Multiplex8+
RESULTS

PATIENT

Name:

Personal ID No.:

PHYSICIAN

Name:

Institution:

Contact:

SAMPLE AND REPORT INFORMATION

FFPE Block ID:

Sample Type:
(site - treatment setting) **Breast - adjuvant**

MDX Order ID: **MDX-PT-733**

Sample receipt date:

Report release date:

Original or revision: **Original**

This sample was examined with the Multiplex8+ test, comprising two examinations developed and validated by this laboratory: RNA-FISH on a tumor section, and NGS (total-RNA sequencing) of tumor tissue isolated by H&E- and RNA-FISH-guided laser capture microdissection. Receptor status is the NGS result against the positivity thresholds shown, which the laboratory established by calibrating NGS expression to immunohistochemistry in a retrospective cohort. The RNA-FISH result is shown as an independent confirmation; where the two disagree, the NGS result stands, as NGS is the reference method against which RNA-FISH was validated. Measurement uncertainty is available on request.

TEST RESULT

RECEPTOR	GENE	NGS OUTPUT (TPM)	NGS POSITIVITY THRESHOLD (TPM)	NGS RESULT	RNA-FISH CONCORDANCE
ER	*ESR1*	4.978	≥5.0	NEGATIVE	DISCORDANCE (RNA-FISH POSITIVE)
PR	*PGR*	0.542	≥1.4	NEGATIVE	CONCORDANCE
HER2	*ERBB2*	26.113	≥154.7 “low” ≥24.325	LOW	CONCORDANCE
Ki-67	*MKI67*	45.233	≥18.9	POSITIVE	CONCORDANCE

LASER CAPTURE MICRODISSECTION



Based on the histological assessment and RNA-FISH biomarker expression, the sample was subjected to laser capture microdissection for further analysis.

COMMENTS & LIMITATIONS

- Due to technical problems with the instrument, usable results from the RNA-FISH analysis were not obtained until the third attempt.

AUTHORISATION

REVIEWED: **APPROVED:** **RELEASED:**

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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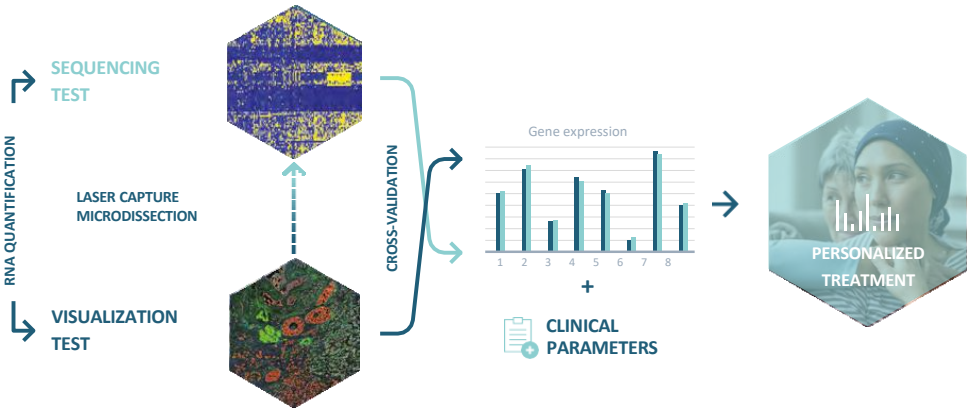
TEST DESCRIPTION

The **Multiplex8+** breast cancer test assesses RNA-based biomarkers by conducting a **VISUALIZATION TEST** that uses RNA fluorescent in situ hybridization **(RNA-FISH)** to visualize a panel of biomarkers. Based on the expression of these biomarkers and the tissue histology, laser capture microdissection is used to dissect out regions of interest. With these tumor-enriched samples, a **SEQUENCING TEST** that utilizes total RNA next-generation sequencing to survey gene expression in a spatially resolved manner, is further carried out. Analytical validation of **Multiplex8+** was conducted on a large retrospective cohort of 1 082 breast tumors.



THE TEST PROVIDES INFORMATION ABOUT:

1. **RECEPTOR STATUS**: for RNA expression of the estrogen receptor, progesterone receptor, HER2 receptor, and Ki-67 measured and cross-validated using both tests.
2. **MOLECULAR SUBTYPE:** based on RNA gene expression tumor biology.
3. **GENE SIGNATURES:** personalized for patients' tumor biology and clinical status.

INTERPRETATION GUIDE

In the following report, each gene/gene signature is given a percentile score, which ranks the expression level in the context of the patients included in our retrospective cohort. For the four main breast cancer biomarkers, estrogen receptor *(ESR1)*, progesterone receptor *(PGR)*, HER2 receptor *(ERBB2)*, and Ki-67 *(MKI67)*, these percentile rankings are in the context of all 1 013 eligible patients. For all other genes/gene signatures, the percentile rankings are in the context of other patients belonging to the same **MOLECULAR SUBTYPE**. For example, for patients classified as Luminal A, the genes and gene signature will receive a percentile score compared to all Luminal A samples in our retrospective validation. The percentile scores do not necessarily imply a given level of sensitivity or resistance to a therapy.

Sample Percentile
Low (1-33)
Medium (<33-66)
High (<66-100)

Percentile groups and ranges

Subtype	No. of patients
Luminal A	432
Luminal B	313
HER2	87
Basal-like	181
All patients	1 013

Number of patients in each molecular subtype and total retrospective cohort that are used to determine percentile rankings

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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RESULTS SUMMARY

A SUMMARY IS PROVIDED BELOW AND ADDITIONAL DETAILS ARE PROVIDED IN THE FOLLOWING PAGES.

MOLECULAR SUBTYPE*

INTRINSIC SUBTYPE	TNBC SUBTYPE
Basal-like	Immunomodulatory (IM)

RELEVANT TREATMENT*

THERAPY	KEY FINDINGS	PREDICTED CLINICAL BENEFIT*
PARP inhibitor (Veliparib), Carboplatin	Gene expression signature	Higher possibility of benefit
Trastuzumab deruxtecan (Enhertu)	Gene expression	Higher possibility of benefit
Sacituzumab govitecan (Trodelvy)	Gene expression	Higher possibility of benefit
Datopotamab deruxtecan (Datroway)	Gene expression	Higher possibility of benefit
5-fluorouracil (5-FU)	Gene expression	Higher possibility of benefit
AKT/PI3K inhibitor (capivasertib/inavolisib/alpelisib)	Gene expression signature	Higher possibility of benefit (off-label, clinical trial)
ADCs in clinical trials	Gene expression	Higher possibility of benefit (off-label, clinical trial)
Gemcitabine and capecitabine	Gene expression	Lower possibility of benefit
Bevacizumab (Avastin)	Gene expression, gene expression signature	Lower possibility of benefit
Methotrexate	Gene expression	Lower possibility of benefit

*Predicted clinical benefit is based on inferences from various data sources. The relationship of Multiplex8+ results to actual clinical benefit has not been validated.

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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MOLECULAR SUBTYPE*

Intrinsic subtype	TNBC subtype[2-4]
Basal-like	Immunomodulatory (IM)

Based on the SEQUENCING TEST, we used a consensus subtyping approach consisting of our proprietary 293-gene molecular subtyping signature, the research-based PAM50 test and the AIMS method to classify the intrinsic molecular subtype [1]. The TNBC subtype, where applicable, was classified according to Lehmann [2-4].

RECEPTOR STATUS

Sample	ESR1	PGR	ERBB2	MKI67
A	–	–	– low	+

Receptor status was determined using both the VISUALIZATION TEST and the SEQUENCING TEST. The table shows results after cross-validation.

INTERPRETATION

- The results from the Multiplex8+ are concordant with the immunohistochemistry findings.

INTERPRETATION*

- The biology of the Basal-like tumor type is consistent with the immunohistochemical and clinical designation.
- The basal-like subtype is similar to TNBC because it often lacks expression of hormone and HER2 receptors. Although patients with basal-like breast cancer respond better to chemotherapy, they are more susceptible to early relapse and have poorer prognosis.
- The immunomodulatory TNBC subtype shows enriched immune gene signatures, including checkpoint inhibitor genes, association with high grade, and shows favorable prognosis[2–4].

GENE SIGNATURE*

- Based on the assigned molecular subtype, and where applicable TNBC subtype, we evaluated several individual genes and gene signatures that demonstrate prognostic and predictive potential in early and advanced/metastatic settings.

Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
Prognosis	HR+_ Prognostic	The prognostic signature is derived from a consensus of three research-based prognostic signatures, including the 21-gene signature GENE21 [5], the 70-gene GENE70 signature [6], and the 50-gene risk of relapse based on subtype alone (ROR-S) signature [7]. The prognostic signatures are intended for early-stage breast cancer patients with ER+/HER2– IHC, lymph node-negative, or 1-3 positive lymph nodes. The score is reported as high, intermediate, or low. Patients with high signature scores are at a greater risk of relapse and may benefit from adjuvant chemotherapy, while patients with low scores have lower risk of relapse and may not benefit from adjuvant chemotherapy.	**High risk**	
	MDX_TNBC_ Risk	MultiplexDX (MDX) 60-gene signature for recurrence-free survival risk prediction for TNBC patients. The signature was developed on 159 TNBC patients from the MDX-BRCA[1] cohort and validated on 148 TNBC patients from the TCGA-BRCA cohort as well as a real-world, multicenter, retrospective cohort of 590 patients diagnosed with early TNBC and treated with neoadjuvant chemotherapy or chemoimmunotherapy.	**Low risk**	

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
Luminal signatures	ESR1	The ESR1 and PGR genes encode for the estrogen (ER) and progesterone (PR) hormone receptors, respectively, which are involved in growth, metabolism, and reproductive functions. High expression of ER/PR is predictive of endocrine therapies and low or negative ER/PR is associated with poor prognosis [8].	**Low (29)**	
	PGR		**Low (23)**	
	ESR1_PGR average	The average gene expression of ESR1 and PGR. Higher expression of hormone receptors is a predictive marker for endocrine therapies.	**Low (28)**	
	E2F4_score	This gene signature assesses activity of the E2F4 transcription factor and its targets. A high E2F4 signature is associated with endocrine resistance to aromatase inhibitors and may predict sensitivity to CDK4/6 inhibitors [9].	**High (70)**	
HER2	ERBB2	The ERBB2 gene encodes the HER2 protein, a receptor tyrosine kinase involved in cell growth/proliferation and is both a prognostic marker and predictive of response to HER2-targeted therapies [8].	**Low (19)**	
	MUC4	Mucin 4 (MUC4) is a glycoprotein that is implicated in resistance to trastuzumab through interactions with the HER2 receptor. High MUC4 is associated with reduced sensitivity to trastuzumab [10].	**High (85)**	
	NRG1	NRG1 codes for neuregulin 1, a ligand of the HER3 receptor. In the phase II NeoSphere trial, high NRG1 gene expression was associated with reduced response to neoadjuvant trastuzumab, but not combination trastuzumab-pertuzumab [11].	**Medium (44)**	
	pSTAT3-GS	A signature that predicts phosphorylation of STAT3 and was found to be predictive of trastuzumab resistance in the FinHer study [12].	**High (90)**	
	Her2 amplicon_ MDX	Proprietary MDX 43-gene signature used to assess HER2 status.	**Medium (41)**	
	Module7_ ERBB2	HER2-signaling signature predictive of response to multiple anti-HER2 treatments in the I-SPY2 trial [13].	**Medium (35)**	
	T-DM1_pred	The trastuzumab emtansine (T-DM1) predictive signature is a Research Use Only classifier that combines 19 genes/gene signature involved in the mechanism of action of T-DM1 and was shown to predict response in the T-DM1 arm of the I-SPY2 trial (https://www.nature.com/articles/s41467-024-55583-2).	**Low (24)**	
Proliferation	AURKA	Aurora Kinase A (AURKA) is a protein coding gene involved in cell proliferation and is an independent prognostic marker in breast cancer.	**Medium (49)**	
	MKI67	MKI67 codes for the marker of proliferation Ki-67 protein, a marker of poor prognosis in ER+/HER2− tumors, but not HER2+ or TNBC tumors. Ki-67 expression is also predictive of sensitivity to neoadjuvant endocrine and chemotherapies [8].	**High (87)**	
	Module11_ proliferation	Proliferation index used in I-SPY2 trial broadly predictive of pathological complete response in hormone receptor positive patients [4].	**High (72)**	
	Proliferation_ MDX	Proprietary MDX 7-gene signature used to assess cellular proliferation and cross-validate MKI67 expression.	**High (92)**	
CDK4/6 inhibitors	CDK4	Cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) are important proteins that regulate cell cycle progression from G1 to S phases. They are the main targets of CDK4/6 inhibitors such as palbociclib (Ibrance), ribociclib (Kisqali), and abemaciclib (Verzenio); however, it is unclear whether their expression predicts CDK4/6 inhibitor sensitivity.	**Medium (50)**	
	CDK6		**Medium (62)**	
	CCNE1	Elevated expression of the G1/S cell cycle regulators, CCNE1, CCND3, and CDKN2D, was associated with resistance to palbociclib (Ibrance) in the single-arm phase II neoadjuvant trial (NeoPalAna) [14].	**High (78)**	
	CCND3		**High (90)**	
	CDKN2D		**High (84)**	
PIK3CA mutations	PIK3CA-GS	A gene signature that is predictive of mutations in the PIK3CA gene and consequently the PI3K inhibitor alpelisib (Piqray). A high PIK3CA-GS score is also associated with activation of the PI3K/AKT pathway and loss of mTORC1 signaling, which may be relevant for response to mTOR inhibitors (e.g., everolimus) [15].	**High (96)**	

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
Chemotherapy	TOP1	The gene encoding DNA topoisomerase I, an enzyme critical for DNA transcription, is a target for anticancer drugs.	High (87)	
	TOP2A	The gene encoding DNA topoisomerase IIa, an enzyme critical for DNA transcription, is a target for anticancer drugs.	Medium (64)	
	RAD51	The DNA repair protein RAD51 homolog 1 (RAD51) is involved in the repair of damaged DNA and is associated with resistance to chemotherapy.	High (96)	
	ERCC1	The DNA excision repair protein ERCC-1 (ERCC1) is involved in the repair of DNA damage and is associated with resistance to chemotherapy.	High (75)	
	TYMS	The Thymidylate Synthetase (TYMS) gene encodes a protein involved in DNA biosynthesis and is the target of the antimetabolite chemotherapy, 5-Fluorouracil [16].	High (87)	
	SLC29A1	SLC29A1 codes for the equilibrative nucleoside transporter 1 (ENT1) protein, which is a nucleoside transporter that is involved in transporting gemcitabine and capecitabine [17].	Low (16)	
	DHFR	Dihydrofolate reductase is an enzyme coded by the DHFR gene and is involved in folate metabolism and cell growth. It is the target of the antimetabolite chemotherapy, methotrexate [18].	Low (32)	
	SLC19A1	SLC19A1 codes for the reduced folate carrier 1 (RFC1) protein, which transports methotrexate into the cell [18].	Low (13)	
	CDK12	The protein product of the Cyclin Dependent Kinase 12 (CDK12) gene regulates transcription, DNA repair pathways, and cell cycle [19].	Low (2)	
	MDX_TNBC_CTx	MultiplexDX (MDX) 45-gene signature predicting pathological complete response (pCR) to neoadjuvant chemotherapy (CTx) for TNBC patients. The signature was developed using 23 studies involving 706 patients with early TNBC who received neoadjuvant therapy and on a real-world, multicenter, retrospective cohort of 590 patients diagnosed with early TNBC and treated with neoadjuvant chemotherapy or chemoimmunotherapy.	pCR positive	
Immune system	PDCD1	PDCD1 codes for the immune checkpoint marker PD-1. PD-1 is the target of pembrolizumab (Keytruda), an immunotherapy approved for the first-line treatment of metastatic TNBC.	High (84)	
	CD274	CD274 codes for the immune checkpoint marker PD-L1. PD-L1 is the target of atezolizumab (Tecentriq), an immunotherapy approved for the first-line treatment of metastatic TNBC.	Medium (65)	
	CTLA4	Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is an immune checkpoint marker.	High (68)	
	MDX_TNBC_ICI	MultiplexDX (MDX) 20-gene signature predicting pathological complete response (pCR) to neoadjuvant immunotherapy (i.e., immune-checkpoint inhibitor, ICI) for TNBC patients. The signature was developed on 60 TNBC patients from the I-SPY2 pembrolizumab (n=29) and durvalumab plus olaparib (n=21) arms and validated on a real-world, multicenter, retrospective cohort of 590 patients diagnosed with early TNBC and treated with neoadjuvant chemotherapy or chemoimmunotherapy.	pCR positive	
DNA damage and repair	VCpred_TN	DNA damage repair / immune signature predictive of response to veliparib (PARP inhibitor) and carboplatin (I-SPY2 trial) [13].	High (87)	
Angiogenesis/ hypoxia	VEGFA	A gene coding for vascular endothelial growth factor, a protein involved in angiogenesis, vasodilation, and endothelial cell growth. VEGF is the target of the drug bevacizumab (Avastin).	Low (16)	
	Hypoxia / Angiogenesis / Inflammatory_ MDX	Proprietary MDX 7-gene signature used to assess hypoxia, angiogenesis, and inflammation. Signature includes genes known to be predictive of response to bevacizumab (Avastin) in the neoadjuvant GeparQuinto trial [22].	Medium (41)	

Multiplex8+ - SUPPLEMENTARY FINDINGS*

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Treatment type/ Pathway	Gene signature	Description	Sample A Percentile	
ADC (antibody-drug conjugate) targets	ERBB2	ERBB2 codes for the protein receptor HER2, which is a target for classical anti-HER2 treatments. Patients with low and ultralow expression of HER2 can be eligible for treatment with the antibody-drug conjugate, trastuzumab deruxtecan (Enhertu) [23].	Low (19)	
	TACSTD2	TACSTD2 codes for Tumor-associated calcium signal transducer 2, also called Trop-2, which is the target of sacituzumab govitecan (Trodelvy) and datopotamab deruxtecan (Datroway), which are antibody-drug conjugates approved for advanced/metastatic HR+/HER2– or TNBC [24,25].	High (93)	
	NECTIN4	Nectin Cell Adhesion Molecule 4 (NECTIN4) is a cell adhesion molecule that is a target for antibody-drug conjugates in clinical trials for breast cancer.	Medium (63)	
	ERBB3	ERBB3 codes for a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. It is under investigation in clinical trials for the antibody-drug conjugate patritumab deruxtecan.	Medium (54)	
	FOLR1	FOLR1 encodes the protein Folate Receptor Alpha, which is an antibody-drug conjugate target under investigation in several phase I and II clinical trials for breast cancer.	High (95)	
	F3	F3 codes for tissue factor, coagulation factor III a target of several antibody-drug conjugates in phase I and II clinical trials.	High (89)	
	SLC39A6	The SLC39A6 gene encodes for the zinc transporter LIV-1, which is highly expressed in luminal breast cancers and is under investigation in several phase I and II clinical trials.	Low (7)	
	CD276	This gene codes for an immune checkpoint marker called CD276 (also known as B7-H3). It is the target of the antibody-drug conjugate (Mirzotamab clezutoclax (ABBV-155) that is in a phase I and II clinical trial for advanced solid cancers, including breast cancer.	Medium (41)	
	VTCN1	V-Set Domain Containing T Cell Activation Inhibitor 1 (VTCN1 also called B7-H4) is an immune checkpoint marker and the target of the antibody-drug conjugate, SGN-B7H4V, which is under investigation in a phase I and II clinical trial for advanced solid cancers, including breast cancer.	High (69)	

Multiplex8+ - INTERPRETATION*

INTERPRETATION*

- In the I-SPY2 study, a high VCpred_TN signature score, which reflects immune activation as well as lack of DNA damage repair, was shown to predict response to veliparib and carboplatin, a finding that was also validated in the BrighTNess study. Since the patient has a high VCpred_TN signature, treatment with PARP inhibitors combined with platinum-based chemotherapy may be beneficial.
- Since the sample is classified as ERBB2-low, the patient may benefit from the antibody-drug conjugate (ADC) trastuzumab deruxtecan (Enhertu), which is approved for HER2-low metastatic breast cancer patients. The cytotoxic payload of this ADC, deruxtecan, targets TOP1, which is expressed at high levels. The expression of both the antigen and cytotoxic payload targets suggest that trastuzumab deruxtecan may be effective in the advanced/metastatic setting.
- The sample has high expression of TACSTD2. TACSTD2 codes for Trop-2, which is the drug target of sacituzumab govitecan (Trodelvy), an ADC approved for the treatment of metastatic TNBC. In the biomarker analysis of the pivotal phase III ASCENT trial, patients with high expression of Trop-2 benefited from sacituzumab govitecan. The cytotoxic payload of this ADC, SN-38, targets TOP1, which is expressed at high levels. Expression of both the ADC target and cytotoxic payload target suggest that sacituzumab govitecan may be effective in the advanced/metastatic setting.

**Supporting literature can be found in the relevant gene description above. This report and its citation coverage are currently being revised.*

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- High levels of TACSTD2 predict sensitivity to the Trop-2 targeting ADC, datopotamab deruxtecan (Datroway), which is approved as a first-line treatment for patients with unresectable or metastatic TNBC, who are not suitable candidates for PD-(L)1 inhibitor therapy. High levels of TOP1, which is the target of the SN-38 cytotoxic payload of datopotamab deruxtecan, supports the expected benefit.
- High levels of TYMS may predict response to 5-fluorouracil and chemotherapies that are metabolized to 5-FU (e.g.,capecitabine).
- A very high PIK3CA-GS (percentile ≥ 90%) may signify the presence of mutations in the PIK3CA or AKT1 gene. In the METABRIC cohort, 16.1% of basal-like patients harbored a PIK3CA mutation. HR+/HER2- patients with PIK3CA mutations may be eligible for treatment with the serine/threonine kinases (AKT) 1, 2, and 3 inhibitor, capivasertib, or the PI3K inhibitors, inavolisib or alpelisib, all administered with fulvestrant. Since none of these treatments are approved for basal-like/TNBC patients, it is considered off-label so it is recommended to explore eligibility for clinical trials. To confirm the presence of PIK3CA mutations, an approved companion diagnostic test is recommended.
- The sample shows high expression of several targets for antibody-drug conjugates, including FOLR1, F3 and VTCN1. These are currently being investigated in clinical trials for triple negative breast cancer.
- Expression levels of nucleoside transporters like SLC29A1 are tied to sensitivity to gemcitabine and capecitabine, with low levels predicting resistance.
- Low expression of VEGFA and medium expression of Hypoxia/Angiogenesis/Inflammatory_MDX suggest the patient may not benefit from bevacizumab (Avastin).
- Low expression of CDK12 suggests the patient would not benefit from methotrexate.

**Supporting literature can be found in the relevant gene description above. This report and its citation coverage are currently being revised.*

REFERENCES

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