Crystal Digital PCR® Assay

Information Sheet

For Research Use Only. Not for use in diagnostic procedures.

Product Name

BRCA1/RAD51C/GSTP1 Methylation Crystal Digital PCR® Assay (R51008)

Description

Detected Targets

Targets	Sample Type	Detection Channels	Multiplex
BRCA1/RAD51C/GSTP1 Methylated/Unmethylated Promoter	DNA	Green/Yellow/Red/Infra-Red	6

The BRCA1/RAD51C/GSTP1 Methylation Crystal Digital PCR® Assay is a 10X assay designed to detect and quantify methylation in the promoters of BRCA1, RAD51C, and GSTP1 using the Ruby Chip. Methylation of the BRCA1, RAD51C, and GSTP1 promoters affects DNA repair mechanisms crucial for genome stability and dysregulation of detoxification pathways, both contributing to tumor initiation, progression, and drug resistance.

Multiplexing Strategy: Color-Combination

This assay relies on the Color-Combination multiplexing strategy proprietary to Stilla Technologies, in which targets are detected, differentiated, and quantified by Crystal Digital PCR® using 2 fluorophores.

The table below indicates with a “X” which channel(s) are used for each target in the assay:

Targets	Blue	Teal	Green	Yellow	Red	Infra-Red	Long-Shift
BRCA1_Unmethylated-DNA				X		X	
BRCA1_Methylated-DNA				X	X		
RAD51C_Unmethylated-DNA					X	X	
RAD51C_Methylated-DNA			X			X	
GSTP1_Unmethylated-DNA			X		X		
GSTP1_Methylated-DNA			X	X			

Components

BRCA1/RAD51C/GSTP1 Methylation Crystal Digital PCR® Assay comprises two reagents: a pool of the assay specific primers and Crystal Flex Probes and a Positive Control. Please refer to the lot specific Certificate of Conformity for characterized concentration, available upon demand to Stilla’s Technical Support team at support-stilla@bio-rad.com.

Component Name	Reference	Concentration	Description
BRCA1/RAD51C/GSTP1 Methylation Crystal Digital PCR® Assay	R51008	10X	Detects methylation of the BRCA1, RAD51C, and GSTP1 promoters
BRCA1/RAD51C/GSTP1 Positive Control	R51008.PC0	10X	Contains: synthetic sequences corresponding to methylated and unmethylated DNAs after bisulfite treatment

Specific Recommendation Regarding Sample Treatment and DNA Input

The assay is designed to detect methylated and non-methylated sequences after bisulfite treatment. Samples must therefore first be subjected to bisulfite treatment. The kit used during assay validations is indicated in section “Consumables Required but Not Provided”.

To ensure optimal performance, it is recommended not to exceed a DNA concentration in the Ruby chamber of 300 cp/µL, which corresponds to 1 ng/µL or 6 ng in the 6 µL of PCR mix prepared.

Thermocycling Programs

On the naica system:

Step		Ramp rate
Step 1	Partition for Ruby Chip	-
Step 2	Temperature 95°C for 180 seconds	1°C/sec
Step 3	Begin Loop for 60 Iterations	-
Step 3.1	Temperature 95°C for 15 seconds	1°C/sec
Step 3.2	Temperature 60°C for 60 seconds	1°C/sec
Step 4	Temperature 58°C for 300 seconds	1°C/sec
Step 5	Release for Ruby Chip	-

On the Nio Digital PCR:

Step		Ramp rate
Step 1	Partition for Ruby Chip	-
Step 2	Temperature 95°C for 180 seconds	1°C/sec
Step 3	Begin Loop for 60 Iterations	-
Step 3.1	Temperature 95°C for 15 seconds	2°C/sec
Step 3.2	Temperature 60°C for 60 seconds	2°C/sec
Step 4	Temperature 58°C for 300 seconds	1°C/sec
Step 5	Release for Ruby Chip	-

Data Acquisition

Download Nio dedicated technical files from bio-rad.com.

- NioProtocol_6C-60X-60°C-60s+58°C300s.nioprotocol (Nio Digital PCR)
- NioAssay_6C_BRCA1-RAD51C-GSTP1_R51008.nioassay (Nio Digital PCR)

Download naica dedicated technical files from bio-rad.com.

- ScanningTemplate_Prism6_BRCA1-RAD51C-GSTP1_R51008.ncx (6-color naica system)

Data Analysis

The following files are embedded in the dedicated scanning files listed above:

- CompensationMatrix_Prism6_BRCA1-RAD51C-GSTP1_R51008.ncm (6-color naica system)
- CompensationMatrix_Nio_BRCA1-RAD51C-GSTP1_R51008.ncm (Nio Digital PCR)
- AnalysisConfiguration_BRCA1-RAD51C-GSTP1_R51008.nca (all systems)

Consumables Required but Not Provided

- Ruby Chip (C16011)
- naica® PCR MIX 10X (R10106)
- Universal Reporters 7 (R42401 200 reactions, R42402 1000 reactions)
- Nuclease-free water
- Bisulfite conversion kit (Example: EZ DNA Methylation-Gold Kit, ref: ZD5005 or ZD5006 from Ozyme)

Instruction for PCR Mix Preparation

To ensure good assay performance, the final concentration of naica® PCR MIX Buffer B should be fixed at 2%. Specific instructions for preparing the PCR mix are given below.

Reagent Name	Initial Concentration	Final Concentration	Volume per reaction (µL)
naica® PCR MIX Buffer A 🟢	10x	1x	0.60
naica® PCR MIX Buffer B 🔴	100%	2%	0.12
Crystal Digital PCR® Assay 🟠	10x	1x	0.60
Crystal Universal Reporter Tube A 🟡	40x	1x	0.15
Crystal Universal Reporter Tube B 🟤	40x	1x	0.15
Nuclease-free water	NA	NA	Variable
Template DNA	**NA**	**NA**	**Variable**
or Positive Control ⚪	**10x**	**1x**	**0.60**
		Total reaction volume (µL)	***6.0***

DNA Digestion

DNA samples with ≥10 kb average length (e.g., genomic DNA) could be fragmented by restriction digestion before partitioning to ensure even distribution of the DNA template during partitioning. Restriction digestion is not required for highly fragmented DNA (e.g., FFPE DNA or circulating DNA). This step could improve assay performance and should be tested utilizing desired samples.

Care must be taken to use restriction enzymes that do not cut within the amplified sequence or the Crystal Flex Probes.

For a list of restriction enzymes compatible with a given Crystal Digital PCR® assay, contact our Technical Support team (support-stilla@bio-rad.com).

Loading Amount

For optimal performance, it is recommended not to exceed a chamber concentration (DNA concentration in the reaction mix) of 1,000 copies/µL. The performance of the assay at higher concentrations is not guaranteed and must be validated by the user.

Representative Data and Instructions for Analysis

Set thresholds for separating positive and negative populations on the 1D plots. To optimize the analysis, the Green/Yellow/Red/Infra-Red thresholds should be set at approximately equal distance from the positive and negative clusters. Examples of results obtained on the 6-color naica® system are given below.

Remark: The threshold can be adjusted on each individual chamber to optimize its placement.

Wet lab testing was carried out using H_2O as a negative control and a positive control consisting of synthetic DNAs corresponding to methylated and unmethylated DNA sequences after bisulfite treatment. hgDNA and methylated DNA standard (CpG Methylated Human Genomic DNA, ref: SD1131 from ThermoFisher) were also tested individually after bisulfite treatment.

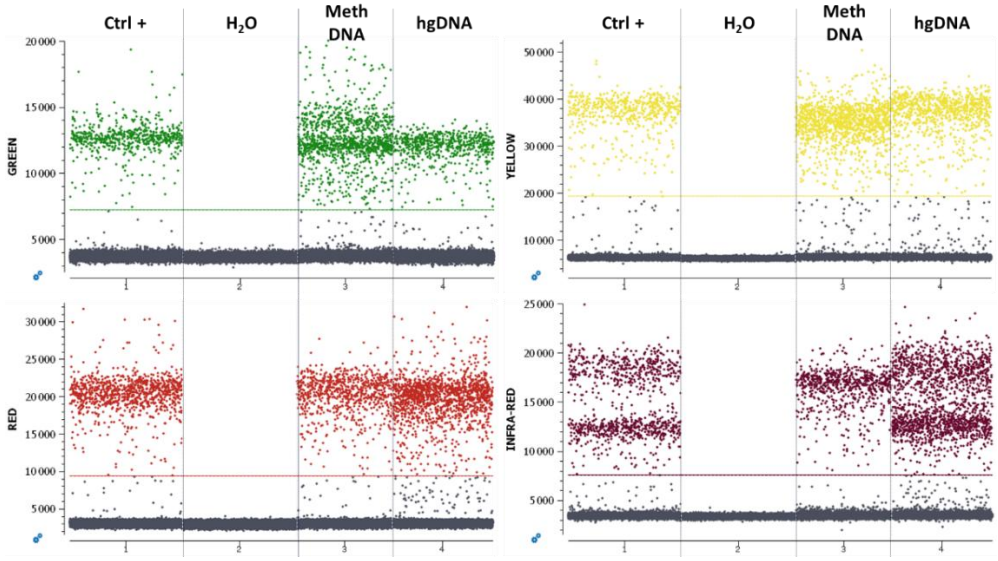


Figure 1: 1D plots obtained during wet lab testing on the 6-color naica® system. *The thresholds should be set at approximately equal distance from the positive and negative clusters.*

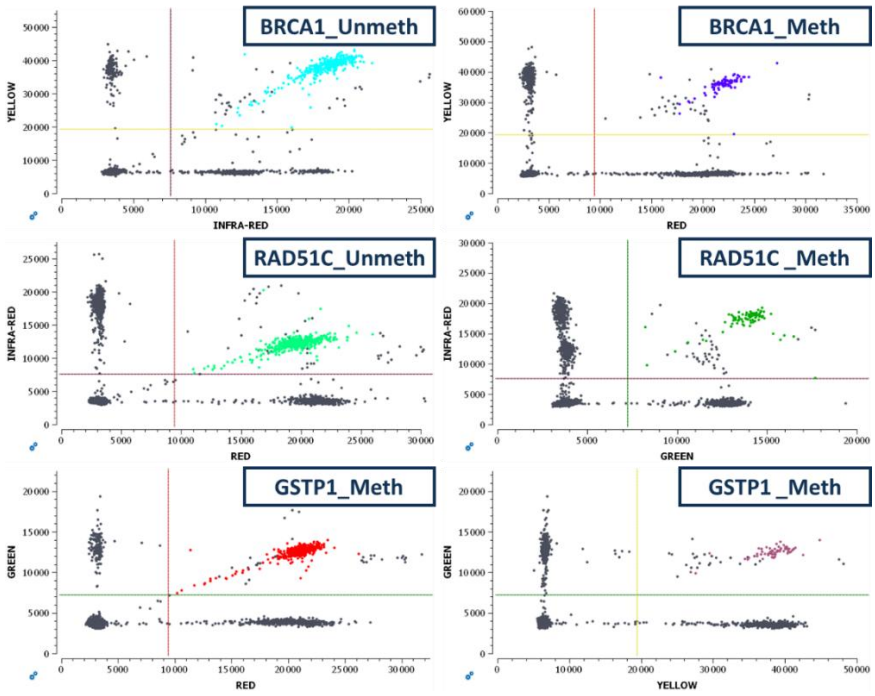


Figure 2: 2D plots obtained during wet lab testing on the 6-color naica® system. *Each target can be visualized as a double-positive population.*

Calculation of methylation percentage

The percentage of methylation of each target can be evaluated as follows:

$$\textbf{\% methylated BRCA1} = \frac{[BRCA1\ meth]}{[BRCA1\ meth]+[BRCA1\ unmeth]} * 100$$

- [BRCA1 meth] = measured methylated BRCA1 concentration
- [BRCA1 unmeth] = measured unmethylated BRCA1 concentration

$$\textbf{\% methylated RAD51C} = \frac{[RAD51C\ meth]}{[RAD51C\ meth]+[RAD51C\ unmeth]} * 100$$

- [RAD51C meth] = measured methylated RAD51C concentration
- [RAD51C unmeth] = measured unmethylated RAD51C concentration

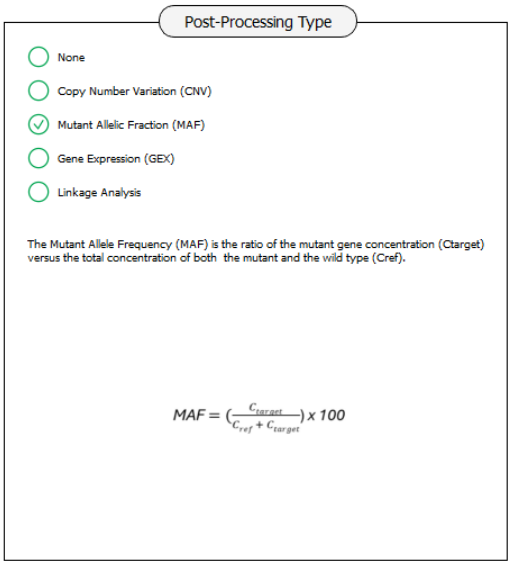
$$\textbf{\% methylated GSTP1} = \frac{[GSTP1\ meth]}{[GSTP1\ meth]+[GSTP1\ unmeth]} * 100$$

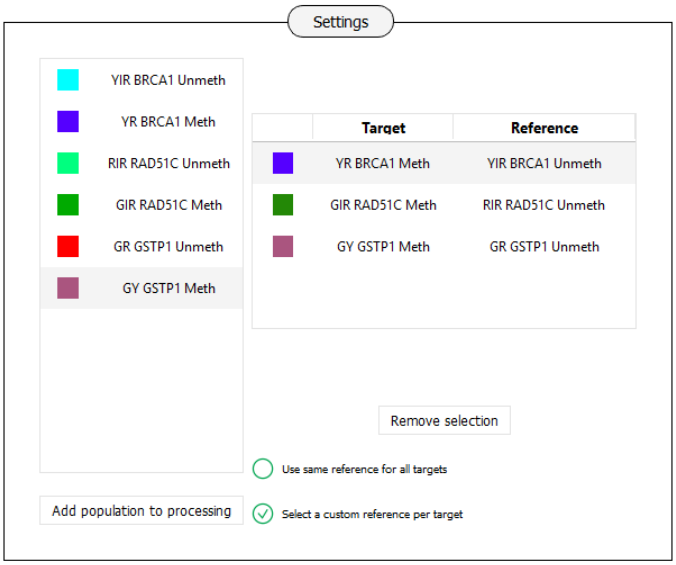
- [GSTP1 meth] = measured methylated GSTP1 concentration
- [GSTP1 unmeth] = measured unmethylated GSTP1 concentration

Remark: The percentage of methylation can be calculated using the Post-Processing feature with NioAnalyzer software by applying the MAF (Mutant Allelic Fraction) function. For more details, see the following paragraph.

Post-Processing (only available with NioAnalyzer software)

To perform a post-processing analysis of the results, click on “Setup” in the “POST PROCESSING” menu and select the appropriate analysis: **Mutant Allelic Fraction (MAF)**. Follow specific instructions for this assay:





Add all “Meth” populations, and select specific reference for each:

- “YIR BRCA1 Unmeth” selected as reference for “YR BRCA1 Meth” population,
- “RIR RAD51C Unmeth” selected as reference for “GIR RAD51C Meth” population,
- “GR GSTP1 Unmeth” selected as reference for “GY GSTP1 Meth” population.

Clicking on apply will launch the calculation. The values will be displayed in the “Results” tab.

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