Laboratory protocols

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Abstract

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Protocol

Step 1.

Trizol Reagents

1. Use mortar and pestle to grind up leaves into powder
2. Pack each 2.0 mL microcentrifuge tube (MCT) to the 0.8mL mark. **Pack and extract two tubes for each sample.**
3. Add 1mL of Trizol reagent and shake to mix; alternatively shake with tissuelyzer for 10sec at 20Hz or vortex for 15 seconds. 1 mL of Tri-reagent is the same thing.
4. Incubate at room temperature (RT) for 5 minutes.
5. Add 200uL chloroform (for each mL of Trizol)
6. Shake with hand vigorously for 15seconds or vortex for 15sec
7. Incubate at RT for 2minutes
8. Centrifuge at 11,000 rcf for 15minutes (Don't exceed 12,000rcf)
9. Transfer the slightly yellowish transparent **upper aqueous phase (720uL)** to a 1.5mL MCT.
10. Add equal volume of phenol-chloroform (680uL). Vortex to mix. Centrifuge for 11,000rcf for 5minutes. ***Note: phenol-chloroform bottle has 2 layers, only the bottom layer is phenol-chloroform. Top layer in the bottle is buffer. MAKE SURE YOU ONLY USE THE BOTTOM LAYER.***
11. Transfer **upper aqueous phase** (700uL) to a new 1.5mL MCT.
12. Repeat step 10; upper aqueous phase is extracted with phenol-chloroform twice.
13. Transfer **upper aqueous phase** (700uL) to a new 1.5mL MCT
14. Add equal volume of chloroform (690 uL). Vortex to mix. Centrifuge for 11,000 rcf for 5 minutes
15. Repeat steps 13 & 14; upper aqueous phase is washed with chloroform twice.
16. Transfer the **upper aqueous phase** (700 uL) to a new 1.5mL MCT
17. Add 500uL of isopropanol (isopropyl alcohol) for each mL of Trizol to precipitate the RNA. Invert tube after adding isopropanol.
18. Incubate at RT for 10 minutes. The RNA precipitates are often invisible before centrifugation.
19. Centrifuge at 11,000rcf for 10minutes. Gel-like pellets are RNA.
    1. ***If pellets are very small, then incubate the tubes in –20C freezer for 1 hr to further precipitate the RNA; invert tubes prior to putting in -20C freezer. Then resume step 18) to centrifuge down the RNA pellet.*
20. Remove supernatants. Pour supernatant slowly, be careful not to lose the pellet.

21. Add **75% ethanol(dilute with DEPC water)** to wash the RNA pellet once. Add at least 1mL of 75% ethanol per mL of Trizol. This step removes salts from the pellet. 75% EtOH = 37.5mL 100% EtOH + 12.5mL DEPC water.
    1. ***If ran out of time to continue the Trizol RNA extraction, it is alright to stop here and store the ethanol+RNA mixture in –20C freezer and continue the extraction some other time.*
22. Mix the RNA pellet + Ethanol by vortexing until pellet dislodge.
23. Centrifuge at 6,000rcf for 5minutes (Do not exceed 7,500 rcf). Pour supernatant slowly, be careful not to lose the pellet.
24. Repeat steps 21-23; pellet is washed with 75% EtOH twice.

1. Removes supernatants. ***Pellets could easily dislodge. Try to pour out most supernatants, short spin for 5sec and use the pipet to remove the rest.***
2. Let the RNA air dry in hood for 15 minutes. *Don't over dry the RNA pellets or else its solubility in water will decrease.*
3. Dissolve the RNA in 60uL of DEPC water. Vortex and do a short spin 10-20s.
4. Incubate the RNA for 10minutes at 55C to help with the dissolving process.
    1. ***If ran out of time to continue, can also incubate the samples overnight at –20C and continue from step 22) another time.*
5. Store the RNA on ice.
6. Weigh the mass of a new 1.5mL MCT for each sample you have.
7. Spin the RNA at maximum RPM for 3 minutes and transfer the supernatants to the new MCT with known mass.
    1. ***Each sample has two MCTs. Combine their supernatants into the MCT prepared in step 30). Try not to pipet the precipitate over.*

1. Do nanodrop to measure the concentration, 260/280 and 260/230. Each nanodrop measurement uses 3uL of RNA.
2. Reweigh the mass of the tube+RNA from step 31). **1g of water 1mL**. Complete a similar table.
    1. Sample Table:

Tube Label	Tube Mass (g)	Tube + RNA Mass (g)	RNA Mass (g)	Volume of RNA (uL) = RNA Mass (g) x 1000	ng/uL	260/280	260/230
T1	0.96784	1.08231	0.11447	114.47	2124.08	2.11	1.78
T2	0.98067	1.09515	0.11448	114.48	1811.75	2.1	1.83
T3	0.9807	1.09411	0.11341	113.41	1577.47	2.11	1.81

1. If the RNA concentration is <1,000ng/uL, vacuum spin the tube. If the RNA concentration is >1,000ng/uL, use the following equation to calculate the amount of DEPC water, V_water, to

add to dilute the RNA.

1. “**V_water = (V1xC1/C2) – V1**”
2. V1= initial volume prior to dilution; ex. For T1, it’s 114.47uL
3. C1= initial RNA concentration prior to dilution; ex. For T1, it’s 2124.08ng/uL
4. C2= Final RNA concentration after dilution = **1,000ng/uL** (for all samples)

Step 2.

miRcute miRNA First-strand cDNA Synthesis

miRcute miRNA First-strand cDNA Synthesis Kit

For first-strand cDNA synthesis from miRNA

TIANGEN

www.tiangen.com

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miRcute miRNA First-strand cDNA Synthesis Kit

Cat. no. KR201

Kit Contents

Contents	KR201-01 25 preps	KR201-02 50 preps
E.coli Poly (A) Polymerase (5 U/μl)	14 μl	28 μl
10x Poly (A) Polymerase Buffer	60 μl	120 μl
5x rATP Solution	120 μl	240 μl
10x RT Primer	60 μl	120 μl
10x RT Buffer	70 μl	150μl
Super pure dNTP (2.5 mM each)	30 μl	60 μl
RNasin (40 U/μl)	30 μl	60 μl
Quant RTase	15 μl	30 μl
RNase-free ddH_2O	1 ml	1 ml
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Storage

miRcute miRNA First-strand cDNA Synthesis Kit should be stored at -20 °C.

Introduction

miRcute miRNA First-strand cDNA Synthesis Kit adds Poly(A) to the 3'-terminal of miRNA and synthesize the first-strand cDNA based on Poly(A) modified miRNA through oligo(dT)-universal tag primed reverse transcription.

The kit contains all the necessary reagents in the process of adding Poly(A) and reverse transcription. The kit can provide high efficient Poly(A) modification and reverse transcription and efficiently synthesize the first-strand cDNA from 20 pg-2 μg total RNA.

Note: This kit has to be used with miRcute miRNA qPCR Detection Kit (SYBR Green) (Cat. no. FP401).

Important Notes

1. Change the disposable gloves frequently to avoid the RNase contaminations from skin.
2. Use RNase-free plastic wares and tips to avoid cross-contaminations.

Protocol

1. Add Poly (A) to the 3' terminal of miRNA

1) Prepare a reaction solution in a pre-cooling tube on ice according to the following table (Add *E.coli* Poly (A) Polymerase in the last step).

Contents	Volume/ Reaction	Final concentration
Total RNA*	-	≤2 μg
E.coli Poly (A) Polymerase (5 U/μl)	0.4 μl	2 U
10× Poly (A) Polymerase Buffer	2 μl	1×
5× rATP Solution	4 μl	1×
RNase-free H_2O	-	-
Total volume	20 μl	

***Total RNA as the template has to contain miRNA in the reaction. miRNA can also be used as template in the reaction (2-5μl miRNA is recommended. Determine the amount according to the abundance of the target miRNA.)**

2) Mix gently by pipetting the solution and centrifuge briefly to remove the drops from walls of tube. Incubate at 37°C for 60 min. The solution can be used directly to the downstream experiments or stored at -20 °C for a short time. For long storage, -80°C is recommended.

2. Reverse transcription of Poly (A) modified miRNA

1) Prepare a reaction solution according to the following table

Contents	Volume
Poly (A) reaction solution	2 μl
10x RT Primer	2 μl
10x RT Buffer	2 μl
Super pure dNTP (2.5 mM each)	1 μl
RNasin (40 U/μl)	1 μl
Quant RTase	0.5 μl
RNase-free ddH_2O	11.5 μl
Total volume	20 μl

2) Mix gently by pipetting the reaction solution and briefly centrifuge to remove drops from the wall of the tube. Incubate at 37°C for 60 min.

The reaction solution including cDNA products can be stored at -20°C or used in downstream quantitative PCR directly.

Ordering Information

RNA Isolation

Product	Size	Cat.no.
miRcute micro RNA Isolation Kit	50 preps	DP501

Real–Time PCR

Product	Size	Cat.no.
miRcute miRNA qPCR Detection Kit (SYBR Green)	50 µl × 50 rxns	DP401

Step 3.

miRcute miRNA qPCR Detection

miRcute miRNA qPCR Detection Kit (SYBR Green)

For detection of miRNA using real-time RT-PCR (SYBR Green I)

TIANGEN

www.tiangen.com

miRcute miRNA qPCR Detection Kit (SYBR Green)

Cat. no. FP401

Kit Contents

Kit Contents	FP401 50 µl × 50 rxns
2× miRcute miRNA Premix (with SYBR and ROX)	1.35 ml
Reverse Primer (10 µM)	55 µl
50× ROX Reference Dye	250 µl
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Storage

miRcute miRNA qPCR Detection Kit should be shipped in dry ice and stored immediately upon receipt at -20℃ in dark condition. Once used, 2× miRcute miRNA Premix (with SYBR and ROX) can be stored at 4℃ for 6 months in dark condition. Reverse Primer (10 µM) should be still stored at -20℃.

Introduction

miRcute miRNA qPCR Detection Kit is designed for miRNA real-time PCR by using SYBR Green I. The kit is composed of 2× miRcute miRNA Premix, 50× ROX Reference Dye and Reverse Primer (10 µM).

2× miRcute miRNA premix (with SYBR and ROX) is developed specifically for quantitative detection of miRNA. The DNA polymerase in this kit is the antibody modified hot-start DNA polymerase, which ensures high specificity, sensitivity and accurate quantitative detection in a wide range combined with the unique buffer.

Note: The kit has to be used with miRcute miRNA First-Strand cDNA Synthesis Kit (Cat. no. KR 201)

Important Notes

1. The kit contains SYBR Green I. Store and prepares PCR reaction in dark condition.
2. Pipet the reaction solution and aliquot with sterile tips and consumable items to avoid contamination.

Reagents and Materials Not Supplied

1. RNase-free ddH_2O
2. Forward primer

Design Principles of Forward Prime

1. Follow the most common design principles of primers.
2. Based on mature miRNA sequence, change U to T to design forward prime.
3. Tm value of Reverse Primer (10 μM) in the kit is about 65°C. Ensure that Tm value of forward prime is basically consistent with supplied Reverse Primer (10 μM).
4. If Tm value of the designed forward prime is too low according to principle 2, it is recommended that several nucleotides (G or C is optional) are added to 5'-terminal. Also one or several nucleotides are added to 3'-terminal, or 5' terminal or 3' terminal are both modified simultaneously.
5. If Tm value of the designed forward prime is too high according to principle 2, remove several nucleotides from 5' or 3' terminal.

Protocol

1. Thaw 2× miRcute miRNA Premix and Reverse Primer (10 μM) at room temperature.
2. Mix the 2× miRcute miRNA Premix thoroughly by inverting gently. Avoid bubbling in the process and centrifuge gently before use.
   Note: The performance will decrease unless the reagents are mixed thoroughly. Do not vortex by shaker.
3. Place all the reagents on ice, and prepare the reaction according to Table A.
   Note: If using PRISM 7000/7300/7700/7900HT, Step one/ Step one plus PCR system from ABI, prepare the reaction according to Table B.

Table A

Components	Volume (50 μl)	Volume (20 μl)	Final Conc.
2× miRcute miRNA Premix (with SYBR and ROX)	25 μl	10 μl	1×
Forward Prime (not supplied)	-	-	200 nM
Reverse Primer (10 μM)	1 μl	0.4 μl	200 nM
miRNA first-strand cDNA	-	-	-
ddH_2O	Up to 50 μl	Up to 20 μl	

Table B

Components	Volume (50 µl)	Volume (20 µl)	Final Conc.
2× miRcute miRNA Premix (with SYBR and ROX)	25 µl	10 µl	1×
Forward Prime (not supplied)	-	-	200 nM
Reverse Primer (10 µM)	1 µl	0.4 µl	200 nM
miRNA first-strand cDNA	-	-	-
50× ROX Reference Dye	4 µl	1.6 µl	5×
ddH_2O	Up to 50 µl	Up to 20 µl	-

Note: Ensure addition of miRNA first-strand cDNA is less than 1/10 volume of real-time PCR reaction. Dilute the cDNA according to actual concentration (Dilution ratio may be 1/10 or 1/100) since high concentration of cDNA will lead to non-specific amplification.

Compatible Real-time Instruments

PRISM 7000/7300/7500/7700/7900HT Real-Time PCR System, Step one/ Step one plus PCR System (Applied Biosystems)
LightCycler (Rocle)
Mx3000P, Mx3005P and Mx 4000 (Stratagene)
Mastercycler ep realplex (Eppendorf)
Line-Gene (Bioer)
Other Real-time instruments

Set up the real-time PCR program according to the procedure outlined in the following table.

2× miRcute miRNA premix (with SYBR and ROX) contains antibody

modified DNA polymerase. The DNA polymerase is activated in a shorter time than other hot-start Taq DNA polymerases. This shorts the reaction time of real-time PCR significantly.

Cycle numbers	Temperature	Time	Comments
1×	94°C	2 min	Initial PCR denaturation
40-45×	94°C	20 sec	Denaturation
	60°C	34 sec	Annealing and extension

Ordering Information

Reverse Transcription

Product	Size	Cat.no.
miRcute miRNA First-Strand cDNA Synthesis Kit	25 rxns	KR201-01
	50 rxns	KR201-02

Step 4.

Relative expression raito of miRNAs

$$\text{Ratio} = \frac{(E_{\text{miRNA}})^{\Delta Ct\ \text{miRNA (average treatment - average control)}}}{(E_{\text{reference gene}})^{\Delta Ct\ \text{reference gene (average treatment - average control)}}}$$