NORGEN BIOTEK CORPORATION

3430 Schmon Parkway
Thorold, ON, Canada L2V 4Y6
Phone: 866-667-4362 • (905) 227-8848
Fax: (905) 227-1061
Email: techsupport@norgenbiotek.com

microRNA (cel-miR-39) Spike-In Kit Product Insert

Product # 59000

The amount of RNA that can be extracted from different biological or clinical samples varies greatly. For example, while a few micrograms of RNA could be easily purified from tissues and cells in excess amounts (such as from a few milligrams of tissue), many liquid biopsy samples may yield very low amounts of RNA. In fact, samples such as urine or plasma may yield 1 - 100 ng or less RNA per 100 µL of sample. Such a range of RNA quantity is often below the detection limit of most commonly used techniques for measuring RNA including nano-spectrophotometry and fluorescent nucleic acid stains. As a result, without properly determined RNA concentration, it becomes very difficult to normalize the starting quantity of RNA used in gene expression studies.

Norgen's microRNA (cel-miR-39) Spike-In Kit offers a quantified synthetic RNA (cel-miR-39) for spike-in during RNA extraction procedures and subsequent normalization in RT-qPCR assays. The amount of cel-miR-39 RNA recovered after RNA extraction is directly correlated with the amount of total RNA recovered. After reverse transcription (such as with Norgen's microScript Reverse Transcription system) of the sample RNA (with spike-in), the level of cel-miR-39 could be determined by subjecting the cDNA generated to quantitative PCR (qPCR) using fluorescent nucleic acid stains such as SYBR Green. A cel-miR-39 specific primer is included in the kit. The level of expression of any target transcripts in different RNA samples can now be normalized to the cel-miR-39 transcript level using standard method such as ΔΔCt relative quantification. In addition, the cel-miR-39 RNA is compatible to library preparation methods (including ligation-based protocols) in Next Generation Sequencing (Small RNA-Seq) workflows. The cel-miR-39 RNA could be used for normalization as well as for tracking library construction efficiency.

Specifications

Component	Product # 59000
cel-miR-39 RNA	10 pmol
cel-miR-39 Forward PCR Primer	1 nmol
Nuclease-Free Water	1.25 mL
Product Insert	1

Storage Conditions

Upon receipt, store Norgen's microRNA (cel-miR-39) Spike-In Kit at -20°C or lower. Avoid multiple freeze-thaw cycles. If needed, prepare smaller working aliquots and store at -20°C or lower.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's microRNA (cel-miR-39) Spike-In Kit is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's Low Abundance RNA Quantification Kit is designed for research purposes only. It is not intended for human or diagnostic use.

Customer-Supplied Reagents and Equipment

- Appropriate Real-Time PCR Instrument
- Nuclease-Free PCR Tubes compatible with PCR Instrument
- SYBR Green I (Life Technologies, Cat# S7563)
- PCR Master Mix (such as Norgen's 2x PCR Master Mix – Cat# 28007)

- Reverse Transcription System (such as Norgen's microScript microRNA cDNA Synthesis Kit – Cat# 54410)
- RNA Purification Kit
    - The kit is compatible with all RNA purification kits that yield high quality, inhibitor-free total RNA (including microRNA)
    - **Recommended Purification Kit:** Norgen Biotek's purification kits for RNA isolation, including:
        - Total RNA Purification Kit – Cat# 17200
        - Plasma/Serum RNA Purification Mini Kit - Cat# 55000
        - Urine Cell-Free Circulating RNA Purification Mini Kit - Cat# 56900
- Disposable powder-free gloves
- Benchtop microcentrifuge
- Micropipettors
- Sterile pipette tips with filters
- PCR tubes
- Ice

Warnings and Precautions

- Follow universal precautions. All patient specimens should be considered as potentially infectious and handled accordingly.
- Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when handling specimens and kit reagents.
- Use sterile pipette tips with filters. Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.
- As contamination of patient specimens or reagents can produce erroneous results, it is essential to use aseptic techniques. Pipette and handle reagents carefully to avoid mixing of the samples.
- Do not use supplies and equipment across the dedicated areas of i) specimen extraction, ii) reaction set-up and iii) amplification/detection. No cross-movement should be allowed between the different areas. Personal protective equipment, such as laboratory coats and disposable gloves, should be area specific.
- Do not substitute or mix reagents from different kit lots or from other manufacturers.
- The presence of PCR inhibitors may cause invalid results.
- Good laboratory practice is essential for the proper performance of this kit. Ensure that the purity of the kit and reactions is maintained at all times, and closely monitor all reagents for contamination. Do not use any reagents that appear to be contaminated.

Instructions for Use

Section 1: Use of cel-miR-39 RNA for RT-qPCR Detection

A. Reconstitution of cel-miR-39 RNA

1. Briefly spin down the contents of the tube containing **cel-miR-39 RNA** (200 x g or ~ 2000 RPM for 1 minute).
2. Add 300 µL of Nuclease-Free Water to the tube to reconstitute the cel-miR-39 RNA. This will give a final concentration of 33 fmol per µL.
3. If needed, make small 30 µL aliquots of the reconstituted RNA. Store working aliquots at -20°C or store at -70°C for long term. Avoid multiple freeze/thaw cycles.

B. Spiking cel-miR-39 RNA during RNA Purification

1. If needed, thaw an appropriate amount of reconstituted cel-miR-39 RNA on ice. Keep the RNA on ice until it is ready to be spiked in.
2. Add 3 μL of cel-miR-39 RNA to the sample during the lysate preparation step. It is highly recommended that the cel-miR-39 RNA be added **after** the addition of the appropriate Lysis Solution of the RNA extraction procedure. It is not recommended that the cel-miR-39 RNA be added directly to the sample prior to RNA extraction. The following examples outline the spiking step using various Norgen RNA extraction products:
   a) **Total RNA Purification Kit (Cat# 17200) –** Add 3 μL of cel-miR-39 RNA to the sample after the addition of the appropriate amount of **Buffer RL** for each type of lysate preparation in **Section 1**.
   b) **Plasma/Serum RNA Purification Mini Kit (Cat# 55000)** - Add 3 μL of cel-miR-39 RNA to the sample after the addition of the appropriate amount of **Lysis Buffer A** in **Step 1**.
   c) **Urine Cell-Free Circulating RNA Purification Mini Kit (Cat# 56900)** - Add 3 μL of cel-miR-39 RNA to the sample after the addition of the appropriate amount of **Lysis Buffer A** in **Step 4.**
3. Proceed with the remaining steps of the RNA extraction procedure according to the kit procedure without modification. Note the volume of final elution.

C. Procedure for First-Strand microRNA cDNA Synthesis

1. If the relative recovery of cel-miR-39 RNA (input versus recovered) is to be determined, prepare an appropriate amount of diluted cel-miR-39 RNA based on the final elution volume of RNA extraction. For example, if the final RNA elution volume is 10 μL, prepare a control cel-miR 39 RNA stock by mixing 3 μL of the original 33 fmol/μL stock (prepared in **Step A2**) with 7 μL of Nuclease-Free Water.

   Note: The diluted cel-miR-39 RNA made will represent the same amount of cel-miR-39 RNA spiked in the RNA extraction but in the same volume as the final RNA elution.

2. If using Norgen's microScript microRNA cDNA Synthesis Kit, set up the First-Strand cDNA Synthesis reaction for the RNA samples in a tube compatible with the thermocycler to be used, as described in **Table 1**. Mix the components by gentle vortexing, or by pipetting up and down a few times.

Table 1. First-Strand cDNA Synthesis Reaction Set-up for RNA samples

Component	Volume per Reaction
Nuclease-Free Water	4 μL
RNA (Sample RNA or Control cel-miR-39 prepared in Step C1)*	5 μL
2x microScript Reverse Transcription Reaction Mix	10 μL
TruScript microRNA Enzyme Mix	1 μL
Total Volume	**20 μL**

* 1 - 5 μL of RNA sample could be used per assay. Maintain the 20 μL reaction volume by reducing the amount of nuclease-free water to be used accordingly.

3. Incubate First-Strand cDNA Synthesis reaction in a thermocycler as described in **Table 2**.

Table 2. Reaction Protocol for First-Strand cDNA Synthesis

Temperature	Time
37°C	30 minutes
50°C	30 minutes
70°C	15 minutes
4°C	Hold

4. Dilute 2 µL of cDNA into 28 µL of nuclease-free water. Use the diluted cDNA for PCR-based quantification in Section D. Un-used and un-diluted cDNA can be stored at -20°C.

D. Quantitative PCR Assay Preparation

Notes Before Use:

- If using Norgen's 2x PCR Master Mix - the 2x PCR Master Mix **does not contain a fluorescent DNA dye**. An appropriate amount of SYBR Green I must be added. For example, with a 10,000x SYBR Green I stock (such as Life Technologies, Cat# S-7563), the user could make a serial dilution down to 10x and use 1 µL for every 20 µL reaction (final concentration = 0.5x)
- The procedure below is based on the use of Norgen's microScript microRNA cDNA Synthesis Kit. The Universal PCR Reverse Primer is provided with the cDNA synthesis kit.
- Add 200 µL of Nuclease-Free Water to the tube to reconstitute the **cel-miR-39 RNA Forward Primer**. If needed, make small 30 µL aliquots of the reconstituted primer. Store working aliquots at -20°C for long term. Avoid multiple freeze/thaw cycles.
- To avoid contamination, add the components to the PCR tubes in the order shown in the tables below (ie: 1) Nuclease-free water; 2) 2x PCR Master Mix; 3) SYBR Green I; 4) Primer Set; and 5) the Sample DNA or Standards).

1. Thaw the Primers, 2x PCR Master Mix and SYBR Green I on ice.
2. Prepare the PCR reaction as shown in Table 3 below. Mix the components by gentle vortexing, or by pipetting up and down a few times. Keep the reaction on ice and away from light prior to performing the Real-Time PCR.

Table 3. qPCR Assay Preparation

PCR Components	Volume Per PCR Reaction
Nuclease-Free Water	4.5 µL
2X PCR Master Mix	10 µL
10x SYBR Green I	1 µL
cel-miR-39 Forward PCR Primer	1 µL
Universal PCR Reverse Primer	1 µL
cDNA (Standard or Sample)	2.5 µL
Total Volume	20 µL

E. PCR Assay Programming

1. Program the thermocylcer according to the program shown in Table 4 below.
2. Run the Real-Time PCR.
3. Record the Ct values of each sample for cel-miR-39. Normalization of the samples can be done by standard ΔΔCt relative quantification using the Ct values of cel-miR-39 and the target transcript.

Table 4. PCR Assay Program

Real-Time PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**95°C**	**3 min**
Cycle 2 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**60°C**	**30 sec**
	Step 3	**72°C**	**45 sec**

Section 2: Use of cel-miR-39 RNA for Next Generation Sequencing Analysis (Small RNA-Seq)

A. Reconstitution of cel-miR-39 RNA

1. Briefly spin down the contents of the tube containing **cel-miR-39 RNA** (200 x g or ~ 2000 RPM for 1 minute)
2. Add 300 μL of Nuclease-Free Water to the tube to reconstitute the cel-miR-39 RNA. This will give a final concentration of 33 fmol per μL.
3. If needed, make small 30 μL aliquots of the reconstituted RNA. Store working aliquots at -20°C or store at -70°C for long term. Avoid multiple freeze/thaw cycles.

B. Spiking cel-miR-39 RNA

1. If needed, thaw an appropriate amount of reconstituted cel-miR-39 RNA on ice. Keep the RNA on ice until it is ready to be spiked in.
2. **For Spike-In during RNA Isolation:** The amount of cel-miR-39 RNA used depends on the amount of endogenous RNA present in the sample. In general, it is recommended to spike in a final concentration of approximately 1% of the total RNA amount. Please note that 33 fmol per μL is approximately equivalent to 250 pg per μL. Hence for an expected RNA amount of 100 ng, it is recommended to use 4 μL of the reconstituted cel-miR-39 RNA. For samples with low RNA abundance such as plasma/serum or urine, the amount of the cel-miR-39 RNA to be spiked will have to be adjusted accordingly. The following examples outline the spiking step using various Norgen RNA extraction products:
   a) **Plasma/Serum RNA Purification Mini Kit (Cat# 55000)**
      i) Dilute 5 μL of the 33 fmol per μL cel-miR-RNA stock with 495 μL of RNase-Free Water to give a 330 amol per μL stock.
      ii) Further dilute 5 μL of the 330 amol per μL cel-miR-RNA stock with 495 μL of RNase-Free Water to give a 3.3 amol per μL stock.
      iii) Add 3 μL of cel-miR-39 RNA (3.3 amol per μL) to the sample after the addition of the appropriate amount of **Lysis Buffer A** in **Step 1**.
      iv) Proceed with the remaining steps of the RNA extraction procedure according to the kit procedure without modification.

b) **Urine Cell-Free Circulating RNA Purification Mini Kit (Cat# 56900)**

i) Dilute 5 μL of the 33 fmol per μL cel-miR-RNA stock with 495 μL of RNase-Free Water to give a 330 amol per μL stock.

ii) Further dilute 5 μL of the 330 amol per μL cel-miR-RNA stock with 495 μL of RNase-Free Water to give a 3.3 amol per μL stock.

iii) Add 3 μL of cel-miR-39 RNA (3.3 amol per μL) to the sample after the addition of the appropriate amount of **Lysis Buffer A** in **Step 4**.

iv) Proceed with the remaining steps of the RNA extraction procedure according to the kit procedure without modification.

3. **For Spike-In during Small RNA-Seq Library Preparation:** The amount of cel-miR-39 RNA used depends on the amount of endogenous RNA present in the sample. In general, it is recommended to spike in a final concentration of approximately 1% of the total RNA amount. Please note that 33 fmol per μL is approximately equivalent to 250 pg per μL. Hence for an expected RNA amount of 100 ng, it is recommended to use 4 μL of the reconstituted cel-miR-39 RNA. Proper quantification of RNA is highly recommended. For samples with low RNA abundance such as plasma/serum and urine, it is recommended to use Norgen's Low Abundance RNA Quantification Kit (Cat# 58900) for RNA quantification.
4. The sequence of the cel-miR-39 RNA provided is: **5' UCACCGGGUGUAAAUCAGCUUG 3'**

Technical Support

Contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362.

Technical support can also be obtained from our website (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

Norgen Biotek Corp.
3430 Schmon Parkway, Thorold, ON Canada L2V 4Y6
Phone: (905) 227-8848
Fax: (905) 227-1061
Toll Free in North America: 1-866-667-4362

