Emory Integrated Genomics Core

Emory Integrated Core Facilities

EIGC.002_Appendix F_Qiagen miRNeasy from Serum/Plasma

Standard Operating Procedure Staff Review Page:

I have read and understand the procedure listed above.

Employee name	Date SOP review complete

Standard Operating Procedure Approval Page:

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Annual Review and Approval

Michael Zwick, PhD
Laboratory Director

Changes Made:
061620: Review, Protocols split out into appendixes. Renumbered SOPs. Previously EIGC.003.

Changes to previous procedures:
041116: Review, LIMS steps updated

Introduction

The miRNeasy Serum/Plasma Kit is designed for purification of cell-free total RNA — primarily miRNA and other small RNA — from small volumes of serum and plasma. This kit may also be used for small volumes of other body fluids (e.g., urine).
When working with serum and plasma samples, we recommend use of a synthetic spike-in control for normalization, such as the miRNeasy Serum/Plasma Spike-In Control. The miRNeasy Serum/Plasma Spike-In Control must be ordered separately (cat. no. 219610). The miRNeasy Serum/Plasma Kit includes a miScript Primer Assay that detects the miRNeasy Serum/Plasma Spike-In Control in real-time PCR.

Principle

The miRNeasy Serum/Plasma Kit combines phenol/guanidine-based lysis of samples and silica-membrane–based purification of total RNA. QIAzol Lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis, to denature protein complexes and RNases, and also to remove most of the residual DNA and proteins from the lysate by organic extraction.

QIAzol Lysis Reagent is added to serum or plasma samples. After addition of chloroform, the lysate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase, while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase.

The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from approximately 18 nucleotides (nt) upwards. The same is then applied to the RNeasy MinElute spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of RNase-free water.

Serum and plasma contain primarily small RNAs, therefore enrichment of miRNAs and other small RNAs in a separate fraction is usually not required.

KIT CONTENTS:

MiRNeasy Serum/ Plasma Kit	(50)
Catalog No	217184
Number of Preps	50
RNeasy MinElute Spin Columns (each packed with 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2.0 ml)	50
QIAzol Lysis Reagent[a]	50 ml
Buffer RWT[a,b]	15 ml
Buffer RPE[c]	11 ml
Ce_Mr-39_1 miScript Primer Assay	(100)
Rnase-Free-Water	10 ml
Quick Start Protocol	1

[a] Contains guanidine salt. Not compatible with disinfecting agents containing bleach; see handbook for safety information.

[b] 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

[c] 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.

miRNeasy Serum/Plasma Spike-In Control Catalog No.	10 pmol 219610
Lyophilized C elegans miR-39 miRNA mimic	10 pmol

Storage

The miRNeasy Serum/Plasma Kit (cat. no. 217184) is shipped at ambient temperature. Store the RNeasy MinElute spin columns immediately at 2–8°C. QIAzol Lysis Reagent can be stored at room temperature (15–25°C) or at 2–8°C. Store the Ce_miR-39_1 miScript Primer Assay at–15°C to –30°C either lyophilized or reconstituted (see next paragraph). Store the remaining components dry at room temperature. All kit components are stable for at least 9 months under these conditions, if not otherwise stated on the label.
To reconstitute Ce_miR-39_1 miScript Primer Assay briefly centrifuge the vial, add 550 µl TE, pH 8.0, and mix by vortexing the vial 4–6 times. This will provide sufficient primer for 100 x 50 µl reactions. We recommend freezing the reconstituted primers in aliquots to avoid repeated freezing and thawing.
The miRNeasy Serum/Plasma Spike-In Control is shipped at ambient temperature. Store at–15°C to –30°C, either reconstituted or lyophilized.

Procedure

Notes before starting

- You will need the following additional equipment and reagents:
  - Chloroform (without added isoamyl alcohol)
  - Ethanol (70%, 80% and 96–100%)*
  - Sterile, RNase-free pipet tips
  - 1.5 ml or 2 ml microcentrifuge tubes
  - Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4°C and at room temperature (15–25°C)

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 –30°C to –15°C or –90 to –65°C in aliquots is recommended. To process frozen lysates, incubate at 37ºC 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 recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA, and the combined QIAzol and RNeasy technologies efficiently remove most of the trace amounts of DNA in plasma and serum. In addition, miScript Primer Assays and most other 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.

This protocol is intended as a guideline for the purification of cell-free total RNA, which primarily includes small RNAs, such as miRNAs, from small volumes (up to 200 µl) of serum and plasma using the miRNeasy Serum/Plasma Kit. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

Buffer RWT may form a precipitate upon storage. If necessary, re-dissolve by warming and then place at room temperature (15–25ºC).

QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.

Except for phase separation (step 7), all protocol and centrifugation steps should be performed at room temperature.

The procedure is suitable for use with either serum samples or plasma samples containing citrate or EDTA. 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 the required volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.

Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 27.

Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use poly-A RNA.

Note: All RNA extraction processes should be performed in the chemical hood.

1. **Prepare serum or plasma or thaw frozen samples.**

2. **Add 5 volumes QIAzol Lysis Reagent (see Table 1. for guidelines). Mix by vortexing or pipetting up and down.**

Table 1. QIAzol Lysis Reagent volumes for various serum/plasma volumes

Serum/plasma (µl)	Protocol step 2: QIAzol Lysis Reagent (µl)	Protocol step 5: chloroform (µl)	Protocol step 7: approx. volume of upper aqueous phase (µl)	Protocol step 8: 100% ethanol (µl)
≤50	250	50	150	225
100	500	100	300	450
200	1000	200	600	900

Note: If the volume of plasma or serum is not limited, we recommend using 100–200 µl per RNA preparation.
Note: After addition of QIAzol Lysis Reagent, lysates can be stored at –70ºC for several months.

3. **Place the tube containing the lysate on the benchtop at room temperature (15–25°C) for 5 min.**

4. **Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.**

5. **Add chloroform of an equal volume to the starting sample to the tube containing the lysate and cap it securely (see Table 2 for guidelines). Vortex or shake vigorously for 15 s.**
   Thorough mixing is important for subsequent phase separation.

6. **Place the tube containing the lysate on the benchtop at room temperature for 2–3 min.**

7. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature if the same centrifuge will be used for the next centrifugation steps.**
   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. See Table 2 for the approximate volume of the aqueous phase.

8. **Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.**

   A precipitate may form after addition of ethanol, but this will not affect the procedure.

9. **Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at 8000 x g (10,000 rpm) for 15 s at room temperature. Discard the flow-through. Reuse the collection tube in step 10.**

10. **Repeat step 9 using the remainder of the sample. Discard the flow-through. Reuse the collection tube in step 11.**

11. **Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm) to wash the column. Discard the flow- through. Reuse the collection tube in step 12.**

12. **Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm) to wash the column. Discard the flow- through. Reuse the collection tube in step 13.**

13. **Pipet 500 µl of 80% ethanol onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.**

    Note: 80% ethanol should be prepared with ethanol (96–100%) and RNase-free water.

    Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

14. **Place the RNeasy MinElute spin column into a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min to dry the membrane. Discard the collection tube with the flow-through.**

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).
    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

15. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.
    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase- free water results in a 12 µl eluate.

Preparation of miRNeasy Serum/Plasma Spike-In Control

The miRNeasy Serum/Plasma Spike-In Control is a C. elegans miR-39 miRNA mimic and is supplied lyophilized at 10 pmol per tube. Reconstitute by adding 300 µl RNase-free water per tube, resulting in a 2 x 1010 copies/µl stock. miRNeasy Serum/Plasma Spike-In Control stock should be stored at –90°C to –65°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –90°C to –65°C.

When working with miRNeasy Serum/Plasma Spike-In Control, first add 4 µl of 2 x 1010 copies/µl miRNeasy Serum/Plasma Spike-In Control stock to 16 µl RNase-free water, resulting in a 4 x 109 copies/µl dilution. If performing purification of RNA from serum and plasma, add 2 µl of the 4 x 109 copies/µl dilution to 48 µl RNase-free water to provide a 1.6 x 108 copies/µl working solution. If generating a standard curve, add 2 µl of the 4 x 109 copies/µl dilution to 78 µl RNase-free water that contains carrier RNA (e.g., 10 ng/µl MS2 [Roche, cat. no. 10 165 948 001] or bacterial ribosomal RNA [Roche, cat. no. 10 206 938 001]) to provide a 1 x 108 copies/µl working solution. These dilutions are summarized in Table below.

Table 2. miScript Serum/Plasma Spike-In Control dilutions

Purpose	Dilution	Concentration (copies/µl)
Stock	Add 300 µl RNase-free water to lyophilized miScript Serum/Plasma Spike-In Control (10 pmol)	2×10^{10}
Dilution	Add 4 µl stock (2×10^{10} copies/µl) to 16 µl RNase-free water	4×10^{9}
Working solution for RNA purification (page 15)	Add 2 µl of 4×10^{9} copies/µl dilution to 48 µl RNase-free water	1.6×10^{8}
Working solution for generation of standard curve (page 29)	Add 2 µl of 4×10^{9} copies/µl dilution to 78 µl RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001)	1×10^{8}

Generation of miRNeasy Serum/Plasma Spike-In Control Standard Curve

This protocol is for generating a real-time PCR standard curve of miRNeasy Serum/Plasma Spike-In Control that is independent of a serum/plasma sample and RNA purification procedure. The standard curve allows estimation of the recovery of miRNeasy Serum/Plasma Spike-In Control when it is added to a serum/plasma sample that is subsequently used for RNA purification (see protocol on page 15).

Important points before starting

- To ensure reproducibility, always use freshly prepared cDNA to generate a standard curve. Perform PCRs for generation of the standard curve and PCRs on RNA from the serum/plasma samples of interest in the same run. Do not store cDNA dilutions for later use.
- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the miRNeasy Serum/Plasma Kit), miScript II RT Kit, miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit **www.qiagen.com/miRNA**.

Procedure

B1. Prepare a 1 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently yet thoroughly.

For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 28.

For dilution of the control, we recommend RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no.10 206 938 001).

B2. Prepare the revaerse transcription reaction on ice according to Table 3.

Table 3.

Component	Volume
MiRNeasy Serum/Plasma Spike-In Control from spep 1 (1x 10^8 copies/µl)	2.2 µl (2.2 x 108 copies/µl)
Total RNA sample[a]	2 µl (~100 ng)
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer[b]	4 µl
10x miScript Nucleics Mix	2 µl
Rnase-free water	7.8 µl
MiScript Reverse Transcriptase Mix	2 µl
Total volume	20 µl

a. Any total RNA sample can be used here to provide a complex RNA background
b. The correct buffer to use depends on the subsequent PCR application. Consult the *miScript PCR System Handbook* for more details.

B3. Gently mix, briefly centrifuge, and then store on ice.

B4. Incubate for 60 min at 37ºC.

B5. Incubate for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and place on ice.

B6. Add 200 µl RNase-free water to the reverse transcription reaction.

This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 1 x 10^6 copies/µl.

B7. Use the diluted reverse transcription reaction to prepare cDNA serial dilutions according to Serial dilutions below.

cDNA serial dilutions

Tube			cDNA	Water	Concentration spike-in control			Use in PCR
1	20	µl	diluted cDNA	20 µl	5 x 105 copies/µl	2	µl	(1 x 106 copies)
2	5	µl	from tube 1	45 µl	5 x 104 copies/µl	2	µl	(1 x 105 copies)
3	5	µl	from tube 2	45 µl	5 x 103 copies/µl	2	µl	(1 x 104 copies)
4	5	µl	from tube 3	45 µl	5 x 102 copies/µl	2	µl	(1 x 103 copies)

B8. Using 2 µl from each tube in Table 4, set up separate PCRs according to Table 6.

We recommend setting up each reaction in triplicate.

Table 4. Reaction set up for real-time PCR

Component	Volume/reaction (384-well)		Volume/reaction (96-well)	Volume/reaction (Rotor-Disc®100)*	
2x QuantiTect® SYBR Green PCR Master Mix	5	µl	12.5 µl	10	µl
10x miScript Universal Primer	1	µl	2.5 µl	2	µl
10x Ce_miR-39_1 miScript Primer Assay	1	µl	2.5 µl	2	µl
RNase-free water	1	µl	5.5 µl	4	µl
Template cDNA from Table 5	2	µl	2 µl	2	µl
Total volume	10	µl	25 µl	20	µl

B9. Mix thoroughly and proceed with PCR using the cycling conditions in Table 7.

Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real-time cyclers. Follow the instructions provided by the supplier.

Table 5. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension§	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles¶		Cycle number depends on the amount of template cDNA and abundance of the target.

B10.Extract CT values for miRNeasy Serum/Plasma Spike-In Control from each reaction.

B11.Generate a standard curve by plotting the log copy number miRNeasy Serum/Plasma Spike-In Control used in each PCR against the mean CT value.

Protocol: Assessment of Recovery of miRNeasy Serum/Plasma Spike- In Control after miRNA Purification

This protocol is a guideline for the addition of miRNeasy Serum/Plasma Spike-In Control to a serum/plasma sample during RNA purification, followed by determination of recovery of miRNeasy Serum/Plasma Spike-In Control by real-time RT-PCR using the standard curve generated in the protocol on page 29.

Important point before starting

- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the miRNeasy Serum/Plasma Kit), miScript II RT Kit, miScript SYBR Green PCR Kit.

Procedure

B1. Prepare a 1.6 x 10^8 copies/µl working solution of miRNeasy Serum/Plasma Spike-In Control. Mix gently yet thoroughly.

For details of preparation of miRNeasy Serum/Plasma Spike-In Control working solution, see Table 3, page 28.

B2. During RNA purification, add 3.5 µl miRNeasy Serum/Plasma Spike-In Control working solution from step B1 (1.6 x 10^8 copies/µl) to the sample after lysis with QIAzol Lysis Reagent (see step 4, page 16). Mix thoroughly.

We recommend addition of miRNeasy Serum/Plasma Spike-In Control after lysis to avoid degradation by endogenous RNases in the sample. This can be modified if desired.

B3. Continue with RNA purification (page 16). After RNA elution in 14 µl RNase-free water (step 15, page 17) miRNeasy Serum/Plasma Spike-In Control is present in the eluate at 4 x 10^7 copies/µl. If a different elution volume is used, calculate the miRNeasy Serum/Plasma Spike-In Control concentration accordingly.

B4. Prepare the reverse transcription reaction on ice according to Table 6.

Table 6. Reverse transcription reaction components

Component	Volume
Purified RNA (containing miRNeasy Serum/Plasma Spike-In Control)	1.5 µl
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer*	4 µl
10x miScript Nucleics Mix	2 µl
RNase-free water	10.5 µl
miScript Reverse Transcriptase Mix	2 µl
Total volume	20 µl

B5. Gently mix, briefly centrifuge, and then store on ice. B6. Incubate for 60 min at 37ºC.

B7. Incubate for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and place on ice.

B8. Add 200 µl RNase-free water to the reverse transcription reaction.

This results in a miRNeasy Serum/Plasma Spike-In Control concentration of 2.7 x 10^5 copies/µl (assuming 100% recovery during RNA purification and reverse transcription).

B9. Set up PCRs according to Table 9.

We recommend setting up each reaction in triplicate

Table 7. Reaction set up for real-time PCR

Component	Volume/reaction (384-well)		Volume/reaction (96-well)		Volume/reaction (Rotor-Disc 100)*	
2x QuantiTect SYBR Green PCR Master Mix	5	µl	12.5 µl	10	µl	
10x miScript Universal Primer	1	µl	2.5 µl	2	µl	
10x Ce_miR-39_1 miScript Primer Assay	1	µl	2.5 µl	2	µl	
RNase-free water	2	µl	6.5 µl	5	µl	
Diluted reverse transcription reaction	1	µl	1 µl	1	µl	
Total volume	10	µl	25 µl	20	µl	

B10. Mix thoroughly and proceed with PCR using the cycling conditions in Table 8.

Note: Perform dissociation curve analysis of the PCR product(s) to verify their specificity and identity. Dissociation curve analysis is an analysis step built into the software of real- time cyclers. Follow the instructions provided by the supplier.

Table 8. Cycling conditions for real-time PCR

Step	Time	Temperature	Additional comments
PCR Initial activation step	15 min	95ºC	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:*†‡			
Denaturation	15 s	94ºC	
Annealing	30 s	55ºC	
Extension§	30 s	70ºC	Perform fluorescence data collection.
Cycle number	40 cycles¶		Cycle number depends on the amount of template cDNA and abundance of the target.

B11. Extract C_T values and determine the mean C_T value for miRNeasy Serum/Plasma Spike-In Control from each reaction.

B12. Compare with the miRNeasy Serum/Plasma Spike-In Control standard curve to determine recovery of miRNeasy Serum/Plasma Spike-In Control.