Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
An economical and sensitive glassmilk-based nucleic-acid extraction approach for SARS-CoV-2 diagnostics

Robert Page, Edward Scourfield, Mattia Ficarelli, Stuart McKellar, Kwok Leung Lee, Thomas J.A. Maguire, Clement Bouton, Maria Jose Lista, Stuart J.D. Neil, Michael H. Malim, Mark Zuckerman, Hannah E. Mischo, Rocio T. Martinez-Nunez

PII: S2666-1667(22)00180-0
DOI: https://doi.org/10.1016/j.xpro.2022.101300
Reference: XPRO 101300

To appear in: STAR PROTOCOLS

Received Date: 12 January 2022
Revised Date: 16 February 2022
Accepted Date: 16 March 2022

Please cite this article as: Page, R., Scourfield, E., Ficarelli, M., McKellar, S., Lee, K.L., Maguire, T.J.A., Bouton, C., Lista, M.J., Neil, S.J.D., Malim, M.H., Zuckerman, M., Mischo, H.E., Martinez-Nunez, R.T., An economical and sensitive glassmilk-based nucleic-acid extraction approach for SARS-CoV-2 diagnostics, STAR PROTOCOLS (2022), doi: https://doi.org/10.1016/j.xpro.2022.101300.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2022
**Step 1**
100 µl swab
100 µl 4% SDS
Mix

**Step 2**
1 min **Mastermix**
Mix 2-propanol, glassmilk and 1.25M NaCl

**Step 3**
2% SDS final concentration

**Step 4**
Incubate 5 min
Discard supernatant
500 µl 70 % ethanol
Spin 7,000 x g 10 sec
Remove all ethanol

**Step 5**
4,500 x g 15 sec
x2

**Step 6**
Air dry 5 min
65°C

**Step 7**
5 min **Bind RNA**
RNA binding to glassmilk

**Step 8**
2 min **Wash x2**
Wash x2 with 70% ethanol
Remove all ethanol

**Step 9**
50 µl H₂O₂ mix
4,500 x g 15 sec
Transfer eluate (RNA) to clean tube

**Step 10**
5 min **Air dry RNA**
Allow glassmilk to dry

**Step 11**
1 min **Elute RNA**
Title: an economical and sensitive glassmilk-based nucleic-acid extraction approach for SARS-CoV-2 diagnostics

Author List: Robert Page1,4, Edward Scourfield2,4, Mattia Ficarelli2, Stuart McKellar2, Kwok Leung Lee2, Thomas J.A. Maguire2, Clement Bouton2, Maria Jose Lista2, Stuart J.D. Neil2, Michael H. Malim2, Mark Zuckerman3, Hannah E. Mischo2,5,*, Rocio T. Martinez-Nunez2,5,6,7**

Affiliations:
1 Dept. Inflammation Biology, School of Immunology and Microbial Sciences. Asthma UK Centre in Allergic Mechanisms of Asthma. Guy’s Campus, King’s College London SE1 9RT, UK
2 Dept. Infectious Diseases, School of Immunology and Microbial Sciences. Guy’s Campus, King’s College London SE1 9RT, UK.
3 South London Specialist Virology Centre. King’s College Hospital (London, UK)
4 These authors contributed equally
5 These authors contributed equally
6 Lead contact
7 Technical contact
* Correspondence: hannah.mischo@kcl.ac.uk
** Correspondence: rocio.martinez_nunez@kcl.ac.uk

Summary

The gold standard protocol for SARS-CoV-2 infection detection remains reverse transcription quantitative polymerase chain reaction (RT-qPCR), which detects viral RNA more sensitively than any other approach. Here, we present Homebrew, a low cost protocol to extract RNA using widely available reagents. Homebrew is as sensitive as commercially available RNA extraction kits. Homebrew allows for sample pooling and can be adapted for automation in high throughput settings.

For complete information on the generation and use of this protocol, please refer to Page et al.,2022.

Before you begin

The protocol below describes the specific steps for using combined nose and throat swabs for SARS-CoV-2 detection employing the Centers for Diseases Control and Prevention (CDC) N1, N2 and RNaseP primers (Integrated DNA Technologies, 10006770) and TaqMan Fast Virus 1-Step Master Mix
All reagents below can be prepared in large batches, batch-tested and stored at ambient temperature (16° - 40° C). In our hands reagents did not lose activity over this temperature scale over more than 12 months storage.

We have made separate sections for testing individual and pooled samples. Details on the sensitivity of our pooling method can be found in Page et al. 2022.

Institutional permissions

Samples for this study were provided under KCL TEST (KCL Ethics Ref: 21150); and as Service Delivery for King’s College Hospital. Samples were combined nose and throat swabs previously tested at KCL TEST or King’s College Hospital as part of a potential service development. All samples were anonymised and assessed after being diagnosed as SARS-CoV-2 positive by the hospital or negative by KCL TEST. We assessed a range of Ct values in order to represent a broad range of viral loads. Positive swabs were inactivated in a Category 3 facility employing 90°C 10 mins in a dry bead bath as per Lista et al., 2021.

Abbreviations: GM: glassmilk; GM-MB: glassmilk master buffer.

Preparation of the glassmilk

This step describes how the matrix for nucleic acid capture is prepared.

Silicon dioxide (Sigma, 342890) once prepared will be referred as glassmilk, GM or silica matrix. The final concentration is 700 mg/mL and thus 21 g will render 30 mL of glassmilk, enough for 3,000 extractions.

Timing: 5h

1.  Suspend 21 g silicon dioxide 325 mesh (Sigma 342890) in 40 mL 10 % HCl in a 50 mL tube. Polypropylene tubes are appropriate.
   
   CAUTION: 10% HCl is highly corrosive. Only use under a fume hood when handling HCl. If diluting from a concentrated stock, add acid to water. This acid wash of silica matrix ensures getting rid of contaminants that may interfere with downstream RT-qPCR (e.g. RNAseP detection due to contamination).
   
   a.  Agitate suspension for 4 hrs on a tube roller. Secure the cap with parafilm to minimise the risk of spillages. If no roller is available move every 10 minutes manually by gently inverting the tube 10 times.
   
   b.  Centrifuge at 2000 g for 5 min
   
   c.  Carefully remove HCl with a pipette and store in appropriate double container for reuse. HCl can be reused for this washing procedure multiple times. Do not dispose of HCl by pouring down the drain with copious amounts of water if it has not been previously neutralized. Spills may be neutralized with sodium bicarbonate or baking soda.

2.  Resuspend the silica pellet with 40 mL single deionised water.
a. Resuspend silica in a 50 mL polypropylene tube by agitation, vortexing at ~ 134 x g or repeated tapping against a surface or hand so no HCl is ‘trapped’ in the pellet
b. Centrifuge at 2000 g for 5 min.
c. Remove wash water and dispose of through the drain. Flush with plenty of water.

3. Repeat step 2 for a total of 6 washes.
4. Before removing the final wash, measure pH of the supernatant with a strip, it should be between 7 and 8. If acidic pH is detected perform more water washes and measure pH after each wash until pH 7-8 is achieved.
5. Remove the supernatant, the pellet should now be white.
6. Resuspend silica in a final volume of 30 mL of H₂O – if possible, use MilliQ or RNase free H₂O.
7. Aliquot in clean 1.5 mL tubes and keep at 16-40°C.

NOTE: we have kept the glass milk for 18 months without loss in performance at temperature ranging form 4-40°C.

Preparation of SDS, NaCl and 70% ethanol

Timing: 15 mins

8. Prepare 1.25M NaCl.
   a. To prepare 500 mL 1.25 M NaCl, weight 36.525 g NaCl (Sigma/Merck S3014-500G) and dissolve in 500 mL H₂O.
   b. Store at 16-40°C. 1.25M NaCl is stable at 16-40°C for at least 6 months.
9. Prepare 70% EtOH
   a. To prepare 500 mL, mix 350 mL pure EtOH (Fisher Scientific, 10644795) and 150 mL H₂O.
   b. Ethanol should be kept in the dark if at all possible (by covering the tube with foil for example), at 16-40°C.
10. Prepare 4% SDS
    a. From a 10% SDS stock, prepare 500 mL by mixing 200 mL 10% SDS (Fisher Scientific, 10552785) with 300 mL H₂O.
    b. Keep SDS solution at 16-40°C.

NOTE: We recommend purchasing a readily dissolved SDS stock.
NOTE: Keep SDS solution at 16-40°C. SDS may precipitate at low temperatures; if precipitates are observed, please warm up and re-dissolve prior to use.
Alternative: SDS can also be prepared from powder. However, this is highly dangerous and needs to be performed under the fume-hood.

Prepare glassmilk master buffer (GM-MB) for individual samples

Timing: 5 mins

Prepare the GM-MB fresh every time prior to use. Follow this table for calculating volumes (in microliter) to prepare enough glassmilk master buffer GM-MB) for all samples. Consider always an
excess of 10% (i.e. prepare enough for 11 samples if you are analyzing 10). The table below shows calculations for one and twenty-four samples.

| Samples # | 10 % added | Glassmilk (µL) | Isopropanol (µL) | NaCl (µL) |
|-----------|------------|----------------|-----------------|-----------|
|           |            | Final 7 µg / sample | Final 65%       | Final 0.4M |
| 1         | N/A        | 10             | 400             | 200       |
| 10        | 11         | 110            | 4,400           | 2,200     |
| 24        | 26.4       | 264            | 10,560          | 5,280     |

**CRITICAL:** Make sure the master buffer is properly mixed and shake vigorously every 3 samples to avoid glassmilk pelleting.

Prepare glassmilk master buffer (GM-MB) for pooled samples

**Timing: 5 mins**
Prepare the GM-MB fresh every time prior to use.

Pooling samples can accelerate processing and considerably reduce the costs of sample processing. We have tested Homebrew and determined that the method allows for pooling of up to 19 negative samples with one positive sample (Page et al., 2022).

For use of the pooled protocol, we found that 20 µl glassmilk is sufficient to capture the nucleic acid material of up to 20 swabs and obtain a clearly visible pellet of glassmilk without losing sensitivity. If using less than 20 swabs, only adjust NaCl and Isopropanol amounts. The glassmilk-master buffer recipe will change to::

| Samples # | 10 % added | Glassmilk (µL) | Isopropanol (µL) | NaCl (µL) |
|-----------|------------|----------------|-----------------|-----------|
| 20        | 22         | 20             | 8,800           | 4,400     |
| 10        | 11         | 20             | 4,400           | 2,200     |
| 40        | 44         | 40             | 17,600          | 8,800     |

**Key resources table**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins |        |            |
| Silicon dioxide 325 mesh | Sigma/Merck | Cat# 342890 |
| Carboxylate modified magnetic SpeedBeads™ | Sigma/Merck | Cat# GE45152105050250 |
| SDS (500 g) | Sigma/Merck | Cat# L3771-500G |
| 10% SDS (1 L) | Fisher Scientific | Cat# 10552785 |
| NaCl (500 g) | Sigma/Merck | Cat# S3014-500G |
| NaI (sodium iodide) | Sigma/Merck | Cat# 383112-100G |
| GITC (Guanidine Isothiocyanate) | Sigma/Merck | Cat# 5120-250GM |
| Item                                                                 | Supplier                      | Cat#/Code         |
|----------------------------------------------------------------------|-------------------------------|-------------------|
| Isopropanol (2.5 L)                                                  | Fisher Scientific              | BP2618-212 2.5L   |
| Absolute ethanol (500 mL)                                            | Fisher Scientific              | 10644795          |
| Nuclease Free Water (500 mL)                                         | Fisher Scientific              | AM9930            |
| **Critical commercial assays**                                       |                               |                   |
| TaqMan Fast Virus 1-Step Master Mix                                  | Thermo Fisher Scientific       | 4444434           |
| 2019-nCov CDC EUA Kit                                                | Integrated DNA Technologies    | 10006770          |
| **Deposited data**                                                   |                               |                   |
| Deposited in Mendeley: doi:10.17632/b2mscbnhmg.1                     |                               |                   |
| **Experimental models: Organisms/strains**                           |                               |                   |
| Severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) | Obtained from Public Health England |                   |
| **Oligonucleotides**                                                 |                               |                   |
| 2019-nCoV_N1 Forward Primer: GAC CCC AAA ATC AGC GAA AT              | CDC                           | 2019-nCoV_N1-F 500nM |
| 2019-nCoV_N1 Reverse Primer: TCT GGT TAC TGC CAG TTG AAT CTG        | CDC                           | 2019-nCoV_N1-R 500nM |
| 2019-nCoV_N1 Probe: FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1        | CDC                           | 2019-nCoV_N1-P 150nM |
| NOTE: This probe spans the N:P13L mutation present in the Omicron variant (B.1.1.529) |                   |                   |
| 2019-nCoV_N2 Forward Primer: TTA CAA ACA TTG GCC GCA AA            | CDC                           | 2019-nCoV_N2-F 500nM |
| 2019-nCoV_N2 Reverse Primer: GCG CGA CAT TCC GAA GAA              | CDC                           | 2019-nCoV_N2-R 500nM |
| 2019-nCoV_N2 Probe: FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1       | CDC                           | 2019-nCoV_N2-P 125nM |
| RNAse P Forward Primer: AGA TTT GGA CCT GCG AGC G                  | CDC                           | RP-F 500nM        |
| RNAse P Reverse Primer: GAG CGG CTG TCT CCA CAA GT                  | CDC                           | RP-R 500nM        |
| RNAse P Probe: FAM – TTC TGA CCT GAA GGC TCT GCG CG – BHQ-1        | CDC                           | RP-P 125nM        |
| **Software and algorithms**                                          |                               |                   |
| GraphPad Prism                                                       |                               |                   |
| **Journal Pre-proof**                                                |                               |                   |

https://www.graphpad.com/scientific-software/prism/
Materials and equipment (optional)

| Reagent                        | Final concentration | Amount          |
|--------------------------------|---------------------|-----------------|
| NaI (sodium iodide)            | 1.25M               | 100 µL/100 µL swab |
| GITC (Guanidine Isothiocyanate) | 1.25M               | 100 µL/100 µL swab |

Keep at 16-40°C.

**CRITICAL:** Both GITC and NaI are toxic. According to their MSDS: GITC has acute oral toxicity Category 4, acute dermal toxicity Category 4, acute Inhalation Toxicity - Dusts and Mists Category 4, skin Corrosion/Irritation Category 1 C and serious Eye Damage/Eye Irritation Category 1; NaI has acute oral toxicity Category 4, acute dermal toxicity Category 4, acute Inhalation Toxicity - Dusts and Mists Category 4, skin Corrosion/Irritation Category 1 C and serious Eye Damage/Eye Irritation Category 1.

**Alternatives:** The homebrew method employs NaCl as its preferred chaotropic. NaCl is not considered hazardous by the 2012 OSHA Hazard Communication Standard (29 CFR 1910.1200). If for some reason NaCl was not available, NaI or GITC can be used instead as per Page et al., 2022.

**Step-by-step method details**

**Note:** when performing this protocol for the first time, we recommend either using spiked swab material with a known positive control (e.g. commercially available or laboratory-grown virus) or using swabs that have previously been tested by conventional extraction methods. Every extraction should include a positive control (in our case we employed laboratory-grown virus) and a negative
extraction control (H₂O). All steps in the extraction are performed at ambient temperature (16-40°C) unless otherwise stated.

We have performed this protocol on swabs from various different sources that have been stored at various temperatures (16-40°C or -80°C) before analysis. As far as we can attest, all different viral transport medias that are currently in circulation allow for successful isolation of viral material. Viral transport medium can be prepared as per CDC recommendation (https://www.cdc.gov/coronavirus/2019-ncov/downloads/Viral-Transport-Medium.pdf), i.e. 2% FBS 100µg/mL Gentamicin 0.5 µg/mL Amphotericin B in Hanks Balanced Salt Solution (HBSS). Please refer to Page et al., 2022 for a more complete characterization of the robustness of this method.

Inactivation and lysis of swab material (individual samples)

This step inactivates potential SARS-CoV-2 viral particles and aids disruption of enveloped viral particles and subsequent solubilization of genetic material.

**Timing: 1 min**

We recommend heat inactivation using a dry bead bath (either 70°C or 90°C for 10 to 30 min) prior to addition of SDS as it allows safe handling of otherwise infectious samples while preserving sensitivity of detection (Lista et al., 2021). Inactivated swabs must ALWAYS be opened in a microbiological safety cabinet class I when possible (class II when I not available) for the safety of the handler. Once swab material is inactivated samples can be handled on a regular bench.

1. Add 100 µL of 4% SDS per 100 µL of swab sample. Scale up appropriately if more swab sample is used. Incubation with SDS is not required.

**CRITICAL:** If swab samples are not heat inactivated, add SDS in a microbiological safety cabinet.  
**CRITICAL:** Check that there are no SDS precipitates. If precipitates are observed, place SDS in a lukewarm bath until precipitates are dissolved.

Inactivation and lysis of swab material (pooled samples)

1. If using the pooled protocol, scale up the amount of 4% SDS accordingly. For example, for 20 samples of 100 µl each, add 20 x 100 µl (=2 mL) 4% SDS.

**CRITICAL:** If swab samples are not heat inactivated, add SDS in a microbiological safety cabinet.  
**CRITICAL:** Check that there are no SDS precipitates. If precipitates are observed, place SDS in a lukewarm bath until precipitates are dissolved.

Mastermix addition and RNA binding (individual samples)

In this step, RNA from the lysed sample binds to glassmilk.

**Timing: 6 mins**
GM-MB contains NaCl as chaotropic agent, isopropanol to increase binding to glassmilk and glassmilk as RNA-binding matrix.

2. Prepare GM-MB buffer as per instructions above. Prepare enough for all samples and consider an extra 10% for pipetting errors.
3. Add 610 µL of GM-MB to 200 µL of lysed swab (100 µL 4% SDS + 100 µL swab sample).
4. Incubate 5 minutes at 16-40°C to allow RNA binding.

Mastermix addition and RNA binding (pooled samples)
Timing: 6 mins

2. Prepare GM-MB buffer as per instructions above. Prepare enough for all samples and consider an extra 10% for pipetting errors. This will contain 20 µL of glassmilk and a multiple per sample of 400 µL isopropanol and 200 µL 1.25 M NaCl. Due to the increased volume, pooled samples are mixed in a falcon tube.
3. Mix well by vortexing.
4. Incubate 5 minutes at 16-40°C to allow RNA binding.

RNA cleaning

In this step, RNA from the lysed samples binds to glassmilk.

Timing: 3 mins

The bound RNA is pelleted and washed with 70% ethanol twice to remove excess proteins in the sample and increase the sensitivity of the PCR.

5. Spin the sample at 4,500 g for 15 seconds.
6. Discard the supernatant by decanting into a waste basin. The pellet will not detach (Movie 1: Decanting).
7. Disaggregate the pellet of GM by tapping the plastic tube against a rack or by vortexing (Figure 1 and Movie 2, Disaggregation by tapping).
   a. If using the pooled protocol, resuspend the pellet from the falcon tube and transfer with a pipette to a microcentrifuge tube. Continue processing as described for individual samples.
8. Add 500 µL of 70% ethanol and mix by flicking the tube.

NOTE: steps 7 and 8 can be inverted, i.e. add 500 µL of 70% ethanol to the GM pellet and then tap the plastic tube against a rack or vortexing to resuspend (Movie 3, GM resuspension with ethanol).

9. Spin the sample at 4,500 g for 15 seconds.
10. Discard the supernatant by decanting (Movie 1, Decanting).
11. Repeat steps 7-10.
12. Spin the tube at 7,000 x g for 15 seconds to allow complete ethanol removal.
13. Remove all remaining 70% ethanol with a pipette.
14. Air dry at 65°C for 5 min in a heat block or equivalent to remove all ethanol from the pelleted GM with bound RNA (Figure 2).

RNA elution

In this step RNA is released from the GM matrix in nuclease-free water.

Timing: 1 min

To elute RNA from the dry GM matrix:

15. Resuspend pellet in 50 µL of nuclease-free water (Movie 4, GM resuspension with water).
16. Spin the sample at 4,500 g for 15 seconds.
17. Take off 45 µL from the supernatant and transfer to a clean tube (Movie 5, Elution).
18. Proceed to downstream detection of viral RNA. We routinely use RT-qPCR but other methods such as RT-LAMP (reverse transcription and loop-mediated isothermal amplification) or CRISPR-based methods can also be employed

Note: We employed 50 µL of nuclease-free water to resuspend the GM matrix. Higher volumes such as 70 µL may be employed but lower volumes will likely not resuspend the GM matrix and/or carry over matrix to the downstream reaction.

Pause point: Extracted RNA can be frozen at this step. Long term storage is recommended at -80 °C but short-term storage (~1 week) can be done at -20 °C.

Reverse Transcription and PCR

This step allows the detection of specific genes from SARSA-CoV-2 in the eluted RNA, utilizing an internal human gene (RNase P) as control of extraction.

Prepare the RT-PCR reaction master mix with the following format. Importantly, always include a non-template control, in which the RNA is replaced with H$_2$O. When possible, prepare the PCR reaction and plate at a different location to that where swabs are handled to reduce risk of contamination. The positive control from the RNA extraction can be employed as positive control in the RT-PCR.

| RT-qPCR reaction master mix (TaqMan Fast Virus 1-Step Master Mix) |
|---------------------------------------------------------------|
| Reagent | Amount/reaction (µl) | 10 reactions + 10 % (µl) including 3 controls* | 24 reactions + 10% (µl) including 3 controls* |
| RNA template | 5 | | |
| Master Mix | 5 | 55 | 132 |
| Pre-mixed primer probe | 1.5 | 16.5 | 39.6 |
| ddH$_2$O | 8.5 | 93.5 | 224.4 |

PCR cycling conditions (Fast mode)
**Steps**

| Steps             | Temperature | Time   | Cycles |
|-------------------|-------------|--------|--------|
| Reverse Transcription | 50 °C       | 5 min  | 1      |
| Denaturation       | 95 °C       | 20 sec | 1      |
| Denaturation       | 95 °C       | 3 sec  | 45 cycles |
| Annealing/Extension | 60 °C       | 30 sec |        |

**NOTE:** * Controls must include positive and negative extraction controls and non-template PCR control.

**NOTE:** For this step pooled and individual samples are treated equally. i.e. both are eluted in 50 µl water and 5 µl are used per technical replicate. For the purpose of establishing this protocol, we performed RT-PCR reactions in technical duplicates, for analytical purposes, it is custom to just perform one technical replicate. The limit of detection for our method, employing this master mix is Ct 36 (Page et al., 2022). Other RT-qPCR conditions may be employed (Lista et al., 2021; Reijns et al., 2020).

**Expected outcomes**

Please refer to Page et al., 2022 for a complete description and troubleshooting of the method, including RNA binding capacity, limitation to isolate human cellular RNA or lack of effect of inhibitors such as blood in the quantification of SARS-CoV-2 in swab samples. Homebrew allows for effective isolation of RNA and not DNA. In Page et al., we systematically compare homebrew to QIAamp Viral RNA Mini Kit (QIAGEN) as per CDC-recommendation. We typically are within 1-3 Ct of the values obtained with RNA isolated using the QIAamp kit. Recovery of pure cellular total RNA is ~30% from a 5 µg preparation (Page et al., 2022).

Although we recommend prior heat inactivation of swabs prior to the addition of SDS, SDS inactivates SARS-CoV-2 at concentrations as low as 0.5% (Patterson et al., 2020). We recommend prior heat inactivation to increase the speed of processing since all steps (including the addition of 4% SDS) can then be performed safely on a bench.

Quantification of SARS-CoV-2 material in RNA isolated from swabs using homebrew has been validated employing the primer-probe and qPCR reagents listed above. According to our parameters, we consider negative/positive/inconclusive/void samples (Table 1):

| Result   | Positive (Ct)                  | Negative (Ct) | Inconclusive (Ct) | Void (Ct) |
|----------|-------------------------------|---------------|-------------------|-----------|
| N1       | <36 regardless of N2 amplification | Undetermined | ≥ 36 and N2 negative |          |
| N2       | <36 regardless of N1 amplification | Undetermined | ≥ 36 and N1 negative |          |
| RNAseP   | < 35                          | < 35          | < 35              | > = 35    |

Inconclusive samples are those in which viral presence is difficult to determine, and void samples are those that have little presence of RNAseP, insufficient to determine a true negative result. In both cases, our recommendation is to ask for a new sample from the same individual, for testing as soon as possible. Void samples may be due to technical errors such as adding variable amounts of GM to each
tube, which are reduced when preparing GM-MB rather than adding components separately. In our experience these are caused by insufficient biological material or wrong storage (e.g. repeated freeze-thaw cycles). We iterate the need for including a positive and a negative extraction control, as specified above. We recommend a positive control with a Ct of around 30 to minimize cross-contamination and demonstrate effective RNA extraction. Negative controls (extraction and RT-qPCR) should not amplify viral targets or RNAseP at all. Small contaminations with RNAseP may occur given its presence in plastics but should be kept at least at 5Ct difference from the maximum allowed.

As shown in Page et al. (Page et al., 2022) we do not see a reduction in sensitivity when pooling up to 20 samples. Pooling is only recommended in low prevalence settings, since positive pools of samples must be reanalyzed to determine which sample(s) are positive amongst the pool, and it depends on the pool size (Cherif et al., 2020). In our case, positive rate should be less than 1 in 20 (5%), otherwise all pools of 20 samples will need to be re-tested given that may will all (statistically) be positive.

**Limitations of the Study**

We have validated our method for SARS-CoV-2 RNA detection from combined nose and throat swab samples, including those that contain inhibitors such as blood (Page et al., 2022). Our method allows for the sub-optimal isolation of cellular RNA from cells and further modifications to the lysis step would be required to improve its efficiency and the quality of isolated RNA. We did not measure the variability between different batches of GM preparations. RNA extraction reproducibility was demonstrated employing serial dilutions of SARS-CoV-2 samples as well as comparable results to gold-standard clinically diagnosed swabs in ~ 100 samples (Page et al., 2022). We recommend anyone starting to use homebrew to carefully validate this protocol with swabs that have already been tested with alternative isolation methods until the user feels confident in our method. Although greatly decreasing the cost per sample, we have used our method with conventional RT-qPCR analysis. Sourcing the reagents for qPCR analysis is in our experience less of a bottleneck, but sufficient provision of reagents should be carefully planned in. Availability of real time thermocyclers is a limitation on the use of homebrew for RT-qPCR, but colorimetric and isothermal methods may still benefit from it (Alcántara et al., 2021; Baba et al., 2021; Joung et al., 2020).

Homebrew employing GM cannot be automated. However, our data employing carboxylated magnetic beads (Page et al., 2022) shows comparable sensitivity, with those being amenable to automatization.

**Troubleshooting**

**Problem 1: GM not resuspending after centrifugation.**

**Potential solution:**

Pull tube over a microtube rack (Movies 1 and 2). This will create a vigorous small shaking that in our experience is most effective to resuspend the GM. Shake vigorously, vortex or flick the tube until resuspended.
Problem 2: Positive extraction control failure

GM contains ethanol in the last step. Carry over ethanol can negatively affect downstream processing i.e., transcription and/or PCR. A void result in the positive extraction control suggests that the extraction has not been efficient, and ethanol carry-over must be the first step to check. It is important to check if more than 50 µL are present in the tube after elution; typically GM absorbs 5 µL of the 50 µL and thus carry-over ethanol can be easily spotted at this stage. Figure 3 depicts a wet (left) vs. a dry (right) GM pellet.

Potential solution:

Air dry the pellet for 2 extra minutes.

Problem 3: multiple void results

We typically consider a failed extraction batch when more than 10% of all samples extracted are ‘void’, i.e. there is no amplification of the internal control (RNAseP in our case). This is likely due to carry-over ethanol or deficient mixing of the GM-MB.

Potential solution:

Repeat the extraction of those samples (with positive and negative extraction controls) ensuring adequate mixing of GM-MB, incubation with the samples and air-drying of the GM pellet.

Ensure that all reagents have been filtered through a 0.2 µm PES membrane to remove contaminating RNases. Repeat extraction with a confirmed positive swab in parallel.

Problem 4: contamination of negative extraction control

Given that PCR is an exponential reaction, it can be relatively easy to contaminate reagents with PCR products.

Potential solution:

To minimize this risk we recommend to perform RNA extractions and RT-qPCR set up in different benches/rooms when possible. Aliquoting of negative and positive controls will also minimize risks of cross-contamination. Use of filter tips is highly recommended. If there is suspected contamination of one of the reagents discard and aliquot from the stock. If there is suspected contamination of the glassmilk, perform an acid wash as in the preparation steps.

Problem 5: GM pellet is over dried

We recommend air drying the GM pellet at 65 °C for 5 minutes (Figure 2).
Potential solution:

If sample appears over dried i.e. the GM pellet does not resuspend (Movie 4, GM resuspension with water), consider warming up water at 65 °C prior to resuspending again. If this does not resuspend the GM pellet, we recommend re-extraction and careful monitoring of the drying process.

Resource availability

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rocio T Martinez-Nunez (rocio.martinez_nunez@kcl.ac.uk).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The datasets underpinning this study are available at Mendeley (doi:10.17632/b2mscbnhmg.1 from Page et al., 2022). Additional Supplemental Items are available from Mendeley Data at https://data.mendeley.com/datasets/t8wfrycwcb5/1. According to UK research councils’ Common Principles on Data Policy, all data supporting this study will be openly available at doi:10.17632/b2mscbnhmg.1 from Page et al., 2022 and https://data.mendeley.com/datasets/t8wfrycwcb5/1. According to Wellcome Trust’s Policy on data, software and materials management and sharing, all data supporting this study will be openly available at doi:10.17632/b2mscbnhmg.1 from Page et al., 2022 and https://data.mendeley.com/datasets/t8wfrycwcb5/1.

Acknowledgments
This work was funded by; a King’s Together Rapid COVID-19 Call award to RTMN and SJDN, and a Huo Family Foundation Award to MHM, RTMN and SJDN. HEM and SM were funded by a Sir Henry Dale Fellowship jointly funded by the Wellcome Trust and the Royal Society (218537/Z/19/Z) to HEM. RTMN was supported by the Wellcome Trust (213984/Z/18/Z). R.P. was supported by the by the (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. MF was supported by the MRC-KCL Doctoral Training Partnership in Biomedical Sciences (MR/N013700/1) and MRC (MR/R50225X/1). This work was supported by the Department of Health via a National Institute for Health Research comprehensive Biomedical Research Centre award to Guy’s and St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust. This research was funded in whole, or in part, by the Wellcome Trust 218537/Z/19/Z and 213984/Z/18/Z. For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.
Author contributions

RP, ES, MF, SM, KL, TJAM, CB, MJL performed experiments. SJDN, MHM and MZ provided intellectual input and samples. HEM and RTMN supervised the project, designed and performed experiments. RP, HEM and RTMN wrote the initial manuscript; all authors were involved in editing the manuscript.

Declaration of interests

The authors declare no competing interests.

References

Alcántara, R., Peñaranda, K., Mendoza-Rojas, G., Nakamoto, J.A., Martins-Luna, J., Del Valle-Mendoza, J., Adaui, V., and Milón, P. (2021). Unlocking SARS-CoV-2 detection in low- and middle-income countries. Cell Rep Methods, 100093.

Baba, M.M., Bitew, M., Fokam, J., Lelo, E.A., Ahidjo, A., Asmamaw, K., Beloumou, G.A., Bulimo, W.D., Buratti, E., Chenwi, C., et al. (2021). Diagnostic performance of a colorimetric RT-LAMP for the identification of SARS-CoV-2: A multicenter prospective clinical evaluation in sub-Saharan Africa. EClinicalMedicine 40, 101101.

Cherif, A., Grobe, N., Wang, X. and Kotanko, P. (2020). Simulation of Pool Testing to Identify Patients With Coronavirus Disease 2019 Under Conditions of Limited Test Availability. JAMA Network Open 3, e2013075.

Joung, J., Ladha, A., Saito, M., Kim, N.-G., Woolley, A.E., Segel, M., Barretto, R.P.J., Ranu, A., Macrae, R.K., Faure, G., et al. (2020). Detection of SARS-CoV-2 with SHERLOCK One-Pot Testing. New England Journal of Medicine 383, 1492-1494.

Lista, M.J., Matos, P.M., Maguire, T.J.A., Poulton, K., Ortiz-Zapater, E., Page, R., Sertkaya, H., Ortega-Prieto, A.M., O'Byrne, A.M., Bouton, C., et al. (2021). Resilient SARS-CoV-2 diagnostics workflows including viral heat inactivation. PLoS One 15, e0256813.

Page, R., Scurfield, E., Ficarelli, M., McKellar, S., Lee, K.L., Maguire, T.J.A., Bouton, C., Lista, M.J., Neil, S.J.D., Malim, M.H., Zuckerman, M., Mischo, H.E., Martinez-Nunez, R.T. A homebrew economical and sensitive glassmilk-based nucleic-acid extraction method for SARS-CoV-2 diagnostics. Cell Reports Methods, 100186.

Patterson, E.I., Prince, T., Anderson, E.R., Casas-Sanchez, A., Smith, S.L., Cansado-Utrilla, C., Solomon, T., Griffiths, M.J., Acosta-Serrano, Á., Turtle, L., et al. (2020). Methods of Inactivation of SARS-CoV-2 for Downstream Biological Assays. The Journal of infectious diseases 222, 1462-1467.

Reijns MAM, Thompson L, Acosta JC, Black HA, Sanchez-Luque FJ, Diamond A, et al. A sensitive and affordable multiplex RT-qPCR assay for SARS-CoV-2 detection. PLoS Biol. 2020;18: e3001030.

Figure titles and legends

Graphical Abstract. Summary of our protocol for COVID-19 testing employing homebrew.
Figure 1. Resuspension of GM pellet by tapping against a tube rack.
Figure 2. Example of air drying GM pellet in a heat block.
Figure 3. Wet vs dry GM pellet. Left-over ethanol is inhibitory to PCR amplification (left). Completely dried pellet is white and defined (right).

Table titles and legends

Table 1. Positive/negative/void/inconclusive thresholds.

Method video titles and legends

Movie 1. Decanting of supernatant. This movie shows that the GM pellet remains attached to the tube, related to step 6.
Movie 2. Disaggregation by tapping. This movie shows resuspension of the GM pellet by tapping the plastic tube against a rack, related to step 7.
Movie 3. GM resuspension with ethanol. This movie shows addition of ethanol to the GM pellet prior to pellet resuspension by tapping the plastic tube against a rack, related to steps 7 and 8 NOTE.
Movie 4. GM resuspension with water. This movie shows the resuspension of the dried GM pellet in 50 μl of water, related to step 15.
Movie 5. Elution. This movie shows the removal of 45 μl of supernatant containing RNA in a clean tube for downstream analysis, related to step 17.
Figure 1. Resuspension of GM pellet by tapping against a tube rack.
Figure 2. Example of air drying GM pellet in a heat block

65°C
5 min
Figure 3. Wet vs dry GM pellet
Highlights:

- Homebrew is as sensitive as commercial RNA extraction kits for SARS-CoV-2 testing;
- Homebrew only requires glassmilk, SDS, NaCl, isopropanol, ethanol and water;
- Cost- and time- effective compared with other RNA extraction techniques
- Homebrew allows for pooling of samples without loss of sensitivity.