Crystal Digital PCR® Assay

Information Sheet

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

Product Name

Mycoplasma Crystal Digital PCR® Assay (R53004)

Description

Detected Targets

Targets	Sample Type	Detection Channels	Multiplex
Extraction control/Mycoplasma	DNA	Blue/Red	2-color (10-plex)

The Mycoplasma Crystal Digital PCR® Assay is a 10X assay designed to detect and quantify mycoplasma species using the Ruby Chip. Mycoplasmas are frequent contaminants of cell cultures and bioprocessing fluids, often compromising product integrity and downstream applications.

The assay aligns with regulatory requirements, including the United States Pharmacopeia Chapter 63 (USP 63), the European Pharmacopoeia Chapter 2.6.7 (EP 2.6.7), and the Japanese Pharmacopoeia Chapter G3 (JP G3), which mandates the detection of 10 specific mycoplasma species. Six key species, namely *M. orale*, *M. hyorhinis*, *M. fermentans*, *M. salivarum* and *A. laidlawii* account for more than 95% of mycoplasma contaminants in cell culture. This assay has been validated on the previously described species and the additional ones recommended by the pharmacopeia*: M. arginini, M. pneumoniae, S. citri, M. synoviae, M. gallisepticum.*

To support assay performance and sample processing quality, the assay includes a spiked-in extraction control to be added to the samples before extraction. This control allows users to determine the extraction yield of the chosen method.

Intended Use

The Mycoplasma Crystal Digital PCR® Assay is intended for the quantification of mycoplasma genomic DNA and estimation of extraction yields.

This assay can be used on DNA extracted from cell culture sample.

Please note:

✓ The extraction method used and sample purity might have an influence on sample compatibility.

✓ Individual sample-type and extraction method compatibilities for digital PCR applications may require a dedicated assay validation by the end user

Assay Configuration

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
Extraction Control	X						
Mycoplasma					X		

Components

Mycoplasma Crystal Digital PCR® Assay comprises 3 reagents: a pool of the assay specific primers and Crystal Flex Probes, a Mycoplasma Positive Control (synthetic DNA, not replicative, not possible to cultivate) and an extraction control. Please refer to the lot specific Certificate of Conformity for characterized concentration, available upon request to our Technical Support Team (support@stilla.fr).

*Mycoplasma positive control and Extraction control should go through more than 10 freeze thaw cycles to ensure the best performance.

Component Name	Reference	Concentration	Description
Mycoplasma Crystal Digital PCR® Assay	R53004	10X	Detects mycoplasma species, including: M. orale, M. hyorhinis, M. fermentans, M. salivarum, A. laidlawii, M. arginini, M. pneumoniae, S. citri, M. synoviae, and M. gallisepticum.
Mycoplasma Positive Control*	R53004.PC0	10X	Contains: Synthetic mycoplasma DNA
Extraction Control*	R53004.EC0	10X	Contains: Synthetic DNA

*For best performances, avoid more than 10 freeze/thaw cycles for the Mycoplasma Positive Control and Extraction Control tubes.

Sample Preparation

The addition of the Extraction Control to the sample just before the DNA extraction is recommended to allow the evaluation of the extraction yield.

- Add 5µL (corresponding to 6000 non-human DNA copies) of the Extraction Control DNA in each sample to be extracted, regardless of the sample volume to extract.
- Start the extraction by following the usual protocol steps.

Thermocycling Programs

On the Nio® Digital PCR:

Step		Ramp rate
Step 1	Partition for Ruby Chip	-
Step 2	Temperature 95°C for 180 seconds	2°C/sec
Step 3	Begin Loop for 40 Iterations	-
Step 3.1	Temperature 95°C for 15 seconds	2°C/sec
Step 3.2	Temperature 60°C for 30 seconds	0.5°C/sec
Step 4	Temperature 58°C for 300 seconds	0.5°C/sec
Step 5	Release for Ruby Chip	-

Image Acquisition

Dedicated scanning file are available on request:

- NioProtocol_3C-40X-60°C-30s_2.nioprotocol (Nio Digital PCR)
- NioAssay_3C_Mycoplasma_R53004.nioassay (Nio Digital PCR)

Image Analysis

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

- CompensationMatrix_Nio_ Mycoplasma_R53004.ncm (Nio Digital PCR)

Consumables Required but Not Provided

- Ruby Chip (C16011)
- PerfeCTa® Multiplex qPCR ToughMix® (Quantabio, references: 95147-250, 95147-01K, 95147-05K)
- Crystal Universal Reporters 3 (R41401 200 reactions, R41402 1000 reactions)
- Nuclease-free water

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)
PerfeCTa® Multiplex qPCR ToughMix®	5x	1x	1.2
Crystal Digital PCR® Assay ●	10x	1x	0.60
Crystal Universal Reporter Tube A ○	40x	1x	0.15
Nuclease-free water	NA	NA	Variable
Sample volume (X)*	**NA**	**NA**	**Variable**
or Positive Control ○	10x	1x	0.60
		Total reaction volume (µL)	*6.0*

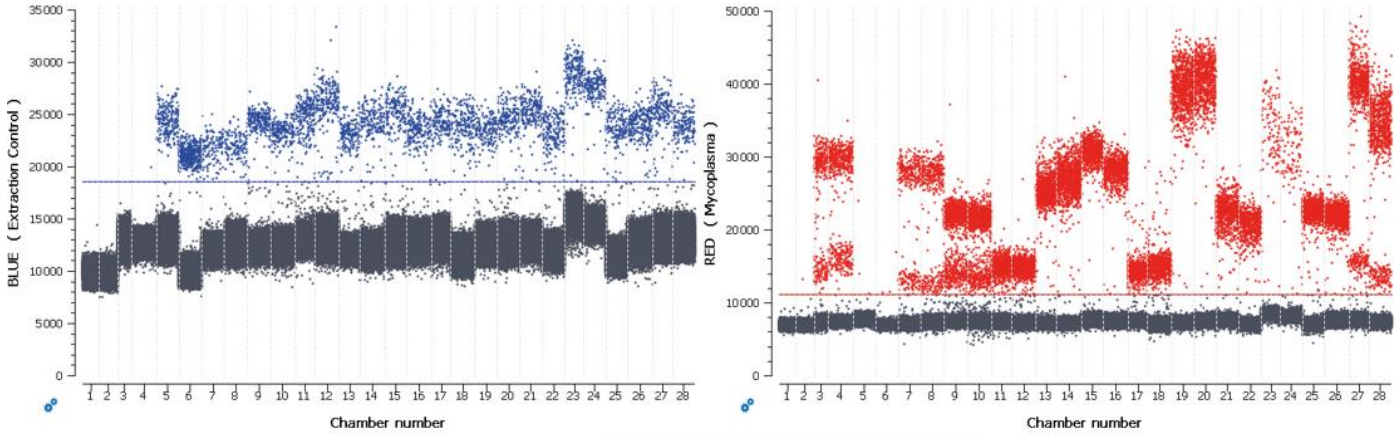
* Sample volume (X) can be between 0.2 and 4 µL for Ruby reaction

Representative Data and Instructions for Analysis

Set thresholds for separating positive and negative populations on the 1D plots. To optimize the analysis, the threshold should be set at just below the lowest positive cluster of the Mycoplasma positive control. Examples of results obtained on the Nio+ are given below. For the Extraction Control, the threshold should be set at approximately equal distance from the positive and negative clusters. Remark: The threshold can be adjusted on each individual chamber to optimize its placement.

Wet lab testing was carried out using genomic DNA extracted from mycoplasma, a positive control consisting of synthetic DNA, a non-template control, and an extraction control alone control (synthetic DNA).

For calculation of the extraction yield, see Annex I.



Chamber ID	Content
1-2	NTC
3-4	Positive Control alone
5-6	Extraction Control Alone
7-8	Positive control + Extraction Control
9-10	M.arginini
11-12	S. citri
13-14	M. fermentans
15-16	M. gallisepticum
17-18	M. hyorhinis
19-20	A. laidlawii
21-22	M. orale
23-24	M. pneumoniae
25-26	M. salivarium
27-28	M. synoviae

Figure 1: 1D plots obtained during wet lab testing on the Nio+. *The thresholds for mycoplasma are set just below the lowest positive cluster on the positive control chamber, and at approximately equal distance from the positive and negative cluster for the extraction control. Some species can present multiples clusters, for example M. arginini.*

Annex: Extraction Yield Calculation

For each sample calculate the extraction yield by using the following formula

$$\text{Extraction yield (\%)} = \left(\frac{observed\ quantification}{\left(\frac{\left(\frac{6000}{Vel} * Svol \right)}{6} \right)} \right) * 100$$

Observed quantification is the quantification given by the Nio Analyzer software for the blue channel in cp/µL for this sample

6000 is the expected number of copies in 5µL of the Extraction Control added to the sample before extraction

Vel is the elution volume post extraction (in µL)

Svol is the extracted sample volume (in µL) added to the PCR Mix (between 0.2 and 4.05µL)

6 is the final volume of the reaction mix before loading in the Ruby Chip

Example of calculation = Addition of 5µL of extraction control to 100µL of cell culture sample pre-extraction. Elution post extraction in 20µL. 4µL of the elution is added to the reaction mix for a total of 6µL. 5µL of the reaction mix with sample is loaded in 1 Ruby Chip chamber. The quantification for this sample provided by Nio Analyzer is 160cp/µL.

$$\text{Extraction yield (\%)} = \left(\frac{160}{\left(\frac{\left(\frac{6000}{20} * 4 \right)}{6} \right)} \right) * 100$$

Extraction yield (%) = 80%

Users must validate in their own experiment conditions and extraction condition the use of Mycoplasma Crystal Digital® PCR Assay as cell debris can potentially lead to inhibition.

Stilla recommends users to define in their own experimental settings the Limit of Blank (LOB) before use by following the method described here: https://www.stillatechnologies.com/digital-pcr/statistical-tools/limit-detection/

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