter Digital Analyzer.
5. If Hybridization Buffer has been stored at cold temperatures and a precipitate is observed, warm tubes at 37°C until salts have dissolved.
6. Do not vortex assay components vigorously to mix, as it may damage the reagents. Mixing should be performed using a pipette.
7. Do not centrifuge Reporter CodeSet faster than 3,000 × *g* for more than 10 seconds. Do not use the "pulse" option to centrifuge. Doing so may precipitate the CodeSet.
8. Maintain hybridization reactions at 65°C until they are ready to be transferred to the Prep Station. Setting the heat block to ramp down to 4°C or placing samples on ice at the end of the hybridization could result in cross-hybridization, which may compromise assay results.
9. Failure to place the strip tubes at 65°C within 15 minutes of adding the Capture ProbeSet could result in cross-hybridization, which may compromise assay results.
10. Failure to initiate processing of the Prep Station within 15 minutes of removing the samples from 65°C could result in cross-hybridization, which may compromise assay results.
11. Ensure the strip tube caps are firmly sealed prior to hybridization in the heat block to prevent evaporation, which may compromise assay results.

6 TRAINING INFORMATION

This assay is intended to be run by professional operators trained in highly complex molecular biology techniques, based on local regulations. Please contact Veracyte for training information specific to running the Prosigna Assay.

7 WASTE HANDLING

See the nCounter Analysis System User Manual for waste handling details specific to the reagents and instruments for use in IVD applications.

See the chosen RNA extraction kit Instructions for use for waste handling and details specific to the RNA extraction reagents.

8 STORAGE AND HANDLING (REAGENTS)

The expiration date for all assay kit components is listed on the barcode label supplied with the CodeSet box as well as on the outer box labeling for all Prosigna components.

- The Prosigna CodeSet box components (Prosigna Reporter CodeSet, Prosigna Capture ProbeSet, and Prosigna RNA Reference Sample) must be stored at -80°C or below.
- The nCounter Cartridges must be stored at or below -20°C.
- The nCounter Prep Plates must be stored at 4°C (2–8°C).
- The nCounter Prep Pack components must be stored at room temperature 15°C–25°C.

9 INSTRUMENTS REQUIRED FOR PROSIGNA

- nCounter Analysis System (Catalog number NCT-SYST-DX) (includes both instruments below)
    - o nCounter Prep Station 5s (Catalog number NCT-PREP-STATION-FLEX)
    - o nCounter Digital Analyzer 5s (Catalog number NCT-DIGITAL-ANALYZER-FLEX)

Refer to the nCounter Analysis System User Manual for additional information.

10 REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

10.1 Materials

1. FFPE RNA Isolation kit (see Section 11.5 for isolation kit requirements if not using Roche FFPET RNA isolation kit or Veracyte FFPE RNA Extraction kit purchased through Veracyte)
2. Hematoxylin and Eosin (H&E)
3. Positively charged glass microscope slides
4. D-Limonene clearing agent (histology grade)
5. 100% Ethanol (Absolute), ACS grade or equivalent (not less than 99.5%)
6. Glycerol, molecular biology grade
7. Nuclease-free water, molecular biology grade[1-2]
8. 10% SDS, molecular biology grade[1]
9. 100% isopropanol[2]
10. Conical tube 50ml[2]
11. Razor Blades (or disposable scalpels)
12. Disposable Microtome Blades
13. 0.5 mL screw-cap[1] and 1.5 or 1.7 mL nonstick RNase-free microcentrifuge tubes
14. RNase-free Micropipette tips with aerosol barrier

[1] Materials required but not provided for RNA extraction using the Roche FFPET RNA Isolation Kit.
[2] Materials required but not provided for RNA extraction using the Veracyte FFPE RNA Extraction kit and also listed in the Instructions For Use of the Veracyte kit.

10.2 Equipment

1. Microtome
2. Water Bath (40°C)
3. Slide Warmer (45°C)
4. Microscope slide drying rack
5. Micropipettes; 2 µL, 20 µL, 200 µL and 1000 µL
6. Mini-centrifuge with a 0.2 mL strip tube rotor and standard 1.5/2.0 mL micorcentrifuge tube rotor
7. Standard bench top micro-centrifuge with a fixed angle rotor that fits 1.5 mL centrifuge tubes
8. Rectangular glass staining dishes with covers (approximate interior dimensions of 3.6 × 2.8 × 2.4" (91 × 71 × 60 mm); quantity of 3 required
9. Slide rack (holds up to ten 3" × 1" (75 × 25 mm) glass slides)
10. Dry heat block, stationary*
11. Bench top vortexer for microcentrifuge tubes
12. Graduated cylinder (suggested size: 100–250 mL)
13. Dissecting Needle or cover glass forceps (angled, non-serrated)
14. Calibrated thermometers (covering the 55°C through 80°C range)
15. Micro-volume UV/Vis spectrophotometer (see specifications below)
16. Heat block with heated lid (see specifications below)
17. Centrifuge with plate microplate adapter (see specifications below)
18. Coplin jar

*Equipment required for RNA extraction with Roche FFPET RNA Isolation kit and Veracyte FFPE RNA Extraction kit.

10.3 Equipment Specifications

Table 1: Full Spectrum Micro-volume UV/Vis spectrophotometer for Nucleic Acid Quantitation

Design Feature	Specifications
Sample Volume Range	1–2 µL
Path Length	1 mm
Wavelength Range	260–280 nm
Wavelength Accuracy or Error	± 1 nm
Spectral Resolution or bandwidth	Less than or equal to 4 nm
Absorbance Precision or random photometric error	0.003 (1 mm path)
Detection Limit	5 ng/µL RNA
Maximum Concentration	≥ 1000 ng/µL RNA

Table 2: Microvolume Photodiode UV-Vis Spectrophotometer for Nucleic Acid Quantitation

Design Feature	Specifications
Sample Volume Range	1–2 µL
Path Length	0.5 mm
Wavelength Range	260 and 280 nm
Spectral Resolution	Less than or equal to 8 nm
Absorbance Accuracy	3% (at 1.05 Abs at 260 nm)
Detection Limit	4 ng/µL RNA
Maximum Concentration	≥ 1000 ng/µL RNA

Table 3: Heat Block with Heated Lid for Assay Hybridization

Design Feature	Specifications
Heat Block Design	• Must fit regular profile, 12 × 0.2 mL keyed strip tubes that are provided as part of the nCounter Prep Pack. ° Heat Blocks designed for Low Profile (LP) and High Profile (HP) tubes are not compatible (also referred to as "rapid" blocks for thermocycling) ° Heat Blocks designed for other types of tubes (e.g., 0.1 mL tubes, 1.5 mL tubes) are not compatible • Must be programmable to hold at a temperature of 65°C • Must hold temperature within ± 1°C of 65°C
Heated Lid Design	• Fixed or adjustable height lids are acceptable • Lid must be programmable to 70°C

Table 4: Centrifuge with microplate carrier to spin nCounter Prep Plates

Design Feature	Specifications
Centrifugation Speed	Minimum of 2000 × *g*
Rotors	4 × 750 mL swinging bucket rotors with microplate carriers (or equivalent) to accommodate SBS-format 96-well microplates
Modes	Acceleration/Deceleration modes

11 SPECIMEN COLLECTION AND PROCESSING

11.1 Tissue Specimen Requirements and Pathology Review

1. The Prosigna Breast Cancer Prognostic Gene Signature Assay should be performed on a formalin-fixed, paraffin-embedded (FFPE) hormone-receptor positive breast tumor tissue specimen that is further specified by a pathologist as one of the following types of invasive breast carcinoma:
    a. Invasive ductal carcinoma
    b. Invasive lobular carcinoma
    c. Invasive carcinoma with ductal and lobular features ("mixed type carcinoma")
    d. No special type (NST) or not otherwise specified (NOS)
2. A pathologist should select the FFPE tumor block with the greatest area of viable invasive breast carcinoma for this test.
3. The test requires unstained slide mounted tissue sections for processing and a corresponding H&E stained slide from the FFPE tumor block.
4. It is recommended that tissue sections for assay processing are cut contiguous to the tissue section cut for H&E staining to ensure that the tumor area identified on the H&E stained slide is representative of the tumor area on the unstained slides.
5. A pathologist must circle the region of viable invasive breast carcinoma on the H&E slide, excluding surrounding non-tumor tissue.
6. A pathologist or trained laboratory technician must estimate the tumor cellularity and tumor surface area within the circled area of the H&E stained slide.
    a. The tumor cellularity percentage on the H&E stained slide must be $\geq$ 10%
    b. The circled tumor surface area on the H&E stained slide must be $\geq$ 4 mm^2
    *Note that tumor cellularity percentage refers to the percentage of viable tumor cells within the circled tumor area.
7. A total tumor surface area of greater than 100 mm^2 is recommended as an input for the test. The following table illustrates the number of slides recommended based on the measured tumor surface area on the H&E stained slide.
8. If the tissue review process shows that the tumor block has insufficient tumor area or insufficient tumor cellularity, then a different block from the same tumor may be assessed. If there are no FFPE blocks which contain sufficient tumor tissue, then the Prosigna Assay should not be run. Please note that for tumors with less than 20 mm^2 surface area, it is more likely that RNA input requirements will not be met.

Table 5: Recommended slide requirements based on tumor surface area

Measured Tumor Surface Area on H&E Stained Slide (mm^2)	Number of unstained slides
4–19	6
20–99	3
$\geq$ 100	1

11.2 Specimen Collection and Storage

1. The following may be performed according to the laboratory's standard operating procedures: tissue collection and formalin fixation, FFPE tumor block handling and storage, and shipping of slide mounted FFPE tissue.
2. Slide mounted FFPE tissue sections must be stored according to the laboratory's standard operating procedures. If storing for longer periods of time (> 30 days), the slides must be stored in a desiccated environment and processed within 9 months to ensure quality of the test results.

11.3 Slide Preparation

1. Using a microtome, cut a 4–5 µm thick section for H&E staining.
2. Using a microtome, cut 10 µm thick sections for use in the Prosigna Assay.
3. Float the sections in a water bath at 40°C.
4. Mount the sections onto positively charged glass microscope slides.
5. Allow slides to air dry.
6. Bake the slides overnight at 45°C.

11.4 Slide Processing

1. Prepare a 3% glycerol working solution by mixing 1.5 mL of glycerol stock with 48.5 mL of molecular grade, nuclease-free water; scale as appropriate. Pour the solution into a Coplin jar for processing slides.
2. Pour approximately 200–250 mL of D-Limonene clearing agent into two staining dishes, ensuring the slides in the slide rack will be completely submerged.
3. Pour approximately 200–250 mL absolute Ethanol (EtOH) into a third staining dish.
4. Place the unstained slide-mounted tissue section(s) into a slide rack.
5. Place the slide rack into the first D-Limonene staining dish and gently agitate the slide rack back and forth for 10–15 seconds. Leave the rack in the first D-Limonene staining dish for a total time of 2 minutes.
6. Move the slide rack from the first D-Limonene staining dish into the second D-Limonene dish. Gently agitate the slide rack back and forth for 10–15 seconds. Leave the slide rack in the second D-Limonene staining dish for a total time of 2 minutes. Be sure all paraffin is removed; otherwise, leave the rack in the second D-Limonene staining dish for about 1 minute longer.
7. Move the slide rack from the second D-Limonene staining dish to the EtOH wash. Gently agitate the slide rack back and forth for 10–15 seconds and remove after 2 minutes.
8. Let slides air dry for 5–10 minutes or until completely dry, and the tissue appears white (this may take longer depending on the size of the tissue).
9. Outline the tumor area on the backside of the unstained slide(s) by aligning it with the corresponding H&E stained slide and transposing the outlined area.
10. Working with one slide at a time, rehydrate tissue on the outlined unstained slide by dipping the slide in the 3% glycerol solution.
11. Remove any excess glycerol from the slide with a lab tissue.
12. When processing multiple slides, the user may allow the slides to dry on a drying rack while rehydrating the other slides.
13. Scrape away any non-tumor tissue surrounding the outlined tumor area with a razor blade or scalpel and discard.
14. Holding one end of the slide and resting the other end on a solid surface at a 45 degree angle, collect the macro-dissected tumor tissue onto the edge of a razor blade. The tissue should easily "curl" onto the razor blade as it is being collected.
15. Repeat previous step for each slide from the same specimen.
    Note: Multiple unstained slides from a single FFPE specimen can be collected onto the same razor blade.

16. Gently slide the tissue sections from the same specimen into a labeled 1.5 mL microcentrifuge tube.
17. If used, clean the dissecting needle or forceps by dipping in D-Limonene for a few seconds and drying between tissue samples.

11.5 Isolation of RNA

Veracyte recommends use of the Roche FFPET RNA isolation kit or the Veracyte FFPE RNA Extraction kit, which have been validated specifically for use with Prosigna.

Other RNA isolation kits may be used to prepare samples for Prosigna if they yield RNA from slide mounted FFPE breast tumor tissue sections which meets the following specifications:

Table 6: RNA Isolation Kit specifications

Metric	Test or Measurement	Specification
RNA concentration	Optical Density at 260 nm	≥ 12.5 ng/µL
RNA total volume (µL)	Total eluted volume	≥ 12 µL
RNA purity	Ratio of optical density at 260 nm to optical density at 280 nm (OD 260/280 nm)	1.7–2.3
DNA Contamination	Genomic DNA content of eluted RNA sample	≤ 1 ng/µL
RNA Integrity	Size distribution of the isolated RNA fragments	≥ 90% of the isolated RNA fragments must be > 100 nucleotides in length

Caution: If an alternative isolation procedure is used in combination with Prosigna assay, this particular workflow must be fully validated by the laboratory before being implemented in routine.

RNA Isolation Procedure:

- If the Roche FFPET RNA Isolation Kit is used, follow the specific instructions for isolation of RNA described below.
- If the Veracyte FFPE RNA Extraction kit is used, follow the instructions for use as provided by Veracyte and then continue to section 11.6.
- If an alternative extraction method is used, follow the validated protocol or protocol given by the manufacturer, and then continue to section 11.6.

Each RNA extraction Kit lot manufactured by Roche or by Veracyte is qualified to produce RNA samples meeting pre-defined specifications for diagnostic gene expression assays. Please reference the chosen RNA extraction Kit Method Sheet / Instructions For Use for appropriate storage, safety and handling instructions.

Preparation of Working Solutions for Roche FFPET RNA Isolation kit

1. Prepare working solutions before proceeding with tissue digests and RNA isolation:

Preparation of Working Solutions	
Reagent	**Procedure**
Wash Buffer I (WB1)*	Add 15 mL 100% Ethanol, store prepared WBI at +15 to +25°C.
Wash Buffer II (WB2)*	Add 80 mL 100% Ethanol, store prepared WBII at +15 to+25°C.
Proteinase K (PK)	Dissolve the lyophilized Proteinase K in 4.5 mL Reagent Preparation Buffer (RPB). Prepare 600 µL aliquots, label and store aliquots at -15 to -25°C.
DNase I (DNase I)*	Dissolve the lyophilized DNase I in 740 µL of Reagent Preparation Buffer (RPB). Prepare 50 µL aliquots, label and store aliquots at -15 to -25°C.

Note: Items marked with an asterisk (*) are not required until after tissue has been digested.

Tissue Digestion for Roche FFPET RNA Isolation kit

1. Thaw a sufficient number of Proteinase K (PK) aliquots. Once thawed, store the aliquots on ice until ready to use.
   Note: One 600 µL PK aliquot contains enough Proteinase K for 4 tissue digests.
2. Add 100 µL of Tissue Lysis Buffer (TLB), 16 µL of 10% SDS, and 120 µL of Proteinase K working solution to sample tubes with tissue.
3. Vortex sample tubes with tissue for several seconds and spin down briefly.
4. Incubate at 55°C overnight (12 hours – 23 hours).

RNA Isolation for Roche FFPET RNA Isolation kit

1. Visually inspect the sample tubes to determine if the tissue is completely digested. A complete digest will appear clear and have little to no tissue in solution.
   a. If digestion is complete, proceed to next step.
   b. If digest is incomplete, add 20 µL of additional Proteinase K and incubate for an additional hour.
2. Briefly spin down all samples (< 30 seconds in a mini centrifuge).
3. Incubate the samples at 80°C for 15 minutes.
4. Prepare or thaw a sufficient number of DNase aliquots. Once thawed, store the aliquots on ice until ready to use.
   Note: One 50 µL DNase aliquot contains enough DNase for 4 RNA isolations.
5. Dilute the DNase in DNase Incubation Buffer (DIB). The required amount for N (number of) RNA isolations is: (N+1) × 90 µL DNase Incubation Buffer + (N+1) × 10 µL DNase. Store the diluted DNase solution on ice until ready to use.
6. Add 325 µL Paraffin Binding Buffer (PBB) and 325 µL absolute Ethanol to each sample and mix by pipetting.
7. Spin down briefly (< 30 seconds in a mini centrifuge).
8. Place a High Pure Filter Tube onto a High Pure Collection Tube.
9. Pipette the sample into the upper reservoir of the filter tube, avoiding any residual tissue.
10. Centrifuge for 30 seconds at 6,000 × *g*.
11. Place High Pure Filter Tube onto a new High Pure Collection tube (discard former collection tube containing the flow-through).
12. Centrifuge for 2 minutes at 16,000 × *g* to completely dry the filter fleece.
13. Place High Pure Filter Tube onto new High Pure Collection Tube (discard former collection tube containing the flow-through).
14. Add 100 µL diluted DNase solution onto the High Pure Filter Tube filter fleece and incubate for 15 minutes at +15 to +25°C.
15. Add 500 µL Wash Buffer I working solution (WB1) to High Pure filter fleece, centrifuge for 20–30 seconds at 6,000 × *g*, discard flow through.
16. Add 500 µL Wash Buffer II working solution (WB2) to High Pure filter fleece, centrifuge for 20–30 seconds at 6,000 × *g*, discard flow through.
17. Add 500 µL Wash Buffer II working solution (WB2) to High Pure filter fleece, centrifuge for 20–30 seconds at 6,000 × *g*, discard flow through.
18. Centrifuge for 2 minutes at 16,000 × *g* to dry filter fleece completely.
19. Place the High Pure Filter Tube into a labeled RNase-free 1.5 or 1.7 mL microcentrifuge tube.
20. Add 30 µL Elution Buffer (EB) to the center of the High Pure filter fleece.
21. Incubate for 1 minute at +15 to +25°C.
22. Centrifuge for 1 minute at 6,000 × *g* to elute RNA from column. Remove and discard the High Pure filter tube.
23. Centrifuge the eluted RNA in the microcentrifuge tube for 2 minutes at maximum speed.
24. Transfer the supernatant to a 0.5 mL screw-cap tube without disturbing any glass fibers that might have washed off of the filter fleece at the bottom of the original tube.

11.6 Measure the RNA Concentration and Quality

1. Measure the concentration of the isolated RNA within the same working day (store at +2 to +8°C) or freeze at -70°C or below until use.
2. Measure the optical density (OD) at 260 and 280 nm of 2 µL of the isolated RNA using a spectrophotometer that meets the defined specifications indicated in 10.3 Equipment Specifications. Avoid pipetting the 2 µL volume from the bottom of the source tube in case any glass fibers remain, which will interfere with the optical density reading.
3. Follow the spectrophotometer manufacturer's instructions for measuring RNA.
4. If any sample fails to meet the minimum RNA purity or concentration metrics (Table 6), centrifuge the sample tube for 1 minute at maximum speed (> 10,000 × *g*), place the tube on ice and repeat the measurement process. If the sample continues to fail either the purity or concentration metric, the RNA sample is not suitable for analysis under the Prosigna Assay procedure. Do not use RNA of insufficient quality or quantity in the Prosigna Assay.
5. The RNA extraction may be repeated if the minimum concentration or minimum purity specifications are not met (Table 6). Users can choose to isolate additional slides from the same FFPE block or choose a separate block from the same patient.

6. If the RNA concentration exceeds 250 ng/µL it must be diluted with molecular grade RNase- and DNase-free water to a target concentration of 200 ng/µL prior to performing the downstream hybridization assay. Use the recorded OD 260/280 ratio result from the undiluted sample to determine if the diluted sample meets the minimum RNA purity of 1.7.
7. Freeze the RNA at -70°C or below if the Prosigna Assay cannot be completed within the same work day.

11.7 Assay Procedure

This assay procedure describes the steps necessary to perform the Prosigna Assay using the nCounter Analysis System. These steps can be summarized into the following categories on two consecutive days:

Day One

- Setup Run Set Identification (RSID) record on web application
- Setup of RNA hybridization with Prosigna CodeSet (30 minute setup, 15–21 hour hybridization)

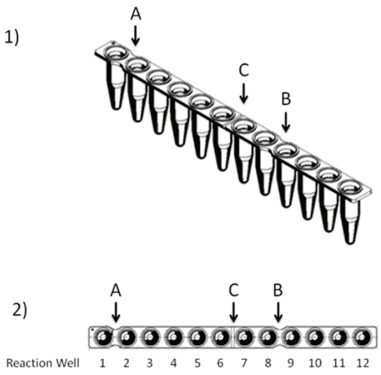
Day Two

- Setup and running of Prep Station (20 minutes of setup, 2–3 hours per run, depending on the number of samples run)
- Setup and scanning of Cartridge on the Digital Analyzer (5 minutes for setup, 2.5–4.5 hours for each Cartridge, depending on the number of samples run)
- Retrieving Report (30 minutes)

Patient Sample Selection and Batch Set Up

1. Determine the patient samples that will be part of the test run. Up to 10 samples can be included in a single batch
    a. Each sample within the batch will be assigned to a unique position within a 12-well strip tube used for hybridization, which is registered as part of the Run Set ID on the instrument (Run Set ID performed through web application software). Note that positions 1 and 2 are reserved for the Reference Sample, and positions 3–12 are for tumor RNA samples.
    b. The illustration below shows side 1) and top views 2) of the strip tube. The strip tubes are asymmetrically keyed between reaction wells 1 and 2 (A) and 8 and 9 (B) to help maintain sample order during processing. The strip tubes are also notched between reaction wells 6 and 7 (C) to facilitate cutting the strip tube if necessary to accommodate standard centrifuge adapters.

Figure 6: Illustration of keyed strip tubes



2. Calculate the amount of RNA and water (when required) to be added to the hybridization reaction for each sample within the batch.
    a. The recommended RNA input is 250 ng for the assay. The acceptable RNA input range for hybridization is 125–500 ng.
    b. Calculate the volume (in microliters) of RNA sample to add to the hybridization reaction by dividing the desired sample input (e.g., 250 ng) by the measured concentration.
    c. If the calculated concentration of the sample is between 12.5 ng/µL and 25 ng/µL, add the maximum volume of 10 µL.
    d. For samples that require less than 10 µL, calculate the volume of water required to generate 10 µL total sample volume.

    Example: For a sample with measured RNA concentration of 85 ng/µL, 2.9 µL of sample is needed for a total mass of 250 ng and 7.1 µL water is required to bring the volume to 10 µL before adding the remaining reagents. In equation: 250 ng ÷ 85 ng/µL = 2.9 µL

Sample Registration and Processing

The user will build a unique Run Set ID for each batch of samples associating the sample IDs with strip tube location (positions 3–12) using the nCounter Analysis System Services web application. The user may refer to the User Manual for instructions on using the nCounter Analysis System Services web application.

1. If the RNA was frozen prior to use, perform the following steps before proceeding:
    a. Completely thaw RNA samples and store on ice.
    b. Centrifuge the thawed sample tube for 1 minute at maximum speed (> 10,000 × *g*) and place back on ice.
2. Choose the appropriate Prosigna test kit size based on the number of patient samples being tested (1, 2, 3, 4 or 10). Remove a tube of each of the following CodeSet kit reagents from the –80˚C freezer to thaw. Store the reagents on ice if not proceeding immediately with the subsequent steps.
    a. Prosigna Reporter CodeSet (Green sticker on cap)
    b. Prosigna Capture ProbeSet (Grey sticker on cap)
    c. Prosigna Reference Sample (No sticker on cap)
3. Remove the CodeSet lot barcode sticker and a test configuration code from the CodeSet box.
4. Using a web browser, log into the IVD nCounter Analysis System web application and select Prosigna as the assay type to begin setting up the digital registration forms.
5. On the Main Page select “Create New Run Set”.
6. The first required field in defining a Prosigna run is the Run Set ID. Enter a unique identifier in the Run Set ID field to identify the batch of samples.
7. Scan or manually enter the Test Configuration Code into the web application. Once this is scanned or entered, it can be discarded.
8. Scan or manually enter the CodeSet Kit Number into the web application.
9. Next, enter the unique sample ID for the sample that will be located in the third position/well of the strip tube into the corresponding sample ID field.
    a. Enter patient RNA Sample IDs using a barcode scanner or manually by entering sample IDs using a keyboard.
    b. After entering in each sample ID, tab over to fill in the required drop down fields (gross tumor size and node status) for the sample before entering the next sample.
        i. Use the number of positive nodes established during the patient’s pathological assessment to select the appropriate nodal category for the test (zero, 1–3, ≥ 4).
        ii. Use the measured gross tumor size or stage established during the patient’s pathological assessment to select the appropriate gross tumor size category for the test (≤ 2 cm or > 2 cm).
    c. Any comments may be entered into the optional Memo field for each sample.

    Note: If any strip tube wells/positions are not required, leave the remaining fields blank. If additional fields are required for more samples, use a different test configuration that accommodates more samples.
10. After completing sample entry, specify which users receive the following:
    a. Status updates for the Prep Station and Digital Analyzer runs.
    b. The notification that the final report is available.
11. Save the completed Run Set.
    a. The Run Set Worksheet may be printed and used for sample traceability and verification purposes.

Hybridization Reaction Procedure

Note: The following steps assume ten (10) patient samples and two (2) reference samples.

Note: Do not spin Reporter CodeSet faster than 3,000 × *g* or for more than 10 seconds and do not "pulse" it to spin. Doing so will cause the centrifuge to reach maximum speed and may spin the CodeSet out of solution.

1. Program the heat block using 30 µL volume, calculated block and lid temperature, and "forever" time setting (or equivalent hold time setting). Set the temperature of the heat block to 65°C and set the heated lid at 70°C.

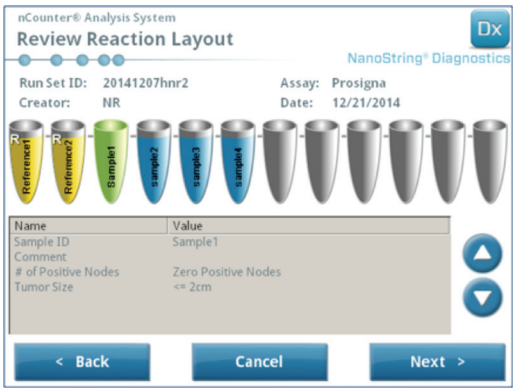
Note: For the following steps, it is critical to maintain the order with which the samples are added to the strip tube, ensuring they match the order on the Run Set ID.

2. Label the provided keyed, 12-well strip tube to distinguish positions 1–6 from positions 7–12 (see strip tube illustration).
3. If necessary, cut the strip tube in half so it will fit into a mini-centrifuge with a strip tube adapter.
4. Pipette 10 µL of Reference Sample into positions 1 and 2 of the keyed strip tube.
5. Pipette the calculated volume of water required for each sample into the respective positions in the keyed strip tube.
6. Pipette the calculated volume of RNA required for each sample into the appropriate position in the keyed strip tube, using a fresh pipette tip for each sample.
7. Once the patient sample has been added to the strip tube, it is recommended to place the sample tube in a sample tube rack, maintaining the order with which the sample was added to the strip tube. This is for verification that samples were added in the intended order after all samples are added to the strip tube.
8. Once all samples have been added to the strip tube, verify that the sample order was maintained in the strip tube (the Run Set Worksheet may be used for verifying sample order).
    a. If needed, edit the Run Set ID using the Web Application software to reflect the sample order in the final layout (refer to nCounter Analysis System User Manual for instructions on editing an existing Run Set ID).
9. After the sample order has been verified, place the individual RNA sample tubes back on ice.
10. Create a master mix containing 130 µL of the Hybridization Buffer and 65 µL of the Reporter CodeSet.
    Note: If the Reporter CodeSet was stored on ice, allow it to equilibrate to room temperature for 1 minute before adding Hybridization Buffer.
11. Mix by pipetting and briefly spin down the master mix.
    Note: Do not ad master mix on ice.
12. Pipette 15 µL of master mix into each of the 12 wells. Use a fresh pipette tip for each well.
    Note: After completion of the **next** step, the strip tube must be placed in the heat block at 65°C within 15 minutes.
13. Add 5 µL Capture ProbeSet to each well, using a new pipette tip for each well.
14. Cap the strip tube wells and mix the reagents by inverting the strip tube several times and flicking with a finger to ensure complete mixing.
15. Briefly spin down the samples in the strip tube in a picofuge or mini-centrifuge (at < 3000 × *g*).
    Note: Use a picofuge that can accommodate a 12-well strip tube, or if necessary a mini-centrifuge that can accommodate cut strip tubes.
16. Place the strip tube(s) in a 65°C heat block with a heated lid. Incubate hybridization assays at 65°C for 15–21 hours. Hybridizations should be left at 65°C until ready for processing on the Prep Station.
    Note: Discard any unused CodeSet.

Processing Samples on the nCounter Prep Station

1. Locate the Prep Station associated with the Digital Analyzer.
2. Remove nCounter Cartridge from –20°C storage and let equilibrate to room temperature for 10–15 minutes in the foil pouch.
    Note: Ensure that components from the same kit lot are used together.
3. When the Cartridge has reached room temperature, remove it from the foil pouch prior to loading the Cartridge onto the Prep Station deck.
4. Remove the nCounter Prep Plate(s) from 4°C storage and let equilibrate to room temperature for 10–15 minutes.
    Note: Only one Prep Plate is required for runs performed using a 1, 2, 3, or 4-test Prosigna kit.
5. Centrifuge the Prep Plates at 2000 × *g* for 2 minutes to collect liquids at the bottom of the wells prior to loading the Prep Plates onto the Prep Station deck.
6. While the Cartridges and Prep Plate(s) come to room temperature, prepare the Prep Station with the nCounter Prep Pack consumables.
7. Using the nCounter Prep Station touch screen interface select the "Diagnostics" button for your assay.
8. Under the Main Menu screen, select the "Process Samples" button on the touch screen interface.
9. Browse the list of available Run Set IDs (RSID) illustrated on the screen to confirm the RSID for the samples currently being processed.
10. Select the RSID by touching the screen and select "Next" on the touch screen interface.
11. On the touch screen interface verify the appropriate RSID was selected by looking at each tube on the screen and cross-referencing the sample information.
    a. Run Set Worksheet may be used here for sample traceability and verification.
    b. If the incorrect RSID was selected, touch the "Back" button and select the correct RSID.
    c. If the RSID was correct but sample entry errors exist, touch the "Back" button and go to a computer work station and edit the RSID through the web application.

Figure 7: Process a Run screen on Prep Station

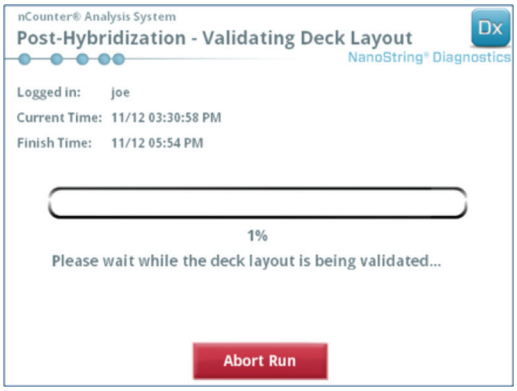


12. In the following several screens you will be prompted to scan the requested Reagent Barcode IDs in the open fields or confirm the deck placement of the required consumables. After performing each task, select "Next" on the touch screen interface to move to the next prompt.
    Note: Only one Prep Plate and one empty heater strip tube are required for runs performed using a 1, 2, 3, or 4-test Prosigna kit. For 1, 2, 3, or 4-test kit runs, load the Prep Plate and empty heater strip tube into their respective front positions (nearest the user) on the Prep Station deck.
13. Remove samples from the heat block.
    Note: Initiate the Prep Station run within 15 minutes of removing the samples from the heat block.
14. Place the strip tube(s) in a picofuge or mini-centrifuge and spin down briefly (at < 3000 × *g*).
15. Gently remove tube caps from the strip tube(s).
16. The notches on the strip tube and guides on the Prep Station should maintain correct order and orientation for the samples.
17. Place the strip tube(s) with the wells in order 1–12 from left-to-right on the nCounter Prep Station deck. If performing a run using a 1, 2, 3, or 4-test kit, only the first half of the strip tube (wells 1–6) needs to be placed in the left side of the sample tube holder on the deck, when applicable. Please note that it is important to use only wells 1–6; the second half of the strip tube (wells 7–12) will not fit into the left half of the holder due to the notched design of the tube.
18. Confirm the strip tubes are seated firmly on the Prep Station and close the metal lid.
19. If the lid does not close properly, you will be prompted to close it

during the validation of the deck layout.

20. Select "Next" on the touch screen interface.
21. Close the instrument door when prompted and select "Next" to start the deck layout validation.
22. If an error occurs, follow the directions associated with the specific error to continue with the deck layout validation.

Figure 8: Post-Hybridization Deck Layout Validation Prep Station



23. After the deck layout has been validated, select "Start Processing" on the touch screen interface.
    Note: If you encounter problems starting the Prep Station, return your hybridized samples to the heat block, but do not exceed the maximum time of 21 hours.
24. Follow prompts on the Prep Station once the run is complete.
25. Once the Prep Station has completed the run, carefully remove the Cartridge from the Prep Station and seal the wells of the Cartridge with the provided Cartridge well adhesive cover.
    Note: Do not let the Cartridge remain unsealed on the Prep Station overnight.
26. If you will not be scanning the samples within the same day, store the Cartridge at 4°C in an opaque box for up to 1 week.

Scanning Cartridge on the nCounter Digital Analyzer

1. Locate the Digital Analyzer that is linked to the Prep Station that processed the samples. Load the Cartridge onto the nCounter Digital Analyzer for scanning.
    a. Open the door to the Digital Analyzer.
    b. Place the Cartridge to be added in an empty slot.
    c. Close door and refer to the touch screen display.
2. The Digital Analyzer touch screen interface has several different graphics to help quickly identify the positional status:
    a. Empty Location: This slot is empty and ready to be loaded with a new Cartridge.
    b. Complete Blue Cartridge: Completed scan.

    DO NOT REMOVE THE FOLLOWING CARTRIDGES:

    a. White Cartridge: This slot contains a Cartridge that is registered but un-scanned.
    b. Partial Blue Cartridge: This slot contains a Cartridge that is in the process of scanning
3. Cartridges that have finished scanning may be removed from the Digital Analyzer.
4. If this is the first Cartridge loaded on the Digital Analyzer touch the "Diagnostics" button, then select "Main Menu" to log in on the Digital Analyzer. If the Digital Analyzer is already scanning Cartridges, proceed to step 9 below.
5. Carefully place the Cartridge into a vacant slot (see positional status guide above) on the Digital Analyzer. The slot and Cartridge are keyed to help ensure the correct orientation. The barcode will be facing up.
6. Lower the slot cover and press on the Cartridge through the opening in the slot lid to ensure that the Cartridge is seated properly.
7. Touch the "Start Counting" button and wait for the scanner to begin the scanning process. You will hear a series of small rhythmic clicking sounds as the Digital Analyzer begins to scan the Cartridge.
8. Confirm that a blue bar appears in the Cartridge position on the screen (within five minutes of starting the scan) indicating that the scan has started.
9. To add a Cartridge to a Digital Analyzer that is already scanning Cartridges, touch "Pause" on the 'Counting Cartridges' screen and wait for the Digital Analyzer to pause the current scan.
10. Open the door to the Digital Analyzer.
11. Place the Cartridge to be added in an empty slot (see positional status guide above).
12. Close the door and touch "Resume".
13. When the scan is complete the software will send the report to the previously specified user email addresses.
14. Upon receiving the email notification, remove the completed Cartridge and dispose according to your institution's guidelines.
    Note: Reports will be generated for successfully completed runs as well as runs with errors associated with data Quality Control (QC). Reports will not be generated in the event of an error that is not associated with data QC. Contact Veracyte Customer Service for assistance if this occurs.
15. Using the link attached to that email, open the web application and download all the test reports associated with the RSID currently being processed.
16. Following Errors: Follow the recommendation outlined in the test report for individual sample or System error.

Note: Individual sample failures are not considered System errors.

12 TROUBLESHOOTING AND TEST FAILURES

Table 7: Error Codes for Test Repeat

Error Code	Failure Description	Recommended Action
5	Scan Failure	Re-run sample with 250 ng of RNA
7	High Signal	Re-spec sample and re-run test with 125 ng of RNA
6	Low Signal	Re-spec sample and re-run test with 500 ng of RNA
30	Low Signal	Re-spec sample and re-run test with 500 ng of RNA
31	Low RNA Signal	Re-spec sample and re-run test with 500 ng of RNA

Reasons to Repeat the Assay:

1. The assay report will identify failing samples and no assay results will be reported. The assay results will be reported in the case of passing samples.
2. The assay report will identify the type of failure and recommended action in the case of a assay failure. The RNA concentration of failing samples may be remeasured and the samples may be re-run (as part of a new batch/RSID) depending on the type of failure and amount of RNA mass that remains in order to obtain an assay result.

13 RESULTS OF THE ASSAY

The Prosigna Assay includes a series of quality control metrics that are automatically applied to each sample during analysis. These metrics evaluate the performance of the assay to determine whether the results fall within expected values. Upon successful analysis of these quality control metrics, the Prosigna Assay gives the following results:

Table 8: Results and output of the Prosigna Assay

Result	Output Values
The Intrinsic Subtype of the Breast Cancer Specimen	Luminal A Luminal B HER2-Enriched Basal-Like
Individual Estimate of the Probability of Distant Recurrence within 10 years	0–100%
Risk of Recurrence (ROR) Score	Integer value on a 0–100 scale
Risk Category	Low, Intermediate, High

13.1 Intrinsic Subtypes

The Intrinsic Subtype of a breast cancer tumor has been shown to be related to prognosis in Early Stage Breast Cancer. On average, patients with a Luminal A tumor have significantly better outcomes than patients with Luminal B, HER2-Enriched, or Basal-like tumors[2,5].

The Intrinsic Subtype is identified by comparing the gene expression profile of 50 genes in an unknown sample with the expected expression profiles for the four intrinsic subtypes. The subtype with the most similar profile is assigned to the unknown sample.

The most common subtypes of breast cancer are the luminal subtypes,

Luminal A (LumA) and Luminal B (LumB). Prior studies suggest that Luminal A comprises approximately 30% to 40% and Luminal B approximately 20% of breast cancers[5]. However, greater than 90% of hormone-receptor positive patients have luminal tumors. The gene expression pattern of these subtypes resembles the luminal epithelial component of the breast tissue[5]. These tumors are characterized by high expression of estrogen receptor (ER), progesterone receptor (PR), and genes associated with ER activation, such as LIV1, GATA3, and cyclin D1, as well as expression of luminal cytokeratins 8 and 18. Luminal A breast cancers exhibit lower expression of genes associated with cell cycle activation when compared to Luminal B breast cancers resulting in a better prognosis.

Prior studies suggest that the HER2-Enriched subtype (HER2-E) comprises approximately 20% of breast cancers[5]. However, HER2-Enriched tumors are generally ER-negative, so only 5% of the tested ER-positive patient population was found to have HER2-Enriched breast cancer. Regardless of ER-status, HER2-Enriched tumors are HER2-positive in the majority of cases with high expression of the ERBB2 cluster, including ERBB2 and GRB7. Genes associated with cell cycle activation are also highly expressed.

Published data suggest that the Basal-like subtype comprises approximately 20% of breast cancers[5]. However, Basal-like tumors are generally ER-negative, so only 1% of hormone receptor-positive patients have Basal-like breast cancer. The Basal-like subtype is almost always clinically HER2-negative and expresses a suite of "basal" biomarkers including the basal epithelial cytokeratins (CK) and Epidermal Growth Factor Receptor (EGFR). Genes associated with cell cycle activation are highly expressed.

13.2 ROR Score

The ROR score is an integer value on a 0–100 scale that is related to an individual patient's probability of distant recurrence within 10 years for the defined intended use population. The ROR score is calculated by comparing the expression profile of 46 genes in an unknown sample with the expected profiles for the four intrinsic subtypes, as described above, to calculate four different correlation values. These correlation values are then combined with a proliferation score and the gross tumor size to calculate the ROR score.

13.3 Probability of 10-Year Distant Recurrence

The ROR scores for 2 cohorts of post-menopausal women with hormone receptor-positive early stage breast cancer were compared to distant recurrence-free survival following surgery and treatment with 5 years of adjuvant endocrine therapy followed by 5 years of observation (see Clinical Performance Section 16.4 for details). These two studies resulted in a model relating the ROR score to the probability of distant recurrence in this tested patient population including a 95% confidence interval.

13.4 Risk Classification

Risk classification is also provided to allow interpretation of the ROR score by using cutoffs related to clinical outcome in tested patient populations.

Table 9: Risk classification by ROR range and nodal status

Nodal Status	ROR Range	Risk Classification
Node-Negative	0–40	Low
	41–60	Intermediate
	61–100	High
Node-Positive (1–3 nodes)	0–15	Low
	16–40	Intermediate
	41–100	High
Node-Positive (≥ 4 nodes)	0–100	High

13.5 Quality Control

Each lot of the Prosigna Assay components is tested using predetermined specifications. All kit-level items are lot tracked, and the critical components contained within each kit are tested together and released as a Prosigna kit lot.

The Prosigna Assay kit includes a series of internal controls that are used to assess the quality of each run set as a whole and each sample individually. These controls are listed below.

Batch Control Set: *In vitro* transcribed RNA Reference Sample

A synthetic RNA Reference Sample is included as a control within the Prosigna Assay kit. The Reference Sample is comprised of *in-vitro* transcribed RNA targets from the 50 algorithm and 8 housekeeping genes. The Reference Sample is processed in duplicate in each Prosigna Assay run along with a set of up to 10 unknown breast tumor RNA samples in a 12 reaction strip tube. The signal from the Reference Sample is analyzed against pre-defined thresholds to qualify the run.

The signal from each of the 50 algorithm genes of the breast tumor RNA sample is normalized to the corresponding genes of the Reference Sample.

Positive Control Set: *in vitro* transcribed RNA targets and corresponding Capture and Reporter Probes

Synthetic RNA targets are used as positive controls (PCs) for the Prosigna Assay. The PC target sequences are derived from the External RNA Control Consortium (ERCC) DNA sequence library[6]. The RNA targets are *in-vitro* transcribed from DNA plasmids. Six RNA targets are included within the assay kit in a 4-fold titration series (128–0.125 fM final concentration in the hybridization reaction) along with the corresponding Capture and Reporter Probes. The PCs are added to each breast tumor RNA sample and Reference Sample tested with the Prosigna Assay. A sample will be disqualified from further analysis if the signal intensities from the PCs do not meet pre-defined thresholds.

Negative control set: exogenous probes without targets

Negative control target sequences are derived from the ERCC DNA sequence library[6]. The probes designed to detect these target sequences are included as part of the assay kit without the corresponding target sequence. The negative controls (NCs) are added to each breast tumor RNA sample and Reference Sample tested with the Prosigna Assay as a quality control measure. The sample will be disqualified from further analysis if the signal intensities from the NCs do not meet pre-defined thresholds.

RNA Integrity Control Set: Housekeeping genes

Capture and Reporter Probes designed to detect 8 housekeeping genes and 50 algorithm genes are included as part of the Prosigna kit. The expression levels of the 8 housekeeping genes are analyzed to determine the quality of RNA extracted from the FFPE tissue sample and input into the Prosigna Assay. The sample will be disqualified from further analysis if the expression levels of the housekeeping genes falls below pre-defined thresholds.

The housekeeping genes are also used to normalize for any differences in the intact RNA amount in a sample prior to Reference Sample normalization.

14 LIMITATIONS OF THE PROCEDURES

1. The Prosigna Assay has been optimized to identify the intrinsic subtype of a breast cancer tumor and the patient's 10-year risk of distant recurrence as an ROR score and risk category, using purified RNA extracted from formalin-fixed, paraffin embedded human breast tissue. Other types of specimens or fixatives have not been tested and should not be used.
2. The performance of the Prosigna Assay was validated using the procedures provided in this package insert only. Modifications to these procedures may alter the performance of the test.
3. Performance characteristics of the Prosigna Assay have been established for postmenopausal women with hormone receptor-positive early stage breast cancer treated with 5 years of adjuvant endocrine therapy. Performance with other treatment regimens or in other patient populations has not been established.
4. If RNA of insufficient quality or quantity is added to the assay, then the Prosigna Assay may be unable to give a valid result and instead will report an assay failure.
5. The interpretation of Prosigna Assay results (intrinsic subtype, ROR score, risk category) should be evaluated within the context of other clinicopathological factors, the patient's medical history and any other laboratory test results.
6. The performance of the Prosigna Assay has been established with RNA meeting the specifications defined in reference to the procedure above. Performance with isolated RNA that does not meet these specifications has not been established.
7. Known interfering substances to the Prosigna Assay include genomic DNA and non-tumor tissue (e.g., normal tissue). Please refer to general assay considerations before starting the procedure. The area of viable invasive carcinoma must be clearly identified by a pathologist prior to running the procedure. Additionally, each RNA sample must be treated with DNase. Prior to proceeding with patient test samples, each new lot of DNase should be tested and qualified per the provided specification when using an isolation kit other than the Roche FFPET RNA isolation kit.

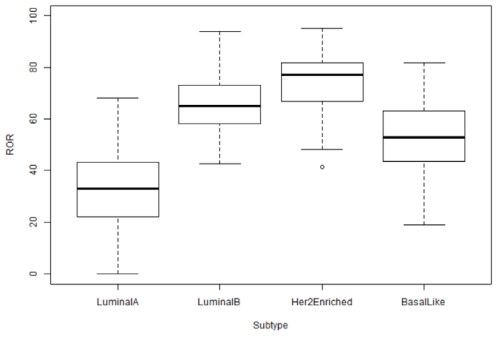
15 EXPECTED VALUES

The Prosigna Assay reports an ROR score (0–100), an intrinsic subtype (Luminal A, Luminal B, HER2-enriched or Basal-like) and risk categorization (Low, Intermediate, or High) for each tumor sample. Based on the two clinical validation studies described below, post-menopausal women with HR+, early stage breast cancer treated with anastrozole or tamoxifen in the ATAC and ABCSG-8 trials, the range and frequency of ROR scores (Figure 10), the continuous relationship of ROR to probability of distant recurrence by nodal status (Figure 11) and distribution of ROR scores by intrinsic subtype (Figure 9) that are expected are shown. Based on these clinical validation studies, the distant recurrence-free survival over 10 years by risk categorization is represented in Figure 12 (Node-Negative patients) and Figure 13 (Node-Positive (1–3 nodes) patients).

15.1 ROR range by Subtype

Figure 9 shows a box plot of ROR Score by Intrinsic Subtype.

Figure 9: Box Plot of ROR Score by Intrinsic Subtype



15.2 Frequency of ROR score by nodal status

The histogram in Figure 10 was generated using a single Cox-model which included ROR score and categorical variables to distinguish the three nodal involvement groups.

Figure 10: Histogram of ROR score and nodal status groups

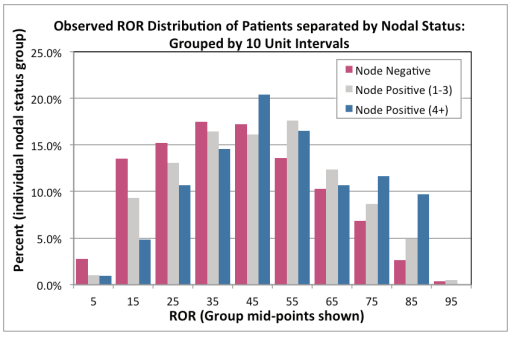
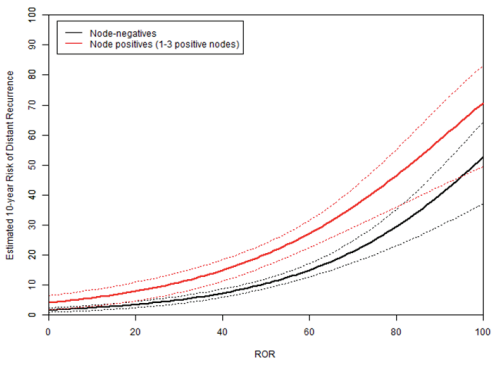


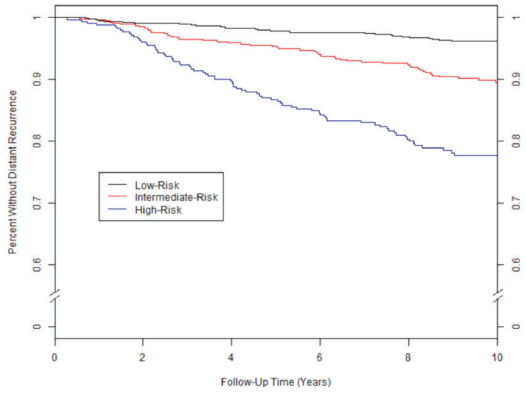
Figure 11: Ten Year Predicted Risk Estimated Within Nodal Status Group



15.3 Distant Recurrence-Free Survival by Risk Categorization

The following data originates from the combined analysis of the TransATAC and ABCSG-8 trials. For allocating patients to risk groups, ROR scores were compared to pre-defined risk thresholds for node-negative or node-positive patients. Figures 12 and 13 show the 10-year distant recurrence-free survival for each risk category group by nodal status.

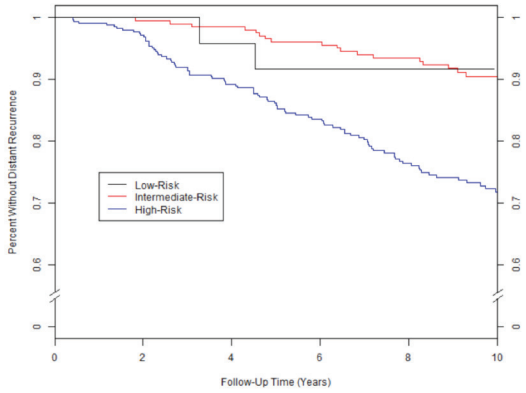
Figure 12: DRFS by Risk Group for Node-Negative Patients



Summary of Data for Figure 12: DRFS by Risk Group for Node-Negative Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	875 (49%)	31	96.2% [94.7% - 97.3%]
Intermediate	551 (31%)	53	89.2% [86.1% - 91.7%]
High	360 (20%)	73	77.7% [72.8% - 81.9%]
Total	*1,786 (100%)*	*157*	

Figure 13: DRFS by Risk Group for Node-Positive (1–3 nodes) Patients



Summary of Data for Figure 13: DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	24 (4%)	2	91.7% [70.6% - 97.8%]
Intermediate	211 (36%)	18	90.4% [85.2% - 93.9%]
High	355 (60%)	87	71.8% [66.3% - 76.6%]
Total	*590 (100%)*	*107*	

Table 10: Ten-year DRFS rates for patients with 4 or more positive nodes

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
High	103	39	57.4% [46.3% - 67.0%]

16 PERFORMANCE CHARACTERISTICS

16.1 Analytical Precision and Reproducibility

In order to estimate overall precision and reproducibility of Prosigna, two studies were conducted and the results combined. The first study conducted was a precision study on the nCounter Analysis System starting with extracted breast tumor RNA, and the second study was a reproducibility study starting from FFPE breast tumor tissue which included pre-analytical factors.

RNA Precision

16.1.1 Study Design

A three-site blinded and randomized comparative study was conducted with the Prosigna Assay on the nCounter Analysis System to assess analytical precision. Five pooled breast tumor RNA samples were generated from archived FFPE specimens for testing at each site. The sample panel represented prototypical gene expression profiles encountered during routine testing and each risk classification group.

Each site completed 18 valid runs (9 runs by each operator, each run consisting of 10 tests) following a familiarization run by each operator (Table 11). Each sample was tested in duplicate during each run at the nominal RNA input level of 250 ng for the assay. Each operator completed one run on a given day per the generally accepted standard for long run methods[7]. The total period of study including familiarization covered more than 4 weeks at each site.

Table 11: Overview of RNA precision study

Study Variable	Numbers
# of breast tumor RNA samples	5
# of sample replicates per run (same Cartridge)	2
# of runs/site	18
# of runs/day	1
# of operators/site	2
# reagent lots/site	3
# of sites	3
Total # of samples tested per site (excluding familiarization)=	*180*
Total # of samples =	*540*

16.1.2 Variance Components Analysis

Table 12 shows the output from the variance components analysis for each panel member. Below the estimated variance is the percent of total variance (in parentheses).

Table 12: Variance Components by Panel Member (pooled RNA sample)

Panel Member by Risk, Subtype	Mean ROR	Variance Component					Total Variance	Total SD
		Lot	Site	Operator	Run	Within-Run		
Low Luminal A	31.4	0.010 (2%)	0.000 (0%)	0.000 (0%)	0.134 (30%)	0.296 (67%)	0.44 (100%)	0.66
Intermediate Luminal B	55	0.105 (18%)	0.000 (0%)	0.000 (0%)	0.046 (8%)	0.426 (74%)	0.576 (100%)	0.76
Intermediate Basal-like	55.4	0.059 (20%)	0.000 (0%)	0.000 (0%)	0.046 (15%)	0.194 (65%)	0.299 (100%)	0.55
High Luminal B	64.8	0.119 (21%)	0.014 (2%)	0.000 (0%)	0.064 (11%)	0.380 (66%)	0.576 (100%)	0.76
High HER2-enriched	76.2	0.165 (37%)	0.000 (0%)	0.000 (0%)	0.000 (0%)	0.277 (63%)	0.442 (100%)	0.66

For all five panel members, the total SD was less than 1 ROR unit on a 0–100 scale. For all panel members, the bulk of the variance came from within-run variance (repeatability). There was almost no site-to-site variance or operator-to-operator variance. A likelihood ratio test for significance of site by panel member demonstrated that the site differences were statistically insignificant ($p > 0.05$). For each lot, the mean ROR scores are less than 1 ROR unit apart for each panel member contributing approximately 20% on average to overall variance.

16.1.3 Concordance of Subtype Call and Risk Classification

For all panel members, there was 100% concordance between the subtype result and the intrinsic subtype of the panel member. For all samples, there was 100% concordance between the measured and expected risk group.

Tissue Reproducibility

16.1.4 Study Design

A three-site blinded and randomized comparative study using replicate breast tumor tissue specimens taken from the same FFPE block were tested on the nCounter Analysis System using the Prosigna Assay. A set of 43 recently banked FFPE breast tumor specimens from Hormone Receptor positive breast cancer patients with confirmed invasive ductal and/or lobular carcinoma were tested as part of the study. All tissue specimens were shipped to the appropriate testing site for processing. The 43 specimens were reviewed independently by three separate pathologists. For each pathology-reviewed tissue sample, a test run consisting of tissue macrodissection, RNA extraction, and testing with the Prosigna Assay was performed by a single operator at each site using the defined assay procedure. The isolated RNA from each of the tissue samples was tested twice in separate assay runs. Three lots of the RNA isolation kit (one per site) and a single lot of assay kit reagents were used in the execution of this study. A single slide was input for RNA extraction when the tumor surface area measured $\geq$ 100 mm^2, and 3 slides were input when the tumor surface measured < 100 mm^2, with a minimum tumor surface area of 4 mm^2 required.

16.1.5 Testing Summary

The call rate for the forty three (43) tissue specimens evaluated at each of the three sites is shown in Table 13.

Table 13: Call Rate at each site

Site	Percent Providing Result	Passing/Total
1	95%	41/43
2	93%	40/43
3	100%	43/43

Forty specimens yielded results at all sites (RNA isolation of one sample at one site required repeating), 1 specimen yielded results at 2 sites, and 2 specimens yielded results at a single site. One hundred percent (100%) of samples passing tissue review and RNA isolation specifications yielded passing results from the Prosigna Assay. The measured tumor surface area for 4/5 RNA isolation failures was $\leq$ 15 mm^2, equaling less than 50 mm^2 total tissue by area input into the test.

The 43 specimens included both node-negative and node-positive patients. The calculated test results from the 43 specimens represent a wide range (94 units) of ROR scores, all 4 intrinsic subtypes, and all risk categories when applying the node-negative or node-positive cutoffs to all specimens. The two samples with results at a single site were excluded from all subsequent statistical analyses as there was no available data for comparing across sites.

16.1.6 Variance Components Analysis

There were no statistically significant ($\alpha = 0.05$) differences across risk categories using a non-parametric Kruskal-Wallis test so the variance components model was fit across all risk-categories simultaneously.

Table 14 shows the results of the variance components analysis using all 41 tissue specimens.

Table 14: Variance Components (Tissue Reproducibility Study)

Variance Components				Total SD
Site	Within Block	Residual	Total	
0.10	7.72	0.51	8.34	2.89

The site component measures systematic site-specific variation, the "Within Block" component measures random variation that differs as a function of tissue specimen review/processing or within FFPE block variation, and the residual variation measures the combined run to run variability and within-run variability in the Prosigna Assay. The site component is very small relative to the random variability within the block indicating that the differences on average between the sites were negligible (< 1% of total variance). The residual variation was consistent with the equivalent variability measured in the RNA-precision study which had fewer samples but more replicate measurements (0.51 variance compared to an average single site within Prosigna reagent lot variance of 0.39 for the RNA-precision study).

Table 15 summarizes the total variability using the sum of the Tissue Processing Variability (site and within block components from Table 14 from this study) as well as the total RNA Processing Variability from the RNA-precision study (averaged across the five tested panel members in Table 12). Pre-analytical factors associated with tissue processing are the primary source of variation for the test (94% of total variance). The total SD including all sources of variation equals 2.9, indicating that the Prosigna Assay is a reliable measure of difference between two ROR values of 6.75 with 95% confidence.

Table 15: Total Variability (Tissue and RNA Processing)

Tissue Processing Variability	RNA Processing Variability	Total Variability	Total SD
7.82	0.47	8.29	2.9

16.1.7 Concordance of Risk-Category and Subtype Classifications

The site-to-site concordance by patient subtype and risk classification (low/intermediate/high risk) is shown in Table 16, where the respective risk cutoffs for the node-negative and node-positive classifications were applied to all specimens. The exact-type 95% confidence intervals are shown in brackets and the number of samples with results at both sites is shown in parentheses. The average concordance is shown in the last column. For each comparison, the concordance was calculated in two steps. First, for each tissue sample, the proportion of the four possible result-pairs (two at site 1 * two at site 2) that agreed was calculated. In the second step, these proportions were averaged across all tissue samples that generated results at both sites in the given comparison.

Table 16: Summary of Concordance of Subtype and Risk-Category by Node Status

Comparison Type	Pairwise Concordance			Average Concordance
	Site 1 vs. Site 2 (n = 40)	Site 1 vs. Site 3 (n = 41)	Site 2 vs. Site 3 (n = 40)	
Subtype	96.3% [86.4%-99.5%]	98.8% [91.0% - 100%]	95% [83.1% - 99.3%]	97%
Risk Category Node-Negative	87.5% [73.2% - 95.8%]	92.7% [80.1% - 98.4%]	90% [76.4% - 97.2%]	90%
Risk Category Node-Positive	88.8% [75.9% - 96.0%]	92.7% [80.1% - 98.4%]	91.3% [79.2% - 97.4%]	91%

For each comparison (subtype and node-negative and node-positive risk categories), the average concordance between sites was at least 90%. There were no samples where the risk category changed from low risk to high risk (or vice versa) between or within sites. There were only two specimens (out of 41) that did not give identical subtypes across all 6 replicates:

1. One specimen had duplicate Luminal A results at one site and duplicate Luminal B results at each of the other two sites.
2. One specimen had duplicate Luminal A results at one site, duplicate HER2-enriched results at another site and one each of Luminal A and HER2-enriched at the third site.

16.2 Sensitivity / RNA input

RNA Input Study Description

The study tested 13 breast tumor RNA samples across three RNA input levels within assay specification (500, 250, and 125 ng) and two additional RNA input levels outside of specification (625, 62.5 ng). Each sample was tested with each kit lot (2 lots total) in a single test run which included duplicate measurements at each level in specification and a single measurement for each level outside of specification. Duplicate blank (i.e., no target) measurements were included in each test run. A single sample was tested with a single lot only.

RNA Input Study Results

All measured blank samples (n = 46) were well below the threshold for signal and yielded a failing test result (0% call rate). All tumor RNA measurements within assay specification (n = 138) yielded a passing test result (100% call rate). One hundred percent (100%) of specimens with input above specification (625 ng) yielded a passing test result. Eighty-three percent (83%) of specimens (10/12) tested at input below specification (62.5 ng) yielded a test result in lot 1 with 100% in lot 2.

The average ROR score for the 13 samples covered a broad range (20–82). The Risk group classification (low/intermediate/high) was 100% concordant across all RNA input levels for the 13 tested samples. Table 17 summarizes the variation in ROR Score as a function of RNA input. The mean ROR score difference between RNA input levels, the SD for the differences and the 90% confidence interval were used for evaluating whether ROR scores generated from different RNA input levels were equivalent to those generated using the target level of 250 ng. To meet the acceptance criterion, the confidence interval had to be completely contained with (-3,3 ROR). At the two levels at the extremes of the assay specification range (125 and 500 ng RNA), the ROR scores were equivalent to those at the target input concentration of 250 ng for each of the two kit lots tested. For each level outside of the assay specification ROR scores were equivalent for one of the lots but not the other.

Table 17: ROR Score Difference Summary. Count equals number of samples included in analysis.

Kit Lot	Mass (ng)	Count	Mean ROR Difference	SD of Difference	Lower Confidence Limit	Upper Confidence Limit
20535	62.5 - 250	10	1.90	2.62	0.54	3.26
	125 - 250	12	0.75	1.23	0.16	1.34
	500 - 250	12	0.04	0.78	-0.33	0.41
	625 - 250	12	-0.13	0.86	-0.53	0.28
20536	62.5 - 250	11	-0.36	3.96	-2.33	1.60
	125 - 250	11	-0.50	3.07	-2.02	1.02
	500 - 250	11	-0.82	3.25	-2.43	0.79
	625 - 250	11	-1.09	4.24	-3.19	1.01

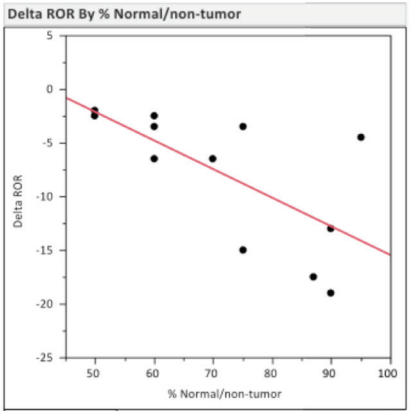
16.3 Interference testing

Adjacent Normal/non-tumor tissue

Adjacent normal/non-tumor breast tissue is commonly present in FFPE breast tumor blocks and can be identified upon pathology review as a distinct area from the area of invasive breast carcinoma. The Prosigna Assay procedure above specifies removal of adjacent normal tissue by macrodissection. To assess the risk of normal tissue contamination to the test results, a total of 13 FFPE breast tumor blocks containing pathologically-confirmed infiltrating ductal carcinoma and approximately 50–95% surrounding normal/non-tumor tissue were tested with and without macrodissection of the surrounding tissue, and the difference in ROR score (delta ROR) was determined.

On average, the ROR of the macrodissected tumor sample was almost 8 ROR units above what was observed when normal/non-tumor tissue was not removed. Figure 14 illustrates that as the amount of normal tissue increases (up to 95% not removed by macrodissection), there is an increasing risk that the reported ROR score will be an underestimated or negatively biased (up to -19 ROR units) point estimate of a patient's risk of recurrence.

Figure 14: Impact of normal/non-tumor tissue on Delta ROR



Necrotic, hemorrhagic, and DCIS tissue interference

To assess the risk of necrotic/hemorrhagic/DCIS contamination to the test results, a total of 11 FFPE breast tumor blocks (3 DCIS, 5 Necrotic, 3 hemorrhagic) containing pathologically-confirmed invasive breast carcinoma and approximately 10–30% of the interferent were tested with and without macrodissection of the interferent, and the difference in ROR score (delta ROR) was determined. At the levels tested, the effect of blood/hemorrhagic, DCIS, and necrotic tissue included in the procedure had negligible impact on the reported ROR score (< 6 ROR units). There was 100% concordance in risk category assignment between the eleven necrotic, hemorrhagic, and DCIS samples with and without macrodissection.

Human genomic DNA

The Prosigna Assay procedure includes removal of human genomic DNA (gDNA) by digestion with DNase I. To assess the risk of gDNA contamination to the test results, ten (10) FFPE breast tumor blocks containing pathologically-confirmed infiltrating ductal carcinoma were tested +/- removal of human genomic DNA, by omitting the DNase step within the procedure. In the samples tested, on average, the ROR score was 4 to 5 units lower in the Low and Intermediate risk groups when gDNA is removed with DNase I (see Table 18). When the samples that were not DNase-treated were subsequently treated with DNase I (post-treat) the ROR scores matched the ROR values originally observed with on-protocol DNase treatment. There is a risk that the reported ROR score will be an overestimated or positively biased (up to 7 ROR units) point estimate of a patient's risk of recurrence in the presence of gDNA. Additionally, the calculated signal for samples without DNase I treatment was significantly ($p < 0.05$) lower than those treated with DNase I due to interference in the absorbance reading used to quantify the amount of RNA prior to testing with the Prosigna Assay.

Table 18: Impact of treatment with DNase on ROR in tumor samples

ROR Category	FFPE Specimens Tested	ROR Difference w/ DNase I - w/o DNase I			ROR Difference w/ DNase I - w/ DNase I (post treat)		
		Mean	Min	Max	Mean	Min	Max
Low	3	-4.0	-6.0	-1.0	0.7	-1.0	3.0
Intermediate	2	-4.5	-7.0	-2.0	1.0	0.0	2.0
High	5	0.4	-1.0	2.0	0.4	-1.0	1.0

16.4 Clinical Performance

Two clinical validation studies were executed to validate the Prosigna Breast Cancer Prognostic Gene Signature Assay. The primary objective of both studies was to validate published observations that the risk of recurrence score (ROR) provides additional prognostic information for distant recurrence-free survival at 10 years over and above standard clinical variables. Also, a secondary objective from both studies aimed to validate previous observations that Luminal A and Luminal B patients have statistically significantly different distant recurrence-free survival at 10 years. Because the entry criteria and results of these two studies were similar, the two databases were combined and analyzed with a prospectively defined analysis plan that had the same objectives as the individual studies

Combined Analysis: Generating risk curves using combined results of the Prosigna Assay from TransATAC and ABCSG-8

A summary of treatment and clinical characteristics from the combined analysis can be found below. For individual study design and analysis information, please see the following sections for Studies 1 and 2, respectively.

Analysis

Table 19: Summary of Treatment and Clinical Characteristics in Combined Analysis of Studies 1 and 2

Characteristic	Value	Node-Negative (n = 1,786)		1–3 Positive Nodes (n = 590)		≥ 4 Positive Nodes (n = 103)	
		Trans ATAC (n = 739)	ABCSG8 (n = 1,047)	Trans ATAC (n = 208)	ABCSG8 (n = 382)	Trans ATAC (n = 54)	ABCSG8 (n = 49)
Treatment	Some Anastrozole	377 (51.0%)	528 (50.4%)	102 (49.0%)	184 (48.2%)	31 (57.4%)	25 (51.0%)
	Tamoxifen Only	362 (49.0%)	519 (49.6%)	106 (51.0%)	198 (51.8%)	23 (42.6%)	24 (49.0%)
Grade	G1	169 (22.9%)	210 (20.1%)	39 (18.8%)	54 (14.1%)	3 (5.6%)	7 (14.3%)
	G2/GX	438 (59.3%)	837 (79.9%)	122 (58.7%)	328 (85.9%)	37 (68.5%)	42 (85.7%)
	G3	132 (17.9%)	0 (0%)	47 (22.6%)	0 (0%)	14 (25.9%)	0 (0%)
Tumor Size	≤ 1 cm	122 (16.5%)	219 (20.9%)	13 (6.2%)	37 (9.7%)	3 (5.6%)	2 (4.1%)
	1–2 cm	420 (56.8%)	568 (54.3%)	83 (39.9%)	193 (50.5%)	15 (27.8%)	18 (36.7%)
	2–3 cm	157 (21.2%)	213 (20.3%)	77 (37.0%)	122 (31.9%)	18 (33.3%)	23 (46.9%)
	> 3 cm	40 (5.4%)	47 (4.5%)	35 (16.8%)	30 (7.9%)	18 (33.3%)	6 (12.2%)
HER2 Status	Negative	649 (87.8%)	984 (94.0%)	186 (89.4%)	367 (96.1%)	47 (87.0%)	46 (93.9%)
	Positive	90 (12.2%)	63 (6.0%)	22 (10.6%)	15 (3.9%)	7 (13.0%)	3 (6.1%)
Recurrences	Distant	79 (10.7%)	91 (8.7%)	50 (24.0%)	64 (16.8%)	31 (57.4%)	10 (20.4%)
	Any	117 (15.8%)	121 (11.6%)	59 (28.4%)	73 (19.1%)	34 (63.0%)	10 (20.4%)
NanoString Intrinsic Subtype	Luminal A	529 (71.6%)	725 (69.2%)	127 (61.1%)	248 (64.9%)	31 (57.4%)	31 (63.3%)
	Luminal B	176 (23.8%)	284 (27.1%)	68 (32.7%)	118 (30.9%)	20 (37%)	16 (32.7%)
	Basal-like	7 (0.9%)	6 (0.6%)	2 (1.0%)	2 (0.5%)	0 (0%)	0 (0%)
	HER2-enriched	27 (3.7%)	32 (3.1%)	11 (5.3%)	14 (3.7%)	3 (5.6%)	2 (4.1%)

Both studies had a treatment arm consisting of 5 years of Tamoxifen. In TransATAC, the other study arm consisted of 5 years of Anastrozole, whereas in the ABCSG-8 study the second arm consisted of 2 years of Tamoxifen followed by 3 years of Anastrozole. When DR was modeled as a function of all the clinical and treatment variables, the treatment did not contribute significantly ($p = 0.66$) as a predictor of DR. The other main differences between these trials were the fact that the TransATAC trial included patients with Grade 3 tumors, and that the overall recurrence rate was higher in the TransATAC study than in the ABCSG-8 study.

Results

Figure 15 shows the 10-year risk of DR as a function of ROR score with 95% confidence bands based on separate Cox-proportional hazards models for each of the node-negative and node-positive (1–3 positive nodes) patient groups.

Figure 15: Ten-year Estimated DR Risk by Node Status with 95% Confidence Intervals

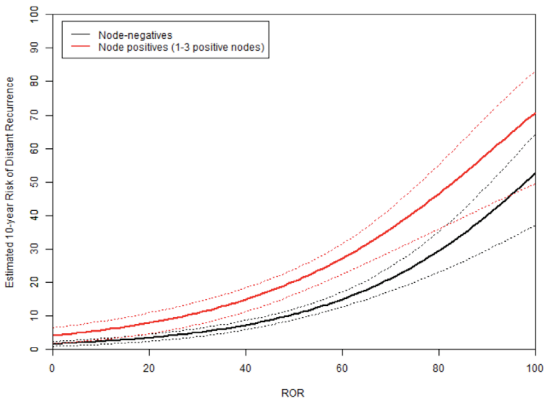
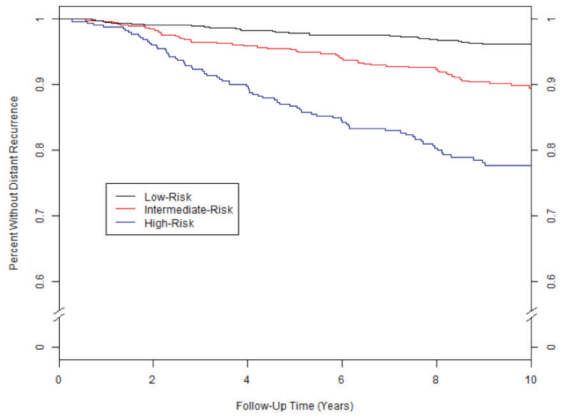


Figure 16 shows the Kaplan-Meier and incidence plots by risk-group for node-negative patients and Figure 17 shows the same plots for node-positive patients with 1–3 positive nodes. In each figure, details of sample sizes, numbers of events and estimated percent without distant recurrence at 10 years are

provided by risk group. In the node-positive patient group, there were very few patients in the pre-defined low-risk groups causing the confidence interval on the Kaplan-Meier curve, and hence the estimate of 10-year DRFS to be very wide.

Figure 16A: DRFS by Risk Group for Node-Negative Patients



Summary of Data for Figure 16A: DRFS by Risk Group for Node-Negative Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	875 (49%)	31	96.2% [94.7% - 97.3%]
Intermediate	551 (31%)	53	89.2% [86.1% - 91.7%]
High	360 (20%)	73	77.7% [72.8% - 81.9%]
Total	*1,786 (100%)*	*157*	

Figure 16B: Incidence by Risk Group for Node-Negative Patients in Five Year Intervals

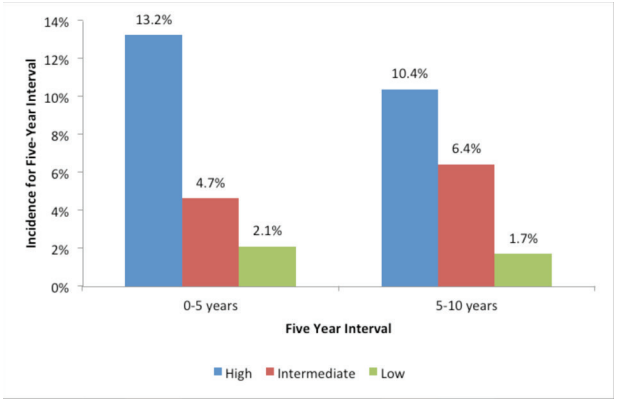
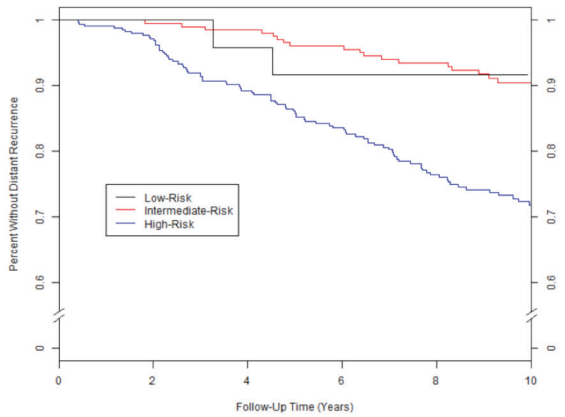


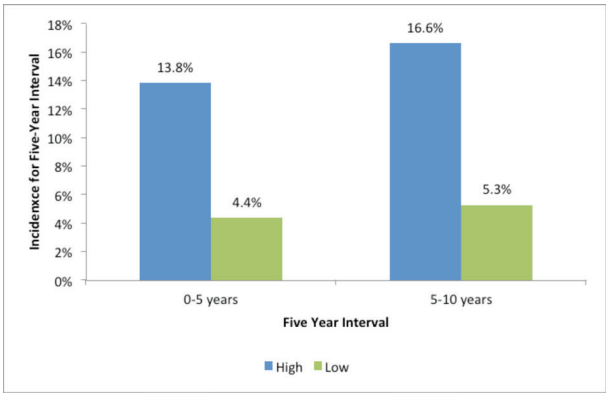
Figure 17A: DRFS by Risk Group for Node-Positive Patients with 1 to 3 Positive Nodes



Summary of Data for Figure 17A: DRFS by Risk Group for Node-Positive Patients with 1 to 3 Positive Nodes

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	24 (4%)	2	91.7% [70.6% - 97.8%]
Intermediate	211 (36%)	18	90.4% [85.2% - 93.9%]
High	355 (60%)	87	71.8% [66.3% - 76.6%]
Total	*590 (100%)*	*107*	

Figure 17B: Incidence by Risk Group for Node-Positive (1–3 nodes) Patients in Five Year Intervals



In Figure 17B, as there were only 24 patients with 2 events in the low-risk node-positive group, these patients have been combined with the intermediate patients for the late recurrence analysis.

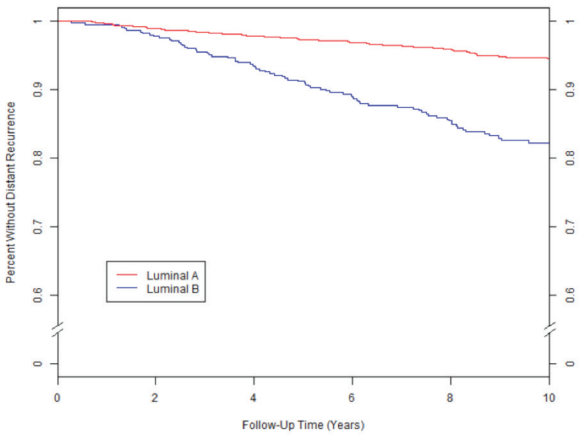
All 103 patients in the combined database with 4 or more positive nodes are classified as being high-risk. Table 20 shows the ten-year DRFS rates for these patients.

Table 20: Ten-year DRFS rates for patients with 4 or more positive nodes

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
High	103	39	57.4% [46.3% - 67.0%]

The majority of the subjects in the combined studies (96%) were either Luminal A or Luminal B. Figure 18 shows a comparison of DRFS by Luminal subtype for node-negative patients.

Figure 18: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Negative Patients

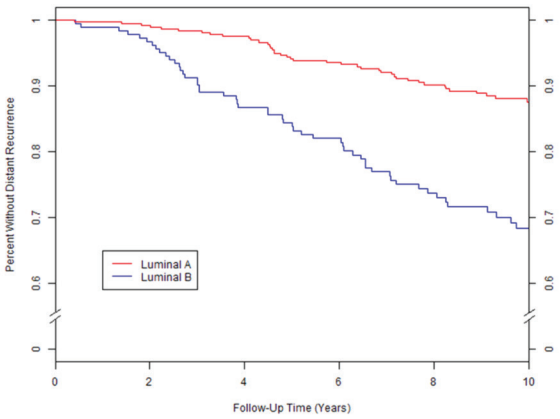


Summary of Data for Figure 18: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Negative Patients

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Luminal A	1254	62	94.6 [93.1 - 95.8]
Luminal B	460	75	81.9 [77.7 - 85.3]
Total	*1,714*	*137*	

Figure 19 shows the same comparison for node-positive patients with 1–3 positive nodes. For both groups, there were significant differences between the DRFS of Luminal A and Luminal B patients.

Figure 19: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Positive Patients with 1–3 Positive Nodes



Summary of Data for Figure 19: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Positive Patients with 1–3 Positive Nodes

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Luminal A	375	41	87.6 [83.5 - 90.8]
Luminal B	186	52	68.3 [60.4 - 75.0]
Total	*561*	*93*	

There were only 98 Luminal-subtype patients in the combined database with 4 or more positive nodes. Table 21 shows the ten-year DRFS rates for these patients who also show a much higher risk when having a Luminal B subtype.

Table 21: Ten-year DRFS rates for patients with 4 or more positive nodes by Luminal Subtype

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Luminal A	62	17	68.3 [53.6 - 79.3]
Luminal B	36	20	38.0 [21.4 - 54.5]
Total	*98*	*37*	

Late Recurrence Analysis

In the previously described combined analysis data, the event rates within each risk group are not constant across the 10-year interval as can be seen in Figures 16B and 17B. To further understand DR in the late recurrence period, a post-hoc retrospective analysis of the combined data described above was conducted for the subset of patients who were free of distant recurrence through five years (a total of 2,163 patients[8]). Of these, 1,605 were node-negative patients and 488 were node-positive patients (1–3 positive nodes). For each node group, the values below the x-axis at year 5 in figures 20 and 21 show the number of patients by risk group at risk at five years, i.e., eligible for the late recurrence analysis.

Table 22 provides a summary of the treatment and clinical characteristics for the node-negative and node-positive (1–3 nodes) patients in the late recurrence analysis.

Table 22: Summary of Treatment and Clinical Characteristics for Late-Recurrence Analysis

Characteristic	Value	Node Negative (n = 1,605)		Node Positive (1–3 Nodes) (n = 488)	
		ABCSG8 (n = 944)	ransATAC (n = 661)	ABCSG8 (n = 311)	TransATAC (n = 177)
Treatment	Some Anastrozole	480	346	153	89
	Tamoxifen Only	464	315	158	88
Grade	Well	192	158	46	36
	Moderate	752	394	265	105
	Poor	0	109	0	36
Tumor Size	≤ 1 cm	204	116	35	11
	1–2 cm	526	376	165	74
	2–3 cm	183	139	90	64
	> 3 cm	31	30	21	28
HER2 Status	Negative	888	590	300	157
	Positive	56	71	11	20
Recurrences	Distant	41	40	28	29
	Any	71	78	37	37
NanoString Intrinsic Subtype	Luminal A	674	488	218	112
	Luminal B	245	150	87	54
	Basal-Like	4	5	0	1
	HER2-Enriched	21	18	6	10

The primary objective was to assess the ability of the ROR Score to provide significant additional prognostic information for DRFS over and above standard clinical variables in years 5 to 10. A null model consisting of CTS alone was compared to an alternate model consisting of CTS and ROR using a likelihood ratio (LR) test. The ROR added statistically significant information for post 5-years DRFS over and above standard clinical variables for all patients ($p < 0.0001$) as well as for node-negative ($p < 0.0001$) and node-positive (1–3 nodes) patients ($p < 0.0001$).

Table 23 shows a summary of the hazard ratios for a 10 point change based on a univariate analysis and on a multivariate analysis which included both the ROR Score and CTS. The hazard ratios for the ROR Score are all significantly different from 1 even after adjustment for CTS. C-indexes are also shown in Table 22. For both groups, the C-index was significantly different from the no-information value of 0.5.

Table 23: Summary of Late Recurrence Testing

Number Positive Nodes	N	Hazard Ratio: 10 point ROR Score change		C-Index with 95% Confidence Intervals		
		Univariate Analysis	Multivariate Analysis	C-Index	Lower	Upper
0	1,605	1.38 [1.23-1.54]	1.29 [1.15-1.46]	70.1%	64.7%	75.5%
1–3	488	1.43 [1.25-1.63]	1.34 [1.16-1.53]	71.1%	64.0%	78.3%

The majority of the patients in the two studies were HER2-negative. Table 24 shows the distribution of HER2-status for the node-negative and the node-positive (1–3 nodes) women. For both groups, over 90% of the women in the studies were HER2-negative.

Table 24: Distribution of HER2-Status by Number of Positive Nodes

Patient Subset	HER2-Status		Total
	Negative	Positive	
Node-Negative Patients	1,478 (92.1%)	127 (7.9%)	1,605
Node-Positive Patients with 1–3 Positive Nodes	457 (93.6%)	31 (6.4%)	488

Table 25 shows a comparison of the multivariate model fitted to all patients in a given node group and the model fitted to all HER2-negtive patients in the group. There are no statistically significant differences.

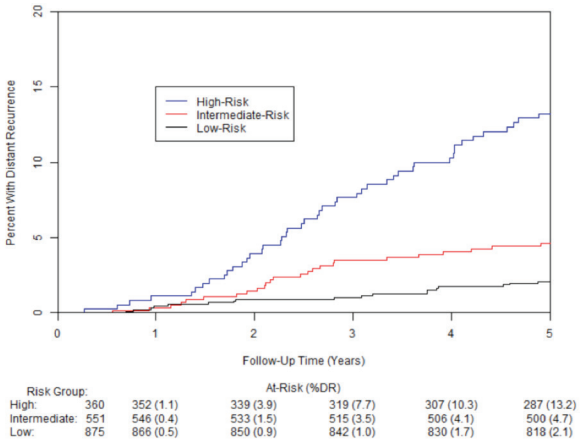
Table 25: Multivariate Hazard Ratios for 10 point ROR Score change: All Patients within Subgroup vs. HER2-Negative Patients Within Subgroup

Number of Positive Nodes	All patients [95% CI]	HER2-Negative patients [95% CI]
Node-Negative Patients	1.29 [1.15-1.46]	1.35 [1.19-1.54]
Node-Positive Patients with 1–3 Positive Nodes	1.34 [1.16-1.53]	1.29 [1.11-1.50]

The comparison across risk groups is further explored in Figures 20 and 21, which show incidence curves for early and late distant recurrence constructed by risk-group in node-negative and node-positive (1–3 nodes) patients, respectively. The incidence curves cover the early recurrence period (in the first 5 years) and late recurrence period (between 5 and 10 years after diagnosis). Immediately below the x-axis each figure displays the number of women at risk and the cumulative incidence. The summary tables below the figures show the confidence intervals for the cumulative DR rate at 5 years or 10 years for those women who were DR-free after the completion of 5 years of treatment. For node-positive (1–3 nodes) patients represented in Figure 21, the Low and Intermediate Risk groups have been combined given the small number of patients in the Low Risk group.

The Low Risk population has a low probability of recurrence between years 5 and 10 after 5 years of endocrine therapy as demonstrated by the cumulative incidence curves and the associated hazard ratios for each risk group. In contrast, the Intermediate and High risk populations have a persistent risk of late distant recurrence after 5 years of endocrine therapy. The difference in outcome between the Intermediate and High Risk node-negative populations is established in the first 5 years (DR rate = 13.2% [9.6% – 16.7%] for High-Risk and 4.7% [2.9% - 6.4%] for Intermediate Risk patients) and persists out to 10 years; however, the recurrence rates for the Intermediate and High Risk groups after 5 years of endocrine therapy are very similar.

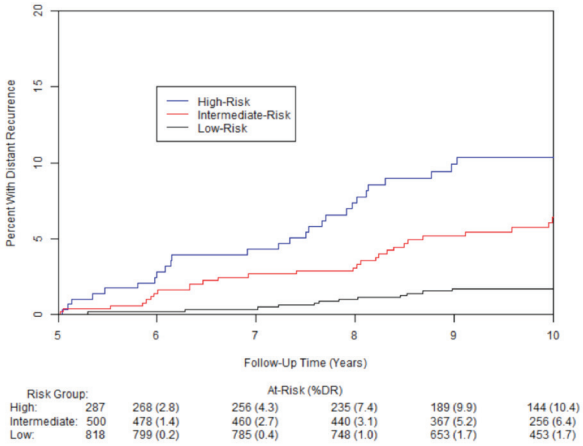
Figure 20A: Incidence Curves for Distant Recurrence by Risk Group from 0–5 years: Node-Negative Patients



Summary of Data for Figure 20A: Incidence Curves for Distant Recurrence by Risk Group from 0–5 years: Node-Negative Patients

DR Rates by Risk Group up to Five Years Completion of Treatment [95% Confidence Intervals]		
High	**Intermediate**	**Low**
13.2% [9.6% - 16.7%]	4.7% [2.9% - 6.4%]	2.1% [1.1% - 3.1%]

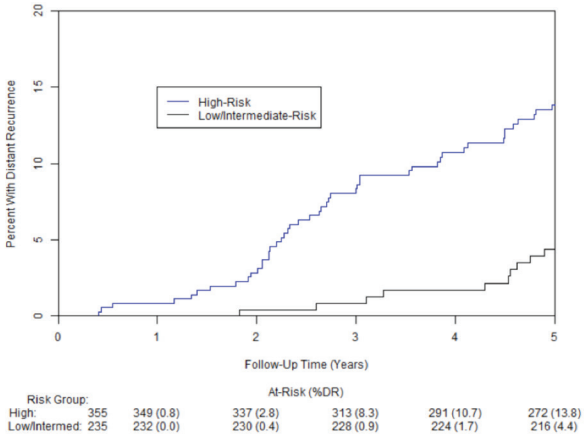
Figure 20B: Incidence Curves for Distant Recurrence by Risk Group from 5–10 years: Node-Negative Patients



Summary of Data for Figure 20B: Incidence Curves for Distant Recurrence by Risk Group from 5–10 years: Node-Negative Patients

DR Rates by Risk Group Five Years after DR-Free Completion of Treatment [95% Confidence Intervals]		
High	**Intermediate**	**Low**
10.4% [6.6% - 14%]	6.4% [4.1% - 8.7%]	1.7% [0.8% - 2.6%]

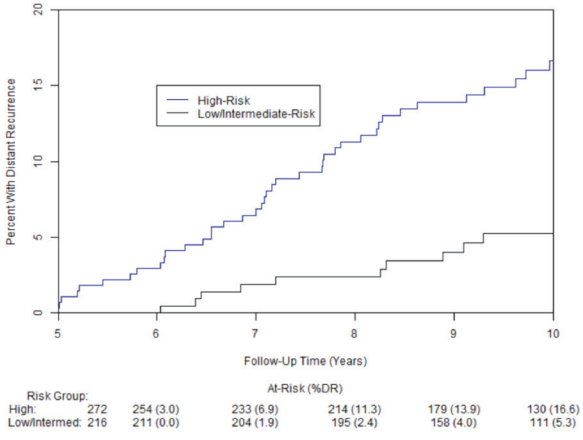
Figure 21A: Incidence Curves for Distant Recurrence by Risk Group from 0–5 years: Node-Positive (1–3 Nodes) Patients



Summary of Data for Figure 21A: Incidence Curves for Distant Recurrence by Risk Group from 0–5 years: Node-Positive (1–3 Nodes) Patients

DR Rates by Risk Group up to Five Years Completion of Treatment [95% Confidence Intervals]	
High	**Low / Intermediate**
13.8% [10.1% - 17.4%]	4.4% [1.7% - 7.0%]

Figure 21B: Incidence Curves for Distant Recurrence by Risk Group from 5–10 years: Node-Positive (1–3 Nodes) Patients



Summary of Data for Figure 21B: Incidence Curves for Distant Recurrence by Risk Group from 5–10 years: Node-Positive (1–3 Nodes) Patients

DR Rates by Risk Group Five Years after DR-Free Completion of Treatment [95% Confidence Intervals]	
High	**Low / Intermediate**
16.6% [11.7% - 21.3%]	5.3% [2.0% - 8.4%]

Combined Analysis Conclusions

The ROR Score was shown to add significant prognostic information in the late recurrence period between 5 and 10 years after diagnosis and above standard clinical variables in the combined study for patients who were distant recurrence-free through five years. Using risk groups defined at baseline for each of the node-count specific cohorts, the risk-groups were shown to classify the full set of patients into groups with significantly different late distant recurrence risk. Both the continuous and ROR Score-based risk group analyses showed similar prognostic information in various subgroups. No material differences were seen between the results using HER2-negative patients compared to all patients.

In each of the TransATAC and ABCSG-8 studies, the ROR had been demonstrated to add significant prognostic information over and above the standard clinical and treatment variables both when included as a continuous measure and when included using three pre-defined risk groups. The two studies had different risk profiles in the sense that the event rate was higher in the TransATAC study than in the ABCSG-8 study: this is clear by comparing the DRFS (%) in the control arms of ATAC (90.8%) and ABCSG8 (92.5%) reported in the literature[9,10]. This analysis combined the data from the two studies with equal weights to generate risk-profiles that are expected to be more generalizable to other patient populations than results from the individual studies.

Study 1: Prediction of risk of distant recurrence in postmenopausal women with node-negative or node-positive, hormone receptor positive early stage breast cancer treated with Arimidex or Tamoxifen: a TransATAC study

Study Design

The clinical validation study was designed to validate that the risk of recurrence score (ROR) provides additional prognostic information for Distant Recurrence-Free Survival (DRFS) over and above standard clinical variables using all available patient samples. This study used RNA isolated from FFPE breast tumor tissue from a subset of the patients who participated in the ATAC trial[11]. The ATAC trial included 9,366 patients across three trial arms (1:1:1) where patients were randomized to receive 5 years of endocrine therapy with 1 mg of anastrozole (i.e., arimidex) plus a tamoxifen placebo, 20 mg of tamoxifen plus anastrozole placebo, or a combination of tamoxifen/arimidex. The combination treatment group was discontinued after the initial analysis because it showed no efficacy or tolerability benefits over tamoxifen alone. A 10 year median follow up of the ATAC trial monotherapy arms was recently reported to satisfy FDA requirements for updated safety and efficacy information[9]. For hormone receptor-positive patients, there was a significant improvement in DFS (HR = 0.86), RFS (HR = 0.79), and DRFS (HR = 0.85) for those patients treated with anastrozole when compared to tamoxifen in this analysis. Absolute differences in distant recurrence free survival between anastrozole and tamoxifen increased over time from 2.7% at 5 years to 4.3% at 10 years. The TransATAC project was initiated in 2002 under the TA/01 protocol to establish a tissue bank from archival histopathology FFPE blocks from ATAC patients retrospectively[11].

A total of 2,006 blocks were obtained from the 4,160 women with hormone-receptor positive breast cancer that were randomized to the monotherapy arms of the ATAC trial. Of those FFPE blocks, 1,372 were collected from patients within the United Kingdom and contained sufficient invasive tumor for analysis using the Genomic Health® Oncotype Dx® test[12]. The Oncotype Dx Recurrence Score® (RS) was determined from the FFPE blocks, and the study results clinically validated the RS for estimating distant recurrence free survival in HR+, post-menopausal breast cancer patients treated with anastrozole or tamoxifen. The remnant RNA from the Oncotype Dx study was shipped to Royal Marsden Hospital in London where it was stored at -70°C. A total of 1,017 patients from the Oncotype Dx study had > 500 ng of RNA remaining and were tested by NanoString as part of the NanoString clinical validation study.

This study used the intrinsic subtypes generated by the assay and evaluated two versions of the ROR score using a pre-defined sequential approach. The two different ROR scores, each of which ranges from 0–100, were calculated by using either all 50 test genes as previously published[2] or a 46-gene subset. In each case, the coefficients were calculated from a Cox model that includes the Pearson correlation to the 50 or 46 genes used to calculate each intrinsic subtype, a proliferation score, and tumor size. All analyses were performed on 10-year follow-up data.

The primary endpoint was distant recurrence-free survival (DRFS). This was defined as the interval from diagnosis until distant recurrence or death due to breast cancer. The secondary endpoint was recurrence-free survival (RFS). This was defined as the interval from diagnosis until first recurrence (local, regional or distant) or death due to breast cancer.

Using all available patient samples, multivariate Cox proportional hazards (PH) models were fitted to evaluate the primary objective in sequential tests of ROR based on 50 and 46 genes. The model included the standard clinical covariates (age, tumor grade, tumor size, nodal status, adjuvant therapy). A Cox model was then fitted and a likelihood ratio test was used to test whether ROR added statistically significant (α = 0.05) additional prognostic information over and above that contained in Clinical Treatment Score (CTS). CTS is an optimized combination of clinicopathological factors developed by the clinical investigator as a measure of standard pathology[12]. The primary analyses were repeated for different patient subsets (all, node-negative, node-positive or HER2 negative only) and endpoints (DRFS or RFS).

For each of the node-negative and node-positive patients, Cox models (excluding CTS) were used to predict the 10-year risk of DR as a function of ROR. Based on these model predictions, three risk-groups were defined as:

Low Risk:	< 10% chance of DR by 10 years
Intermediate Risk:	10 - 20% chance of DR by 10 years
High Risk:	> 20% chance of DR by 10 years

Analysis

Kaplan-Meier plots were generated for each risk group. Likelihood ratio tests (used for comparing fit of two statistical models) as described in the primary analysis were performed for Genomic Health's Oncotype Dx test (RS, Recurrence Score) and the principal investigator's immunohistochemistry-based test (IHC4). These results were compared to those obtained for ROR to determine the extent to which each scoring system provides additional prognostic information over and above the CTS. The IHC4 results will not be discussed further as they are difficult to compare with the other assays because the IHC4 test was trained using the TransATAC study data.

Table 26: Summary of Demographics and Clinical Characteristics

Characteristic	Current Study (n = 1,007)		Initial Study from which RNA obtained (n = 1,231)	Single Agent Arms of ATAC Not included (n = 2,929)
	# Patients	% Patients		
Nodal Status				
Negative	701	70%	71%	68%
Positive	268	27%	25%	25%
Unknown	38	4%	4%	7%
Tumor Size				
≤ 1 cm	138	14%	67%	70%
1–2 cm	523	52%		
2–3 cm	253	25%	33%	30%
> 3 cm	93	9%		
Tumor Grade				
Well	213	21%	27%	25%
Moderate	601	60%	57%	59%
Poor	193	19%	16%	17%
Age				
Mean	64.4 yrs		64.3	66.1

Table 27: Additional Clinical Characteristics

Characteristic	Number of Patients	% of Patients
Subtype		
Basal-Like	9	1%
HER2-Enriched	41	4%
Luminal A	692	69%
Luminal B	265	26%
Treatment		
Anastrozole	513	51%
Tamoxifen	494	49%
Recurrences		
Any	210	21%
Distant	160	16%
HER2 Status		
Negative	888	88%
Positive	119	12%

Results

Primary analysis testing demonstrated that ROR score provides additional prognostic information for distant recurrence free survival over and above standard clinical variables (CTS). All reported ROR data that follows is based on 46 genes, as this is the basis for ROR as reported by the Prosigna Assay.

Table 28: Primary Analysis testing of ROR

Null Model	Alternate Model	ΔLR χ^2	χ^2 p-value
CTS	CTS + ROR	34.21	P < 0.0001

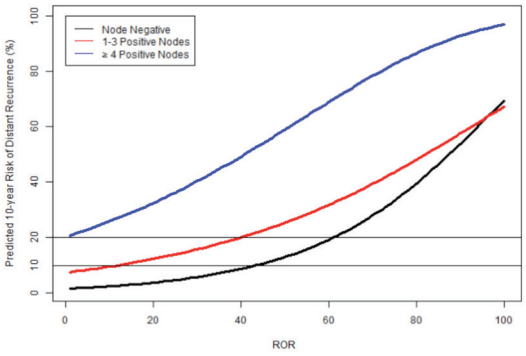
Secondary analyses demonstrated that ROR is significantly related to distant recurrence-free survival and adds prognostic information beyond CTS in multiple clinically relevant subgroups.

Table 29: Repeat of Primary Analysis Testing for Pre-Defined Subgroups

Subject Group	End-Point	# Patients	# Events	CTS+ROR vs. CTS	
				ΔLR χ^2	χ^2 p-value
All	DRFS	1007	160	34.2	< 0.0001
	RFS	1007	210	31.2	< 0.0001
HER2-Negatives	DRFS	888	131	28.9	< 0.0001
	RFS	888	179	26.9	< 0.0001
Node-Negatives	DRFS	739	79	25.0	< 0.0001
	RFS	739	117	21.5	< 0.0001
Node-Positives	DRFS	268	81	9.3	0.0023
	RFS	268	93	10.6	0.0011
HER2-Negative Node-Negatives	DRFS	649	62	24.6	< 0.0001
	RFS	649	98	20.8	< 0.0001

Primary and secondary analyses showed that ROR was continuously related to DRFS in all patients and in all subgroups.

Figure 22: Ten year predicted Distant Recurrence Risk estimated by analysis of ROR Score within Nodal Status Group



Secondary analyses demonstrated that the Luminal A and Luminal B subtypes had statistically significantly different outcomes within each subgroup of patients defined by nodal status.

Figure 23: Kaplan-Meier curves for DRFS for Node-Negative patients by Intrinsic Subtype

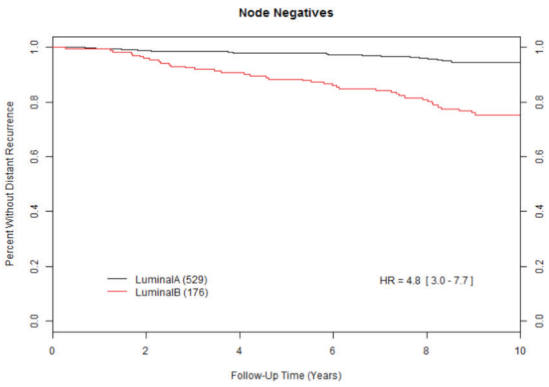
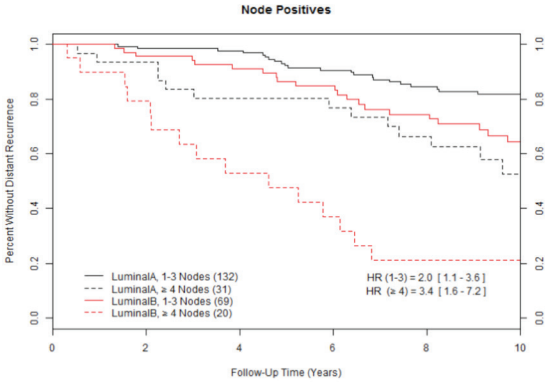
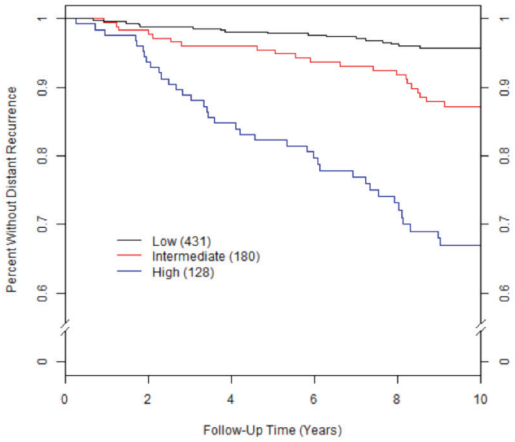


Figure 24: Kaplan-Meier curves for DRFS for Node-Positive patients by Intrinsic Subtype



Figures 25 and 26 demonstrate that within each nodal category, the absolute clinical risk of those patients predicted to be low risk was substantially different from the absolute clinical risk of the patients predicted to be high risk: the patients predicted to be low risk had observed 10-year DR rates of less than 10% while the patients predicted to be high risk had observed 10-year DR rates of greater than 30%.

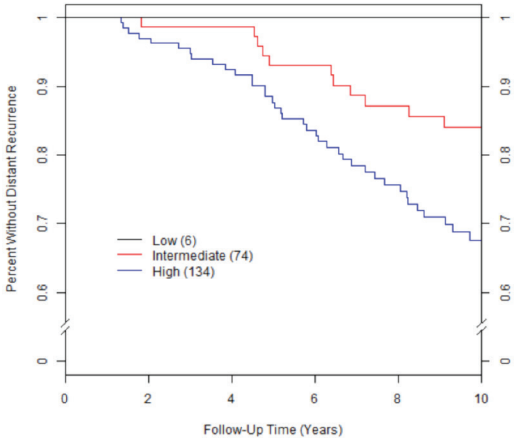
Figure 25: DRFS by Risk Group for Node-Negative Patients excluding CTS



Summary of Data for Figure 25: DRFS by Risk Group for Node-Negative Patients excluding CTS

Risk Group	Number of Patients (%)	Number of Events	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	431 (58%)	17	96% [94% - 98%]
Intermediate	180 (24%)	22	86% [81% - 92%]
High	128 (17%)	38	67% [59% - 76%]
Total	*739 (100%)*	*77*	

Figure 26: DRFS by Risk Group for Patients with 1–3 Positive Nodes Without CTS



Summary of Data for Figure 26: DRFS by Risk Group for Patients with 1–3 Positive Nodes Without CTS

Risk Group	Number of Patients (%)	Number of Events	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	6 (3%)	0	100% [N/A]
Intermediate	74 (35%)	11	84% [76% - 93%]
High	134 (63%)	38	68% [59% - 77%]
Total	*214 (100%)*	*49*	

Comparison of ROR with RS

Of the 1,007 samples with ROR scores, Oncotype Dx test results were available for all 1,007 samples but IHC results were only available for 940 samples. To allow comparison of all three tests, results in this section are based on the 940 samples that had test results for all three methods (however IHC4 is not reported here). The likelihood ratio tests are presented for the addition of a single variable, so for added information to be statistically significant ($\alpha = 0.05$), the change in the 1-degree of freedom χ^2 statistic must be greater than 3.84. The figures below show the information added when the prognostic test is added to another prognostic test plus CTS in sequence. At each addition, the added information is measured by the change in χ^2.

ROR added to RS in addition to CTS: prognostic information

Figure 27: Prognostic Information for DRFS beyond CTS in All Patients (n = 940)

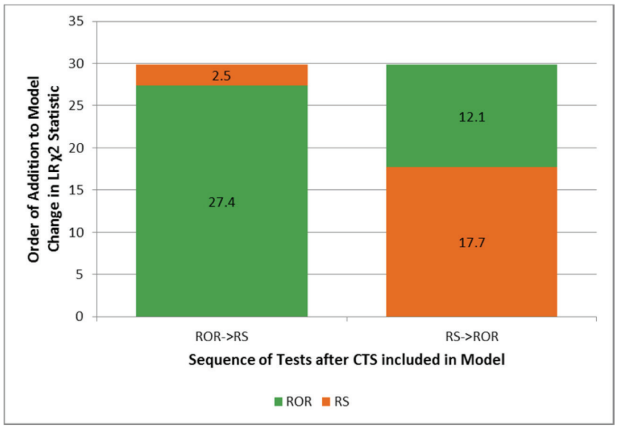


Figure 28: Prognostic Information for DRFS beyond CTS in Node-Negative Patients (n = 683)

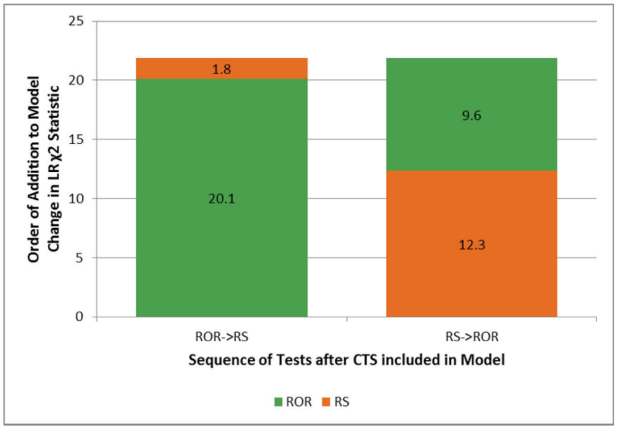


Figure 29: Prognostic Information for DRFS beyond CTS in Node-Positive Patients (n = 257)

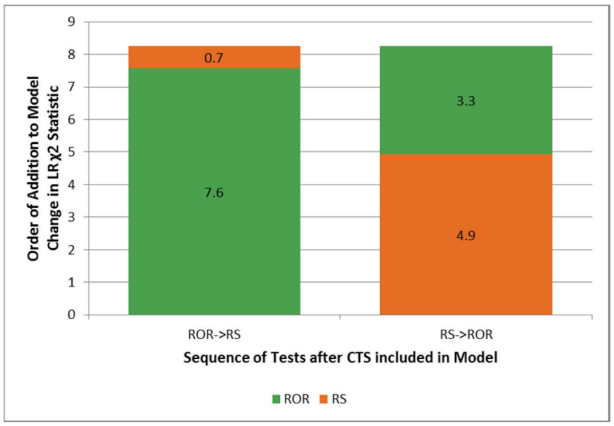
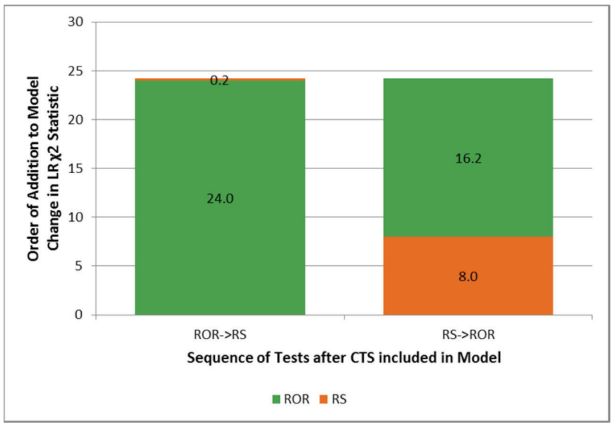


Figure 30: Prognostic Information for DRFS beyond the CTS in Node-Negative HER2-Negative Patients (n = 649)



Figures 27 through 30 show the information added beyond the CTS when the two prognostic tests are added in sequence. At each addition, the added information is measured by the change in the χ^2 statistic. For example, when ROR was the first test added after the inclusion of CTS (all patient data), the change in the χ^2 statistic was 27.4. With CTS and ROR in the model, adding RS yielded a change in the χ^2 statistic of 2.5, which is not significant (critical value for χ^2 test with 1 degree of freedom is 3.84); i.e., once CTS and ROR are both in the model, RS does not add significant information. However, if RS was the first test added, there was still information contained in the ROR that was not included in the combination of CTS and RS. Both tests show prognostic significance when added to CTS in node-positive patients, but neither of the tests shows significance as a second added test, possibly because of the smaller sample size. For the node-negative HER2-negative patient subset, RS does not add significant prognostic information to the CTS + ROR. On the other hand, ROR adds significant prognostic information to CTS + RS.

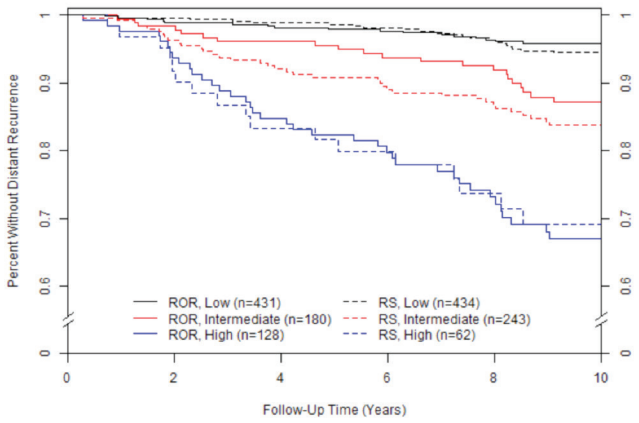
ROR vs. RS: outcome of risk groups

In order to compare how the two tests separated patients according to risk, risk groups were defined based on each test's estimate of the risk of distant recurrence at 10 years within the TransATAC population. Risk score thresholds to define the risk groups were chosen for each test based on the results of our TransATAC study in order to define risk groups that contain patients with the same risk. In order to achieve these comparable risk groups, the cut points used for Oncotype DX were different than those used by Genomic Health. For each test, the low risk group was prospectively defined as patients with less than a 10% estimated risk of recurrence. For each test, the intermediate risk group was prospectively defined as patients with between a 10% and 20% estimated risk of recurrence. For each test, the high risk group was prospectively defined as patients with greater than a 20% estimated risk of recurrence. The figure below summarizes the sizes and outcomes of the risk groups defined by each test.

Figure 31 illustrates the result that Prosigna assigned 26% fewer patients to the intermediate risk group than did Oncotype DX (180 patients vs. 243 patients). In addition, Prosigna assigned more patients to the high risk group than did Oncotype DX; however, the low risk and high risk groups defined by each test have similar outcomes as illustrated by the overlapping Kaplan-Meier curves.

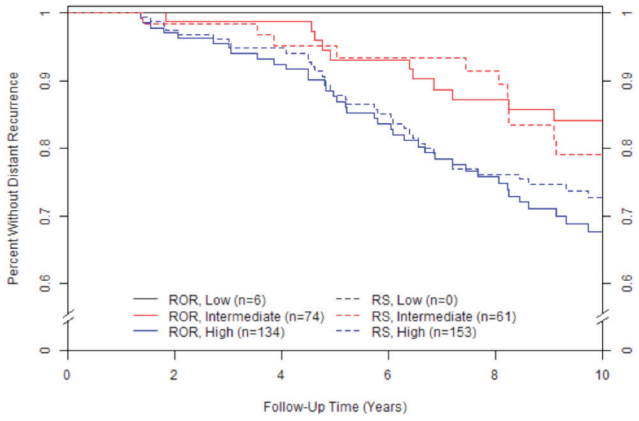
This observation led the independent investigators of our TransATAC study to conclude that Prosigna assigned fewer patients to the intermediate risk group than Oncotype DX RS, with equivalent or higher separation between the low and high risk groups.

Figure 31: Prosigna's ROR score identified substantially more high risk patients and fewer intermediate risk patients than Oncotype DX's RS score for node-negative patients.



When using ROR alone in node-positive patients with 1–3 positive nodes, there were 6 patients predicted to have a < 10% risk of distant recurrence. None of these patients had events over the course of the study. One of these patients was observed for 7.9 years and all others had no DR in at least 9.9 years of follow-up indicating that the node-positive patients predicted to be low-risk indeed were low-risk. The log-rank tests were not used for comparison as there was no low-risk group for RS.

Figure 32: Comparing 10-Year DRFS Risk-Group Classification without using CTS: Node-Positive Patients (1–3 nodes) (ROR vs. RS)



Clinical Study 1 Conclusions

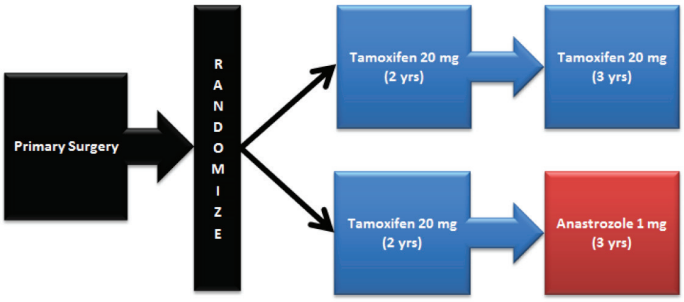
The primary analysis showed that ROR added significant prognostic information beyond that of the standard clinical covariates (CTS) in all patients and in all predefined clinically relevant subgroups. ROR was shown to subdivide patients into 3 risk groups, which have statistically significantly different outcomes in the node-negative patients. The Luminal A and Luminal B intrinsic subtypes were shown to have significantly different DRFS and RFS irrespective of nodal status. In comparison with the prognostic indicator RS (21-Gene Recurrence Score from Oncotype Dx), the ROR added prognostic information beyond RS in all patients and in clinically relevant subgroups. Further, in the node-negative group, ROR doubled the number of patients assigned to the high risk group, and substantially reduced the number of patients assigned to the intermediate risk group, without reducing the differences in outcomes between the low and high risk groups when compared with RS.

Study 2: Prognosis for Hormone Receptor-Positive, Post-menopausal Breast Cancer Patients Receiving Adjuvant Systemic Endocrine Therapy Alone Using the Prosigna Assay: an ABCSG-8 study

Study Design

The study cohort consists of FFPE breast tumor tissue samples retrospectively collected and archived in the ABCSG tumor bank from patients enrolled between 1996 and 2004 in the ABCSG-8 trial[13]. A total of 3,901 post-menopausal women with HR+, early stage breast cancer were randomized prior to treatment to two years of adjuvant Tamoxifen followed by three years of Arimidex® (anastrozole) or five years of adjuvant Tamoxifen. The treatment structure of the trial is shown in Figure 33.

Figure 33: Schematic of the ABCSG-8 trial Study Design



The validation cohort represents the fraction of the evaluable ABCSG-8 cohort for which tissue specimens could be collected from the retrospectively archived ABCSG tumor bank and for which informed consent could be obtained, or the patient was deceased. Patients who meet the eligibility criteria for the original trial were only excluded either because tissue was unavailable for NanoString's assay to be performed or the patient could not be re-consented. All samples with a tumor block and patient consent available were tested as part of this study.

This study used the intrinsic subtypes generated by the assay and evaluated ROR score using a pre-defined analysis plan. The ROR score, which ranges from 0–100, was calculated by using a 46-gene subset from the 50 test genes previously published[2]. The coefficients for ROR were calculated from a Cox model that includes the Pearson correlation to the 46 genes used to determine each intrinsic subtype, a proliferation score, and gross tumor size. All analyses were performed on maximal follow-up data.

The primary endpoint was distant recurrence-free survival (DRFS). This was defined as the interval from diagnosis until distant recurrence or death due to breast cancer. The secondary endpoint was recurrence-free survival (RFS). This was defined as the interval from diagnosis until first recurrence (local, regional, or distant) or death due to breast cancer.

Using all available patient samples, multivariate Cox Proportional Hazards (PH) models were fitted to evaluate the primary objective in sequential tests of ROR. The model included the standard clinical covariates (age, tumor grade, gross tumor size, nodal status, adjuvant therapy). A Cox model was then fitted and a likelihood ratio test was used to test whether ROR added statistically significant (α = 0.05) additional prognostic information over and above that contained in Clinical Treatment Score (CTS). CTS is an optimized combination of clinicopathological factors developed as a measure of standard pathology[12]. The primary analyses were repeated for different patient subsets (all, node-negative, node-positive, or HER2 negative only) and endpoints (DRFS or RFS).

Analysis

A sequential approach was used in which the primary scientific objective was to demonstrate that ROR adds significant prognostic information over and above the standard clinical variables. The primary objective added an additional requirement to demonstrate that categorical risk classification into one of three groups (low/intermediate/high) adds significant prognostic information over and above the standard clinical variables. To meet this requirement, both of the following had to be demonstrated:

- Show that the continuous ROR score adds prognostic value over and above the standard clinical variables
- If the null hypothesis of no prognostic information is rejected, show that the ROR- based risk categories add prognostic value over and above the standard clinical variables

Using all available patient samples, multivariate Cox Proportional Hazards (PH) models were fitted to evaluate the primary objective in sequential tests of ROR followed by pre-defined ROR-based risk categories. The models included the following categorical standard clinical covariates (with possible values):

- Age (≥ 65 or < 65)
- Grade (G1 or G2/GX)
- Gross Tumor Size (T1, T2/T3)
- Nodal Status (N0, N+(1–3), N+(≥ 4))
- Adjuvant Therapy (Tamoxifen alone, or Tamoxifen → Anastrozole)

where N0 denotes node-negative patients, N+(1–3) denotes node-positive patient with 1–3 positive nodes, and N+(≥ 4) denotes node-positive patients with 4 or more positive nodes. T1 indicates tumor ≤ 2 cm across, T2 indicates tumor greater than 2 cm but no more than 5 cm across, and T3 indicates tumor more than 5 cm across. There were only 14 T3 specimens in the study, so these were combined with the T2 specimens. The well-differentiated (G1) tumors were compared to the combination of moderately differentiated (G2) and GX lobular tumors. GX lobular tumors were treated as G2 tumors for the purpose of analysis because G2 tumors are the most common grade in this intended use patient population.

These covariates are entered into the model in the form of a Clinical Treatment Score (CTS). To obtain the CTS, the following model was fitted:

$$\lambda(t) = \lambda_0(t)\exp(\sum_j z_j \gamma_j)$$

Where the z's represent the clinical and treatment variables listed above and the CTS was defined using the estimates of the γ's obtained above; i.e., $CTS = \sum_j z_j \hat{\gamma}_j$.

The proportional hazards assumption was tested using the Schoenfeld residuals.

Patients included in the validation study had similar characteristics to those in the original ACBSG-8 study.

Table 30: Summary of Clinical Characteristics

Characteristic	Value	Included (n = 1,478)		Not Included (n = 2,236)		Total (n = 3,714)	
		#	%	#	%	#	%
Treatment	Tamoxifen Only	741	50.1%	1108	49.3%	1,849	49.8%
	Tamoxifen → Anastrozole	737	49.9%	1128	50.2%	1,865	50.2%
ER Status	Negative	14	0.9%	32	1.4%	46	1.2%
	Positive	1,464	99.1%	2,199	98.3%	3,663	98.6%
	Unknown	0	0.0%	5	0.2%	5	0.1%
Grade	G1	271	18.3%	468	20.8%	739	19.9%
	G2	1,152	77.9%	1659	73.9%	2,811	75.7%
	GX	55	3.7%	109	4.9%	164	4.4%
Nodal Status	N0	1047	70.8%	1723	76.7%	2,770	74.6%
	N+(1–3)	382	25.8%	449	20.0%	831	22.4%
	N+(≥ 4)*	49	3.3%	64	2.8%	113	3.0%
PgR Status	Negative	260	17.6%	424	18.9%	684	18.4%
	Positive	1,218	82.4%	1,805	80.4%	3,023	81.4%
	Unknown	0	0.0%	7	0.3%	7	0.2%
Tumor Stage	T1	1,037	70.2%	1745	77.7%	2,782	74.9%
	T2	427	28.9%	472	21.0%	899	24.2%
	T3	14	0.9%	19	0.8%	33	0.9%
Age	Median	63		NA		64	
	Range	41-79				41 – 80	

* Includes one patient with > 9 positive nodes

Table 31: Additional Clinical Characteristics

Characteristic	Value	Number of Patients	% of Patients
NanoString Intrinsic Subtype	LuminalA	1,004	67.9%
	LuminalB	418	28.3%
	HER2-Enriched	48	3.2%
	BasalLike	8	0.5%
Recurrences	Distant	155	10.5%
	Any	194	13.1%
HER2 Status	Negative	1,397	94.5%
	Positive	77	5.2%
	Unknown	4	0.3%

Results

Of the 1,620 tissues available for testing, 25 (1.5%) did not pass pre-defined pathology review for adequate tumor, 73 of the 1,595 tissue samples (4.6%) with viable invasive tissue did not pass pre-defined QC specifications for extracted RNA, and 44 of the 1,522 RNA samples (2.9%) failed assay QC specifications for Prosigna results, leaving a total of 1,478 (91.2%) available for analysis.

Of the 1,478 patients available for analysis, 155 had distant recurrences and 194 had local or distant recurrence or death due to breast cancer. The median follow-up for the trial was 10 years.

Primary analysis testing demonstrated that ROR score provides significant additional prognostic information for distant recurrence free survival over and above standard clinical variables (CTS).

Table 32: Summary of Primary Analysis Testing

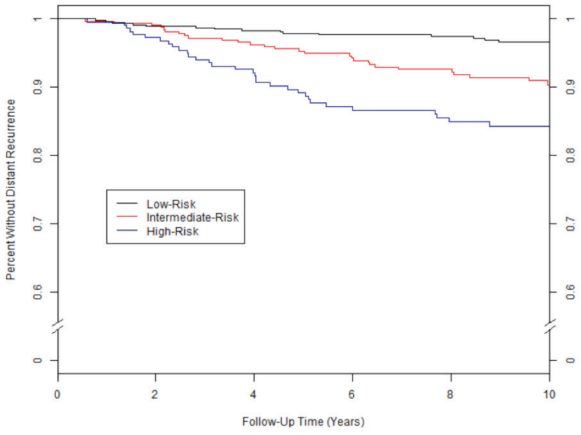
Null Model	Alternate Model	ΔLR χ^2	χ^2 Critical Value(Degrees of freedom)	χ^2 p-value
CTS	CTS + ROR	53.49	3.84 (df = 1)	p < 0.0001
CTS	CTS + Risk Group	34.12	5.99 (df = 2)	p < 0.0001

Secondary analyses demonstrated that ROR is significantly related to distant recurrence free survival and adds prognostic information beyond CTS in multiple clinically relevant subgroups.

Table 33: Repeat of Primary Analysis Testing for Pre-Defined Subgroups

Subject Group	# Patients	# Events	CTS+ROR vs. CTS	CTS+Risk-Group vs. CTS
			ΔLR χ^2 (Crit. Value= 3.84)	ΔLR χ^2 (Crit. Value = 5.99)
All	1,478	155	53.49	34.12
HER2-negative	1,397	145	47.50	29.94
N0	1,047	86	25.57	23.36
N0, HER2-negative	984	79	21.69	20.32
N+(1–3)	382	59	25.99	19.94
N+(1–3), HER2-negative	367	56	22.75	18.75

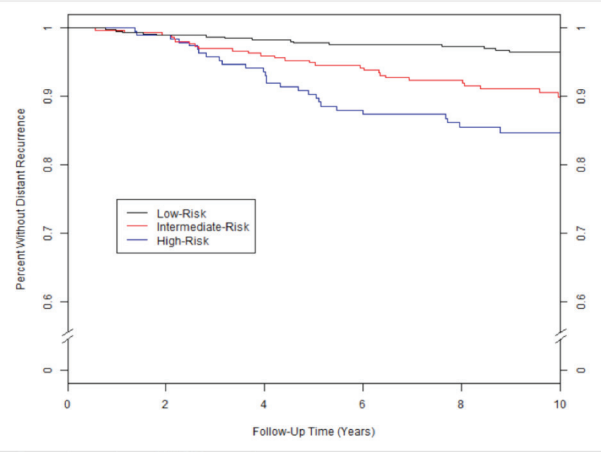
Figure 34: DRFS by Risk Group for Node-Negative Patients



Summary of Data for Figure 34: DRFS by Risk Group for Node-Negative Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	487 (47%)	15	96.6% [94.4% - 97.9%]
Intermediate	335 (32%)	28	90.4% [86.3% - 93.3%]
High	225 (21%)	32	84.3% [78.4% - 88.6%]
Total	*1,047 (100%)*	*75*	

Figure 35: DRFS by Risk Group for HER2-Negative Node-Negative Patients

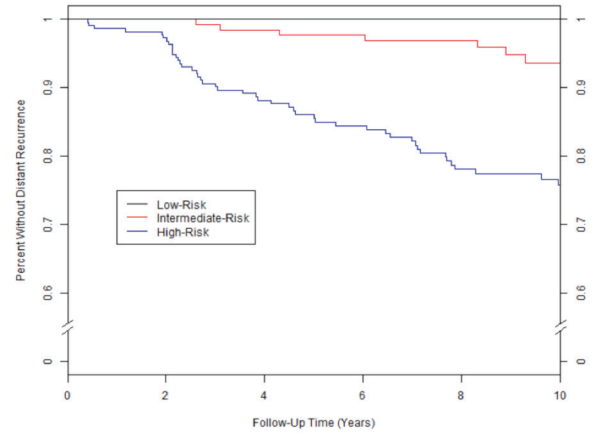


Summary of Data for Figure 35: DRFS by Risk Group for HER2-Negative Node-Negative Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	474 (48%)	15	96.5% [94.3% - 97.9%]
Intermediate	311 (32%)	27	90% [85.6% - 93.1%]
High	199 (20%)	27	84.7% [78.4% - 89.3%]
Total	*984 (100%)*	*69*	

Figure 36 shows the Kaplan-Meier plots by risk-group for node-positive (1–3 nodes) patients and Figure 37 shows the same plots for node-positive (1–3 nodes) HER2-negative patients. The results with and without the HER2-positive patients are similar.

Figure 36: DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

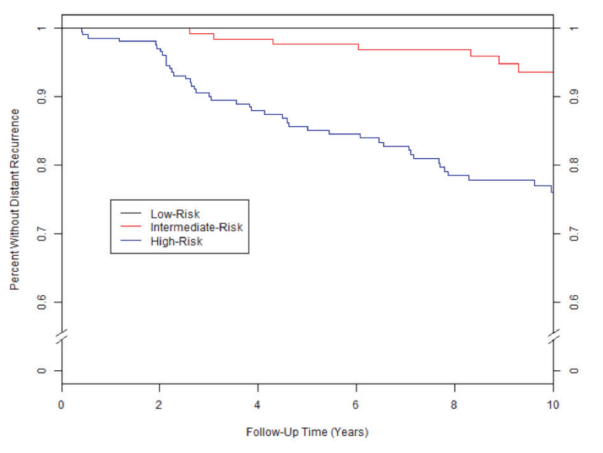


Summary of Data for Figure 36: DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	15 (4%)	0	100% [78.2% - 100%]*
Intermediate	143 (37%)	7	93.6% [86.9% - 97%]
High	224 (59%)	46	75.8% [68.9% - 81.4%]
Total	*382 (100%)*	*53*	

* Confidence Interval estimated using Clopper-Pearson Method

Figure 37: DRFS by Risk Group for HER2-Negative Node-Positive (1–3 nodes) Patients



Summary of Data for Figure 37 DRFS by Risk Group for HER2-Negative Node-Positive (1–3 nodes) Patients

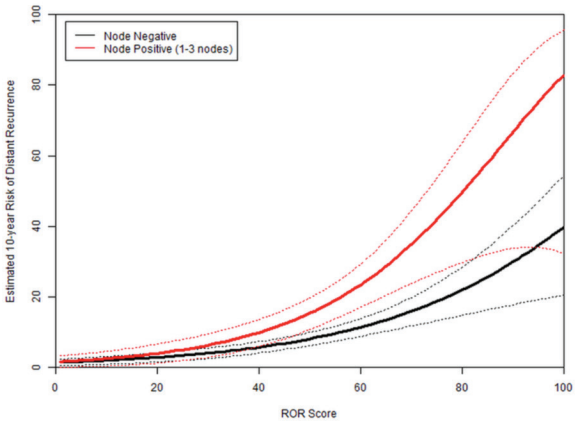
Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	15 (4%)	0	100% [78.2% - 100%]*
Intermediate	142 (39%)	7	93.6% [86.8% - 96.9%]
High	210 (57%)	43	76.1% [69.0% - 81.8%]
Total	*367 (100%)*	*50*	

* Confidence Interval estimated using Clopper-Pearson Method.

Relationship between ROR and Risk Prediction

Figure 38 shows the 10-year risk of DR as a function of ROR score with 95% confidence intervals based on separate Cox proportional hazards models for each of the node-negative and node-positive (1–3 nodes) patient groups. For the node-positive (1–3 nodes) patients, the proportional hazards assumption was violated when fitting across the entire range. The curve shown here for the node-positive (1–3 nodes) patients uses node-positive (1–3 nodes) patients with ROR scores in the range of 0–80, for which the proportional hazards assumption was met.

Figure 38: Ten year Estimated DR Risk by Nodal Category with 95% Confidence Intervals



Within each subgroup, the absolute clinical risk of those patients assigned to the low risk category was substantially different from the absolute clinical risk of the patients assigned to the high risk category.

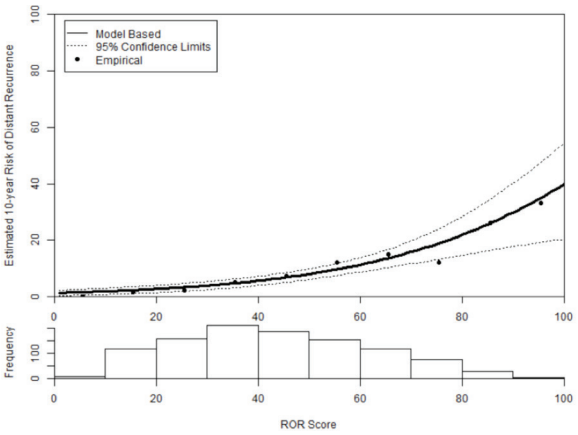
Table 34 shows the distribution of Node-Negative patients by 10-unit ROR bins. Also shown is the 10-year DR risk.

Table 34: Distribution of Node-Negative patients by 10-unit ROR Range

ROR Range	Number of Patients	Percent of Patients	10-year DR Risk (Empirical)
1–10	7	0.7%	0.0%
11–20	116	11.1%	1.8%
21–30	155	14.8%	2.5%
31–40	209	20.0%	5.1%
41–50	183	17.5%	7.5%
51–60	152	14.5%	12.1%
61–70	116	11.1%	15.0%
71–80	77	7.4%	12.3%
81–90	28	2.7%	26.1%
91–100	4	0.4%	33.3%
Total	*1,047*	*100%*	

Figure 39 shows the model-based curve for the Node-Negative patients together with the empirically estimated 10-year survival rates for the 10 bins where each bin consists of all patients within the 10-unit ROR ranges (1–10, 11–20 etc.). Below the curve is a histogram showing the frequency distribution by bin.

Figure 39: Comparison of Model-Based and Empirical Estimates of Ten-Year DR Risk for Node-Negative Patients with Distribution of ROR Scores shown below



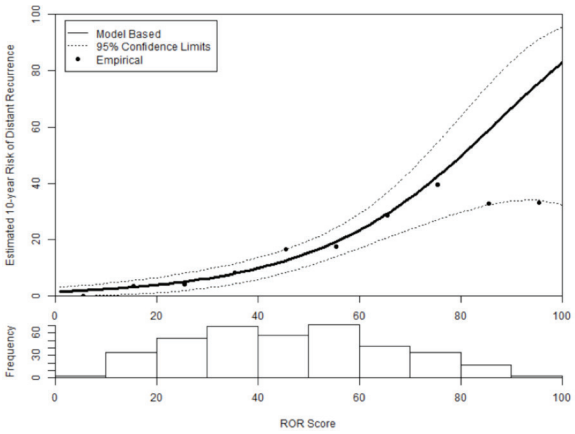
For the node-negative patients, the proportional hazards model-based estimates were similar to the empirical estimates across the entire range. Table 35 shows the distribution of node-positive (1–3 nodes) patients by 10-unit ROR bins. Also shown is the 10-year DR risk.

Table 35: Distribution of Node-Positive (1–3 nodes) patients by 10-unit ROR Range

ROR Range	Number of Patients	Percent of Patients	10-year DR Risk (Empirical)
1–10	3	0.8%	0.0%
11–20	34	8.9%	3.6%
21–30	53	13.9%	4.1%
31–40	68	17.8%	8.5%
41–50	57	14.9%	16.7%
51–60	71	18.6%	17.8%
61–70	42	11.0%	28.9%
71–80	34	8.9%	39.5%
81–90	17	4.5%	33.0%
91–100	3	0.8%	33.3%
Total	*382*	*100%*	

Figure 40 shows the model-based curve (using Node-Positive (1–3 nodes) patients with ROR scores ≤ 80) for the node-positive (1–3 nodes) patients together with the empirically estimated 10-year survival rates for the 10 bins where each bin consists of all patients within the 10 ROR ranges (1–10, 11–20, etc.). Below the curve is a histogram showing the frequency distribution by bin.

Figure 40: Comparison of Model-Based and Empirical Estimates of Ten-Year DR Risk for Node-Positive (1–3 nodes) Patients with Distribution of ROR Scores shown below



Both Table 35 and Figure 40 show the flattening of the observed 10-year risk at the top of the ROR range that led to the failure of the proportional hazards assumption. However, it should be noted that the sample sizes in the two bins above 80 were both small for the node positive (1–3 nodes) patients (17 patients from 81–90 and only 3 from 91–100).

Comparison of Luminal A and Luminal B Intrinsic Subtypes

The majority of the subjects in the study (96%) were either Luminal A or Luminal B, which was not unexpected as these intrinsic subtypes predominate in hormone receptor-positive patients[12].

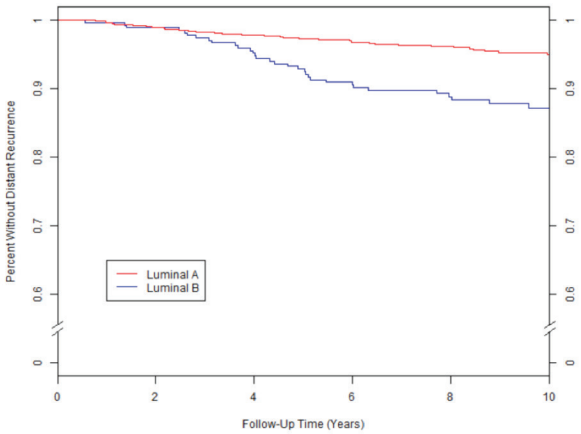
Table 36 shows the results of the likelihood ratio test for showing the additional prognostic value for DRFS that the Luminal A/Luminal B distinction adds beyond the CTS. The table also shows the hazard ratio comparing Luminal A to Luminal B patients. There was a significantly lower risk of distant recurrence for Luminal A patients in all three groups.

Table 36. Likelihood Ratio Test for DRFS Prognostic Value of Luminal Subtypes

Subgroup	# Patients	# Events	ΔLR χ^2	χ^2 p-value	Hazard Ratio of LumA : LumB (95% CI)
All	1,422	135	24.42	< 0.0001	0.42 [0.30 – 0.59]
N0	1,009	74	9.68	0.0019	0.47 [0.30 – 0.75]
N+(1–3)	366	51	14.94	0.0001	0.33 [0.19 – 0.58]

Figure 41 shows a comparison of DRFS by Luminal subtype for Node-Negative patients, Figure 42 shows the same comparison for node positive (1–3 nodes) patients. For both groups, there were significant differences between the DRFS of Luminal A and Luminal B patients.

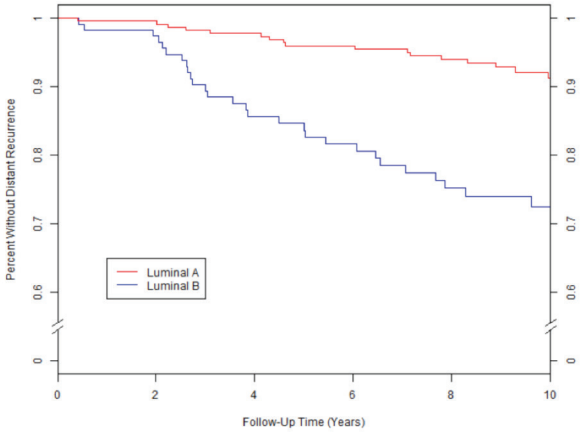
Figure 41: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Negative Patients



Summary of Data for Figure 41: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Negative Patients

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Luminal A	725	32	95.1% [93.4% - 96.3%]
Luminal B	284	32	87.2% [83.2% - 90.3%]
Total	1,009	*64*	

Figure 42: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Positive (1–3 nodes) Patients



Summary of Data for Figure 42: Kaplan-Meier curves for DRFS by Intrinsic Subtype for Node-Positive (1–3 nodes) Patients

Risk Group	Number of Patients	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Luminal A	248	17	91.3% [87.2% - 94.2%]
Luminal B	118	28	72.5% [64.2% - 79.1%]
Total	366	*45*	

Table 37 shows a table of 10-year RFS rates by luminal subtype for the node-negative and node-positive (1–3 nodes) nodal groups.

Table 37: Ten-year RFS rates by Nodal Group and Luminal Subtype

Node Status	Luminal Subtype	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
N0	Luminal A	725 (72)	44	93.0% [91.1% - 94.5%]
	Luminal B	284 (28)	44	82.2% [77.6% - 85.9%]
N+(1–3)	Luminal A	248 (68)	21	89.1% [84.7% - 92.4%]
	Luminal B	118 (32)	30	71.6% [62.2% - 77.4%]

For each of node-negative and node-positive (1–3 nodes) patient populations, the difference between Luminal A and Luminal B patients was significant.

Clinical Study 2 Conclusions

The ROR was demonstrated to add significant prognostic information over and above the standard clinical and treatment variables both when included as a continuous measure and when included using three pre-defined risk groups. The low-risk group had 10-year DRFS well above 90% as anticipated. The high-risk group had 10-year DRFS of 80% which was higher than anticipated—it had been expected to be demonstrably lower than 80%. The cutoffs that were used to define risk-groups were based on the TransATAC cohort, which is higher risk than the current cohort, leading to an overall lower risk "high-risk group" than was anticipated. The ROR (continuous and risk-group based) showed similar prognostic information in various subgroups. The continuous risk model closely fit the empirical rates of recurrence in both node-negative and node-positive (1–3 nodes) patient populations. Most patients (96%) in the study had tumors of one of two luminal subtypes (Luminal A or Luminal B). In all nodal status groups, the Luminal A/Luminal B distinction added prognostic information regarding DRFS.

Combined Clinical Studies Summary

Results are generalizable for distributed use as samples were sent to and analyzed in different labs in the two clinical validation studies. The ROR was demonstrated to add significant prognostic information for 10 year DRFS over and above the standard clinical and treatment variables both when included as a continuous measure and when included using three pre-defined risk groups. Additionally, in a post-hoc analysis, the ROR added significant information for post 5-years DRFS over and above standard clinical variables for all patients. The ROR (continuous and risk-group based) showed similar prognostic information in various subgroups. Limited analyses were also performed using RFS. The ROR classes were also able to define three risk groups with distinct RFS. For both studies, there were significant differences between the DRFS of Luminal A and Luminal B subgroups, independent of nodal status.

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18 SYMBOLS AND DEFINITIONS

- Manufacturer

EC REP - Authorized Representative in the European Community

IVD - *In vitro* diagnostic medical device

- Consult Instructions for Use

CE - CE Mark

LOT - Batch code / Lot number

REF - Catalogue or Reference number

- Contains sufficient for <n> tests

- Temperature range storage conditions

- Lower limit of temperature storage conditions

- Upper limit of temperature storage conditions

- For Use by / Expiry Date

- This side-up

Room Temp. = Room Temperature
HYB = Hybridization

Regulatory Disclaimer
For *in vitro* diagnostic use.

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