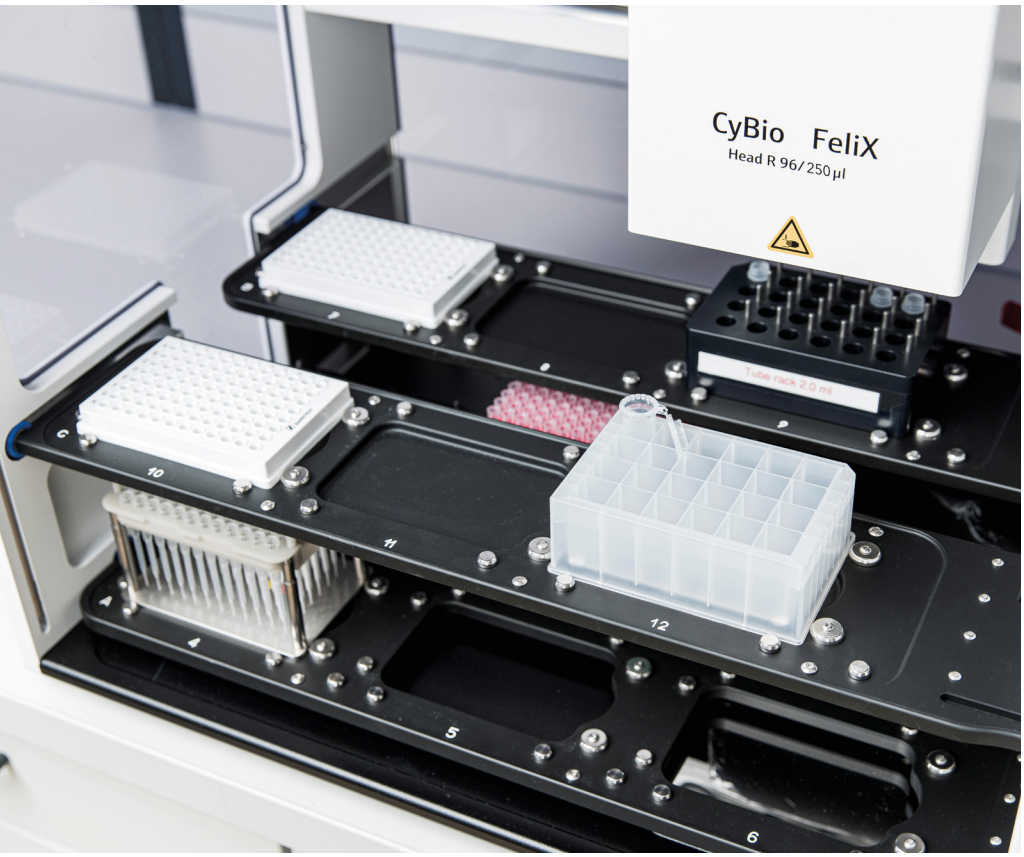
AppNote: Nucleic Acid Extraction and Amplification



Challenge

Automation of a method that combines lysis of sample material and amplification of nucleic acids as well as the detection of SARS-CoV-2 for fast and flexible generation of results.

Solution

A modular script for the automation of LumiraDx SARS-CoV-2 RNA STAR Complete kit within Application Studio allows for convenient and flexible extraction and reaction plate preparation with CyBio FeliX. Target and control signal detection with qTOWER^3 delivers results in 20 min for up to 96 samples.

Automated solution for the LumiraDx SARS-CoV-2 RNA STAR Complete Kit for rapid non-isothermal nucleic acid amplification

Introduction

Streamlining workflows is essential when time and capacities are limited. When trying to detect nucleic acids in sample materials, there are two main steps that need be performed, both of which can be very laborious and time consuming: first, extracting nucleic acids from the sample material, second, amplifying and detecting the extracted nucleic acids. The first step usually involves a multi-step process resulting in a solution containing nucleic acids. The second step is most often accomplished by conventional real-time PCR, which ideally doubles the target sequence within the sample with each temperature cycle.

The LumiraDx SARS-CoV-2 RNA STAR Complete kit by LumiraDx in combination with Analytik Jena devices condenses both steps to their essentials, effectively saving end users a lot of time and effort. The sample preparation for extraction of nucleic acids is reduced to a single step in which samples or controls are mixed with an extraction buffer. The second step is realized using a direct amplification method that utilizes a complex cocktail of enzymes and cofactors which are then mixed with the extracted samples.

Compared to conventional real-time PCR, the time to detection within a thermal cycler is drastically reduced, due to the qSTAR (qualitative selective temperature amplification reaction) approach[1]. This innovative and patented nucleic acid amplification technique (NAAT) cuts the time for amplification down to just 20 minutes. This is achieved by using a nicking enzyme in addition to the two enzymes used in conventional RT-PCR. qSTAR also enables faster cycling via a process called “temperature-gating”, which reduces the temperature delta (~57°C- ~63°C) during the reaction. This process enables the enzymes to work at their optimum, with one being preferred over the other depending on the current temperature. Like conventional PCR, qSTAR works by amplifying cDNA derived from viral RNA, allowing for the detection of SARS-CoV-2 present in the sample.

In qSTAR, the additional DNA nicking enzyme cleaves newly created DNA, generating more target DNA template while the polymerase is working in parallel. The ability to rapidly increase the amount of DNA is what makes qSTAR faster than other NAATs and is unique in the market.
Extraction and amplification can efficiently be realized by Analytik Jena instruments with maximum convenience for the customer (Figure 1). A modular script allows for automated preparation of plates with Extraction Buffer and the Reaction Mix (enzymes and cofactors) by CyBio FeliX, minimizing the time to result upon addition of samples. In addition, the speed and temperature accuracy of Analytik Jena's thermal cycler qTOWER^3 provides the optimal environment for efficient target amplification and generation of results within a minimal time span.

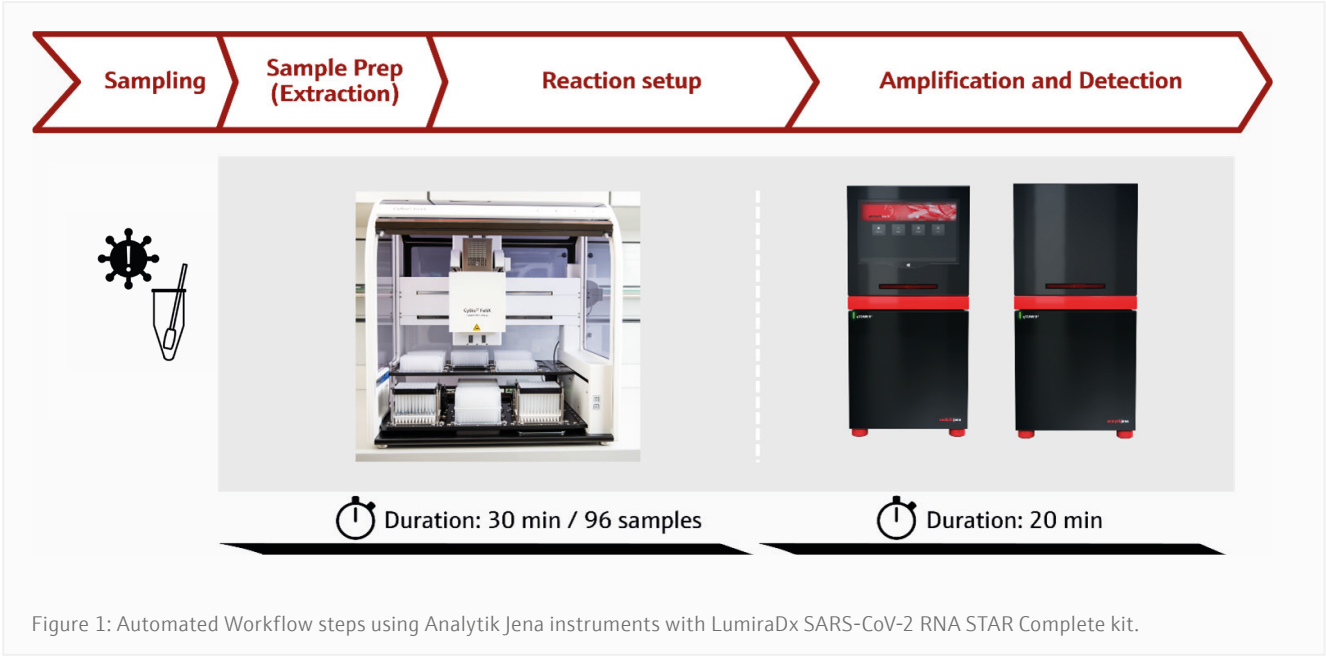


Figure 1: Automated Workflow steps using Analytik Jena instruments with LumiraDx SARS-CoV-2 RNA STAR Complete kit.

Materials and Methods

Samples, reagents and consumables

- SARS-CoV-2 RNA STAR Complete kit (L018180130096, LumiraDx)
- 96 Well PCR Plate (0.2 mL; LP), full-skirted, white (844-70038-S, Analytik Jena)
- Optical sealing foil (77 x 140 mm), 5 pcs (846-050-258-5D, Analytik Jena)
- CyBio TipBox 96/250 µL (with Filter Tips) (OL3811-25-937-F, Analytik Jena)
- CyBio RoboTipTray 96/250 µL (with Filter Tips) (OL3810-25-669, Analytik Jena)
- Waste bag; for use with Waste Box (10-406-342, Analytik Jena)
- 24-well plate (24 PP Large Volume Reservoir, pyramid bottom, Axygen)
- 1.5 mL Eppendorf Tubes® 3810X, (0030 125 150, Eppendorf)
- 5.0 mL Eppendorf Tubes® with snap cap, (0030 119 401, Eppendorf)

Instrumentation

- CyBio FeliX Basic Unit with Enclosure (OL5015-24-100, Analytik Jena)
  - CyBio Head R 96/250 µL (OL3316-14-850, Analytik Jena)
  - 1-Channel Adapter; Head R96 (OL3317-14-300, Analytik Jena)
  - Adapter 24 with passive cooling function for 24x 1.5 mL or 2.0 mL tubes (844-00136-0, Analytik Jena)
  - 40 mm Height Adapter (844-00445-0, Analytik Jena)
  - 70 mm Support (OL3317-11-110, Analytik Jena)
  - Waste Box (844-00430-0, Analytik Jena)
- qTOWER^3 qPCR device (Analytik Jena) in appropriate system configuration:
  - basic unit with color module 1 to detect FAM (230V: 844-00553-2; 115V: 844-00553-4; 100V: 844-00553-5)
  - color module 4 for ROX detection (844-00523-0)
- Pipettes, complete set (10 µL, 100 µL, 200 µL, 1000 µL pipettes, Eppendorf)

Methods

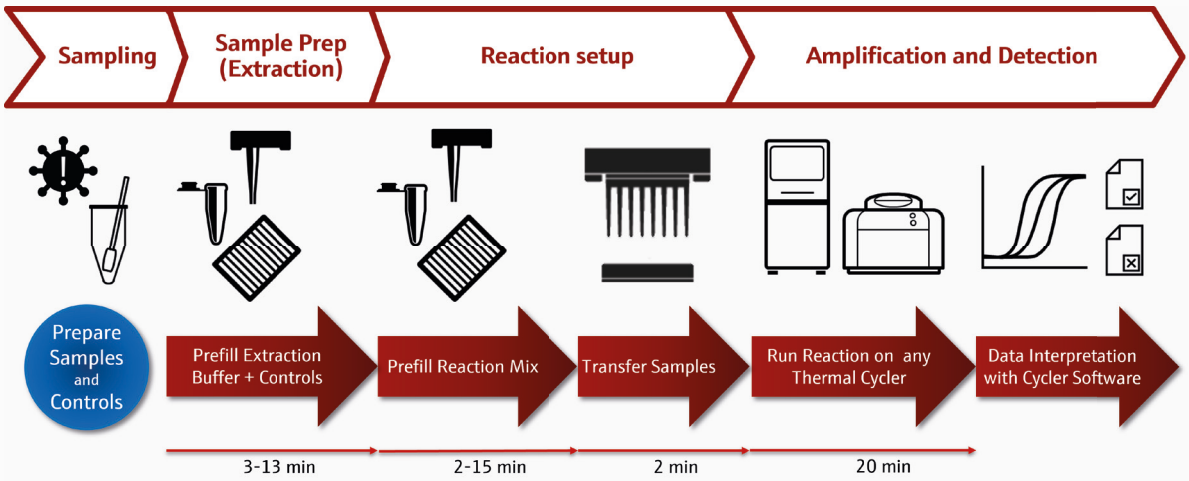


Figure 2: Workflow automation: Modular system of automated steps (red arrows) of the SARS-CoV-2 RNA STAR Complete kit with Analytik Jena devices – CyBio FeliX for sample preparation and reaction setup, qTOWER^3 family for amplification and detection.

The initial sample preparation and the pre-dilution of controls are done manually as described in the IFU of SARS-CoV-2 RNA STAR Complete kit (LumiraDx).[2] Subsequent process steps are automated and are executed in a modular workflow (Figure 3). The three automated protocols, "Prefill Extraction Buffer + Controls" (1), "Prefill Reaction Mix" (2), and "Transfer of Samples" (3), make up 5 distinct modules (A-E, Figure 3) which can be accessed via the software (Application Studio). Manual interventions are necessary at the transitions between protocols 1 and 2 as well as between protocols 2 and 3. Both transitions also represent safe stopping points, meaning that the workflow may be discontinued here and plates may be stored refrigerated for later use. Given that evaporation is prevented by covering the plates appropriately, the Extraction Buffer can be stored refrigerated (2-8°C) up to the date of expiry, and the prepared Reaction Mix is stable for up to 4 hours at 2-8°C.

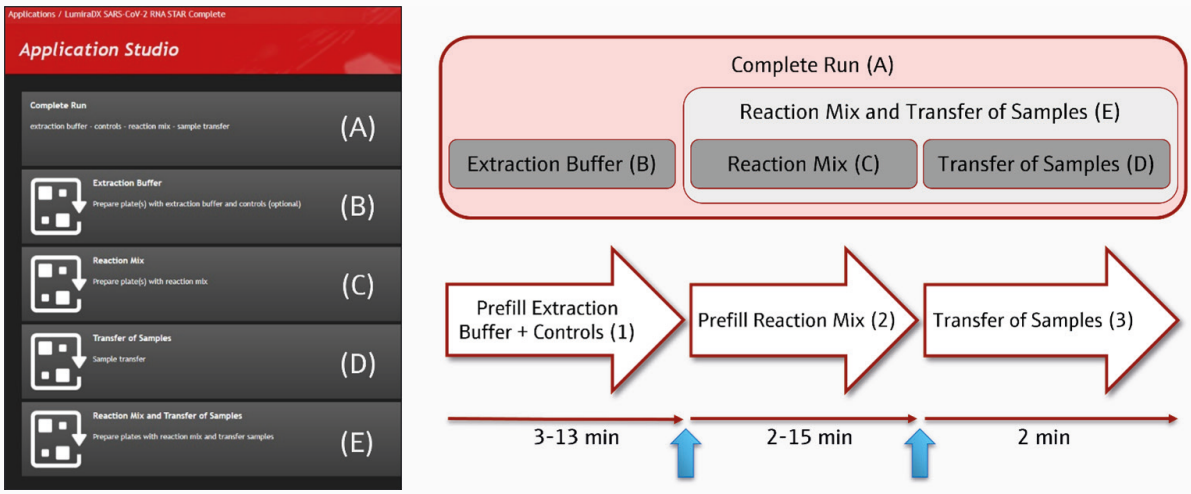


Figure 3: Overview of the software modules to automate steps of the SARS-CoV-2 RNA STAR Complete kit with the CyBio FeliX
Left: Module selection in Application Studio software. Right: Nesting of modules and their respective run times. A – Complete Run includes protocols 1 – 3: preparation of extraction plate(s), preparation of reaction plate(s) and sample transfer from extraction to reaction plate(s); B – Extraction Buffer: distribution of Extraction Buffer and optionally controls to the extraction plate(s) (protocol 1); C – Reaction Mix: distribution of Reaction Mix to the reaction plate(s) (protocol 2); D – Transfer of Samples from extraction plate(s) to reaction plate(s) (protocol 3); E – Reaction Mix and Transfer of Samples includes protocols 2 and 3: distribution of Reaction Mix to reaction plate(s) and subsequent transfer of samples from extraction plate(s) to reaction plate(s). Blue arrows indicate safe stopping points at which prepared plates can be sealed and stored at 2-8°C for later use (Extraction Buffer: storage until date of expiry; Reaction Mix: 4 hours).
Modules A and E can process up to 2 plates in parallel, modules B-D process up to 3 plates in parallel.

Module A: Complete Run

The complete run includes all three automated protocols, ranging from Extraction Buffer prefilling to the transfer prepared samples into the amplification Reaction Mix. The final product is a PCR plate ready for sealing and execution of the amplification reaction in a thermal cycler. The initial deck layout for a complete run is shown in Figure 4. The complete process has the capacity to prepare up to two 96-well plates per run and allows for the preparation of partially filled plates (8-96 samples). If sample logistics and/ or testing strategy requires more flexibility of sample handling, the modularity of the script also allows the three main automation protocols to be carried out as individual modules (see Figure 3). For details on the individual automation protocols, please refer to the protocol description given in the following sections.

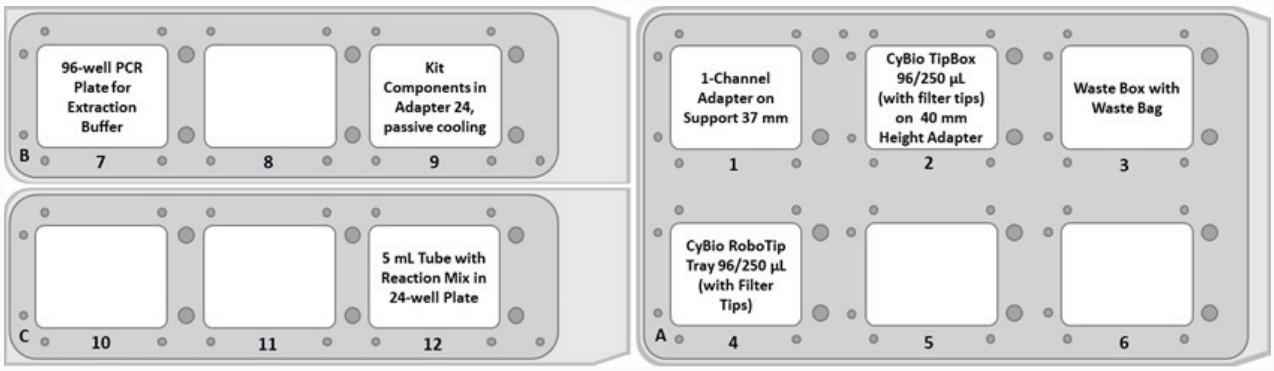


Figure 4: Exemplary graphical deck layout at the beginning of a complete run (module A) in which one plate is to be processed. The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right.

Protocol 1: Preparation of Extraction Plates

This automation protocol executes the prefilling of extraction plates. The software offers the choice between manual or automated addition of controls to the extraction plate. The software also guides the user through the setup of the deck layout (Figure 5) as well as the volumes of reagents needed for the individually chosen sample number (between 8 and 96 samples per plate). Integrated into the complete run (module A), protocol 1, i.e., the prefilling of up to two 96-well extraction plates, is immediately followed by the manual addition of 26 µL of sample and controls, if applicable, to the individual wells of the extraction plate(s). Subsequently, the workflow proceeds to protocols 2 and 3 as described in the following sections.

Within module B, protocol 1 can be used to prefill up to three 96-well plates with Extraction Buffer, which may be stored under refrigerated conditions for later use. Before proceeding to subsequent steps, samples and optionally controls are added manually to each well of the prefilled extraction plate(s). The extraction plate can then be further processed using protocol 3 as part of modules D or E.

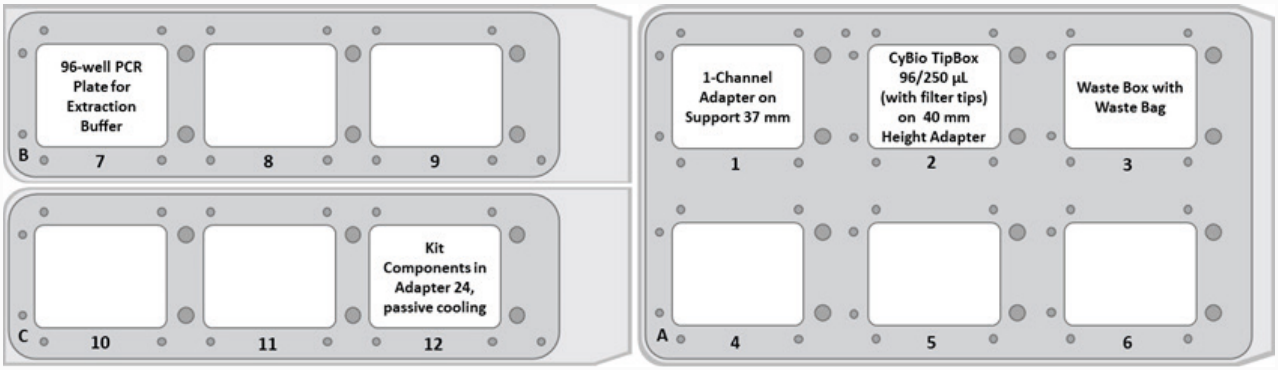


Figure 5: Exemplary graphical deck layout for the stand-alone preparation/ prefilling of one extraction plate (module B). The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right.

Protocol 2: Preparation of Reaction Plate

The Reaction Mix consists of 3 components (Salt Mix, IC/P Mix, Master Mix) which have to be combined manually according to the IFU of SARS-CoV-2 RNA STAR Complete kit (LumiraDx)[2]. To assist the user, software prompts also provide a calculation of the respective volumes needed according to the chosen number of samples.

The automation protocol 2 is part of three of the software modules:

1. as the second protocol of the complete run (module A)
2. as a stand-alone module to prepare reaction plates for cooled storage and later use (module C, Figure 6)
3. as the first protocol of the workflow completion using prefilled extraction buffer plate(s) (module E)

Due to the efficiency of its distribution to the reaction plate(s), the Reaction Mix does not have to be chilled while on the CyBio FeliX deck. However, single components of the Reaction Mix should be stored according to the kit instructions immediately prior to and after use. The capacity of the reaction plate preparation process is two 96-well plates as part of the complete run (module A) and the workflow completion process (module E), respectively. In the context of the stand-alone preparation (module C), three 96-well plates can be processed.

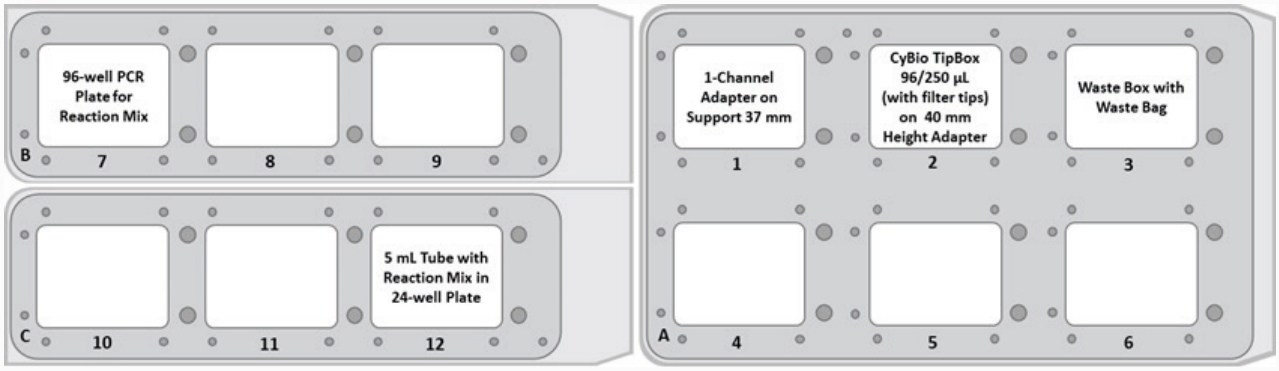


Figure 6: Exemplary graphical deck layout for the stand-alone preparation of one reaction mix plate (module C). The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right.

Protocol 3: Transfer of Samples

The mix of samples and Extraction Buffer is transferred from the extraction plate(s) to the prepared reaction plate(s) in order to complete the automated process. The resulting plate(s) is/are ready for sealing and amplification in the thermal cycler.

The software implements this last automation protocol in the context of three of the modules:

1. as the third protocol of the complete run (module A)
2. as a stand-alone module to combine the Reaction Mix and the prepared samples (module D, Figure 7)
3. as the second protocol of the workflow completion using prefilled extraction buffer plates (module E)

The capacity of the reaction plate preparation process is two 96-well plates as part of the complete run (module A) and the workflow completion process (module E), respectively. In the context of the stand-alone preparation (module D), three 96-well plates can be processed.

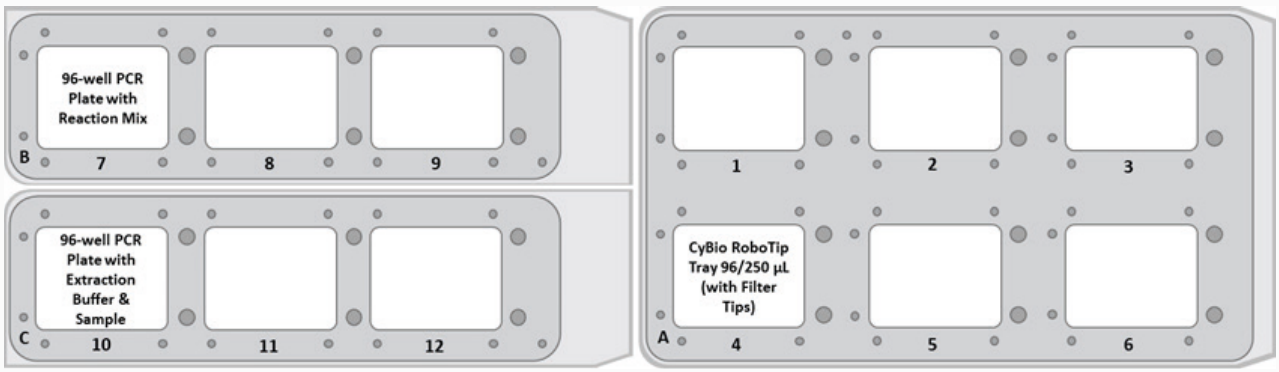


Figure 7: Exemplary deck layout for the transfer of samples from one extraction plate to one reaction mix plate (module D). The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right.

Target amplification protocol

The prepared and sealed reaction plates are transferred to the qTOWER³ real-time thermal cycler. The temperature-time protocol is programmed according to the instructions of the of SARS-CoV-2 RNA STAR Complete kit (Figure 8). Signals were detected using color module 1 for FAM, the fluorophore labelling the target sequence of SARS-CoV-2, and color module 4 for ROX, the fluorophore labelling the internal control sequence (see Figure 9).

Lid temp. °C: 100 Preheat lid Device: qTOWER³G

	4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
25x[	1		30,0	00:10	--	---	--,-	---	2,0
	2		51,0	03:00	--	---	--,-	---	2,0
	3		61,5	00:01	--	---	--,-	---	2,0
	4	◆	54,0	00:10	3	24	--,-	---	2,0

Figure 8: Temperature profile. Amplification using SARS-CoV-2 RNA STAR Complete kit (LumiraDx) on qTOWER³ (Analytik Jena). Data acquisition occurs in step 4.

Pos.	Channel	Dye	Gain	Measurement	Pass. Ref.
1	Blue	FAM	5	◆	
2	Green	JOE	5		
3	Yellow	TAMRA	5		
4	Orange	ROX	5	◆	
5	Red	Cy5	5		
6	NIR1	Cy5.5	5		

Figure 9: Scan settings. For detection of the FAM-labeled target sequence of SARS-CoV-2 and ROX-labeled internal control with the qTOWER³ (Analytik Jena) using the SARS-CoV-2 RNA STAR Complete kit (LumiraDx).

Experimental Test Setup

For the test setup, the positive control provided with the SARS-CoV-2 RNA STAR Complete kit was diluted 1:4. The data presented in this Application Note was generated using this diluted positive control. It was treated like a patient sample for the purpose of demonstrating the homogeneity of the run as a quality indicator for the automated processing. Positive sample material was spotted alternating with negative control to verify absence of cross contamination.

For columns 1-9 (=72 sample positions), the setup was done by CyBio FeliX using the complete protocol (A). While the automated preparation of the reaction plate was running, samples were added manually to the extraction plate. As an on-plate head-to-head comparison with the manual use of the kit, the six samples present in column 12 were prepared entirely manually (Figure 10).

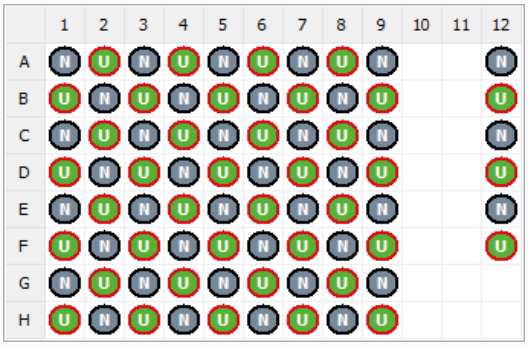


Figure 10: Sample distribution for the test setup. Diluted positive control medium (1:4 dilution from stock) of the SARS-CoV-2 RNA STAR Complete kit (LumiraDx) was distributed in a checkerboard pattern across the sample plate and defined as unknown sample (U). These were interspersed with the negative control medium (N). Columns 1-9: automated distribution of Extraction Buffer and Reaction Mix as well as transfer of samples from the extraction plate to the reaction plate (protocol A). Column 12: manual setup according to the kit IFU.

Results and discussion

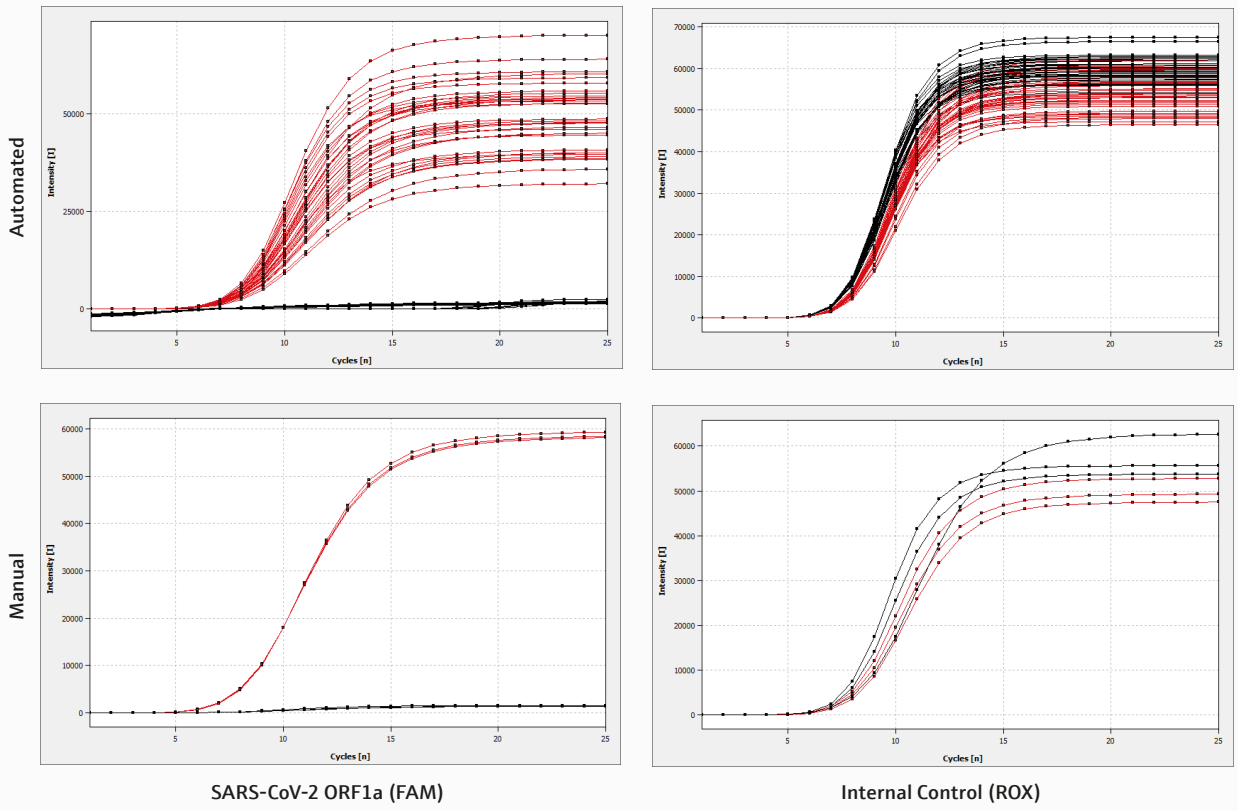


Figure 11: Amplification results: The top panels show the results of the automated test setup using the CyBio FeliX (columns 1-9). The bottom panels show the results from column 12, which were generated with the manually prepared setup (see Figure 10). Amplification curves of the ORF1a SARS-CoV-2 target sequence (left) as well as the internal control (right) are shown for the positive control medium (PCM, red) and the negative control medium (NCM, black).

Table 1: Average Ct values of amplification curves shown in Figure 11

Reaction Setup	Sample Type	ORF1a of SARS-CoV-2		Internal Control	
		Mean Ct	SD	Mean Ct	SD
Automated	PCM	7,16	0,20	7,13	0,05
	NCM	No Ct	-	6,80	0,05
Manual	PCM	6,97	0,05	7,24	0,16
	NCM	No Ct	-	7,06	0,14

The automated preparation of the plate with Extraction Buffer and Reaction Mix yielded results that were very homogeneous with respect to the Ct values for the target (ORF1a of SARS-CoV-2) and the control sequence (Figure 11 top panel, Table 1). These results were within the same range as those of the manually prepared wells (Figure 11 bottom panel, Table 1), showing only slightly higher Ct values. Both were measured within the same run on qTOWER^3. The target sequence (Figure 11, left panel) was only detected in wells with positive control medium (PCM, red curves in Figure 11) whereas the control sequence (Figure 11, right panel) was detected in all wells. Due to the qSTAR[1] approach, Ct values generated with the SARS-CoV-2 RNA STAR Complete kit are markedly lower than those obtained with conventional real-time PCR assays.

Conclusion

Fast and high-throughput solutions for infection diagnostics are in high demand during pandemic events to identify infection clusters and to prevent spreading events. Fast turnaround, process efficacy and application security are key characteristics needed for such solutions. This application note demonstrates how the interplay of kit chemistry, hardware and software can address customers' needs for fast, reliable, and flexible sample processing:

(I) The LumiraDx SARS-CoV-2 RNA STAR Complete Kit effectively eliminates a time-consuming separate extraction procedure from swab samples and allows very fast amplification of target sequences.

(II) Process automation on the Analytik Jena CyBio FeliX prepares plates with Extraction Buffer or Reaction Mix, either for storage or immediate use. The modular setup of the Application Studio software allows each automated step of the procedure to be run separately, processing up to 3 plates in parallel (288 samples plus positive/negative controls). This enables customers to maximize their throughput while also efficiently using times of low sample influx. The workflow is very flexible with respect to the sample number to be processed, any number between 8 and 96 samples per plate is possible.

With its minimal tip consumption, the automated workflow is preserving resources: It uses only 1 tip each for the distribution of both, Extraction Buffer and Reaction Mix, for the preparation of up to 3 plates. For the sample transfer, one tip per sample is required.
The data presented here show that the automated preparation of plates not only increases process efficacy and adds flexibility and convenience to the user but leads to highly homogeneous results of the same quality as manual kit handling. Also, the amplification using qTOWER^3 was shown to be efficient, with highly reproducible and homogenous results across the whole reaction plate.

References

[1] Lumira Dx Fast lab solutions: https://www.lumiradx.com/de-de/was-wir-tun/diagnostik/fast-lab-solutions/

[2] IFU LumiraDx SARS-CoV-2 RNA STAR Complete: https://www.lumiradx.com/assets/pdfs/fast-lab-solutions/sars-cov-2-rna-star-complete-instructions-for-use-ce_uk.pdf?v=3

Analytik Jena thanks LumiraDx for material and information.

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