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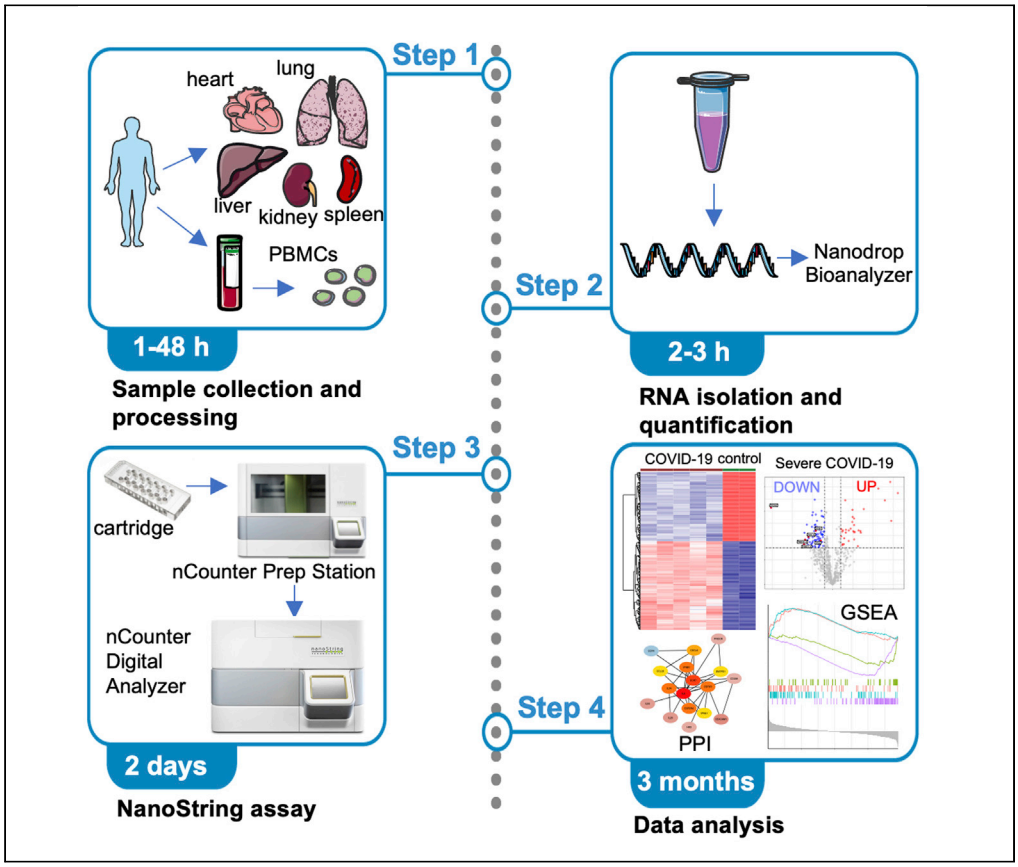
Protocol

Transcriptome profiling of PBMCs and formalin-fixed autopsy tissues from COVID-19 patients



Here we present an optimized protocol for transcriptome profiling of COVID-19 patient samples, including peripheral blood mononuclear cells (PBMCs) and formalin-fixed paraffin-embedded tissue samples obtained from the lung, liver, heart, kidney, and spleen, with the matched controls. We describe RNA extraction and subsequent transcriptome analysis using NanoString technology of the patient samples. The protocol provides information about sample preparation, RNA extraction, and NanoString profiling and analysis. It can be also applied to differentiated Th17 and Treg subsets or formalin-fixed colon tissue samples.

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Highlights

We present a protocol for RNA isolation and transcriptome analysis using NanoString

The protocol is applicable to PBMC and formalin fixed paraffin-embedded tissue samples

We inform about sample preparation, RNA extraction and NanoString profiling

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Transcriptome profiling of PBMCs and formalin-fixed autopsy tissues from COVID-19 patients

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SUMMARY

Here we present an optimized protocol for transcriptome profiling of COVID-19 patient samples, including peripheral blood mononuclear cells (PBMCs) and formalin-fixed paraffin-embedded tissue samples obtained from the lung, liver, heart, kidney, and spleen, with the matched controls. We describe RNA extraction and subsequent transcriptome analysis using NanoString technology of the patient samples. The protocol provides information about sample preparation, RNA extraction, and NanoString profiling and analysis. It can be also applied to differentiated Th17 and Treg subsets or formalin-fixed colon tissue samples. For complete details on the use and execution of this protocol, please refer to Wang et al. (2021).

BEFORE YOU BEGIN

The protocol below has been validated for the RNA extraction and NanoString profiling of peripheral blood mononuclear cells (PBMCs), obtained from patients with COVID-19 at different stages of disease (severe, moderate, convalescent) and healthy controls; and from formalin-fixed paraffin-embedded (FFPE) tissue samples, obtained at autopsy from lung, liver, heart, kidney and spleen of deceased COVID-19 patients and matched controls. We have also used this protocol for T cell subsets like Th17 and regulatory T cells, differentiated from CD4 lymphocytes and other FFPE tissue, like colon, with comparable results.

The studies described in this protocol received IRB approval (IRB # 2020P000675) by the Committee of Clinical Investigations, Beth Israel Deaconess Medical Center (Boston, MA).

PBMC isolation and storage

Timing: 1 h, it takes longer if more than 6 samples are processed at the same time

1. Collect 10–15 mL peripheral blood in plastic whole blood tubes with spray-coated K2EDTA (BD Vacutainer, BD, Franklin Lanes, NJ). Keep the sample at room temperature (20°C–22°C) until processing.

2. Gently layer the blood onto 5–7.5 mL Ficoll-Paque Plus (GE Healthcare Life Sciences, Pittsburgh, PA) in a 15 mL conical tube.
3. Centrifuge at 600×*g* at room temperature for 20 min. Make sure that the centrifuge deceleration is set to '0' to avoid disruption of layers obtained after centrifugation.
4. Collect the PBMC layer at the interface between plasma and Ficoll-Paque Plus and transfer it into a new 15 mL conical tube.
5. Wash the cell suspension with 10 mL of 1xPBS (Thermo Fisher Scientific, Waltham, MA) and centrifuge at 300×*g* at room temperature for 5 min.
6. Discard the supernatant and flick the tube to detach the cell pellet. Repeat step 5.
7. Resuspend the cell pellet using 1–2 mL 1xPBS. Check the cell viability using Trypan Blue Stain 0.4% (Thermo Fisher Scientific).
8. Centrifuge at 300×*g* at room temperature for 5 min to pellet the cells.
9. Resuspend the cells at 1 × 10^6/mL fetal bovine serum supplemented with 10% dimethyl sulfoxide (DMSO) (Fisher Scientific, Waltham, MA).
10. Transfer the cell suspension into a 1.2 mL cryovial and store at −80°C. When longer storage is planned, transfer the sample into −140°C or liquid nitrogen between 1 to 7 days after storage at −80°C.

Note: We recommend processing samples within 6 h from the collection.

Note: When checking cell viability, we suggest diluting Trypan Blue Stain 0.4% at 1:1 with 1xPBS, and then use 1:1 Trypan Blue/cell suspension dilution for counting.

Note: We recommend transferring samples to −140°C or liquid nitrogen 24 h after storage at −80°C. However, please note that we did not observe significant changes in cell viability if storage at −80°C is prolonged for up to 7 days.

Note: When processing PBMC samples obtained from COVID-19 infected individuals, procedures are carried out in a Biosafety Level 2 with Enhanced Biocontainment Practices (BSL-2+) facility. Although the steps for sample processing are the same as indicated above, it is important that laboratory personnel strictly adhere to the safety measures outlined below: 1) Personal Protective Equipment: laboratory personnel should wear disposable lab coats or cloth lab coats with disposable sleeves, double gloves (latex over nitrile or nitrile over nitrile). Face protection shields, or goggles and a surgical mask must be worn (Figure 1). Personnel should wash their hands after removing gloves. 2) Biosafety cabinet: this should be turned on 5 min before initiating the work and will remain on at least 5 min after the work is ended. A waste container (i.e., small biohazard bag or pipette tray) should be placed inside the cabinet, so that personnel do not have to remove waste frequently while working. At the end of the work session all the supplies that have been used inside the cabinet should be disinfected before being removed from the hood. The interior walls of the cabinet should be disinfected too. We recommend using 10% bleach, followed by 70% ethanol (Fisher Scientific) to disinfect suppliers and surfaces. 3) Centrifuge: the centrifuge inside the BSL-2+ facility should be cleaned and disinfected with 10% bleach followed by 70% ethanol after the work session has ended. 4) Waste disposal: Liquid waste: all liquid waste generated during the work session should be immediately decontaminated by mixing with 10% bleach for at least 30 min. The solution is then disposed of in the sink, which should be washed and decontaminated thereafter. Solid waste: the solid waste generated during a work session (i.e., pipettes, vials, tubes, tips) must be placed into a beaker containing 10% bleach and left soak for at least 15 min before being removed from the hood. Decontaminated waste and used gloves should be collected in a small biohazard bag inside the biosafety cabinet and then disposed of in a larger biohazard bag outside the cabinet when the work is completed.

Figure 1. Personal protective equipment (PPE) worn in a Biosafety Level 2 with Enhanced Biocontainment Practices (BSL-2+) facility when processing PBMC samples obtained from COVID-19 infected individuals
PPE includes disposable lab coat, surgical mask, goggles and double gloves (depicted nitrile over nitrile).

⚠ CRITICAL: If outer gloves become contaminated while working inside the biosafety cabinet, keep your hands inside the cabinet, remove the contaminated outer gloves and dispose them in the small biohazard bag inside the hood.

PBMC thawing

Timing: approximately 1 h, it takes longer if more than 6 samples are thawed at the same time

11. Thaw the cryovials containing the PBMC solution in a water bath at 37°C.
12. Transfer the cell suspension into a 15 mL conical tube containing 10 mL ice-cold 1xPBS and centrifuge at 300×*g* at 4°C for 5 min.
13. Discard the supernatant and flick the tube to detach the cell pellet. Repeat the wash by adding 10 mL ice-cold 1xPBS and centrifuge at 300×*g* at 4°C for 5 min.
14. Discard the supernatant. Make sure that supernatant is removed completely to avoid interference with the next steps of RNA isolation.

Note: We recommend gentle shaking of the cryovials when in the water bath. We also recommend thawing no more than 12 PBMC samples at once to minimize the time thawed PBMCs are left at room temperature.

⚠ CRITICAL: Do not leave the cryovials in the water bath at 37°C after thawing as this would decrease cell viability.

Collection of biopsies at autopsy and storage

Timing: approximately 2 days

15. Ultrasound guided biopsies are collected at autopsy from the lung, heart, kidney, liver and spleen.
16. Place the biopsies in a small tube containing 10% Buffered Formalin Phosphate (Fisher Scientific), immediately after collection.
17. Place the biopsies into Processing and Embedding plastic cassettes (Fisher Scientific) and keep them in a container with 10% Buffered Formalin Phosphate on gentle shaking.
18. 24 h later, place the cassettes in a tissue processor.
19. We recommend the following protocol for tissue processing:
    a. Step 1: 70% ethanol for 40 min
    b. Step 2: 80% ethanol for 40 min
    c. Step 3: 95% ethanol for 50 min
    d. Step 4: 95% ethanol for 50 min
    e. Step 5: 100% ethanol for 50 min
    f. Step 6: 100% ethanol for 50 min
    g. Step 7: 100% ethanol for 50 min
    h. Step 8: xylene (StatLab, McKinney, TX) for 50 min
    i. Step 9: xylene for 43 min
    j. Step 10: xylene for 50 min
    k. Step 11: paraffin (Paraplast Plus, McCormick Scientific, Hunt Valley, MD) for 45 min
    l. Step 12: paraffin for 45 min.
20. Once the tissue processing has ended, proceed with paraffin embedding:
    a. Turn on the paraffin wax machine
    b. Coat the bottom layer of a disposable base mold (StatLab) with melted paraffin
    c. Take the sample and place it on top of the base mold. Orient it as you wish and add more paraffin
    d. Place the cassette on top of the mold and completely fill the mold
    e. Place the mold with the sample on the 'cold side' of the embedding station. The paraffin will solidify in 10–15 min
    f. Remove the embedded sample from the mold
21. Keep FFPE samples at 4°C until cutting.

Note: Fixation should not exceed 24 h. Longer fixation time may result in nucleic acid fragmentation and lower performance in downstream applications.

Alternatives: The Shandon Citadel Tissue Processor 2000 (Thermo Fisher Scientific) and the Shandon HistoCentre 2 (Thermo Fisher Scientific) were used as tissue processor and paraffin embedding machine respectively; however, comparable tissue processors might be also found from Leica Microsystems (Buffalo Grove, IL) or Histo-Line Laboratories (Pantigliate, Milan, Italy). Similar paraffin embedding machines are also found from Leica Microsystems and Sakura Finetek USA (Torrance, CA).

Note: Samples should be thoroughly deparaffinized before embedding because residual formalin can inhibit proteinase K digestion.

Obtaining tissue slices from paraffin embedded blocks

Timing: 1 h for 12 samples

22. Before cutting the tissue slices, make sure to trim the area around the sample with a scalpel, to remove the excess of paraffin.
23. Obtain 3 tissue slices of 15 μm each using a microtome.
24. Leave the tissue slices at 4°C until RNA extraction.

Note: The number of tissue slices to be obtained for effective RNA isolation may vary considerably across samples. We recommend performing a trial to define the exact number of slices to cut, in order to obtain enough RNA for the subsequent steps of the protocol. However, avoid cutting and pooling too many slices as paraffin removal in these cases might be challenging and could affect RNA purity.

⚠ CRITICAL: RNA should be extracted within 12 h after the slices are cut to minimize the risk of dehydration.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
COVID-19 PBMC samples (human, 24–81 years, 20 females, 23 males)	Massachusetts General Hospital and Brigham's and Women's Hospital	https://www.massgeneral.org https://www.brighamandwomens.org
COVID-19 and non-COVID-19 FFPE samples (human, 58–91 years, 4 females, 3 males)	Beth Israel Deaconess Medical Center	https://www.bidmc.org
Healthy control PBMC samples (human, 26–55 years, 4 females, 6 males)	Blood Donor Center at Boston Children's Hospital	https://www.childrenshospital.org/ways-to-help/donate-blood
Chemicals, peptides, and recombinant proteins		
K2 EDTA (K2E) Plus Blood Collection Tubes	BD Vacutainer	Cat. # 366643
Ficoll-Paque Plus	GE Healthcare Life Sciences	Cat. # GE17-1440-02
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	Cat. # BP231-100
Trypan Blue Stain 0.4%	Thermo Fisher Scientific	Cat. # T10282
10% Buffered Formalin Phosphate	Fisher Scientific	Cat. # SF100-4
Xylene	StatLab	Cat. # 8400-1
Paraplast Plus - Tissue Infiltration/ Embedding Medium	McCormick Scientific	Cat. # 39502004
β-mercaptoethanol	Sigma-Aldrich	Cat. # M3148
Isopropanol Molecular Biology Grade	Fisher Scientific	Cat. # BP26181
Critical commercial assays		
RNeasy Mini kit (50)	QIAGEN	Cat. # 74104
AllPrep DNA/RNA FFPE kit	QIAGEN	Cat. # 80234
nCounter CAR-T Characterization Panel	NanoString	Cat. # XT-CSO-CART1-12
Coronavirus Panel Plus	NanoString	n/a
Deposited data		
NanoString raw data	Wang et al. (2021); Mendeley Data	https://doi.org/10.17632/mkmv3mgz86.1
Software and algorithms		
nSolver Analysis Software, 4.0	NanoString Technologies	https://www.nanostring.com/products/analysis-solutions/ncounter-advanced-analysis-software/
R software, 4.0.2	R	https://cran.r-project.org
GraphPad Prism, 9.3.0	GraphPad Software	https://www.graphpad.com
Other		
Thermolyne 16500 Dri Bath	Marshall Scientific	Cat. # TH-16500
SureSeal S MicroTubes	MTC Bio	Cat. # C2000
NanoDrop 1000 Spectrophotometer, 3.7.0	Thermo Fisher Scientific	n/a
2100 Bioanalyzer	Agilent Technologies	Cat. # G2939BA
C1000 Touch Thermal Cycler with dual 48/48 Fast Reaction Module	Bio-Rad Laboratories	Cat. # 1851148
nCounter MAX Analysis System	NanoString Technologies	https://www.nanostring.com/products/ncounter-analysis-system/max-analysis-system/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Disposable Lab Coat	Fisher Scientific	Cat. # 12-893-0063A
Fisherbrand Disposable Face Mask	Fisher Scientific	Cat. # 12-888-001A
Bollé Safety Override OTG Goggles	Fisher Scientific	Cat. # 18-072-109
Nitrile Powder Free Examination Gloves	Nest	Cat. # 902021

MATERIALS AND EQUIPMENT

For the deparaffinization step, a thermal mixer or, alternatively, a heating block or water bath capable of incubation at 56°C, 80°C and 90°C is needed.

For RNA extraction, a conventional benchtop microcentrifuge is needed. When isolating RNA from FFPE samples, we recommend using SureSeal S MicroTubes (MTC Bio, Sayreville, NJ) or locks for reaction tubes. RNA quantification was determined using NanoDrop Spectrophotometer, whereas RNA integrity was verified using Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA).

For hybridization, the C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used, although other thermocyclers (e.g., from Qiagen) are also compatible. The nCounter MAX Analysis System (NanoString Technologies, Seattle, WA) was used for the NanoString assay.

> *Alternatives:* The Agilent TapeStation (Agilent Technologies) or the QIAxcel Advanced System (Qiagen, Germantown, MD) can be used instead of Agilent Bioanalyzer.

STEP-BY-STEP METHOD DETAILS

RNA isolation

> Timing: approximately 1 h for PBMC samples and 2 h for FFPE samples

This step will enable RNA isolation from PBMC and FFPE samples. The protocols and timing below are described for 12 samples. This corresponds to the number of samples that can be run in one NanoString cartridge. Please note that steps 1 and 2 under 'RNA isolation' can be carried out independently as they refer to RNA isolation of PBMC and FFPE samples respectively (Figure 2).

RNA is isolated using the RNeasy Mini Kit (Qiagen) when using PBMC samples and the AllPrep DNA/RNA FFPE Kit (Qiagen) when using FFPE samples, according to the manufacturer's protocols (please refer to https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en for RNeasy Mini Kit; and to https://www.qiagen.com/us/resources/resourcedetail?id=3ff86eb0-d95b-4941-95c3-252ad30fe3e6&lang=en for AllPrep DNA/RNA FFPE Kit). Changes from the original protocols have been indicated.

> *Note:* For both PBMC and FFPE samples, we recommend including control and patients' samples in the same run. This will enable avoiding a batch effect potentially confounding the analysis of results. This recommended experimental setup will also enable assessing whether differences between control and patients' samples are consistent between runs.

1. RNA isolation from PBMC samples
   Reagents preparation for RNeasy Mini Kit: Before starting RNA isolation from PBMC samples, we recommend i) adding 10 μL β-mercaptoethanol (Sigma Aldrich, St. Louis, MO) to 1 mL of RLT

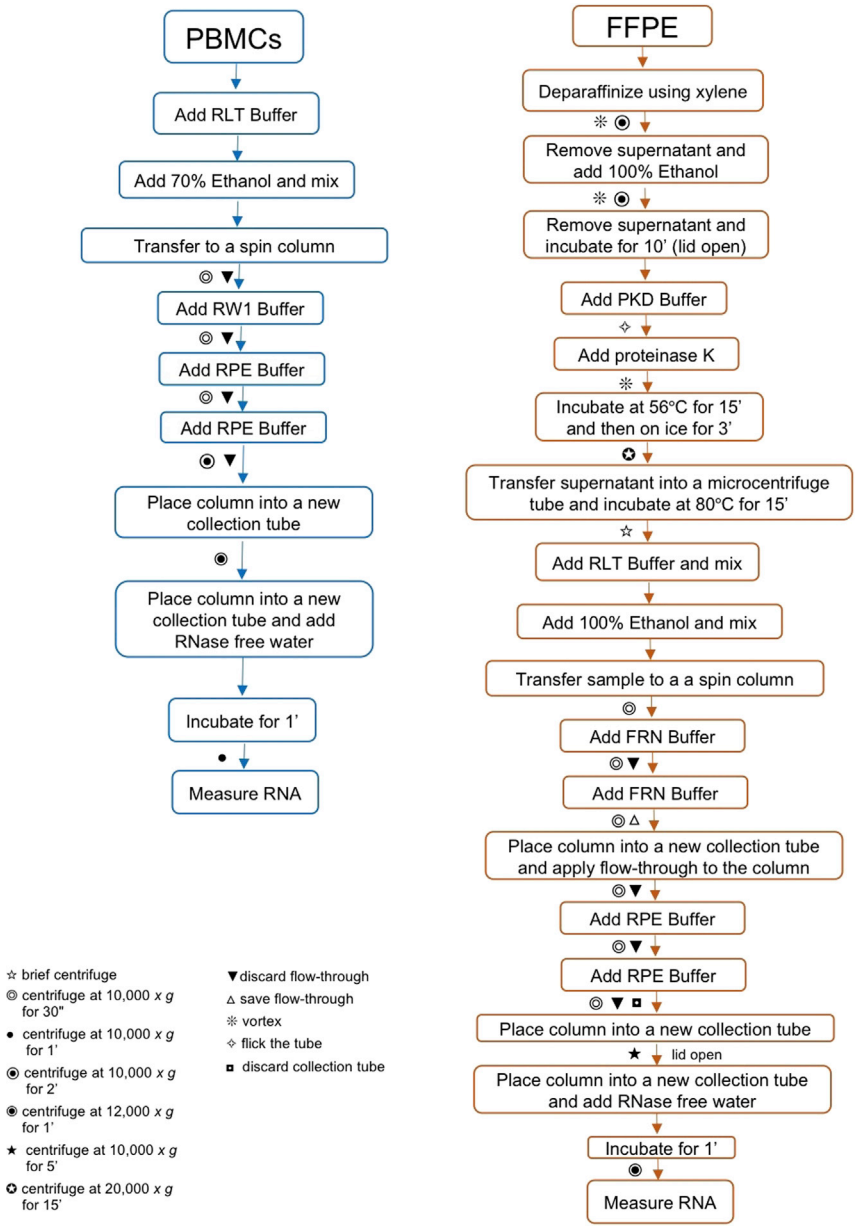

Figure 2. Schematic of RNA isolation from PBMC and FFPE samples

Buffer (included in the kit) before use; ii) adding 4 volumes of 100% ethanol to RPE Buffer, which is supplied as a concentrate in the kit; iii) preparing 70% ethanol.

⚠ CRITICAL: β-mercaptoethanol should be added to RLT Buffer under a fume hood while wearing appropriate protective clothing.

Note: Buffer RLT containing β-mercaptoethanol can be stored at room temperature for up to one month without being degraded.

Note: The procedure described here is optimized for 5–10 × 10^6 PBMCs per sample. For samples with lower cell numbers, add half of the volumes indicated below.

a. Add 600 μL RLT Buffer and pipet to resuspend the pellet. Do not centrifuge.
b. Add 600 μL 70% ethanol to the homogenized cell pellet and mix by pipetting.
c. Transfer 600 μL sample to a RNeasy spin column placed in a 2 mL collection tube, supplied with the kit.

Note: We recommend adding no more than 600 μL sample to avoid unnecessary spillage when closing the lid.

d. Centrifuge at 10,000×*g* for 30 s. Discard the flow-through and re-use the collection tube.
e. Centrifuge the remaining 600 μL sample at 10,000×*g* for 30 s, using the same RNeasy spin column. Discard the flow-through after centrifugation.
f. Add 700 μL RW1 Buffer to the column and carefully close the lid. Centrifuge at 10,000×*g* for 30 s and subsequently discard the flow-through. Please re-use the same collection tube.

Note: After centrifugation carefully remove the column from the collection tube to avoid the column getting in contact with the discarded flow-through.

g. Add 500 μL RPE Buffer to the column and spin at 10,000×*g* for 30 s. This step enables washing the column membrane. After discarding the flow-through, please re-use the collection tube.
h. Add 500 μL RPE Buffer to the column and spin at 10,000×*g* for 2 min. This centrifugation enables drying the spin column membrane from ethanol, which may interfere with subsequent steps. Remove the spin column and discard the flow-through.
i. Place the column into a new 2 mL collection tube (supplied with the kit) and centrifuge at 12,000×*g* for 1 min. This step enables removal of residual RPE Buffer.
j. Place the column into a new 1.5 mL collection tube (supplied with the kit) and add 25 μL RNase free water to the column membrane. After closing the lid, leave the columns on the bench for 1 min and then centrifuge at 10,000×*g* for 1 min. This step will enable RNA elution.

Note: The volume of RNase free water depends on the initial number of cells. For large cell numbers, the volume of water applied to the column should be adjusted.

2. RNA isolation from FFPE samples

Reagents preparation for AllPrep DNA/RNA FFPE kit: Before starting the protocol, we recommend preparing i) RLT Buffer by adding 10 μL β-mercaptoethanol/mL Buffer; ii) FRN Buffer by addition of 42 mL 100% isopropanol to the 14 mL concentrate (then tick the box on the bottle to indicate that isopropanol addition has been made); if precipitation is noted for RLT and/or FRN Buffer, we recommend dissolving by warming the solution with gentle agitation; iii) RPE Buffer by adding 4 volumes of ethanol (i.e., 44 mL) to the bottle containing 11 mL concentrate RPE Buffer.

Note: Shake the bottles before use to ensure mixing of the solutions.

a. Deparaffinize using xylene; add 1 mL xylene and vortex vigorously for 10 s, then centrifuge at 10,000×*g* for 2 min.
b. Remove the supernatant by either pipetting without disturbing the pellet, or, alternatively, by pouring it into a fresh tube. We prefer this second option.
c. Add 1 mL 100% ethanol, vortex and centrifuge at 10,000×*g* for 2 min.
d. Remove the supernatant as indicated in 'b'.
e. Incubate at room temperature for 10 min with the lid open.
f. Add 150 μL PKD Buffer and flick the tube to loosen the pellet. Add 10 μL proteinase K and vortex.
g. Incubate at 56°C for 15 min.
h. Incubate on ice for 3 min.

i. Centrifuge at 20,000×*g* for 15 min.

Note: During centrifugation, set the heating block at 80°C.

j. Transfer the supernatant without disturbing the pellet into a new 2 mL safe-lock microcentrifuge tube for RNA purification.
k. Incubate the supernatant at 80°C for 15 min.

Note: Do not exceed the 15-min incubation. If the heat block has not reached the 80°C yet, the sample should be left at room temperature while the desired temperature is achieved.

l. Briefly centrifuge to remove the drops from the lid.
m. Add 320 µL RLT Buffer and mix by pipetting.
n. Add 1,120 µL 100% ethanol and mix by pipetting.
o. Transfer 700 µL sample (including precipitates that may have formed) to a RNeasy MinElute spin column, which is placed in a 2 mL collection tube (supplied with the kit). Centrifuge at 10,000×*g* for 30 s. Repeat the step until the entire sample volume has passed through the column.
p. Add 350 µL FRN Buffer to the column and centrifuge at 10,000×*g* for 30 s and discard the flow-through.

Note: Although indicated in the manufacturer's protocol, we do not perform DNase treatment. However, we recommend you test this additional step in your experiment. For example, if the analysis of RNA integrity indicates strong genomic DNA contamination, DNAse treatment step becomes necessary.

q. Add 500 µL FRN Buffer to the column and centrifuge at 10,000×*g* for 30 s. Save the flow-through as this contains RNA.
r. Place the column into a new 2 mL collection tube (supplied with the kit) and apply the flow-through to the column. Centrifuge at 10,000×*g* for 30 s and discard the flow-through.
s. Add 500 µL RPE Buffer to the column, centrifuge at 10,000×*g* for 30 s and discard the flow-through.
t. Add 500 µL RPE Buffer to the column and centrifuge at 10,000×*g* for 30 s to wash the column membrane. After centrifugation, discard the collection tube and the flow-through.
u. Place the column into a new 2 mL collection tube (supplied with the kit) and centrifuge at 10,000×*g* for 5 min keeping the lid open.
v. Place the column into a new 1.5 mL collection tube (supplied) and add 20 µL RNase free water to the column membrane. Close the lid and incubate at room temperature for 1 min.
w. Centrifuge at 12,000×*g* for 1 min.

Note: When adding water to the column membrane, please consider that the dead volume in the column is 2 µL.

RNA quantification and purity assessment

Timing: 1 h

In this step, measurement of RNA concentration will be carried out using NanoDrop Spectrophotometer. RNA integrity will be evaluated using Agilent Bioanalyzer.

3. Measure the concentration of RNA isolated from PBMC and/or FFPE samples using NanoDrop Spectrophotometer:

a. Bring the RNA samples along with RNase free water used to elute them on ice to the NanoDrop Spectrophotometer Station.
b. Clean the sample reader with molecular grade water and wipe with a KimWipe.
c. Load 1.2 μL of RNase free water and initialize the system.
d. Select 'RNA' and click 'blank'.
e. Load 1.2 μL of RNA sample and click 'measure'.
f. Record the A260/280 and A260/A230 ratios along with the RNA concentration (ng/mL).
g. Wipe the sample reader between samples with a clean KimWipe and repeat steps e-f.

Note: RNA samples with a A260/A280 ratio ranging from 1.8 to 2.1 are considered of good purity. A A260/A280 ratio lower than 1.8 may indicate DNA or protein contamination. This is, however, more frequent when other protocols of RNA isolation are applied (e.g., TRizol). The optimal A260/A230 ratio should be above 2. Lower values are indicative of contamination with the wash solutions, phenols and proteins. Troubleshooting 1. Troubleshooting 2.

4. Check RNA integrity using Agilent Bioanalyzer. 2.5 μL per sample is used.

Note: RNA integrity is assessed as an additional measure of the sample RNA quality. The Distribution Value 200 (DV200) is the percentage of RNA fragments longer than 200 nucleotides. The lower is the DV200 value, the more degraded is the RNA in the sample. Reportedly, DV200 < 30% are considered being degraded for further experiments (Wimmer et al., 2018); values of DV200 between 50% and 70% are of acceptable quality but might require higher input volumes for transcriptome analysis (Wimmer et al., 2018). DV200 values higher than 70% are usually associated with high RNA quality (Wimmer et al., 2018). Based on this evidence, we recommend using samples with DV200 higher than 70% to maximize the chances of success in the NanoString assay. For additional information on how the DV200 is calculated, please refer to https://www.agilent.com/en/promotions/dv200-determination.

NanoString assay

Timing: approximately 2 days

NanoString technology enables detection and counting of hybridized transcripts using barcodes. In this protocol step, RNA hybridization and post-hybridization processing are carried out as per manufacturer's instructions at https://www.nanostring.com/support-documents/ncounter-xt-assay-user-manual/. (Figure 3).

Day 1

5. Pre-heat the thermocycler to 65°C (lid temperature 70°C). Program the thermocycler for 18 μL volume.
6. Thaw the Reporter Code Set (nCounter CAR T Characterization Panel), the Capture Probe Set (nCounter CAR T Characterization Panel), PLUS COVID-19 Reporter and PLUS COVID-19 Capture (Coronavirus Panel Plus). Thawing is carried out at room temperature. Invert or flick the tubes several times and spin at less than 1,000×*g* for less than 30 s.

⚠ CRITICAL: Do not vortex or pipette vigorously as this may shear the Reporter Code Set. When using the microcentrifuge to spin down the tube, please do not use speed higher than 1,000×*g* for more than 30 s. Pulse centrifugation should be avoided too.

7. Add 28 μL of PLUS COVID-19 Reporter to Reporter Code Set. Invert or flick several times and spin down tubes, as indicated in step 6.

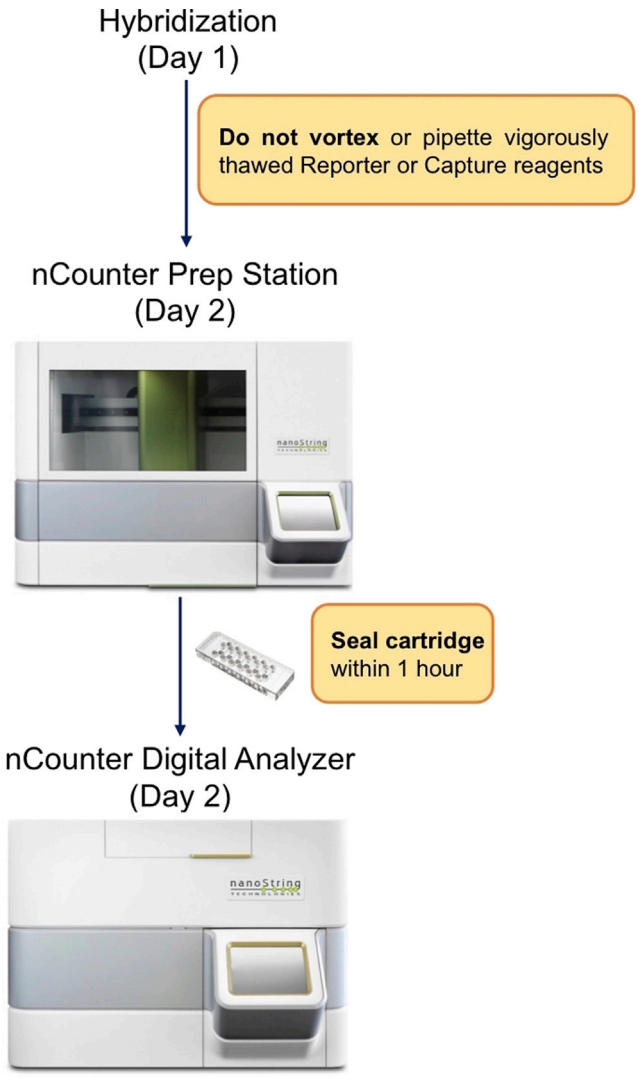

Figure 3. Schematic of NanoString assay procedure. Critical steps are highlighted

8. Prepare a master mix by adding 70 μL of hybridization Buffer to the tube containing PLUS COVID-19 Reporter and Reporter Code Set. The Reporter Code Set should not be removed from the tube.
9. After labeling tubes, add 10 μL of the master mix into each tube.
10. Add 100 ng of RNA sample per tube. The volume should not exceed 5 μL; if less, adjust with RNase free water. The total volume per tube should be 15 μL.
11. Add 14 μL of Capture PLUS Reagent to the thawed aliquot of Capture Probe Set. Invert several times to mix and spin down reagents.
12. Add 3 μL of Capture Probe Set and Capture Plus Reagent mix into each tube immediately before placing it at 65°C.

Note: It is important minimizing the time between the addition of the Capture Probe Set and the incubation at 65°C as this will improve assay sensitivity.

13. Incubate in the thermocycler at 65°C for 18 h. Ramp the reaction down to 4°C (see table) and process the following day.

Note: We recommend not leaving the samples at 4°C for more than 24 h as this may result in increased background.

Temperature (lid)	Volume	Time
65°C (70°C)	18 μL	18 h*

*This includes 16 h hybridization and 2 h at 4°C.

Note: It is recommended not hybridizing more than 12 samples at the same time.

Day 2

14. Remove the samples from the thermocycler and spin them down.

Note: Make sure that no evaporation has occurred, and all samples have the same volume.

15. Take the two 96-well plates (supplied with the NanoString kit) from the refrigerator at 4°C and spin them down at 2,000×*g* for 2 min. Remove the cartridge from −20°C freezer.
16. Bring the samples, 96-well plates, tips and cartridge to the nCounter Prep Station.
17. Prepare the nCounter Prep Station based on the manufacturer's instructions, shown on the display. Please also refer to the manual https://www.nanostring.com/wp-content/uploads/2020/12/MAN-C0035_nCounter_Analysis_System_MAX_FLEX.pdf
18. Follow the instructions on the screen without changing any setting. The run is already pre-set for 'high sensitivity'.
19. Place the plates on the deck with labels forward. Discard the lids.
20. Remove the foil from the cartridge. Before loading the cartridge, make sure the bottom is not cracked or damaged. Place the cartridge under the electrode fixture with the notch in the back and the barcode on the right. The right side should bounce up and down after applying a light pressure from your finger. Lower the electrode fixture to insert the electrodes in the cartridge.

Note: When loading the tips (supplied with the kit and kept at room temperature until use), you can facilitate the procedure by covering them with a plate lid.

21. Place the tip sheaths in the slot in front of the tip rack.
22. Place empty strip tubes, provided with the kit, into the heater block.
23. Uncap the sample strip tubes and place them into the metal tube holder.
24. Close the heater lid and click 'Start Processing'. The processing will take approximately 3 h.

Note: We recommend waiting for 10 min until the 'System Processing' message appears on the display (this indicates that the validation automatically carried out by the machine is completed and the run is starting).

25. After 3 h, remove the cartridge from the nCounter Prep Station and seal it with the clear adhesive film (supplied with the kit).

⚠ CRITICAL: Make sure to seal the cartridge within 1 h after processing, to avoid evaporation and subsequent data loss.

Note: Cartridges can be scanned immediately after processing or stored in the dark at 4°C for up to 1 week.

26. Clean the nCounter Prep Station following the instructions shown on the display and proceed to the nCounter Digital Analyzer.
27. Retrieve the NanoString supplied USB from −80°C. The CodeSet from NanoString is shipped with a Reporter Library File (RLF) that can be found on the NanoString USB inside the −80°C CodeSet box. The RFL contains the barcode definitions for the group of probes included in the CodeSet.
28. Insert the NanoString supplied USB into the nCounter Digital Analyzer (port on the right-hand side of the Digital Analyzer touch screen) and follow the instructions on the screen and as indicated in the manufacturer's protocol https://www.nanostring.com/wp-content/uploads/2020/12/MAN-C0035_nCounter_Analysis_System_MAX_FLEX.pdf
29. Click on 'Upload Files' and then 'Upload RLF'.

Note: This is done for the first run of each NanoString panel. RLF files are saved in the analyzer).

30. Click on 'Start Counting' and select a stage position on the touch screen.
31. Place the cartridge in the selected stage position by holding it lengthwise. Close the magnetic clip and push down to ensure the cartridge is flat.
32. Under Cartridge Definition Mode, select 'Create New'. This option is for use with generic sample names. If specific names are used, please refer to 'Creating a CDF section' in the nCounter Analysis System Manual https://www.nanostring.com/wp-content/uploads/2021/02/MAN-C0035-08-nCounter-Analysis-System-User-Manual.pdf
33. Enter a cartridge ID.
34. Select 'fields of view (FOV) count' for scanning.

Note: Higher FOV counts result in longer scanning time and generate less noisy data. In our protocol, the maximum FOV count (i.e., 555) was used.

35. Click 'Samples' and select all sample tubes.
36. Enter a 'Sample ID'; individual samples will be differentiated by lane numbers.
37. Click 'Library RLF' and select the RLF that corresponds to the CodeSet being run.
38. Click 'Done' to return to the Cartridge Information Screen; click 'Done' again to return to the Select Stage Position Screen; and then 'Done' for the third time, if no additional scans are planned.
39. Click 'Start'.

Note: The run will take approximately 5 h. The cartridge can be left in the counter until the next day and then placed at 4°C, covered by aluminum foil. This would enable scanning the cartridge again, if needed.

40. Once the scan has completed, insert a USB drive into the Digital Analyzer USB port.
41. Click 'Download data' from the touch screen.
42. Select the file corresponding to the cartridge ID and click 'Download data' and export Reporter Code Count (RCC) files.

EXPECTED OUTCOMES

For a successful experimental outcome, we recommend using 100 ng RNA per sample. In the case of PBMCs, fresh or recently collected samples are preferable to maximize the chances of obtaining higher RNA concentrations. Selection of RNA samples with DV200 higher than 70% at the bioanalyzer further enhances the chances of a successful run. By applying these parameters, we were able to show significant differences in gene metabolism, TCR repertoires and *ENTPD1/CD39* (Savio et al., 2020; Longhi et al., 2021; Wang et al., 2021) levels between COVID-19 impacted organs and

non-COVID-19 controls (Wang et al., 2021). When considering PBMCs, we noted that samples obtained from severe COVID-19 patients displayed limited TCR repertoires and lower *ENTPD1/CD39* levels compared to moderate COVID-19 patients, convalescents and healthy controls (Wang et al., 2021).

Importantly, NanoString data were validated using other approaches, namely flow cytometry, qPCR and immunohistochemistry (Wang et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

RCC files are exported from the nCounter Digital Analyzer and initially analyzed using nSolver Advanced Analysis software, based on the manufacturer's instructions (https://www.nanostring.com/wp-content/uploads/2020/12/MAN-10030-03_nCounter_Advanced_Analysis_2.0_User_Manual.pdf). As indicated in the original manuscript (Wang et al., 2021), further analysis of normalized data, differentially expressed genes (DEGs) and gene set scores, was conducted using R software (version 4.0.2). DEGs were defined based on P value $\leq 10^{-4}$ when analyzing samples from organs; and P value $\leq 10^{-3}$ when analyzing PBMC samples. Different thresholds were applied because we noted more substantial differences in the organs than PBMCs, between COVID-19 and control samples. False discovery rate was controlled by the Benjamini-Yekutieli method (Benjamini and Yekutieli, 2001). When comparing mRNA levels in the organs of COVID-19 patients and controls, unpaired *t* test was used; when considering PBMCs, comparisons of mRNA levels between patients at different disease stage and controls were performed using one-way ANOVA test, followed by Tukey's multiple comparison test. $P \leq 0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism, version 9.3.0 (GraphPad Software, San Diego, CA).

LIMITATIONS

The NanoString assay described here enables obtain a comprehensive gene profiling of PBMCs and FFPE samples. Using this approach, correlations between gene sets can be performed; however, the technique does not enable obtain data at the single cell level. If analyses at the single cell level are required, single-cell RNAseq techniques are preferable, especially when analyzing whole tissue samples (Gohil et al., 2021; Delorey et al., 2021). In this regard, the recent introduction of GeoMx Digital Spatial Profiler has enabled whole transcriptome profiling at the single cell level in a spatial context, providing critical information about the expression of key targets specific to a certain tissue region (Delorey et al., 2021; Kulasinghe et al., 2021; Park et al., 2021).

NanoString enables identifying gene pathways that may be altered during disease. However, analysis at the functional level should be incorporated to determine whether alterations at the gene level impact cell function.

TROUBLESHOOTING

Problem 1 (step 3)

Low RNA concentration after isolation from PBMCs and/or FFPE samples.

Potential solution

In the presence of low RNA yield, increase the initial cell number or the number of paraffin embedded slices. We recommend not to exceed 4-5 slices per sample as excess of paraffin is hard to remove.

Problem 2 (step 3)

Low A260/A230 ratio.

Potential solution

As this might be due to contamination with washing buffers, we recommend being careful when performing the spin column washes, especially when removing the spin column from the collection tube containing the flow-through.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Maria Serena Longhi (mlonghi@bidmc.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The NanoString bulk transcriptomic data generated during this study are available from Mendeley data at https://doi.org/10.17632/mkmv3mgz86.1.

This study did not generate code.

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AUTHOR CONTRIBUTIONS

M.V., N.W., A.K., and J.J.G. performed the experiments, analyzed data, and drafted the manuscript. M.S.L. wrote the manuscript and acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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