COVID-19 RT-qPCR Testing Pipeline

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This protocol describes the complete setup of the VCDI COVID-19 testing pipeline, from self-sampling, via primary sample processing, RNA preparation and RT-qPCR to the administration of samples and test results. A first version of this pipeline has been applied in several SARS-CoV-2 screening projects, including the routine monitoring of ~800 staff members at several partner institutes. In parallel, we are constantly optimizing protocol steps (e.g. to further increase the throughput of our pipeline), so please check back our website for updates.

1 Sampling Procedure

During the early phase of SARS-CoV-2 infections, which is often asymptomatic but highly infectious, high virus titers can be detected in the **back of the throat**. As an alternative to nasopharyngeal swabs, SARS-CoV2 RNA can be detected in saliva, sputum or throat wash samples that can be easily obtained through self-sampling. For routine COVID-19 screening, we have established and validated a simple, safe and well-tolerable **gargling procedure** that can be performed at home, thereby eliminating the logistical burden and infection risk associated with centralized sampling. Compared to other self-sampling methods (saliva, sputum), the gargling procedure is highly standardized and produces samples of comparable general quality to nasopharyngeal swab samples. A shortcoming is that the gargling procedure leads to the production of **potentially infectious aerosols**, which are not a major concern in a home environment but can pose an infection risk in public closed spaces.

The general utility and sensitivity of throat wash samples for RT-qPCR-based detection of respiratory virus infections has been demonstrated previously, e.g. by Bennet et al. (*Journal of Virological Methods*, 2017), who reported a higher sensitivity of throat wash samples compared to swabs. After adapting and optimizing a throat wash procedure for SARS-CoV2 detection, we have validated our protocol side-by-side with nasopharyngeal swabs in COVID-19 patients and healthy controls (*Fritsche-Polanz, Zuber & Födinger et al., manuscript in preparation*). SARS-CoV2 RNA was detected in all throat wash samples of patients with active SARS-CoV2 infections, in some cases with higher and in other cases with lower sensitivity compared to nasopharyngeal swabs. Sensitivity differences were pronounced in cases with particularly high virus titers in either of the two sample types, but in all cases active SARS-CoV2 infections could be confidently detected using both sampling methods.

General considerations for sampling

Home sampling vs. centralized sampling

- Given the increasing demand and capacity for large-scale SARS-CoV2 screening, primary sampling and sample processing procedures are becoming the major bottleneck for scaling up testing.
- The current standard test (i.e. nasopharyngeal swabs) is not suitable for large-scale virus surveillance for two reasons: 1. It needs to be performed by medical professionals, which poses a substantial logistical and financial burden. 2. The procedure is unpleasant and hard to tolerate on a routine basis, which reduces testing compliance.
- In addition, centralized sampling (e.g. at institutions or companies) poses not only a logistical burden but also an infection risks at the sampling site.

- Validated self-sampling methods such as throat washes or saliva/sputum sampling open the opportunity to perform the sampling procedure in a decentralized way (e.g. at home). In addition to reducing logistical burdens and infection risks, self-sampling procedures can involve basic processing steps (e.g. sample transfer into vials that can be processed in an automatized way), which are tedious in the laboratory and a major bottleneck for scaling up testing.
- The main disadvantage of self-sampling procedures is that they depend on the full cooperation the tested person, since the sample quality and identity behind samples cannot be controlled. However, given the current demand for large-scale testing, the benefits of self-sampling outweigh these limitations.

Comparison of sampling methods

Sampling method	Advantages	Disadvantages
Nasopharyngeal swab	- standardized procedure - suitable for centralized sampling - comparable sample quality	- **requires medical professional** (self-sampling not possible) - **unpleasant** (not ideal for routine monitoring)
Saliva/sputum	- self-sampling possible - suitable for centralized and home sampling	- procedure hard to standardize, not easy for some people - **highly variable sample amount and quality** - sample processing can be complicated (sputolysis)
Gargling/throat wash	- standardized procedure - suitable for home-sampling - self-sampling possible - comparable sample quality	- **produces aerosols** that pose an infection risk! not recommended for centralized sampling in closed spaces - sample quality depends on the cooperation of the test person to strictly follow the protocol - sample processing requires sputolysis in some cases

Table 1 | Comparison of different sampling methods

Sample buffer for obtaining and transporting throat wash samples

- As standard buffer for gargling, transport and short-term storage of throat wash samples we recommend the use of Hanks' Balanced Salt solution (HBSS with Ca/Mg, without phenol red; for buffer composition see https://www.thermofisher.com/at/en/home/technical-resources/media-formulation.153.html) for several reasons:
  - HBSS is well-known to preserve intact cells (as a major source of viral RNA) at room temperature and +4°C for many hours. For this reason, HBSS is used as standard buffer in Viral Transport Medium (VTM), which is basically HBSS supplemented with several components (FBS, antibiotics/antimycotics) that are dispensable for RNA sampling. We have tested the stability of HBSS gargle samples at +4°C and room temperature for RT-qPCR-based SARS-CoV2 testing and did not observe any sensitivity loss over 24 hours storage.
  - HBSS is safe to use in humans, recommended in various guidelines for throat washes, and can be produced and/or aliquoted by regular pharmacies as gargle solution for use in humans (including children)
  - HBSS contains D-glucose (dextrose) and thus tastes better than NaCl or PBS, making it more suitable for extended gargling.
  - HBSS stock solution should be sterilized and aliquoting should ideally be performed under sterile conditions. Non-sterile aliquots should not be stored at room temperature for extended time periods.
  - If HBSS cannot be obtained, gargling with 0.9% NaCl might be an alternative. However, we have not evaluated sample quality and stability under these conditions, and one must assume that cells are less well preserved than in HBSS.

Protocol

Test participants receive a **test kit** (Figure 1) containing:

- 50 ml Falcon tube with 5-10 ml HBSS buffer (we so far use 10 ml, but 5 ml are likely sufficient and more suitable for children).
- code-labelled sample tube (5 ml or 1 ml, depending on the sample processing setup)
- disposable plastic pipettor
- instructions

Perform the following procedure as the **first thing in the morning before brushing teeth and before breakfast:**

1. Take the HBSS gargle buffer in your mouth and gargle intensely in the back of your throat for 1 minute (short breaks for breathing are of course allowed).
2. Spit the entire gargle solution back into the 50 ml Falcon tube. Leave it open and place it somewhere upright (e.g. into a glass our cup).
3. Wash hands with soap!
4. Let the tube stand for at least 30 minutes so that sediment settles on the bottom.
5. Open the sample tube and use the pipettor to transfer sample material from the bottom of the 50 ml Falcon tube into the sample tube. For 5 ml sample tubes transfer 3-4 ml, for 1 ml sample tubes transfer ~0.75 ml (always taken from the bottom of the 50 ml Falcon tube).
6. Close the 5 ml sample tube and put it in a sealable plastic bag. Dispose the 50 ml Falcon tube and the pipettor.
7. Wash hands with soap!
8. Note your sample code (e.g. by writing it down or taking a photo). Without the sample code you will not be able to obtain your test results! Keep a record of all your sample codes.
9. Take the sample to work, disinfect then outside and submit (designated refrigerator).

Figure 1 | Our test kit for gargle sampling at home

2 Sample preparation

General considerations

Even with self-sampling procedures in place, the processing of primary samples is a labor-intense step that can become the main bottleneck for scaling up screening operations. Until the virus is inactivated (e.g. by adding an appropriate lysis buffer), **samples must be considered potentially infectious** and all steps must be carried out in a BSL2 laboratory. Personal protection (gloves, FFP2/3 mask, protective goggles, lab coat) must be worn at all times and open samples should only be handled in a safety cabinet (laminar flow hood). Depending on available equipment, sample processing can be performed manually or automated:

- **Manual sample processing** (following the protocol below) is suitable for handling small to medium numbers of samples (up to 200 per day). Use of machine-readable barcodes for sample and plate administration increases the achievable throughput, yet human pipetting errors remain a major challenge for processing many samples. As starting material, we recommend 3-4 ml of throat-wash samples submitted in 5 ml tubes.
- **Automated sample processing** can dramatically reduce the time required for sample processing and human pipetting errors but requires special devices (e.g. de-capping and liquid handling robotics). In our preferred setup (Figure 5), users provide 0.75 ml throat wash samples in 1 ml Matrix tubes, which can be assembled in a 96-well format, registered by scanning QR-codes (on the bottom of Matrix tubes), further processed in a 96-well format using de-capper and liquid handling robots, and directly transferred to deep-well plates for automated RNA extraction. This way, >400 samples can be registered and processed per hour. As soon as final optimizations are completed, a detailed protocol will be made available.

Processing of throat-wash samples

Direct sample usage vs. cell enrichment in throat-wash samples

- Throat-wash samples contain a mix of saliva, oropharyngeal mucus and epithelial cells, and if done properly, intensive gargling over 1 min samples a lot of epithelial cells from relevant regions.
- All these components can contain SARS-CoV2 RNA, either as free viral particles or in form of pre-cursor RNAs transcribed in infected cells. The latter fraction can be easily enriched by centrifuging samples (e.g. 3500 x g for 5 min) and extracting RNA only from cell pellets.
- We have benchmarked the sensitivity of SARS-CoV2 detection in RNA isolated from enriched cells (pelleted from 3.5 ml gargle sample) vs. direct sampling (100 µl gargle sample) side-by-side and observed a small but significant sensitivity gain after cell enrichment (on average 1.8 cycles, p=0.001).
- However, given that (1) the sensitivity gain is fairly small, (2) pelleting is more laborious and hard to implement in automated processing, and (3) large cell pellets can interfere with virus inactivation, RNA extraction and RT-qPCR, we prefer not to pellet cells but only enrich for cellular components during primary sample transfer (see section 1 above) and prior to sample lysis.
- Specifically, we enrich for cells either by letting samples stand upright for 30 min or using brief centrifugation (500 x g, 30 sec), and then sample from the bottom of the sample tube (as detailed below).

Sample quality and potential problems with throat-wash samples

- Although gargling provides a quite standardized sampling method, the cell content and other relevant properties of throat wash samples can differ quite dramatically.

- One major problem are remnants of food, and we therefore strongly recommend to self-sample directly after waking up before breakfast and before brushing teeth (to ensure sufficient cell sampling).
- Another problem are substantial differences in the viscosity of throat wash samples, which can cause problems during sample processing (i.e. samples cannot be pipetted and/or mucous threads pose a cross-contamination risk). This problem can be easily solved by adding fresh DTT (5-10 mM final concentration) and incubation for 10-15 min prior to further processing (DTT quickly dissolves mucus by reducing disulfide bonds).

Stability and preservation of throat-wash samples in HBSS

- We have tested the stability SARS-CoV2 in HBSS throat-wash samples from COVID-19 patients and did not observe any decline in assay sensitivity upon storage of samples at +4°C or room temperature over 24 hours. In preliminary tests, HBSS samples are even stable upon storage at +4°C over up to 5 days.
- This high sample stability has several implications:
    - Home sampling is feasible and even extended transport times are not a major concern (we are currently testing stability over extended periods of time to assess whether sample shipment via mail would be feasible).
    - It is feasible to organize screening pipelines in a way that all samples deposited until a certain time (e.g. 11:00 a.m. at our institute) are processed and analysed on the same day, while all samples submitted later remain in the submission fridge and will be analysed on the next day.
    - To generate a backup of each sample for re-testing, which we highly recommend, it is feasible to just keep 50% of the original or partially processed sample in the fridge for 24 hours.

Protocol

The following describes our established protocol for manual processing of throat wash samples for RNA extraction using magnetic bead separation on a KingFisher robot. Samples are collected as 5 ml tubes each containing 3-4 ml throat-wash sample in a submission fridge directly in centrifuge buckets. A detailed protocol describing automated sample processing (Figure 5) will be provided shortly.

RNA lysis buffer

Caution! Contains GITC, which is highly toxic and can form a hazardous gas upon contact with acids!

80 mM Tris pH 6.4
5.7 M guanidinium isothiocyanate (GITC)
35 mM EDTA
2% Triton X-100

- Prepare as stock solution and store at room temperature.
- Prior to every use, **freshly add DTT** (stored @ -20°C) to a **final concentration of 56 mM** (e.g. for a 96-well plate add 1 ml 2M DTT to 35 ml RNA lysis buffer). DTT supplemented lysis buffer can be stored for up to 24h at RT.
- This RNA lysis buffer inactivates SARS-CoV2 and is compatible with various RNA extraction protocols.
- Lysed samples can be stored @ -20°C for several days without any loss in SARS-CoV2 detection sensitivity.

All steps must be carried out in a BSL2 laboratory. Personal protection (gloves, FFP2/3 mask, protective goggles, lab coat) should be worn at all times and all protocol steps involving open samples should be performed in a biosafety cabinet (laminar flow hood).

1. Put on personal protection equipment (gloves, FFP2/3 mask, protective goggles, lab coat) and collect samples from sample submission fridge.
2. Disinfect all sample tubes from the outside.
3. Make sure all sample lids are properly closed and centrifuge samples briefly at 500 x g for 30 secs to spin down liquid on the lid.
4. Add 12.5 µl 2M DTT to each sample (5-10 mM final concentration) to reduce viscosity, vortex briefly, and incubate for approx. 10 mins at RT.
5. Make sure all lids are properly closed and centrifuge samples briefly at 500 x g for 30 secs. This will spin down residual liquid on the lid and enrich cells on the bottom.
6. Transfer and register samples in a rack that copies the final 96-well plate layout. Registration can be done manually (by writing down sample IDs on a 96-well sheet) or by barcode scanning (using a suitable scanner and software solution).
7. Prefill a 96 deep-well plate with 350 µl RNA lysis buffer (with freshly added DTT; recipe see above) at all sample positions. Reserve some positions for process controls.
8. Gently loosen pelleted cells on the bottom of sample tubes by gently pipetting up and down 1-2 times. Transfer 100 µl from the bottom of sample tubes into the 96 deep-well plate pre-filled with 350 µl RNA lysis buffer. Try not to get in contact with the well walls. Mix sample and Lysis buffer well by pipetting up and down 10 times. The lysis buffer inactivates the SARS-CoV2 virus. However, care must be taken as not every area of the deep well plate may be sufficiently exposed to lysis buffer.
9. Add positive and negative controls to the selected positions:

   Suitable negative controls:
   - **450 µl empty lysis buffer** and/or
   - **450 µl negatively tested lysate** (stored at -20°C)

   Suitable positive controls:
   - **450 µl positively tested lysate** (stored at -20°C) and/or
   - **450 µl lysate supplemented with 5000 copies synthetic SARS-CoV2 RNA** (Twist Bioscience, SKU: 102024)

10. Before proceeding with RNA extraction, make sure each sample has been incubated with lysis buffer for at least 10 mins at room temperature to complete the lysis process.
11. Seal plate with adhesive foil, spray with 70% ethanol, and proceed to RNA extraction. Alternatively, lysates can be safely stored @-20°C for several days without any loss in SARS-CoV2 detection sensitivity.
12. Store remaining sample material in original sample tubes (preferably in a format that copies the layout of the corresponding deep-well plate) at 4°C for potential retesting of individual samples.
13. Clean up the hood and start UV-decontamination.

3 RNA preparation

General considerations

- Various RNA isolation methods can be used for sensitive SARS-CoV2 detection from swab, sputum and throat-wash samples, including standard Trizol-based extractions and various commercial kits (e.g. QIAamp Viral RNA Mini Kit). While these standard protocols are suitable for small sample numbers, they quickly become a bottleneck in larger screening operations.
- For processing higher sample numbers, we have established an automated RNA extraction method based on carboxylated magnetic bead separation (adapted from Boom et al., *Journal of Clinical Microbiology*, 1990), which we implemented on a KingFisher Flex magnetic particle processor (detailed protocol below). Of note, the protocol should be applicable to any liquid handling robot with minor additional equipment (96-well magnet plate, heat block).
- To bypass the need for extracting highly purified RNA, several protocols have been described for quick RNA extraction or direct use of cell lysates in RT-qPCR reactions. While such faster protocols can detect high virus titers (i.e. highly infectious patients), they suffer from a substantial loss in sensitivity due to interference of impurities and buffer components with the RT and qPCR reaction, as well as the limited sample volume that can be assayed. Consequently, these methods are less suitable for detecting very early and delayed stages of SARS-CoV2 infections, which can be identified most sensitively using highly purified and concentrated RNA.
- In our experience, the most sensitive rapid extraction method is a simple protocol described by Ladha et al. involving cell lysis using Quick Extract™ Solution (Lucigen, QE09050) followed by direct use of the lysate in RT-qPCR (https://www.medrxiv.org/content/10.1101/2020.05.07.20055947v1.full.pdf). Based on preliminary comparisons, use of this protocol results in a sensitivity loss of only ~3 qPCR cycles compared to standard RNA extraction. In our current view, the protocol should be considered as a simple alternative for high-throughput screening of individual samples, while pool-based screening and diagnostic assays should be based on purified RNA.

Automated RNA extraction using magnetic-bead separation on KingFisher Flex

This protocol describes the RNA isolation from 100 µl of samples (throat-wash as described above, sputum, eluate of nasal/oropharyngeal swaps) using magnetic-bead separation on a KingFisher Flex for subsequent SARS-CoV2 RT-qPCR testing. Using this protocol, RNA can be extracted from ~93 samples with ~1 hour or ~30 minutes (when omitting the DNAse digest step).

Reagents and material (store at room temperature if not indicated differently)

- DTT / 1,4-Dithiothreitol (Roche #10 708 984 001; store as 2M aliquots at -20°C)
- Magnetic beads (GE healthcare #65152105050450 or equivalent)
- DNase I (RNase-free) (NEB M0303S or equivalent, store at -20°C)
- RNAse free H_2O
- KingFisher 96-Deep well plates V-bottom (Thermo #95040450)
- KingFisher 96-tip combs (Thermo #97002534)
- Ethanol
- Filtered tips
- Sealing foils

- **Wash buffer I** (Caution! Contains toxic guanidine HCl)

	For 1 l
4.7 M guanidine HCl	449 g guanidine HCl
10 mM Tris pH 6.6	100 ml 1M Tris pH 6.6
60% ethanol	600 ml
	Fill up to 1l with H_2O

- **Wash buffer II**

	For 1 l
20mM NaCl	4 ml 5M NaCl
2 mM Tris pH 7.5	2 ml 1M Tris pH 7.5
80% EtOH	800 ml ethanol
	Fill up to 1l with H_2O

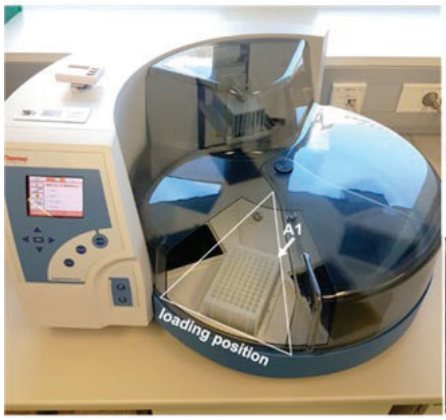
- **10x DNase I buffer**

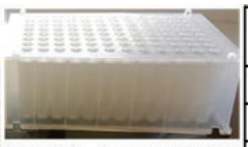
	For 1 l
25mM $MgCl_2$	25 ml 1M $MgCl_2$
100 mM Tris pH 7.6	100 ml 1M Tris pH 7.6
5 mM $CaCl_2$	5ml 1M $CaCl_2$
	Fill up to 1l with H_2O

- **Warning!** Lysis buffer contains guanidinium isothiocyanate (GITC), which is highly toxic and can form very toxic gas in contact with acids.
- **Warning!** Wash Buffer I contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. Do not add bleach or acidic solutions to the sample-preparation waste.

Basic information KingFisher Flex

- The KingFisher System (Figure 2) has magnetic rods fixed in a 96-well format and can isolate RNA from up to 96 samples at once. Instead of changing the liquids (e.g. wash buffers etc.) in the well containing the beads, the robot transfers the beads into different plates holding the needed liquid. Therefore, one 96-well plate per reagent is needed. If less than 96 samples will be prepared, add samples in the sequence of your choice, but make sure that the same wells are filled in all other reagent plates as well).





Comb in deep well plate

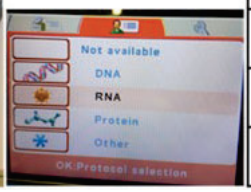


Plate name	Reagent	Volume in µl
Comb	Comb in stand	
Elution	RNase free H2O	50
Wash 2b	Wash Buffer II	700
Wash 1	Wash Buffer I	900
Dnase digest	DNase buffer/DNaseI	200
Wash 2a	Wash Buffer II	700
Sample	Lysed sample/ beads/ethanol	450/20/ 400

Figure 2 | KingFisher Flex system, required buffers with volumes per well

Protocol

- We recommend preparing stock reagents and reagent plates in a different room than the sample preparation and qPCR room to prevent cross contaminations.
- For each extraction, prepare the following KingFisher deep-well plates containing the indicated solutions in all sample positions. Upon preparation, seal all plates with adhesive foil and label them. Note that KingFisher deep-well plates have an orientation and make sure position that A1 is always at the top left (mark this position with a pen).

  1. Sample plate: Sample lysates containing 100 µl of sample in 350 µl Lysis buffer. The preparation of sample plates is described above. Please make sure to include suitable positive and negative controls (as described above).

  2. Wash 1 plate: 900 µl Wash buffer I (check for and dissolve possible precipitates at 37°C)

  3. Wash 2a plate: 700 µl Wash buffer II

  4. Wash 2b plate: 700 µl Wash buffer II

  5. Elution plate: 50 µl RNase-free H_2O

  6. DNAse digest plate: Freshly prepare a mastermix containing for each sample: 21 µl 10 x DNase I buffer, 5 µl DNAse I (RNase free) and 184 µl RNase free H_2O. Add 200 µl of this mastermix to all relevant wells. Of note, there is an option to skip the DNAse digest step in the protocol (see below).

- Put a KingFisher 96-tip comb into a KingFisher deep well plate. Optionally, this plate can be reused since it serves only as a holder for the comb. Make sure to keep it RNase-free and discard if in doubt.
- For sample processing, wear personal protection equipment (gloves, FFP2/3 mask, protective goggles, lab coat) and apply general precautions for working with RNA like using disposable RNase free reagents and plasticware and working quickly.
- If samples were frozen after lysis precipitates will occur after thawing. We did not observe detrimental effects as precipitates will be resuspended within the first seconds of the binding step/mixing. Mixing the samples (using KingFisher) before adding the ethanol/bead solution guarantees proper RNA isolation.
- Carefully add 420 µl Ethanol-Bead suspension to the wall of each well containing lysed samples and control wells.
- Unseal all plates and start the KingFisher program **VCDI-COVID19-RNA** (the program is available upon request; please contact Robert.Heinen@gmi.oeaw.ac.at)
- The device will ask you to put the plates into the loading position sequentially and will instruct you which of the plates to load. Check the correct orientation of the plate. Confirm each loading step by pressing START. After the machine starts close the sliding door.
- After approx. 25 mins (end of DNase I reaction) the program pauses and will present the DNase I digest plate at the loading position.
- Carefully add 200 µl ethanol to the wall of each well and press the “START” button to continue. Do not press the button before you added the ethanol.
- The program is finished after a total of 55 mins. Remove plates from the device (similar process as the loading procedure), seal the elution plate and store RNA at -80°C or use immediately for downstream processes.
- Aspirate buffers from deep wells and discard plates. Buffers from Sample plate and the Wash 1 plate should be put into separate designated waste bins!

Additional notes and options

- Depending on the sample quality and buffer, some beads might remain in the elution. In our experience, this does not influence subsequent applications, however, try to prevent pipetting those as much as possible.
- Even though the system is capable to isolate RNA with very high purity, there might be some gDNA impurities in throat-wash samples. However, those impurities do not appear to interfere with RT-qPCR reactions (tested for CDC-N1 and CDC-N2 primers).
- To further increase throughput and reduce consumable costs, the **DNAse I digest step can be skipped**, which dramatically reduces the processing time (from ~55 min to ~33 min per plate). We have carefully tested this simplified protocol and found that elimination of the DNAse I step neither reduces RNA yields, nor the sensitivity of RT-qPCR SARS-CoV2 detection using our standard primer/probe sets (CDC-N1 and CDC-N2). In case other primers are preferred, we recommend testing their performance with and without the DNAse I step. Importantly, commonly used human RNAseP primers (recommended by the CDC/EUA as an internal RNA extraction control) are not informative without DNAse I treatment, because both primers bind in Exon 1 and will amplify from both gDNA and cDNA templates. We recommend replacing these by exon-junction spanning primer pairs for RNAseP, which we have established (details below). A modified KingFisher program without DNAse I treatment (**VCDI-COVID19-RNA-DD)** is available upon request (contact Robert.Heinen@gmi.oeaw.ac.at).

4 RT-qPCR assay

General considerations

- Preferred are One-step RT-qPCR reactions with transcript-specific RT priming and dual-labelled hydrolysis probes (TaqMan).
- The choice of One-step kit is critical for sensitivity and at the same time a major cost factor. We recommend the NEB Luna Universal Probe One-Step RT-qPCR Kit (E3007), which is attractively priced and performs among the most sensitive One-step kits we tested.
- TaqMan RT-qPCR assays can be run in different formats (96/384-well, single-color or multiplexed). Based on our preliminary testing, we have observed that upscaling to a 384-well format can reduce the performance of some primer/probe sets (e.g. E_Sarbeco), so we currently prefer the 96-well format and are implementing multiplexed assays as an alternative measure to reduce costs and machine time (details below).
- Contaminations with RNA or DNA templates (from positive samples, controls, or RT-qPCR products) are a major threat to the operation! To minimize the risk of contamination we recommend:
    - A strict separation of low- and high-copy number areas, ideally in separate rooms with completely separate equipment (see details below).
    - We strongly advice against the use of plasmid-based positive controls as these pose a significant contamination risk. Instead, previously positively tested RNA extracts or synthetic SARS-CoV2 RNA (Twist Bioscience, SKU: 102024) diluted to a low copy number (50-500 copies/µl) are recommended.
    - Never open already amplified RT-qPCR plates in low-copy areas or clean rooms!

Primer-probe sets for SARS-CoV2 detection

To establish a highly sensitive and specific SARS-CoV2 detection assay, we have analysed and experimentally benchmarked various primer-probe sets that have been recommended by three leading institutions: (1) Charité in Germany (Corman, et al., *Euro Surveillance*, 2020), (2) Hong-Kong University/HKU (Chu et al., *Clinical Chemistry*, 2020), and (3) the CDC (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html). We found that established primer-probe sets vastly differ in their sensitivity and specificity for SARS-CoV2. Moreover, re-analysis of available SARS-CoV2 sequences (https://nextstrain.org/ncov/global) revealed that several primer and probe binding sites are affected by SNPs. Although these occur at low frequencies, SNP variants should be considered, and we recommend using at least two independent SARS-CoV2-specific primer-probe sets. Primer-probe sets detecting the N-gene appear to be highly sensitive, in line with the high abundance of N-gene transcripts in SARS-CoV2-infected cells (Kim et al., *Cell*, 2020; Blanco-Melo, *Cell*, 2020). We recommend the following primer-probe sets for sensitive SARS-CoV2 detection (in order of preference):

Name *(target)*	Primer/probe ID	Primer	Comments
CDC-N1 *(N gene)*	CDC-N1-F CDC-N1-R CDC-N1-P	GACCCCAAAATCAGCGAAAT TCTGGTTACTGCCAGTTGAATCTG **FAM**-ACCCCGCATTACGTTTGGTGGACC-**BHQ1**	Very sensitive; SARS-CoV2-specific; low false-positive rate
IMP-ORF1b *(Orf1b)*	IMP-ORF1b-F IMP-ORF1b-R IMP-ORF1b-P	TGGGGTTTTACAGGTAACCT AACACGCTTAACAAAGCACTC **TexasRed**-TAGTTGTGATGCAATCATGACTAG-**BHQ1**	SARS-CoV2-specific version of HKU-ORF1b-nsp14; very sensitive; low false-positive rate
CDC-N2 *(N gene)*	CDC-N2-F CDC-N2-R CDC-N2-P	TTACAAACATTGGCCGCAAA GCGCGACATTCCGAAGAA **FAM**-ACAATTTGCCCCCAGCGCTTCAG-**BHQ1**	Very sensitive; SARS-CoV2-specific; false positives in presence of genomic DNA
E_Sarbeco *(E gene)*	E_Sarbeco-F E_Sarbeco-R E_Sarbeco-P	ACAGGTACGTTAATAGTTAATAGCGT ATATTGCAGCAGTACGCACACA **TexasRed**-ACACTAGCCATCCTTACTGCGCTTCG-**BHQ1**	Sensitive; not SARS-CoV2 specific; reduced sensitivity in 384-well format

Table 2 | Preferred primer-probe sets for sensitive SARS-CoV2 detection. Indicated fluorophores and quenchers are a suggestion and can be modified depending on the instrument setup and vendor. -F, forward primer; -R, reverse primer; -P, TaqMan probe. Primers CDC-N1 and CDC-N2 were purchased as 2019-nCov CDC EUA Kit (Integrated DNA Technologies, Inc.) and used at the recommended concentration. All other primer/probe sets were used at final concentrations of 500 nM for primers and 250 nM for probes.

The following primer-probe sets were tested and excluded from further usage due to the indicated reasons:

Name *(target)*	Source	Reason for exclusion
CDC-N3 *(N gene)*	CDC	High false positive rate.
HKU-N *(N gene)*	HKU	Limited sensitivity. Not SARS-CoV2-specific.
HKU-ORF1b-nsp14 *(Orf1b)*	HKU	Not SARS-CoV2-specific (was modified to SARS-CoV2-specific IMP-ORF1b)
RdRP_SARSr *(RdRP)*	Charité	Low sensitivity.

Primer-probe sets for controlling sample quality and RNA extraction

Primer-probe sets detecting a human mRNA expressed in sampled epithelial cells are very useful to internally control for (1) the cell content and overall quality of the sample, (2) the RNA extraction process, (3) interference with the RT-qPCR reaction. The most widely used primer-probe set detect human RNAseP (RPP30), a housekeeping gene expressed at intermediate levels that are in the range of average abundances of SARS-CoV2 RNA in infected patients. RNAseP control primers recommended by the CDC bind in the first exon of RPP30 and do not distinguish RNA and DNA templates, limiting their value for assessing RNA quality in the presence of genomic DNA (e.g. after DNAse-I free RNA extraction or quick extraction protocols).

We therefore established two alternative primer-probe sets that span exon-junctions and large introns and allow for sensitive and specific detection of RNA templates (Table 3). In contrast to CDC-RP, both alternative RNAseP primer-probe sets show no signal when the RT step is omitted (Figure 3) and no change in signal when the DNAse-I step was omitted during RNA extraction, demonstrating that they only detect RNA templates. The relative performance apears to depend on the One-step RT-qPCR kit used - we therefore recommend testing both primer-probe sets when using kits other than Luna Universal Probe One-Step RT-qPCR Kit (NEB E3007).

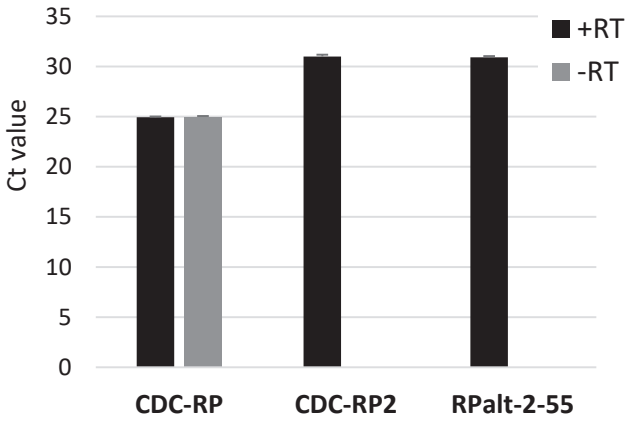


Figure 3 | Direct comparison of RP control primers on RNA sample containing gDNA. qPCR was conducted on a sample treated with and without reverse transcriptase (RT)

Name *(target)*	Primer/probe ID	Primer	Comments
CDC-RP *(RPP30)*	RP-F RP-R RP-P	AGATTTGGACCTGCGAGCG GAGCGGCTGTCTCCACAAGT **FAM**-TTCTGACCTGAAGGCTCTGCGCG–**BHQ1**	Original set recommended by the CDC. Not exon-junction spanning, cannot distinguish RNA/DNA templates.
CDC-RP2 *(RPP30)*	RP-F RP-R2 RP-P	AGATTTGGACCTGCGAGCG GCAACAACTGAATAGCCAAGGT **HEX–**TTCTGACCTGAAGGCTCTGCGCG**–BHQ1**	Forward primer and probe identical to CDC-RP, but uses a reverse primer in exon 2, spanning a 2.8 kb intron. Works well with the Luna One-Step kit (NEB E3007).
RPalt-2-55 *(RPP30)*	RPalt-2-55-F RPalt-2-55-F RPalt-P	CCATTTTACTTCAAAAGACCTC AGGGCACTGGAAATTGTAT **HEX-**AGCCCTGCTATCAAAGACTCCACA**-BHQ1**	Spans a 8.9 kb intron between exon 6 and exon 7. More sensitive than CDC-RP2 with some One-Step RT-qPCR kits.

Table 3 | Primer-probe sets for controlling sample quality and RNA extraction. Indicated fluorophores and quenchers be modified depending on the instrument setup and vendor. -F, forward primer; -R, reverse primer; -P, TaqMan probe.

Recommendations for multiplexing and pooling

Multiplexed assays using different fluorophores can increase the throughput and help saving reagents. The performance of multiplexed primer-probe sets should be thoroughly tested in comparison to single-probe assays. We have tested and recommend the following two primer-probe pairs: (1) CDC-N1/FAM and CDC-RP2/HEX, (2) CDC-N2/FAM and IMP-ORF1b/TexasRed. For routine screening, these pairs can be run in two separate plates, which serves as additional quality control and delivers data on three very sensitive, SARS-CoV2-specific primer-probe sets and the RNAseP internal control. For boosting the throughput, the second pair can be omitted.

Another option for increasing the throughput of RTqPCR-based screening is the pooling of primary samples (e.g. directly or at the lysis step). In our experience, pooling of five samples reduces the sensitivity of the assay by 2-3 cycles, pooling of ten samples by 3-5 cycles. We currently consider pools of 5 as the best option for balancing sensitivity and throughput. For analyzing pooled samples in multiplexed assays, the RNAseP internal control can be omitted in favor of multiple sensitive SARS-CoV2-specific primer-probe sets. Positive and negative process controls should of course be included as separate samples.

Protocol

The following describes our current standard protocol for routine COVID-19 screening, which is based on primers and procedures recommended by the CDC/EUA (https://www.fda.gov/media/134922/download). Conveniently, the respective primer/probe sets are provided as 2019-nCov CDC EUA Kit from Integrated DNA Technologies (IDT) at relatively low costs (https://www.idtdna.com/pages/landing/coronavirus-research-reagents/cdc-assays). However, the provided probes are uniformly labelled with FAM precluding multiplexed assays, and the RNAseP primer pair binds in the same exon and cannot distinguish DNA and RNA templates. Therefore, we have recently implemented multiplexed assays using 2 primer/probe sets per plate and alternative RNAseP primer pairs spanning large introns (see above). Depending on the primer stock concentration, another possible optimization is the use of higher template volumes (e.g. 7.5 µl or 10 µl RNA) instead of adding H_2O to the mastermix. This results in a significant sensitivity gain of 1-2 cycles, which is particularly relevant for pool-based analyses.

Reagents

- Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs E3007)
  - 2x Luna® Universal Probe One-Step Reaction Mix (store at -20°C)
  - 20x Luna® WarmStart® RT Enzyme Mix (store at -20°C)
  - Nuclease-free water (store at -20°C)
- 2019-nCov CDC EUA Kit (Integrated DNA Technologies, Inc.)
  - Premixed N1, N2 and RP assay primers and probe, each containing 2 primers (6.7 µM) and 1 probe (1.7 µM) at CDC's recommended working concentration (store at -20°C)
- Twist SARS-CoV-2 RNA Control (Twist Bioscience) or RNA control from positive patient
- Full-skirted 96 well plate
- Adhesive PCR plate seal
- Filter pipette tips
- Eppendorf tubes (2 ml)

Mastermix preparation in clean room / clean workbench

- All original kit reagents, working aliquots, and master mixes should be prepared and handled in a clean room (minimally in a separate template-free laminar-flow or PCR workbench), in which RNA or other possible PCR templates are introduced at no point.
- Prepare working aliquots to avoid contamination of kit stocks and keep enzymes, primer and probe mixes and reaction master mixes cool at all times.

- Prepare a reaction master mix for all three assays according to the following protocol:

Reaction mix (N=1) for CDC N1, N2, and RP assays		
Nuclease-free water	2.5 µL	• If number of samples and controls (n) equals 1 - 14, then N=n+1 • If number of samples and controls (n) is 15 or greater, then N=n+2 • For a 96-well plate, N=100.
2x Luna® Universal Probe One-Step Mix	10.0 µL	
Respective Primers/Probe premix	1.5 µL	
20x Luna® WarmStart® RT Enzyme Mix	1.0 µL	

- Setup the 96-well reaction plate by dispensing 15 µl of mastermix into all sample and control wells. Seal the mastermix plate and transfer to the low copy room / low-copy workbench for sample addition.

Sample addition in low-copy room / low-copy workbench

- Sample RNA and all positive and negative controls should ideally be added in a separate low-copy room (minimally in a separate template-free laminar-flow or PCR workbench).
    - Add 5 µl of synthetic or positive sample RNA to positive control well
    - Add 5 µl of nuclease-free water to negative control well
    - Add 5 µl of sample RNA to all remaining wells
    - Seal plate with adhesive PCR plate seal

High-copy room

- Move assay plate to high-copy room, where cyclers are located, and run the following PCR protocol:

Step	Number of cycles	Temperature	Time
RT reaction	1	55°C	10 min
Polymerase activation	1	95°C	1 min
Amplification	45	95°C	10 sec
		55°C	30 sec

- fluorescence data should be acquired during the 55°C incubation step.

Analysis

Once the PCR protocol is finished, analyze the data using CFX Maestro or CFX Manager or any other suitable software. A detailed guideline on RT-qPCR analysis can be found in the EUA-CDC protocol (https://www.fda.gov/media/134922/download). In brief, Ct values <40 are considered positive and interpreted in the following way:

- If both N1 and N2 are positive, the sample is considered positive (regardless of the RP result).
- If only N1 or N2 is positive, the sample is flagged as suspect (regardless of the RP result) and should be re-assayed using the same and/or additional primer-probe sets.
- If the N1 and N2 assays are negative, but the RP assay is positive, the sample if considered negative.
- If all assays are negative, the sample is considered inconclusive and re-assayed. If all assays are negative twice, the sample is considered failed.
- If no signals in positive controls are observed, the run is considered inconclusive.
- If signals in negative controls are observed, the run is considered inconclusive.

We recommend confirming all positive (N1 and N2 positive) and all suspicious (N1 or N2 positive) tests by re-running remaining material of the same sample (see 2. Sample preparation) through the entire pipeline.

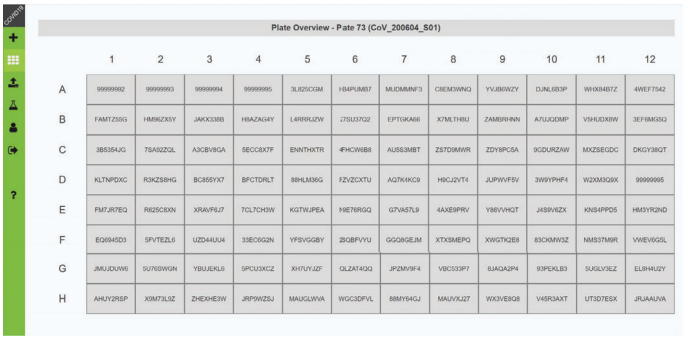
5 Sample administration and data management

To administer samples and test results, the team of Thomas Micheler at VBCF has developed a web-based COVID-19 testing database solution that supports sample registration and tracking through all steps of the experimental pipeline, storage and approval of all test results, and an anonymous user administration with anonymous result retrieval through a web interface (https://www.vbcf.ac.at/myvbcf-sim/covid19-application). Here is a brief description of key features and the general workflow:

1. To participate in COVID-19 testing, all users fill a general consent form (detailing sample and data handling, as well as procedures in case of a positive test result). Upon declaring their consent, users are provided with an anonymous User ID/Password combination that provides access to the database frontend.
2. For testing, users obtain a testing kit that contains a sample tube, which is labeled with a 8-digit sample code and a QR code. Labels and codes are produced by the COVID-19 database and can be printed from there. Users must note the 8-digit sample code (e.g. using a photo) for result retrieval.
3. Upon sample submission, all collected sample tubes are registered in the database using a QR code scanner and assigned to a 96-well sample plate in a layout that is kept throughout the assay (Figure 4).
4. Upon registration and primary processing of samples, the layout of the 96-well sample plate is exported for easy import into the RT-qPCR software (CFX Maestro).
5. Upon completion of the RT-qPCR assay, results (i.e. Cq values and optional notes on individual results) are exported from the RT-qPCR software and imported into the database.
6. Results are reviewed and interpreted by experienced personnel and then released. Communicated results include the Cq values (e.g. N1, N2, RP), an interpretation of the test result and sample quality, and optional comments (e.g. to provide further interpretations and instructions in case of positive or ambiguous results).
7. Users obtain their test results by logging into the website and entering their 8-digit sample ID (Figure 4). Upon entry, the respective sample ID is permanently affiliated with the given user, so each user can review all previous test results.

For more information, please visit https://www.vbcf.ac.at/myvbcf-sim/covid19-application.

Sample registration and plate administration



Online result retrieval

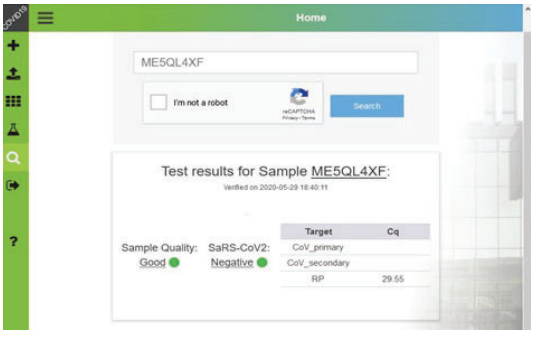


Figure 4 | COVID-19 sample and data management system (snapshots)

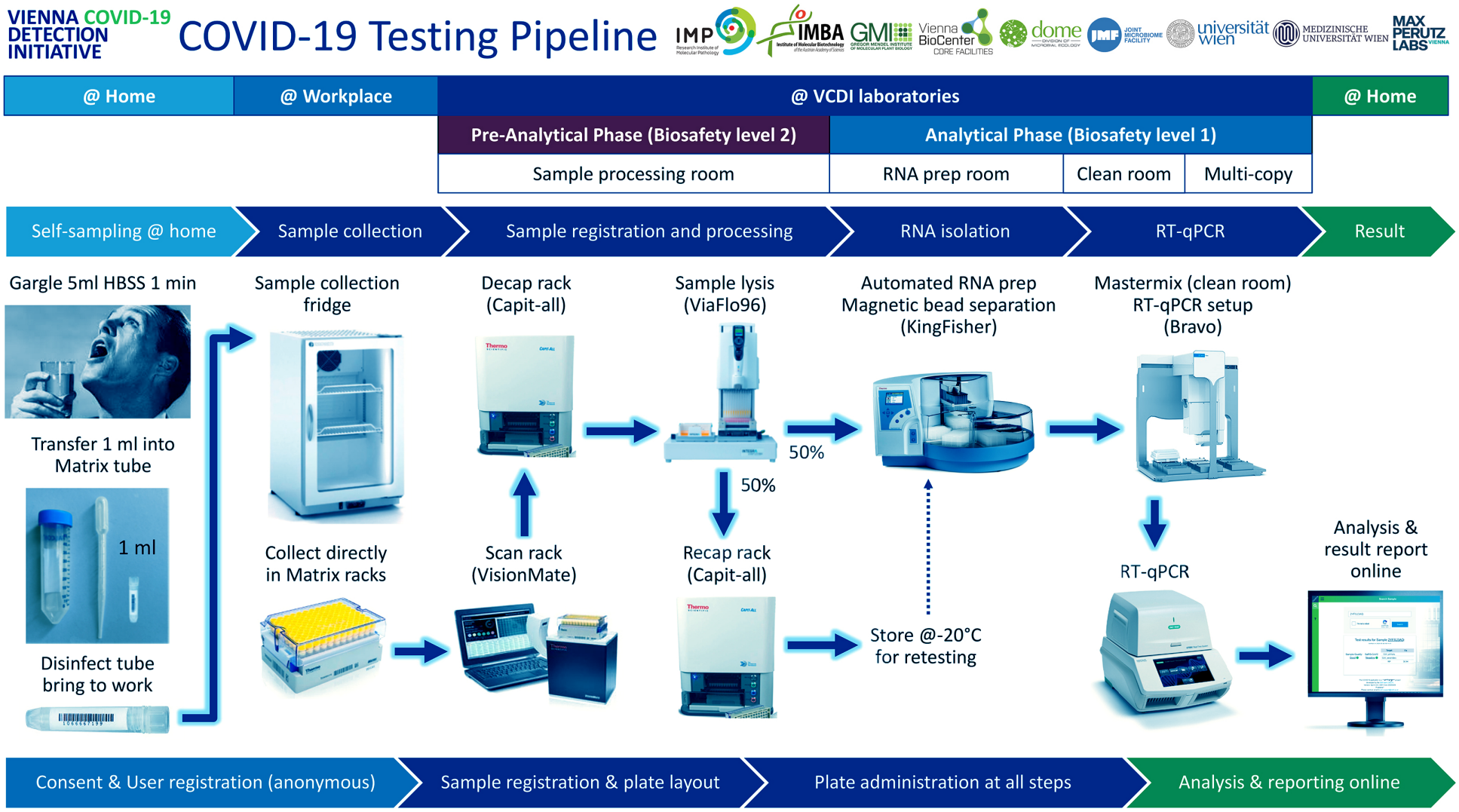


Figure 5 | Schematic of our automated SARS-CoV2 RT-qPCR-based screening pipeline