Application Note

Small-scale automatic robot handling line for molecular pathogen diagnostics

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Abstract

Here we present an integrated small scale fully automatic diagnostic robot handling line for the purification, detection, and quantification of human pathogens. First, sample barcodes were scanned, and nucleoid acids were purified with the Roche MagNA Pure 24 system. For the PCR setup a modified version of BRAND's Liquid Handling Station (LHS) with new Middleware in combination with an new LHS control software was established. This specific software package can automatically setup PCRs with up to eleven different Mastermixes on one plate. Furthermore, it adds all control reactions to the PCR setup and creates a PCR plate layout, which can be loaded into the LightCycler 480 software. The automatic sample barcode pass through, and the PCR setup helps to avoid sample mix-up while pipetting with the LHS ensures a reproducible and pipetting error-free sample amplification. Besides, the automatic robot handling line frees precious technicians' labor time, reducing laboratory sample costs.

Detection of pathogens and determination genome amounts by qPCR or RTqPCR are standard tools in molecular diagnostics. Recently, complete automatic diagnostic systems, which purify and detect pathogens in high throughput scenarios, such as blood bank tests, were brought to market. However, small systems for less than 25 samples were not available and forced diagnostic laboratories to setup PCR reactions by hand. This project aims to establish a small scale fully automatic robot handling line for diagnostic laboratories by linking an RNA/DNA purification system to a pipetting robot for PCR preparation and finally to a qPCR-cycler (Figure 1).

The Roche MagNa Pure 24 system was selected as a fully automatic nucleotide acid purification system, which is capable of tracking sample ID and barcodes. Furthermore, it can be integrated into the lab information system and delivers result files back to the system. The Liquid Handling Station (LHS) from BRAND was chosen as a pipetting device for the PCR setup. Due to its small size, it helps saving lab space, and since it contains just a few moving parts, it provides low maintenance costs. Both, the MagNa Pure 24 and the LHS are walkaway devices, which on the one hand free technicians' labor time and on the other hand, ensure high reproducibility of sample purification and of the PCR setup. Finally, the Roche LightCycler 480 was selected as diagnostic PCR Cycler with six different channels to perform complex diagnostic multiplex PCRs. Roche LightMix® Modular assays were chosen as PCR reagents, due to their extensive collection for the diagnostics of all significant pathogens.

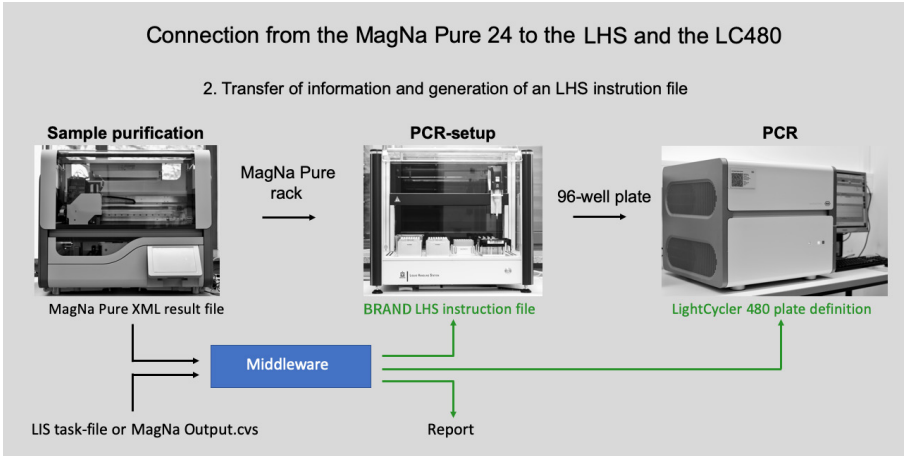

Figure 1: Automatic diagnostic robot handling line for molecular pathogen diagnostics. The RNA/DNA eluates are transferred to the LHS by placing the MagNa Pure elution-rack directly in the LHS. A newly developed middleware software connects the MagNa Pure 24 to both the LHS and the LightCycler 480. (Pictures: J. Bodem)

To standardize the LHS setup and to avoid incorrect operations, fixed positions for the pipette tip racks, for the sample racks and the PCR reaction mixes were defined (Figure 2). Furthermore, the areas of the PCR reaction mixes, positive and negative controls were defined and color-coded within the "PCR reaction-mix"-rack (Figure 2). The color-coding allowed the selection of similar colored tubes available from BRAND and included in the Roche edition of the LHS for PCR mix setup. The match of the tube and adapter colors will further reduce the risk of tube misplacement. The volume of the positive reaction was set to 23 µl, whereas the negative control contains 100 µl of PCR water. This allows the storage of pre-mixed positive controls and negative controls. With this setup, the 24 samples of a single MagNa Pure run can be analyzed in up to eleven distinct multiplex PCR reactions on one plate offering unique flexibility for the PCR setup.

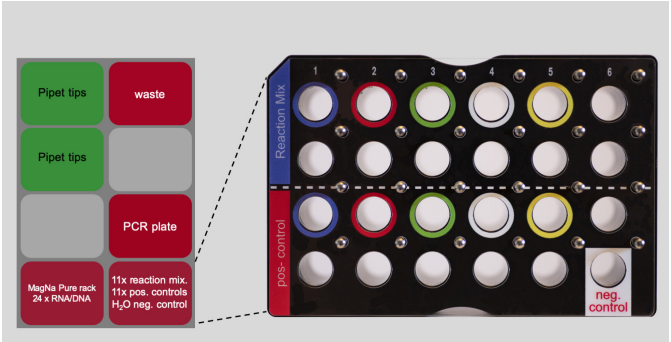

Figure 2: The LHS can setup up to eleven different multiplex-PCRs on one PCR plate. The positions of the racks within the LHS are defined to avoid mistakes (left panel). The PCR reaction mixtures and corresponding controls are placed into the block at defined positions. Both the volumes of the positive (23 µl) and negative (100 µl) controls are pre-defined. Thus, these controls can be aliquoted and stored. (Pictures: J. Bodem)

First analyses showed that two layers of connections from purification to amplification had to be established. First, the risk of sample confusion during sample transfer from the MagNA Pure to the LHS system and from the LHS to the LightCycler had to be eliminated, and secondly, automatic LHS programming and the LC480 plate layout using the MagNa Pure output files had to be created.
To eliminate sample mix-up inadvertently during the transfer from the MagNa Pure to the LHS a new LHS adapter, which allows placing the MagNa Pure elution rack directly in LHS, was constructed by BRAND (Figure 3). With this adapter, the MagNa Pure sample rack containing three 8-well strips can be used for the setup of the PCR reactions. These reactions are directly pipetted into a Roche 96-well PCR plate.

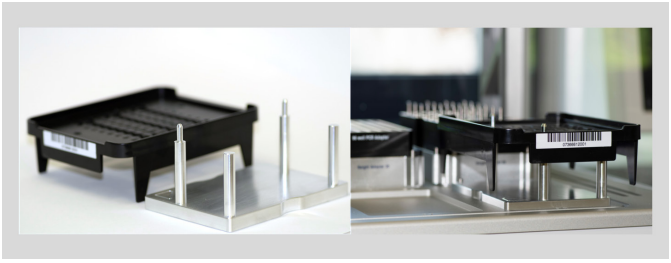

Figure 3: The MagNa Pure elution-rack (black) is directly placed into the LHS via a special adapter plate (metal). To eliminate sample exchange inadvertently, the purified DNA/RNAs are transferred by placing the sample rack into the LHS at position 8. (Pictures: J. Bodem)

The second line of connection is mediated by the middleware software package. The pilot program was created by an external software engineer (Sebastian Arnd, software engineer, Berlin). The goal of the software development was to generate an instruction file for the LHS based on the MagNa Pure output file, a test definition-file, and a task file, in which the test request for the individual samples are defined. Both the test and the task-file were kept as simple as possible. The test-definition-file is not accessible by the user but can be adapted by system administrators. The software creates two output files. The first is used by the LHS control software and defines the pipetting steps (Figure 1). Furthermore, positive and negative controls are automatically added by the software to all PCRs used in the PCR-setup. The second output file is transferred to the LightCycler480 control computer and defines the plate layout for the PCR analyses. Also, the software calculates the amounts of PCR mixes and provides this information to the user. In summary, the new middleware software automatically creates a robot control file, adds all necessary controls, and generates a LightCycler480 plate layout (Figure 1).

To optimize the LHS to the LightMix® Modular Respiratory Syncytial kit or Influenza kit, we used viral RNAs from cell culture supernatants from either RSV or Influenza virus-infected cells and modulated the uptake velocity. All reactions were performed 6times from the same RNA on the Roche LightCycler480 qPCR machines. Quality of the RTqPCRs was ensured by following the MIQE guidelines [Bustin, S. A. et al. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55, 611–622. http://doi.org/10.1373/clinchem.2008.112797)].

In brief: First, the recursion coefficient of the relative-standard curve r^2 had to be >0.95. Second, sixlett assays with a standard deviation of >0.5 were excluded from the analyses. The settings of the LHS parameters for pipetting were optimized for Roche LightMix® Assays. At the optimal uptake velocity of 0.8 mm/s, the standard deviation of the Cq value of the six-lett was determined below 0.2. The blowout velocity was optimized to 0.8 mm/s in similar assays. Also, the blowout during prewetting of the pipet tips was set to "blowout above liquid".

In the following trial period, more than 5.000 PCR reactions were pipetted in triplet assays to analyze to determine the standard deviation of the Cq values. This standard deviation was always 0.2 > standard deviation > 0.05 showing the precision of the PCR setup. Our quality control assays also included PCRs in a checkerboard pattern, where positive and negative alternate on the PCR plate to analyze DNA/RNA contaminations during PCR setup. Again, RSV with a Cq of less than 18 was used as positive samples. Also, we either used the Influenza virus alone or in multiplex PCRs with RSV, but we never observed any contamination by the LHS in all assays analyzed.

After these preliminary contamination tests, routine RTqPCR were performed using defined cell culture-derived viruses. All infections were performed in triplicates, and viral genomes were purified from centrifuged (2000 rpm, 5 min) cell culture supernatants with the MagNa Pure 24 systems. L3/S3 viruses were inactivated prior purification with the MagNA Pure LC Total Nucleic Acid Isolation Kit - Lysis/Binding Buffer as described by Roche. RNAs were eluted in 100µl. RTqPCRs were performed with the Roche RNA Process Control Kit and the respective LightMix® kits in triplicate PCR assays to ensure quality control.

In this study Chikungunya, Dengue, Enteroviruses (Polio vaccine strain, Enterovirus-71), Influenza, Rubella, Zika viruses were successfully quantified, and the assays were validated indicating that the system from RNA extraction with the MagNa Pure 24 to genome quantification with the LHS as PCR setup device leads to highly reproducible results. Since all these reactions were performed in triplicates, we were able to analyze the standard deviation of Cq values, which were between 0.05 to 0.2. These values are below the standard deviation usually achieved by manual pipetting.

In conclusion, a modified version of the LHS closes the gap between nucleoid acid purification and amplification with the LightCycler 480. Two different version of the LHS (Table 1) could be considered with all required material including a modified block for PCR mixtures, and in case of a MagNa Pure version the sample rack adapter, and the middleware software package should be included.

	LHS MagNa Pure	LHS LightMix®
Adapter for MagNa elution rack	✔	x
Coloured reaction mix rack	✔	✔
Middleware software	✔	x
Predefined templates for contamination control	✔	✔

Table 1: Components of possible LHS versions for the MagNa Pure or LightMixes®

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