# EZ-press Cell to cDNA Kit for microRNA

## Catalog No.: EZB-miRT1

## Description

The EZBioscience® EZ-press Cell to cDNA Kit for microRNA is directly used for reverse transcription fast and conveniently from cultured cell lysates. The cells are first lysed in the Cell Lysis Buffer to release the microRNA. Then, cell lysates are treated with gDNA Remover to eliminate the contaminating genomic DNA. Finally, Polyadenylation and Reverse transcription are completed in one step in the same reaction system.

The kit contains 6 tubes of reagents: Cell Lysis Buffer, gDNA Remover, miRNA RT Enzyme Mix, 4× miRNA RT Buffer, a forward U6 primer, and a universal qPCR reverse primer (Universal 3' qPCR Primer), which can be used for the quantitative detection of all miRNAs. So only a specific forward primer for each target miRNA is needed for qPCR reactions.

The miRNA RT Enzyme Mix mainly contains *E.coli* Poly(A) Polymerase, reverse transcriptase, and RNase Inhibitor. Among them, *E.coli* Poly(A) Polymerase not only has high efficiency of adding poly(A) tail, but also specifically recognizes single-stranded mature miRNA, thus avoiding further reverse transcription reaction of miRNA precursor with double-stranded structure. The M-MLV mutant reverse transcriptase has strong anti-interference ability and amplification ability, and the amplification efficiency is particularly good.

4× miRNA RT Buffer contains all the raw materials and primers for miRNA polyadenylation and reverse transcription reaction, including Oligo (dT)-universal tag primer, buffer and dNTPs, and has been carefully optimized to ensure polyadenylation and the reverse transcription are simultaneously performed efficiently.

## Components

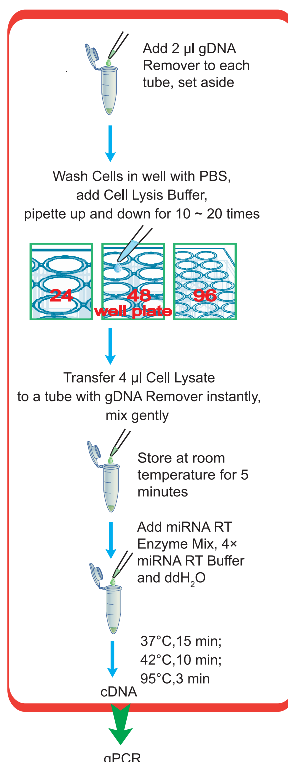
| Components | EZB-miRT1 (50 Rxns) |
|---|---|
| Cell Lysis Buffer | 11 ml |
| gDNA Remover | 110 µl |
| miRNA RT Enzyme Mix | 110 µl |
| 4× miRNA RT Buffer | 275 µl |
| Universal 3' qPCR Primer (10 µM) | 750 µl |
| U6 Primer (10 µM ) | 150 µl |
| Nuclease free ddH$_2$O | 1 ml |

## Storage

Store at -20°C.

## Protocol

### Experimental Procedure Overview:




### Preparation of cells

Cells are recommended to be passaged in 24, 48, or 96 well cell culture plates, and should have a confluence of more than 50% before use.

Thaw the ***Cell Lysis buffer***, invert or flick the tube several times to **mix thoroughly** (**do not use vortex**), and place the tube at room temperature before use.

### Cell Lysis

1. **First, add 2 µl gDNA Remover to each tube before starting cell lysis.**

**2A. For adherent cells:**

a) Aspirate the culture medium from the wells, Wash the wells with PBS (~200 µl/well).

b) Lysis cells in turn:
Remove PBS in one well as completely as possible without disturbing the cells. Add Cell Lysis Buffer to this sample with the proportion of 50 µl Cell Lysis Buffer/1×10$^5$ cells, pipette up and down for 10 times to lyse the cells (be avoid of producing too much bubbles). [Note: Cells should be lysed by pipetting up and down, until no cells residues could be seen at the bottom of the wells]. Transfer 4 µl of the cell lysate to a tube with 2 µl gDNA Remover immediately and mix gently, placed on ice.

c) Treat the remaining sample with the above procedure, one cell sample at a time.

**2B. For suspension cells:**

a) The cell suspension is collected into a RNase-free centrifuge tube, centrifuged at 500 g for 3 minutes to remove the medium, and then the cells are suspended in PBS and counted.

b) For cells with similar size to B and T cells, aspirate PBS suspension containing 1×10$^5$ to 3×10$^5$ into a new 1.5 ml centrifuge tube for each sample, centrifuge cells at 500 g for 3 minutes, then aspirate the PBS carefully without disturbing the cell pellets; then add 10 ~ 30 µl Cell Lysis Buffer to one sample, pipetting up and down for 10 times to lyse the cells.
For cells with similar size to macrophages and CHO cells, aspirate PBS suspension containing 1×10$^5$ to 3×10$^5$ into a new 1.5 ml centrifuge tube for each sample, centrifuge cells at 500 g for 3 minutes, aspirate the PBS carefully without disturbing the cell pellets; then add 50 ~ 150 µl Cell Lysis Buffer to one sample, pipetting up and down for 10 times to lyse the cells

c) After cells are lysed thoroughly, transfer 4 µl of the cell lysate to a tube with 2 µl gDNA Remover immediately and mix gently, placed on ice.

3. **After all samples completely treated, Incubate at room temperature (19 ~ 27°C) for 5 minutes.**

4. **Put the cell lysates on ice; carry out the RT reactions as soon as possible (no more than 30 min).**

### Reverse Transcription

5. **Add miRNA RT Enzyme Mix, 4× miRNA RT Buffer and ddH$_2$O to the cell lysates**:

| Components | Volume (20 µl) |
|---|---|
| Cell lysates | 6 µl |
| miRNA RT Enzyme Mix | 2 µl |
| 4x miRNA RT Buffer | 5 µl |
| Nuclease free ddH$_2$O | 7 µl |

6. **Once assembled, mix reactions gently, then centrifuge briefly to collect the contents at the bottom of the reaction tube. Perform the reverse transcription reaction at 37°C for 15 minutes, 42°C for 10 minutes, then 95°C for 3 minutes.**

**Note:**

1. Read the manual carefully before use to ensure the experiments are done right.
2. The lysis capacity of 50 µl Cell Lysis Buffer is about 1×10$^5$ cells, varies according to the cell type. To achieve the best results, it is strongly

recommended to do a pilot experiment to determine the best number of cells.

3. Cells cultured in 96, 48, 24 well cell culture plates with the cell number of not more than $4×10^5$ can be used for lysis directly after PBS washing. When doing with cells more than $4×10^5$/well (or dish), follow the procedure 2B.

4. The cell lysate is incompatible with any other RT reagents commonly used (no or few cDNA can be produced using other RT reagents). So the cell lysates **MUST** be reverse transcribed using the specially optimized RT reagents supplied in this kit.

## Real-time PCR

The reverse transcription products can be used in PCR reactions immediately without need to dilute, or diluted based on the abundance of gene expression; if not used immediately, it can be stored in aliquots at -80°C for use within 3 month. Avoid repeated freeze-thaw cycles. Foward primer is specific designed according to the sequence of targeted miRNA. Reverse primer is miR-Q Reverse Primer.

Set up the reactions as the following table with using undiluted reverse transcription products:

| Components | Volume (20 µl) |
|---|---|
| 2× SYBR Green qPCR Master Mix | 10 µl |
| Template | 0.4 µl |
| miRNA specific Forword primer (10 µM) | 0.4 µl |
| miR-Q Reverse Primer(10 µM) | 0.4 µl |
| ROX Reference Dye* | 0.4 µl |
| $ddH_2O$ | 8.4 µl |

*The usage of ROX depends on the real-time PCR instrument used.

Then set the real-time PCR amplification program according to the product manual of the qPCR Kit, and run the reaction.

### Cell types tested by EZ-press Cell to cDNA Kit for microRNA

| Cell Name | Growth | Species | Source Tissue |
|---|---|---|---|
| A431 | Adherent | *H. sapiens* | Epidermoid Carcinoma |
| A549 | Adherent | *H. sapiens* | Lung Carcinoma |
| CHO-K1 | Adherent | *Hamster* | Ovary |
| COS-7 | Adherent | *Monkey* | Kidney |
| HEK-293T | Adherent | *H. sapiens* | Kidney |
| HeLa | Adherent | *H. sapiens* | Cervical Adenocarcinoma |
| HepG2 | Adherent | *H. sapiens* | Liver Carcinoma |
| Jurkat | Suspension | *H. sapiens* | Acute T-Cell Leukemia |
| NCI-H446 | Adherent | *H. sapiens* | Small Cell Lung Cancer |
| NIH-3T3 | Adherent | *M. musculus* | Embryonic Fibroblast |

*Cell types tested are listed in this table. When use other cells that are not included in this table, it is suggested optimizing the cell numbers to get better results.*

## Representative experimental results

cDNA synthesized by **EZBioscience®** EZ-press Cell to cDNA Kit for microRNA were evaluated by real-time qPCR with 3 pairs of primers (in A549 cells). As shown in the following figure, the amplification curve, melt curve and Ct value of these microRNAs were very good, indicating that the expression of microRNA can be well detected using this kit.

## Troubleshooting

**1. The Ct value of reference gene is too large or no amplification.**

**a.** Make sure the reagents and primers are reliable. Fresh reagents are preferred. The solutions of primers should be stored in aliquots to avoid contamination;

**b. Make sure the experimental process is correct, e.g.:**

**1)** Mix the Cell Lysis Buffer before use;

**2)** Wash the cells with PBS before cell lysis and aspirate the PBS as clear as possible;

**3)** Add the Cell Lysis Buffer and gently pipette 10 times. The cells must be completely covered by the Cell Lysis Buffer;

**4)** Mix all the reactions thoroughly before start;

**5)** Make sure the cell lysates are reverse transcribed using the RT reagents supplied in this kit.

**c.** In most conditions, be sure the cell number loaded is not more than $1×10^5$ per 80 µl Cell Lysis Buffer, if the result is still not so good, decrease the cell number by half, or double the volume of Cell Lysis Buffer used and try again;

**d.** Remove PBS as completely as possible before adding the Cell Lysis Buffer to cells;

**e.** Make sure there are really enough cells left after PBS washing, especially when the cell number is less than 10000. For small amount of cells (< 10000), use 5 ~ 10 µl Cell Lysis Buffer.

**2. The reference gene can be amplified normally, but genes of low expression level cannot.**

**a.** Try to use more cells, use 50 µl Cell Lysis Buffer per $1×10^5$ cells;

**b.** Make sure the primers are right, without contamination or degradation, and the target fragment can be amplified in the qPCR reaction properly.

**c.** If the Ct value is just slightly larger than your expectation, try to transfer 20 ~ 100 µl cell lysates to a new tube, add ethanol to the cell lysates (final ethanol concentration 70%), and centrifuge at 12000 rpm for 2 minutes to precipitate the RNA. Discard the supernatant and air dry for 5 minutes. Dissolve in 4 µl Cell Lysis Buffer and 2 µl gDNA Remover, and incubate at room temperature (19 ~ 27°C) for 5 minutes.

**3. Some genes' amplification plot or melt curve appears abnormal.**

**a.** Decrease the cell number, or increase the volume of Cell Lysis Buffer to lyse the cells completely. For most conditions, the cell number is not more than $1×10^5$ per 50 µl Cell Lysis Buffer;

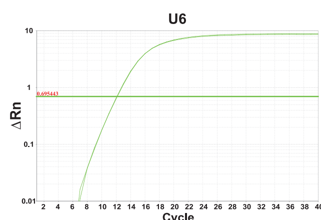
**b.** Decrease the volume of cell lysates used in RT reactions, e.g. add 2 µl of cell lysates to each 20 µl RT reaction instead of 4 ul cell lysate;

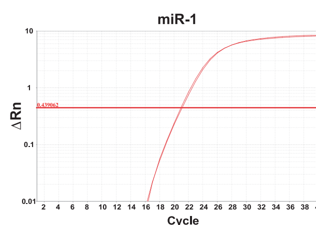
**c.** Decrease the volume of cDNA used in qPCR, e.g. add 0.4 µl of cDNA to the 20 µl qPCR reactions instead of 0.8 µl cDNA;

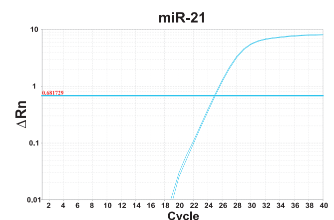
**d.** Make sure the reagents are reliable;

**e.** Make sure the primers are not contaminated or degraded. The volume of primers can be increased.

**Amplification Plot**




**Melt Curve Plot**