February 2019

miRNeasy 96 Advanced QIAcube HT Kit Handbook

For automated purification of total RNA, including miRNA, from serum and plasma samples using the QIAcube HT Prep Manager Software

Sample to Insight

QIAGEN

Contents

Kit Contents

miRNeasy 96 Advanced QIAcube HT Kit	**(5)**
Catalog no.	**217261**
Number of preps	**480**
Buffer RPL*	2 x 20 ml
Buffer RPP	2 x 8 ml
Buffer RWT*†	2 x 80 ml
Buffer RPE‡	2 x 65 ml
RNase-Free Water	3 x 30 ml
TopElute Fluid	60 ml
Quick-Start Protocol	1
RNeasy® 96 Plates	5
Collection Microtube Rack	5
Caps for Collection Microtubes (55 x 8)	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See Safety Information section.

† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96%–100%) as indicated on the bottle to obtain a working solution.

‡ Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96%–100%) as indicated on the bottle to obtain a working solution.

QIAcube HT Plasticware	(480)
Catalog no.	950067
Number of preps	480
S-Blocks	5
Filter-Tips OnCor C	9 x 96
Tape pad	1
Elution Microtube RS (EMTR)	5
8-well Strip Caps for EMTR	120

Note: The following components can also be ordered separately: S-Blocks, Elution Microtubes RS (including caps for strips), and TopElute Fluid. Please see Ordering Information on page 45.

Shipping and Storage

The miRNeasy 96 Advanced QIAcube HT Kit (cat. no. 217261) is shipped at ambient temperature. Store all components dry at room temperature. All kit components are stable for at least 9 months under these conditions.

Intended Use

The miRNeasy 96 Advanced QIAcube HT Kit is intended for the automated extraction of miRNA using the QIAcube HT instrument with the Prep Manager Software. The miRNeasy 96 Advanced QIAcube HT Kit is intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in a convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION 	CAUTION: DO NOT add bleach or acidic solution directly to the sample preparation waste.

Buffer RWT contains guanidine thiocyanate, which can form highly reactive compounds when combined with bleach. If liquid containing these solutions is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of miRNeasy 96 Advanced QIAcube HT Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Interest in smaller RNA species, such as miRNA, has increased over the past years as researchers understand the regulatory role of small non-coding RNAs. The miRNeasy 96 Advanced QIAcube HT Kit is designed for automated high throughput purification of cell-free total RNA – primarily miRNA and other small RNA – from small volumes of serum and plasma on the QIAcube HT.

When working with serum and plasma samples, we recommend the use of a synthetic spike-in control to provide a control for the quality of the RNA isolation, such as the RNA Spike-in Kit, for RT, which must be ordered separately (cat. no. 339390).

The miRNeasy 96 Advanced QIAcube HT Kit offers a phenol-free protocol to isolate high yields of cell-free total RNA including miRNA from up to 200 µl of human, mouse, and rat serum or plasma.

Principle and Procedure

The miRNeasy 96 Advanced QIAcube HT Kit combines guanidine-based lysis of samples, an inhibitor removal step and silica-membrane–based purification of total RNA. Buffer RPL, included in the kit, contains guanidine thiocyanate as well as detergents, designed to facilitate lysis, to denature protein complexes and RNases. Therefore RNA in samples lysed in buffer RPL are stable and protected from degradation.

Buffer RPL is added to serum or plasma samples. After thoroughly mixing to ensure a complete lysis, Buffer RPP is added to precipitate inhibitors (mostly proteins which are highly concentrated in serum/plasma samples) by centrifugation.

The supernatant containing the RNA is transferred into a new S-Block. This step can be automated on the QCHT. Afterwards the QCHT is loaded according to the instruction and the isolation protocol started. Here, isopropanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The sample is then applied to the RNeasy 96 Plate, where the total RNA binds to the membrane and all contaminants are efficiently washed away. High-quality RNA is eluted in RNase-free water.

Real-time RT-PCR detection of miRNAs using the miRCURY LNA miRNA PCR system

In general, real-time RT-PCR is recommended to accurately quantify yields of miRNA. The miRCURY LNA miRNA PCR system allows sensitive and specific quantification and profiling of miRNA expression using SYBR® Green-based real-time PCR. The miRCURY LNA miRNA PCR system comprises the miRCURY LNA RT Kit, the miRCURY LNA SYBR® Green PCR Kit, miRCURY LNA miRNA PCR Assay, and miRCURY LNA PCR Panels. It covers all the steps of miRNA quantification, from conversion of RNA into cDNA to real-time PCR detection of miRNAs and straightforward data analysis. The RNA Spike-In Kit enables quality control of the RNA isolation, cDNA synthesis, and PCR amplification steps of miRCURY LNA miRNA qPCR experiments.

Individual assays for mature miRNAs for a variety of different species can be ordered on GeneGlobe® (**www.qiagen.com/GeneGlobe**). Alternatively, for high-throughput experiments, miRCURY LNA PCR Panels enable rapid profiling of the complete miRNome. Other Focus panels like the miRCURY LNA miRNA Serum/Plasma Focus PCR Panel are available for the detection of mature miRNAs. Find out more about the miRCURY LNA miRNA PCR System at **www.qiagen.com**.

Description of protocols

This handbook contains one protocol for the purification of cell-free total RNA, including miRNA, from serum or plasma using the miRNeasy 96 Advanced QIAcube HT Kit (see Protocol: Purification of Total RNA, Including miRNA, from Serum and Plasma). In addition, guidelines are provided in the appendices for collection, preparation, and storage of samples, and for storage, quantification, and determination of quality of RNA.

Equipment and Reagents Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Ethanol (80%, and 96% –100%)*
- Isopropanol (100%)
- Sterile, RNase-free pipet tips
- Centrifuge 4–16KS
- QIAcube HT Instrument
- QIAcube HT Prep Manager Software
- QIAcube HT Reagent troughs (70 ml and 170 ml)
- QIAcube HT Plasticware (cat.no. 950067)
- Disposable gloves
- RNA Spike-in Kit, for RT (cat no. 339390)
- Equipment and tubes for serum/plasma collection and separation
- For serum: primary blood collection tube(s) without anticoagulants such as EDTA or citrate
    - For plasma: primary blood collection tube(s) containing EDTA as anticoagulant
    - Conical tube(s)
    - Refrigerated centrifuge with a swinging bucket rotor and fixed-angle rotor

* Do not use denatured alcohol, which contains other substances such as methanol and methylethylketone.

Centrifuge 4–16KS

For optimal handling, QIAGEN, in cooperation with the centrifuge manufacturer Sigma Laborzentrifugen GmbH, has developed a centrifugation system consisting of the Plate Rotor 2 x 96, and the refrigerated table-top Centrifuge 4–16KS. A temperature of 4°C is necessary during phase separation for optimal removal of genomic DNA.

For further information about the centrifuge and rotor, please contact QIAGEN or your local distributor.

Warning: Do not centrifuge the Plate Rotor 2 x 96 metal holders without the RNeasy 96 plates and S-Blocks, collection microtubes, or elution microtubes. If unsupported, the holders will collapse under high g-force. Therefore, remove the holders during test runs.

Standard 96-well microplates may be centrifuged in the holders if a g-force of *500 x g* is not exceeded.

Important Notes

Volume of starting material

The preferred volume of starting material is set to 200µl. This is usually enough to detect also low abundance miRNA in serum/plasma samples. If using less amounts of serum/plasma the volume needs to be filled up to 200µl using TBS.

Yields of total RNA achieved with the miRNeasy 96 Advanced QIAcube HT Kit vary strongly between different plasma samples. However, they are usually too low for quantification by OD measurement. Instead RNA isolation quality can be checked using a Spike-in control. (RNA Spike-In Kit, for RT (cat no. 339390)

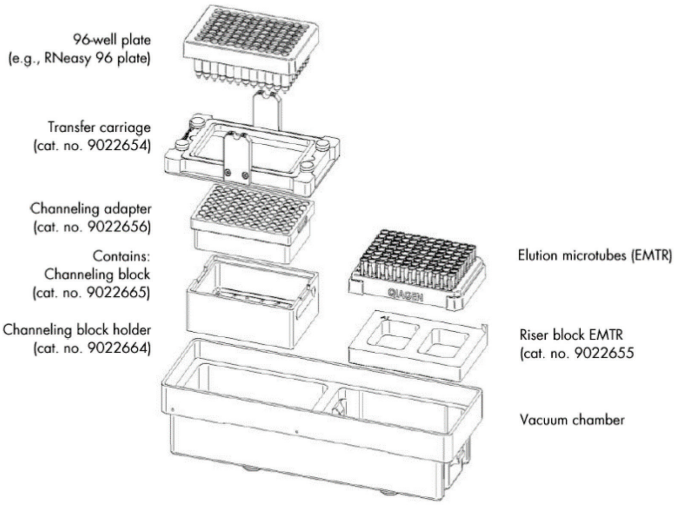
TopElute Fluid

TopElute Fluid is used during elution of nucleic acids from the QIAamp 96 Plate membranes. It enables application of a stable and high vacuum and results in equal eluate volumes. In addition, TopElute Fluid eliminates the formation of drops of elution buffer at the outlet nozzles of the QIAamp 96 Plates.

Assembling the vacuum chamber

Figure 3 illustrates the assembly of the vacuum chamber. For further information, please refer to the *QIAcube HT User Manual*.

1. Insert the channeling block holder into the left (waste) chamber of the vacuum chamber.
2. Press firmly on the sides of the channeling block holder to seat it in the chamber, sealing the O-ring on the spigot into the drain.
3. Place the channeling block into the channeling block holder.
4. Place the RNeasy 96 Plate in the transfer carriage. Load the carriage with the RNeasy 96 Plate into the left (waste) chamber of the vacuum chamber.
5. Ensure that the carriage is positioned to the left inside the vacuum chamber.
6. Place the riser block EMTR in the right (elution) chamber of the vacuum chamber with the pin of the riser block EMTR in the top right position.
7. Load EMTR (without lids) into the elution chamber.



Processing fewer than 96 samples per run

If processing fewer than 96 samples, you can reuse the unused portions of the RNeasy 96 Plates, S-Blocks, and Elution Microtubes RS up to three times.

We recommend using fresh plasticware for every run. If reusing, take extreme care to prevent cross-contamination.

- At a minimum, process at least 24 samples next to each other.
- Process samples together in increments of 8.
- Cover unused wells of the S-Block and RNeasy 96 Plate with adhesive tape.
- Store plates to ensure separation of the outlet nozzles under the plate – in S-Blocks used in the same run or in a fresh 96-well microtiter plate, for example.

Using the UniSp2, UniSp4, UniSp5 RNA Spike-in Mix in RNA Isolation

For further details refer to the *RNA Spike-in Kit, for RT Handbook* at **www.qiagen.com/HB-2433.**

Resuspension of the RNA isolation spike-in mix (UniSp2, UniSp4 and UniSp5)

1. Spin down the vial before opening.
2. Resuspend the spike-in mix by adding 80 µl nuclease-free water to the vial. Leave for 20 minutes on ice to properly dissolve the RNA pellet.
3. Mix by vortexing and briefly centrifuge. Store in aliquots at –30 to –15°C to avoid repeated freeze-thaw cycles.
4. Prior to starting the RNA isolation procedure, add 1 µl of this UniSp2, UniSp4, and UniSp5 RNA spike-in mix per RNA prep to the lysis buffer.

Important note: The RNA spike-in mix must be combined with the lysis buffer before mixing with the sample. If it is added directly to the sample, it may be rapidly degraded.

Note: Do not add the Spike-In Control to your samples.

Protocol: Purification of Total RNA, Including miRNA, from Serum and Plasma

This protocol is for the purification of cell-free total RNA, which primarily includes small RNAs such as miRNAs, from small volumes (50–200 µl) of serum or plasma samples using the miRNeasy 96 Advanced QIAcube HT Kit with the QIAcube HT Prep Manager software. Download the corresponding protocol at **www.qiagen.com**. The protocol is not compatible with QCHT 4.17 software.

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage, page 35.

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –80 to –20°C or in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- DNase I digestion is not necessary for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA. In addition, assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum. Nevertheless, an optional DNase-digest can be selected during protocol set up.
- Buffers RPL and RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- Buffers RPL and RWT contain a guanidine salt and are therefore not compatible with reagents containing bleach.

- Equilibrate buffers to room temperature (15–25°C) before starting the protocol.
- All steps should be performed at room temperature (15–25°C). Work quickly.
- Plasma samples containing heparin should not be used because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add 260 ml ethanol (96%–100%) to Buffer RPE and 160 ml to each bottle of Buffer RWT to obtain a working solution.
- Prepare a working solution of the UniSp2, 4 and 5 spike-ins from the RNA Spike-in Kit, for RT as described in Using the UniSp2, UniSp4, UniSp5 RNA Spike-in Mix in RNA Isolation, page 16.
- All centrifugation steps in the protocol are performed in a centrifuge (Centrifuge 4–16KS).
- Prepare a serum, plasma, or thaw frozen samples. Up to 200 µl human, mouse, or rat plasma can be used. For starting volumes that are less than 200 µl, please fill up the volume to 200 µl with TBS.
- If you use the protocol on the QIAcube HT for the first time please make the Collection Microtube Rack and the RNeasy Plate available as described here: Go to Configuration/Labware Manager/ Plates and select the plates and add them. Further information about the labware manager can be found in the *QIAcube HT User Manual* (**www.qiagen.com/HB-2120**).

Procedure

Preparing the samples

1. Transfer 200 µl serum or plasma per well into a Collection Microtube Rack (CMTR rack). If using less than 200 µl starting material, please fill-up the volume with buffer TBS.
2. Add 60 µl Buffer RPL and close the tubes with caps. Vortex thoroughly, and leave at room temperature for 3 min. Centrifuge shortly to spin down lysate. Optional: RNA spike-in control may be added at this point. Add 1 µl UniSp2, UniSp4, and UniSp5 RNA Spike-in mix. Mix thoroughly.
3. Add 20 µl Buffer RPP. Securely cap the homogenates using the collection microtube caps. Mix vigorously by vortexing for at least 10 s. Incubate for 3min at room temperature.

   Important: Thorough mixing is important for subsequent phase separation.
4. Centrifuge at 6,000 rpm (approximately 5,600 x g) for 3 min at room temperature to remove precipitate.

   Note: Supernatant should be clear and colorless.
5. Transfer supernatant (~230 µl) to a new S-Block.

   Note: Transfer procedure of step 5 can be carried out by QIAcube HT instrument if the starting volume is 200 µl. Select **Transfer Protocol** under **Setup/Selected protocol**. For more details, see Transfer Protocol on page 20.

   Starting volumes less than 200 µl that have been filled up with TBS need to be manually transferred. If an automated transfer for lower starting volumes is desired, please contact QIAGEN Technical Services.

Transfer Protocol

The Transfer Protocol is for the automated transfer of the supernatant from the Collection Microtube Block into a fresh S-block.

Note: This protocol can only be done if 200 µl Serum/Plasma is used at the beginning as starting volume.

1. Start the QIAcube HT Prep Manager software. Click the **Home** icon in the main toolbar.
2. Select miRNeasy 96 Advanced from the **Create Experiment** list. Follow the instructions in the wizard and fill in all required fields.
3. In the **Setup** step, select **Sample type** and **Pre-treatment** for documentation.
4. In the **Selected protocol** field, select the protocol: **Transfer protocol**.
5. Define samples in the **Labware selection** step. See step 5 of miRNeasy 96 Advanced Protocol on the QIAcube HT.
   Note: Check that the Input Labware is a Collection Microtube Rack. If not, change to a Collection Microtube Rack. For further details, refer to *QIAcube HT Prep Manager User Manual*.
6. Arrange samples to the output plate in the **Assignment** step.
   Note: The instrument must be switched on and connected to the software before entering the **Worktable** step.
7. Follow the instructions on the virtual worktable to prepare the workbench of the instrument.
8. Use the Collection Microtube Rack as it comes from the manual pretreatment. Do not add any liquid to the block.
9. Click **Save** before running the setup.
10. Click **Start Run**.
11. When the protocol is finished, start with the "miRNeasy 96 Advanced Protocol".
12. Create a report and save an output file if required.

miRNeasy 96 Advanced Protocol on the QIAcube HT

1. Start the QIAcube HT Prep Manager software. Click the **Home** icon in the main toolbar.
2. Select miRNeasy 96 Advanced from the **Create Experiment** list. Follow the instructions in the wizard and fill in all required fields.
3. In the **Setup** step, select **Sample type** and **Pre-treatment** for documentation.
4. In the **Selected protocol** field, select **miRNeasy 96 Advanced protocol**.
5. Perform these steps in Input Labware window:

   5a. Check the appropriate box based on how you want to use sample IDs.

   - To manually enter sample IDs, check the **Use sample IDs or existing sample input file** box. Then, select whether to enter the sample IDs or load the existing sample file.
   - To automatically generate sample IDs, check the **Auto-generate sample IDs for all samples** box.

   5b. Click **Define samples**.

6. Arrange samples to the output plate in the **Assignment** step.

   Note: The instrument must be switched on and connected to the software before entering the **Worktable** step.
7. Follow the instructions on the virtual worktable to prepare the workbench of the instrument.
8. Add the appropriate sample volume to the selected S-Block wells as indicated in the virtual **Worktable**.
9. Click the **Save** button before running the setup.
10. Click **Start Run**.

    Important: The protocol includes an optional vacuum performance check after the first application of vacuum. This vacuum performance check can be activated during

experimental setup. If activated, the software will show a dialog box that needs to be confirmed after the application of vacuum is completed.

11. When the protocol is finished, cover the Elution Microtube Rack (EMTR) with the lid, and remove it from the elution chamber.

    Note: If Top Elute Fluid was used, elution microtubes of the EMTR will contain two liquid phases. RNA will be in the lower phase covered by Top Elute.

    Important: Please ensure that you only take the eluate from below the top layer for downstream applications.

12. Create a report and save an output file if required.

13. Follow the cleaning procedure.

Cleaning the instrument after completing a run

Follow the instructions in the QIAcube HT Prep Manager Software for cleaning the instrument after a run.

1. Cover the output plate with the lid and remove it from the worktable.
2. Cover tip racks that contain only unused tips with the lid and remove them from the worktable.
3. Cover fractions of partly used tip racks with an adhesive tape. Then, cover the tip racks with the lid and remove from the worktable. Discard empty tip racks.
4. If the run has been stopped and the instrument did not remove all used tips, remove them now and discard them.
5. Remove all reagent troughs and discard them.

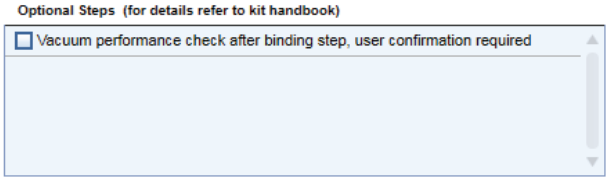
   Note: We recommend not reusing reagents for multiple runs.
6. Remove the input plate.
7. Discard the RNeasy 96 plate or keep partially used RNeasy 96 plates for subsequent reuse. In this case, cover used fractions with an adhesive tape.
8. Remove the tip chute and all adapters from the worktable. Remove the carriage, channeling adapter, and riser block from the vacuum chamber. Clean all parts as described in the *QIAcube HT User Manual*.
9. Discard the tip disposal box.
10. Clean any reagents that may have spilled on the instrument worktable or vacuum chamber with a damp cloth.
11. Discard all waste according to local safety regulations.

Note: For all further cleaning and maintenance operations, see the *QIAcube HT User Manual* for detailed instructions.

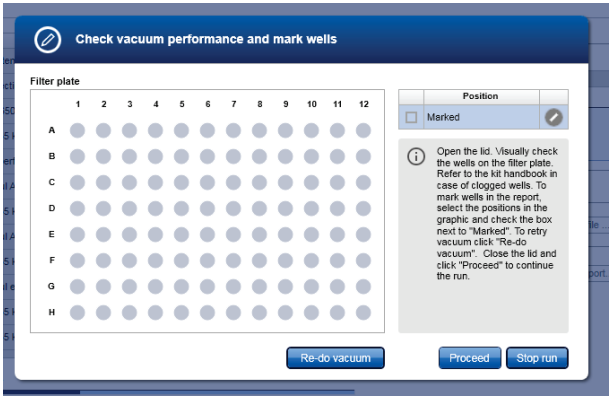
Optional steps

Vacuum performance check

Using the vacuum performance check option results in one manual intermediate step after the binding process. This optional setting allows the user to check whether all the liquid has passed through the membranes. By default, this step is not activated.



If the vacuum performance check step is activated, the instrument will pause after the binding step. The intermediate step allows to evaluate if all liquid has passed through the membranes. If liquid remains on the surface, re-apply vacuum (**Re-do vacuum**), if not, continue with the procedure (**Proceed**).



1. Open the instrument lid.

   Note: The lid sensor is disabled during the vacuum performance check, allowing to the user to observe the wells.

2. Check the wells on the RNeasy 96 plate for any remaining liquid.

   If no liquid is visible in the wells after the vacuum step, click **Proceed**.

   If liquid remains in the wells, click **Re-do vacuum** to apply the same vacuum pressure again. The vacuum will be activated for a certain time or until you click the **Stop vacuum** button.

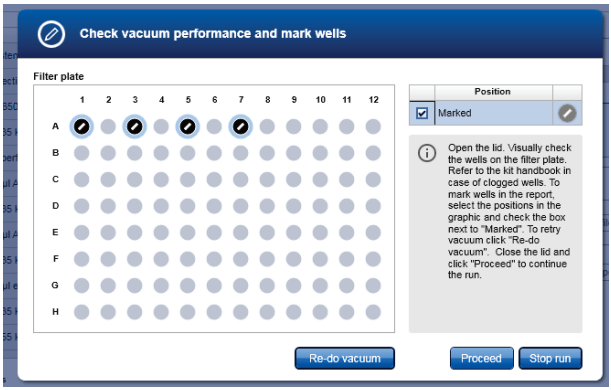
3. Mark any well that is clogged or not empty in the dialog box that appears. This information will be included in the run report. To mark a well, select a position in the dialog box.

   In selecting multiple positions, perform one of the following steps:

- To select multiple adjacent positions, press the Shift key on your keyboard as you click. You can also drag the mouse to select adjacent positions in a rectangle.
- To select multiple, nonadjacent positions, press the Ctrl key on your keyboard as you click.

  In the position table at the right, check the box next to **Marked**. The selected position on the RNeasy 96 plate will be displayed as marked.

  Note: To unmark a position, select the position and uncheck the box next to **Marked**.



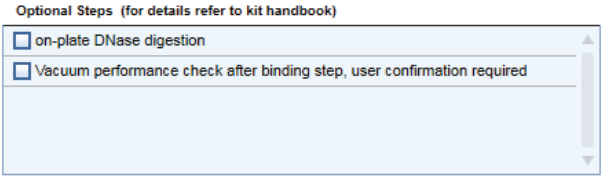
4. If liquid still remains in any well, manually remove the liquid using a pipet.
5. After the instrument has added additional reagents, open the hood to pause the run. Check to see whether the affected well is still blocked. If so, manually remove the liquid from the affected well using a pipet.
6. Click **Proceed** to continue the run, or click **Stop run**.

   If you click Stop run, a confirmation message appears . Click **Cancel** to close the message, or click **OK** to stop the run.

DNase digestion

Generally, DNase digestion is not required, because the RNeasy 96 technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA.

If additional DNase digestion should be carried out during the run, check the option **on-plate DNase digestion**. See Appendix C: Storage, Quantification, and Determination of Quality of RNA on page 43 for more details.



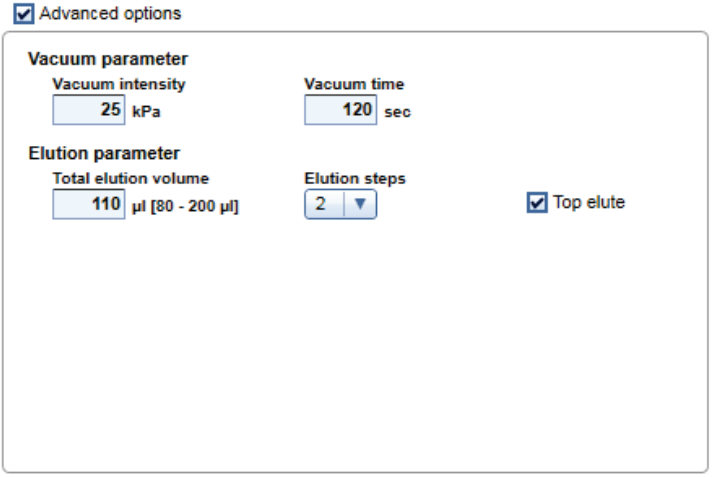
Note: Two RNase-Free DNase Sets are required for a single run with 96 samples, if performing the optional DNase digestion step. RNase Free DNase Sets (50) (cat. no. 79254) can be ordered in **www.qiagen.com**.

Advanced options

Important: QIAGEN does not recommend modifying any of the parameters found in the Advanced options section.

These parameters have been optimized for each QIAcube HT Kit to guarantee accurate and valid experiment results. Please note that any changes to these options are carried out at your own risk.

Note: A warning icon and a corresponding warning message will be displayed if you change any parameter. The warning text contains the recommended value. If you return to the recommended value, the warning message will disappear.



Vacuum parameter

In the **Vacuum parameter** section, it is possible to change two parameters: vacuum intensity and vacuum time. The default settings are 25 kPa for the vacuum intensity.

The vacuum intensity can be changed from 25 kPa to 70 kPa. Changing the vacuum intensity parameter only affects the vacuum intensity following the binding step. All other vacuum steps will be unaffected.

The vacuum time can be changed between 60–360 sec. Changing the vacuum time parameter only affects the vacuum time following the binding step. All other vacuum steps will be unaffected.

Elution parameter

In the **Elution parameter** section, it is possible to change the total elution volume and the elution step. The recommended values for these parameters are shown in the QIAcube HT Prep Manager Software. The total elution volume can be changed to another value within the defined range.

Changing the number of elution steps will affect the elution buffer distribution, incubation pause, and vacuum step(s) without influencing the total amount of elution volume.

TopElute

TopElute Fluid is used during elution of nucleic acids from the RNeasy membrane. It enables application of a stable and high vacuum and results in equal eluate volumes. In addition, TopElute Fluid eliminates the formation of drops of elution buffer at the outlet nozzles of the RNeasy 96 plates. By default, the Top Elute option is checked. In case TopElute Fluid should not be used during the run, uncheck the **TopElute** option under **Advanced** options.

Important: Changing the usage of TopElute Fluid is not recommended or tested by QIAGEN.

Note: TopElute Fluid might be found as a top layer over the elution buffer. It is inert and has no effects on downstream applications.

Important: Please ensure that you only take the eluate from below the top layer.

Troubleshooting Guide

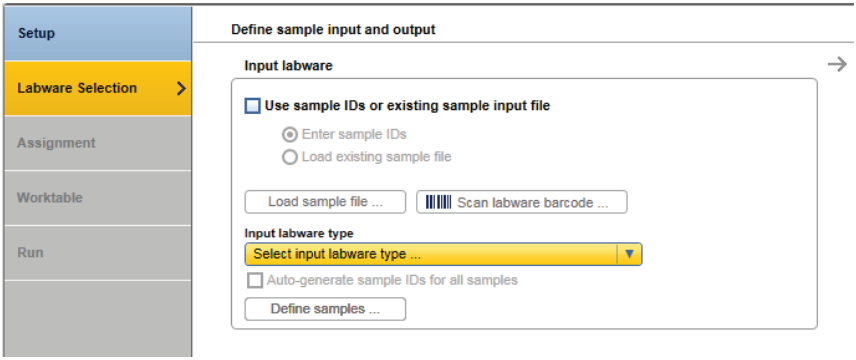
This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit **www.qiagen.com**).

		Comments and suggestions
Clogged column		Please make sure not to transfer any of the pellet into the new S-Block. If using the automated transfer, adjustments need to be made. In this case, please contact the Application Lab. Do not use more than 200 µl starting material.
Low miRNA yield or poor performance of miRNA in downstream applications		
a)	Low quality starting material	The RNA content may vary from sample to sample. Please make sure that samples were treated appropriately before staring the isolation process.
RNA degraded		
a)	Sample inappropriately handled	Perform the protocol quickly, especially the first few steps. See "Appendix C: Storage, Quantification, and Determination of Quality of RNA" (page 43).
b)	RNase contamination	Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Make sure not to introduce any RNases during the procedure or later handling. See "Appendix B: General Remarks on Handling RNA" (page 40). Do not put RNA samples into a vacuum dryer that has been used in DNA preparations where RNases may have been used.
RNA does not perform well in downstream experiments		
a)	Salt carryover during elution	Ensure that Buffer RPE is at 15–25°C.
b.)	Inhibitors	Do not use more than 200 µl. Do not use Heparin Tubes.

		Comments and suggestions
Contamination by genomic DNA		
a)	Sample inappropriately handled	Invert tubes gently to mix contents after blood collection. Vigorous mixing or shaking can promote hemolysis. Generate plasma as quickly as possible after blood collection. Long delays can promote hemolysis or apoptotic cell death. Perform the second centrifugation or filtration before freezing the plasma, if possible. See Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage for details.

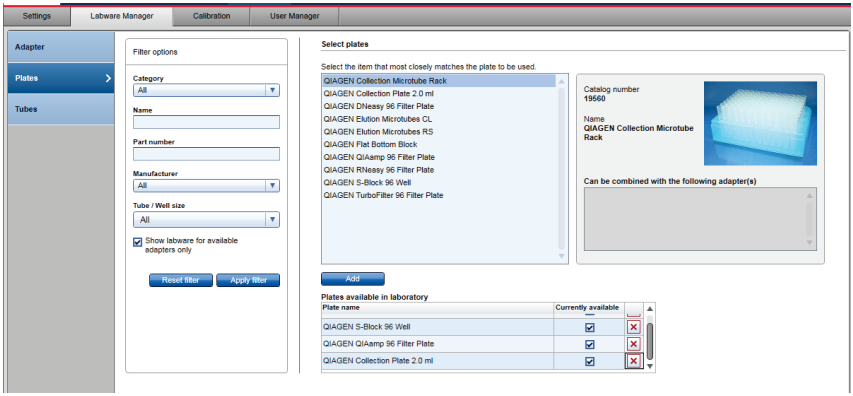
Instrument

Error message in the Transfer Protocol / Transfer Protocol could not be started



If the Collection Microtube Rack is not available in the Labware Manager, the **Input labware type** field is marked as yellow.

Go to the Labware Manager and add the QIAGEN Collection microtube rack. Click **Add** then **Save**. Open the miRNA Advanced experiment again. Verify if the input plate is valid.



miRNeasy 96 Advanced Protocol could not be scheduled

Check if RNeasy 96 Plate was added in Labware Selection. Go to the Labware Manager, and add the QIAGEN RNeasy 96 Plate. Click **Add**, then **Save**. Open the miRNA Advanced experiment again. Verify if the input plate is valid.

Appendix A: Recommendations for Serum and Plasma Collection, Separation, and Storage

In order to isolate circulating, cell-free nucleic acids from whole blood samples, we recommend following these protocols. The protocols include an initial low *g*-force centrifugation step to separate cells from plasma or serum followed by a high *g*-force centrifugation or filtration step to remove all remaining cellular debris. The latter centrifugation step significantly reduces the amount of cellular or genomic DNA and RNA in the sample. Because of the much higher abundance of RNA in cells, even small amounts of cellular debris can have a very significant effect on RNA profiling of cell-free fluids. The sooner after blood collection this removal of cellular materials is performed, the lower the risk of additional background from blood cell-derived nucleic acid released in vitro. Use of gel barrier tubes generally results in fewer residual cells.

The speed at which the second centrifugation step is performed will influence the recovery of different types of nucleic acid. Centrifugation at medium speed (e.g. 3,000 x g) will effectively remove cellular material, including thrombocyte fragments and apoptotic bodies. Centrifugation at higher speed (e.g. 16,000 x g) may in addition remove intact chromatin from ruptured blood cells, but may also remove larger extracellular vesicles that may contain cell-free nucleic acid (especially mRNA).

Syringe filters with 0.8 µm pore size (e.g. Sartorius® Minisart® NML (cat. no. 16592) or Millipore® Millex®-AA (cat. no. SLAA033SB)) remove remaining cell fragments and debris strictly based on size, irrespective of density. These filters have dead volumes of around 100–200 µl.

Procedure: Plasma Separation and Storage

1. Collect whole blood in BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA (or any other primary blood collection tube containing EDTA as anticoagulant). Store tubes at room temperature (15–25°C) or 4°C and process within 1 hour.

   Note: Do not use heparin-containing blood collection tubes as this anticoagulant can interfere with downstream assays, such as RT-PCR.

2. Centrifuge blood samples in primary blood collection tubes for 10 min at 1900 x *g* (3000 rpm) and 4°C using a swinging bucket rotor.

3. Carefully transfer the upper (yellow) plasma phase to a new tube (with conical bottom) without disturbing the intermediate buffy coat layer (containing white blood cells and platelets). Normally up to 4–5 ml plasma can be obtained from 10 ml of whole blood.

   Note: Carryover of white blood cells and platelets from the buffy coat layer is the most likely source of cellular miRNA/RNA contamination in plasma.

   Note: Plasma can be used for cell-free nucleic acid purification at this stage. However, an additional Filtration or centrifugation will remove additional cellular debris and minimize contamination of cell-free nucleic acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge plasma samples in conical tubes for 15 min at 3,000 x g (or 10 min at 16,000 x g — see above) and 4°C or pass through a 0.8 µm filter (see recommendations above). This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet (which forms a smear along the outer side / bottom of the centrifugation tube).

6. Store at 2–8°C until further processing, if plasma will be used for nucleic acid purification on the same day. For longer storage, keep plasma frozen in aliquots at –90 to –65°C.

7. Before using frozen plasma for nucleic acid purification, thaw at room temperature (15–25°C). Optional: To remove cryoprecipitates, centrifuge thawed plasma samples for 5 min

at 3,000 x *g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Procedure: Serum Separation and Storage

1. Collect whole blood in a primary blood collection tube with or without clot activator, but without anticoagulants such as EDTA or citrate (e.g., Sarstedt S-Monovette® Serum-Gel 9 ml tubes, cat. no. 02.1388). For complete clotting, leave tubes at room temperature (15–25°C) for 10 min to 1 h.

   Note: Tubes with clot activator can be processed after 10 min clotting time, while tubes without clot activator should be stored for at least 30 min at room temperature to allow clotting to take place.

2. Centrifuge tubes for 10 min at 1900 x *g* (3000 rpm) and 4°C using a swinging bucket rotor.

   Note: If using Sarstedt S-Monovette Serum-Gel 9ml tubes, a gel bed will form between the upper serum phase and the lower cellular phase, facilitating recovery of serum.

3. Carefully transfer the upper (yellow) serum phase to a new tube (with conical bottom) without disturbing the pellet containing cellular material. Normally up to 3–5 ml serum can be obtained from 10 ml of whole blood.

   Note: Prevent transfer of cellular material from the lower phase.

   Note: Serum can be used for cell-free nucleic acid purification at this stage. However, an additional filtration or centrifugation will remove additional cellular debris and minimize contamination acids by gDNA and RNA derived from damaged blood cells.

4. Centrifuge serum samples in conical tubes for 15 min at 3,000 x g (or 10 min at 16,000 x g — see above) and 4°C or pass through a 0.8 µm filter (see recommendations above). This will remove additional cellular nucleic acids attached to cell debris.

5. Carefully transfer cleared supernatant to a new tube without disturbing the pellet (which forms a smear along the outer side of the centrifugation tube).

6. Store at 2–8°C until further processing, if serum will be used for nucleic acid purification on the same day. For longer storage, keep serum frozen in aliquots at –90 to –65°C.

7. Before using frozen serum for nucleic acid purification, thaw at room temperature (15–25°C). Optional: To remove cryoprecipitates, centrifuge thawed serum samples for 5 min at 3,000 x *g* and 4°C or pass through a 0.8 µm filter. Transfer supernatant to a new tube, and begin nucleic acid purification protocol.

Appendix B: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to degrade RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipets and electrophoresis tanks), use of a Decontamination Solution is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 41), or rinse with chloroform* if the

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material data sheets (SDSs), available from the product supplier.

plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for 4 hours or more (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions)* should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer.

Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

Appendix C: Storage, Quantification, and Determination of Quality of RNA

Storage of DNA/RNA Eluates

Purified DNA/RNA may be stored at –30 to –15°C to or –90 to –65°C to in RNase-free water. Under these conditions, no degradation of DNA or RNA is detectable after 1 year.

Quantification of RNA

The concentration of ccfRNA should not be determined by spectrophotometric quantification, because the amounts present in serum and plasma are usually too low for reliable measurements. Small amounts of DNA and RNA can best be quantified using quantitative PCR / RT-PCR. Fluorometric quantification (e.g. using Qubit) is often unreliable for short nucleic acid fragments.

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While miRNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample. However, serum, plasma, and other cell-free body fluids contain very little DNA.

For analysis of very low-abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in gene expression analysis real-time RT-PCR applications, such as with ABI PRISM and LightCycler instruments, we recommend designing primers that

anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Primer Assays from QIAGEN are designed for SYBR® Green based real-time RT-PCR analysis of RNA sequences (without detection of genomic DNA) where possible (the assays can be ordered online at **www.qiagen.com/GeneGlobe**). For real-time RT-PCR assays where amplification of genomic DNA cannot be avoided, we recommend using the QuantiTect Reverse Transcription Kit for reverse transcription. The kit integrates fast cDNA synthesis with rapid removal of genomic DNA contamination.

Alternatively, gene expression analysis can be performed using QuantiFast® Probe Assays and the QuantiFast Probe RT-PCR Plus Kit, which includes an integrated genomic DNA removal step.

Integrity of RNA

Cell-free RNA from serum or plasma consists mainly of small RNAs of less than 100 nucleotides. Appearance of rRNA bands is usually indicative of contamination by cells or cell debris. Therefore RNA integrity cannot be analyzed by denaturing agarose gel electrophoresis and ethidium bromide* staining or by using the QIAxcel® system or Agilent 2100 Bioanalyzer.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Ordering Information

Product	Contents	Cat. no.
miRNeasy 96 Advanced QIAcube HT Kit	Contains Buffer RPL, Buffer RPP, Buffer RWT, Buffer RPE, RNase-free water, TopElute Fluid, RNeasy 96 Plates, Collection Microtube Rack, and caps for Collection Microtube (55 x 8)	217261
QIAcube HT plastic ware	Contains S-Blocks, Filter-Tips OnCor C, Tape pad, Elution Microtube RS, and 8-well Strip Caps for Elution Microtube RS	950067
QIAcube HT System	Robotic workstation with UV lamp, HEPA filter, laptop, QIAcube HT operating software, startup pack, installation and training, 1 year warranty on parts and labor	9001793
Reagent Trough (with lid), 70 ml	Box of 10 plus lid; Liquid reservoirs; Used with Reagent Trough R1 Position Plate and Reagent Trough SBS Plate	990554
Reagent Trough (with lid), 170 ml	Box of 20 plus lid; Liquid reservoirs; Used with Reagent Trough R1 Position Plate and Reagent Trough SBS Plate	990556
S- Blocks (24)	96-well blocks with 2.2 ml wells, 24 per case	19585
Centrifuge 4–16 KS	Refrigerated universal laboratory centrifuge with brushless motor (220–240 V, 50/60Hz)	81610

RNA Spike-In Kit, for RT	Contains the UniSP2, UniSP4, and UniSP5 RNA Spike-In Template Mix and the cel-miR-39-3p RNA Spike-In Template	339390
RNase Free DNase Sets (50)	1500 Kunitz units RNase-free DNase I, RNase-free Buffer RDD, and RNase-free water	79254

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

R1	Initial release
02/2019	

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