Agendia NV.

MammaPrint® and BluePrint® Breast Cancer Recurrence and Molecular Subtyping Kit – Package Insert

Targeted sequencing of RNA from formalin-fixed, paraffin-embedded tissue sections to assess breast cancer risk of recurrence and molecular subtype

Version
M-ROW-133-V6 (2021SEP)

Contents

MammaPrint® BluePrint®

Breast Cancer Recurrence and Molecular Subtyping Kit

For professional and laboratory use

For use with RNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissue

CAREFULLY READ ALL INSTRUCTIONS PRIOR TO USE.

Intended Use

The MammaPrint BluePrint Breast Cancer Recurrence and Molecular Subtyping Kit (MammaPrint BluePrint Kit) is an *in vitro* diagnostic test using target enrichment next-generation sequencing (NGS) technology for gene expression on formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue samples.

The 70-gene MammaPrint test is intended to distinguish patients that are at a Low Risk or High Risk to develop distant metastases within 5 years of diagnosis [1] [2] [3]. The BluePrint 80-gene test is intended to assess the molecular subtype of breast cancer and determines if tumors are Luminal-type, HER2-type, or Basal-type [4].

The MammaPrint BluePrint Kit is indicated for use in female breast cancer patients with Stage I or II invasive breast cancer, tumor size ≤ 5.0 cm, lymph node negative or 1-3 lymph node positive disease. The test is used independent of estrogen receptor status (ER+/-) [5]. The MammaPrint test is a prognostic marker only and can be used in conjunction with traditional clinicopathological factors. The MammaPrint BluePrint Kit is performed on the Illumina® MiSeq® Sequencer System and results are analyzed using the Agendia Data Analysis Pipeline Tool (ADAPT).

Summary and Explanation of the Test

The MammaPrint BluePrint Kit provides an individualized Low or High Risk of disease recurrence result as well as an individualized determination of the molecular subtype of a tumor.

MammaPrint determines the activity of 70 genes in a tumor sample, resulting in an expression profile or “fingerprint” of the tumor. Using a proprietary algorithm, the gene expression profile is used to calculate the MammaPrint Index (MPI), which indicates the prognostic profile for risk of recurrence of breast cancer.

BluePrint analysis determines the activity of 80 genes in a tumor sample, resulting in three expression profiles. Using a proprietary algorithm, the three gene expression profiles are used to calculate BluePrint indices which are used to determine the molecular subtype of the sample: Luminal-type, HER2-type, or Basal-type. The genes and scoring algorithms used for the MammaPrint BluePrint Kit are identical to those used for the MammaPrint and BluePrint test performed in the central Agendia laboratories on microarray ([1] [2] [3] [6] [7] [8]).

Principle of the Procedure

The MammaPrint BluePrint Kit uses capture sequencing to determine gene expression in RNA isolated from FFPE tissue with a tumor cell content of at least 30%.

The kit enables the preparation of targeted NGS libraries from FFPE RNA using the Agilent SureSelect^{XT} RNA target enrichment system in the absence of a ribosomal depletion step. The target enrichment workflow utilizes ultra-long 120-mer biotinylated cRNA baits to capture MammaPrint and BluePrint genes, enriching them from an NGS genomic fragment library. Read count data generated from the sequencing output (in FASTQ format) is used to assess the expression levels of MammaPrint and BluePrint profiles and report out test results.

Sequencing output is securely transferred to Agendia's web portal and analysis is performed using the Agendia Data Analysis Pipeline Tool (ADAPT). The MammaPrint test result includes the MPI, which is reported on a scale of -1.000 to +1.000 and determines the sample's prognostic profile: Low Risk (MPI greater than +0.000) or High Risk (MPI equal to or less than 0.000). The BluePrint test results include three BluePrint indices and the highest index determines the sample's molecular subtype.

Reagents

Reagents Provided

Catalog #931280 has been configured for up to 16 reactions.

MammaPrint BluePrint NGS RNA Library Prep (Pre-PCR) Catalog #931281 [Box 1 of 4]		**-20°C**
Component	**Cat #**	**Volume**
Agendia NGS Fragmentation Mix	931281-01	304 µL
Agendia NGS 1st Strand Master Mix	931281-02	140 µL
Agendia NGS 2nd Strand + End Repair Enzyme Mix	931281-03	400 µL
Agendia NGS 2nd Strand + End Repair Oligo Mix	931281-04	80 µL
Agendia NGS dA Tailing Master Mix	931281-05	320 µL
Agendia NGS Oligo Adaptor Mix	931281-06	80 µL
Agendia NGS Ligation Master Mix	931281-07	80 µL
Agendia NGS Forward PCR Primer	931281-08	60 µL
Agendia NGS PCR Master Mix	931281-09	800 µL
Agendia NGS Uracil DNA Glycosylase (UDG)	931281-10	16 µL
Agendia NGS Reverse PCR Primer	931281-11	16 µL
Agendia NGS Nuclease-Free Water	931282-07	2.4 mL

MammaPrint BluePrint NGS Target Enrichment (Post-PCR Box 1) Catalog #931282 [Box 2 of 4]		**Room Temp**
Component	**Cat #**	**Volume**
Agendia NGS Hyb 1	931282-01	400 µL
Agendia NGS Hyb 2	931282-02	1.25 mL
Agendia NGS Hyb 4	931282-03	1.25 mL
Agendia NGS Binding Buffer	931282-04	13.2 mL
Agendia NGS Wash Buffer 1	931282-05	8 mL
Agendia NGS Wash Buffer 2	931282-06	24 mL
Agendia NGS Nuclease-Free Water	931282-07	2.4 mL
Agendia NGS Elution Buffer	931282-08	5.8 mL
Agendia NGS Neutralization Buffer	931282-09	960 µL
MammaPrint BluePrint Package Insert	931282-10	1x

MammaPrint BluePrint NGS Target Enrichment (Post-PCR Box 2) Catalog #931283 [Box 3 of 4]		**-20°C**
Component	**Cat #**	**Volume**
Agendia NGS Indexing Block 1	931283-01	45 µL
Agendia NGS Block 2	931283-02	45 µL
Agendia NGS Indexing Block 3	931283-03	12 µL
Agendia NGS RNase Block	931283-04	18 µL
Agendia NGS Hyb 3	931283-05	160 µL
Agendia NGS Post-Capture PCR Primer	931283-06	16 µL
Agendia NGS PCR Master Mix	931281-09	800 µL
Agendia NGS 8bp Index Plate*	931283-07	12 µL

MammaPrint BluePrint NGS Panel Catalog #931284 [Box 4 of 4]		**-80°C**
Component	**Cat #**	**Volume**
MammaPrint BluePrint NGS Baits Library	931284	36 µL

* Index sequences can be found in Appendix A: Nucleotide Sequences of MammaPrint BluePrint NGS Indexes

Reagents and Equipment Required, Not Provided

Reagent	Manufacturer, Catalog Number
RNA Isolation RNeasy FFPE Kit	QIAGEN, 73504
Actinomycin D	From Streptomyces sp. (Sigma-Aldrich, A1410)
Dynabeads® MyOne™ Streptavidin T1	Invitrogen, 65601 or 65602
Agencourt AMPure XP	Beckman Coulter Genomics, A63880 or A63881 or A63882
MiSeq Reagent Kit v3 Kit (150-cycle)	Illumina, MS-102-3001
PhiX Control Kit V3	Illumina, FC-110-3001
Dimethyl sulfoxide	Molecular biology grade (Sigma-Aldrich, D8418)
Buffer EB	QIAGEN, 19086
Ethanol (EtOH), 100%	Molecular Biology grade (VWR, 1085430250)
Tween 20	Non-ionic (Sigma-Aldrich, P7949)
Nuclease-free water	Nuclease-free, deionized, no chemical additives (QIAGEN, 129114)
Sodium hydroxide (NaOH)	1N, Molecular Biology grade (Sigma-Aldrich, 72068)
Xylene	Any available
Histo-Clear	National Diagnostics, HS-200

Equipment	Minimum Specifications
Nucleic acid fragment analysis platform and consumables	RNA DV200 200 nt-4000 nt DNA 150 bp-550 bp (Quantitative sensitivity 0.5-50 ng/µL) DNA 150 bp-700 bp (Quantitative sensitivity 5-500 pg/µL)
Vortex mixer	Any available
Centrifuge	For 1.5 mL/0.5 mL tubes
Plate centrifuge	Fits 0.8 mL MIDI plates
Vacuum centrifuge	Temp range: 15°C to 45°C
Heat blocks	37°C for 0.8 mL MIDI plate 65°C for 1.5/2 mL tube
Thermal mixer	27°C and 65°C 1200-1400 rpm Fits 0.2 mL 8-strip tubes
Timer	NIST traceable
Magnetic stand	Fits 0.80 mL plates (Life Technologies, AM10027) Fits 0.2 mL 8-strip tubes (Life Technologies, 12331D) Fits 1.5/2 mL tubes (Life Technologies,12321D)
Thermal cycler	Heated lid: 105°C Temp range: 4°C-105°C
Single channel pipettes	1 µL – 1000 µL
8-strip tubes/spinner	Any available
Multi-channel pipettes *(optional)*	1 µL – 1000 µL
Repeater pipettes *(optional)*	1 µL – 10 mL
MiSeq system	Illumina, SY-410-1003 or Illumina, DX-410-1001 RUO mode

Warnings and Precautions

- For *in vitro* diagnostic use.
- This device is for use by healthcare professionals only.
- The results provided by the MammaPrint BluePrint Breast Cancer Recurrence and Molecular Subtyping Kit are indicated for use by physicians as a prognostic marker only along with standard clinical-pathological factors. The test is not intended to determine the outcome of disease, nor to suggest or infer an individual patient's response to therapy.
- Do not use kit contents beyond the expiration date printed on the outside of the box.
- Do not interchange assay components from different kit lots. Note that kit lots are identified on the outer box label.
- Store the kit components at the specified temperatures in designated pre-amplification and post-amplification areas.
- To obtain accurate results, you must follow the Test Procedure instructions exactly. Failure to follow the instructions, modification to the test system instructions, or use of reagents or instruments not recommended by Agendia for deparaffinization, RNA isolation, target enrichment or sequencing may invalidate the test results.
- Wear appropriate personal protective equipment (PPE) when working in the laboratory.
- Inadequate or poor-quality RNA may yield incorrect results.
- Seek specific training or guidance if you are not experienced with RNA isolation or next-generation sequencing procedures.
- NOTE: The Agendia NGS Hyb 1 reagent and Agendia NGS Neutralization Buffer contain potentially  hazardous materials and will cause serious eye and skin irritation. Wear protection gloves, clothing, eye and face shield protection. Wash hands thoroughly after handling. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do. Continue rinsing.
- Handle used reagents as chemical waste and discard in accordance with applicable regional, national and local laws and regulations. For environmental, health and safety information, refer to the Safety Data Sheets (SDS) located at www.agendia.com/diagnostic-products.
- Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink or smoke in designated laboratory work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- Actinomycin D is obtained as a solid and prepared at 4 µg/µL concentration in DMSO then stored in 3 µL single-use aliquots at –20°C, protected from light. The aliquots may be stored for up to one year before use. The 4 µg/µL Actinomycin D in DMSO is diluted with water, immediately before use, to a final Actinomycin D concentration of 120 ng/µL.
- NOTE: Actinomycin D used in step 2 of the procedure is hazardous – acute toxicity: oral, dermal and  inhalation.

Storage and Handling

Kit contents are stable until the expiration date printed on the outer box label.

Store each box at the following temperatures:

- Box 1 and Box 3: -20°C between -15°C and -25°C
- Box 2: Room temperature (RT) (between 15°C and 30°C). Store out of direct sunlight.
- Box 4: -80°C between -75°C and -85°C

Reagents are stable for a maximum of 5 freeze/thaw cycles that occur before the specified expiration date on the box.

Before use, vortex vigorously and then visually inspect to ensure that no precipitates are present.

Make sure to prepare 0.2 N NaOH fresh daily, this is stable for up to 12 hours.
Prepare 70% ethanol fresh daily.

Adhere to the following best practice when handling PCR Clean-Up AMPure XP Beads and Library Streptavidin Beads:

- The PCR Clean-Up AMPure XP Beads should never be frozen
- Allow the AMPure XP beads to reach room temperature for at least 30 minutes before use
- Immediately prior to use, vortex the beads until well-suspended and color appears homogeneous
- Thoroughly mix the sample after the Streptavidin beads are added by pipetting up and down 10 times
- Incubate the bead/sample mixture at room temperature for the entire duration indicated

PCR contamination may cause inaccurate and unreliable results. To prevent contamination, ensure that pre-amplification and post-amplification areas have dedicated equipment (e.g., pipettes, pipette tips, vortexer and centrifuge).

Avoid cross-contamination. Use fresh pipette tips between samples and between dispensing reagents. Mix samples with a pipette and centrifuge the plate when indicated. Do not vortex the plates unless otherwise specified. Use aerosol-resistant tips to reduce the risk of amplicon carry-over and sample-to-sample cross-contamination.

Specimen Collection and Preparation for Analysis

Select the FFPE tumor block for each specimen to be processed by using a tissue sample that contains the greatest amount of invasive carcinoma and is morphologically consistent with the submitted diagnosis. The selected FFPE tumor block should not be older than 5 years.

Subsequently, for each tissue block, 10 slides of 5 µm will be sectioned with one 5 µm serial section on each slide. It is recommended to use charged slides to reduce the chance of sections falling off the slide. One slide will be used for hematoxylin and eosin (H&E) staining to determine the tumor cell percentage and the remaining slides, depending on the size of the tissue, can all or partly be used for the RNA isolation. Deparaffinization needs to be performed using either Xylene or Histo-Clear[1].

The invasive tumor cell percentage must be at least 30% as this is required to obtain valid results. When needed and possible, a macro dissection can be performed to avoid large areas of *in-situ* carcinoma, necrosis, adipose tissue, stroma and/or hemorrhage as these will decrease the overall invasive tumor cell percentage.

[1] Histo-Clear II has not been validated for use with the MammaPrint BluePrint Kit.

Quality Control

Perform appropriate equipment calibration and maintenance on equipment used in the laboratory processes in accordance with your laboratory's standard Quality Control requirements.

Analytical Quality Assessment

QC 1: Quality assessment of purified, FFPE total RNA

This QC assesses quality of the FFPE total RNA based on the DV200 metric.

The DV200 is measured as the percentage of RNA fragments with a length between 200 nt and 4000 nt.

QC 2: Quality assessment of amplified, adaptor-ligated cDNA libraries

This QC assesses quality (cDNA fragments must fall in the right size range, i.e. between 150 to 550 bp) and quantity (ng/µL) of the adaptor-ligated cDNA library.

QC 3: Quality assessment of amplified, target-enriched indexed libraries

This QC assesses quality (cDNA fragments must fall in the right size range, i.e. between 150 to 700 bp), quantity (pg/µL) and molarity (pmol/L) of the amplified, target-enriched indexed library.

Assay Controls

Good laboratory practice suggests that control material should be evaluated to detect technical procedural differences in the user's laboratory that may produce significant variability or inaccuracies in results.

It is recommended that prior to initial use of this test in the user's laboratory, the performance of the test should be verified by testing several samples with known performance outcomes.

Test Procedure

Figure 1 provides an overview of the procedure.

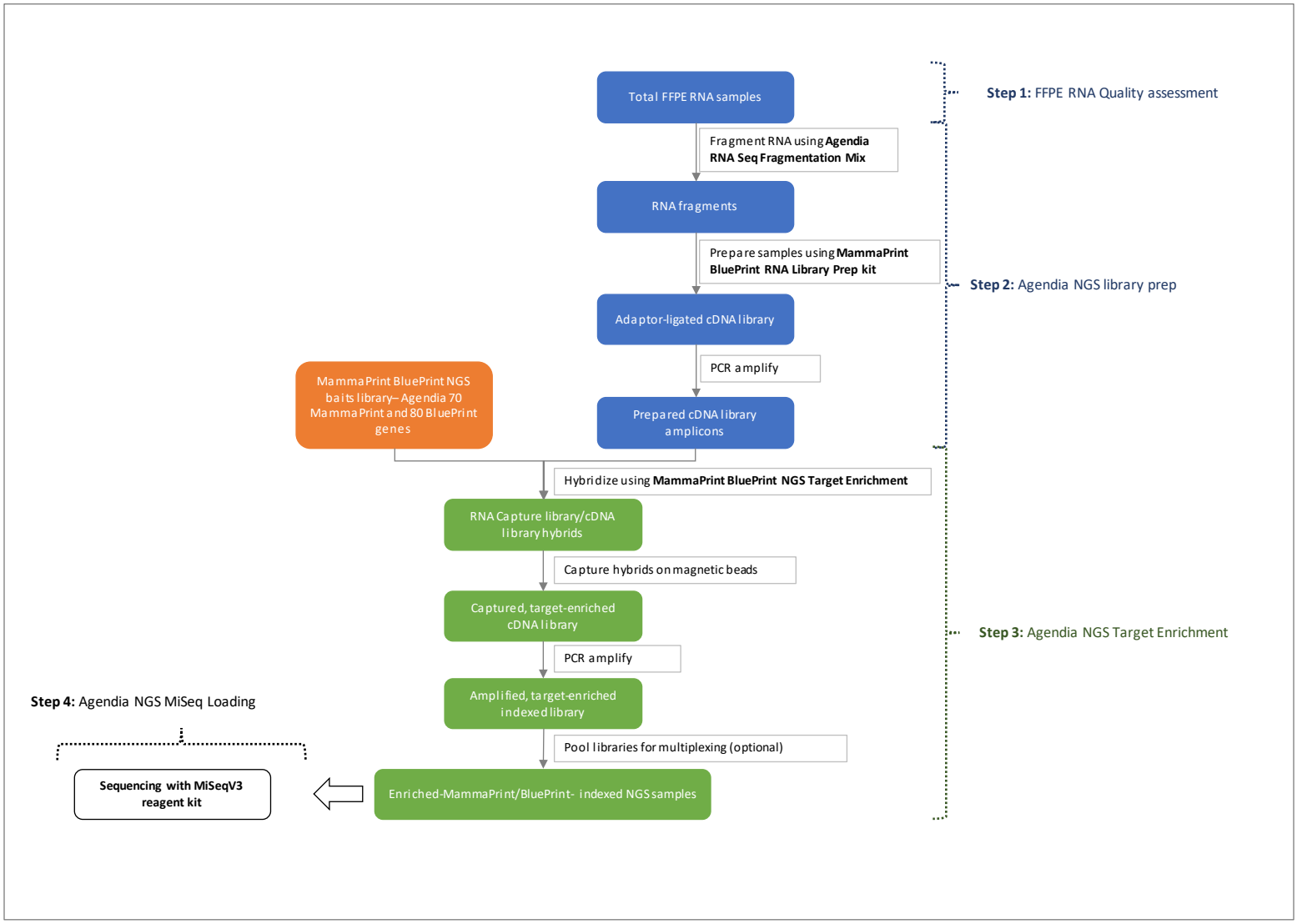

Figure 1: Overview of MammaPrint BluePrint Kit procedure

Step 1: FFPE RNA Quality Assessment and Preparation

RNA isolation is performed using QIAGEN RNeasy FFPE Kit in accordance with the manufacturer's instructions for use. Isolated FFPE Total RNA must have 260/280 and 260/230 absorbance ratio values close to 2.0 for both ratios. Ratios with significant deviation from 2.0 may indicate the presence of organic or inorganic contaminants, which may require further purification or may indicate that the sample is not suitable for use with the MammaPrint BluePrint Kit.

Before you begin, prepare total RNA from each sample in nuclease-free water.

FFPE RNA Quality Assessment

1.1 Assess the DV200 value for FFPE RNA samples using a suitable nucleic acid fragment analysis platform.

Aliquot 200 ng of RNA per sample for Standard, Good to Medium and Poor samples.

If 200 ng is not available, aliquot the amount of RNA indicated in the table below.

Category	Distribution Value (DV200)	Amount RNA Required for Library Prep
Standard	≥70% above 200 nt	100 ng
Good to Medium	≥50% above 200 nt	150 ng
Poor	≥20% above 200 nt	200 ng
Not recommended	<20% above 200 nt	Not recommended

Step 2: Agendia NGS Library Preparation

RNA Fragmentation and Primer Annealing

2.1	Thaw ***Agendia NGS Fragmentation Mix*** at RT, then place on ice.
2.2	Thaw ***Agendia NGS First Strand Master Mix*** on ice.
2.3	Use a vacuum centrifuge (≤45°C) to lyophilize the aliquoted FFPE RNA. *Do not overdry*.
2.4	Vortex ***Agendia NGS Fragmentation Mix*** for 10 seconds. Re-suspend FFPE RNA in 19 µL ***Agendia NGS Fragmentation Mix***. Vortex and briefly centrifuge.
2.5	Run the following thermal cycler program for Standard and Good/Medium RNA quality samples: Heat lid at 95°C. 1. 2 minutes at 94°C 2. 3 minutes at 65°C 3. At least 1 minute at 4°C Keep at 4°C or on ice until ready to proceed.
2.6	Run the following thermal cycler program for Poor RNA quality samples: Heat lid at 95°C 1. 5 minutes at 65°C 2. At least 1 minute at 4°C Keep at 4°C or on ice until ready to proceed.

Synthesis of First Strand cDNA

2.7	Prepare fresh 120 ng/µL ***Actinomycin D*** dilution according to the table below. This volume is sufficient for 96 reactions.	
	Agendia NGS Nuclease-Free Water	97 µL

	Actinomycin D (4 µg/µL in DMSO)	3 µL
	Total volume	100 µL
	Vortex mix, briefly spin, keep at RT and protect from light.	
2.8	Prepare ***Agendia NGS First Strand Synthesis Mix*** according to the table below. Calculate for 1 additional reaction. **Note:** Vortex First Strand Master Mix for 10 seconds before combining reagent components.	
	Reagent	**Volume per Reaction**
	Actinomycin D (120 ng/µL in H2O)	0.5 µL
	Agendia NGS First Strand Master Mix	8.0 µL
	Total volume	8.5 µL
	Vortex mix, briefly spin and keep on ice.	
2.9	On ice, add 8.5 µL ***Agendia NGS First Strand Synthesis Mix*** to each well of a new First Strand cDNA plate.	
2.10	Transfer fragmented FFPE RNA into the wells of the First Strand cDNA plate. Seal the plate, vortex, and briefly centrifuge.	
2.11	Run the following thermal cycler program: Heat lid at 95°C. 1. 10 minutes at 25°C 2. 40 minutes at 37°C 3. At least 3 minutes at 4°C Keep at 4°C or on ice until ready to proceed.	

Synthesize Second Strand cDNA and Repair Ends		
2.12	Prepare ***Second Strand Synthesis and End Repair Mix*** according to the table below. Calculate for 1 additional reaction. **Note:** Vortex each reagent for 5 seconds before combining.	
	Reagent	**Volume per Reaction**
	Agendia NGS Second Strand + End Repair Enzyme Mix	25.0 µL
	Agendia NGS Second Strand + End Repair Oligo Mix	5.0 µL
	Total Volume	30.0 µL
	Vortex mix, briefly spin and keep on ice.	
2.13	Add 30 µL ***Second Strand Synthesis and End Repair Mix*** to each well.	
2.14	Seal the plate, vortex, and briefly centrifuge.	
2.15	Run the following thermal cycler program: Do not use heated lid. If heated lid cannot be disabled, the program should be run with the lid open. 1. 60 minutes at 16°C 2. At least 3 minutes at 4°C Keep at 4°C or on ice until ready to proceed.	

Purify Synthesized cDNA Using AMPure XP beads	
2.16	Allow ***AMPure XP beads*** to equilibrate to room temperature for at least 30 minutes. Vortex bead suspension until homogeneous. If proceeding to **Adenylate cDNA 3'Ends**, thaw ***Agendia NGS dA Tailing Master Mix*** on ice.
2.17	Add 108 µL homogeneous bead suspension to each well of a new 0.8 mL 96-well MIDI plate.
2.18	Transfer 57.5 µL sample mix to respective well of the 0.8 mL 96-well MIDI plate.
2.19	Seal the plate, vortex, and briefly centrifuge.
2.20	Incubate samples for 5 minutes at RT.
2.21	Place plate on magnetic stand at RT for at least 5 minutes.
2.22	With the plate on the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
2.23	With the plate on the magnetic stand, dispense 200µL fresh 70% ethanol into each well.
2.24	Wait 10 seconds (or until solution is clear) to allow any disturbed beads to settle, then carefully remove the ethanol.
2.25	Repeat for a total of 2 washes.
2.26	If needed, briefly centrifuge MIDI plate, return plate to magnetic stand, and then remove remaining ethanol droplets with a pipette.
2.27	Dry samples on the heat block at 37°C for 3 minutes. Do not overdry, but ensure that all ethanol is removed.
2.28	Add 21.5 µL nuclease-free water to each sample well.
2.29	Seal the plate, vortex well and briefly centrifuge plate to collect liquid.
2.30	Incubate 2 minutes at RT.
2.31	Place MIDI plate on magnetic stand and incubate for 5 minutes or until solution is clear.
2.32	Remove 20 µL of cleared supernatant and add to a new 0.2 mL 96-well plate.
Stopping point: If not continuing to the next step, seal the plate and store at –20°C.	

Adenylate cDNA 3' Ends		
2.33	Thaw ***Agendia NGS dA Tailing Master Mix*** on ice. Vortex ***Agendia NGS dA Tailing Master Mix*** for 15 seconds at high speed. Add 20 µL to each well of the plate containing 20 µL of cleared supernatant. Vortex mix, briefly spin and keep on ice.	
2.34	Run the following thermal cycler program: Do not use heated lid. If heated lid cannot be disabled, the program should be run with the lid open. 1. 30 minutes at 37°C 2. At least 3 minutes at 4°C Keep at 4°C or on ice until ready to proceed.	
Ligate Adaptors		
2.35	Thaw ***Agendia NGS Ligation Master Mix*** in ice. Thaw ***Agendia NGS Oligo Adaptor Mix*** on ice. Prepare ***Adaptor Ligation Mix*** according to the table below. **Note:** Vortex each reagent for 10 seconds.	
	Reagent	**Volume per Reaction**
	Agendia NGS Ligation Master Mix	5.0 µL
	Agendia NGS Oligo Adaptor Mix	5.0 µL
	Total volume	10.0 µL
	Vortex mix, briefly spin and keep on ice. For small sample batches, reagents components can be added individually to each sample well. When adding individually, pipette Agendia NGS Ligation Master Mix slowly to ensure full volume is dispensed.	

2.36	Place Adenylate/Ligation plate on ice, then add 10 µL of the ***Adaptor Ligation Mix*** to each sample well. Seal plate, vortex, and briefly centrifuge.

2.37	Run the following thermal cycler program: Do not use heated lid. If heated lid cannot be disabled, the program should be run with the lid open. 1. 15 minutes at 20°C 2. At least 3 minutes at 4°C Keep at 4°C or on ice until ready to proceed.

Purify Adaptor Ligated cDNA Using AMPure XP beads	
2.38	Allow ***AMPure XP beads*** to equilibrate to room temperature for at least 30 minutes. Vortex bead suspension until homogeneous. Thaw ***Agendia NGS PCR Master Mix*** at RT. Place in ice when thawed. Thaw ***Agendia NGS Uracil DNA Glycosylase (UDG), Agendia NGS Forward PCR Primer*** and ***Agendia NGS Reverse PCR Primer*** in ice.
2.39	Add 90 µL homogeneous bead suspension to each well of a new 0.8 mL 96-well MIDI plate.
2.40	Transfer 50 µL sample mix to respective well of the 0.8 mL 96-well MIDI plate.
2.41	Seal the plate, vortex, and briefly centrifuge.
2.42	Incubate samples for 5 minutes at RT.
2.43	Place plate on magnetic stand at RT for at least 5 minutes.
2.44	With the plate on the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
2.45	With the plate on the magnetic stand, dispense 200 µL fresh 70% ethanol into each well.
2.46	Wait 10 seconds (or until solution is clear) to allow any disturbed beads to settle, then carefully remove the ethanol.
2.47	Repeat for a total of 2 washes.
2.48	If needed, briefly centrifuge MIDI plate, return plate to magnetic stand, and then remove remaining ethanol droplets with a pipette.
2.49	Dry samples on the heat block at 37°C for 3 minutes. Do not overdry the samples, but ensure that all ethanol is removed.
2.50	Add 23 µL nuclease-free water to each sample well.
2.51	Seal the plate, vortex well and briefly centrifuge plate to collect liquid.
2.52	Incubate 2 minutes at RT.
2.53	Place MIDI plate on magnetic stand and incubate for 5 minutes or until solution is clear.
2.54	Remove 22 µL of cleared supernatant and add to a new 0.2 mL 96-well plate.

Amplification of Adaptor-Ligated cDNA Library		
2.55	Prepare ***Pre-Capture PCR Mix*** on ice according to the table below. **Note:** Vortex ***Agendia NGS PCR Master Mix*** reagent for 30 seconds before combining.	
	Reagent	**Volume per Reaction**
	Agendia NGS PCR Master Mix	25.0 µL
	Agendia NGS Uracil DNA Glycosylase (UDG)	1.0 µL
	Agendia NGS Forward PCR Primer	1.0 µL

	Agendia NGS Reverse PCR Primer	1.0 µL
	Total volume	28.0 µL
	Vortex mix, briefly spin and keep on ice.	
2.56	Add 28 µL of the ***Pre-Capture PCR Mix*** to each sample well. Seal plate, vortex, and briefly centrifuge.	
2.57	Run the following thermal cycler program: Heat lid at 95°C. 1. 15 minutes at 37°C 2. 2 minutes at 95°C 3. 30 seconds at 95°C 4. 30 seconds at 65°C 5. 1 minute at 72°C 6. Repeat steps 3-5 for a total of 14 cycles 7. 5 minutes at 72°C 8. At least 3 minutes at 4°C Keep at 4°C or on ice until ready to proceed.	

Purify Amplified Adaptor Ligated cDNA Using AMPure XP beads	
2.58	Allow ***AMPure XP beads*** to equilibrate to room temperature for at least 30 minutes. Vortex bead suspension until homogeneous.
2.59	Add 90 µL homogeneous bead suspension to each well of a new 0.8 mL 96-well MIDI plate.
2.60	Transfer 50 µL sample mix to respective well of the 0.8 mL 96-well MIDI plate.
2.61	Seal the plate, vortex, and briefly centrifuge.
2.62	Incubate samples for 5 minutes at RT
2.63	Place plate on magnetic stand at RT for at least 5 minutes.
2.64	With the plate on the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
2.65	With the plate on the magnetic stand, dispense 200 µL fresh 70% ethanol into each well.
2.66	Wait 10 seconds (or until solution is clear) to allow any disturbed beads to settle, then carefully remove the ethanol.
2.67	Repeat for a total of 2 washes.
2.68	If needed, briefly centrifuge MIDI plate, return plate to magnetic stand, and then remove remaining ethanol droplets with a pipette.
2.69	Dry samples on the heat block at 37°C for 3 minutes. Do not overdry the samples but ensure that all ethanol is removed.
2.70	Add 26 µL nuclease-free water to each sample well.
2.71	Seal the plate, vortex well and briefly centrifuge plate to collect liquid.
2.72	Incubate for 2 minutes at RT.
2.73	Place MIDI plate on magnetic stand and incubate for 5 minutes or until solution is clear.
2.74	Remove 25 µL of cleared supernatant and add to a new 0.2 mL 96-well plate.
Stopping point: If not continuing to the next step, seal the plate and store at –20°C.	
Quantify and Normalize Amplified Adaptor-Ligated cDNA	
2.75	Quantify the Amplified Adapter Ligated Pre-Capture library using a suitable nucleic acid fragment analysis platform in the region between 150bp-550bp. Aliquot a total of 200 ng cDNA pre-capture library.

2.76	Using a vacuum centrifuge, lyophilize the 200 ng cDNA pre-capture library and re-constitute in 3.4 µL nuclease-free water. *Do not overdry*. Vortex well and briefly centrifuge.
Stopping point: If not continuing to the next step, store the tubes at –20°C.	

Step 3: Agendia NGS Target Enrichment

Hybridize Library

3.1 Prepare an ice bucket and thaw reagents as described below:

Mix A Hyb Buffer	Mix B Prepped Library	Mix C Capture Library
Agendia NGS Hyb #1 **RT**	***Agendia NGS Indexing Block #1*** **4°C (ice)**	***Agendia NGS RNase Block*** **Remove from -20°C only when making Mix C**
Agendia NGS Hyb #2 **RT**	***Agendia NGS Block #2*** **4°C (ice)**	***MammaPrint BluePrint NGS Baits Library*** **4°C (ice)**
Agendia NGS Hyb #3 **RT**	***Agendia NGS Indexing Block #3*** **4°C (ice)**	
Agendia NGS Hyb #4 **RT**		

3.2 Prepare **Library Mix A** according to **table below**, into a 1.5 mL tube at RT.

Reagent	Volume for 1 reaction
Agendia NGS Hyb #1	6.63 µL
Agendia NGS Hyb #2	0.27 µL
Agendia NGS Hyb #3	2.65 µL
Agendia NGS Hyb #4	3.45 µL
Total volume	**13.0 µL**

Gently vortex mix, briefly spin and keep at RT.

3.3 Prepare a new 8-strip tube and label as **"A",** then aliquot 13 µL per sample of ***Library Mix A*** into each well.

Keep strip tubes at RT.

3.4 Prepare ***Library Mix B*** according to **table below**, into a 0.5 mL tube on ice.

Reagent	Volume for 1 reaction
Agendia NGS Indexing Block #1	2.5 µL
Agendia NGS Block #2	2.5 µL
Agendia NGS Indexing Block #3	0.6 µL
Total volume	**5.6 µL**

Gently vortex mix, briefly spin and keep on ice.

3.5 Prepare a new 8-strip tube and label as **"B"**, then

a. Aliquot 5.6 µL of ***Library Mix B*** into each well**.**
b. Add 3.4 µL of sample into each of their individual well.
c. Gently mix by pipetting up and down 10 times.

3.6 Place the 8-strip tube containing ***Library Mix B and sample*** onto a thermocycler and run the following program:

Heat lid at 105°C.; volume set at **29 µL**, if applicable

1. 5 minutes at 95°C
2. 5 minutes at 65°C
3. Hold at 65°C

<table>
<tr><td>3.7</td><td>Prepare Library Mix C according to table below into a 0.5 mL tube in 4°C ice.

<table><tr><th>Reagent</th><th>Volume for 1 reaction</th></tr><tr><td>Agendia NGS Nuclease-free water</td><td>4.5 µL</td></tr><tr><td>Agendia NGS RNase Block</td><td>0.5 µL</td></tr><tr><td>MammaPrint BluePrint NGS Baits Library</td><td>2.0 µL</td></tr><tr><td>Total volume</td><td>7.0 µL</td></tr></table>
Gently mix by pipetting up and down. Ensure there are no bubbles.

Note: Prepare Mix C near the 5-minute mark at 65°C of step 3.6. Keep the mixture at RT only briefly, until adding Mix A to Mix C. Do not keep solutions containing the MammaPrint BluePrint NGS Baits Library at RT for extended periods.</td></tr>
<tr><td>3.8</td><td>Label a new 8-strip tube as “C” and add 7 µL of Library Mix C into each well of the 8-strip tube.</td></tr>
<tr><td>3.9</td><td>Pipette 13 µL of Library Mix A into Library Mix C 8-strip tubes. Mix well by vortexing for 5 seconds and briefly centrifuge. Keep mixture at RT briefly, until use in step 3.10.</td></tr>
<tr><td>3.10</td><td>While thermocycler is still holding at 65°C, pipette everything from Library Mix A+C into sample wells containing Library Mix B. Ensure the entire contents of Library Mix A+C is transferred into Library Mix B.</td></tr>
<tr><td>3.11</td><td>Gently mix by pipetting up and down 10 times and close lid. If bubbles are present, perform a quick spin.</td></tr>
<tr><td>3.12</td><td>Ensure all tube lids are sealed. Close the lid of the thermocycler and run the following program:

1. 17-24 hours at 65°C
2. Hold at 65°C</td></tr>
</table>

<table>
<tr><th colspan="2">Prepare Streptavidin Beads</th></tr>
<tr><td>3.13</td><td>Vortex and aliquot 650 µL Agendia NGS Wash Buffer 2 per sample into 2 mL tubes.</td></tr>
<tr><td>3.14</td><td>Put aliquoted tubes into a 65°C heat block for at least 30 minutes.</td></tr>
<tr><td>3.15</td><td>Vortex the Dynabeads MyOne Streptavidin T1 beads for at least 30 seconds to break up bead clumps.</td></tr>
<tr><td>3.16</td><td>Aliquot 50 µL of beads per sample into 2 mL tubes. (Maximum of 200 µL beads, for 4 samples, per tube)</td></tr>
<tr><td>3.17</td><td>Wash the Streptavidin beads:

a. Add 200 µL of Agendia NGS Binding Buffer PER SAMPLE in the 2 mL tube. (Maximum of 800 µL buffer, for 4 samples, per tube)
b. Mix the beads on a vortex mixer for 5 seconds and briefly centrifuge.
c. Put the 2.0 mL tubes onto a magnetic separator device for 2 minutes and allow the solution to clear.
d. Remove and discard the supernatant.
e. Repeat steps a to d for a total of 3 washes.</td></tr>
<tr><td>3.18</td><td>Re-suspend the beads in 200 µL per sample of Agendia NGS Binding Buffer and aliquot them in 8-tube PCR strips.</td></tr>
</table>

<table>
<tr><th colspan="2">Capture Hybrids Using Streptavidin Beads</th></tr>
<tr><td>3.19</td><td>While maintaining the samples at 65°C on the thermocycler, transfer 29 µL from Hybridization Library 17-24 hours incubation into 8-strip tube containing 200 µL of washed streptavidin beads (Keep washed Streptavidin beads at RT).</td></tr>
<tr><td>3.20</td><td>Cap the 8-tube strip and mix by inverting, and then quick spin.</td></tr>
</table>

3.21	Incubate the sample on the thermal mixer at 27°C at 1400 rpm for 30 minutes. **Note:** Check beads for clumping after 5 minutes. If bead clusters have formed, quickly vortex the tube. After the 30 minutes incubation, set the thermal mixer to 65°C.
3.22	Briefly centrifuge tubes.
3.23	Put the plate on a magnetic separator for 2 minutes to collect the beads from the suspension.
3.24	Remove and discard the supernatant.
3.25	Re-suspend the beads in 200 µL of ***Agendia NGS Wash Buffer 1*** by mixing on a vortex mixer for 5 seconds.
3.26	Incubate the samples for 15 minutes at RT.
3.27	Separate the beads and buffer on a magnetic separator for 2 minutes and remove the supernatant.
3.28	Wash the beads with ***Agendia NGS Wash Buffer 2***: a. Re-suspend the beads in 200 µL of 65°C prewarmed Agendia NGS Wash Buffer 2. b. Cap the tubes and mix on a vortex mixer for 5 seconds to re-suspend the beads. Briefly spin down. c. Incubate the tubes on a thermal shaker for 10 minutes at 65°C and at 1200 rpm. Invert the tubes to mix occasionally if you notice that the beads settle. d. Briefly spin the tubes in a centrifuge or mini-plate spinner. e. Put the tubes on a magnetic separator for 2 minutes. f. Remove and discard the supernatant. g. Repeat steps a to f for a total of 3 washes. h. At the completion of the 3rd wash, make sure all of the wash buffer has been removed.
3.29	Vortex the beads in 31.5 µL of ***Agendia NGS Elution Buffer*** for **5 seconds** to re-suspend the beads. Briefly spin down.
3.30	Incubate the samples for 10 minutes at RT.
3.31	Separate the beads and buffer on the magnetic separator for 2 minutes.
3.32	Transfer 30 µL supernatant to a fresh tube/plate. Discard the beads.
3.33	Add 30 µL of ***Agendia NGS Neutralization Buffer*** to the captured cDNA library.

Purify the Captured Library with AMPure XP beads	
3.34	Allow ***AMPure XP beads*** to equilibrate to RT for at least 30 minutes. Vortex bead suspension until homogeneous. If proceeding to "**Amplify the Captured Libraries to Add Index Tags**", thaw ***Agendia NGS PCR Master Mix***, ***Agendia NGS Post-Capture PCR Primer***, and ***Agendia NGS 8bp Index Plate*** and place them in ice.
3.35	Add 108 µL homogeneous bead suspension to each well of a new 0.8 mL 96-well MIDI plate.
3.36	Add the 60 µL sample mix from step 3.33.
3.37	Seal the plate, vortex, and briefly centrifuge.
3.38	Incubate samples for 5 minutes at RT
3.39	Place plate on magnetic stand at RT for at least 5 minutes.
3.40	With the plate on the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
3.41	With the plate on the magnetic stand, dispense 200 µL fresh 70% ethanol into each well.

3.42	Wait 10 seconds (or until solution is clear) to allow any disturbed beads to settle, then carefully remove the ethanol.
3.43	Repeat for a total of 2 washes.
3.44	If needed, briefly centrifuge MIDI plate, return plate to magnetic stand, and then remove remaining ethanol droplets with a pipette.
3.45	Dry samples on the heat block at 37°C for 3 minutes. Do not overdry the samples, but ensure that all ethanol is removed.
3.46	Add 36 µL nuclease-free water to each sample well.
3.47	Seal the plate, vortex well and briefly centrifuge plate to collect liquid.
3.48	Incubate for 2 minutes at RT.
3.49	Place MIDI plate on magnetic stand and incubate for 5 minutes or until solution is clear.
3.50	Remove 35 µL of cleared supernatant and add to a new 0.2 mL 96-well plate or new tubes.
Stopping point: If not continuing to the next step, seal the plate and store at –20°C.	

Amplify the Captured Libraries to Add Index Tags

3.51

Thaw ***Agendia NGS PCR Master Mix***, ***Agendia NGS Post-Capture PCR Primer***, and ***Agendia NGS 8bp Index Plate*** and place them in ice.
Note: ***Agendia NGS PCR Master Mix*** is viscous, vortex for **30 seconds** before adding PCR primers.

Prepare ***Post-Capture PCR Mix*** according to **table below** into a 1.5 mL tube in ice.

Reagents	**Volume for 1 reaction**
Agendia NGS PCR Master Mix	25 µL
Agendia NGS Post-Capture PCR Primer	1 µL
Total Volume	**26 µL**

Gently vortex and spin the mix. Keep on ice.

3.52

For each sample to amplify, place 26 µL of the ***Post-Capture PCR Mix*** into a PCR plate well.

3.53

Add 5 µL of the appropriate ***indexing primer*** (from the ***Agendia NGS 8bp Index Plate*****)** to each well containing the Post-Capture PCR Mix.
Use a different indexing primer for each sample to be sequenced in the same lane.

3.54

Add 19 µL of purified library from step 3.50 to each well containing the ***Post-Capture PCR Mix***.
Mix by pipetting up and down 10 times. Briefly centrifuge plate.

3.55

Place the PCR plate in a thermocycler. Run the following thermocycler program:

Heat Lid at 105°C

1. 2 minutes at 95°C
2. 30 seconds at 95°C
3. 30 seconds at 57°C
4. 1 minute at 72°C
5. Repeat steps 2-4 for a total of 12 cycles
6. 5 minutes at 72°C
7. Hold at 4°C

Purify the Amplified Captured Libraries Using AMPure XP Beads.	
3.56	Allow ***AMPure XP beads*** to equilibrate to RT for at least 30 minutes. Vortex bead suspension until homogeneous.
3.57	Add 90 µL homogeneous bead suspension to each well of a new 0.8 mL 96-well MIDI plate.
3.58	Transfer 50 µL sample mix to respective well of the 0.8 mL 96-well MIDI plate.
3.59	Seal the plate, vortex, and briefly centrifuge.
3.60	Incubate samples for 5 minutes at RT
3.61	Place plate on magnetic stand at RT for at least 5 minutes.
3.62	With the plate on the magnetic stand, carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
3.63	With the plate on the magnetic stand, dispense 200 µL fresh 70% ethanol into each well.
3.64	Wait 10 seconds (or until solution is clear) to allow any disturbed beads to settle, then carefully remove the ethanol.
3.65	Repeat for a total of 2 washes.
3.66	If needed, briefly centrifuge MIDI plate, return plate to magnetic stand and remove remaining ethanol droplets with a pipette.
3.67	Dry samples on the heat block at 37°C for 3 minutes. Do not overdry the samples, but ensure that all ethanol is removed.
3.68	Add 22.5 µL ***Buffer EB*** to each sample well.
3.69	Seal the plate, vortex well and briefly centrifuge plate to collect liquid.
3.70	Incubate for 2 minutes at RT.
3.71	Place MIDI plate on magnetic stand and incubate for 5 minutes or until solution is clear.
3.72	Remove 21 µL of cleared supernatant to a new 96-well plate or new tubes.
Stopping point:, If not continuing to the next step, seal the plate and store the 21 µL **indexed** libraries at -20°C.	

QC 3: Quality assessment of amplified, target-enriched indexed libraries

Confirm the size distribution of each amplified, captured, indexed library using a suitable nucleic acid fragment analysis platform. The fragment size distribution should be 150-700 bp. For accurate quantification, make sure that the concentration falls within the linear range of the assay (5-500 pg/µL).

Stock Molarity [pmol/L]	**Molarity Target [nM]**
≥ 4000	4
2000-3999	2
1000-1999	1
<1000	Do not run, failed sample

Step 4: Agendia NGS MiSeq Loading

Preparation of Sample Sheet

4.1

Prepare MiSeq Sample Sheet in comma separated value (CSV) format according to the instructions below. This is a 150 bp single-end protocol.
Please refer to support.illumina.com/downloads/miseq_sample_sheet_quick_reference_guide_15028392.html for general instructions. An example of a MiSeq Sample Sheet can be found here: www.agendia.com/diagnostic-products/resources

Under **[Header]**

Investigator Name	Required, *user supplied*
Project Name	Required, *user supplied*
Experiment Name	Required, *user supplied*
Date	Required
Workflow	Required **[GenerateFASTQ]**
Assay	Required **[SureSelect]**
Chemistry	Required **[Default]**

Under **[Reads]: 150**

Under **[Settings]**

OnlyGenerateFASTQ	**1**
FilterPCRDuplicates	**0**

Under **[Data]:**

Sample ID	Sample Name	Sample Plate	Sample Well	Sample Project	Index	I7_Index_ID
Required, *user supplied*	Optional	Optional	Optional	Optional	Required **[Index Sequence]**	Optional

Note: Every sample must have a unique "Sample ID"; The "Sample Name" will be contained in the FASTQ file names

Save sheet. The software will prompt user to upload the Sample Sheet after flow cell and reagents are loaded into the sequencer.

4.2 Prepare reagent cartridge according to Illumina's recommendations.

Pool Final Libraries for Multiplexed Sequencing

4.3 Sequencing protocol used (**1 nM**, **2 nM** or **4 nM)** depends on the samples on the prepared Sample Sheet. Dilute each sample individually to the Molarity Target (i.e 1 nM, 2 nM, or 4 nM). If samples of varying molarity targets need to be combined into one MiSeq run, dilute all samples to the lowest common molarity target and pool.

4.4 Add 5 µL from each sample into a single 1.5 mL tube.

4.5 Vortex, briefly centrifuge and place in ice.

Denature Pooled cDNA Library

4.6 Prepare a fresh tube of 0.2 N NaOH (0.2 N NaOH is stable for a maximum of 12 hours)
Denature the pooled library according to the sequencing protocol used:

	1 nM	**2 nM**	**4 nM**
Pooled library	10 µL	5 µL	5 µL
0.2 N NaOH	10 µL	5 µL	5 µL

4.7 Vortex briefly to mix and centrifuge at 280 × g for 1 minute at RT.

4.8 Incubate at RT for **5 minutes**.

Dilute Denatured cDNA Library

4.9 Dilute the denatured cDNA library with pre-chilled HT1 buffer according to the sequencing protocol used:
Note: Invert HT1 buffer to mix.

	1 nM	**2 nM**	**4 nM**
Denatured cDNA library	20 µL	10 µL	10 µL
HT1	480 µL	490 µL	990 µL
Concentration	20 pM	20 pM	20 pM

4.10 Invert to several times to mix, briefly centrifuge and place on ice.

4.11 In a new 1.5 mL tube, further dilute the denatured library from step 4.10 according to the sequencing protocol to give the desired final input concentration.

	1 nM	**2 nM**	**4 nM**
Denatured DNA	500 µL	500 µL	450 µL
HT1	167 µL	167 µL	150 µL
Final concentration	15 pM	15 pM	15 pM

4.12 Invert several times to mix and then pulse centrifuge. Place in ice until ready to load into the reagent cartridge.

Combine Sample Library and PhiX Control (Optional)

4.13 Prepare 20 pM PhiX according to Illumnina's MiSeq Protocol or thaw 20 pM PhiX library (if previously prepared) on ice. Invert to mix and briefly centrifuge.

4.14 Combine the following volumes of PhiX control and sample library into a 1.5 mL tube:
a. 6 µL Denatured, diluted 20 pM PhiX library
b. 594 µL Denatured, diluted sample library (if no PhiX is added, add 600 µL denatured, diluted sample library)

4.15 Invert several times to mix and briefly centrifuge. Set aside in ice until ready to load into the reagent cartridge.

4.16 On the Welcome screen of the software interface, select "Sequence" to start the sequencing run set-up steps.

4.17 Load the MiSeq reagent cartridge with Sample Library and PhiX control. Proceed with sequencing per Illumina's MiSeq protocol to generate FASTQ files.

Step 5: Analysis of MiSeq Output

The FASTQ files generated by the MiSeq sequencer will be processed by the Agendia Data Analysis Pipeline Tool (ADAPT). ADAPT is a high-performance and data security compliant cloud-based genomics analysis platform. It delivers integrated data interpretation of samples processed in the MammaPrint BluePrint Kit.

For a detailed explanation, please refer to the ADAPT User Guide, available at www.agendia.com/ADAPT. The use of ADAPT is briefly described as follows:

Initial Setup	
1	Create an ADAPT Account
2	Install the Agendia Service Connector
3	Customize ADAPT Reports (optional)
Using ADAPT	
4	Upload FASTQ Files
5	Analyze Data
6	Download Reports

Results

The user will receive two documents per sample – the Technical Report and the Explanation of Results. The Technical Report will have information about the sample and the ADAPT processing, including quality control information and the MammaPrint BluePrint Kit results which include the MammaPrint Index (MPI), the determination of risk of recurrence (High Risk or Low Risk), and the BluePrint outcome (Luminal-type, HER2-type, or Basal-type). Refer to the Interpretation of Results section for more detailed information. The Explanation of Results explains the test results in the context of published clinical data.

Interpretation of Results

A test result is considered valid only if the Overall Assessment field on the Technical Report says “Pass”. If any of the quality control metrics fail, the Overall Assessment will also indicate “Fail”. If the Overall Assessment says “Fail”, the Technical Report will show "Unable to provide result for this specimen" in the Test Results section and the Explanation of Results document will not be provided. The testing laboratory may choose to retest the sample to see if the subsequent result will result in a valid test result.

MammaPrint

The MammaPrint result is provided as a binary result and can be either “Low Risk” or “High Risk” for risk of recurrence. The prognostic profile (Low Risk, High Risk) of the sample is determined by calculating the MPI on a scale of -1.000 to +1.000 (MammaPrint FFPE reportable range; Figure 2). High Risk results are those results that are equal to or below 0.000 whereas Low Risk results are those above 0.000. If the MammaPrint Index (MPI) falls within a pre-defined area around the classification cut-off between -0.0575 and +0.0575, the classification accuracy is less than 90%.

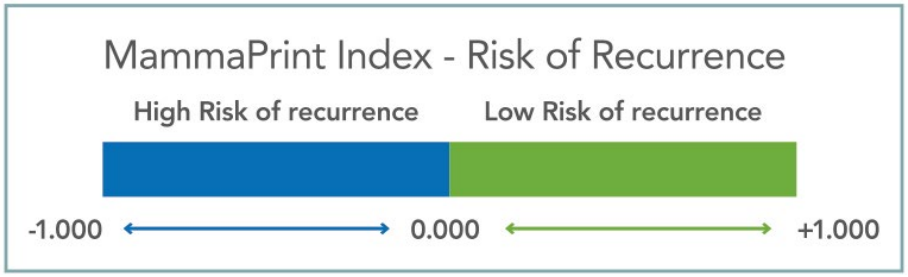

Figure 2: MammaPrint Index

BluePrint

BluePrint is a molecular subtyping assay that classifies breast cancer into three distinct subtypes: Luminal-type, HER2-type and Basal-type by determining the mRNA levels of 80 genes that best discriminate among these 3 distinct molecular subtypes. Each of these subtypes have marked differences in long-term outcome and response to (neo)-adjuvant chemotherapy [9] [10]. Combining MammaPrint and BluePrint allows patients to be stratified into the following subgroups: luminal-type/MammaPrint Low Risk (similar to luminal A); Luminal-type/MammaPrint High Risk (similar to luminal B); HER2-type and Basal-type.

Limitations of the Procedure

- The MammaPrint BluePrint Breast Cancer Recurrence and Molecular Subtyping Kit has only been validated for use with FFPE breast cancer tumor tissue from female patients. Testing of other specimen types or other preservation methods has not been evaluated.
- The RNeasy FFPE kit has been validated for use in this test. Use of other RNA isolation kits has not been evaluated.
- The MammaPrint BluePrint Kit has been validated in combination with Illumina MiSeq V3 reagents for 150 cycles. Use of other DNA sequencers or other reagents has not been evaluated.
- A MammaPrint Low Risk result does not guarantee that the breast cancer will not recur within five years. Similarly, a High Risk result does not guarantee that the breast cancer will recur. Test results should be used in conjunction with clinicopathological factors.
- The MammaPrint BluePrint Kit results are indicated for use by physicians as a prognostic marker only in addition to standard clinical-pathological factors. The test is not intended to determine the outcome of disease, nor to suggest or infer an individual patient's response to therapy.

Expected Values

MammaPrint

Clinical data from population-based studies has demonstrated the clinical utility of the MammaPrint test in the intended use population. MammaPrint has been clinically validated in a prospective clinical trial for use in early-stage (I and II) breast cancer patients regardless of Estrogen Receptor (ER) or HER2 status, with a tumor size ≤ 5.0 cm, and 0-3 positive lymph nodes (LN0-3), with no special specifications for nodal micrometastases. [14] The primary analysis demonstrated that withholding chemotherapy from Clinically-high risk/Genomic-MammaPrint-low risk (C-high/G-low) patients does not detrimentally impact outcome. No significant benefit for adjuvant systemic chemotherapy at 5-year was observed for MammaPrint Low Risk patients, also when presented with 1-3 positive lymph nodes [14]. From these and other published studies [7] [12] [13] [15] [16] [17] [18], it has been shown that the MammaPrint test improves prediction of clinical outcome in women with early-stage breast cancer.

BluePrint

Basal-type breast cancers are characterized by gene expression of the basal/myoepithelial cells of origin. The Basal-type cancers are typically triple-negative for ER, PR and HER2 (basal-like) with a specific gene expression profile. Hormone therapy and anti-HER2 therapies, such as trastuzumab and lapatinib, are not believed to be effective against these cancers, although chemotherapy is thought to be helpful.

Luminal-type breast cancers are characterized by gene expression of the luminal epithelial cells that line the breast ducts and glands. The Luminal-type cancers are typically hormone receptor positive tumors and are likely responsive to hormonal therapy. Patients classified as MammaPrint Low Risk and Luminal-type can be expected to have a clinical course similar to luminal A, usually treated with hormonal therapy, whereas those with a MammaPrint High Risk and Luminal-type, can be expected to have a clinical course similar to Luminal B patients who usually benefit from more aggressive treatment which may include chemotherapy.

The HER2-type breast cancers are characterized by amplification or over-expression of the HER2 locus and are typically HER2-positive tumors by IHC or FISH (HER2/neu positive). These cancers tend to grow more rapidly and may recur, although they can often be treated with targeted therapies such as trastuzumab and lapatinib.

Performance Characteristics

In order to estimate the precision, reproducibility and inter-laboratory reproducibility of the MammaPrint BluePrint Breast Cancer Recurrence and Molecular Subtyping Kit, analytical and clinical validation studies were conducted, and the results are presented below.

MammaPrint

Analytical Performance

Concordance between the MammaPrint test on NGS and the currently marketed MammaPrint FFPE on microarray technology was assessed using RNA from 85 FFPE samples. All testing was performed at Agendia's central laboratory in Amsterdam, The Netherlands. Assay performance was determined by calculating the positive percent agreement (PPA), the negative percent agreement (NPA) and the overall concordance between the two tests. PPA, NPA and overall concordance was 100%, 94% and 98%, respectively.

Reproducibility of the MammaPrint test on NGS was assessed over time using RNA isolated from three FFPE tissue samples that represented both MammaPrint risk categories (MammaPrint High Risk and Low Risk). Samples were analyzed multiple times over different days by multiple operators at Agendia's central laboratories in Amsterdam, The Netherlands and Irvine, California, USA. Per day a single run was performed: Sample 1 had 25 measurements, Sample 2 had 17 measurements and Sample 3 had 14 measurements. The median relative reproducibility based on the MammaPrint index was 98%.

Reproducibility was assessed between two isolations of RNA obtained from the same FFPE tissue sample, for a total of 43 samples. The two isolations of the 43 tissue samples were analyzed on the same day at Agendia's central laboratory in Amsterdam, The Netherlands. Concordance of MammaPrint results between the first and second isolation using these 43 samples was 98%.

Inter-laboratory reproducibility was assessed at two external European sites and Agendia's central laboratory in Amsterdam, The Netherlands. In total, RNA isolated from 16 FFPE samples was shipped to the three sites for testing. The 16 samples were divided over at least two operators at each site. Inter-laboratory reproducibility was determined between the two external sites and Agendia. Overall concordance was 100%.

Clinical Performance

The MammaPrint test on NGS clinical performance characteristics was assessed using a study cohort of 316 FFPE breast tumor tissue samples retrospectively collected and archived from breast cancer patients with Stage I or Stage II disease, tumor size $\leq$ 5.0 cm and lymph node negative or 1-3 lymph node positive enrolled between 2004 and 2006. To support clinical performance of the MammaPrint test, the 316 samples were evaluated with 5-year outcome data for Distant Recurrence Free Interval (DRFI), which is the time until the diagnosis of distant metastasis or death from breast cancer. As expected, these data demonstrated a significant difference between the MammaPrint High and Low Risk groups for the 5-year

DRFI (LogRank p=0.002). Importantly, the clinical performance of MammaPrint test on NGS for both High and Low Risk groups in this study cohort was statistically equivalent (High Risk p=0.83, Low Risk p=0.44) to the performance of the currently marketed MammaPrint FFPE on microarray technology.

Lastly, a field correlation study was conducted at two independent field European sites. Breast cancer samples were prospectively collected from 95 patients in the intended use population (i.e., Stage I or Stage II disease, tumor size $\leq$ 5.0 cm and lymph node negative or 1-3 lymph node positive). These samples were processed at the field sites on MammaPrint test on NGS and part of the tissue was shipped to Agendia's central laboratory in Amsterdam, The Netherlands for testing on MammaPrint test on NGS as well as the currently marketed MammaPrint FFPE on microarray technology. Assay performance was assessed by comparing the MammaPrint test NGS results obtained at the field sites to the MammaPrint test on NGS and to MammaPrint FFPE results obtained at Agendia. Concordance between MammaPrint test on NGS performed in the field to MammaPrint test on NGS performed at Agendia based on 86 samples was 93%. Similarly, concordance between MammaPrint test on NGS performed in the field to MammaPrint FFPE on microarray performed at Agendia was 91%.

BluePrint

Analytical Performance

Concordance between the BluePrint test on NGS and the currently marketed BluePrint FFPE on microarray technology was assessed using 98 FFPE RNA samples. All testing was performed at Agendia's central laboratory in Amsterdam, The Netherlands. Assay performance was determined by calculating the overall concordance between the two tests, which was 100%.

Reproducibility of BluePrint test on NGS was assessed over time using RNA isolated from three FFPE tissue samples that represented the different outcome levels of BluePrint: Luminal-type, HER2-type and Basal-type. Samples were analyzed multiple times over different days by multiple operators at Agendia's central laboratories in Amsterdam, The Netherlands and Irvine, California, USA. Per day a single run was performed: Sample 1 had 25 measurements, Sample 2 had 17 measurements and Sample 3 had 14 measurements. The median relative reproducibility in the BluePrint index for Luminal-type was 98%; for HER2-type was 98%; and for Basal-type was 98%.

Reproducibility was assessed between two isolations of RNA obtained from the same FFPE tissue, for a total of 43 samples. The two isolations of the 43 tissue samples were analyzed on the BluePrint test on NGS on the same day. Concordance between the first and second isolation of these 43 samples was 100%.

Inter-laboratory reproducibility was assessed at two external European sites and Agendia's central laboratory in Amsterdam, The Netherlands. RNA isolated from 16 FFPE samples were shipped to the three sites for testing. The 16 samples were divided over at least two operators at each site. Inter-laboratory reproducibility was determined between the two external sites and Agendia. Overall concordance was 100%.

Clinical Performance

A field correlation study was conducted at two independent European field sites. Breast cancer samples were prospectively collected from 95 patients in the intended use population (i.e., Stage I or Stage II disease, tumor size $\leq$ 5.0 cm and lymph node negative or 1-3 lymph node positive). These samples were processed at the field sites on BluePrint kit test on NGS and part of the tissue was shipped to Agendia's central laboratory in Amsterdam, The Netherlands for testing on BluePrint test on NGS as well as the currently marketed BluePrint FFPE on microarray technology. Assay performance was assessed by comparing the BluePrint test on NGS results obtained at the field sites to the BluePrint test on NGS and BluePrint FFPE results obtained at Agendia. Concordance between BluePrint test on NGS performed in the field to BluePrint test on NGS performed at Agendia including 86 samples was 100%. Similarly, concordance between BluePrint test on NGS performed in the field to BluePrint FFPE on microarray performed at Agendia was 98%.

Assistance

If you have any questions regarding the use of this product, please contact Agendia Customer Service at customerservice@agendia.com or by telephone at +31 (0) 20 462 1510, Monday to Friday from 08:30 to 17:00 (GMT/UTC +1).

Bibliography

[1] L. J. van 't Veer, H. Dai, M. J. van de Vijver, Y. D. He, A. A. M. Hart, M. Mao, H. L. Peterse, K. van der Kooy, M. J. Marton, A. T. Witteveen, G. J. Schreiber, R. M. Kerkhoven, C. Roberts, P. S. Linsley, R. Bernards, and S. H. Friend, "Gene expression profiling predicts clinical outcome of breast cancer," *Nature*, vol. 415, pp. 530–536, 2002.

[2] M. J. van de Vijver, Y. D. He, L. J. van 't Veer, H. Dai, A. A. M. Hart, D. W. Voskuil, G. J. Schreiber, J. L. Peterse, C. Roberts, M. J. Marton, M. Parrish, D. Atsma, A. Witteveen, A. Glas, L. Delahaye, T. van der Velde, H. Bartelink, S. Rodenhuis, E. T. Rutgers, S. H. Friend, and R. Bernards, "A gene-expression signature as a predictor of survival in breast cancer," *N. Engl. J. Med.*, vol. 347, no. 25, pp. 1999–2009, 2002.

[3] A. M. Glas, A. Floore, L. J. M. J. Delahaye, A. T. Witteveen, R. C. F. Pover, N. Bakx, J. S. T. Lahti-Domenici, T. J. Bruinsma, M. O. Warmoes, R. Bernards, L. F. A. Wessels, and L. J. van 't Veer, "Converting a breast cancer microarray signature into a high-throughput diagnostic test.," *BMC Genomics*, vol. 7, p. 278, 2006.

[4] O. Krijgsman, P. Roepman, W. Zwart, J. S. Carroll, S. Tian, F. A. de Snoo, R. A. Bender, R. Bernards, and A. M. Glas, "A diagnostic gene profile for molecular subtyping of breast cancer associated with treatment response," *Breast Cancer Res. Treat.*, vol. 133, no. 1, pp. 37–47, 2012.

[5] S. Mook, M. K. Schmidt, B. Weigelt, B. Kreike, I. Eekhout, M. J. van de Vijver, A. M. Glas, A. Floore, E. J. T. Rutgers, and L. J. van 't Veer, "The 70-gene prognosis signature predicts early metastasis in breast cancer patients between 55 and 70 years of age," *Ann. Oncol.*, vol. 21, no. 4, pp. 717–722, 2010.

[6] M. Buyse, S. Loi, L. van 't Veer, G. Viale, M. Delorenzi, A. M. Glas, M. Saghatchian d'Assignies, J. Bergh, R. Lidereau, P. Ellis, A. Harris, J. Bogaerts, P. Therasse, A. Floore, M. Amakrane, F. Piette, E. Rutgers, C. Sotiriou, F. Cardoso, and M. J. Piccart, "Validation and Clinical Utility of a 70-Gene Prognostic Signature for Women With Node-Negative Breast Cancer," *JNCI J. Natl. Cancer Inst.*, vol. 98, no. 17, pp. 1183–1192, 2006.

[7] C. A. Drukker, J. M. Bueno-de-Mesquita, V. P. Retèl, W. H. van Harten, H. van Tinteren, J. Wesseling, R. M. H. Roumen, M. Knauer, L. J. van ’t Veer, G. S. Sonke, E. J. T. Rutgers, M. J. van de Vijver, and S. C. Linn, “A prospective evaluation of a breast cancer prognosis signature in the observational RASTER study,” *Int. J. Cancer*, vol. 133, no. 4, pp. 929–936, 2013.

[8] I. Beumer, A. Witteveen, L. Delahaye, D. Wehkamp, M. Snel, C. Dreezen, J. Zheng, A. Floore, G. Brink, B. Chan, S. Linn, R. Bernards, L. van ’t Veer, and A. Glas, “Equivalence of MammaPrint array types in clinical trials and diagnostics,” *Breast Cancer Res. Treat.*, vol. 156, no. 2, pp. 279–287, 2016.

[9] S. Glück, F. de Snoo, J. Peeters, L. Stork-Sloots, and G. Somlo, “Molecular subtyping of early-stage breast cancer identifies a group of patients who do not benefit from neoadjuvant chemotherapy,” *Breast Cancer Res. Treat.*, vol. 139, no. 3, pp. 759–767, 2013.

[10] K. R. Hess, K. Anderson, W. F. Symmans, V. Valero, N. Ibrahim, J. A. Mejia, D. Booser, R. L. Theriault, A. U. Buzdar, P. J. Dempsey, R. Rouzier, N. Sneige, J. S. Ross, T. Vidaurre, H. L. Gómez, G. N. Hortobagyi, and L. Pusztai, “Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer,” J. Clin. Oncol., vol. 24, no. 26, pp. 4236–4244, 2006.

[11] G. Viale, F. A. de Snoo, L. Slaets, J. Bogaerts, L. van ’t Veer, E. J. Rutgers, M. J. Piccart-Gebhart, L. Stork-Sloots, A. Glas, L. Russo, P. Dell’Orto, K. Tryfonidis, S. Litière, F. Cardoso, and MINDACT investigators, “Immunohistochemical versus molecular (BluePrint and MammaPrint) subtyping of breast carcinoma. Outcome results from the EORTC 10041/BIG 3-04 MINDACT trial.,” *Breast Cancer Res. Treat.*, 2017.

[12] K. Yao, R. Goldschmidt, M. Turk, J. Wesseling, L. Stork-Sloots, F. de Snoo, and M. Cristofanilli, “Molecular subtyping improves diagnostic stratification of patients with primary breast cancer into prognostically defined risk groups,” *Breast Cancer Res. Treat.*, vol. 154, no. 1, pp. 81–88, 2015.

[13] L. J. Esserman, C. Yau, C. K. Thompson, L. J. van ’t Veer, A. D. Borowsky, K. A. Hoadley, N. P. Tobin, B. Nordenskjöld, T. Fornander, O. Stål, C. C. Benz, and L. S. Lindström, “Use of Molecular Tools to Identify Patients With Indolent Breast Cancers With Ultralow Risk Over 2 Decades,” *JAMA Oncol.*, 2017.

[14] F. Cardoso, L. J. van’t Veer, J. Bogaerts, L. Slaets, G. Viale, S. Delaloge, J.-Y. Pierga, E. Brain, S. Causeret, M. DeLorenzi, A. M. Glas, V. Golfinopoulos, T. Goulioti, S. Knox, E. Matos, B. Meulemans, P. A. Neijenhuis, U. Nitz, R. Passalacqua, P. Ravdin, I. T. Rubio, M. Saghatchian, T. J. Smilde, C. Sotiriou, L. Stork, C. Straehle, G. Thomas, A. M. Thompson, J. M. van der Hoeven, P. Vuylsteke, R. Bernards, K. Tryfonidis, E. Rutgers, and M. Piccart, “70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer,” *N. Engl. J. Med.*, vol. 375, no. 8, pp. 717–729, 2016.

[15] S. Mook, M. K. Schmidt, G. Viale, G. Pruneri, I. Eekhout, A. Floore, A. M. Glas, J. Bogaerts, F. Cardoso, M. J. Piccart-Gebhart, E. T. Rutgers, and L. J. van ’t Veer, “The 70-gene prognosis-signature predicts

disease outcome in breast cancer patients with 1-3 positive lymph nodes in an independent validation study," Breast Cancer Res. Treat., vol. 116, no. 2, pp. 295–302, 2009.

[16] L. J. van 't Veer, C. Yau, N. Y. Yu, C. C. Benz, B. Nordenskjöld, T. Fornander, O. Stål, L. J. Esserman, and L. S. Lindström, "Tamoxifen therapy benefit for patients with 70-gene signature high and low risk," *Breast Cancer Res. Treat.*, 2017.

[17] J. M. Bueno-de-Mesquita, W. H. van Harten, V. P. Retel, L. J. van 't Veer, F. S. van Dam, K. Karsenberg, K. F. Douma, H. van Tinteren, J. L. Peterse, J. Wesseling, T. S. Wu, D. Atsma, E. J. Rutgers, G. Brink, A. N. Floore, A. M. Glas, R. M. Roumen, F. E. Bellot, C. van Krimpen, S. Rodenhuis, M. J. van de Vijver, and S. C. Linn, "Use of 70-gene signature to predict prognosis of patients with node-negative breast cancer: a prospective community-based feasibility study (RASTER)," *Lancet Oncol.*, vol. 8, no. 12, pp. 1079–1087, 2007.

[18] B. S. Wittner, D. C. Sgroi, P. D. Ryan, T. J. Bruinsma, A. M. Glas, A. Male, S. Dahiya, K. Habin, R. Bernards, D. A. Haber, L. J. van 't Veer, and S. Ramaswamy, "Analysis of the mamma print breast cancer assay in a predominantly postmenopausal cohort," *Clin. Cancer Res.*, vol. 14, no. 10, pp. 2988–2993, 2008.

[19] L. J. Delahaye, D. Wehkamp, A. N. Floore, R. Bernards, L. J. van 't Veer, and A. M. Glas, "Performance characteristics of the MammaPrint breast cancer diagnostic gene signature," *Per. Med.*, vol. 10, no. 8, pp. 801–811, 2013.

[20] A. Sapino, P. Roepman, S. C. Linn, M. H. J. Snel, L. J. M. J. Delahaye, J. van den Akker, A. M. Glas, I. M. Simon, N. Barth, F. A. de Snoo, L. J. van 't Veer, L. Molinaro, E. M. J. J. Berns, J. Wesseling, L. B. Riley, D. Anderson, B. Nguyen, and C. E. Cox, "MammaPrint Molecular Diagnostics on Formalin-Fixed, Paraffin-Embedded Tissue.," *J. Mol. Diagn.*, vol. 16, no. 2, pp. 190–197, 2014.

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	Batch code	Indicates the manufacturer's batch code so that the batch or lot can be identified.
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	Temperature limit	Indicates the temperature limits to which the medical device can be safely exposed.
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	Consult instructions for use	Indicates the need for the user to consult the instructions for use.
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	*In vitro* diagnostic medical device	Indicates a medical device that is intended to be used as an in vitro diagnostic medical device.

Appendix A: Nucleotide Sequences of MammaPrint BluePrint NGS 8bp Indexes

Table 1: Nucleotide Sequences of MammaPrint BluePrint Kit Indexes A01 to H04

Index (plate coordinate)	Sequence
A01	ATGCCTAA
B01	GAATCTGA
C01	AACGTGAT
D01	CACTTCGA
E01	GCCAAGAC
F01	GACTAGTA
G01	ATTGGCTC
H01	GATGAATC
A02	AGCAGGAA
B02	GAGCTGAA
C02	AAACATCG
D02	GAGTTAGC
E02	CGAACTTA
F02	GATAGACA
G02	AAGGACAC
H02	GACAGTGC
A03	ATCATTCC
B03	GCCACATA
C03	ACCACTGT
D03	CTGGCATA
E03	ACCTCCAA
F03	GCGAGTAA
G03	ACTATGCA
H03	CGGATTGC
A04	AACTCACC
B04	GCTAACGA
C04	CAGATCTG
D04	ATCCTGTA
E04	CTGTAGCC
F04	GCTCGGTA
G04	ACACGACC
H04	AGTCACTA