DB-1206

Automated RNA Isolation Kit for Agilent Bravo

User manual

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DIANA Biotechnologies s.r.o., Nad Safinou II 366, 25250 Vestec, Czech Republic
Email: support@dianabiotech.com, Web: www.dianabiotech.com

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1 Preface

1.1 Purpose and kit usage

Automated RNA Isolation Kit for Agilent Bravo is designed for isolation of total RNA from biological samples using an Agilent Bravo automated liquid handling station. This kit is validated for isolation of SARS-CoV-2 (COVID-19) RNA from clinical samples with subsequent detection of viral RNA strand by RT-PCR method. A *COVID-19 Multiplex RT-PCR Kit* (cat.no DB-1211) is recommended RT-PCR kit to be used for this purpose.

This kit has been validated for isolation of SARS-CoV-2 viral RNA from phosphate buffered saline or from Copan Universal Transport Media, both clean or with addition of diluted human sputum (10%), saliva (10%) or blood (1%). Moreover, it has been validated for isolation of RNA from intact cultivated SARS-Cov-2 virus in DMEM medium with 2% fetal bovine serum. It has been also validated for isolation of RNA from SARS-Cov-2 virus in clinical samples: (i) human nasopharyngeal swabs collected to viral transport medium and (ii) human saliva. However, it is the sole responsibility of the end user to validate the isolation efficiency for his/her particular purpose using positive and negative controls.

The kit contains reagents and other consumables in a format which is intended to be used by running the "RNA Isolation protocol" on Agilent Bravo (described in section 4). In general, the kit can be used to isolate RNA from different types of biological material. Isolated RNA can be then used for different types of downstream analysis, most common being RT-PCR analysis. For this purpose, RT-PCR Master Mix Reservoir is included in the kit in-order-to be used together with the "Assay plate preparation protocol" on Agilent Bravo to prepare the PCR plates (Assay plate; described in section 5). Both protocols, for RNA Isolation and Assay plate preparation, are part of *Agilent Bravo installation package for Automated RNA Isolation kit* (cat.no. DB-1214).

This kit is designed for research use only and not for *in vitro* diagnostics (IVD). If used within IVD diagnostic workflow together with CE IVD marked RT-PCR diagnostic kits, it is the sole responsibility of the user to validate the RNA isolation process.

1.2 Usage of controls in the kit

For a proper usage in diagnostic practice it is mandatory to use proper controls described in section 3.9.

1.3 General remarks

Good laboratory practice should be followed to avoid sample cross-contamination, including the use of disposable filtered pipetting tips. Handling of samples and positive controls should be spatially separated from handling of the kit stock solutions to avoid their contamination. For the same reason, do not pipette solutions directly from storage bottles, pour the required volume into a suitable container beforehand, and NEVER return solutions back to storage bottles.

1.4 Safety notice

This kit is designed for professional use only. Follow the general principles of chemical safety. Wear protective equipment (gloves, goggles) and avoid contact with chemicals. Refrain from eating or drinking in laboratory areas. Dispose the waste in designated containers and do so in accordance with the applicable legislation.

Lysis Buffer Concentrate and Wash 1 Buffer Concentrate contain Guanidium Isothiocyanate, which is toxic. Acids (including concentrated solution of bleach) must not be added to these solutions or their waste bottles! Otherwise it could result in a formation of a toxic gas.

Bead solution, Wash buffer 2 and Elution buffer contain azide of up to 0.05%, which is toxic and upon contact with acids produces toxic gas. Wash buffer 1 contain 20% ethanol, wash buffer 2 contain 80% ethanol, which is flammable. The material safety data sheet (MSDS) will be provided on request.

If you work with biological samples, pay attention to the safety rules for working with infectious biological material, use appropriate protective equipment (shield, respirator) and work with samples only in designated biohazard boxes or designated areas. Work with infectious samples in BSL2 + or BSL3 laboratories. Dispose of infectious waste in accordance with the applicable legislation.

Continuously check that the work area is free of spilled solutions, chemicals, and biological samples. In the event of a spill, decontaminate the work area immediately.

2 List of Materials

2.1 Required laboratory equipment

- Calibrated handheld single-channel pipette (200 µL and 1000 µL)
- Calibrated handheld multichannel pipette (200 µL)
- Benchtop centrifuge for standard SBS microplates
- Benchtop centrifuge for 1.5/2.0 mL tubes
- Test tube shaker/vortex
- Gloves and other personal protective equipment
- Recommended: automated plate sealer

2.1.1 Agilent Bravo automated liquid handling station

This kit is designed to be run at Agilent Bravo liquid handling station equipped with a pipetting head 96ST, with 6 deck platepads and 3 alignment stations, and with installed protocols for "RNA isolation" and "Assay plate preparation", which are part of the *Agilent Bravo installation package for Automated RNA Isolation kit* (cat.no. DB-1214).

The provided manual expects from the user the basic knowledge of how to operate Agilent Bravo station. The original manuals in PDF forms for Agilent Bravo are provided during the Agilent Bravo installation. At a minimum, we advise each user to get familiar with the Quick Guide Manual (G5409-90020A_EN).

2.2 Required material not included in the kit

- 100% Isopropanol
- Disposable pipette tips (filter tips recommended)
- Empty original Agilent Bravo tip box (part of Installation package for automated RNA Isolation (cat No DB-1214))
- Optional: sealing foils to seal the ELUTION plate for long-term storage
- Optional – only required for downstream RT-PCR analysis of isolated RNA: 96-well PCR plate[1] suitable for your qPCR instrument (Assay plate) and corresponding optical seal.

[1] – provided protocol for Agilent Bravo for preparation of the Assay plate and mixing the eluted RNA with RT-PCR Master Mix is validated for 4ti-0951 PCR plate (4ti-tude) and will work for any plate with identical well geometry (i.e. any dual-composite Framestar plates). For plates with different geometry, the protocol must be validated and may need to be adjusted (in that case kindly contact us).

2.3 Kit components

Components	Amount	Storage temp.	Box no. [7]	Vial cap color
Lysis Buffer Concentrate [1,2]	1x 110 mL	18-30 °C	1	
REAGENT plate [1,2]	10 pieces	18-30 °C	1 [8]	
ELUTION plate [3]	10 pieces	18-30 °C	1 [8]	
WASTE plate [3]	10 pieces	18-30 °C	1 [8]	
LYSIS&SAMPLE plate [3]	10 pieces	18-30 °C	1 [8]	
Lidded RT-PCR Master Mix reservoir [3]	1 piece	18-30 °C	1	
50 mL tube for lysis buffer [3]	10 pieces	18-30 °C	1 [8]	
25 mL reservoir for lysis buffer [3]	10 pieces	18-30 °C	1 [8]	
Agilent Bravo 30 µL tips (384)	10 boxes	18-30 °C	1	
Agilent Bravo 70 µL tips (384)	10 boxes	18-30 °C	1	
Labels for non-labeled consumabels [2,4]	1 box	18-30 °C	1	
General adhesive seal	20 pieces	18-30 °C	1	
Pre-pierced seal	10 pieces	18-30 °C	1	
BEAD plate [2]	10 pieces	2-8 °C [5]	2	
Lysis Enhancer	2x 650 µL	-20 °C [6]	3	
RNA Carrier	2x 650 µL	-20 °C [6]	3	
DTT Concentrate	2x 650 µL	-20 °C [6]	3	

[1] – **Store in dark compartment**
[2] – Before use - read section 3 (Preparation of reagents for RNA isolation)
[3] – Empty plastic provided along with the kit
[4] – Labels intended for labeling of the tip box (30 µL, 70 µL - EMPTY tip box) and the Assay plate
[5] – **Do not freeze (frozen beads cannot be used)**
[6] – Avoid repeated freeze-thaw cycles (if you intend to use the component vials repeatedly, divide them into single-use aliquots during their first use and store at -20°C upon use).
[7] – Kit is divided into 3 boxes for shipping purposes
[8] – All these parts are packaged along in one box – there is one piece of each item in the box

2.4. Stability of the kit components

2.4.1 Lysis buffer

The lysis buffer, prepared according to the instructions written in section 3.7, is stable for 8 hours at laboratory temperature.

2.4.2 DTT Concentrate, Lysis Enhancer and RNA Carrier

Try to limit the freeze/thaw cycles to minimum and also try to limit the time at which the solutions are kept outside of a freezer. Never exceed the number of freeze/thaw cycle more than 4 times. In case of repeated need of small amounts of the solutions, aliquot them and freeze them.

2.4.3 BEAD plate

Span down BEAD plate is stable for at least 8 hours at laboratory temperature.

3 Preparation of reagents for RNA isolation

3.1 General notes

- Do not use any parts of the kit, which are apparently damaged, or thawed. Keep them for a future reclamation and contact the manufacturer.
- Incorrect handling of all parts of kits and deviation from the protocols described in this manual may negatively affect the results.
- Use the same version of the Manual (see header), as is indicated on the kit label.
- Do not use the kit after its expiry date (see the kit label)
- Do not combine solutions from different kits if they have different lot numbers (lot number are printed on the kit label)
- All frozen solutions should be, first fully thawed, then span down, shortly mixed (a short vortexing is sufficient) and then span down again. After this they can be used.
- Make sure that all solution in the pre-pipetted plates stays at the bottom of the wells when peeling of the seal. Avoid any quick handling to prevent spilling.

3.2 Collection of required reagents for RNA isolation

1. Take one tube of **Lysis Enhancer** (violet cap), **RNA Carrier** (blue cap) and **DTT Concentrate** (yellow cap) out of the freezer. Let all tubes thaw. Leave the DTT Concentrate at room temperature (the solution will solidify if placed on ice). Put Lysis Enhancer and RNA Carrier on ice.
2. Take the **BEAD** plate out of the fridge.
3. Take out one package of the isolation kit, which contains most of the reagents stored at room temperature needed for one RNA isolation (96 tests): **REAGENT** plate, **ELUTION** plate, **WASTE** plate, **LYSIS&SAMPLE** plate, 50 mL tube, 25 mL reservoir.
4. Prepare the RNA isolation control and optionally the positive control. If you are using the *COVID-19 Multiplex RT-PCR Kit* (cat.no. DB-1211) take tube RNA Isolation control (red cap number 5) and Positive control (yellow cap number 4) out of the freezer. Let both tubes thaw and keep them on ice.

3.3 Labelling of tip boxes for Agilent Bravo

There are labels in the kit intended for labeling of the tip boxes. Tips are supplied in the original manufacturer packaging.

1. Label the tip box with 30 µL tips with blue circle with a label saying „tip30 tip box“.
2. Label the tip box with 70 µL tips with blue circle with a label saying „tip70 tip box“.

Note: We recommend labelling all tip boxes upon kit opening.

3. Use the label „EMPTY tip box“ for the empty tip box. An EMPTY tip box is created at the end of each Assay plate preparation protocol (described in section 5.3.4).

3.4 Inspection of Lysis Buffer Concentrate

The "Lysis Buffer Concentrate" can form precipitate during shipment or storage below 18°C. Therefore, please inspect the bottle for visible precipitation before each use. If precipitate is present – place bottle into thermal incubator set to 37 °C for 30-60 minutes and mix afterwards by turning bottle upside down several times – precipitate should dissolve, and stock solution should not precipitate if stored at room temperature.

3.5 REAGENT plate and BEAD plate preparation

1. Spin **REAGENT plate** at 200g for 1 minute before use.
2. Spin **BEAD plate** at 200g for 1 minute before use.

Note: Use proper balance plates for both BEAD and REAGENT plate. Do not spin only these two plates since they have different weight. Appropriate counter-balance plates are part of Agilent Bravo installation package for Automated RNA Isolation kit (cat.no. DB-1214).

3.6 Sample preparation

All samples must be treated as potentially infectious material prior to addition of the lysis buffer and must be handled accordingly. Use proper personal protective equipment and work only in an area designated for a work with infectious material.

3.6.1 Nasopharyngeal swabs

For RNA isolation from a nasopharyngeal swab in virus transport medium vortex the swab in a sealed vial for 1 minute to release biological material and then spin down briefly.

3.6.2 Other type of biological matrix

For RNA isolation from other types of biological samples apply standard sample preparation steps accordingly.

***Optional**: The sample can be heat-inactivated for 10-30 minutes at 65°C to reduce infectivity. However, possible impacts of the heat inactivation on the RNA recovery must be evaluated for each analyzed RNA type and biological matrix. For SARS-Cov-2 detection from nasopharyngeal swabs, we observed 1-3 cycles signal decrease.*

3.7 Lysis buffer preparation

1. The lysis buffer should be prepared fresh in the amount required for an immediate use.

Note: After the all components of the lysis buffer are mixed together the buffer is stable at least for 8 hours at 25°C and therefore it is possible to prepare the buffer in the morning for the whole day. It is also possible to prepare the whole plate LYSIS&SAMPLE with aliquoted lysis buffer, however it is essential that the plate is properly sealed using an adhesive seal.

2. Components Lysis Enhancer, RNA Carrier and DTT Concentrate are frozen and need to be thawed completely before use, until clear solution is formed. To ensure homogeneity of thawed stock solutions always spin down all fluids to the vial bottom, vortex and spin down once again before pipetting the solution from each vial.

Note: In case the lysis buffer is prepared several times during one day, leave the thawed Lysis Enhancer and RNA carrier on ice and DTT Concentrate at room temperature. Each vial contains enough solution

for 5 RNA isolations (5x96 samples). For better overview we recommend marking the vials for how many times was each solution used. Place the used solution back to - 20 C after use.

3. Prepare the RNA isolation control (not part of this kit). This control is usually part of the RT-PCR kits (follow the procedure for preparation written in the RT-PCR kit manual).
4. For isolation of 96 samples follow the next lines (also summarized in table 1):
    a) Pipet on the side of provided 50 mL tube **9 ml of lysis buffer concentrate** (in case you are using 1000 µL pipet, use reverse-pipetting technique).
    b) Add **110 µL of Lysis Enhancer** (violet cap 🟣) using a new pipet tip into the same tube. Wash the tip once with the solution in the tube and throw it away.
    c) Add **110 µL of RNA Carrier** (blue cap 🔵) using a new pipet tip into the same tube. Wash the tip once with the solution in the tube and throw it away.
    d) Add **110 µL of DTT concentrate** (yellow cap 🟡) using a new pipet tip into the same tube. Wash the tip once with the solution in the tube and throw it away.
    e) Add **8.2 ml of 100% isopropanol**. In case you are using 1000 µL, **DO NOT** use reverse-pipetting technique.
    f) Close the tube and turn it upside down several times to ensure proper mixing (**before adding the isolation control**).
    g) **OPTIONAL**: if you want to have a negative control for RNA isolation control, take out 140 µL of the solution and pipet it into one of the wells on LYSIS&SAMPLE plate (do not add lysis buffer with isolation control into this well).
    h) Add **126 µL RNA Isolation control** into the same tube (the isolation control must be mixed well before this operation). Wash the tip once with the solution in the tube and throw the tip away. In case you are not using the isolation control, skip this step (for use of this kit in *in vitro* diagnostics the control is mandatory).
    i) Close the tube and properly mix the lysis buffer by turning the tube upside down several times.

Table 1: List of all chemicals needed for preparation of lysis buffer and summary of the preparation instructions

Order of addition	Component	Volume [µl]	Vial cap color
		1 plate (96 samples)	
1	Lysis Buffer Concentrate	9 000	
2	Lysis Enhancer [1]	110	
3	RNA Carrier [1]	110	
4	DTT Concentrate	110	
5	100 % Isopropanol [2]	8 200	
turn several times upside down to ensure **thorough mixing**			
6	RNA Isolation control [1,2,3]	126	
turn several times upside down to ensure **thorough mixing**			

[1] – Upon thawing keep on ice
[2] – Not part of the RNA isolation kit
[3] – If you are not using the isolation control, skip this step

*Note 1 : In case you are using the recommended isolation control from COVID-19 Multiplex RT-PCR Kit (cat.no. DB-1211) as an isolation control use undiluted solution from vial **Isolation control** (red cap 🔴,*

with number 5). If you are using different isolation control, follow the manufacturer instructions written in the corresponding manual and dilute to the final volume of 126 µL using PCR-grade water.

Note 2: The indicated volumes in table 1 already account for the void volume of the liquid (the volume should be sufficient for analysis of 126 samples, therefore it is not needed to increase the volume any further). The hereby described preparation of the lysis buffer is sufficient for isolation of RNA from 96 samples using one LYSIS&SAMPLE plate. If you require to prepare a different amount of lysis buffer, adjust the volumes accordingly.

5. Pour the prepared lysis buffer into 20 mL plastic reservoir (part of the kit) and dispense **140 µL of lysis buffer to each well of the LYSIS&SAMPLE plate** using 200 µL multichannel pipet.

IMPORTANT: *The pipetting of 140 µL into each well must be done relatively quickly (ideally within 1-2 minutes after the lysis buffer was poured into the plastic reservoir). The sole reason for this is that if done too slowly, the lysis buffer can change composition due to evaporation. It is also possible to pipet the solution in the plate using an electronic multichannel pipet or any pipetting system that is able to dispense precisely the required volume directly out of the 50 mL tube to limit the evaporation.*

6. If you are going to add samples into the LYSIS&SAMPLE plate within 30 minutes after its preparation cover the plate with the pre-pierced foil. In case the samples will be added later, cover the plate first using adhesive foil (thus prepared plate can be stored for up to 8 hours). Then right before adding the samples, replace the adhesive foil with the pre-pierced foil.

3.8 Sample addition into LYSIS&SAMPLE plate

1. Pipet **60 µL of sample into each corresponding well in the LYSIS&SAMPLE plate** through the pre-pierced seal. **Pipet the sample directly into the solution in the well, not on the wall of the well.**

Note: Avoid repeated up-and-down or fierce pipetting, which can cause foaming and bubbling. Mixing of sample with lysis buffer is not needed since it will be done during the protocol at the Bravo station.

2. Use the same procedure to pipet all samples into the LYSIS&SAMPLE plate.
3. In the next step add negative control and only after that add the positive control.
4. At the end cover the LYSIS&SAMPLE plate with a new adhesive seal (part of the kit).
5. The overall bench time work with the LYSIS&SAMPLE plate, which is covered with the pre-pierced seal, **should not exceed 2 hours**.
6. The lysis buffer inactivates the sample, however it is strongly recommended to treat all samples as still potentially infectious (especially before the samples are properly mixed with the lysis buffer during RNA isolation protocol). Therefore, after covering the plate with the new adhesive seal, we recommend wiping the whole plate with either isopropanol or ethanol. Make sure that during the whole process the solutions in LYSSI&SAMPLE plate stays at the bottom of the wells (do not turn the plate upside down or swing it violently).
7. Spin down the plate at 200x g for 1 minute.

Note: Use a proper counter-balance during the centrifugation. A proper balance plate is provided as part of the Agilent Bravo installation package for Automate RNA Isolation Kit (cat.no. DB-1214).

3.9 Kit controls

3.9.1 Mandatory controls for usage in *in vitro* diagnostics

RNA isolation efficacy control (RNA isolation control): An external control must be added into each sample before isolation. This control is detected in the downstream RT-PCR analysis. Using this control, efficacy of the RNA isolation is revealed and at the same time undesirable effects, such as inhibition of RT-PCR reaction, are excluded. Exact use of RNA isolation control is described in section 3.7 and in table 1. This control is usually part of RT-PCR kits and detailed instructions for data interpretation should be described in the manual of the RT-PCR kit. We recommend using the RNA Isolation control (red cap 🔴 vial number 5) from the *COVID-19 Multiplex RT-PCR Kit* (cat.no. DB-1211) together with this RNA isolation kit.

Negative control of RNA isolation: Add into one well of LYSIS&SAMPLE plate either a known negative sample or blank sample medium instead of test sample to make sure that there was no contamination by the target RNA or DNA. This control should be mixed after manipulations with all test samples.

Positive control of RNA isolation: Add into one well of LYSIS&SAMPLE plate a known positive sample or target RNA instead of test sample to verify that RNA isolation and RT-PCR reaction proceeded correctly. The target RNA can be part of the RT-PCR kit (*e.g.* Positive control, yellow cap 🟡 vial number 4 in *COVID-19 Multiplex RT-PCR Kit,* cat.no. DB-1211). This control can be replaced with a RT-PCR control. In that case add the target RNA directly into RT-PCR mix as if it was a sample.

3.9.2 Optional controls

Negative control of RNA isolation control: RNA Isolation control, which is used to monitor the efficacy of RNA isolation, can potentially contaminate other solutions and buffers. Therefore, we recommend to add one more isolation control with negative sample or blank medium instead of test sample, into which the RNA isolation control **was not** added (it is necessary to prepare a separate lysis buffer for this one isolation, which does not contain the RNA isolation control, or pipet out an aliquot before the RNA is added, see section 3.7). To prove that no contamination occurred during the solution preparations there should be negative RT-PCR result in the RNA isolation control channel for this isolation.

4 RNA Isolation protocol

RNA Isolation protocol on Agilent Bravo performs simultaneous RNA isolation from 96 samples and yields 18 µL of isolated RNA in ELUTION plate. The well positions in LYSIS&SAMPLE plate are preserved during the protocol and are identical to the well positions in the ELUTION plate.

4.1 General notes

1. To ensure proper efficacy of the RNA Isolation protocol it is essential that the Agilent Bravo automated liquid handling station is installed following manufacturer's instruction by service technician authorized by Agilent technologies.
2. The testing of Agilent Bravo station and import of RNA Isolation protocol must be done by personnel trained by DIANA Biotechnologies s.r.o. following the instructions provided in *Agilent Bravo installation package for Automated RNA Isolation kit* (cat.no. DB-1214).
3. We highly recommend performing regular maintenance of Agilent Bravo station by authorized service for long-term good performance
4. Protocol should be done at room temperature (app. 18-25 °C).

4.2 Running RNA isolation protocol on Agilent Bravo

4.2.1 Set-up Agilent Bravo station

1. Switch ON the Agilent Bravo station (the switch is located at top-right side).
2. Open the file shortcut **DIANA_RNA_Isolation** located on the desktop.

Note The shortcut on desktop with abovementioned name should be created during the installation. If you cannot locate the shortcut, you can find the protocol file in folder C:\DIANA Biotech\Bravo files\Protocol Files under the name DIANA_RNA_isol_DB-1206_v2.x (if there are more files with this name choose the most recent version of the protocol file).

3. Initialize Agilent Bravo station and log in into the VWorks program.

Note: When initialized for the first time upon switching on, you will receive two messages during the initialization. For first message (question whether there is a plate present inside gripper) press "Ignore and continue…" if there is no plate present in the gripper. For second message (question whether there are tips with liquid present on the Bravo head) press "Retry" if there are no tips with liquid present on the Bravo head.

4. The VWForms window (screenshot shown in Figure 1) will appear automatically.
5. Fill out the fields "Experiment ID", "Operator Name" and "Elution plate ID" (the Elution plate ID can be found on the side of the ELUTION plate and it is unique for each plate)

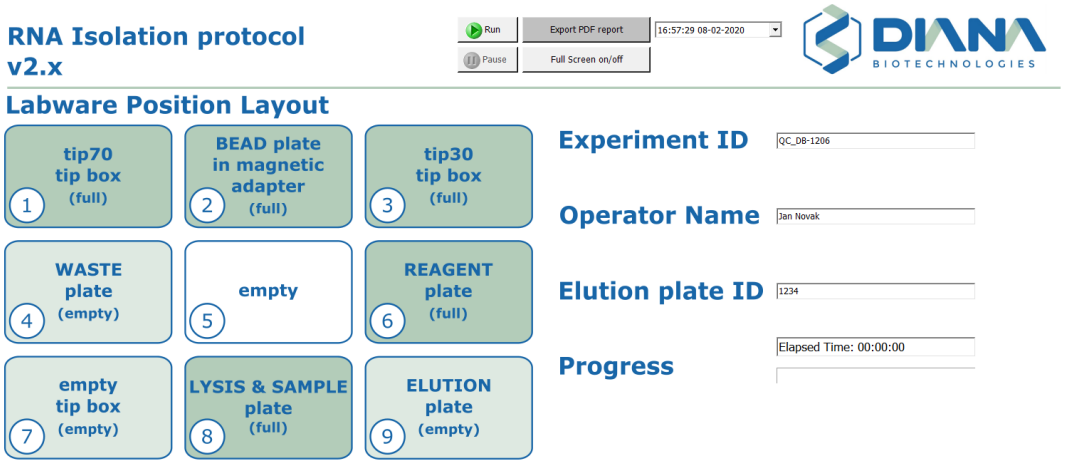

Figure 1: Screenshot of VWForms window of RNA Isolation protocol.

4.2.2 Place proper labware into Agilent Bravo

Start loading the Bravo station with labware from back positions (1, 2, and 3) and move forward. Avoid any manipulations above opened tip boxes and plates.

Perform next step with new pair of clean gloves (especially not with gloves that you used during LYSIS&SAMPLE plate preparation) to prevent potential contaminations. Start the RNA isolation protocol as soon as possible (MAX 10 minutes) after peeling off the seal from REAGENT plate. Start with the next steps after you have prepared LYSIS&SAMPLE plate and fill it with all samples and controls (see sections 3.7, 3.8, and 3.9).

1. Follow the "Labware Position Layout" shown in the screen (see Figure 1). The orientation of Bravo station is such that positions 7,8 and 9 are the user-facing front positions.
2. Place clean **tip70 tip box in position 1** and clean **tips30 tip box** in **position 3**. Remove the lids from the boxes.
3. Carefully peel off the seal from **BEAD** plate (make sure not to spill any liquid).
4. Place the **BEAD** plate in **position 2** into the ***Magnetic Adapter*** located at this position.
5. Peel off the seal from **WASTE** plate and place it in **position 4**.
6. Carefully peel off the seal from **REAGENT** plate (make sure not to spill any liquid).
7. Place the **REAGENT** plate in **position 6**.
8. Place **EMPTY tip box** in **position 7** and remove its lid.
9. Peel off the seal from **ELUTION** plate and place it in **position 9**.
10. Carefully peel off the seal from **LYSIS&SAMPLE** plate and place it in **position 8**.

⚠ ***IMPORTANT**: Discard the seal into the biohazard waste. Peeling off the foil must be done slowly to minimize the risk of cross-contamination.* ***Always put LYSIS&SAMPLE plate as the last plate into the Bravo station.***

4.2.3 Run the RNA Isolation protocol at Bravo

Before starting a run, carefully check that:

- all plates, tip boxes and magnet adaptor are oriented in a way that their labels are facing the operator
- all plates, tip boxes and magnet adaptor are at the right positions (position of each labware is written in circle on its label)
- all plates, tip boxes and magnet adaptor sit precisely in their designated position
- there are no lids or foils present on plate or tip boxes

- **if these guidelines are not followed the RNA isolation protocol will not work properly and it can even lead to the damage the Agilent Bravo station**

Start the RNA isolation protocol following instructions below:

1. Press the "Run" button 

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2. After clicking "Run" a new window pops up on the screen providing more details about the protocol and tips usage. Click "Finish" button at bottom right part of this window.
3. New message appears that summarizes the layout of each labware on its designated position. Check that the layout corresponds to the physical layout of your setup.
4. Check again that all lids are removed, and all seals are peeled off.
5. Click "Continue" button which starts the RNA Isolation protocol.
6. **IMPORTANT: Observe movements of Bravo station during the start (app. 1 min) of the protocol to ensure that it operates properly, the following steps should be performed:**
   i. Loading the tips from tip70 tip box in position 8
   ii. Aspiration of liquid from BEAD plate in position 2
   iii. Dispense of liquid into WASTE plate in position 4
7. The RNA Isolation protocol comprises of following steps:
   i. Sample and lysis buffer inside LYSIS&SAMPLE plate are properly mixed and then transferred to the magnetic beads into BEAD plate,
   ii. Sample is removed after incubation (during which the viral RNA is bound to the beads) from the beads and transferred into the WASTE plate,
   iii. Magnetic beads are then washed sequentially three times and dried afterwards,
   iv. In the last step elution buffer is added to the beads and upon incubation the solution with eluted viral RNA is transferred to the ELUTION plate.
8. RNA Isolation protocol takes 40 minutes and does not require operator's intervention.
9. After protocol finishes click "Export PDF report" button to create PDF report of the protocol that will include name of the used Bravo protocol, time, "Experiment ID", "Operator Name", and "Elution plate ID".
10. The result of RNA isolation protocol is 18 µL isolated RNA in each well of ELUTION plate.

***IMPORTANT**: Using Assay plate preparation protocol on Agilent Bravo will consume 4.5 µL of isolated RNA for one RT-PCR reaction. Therefore, isolated RNA obtained by this protocol is sufficient for three independent RT-PCR reactions. For long-term storage keep the ELUTION plate sealed with freeze-resistant foil and place it into -80 °C.*

4.2.4 Labware clean-up after RNA Isolation protocol

The following instructions describe the labware clean-up in case the Assay plate preparation protocol directly follows the isolation protocol. In case the RT-PCR Assay plate preparation will not be done on Agilent Bravo, follow the instructions described in Appendix (section 9.2).

1. Place a lid on the **EMPTY tip box** and discard it from **position 7**.

IMPORTANT: Not discarding the EMPTY tip box from position 7 and running the subsequent Assay plate preparation protocol may severally damage the Agilent Bravo station!!

2. Carefully take out from Bravo station and discard **WASTE** plate from **position 4**.
3. Carefully take out from Bravo station and discard **LYSIS&SAMPLE** plate from **position 8**.

***IMPORTANT**: LYSIS&SAMPLE plate and WASTE plate may contain viral RNA and thus must be discard in a way that minimizes the risk of workspace contamination. We recommend to re-seal the plates or to place them into water-leak resistant container.*

4. Close the VWorks program (if asked, do not save the VWForms file).
5. Continue with the preparation of Assay plate described in section 5.

5 Assay plate preparation protocol

This protocol is a recommended as a direct follow-up to the RNA isolation protocol (described in section 4). The protocol dispenses 13.5 µL RT-PCR master mix into 96-well PCR plate (Assay plate) and then transfers 4.5 µL of the isolated RNA from ELUTION PLATE into Assay plate. The outcome of the protocol is Assay plate, which upon sealing, is ready to be analyzed in the real-time PCR cycler.

Note: Used RT-PCR master mix must be prepared for preparation of reaction in ratio 3:1 (i.e. mixing 15 µL of master mix with 5 µL of sample). In Assay plate preparation protocol the reaction is mixed in 10% lower volume (i.e. mixing 13.5 µL of master mix with 4.5 µL of sample) in order to compensate for the dead volume that is required due to automation of the whole process.

5.1 General notes

1. It is essential that the Agilent Bravo automated liquid handling station is installed following manufacturer's instruction by service technician authorized by Agilent technologies to ensure proper efficacy of the Assay plate preparation protocol.
2. The testing of Agilent Bravo station and proper installation of the Assay plate preparation protocol must be done by personnel trained by DIANA Biotechnologies s.r.o. following the instructions provided in *Agilent Bravo installation package for Automated RNA Isolation kit* (cat.no. DB-1214).
3. For long-term good performance, we highly recommend performing a regular maintenance of the Agilent Bravo station by authorized service.
4. Protocol should be done at room temperature (app. 18-25 °C).

5.2 Preparation of RT-PCR Master Mix reservoir

1. To minimize risk of RT-PCR master mix contamination, perform the following steps in area that is physically separated from the area where RNA isolation, sample handling or positive control handling is done.
2. Prepare 1.6 mL of RT-PCR master mix along manufacturer's instruction in a concentration that is calculated to be mixed in the RT-PCR reaction in ratio 1:3 (*e.g.* mixing 15 µL of master mix with 5 µL of sample). The required volume accounts for the dead volume and should be reachable by using common RT-PCR mixes for 100 reactions, which contains approximately 10 % volume overfill.

***COVID-19 Multiplex RT-PCR Kit (cat.no DB-1211) is recommended RT-PCR kit in combination with this RNA isolation protocol.** In case you use the 100rxn kit size (cat.no DB-1211-100rxns), mix whole volumes of components 1, 2, and 3. Each vial contains app. 550 µL, therefore the final obtained volume is 1650 µL of RT-PCR master mix. In case you are using the 1000rxn kit size (cat.no DB-1211-1000rxns), mix 533 µL of each component 1, 2, and 3 in new clean 2 mL vial to obtain 1600 µL of RT-PCR master mix. Alternatively, you can mix whole volume (5.5 mL)*

of each component together and then prepare ten 1600 µL aliquots of ready-to-use RT-PCR master mix. Thus prepared RT-PCR Master Mix is stable on ice for several hours and for one month at -80°C (it MUST be placed into -80°C right after its preparation). More information about stability of RT-PCR master mix and its components can be found in the manual for COVID-19 Multiplex RT-PCR Kit.

3. Peel off the foil from one column (eight wells) of RT-PCR Master Mix Reservoir.
4. Dispense 180 µL of RT-PCR master mix into each of eight freshly uncovered wells. To ensure precise pipetting, you must use 200 µL pipette and reverse pipetting method.
5. Check that there are no bubbles at the bottom or on surface of the wells.
6. Cover the RT-PCR Master Mix Reservoir with provided lid and transport it into the Agilent Bravo station.

5.3 Running RT-PCR Assay plate preparation on Agilent Bravo

5.3.1 Set-up Agilent Bravo station

1. The following instructions are meant to be performed directly after RNA isolation protocol and utilize the tip boxes and ELUTION plate that both stay inside Bravo station after RNA isolation.
2. Open the file shortcut **DIANA_Assay_plate_prep** located on the desktop.

Note: The shortcut on desktop with abovementioned name should be created during the installation. If you cannot locate the shortcut, you can find the protocol file in folder C:\DIANA Biotech\Bravo files\Protocol Files under the name DIANA_Assay_plate_prep_DB-1206_v2.x (if there are more files with this name choose the most recent version of the protocol file).

3. Initialize Agilent Bravo station and log in into the VWorks program.
4. The VWForms window (screenshot shown in Figure 2) will pop up automatically.

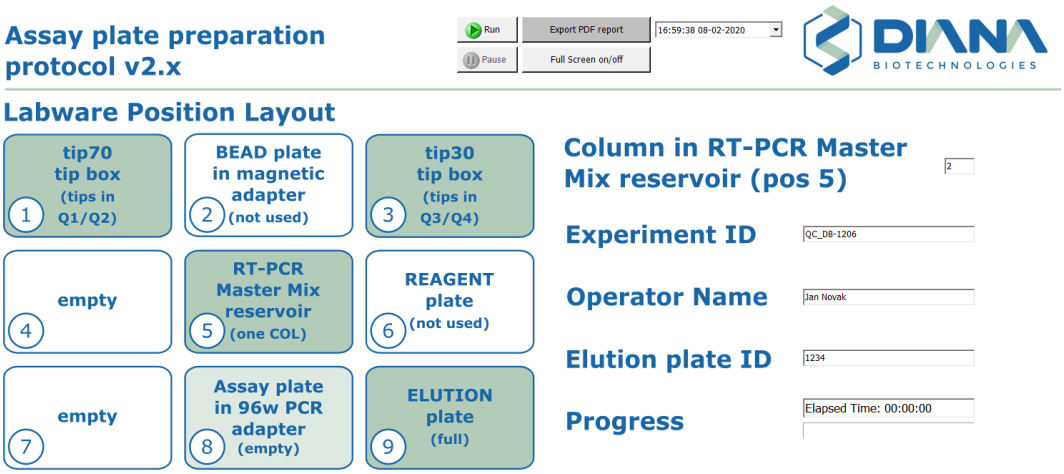


Figure 2: Screenshot of VWForms window of Assay plate preparation protocol. The Labware Position Layout represents the labware distribution of Assay plate preparation which is done as a direct follow-up of RNA isolation protocol and plate and tip boxes are cleaned-up as described in section 4.2.4.

5. Fill out the field „Column in RT-PCR Master Mix reservoir (pos 5)“. The entered value must be integer in a range of 1-12 that reflect the column number of RT-PCR Master Mix Reservoir where the RT-PCR master mix has been dispensed. **Entering correct column number value is crucial for proper function of the protocol.**

6. Fill out the fields “Experiment ID”, “Operator Name” and “Elution plate ID” (the Elution plate ID can be found on the side of the ELUTION plate and is unique for each plate)

5.3.2 Place proper labware into Agilent Bravo

Start loading the Bravo station with labware from back positions (1, 2, and 3) and move forward. Avoid the movements above opened tip boxes and plates.

The following instructions should be followed if the RNA Isolation protocol was done beforehand and the plates and tip boxes were cleaned-up as described in section 4.2.4. In that case, following plates and tip boxes should be already present inside Bravo station:

a) **tip70 tip box** in **position 1** (contains used 30 µL tips in quadrants 1 a 2),
b) **BEAD** plate in **position 2** (will not be used during this protocol),
c) **tip30 tip box** in **position 3** (contain clean 30 µL tips in quadrants 3 a 4),
d) **REAGENT** plate in **position 6** (will not be used during this protocol),
e) **ELUTION** plate in **position 9**

Note: If the Assay plate preparation protocol is used apart from the RNA isolation protocol then the following labware must be first placed inside Bravo station: i) tip box with empty positions in quadrants 3 and 4 in position 1, ii) tip box with clean 30 µL tips in quadrants 3 a 4 in position 3, and iii) ELUTION plate (without any lid or foil) in position 9.

1. Place lidded **RT-PCR Master Mix Reservoir** in **position 5**.
2. **IMPORTANT**: Remove the lid from RT-PCR Master Mix reservoir.
3. Place **96w PCR adapter** (cat.no. G5498B#G013) in **position 8**.
4. Place clean PCR plate (**Assay plate**) into the 96w PCR adapter in **position 8**.

⚠ ***IMPORTANT:*** *Mark visibly the front part of the Assay plate before you place it into the Bravo station to avoid accidental turn of the plate by 180°. For Assay plate labelling you can advantageously use the „Assay plate in 96w PCR adapter“ label that is provided with the kit.*

5.3.3 Run the Assay plate preparation protocol

Before starting a run, carefully check that:

- all plates, tip boxes and magnet adaptor are oriented in a way that their labels are facing the operator
- all plates, tip boxes and magnet adaptor are in the right positions (position of each labware is written in circle on its label)
- all plates, tip boxes and magnet adaptor sit precisely in their designated position
- there are no lids or foils present on plate or tip boxes
- **if these guidelines are not followed the RNA isolation protocol will not work properly and it can even lead to the damage the Agilent Bravo station**

Start the Assay plate preparation protocol following instructions below:

1. Press the “Run” button 

.
2. After clicking “Run” a new window pop up on the screen providing more details about the protocol and tips usage. Click “Finish” button at bottom right part of that window.
3. New message appears that summarizes the layout of each labware on its designated position. Check that the layout corresponds to the physical layout of your experiment.
4. Check that all lids are removed, and all seals are peeled off (only the seal over the clean yet unused column at RT-PCR Master Mix Reservoir are present) and press “Continue”.

5. New message appears to check once more that you have filled correct column number of the RT-PCR Master Mix Reservoir into the VWForms window. **Filling correct column number is crucial for proper function of the protocol.**
6. Click "Continue" button which starts the Assay plate preparation protocol.
7. **IMPORTANT: Observe movements of Bravo station at the beginning of the run (app. 1 min) to ensure that it operates properly, the following steps should be performed:**
    i. Loading one column (eight tips) from tip30 tip box in position 3
    ii. Aspiration of liquid from correct column of RT-PCR Master Mix reservoir in position 5
8. The Assay plate preparation protocol comprises of following steps:
    i. Dispense of 13.5 µL of RT-PCR master mix from one column of RT-PCR Master Mix Reservoir into Assay plate
    ii. Transfer of 4.5 µL isolated RNA from ELUTION plate into RT-PCR master mix in the Assay plate and subsequent mixing.
9. Assay plate preparation protocol takes approximately 5 minutes to finish.
10. After protocol finishes click "Export PDF report" button to create PDF report of the protocol that will include name of the used Bravo protocol, time, and all information you filled in in step 5.3.1.
11. Carefully take out the **Assay plate** from **position 8** and seal it with optically transparent foil which is compatible with your real-time cycler. The Assay plate is now ready to be placed into the real-time cycler for measurement.

5.3.4 Labware clean-up after Assay plate preparation protocol

1. Carefully take out the **ELUTION plate** from **position 9**. For long-term storage seal the plate with freezing-resistant foil and keep the plate at -20 °C (storage up to 30 days) or -80 °C (storage longer than 80 days).
2. Lid and carefully take out the **RT-PCR Master Mix Reservoir** from **position 5**.
3. **IMPORTANT**: Keep the RT-PCR Master Mix Reservoir for further use (reservoir can be used up to 12 times)
4. Take out and discard the **REAGENT plate** from **position 6**.
5. Lid and discard the **tip70 tip box** from **position 1**.
6. Take out and discard the **BEAD plate** from **position 2**.

***IMPORTNAT**: BEAD plate may contain viral RNA and thus must be discard in a way that minimizes the risk of workspace contamination. We recommend to re-seal the plates or to place them into water-leak resistant container.*

7. Take out **tip30 tip box** from **position 3**. Throw out the clean left-over tips from the box.
8. **IMPORTANT**: Keep the **tip30 tip box** and use it as **EMPTY tip box** in next RNA Isolation protocol. Re-label the "tip30 tip box" to "EMTPY tip box" using the provided label.
9. Close the VWorks program (if asked, do not save the VWForms file).
10. Switch OFF the Agilent Bravo station if you are not planning to use it for the rest of the day.

6 Legal notice

To the fullest extent provided by the laws of your jurisdiction, DIANA Biotechnologies, s.r.o. disclaims any liability for any direct and/or indirect damage related to or arising from this document or its use and any direct and/or indirect damage related to or arising from Automated RNA Isolation Kit for Agilent Bravo and its use.

In case of use of Automated RNA Isolation kit for Agilent Bravo for *in vitro* diagnostics together with CE IVD certified RT-PCR kits, liability for the RNA isolation process validation shall be borne by the user.

DIANA Biotechnologies s.r.o. is the exclusive holder of all the protocols for RNA isolation and RT-PCR plate preparation using the Agilent Bravo pipetting station as referred to in this manual. Providing the protocols to third parties without the express consent of an authorized agent of DIANA Biotechnologies s.r.o. is prohibited.

7 List of compatible kits

REF DB-1211-100rxns COVID-19 Multiplex RT-PCR Kit (100 tests)

REF DB-1211-1000rxns COVID-19 Multiplex RT-PCR Kit (1000 tests)

8 Used graphical symbols

	Manufacturer
	Date of Manufacture
	Caution
	Lot Number
	Operator´s manual, operating instructions
	Catalogue Number
	Lower limit of temperature
	Temperature limit
	Upper limit of temperature
	Do not use if package is damaged
	Use by date
	Do not reuse
	Keep away from sunlight

9 Appendix

9.1 Quadrants layout in 384-well plate

384-well plate can be virtually divided in four quadrants. Each quadrant comprises 96 wells that have identical layout as in 96-well plate. The numbering of the quadrants in 384-well plate is from 1 to 4 moving from left to right and from top to bottom, unless otherwise stated. Figure 3 illustrates the layout of quadrants in 384-well plate.

384wp	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
C	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
D	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
E	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
F	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
G	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
I	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
J	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
K	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
L	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
M	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
N	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4
O	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
P	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4	3	4

Figure 3: Quadrant layout in 384-well plate. Quadrant 1 (Q1), colored blue, comprises wells A1, A3, …, O21, O23; quadrant 2 (Q2), colored green, comprises wells A2, A4, … , O21, O24; quadrant 3 (Q3), colored red, comprises wells B1, B3, B5, …, P21, P23; and quadrant 4 (Q4) comprises wells B2, B4, B6, ..., P22, P24.

9.2 Clean-up of Agilent Bravo station in case the Assay plate is not prepared in the station

1. Carefully take out the **ELUTION plate** from **position 9**. For long-term storage seal the plate with freezing-resistant foil and keep it at -20 °C (storage up to 30 days) or -80 °C (storage longer than 80 days).
2. Lid and discard the **EMPTY tip box** from **position 7**.
3. Carefully take out from Bravo station and discard **LYSIS&SAMPLE** plate from **position 8**.
4. Carefully take out from Bravo station and discard **WASTE** plate from **position 4**.
5. Take out and discard the **REAGENT plate** from **position 6**.
6. Take out and discard the **BEAD plate** from **position 2**.

⚠ ***IMPORTNAT**: BEAD plate may contain viral RNA and thus must be discard in a way that minimizes the risk of workspace contamination. We recommend to re-seal the plates or to place them into water-leak resistant container.*

7. Lid and discard the **tip70 tip box** from **position 1**.
8. Take out **tip70 tip box** from **position 1**. Throw out the tips from the box.
9. **IMPORTANT**: Keep the **tip70 tip box** and use it as **EMPTY tip box** in next RNA Isolation protocol. Re-label the “tip70 tip box” to “EMTPY tip box” using the provided label.
10. Lid and keep the **tip30 tip box** that contains clean tips in quadrants 3 and 4. These tips can be used for one more RNA isolation protocol, but the box **MUST BE ROTATED by 180°** (clean tips must be present in quadrants 1 and 2).
11. Close the VWorks program (if asked, do not save the VWForms file).
12. Switch OFF the Agilent Bravo station if you are not planning to use it for the rest of the day

Summary protocol

Summary of all needed reagents

1. **Lysis buffer preparation**: Lysis Buffer Concentrate and Isopropanol (MIN 10 mL each); Lysis Enhancer, RNA Carrier a DTT Concentrate (MIN 110 µL each); 126 µL RNA Isolation control; 1x 50 mL tube; 1x 25 mL reservoir; 2x adhesive foil; 1x pre-pierced foil
2. **Plates** (one plate of each type): BEAD plate, REAGENT plate, ELUTION plate, WASTE plate, LYSIS&SAMPLE plate; at least one sealed column of RT-PCR Master Mix Reservoir (all plates are included in the kit); PCR plate (not part of the kit)
3. **Tips**: 1x tip70 tip box (green), 1x tip30 tip box (blue), 1x EMPTY tip box

Preparation of reagents for RNA isolation

1. Mix the Lysis Buffer Concentrate bottle and visually inspect if there is no precipitate present. If there is any, place the bottle into 37 °C for at least 30 minutes. Afterwards, mix again and check that the precipitate dissolved.
2. Spin down BEAD plate a REAGENT plate 200x g for 1 minute.
3. Upon thawing, spin, vortex, and spin again all reagents that are stored at -20°C.
4. Prepare lysis buffer in 50 mL tube according to the table below. Keep the order of addition of all reagents as stated in the table. Mix the tube thoroughly both BEFORE and AFTER the addition of the Isolation Control.

Table: List of reagents needed for lysis buffer preparation

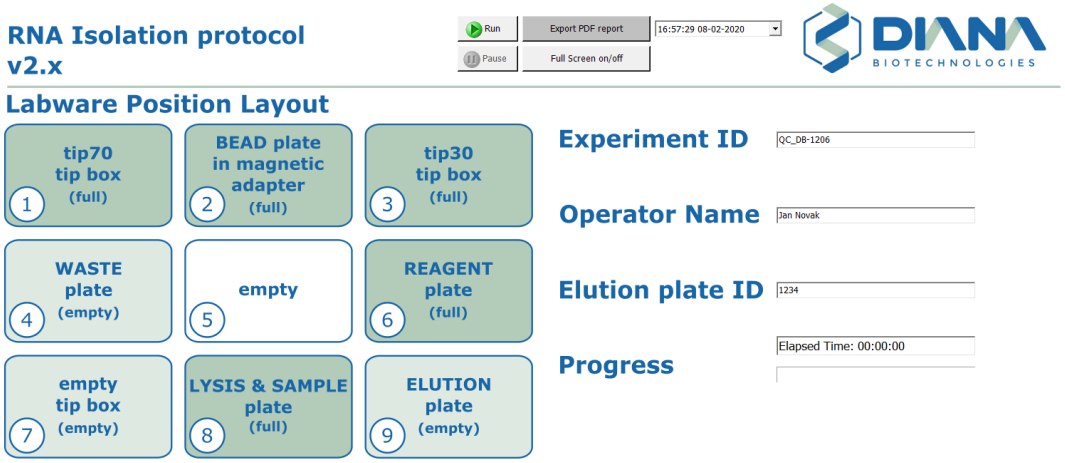
Order of addition	Component	Volume (µl)	Vial cap color
		1 plate (96 samples)	
1	Lysis Buffer Concentrate	9 000	
2	Lysis Enhancer	110	
3	RNA Carrier	110	
4	DTT Concentrate	110	
5	100 % Isopropanol	8 200	
turn several times upside down to ensure **thorough mixing**			
6	RNA Isolation Control	126	
turn several times upside down to ensure **thorough mixing**			

5. Dispense 140 µL of prepared lysis buffer in LYSIS&SAMPLE plate and seal the plate with pre-pierced seal, which is part of the kit.
6. Prepare the samples for RNA isolation including appropriate controls.
7. Through the pre-pierced foil, dispense 60 µL of each sample straight into the lysis buffer to the bottom of the LYSIS&SAMPLE plate (do not pipette the sample on the well wall). After the addition of last sample, seal the LYSIS&SAMPLE plate with adhesive foil, which is part of the kit.
8. Spin the LYSIS&SAMPLE plate 200x g for 1 minute.

Note: Total working time of LYSIS&SAMPLE plate sealed only with pre-pierced foil should never exceed 2 hours. If the LYSIS&SAMPLE plate with dispensed lysis buffer is sealed with adhesive foil the plate is stable for 8 hours at room temperature.

RNA Isolation protocol on Agilent Bravo

1. Switch ON the Agilent Bravo station.
2. Open the file shortcut **DIANA_RNA_Isolation** located on your desktop.
3. Initialize Agilent Bravo station and log in into the VWorks program.
4. Place the corresponding plates and tip boxes into the Agilent Bravo station as indicated in the scheme that appears after opening DIANA_RNA_isolation protocol (shown in figure below). In case the plates are sealed, first peel off the seal and then put the plate into the Bravo station. Remove the lid from the tip boxes after placing them into Bravo station.



5. Fill in the fields Experiment ID", "Operator Name" and "Elution plate ID".
6. Press "Run", then „Finish" button and then „Continue" button.
7. RNA Isolation protocol starts and will take app. 40 minutes to finish.
8. **Observe the pipetting station at the beginning (approx. 1 min) of the protocol to ensure it works properly. Bravo should load tips from tip70 tip box and transfer liquid from BEAD plate to WASTE plate.**
9. **IMPORTANT**: Discard lidded EMPTY tip box, LYSIS&SAMPLE plate, and WASTE plate.
10. By pressing „Export PDF report" create report of the protocol.
11. Close the VWorks program (if asked do not save the VWForms file).

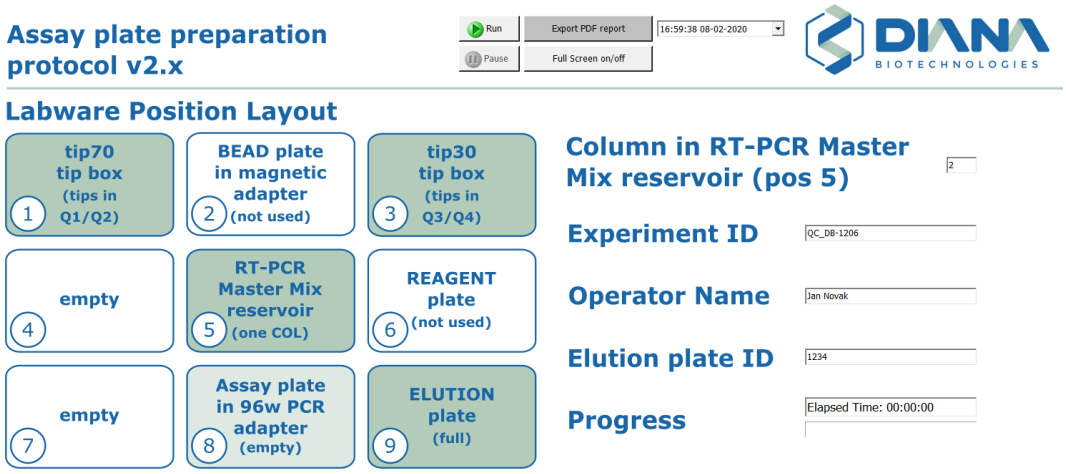
RT-PCR master mix reservoir preparation

1. Upon thawing - spin, vortex, and spin again all reagents that are stored at -20°C.
2. Prepare 1.5-1.6 mL of RT-PCR Master Mix following the manufacturer's instructions (applicable for reaction that are mixed in ratio 15 µL master mix and 5 µL sample).
3. Peel off one strip (eight-well column) from RT-PCR Master Mix Reservoir and dispense 180 µL of prepared RT-PCR master mix into each well.

COVID-19 Multiplex RT-PCR Kit (cat.no DB-1211) is recommended RT-PCR kit in combination with this RNA isolation protocol. *In case you use the 100rxn kit size (cat.no DB-1211-100rxns), mix whole volumes of components 1, 2, and 3. Each vial contains app. 550 µL, so you obtain 1650 µL of RT-PCR master mix. In case you use the 1000rxn kit size (cat.no DB-1211-1000rxns), then mix 533 µL of each component 1, 2, and 3 in new clean 2 mL vial to obtain 1600 µL of RT-PCR master mix. Alternatively, you can mix whole volume (5.5 mL) of each component together and then prepare ten 1600 µL aliquots of ready-to-use RT-PCR master mix. Prepared RT-PCR master mix is stable on ice for several hours and for one month at -80°C (it MUST be placed into -80°C right after its preparation). More information about stability of RT-PCR master mix and its components can be found in the manual for COVID-19 Multiplex RT-PCR Kit.*

RT-PCR Assay plate preparation protocol

1. The protocol described below should be done right after finishing the RNA isolation protocol and it utilizes tip boxes and ELUTION plate from RNA isolation protocol.
2. Open the file shortcut **DIANA_Assay_plate_prep** located on your desktop.
3. Initialize Agilent Bravo station and log in into the VWorks program.
4. Place corresponding plates and tip boxes into the Bravo station as indicated in the scheme that appears after opening DIANA_RNA_isolation protocol (shown in the figure below).
   a) Place lidded RT-PCR Master Mix reservoir to position 5 and then **REMOVE** its lid.
   b) Place clean empty Assay plate in 96w PCR adapter to position 8. Before inserting the plate into Bravo station, label it from front side, preferentially by the provided label „Assay plate in 96w PCR adapter“.



7. Fill in the fields “Column in RT-PCR Master Mix reservoir (pos 5)“, “Experiment ID”, “Operator Name”, and “Elution plate ID”.
8. **Before running protocol check again that position 7 in Bravo station is EMPTY!**
9. Press “Run”, then „Finish“ button and then twice „Continue“ button.
10. Assay plate preparation protocol starts and will take app. 5 minutes to finish.
11. **Observe the pipetting station at the beginning (approx. 1 min) of the protocol to ensure it works properly. Bravo should load eight tips (one column) from tip30 tip box and aspirate liquid from one column of the RT-PCR Master Mix Reservoir.**
12. Afterwards, take out the Assay plate from position 8, seal it with optically transparent sealing foil. The plate is now ready for the analysis in real-time PCR cycler.
13. Take out the ELUTION plate from position 9. For long-term storage seal the plate with freezing resistant foil and keep at -20-80 °C.
14. Lid and take out the RT-PCR Master Mix reservoir from position 5. Keep the reservoir for next Assay plate preparation protocol (can be used up to 12 times).
15. Carefully take out and discard lidded tip70 tip box, BEAD plate, and REAGENT plate.
16. Carefully take out and throw out all unused/clean tips from tip30 tip box. KEEP the box and use it as EMPTY tip box in next RNA Isolation protocol. Re-label the “tip30 tip box” to “EMTPY tip box” using the labels provided in the kit.
17. By pressing „Export PDF report“ create report of the protocol.
18. Close the VWorks program (if asked do not saved the VWForms file).
19. If the Agilent Bravo station is not used for the rest of the day switch the station OFF.