Note: *for laboratory research use only.*

microRNA Extraction Kit

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I. Kit Content, Storage, and Stability

Content	Storage	50 preps	100 preps
Buffer MRL	4℃ in the dark	55 ml	110 ml
Buffer RW	RT	15 ml	25 ml
		Add the ration ethanol before use.	
RNase-free H_2O	RT 10 ml		20ml
70% ethanol	RT	9ml RNase-free H_2O	9ml RNase-free H_2O×2
		Add the ration ethanol before use.	
RNase-free Spin-column RA	RT 50		100
RNase-free Spin-column RB	RT 50		100
Collection Tube （2ml）	RT 50		100

All reagents will be stable for 12 months when stored properly.

Notes:

1. ***Please add proper volume ethanol to buffer RW and 70% ethanol before use, vortex adequately, and then tick on the blocks, avoiding multi-adding!***
2. All reagents should be clear, which may precipitate under low temperature, then incubate at 37 for a moment **until clear**, then cool down to RT for use.
3. Some reagents will form precipitation, which affects RNA yield, under improper low temperature (4℃ or -20℃) condition. So the transportation and saving of reagents are under room temperature (15℃-25℃). Buffer RL can be transported at RT, then keep it at **4**℃ **in the dark** upon arrival.
4. Please keep the lids of all reagents tightly after use, preventing reagents evaporation, oxidation and pH change.

II. Principle

The Kit applies improved guanidine thiocyanate-phenol one-step method to lyse samples and inactivate ribonucleases， then genomic DNA and 18 and 28s RNA are removed by the first spin-column. Then microRNA (including miRNA, snRNA,and other RNA less than 200bp) is absorbed by the second spin-column. After a serial of

elution- centrifugation steps to remove cellular metabolite and proteins etc, finally microRNA will be eluted from silica membrane using low salt RNase-free water.

III. Features

1. The silica membranes in the spin-column are produced by the world-famous company. There is little variation among different spin-columns, so repeatability of tests is reliable!
2. MicroRNA can be eluted from silica membrane without ethanol precipitation and dissolve.
3. The buffer MRL contains some special components can clean up the contamination of genomic DNA efficiently.
4. Multi-elution can ensure high-purity microRNA.

IV.Notes

1. ***For preventing RNA degradation, all the centrifugation steps should be made under -4 , excluding having special notes,*** suggest using up to 13,000 rpm traditional centrifuge, for example Eppendorf 5415C and the similar.
2. Buffer RL and Buffer RE contain the stimulating compounds; please wear latex gloves, avoiding skin, eyes and cloth to be contaminated. ***If that, please wash with use water or physiological saline.***
3. Due to the prevalence of RNases, wear gloves at all times and change them whenever may have been contacted, please follow standard laboratory procedures of "Molecular Clone" rules.

* Wear gloves in whole process. There are many bacteria on the skin of our hands, which will be the main source of RNase affecting RNA extracting.

* Whenever possible, sterile disposable plasticware should be used for handling RNA, avoiding contaminating by public equipments

* Treat non-disposable glassware and plasticware before use to ensure that it is RNase-free. Bake glassware at 200°C overnight, and thoroughly rinse plasticware with 0.1N NaOH, 1mM EDTA followed by RNase-free water.

4. Considering environmental problem, the kit doesn't have chloroform, please prepare it by yourself.
5. The integrity of purified RNA may be determined by denaturing agarose gel electrophoresis (or agarose gel electrophoresis). The ratio of ~5Kb（28S）to ~2Kb（18S）ribosomal RNA should be approximately 2:1 by ethidium bromide staining.
6. The routine method to determine the yield and purity of RNA is spectrophotometry (OD_{260}/OD_{280}). Please dissolute RNA by TE, water will make OD_{280}higher because of lower ion intensity and PH.
7. The sample, which had mixed with Buffer MRL and homogenized, but without chloroform, can store under –60℃-70 for a month even more.

V. Procedure

Please add proper volume ethanol to buffer RW and bottle of 70% ethanol before use.

1. Homogenization
    a. Tissues
    Please homogenize tissue in an appropriate volume of Buffer MRL (50-100mg/mL) until no visible tissue; for tissue in liquid nitrogen, grind the tissue into a fine powder using a mortar and pestle. Pay attention to the volume of sample should not beyond 1/10 total volume of Buffer MRL.
    b. Adherent cells
    You can directly append an appropriate volume Buffer MRL to the culture plate, and completely lysis cell by pipetting. The volume of Buffer MRL is decided by the area of culture plate, about 10cm^2 per 1ml. In common situation, 1mL Buffer MRL is enough. Once appending not enough Buffer RL, it's possible to contaminated genomic DNA.
    c. Suspending cells
    Harvesting cells in Buffer MRL (animal, plant, fungus, maximum 5~10×10^6cells per 1mL; bacteria, maximum 1×10^7cells per 1mL) in a micro centrifuge tube by centrifuging.
2. Mix vigorously and incubate for 5 minutes under to lysate ribosomal particle comptelely.
3. **Alternative step** Centrifuge mixture at 12,000g for 10 minutes in a microfuge at 4°C. Remove upper, aqueous phase to a clean, sterile, DEPC-treated eppendorf tube.
    When the sample is rich in proteins, fats, amylase and other extracellular substances (eg, muscle), or fatty tissue and plant tuber, it need another procedure.
4. Add 200µl chloroform per 1mL Buffer MRL, lid tightly and mix vigorously 15 sec, then incubate for 3 minutes under RT.
5. Centrifuge mixture at 12,000g for 10 minutes in a microfuge at 4°C. Remove upper, aqueous phase that RNA is present to a clean, sterile, DEPC-treated eppendorf tube.
6. Precipitate the aqueous phase by the addition of an equal volume (500µl) of 70% ethanol **(please check if or not add ethanol!),** mix gently then maybe appear the flocculated precipitate.
7. Put last step solution and the flocculated precipitate into a Spin-column RA (place the spin-column to collection tube).
8. Centrifuge at 10,000rpm for 45 sec, collect flow-through (including micro RNA)**,** check the volume of flow-through **(please more exactly).** Add 70% ethanol (2/3 times volume of flow-through) and mix gently, then put this mixed solution into a Spin-column RB, centrifuge at 10,000g for 30 sec in a

If you want separate macro RNA (18s and 28s), can gain from the spin column RA following steps:

9. Add 700μl buffer RW（**please check if or not add ethanol!**），centrifuge at 12,000g for 60 sec, and discard flow-through.
10. Add 500μl buffer RW, centrifuge at 12,000g for 60 sec ， discard flow-through liquid.
11. Place spin-column RB back to collection tube，centrifuge at 12,000g for 2 min, and discard supernatant as far as possible to avoid ethanol inhibits the down-stream procedures.
12. Take the Spin-column RB out, then put a new RNase-free tube, add 60-80μl RNase-free water (having been incubated at 65-70), put for 2 min at RT, centrifuge at 12,000g for 1 min. Keep micro RNA at -20℃ or -80℃.

Ⅵ.Trouble shooting

Problem	Possible Reason	Advices
Low microRNA	Tissue not homogenized thoroughly	For tissue in liquid nitrogen, grind the tissue into a fine powder, after add buffer RL, then completely lyse cells by pipetting or vortexing; for the fresh tissue or plant tissue, grind the tissues in Buffer MRL using a mortar and pestle.
	RNA degraded	Take fresh samples for isolation.
	The sample is poor in RNA	There are different RNA percentage in all kinds' tissues and cells. So some "poor RNA" samples need more amount for homogenization.
	Beyond the binding maximum of silica membrane	Using multiple spin-columns RA for the same sample.
	Ethanol not added to Buffer RW	***Add the ration ethanol before use.***

OD_{260}/OD_{280} <1.6	Dissolute RNA by water, which will make OD_{280} higher because of lower ion intensity and PH.	Please dissolute RNA by TE for spectrophotometry.
	Contaminated by proteins and phenol	Don't take middle and lower phases in Step5 and ensure to do step8.
Genomic DNA contamination	Initial sample beyond the coping range of Buffer MRL	Selecting appropriate amount sample.
	Sample contains some chemical solvent (such as ethanol, DMSO etc), intensive buffer or alkaline solution.	Avoid these substances.
	May extract middle phases in Step5.	Don't take middle phases in Step5.
RNA degradation and integrity not well	non-disposable glassware and plasticware not treated before use.	Treat non-disposable glassware and plasticware before use to ensure that it is RNase-free
	Samples were not properly prepared or stored. RNA may have been degraded during sample preparation.	To halt RNA degradation within samples, immediately homogenize sample with Buffer MRL. Or freeze samples immediately in liquid nitrogen and store at -70°C if they cannot be immediately processed.
	RNA not stored under –60℃-70℃	Store under –60℃-70℃
	RNA degradation in process.	It is essential to work quickly during sample preparation. Maintain the sample lysate at 4°C during preparation.
Down-stream RT-PCR not successful.	Forget to do step11, or when take the spin-column out, touching some flow-through including ethanol carelessly. Finally ethanol inhibits RT-PCR	Ensuring to do step11, take the spin-column out carefully, and then put outside for a few minutes for ethanol evaporation.

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