220-plex microRNA expression profile of a single cell

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Here we describe a protocol for the detection of the microRNA (miRNA) expression profile of a single cell by stem-looped real-time PCR, which is specific to mature miRNAs. A single cell is first lysed by heat treatment without further purification. Then, 220 known miRNAs are reverse transcribed into corresponding cDNAs by stem-looped primers. This is followed by an initial PCR step to amplify the cDNAs and generate enough material to permit separate multiplex detection. The diluted initial PCR product is used as a template to check individual miRNA expression by real-time PCR. This sensitive technique permits miRNA expression profiling from a single cell, and allows analysis of a few cells from early embryos as well as individual cells (such as stem cells). It can also be used when only nanogram amounts of rare samples are available. The protocol can be completed in 7 d.

INTRODUCTION

miRNAs comprise a large family of small non-coding RNAs with diverse and important functions during vertebrate development[1–4]. Generating expression profiles of miRNAs for specific cells and tissues is a prerequisite for understanding their function. A number of strategies have been developed to obtain miRNA expression profiles[5–19]. Due to their extremely small size, most current miRNA profiling methods are not highly sensitive, and they usually require microgram quantities of total RNA for analysis, which correspond to hundreds of thousands of cells. Most methods also have a relatively narrow linear dynamic range, usually <3 logs. However, by taking advantage of real-time PCR amplification, our miRNA expression profiling method can be used to generate miRNA profiles from <1 ng total RNA, which is thousands of times more sensitive than other commonly used miRNA profiling methods[20–22]; it is easy to cover a linear dynamic range of 6 logs by this method. The method is also specific for the detection of mature miRNAs. This approach can therefore be used to obtain miRNA expression profiles from limited amounts of material (such as from an early embryo), and, indeed, from individual stem cells and blastomeres (for example, from cloned embryos)[23]. We expect that the method could also be adapted for other small RNAs, such as repeat-associated small-interfering RNAs (rasiRNAs) and trans-acting siRNAs (tasiRNAs), by designing appropriate primers and probes following the same principles of design as for our miRNA set[24,25].

Appropriate methods are required for the isolation of single cells from early embryos and tissues. Here we describe the method for the isolation of single embryonic stem (ES) cells. This protocol could be modified to prepare single cells from other sources. The whole method has been described elsewhere[20–22], and the schematic strategy of the method can be found in Figure 1 of ref. 21. Here we provide a detailed step-by-step protocol for the method.

MATERIALS

REAGENTS

- BSA (Sigma; cat. no. B 8894)
- PBS (Gibco; pH 7.2)
- Molecular biology grade H_2O (Eppendorf)
- BSA–PBS solution (0.1% (wt/vol) or 1 mg ml^{-1} BSA in PBS)
- EDTA–PBS solution (0.038% (wt/vol) or 0.38 mg ml^{-1} EDTA in PBS)
- 1× trypsin–EDTA solution (Gibco; 0.25% (wt/vol) or 2.5 mg ml^{-1} trypsin, 0.038% (wt/vol) or 0.38 mg ml^{-1} EDTA)
- 220-plex reverse primers (RPs; 200 nM; the sequence of these primers are listed in ref. 21; Integrated DNA Technologies Inc.)
- 220-plex forward primers (FPs; 450 nM; the sequence of these primers are listed in ref. 21; Integrated DNA Technologies Inc.)
- Universal RP (URP; 100 μM; the sequence is: 5′-CTCAAGTGTCGTGGAGTCGGCAA-3′; Integrated DNA Technologies Inc.)
- 100 mM dNTP (ABI)
- 100 mM $MgCl_2$ (ABI)
- 2× TaqMan® Universal PCR Master Mix (2× UMM) without AmpErase® UNG (ABI, cat. no. 4324018)
- RNase inhibitor (20 U μl^{-1}; ABI, cat. no. N8080119)
- Moloney murine leukemia virus (MMLV) reverse transcriptase (50 U μl^{-1}; ABI: high capacity cDNA achieve kit, cat. no. 4322171)
- AmpliTaqGold DNA polymerase (5 U μl^{-1}; ABI, cat. no. 4311806)
- 1-plex FP (5 μM) plus TaqMan probe (1 μM) mix: the sequences of this primer and probe are listed in ref. 21; Integrated DNA Technologies Inc.)
- Mouse embryonic fibroblasts (MEFs)

EQUIPMENT

- Prism 7000 SDS (ABI) or any other real-time PCR instruments compatible with TaqMan probe-directed real-time PCR assays

REAGENT SETUP

TaqMan probes These contain FAM-labeled dye that might have potential toxicity.

MEFs These were prepared from embryonic day (E) 13.5 Dicer$^{flox/flox}$ mouse embryos and cultured in DMEM medium with 15% (vol/vol) FCS[26]. Dicer$^{flox/flox}$ MEFs were transformed by retroviral transduction with large T antigen. The cells were then treated twice with adeno-Cre and seeded at low density. The Dicer$^{-/-}$ individual clones were picked after 3 wk. The Dicer$^{-/-}$ identity of these MEFs was confirmed by genotyping.

PROCEDURE

Preparation of single cells

1| In order to pick up and transfer individual cells, ideally use a micropipette attached to, and controlled by, a mouth tube, which is commonly used for manipulating early mouse embryos under a dissection microscope. To commence, pick up an ES cell colony with a micropipette. Transfer it to a drop of EDTA–PBS and incubate at room temperature (20–30 °C) for 10 min. Then, transfer the ES cell cluster to a drop of trypsin–EDTA solution for 10 min at 37 °C.

● TIMING

2| Transfer the cell cluster to a drop of BSA–PBS, and then to another drop of BSA–PBS. Gently pipette 20–30 times with a heat-polished Pasteur pipette or microcapillary until the cell cluster dissociates into single cells. Transfer a proportion (i.e., numbers that are sufficient for later analysis) of the single cells to another drop of BSA–PBS.

▲ CRITICAL STEP The methods used for obtaining single-cell suspensions from different tissues will need to be established, and will depend on the nature of the tissue used for analysis. Provided they lead to isolation of healthy intact single cells, any suitable techniques and enzymes can be used to disaggregate the tissues.

? TROUBLESHOOTING

Lysis of single cells and reverse transcription

3| Prepare a RT master mix containing the following:

	X1	X20X1.1
H_2O	3.61 μl	79.42 μl
10× cDNA archiving kit buffer	0.5 μl	11 μl
220-plex RPs (200 nM)	0.125 μl	2.75 μl
RNase inhibitor (20 U μl^{-1})	0.065 μl	1.43 μl
Total volume	4.3 μl	94.6 μl

Mix gently but thoroughly.

▲ CRITICAL STEP The final concentration of 220-plex RPs should be 1–5 nM depending on the nature of the sample.

● TIMING

4| Prepare an RT reaction medium by adding 4.3 μl RT master mix to each thin-walled PCR Eppendorf tube on ice. Pick and transfer a single cell into each tube. It is critical to ensure that the amount of BSA–PBS carry over with each cell is minimal. Centrifuge at 9,000*g* for 10 s, followed by treatment of the samples at 95 °C for 5 min. Place on ice.

▲ CRITICAL STEP It is important to ensure that the cell to be analyzed has actually been transferred into the reverse-transcription reaction buffer from the drop of BSA–PBS with the help of a micropipette.

▲ CRITICAL STEP It is important not to use the same capillary tube repeatedly for the transfer of single cells.

▲ CRITICAL STEP It is important to include, as a control, a sample without a single cell, in which only BSA–PBS solution equivalent to the volume transferred with a single cell is added to a reverse-transcription reaction tube. This control will also ensure that the drop of BSA–PBS is not contaminated.

? TROUBLESHOOTING

5| Prepare an enzyme mix containing the following:

	X1	X20X1.1
RNase inhibitor (20 U μl^{-1})	0.065 μl	1.43 μl
MMLV RT (50 U μl^{-1})	0.335 μl	7.37 μl
dNTP (100 mM; with dTTP)	0.25 μl	5.5 μl
Total volume	0.65 μl	14.3 μl

Add 0.65 μl enzyme mix into each tube, mix evenly by vortexing the samples briefly and then spin down the samples by centrifuging at 9,000*g* for 10 s.

● TIMING

6| Perform the RT reaction by performing the following cycles: first, 16 °C for 30 min; then, 20 °C for 30 s, 42 °C for 30 s and 50 °C for 1 s for 60 cycles; then, 85 °C for 5 min (to inactivate RT); and, finally, 4 °C prior to the next step.

Pre-PCR

7| Prepare a pre-PCR master mix by combining the following:

● TIMING

	X1	X20X1.1
2× UMM	12.5 μl	275 μl
220-plex FPs (450 nM)	2.78 μl	61.16 μl
H_2O	0.72 μl	15.84 μl
$MgCl_2$ (100 mM)	0.5 μl	11 μl
dNTP (100 mM)	1 μl	22 μl
URP (100 μM)	1.25 μl	27.5 μl
AmpliTaqGold polymerase (5 U μl^{-1})	1.25 μl	27.5 μl
Total volume	20 μl	440 μl

8| Add 20 μl pre-PCR master mix into each RT reaction tube. Mix evenly by vortexing and then spin down the samples by centrifuging at 9,000*g* for 10 s.

9| Perform the pre-PCR reaction by the following cycles: first, 95 °C for 10 min (to activate the AmpliTaqGold polymerase); then, 55 °C for 2 min; then, 95 °C for 1 s and 65 °C for 1 min for 18 cycles; and, finally, save at 4 °C.
▲ **CRITICAL STEP** The number of initial PCR cycles can be reduced to 10–18 if the amount of material available is >15 pg (or if more than a single cell is used) to detect miRNAs.
■ **PAUSE POINT** The pre-PCR product can be stored for months at –80 °C.

10| Dilute the pre-PCR product to give a 1:4 dilution (i.e., 25 μl pre-PCR product plus 75 μl H_2O).
▲ **CRITICAL STEP** The dilution of the initial PCR product can be variable. Use of between 1/1,000 and 1/10,000 of the initial PCR product for a 10 μl real-time PCR reaction will produce reliable results.
● **TIMING**

Real-time PCR for quantification

11| Prepare a TaqMan master mix containing the following:

	X1	X220X1.1X1.1
2× UMM (no UNG)	5 μl	1331 μl
URP (100 μM)	0.1 μl	26.62 μl
1:4 diluted pre-PCR product	0.1 μl	26.62 μl
H_2O	2.8 μl	745.36 μl
Total volume	8 μl	2129.6 μl

For the PCR reaction, combine 17.6 μl TaqMan master mix and 4.4 μl 1-plex FP (5 μM) plus TaqMan probe (1 μM), to give a total volume of 22 μl.
▲ **CRITICAL STEP** The final concentration of FP, URP and TaqMan probe in the real-time PCR reaction can also be variable. For example, between 1 μM FP plus 1 μM URP plus 0.2 μM probe, and 0.25 μM FP plus 0.25 μM URP plus 0.05 μM probe can be used, all of which work robustly.
? TROUBLESHOOTING

12| For the standard, prepare the following PCR master mix:

	X1	X40X1.1
2× UMM (no UNG)	5 μl	220 μl
H_2O	1.9 μl	83.6 μl
URP (100 μM)	0.1 μl	4.4 μl
1-plex FP (5 μM) plus TaqMan probe (1 μM)	2 μl	88 μl
Total volume	9 μl	396 μl

For the PCR reaction of standards, combine 19.8 μl PCR master mix and 2.2 μl standard cDNA sample to give a total volume of 22 μl.

13| Mix thoroughly and add 10 μl to each well of a 96-well plate (**Fig. 1**). In the plate, samples S1–S4 represent the standard samples with serial 10-fold dilution. NTC represents the no template control reaction. Samples A1–A43 represent the samples for assaying the miRNA expression. Each assay is duplicated. Ideally, assays for the same miRNA in different samples should be run on the same plate to reduce potential variations between different plates.

14| Load the plate into the ABI Prism 7000 SDS, or any other real-time PCR instruments compatible with the TaqMan probe-directed real-time PCR assay. Run the following real-time PCR program: 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min for 40 cycles.

? TROUBLESHOOTING

● **TIMING**

Steps 1,2: 30 min.
Steps 3,4: 1 h.
Steps 5,6: 4 h.
Steps 7–9: 1.5 h.
Steps 10–14: Time required is dependent on sample numbers. For example, for each 96-well plate (one round of real-time PCR) it is ~3 h. For six samples (three single cells of one cell type versus three single cells of the other cell type), it takes ~6 d to analyze all 220 miRNAs in duplicate.

? TROUBLESHOOTING

See **Table 1**.

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
A25	A26	A27	A28	A29	A30	A31	A32	A33	A34	A35	A36
A25	A26	A27	A28	A29	A30	A31	A32	A33	A34	A35	A36
A37	A38	A39	A40	A41	A42	A43	S1	S2	S3	S4	NTC
A37	A38	A39	A40	A41	A42	A43	S1	S2	S3	S4	NTC

Figure 1 | Layout of the 96-well plate. For definitions, see Step 13.

TABLE 1 | Troubleshooting table.

PROBLEM	SOLUTION
Picking buffer-only control shows miRNA expression signal.	Make sure the BSA–PBS drop holding dissociated single cells is not contaminated by lysed cells. Be sure to use a clean capillary tube to pick each individual cell. Aliquot all reagents for the RT and pre-PCR step into small batches. Each batch should be used only once.
House-keeping miRNA (e.g., miR-16) shows significant variations between single cells in the same batch of the same cell type.	Make sure the time interval between dissociation and picking individual cells is as short as possible (ideally <30 min).
NTC for real-time PCR shows positive signal.	Real-time PCR is an extremely sensitive assay. It is essential therefore to keep all the reagents as clean as possible. Aliquot all reagents for real-time PCR into small batches. Discard a batch in case of problems and use a new batch.
Sensitivity of the real-time PCR assay decreases significantly.	The TaqMan probe is sensitive to light as well as to freeze–thaw cycles. Reagents containing TaqMan probe should be stored in the dark at 4 °C, and the exposure of the reagent to light should be kept as short as possible. Avoid repeated freeze–thaw of the TaqMan probe.
Whole-set assay of miRNAs shows significant variations between single cells from the same batch of the same cell type.	The standard volume for real-time PCR is 50 μl. To reduce the cost of the assay, we use 10 μl real-time PCR reagents. This works robustly in our hands. However, in case of problems and significant variability, the volume of real-time PCR can be increased to 20 μl.

ANTICIPATED RESULTS

Initially, we determined that the initial PCR step did not introduce any bias into the miRNA expression profiling. We compared the expression of miR-16 in ES cells with and without the initial PCR step, and found that 18 cycles of the initial PCR step did not bias the measurement of miR-16 expression levels (**Fig. 2**). Compared with the assay without the initial PCR step, 18 cycles of this PCR step increased the sensitivity of the assay as expected. The expected difference in the threshold cycle (Ct) values should be equal to the number of initial PCR cycles minus the dilution factor. As 18 cycles of the initial PCR-amplified cDNAs were diluted 4,000-fold ($2^{12} = 4{,}096$), the expected Ct value difference of RT only and 18 cycles of the initial PCR step was 18–12 = 6. The average Ct value difference was measured to be 6.01 ± 0.14 (**Fig. 2**).

We also determined whether the miRNA profiling assay could be carried out directly on cell lysate following heat treatment of the samples at 95 °C for 5 min. This was found to give a reliable expression profile. We compared the miRNA expression profiles of whole-cell lysate of 10,000 ES cells with 1 ng (1,000 pg) of purified total RNA of ES cells. We found that the miRNA expression profile of whole-cell lysate paralleled the results from purified total RNA (**Fig. 3**). This result indicates that genomic DNA in the cell lysate does not interfere with miRNA detection. Thus, heat-treated whole-cell lysate miRNA profiling is a reliable method of obtaining the miRNA expression profile.

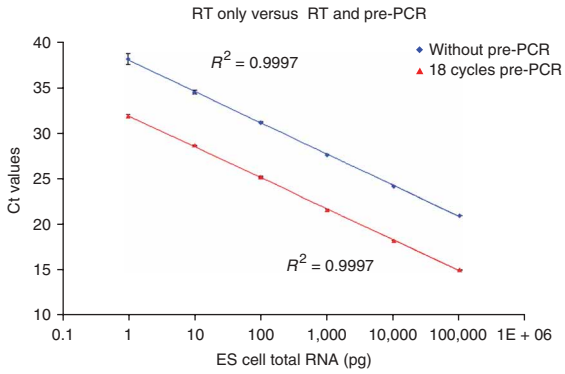

Figure 2 | Titration curves of miR-16 in ES-cell total RNA with (red triangles) and without (blue diamonds) pre-PCR amplification. R^2 represents the linear correlation coefficient. The SD is shown as an error bar for each data point.

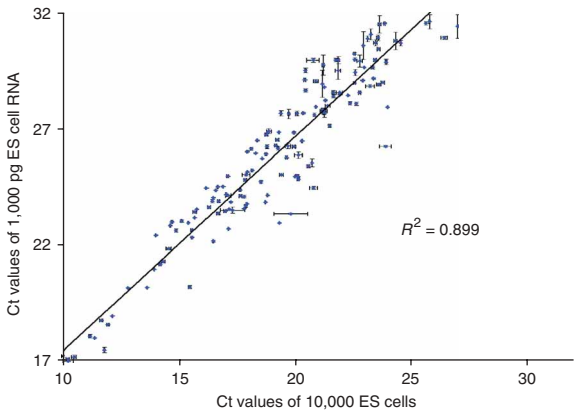

Figure 3 | Expression profile of 220 mouse miRNAs for whole-cell lysate of 10,000 ES cells and 1,000 pg total RNA purified from ES cells. The results are plotted as correlation scatter plots with the Ct values for 1,000 pg purified total RNA on the *y*-axis and the Ct values of 10,000 ES whole cells on the *x*-axis. R^2 represents the linear correlation coefficient. The SD is shown as an error bar for each data point.

We also ran the assay on synthesized miR-16 and established its standard curve. This permitted us to deduce the relative abundance of all miRNAs (**Fig. 4**).

To confirm the specificity of the assay, and to discriminate between mature miRNAs, the corresponding genomic loci, primary miRNA transcripts and precursor miRNAs, we ran a 220-plex assay on Dicer wild-type ($Dicer^{Flox/Flox}$) and Dicer-knockout ($Dicer^{-/-}$) single MEFs (**Fig. 5**). It is known that in Dicer-knockout cells, the mature miRNAs disappear, while the pri-miRNAs and pre-miRNAs will be unaffected or might even accumulate[27–30]. We found that in the assay of the 215 miRNAs in Dicer-knockout MEFs, 177 showed a Ct value of 40 (which implies absence of a detection signal), while 25 showed a low signal with a relative abundance of less than 1% of the corresponding value detected in Dicer wild-type MEFs. Thus, for the 202 miRNAs (94%), the assay could reliably discriminate between mature miRNAs from the corresponding genomic loci, primary miRNA transcripts and precursor miRNAs. This was the case even under the conditions where pri-miRNAs and pre-miRNAs accumulated and reached levels that significantly exceeded those in normal wild-type cells[27–30]. The 13 miRNAs (6%) that showed background signal in Dicer-knockout MEFs of $>1\%$ compared with Dicer wild-type MEFs were as follows: miR-34a, miR-129, miR-139, miR-140*, miR-150, miR-151, miR-188, miR-223, miR-298, miR-323, miR-324-3p, miR-382 and let-7i.

The majority of known miRNAs have at least a two-base difference between them[12]. Furthermore, no two miRNAs have sequences that are closer than those within the let-7 family. To determine the power of discrimination of our 220-plex method, we used synthetic let-7a templates (100 pM to 100 fM) as the inputs for the RT step and pre-PCR to amplify the cDNA products, followed by analysis of the let-7 family targets (**Table 2**). We found that the 220-plex assay could discriminate perfectly between miRNA sequences with two or more base differences. However, there was a 75% crossing reaction between let-7a and let-7f, which differed by only one base at position 10 from the 3′ end. We could increase the specificity of detection by reducing the overlap between the RPs and miRNA targets, but this would also reduce the efficiency of RT. To achieve single-cell miRNA

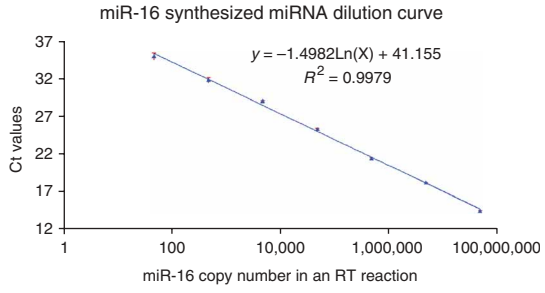

Figure 4 | Titration curve of synthesized miR-16. R^2 represents the linear correlation coefficient. The SD is shown as an error bar for each data point.

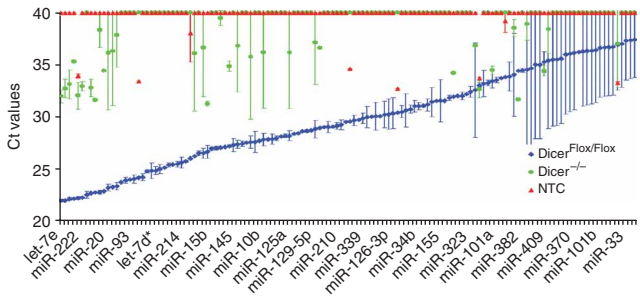

Figure 5 | miRNA expression profile of $Dicer^{Flox/Flox}$ and $Dicer^{-/-}$ MEF single cells. A Ct value of 40 means that there was no detectable signal after 40 cycles of real-time PCR amplifications. The SD is shown as an error bar for each data point.

TABLE 2 | Specificity of the 220-plex miRNA expression profiling assay.

Ratios	Mouse miRNA sequences	100 pM let-7a	10 pM let-7a	1 pM let-7a	100 fM let-7a
let-7a	UGAGGUAGUAGGUUGUAUAGU	1.00	1.00	1.00	1.00
let-7b	UGAGGUAGUAGGUUGUGUGGUU	0.00	0.00	0.00	0.00
let-7c	UGAGGUAGUAGGUUGUAUGGUU	0.00	0.00	0.00	0.00
let-7f	UGAGGUAGUAGAUUGUAUAGU	0.66	0.75	0.86	0.72

The expression levels of the let-7a assay at each concentration were normalized as 1. The bases indicated in bold are those that differ from the sequence of let-7a.

profiling, we chose to design multiplex assays that had higher sensitivity, while maintaining the power of discrimination at the level of a two-base difference.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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