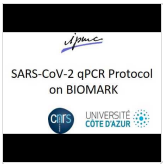


Apr 15, 2021

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

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

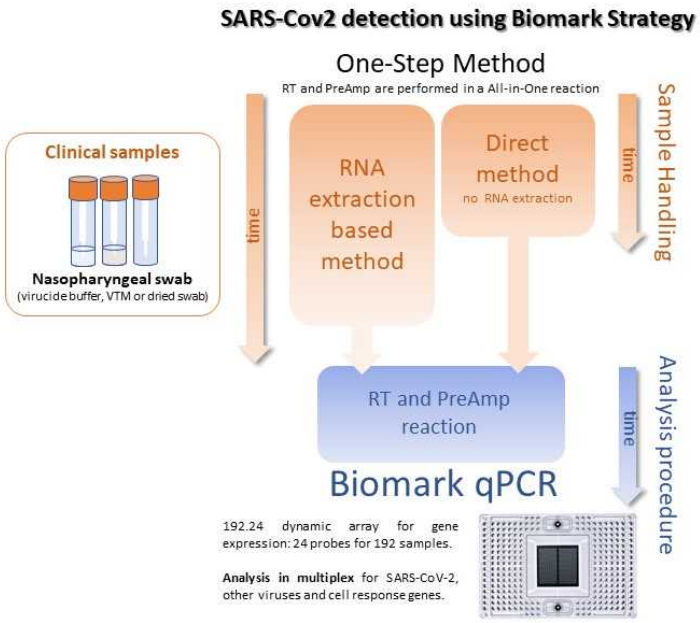
Julien Fassy[1], Caroline Lacoux[1], David Rouquié[2], Jean Louis Nahon[3], Pascal Barbry[1], Laure-Emmanuelle Zaragosi[1], Bernard Mari[1]

[1]Université Côte d'Azur, CNRS, IPMC, FHU-OncoAge, Valbonne, France; [2]Bayer Crop Science, Valbonne, France; [3]Université Côte d'Azur, CNRS, IPMC, Valbonne, France

1 Works for me dx.doi.org/10.17504/protocols.io.bnx4mfqw

Laure-Emmanuelle Zaragosi
Institut de Pharmacologie Moléculaire et Cellulaire

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

ATTACHMENTS

GE_24.192_TaqMan_pr_101-7571A2.pdf

DOI

dx.doi.org/10.17504/protocols.io.bnx4mfqw

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

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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 One-Step qPCR Protocol on BIOMARK. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

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

FORK NOTE

FORK FROM

Forked from IPMC SARS-CoV-2 Two-Step qPCR Protocol on BIOMARK, Laure-Emmanuelle Zaragosi

CREATED

Oct 25, 2020

LAST MODIFIED

Apr 15, 2021

PROTOCOL INTEGER ID

43740

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 and a human RNP control 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

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

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.

Sample processing upon arrival

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

Transfer the totality of the transport medium into a 2 mL cryotube and stored at -80°C in a hermetically sealed body bag.

If performing RNA extraction prior to RT-PCR, proceed to steps 2 to 8.
If performing RT-PCR on samples without RNA extraction, go directly to step 9.

RNA extraction with QIAamp Viral RNA Mini Kit Qiagen

2 See manufacturer's instructions in the following file.

HB-0354-007_HB_QA_Viral_RNA_Mini_0720_WW.pdf

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

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

3 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.

4 Incubate for 10 min **Room temperature**

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

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

7 Go to Qiacube. **Elution volume: default 60 µL of AVE buffer.**
See Qiacube protocol in the attached file.
Virus_QIAampViralRNA_BodyFluid_ManualLysis_V2.pdf

8 Extracted samples can be stored at -20 °C (or -80 °C) for further processing.
Proceed to step 10.

Direct RTqPCR on sample

9 If performing RT-qPCR without prior RNA extraction and purification, starting samples in Viral Transport Medium or in 10mM Tris-HCl pH 6.8 and 1mM EDTA, proceed to either of the following:
- heat inactivate only (substep 9.1).
- treat with QuickExtract DNA extraction DNA solution (from Lucigen) or 0,5% Tween 20 and 2mg/mL Proteinase K (substeps 9.2 and 9.3).

9.1 If performing direct heat inactivation only:

Transfer 25µl of sample in a 0.2 mL PCR-grade tube and incubate 5 min at 95°C in a thermo cycler. Then, keep the treated sample at +4°C until the One Step RT-PreAmp reaction (proceed to Step 10).

9.2 If performing treatment using QuickExtract DNA extraction solution or Tween 20/Proteinase K:

Prepare a 2X master mix solution for the Tween 20/Proteinase K treatment (using 2mg/mL proteinase K in 0,5% tween 20 as final conditions), as indicated in the following table:

Components	Volume (µL)	Final concentration
100mM Tris HCl pH6.8	120	20 mM
50 mM EDTA	24	2 mM
20% Tween20	30	1%
10 mg/mL Proteinase K	240	4mg/mL
H2O	186	
final volume	600	

Tween 20/Proteinase K 2X Master Mix preparation

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

In 0.2mL PCR-grade tubes, combine 25 µL of the 2X Tween 20/ProteinaseK master mix or 25 µL of QuickExtract DNA extraction solution with 2 5µL of the sample.
Pipet up and down to mix well and spin down quickly.

9.3 Incubate the tube at 65°C for 10 min, 95°C for 5 min.
Do not incubate tubes directly at +4°C but instead let the treated samples at room temperature (optional: you can store tube on ice afterwards).

Samples are ready to use, proceed to step 10.

One-step RT and pre-amplification with Taqman assays

10 *Pool primer and probes :*
- **Intermediate solution**: mix together primers and probe for each target gene (substep 10.1)
- **Pooled Taqman assay mix**: pool **intermediate solution** (each assay) into a final pooled assay (substep 10.2)

10.1 Prepare and 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	

10.2

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

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

11 *Prepare One-step Reverse transcription and pre-amplification Pre-Mix :*

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

Components are from the Cells Direct One-Step qRT-PCR kit (Invitrogen, cat. no. 46-7200).

In a DNA-free hood, prepare the following premix:

Component	Volume for 1 reaction(µL)	Vol. for 48 Reactions* (µL)	Vol. for 96 Reactions* (µL)	Vol. for 192 Reactions* (µL)
Pooled Taqman assay mix (step 10.2)	6.25	330	660	1320
Superscript III RT/ Platinum Taq	0.5	26.4	52.8	105.6
2x Reaction mix	12.5	660	1320	2640
Water	0.25	13.2	26.4	52.8

Pre-mix for One-step Reverse transcription and pre-amplification

* Includes 10% overage.

12 In a PCR plate, aliquot 19.5 µL of pre-mix for each sample.

13 Remove the plate from the DNA-free hood and add 5.5 µL of sample to each well containing pre-mix, making a total volume of 25 µL. If raw sample if used, this step should be performed in a BSL2 laboratory.

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

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

	Condition	Temperature	Time
	Hold	42 °C	15 min
	Hold	95 °C	2 min
20 cycles	Denaturation	95 °C	15 s
	Annealing	60 °C	2 min
	Hold	4 °C	∞

Thermal cycler protocol for preamplification

16 After cycling, dilute the reaction 1:5 by adding 100 µL Dilution Reagent (Fluidigm PN 100-8726) to the 25 µL reaction volume for a total volume of 125 µL.

Gene Expression with the 192.24 IFC Using Standard TaqMan Assays

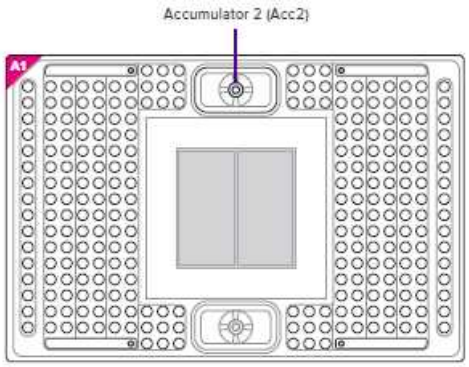
17 Manufacturer's instructions for general 192.24 IFC protocol

192.24_GE_TaqMan-Std_qr_100-6170.pdf

Prepare the IFC

Citation: Julien Fassy, Caroline Lacoux, David RouquiÃÂ©, Jean Louis Nahon, Pascal Barbry, Laure-Emmanuelle Zaragosi, Bernard Mari (04/15/2021). IPMC SARS-CoV-2 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw

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

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

19 *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 (from step 10.1)	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

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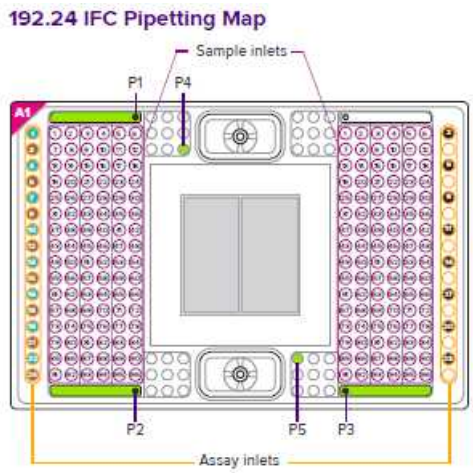
Component	Vol. per inlet (µL)	Vol. per inlet with overage (µL)	Sample pre-mix for 192.24 with overage* (µL)
TaqMan Universal PCR Master Mix (2X) (Life Technologies PN 4304437)	1.5	2.0	480.0
20X GE Sample Loading Reagent (Fluidigm PN 100-7610)	0.15	0.2	48.0

Volumes for sample premix preparation

21 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.

22 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.

23 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).



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

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

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

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

28 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.

29 Start data collection on Biomark HD

Biomark HD

Fluidigm **BMKHD-BMKHD**

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

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

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

33 Place the loaded IFC into the Biomark HD.

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

35 Click Load.

36 Verify IFC barcode and IFC type.

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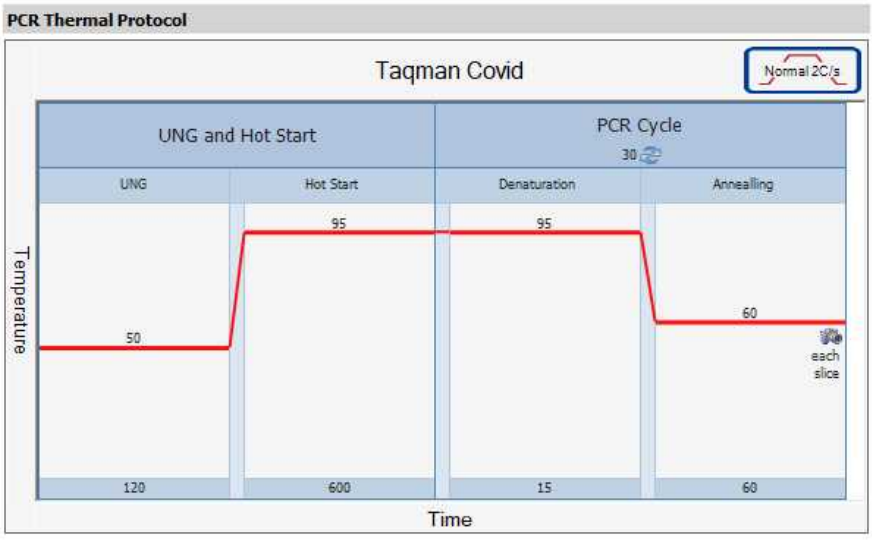
37 Choose project settings (if applicable). Click Next.

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

39 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.

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



41 Confirm Auto Exposure is selected. Click Next.

42 Verify IFC run information

43 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 One-Step qPCR Protocol on BIOMARK. https://dx.doi.org/10.17504/protocols.io.bnx4mfqw