Tumor BRACAnalysis® Technical Specifications
Effective Date: February 2017

Description of Analysis

The Myriad Tumor *BRACAnalysis*® test consists of sequencing and large rearrangement analyses of the *BRCA1* and *BRCA2* genes using next generation sequencing (NGS). This analysis is performed on genomic DNA isolated from formalin fixed, paraffin embedded (FFPE) tumor tissue, including primary or metastatic ovarian cancer and breast cancer.

DNA sequence analysis

BRCA1: Full sequence determination of approximately 5,400 base-pairs comprising 22 coding exons and approximately 750 adjacent base pairs in the non-coding intervening sequences (introns) is performed. Exons 1 and 4, which are non-coding, are not analyzed. The wild-type *BRCA1* gene encodes a protein composed of 1,863 amino acids.

BRCA2: Full sequence determination of approximately 10,200 base-pairs comprising 26 coding exons and approximately 900 adjacent base pairs in the non-coding intervening sequence (introns) is performed. Exon 1, which is non-coding, is not analyzed. The wild-type *BRCA2* gene encodes a protein composed of 3,418 amino acids.

The non-coding intronic regions of *BRCA1* and *BRCA2* that are analyzed typically do not extend more than 20 base pairs proximal to the 5' end and 10 base pairs distal to the 3' end of each exon.

Large rearrangement analysis

Genomic DNA derived from FFPE tumor tissue is analyzed by NGS dosage analysis to determine copy number abnormalities indicative of deletion or duplication mutations. Coding exons of *BRCA1*/*BRCA2* and limited flanking intron regions are examined for evidence of deletions and duplications (see Limitations of method section for any exceptions). Large rearrangement detection utilizes the number of reads that map to each nucleotide normalized to the run median depth of coverage of the same nucleotide.

Description of Method

Acceptable sample types are FFPE tumor tissue from blocks or slides of primary or metastatic ovarian cancer and breast cancer. The portion of the tumor should measure at least 5x5mm and contain 20% or greater tumor cellularity determined using the adjacent hematoxylin and eosin stained (H&E) section. In cases where blocks are not available, optimal tumor BRACAnalysis® testing requires 5-11 unstained slides sectioned in the following order: one 2-5 µm tissue section mounted on a charged slide for H&E staining followed by 4-10 consecutive 10µm unstained sections mounted on uncharged slides for macrodissection of tumor. Patient DNA is extracted and purified from the tumor specimen, assigned a unique bar-code for robotic-assisted continuous sample tracking, and submitted for molecular testing.

Tumor DNA sequence analysis by next-generation sequencing (NGS): The samples are prepared through a hybridization-based target-enrichment strategy for subsequent NGS. Fragmented patient tumor DNA is ligated to specific adaptors and hybridized to biotinylated RNA library 'baits' for selective capture of *BRCA1* and *BRCA2*. The captured DNA is amplified, purified and diluted for loading on the NGS instrument. Samples are loaded on the NGS Illumina instrument, and the criteria for both sequencing and large arrangement calls is 99% of the bases must have ≥ 100 reads. Average coverage exceeds 500X.

NGS data analysis and confirmation: A combination of commercial and laboratory-developed software is used for next-generation sequencing data processing, which includes base-calling, alignment, variant identification, annotation, and quality metrics. Genetic variants are reviewed by computer software and human reviewers. The minimum depth of coverage used for sequence and LR determination by NGS is 50x per base.

Performance Characteristics

Analytical specificity: The incidence of a false report of a genetic variant or mutation resulting from technical error or errors in specimen handling and tracking is estimated from validation studies to be less than one percent (<1%). The validation studies demonstrated 100% concordance with the reference results, with a 95% lower confidence limit of 0.999997.

Analytical sensitivity: Failure to detect a genetic variant or mutation in the analyzed DNA regions may result from errors in specimen handling and tracking, hybridization, 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 *BRCA1* and *BRCA2* was demonstrated to be 100% with a 95% lower confidence limit of 0.993817, based on complete concordance of results from 54 individual anonymized breast and ovarian resection tumor DNA samples collectively carrying 483 sequence variants which were confirmed by a second laboratory (Myriad Genetic Laboratories in the USA). The lower limit of detection was determined to require a minimum of 20% tumor content, based on a 'wild-type' cell line embedded in FFPE titrated with 50 known variants contained in six individual cell lines embedded in FFPE. All samples that were previously identified by alternative methods to be positive for deletions or duplications in *BRCA1/BRCA2* were correctly identified for large rearrangements using NGS dosage analysis.

Test reproducibility: Analytical validation studies included a reproducibility study for NGS. In a separate study, 4 samples (two breast and two ovarian primary re-section samples) were analyzed by the tissue BRACAnalysis® assay across three batches. Each batch contained at least three replicates per sample. This study demonstrated 100% intra-run and inter-run reproducibility with a 95% lower confidence limit of 0.999957.

Limitations of method: Unequal allele amplification may result from rare sequence changes under hybridization sites. There

may be uncommon genetic abnormalities such as specific insertions, inversions, and certain regulatory mutations that will not be detected by the tumor BRACAnalysis® assay. The detection of large rearrangement deletions and duplications is dependent on the quality of the submitted specimen. Large rearrangements restricted to the non-coding exon 1 or exons 1 and 2 of the *BRCA1* and *BRCA2* genes are not assessed by the tumor BRACAnalysis® assay. Other terminal duplications are reported as variants of uncertain significance. This analysis, however, is believed to rule out the majority of abnormalities in the genes analyzed.

Description of Nomenclature:
All mutations and genetic variants are referenced to cDNA positions on their respective primary transcripts and named according to the HGVS convention (J Mol Diagn. 2007 Feb;9(1):1-6). Transcript IDs are indicated on patient reports with their associated variants.

BRCA1	NM_007294.3
BRCA2	NM_000059.3

Interpretive Criteria:

Overall Interpretations

"Tumor BRCA1/BRCA2 Mutation Status: Positive"
The test results demonstrate the presence of a deleterious or suspected deleterious sequencing mutation or large rearrangement.

"Tumor BRCA1/BRCA2 Mutation Status: Special Interpretation"
The test results demonstrate the presence of a genetic variant with a complex clinical interpretation. Specific interpretations are provided for each variant in the patient report.

"Tumor BRCA1/BRCA2 Mutation Status: Negative"
The test results do not demonstrate the presence of a deleterious or suspected deleterious sequencing mutation or large rearrangement. This category includes genetic variants for which published data demonstrate absence of substantial clinical significance and truncating mutations in *BRCA2* that occur at and distal to amino acid 3.326 (Mazoyer S et al., *Nature Genetics* 1996, 14:253-254). It also includes variants in the protein-coding region that neither alter the amino acid sequence nor are predicted to significantly affect exon splicing, and base pair alterations in non-coding portions of the gene that have been demonstrated to have no pathogenic effect on the length or stability of the mRNA transcript. There may be uncommon genetic abnormalities in *BRCA1* and *BRCA2* that will not be detected by the tumor BRACAnalysis® assay (see **Limitations of method**).

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. Deletions and duplications of an entire exon(s) identified by the tumor BRACAnalysis® test may also be interpreted to be deleterious. Deleterious large genomic rearrangements include single exon and multi-exonic deletions that are out-of-frame. Out-of-frame single or multi-exonic duplications are classified as deleterious if the orientation is determined to be in tandem and head-to-tail. In- frame deletions/duplications are interpreted on an individual basis and the specific evidence supporting the classification of these mutations is included in the individual patient report.

"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 in the patient report.

"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.

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 within a patient's test result, an amended report will be provided by Myriad Genetic Laboratories.