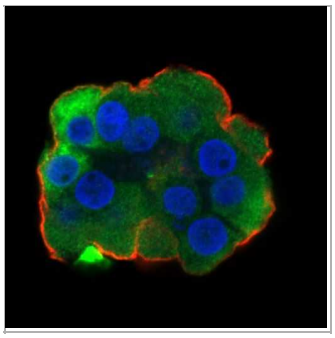

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RNA Extraction and RT-qPCR of Human Lung Organoids V.3

Peer-reviewed method

In 1 collection

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DISCLAIMER

Informed written consent was obtained from all volunteers and the study was approved by the Charité Ethics Committee (project 451, EA2/079/13).

ABSTRACT

This protocol describes the RNA extraction from human alveolar-like organoids followed by the perfomance of a RT-qPCR to quantify the amount of hostfactors (*ACE2*, *TMPRSS2*, and *FURIN*) of SARS-CoV-2. The protocol contains detailed steps for the lysis of human alveolar-like organoids, as wells as the RNA extraction followed by the reverse transcription into cDNA. In the next section the protocol describes in detail how to quantify the gene expression with the TaqMan gene expression kit to obtain donor dependent C_t values.

GUIDELINES

This protocol describes the processing of human alveolar-like organoids which have been grown according to Youk et al., 2020. https://doi.org/10.1016/j.stem.2020.10.004.

MATERIALS TEXT

A	B	C	D
Substance	Company	Order number	Concentration
GlutaMax 100x	invitrogen	35050-038	5 mL/500 mL medium
Hepes	invitrogen	15630-056	5 mL/500 mL medium
Advanced DMEM/F12	invitrogen	12634-034	1x

Composition of base medium

A	B	C
Substance	Company	Order number
High Capacity cDNA Reverse Transcription Kit	Applied biosciences	4368814
RNeasy Mini Kit (50)	Qiagen	74104
Gene Expression Master Mix	AppliedBiosystems by ThermoFisher Scientific	4369510
Ambion™ Nuclease Free Water	Invitrogen By ThermoFisher Scientific	AM 9937
RLT buffer	Qiagen	7921
Bioanalyzer RNA Analysis	Agilent	G2939BA
Agilent RNA 6000 Nano Kit	Agilent	5067-1511
Biometra TRIO PCR Cycler	analytikjena	846-2-070-720
β-Mercaptoethanol	Merck	M6250

Reagents, kits and devices for RNA extraction and qPCR

A	B	C
TaqMan Gene Expression Assay	ThermoFisher Scientific	4331182
	ACE2	Hs01085333_m1
	TMPRSS2	Hs01122322_m1
	FURIN	Hs00965485_g1
	GAPDH	Hs02758991_g1
	ACTIN	Hs01060665_g1

TaqMan Gene Expression Assay and primers

SAFETY WARNINGS

If working with SARS-CoV-2 infected material please be sure to work at biosafety level 3 (BSL3) until step 7.

BEFORE START INSTRUCTIONS

To avoid contaminations RNAase/DNAase free reagents, consumables and filter tips must be used at all steps.
Please use RNA low bind tubes.

Organoid lysis for RNA isolation

1 Grow your 3D model as described.

CITATION

Youk J, Kim T, Evans KV, Jeong YI, Hur Y, Hong SP, Kim JH, Yi K, Kim SY, Na KJ, Bleazard T, Kim HM, Fellows M, Mahbubani KT, Saeb-Parsy K, Kim SY, Kim YT, Koh GY, Choi BS, Ju YS, Lee JH (2020). Three-Dimensional Human Alveolar Stem Cell Culture Models Reveal Infection Response to SARS-CoV-2.. Cell stem cell.
LINK
https://doi.org/10.1016/j.stem.2020.10.004

2 Remove the entire organoid medium from the wells.

3 Add 1 mL ice-cold base medium and collect Cultrex with organoids in a 2 mL tube and flush well with additional 1 mL base medium and collect also.

4 To disolve the Cultrex place the tube at 4 °C for 00:05:00 . 5m

5 Centrifuge at 900 x g, 4°C, 00:05:00 . 5m

6 Carefully remove supernatant and add 350 µL RLT Puffer + β-Mercaptoethanol to the organoids (pellet).

7 Place supernatant in a 1.5 mL safelock tube.

Safety information

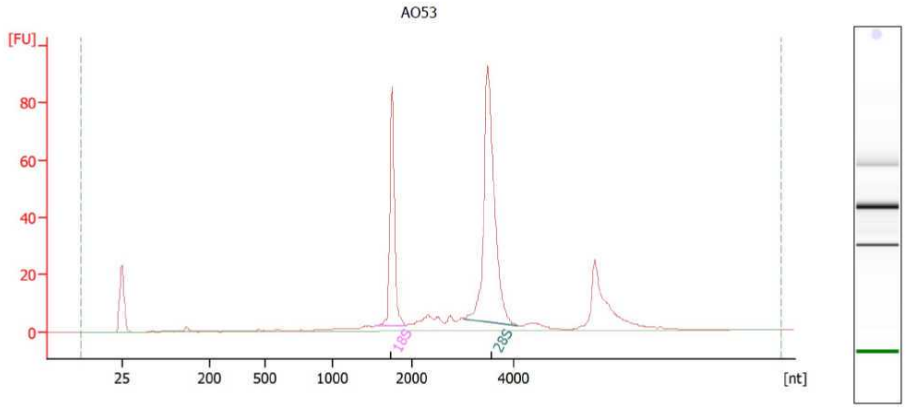
In case the organoids were infected with a virus they can now be transported out of the biosafety level 3 (BSL3) laboratory

Isolate RNA using „RNeasy Mini Kit" from Qiagen (Cat No./ID 74104).

Note

This step represents the RNA isolation protocol based on the „RNeasy Mini Kit" from Qiagen (Cat No./ID 74104) - no changes have been made to the protocol included in the kit

8 After isolation, measure RNA quality via RIN (RNA Integrity Number) using 2100 Bioanalyzer instrument (manufacturer's instructions, no changes have been made to the protocol included in the kit "Agilent RNA 6000 Nano Kit"). High-quality RNA will contain an RIN of at least 8. All samples used in this protocol had a RIN of 10.



Overall Results for sample 1 : AO53

RNA Area:	392.8	RNA Integrity Number (RIN):	10 (B.02.08)
RNA Concentration:	176 ng/µl	Result Flagging Color:	
rRNA Ratio [28s / 18s]:	2.1	Result Flagging Label:	RIN:10

Fragment table for sample 1 : AO53

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,539	1,930	75.0	19.1
28S	3,025	4,076	158.2	40.3

Examplary RNA integrity check using a Bioanalyzer. This RIN example contains a RIN of 10 (completely intact).

2h 15m

Preparation of 2x RT master mix and cDNA reverse transcription

9 All steps On ice .

10 To quantify the amount of RNA before reverse transcription use 1.5 µL of your RNA solution using the NanoDrop.

11 Allow the kit components to thaw on ice (high capacity cDNA reverse transcription kit, Applied biosciences).

12 Calculate the volume of components needed to prepare the required number of reactions.

13 Prepare the RT master mix on ice.

14 One reaction needs:

A	B
Reagent	Amount (µL)
10x RT Buffer	2
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2
MultiScribe™ RT	1
Nuclease-free H2O	4.2
TOTAL	10

Master mix (reverse transcription)

15 According to NanoDrop-measurement prepare an RNA/water solution containing 1 µg RNA/ 10 µL water in a thin wall PCR tube. If RNA concentration is low, 500 ng RNA/ 10 µL water is also sufficient.

16 Include RT control: use water instead of RNA, run one control with each primer.

17 Add 10 µL of 2x reverse transcription master mix to each tube.

18 Use the following program for reverse transcription:

A	B	C
Step	Temperature	Duration
1	25°C	10 min
2	37°C	2 h
3	85°C	5 min
4	4°C	infinite

Program for Reverse Rranscription in a Thermal Cycler

19 Add 80 µL H_2O to each tube (cDNA).

20 Use the cDNA directly for qPCR or store at -20 °C.

14m 15s

TaqMan - quantitative PCR

21 Prepare the qPCR master mix. Calculate the volume of components needed to prepare the required number of reactions for each Gene expression assay.

A	B
Reagent	Amount (µL)
Gene Expression Master Mix	10
H2O	4
TaQMan Gene Expression Assay	1

A	B
Total	15

TaqMan Gene Expression Assay Master Mix (1 Reaction)

22 Perform qPCR, pipetting 15 µL of respective TaqMan gene expression assay master mix to the bottom of the well, then pipet 5 µL of cDNA on the upper wall of the well.

23 Seal the plate with a Clear Adhesive Film.

30s

24 Centrifuge the plate for 1200 x g, 00:00:30 .

25 Important quality controls:
Run each sample in duplicates.
Always run house keeping genes (e.g., *GAPDH* and *ß-ACTIN*) and gene of interest on the same plate.
Include water control for each primer.
Include RT control from previous step.

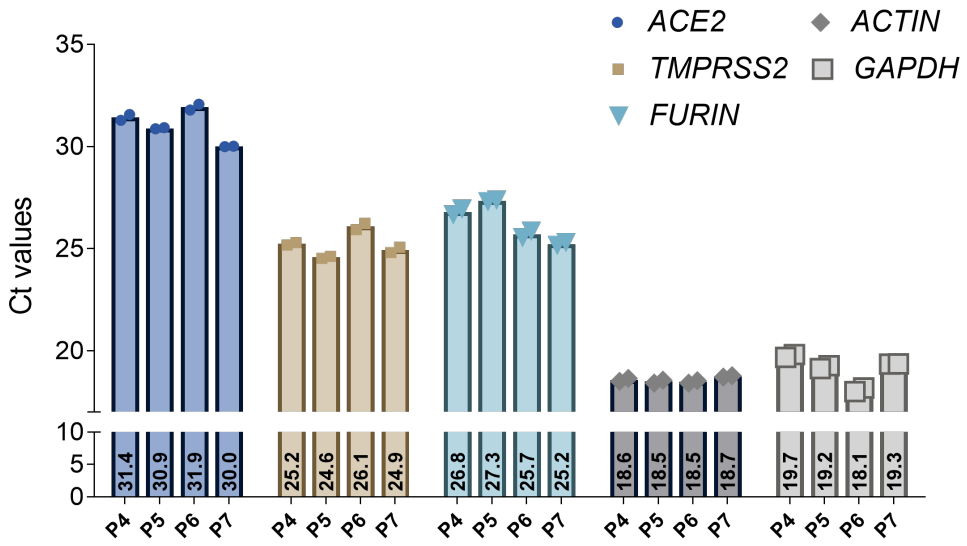
26 Run the qPCR reaction in a Thermal Cycler:

A	B	C	D
Stage	Temperature	Duration	Repetitions
1	50°C	2 min	1
2	95°C	10 min	1
3	95°C	15 s	40
4	60°C	1 min	40

qPCR Program (PCR Volume = 20 µL)

Analysis

27 **Exemplary result:**



Examplary mRNA expression (Ct values) of *ACE2, TMPRSS2,* and *FURIN* in human alveolar-like organoids (*GAPDH* and *ß-ACTIN* serve as reference genes, 4 donors)