Supplementary. General Experiment

Part. 1 Summary of our laboratory experiment

PCR

Common taq enzymes are used for colony identification and high fidelity enzymes are used to obtain accurate DNA fragments. the PCR process pays attention to the setting of annealing temperature and extension time. Please refer to the instructions of each enzyme for the specific system preparation and program setting.

Enzyme digestion, T4 ligation, homologous recombination

For the system, please refer to the instruction manual of each reagent. Note that the DNA samples need to be purified before T4 ligation or homologous recombination.

Transformation

The receptor cells we use are DH5a (for preservation and amplification of plasmids), BL21 (DE3) (for protein expression), shuffle T7 (for protein expression), and Rosetta gamma2 (for protein expression). The steps of transformation were all done by heat-strike method. DNA was first added to the receptor cells and left on ice for 30min, then heat-strike for 45-90s at 42 degrees, then placed on ice again for 2min, 700ul of antibiotic-free medium was added to recover at 37 degrees for 1h, centrifuged to discard most of the medium, and the remaining 100ul or so of medium was resuspended and coated on the plate with antibiotics.

Agarose gel electrophoresis

(1) Prepare 1% agarose gel containing nucleic acid dye (if the target fragment is small, increase the concentration of the gel appropriately. Conversely, reduce the concentration of the gel.
(2) Mix the DNA sample and Loading buffer and add to the spiked wells.
(3) Electrophoresis with 100V for 30min.
(4) After electrophoresis, remove the gel and observe and take pictures.

SDS-PAGE Komas blue staining

(1) Assemble the gel plate, first prepare the appropriate concentration of separation gel (commonly 10%, 12.5%), flatten the liquid surface with anhydrous ethanol, and then prepare 5% concentrated gel.

(2) After the gel solidifies, assemble the electrophoresis device and add the pre-cooled electrophoresis buffer (fresh buffer is used in the inner tank and recycled buffer can be used in the outer tank)
(3) Add protein samples.
(4) Electrophoresis at 60V for 30 min (until the bromophenol blue band migrates to the dividing line), then electrophoresis at 80V until the end
(5) Remove the plate, rinse the cut separation gel with deionized water, and then add Komas Brilliant Blue fast staining solution, stain for more than 10 min until the bands are clear, discard the staining solution, and wash with deionized water 3 times.

Western blot

(1) Cut off the destination position of the completed electrophoresis SDS-PAGE gel and use PVDF membrane. Transfer membrane (transfer conditions: such as for 55kD molecular weight protein can use 235mA constant current transfer membrane 1h conditions, as a benchmark, if the protein molecular weight is too large or too small need to adjust the current or transfer time)
(2) PVDF membranes were rinsed once with TBST after membrane transfer, and then 5% BSA was added to close the membrane at room temperature for 1h
(3) Antibody incubation: incubate the primary antibody at 4 degrees overnight (recommended) or 3-4h at room temperature, then wash 3 times with TBST (10min each time, the same later), incubate the HRP-coupled secondary antibody for 1h at room temperature, and wash three times with TBST. (Refer to the antibody instructions for the antibody dilution ratio. If you need to incubate three antibodies, refer to the following steps: add scFv-His primary antibody and incubate overnight at 4 degrees, wash three times with TBST, add anti-His antibody and incubate at room temperature for 3h, wash three times with TBST, incubate HRP-coupled secondary antibody at room temperature for 1h, wash three times with TBST)
(4) Add freshly prepared exposure solution for exposure, avoid exposing to the daylight for too long.

Cell lines

HEK293t, HepG2, PBMC

Complete growth medium for cells

HEK293t and HepG2：

89% DMEM (Gibco, C11885500BT) + 10% Fetal Bovine Serum (Gibco, 10099141C) +1% Penicillin-Streptomycin Solution (Merck, TMS-AB2-C)

PBMC:

89% RPMI1640 (Gibco, C11875500BT) + 10% Fetal Bovine Serum (Gibco, 10099141C) +1% Penicillin-Streptomycin Solution (Merck, TMS-AB2-C)

Cell resuscitation

1. Rapidly thaw cells by placing the cryovial in a 37°C water bath, swirling gently. Remove the cryovial from the water bath when only a few ice crystals are remaining.
2. Sterilize the cryovial by rinsing with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Using a 1-mL pipette, transfer thawed cells drop-wise into 9 mL pre-warmed complete growth medium in a 15-mL centrifuge tube. Gently pipette the cells up and down several times to mix thoroughly.
4. Centrifuge the cell suspension at 1000rpm for 3 minutes.
5. Carefully aspirate the supernatant and discard, leaving the cell pellet.
6. Gently resuspend the cell pellet in 2 mL fresh pre-warmed complete growth medium, and transfer cell suspension into a 100-mm Petri dish (Corning, 430167) containing 8 mL fresh pre-warmed complete growth medium. Cells should be seeded at a density of 5 x 10^5 cells/mL.
7. Place the Petri dish in a 37°C incubator with 5% CO_2.

Subculturing procedure

1. Remove and discard the spent cell culture media from the Petri dish.
2. Wash cells using pre-warmed 1× PBS buffer (approximately 2 mL per 10 cm^2 culture surface area). Gently add pre-warmed 1×PBS buffer to the side of the Petri dish opposite the attached cell layer to avoid disturbing the cell layer, and rock the Petri dish back and forth several times.
3. Remove and discard the wash solution from the Petri dish.
4. Add the pre-warmed 2 mL 0.25% trypsin-EDTA (Gibco, 25200056) to the side of the Petri dish (enough to cover the cell layer). Gently rock the Petri dish to get complete coverage of the cell layer.
5. Incubate the Petri dish at room temperature for approximately 2 minutes (2 minutes for HepG2 and 1 minute for HEK293t).
6. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds.
7. When ≥ 90% of the cells have detached, tilt the Petri dish for a minimal length of time to allow the cells to drain. Add 5 mL pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.
8. Transfer the cells to a 15-mL conical tube and centrifuge then at 1000rpm for 3 minutes.
9. Resuspend the cell pellet in 2 mL pre-warmed complete growth medium, add cells to a new Petri dish containing 8 mL fresh complete growth medium. Remove a sample for counting when needed.

Note: For PBMCs, skip step 2~7.

Cryopreservation

1. Prepare 2X freezing medium (complete growth medium supplemented with 15% DMSO) and store at 4°C until ready to use.

2. Dissociate cells using methods in “Subculturing procedure” step 1~7 Determine the viable number of cells and percent viability. Calculate the required volume of freezing medium based on the desired viable cell density per vial.
3. Centrifuge the cell suspension at 1000rpm for 3 minutes. Carefully aspirate & discard supernatant.
4. Resuspend the cell pellet in complete growth medium (about 500 µL), and then add equal volume of the cold 2X freezing medium (prepared in step 1).
5. Transfer the cell suspension into cryovials (2 mL/vial). Continue to gently mix the cell suspension to avoid cell clumping and to keep the suspension at a homogeneous state.
6. Freeze the cells at 4°C for 10 minutes, and then transfer the cryovials to -20°C for 30 minutes. And finally store cells at -80°C.
Note: The cells should not be left at -80°C for more than 24 to 48 hours. Once at -80°C, frozen cryovials should be transferred to the vapor phase of liquid nitrogen for long-term storage. However, because of the limitation of our facilities, we store cells in -80°C and the time between we cryopreserve and resuscitate cells would not be longer than 3 months.

Part .2 Instruction manual of common experiment kits.

Annexin V-FITC/PI Apoptosis Detection Kit

The kit comes from Vazyme Biotech Co., Ltd.

1. sample staining

(i) Induce apoptosis according to experimental requirements. The assay samples should contain untreated cell samples as negative controls. In addition, the experimental groups need to be taken for Annexin V-FITC and PI single staining for regulatory compensation when performing the flow assay, respectively.

(ii) **Collection of cells:** collect 1 - 5 × 105 cells in suspension: centrifuge at 1000 rpm, 4 °C for 5 min and discard the medium supernatant.

(iii) **Walled cells:** digest the cells with EDTA-free trypsin, terminate the digestion and collect the cells, centrifuge at 1000 rpm, 4°C for 5 min, discard supernatant. (Wash the cells: Wash the cells twice with pre-cooled PBS, centrifuge at 1000 rpm, 4°C for 5 min each time, and discard the supernatant).

(iv) **Cell resuspension:** Add 100 µl of 1 × Binding Buffer and gently blow well to a single cell suspension.

(v) **Cell staining:** Add 5 µl Annexin V-FITC and 5 µl PI Staining Solution and blow gently; incubate for 10 min at room temperature (20~25℃), protected from light; add 400 µl 1 × Binding Buffer and mix gently. After staining, the samples were detected by flow cytometry within 1 h.

2. Sample analysis

(i) Flow Cytometry Analysis

The excitation wavelength of the flow cytometer is 488 nm; the green fluorescence of FITC is detected on the FL1 channel; the red fluorescence of PI is detected on the FL2 or FL3 channel. It is recommended to use FL3 and collect 10,000 events for each sample. Use FlowJo and other software for data analysis, FL1 is the abscissa, FL3 is the ordinate, the yin and yang limits of the two fluorescence parameters are determined according to the FITC and PI fluorescence values, and the cross gate is delineated. In a typical experiment, cells can be divided into three subgroups: living cells are double negative (Annexin V-FITC - /PI -); early apoptotic cells are single positive for Annexin V-FITC (Annexin V-FITC + /PI -) ; Late apoptotic cells are double positive for Annexin V-FITC and PI (Annexin V-FITC + /PI +).

(ii) **Fluorescence microscopy analysis**

Drop a drop of the stained cell suspension on a glass slide, cover the cells with a cover glass, and observe under a fluorescent microscope. Observed under a fluorescence microscope with a two-color filter: the fluorescence signal of Annexin V-FITC is green, and the fluorescence signal of PI is red.

Cell Counting Kit-8 (CCK8)

The kit comes from APExBIO.

1. Required equipment and materials

Microplate reader (450 nm filter)
96-well plates
10 μl, 100-200 μl multichannel pipette
Cell CO_2 incubator

2. Cell viability detection

(i) Inoculate the cell suspension (100 μl/well) in a 96-well plate. Pre-incubate in a humidified incubator for a certain period of time (for example, at 37°C, 5% CO2).
(ii) Add 10 μl of CCK-8 solution to each well of the plate. Be careful not to introduce air bubbles into the hole as they will interfere O.D. value detection.
(iii) Place the culture plate in the incubator and incubate for 1-4 hours.
(iv) Use a microplate reader to measure the absorbance at 450 nm.

Note: If you do not measure the absorbance immediately, add 10μl of 1% w/v SDS or 0.1 M HCl to each well, cover and store in the dark at room temperature. No change in absorbance will be observed within 24 hours.

3. Cell proliferation/toxicity detection

(i) Inoculate 100 μl of cell suspension (about 5000 cells/well) in each well of a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (for example, at 37°C, 5% CO_2).
(ii) Add 10 μl of different concentrations of the test substance to the well.

(iii) Incubate the culture plate in the incubator for an appropriate length of time (for example, 6, 12, 24 or 48 hours).

(iv) Add 10 μl of CCK-8 solution to each well of the plate. Be careful not to introduce air bubbles into the hole as they will interfere O.D. value detection.

(v) Incubate the culture plate in the incubator for 1-4 hours.

(vi) Before reading the plate, you can gently mix on a shaker. Then use a microplate reader to measure the absorbance at 450 nm.

Note: If you do not measure the absorbance immediately, add 10μl 1% w/v SDS or 0.1 M HCl to each well, cover it and leave it at room temperature and keep away from light. No change in absorbance will be observed within 24 hours.

4. Data analysis

There are several methods for statistical analysis. You can choose to use O.D. value or cell number. We provide one of these methods.

Cell survival rate (%) = [(As-Ab)/(Ac-Ab)]×100 Inhibition rate (%)= [(Ac-As)/(Ac-Ab)]×100

As = Absorbance of experimental wells (absorbance of wells containing cells, culture medium, CCK-8 and test compound)

Ab = Absorbance of blank wells (absorbance of wells containing medium and CCK-8)

Ac = absorbance of control wells (absorbance of wells containing cells, culture medium and CCK-8)

5. Make a standard curve

(i) Count the number of cells in the cell suspension on the cell counter

(ii) Use the culture medium to dilute the cell suspension in an equal ratio to a concentration gradient, usually 5-7 concentration gradients, and several replicate wells in each group. Then inoculate the cells. (Pay attention to the number of cells in each well. If you dilute the cell suspension in a tube, carefully mix the cells again before adding to the wells of the culture plate. The volume of cell suspension in each well should be the same.)

(iii) Incubate until the cells adhere to the wall (usually 2-4 hours), and then add 10 μL of CCK-8 for every 100 μL of medium. Continue to incubate

(iv) Measure the absorbance at 450nm with a microplate reader for 1-4 hours. Make a line with the number of cells as the X-axis coordinate, and the O.D. value is Y

The standard curve of the axis coordinates.

The number of cells in the sample to be tested can be determined based on this curve. The prerequisite for using this standard curve is that the culture and detection conditions are the same.

IFN-α ELISA Kit

The kit comes from Dakewe Biotech Co., Ltd.

1. Required equipment and materials

- Microplate reader (recommend to warm up in advance by referring to the instructions for use of the instrument)
- Micro-dosing device and tip: P10, P50, P100, P200, P1000
- Distilled or deionized water
- Brand new filter paper
- Vortex oscillator and magnetic stirrer

2. Experimental Procedure

(i) Before use, mix all reagents thoroughly to avoid foaming.

(ii) Determine the number of slats required according to the number of experimental holes (blank and standard). Samples (including standards) and blanks should be replicated.

(iii) Add sample: add 100 μL/well to the diluted Cytokine standard to the standard well, 100 μL/well to add the sample to the sample well, and 100 μL/well to add Dilution buffer R (1×) to the blank control well. Cover with sealing film and incubate at room temperature (18℃-25℃) for 2 hours.

(iv) Wash the plate: deduct the liquid in the well, add 300 μL/well to 1×Washing buffer working solution; leave it for 1 minute and discard the liquid in the well. Repeat 4 times, buckle dry on the filter paper each time.

(v) Add detection antibody: 100 μL/well add Biotinylated antibody working solution.

Cover with sealing film and incubate at room temperature (18-25°C) for 1 hour.

(vi) Wash the plate: Repeat step 4.

(vii) Add enzyme: 100 μL/well add Streptavidin-HRP working solution. Cover with sealing film and incubate at room temperature (18-25°C) for 30 minutes.

(viii) Wash the plate: Repeat step 4.

(ix) Color development: Add TMB to 100 μL/well, and incubate at room temperature (18-25°C) in the dark for 5-30 minutes. The reaction is terminated according to the color depth (dark blue) in the well. Usually 10-20 minutes of color development can achieve good results.

(x) Stop the reaction: 100 μL/well quickly add Stop solution to stop the reaction.

(xi) Read the plate: within 10 minutes after termination, use the measurement wavelength to read the value at 450 nm. It is recommended to read the plate at the same time as the measurement wavelength 450 nm, the reference wavelength or the reference wavelength 610-630 nm, and the measurement results will be more accurate.

3. Result analysis

(i) It is recommended to fit the curve coordinate logarithm or natural number. The fitting equation is usually a straight line, a quadratic equation and a four-parameter equation. The best standard curve is selected through various application software fittings, and the corresponding concentration is found according to the OD value of the sample.

(ii) The diluted sample should be multiplied by the dilution factor when calculating the concentration.

ExFect Transfection Reagent

This protocol comes from Vazyme Biotech Co., Ltd.

1. Required equipment and materials

- Plasmid DNA extraction: recommended for endotoxin-free plasmid large-scale extraction kit (FastPure EndoFree Plasmid Maxi Kit, Vazyme #DC202);
- Cell seeding: cell culture medium, FBS, pancreatin, hemocytometer, cell culture plate, etc.;
- ExFect/DNA complex formation: It is recommended to use opti-MEM or other serum-free, anti-bibial cell culture medium, 1.5 ml EP tube, etc.; other materials: PBS, pipette, pipette, 15 ml centrifuge tube, T- 75 cell culture flask, carbon dioxide cell incubator, etc.

2. Factors affecting transfection efficiency

(i) Cell culture medium: ExFect Transfection Reagent is suitable for most commonly used cell culture media, and there is no need to change the medium before and after transfection. The presence of serum does not affect the transfection efficiency.

(ii) Cell density during transfection: Generally speaking, transfection can achieve higher transfection efficiency when the cell density reaches 60%-80%. However, the optimal transfection density of different cells is not the same. Therefore, when transfecting a certain cell for the first time, the optimal transfection density of the cell can be confirmed through preliminary experiments.

(iii) Plasmid DNA purity and concentration: The DNA used for cell transfection should have high purity and no endotoxin residue (recommend to use endotoxin-free plasmid large-scale extraction kit, Vazyme #DC202). The amount of DNA in a 24-well plate is recommended to be 0.5-1 μg per well. Please refer to Table 1 (07-2) for the recommended amount of DNA for different culture volumes.

(iv) The ratio of ExFect to DNA: When transfecting certain cells for the first time, the recommended ratio of ExFect to DNA is 2:1, that is, 1 μg DNA uses 2 μl ExFect. The dosage of ExFect used to transfect 1 μg DNA can be adjusted between 1-5 μl to obtain the best transfection efficiency.

(v) Cell culture time after transfection: For most cell experiments, it can be cultured for 24-48 h. If the experimental situation is special, it can be optimized between 12-72 h.

3. Adjustment of transfection system

Table. Recommended initial transfection conditions for different culture systems

		96	48	24	12	6	10 cm Dish
Surface Area (cm^2) *		0.35	1.0	1.9	3.8	9.6	59
Complex Formation Reaction	Serum-free Media (μl)	20	50	100	200	400	2,000
	ExFect (μl)	0.4	1	2	4	10	60
	1 μg/μl plasmid (μl)	0.2	0.5	1	2	5	30
Complete Growth Media (ml)		0.10	0.25	0.50	1.0	2.0	10

* Data from Greiner tissue culture plates and Falcon 10 cm dishes。

4. Transient cell transfection operation process Cell seeding

(i) Passage the cells about 24 hours before transfection, the seeding density is about 0.3-1 × 105 cells/well.

(ii) Incubate overnight. ExFect/DNA complex formation

(iii) Add 50 μl opti-MEM serum-free medium to a 1.5 ml sterile centrifuge tube, and add an appropriate amount of ExFect (1-5 μl/μg DNA, see Table 1), and mix gently with a pipette.

(iv) Add 50 μl opti-MEM to a 1.5 ml sterile centrifuge tube, and add an appropriate amount of DNA (0.5-1 μg DNA/well, see Table 1), and mix gently with a pipette.

(v) Add ExFect-opti-MEM dropwise to DNA-opti-MEM and mix gently with a pipette. After standing at room temperature for 15-20 minutes, it can be used for transfection.

Note: Try to use the ExFect/DNA complex solution formed within 30 minutes.

Transfection

(i) Add the ExFect/DNA complex mixture dropwise to the culture medium, and gently shake the petri dish to disperse ExFect/DNA evenly.

Note: In special circumstances, fresh serum-containing medium can be replaced before the start of transfection to prevent cell death caused by too high cell density and insufficient nutrition in the culture stage after transfection.

(ii) Incubate overnight for 24-48 h.

Note: If you need to change the fresh medium after transfection, please change it 4-12 h after adding ExFect/DNA complex.

(iii) Collect cells for follow-up experiments.

Lipofectamine™ 3000 Reagent Kit

The kit comes from ThermoFisher Scientific.

1. Protocol Outline

A. Plate cells so they will be 70–90% confluent at the time of transfection.

B. Prepare plasmid DNA-lipid complexes (recommend 2 doses of lipid).

C. Add DNA-lipid complexes to cells.

2. Transfection Amounts

Component	96-well	24-well	6-well
DNA per well	100 ng	500 ng	2500 ng
P3000™ Reagent per well	0.2 µL	1 µL	5 µL
Lipofectamine™ 3000 Reagent per well	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL

3. Experimental Procedure

Day0: Seed cells to be 70–90% confluent at transfection

Day1:

1. Dilute Lipofectamine™ 3000 Reagent in Opti-MEM™ Medium (2 tubes) – Mix well
2. Add Diluted DNA to each tube of Diluted Lipofectamine™ 3000 Reagent (1:1 ratio)
3. Add DNA-lipid complex to cells

Day2-4: Observation under the microscope

MiPure Cell / Tissue miRNA Kit

The kit comes from Vazyme Biotech Co., Ltd.

1. Experiment preparation

The key to RNA extraction is to prevent RNase contamination. RNase is ubiquitous in the environment and is extremely stable. Trace amounts of RNase can quickly degrade RNA. Therefore, protective measures need to be taken from the following aspects:

a. Wear disposable clean gloves; operate in a separate clean area; wear a mask and avoid speaking during the operation. The above methods can effectively prevent the RNase contamination in the sweat and saliva of the experimenter.
b. Use RNase-free experimental equipment, including pipette tips and centrifuge tubes. The equipment used in RNA experiments should be used exclusively, not for other experiments.
c. All reagents used in RNA experiments should be dedicated to avoid cross-contamination after mixed use. After RNase-free ddH2O is recommended to install

Sample processing

The homogenization of the sample is a necessary step for all RNA extraction. By pipetting or homogenizing, the cells or tissue masses are quickly dispersed, and the cell wall and cytoplasmic membrane are ruptured, so that the nucleic acid is released into the lysis solution. Insufficient homogenization may result in decreased RNA yield and purity. Several common sample homogenization treatment methods are listed below:

(i) Liquid nitrogen treatment

Cut an appropriate amount of tissue and weigh it, place it in a pre-cooled mortar, quickly add liquid nitrogen to grind the tissue into powder, and then pour the powder into the pre-cooled centrifuge tube (Note: Pre-cool the centrifuge tube, otherwise the sample will be poured , The boiling of liquid nitrogen will cause sample loss). After the liquid nitrogen has completely evaporated, add an appropriate amount of RNA Isolater and vortex to mix. Since liquid nitrogen grinding can only play a role in breaking up the sample, it is best to homogenize with a syringe or a mechanical homogenizer to reduce the viscosity of the lysate.

(ii) Mechanical homogenizer homogenization

The mechanical homogenizer can efficiently homogenize most tissues and cells, and at the same time play a role in breaking up and homogenizing. Put the sample in a suitable 1-5 ml glass tube or centrifuge tube, add RNA Isolater, insert the rotor into the RNA Isolater, homogenize at low temperature and high speed intermittently, each time 10-30 sec, the same time interval until the sample is completely homogenized. When using a mechanical homogenizer, the general tissue can achieve the ideal homogenization effect within one minute.

Most mechanical homogenizers have rotors of different sizes. Smaller volumes of lysate are suitable for smaller rotors.

(iii) Glass homogenizer

Transfer the sample and appropriate amount of lysate to a glass homogenizer, and push up and down until the tissue mass is sufficiently broken up.

2. Scheme 1. Extraction of small cell RNA

This scheme is suitable for the enrichment of small RNA (<200 nt) from less than 5 × 106 cultured cell samples. If you don't need to remove macromolecules
RNA (>200 nt), you can extract total RNA (including large molecule RNA and small molecule RNA) after step 5 operation followed by scheme 4.

(i) Collect cells

a. Suspension culture cells: Calculate the number of cells, collect the cells by centrifugation at 300 g for 5 min, carefully discard the culture medium, and proceed according to step 2.

b. Adherent cells: Adherent cells can be lysed directly in a culture flask/dish, or they can be collected by centrifugation after trypsinization.

Note: Direct lysis: Calculate the number of cells, completely discard the culture medium, and proceed as in step 2.

Note: Trypsin digestion treatment: count the number of cells, discard the culture medium, add an appropriate amount of PBS to wash the cells, discard the PBS, and then add 0.1-0.25%
Trypsin's PBS was used to digest the cells. After the cells fall off the wall, add the serum-containing culture medium, transfer to a centrifuge tube, and centrifuge at 300 g for 5 min. Collect the cells and proceed according to step 2.

(ii) Add 1 ml RNA Isolater to the cell sample. Vortex or pipette to treat the cell pellet.

Note: Cells collected by centrifugation: first flick to loosen the cells, then add 1 ml RNA Isolater, and pipette 10-15 times to completely break up the cells. Direct lysis of adherent cells: After completely discarding the culture medium, add 1 ml RNA Isolater to the culture flask or dish. Use a gun to blow the cells off the wall completely, collect the lysate, and transfer to a centrifuge tube.

(iii) Leave at room temperature for 2-3 minutes to fully lyse the cells.

Note: At this time, the sample can be stored at 2-8^{o} C for one week, and at -20^{o} C to -70^{o} C for more than six months.

(iv) Add 200 µl of chloroform to the lysis buffer. Shake vigorously by hand for 15 sec and let stand at room temperature for 3 min.

Note: Using vortex instead of oscillation may bring in genomic DNA contamination.

(v) Centrifuge at 12,000 rpm (13,400 × g) for 15 min at 4°C. Pipette 500 µl of the supernatant into a new 1.5 ml centrifuge tube. Follow steps 6-16 to enrich small RNA, or extract total RNA (including small RNA and large RNA) according to Scheme 4.

Note: Please carefully suck the supernatant water phase to avoid sucking the middle and lower organic phases to affect the subsequent extraction results.

(vi) Add 160 µl of absolute ethanol to the supernatant, vortex and mix for 10 sec.(The

following centrifugation is performed at room temperature.)

(vii) Place the MiPure RNAspin Column into the 2 ml Collection Tube. Transfer the above mixture to RNA column. Centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

Note: If you need to extract large molecular RNA, keep the RNA column and extract large molecular RNA (>200 nt) according to Scheme 3.

(viii) Add 0.9 times the volume of absolute ethanol to the filtrate, pipette and mix 3-5 times.

Example: If the volume of the filtrate is 640 μl, 576 μl of absolute ethanol needs to be added.

(ix) Put the miRNA column (MiPure miRNA Column) into a 2 ml collection tube. Transfer half the volume of the mixed solution to the miRNA column. Centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(x) Discard the filtrate and put the miRNA column back into the collection tube. Transfer the remaining mixture to the miRNA column and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(xi) Discard the filtrate and put the miRNA column back into the collection tube. Add 500 μl Buffer miRW1 (alcohol already added!) to the miRNA column, place it at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(xii) Discard the filtrate and put the miRNA column back into the collection tube. Add 500 μl Buffer miRW2 (alcohol already added!) to the miRNA column, place it at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(xiii) Discard the filtrate and put the miRNA column back into the collection tube. Add 500 μl of 80% ethanol (freshly prepared with RNase-free ddH2O) to the miRNA column, place at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(xiv) Discard the filtrate and return the miRNA column to the collection tube. Centrifuge the empty column at 12,000 rpm (13,400 × g) for 2 min, and spin dry the matrix of the miRNA column. This step can completely remove the residual ethanol in the miRNA column.

(xv) Transfer the miRNA column to a new 1.5 ml centrifuge tube. Dry at room temperature for 2-5 minutes, and add 30-50 μl RNase-free ddH2O to the center of the miRNA column membrane. Let stand at room temperature for 2 min. Centrifuge at 12,000 rpm (13,400 × g) for 1 min to collect the filtrate.

Note: The minimum elution volume of MiPure miRNA Column is 30 μl. If it is less than 30 μl, the elution efficiency of RNA will decrease.

(xvi) Discard the miRNA column and store the collected miRNA samples at -70°C.

3. Scheme 2. Extraction of small RNA from animal tissues

This protocol is suitable for the enrichment of small RNA (<200 nt) from animal tissues less than 100 mg. If you don't need to remove large RNA (>200 nt), you can extract total RNA (including large molecule RNA and small molecule RNA) according to Scheme 4 after step 6.

a. Tissue dosage The tissue dosage is a key factor in RNA yield and purity. The tissue dosage of the kit can be as low as 0.01 mg, but the maximum tissue dosage depends on the content of RNA, protein and impurities in the sample.

Note: Animal brain tissue and adipose tissue have low RNA content, and the maximum amount of tissue can reach 100 mg;

Note: Animal liver, spleen, kidney, thymus, etc. are rich in RNA, and the amount of tissue should not exceed 20 mg;

Note: The heart, muscle, and skin contain moderately abundant RNA, and the amount of tissue should not exceed 50 mg.

*MiPure miRNA Column has a binding capacity of 200 μg. Excessive tissue dosage will cause RNA degradation and contamination of large molecular RNA. If there is no relevant information about the tissue to be treated, it is recommended that the first initial dosage is 30 mg, and the dosage of the tissue can be increased or decreased according to the results obtained.

b. **Tissue** lysis and homogenization: Add 1 ml RNA Isolater to the tissue volume of 10-100 mg.

Note: Select appropriate homogenization tools for homogenization, refer to "Sample Handling" on page 5 for details.

c. Leave the tissue at room temperature for 2-3 minutes to fully lyse the tissue.

d. (Optional) Centrifuge at 12,000 rpm (13,400 × g) at 4°C for 10 min. Carefully pipette the supernatant into a new centrifuge tube.

Note: When processing fat samples, a layer of grease will float on the surface of the solution after centrifugation. Carefully transfer the lower layer of liquid to a new tube.

e. Add 200 μl of chloroform to the lysate or supernatant. Shake vigorously by hand for 15 sec and let stand at room temperature for 3 min.

Note: Replacing vibration with vortex may introduce genomic DNA contamination.

f. Centrifuge at 12,000 rpm (13,400 × g) for 15 min at 4°C. Pipette 500 μl of supernatant into a new 1.5 ml centrifuge tube.

Note: Please carefully suck the supernatant water phase to avoid sucking the middle and lower organic phases and affect the subsequent extraction results.

Follow steps 6-16 of Protocol 1 to enrich small RNA or follow Protocol 4 to extract total RNA (including small RNA).

4. Scheme 3: Extraction of large molecule RNA

This program is suitable for extracting large molecular RNA from various samples.

(i) Take the MiPure RNAspin Column combined with the macromolecular RNA in Protocol 1 or 2 and place it in a 2 ml collection tube.

(ii) Add 500 μl Buffer miRW1 (alcohol already added!) to the column, leave it at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(iii) Discard the filtrate and put the RNA column back into the collection tube. Add 500 μl Buffer miRW2 (alcohol has been added!) to the column, leave it at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(iv) Discard the filtrate and put the RNA column back into the collection tube. Add 500 μl of 80% ethanol (freshly prepared with RNase-free ddH2O) to the column, place at room temperature for 1 min, and centrifuge at 12,000 rpm (13,400 × g) for 30 sec.

(v) Discard the filtrate and put the RNA column back into the collection tube. Centrifuge the empty column at 12,000 rpm (13,400 × g) for 2 min, and spin dry the matrix of the RNA column.

(vi) Transfer the RNA column to an RNase-free 1.5 ml centrifuge tube. Dry at room temperature for 2-5 minutes, add 30-50 μl RNase-free ddH2O to the center of the RNA column membrane, and let it stand at room temperature for 2 minutes. Centrifuge at 12,000 rpm (13,400 × g) for 1 min.

(vii) (Optional) Add another 30-50 μl RNase-free ddH2O to the center of the RNA column membrane and let it stand at room temperature for 2 minutes. 12,000 rpm(13,400 × g) Centrifuge for 1 min.

Note: The minimum elution volume of MiPure RNAspin Column is 30 μl. If it is less than 30 μl, the elution efficiency of RNA will decrease. If the RNA yield exceeds 30 μg, it is recommended to perform the second elution in step 7 to obtain a higher yield.

(viii) Discard the RNA column, and store the large RNA collected by centrifugation at -70°C.

FastPure® Cell/Tissue Total RNA Isolation Kit

The kit comes from Vazyme Biotech Co., Ltd.

1. Required Materials and Equipments

Absolute ethanol, 50% ethanol, 1.5 ml RNase-free centrifuge tube, RNase-free pipette tip, etc.

2. Sample processing animal tissue

(i) Homogenization treatment: Take fresh tissue, add 500 μl Buffer RL (350 μl Buffer RL for liver tissue) every 10-20 mg, and homogenize with a glass homogenizer or electric homogenizer until there is no obvious tissue mass.

Note: The homogenization is performed on ice to prevent the instantaneous rise in local temperature from causing RNA degradation.

(ii) Liquid nitrogen grinding: transfer the ground powder with liquid nitrogen to Buffer RL immediately, add 500 μl Buffer RL (350 μl Buffer RL for liver tissue) every 10-20 mg, and vortex until there is no obvious powder cluster.

Note: The samples after homogenization or liquid nitrogen grinding can be stored at -85 ~ -65℃ if they are not extracted immediately.

Cultured cells

(i) **Adherent cells:** no need to digest, after aspirating the cell culture supernatant, you can use Buffer RL immediately in the culture dish

Digestion, lysis; or trypsinization and centrifugation to collect cells and add Buffer RL, add 500 μl Buffer RL for every <5 × 106 cells, vortex and shake until there are no obvious cell clusters.

(ii) **Suspension cells:** collect cells directly by centrifugation, add Buffer RL, add 500 μl Buffer RL for every <5 × 106 cells, vortex and shake until there are no obvious cell

clusters.

Note: If the sample after cell lysis is not extracted immediately, it can be stored at -85 ~ -65℃.

3. RNA extraction

The following processes are all carried out in an environment free from RNase pollution

(i) Transfer the lysed sample to FastPure gDNA-Filter Columns Ⅲ (FastPure gDNA-Filter Columns Ⅲ has been placed in the collection tube), and centrifuge at 12,000 rpm (13,400 × g) for 30 sec. Discard FastPure gDNA-Filter Columns III, and collect the filtrate.

(ii) Add 0.5 times the filtrate volume of absolute ethanol to the filtrate (for liver tissue samples, add 1 times the filtrate volume of 50% ethanol), and mix well.

Note: After adding ethanol, the solution appears turbid or flocculent, which is a normal phenomenon. After shaking and mixing, you can directly proceed to the next step.

(iii) Transfer all the mixture from step 2 to FastPure RNA Columns Ⅲ (FastPure RNA Columns Ⅲ has been placed in the collection tube), centrifuge at 12,000 rpm (13,400 × g) for 30 sec, and discard the filtrate.

(iv) Add 700 μl Buffer RW1 to FastPure RNA Columns Ⅲ, centrifuge at 12,000 rpm (13,400 × g) for 30 sec, and discard the filtrate.

(v) Add 700 μl Buffer RW2 (alcohol has been added) to FastPure RNA Columns Ⅲ, centrifuge at 12,000 rpm (13,400 × g) for 30 sec, and discard the filtrate.

(vi) Add 500 μl Buffer RW2 (alcohol has been added) to FastPure RNA Columns Ⅲ, 12,000 rpm. Centrifuge (13,400 × g) for 2 min, and carefully remove the adsorption column from the collection tube to avoid contact with the filtrate and cause contamination.

(vii) (Optional) If the adsorption column has liquid residue or comes in contact with the filtrate, discard the filtrate, put FastPure RNA Columns Ⅲ back into the collection tube, and leave it at 12,000 rpm (13,400 × g) for 1 min to prevent ethanol contamination.

(viii) Carefully transfer the adsorption column to a new RNase-free Collection Tubes 1.5 ml centrifuge tube, add 50-200 μl RNase-free ddH2O dropwise to the center of the adsorption column, and let stand at room temperature for 1 min, 12,000 rpm (13,400 × g) Centrifuge for 1 min to elute RNA.

Note: In order to increase the yield, RNase-free ddH2O can be preheated at 65℃ in advance, after being dropped into the membrane, let it stand at room temperature for 2-5 min; or after centrifugation for secondary elution.

(ix) The extracted Total RNA can be used directly in downstream experiments or stored at -85 ~ -65℃.

FastPure® Plasmid Mini Kit

The kit comes from Vazyme Biotech Co., Ltd.

1. Required Materials and Equipments

1.5 ml sterile centrifuge tube, absolute ethanol

2. Experimental Procedure

(i) Take 1-5 ml of the bacterial solution cultured overnight (12-16 h), add it to a centrifuge tube (provided by yourself), and centrifuge at 10,000 rpm (11,500 × g) for 1 min. Discard the culture medium and buckle upside down on absorbent paper to suck up the remaining liquid.

(ii) Add 250 μl of Buffer P1 to the centrifuge tube with bacterial pellet (please check if RNase A has been added to Buffer P1), and mix well with a pipette or vortex.

Note: The complete resuspension of bacteria in this step is critical to the yield, and there should be no clumps of bacteria after resuspension. If there are bacteria clumps that are not thoroughly mixed, it will affect the lysis, resulting in low extraction volume and purity.

(iii) Add 250 μl Buffer P2 to step 2, and gently mix upside down for 8-10 times to fully lyse the bacteria.

Note: TMix gently by inverting. Vortexing will cause genomic DNA fragmentation, resulting in genomic DNA fragments mixed in the extracted plasmid. At this time the solution became viscous and translucent, indicating that the bacteria had been fully lysed. Do not use more than 5 minutes to avoid damage to the plasmid. If the solution does not become clear, the lysis may be incomplete due to too much bacteria, so the amount of bacteria should be appropriately reduced.

(iv) Add 350 μl Buffer P3 to step 3, and immediately gently invert 8-10 times to allow the solution to completely neutralize Buffer P2.

A white flocculent precipitate should appear at this time. Centrifuge at 12,000 rpm (13,400 × g) for 10 min.

Note: TBuffer P3 should be inverted and mixed immediately after adding to prevent local precipitation from affecting the neutralization effect. If there are tiny white precipitates in the supernatant, centrifuge again and take the supernatant.

(v) Place the FastPure DNA Mini Columns in the Collection Tube 2 ml collection tube. Transfer the supernatant from step 4 to the adsorption column carefully with a pipette, taking care not to suck the precipitate, and centrifuge at 12,000 rpm (13,400 × g) for 30-60 sec. Pour off the waste liquid in the collection tube, and put the adsorption column back into the collection tube.

(vi) (Optional) Add 500 μl Buffer PW1 to the adsorption column. Centrifuge at 12,000 rpm (13,400 × g) for 30-60 sec. Discard the waste liquid and put the adsorption column back into the collection tube.

Note: TIf the host bacteria are end A+ (TG1, BL21, HB101, JM series, ET12567, etc.), these host bacteria contain a large amount of nuclease and are easy to degrade plasmid DNA. This step is recommended. If the host bacteria is endA-host bacteria (DH5α, TOP10, etc.), this step can be omitted.

(vii) Add 600 μl Buffer PW2 (please check if it has been diluted with absolute ethanol) to the adsorption column. Centrifuge at 12,000 rpm (13,400 × g) for 30-60 sec. Discard the waste liquid and put the adsorption column back into the collection tube.

(viii) Repeat step 7.

(ix) Put the adsorption column back into the collection tube. Centrifuge at 12,000 rpm (13,400 × g) for 1 min to dry the adsorption column. The purpose is to completely remove the residual rinsing liquid in the adsorption column.

Note: TEthanol residue in the rinsing solution will affect downstream enzyme reactions, such as enzyme digestion, enzyme chain, PCR, etc. This step cannot be omitted.

(x) Place the adsorption column in a new sterile 1.5 ml centrifuge tube. Add 30-100 μl Elution Buffer to the center of the membrane of the column adsorption column. Let stand at room temperature for 2 min, and centrifuge at 12,000 rpm (13,400 × g) for 1 min to elute DNA.

Note: TThe elution volume should not be less than 30 μl. If it is less than 30 μl, the elution efficiency will decrease. If you need to obtain the highest yield, preheat the Elution Buffer to 55° C to improve the elution efficiency. In addition, the solution obtained by centrifugation can be added to the centrifugal adsorption column again, and step 10 can be repeated. If you need to use ddH2O for subsequent sequencing, make sure that the pH of ddH2O is within the range of 7.0-8.5. A pH lower than 7.0 will reduce the elution efficiency.

(xi) Discard the adsorption column and store the DNA product at -20°C to prevent DNA degradation.

qRT-PCR: EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix

Step 1. Prepare the RT-PCR mix

Component	Volume
Total RNA/mRNA	0.1 ng – 5 μg/ 10pg – 500 ng
Random Primer(N9)(0.1 μg/ μL)	1 μL
2x ES Reaction Mix	10 μL
EasyScript RT/RI Enzyme Mix	1 μL
gDNA Remover	1 μL
RNase-free Water	Variable
Total volume	20 μL

Step 2. RT-PCR

To get the product for qPCR, as for samples with Random Primer, they are incubated firstly under 25℃ for 10minutes and later under 42℃ for 15minutes.

Later, heat under 85℃ for 5 seconds to inactivate EasyScript RT/RI and gDNA remover.

Step 3. qPCR

Component	Volume	Final Concentration
Template	Variable	As required
Forward Primer (10 μM)	0.4 μL	0.2 μM
Reverse Primer (10 μM)	0.4μL	0.2 μM
2x TransStart Top/Tip Green qPCR SuperMix	10 μL	1x
Passive Reference Dye (50x) (optional)	0.4 μL	1x
Nuclease-free Water	Variable	-
Total volume	20 μL	-

Three Steps Method	
Temperature (℃)	Time (second)
94	30
94	5 (40-45 cycles)
50-60	15 (40-45 cycles)
72	10 (40-45 cycles)