Crystal Digital PCR® Assay

Information Sheet

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

Product Name

MGMT Methylation Crystal Digital PCR® Assay (R51053)

Description

Detected Targets

Targets	Sample Type	Detection Channels	Multiplex
MGMT Methylated/Unmethylated Promoter	DNA	Blue/Green	2

The MGMT Methylation Crystal Digital PCR® Assay is a 10X assay designed to detect and quantify methylation in the promoter of the MGMT gene using the Ruby Chip. MGMT plays critical roles in DNA repair mechanisms: it is involved in the direct reversal of DNA alkylation damage. Promoter hypermethylation is the most common mechanism of gene silencing, leading to loss of function. This epigenetic inactivation contributes significantly to genomic instability and uncontrolled cell proliferation in a variety of cancers.

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
MGMT Unmethylated DNA	X						
MGMT Methylated DNA			X				

Components

MGMT 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
MGMT Methylation Crystal Digital PCR® Assay	R51053	10X	Detects methylation of the MGMT promoter
MGMT Positive Control	R51053.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”.

Thermocycling Programs

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_3C-60X-60°C-60s+58°C300s.nioprotocol
- NioAssay_3C_MGMT_R51053.nioassay

Data Analysis

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

- UniversalCompMatrix_3C_Prism6-Nio.ncm
- AnalysisConfiguration_MGMT_R51053.nca

Consumables Required but Not Provided

- Ruby Chip (C16011)
- naica® PCR MIX 10X (R10106)
- Crystal Universal Reporters 3 (R41401 200 reactions, R41402 1000 reactions)
- Nuclease-free water
- Bisulfite conversion kit (Example: EZ DNA Methylation-Lightning Kit, ref: ZD5030 or ZD5030-E from Ozyme)

Instruction for PCR Mix Preparation

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%	4%	0.24
Crystal Digital PCR® Assay	10x	1x	0.60
Crystal Universal Reporter Tube A	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 Blue/Green thresholds should be set above the negative cluster. Examples of results obtained on the Nio™+ 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. Synthetic DNAs, hgDNA and methylated DNA standard (CpG Methylated Human Genomic DNA, ref: SD1131 from ThermoFisher) were also tested individually after bisulfite treatment.

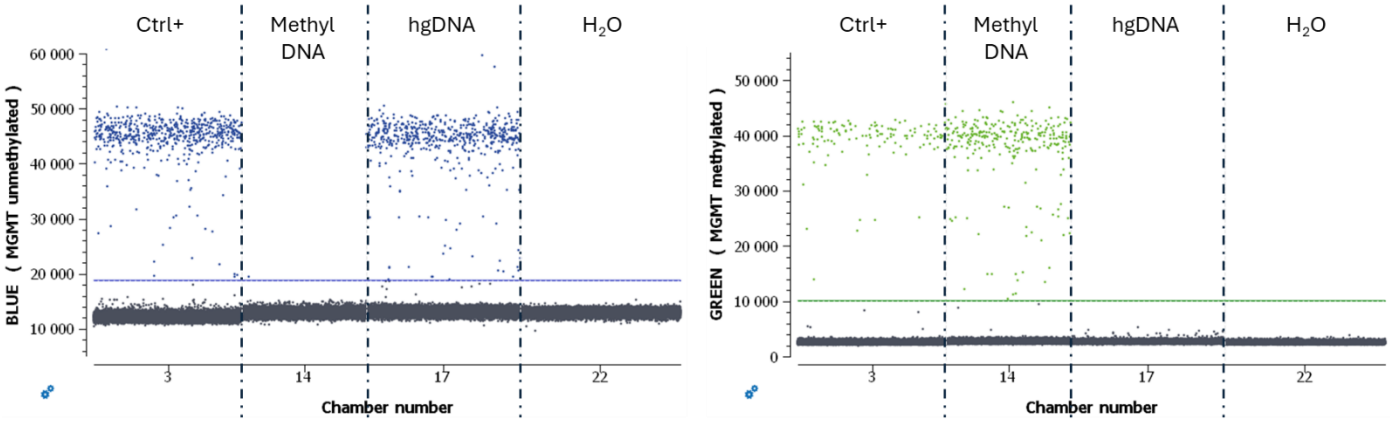


Figure 1: 1D plots obtained during wet lab testing on the Nio™+. *The thresholds should be set above negative cluster.*

Calculation of methylation percentage

The percentage of methylation of MLH1 can be evaluated as follows:

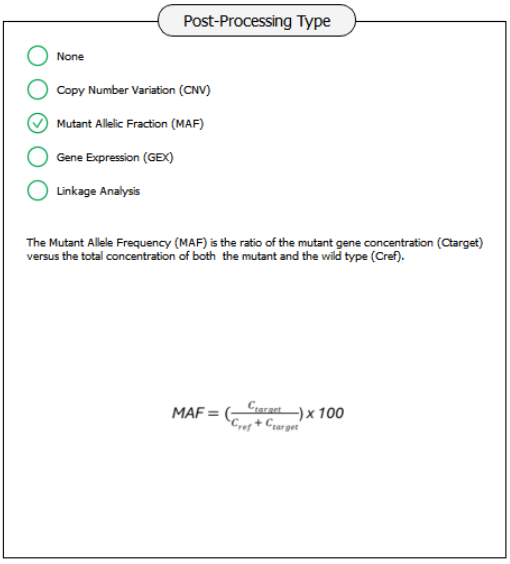
$$\textbf{\% methylated MGMT} = \frac{[MGMT\ meth]}{[MGMT\ meth]+[MGMT\ unmeth]} * 100$$

- [MGMT meth] = measured methylated MGMT concentration
- [MGMT unmeth] = measured unmethylated MGMT 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:



All populations should be added to processing, and “G_MGMT Unmethylated” selected as reference.

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

Stilla Technologies
F-94800 Villejuif, FRANCE