METHOD ARTICLE

Laboratory protocol for the digital multiplexed gene expression analysis of nasopharyngeal swab samples using the NanoString nCounter system [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

This paper describes a laboratory protocol to perform the NanoString nCounter Gene Expression Assay from nasopharyngeal swab samples.
It is urgently necessary to identify factors related to severe symptoms of respiratory infectious diseases, such as COVID-19, in order to assess the possibility of establishing preventive or preliminary therapeutic measures and to plan the services to be provided on hospital admission. At present, the samples recommended for microbiological diagnosis are those taken from the

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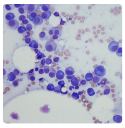
Approval Status ✓ ✓

	1	2
version 2 (revision) 30 Sep 2022	✓ view	✓ view
version 1 02 Feb 2022	✗ view	? view

upper and/or the lower respiratory tract.
The NanoString nCounter Gene Expression Assay is a method based on the direct digital detection of mRNA molecules by means of target-specific, colour-coded probe pairs, without the need for mRNA conversion to cDNA by reverse transcription or the amplification of the resulting cDNA by PCR. This platform includes advanced analysis tools that reduce the need for bioinformatics support and also offers reliable sensitivity, reproducibility, technical robustness and utility for clinical application, even in RNA samples of low RNA quality or concentration, such as paraffin-embedded samples. Although the protocols for the analysis of blood or formalin-fixed paraffin-embedded samples are provided by the manufacturer, no corresponding protocol for the analysis of nasopharyngeal swab samples has yet been established. Therefore, the approach we describe could be adopted to determine the expression of target genes in samples obtained from nasopharyngeal swabs using the nCOUNTER technology.

Keywords
respiratory infection, nasopharyngeal swab, gene expression, Immunology, Digital RNA quantification



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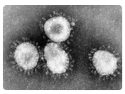

This article is included in the Coronavirus collection.

1. **Jan Weber**, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic

2. **Tara K. Sigdel**, University of California San Francisco, San Francisco, USA

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: García Aranda M: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **López-Rodríguez I**: Investigation, Methodology; **García-Gutiérrez S**: Conceptualization, Funding Acquisition, Resources; **Padilla-Ruiz M**: Formal Analysis, Methodology; **de Luque V**: Investigation, Methodology; **Hortas ML**: Investigation, Methodology; **Diaz T**: Investigation, Methodology; **Álvarez M**: Formal Analysis, Investigation, Methodology; **Barragan-Mallofret I**: Conceptualization, Formal Analysis, Investigation, Methodology, Supervision, Writing – Review & Editing; **Redondo M**: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing;

Competing interests: No competing interests were disclosed.

Grant information: This research was supported by grants awarded to Dr. Maximino Redondo from Instituto de Salud Carlos II (REDISSEC-COV20/00459) and Consejería de Economía, Conocimiento, Empresas y Universidad de la Junta de Andalucía (CV20-76497_COVID) as well as by a donation from Fundación Fuerte (Marbella, Spain) to the Research and Innovation Unit at the Hospital Costa del Sol. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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First published: 02 Feb 2022, **11**:133 https://doi.org/10.12688/f1000research.103533.1

Introduction

Nasopharyngeal swabs as valuable biospecimens

Nasopharyngeal swabs are essential for the accurate diagnosis of respiratory infectious diseases such as coronavirus disease 2019 (COVID-19), which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The samples currently recommended for the microbiological diagnosis of respiratory infectious diseases are those obtained from the upper respiratory tract (nasopharynx and oropharynx) and/or the lower respiratory tract, such as sputum, endotracheal aspirates, bronchoalveolar lavages or bronchial aspirates, especially in patients with severe respiratory disease.

Although specimen collection can be an uncomfortable procedure for the patient it provides a valuable mixture containing biological material both from the infectious agent and from the patient. The high viral load obtained,[1] the simplicity of the procedure involved and the ready availability of this type of sample in laboratories performing routine microbiological analyses make surplus biospecimens a valuable source of biologic material for conducting molecular or genetic studies of the infectious agent and the host.

Digital multiplexed RNA quantification

In recent years, the study of selected genes by real-time PCR or genome-wide gene expression microarray analysis has been used in genetic research to detect associations between specific gene expression profiles and particular diseases. Within these technologies, the nCOUNTER® platform (NanoString Technologies, Seattle, WA) delivers direct and multiplexed measurement of gene expression, providing digital readouts of the relative abundance of mRNA transcripts simultaneously[2] in a single assay, without the need for cDNA conversion or amplification of target RNA. This platform, which offers reliable sensitivity, reproducibility, technical robustness and utility for clinical application,[3] is also capable of analysing RNA samples of poor quality such as fragmented RNA (35 to 50-base target-specific sequences) or cell lysates with total RNA concentrations as low as 100 ng,[4] as is foreseeably the case with samples from nasopharyngeal swabs.

The nCOUNTER Human Immunology V2 CSO panel, which facilitates the study of 594 genes, including the major classes of cytokines, chemokine ligands, interferons and their receptors, the TNF-receptor superfamily, the KIR family genes and genes involved with the anti-fungal immune response, is recommended for the study of the immune response to infectious disease in samples with fragmented RNA or low RNA inputs.[5] This panel can also be combined with an additional panel of 55 genes related to the human inflammatory response. Although the protocols for the study of blood or formalin-fixed paraffin-embedded samples are well known and provided by the manufacturer, no protocol for the analysis of nasopharyngeal swab samples has yet been established.

Protocol

Patients

Our study will include 250 patients admitted to the Hospital Costa del Sol (Marbella, Spain) with severe COVID-19 and positive PCR results for SARS-CoV-2. To participate in the study, all patients will receive a patient information sheet and will be asked to sign the corresponding informed consent form.

Obtention of biologic samples

The procedure for the routine determination of SARS-CoV-2 by PCR includes taking a nasopharyngeal sample with the sterile, fine, flexible swab that is included in the specific respiratory sampling kit for viruses. According to the protocol stipulated by the Spanish Ministry of Health,[1] during sampling, the swab must be introduced through the nostril, parallel to the palate, to a depth equal to the distance from the nostrils to the outer opening of the ear. The swab should be maintained inside the nostril for five seconds to allow absorption of the secretions and should then be withdrawn slowly while making 180° rotations. After taking the sample, the swab should immediately be placed in a sterile tube with 2-3 ml of viral transport medium and kept refrigerated at +4°C until it is analysed at the molecular biology and microbiology laboratory.

Various kits are currently marketed for the collection, transport, and maintenance of clinical samples until the laboratory analysis is performed, some of which include transport media with an inactivator. In the subsequent analysis of the results, it must be considered whether the use of one or other means of transport might affect the final result.

We have performed a local validation study of a subset of samples to confirm that the viral transport media brands used in our laboratory (δ-Swab™; UTM™-Viral Transport Media; Viroclinics Biosciences, Mwe Viral Transport Media, Vircell Transport Media) do not compromise the results obtained.

Heat inactivation protocol

To safely handle biological samples, they must first be inactivated. With samples obtained from nasopharyngeal swabs for molecular analysis, this is usually accomplished by the addition of a chemical quencher or by heat treatment.

Given the low concentration of genetic material in nasopharyngeal swabs, together with the high presence of biologic contaminants in upper respiratory airways, we recommend heat-treatment inactivation. Various techniques have been described to perform this task without affecting the integrity of the RNA, including inactivation at 56°C for 30 minutes, at 65°C for 15 minutes, at 95°C for 5 minutes or at 98 °C for 2 minutes.[6,7]

Before processing the samples, it is necessary to ensure that thermal inactivation does not impair RNA integrity, which can be performed by comparing the performance of RT-PCR analysis for SARS-CoV-2 after treating a set of samples to each of the heat inactivation protocols. In our study, we performed a local validation of a subset of samples with a volume of 400 µl, which confirmed that RT-PCR sensitivity is not compromised by heat inactivation at 98°C for 2 minutes.

Nucleic acid extraction protocol

We performed the local validation of a subset of samples to assess the performance of both the manual and the automated procedures considered. In the first case, the extraction was performed using the RNEasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, using an initial sample volume of 500 µl and a final volume of 10 µl. For automated extraction, we tested the Biorobot EZ1, also obtained from Qiagen, with an initial sample volume of 200 µl and a final volume of 60 µl. Finally, RNA extraction was performed in the MagCore robot (Magcore Lamination India Pvt. Ltd), with an initial sample volume of 200 µl or 400 µl and a final volume of 40 µl.

NanoString input recommendations stipulate a total RNA concentration range of 100-125 ng and specific purity ratios of absorbance measured by spectrophotometry at 260, 280 and 230nm. Since aromatic proteins have a strong UV absorbance at 280 nm, the A260/280 ratio is generally used to assess protein contamination in a nucleic acid sample. A260/280 ratios under 1.7 indicate the presence of contaminants that can affect the result, being the A260/280 ratio of ~2.0 as the generally accepted as "pure" for RNA. In such a manner, the A260/230 ratio is used to reveal the presence of organic contaminants such as phenol or TRIzol. Generally acceptable A260/230 ratios are those in the range of 2.0-2.2.

In our local validation study, we obtained varying results for RNA concentration and purity (Table 1). As can be seen, the eluates obtained by manual extraction had neither the concentration nor the purity required by the equipment. Automated extraction with the Qiagen EZ1 kit also produced aliquots of insufficient purity, which could invalidate the analysis results in the nCounter. Finally, using the MagCore equipment, for the same final eluate volume of 40 µl, initial sample volumes of 200 µl and 400 µl were tested, with the latter obtaining the best results.

Table 1. Performance of four different ARN extraction procedures.

Procedure	Kit name	Sample input	RNA Concentration (ng/µl)	A260/280	A260/230
Manual	RNEasy (Qiagen®)	Initial sample volume, 500 µl. Final eluate volume, 10 µl.	7.03 (SD 9.53)	1.25 (SD 0.39)	0.22 (SD 0.25)
Automated	Kit EZ1 Virus Mini Kit v2.0 de Qiagen®	Initial volume 400 µl. Final volume 60 µl.	259.34 (SD 66.49)	3.20 (SD0.26)	0.84 (SD 0.17)
	Kit MagCore® Viral Nucleic Acid Extraction Kit Cartridge Code 203 (HF16, Compact)	Initial sample volume, 200 µl. Final eluate volume, 40 µl.	28.39 (SD13.47)	2.14 (SD 0.36)	1.12 (SD 0.51)
	Kit MagCore® Viral Nucleic Acid Extraction Kit Cartridge Code 203 (HF16, Compact)	Initial sample volume, 400 µl. Final eluate volume, 40 µl.	43.60 (SD 36.1)	1.90 (SD 0.13)	1.53 (SD 0.40)

The A260/280 ratio is used to assess protein contamination. For pure RNA, the recommended A260/280 ratio should be ~2.0. The A260/230 ratio is used to assess the presence of organic contaminants. A260/230 ratios under 1.8 indicate the presence of contaminants. For RNA, the recommended A260/230 ratio should also be ~2.0. SD (Standard deviation).

Gene expression

We performed a local validation study in a subset of purified RNA aliquots from heat-inactivated nasopharyngeal swabs, in order to evaluate the performance of the NanoString nCounter platform in analysing the expression of our target genes, and obtained satisfactory results.

We followed the manufacturer's instructions, using 100 ng of total RNA. In summary, the protocol consists of the following steps:

1. Preheat the thermocycler to 65°C. Thaw the kit reagents and samples for 30 minutes.
2. Add 70 µl of hybridisation buffer + 28 µl of Reporter Plus to the Reporter Codeset tube. Mix gently.
3. Aliquot 10 µl of Master Mix to each well of the 12-tube strip.
4. Add 5 µl of the samples to each corresponding well (RNA concentration should be 100 µg/µl. Dilute out-of-range samples with RNAse-free water).
5. Spin the Capture ProbeSet and Capture Plus tubes.
6. Add 14 µl of Capture Plus to the Capture ProbeSet tube to create the Master Capture. Mix gently and spin the Master Mix.
7. Add 3 µl of Master Capture to each well of the 12-tube strip.
8. Cover the tube strip with a corresponding plug strip. Mix gently and spin slowly so that the entire volume drops to the bottom of the well, leaving no bubbles.
9. Use the thermocycler to perform hybridisation with the reporter and capture probes that carry the fluorescent signals, for 16-24 hours.
10. Combine pairs of probes specific for the selected genes with a series of internal controls to form a molecular barcoding, or CodeSet, allowing downstream digital detection.
11. Remove excess probes, align the probe/target complexes, immobilise them in the nCounter Cartridge, and then insert them into the nCounter Digital Analyser for data collection.

Data analysis

The differential expression analysis data model preferentially applies the optimal statistical method per gene given the following variable distribution: 1) Mixture negative binomial model, 2) Simplified negative binomial model, 3) Log-linear model, in that order. FDR p-value adjustment will be performed according to the Benjamini-Hochberg method. All samples will be normalised using the geometric mean of the housekeeping genes.

Conclusions

COVID-19 is a major global health problem, making it necessary to develop tools to optimise healthcare and facilitate personalised treatment. From a clinical perspective, the identification of gene transcripts related to the poor prognosis of patients hospitalised with SARS-CoV-2 has undeniable practical value. This complementary information would be straightforward to design multiplexed panels and prediction tools that can be incorporated into computers used in daily practice, helping clinicians predict and identify possible outcomes and facilitating decision-making in this respect.

Our study may also provide useful information to help establish the protocols of other studies based on the analysis of nasopharyngeal swab samples using the NanoString nCOUNTER platform.

Data availability

Normalisation, differential expression analysis and pathway analysis can be performed with Nanostring nCounter nSolver™ 4.0 (RRID:SCR_003420), using the Nanostring Advanced Analysis Module 2.0 plugin and following the Nanostring Gene Expression Data Analysis Guidelines. Advanced Analysis Module 2.0 software uses open-source R programs for quality control, normalisation, differential data analysis, pathway scoring and gene-set enrichment analysis.

Author contributions

MGA, SGR, IBM, MR conceptualization of the study. SGR, MGA, MR funding acquisition. MGA, ILR, TD, VDL, IBM, MA, MLH investigation and methodology. MPR, IBM, MGA, MA contributed to data analysis. MGA, IBM, MR supervised the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics

Our Institutional Review Board (CEI Costa del Sol exp.003_JUL20_PI-IMMU-COVID19) approved this study in July 2020. All patients will be informed of the study and invited to participate. All participation will be subject to the provision of informed written consent.

Acknowledgments

We thank Ms Alicia Aguilera and Ms Belén Sojo for their excellent technical assistance.

References

1. Ministerio de Sanidad, C.y.B.S: **Documento técnico: toma y transporte de muestras para diagnóstico por PCR de SARS-CoV-2 (technical document: Collection and transport of samples for PCR diagnosis of SARS-CoV-2). Health, Ed.** 2020.
2. NanoString: **nCounter(R) Analysis System and FFPE Samples for Gene Expression Analysis.** 2012.
3. Veldman-Jones MH, Brant R, Rooney C, *et al.*: **Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples.** *Cancer Res.* 2015; **75**: 2587–2593.
PubMed Abstract | Publisher Full Text
4. Kulkarni MM: **Digital multiplexed gene expression analysis using the NanoString nCounter system.** *Curr. Protoc. Mol. Biol.* 2011; Chapter 25: Unit25B.10.
PubMed Abstract | Publisher Full Text
5. NanoString: **nCounter® Immunology Panel.** (accessed on December 21, 2021).
Reference Source
6. Batéjat C, Grassin Q, Manuguerra J-C, *et al.*: **Heat inactivation of the severe acute respiratory syndrome coronavirus 2.** *J. Biosaf. Biosecur.* 2021; **3**: 1–3.
PubMed Abstract | Publisher Full Text
7. Auerswald H, Yann S, Dul S, *et al.*: **Assessment of inactivation procedures for SARS-CoV-2.** *J. Gen. Virol.* 2021; **102**.
PubMed Abstract | Publisher Full Text

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Current Peer Review Status: ✗ ?

Version 1

Reviewer Report 22 March 2022

https://doi.org/10.5256/f1000research.113351.r122366

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Tara K. Sigdel
Division of MultiOrgan Transplantation, Department of Surgery, University of California San Francisco, San Francisco, CA, USA

The method on the digital multiplexed gene expression analysis of nasopharyngeal swab samples using the NanoString nCounter system is adequately presented in the manuscript. This could be of interest to the readers of F1000Research and researchers working with gene expression of immune signature and SARS-CoV-2.

I find the rationale of the protocol adequate. I find the protocol not quite complete. Such as:

1. The authors should provide information on any failure in extracting enough total RNA as demanded by the method.

2. In real-life situations it is hard to get enough RNA from 100% samples. If it was the case then it has to be stated clearly.

3. There is some ambiguity on the amount of total RNA. From the manuscript it appears that only 100 ng is needed. The following statement is contradicting:

   "4. Add 5 µl of the samples to each corresponding well (RNA concentration should be 100 µg/µl. Dilute out-of-range samples with RNAse-free water)."

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Organ transplantation, kidney

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Mar 2022

Marilina García Aranda, Hospital Costa del Sol, Marbella, Spain

We thank the reviewer for the constructive comments which will help us to improve the quality of our work. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for acceptance.
Please find below our point-to-point responses to the comments.

The method on the digital multiplexed gene expression analysis of nasopharyngeal swab samples using the NanoString nCounter system is adequately presented in the manuscript. This could be of interest to the readers of F1000Research and researchers working with gene expression of immune signature and SARS-CoV-2.

I find the rationale of the protocol adequate. I find the protocol not quite complete. Such as:

1. The authors should provide information on any failure in extracting enough total RNA as demanded by the method. In real-life situations it is hard to get enough RNA from 100% samples. If it was the case then it has to be stated clearly.

   We agree with the Reviewer and will include the success rate of the extractions performed with the MagCore robot: The success rate of extractions performed with the MagCore robot has been 86%.

2. There is some ambiguity on the amount of total RNA. From the manuscript it appears that only 100 ng is needed. The following statement is contradicting:
   "4. Add 5 µl of the samples to each corresponding well (RNA concentration should be 100 µg/µl. Dilute out-of-range samples with RNAse-free water)."

We agree with the reviewer and apologize for error. We will modify the corresponding paragraphs and will also include additional information:
NanoString input recommendations stipulate a total RNA amount range of 100-300 ng and specific ratios of absorbance at 260nm and 280nm (A260/280) measured with NanoDrop within the range 1.8-2nm. Given the characteristics of the samples, we decided to hybridize the nCounter probes with 100 ng to 200 ng of total RNA per assay, reason why we only assessed gene expression on eluates with total RNA concentration > 2.5 ng/µl measured with the bioanalyzer.
Samples were prepared following manufacturer's instructions. When necessary, we diluted samples in order to obtain 100-200ng of total RNA to each corresponding well. Time of hybridization was set between 16 and 21 hours. Results were obtained with the nCounter Prep Station and Digital Analyzer set at high sensitivity.

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 February 2022

https://doi.org/10.5256/f1000research.113351.r122369

Jan Weber
IOCB Gilead Research Center, Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague, Czech Republic

Authors here described their protocol for RNA preparation from nasopharyngeal swabs for subsequent analysis using the NanoString nCounter Gene Expression Assay. This article is a combination of method development with a description of a study on 250 SARS-CoV-2 positive patients with severe COVID-19.

Major issues

1. The major criticism here is that readers will not be able to assess if the RNA preparation from nasopharyngeal swab and subsequent evaluation by NanoString platform delivered good and reliable data. Reviewer assumes that it will be part of future publication, but without this data it is difficult to judge that nasopharyngeal swabs can be used as source material for this platform. Ideally, authors can post a preprint into archives and put link here.

2. Authors compared manual RNEasy kit that purifies total RNA (usually from cells, tissue, etc.) with automated kits specialized for purification of viral nucleic acid. Authors should have used e.g. QIAamp MinElute Virus kit or similar for a fair comparison.

3. Authors mentioned the nCounter Human immunology V2 CSO panel that can analyze 594 host genes. For this panel, total RNA isolation kit would be a better choice.

4. Did authors test the platform also with the RNAs purified from EZ1 Virus Mini kit? The A260/A230 ratio is not very good, but high A260/A280 does not necessarily indicate contaminants. Did authors get chance to check the whole UV range spectrum?

5. It would be beneficial to show more data about different viral transport medium, heat inactivation, different nucleic acid extraction protocol and compare their impact on results from several internal controls, rather than showing manufacturer's instruction for NanoString assay. Was there really any change in the protocol in the case of RNA from nasopharyngeal swabs? If yes, please stress the differences only.

Minor issues:

1. Authors should change the future tense to past tense when describing their study in paragraphs "Patients", "Data analysis" and "Ethics".

2. Table 1 correct ARN to RNA.

3. Include number of samples "n" per each isolation method in the Table 1.

4. Make clear protocol point no. 4. At the protocol beginning, it is mentioned using 100ng of total RNA, but no.4 talks about using 5µl of sample and RNA concentration 100µg/µl.

5. In the protocol include temperature and other details (such as conditions of each spin).

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology and molecular biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 29 Mar 2022

Marilina García Aranda, Hospital Costa del Sol, Marbella, Spain

We thank the reviewer for taking the time and effort necessary to review the manuscript and sincerely appreciate all valuable comments and suggestions which will help us to improve the quality of the paper. Please find below our point-by-point responses to each of the comments:

Authors here described their protocol for RNA preparation from nasopharyngeal swabs for subsequent analysis using the NanoString nCounter Gene Expression Assay. This article is a combination of method development with a description of a study on 250 SARS-CoV-2 positive patients with severe COVID-19.

Major issues

1. The major criticism here is that readers will not be able to assess if the RNA preparation from nasopharyngeal swab and subsequent evaluation by NanoString platform delivered good and reliable data. Reviewer assumes that it will be part of future publication, but without this data it is difficult to judge that nasopharyngeal swabs can be used as source material for this platform. Ideally, authors can post a preprint into archives and put link here.

   We agree with the reviewer. However, we would like to point out that, as a pre-protocol study, we only have preliminary results. We are currently generating additional data and we will be able to stablish more consolidated conclusions in the protocol paper.

2. Authors compared manual RNEasy kit that purifies total RNA (usually from cells, tissue, 0etc.) with automated kits specialized for purification of viral nucleic acid. Authors should have used e.g. QIAamp MinElute Virus kit or similar for a fair comparison.

   *We agree with the reviewer, however, due to shortage of RNA extraction kits during the COVID-19 pandemic, we were not able to use the QIAmp MinElute Virus kit. Instead, we decided to use RNeasy Mini kit based on previously published papers reporting its use as a valid alternative strategy for viral detection in sputum (*DOI: 10.1016/s0166-0934(01)00284-1) *or nasopharyngeal samples (* https://www.biorxiv.org/content/10.1101/2020.03.20.001008v1.full.pdf), *Besides, the RNeasy Mini Kit is one of the most common commercial kits and the Gold Standard for RNA extraction. We will modify the corresponding paragraphs in the text accordingly.*

3. Authors mentioned the nCounter Human immunology V2 CSO panel that can analyze 594 host genes. For this panel, total RNA isolation kit would be a better choice.

   We agree with the reviewer. Indeed, we used RNEasy kit that purifies total RNA. We will

modify the corresponding paragraph to make this point clearer.

4. Did authors test the platform also with the RNAs purified from EZ1 Virus Mini kit? The A260/A230 ratio is not very good, but high A260/A280 does not necessarily indicate contaminants. Did authors get chance to check the whole UV range spectrum?

   We are sorry we did not. Despite its affordability, rapidity and ease of use, the NanoDrop usually does not give perfect and reliable readings, especially in samples with contaminants. For this reason, we measured the A260/280 ratios to get a general idea about the best RNA extraction method within the equipment available in the molecular biology laboratory of our hospital. Once we chose the method that best suited our criteria of 260/280 ratios (1.80-2.0 nm), we assessed the quality and quantity of RNA eluates with the Agilent 2100 Bioanalyzer. We will include a new table with the corresponding results in the manuscript.

5. It would be beneficial to show more data about different viral transport medium, heat inactivation, different nucleic acid extraction protocol and compare their impact on results from several internal controls, rather than showing manufacturer's instruction for NanoString assay. Was there really any change in the protocol in the case of RNA from nasopharyngeal swabs? If yes, please stress the differences only.

   Following the recommendations of the Reviewer, we have added information regarding the impact of transport medium and the heat inactivation protocol. While the former did not influence the experiment performance, the heat inactivation protocol was selected based on the reported standards (DOI: 10.1099/jgv.0.001539). Regarding our in house modifications of the manufacturers protocol to adapt it to this specific type of sample, as the reviewer emphasizes, it is important to make them clear in the manuscript. Therefore, we have modified it including and highlighting the optimized processes. This is of high relevance given that RNA from remnant nasopharyngeal exudates is present at very low concentrations and is highly degraded; indeed the RNA integrity number was less than 6 and the DV200 was less than 30% in most of the samples, what would make them inadequate for RNAseq. These samples are also highly heterogeneous, present great variability from patient to patient, and have been transported with several types of transportation medium. Also sampling procedure varies from center to center. Moreover, the fact that only diagnostic remnants were used implied that only limited volumes were available. For these reasons, the optimization of such a method that detects enough number of genes with this type of diagnostic residual samples is of high importance for performing research in the field.

Minor issues:

1. Authors should change the future tense to past tense when describing their study in paragraphs "Patients", "Data analysis" and "Ethics".

   The original version of the manuscript corresponds to the development phase of the technique, which was carried out prior to patient recruitment, reason why "Patients" and "Ethics" sections were written in future tense. We will modify the corresponding paragraphs accordingly.

2. Table 1 correct ARN to RNA.

   We apologize for the mistake and will correct the typo in the new version of the manuscript.

3. Include number of samples “n” per each isolation method in the Table 1.

   We appreciate the Reviewer’s suggestion, and will include the corresponding information in the new version.

4. Make clear protocol point no. 4. At the protocol beginning, it is mentioned using 100ng of total RNA, but no.4 talks about using 5µl of sample and RNA concentration 100µg/µl.

   We apologize for the mistake and will change the corresponding paragraph.

5. In the protocol include temperature and other details (such as conditions of each spin).

   We agree with the Reviewer’s suggestion and will modify the protocol accordingly.

Competing Interests: No competing interests were disclosed.