Myriad BRAC*Analysis*®, COLARIS®, COLARIS *AP*®, and MyRisk® Hereditary Cancer Technical Specifications

Myriad Genetic Laboratories

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TEST RESULTS SHOULD BE USED ONLY AFTER REVIEW OF THE FOLLOWING SPECIFICATIONS

Description of Analysis:

All test offerings covered by this document include germline DNA-based next generation sequencing (NGS) analysis and/or copy number variation (CNV) testing of genes associated with hereditary cancer syndromes (Table 1).[i] The genes analyzed by these tests are as follows:

- Myriad BRAC*Analysis*® analyzes *BRCA1* and *BRCA2*.
- Myriad COLARIS® analyzes *EPCAM, MLH1, MSH2, MSH6, MUTYH,* and *PMS2*.
- Myriad COLARIS *AP*® analyzes *APC* and *MUTYH* genes.
- Myriad MyRisk® analyzes 48 genes: *APC, ATM, AXIN2, BAP1, BARD1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A* (*p16* and *p14ARF*), *CHEK2, CTNNA1, EGFR, EPCAM, FH, FLCN, GREM1, HOXB13, MEN1, MET, MITF, MLH1, MSH2, MSH3, MSH6, MUTYH, NTHL1, PALB2, PMS2, POLD1, POLE, PTEN, RAD51C, RAD51D, RET, SDHA, SDHB, SDHC, SDHD, SMAD4, STK11, TERT, TP53, TSC1, TSC2, VHL*.

For all tests, the genes of interest are analyzed for coding regions and flanking non-coding intronic regions, typically 20 base pairs (bp) before and 10 bp after each exon, though the exact region may be adjusted based on the presence of either potentially significant variants or highly repetitive sequences. The MyRisk® comprehensive test also produces a combined RiskScore® result for breast cancer risk assessment in women of all ancestries who meet eligibility criteria. The RiskScore® result is generated by taking clinical and family history data, in the form of a Tyrer-Cuzick score, together with data generated from up to 149 weighted genetic markers throughout the genome to assess breast cancer risk and global ancestry.

Description of Method:

Patient samples are assigned a unique barcode for robotic-assisted continuous sample tracking. Genomic DNA is extracted and purified from peripheral blood samples, saliva samples, or fibroblast samples submitted for molecular testing.

DNA sequence analysis by NGS

All test offerings mentioned herein follow the same laboratory process. The samples are prepared through a hybridization capture-based target enrichment strategy for subsequent next generation sequencing. Aliquots of patient genomic DNA are fragmented. The fragmented DNA is built into a library by ligation of sequencing adaptors containing unique patient indices. This library is purified and then enriched for targets of interest through hybridization to a set of biotinylated probes, which are then captured on streptavidin coated beads. Indexed samples are then pooled and loaded onto massively parallel next generation sequencers for paired-end sequencing. Probe design and NGS data analysis were optimized for genes with known pseudogene regions, with additional confirmatory testing as needed.

NGS Data Analysis and Confirmation

A combination of open source and laboratory developed software is used for NGS data processing, which includes base-calling, alignment, variant identification, annotation, and generation of quality metrics. Genetic variants identified in the genes for each test offering are reviewed by computer software and human reviewers. Regions included in the comprehensive test design that are not part of the test ordered are masked during processing and are not reviewed for test interpretation or reporting. Regions that do not meet required NGS quality and coverage metrics are independently confirmed, generally with repeat NGS. Germline heterozygous sequence variants identified by NGS have allele frequencies between approximately 30% and 70%; homozygous sequence variants have allele frequencies above approximately 90%. NGS variants with intermediate frequencies meeting quality metrics may be reported. Putative NGS variants below approximately 10% allele frequencies are not called. Extensive validation and empirical data have demonstrated that heterozygous sequence variants identified by NGS can safely forego routine confirmation. Technically challenging mutations such as copy number variations, structural variations (SV), and mutations in paralogous regions of *PMS2/PMS2CL* are confirmed with repeat testing. Where necessary due to *PMS2* gene conversion, nonspecific NGS is performed with modified thresholds and any potentially actionable variants are confirmed by site-specific Sanger sequencing nested from long-range PCR products.

Copy Number Variation Analysis

Patient samples undergo NGS dosage analysis to determine copy number abnormalities indicative of deletion or duplication mutations. NGS dosage analysis uses normalized read counts to determine gene copy number. Pseudogenes are avoided through assay design and alignment quality filters for NGS data analysis. Promoter regions of all genes undergoing full gene sequence analysis are also analyzed for gross copy number variation (deletions or duplications). For NGS dosage analysis, the normalized ratio of each region of interest is compared across patients to identify regions of altered copy number. Limited analysis is also performed for additional structural variants and is included concurrently with the comprehensive CNV analysis for deletions and duplications. Test offerings that include *BRCA2* are evaluated for an Alu insertion in exon 3 (c.156_157insAlu), which is a Portuguese founder mutation. Test offerings that include *MSH2* are evaluated for a 10 Mb inversion mutation involving exons 1-7. Additional transposon insertion detection is accomplished by a combination of direct analysis of NGS reads for inserted transposon sequence and by monitoring for the impacts of read misalignment due to large foreign sequence insertion. Due to the potential location and complexity of transposon insertions, not all events may be detected. Patient samples positive for CNVs or SVs are confirmed by repeat testing using one or more methods, which can include NGS dosage analysis, Multiplex Ligation-dependent Probe Amplification (MLPA), or PCR analysis.

RiskScore® Analysis

The MyRisk® test offering assesses allele status at up to 149 genome-wide markers during NGS sequencing by automated variant analysis, including 56 ancestry-informative markers, and 93 markers that are associated with risk of breast cancer.[ii] RiskScore® is not reported if 6 or more ancestry-informative markers, or 3 or more breast cancer markers, fail analysis. These data are weighted and combined with a Tyrer-Cuzick risk score based on personal and family history data.[iii] RiskScore® results are calculated for eligible women of all ancestries between the ages of 18-84 years and without a personal history of breast cancer, LCIS, hyperplasia, atypical hyperplasia, or a breast biopsy of unknown results. RiskScore® results are also calculated to modify breast cancer risk in monoallelic *CHEK2* mutation carrier women only of European ancestry. Otherwise, RiskScore® results are not calculated if a woman has a mutation in a high penetrance breast cancer risk gene or has a blood relative with a known mutation in a high penetrance breast cancer risk gene (*BRCA1*, *BRCA2*, *TP53*, *PTEN*, *CDH1*, *PALB2*, *ATM* c.7271T>G, or biallelic *CHEK2*). RiskScore® eligible patients will also receive breast cancer risk estimates based solely on the Tyrer-Cuzick model, except for those who carry monoallelic *CHEK2* mutations.

Single Site Analysis

When single site testing is ordered for a variant, analysis will be performed for the specified gene, as appropriate (Table 1). Single gene analysis will be performed on acceptable sample types using NGS by sequence and/or NGS dosage analysis. In some cases, long-range PCR analysis and/or sequencing of the resulting PCR product can be used to detect specific, previously reported variants. Single site reports will clearly indicate whether the familial variant was identified in the patient. If additional reportable variants are found in the analyzed gene, those will also be included in the single site report.

Performance Characteristics:

Analytical specificity

The incidence of a false report of a genetic variant or mutation resulting from technical error is considered negligible (see below). The incidence of a false report of a clinically significant genetic variant or mutation resulting from errors in specimen handling and tracking was assessed by performing a comparison of all eligible variants in 6,882 samples that underwent at least two independent DNA extractions and cycles of NGS testing. No evidence of sample switches was found during this comparison; therefore, the incidence of a false report of a clinically significant genetic variant or mutation is estimated to be <0.00003% (upper bound of the 95% CI).

Analytical sensitivity

Failure to detect a genetic variant in the analyzed DNA regions may result from errors in specimen handling and tracking, amplification and sequencing reactions, or computer-assisted analysis and data review. The rate of such errors is estimated from validation studies to be less than one percent (<1%). The analytical sensitivity of next-generation sequencing for Table 1 genes[i] was 100% (>99.99%-100%, 95% C.I.) and the analytical specificity was 100% (>99.99%-100%, 95% C.I.) based on complete concordance of heterozygous and homozygous germline variant detection in comparative studies to validated reference methods. These studies were performed on 7,174 samples originating either from de-identified samples extracted from blood, saliva, or fibroblast, or well-characterized external reference samples (6 NIST Genome in a Bottle Consortium (GIAB), 2 Illumina platinum genomes (with one sample in common to GIAB samples), and 24 Broad Institute 1000 Genomes). A total of 339,810 heterozygous or homozygous sequence variants were successfully identified in these validation studies.

Copy Number Variation Validation

Validation studies for CNV detection using NGS dosage analysis were performed using DNA samples extracted from blood, saliva, and fibroblast samples. These samples included 564 that had previously tested positive for CNV mutations, which were all successfully detected by NGS dosage analysis for the Table 1 genes. All reviewable results for CNVs were 100% concordant with the expected mutations.

Concurrent with comprehensive CNV validation, samples positive for a variety of previously identified transposon element insertions were also tested using NGS analysis. All 90 samples reviewed were 100% concordant with the expected transposon insertion mutations.

Test reproducibility

Reproducibility and accuracy were assessed using 4 well-characterized references samples from NIST Genome in a Bottle Consortium or Illumina platinum genomes as well as de-identified previously tested samples. These samples were processed by NGS in triplicate within a batch and then repeated across three independent batches, to assess intra-batch and inter-batch assay reproducibility. All reviewable sequence results were 100% concordant.

Limitations of method

Sequence and copy number variants with allele frequencies below that of heterozygous germline variants may not be detected. Unequal allele frequencies in germline testing may result from certain DNA contexts, including repetitive or low complexity sequences. The presence of pseudogenes, non-reference paralogous sequences, or gene conversion may complicate the detection of sequencing and CNV mutations, potentially leading to decreased sensitivity and specificity in certain genes such as *PMS2* and *SDHA*. There may be uncommon genetic variants such as specific insertions, inversions, and certain regulatory mutations that will not be detected. However, the analysis is believed to identify the majority of germline variants in the gene regions analyzed. Genetic testing results on blood or saliva samples may not reflect the germline genetic status of patients with a hematologic malignancy, or patients who underwent allogeneic bone marrow transplants. In rare cases, testing blood or saliva derived DNA may identify somatic sequence variants that display allele frequencies within the expected range for heterozygous germline variants (e.g., in the *TP53* gene). In such cases, please contact Medical Services to discuss re-submission of an appropriate sample type.

Description of Nomenclature:

All sequencing mutations and genetic variants are referenced to cDNA positions on their respective primary transcripts and named according to the HGVS convention.[iv] The reference sequence used for variant naming is hg19/GRCh37. Transcript IDs for genes with clinically actionable variants are indicated on patient reports with their associated variants (Table 1). Allele differences have been documented at a limited number of nucleotide locations, based on the major/minor alleles observed upon testing and reference sequences used historically at Myriad Genetic Laboratories.

Interpretive Criteria:

Functional Variant Interpretations

A functional interpretation is assigned to each variant identified. This interpretation reflects whether the variant is predicted to result in a significant change to normal protein production and/or function and is based, in part, on the ACMG guidelines for classifying variants.[v] It may not necessarily reflect cancer risk (see Clinical Variant Interpretations).

"Deleterious mutation": Includes most nonsense and frameshift mutations that occur at/or before the last known deleterious amino acid position of the affected gene. In addition, specific missense mutations and non-coding intervening sequence (IVS) mutations are recognized as deleterious on the basis of data derived from linkage analysis of high-risk families, functional assays, biochemical evidence, statistical evidence, and/or demonstration of abnormal mRNA transcript processing.

"Genetic variant, suspected deleterious": Includes genetic variants for which the available evidence indicates a high likelihood, but not definitive proof, that the mutation is deleterious. The specific evidence supporting an interpretation will be summarized for individual variants on the Genetic Test Result.

"Genetic variant of uncertain significance": Includes missense variants and variants that occur in analyzed intronic regions whose functional significance has not yet been determined, as well as nonsense and frameshift mutations that occur distal to the last known deleterious amino acid positions of the affected genes.

"Genetic variant, favor polymorphism" and "Genetic variant, polymorphism": Includes genetic variants for which available evidence indicates that the variant is highly unlikely to alter protein production and/or function or contribute substantially to cancer risk. Variants of this type are not reported.

In the case of genes with recessive risk transmission (*MSH3, MUTYH,* and *NTHL1*), these interpretations may be modified depending on the ability to determine whether the mutations are on opposite alleles. Two mutations detected may be labelled "Positive for two mutations" or "Positive for two mutations, clinical significance uncertain" depending on whether test data can or cannot confirm that the mutations are on opposite alleles, respectively. If a single mutation is detected in one

of these genes, an interpretation of "Carrier for a clinically significant mutation of a recessive condition" may be applied.

Clinical Variant Interpretations

A clinical interpretation is assigned to each variant identified. This interpretation reflects whether the variant is predicted to be associated with significantly increased risk for one or more cancer types.

"High Cancer Risk": Includes genetic variants for which absolute cancer risk is predicted to be higher than ~5% with a ~3-fold or higher increased relative risk over that of the general population. Strong data is available to support gene-specific risk estimates, although actual variant-specific risks may differ.

"Elevated Cancer Risk": Includes genetic variants for which there is sufficient data to indicate that the specific variant increases risk for one or more cancers over that of the general population. These risks may be lower than those conveyed by "High Cancer Risk" variants or may be supported by less solid, but still significant, data.

"Clinical Significance Unknown": Includes genetic variants for which there is insufficient data to determine whether the variant is associated with increased cancer risk.

"Clinically Insignificant": Includes genetic variants for which available evidence indicates that the variant is highly unlikely to significantly contribute to cancer risk. Variants of this type are not reported.

"Special Interpretation": Includes genetic variants with more complex clinical interpretations. Specific interpretations will be provided for each variant on the Genetic Test Result.

"Carrier Interpretation": Includes functionally deleterious or suspected deleterious genetic variants in autosomal recessive genes, for which there is no known cancer risk when found in the heterozygous state. However, the biological children of patients provided with a 'carrier' classification are at risk for an autosomal recessive condition if the other parent is also a carrier of a pathogenic variant in the same gene. Screening the other biological parent of any children for variants within the same gene and genetic counseling to discuss reproductive risks may be appropriate.

Summary Interpretations

"Clinically significant mutation identified": Includes Genetic Test Results in which one or more genetic variants, which are associated with the potential to alter medical intervention, were identified."

"No clinically significant mutation identified": Includes Genetic Test Results in which either no genetic variants were identified, or all identified variants were classified as "Clinical Significance Unknown" or "Clinically Insignificant."

"Carrier for a clinically significant mutation of a recessive condition": Includes Genetic Test Results in which one or more genetic mutations were identified in the heterozygous state in a gene for which two mutations are required to manifest a cancer or non-cancer phenotype. There are no known cancer risks associated with carrying a single gene mutation.

"Mutation identified with special interpretation": Includes Genetic Test Results in which one or more genetic mutations have complex clinical interpretations that may or may not be related to cancer.

Change of interpretation and issuance of amended reports

The classification and interpretation of all variants identified in the assay reflect the current state of scientific understanding at the time the report is issued. In some instances, the classification and interpretation of such variants may change as new scientific information becomes available. Whenever there is a clinically significant change in the classification of a variant, an amended report will be provided by Myriad Genetic Laboratories. Amended reports may not be issued for RiskScore® changes resulting from changes in personal and/or family clinical history. Reports that are amended for reasons outside of RiskScore® and issued more than 30 days after the original report date, may not include RiskScore® or Tyrer-Cuzick breast cancer risk estimates since clinical variables that affect these estimates may change over time.

Analysis and Transcript Usage:

Gene features analyzed in Table 1 genes

Testing includes germline analysis for the genes and transcripts listed in Table 1. Unless otherwise specified, all coding regions and flanking non-coding regions are analyzed for sequence variation. Analysis of flanking intronic regions typically does not extend more than 20 bp before and 10 bp after each exon, though the exact region may be adjusted based on the presence of either potentially significant variants or highly repetitive sequences. Coding regions and proximal promoter regions near the transcription start sites are analyzed for large deletions or duplications. Specific genes are tested only for sequence and/or CNVs within limited regions (Table 1). Limited clinically relevant regions are included for *EGFR* (sequencing and CNV analysis of exons 18-21), *RET* (sequencing and CNV analysis of exons 5, 8, 10, 11, and 13-16), and *MITF* (sequencing of position c.952). Terminal *EPCAM* deletions that affect the adjacent *MSH2* gene expression are associated with Lynch syndrome; only CNV analysis of the last two exons of *EPCAM* is routinely performed. At least three unique duplications, which have been observed in the literature, leading to changes in expression of GREM1 have been reported in patients with Hereditary Mixed Polyposis Syndrome (HMPS). CNV analysis of *GREM1* includes the upstream region overlapping the adjacent gene *SCG5*. *MSH3* exon 1 contains a long polyalanine repeat that can interfere with variant calling; therefore, *MSH3* analysis excludes c.121 to c.237. Mutations in the exonuclease domains of *POLD1* and *POLE* are associated with increased risk of hereditary colorectal cancer and polyposis; only sequence analysis of the exons encompassing the exonuclease domains of these genes is performed (*POLD1* c.841 to c.1686, *POLE* c.802 to c.1473). Limited promoter regions in selected genes undergo sequence analysis including *TERT* (c.-71 to c.-1), and *APC* Promoter 1B [c.-195 to c.-190 and c.-125 (NM_001127511.3) associated with gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS)]. A selected deep intronic region in *VHL* intron 1 (c.340+556 to c.340+827) undergoes sequence analysis. In some cases, RiskScore® results may not be included with the test per healthcare provider or payer request. Regions included in the comprehensive test design but that are not part of the test ordered will be masked during processing and will not be reviewed for test interpretation or reporting.

Table 1: Comprehensive MyRisk® genes, transcript IDs, and analysis summary (see text for details)

Gene Name	Transcript ID	Analysis Description
APC	NM_000038.5	Full gene, sequence and CNV, Promoter 1B CNV and sequence of c.-195 to c.-190 and c.-125 (NM_001127511.3)
ATM	NM_000051.3	Full gene, sequence and CNV
AXIN2	NM_004655.3	Full gene, sequence and CNV
BAP1	NM_004656.4	Full gene, sequence and CNV
BARD1	NM_000465.3	Full gene, sequence and CNV
BMPR1A	NM_004329.2	Full gene, sequence and CNV
BRCA1	NM_007294.3	Full gene, sequence and CNV
BRCA2	NM_000059.3	Full gene, sequence and CNV
BRIP1	NM_032043.2	Full gene, sequence and CNV
CDH1	NM_004360.3	Full gene, sequence and CNV
CDK4	NM_000075.3	Full gene, sequence and CNV
CHEK2	NM_007194.3	Full gene, sequence and CNV
CTNNA1	NM_001903.5	Full gene, sequence and CNV
EGFR	NM_005228.5	Exons 18-21, sequence and CNV
EPCAM	NM_002354.2	Exons 8-9, CNV
FH	NM_000143.3	Full gene, sequence and CNV
FLCN	NM_144997.7	Full gene, sequence and CNV
GREM1	NM_013372.6	Full gene, including expanded upstream region overlapping *SCG5*, CNV
HOXB13	NM_006361.5	Full gene, sequence
MEN1	NM_130799.2	Full gene, sequence and CNV
MET	NM_000245.3	Full gene, sequence and CNV
MITF	NM_000248.3	Evaluated for c.952 only, sequence
MLH1	NM_000249.3	Full gene, sequence and CNV
MSH2	NM_000251.2	Full gene, sequence and CNV
MSH3	NM_002439.4	Full gene excluding c.121 to c.237, sequence and CNV
MSH6	NM_000179.2	Full gene, sequence and CNV
MUTYH (alpha3) *MUTYH (alpha5)*	NM_001048171.1 NM_001128425.1	Full gene, sequence and CNV Full gene, sequence and CNV
NTHL1	NM_002528.6	Full gene, sequence and CNV
p14ARF	NM_058195.3	Full gene, sequence and CNV
p16	NM_000077.4	Full gene, sequence and CNV
PALB2	NM_024675.3	Full gene, sequence and CNV
PMS2	NM_000535.5	Full gene, sequence and CNV
POLD1	NM_002691.3	Exonuclease region only (c.841 to c.1686), sequence
POLE	NM_006231.3	Exonuclease region only (c.802 to c.1473), sequence
PTEN	NM_000314.4	Full gene, sequence and CNV
RAD51C	NM_058216.2	Full gene, sequence and CNV
RAD51D	NM_002878.3	Full gene, sequence and CNV
RET	NM_020975.6	Exons 5, 8, 10, 11, and 13-16, sequence and CNV
SDHA	NM_004168.4	Full gene, sequence and CNV
SDHB	NM_003000.2	Full gene, sequence and CNV
SDHC	NM_003001.3	Full gene, sequence and CNV
SDHD	NM_003002.4	Full gene, sequence and CNV
SMAD4	NM_005359.5	Full gene, sequence and CNV
STK11	NM_000455.4	Full gene, sequence and CNV
TERT	NM_198253.2	Promoter region only (c.-71 to c.-1), sequence
TP53	NM_000546.5	Full gene, sequence and CNV
TSC1	NM_000368.4	Full gene, sequence and CNV
TSC2	NM_000548.5	Full gene, sequence and CNV
VHL	NM_000551.3	Full gene, sequence and CNV, including a portion of intron 1 (c.340+556 to c.340+827)

https://www.ncbi.nlm.nih.gov/refseq/

i. Judkins *et al*., (2015). Development and analytical validation of a 25-gene next generation sequencing panel that includes the *BRCA1* and *BRCA2* genes to assess hereditary cancer risk. *BMC Cancer*, 15:215. PMID: 25886519

ii. Hughes *et al*., (2022). Development and Validation of a Breast Cancer Polygenic Risk Score on the Basis of Genetic Ancestry Composition. *JCO Precis Oncol*., 6:e2200084. PMID: 36331239

iii. Tyrer *et al*., (2004). A breast cancer prediction model incorporating familial and personal risk factors. *Stat Med*. 23(7):1111-30. PMID: 15057881

iv. Ogino *et al*., (2007). Standard mutation nomenclature in molecular diagnostics: practical and educational challenges. *J Mol Diagn*. 9(1):1-6. PMID: 17251329

v. Richards *et al*., (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 17(5):405-424. PMID: 25741868