MicroRNA expression profiling of single whole embryonic stem cells

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ABSTRACT

MicroRNAs (miRNAs) are a class of 17–25 nt non-coding RNAs that have been shown to have critical functions in a wide variety of biological processes during development. Recently developed miRNA microarray techniques have helped to accelerate research on miRNAs. However, in some instances there is only a limited amount of material available for analysis, which requires more sensitive techniques that can preferably work on single cells. Here we demonstrate that it is possible to analyse miRNA in single cells by using a real-time PCR-based 220-plex miRNA expression profiling method. Development of this technique will greatly facilitate miRNA-related research on cells, such as the founder population of primordial germ cells where rapid and dynamic changes occur in a few cells, and for analysing heterogeneous population of cells. In these and similar cases, our method of single cell analysis is critical for elucidating the diverse roles of miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are a large family of small non-coding RNAs that consist of more than two hundred known members in the mammalian genomes (1). Recently, these non-coding RNAs have been shown to be of great importance in a wide variety of biological processes including cell cycle regulation, apoptosis, cell differentiation, maintenance of stemness and imprinting (2). Profiling of miRNAs in individual cells is prerequisite in some instances where there is inherent variability amongst the cells, or because only a few such cells are available for analysis in early developing embryos. A number of laboratories have independently developed microarray technique for miRNAs (3–15), although this approach is

technically challenging because of the extremely small size of mature miRNAs (17–25 nt).

A relatively recent technical innovation has been to use a bead-based flow cytometric miRNA expression profiling method (16). While this approach provides more accurate information, it requires isolation of total RNAs and the removal of genomic DNA, followed by recovery of small RNA fragments from this sample. Therefore, although the sensitivity of the method is high and requires only 5 ng of material, it is not amenable for analysis of single cells. As a result, majority of the studies have been on miRNA expression profiling of tissues or organs, which contain a mixture of different cell types. In some instances, it is possible to adopt FACS sorting to obtain larger numbers of relatively homogenous cells. However, this is not always possible, for example, where few cells are available from early embryos. It is also the case that seemingly uniform cells, such as stem cells, may indeed differ from each other, which can only be judged by analysis of single cells. For this reason, it is highly desirable to develop efficient methods that provide unambiguous miRNA profile of individual specific cell types. In this regard, the recently developed method by Chen *et al.* (17) using a looped real-time PCR-based technique to detect expression of miRNAs is potentially helpful. With this approach they can cover at least 7 log of expression range that is accurate and specific for mature miRNA, which can clearly discriminate between mature miRNA and corresponding primary miRNA and precursor miRNA. Here we describe a multiplex format of this technique (Figure 1) that can effectively generate miRNA expression profile of individual cells.

MATERIALS AND METHODS

Reverse transcription

One microlitre of total RNA or cell lysate was used as template for a 5 µl reaction. RT reaction is carried out according to the

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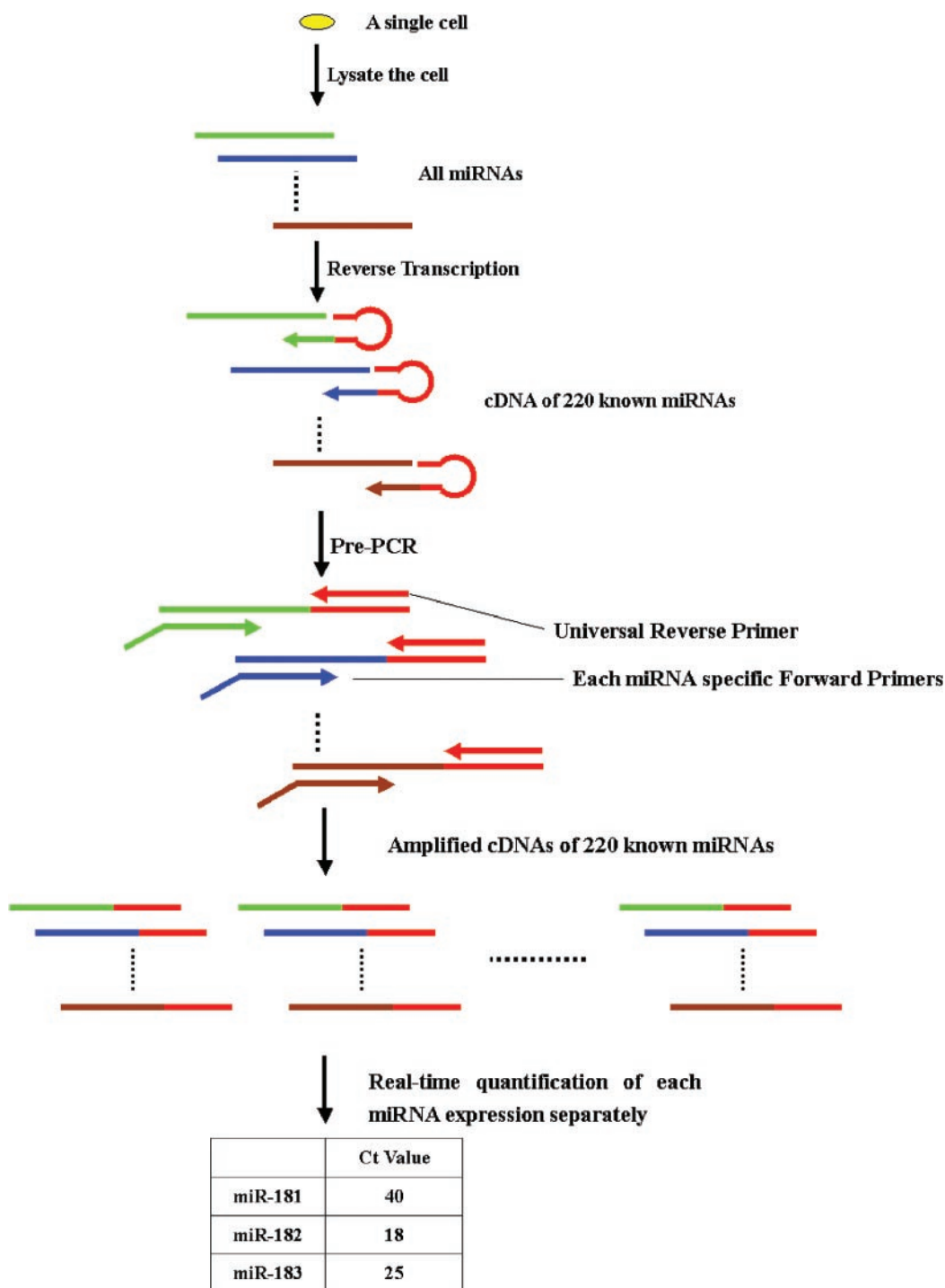
Figure 1. Schematic representation of real-time PCR-based 220-plex miRNA expression profiling method.

manufacture's suggestions using the ABI high capacity cDNA archive kit (CN: 4322171). All primers and probes are designed based on miRNA sequences released by the Sanger Institute (1) (Supplementary Table 2). The primer and probe design was according to Chen *et al*. (17). For example, for miR-16, the miRNA sequence is 5′-UAGCAGCAC-GUAAAUAUUGGCG-3′.

The reverse primer is 5′-CTCAACTGGTGTCGTGGAGT-CGGCAATTCAGTTGAGCGCCAATA-3′.

The forward primer is 5′-ACACTCCAGCTGGGTAGC-AGCACGTAAATA-3′.

The TaqMan Probe is (6-FAM)TTCAGTTGAGCGCCA-ATA(MGB; MGB is a minor grove binder with non fluorescent quencher).

For miR-293, the miRNA sequence is 5′-AGUGCCGCA-GAGUUUGUAGUGU-3′.

The reverse primer is 5′-CTCAACTGGTGTCGTGGAG-TCGGCAATTCAGTTGAGACACTACA-3′.

The forward primer is 5′-ACACTCCAGCTGGG AGTG-CCGCAGAGTTTG -3′.

The TaqMan Probe is (6-FAM)TTCAGTTGAGACAC-TACA (MGB).

Briefly, mixtures of 5 nM of each of the 216 miRNA specific reverse primer together with 1.3 U RNase Inhibitor, 16.75 U MMLV RT and 25 M dNTP were used for each RT reaction. The potential non-specific interactions between the looped primers are reduced by using 10-fold less looped primer concentration compared with amounts used in 1-plex looped RT–PCR assay (17) (i.e. 5 nM of each primer instead of 50 nM). We carried out dilution experiments to show that the optimal looped primer concentration is between 1 and 5 nM. All 216 miRNAs were converted into corresponding cDNAs in one RT reaction. A pulsed RT reaction condition was used to increase RT efficiency and further reduce non-specific interactions between primers for different miRNAs. The pulsed RT reaction condition gives 0.5–1 lower Ct value which means better detection sensitivity compared with non-pulsed condition used in 1-plex looped RT–PCR assay (17). However, there is no amplification of the miRNA cDNAs at this step. The reaction condition was as follows: 16°C for 30 min, followed by 60 cycles at 20°C for 30 s, 42°C for 30 s and 50°C for 1 s. Finally, we used 85°C for 5 min to inactivate MMLV RT. The 220-plex miRNA assay is commercially available to any end users of this method.

Pre-PCR

RT product (5 μl) was used as template for a 25 μl PCR. Briefly, 50 nM of each of the 216 miRNA's Forward Primers, 1× TaqMan Universal Master Mix (ABI), 4 mM dNTP, 2 mM $MgCl_2$, 5 μM Universal Reverse Primer, 6.25 U AmpliGold Taq (ABI) were used for each Pre-PCR. The condition for the PCR is 95°C for 10 min, 55°C for 2 min, followed by 18 cycles of 95°C for 1 s and 65°C for 1 min. Pre-PCR is an essential step for the 220-plex assay, since without this step there is significant loss of detection sensitivity, and most miRNAs will not be detectable except for those that are expressed at high levels in single cell inputs.

Real-time PCR

Two microlitres of 1:400 diluted Pre-PCR product was used for a 20 μl reaction. All reactions were duplicated. Because the method is very robust, we find that duplicate samples are sufficient and accurate enough to obtain values for miRNA expression levels. TaqMan universal PCR master mix of ABI was used according to manufacture's suggestion. Briefly, 1× TaqMan Universal Master Mix (ABI), 1 μM Forward Primer, 1 μM Universal Reverse Primer and 0.2 μM TaqMan Probe was used for each real-time PCR. The conditions used were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. All the reactions were run on ABI Prism 7000 Sequence Detection System.

Cell culture

ES cells were cultured in Glasgow medium [Plus 15% fetal calf serum (GIBCO) and 1000 U/ml LIF]. The ES cells were undifferentiated as judged from their morphology and Oct4-GFP expression (18).

Cell lysate

Cell number was counted by using hemocytometer. After counting, ES cells were re-suspended in phosphate-buffered saline (PBS) at expected concentrations. Then all miRNA were released from cells by treating cell suspension at 95°C for 5 min. For single cell analysis, ES cells were digested by trypsin for 10 min at room temperature into single cell suspension. Then single cells were picked with a micro capillary under a dissection microscope. After that, the single cells were washed in 0.1% BSA in PBS twice. Subsequently the single cells were individually picked and introduced into RT reaction solution (without RT and dNTP) and treated at 95°C for 5 min. Finally, RT, RNase Inhibitor and dNTP were added for RT reaction.

Isolation of total RNA

MirVana miRNA isolation kit (Ambion) was used to isolate total RNA (including miRNAs) according to the manufacturer's protocol. All the places where we refer to total RNA, the RNA was isolated using the mirVana kit. Where we refer to cell lysates, the cells were disrupted by treatment at 95°C for 5 min, and the sample was directly used as template to run 220-plex assay.

RESULTS

First, we tested the sensitivity of the looped real-time PCR-based miRNA expression profiling method in multiplex format. For this purpose, we diluted in a step-wise manner, the total RNA obtained from ES cells from 1 μg to 0.01 pg. The samples were subjected to RT reaction in 220-plex involving 18 cycles of amplification to generate enough templates for the subsequent miRNA real-time quantification for all of the 220 miRNAs. The analysis showed that this approach works accurately for 8 log range of expression for miR-16, which is effective with 1 μg to 0.01 pg of total RNA (Figure 2a). As the usual amount of total RNA in a cell is around 15 pg, this strongly suggests that the method is sensitive enough for single cell miRNA expression profiling.

To confirm the accuracy of the assay for isolated total RNA, we also tested step-wise 2-fold dilution of ES cell RNA from 1024 to 1 pg, which again showed that the method works accurately for miR-16, which we confirmed was also the case for miR-293 expression (Figure 2b). These experiments suggest that the method is sensitive and accurate for the analysis of total RNA from single cells.

For the analysis of single cells, it is desirable and essential for the method to work on the whole cell lysate rather than on purified total RNA. To test for this possibility, we diluted whole cell lysate from 10 000 cells in a step-wise manner down to the level of material expected from one cell. This analysis showed that we could indeed detect expression of miR-16 accurately down to a single cell level in samples from whole cell lysates (Figure 2c). We confirmed the precision of the method for whole cell lysate by 2-fold progressive dilution of cell lysate from 1024 cells down to 1 cell (data not shown). Indeed, our analysis showed that for miR-293, the method works down to an equivalent of 0.125 of the material
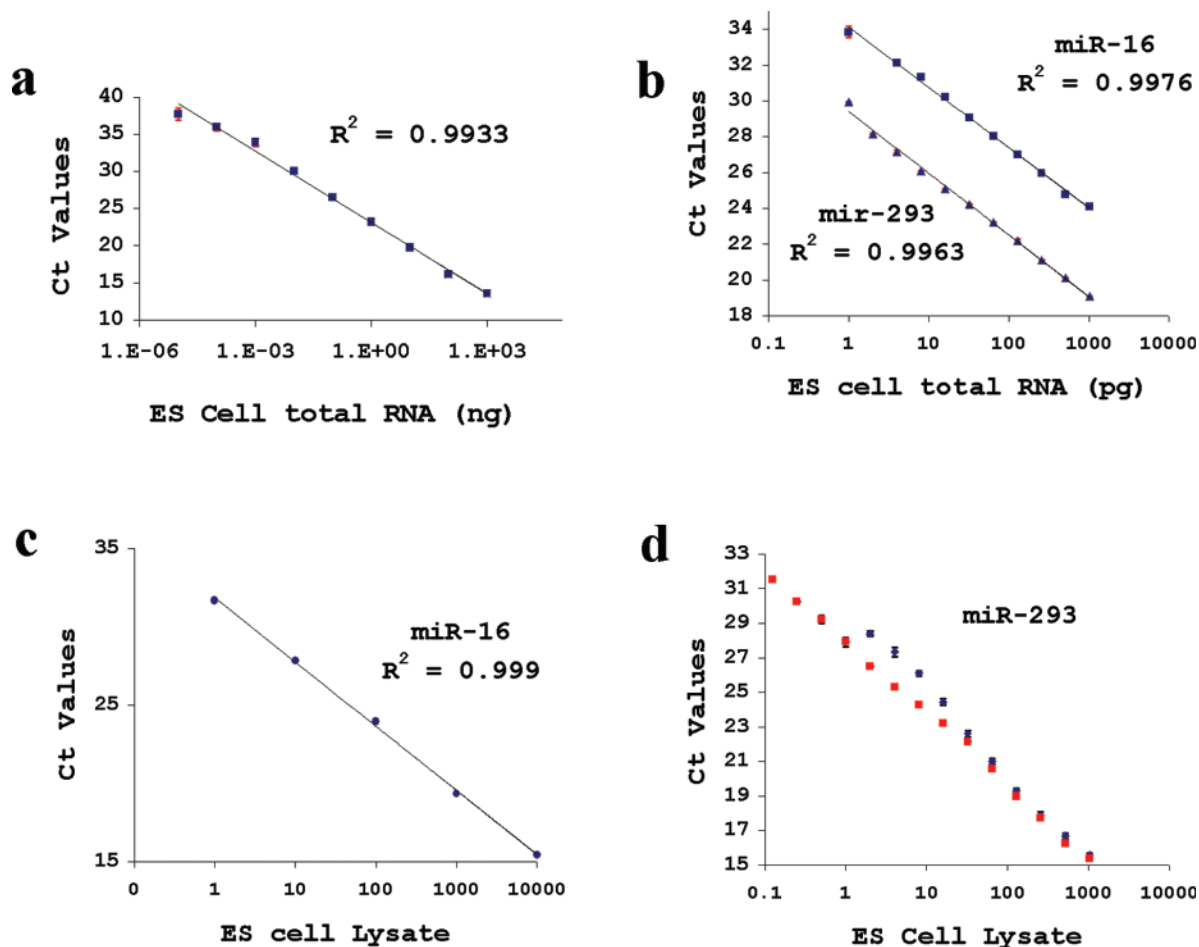
Figure 2. The sensitivity and accuracy of the miRNA expression profiling method. (**a**) Step-wise 10-fold dilution of purified total RNA from ES cells, from 1 μg (1000 ng) to 0.01 pg (10 fg). (**b**) Step-wise 2-fold dilution of purified total RNA from ES cell lysate, from 1024 to 1 pg. Squares represent mir-16, and triangles represent mir-293. (**c**) Step-wise 10-fold dilution of whole cell lysate from 10 000 cells to 1 cell. (**d**) Step-wise dilution by 2-fold of ES cell lysate (represented by red squares) and step-wise dilution of intact ES cells (represented by blue diamonds) from 1024 cells to 1 cell for mir-293. Cell lysates were diluted step-wise by 2-fold, while intact cell suspension was progressively diluted by 2-fold, to prepare the samples for analysis. Ct = threshold cycle. Ct 40 means that there was no detectable signal after 40 cycles of PCR amplifications. R^2 = linear correlation coefficient.

available in a single cell (Figure 2d), which shows that the method is accurate and sensitive enough for the detection of miRNA from single cells.

While the method is clearly sensitive for detection of miRNA from single cells, it is important to establish if it can indeed work directly on individual handpicked cells. For this purpose, we first manually picked 16 single ES cells, which were lysed together, and the sample was diluted to generate amounts of material that is equivalent to that present in a single cell. The measurement of miR-16 and miR-293 showed the expected levels of miRNA (Figure 3a and b). We next picked 1, 2, 4, 8 and 16 single ES cells for analysis, which also showed the expected levels of miR-16 and miR-293 in these samples (Figure 3a and b). Although we did detect variations between two independent batches of samples, we suspect that this could be due to inherent variations in the levels of miRNA expression in ES cells.

To determine the extent to which individual cells may differ from each other, we picked 15 single ES cells that were analysed separately. We observed ∼2-fold difference in miR-16 amongst these 15 single ES cell samples (Figure 3c). These

data suggest that the variations between independent batches of 1–16 single whole cells reflects an inherent variations in the levels of miRNA expression in single ES cells.

Finally, we asked if the method we have described could be used to obtain a comprehensive miRNA expression profile of single cells. For this purpose, we picked 5 single ES cells and analysed 24 miRNAs that show high, medium or low levels of expression in ES cells. We found that for all the 5 single ES cells analysed individually, the 24 miRNAs expression profile accurately reflect the expected levels of expression (Figure 4). These samples were then analysed for all the 220 miRNAs, and we found that the method works reliably for such a comprehensive miRNA expression profile of individual cells (Supplementary Figure 1 and Supplementary Table 1). We also compared our expression profiling data with published ES cell miRNA cloning data (Table 1). In principle, our single cell miRNA profiling data correlate well with the cloning and northern blot data although the published cloning frequencies are very low (19). This further proved that our expression profiling method works reliably for single whole ES cells.
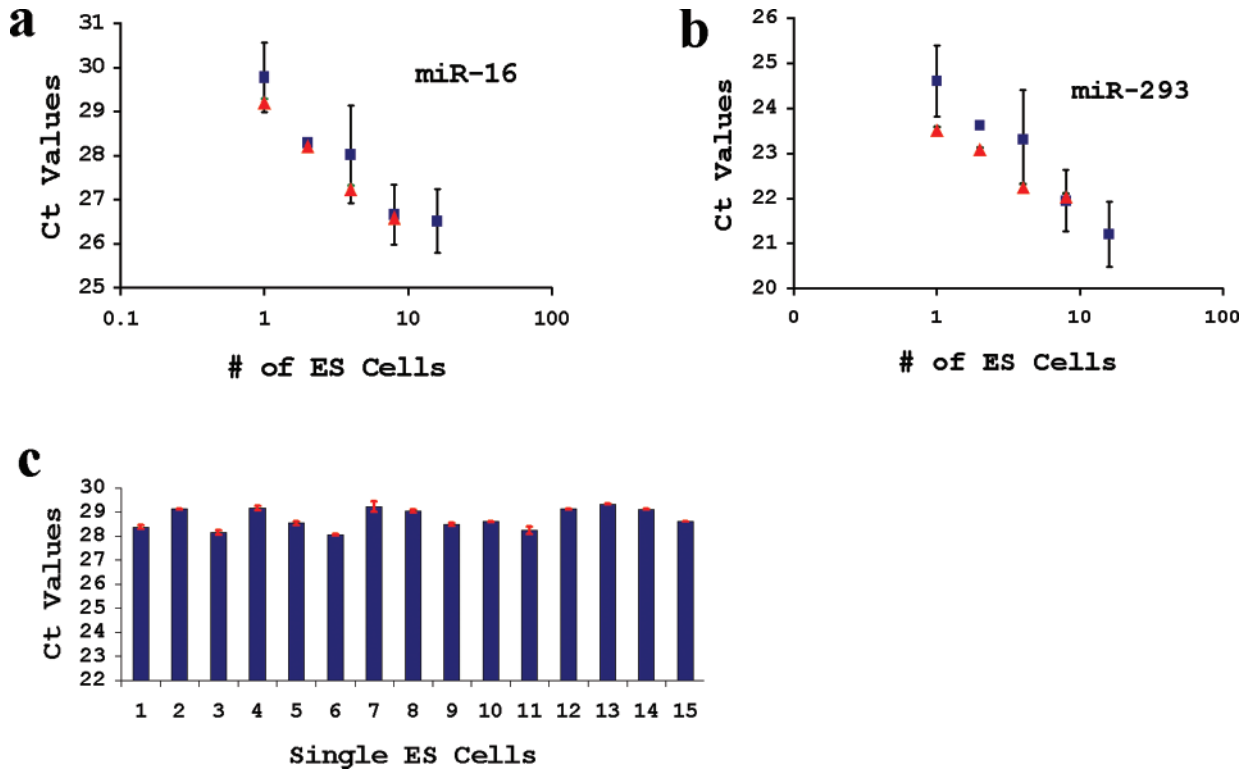
Figure 3. Expression profile of manually picked single ES cells. (**a**) Between 1 and 16 cells were picked individually (represented by squares), or 16 cells were picked together and subjected to lysis, followed by step-wise 2-fold dilution (represented by triangles) for analysis of miR-16. (**b**) The same batch of 1–16 cells was treated as above and analysed for the expression of miR-293. The error bars around the squares and triangles represent SDs between values for the 1–16 cells. (**c**) Expression of miR-16 in 15 single ES cells. The error bars around the columns represent SDs for values of each single cell.
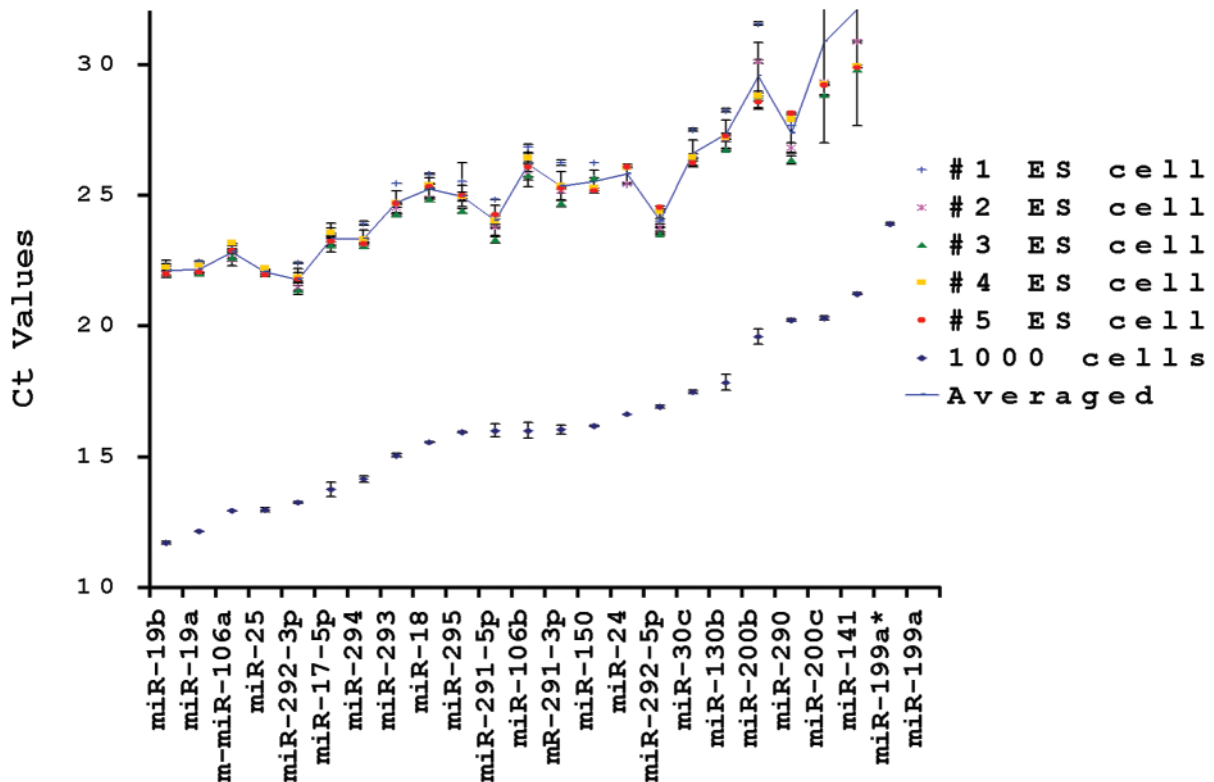
Figure 4. Five individual ES cells were picked and analysed for 24 miRNAs.

Table 1. Comparing our single ES cell miRNA profiling data with the published analysis by northern blot and cloning of miRNAs from mouse ES cells (19)

	Name	With northern blot data	Cloned from undifferentiated ES cells	Cloned from ES cell's differentiated progenies	Detected in undifferentiated single ES cells in our assay
1	miR-106a	NA	Yes		Yes
2	miR-127	NA	Yes		Yes
3	miR-130b	NA	Yes		Yes
4	miR-141a	NA	No	Yes	No
5	miR-15b	NA	Yes		Yes
6	miR-16	Yes	Yes		Yes
7	miR-18	NA	Yes		Yes
8	miR-19b	Yes	Yes		Yes
9	miR-20	NA	Yes		Yes
10	miR-21	Yes	Yes		Yes
11	miR-290	Yes	Yes		Yes
12	miR-291-as	Yes	Yes		Yes
13	miR-291-s	NA	Yes		Yes
14	miR-292-as	Yes	Yes		Yes
15	miR-292-s	NA	Yes		Yes
16	miR-293	Yes	Yes		Yes
17	miR-294	Yes	Yes		Yes
18	miR-295	Yes	Yes		Yes
19	miR-296	NA	No	Yes	No
20	miR-298	NA	No	Yes	No
21	miR-301	Yes	No	Yes	No
22	miR-302	Yes	No	Yes	No
23	miR-34b	NA	No	Yes	No
24	miR-92	Yes	Yes		Yes
25	miR-93	Yes	Yes		Yes
26	miR-96	NA	No	Yes	No
27	miR-99b	NA	No	Yes	No

In our assay, miRNAs with relative copy number of >100 per cell (or Ct value of <30.2; we used synthesized miR-16 to prepare a standard curve and deduced the relative copy number of all measured miRNAs) are considered to be expressed at significant levels. NA, data not available.

DISCUSSION

The method we describe here for the detection of miRNA using a PCR-based miRNA analysis is sensitive enough to generate a miRNA expression profile of single cells. Efficient and reliable detection of miRNAs is the first essential step towards understanding their potential roles in specific cases. Together with the recent development of a real-time PCR-based miRNA precursor profiling method, it should be possible to study the regulation and processing of miRNA precursors into mature miRNAs (20). In many previous studies, total RNA extracted from a large number of possibly heterogeneous cells has been used for analysis. Even 'pure' cultured cells can show inherent variations. The approaches used previously have hitherto been necessary since nearly all known miRNA profiling methods need microgram amounts of total RNA (3,4,7–10,12,14), which is required for the fractionation of small RNAs before subsequent analysis (3,4,6–8,10). Such an approach is clearly not compatible with miRNA expression analysis of a single cell.

It is important to consider if in our method the use of the initial PCR amplification of the sample occurs while retaining the relative differences in the original population of miRNAs present in single cells. Our analysis shows that when comparing the initial PCR amplification involving 14, 18 and 22 cycles, we obtained representative and unbiased amplification that accurately reflects the miRNA profile provided <20 PCR amplification cycles are used. However, the use of >20 PCR cycles does result in a loss of representative amplification of the sample (F. Tang, K. Lao, M.A. Surani, unpublished data),

so it is important not to exceed this limit. We can also rule out variable amplification efficiency that may occur due to the primers used for the detection of individual miRNAs because our combined comparisons of results from 5 single ES cells and 1000 ES cells showed that the amplification efficiency of primers for most if not all miRNAs are similar. To our knowledge, no miRNA expression analysis method can guarantee identical detection efficiency for all miRNAs due to the extreme small size of mature miRNAs. For a typical hybridization-based miRNA profiling method, it is usually necessary to have 5–20 μg total RNA (3,7–10,12,14). In contrast, our method is extremely sensitive and can be used routinely for the analysis of single cells (0.015 ng total RNA).

The method we describe here should prove useful in many cases. For example, specification of cells, such as primordial germ cells in very early embryos occurs in just 40 cells (21). To understand the role that miRNA may play in this process requires analysis at the single cell level. Other relatively rare cells such as some stem cells in adults also require analysis of single cells. Tumours also often consist of a heterogeneous group of cells so it is desirable to analyse them individually to determine what role miRNA plays in cancers (22). We propose that the method we have described here will help to advance an understanding of the functions of miRNAs generally.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

REFERENCES

1. Griffiths-Jones,S. (2004) The microRNA Registry. *Nucleic Acids Res.*, **32**, D109–D111.
2. Ambros,V. (2004) The functions of animal microRNAs. *Nature*, **431**, 350–355.
3. Krichevsky,A.M., King,K.S., Donahue,C.P., Khrapko,K. and Kosik,K.S. (2003) A microRNA array reveals extensive regulation of microRNAs during brain development. *RNA*, **9**, 1274–1281.
4. Nelson,P.T., Baldwin,D.A., Scearce,L.M., Oberholtzer,J.C., Tobias,J.W. and Mourelatos,Z. (2004) Microarray-based, high-throughput gene expression profiling of microRNAs. *Nature Methods*, **1**, 155–161.
5. Thomson,J.M., Parker,J., Perou,C.M. and Hammond,S.M. (2004) A custom microarray platform for analysis of microRNA gene expression. *Nature Methods*, **1**, 47–53.
6. Baskerville,S. and Bartel,D.P. (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA*, **11**, 241–247.
7. Liang,R.Q., Li,W., Li,Y., Tan,C.Y., Li,J.X., Jin,Y.X. and Ruan,K.C. (2005) An oligonucleotide microarray for microRNA expression analysis based on labeling RNA with quantum dot and nanogold probe. *Nucleic Acids Res.*, **33**, e17.
8. Barad,O., Meiri,E., Avniel,A., Aharonov,R., Barzilai,A., Bentwich,I., Einav,U., Gilad,S., Hurban,P., Karov,Y. *et al.* (2004) MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Res.*, **14**, 2486–2494.
9. Babak,T., Zhang,W., Morris,Q., Blencowe,B.J. and Hughes,T.R. (2004) Probing microRNAs with microarrays: tissue specificity and functional inference. *RNA*, **10**, 1813–1819.
10. Miska,E.A., Alvarez-Saavedra,E., Townsend,M., Yoshii,A., Sestan,N., Rakic,P., Constantine-Paton,M. and Horvitz,H.R. (2004) Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol.*, **5**, R68.
11. Calin,G.A., Liu,C.G., Sevignani,C., Ferracin,M., Felli,N., Dumitru,C.D., Shimizu,M., Cimmino,A., Zupo,S., Dono,M. *et al.* (2004) MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc. Natl Acad. Sci. USA*, **101**, 11755–11760.
12. Sun,Y., Koo,S., White,N., Peralta,E., Esau,C., Dean,N.M. and Perera,R.J. (2004) Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Res.*, **32**, e188.
13. Sioud,M. and Rosok,O. (2004) Profiling microRNA expression using sensitive cDNA probes and filter arrays. *Biotechniques*, **37**, 574–576, 578–580.
14. Liu,C.G., Calin,G.A., Meloon,B., Gamliel,N., Sevignani,C., Ferracin,M., Dumitru,C.D., Shimizu,M., Zupo,S., Dono,M. *et al.* (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc. Natl Acad. Sci. USA*, **101**, 9740–9744.
15. Sempere,L.F., Freemantle,S., Pitha-Rowe,I., Moss,E., Dmitrovsky,E. and Ambros,V. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.*, **5**, R13.
16. Lu,J., Getz,G., Miska,E.A., Alvarez-Saavedra,E., Lamb,J., Peck,D., Sweet-Cordero,A., Ebert,B.L., Mak,R.H., Ferrando,A.A. *et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.
17. Chen,C., Ridzon,D.A., Broomer,A.J., Zhou,Z., Lee,D.H., Nguyen,J.T., Barbisin,M., Xu,N.L., Mahuvakar,V.R., Andersen,M.R. *et al.* (2005) Real-time quantification of microRNAs by stem–loop RT–PCR. *Nucleic Acids Res.*, **33**, e179.
18. Ying,Q.L., Nichols,J., Chambers,I. and Smith,A. (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*, **115**, 281–292.
19. Houbaviy,H.B., Murray,M.F. and Sharp,P.A. (2003) Embryonic stem cell-specific microRNAs. *Dev. Cell*, **5**, 351–358.
20. Jiang,J., Lee,E.J., Gusev,Y. and Schmittgen,T.D. (2005) Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res.*, **33**, 5394–5403.
21. Saitou,M., Barton,S.C. and Surani,M.A. (2002) A molecular programme for the specification of germ cell fate in mice. *Nature*, **418**, 293–300.
22. Wang,J.C. and Dick,J.E. (2005) Cancer stem cells: lessons from leukemia. *Trends Cell Biol.*, **15**, 494–501.