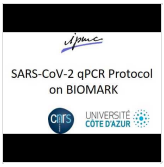

Apr 15, 2021

IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK

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1 Works for me dx.doi.org/10.17504/protocols.io.bd3ii8ke

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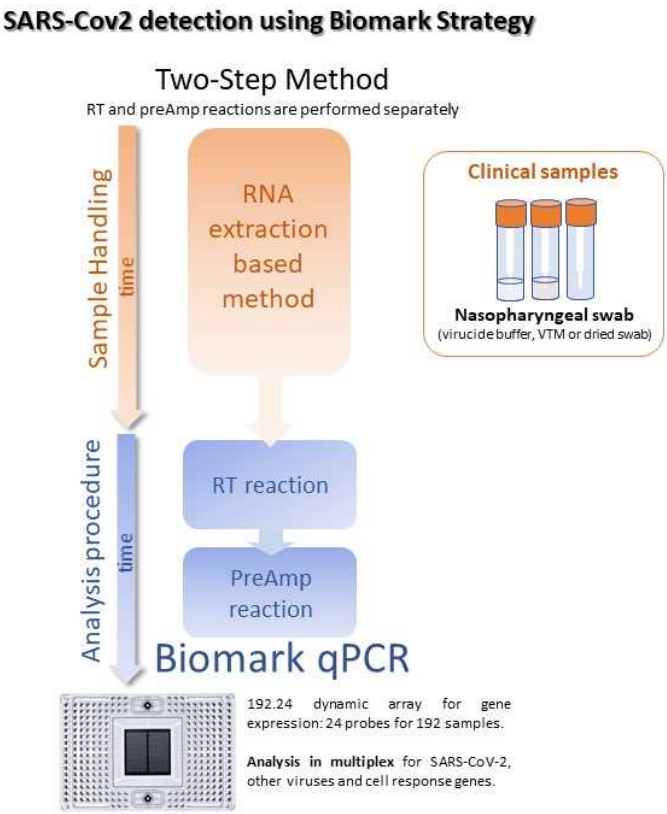
ABSTRACT



EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0243333

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Fassy J, Lacoux C, Leroy S, Noussair L, Hubac S, Degoutte A, Vassaux G, Leclercq V, Rouquié D, Marquette C, Rottman M, Touron P, Lemoine A, Herrmann J, Barbry P, Nahon J, Zaragosi L, Mari B (2021) Versatile and flexible microfluidic qPCR test for high-throughput SARS-CoV-2 and cellular response detection in nasopharyngeal swab samples. PLoS ONE 16(4): e0243333. doi: 10.1371/journal.pone.0243333

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

ATTACHMENTS

GE_24.192_TaqMan_pr_101-7571A2.pdf

DOI

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PROTOCOL CITATION

Julien Fassy, Caroline Lacoux, David Rouquié, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari 2021. IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Fassy J, Lacoux C, Leroy S, Noussair L, Hubac S, Degoutte A, Vassaux G, Leclercq V, Rouquié D, Marquette C, Rottman M, Touron P, Lemoine A, Herrmann J, Barbry P, Nahon J, Zaragosi L, Mari B (2021) Versatile and flexible microfluidic qPCR test for high-throughput SARS-CoV-2 and cellular response detection in nasopharyngeal swab samples. PLoS ONE 16(4): e0243333. doi: 10.1371/journal.pone.0243333

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PROTOCOL INTEGER ID

34634

GUIDELINES

- This protocol has been **validated using an *in vitro* transcribed RNA positive control** (from a plasmid: 2019-nCoV_N_Positive Control, ref 10006625, IDT) and **clinical transport media from SARS-CoV-2 diagnosed-patients**.

The assay has been set up using the **CDC's recommended set of primers / probes (CDC 2019-nCoV primer/probe set: N1, N2 and a human RNP control).**

Titration of the **fully synthetic SARS-CoV-2 RNA control** shows a sensitivity of around **7 copies.** Dilution assay of an RNA sample from a strong positive patient indicates a limit at around 20 CT at a dilution of 10^6.

We have validated additional set of primers / probes and we recommend the use of the **3 SARS-CoV-2 probes presented in the table, below.**

- The protocol is based on the use of the **192.24 IFC.** However, it can also be used on all Biomark IFC and can be adapted for anay number of primer / probe sets and samples.

- Regarding the **extraction step**, we have opted for the **QIAamp Viral RNA Mini Kit.** We have also validated a degraded solution using the **miRNeasy Serum / Plasma Advanced Kit (Qiagen)** due to the unavailability of the QIAamp DSP Viral RNA Mini Kit at the beginning of the project. One optional step has been added to the miRNeasy Serum / Plasma Advanced Kit, using yeast t-RNA as a carrier RNA.

- Since our probes are designed with **5' 6-FAM/3'BHQ-1** chemistry, we have integrated this parameter in the biomark software for the detection. The parameter "FAM/MGB" also looks compatible for detection of these probes according to Fluidigm.

Charité, Germany	E gene / E_Sarbeco (Charité)	E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT
		E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA
		E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG
China CDC	Orf1 / Rdrp gene	ORF1ab-F	CCC TGT GGG TTT TAC ACT TAA
		ORF1ab-R	ACG ATT GTG CAT CAG CTG A
		ORF1ab-P	CCG TCT GCG GTA TGT GGA AAG GTT ATG G
Japan	N Gene	NIID_2019-nCOV_N_F2	AAA TTT TGG GGA CCA GGA AC
		NIID_2019-nCOV_N_R2	TGG CAG CTG TGT AGG TCA AC
		NIID_2019-nCOV_N_P2	ATG TCG CGC ATT GGC ATG GA
USA CDC	RnaseP	CDC-RP-F	AGATTTGGACCTGCGAGCG
		CDC-RP-R	GAGCGGCTGTCTCCACAAGT
		CDC-RP-P	TTCTGACCTGAAGGCTCTGCGCG

List of recommended probes

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MATERIALS TEXT

PHAGO'SPRAY DM* **Contributed by users Catalog #60416**

Dilution Reagent **Fluidigm Catalog #PN 100-8726**

Preamp Master Mix **Fluidigm Catalog #100-5744**

2X Assay Loading Reagent **Fluidigm Catalog #100-7611**

TaqMan Universal PCR Master Mix (2X) **Life Technologies Catalog #4304437**

20X GE Sample Loading Reagent **Fluidigm Catalog #100-7610**

192.24 Dynamic Array™ IFC for Gene Expression **Fluidigm Catalog #100-6265**

Biomark Control Line Fluid 192.24 & 24.192 **Fluidigm Catalog #100-4058**

2019-nCoV CDC EUA Kit **IDT Catalog #10006606**

QIAamp Viral RNA Mini Kit **Qiagen Catalog #52906**

SAFETY WARNINGS

The clinical specimens must be autoclaved and discarded appropriately.
First steps of RNA extractions must be performed in a BSL2 laboratory, as indicated in the protocol, with strict respect of biosafety guidelines.

BEFORE STARTING

The batch number of each reagents must be recorded for each analyzed sample.

RNA Extraction with QIAamp Viral RNA Mini Kit Qiagen

1

To be performed in the appropriate biosafety conditions (BSL2 laboratory)

Transfer the totality of the transport medium into a 2 mL cryotube.

OPTIONAL: in the case of a non-virucide transport medium, heat the sample for 10 min at 65°C. This step should strongly decrease the virus infectivity but might not end up in total inactivation. The effect of heat inactivation may lead to a decrease of test sensitivity depending on the type of swab transport medium.

Manufacturer's instructions in the attached file.

HB-0354-007_HB_QA_Viral_RNA_Mini_0720_WW.pdf

OPTIONAL: Addition of carrier RNA (provided in the kit) to Buffer AVL to improve yield. Follow the table provided in the handbook to prepare a solution containing 5.6 µg of carrier RNA per sample. We have compared protocols containing

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

or not this step and did not find any significant differences regarding the sensitivity of detection.

Transfer **140 µL** sample into a 1.5 mL microcentrifuge tube.

2 Add 560 Buffer AVL. Close the tube caps and vortex for >15 s.

Note: If using a volume of sample other than 140 µL, increase the amount of Buffer AVL proportionally.

3 Incubate for 10 min **Room temperature**

4 Briefly centrifuge the tube to remove drops from the inside of the lid

5 Tubes can be thouroughly wiped with PHAGO'SPRAY ND (Phagogene) or Virospray (Steriscienses) and taken out the BSL2 laboratory.

6 Go to Qiacube. **Elution volume: default 60 µL**
See Qiacube protocol in the attached file.
Virus_QIAampViralRNA_BodyFluid_ManualLysis_V2.pdf

7 Extracted samples can be stored at -20 °C (or -80 °C) for further processing.

cDNA Preparation with Reverse Transcription Master Mix

8 Thaw all reagents on ice. Briefly vortex and centrifuge the reagents before using.

Manufacturer's instructions in the attached file.
cDNA-Prep-RT-MM_qr_100-6472.pdf

9 In a PCR plate (on ice), prepare a pre-mix of the Reverse Transcription Master Mix and water as indicated in the following table.

Component	Volume per Reaction (µL)	Volume for 48 Reactions* (µL)	Volume for 96 Reactions* (µL)	Volume for 192 Reactions* (µL)
Reverse Transcription Master Mix	1.0	52.8	105.6	211.2
RNase-free water	2.0	105.6	211.2	422.4

Premix volumes for reverse transcription

* Includes 10% overage for ease of pipetting.

Add 2 µL of RNA to each well containing pre-mix, to reach a total volume of 5 µL.

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

10

11 Properly seal and gently vortex to mix the reverse transcription reactions.

12 Centrifuge the reactions **1000 rpm, Room temperature , 00:01:00**

13 Place in a standard thermal cycler.
Incubate using the following protocol:

Condition	Temperature	Time
Hold	25 °C	5 min
Hold	42 °C	30 min
Hold	85 °C	5 min
Hold	4 °C	∞

Thermal cycler protocol for reverse transcription

Fluidigm Preamp Master Mix and TaqMan Assays

14 *Pool primer and probes with 2 steps:*
- **Intermediate solution**: mix together primers and probe for each target gene (substep 14.1)
- **Pooled Taqman assay mix**: pool the **intermediate solution** into a final pooled assay (substep 14.2)

14.1 Prepare an **intermediate solution** by mixing primers and probe for each target, with the following volumes:

Component	Volume (µL)	Final concentration
Forward primer (100 µM)	16.77	6.7 µM
Reverse primer (100 µM)	16.77	6.7 µM
Probe (100 µM)	4.25	1.7 µM
Water	212.21	
Total Volume	250	

14.2 Pool each **intermediate solution** into a **pooled Taqman assay mix** (0,2X) using TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) so that each primer is at a final concentration of 180 nM, and each probe at 50 nM.

This step should be adapted depending on the number of primers/probes to be used in the final pooled assay (ideally 8 or 12 sets of primers for a 192.24 IFC).

The chart below provides an example using 4 assays used to detect SARS-CoV-2

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

Component	Volume (µL)
Intermediate solution (6.7/1.7 µM)	6 (each assay) x 4
TE	176
Total	200

Pooled Taqman assay mix (0,2X)

Note: Volume can be adjusted proportionally based on the number of samples to be amplified

GE_Preamp-MM_TaqMan_qr_100-5876C2.pdf

15 *Prepare Pre-Mix and Samples:*
In a DNA-free hood, prepare the sample pre-mix for the reactions as shown in the following table:

Component	Vol. per Reaction (µL)	Vol. for 48 Reactions* (µL)	Vol. for 96 Reactions* (µL)	Vol. for 192 Reactions* (µL)
Preamp Master Mix (Fluidigm PN 100-5744)	1.00	52.8	105.6	211.2
Pooled TaqMan assay mix (0.2X)	1.25	66.0	132.0	264.0
Water	-	-	-	-

Premix volumes for preamplification

* Includes 10% overage.

16 In a PCR plate, aliquot 2.25 µL of pre-mix for each sample.

17 Remove the plate from the DNA-free hood and add 2.75 µL of cDNA to each well containing pre-mix, making a total volume of 5 µL.

18 Mix the reactions by briefly vortexing, and then centrifuge **1000 rpm, Room temperature , 00:01:00**

19 Place the plate in the thermal cycler and cycle using the following table as a guide:

	Condition	Temperature	Time
	Hold	95 °C	2 min
20 cycles	Denaturation	95 °C	15 s
	Annealing/extension	60 °C	2 min

Thermal cycler protocol for preamplification

20 After cycling, dilute the reaction 1:5 by adding 20 µL Dilution Reagent (Fluidigm PN 100-8726) to the final 5 µL reaction

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volume for a total volume of 25 µL.

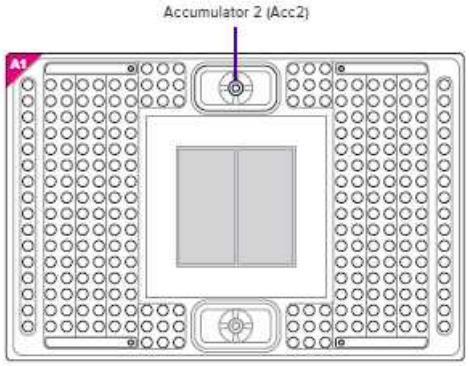
Gene Expression with the 192.24 IFC Using Standard TaqMan Assays

21 Manufacturer's instructions for general 192.24 IFC protocol

192.24_GE_TaqMan-Std_qr_100-6170.pdf

Prepare the IFC

Inject control line fluid into accumulator 2 (Acc2) on the IFC.



Manufacturer's intruction for control line fluid loading

Control+Line+Fluid_Loading_qr_68000132r07.pdf

22 Remove and discard the blue protective film from the bottom of the IFC.

23 *Prepare 10X Assays*

In a DNA-free hood, prepare aliquots of 10X assays using volumes in the following table. Scale up appropriately for multiple runs.

Component	Vol. per inlet (µL)	Vol. per inlet with overage (µL)	Vol. for 50 µL stock
Intermediate solution (step 14.1)	1.5	2.0	25.0
2X Assay Loading Reagent (Fluidigm PN 100-7611)	1.5	2.0	25.0
Total	3.0	4.0	50.0

Volumes for preparation of 10x-concentrated assays
Final concentration (at 10X): primers, 3,35 µM; probe, 0,85 µM

24 *Prepare Sample Pre-Mix and Samples*

Combine components in the following table to make sample pre-mix and final sample mixture. Scale up appropriately for multiple runs.

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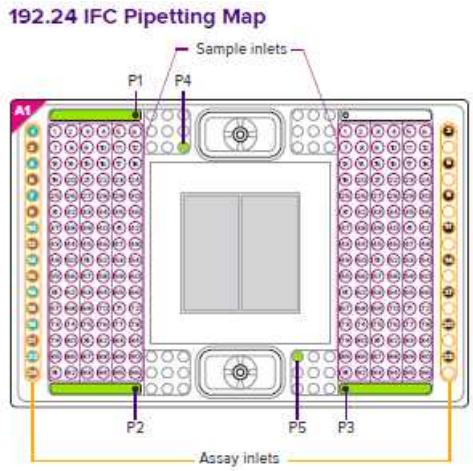
Component	Vol. per inlet (µL)	Vol. per inlet with overage (µL)	Sample pre-mix for 192.24 with overage* (µL)
TaqMan Universal PCR Master Mix (2X) (Life Technologies PN 4304437)	1.5	2.0	480.0
20X GE Sample Loading Reagent (Fluidigm PN 100-7610)	0.15	0.2	48.0

Volumes for sample premix preparation

25 In a DNA-free hood, combine the TaqMan Universal PCR Master Mix with the GE Sample Loading Reagent in a 1.5 mL sterile tube—enough volume to fill an entire IFC. 2.2 µL of this sample pre-mix can then be aliquoted for each sample.

26 Remove these aliquots from the DNA-free hood and add 1.8 µL of cDNA to each, making a total volume of 4 µL in each aliquot.

27 Pipet 3 µL of each assay and 3 µL of each sample into the respective inlets on the IFC (see the 192.24 IFC pipetting map).



28 Pipet 150 µL of Actuation Fluid into the P1 well on the IFC.

29 Pipet 150 µL of Pressure Fluid into the P2 and P3 wells on the IFC.

30 Pipet 20 µL of Pressure Fluid into the P4 and P5 wells on the IFC.

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31 Blot the carrier surface with a dry, lint-free cloth.

32 Prime IFC on the Controller.

Place the IFC into the instrument and run the load script:
- Juno:Load Mix 192.24 GE
- RX: Load Mix (169x)

IMPORTANT : Start IFC run within 1 hour of loading samples.

33 Remove any dust particles or debris from the IFC surface with clear tape.

34 Start data collection on Biomark HD

Biomark HD
Fluidigm **BMKHD-BMKHD**

34.1 Double-click the Data Collection icon on the desktop.

34.2 Click Start a New Run.
Ensure that the status indicators for the lamp and the camera are green.

34.3 Place the loaded IFC into the Biomark HD.

34.4 Choose project settings (if applicable). Click Next.

34.5 Click Load.

34.6 Verify IFC barcode and IFC type.

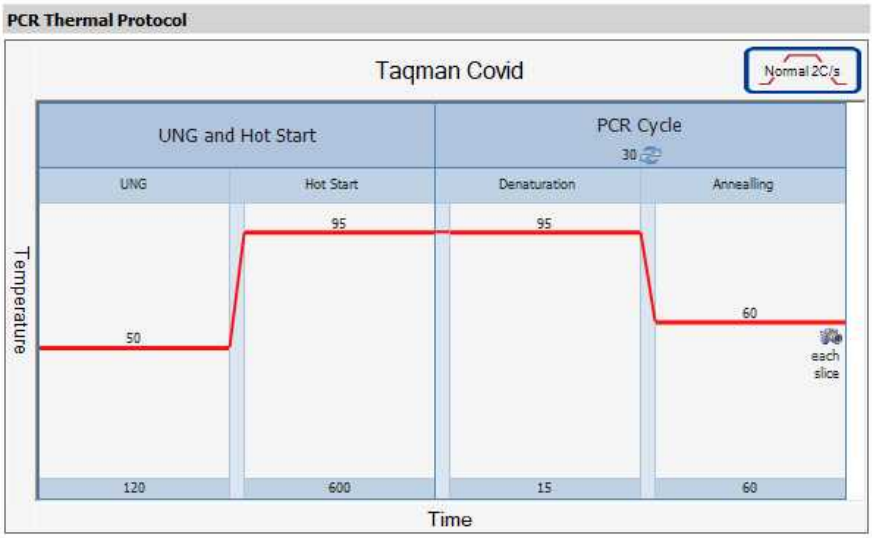
Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke

34.7 Choose project settings (if applicable). Click Next.

34.8 Provide a name and select a file storage location for a new IFC run, or browse to select a predefined run file. Click Next.

34.9 Choose the application, reference, and probes:
a) Application type: Gene Expression
b) Passive reference: ROX
c) Assay: Single probe
d) Probe type: if using a dark quencher choose FAM-Non fluo (Need to be added manually into the Biomark) or FAM-MGB.
e) Click Next.

34.10 Browse to and choose the thermal protocol: below the thermal protocol (Taqman Covid) we have used:



34.11 Confirm Auto Exposure is selected. Click Next.

34.12 Verify IFC run information

34.13 Click Start Run

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bd3ii8ke