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Prosigna.
BREAST CANCER ASSAY

Package Insert
Prosigna® Breast Cancer Prognostic Gene Signature Assay

IVD

Version 02, created 2022-12

Σ
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

Caution: Federal law restricts this device to sale by or on the order of a physician

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1 INTENDED USE / INDICATIONS FOR USE

The Prosigna® Breast Cancer Prognostic Gene Signature Assay is an in vitro diagnostic assay which is performed on the IVD enabled nCounter® system using formalin-fixed paraffin-embedded (FFPE) breast tumor tissue previously diagnosed as invasive breast carcinoma. This qualitative assay utilizes gene expression profiling on the PAM50 gene set to generate an intrinsic molecular subtype* (luminal A, luminal B, HER2-enriched, or basal-like) and a proliferation score* which are weighted together with tumor size to provide a risk of recurrence score (ROR), and then lymph node status to generate an associated risk category. The risk category and ROR score are intended to be used to assess a patient's risk of distant recurrence of disease. The intrinsic molecular subtype and proliferation score are not intended to be used for treatment decisions.

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

1. 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.

2. 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), Stage II breast cancer to be treated with adjuvant endocrine therapy alone, when used in conjunction with other clinicopathological factors. The device is not intended for patients with 4 or more positive nodes.

* *The intrinsic molecular subtype and proliferation score are only reported as constituent components of the ROR. Their correspondence with histopathological subtyping is not established.*

2 SUMMARY OF THE TEST SYSTEM

The nCounter® Dx 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 in the PAM50 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. In addition, a Reference Sample is included in the Prosigna kit 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 slide, and isolates RNA from the tissue. The isolated RNA is then tested on the nCounter® Dx Analysis System to provide test results including the Risk of Recurrence (ROR) Score and risk category.

2.1 Principles of the nCounter® Dx Analysis System

The nCounter® Dx 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

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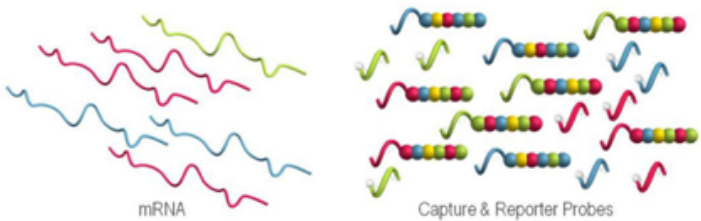
After hybridization, all of the sample purification steps are automated on the nCounter Prep Station. First, excess Capture and Reporter Probes are removed (Figure 2) using successive magnetic bead capture steps followed by binding of the probe-target complexes to random locations on the surface of the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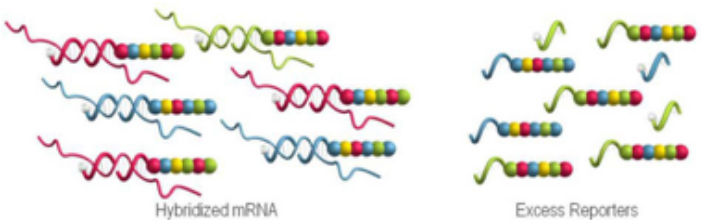
Figure 2: Remove Excess Reporters

Hybridized mRNA | Excess Reporters

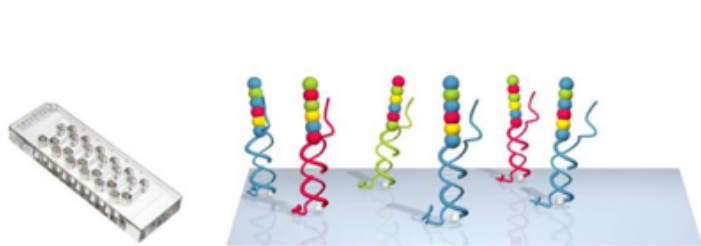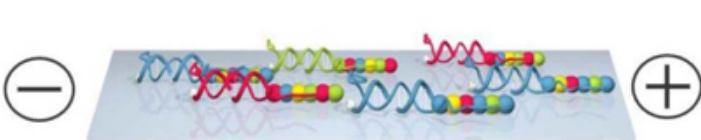**Figure 3: Bind Hybridized Reporters to Surface of Cartridge**

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® Dx 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 PAM50 molecular subtypes determined by the tumor's molecular profile[3]. The prototypical gene expression profiles (e.g. centroids) of the four PAM50 molecular subtypes were retrained on the nCounter® Dx 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 four PAM50 centroids.

The algorithm reports a risk category (low, intermediate, or high) based on both ROR Score, and nodal status. The ROR Score is reported on a 0–100 scale, 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 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 PAM50 centroid, a proliferation score that is derived from the expression levels of a subset of the genes related to proliferation, 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 reported based on cut-offs by nodal status for ROR Score which were validated 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 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.

Catalog 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

To be used in conjunction with the Roche FFPET RNA Isolation Kit (Roche-FFPET-025) provided by NanoString.

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 Covers — *adhesive films*
nCounter Tip Sheaths — *6–well tip holders*

4 WARNINGS AND PRECAUTIONS

1. Prosigna is not indicated as a standalone test to determine the outcome of disease, nor to suggest an individual patient's likely response to any specific therapy. Results should be taken in the context of other relevant clinico-pathological factors and standard practice of medicine.
2. For In Vitro diagnostic use.
3. This assay is intended to be run by operators trained in molecular biology techniques in high complexity CLIA labs.
4. 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.
5. Any remnant reagents should not be reused in the Prosigna assay.

6. Discard any reactions with compromised hybridization times or temperatures.
7. 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.
8. Failure to store reagents under the conditions stated on the label could adversely affect assay performance.
9. Always wear gloves while handling reagents and samples.
10. Avoid RNase contamination, which may negatively affect the quality of the results.
11. 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.
12. Never pipet by mouth.
13. Avoid reagent contact with eyes, skin and mucous membranes.
14. 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.
15. 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.
16. Additional instrument-specific disposal information can be found in the nCounter® Dx Analysis System User Manual and Service Manuals for the Prep Station and Digital Analyzer.
17. Material Safety Data Sheet information for Reporter CodeSet, Capture ProbeSet, Hybridization Buffer and Prep Plates can be found at www.prosigna.com.
18. All hazardous materials should be disposed of according to your institution's guidelines for hazardous disposal.
19. Any unused CodeSet should be discarded.
20. If a patient's gross 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 mis-classification).
21. 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).
22. 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 an 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.

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 or 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 laboratory personnel trained in molecular biology techniques in high complexity CLIA labs. Please contact Veracyte for training information specific to running the Prosigna Assay.

7 WASTE HANDLING

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

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 stability of a Prosigna kit lot is currently 8 months.

- 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 (15–25°C).

9 INSTRUMENTS REQUIRED FOR PROSIGNA

- nCounter® Dx Analysis System (Catalog numbers 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® Dx Analysis System User Manual for additional information.

10 REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

10.1 Materials

1. Hematoxylin and Eosin (H&E)
2. Positively charged glass microscope slides
3. D-Limonene clearing agent (histology grade)
4. 100% Ethanol (Absolute), ACS grade or equivalent (not less than 99.5%)
5. Glycerol, molecular biology grade
6. Molecular grade, nuclease-free water
7. 10% SDS, molecular biology grade
8. Razor Blades (or disposable scalpel)
9. Disposable Microtome Blades
10. 0.5 mL screw cap and 1.5 or 1.7 mL nonstick RNase-free microcentrifuge tubes
11. RNase free Micropipette tips with aerosol barrier

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 a standard 1.5/2.0 mL microcentrifuge 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 cover (approximate inside dimensions of 3.6 x 2.8 x 2.4" (91 x 71 x 60 mm)); quantity of 3 required
9. Slide rack (holds up to ten 3" x 1" (75 x 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 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 microplate adapter (see specifications below)
18. Coplin jar

10.3 Equipment Specifications

Table 1a: Full-Spectrum Microvolume 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 1b: Microvolume Photodiode UV-Vis Spectrophotometer for Nucleic Acid Quantitation

Design Feature	Specifications
Sample Volume Range	1–2µL
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 2: Heat Block with Heated Lid for Assay Hybridization

Design Feature	Specifications
Heat Block Design	• Must fit regular profile, 12 x 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 3: Centrifuge with microplate carrier to spin nCounter Prep Plates

Design Feature	Specifications
Centrifugation Speed	Minimum of 2000 x *g*
Rotors	4 x 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 ≥ 10%*
    b. The circled tumor surface area on the H&E stained slide must be ≥ 4 mm².
    *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 mm² 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 mm² surface area, it is more likely that RNA input requirements will not be met.

Table 4. Recommended slide requirements based on tumor surface area

Measured Tumor Surface Area on H&E Stained Slide (mm²)	Number of unstained slides
4–19	6
20–99	3
≥ 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 slides overnight at 45°C.

11.4 Slide Processing

1. Prepare a 3% glycerol working solution by mixing 1.5 mL of the 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 Preparation of Reagents for RNA Isolation

See Roche FFPET RNA Isolation Kit Package Insert for Contents

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.

11.6 Isolation of RNA

Please reference the Roche FFPET RNA Isolation Kit Package Insert for appropriate storage, safety and handling instructions. Please utilize the following specific instructions for isolation of RNA for the Prosigna Assay as they have been validated specifically for use with Prosigna.

Tissue Digestion

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, 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

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, 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 microfuge 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 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.
25. 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.

11.7 Measure the RNA Concentration and Quality

1. 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.
2. Follow the spectrophotometer manufacturer's instructions for measuring RNA.

3. If any sample fails to meet the minimum RNA purity or concentration metrics, 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.
4. The RNA extraction may be repeated if the minimum concentration or minimum purity specifications are not met (Table 5). Users can choose to isolate additional slides from the same FFPE block or choose a separate block from the same patient.
5. If the concentration of RNA 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.
6. Freeze the RNA at −70°C or below if the Prosigna Assay cannot be completed within the same work day.

Table 5. RNA Quality Control Specifications

Quality Control	Quality Control Specification
RNA concentration	≥ 12.5 ng/µL
RNA purity	A_{260}/A_{280} ratio between 1.7 and 2.3

11.8   Assay Procedure

This assay procedure describes the steps necessary to perform the Prosigna assay using the nCounter® Dx 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
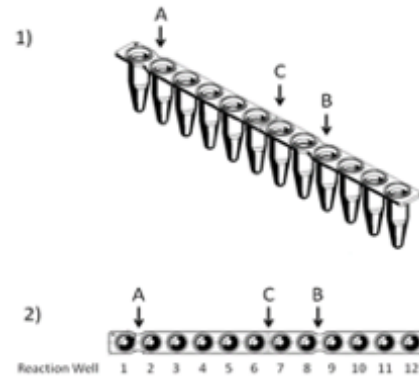
2. Calculate the amount of RNA and water (when required) to be added to the hybridization reaction for each sample within the batch.
   a. The recommended RNA input is 250 ng for the assay. The acceptable RNA input range for hybridization is 125–500 ng.
   b. Calculate the volume (in microliters) of 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® Dx Analysis System Services web application. The user may refer to the User Manual for instructions on using the nCounter® Dx 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® Dx 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 (0, 1–3).
   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 the number of samples to be tested exceeds the number of positions available in the RunSet ID, use a different test configuration code 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 are 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® Dx 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 add the Capture ProbeSet to the master mix AND do not store completed 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 tubes 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 a 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: Be sure to 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 Cartridge 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. 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
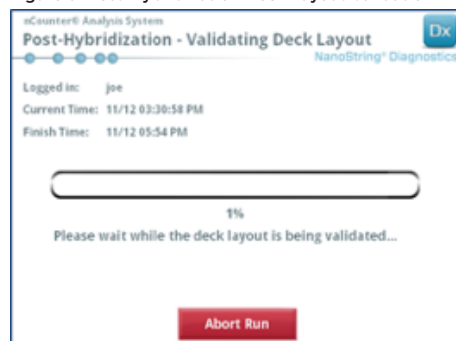
23. After the deck layout has been validated, select "Start Processing" on the touch screen interface.
Note: If you encounter problems starting the Prep Station, return your hybridized samples to the heat block, but do not exceed the maximum time of 21 hours.
24. Follow prompts on the Prep Station once 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:
    c. White Cartridge: This slot contains a Cartridge that is registered but un-scanned.
    d. 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 Step 2 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 Step 2 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 NanoString 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 6: 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 an 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 7: Results and output of the Prosigna Assay

Result	Output Values
ROR Score	Integer value on a 0–100 scale
ROR Subcomponent: Dominant subtype	Subtype with highest Pearson's Correlation
ROR Subcomponent: Proliferation Score	Numerical value on a -2.00 to +2.00 scale
ROR Subcomponent: Gross Tumor Size	≤ 2cm or > 2cm
Risk Category (Node-Negative)	Low, Intermediate, High
Risk Category (1–3 Positive Nodes)	Low, High

13.1   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 profiles of 46 genes in an unknown sample with the four PAM50 centroids[2], 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. The patient test sample is assigned the subtype with the highest Pearson's correlation. The proliferation score is a relative assessment of proliferation using a subset of the 46 genes with a summary score between -2.00 and 2.00. The gross tumor size is taken from the test requisition and a dichotomous value for ≤ 2cm or > 2cm is used in the algorithm. The values for these subcomponents are reported to support understanding the ROR result.

13.2   Probability of 10-Year Distant Recurrence

The ROR Scores for a cohort 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). This study resulted in a model relating the ROR Score to the probability of distant recurrence in two tested patient populations (node-negative and node-positive) including a 95% confidence interval.

13.3   Risk Classification

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

Table 8: Risk classification by ROR Score range and nodal status

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

13.4   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 NanoString 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[5]. 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 (NC) target sequences are derived from the ERCC DNA sequence library[5]. 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 level 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 use 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 (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.

15   EXPECTED VALUES

The Prosigna Assay reports a ROR Score (0–100), and risk categorization (Low, Intermediate or High) for each tumor sample. Based on the clinical validation study described below, post-menopausal women with HR+, early stage breast cancer treated with 5 years of Tamoxifen or 2 years of Tamoxifen followed by 3 years of Anastrozole (ABCSG-8 trial), the distant recurrence-free survival over 10 years by risk categorization is represented below.

15.1 Distant Recurrence-Free Survival by Risk Categorization

For allocating patients to risk groups, ROR Scores were compared to predefined risk thresholds for node-negative or node-positive patients. The figures below show the 10-year distant recurrence-free survival (DRFS) for each risk category group by nodal status.
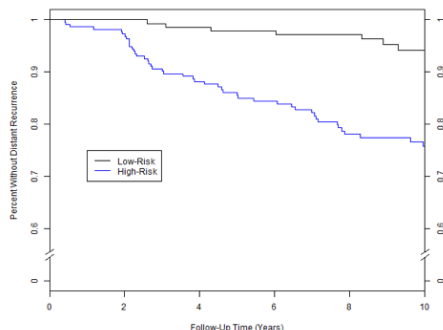
Figure 9. DRFS by Risk Group for Node-Negative Patients
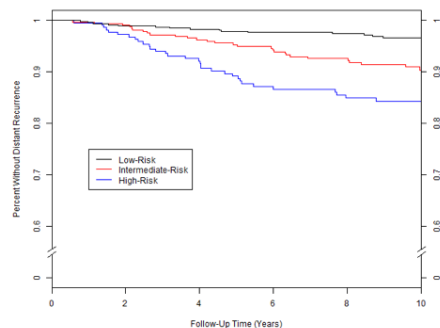
Summary of Data for Figure 9: DRFS by Risk Group for Node-Negative Patients

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 10. DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

Summary of Data for Figure 10: 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	158 (41%)	7	94.2% [88.1%–97.2%]
High	224 (59%)	46	75.8% [68.9%–81.4%]
Total	382 (100%)	53	

Three risk-groups were defined based on predicted risk in the transATAC study[6]:

- Low Risk: < 10% predicted risk of DR by 10 years
- Intermediate Risk: 10–20% predicted risk of DR by 10 years
- High Risk: > 20% predicted risk of DR by 10 years

For the node-negative patients, the cutoffs to define the Low, Intermediate, and High risk groups were set at 40 and 60. For the patients with 1–3 positive nodes, a single cutoff of 40 was set to define two risk categories, Low risk and High risk. The analysis for node-positive patients was limited to two risk categories since the ABCSG-8 study included a smaller number of node-positive patients (n=382) relative to the number of node-negative patients (n=1,047). This study design resulted in sufficient patients in all risk categories to provide a precise estimate of the group's survival in the ABCSG-8 study.

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® Dx 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® Dx 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.

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 9). 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 9. 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 10 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 10. Variance Components by Panel Member (pooled RNA sample)

Panel Member	Mean ROR Score	Variance Component					Total Variance	Total SD
		Lot	Site	Operator	Run	Within-Run		
Sample 1	31.4	0.010 (2%)	0.000 (0%)	0.000 (0%)	0.134 (30%)	0.296 (67%)	0.44 (100%)	0.66
Sample 2	55	0.105 (18%)	0.000 (0%)	0.000 (0%)	0.046 (8%)	0.426 (74%)	0.576 (100%)	0.76
Sample 3	55.4	0.059 (20%)	0.000 (0%)	0.000 (0%)	0.046 (15%)	0.194 (65%)	0.299 (100%)	0.55
Sample 4	64.8	0.119 (21%)	0.014 (2%)	0.000 (0%)	0.064 (11%)	0.380 (66%)	0.576 (100%)	0.76
Sample 5	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 Score 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 Score unit apart for each panel member contributing approximately 20% on average to overall variance.

Tissue Reproducibility

16.1.3 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® Dx 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 Roche FFPET 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 ≥ 100 mm², and 3 slides were input when the tumor surface measured < 100 mm², with a minimum tumor surface area of 4 mm² required.

16.1.4 Testing Summary

The call rate for the forty three (43) tissue specimens evaluated at each of the three sites is shown in Table 11 .

Table 11. 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 ≤ 15 mm², equaling less than 50 mm² 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, gene expression profiles, 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.

RNA yields per slide mounted tissue section were highly correlated (r > 0.9) when repeat isolations were performed from the same FFPE blocks across 3 sites, 3 operators, and 3 RNA extraction lots using the FFPET RNA extraction kit. Similarly, the mean RNA purity (A260/A280) at each site was close to a theoretically pure RNA measurement of 2.0 (2.0 at two sites and 1.9 at the other site) with a SD of 0.1, validating the RNA isolation procedure and specifications for the Prosigna Assay.

16.1.5 Variance Components Analysis

There were no statistically significant (α = 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 12 shows the results of the variance components analysis using all 41 tissue specimens.

Table 12: Variance Components (Tissue Reproducibility Study)

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

*total SD (imprecision) using only the first RNA replicate at each site is 2.91 (SD range of 0.0–7.6)

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 13 summarizes the total variability using the sum of the Tissue Processing Variability (site and within block components from Table 12 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 10). 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 Scores of 6.75 with 95% confidence.

Table 13: Total Variability (Tissue and RNA Processing)

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

16.1.6 Concordance of Risk-Category Classifications

The site-to-site concordance by patient risk classification (Low/Intermediate/High risk) is shown in Table 14, 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 are 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 14: Summary of Concordance of Risk-Category by Node Status

Node Status	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)	
Node-Negative	87.5% [73.2%–95.8%]	92.7% [80.1%–98.4%]	90% [76.4%–97.2%]	90%
Node-Positive	90.0% [76.9%–96.0%]	95.1% [83.9%–98.7%]	95.0% [83.5%–98.6%]	93%

For each comparison (node-negative and node-positive risk categories), the average concordance between sites was at least 90%.

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 15 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 within (–3, 3 ROR Score). 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 15: ROR Score Difference Summary. Count equals number of samples included in analysis.

| Kit Lot	Mass (ng)	Count	Mean ROR Score 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 was determined.

On average, the ROR Score of the macrodissected tumor sample was almost 8 units above what was observed when normal/non-tumor tissue was not removed. Figure 11 below 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 Score units) point estimate of a patient's risk of recurrence (Figure 11).

Figure 11. Impact of normal/non-tumor tissue on ROR Score
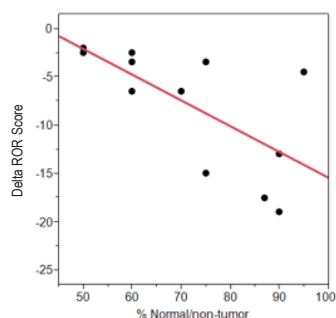
Necrotic, hemorrhagic, and DCIS tissue interference

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 Score) 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 Score 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 w/DNase I (see Table 16). When the samples that were not DNase-treated were subsequently treated with DNase I (post-treat) the ROR Scores matched the ROR Score 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 Score 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 16. Impact of treatment with DNase on ROR Score in tumor samples

| ROR Score Category	FFPE Specimens Tested	ROR Score Difference w/ DNase I – w/o DNase I			ROR Score 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

Prognosis for Hormone Receptor-positive, Post-menopausal Breast Cancer Patients Receiving Adjuvant Systemic Endocrine Therapy Alone Using the NanoString 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[8]. 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 12.

Figure 12. Schematic of the ABCSG-8 trial Study Design
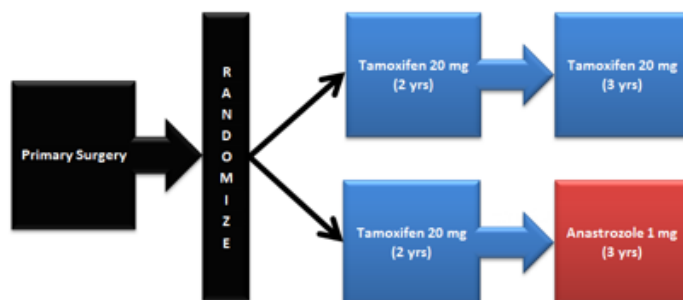
The validation cohort represents the fraction of the evaluable ABCSG-8 cohort for which tissue specimens could be collected from the retrospectively archived ABCSG tumor bank and for which informed consent could be obtained, or the patient was deceased. Patients who meet the eligibility criteria for 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 evaluated the prognostic value of the ROR Score in the validation cohort using a pre-defined analysis plan (see Analysis section below for details). The ROR Score, which ranges from 0–100, was calculated by using a 46-gene subset from the 50 test genes as previously published[2]. The coefficients for the ROR Score were calculated from a Cox model that included the Pearson correlation to the 46 genes used to determine the PAM50 molecular 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.

Using all available patient samples, multivariate Cox proportional hazards (PH) models were fitted to evaluate the primary objective in sequential tests of ROR Score. 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 Score 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[9]. The primary analyses were repeated for different patient subsets (all, node-negative, node-positive, or HER2 negative only) and endpoints (DRFS).

Analysis

A sequential approach was used in which the primary scientific objective was to demonstrate that ROR Score 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 Score 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 Score followed by pre-defined ROR Score-based risk categories. The model included the following categorical standard clinical covariates (with possible values):

- Age ($\geq$ 65 or < 65)

- Grade (G1 or G2/GX)

- Gross Tumor Size (T1, T2/T3)

- Nodal Status (N0, N+ (1–3), N+ ($\geq$ 4))

- Adjuvant Therapy (Tamoxifen alone, or Tamoxifen $\rightarrow$ Anastrozole)

where N0 denotes node-negative patients, N+ (1–3) denotes node-positive patients with 1–3 positive nodes and N+ ($\geq$ 4) denotes node-positive patients with 4 or more positive nodes. T1 indicates tumor $\leq$ 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 (as per the pre-defined analysis plan) 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 17. 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+ ($\geq$ 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%	1,745	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 18. Additional Clinical Characteristics

Characteristic	Value	Number of Patients	% of Patients
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 recurrences 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 19. 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 Score	53.49	3.84 (df = 1)	p < 0.0001
CTS	CTS + Risk Group	34.12	5.99 (df = 2)	p < 0.0001

*ΔLR is used to denote twice the difference of the log likelihoods when comparing two models, e.g., CTS and CTS + ROR Score. The statistic has an approximate χ^2 distribution.

The following table shows the coefficients and hazard ratios (HRs) for the proportional hazards (PH) modeling DRFS as a function of the standard clinical and treatment variables and continuous ROR Score. ROR Score remains a highly significant predictor of DRFS.

Table 20. PH Model for Clinical and Treatment Variables and Continuous ROR Score: All Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	0.098	0.5486	1.10	0.8–1.52
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	−0.073	0.6504	0.93	0.68–1.28
Grade (G2 or GX vs. G1)	0.501	0.0886	1.65	0.93–2.94
Nodal Status (N+ (1–3) vs. N0)	0.556	0.0014	1.74	1.24–2.45
Nodal Status (N+ (≥ 4) vs. N0)	0.908	0.0072	2.48	1.28–4.81
Tumor Stage (T2 or T3 vs. T1)	0.521	0.0024	1.68	1.2–2.36
ROR Score	0.033	< 0.0001	1.03*	1.02–1.04

*Hazard Ratio associated with a 1-unit increase in ROR Score

The following table shows the model using the ROR Score risk groups. Both the Intermediate and High risk groups are significantly different from the Low risk group.

Table 21. PH Model for Clinical and Treatment Variables and ROR Score Risk Groups: All Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	0.142	0.3860	1.15	0.84–1.59
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	−0.070	0.6653	0.93	0.68–1.28
Grade (G2 or GX vs. G1)	0.560	0.0566	1.75	0.98–3.11
Nodal Status (N+ (1–3) vs. N0)	0.131	0.4727	1.14	0.80–1.63
Nodal Status (N+ (≥ 4) vs. N0)	0.180	0.6065	1.20	0.60–2.38
Tumor Stage (T2 or T3 vs. T1)	0.649	0.0001	1.91	1.38–2.65
ROR Score Risk Group (High vs. Low)*	1.539	< 0.0001	4.66	2.66–8.18
ROR Score Risk Group (Intermediate vs. Low)*	0.808	0.0062	2.24	1.26–4.00

*The model was calculated based on the original pre-specified hypothesis in ABCSG-8 which included 3 risk categories for both node-negative and node-positive (1–3 nodes) patients.

These data have also been provided separately for node-negative and node-positive (1–3 nodes) patients. The continuous ROR Score remains the most highly significant predictor of DRFS.

Table 22. PH Model for Clinical and Treatment Variables and Continuous ROR Score: Node-Negative Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	0.142	0.5212	1.15	0.75–1.78
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	-0.189	0.3827	0.83	0.54–1.27
Grade (G2 or GX vs. G1)	0.452	0.2098	1.57	0.78–3.19
Tumor Stage (T2 or T3 vs. T1)	0.539	0.0186	1.71	1.09–2.68
ROR Score	0.030	< 0.0001	1.03	1.02–1.04

Table 23. PH Model for Clinical and Treatment Variables and ROR Score Risk Groups: Node-Negative Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	0.162	0.4621	1.18	0.76–1.81
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	−0.203	0.3496	0.82	0.53–1.25
Grade (G2 or GX vs. G1)	0.489	0.1736	1.63	0.81–3.3
Tumor Stage (T2 or T3 vs. T1)	0.600	0.0076	1.82	1.17–2.83
ROR Score Risk Group (High vs. Low)	1.391	< 0.0001	4.02	2.21–7.3
ROR Score Risk Group (Intermediate vs. Low)	0.957	0.0015	2.60	1.44–4.71

Table 24. PH Model for Clinical and Treatment Variables and Continuous ROR Score: Node-Positive (1–3 nodes) Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	−0.021	0.9381	0.98	0.57–1.67
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	−0.035	0.8938	0.97	0.58–1.61
Grade (G2 or GX vs. G1)	1.173	0.1044	3.23	0.78–13.3
Tumor Stage (T2 or T3 vs. T1)	0.321	0.2501	1.38	0.8–2.39
ROR Score	0.037	< 0.0001	1.04	1.02–1.05

Table 25. PH Model for Clinical and Treatment Variables and ROR Score Risk Groups: Node-Positive (1–3 nodes) Patients

Variable	DRFS			
	Coefficient	p-value	HR	HR 95%CI
Age Category (Age ≥ 65 vs. Age < 65)	−0.009	0.9730	0.99	0.58–1.69
Treatment (Tamoxifen → Anastrozole vs. Tamoxifen Only)	−0.002	0.9946	1.00	0.6–1.66
Grade (G2 or GX vs. G1)	1.244	0.0850	3.47	0.84–14.28
Tumor Stage (T2 or T3 vs. T1)	0.507	0.0591	1.66	0.98–2.81
ROR Score (High vs. Low)	1.435	0.0002	4.20	1.97–8.94

In all cases, age, treatment and grade are not significant when other variables are included in the model. ROR Score (continuous or risk-group) is a statistically significant predictor of DRFS in all cases. Tumor stage was a significant predictor for the node-negative patients but not for the node-positive patients.
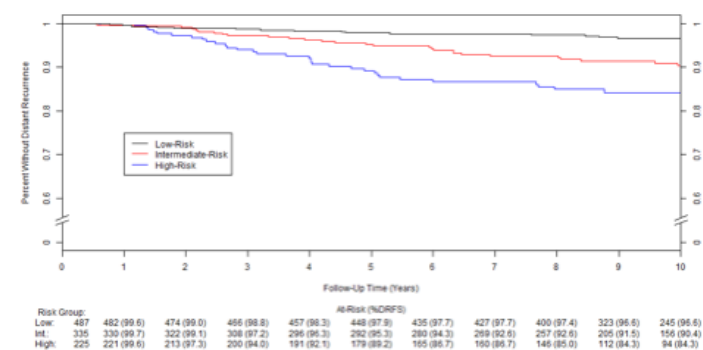
Table 26. Repeat of Primary Analysis Testing for Pre-Defined Subgroups

Subject Group	# Patients	# Events	CTS+ROR Score vs. CTS	CTS+Risk-Group vs. CTS
			ΔLR χ² (Crit. Value = 3.84)	ΔLR χ² (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 and HER2-negative*	984	79	21.69	20.32
N+ (1–3)	382	59	25.99	19.94
N+ (1–3) and HER2-negative*	367	56	22.75	18.75

*When HER2-positive patients were removed from the analysis population, the results were very similar to analysis with all patient data (HER2-positive and HER2-negative patients)

Figure 13 shows the Kaplan-Meier plots by risk-group for Node-Negative patients. The x-axis shows the number of patients and the percent without a distant recurrence (DR) event for each risk group. The observed incidence of distant recurrence in the Low risk node-negative patients remains consistently low over the 10 year evaluation period. Similar DR rates for these patients were observed between the first 5 years, while patients were receiving endocrine therapy, and years 5 and 10 after diagnosis, following completion of endocrine therapy. A total of 2.1% of Low risk node-negative subjects had a DR event through 5 years after diagnosis and 1.3% of subjects had a DR event between years 5 and 10 after diagnosis (3.4% total 10-year risk of recurrence). The incidence of distant recurrence in the Intermediate risk node-negative patients also appeared similar between the first 5 years and 5 and 10 years after diagnosis, though at a consistently higher rate than the Low risk population. A total of 4.7% of these subjects had a DR event through 5 years after diagnosis and 4.9% of subjects had a DR event between years 5 and 10 after diagnosis (9.6% total 10-year risk of recurrence). The incidence of distant recurrence in the High risk node-negative patients appears more variable over the 10 year evaluation period. It was observed that 10.8% of these subjects had a DR event through 5 years after diagnosis and 4.9% of subjects had a DR event between years 5 and 10 after diagnosis (15.7% total 10-year risk of recurrence).

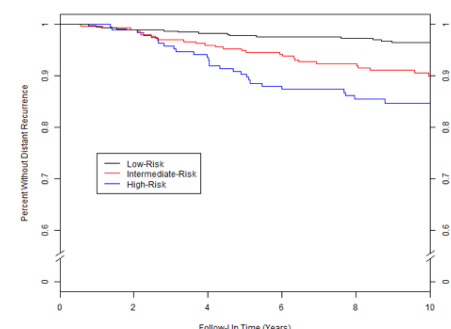**Figure 13. DRFS by Risk Group for Node-Negative Patients**

Risk Group:
Low: 487 482 (99.6) 474 (99.0) 466 (98.8) 457 (98.3) 448 (97.9) 435 (97.7) 427 (97.7) 400 (97.4) 323 (96.6) 245 (96.6)
Int: 335 330 (99.7) 322 (99.1) 308 (97.2) 296 (96.3) 292 (95.3) 280 (94.3) 269 (92.6) 257 (92.6) 205 (91.5) 156 (90.4)
High: 225 221 (99.6) 213 (97.3) 200 (94.0) 191 (92.1) 179 (89.2) 165 (86.7) 160 (86.7) 146 (85.0) 112 (84.3) 94 (84.3)

Figure 15. DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

Risk Group:
Low: 158 151 (100.0) 146 (100.0) 142 (99.3) 139 (98.5) 136 (97.9) 132 (97.9) 130 (97.2) 125 (97.2) 93 (95.3) 69 (94.2)
High: 224 217 (98.7) 208 (97.3) 186 (90.6) 171 (88.2) 160 (86.1) 153 (84.4) 142 (82.2) 129 (78.1) 105 (77.5) 86 (75.8)

Summary of Data for Figure 13: 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 14 shows the plots for Node-Negative HER2-negative patients. The results with and without the HER2-positive patients are similar.

Figure 14. DRFS by Risk Group for HER2-Negative Node-Negative Patients

Summary of Data for Figure 14: 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 15 shows the Kaplan-Meier plots by risk-group for Node-Positive (1–3 nodes) patients. The x-axis shows the number of patients and the percent without a DR event for each risk group. The incidence of distant recurrence in node-positive (1–3 nodes) patients remains consistently lower in the Low risk group than in the High risk group. In the Low risk group, 2.1% of patients had a DR through 5 years after diagnosis and 3.7% had a distant recurrence event between years 5 and 10 after diagnosis (5.8% total 10 year risk of recurrence). In the High Risk Node-Positive (1–3 nodes) patients the incidence of distant recurrence is 13.9% through 5 years after diagnosis and 10.3% between years 5 and 10 after diagnosis (24.2% total 10-year risk of recurrence).
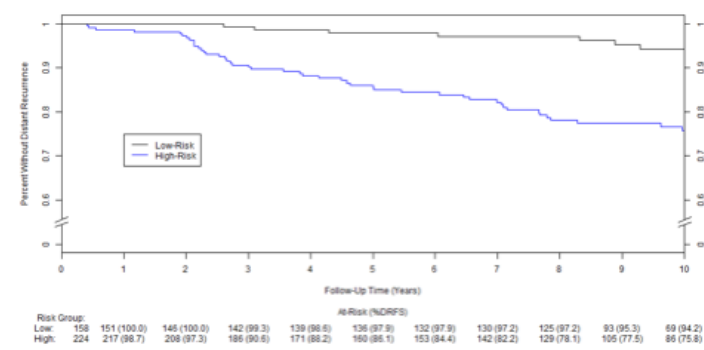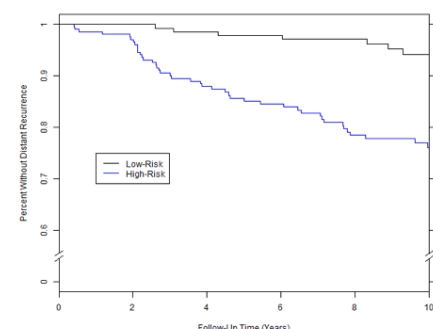
Summary of Data for Figure 15: DRFS by Risk Group for Node-Positive (1–3 nodes) Patients

Risk Group	Number of Patients (%)	Number of Events Through 10 Years	Estimated Percent Without Distant Recurrence at 10 years [95% CI]
Low	158 (41%)	7	94.2% [88.1%–97.2%]
High	224 (59%)	46	75.8% [68.9%–81.4%]
Total	*382 (100%)*	*53*	

Figure 16 shows the plots for Node-Positive (1–3 nodes) HER2-negative patients.

Figure 16. DRFS by Risk Group for HER2-Negative, Node-Positive (1–3 nodes) Patients

Summary of Data for Figure 16: 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	157 (43%)	7	94.2% [88.0%–97.2%]
High	210 (57%)	43	76.1% [69.0%–81.8%]
Total	*367 (100%)*	*50*	

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.

For both node-negative and node-positive (1–3 nodes) patients, the event rates within some risk groups were not constant across the 10-year interval (see figures 13 and 15 above). Based upon post-hoc analysis of the 10 year data, for High risk patients more DR events appeared to occur in the first 5 years of the overall 10-year risk period, while Intermediate and Low risk patients showed similar rates of DR in between the first 5 years and years 5 to 10 of the ABCSG-8 study. The ROR Score provided insight into the probability of DR events in the first 5 years after diagnosis, while patients were receiving endocrine therapy, and years 5 and 10 after diagnosis, following completion of endocrine therapy.
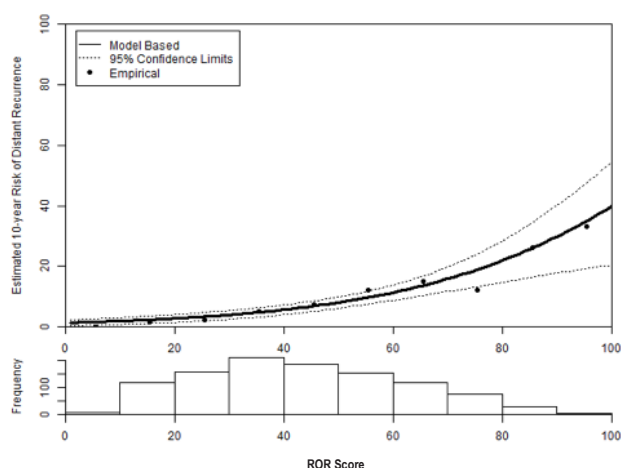
Table 27 shows the distribution of Node-Negative patients by 10-unit ROR Score bins where each bin consists of all patients within the 10-unit ROR Score ranges (1–10, 11–20, etc.). Also shown is the 10-year DR risk with 95% confidence limits estimated using only data from patients within each bin.

Table 27. Distribution of Node-Negative patients by 10-unit ROR Score Range

ROR Score Range	Number of Patients	Percent of Patients	10-year DR Risk (Empirical) with 95% Confidence Limits
1–10	7	0.7%	0% [N/A]
11–20	116	11.1%	1.8% [0%–4.3%]
21–30	155	14.8%	2.5% [0%–5.2%]
31–40	209	20.0%	5.1% [2.0%–8.1%]
41–50	183	17.5%	7.5% [3.3%–11.6%]
51–60	152	14.5%	12.1% [6.2%–17.6%]
61–70	116	11.1%	15% [7.6%–21.8%]
71–80	77	7.4%	12.3% [4.4%–19.6%]
81–90	28	2.7%	26.1% [7.3%–41.1%]
91–100	4	0.4%	33.3% [0%–70.0%]
Total	*1,047*	*100%*	

Figure 17 shows the model-based curve for the Node-Negative patients together with the empirically estimated 10-year DR Risk for the 10 bins. The confidence limits shown in Figure 17 were estimated based on the fitted model that included all Node-Negative patients. Below the curve is a histogram showing the frequency distribution by bin.

Figure 17. 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 28 shows the distribution of Node-Positive (1–3 nodes) patients by 10-unit ROR Score bins. Also shown is the 10-year DR risk with 95% confidence limits estimated using only the data from patients within each bin.

Table 28. Distribution of Node-Positive (1–3 nodes) patients by 10-unit ROR Score Range

ROR Score Range	Number of Patients	Percent of Patients	10-year DR Risk (Empirical) with 95% Confidence Limits
1–10	3	0.8%	0% [N/A]
11–20	34	8.9%	3.6% [0%–10.2%]
21–30	53	13.9%	4.1% [0%–9.6%]
31–40	68	17.8%	8.5% [0%–16.2%]
41–50	57	14.9%	16.7% [5.1%–27.0%]
51–60	71	18.6%	17.8% [6.8%–27.4%]
61–70	42	11.0%	28.9% [11.7%–42.7%]
71–80	34	8.9%	39.5% [18.9%–54.9%]
81–90	17	4.5%	33% [4.5%–53.0%]
91–100	3	0.8%	33.3% [0%–70.0%]
Total	*382*	*100%*	

Figure 18 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 8 bins where each bin consists of all patients within the 10 ROR Score ranges (1–10, 11–20, etc.). Due to the small sample size of Node-Positive (1–3 nodes) patients with scores greater than 80 in the clinical validation study, the exact relationship of the ROR Score to probability of distant recurrence could not be established with a retrospective model fitting. The confidence limits shown in Figure 18 were estimated based on the fitted model that included all Node-Positive (1–3 nodes) patients with ROR Score ≤ 80. Below the curve is a histogram showing the frequency distribution by bin.

Figure 18. 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.
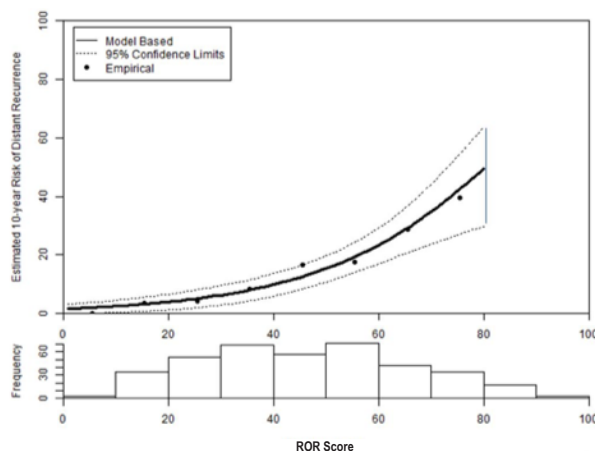
Clinical Study Summary

Results are generalizable for distributed use as all samples were sent to, and all tests were performed at, an independent academic pathology laboratory. The ROR Score 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 pre-defined risk groups of both node-negative and node-positive patients. The ROR Score (continuous and risk-group based) showed similar prognostic information in various subgroups.

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18　SYMBOLS AND DEFINITIONS

-Manufacturer

IVD -*In Vitro* Diagnostic Medical Device

-Consult Instructions for Use

LOT -Batch code / Lot number

SN -Serial 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

-Date of manufacture

Room Temp. = Room Temperature
HYB = Hybridization

Regulatory Disclaimer
For *In Vitro* Diagnostic Use.

19　CONTACT INFORMATION

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