METHOD ARTICLE

LABORATORY PROTOCOL FOR THE DIGITAL MULTIPLEXED GENE EXPRESSION ANALYSIS OF NASOPHARYNGEAL SWAB SAMPLES USING THE NANOSTRING N COUNTER SYSTEM [VERSION 2; PEER REVIEW: 2 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 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.
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Competing interests: No competing interests were disclosed.

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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

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.

Nasopharyngeal swabs provide a valuable mixture containing biological material both from the infectious agent and from the patient. The high viral load obtained, 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 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, is also capable of analyzing RNA samples of poor quality such as fragmented RNA (35 to 50-base target-specific sequences) or cell lysates with RNA concentrations as low as 100 ng, as is foreseeable 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. 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 included 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 received a patient information sheet and were 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, 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 analyzed at the molecular biology and microbiology laboratory. In case of delay in processing, samples may also be frozen at -80°C until further analysis.
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, we also considered whether the use of one or other viral transport mediums affected the final result.

**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, we ensured that thermal inactivation did not impair RNA integrity, 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 experiment, all samples were inactivated at 98°C for 2 minutes as previously described.

**Nucleic acid extraction protocol**
Following viral inactivation, RNA was immediately extracted. Given the novelty of the protocol, we performed a local validation of a subset of samples to assess the performance of both the automated and manual RNA extraction procedures.

For automated extraction, we tested the performance of the Biorobot EZ1 (Qiagen, Hilden, Germany), with an initial sample volume of 200 μl and a final eluate volume of 60 μl; and the MagCore robot (Magcore Lamination India Pvt. Ltd), with initial sample volumes of 200 μl or 400 μl and a final eluate volume of 40 μl. Although these kits are not the most optimal methods to allow for the detection of host genes, our preliminary results demonstrate their validity to study host gene expression.

With respect to the manual RNA extraction procedure, and due to supply shortages during the COVID-19 pandemic, we were not able to use the recommended QIAMp MinElute Virus extraction kit (Qiagen). Instead, and according to previously published papers in this field, we tested the RNEasy Mini Kit (Qiagen), which is one of the most common commercial kits and the Gold Standard for RNA extraction, as an alternative strategy for viral detection in sputum8 or nasopharyngeal samples.9 We processed the samples according to manufacturer’s instructions, using an initial sample volume of 500 μl and a final volume of 10 μl.

| Procedure               | Kit name                                      | N  | Sample input                          | RNA Concentration (ng/μl) | A260/280 | A260/230  |
|------------------------|-----------------------------------------------|----|--------------------------------------|---------------------------|----------|-----------|
| Manual                 | RNEasy (Qiagen®)                              | 12 | Initial sample volume, 500 μl. Final eluate volume, 10 μl. | 7.03 (SD 9.53)            | 1.25     | 0.22 (SD 0.25) |
| Automated              | Kit EZ1 Virus Mini Kit v2.0 de Qiagen®         | 14 | Initial volume 400 μl. Final volume 60 μl. | 259.34 (SD 66.49)         | 3.20     | 0.84 (SD 0.17) |
|                        | Kit MagCore® Viral Nucleic Acid Extraction Kit Cartridge Code 203 (HF16, Compact) | 16 | Initial sample volume, 200 μl. Final eluate volume, 40 μl. | 37.30 (SD 9.18)           | 2.08     | 1.12 (SD 0.38) |
|                        | Kit MagCore® Viral Nucleic Acid Extraction Kit Cartridge Code 203 (HF16, Compact) | 16 | Initial sample volume, 400 μl. Final eluate volume, 40 μl. | 49.17 (SD 26.54)          | 1.87     | 1.40 (SD 0.29) |

SD: Standard deviation.
**Table 2.** Results of RNA concentration and absorbance ratios measured with NanoDrop 2000, RNA integrity and concentration measured with Agilent Bioanalyzer and nCounter success rate and performance for each of the tested viral transport media brands.

| Transport media       | N   | NanoDrop 2000 | Agilent Bioanalyzer | nCounter NanoString |
|-----------------------|-----|---------------|---------------------|---------------------|
|                       |     | Concentration (ng/μl) | A260/280 | A260/230 | Concentration (pg/μl) | DV200 | Panel validity | Panel Performance |
| Copan UTM-RT          | 37  | 19.49 (SD 46.54) | 1.89 (SD 46.54) | 0.80 (SD 46.54) | 7814.27 (SD 8514.47) | <30%: 29.73% 30-50%: 29.73% 50-70%: 13.51% >70%: 29.73% | 70.27% | Excellent: 76.92% Very good: 19.23% Good: 3.85% |
| Deltaswab VicUM       | 8   | 39.93 (SD 20.46) | 1.89 (SD 0.23) | 1.18 (SD 0.37) | 7467.76 (SD 13984.33) | <30%: 25.00% 30-50%: 25.00% 50-70%: 12.50% >70%: 37.50% | 62.50% | Excellent: 80.00% Very Good: 20.00% |
| Deltaswab Virus       | 54  | 11.44 (SD 19.53) | 1.87 (SD 0.10) | 0.47 (SD 0.58) | 7814.27 (SD 10181.38) | <30%: 38.89% 30-50%: 14.81% 50-70%: 3.70% >70%: 42.59% | 66.67% | Excellent: 83.33% Very Good: 11.11% Good: 5.56% |
| Sigma Virocult        | 20  | 24.80 (SD 25.71) | 1.86 (SD 0.03) | 0.68 (SD 0.34) | 19425.12 (SD 12966.48) | <30%: 35.00% 30-50%: 30.00% 50-70%: 5.00% >70%: 10.00% | 70.00% | Excellent: 78.57% Very Good: 14.29% Good: 7.14% |
| Vircell               | 6   | 10.53 (SD 5.27) | 1.86 (SD 0.06) | 0.44 (SD 0.17) | 5810.98 (SD 3414.83) | <30%: 83.33% >70%: 50.00% | 50.00% | Excellent: 66.67% Very Good: 0.00% Good: 33.33% |
| None (bronchoaspirates)| 6  | 56.66 (SD 150.03) | 1.81 (SD 0.12) | 0.91 (SD 0.63) | 11885.32 (SD 12894.06) | 30-50%: 16.67% 50-70%: 33.33% >70%: 50.00% | 83.33% | Excellent: 100% |

All of the studied viral transport media comply with the European Union’s (EU) mandatory conformity certification and are authorized for use in clinical laboratories. A260/280 and A260/230: Absorbance ratios measured with NanoDrop 2000. DV200: Percentage of fragments > 200 nucleotides, measured with the Agilent Bioanalyzer. nCounter performance: Excellent (at least 3 housekeeping genes with more than 100 lectures; Very Good (at least 2 housekeeping genes with more than 100 lectures and at least 1 housekeeping gene with more than 70 lectures), Good (1 housekeeping gene with more than 100 lectures and at least 2 housekeeping genes with a minimum of 70 lectures). SD: Standard Deviation.
Given its affordability, rapidity and ease of use, we firstly carried out a general assessment of eluates sample quality and concentration with the NanoDrop™2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). NanoString input recommendations stipulate a total RNA concentration range of 100-130 ng and specific ratios of absorbance at 260 nm and 280 nm (A260/280) measured with NanoDrop within the range 1.80-2 nm. 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 usually 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 normally used to reveal the presence of organic contaminants such as phenol or TRIzol. Generally acceptable A260/230 ratios are those in the range of 1.8-2.2.

In our local validation study, we obtained varying results for RNA concentration and absorbance ratios (Table 1) for each of the studied procedures. As can be seen, eluted samples 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 elution volume of 40 μl, initial sample volumes of 200 μl and 400 μl were tested, with the latter obtaining the best results. The success rate of extractions performed with the MagCore robot was 86%.

Once we decided to use the MagCore extraction method, we performed a local validation study to assess RNA concentration and purity measured with NanoDrop™2000 spectrophotometer as well as RNA concentration and integrity measured with the Agilent 2100 System Bioanalyzer (Agilent Scientific Instruments Inc., Santa Clara, California) of a subset of 131 purified RNA aliquots from heat-inactivated nasopharyngeal swabs (2 minutes at 98°C) processed with the MagCore robot (initial sample volume of 400 μl and a final elute volume of 40 μl), considering the different viral transport media brands in which patient samples were preserved (Deltaswab ViCUM®, Deltaswab Virus®, Sigma Virucid® Mwe; Vircell Transport Medium; Copan UTM®-RT) (Table 2).

**Gene Expression**

After heat inactivation and RNA extraction, RNA eluates were stored at -80°C until further processing. Samples were prepared following manufacturer’s instructions and diluted, in the case of the most concentrated samples, so that the final volume of 5 μl per well contained 150-200 ng of total RNA. Samples were hybridized 22 hours at +65°C and stored at +4°C until digital readout. Results were obtained with the nCounter Prep Station and Digital Analyzer set at high sensitivity. Performance of the NanoString platform and panel validity was assessed with respect to positive and negative controls as well as to the number of readings of housekeeping genes (Table 2).

**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 results are normalized using the geometric mean of the housekeeping genes.

**Limitations of the study**

Our study had several strengths, as being the first study in to set up a protocol for the digital multiplexed gene expression analysis of nasopharyngeal exudates using the NanoString nCounter System. On the other hand, and apart from the lack of previous studies on this topic, our study also had other limitations: Since our study is based on the analysis of diagnostic surpluses, we have only been able to analyze a certain number of samples preserved in each viral transport medium. We neither know which of the original nasopharyngeal samples were stored at -80°C and subsequently thawed before performing the diagnostic PCR during the care process, which could explain why RNA in some samples was quite degraded. Finally, reagents shortage during the first and second waves of the COVID-19 pandemic, has also conditioned the techniques used.

**Conclusions**

COVID-19 is a major global health problem, making it necessary to develop tools to optimize healthcare and facilitate personalized treatment. From a clinical perspective, the identification of gene transcripts related to the poor prognosis of patients hospitalized 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 RNA analysis from nasopharyngeal swab samples using the NanoString nCOUNTER platform.
Data availability
Normalization, 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 plugin https://www.nanostring.com/products/analysis-solutions/ncounter-advanced-analysis-software/ and following the NanoString Gene Expression Data Analysis Guideliness. Advanced Analysis Module 2.0 software uses open-source R programs for quality control, normalization, 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 were informed of the study and invited to participate. All participation was subject to the provision of informed written consent.

Acknowledgments
We thank Ms Isabel María Luque Bardón for her support. We also thank Alicia Aguilera at Hospital Costa del Sol and Belén Sojo and Beatriz Martinez at Centro de Investigaciones Médico Sanitarias (CIMES), for their 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 O, Manuguerra JC, 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
8. Xiang X, Qiu D, Hegele RD, et al.: Comparison of different methods of total RNA extraction for viral detection in sputum. J. Virol. Methods. 2001; 94: 129–136. PubMed Abstract | Publisher Full Text
9. Bruce EA, Tighe S, Hoffman JJ, et al.: RT-qPCR DETECTION OF SARS-CoV-2 RNA FROM PATIENT NASOPHARYNGEAL SWAB USING QIAGEN RNEASY KITS OR DIRECTLY VIA OMISSION OF AN RNA EXTRACTION STEP. bioRxiv. 2020. Publisher Full Text
Open Peer Review

Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 28 October 2022

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

The authors have adequately responded to my comments/concerns. I do not have further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Organ transplantation, kidney, Biomarker, Transcriptomics, Proteomics

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.

Reviewer Report 21 October 2022

https://doi.org/10.5256/f1000research.138941.r152139

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

Authors have satisfactorily addressed most of my concerns and explained why they used less than optimal approach. They included a paragraph with limitations that explains the large variations in RNA quantity and quality. It is clear that more samples and prepared in more controlled approach are necessary before we can conclude that nasopharyngeal swabs can be used for NanoString
nCounter Gene Expression Assay.

Minor issues:
1. In Table 2 Copan UTM-RT row, put correct SD for A260/280 and A260/280 ratios.

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 confirm that it is of an acceptable scientific standard.

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.

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**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.
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.

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Author Response 29 Mar 2022

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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 establish more consolidated conclusions in the protocol paper.

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.

   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 QIAamp 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.
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