Global single-cell cDNA amplification to provide a template for representative high-density oligonucleotide microarray analysis

Kazuki Kurimoto[1], Yukihiro Yabuta[1], Yasuhide Ohinata[1] & Mitinori Saitou[1,2,3]

[1]Laboratory for Mammalian Germ Cell Biology, Center for Developmental Biology, RIKEN Kobe Institute, 2-2-3 Minatojima-Minamimachi, Chuo-ku, Kobe 650-0047, Japan. [2]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, 4-1-8 Hon-cho, Kawaguchi, Saitama 332-0012, Japan. [3]Laboratory of Molecular Cell Biology and Development, Graduate School of Biostudies, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan. Correspondence should be addressed to M.S. (saitou@cdb.riken.jp.

Published online 29 March 2007; doi:10.1038/nprot.2007.79

We describe here a protocol for the representative amplification of global mRNAs from typical single mammalian cells to provide a template for high-density oligonucleotide microarray analysis. A single cell is lysed in a tube without purification and first-strand cDNAs are synthesized using a poly(dT)-tailed primer. Unreacted primer is specifically eliminated by exonuclease treatment and second strands are generated with a second poly(dT)-tailed primer after poly(dA) tailing of the first-strand cDNAs. The cDNAs are split into four tubes, which are independently directionally amplified by PCR, and then recombined. The amplified products (~100 ng) show superior representation and reproducibility of original gene expression, especially for genes expressed in more than 20 copies per cell, compared with those obtained by a conventional PCR protocol, and can effectively be used for quantitative PCR and EST analyses. The cDNAs are then subjected to another PCR amplification with primers bearing the T7 promoter sequence. The resultant cDNA products are gel purified, amplified by one final cycle and used for isothermal linear amplification by T7 RNA polymerase to synthesize cRNAs for microarray hybridization. This protocol yields cDNA templates sufficient for more than 80 microarray hybridizations from a single cell, and can be completed in 5–6 days.

INTRODUCTION

The developmental programme generates a highly complex array of diverse cell types from a single fertilized oocyte. In turn, the resultant cells comprising our bodies dynamically alter their properties in response to environmental stimuli to maintain homeostasis. It is often the case that the activities of a small number of key cell types regulate profoundly important aspects of these biological processes. Therefore, the development of methodologies that enable quantitative measurements of the functions of small numbers of cells, ideally single cells, represents a critical challenge in any modern biological study.

Differential gene expression is a key property of a cell's function. The recent completion of the genome sequencing of many organisms and the development of microarray platforms encompassing whole-genome information[1], among many other endeavors including expressed sequence tag[2,3] analyses and serial analysis of gene expression[4], have provided unprecedented opportunities for analyzing genome-wide gene expression profiles in various biological settings. Exponential PCR amplification and multiple-round linear amplification of starting mRNA molecules have been utilized as two of the primary methods for monitoring the genome-wide gene expression of small numbers of key cell types[5–9]. The exponential procedure comprises the synthesis of the first-strand cDNAs by reverse transcription (RT) using an oligo(dT)-tailed primer, tagging of the RT products with poly(dA) at their 3′ ends and their amplification by PCR using the identical primer for the initial cDNA synthesis, resulting in nondirectional amplification products[5,6,9]. In contrast, the linear method synthesizes cDNAs by RT using a primer with the T7 promoter sequence tagged with oligo(dT), and amplifies cRNA directionally by multiple rounds of *in vitro* transcription (IVT) using T7 RNA polymerase[7,8]. These two methods are essentially complementary in their amplification quality, with each method offering critical advantages and disadvantages[10–12]. We have recently developed a cDNA amplification protocol consisting of a small number of directional PCR cycles followed by one round of linear amplification that generates representative, by-product-free cRNA that is immediately applicable to any commercially available microarray platform[13]. The directional PCR products can also be used for any other gene expression analyses such as quantitative(Q)-PCR and EST analyses. Some of the major technical modifications that distinguish our protocol from conventional PCR methods include (i) specific elimination of the unreacted primer for the first-strand cDNA synthesis by exonuclease I, thereby reducing the generation of reaction by-products, which occupy 28–70% of the PCR product in the conventional methods[12], (ii) splitting of the first-strand cDNA product into four tubes for subsequent independent PCR amplification followed by recombination of the independently amplified materials, thereby reducing the stochastic error associated with PCR, (iii) designing an original primer pair that enables highly efficient directional PCR amplification in a small number of cycles, thus preserving sense–antisense orientation and allowing subsequent isothermal linear amplification by T7 RNA polymerase using general protocols widely supported by commercially available microarray systems (e.g., Affymetrix GeneChip). Although both of the primers bear $(dT)_{24}$ at their 3′ termini, thus carrying a risk of misannealing with poly(dA) tails of both cDNA ends and resulting in non-directional amplification, the high annealing temperature for PCR (67 °C; see Steps 34, 43 and 53) prevents possible misannealing by the $(dT)_{24}$

portion (the melting temperature of the $(dA)_{24}$-$(dT)_{24}$ hybrid is calculated at around 50 °C). Indeed, we sequenced 40 different clones from the cDNAs amplified from 10 pg embryonic stem (ES) cell total RNA and verified that all of them preserved sense–antisense orientation[13].

These improvements culminate in quantitative and reproducible amplification performance highly superior to that obtained by the conventional methods[5,13,14]. The performance of the method was estimated by using 10 pg total RNA purified from ES cells[13] (any other cell type can also be used as a source of RNA for the control amplification). With our protocol, as many as 65% and 89% of the genes examined were represented within two- and fourfold differences, respectively, from the unamplified control, whereas only 7% and 22% were so represented when the conventional method was used[13]. Furthermore, microarray analyses showed that the rates of false positives and negatives were as low as 3% and 6%, respectively, for genes expressed at levels above 20 copies per cell[13]. Even at an expression level as low as five copies per cell, more than half of expressed genes were successfully detected and approximately 70% of detected genes were truly expressed, underscoring the sensitivity and accuracy of the method[13]. Furthermore, our method has a key advantage over multiple-round linear amplifications in that it generates abundant cDNA products from a single experiment (using only ~1/80 of the initial 20 cycle amplification product for a microarray analysis (see Steps 40–60)), enabling a variety of quality assessments and multiple microarray hybridizations, and thereby broadening the method's applicable capacity.

It is highly recommended to include spike RNAs as an amplification control and as a measure to estimate the expression levels of genes of interest. Spike RNAs are artificially poly(A)-tailed RNAs that are not encoded in the genome of the organism of the research (e.g., genes from *Baccilus subtilis* as a spike RNAs for gene expression analysis in mice). The rate of successful amplification, determined by quantitative amplifications of spike RNAs of ≥20 copies per cell, is estimated to be more than 90% (17/18) for amplifications from the 10 pg ES cell total RNA and ~75% (366/488) for amplifications from real single cells (data not shown); the lower apparent success rate for the latter versus the former may result from possible degradation of the spike RNAs in the cell lysis buffer, which is incubated on ice during isolation of the single cells (~1.5 h) (Steps 2–10).

The average length of the amplified cDNA products without poly(dA) tract and primer sequences is approximately 700 bps. Almost all of the cDNA products bear the bona fide transcript ends. The probes of commercially available microarray slides are generally located at the 3′ ends of the transcripts. For example, 89% of the probes on the GeneChip Mouse Genome 430 2.0 array (Affymetrix) are located within 600 bps of the 3′ ends of the transcripts. Therefore, a 3′ restriction of the amplified product is not a major drawback for microarray or Q-PCR analysis[13]. However, the amplified products may not be used for analyses that strictly require full-length cDNAs, such as splice variant and transcription start site analyses.

We have demonstrated the power of the method by showing that it unambiguously detects, for the first time, the presence of two distinct populations of cells in the inner cell mass (ICM) of embryonic day (E) 3.5 mouse blastocyst[13]. Subsequently, a similar conclusion was made by another group using different approaches[15]. This independent conclusion emphasizes the accuracy of our method. Clearly, our method is applicable to a wide range of biological/biomedical procedures that require resolution at the single-cell level. Examples include accurate examination of gene expression properties of rare tissue stem cells, the identification of functionally distinct subpopulations among seemingly identical cell populations in development and adults, molecular taxonomies of neurons, exploration of gene expression dynamics associated with cell fate specification in development and adults in both normal and perturbed (diseased or regenerating) conditions, analysis of the gene expression response of cells to various stimuli (e.g., environmental stimuli, nutrient, drug administration and cytokines), molecular taxonomies and drug discoveries for cancer cells, and potential identification of cancer stem cells.

This method is designed for the amplification of mRNA from typical single mammalian cells containing total RNA of ~10 pg per cell, and should be applicable to any type of cells with similar total RNA content (e.g., *Caenorhabditis elegans*, 200 pg per embryo for 4- to ~190-cell embryos; *Drosophila melanogaster*, ~2 pg cell^{-1} for adults, ~4.5 pg cell^{-1} for embryos 24 h after hatching; typical chicken cells, ~10–30 pg cell^{-1}; *Xenopus laevis*, ~77 pg cell^{-1} for stage 40–41 embryos)[16–21], with appropriate control experiments. When applied to cells with smaller RNA amounts or to subcellular materials, further methodological optimization, including an increase in the number of initial PCR cycles, may be required. Here, we describe the detailed step-by-step protocol of our method, which is schematically represented in **Figure 1**.

MATERIALS

REAGENTS

- Plasmid pGIBS-Lys contained in *E. coli* DH5, freeze-dried (American Type Culture Collection (ATCC); 87482)
- Plasmid pGIBS-Phe contained in *E. coli* DH5, freeze-dried (ATCC; 87483)
- Plasmid pGIBS-Thr contained in *E. coli* DH5, freeze-dried (ATCC; 87484)
- Plasmid pGIBS-Dap contained in *E. coli* DH5, freeze-dried (ATCC; 87486)
- BSA (Sigma; A-2153)
- EDTA (Wako; 345-01865)
- 1× PBS (pH 7.2) (Gibco; 14249-95)
- Dulbecco's modified Eagle's medium (DMEM) (Gibco; 119995-065)
- Trypsin-EDTA (1×) (Gibco; 25300-054)
- Deionized distilled water (DDW) (Gibco; 15230-162)
- V1$(dT)_{24}$ primer, V3$(dT)_{24}$ primer, T7V1 primer (each 1 μg μl^{-1}, dissolved in DDW) and specific primer pairs (mixture of forward and reverse primers; 5 μM each) to validate the amplification quality by Q-PCR are purchased from Hokkaido System Science (Sapporo, Japan) or Operon Biotechnology. Sequences are described in ref. 13
- Prime RNase Inhibitor (30 U μl^{-1}; Eppendorf; 0032 005.357)
- RNAguard RNase Inhibitor (20–40 U μl^{-1}; GE Healthcare; 27-0815-01)
- SuperScript III reverse transcriptase (200 U μl^{-1}; Invitrogen; 18080-044; with DTT, 0.1 M)
- T4 gene 32 protein (5–6 mg ml^{-1}; Roche; 972 983)
- Exonuclease I (5 U μl^{-1}; Takara; 2650A; with 10× exonuclease I buffer)
- TdT, recombinant (15 U μl^{-1}; Invitrogen; 10533-065)
- RNaseH (2 U μl^{-1}; Invitrogen; 18021-014)

- Takara ExTaq Hot Start Version (5 U μl^{-1}, Takara; RR006A; with 10× ExTaq buffer and dNTP mix, 2.5 mM each of dATP, dCTP, dGTP and dTTP)
- dATP, 100 mM (GE Healthcare; 27-2050-01)
- Nonidet P-40 SP (Nakalai Tescque; 23640-65)
- GeneAmp 10× PCR buffer II (Applied Biosystems; N8080010; with $MgCl_2$ solution, 25 mM)
- Qiaquick PCR purification kit (Qiagen; 28106)
- RNeasy kit (Qiagen; 74104)
- Qiaquick gel extraction kit (Qiagen; 28704)
- GeneChip Sample Cleanup Module (Affymetrix; 900371)
- MEGAscript T3 kit (Ambion; 1338)
- MEGAscript T7 kit (Ambion; 1334)
- SYBR Green PCR Mastermix (Applied Biosystems; 4334973)

EQUIPMENT

- 0.5 ml thin-walled PCR tube with flat cap (Applied Biosystems; N801-0737)
- 8-well 0.2 ml PCR tube band without cap (Greiner Bio-One; 050905-043)
- 0.2 ml PCR tube with cap (Greiner Bio-One; 050210-179)
- Thermo Minder 50 mini water bath (TAITEC)
- GeneAmp PCR System 9700 (Applied Biosystems)
- Model P-97/IVF micropipette puller (Sutter Instrument)
- Micro Forge MF-900 (Narishige)
- Millex-HV PVDF 0.45 μm filter (Millipore; SLHV J13 SL)
- Mouth piece
- Silicon tubes
- Gas burner
- Borosilicate glass capillary (Sutter Instrument; OD: 1.0 mm, ID: 0.58 mm, length: 10 cm; B100-58-10)
- Mupid-2plus submarine-type gel electrophoresis system (Takara; AD110)
- ABI PRISM 7900 real-time PCR system (Applied Biosystems)
- MicroAmp Optical 384-well reaction plate (Applied Biosystems; 4309849)
- MicroAmp Optical Adhesive Film (Applied Biosystems; 4311971)

REAGENT SETUP

Spike RNAs We use the following four spike RNAs supported by the GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix): Lys, Dap, Phe, and Thr (Accession numbers: X17013, L38424, M24537, X04603, respectively; ATCC). Use the *Escherichia coli* strains bearig plasmids encoding these spike RNAs (pGIBS-Lys, pGIBS-Dap, pGIBS-Phe, pGIBS-Thr, respectively). Cut plasmids with NotI and transcribe the linearized plasmids (0.8 μg) with the MEGAscript T3 kit in a 20-μl scale according to the manufacturer's instructions. The typical total yield of the RNAs is approximately 100 μg. Prepare the spike RNA mixture with *Lys*, *Dap*, *Phe* and *Thr* RNAs at 1,000, 100, 20 and 5 copies, for these genes, respectively, per microliter (1-cell amount per μl). The final concentration of each spike RNA should be as follows: *Lys*, 5.50×10^{-4} pg μl^{-1}; *Dap*, 1.01×10^{-4} pg μl^{-1}; *Phe*, 1.45×10^{-5} pg μl^{-1}; and *Thr*, 5.45×10^{-6} pg μl^{-1} (calculation based on the approximate sizes of the spike RNAs (*Lys*: 1.0 kb; *Dap*: 1.84 kb; *Phe*: 1.32 kb and *Thr*: 1.98 kb)). Prepare a high-concentration stock mixture (e.g., *Lys*: 4.23×10^{-3} μg μl^{-1}; *Dap*: 7.69×10^{-4} μg μl^{-1}; *Phe*: 1.1×10^{-4} μg μl^{-1}; and *Thr*: 4.13×10^{-5} μg μl^{-1}) and dilute it with DDW (e.g., by 7.69×10^6-fold) to prepare the 1-cell amount per μl spike RNA mixture. Add the 1-cell amount per μl spike RNA mixture to the cell lysis buffer (see Step 1) for both positive controls (see also Step 10) and real single-cell cDNA amplifications.

▲ **CRITICAL** Store the spike RNA mixture at −80 °C. Avoid freezing and thawing more than once.

Control RNA We use total RNA purified from ES cells with the RNeasy kit. Add the spike RNA mixture of 1×10^5-cell amount to 1 μg μl^{-1} total RNA at the ratio of 1:1 (v/v), and synthesize the first-strand cDNA from 1 μg of total RNA with SuperScript III reverse transcriptase according to the manufacturer's instruction. Purify the first-strand cDNA with the PCR purification kit (final elution with 50 μl EB buffer). To make the starting material for control amplification, serially dilute the purified total RNA with DDW to concentrations of 2.5 ng μl^{-1}, 250 pg μl^{-1} and 25 pg μl^{-1} (equivalent to 2.5 cells μl^{-1}) and store at −80 °C.

PBS-BSA (0.2 mg ml^{-1}) Dissolve BSA in PBS at 0.2 mg ml^{-1} and sterilize it by autoclaving. Store the solution at 4 °C.

PBS-EDTA (1 mM) Make 500 mM EDTA solution (pH 8.0) and add it to PBS at 1:500. Store the solution at 4 °C.

DMEM-BSA Dissolve BSA in MilliQ H_2O at 100 mg ml^{-1}, sterilize the solution by filtration (0.45 μm PVDF filter) and add it to DMEM at 1:100. Store the solution at 4 °C.

EQUIPMENT SETUP

Mouth pipette Using borosilicate glass capillaries, make micropipettes with diameters appropriate for picking up single cells. Pull a glass capillary using the micropipette puller (settings: P = 300, HEAT = 850, PULL = 30, VEL = 120 and TIME = 200) and cut the pulled capillary using the micro-forge so that the capillary diameter fits to a single-cell collection (e.g., ~30 μm for ICM cells from mouse blastocysts). Assemble the mouth pipette as shown in **Figure 2.** ▲ **CRITICAL** It is necessary to make at least the same number

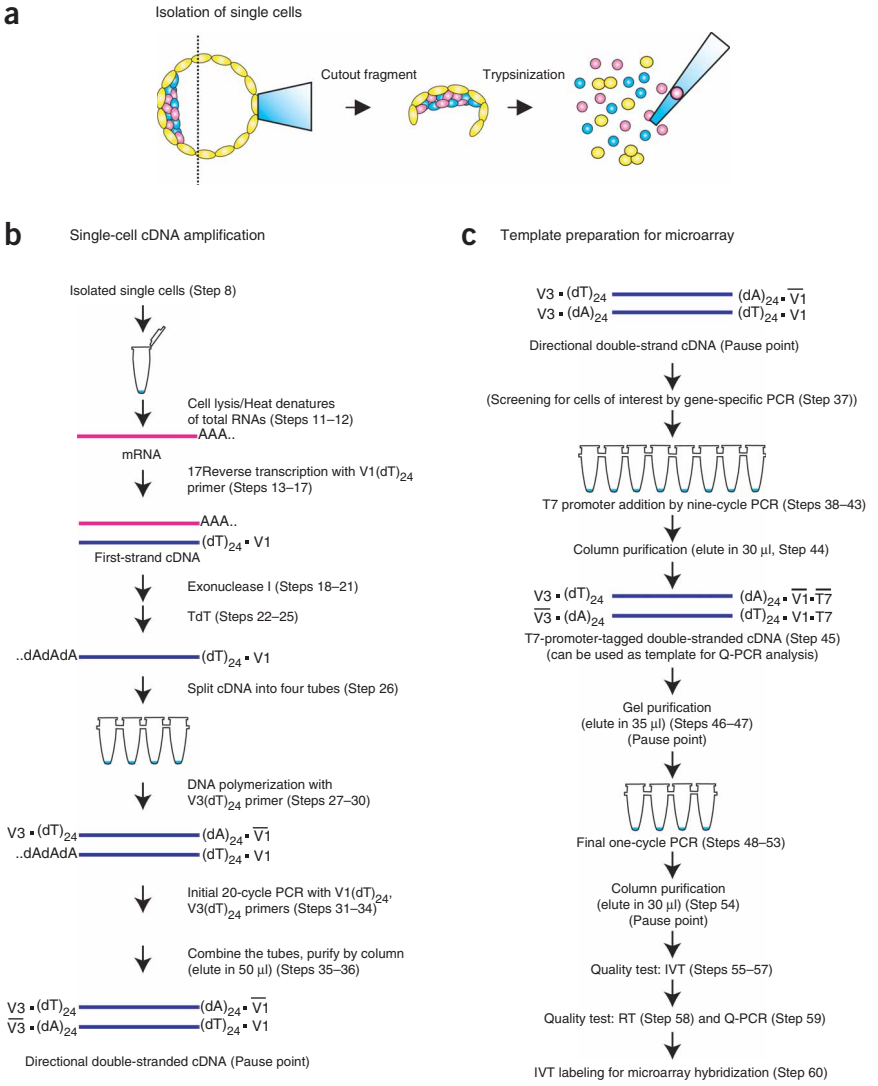

Figure 1 | Schematic representation of the step-by-step protocol. (**a**) Isolation of single cells. Epiblast-like cells, primitive endoderm-like cells and trophectoderm cells are represented by blue, pink and yellow circles, respectively. (**b**) Protocol of the single-cell cDNA synthesis and initial PCR amplification. (**c**) Protocol of the template preparation for microarray hybridization.

of micropipettes as the number of cells picked up (see also Step 7). ▲ CRITICAL Any set of equipment with which researchers can pick up single cells can alternatively be used.

Glass knife We use glass knives for dissecting blastocysts or tissue fragments of interest from mouse embryos. Heat the central part of microcapillaries with a gas burner to melt away the cavities and thus form a solid portion. Pull the resulting capillaries using the micropipette puller at the above settings.

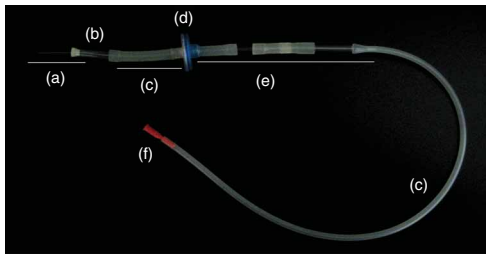

Figure 2 | A mouth pipette. The mouth pipette was assembled with the following parts: (**a**) micropipette (ID 30 μm), (**b**) adapter for micropipette, (**c**) silicone tube, (**d**) PVDF filter (0.45 μm), (**e**) adapter for the filter and silicon tube (made from a Pasteur pipette and a silicon tube) and (**f**) mouthpiece.

PROCEDURE

▲ CRITICAL STEP To perform the experiments efficiently, we usually carry out the procedure with two or more persons; one picks up single cells and the others perform cDNA synthesis and amplification. We typically perform 20 amplifications (19 single cells plus one negative control) per experiment.

▲ CRITICAL STEP All the procedures are essentially carried out in the PCR buffer[9,13] with the intention of making the PCR amplification as efficient as possible; the TdT mastermix (Step 22) and cell lysis buffer (Step 1) are based on the PCR buffer. The exonuclease mastermix (which is based on exonuclease I buffer (Step 18)) occupies only 1/6 volume in the reaction mixture (Step 19) and the exonuclease reaction works well in the PCR buffer in our experience.

▲ CRITICAL STEP For mastermix preparation, we add reagent volumes ×1.1 per reaction to allow a little excess to compensate for pipetting error.

Prepare cell lysis buffer

1| Prepare cell lysis buffer mastermix on ice as follows:

Number of reactions	×1	×20
10× PCR buffer II (without $MgCl_2$)	0.45 μl	9.0 μl
$MgCl_2$ (25 mM)	0.27 μl	5.4 μl
NP40 (5%)	0.45 μl	9.0 μl
DTT (0.1 M)	0.225 μl	4.5 μl
Prime RNase Inhibitor	0.045 μl	0.9 μl
RNAguard RNase Inhibitor	0.045 μl	0.9 μl
$V1(dT)_{24}$ primer (10 ng μl^{-1})	0.09 μl	1.8 μl
dNTP mix (2.5 mM each)	0.09 μl	1.8 μl
1-cell amount per μl spike RNA mixture	0.945 μl	18.9 μl
DDW	1.89 μl	37.8 μl
Total volume	4.5 μl	90 μl

▲ CRITICAL STEP Add Prime RNase Inhibitor and RNAguard RNase Inhibitor before adding the 1-cell amount per μl spike RNA mixture.

▲ CRITICAL STEP Start the cDNA synthesis procedure as quickly as possible (within 2 h) after the preparation of the cell lysis buffer.

2| Aliquot 4.5 μl cell lysis mastermix into a separate 0.5 ml thin-walled PCR tube for each cell/reaction and store on ice during cell isolation.

Isolation of single cells

3| Isolate blastocysts in DMEM-BSA, then cut a fragment containing the ICM using a glass knife at room temperature (20–25 °C) (see **Fig. 1a**).

▲ CRITICAL STEP As an example, we describe our procedure for preparing single-cell suspensions from ICM of mouse E3.5 blastocysts. However, the procedure to prepare single-cell suspensions depends on the tissues or cells to be analyzed. Any procedure that enables quick and intact preparation of single-cell suspensions would be acceptable (see refs. 5, 22–26).

4| Transfer the dissected fragment into PBS–EDTA and incubate for 5 min at room temperature (20-25 °C).

5| Transfer the dissected fragment into trypsin-EDTA (1×) and incubate for 7 min at 37 °C.

6| Transfer the trypsin-treated fragment into PBS-BSA and dissociate it into single cells by gently pipetting with the mouth pipette (see **Fig. 2**).

7| Using the mouth pipette, transfer a single cell into a 0.5 ml thin-walled PCR tube containing the cell lysis buffer (see Step 2). The volume of cell suspension to be transferred should be less than 0.5 µl.
▲ CRITICAL STEP It is important to collect all the single cells as quickly as possible (within 1 h once the cells are suspended in PBS-BSA).
▲ CRITICAL STEP To avoid contamination, use one capillary for picking up one single cell only. Do not reuse the microcapillaries.

8| Centrifuge the tube immediately at $6.7 \times 10^3 g$ for 15 s at room temperature and keep on ice.

9| Prepare one tube of cell lysis buffer into which only suspension solution without cells is loaded as a negative control for amplification.

10| Prepare the positive control for amplification using control RNA (see REAGENT SETUP) as follows: add a 0.4 µl aliquot of diluted total RNA (25 pg μl^{-1}) into a tube containing 4.5 µl of cell lysis buffer on ice and immediately centrifuge at $6.7 \times 10^3 g$ for 15 s. We perform this step separately from real single-cell amplifications.
▲ CRITICAL STEP To properly assess whether or not the amplification protocol works for researchers, it is important to perform positive control amplifications sufficient for a statistical analysis (e.g., eight samples per experiment).
▲ CRITICAL STEP This step is optional, but we strongly recommend performing this step to establish the protocol before starting with real single cells.

Cell lysis and denaturation of total RNA

11| For lysing single cells and denaturing total RNA, incubate the tubes at 70 °C for 90 s in a water bath.

12| Place the tubes on ice immediately for 1 min and centrifuge them at $16.1 \times 10^3 g$ for 10 s at room temperature.

Reverse transcription

13| Prepare the RT mastermix on ice as follows:

Number of reactions	×1	×20
SuperScriptIII	0.2 µl	4.0 µl
RNAguard RNase Inhibitor	0.033 µl	0.66 µl
T4 gene 32 protein	0.067 µl	1.34 µl
Total volume	0.3 µl	6.0 µl

▲ CRITICAL STEP Chill the tube on ice before use.

14| Aliquot 0.3 µl RT mastermix into each tube (from Step 12) and mix by tapping gently and thoroughly.

15| Incubate the tubes in a water bath at 50 °C for 5 min for first-strand synthesis.

16| Heat-inactivate the reaction by incubating the tubes at 70 °C for 10 min.

17| Place the tubes on ice immediately for 1 min and centrifuge them at $16.1 \times 10^3 g$ for 10 s at room temperature.
▲ CRITICAL STEP For representative amplification of global cDNAs by PCR, it is important to keep the cDNA length short. RT by Superscript III under the above conditions typically yields cDNAs with an average length of ~700 bp, short enough for subsequent representative amplification and long enough for microarray hybridization[13].

Exonuclease I treatment

18| Prepare exonuclease I mastermix on ice as follows:

Number of reactions	×1	×20
10× exonuclease I buffer	0.1 µl	2.0 µl
DDW	0.8 µl	16.0 µl
Exonuclease I	0.1 µl	2.0 µl
Total volume	1.0 µl	20.0 µl

▲ CRITICAL STEP Mastermix can be prepared in 1.5-ml tubes in advance and chilled on ice until use.
▲ CRITICAL STEP Add exonuclease I immediately before use.

19| Add 1.0 µl exonuclease I mastermix into each tube (from Step 17) and mix by tapping gently and thoroughly.

20| Incubate the tubes in a thermal cycler at 37 °C for 30 min to eliminate the unreacted first-strand primer and then at 80 °C for 25 min for the inactivation of exonuclease I.

21| Place the tubes on ice immediately for 1 min and centrifuge them at 16.1 × $10^3 g$ for 10 s at room temperature.

Poly(dA) addition

22| Prepare TdT mastermix on ice as follows:

Number of reactions	×1	×20
10× PCR buffer II (without $MgCl_2$)	0.6 µl	12.0 µl
$MgCl_2$ (25 mM)	0.36 µl	7.2 µl
ATP (100 mM)	0.18 µl	3.6 µl
DDW	4.26 µl	85.2 µl
RNaseH	0.3 µl	6.0 µl
TdT	0.3 µl	6.0 µl
Total volume	6 µl	120 µl

▲ **CRITICAL STEP** Mastermix can be prepared in 1.5 ml tubes in advance and chilled on ice until use.
▲ **CRITICAL STEP** Add TdT and RNase H immediately before use.

23| Add 6.0 µl TdT mastermix into each tube (from Step 21) and mix by tapping gently and thoroughly.

24| Incubate the tubes in a water bath at 37 °C for 15 min for poly(dA) addition reaction and then 70 °C for 10 min to inactivate the TdT.

25| Place the tubes on ice immediately for 1 min and centrifuge them at 16.1 × $10^3 g$ for 10 s at room temperature.

Second-strand cDNA synthesis

26| Split the product from Step 25 into four empty 0.2 ml PCR tubes (3 µl each).
▲ **CRITICAL STEP** To reduce intrinsic variation associated with PCR amplification, perform PCR amplification in four independent tubes and recombine the products afterwards (averaging four samples reduces variance by half).

27| Prepare PCR mastermix I on ice as follows:

Number of reactions	×1	×20
10× ExTaq buffer	7.6 µl	152 µl
DNTP mix (2.5 mM each)	7.6 µl	152 µl
$V3(dT)_{24}$ primer (1 µg µl^{-1})	1.52 µl	30.4 µl
DDW	58.52 µl	1,170.4 µl
ExTaq Hot Start Version	0.76 µl	15.2 µl
Total	76 µl	1,520 µl

▲ **CRITICAL STEP** Mastermix can be prepared in 1.5-ml tubes in advance and chilled on ice until use.
▲ **CRITICAL STEP** Add ExTaq HS immediately before use.

28| Add 19.0 µl PCR mastermix I into each tube (from Step 26) and immediately put it on ice.

29| Centrifuge the tubes at 2 × $10^3 g$ for a few seconds at room temperature and perform one round of PCR for second-strand synthesis as follows: 95 °C for 3 min, 50 °C for 2 min and 72 °C for 3 min.

30| Place the tubes on ice for 1 min and centrifuge them at 2 × $10^3 g$ for a few seconds.

20-cycle PCR for initial cDNA amplification

31| Prepare PCR mastermix II on ice as follows:

Number of reactions	×1	×20
10× ExTaq buffer	7.6 µl	152 µl
dNTP mix (2.5 mM each)	7.6 µl	152 µl
$V1(dT)_{24}$ primer (1 µg µl^{-1})	1.52 µl	30.4 µl
DDW	58.52 µl	1,170.4 µl
ExTaq Hot Start Version	0.76 µl	15.2 µl
Total	76 µl	1,520 µl

▲ **CRITICAL STEP** Master mix can be prepared in 1.5-ml tubes in advance and chilled on ice until use.
▲ **CRITICAL STEP** Add ExTaq HS immediately before use.

32| Add 19.0 µl PCR mastermix II into each tube (from Step 30) on ice.

33| Add a drop of mineral oil into each tube, centrifuge the tube at 2 × $10^3 g$ for a few seconds at room temperature, then immediately place it on ice.
▲ **CRITICAL STEP** We recommend the addition of mineral oil to avoid evaporation of the reaction mixture even with the thermal cyclers equipped with heat covers.

34| Perform 20-cycle PCR as follows: 95 °C for 30 s, 67 °C for 1 min and 72 °C for 3 min (+6 s for each cycle) (for 20 cycles), followed by 72 °C for 10 min.

35| Mix together the amplified cDNA that was split into four tubes at Step 26.

36| Purify the cDNA with the Qiaquick PCR purification kit, according to the manufacturer's instructions (final elution with 50 µl EB buffer).
■ **PAUSE POINT** The amplified single-cell cDNAs can be stored at −80 °C for more than one year.

Screening for cells of interest
37| This step is optional, but to identify cells of interest, perform 30-cycle PCR with gene-specific primers of your interest, using 0.4 µl PCR product as template. Analyze PCR products with agarose gel electrophoresis.

T7 promoter addition by nine-cycle PCR
38| The following steps are performed to prepare templates for IVT labeling procedures supported by commercial microarray systems (e.g., Affymetrix). For T7 promoter addition, prepare PCR mastermix III on ice as follows:

Number of reactions	**×1**	**×8**
10× ExTaq buffer	5 µl	40 µl
DNTP mix (2.5 mM each)	5 µl	40 µl
T7V1 primer (1 µg µl^{-1})	1 µl	8.0 µl
V3(dT)$_{24}$ primer (1 µg µl^{-1})	1 µl	8.0 µl
DDW	36.87 µl	294.96 µl
ExTaq Hot Start Version	0.5 µl	4.0 µl
Total volume	49.37 µl	394.96 µl

39| Mix the mastermix gently and keep it on ice.

40| Add 0.63 µl of 20-cycle PCR product (from Step 36) into each of eight 0.2-ml PCR tubes (5.04 µl in total).
▲ **CRITICAL STEP** The aim of this step is to obtain sufficient PCR products for their efficient recovery by gel purification (Steps 46 and 47). We perform this step using an eight-well 0.2-ml PCR tube band for one sample, which in total gives a good final cDNA yield for gel purification. If gel purification is not required (e.g., researchers would like to examine gene expression profiles of many samples only by Q-PCR), nine-cycle PCR at this step can be performed using only one tube (i.e., at a 50-µl scale).

41| Add 49.37 µl PCR mastermix III into each tube and add a drop of mineral oil.

42| Centrifuge the tubes at 2 × $10^3 g$ at room temperature and place them on ice.

43| Perform nine-cycle PCR as follows: 95 °C for 5 min 30 s, 64 °C for 1 min and 72 °C for 5 min 18 s (for one cycle); 95 °C for 30 s, 67 °C for 1 min and 72 °C for 5 min 18 s (+6 s for each cycle) (for eight cycles); followed by 72 °C for 10 min.

44| Mix together the PCR product in each of the eight tubes , and then purify them with the Qiaquick PCR purification kit, according to the manufacturer's instructions, eluting with 30 µl EB buffer.
■ **PAUSE POINT** cDNAs can be stored at −20 or −80 °C for more than one year.
▲ **CRITICAL STEP** The standard IVT labeling protocol supported by commercial microarray systems such as Affymetrix needs a relatively large quantity of template cDNAs, equivalent to a few micrograms of total RNA. It is important to prepare an adequate quantity of cDNAs for the microarray experiment.

45| This step is optional, but we recommend examining the quality of the amplification by Q-PCR using the Step-44 product. In addition, this allows an insight into the properties of cells of interest and their gene expression profiles to be obtained before applying single-cell cDNAs to microarrays. This aids decisions on which cells should be scrutinized by miroarray analysis. To perform this, dilute the Step-44 product with H_2O 1:40 (1:20 if Step 40 has been performed in one tube) and use 2 µl as a template for a 10 µl per well-scale Q-PCR. Duplicate each reaction and take the average of the resultant threshold cycles (C_t).

Gel purification

46| Apply the whole step 44 product to 2% agarose gel and run electrophoresis in 0.5× TAE at 100 V for 10 min.
▲ **CRITICAL STEP** Large gel volumes result in inefficient recovery. Make the agarose gel as thin as possible (e.g., 20 ml agarose solution for a 110 × 60 mm gel). Longer electrophoresis also results in a cDNA-containing gel fragment with large volume, and therefore we recommend electrophoresis does not exceed 10 min.

47| Visualize the DNA under UV light (wavelength 365 nm) and cut out fragments containing cDNA smears (longer than 250 bps) from the gel. Purify the DNA with the QiaQiuck gel extraction kit, following the manufacturer's instructions, eluting with 35 μl buffer EB.
▲ **CRITICAL STEP** The PCR product still contains some by-products derived from nonspecific amplification of primers themselves, which form a clear band of smaller than 200 bps. Note that the purpose of the gel purification step is to remove these by-products. As an average length of the V3-poly(dT) plus poly(dA)-V1T7 portions in amplified cDNAs is estimated to be approximately 165 bp long[13], removal of cDNA products less than 250 bps results in the loss of only those gene products that are less than 85 bp long, if any.
▲ **CRITICAL STEP** Gel purification may also be necessary when the application of the cDNAs to cloning and sequencing analyses (e.g., EST, subtraction analyses) is desired. If gel purification is omitted, by-products having low molecular weights may be cloned predominantly.

Final one-cycle PCR

48| Prepare PCR mastermix IV on ice as follows:

Number of reactions	×1	×4
10× ExTaq buffer	5 μl	20 μl
dNTP mix (2.5 mM)	5 μl	20 μl
T7V1 primer (1μg μl^{-1})	1 μl	4.0 μl
V3(dT)24 primer (1μg μl^{-1})	1 μl	4.0 μl
DDW	35.3 μl	141.2 μl
ExTaq Hot Start Version	0.5 μl	2.0 μl
Total volume	47.8 μl	191.2 μl

49| Mix mastermix gently and keep it on ice.

50| Add 2.2 μl of the Step-47 product into each of four 0.2 ml thin-walled PCR tubes (8.8 μl in total).

51| Add 47.8 μl PCR mastermix IV into each tube and add a drop of mineral oil.

52| Centrifuge the tubes at 2 × $10^3 g$ at room temperature and place them on ice.

53| Perform one-cycle PCR as follows: 95 °C for 5 min 30 s, 67 °C for 1 min and 72 °C for 16 min (one cycle).

54| Purify the cDNA with either the Qiaquick PCR purification kit or the GeneChip Sample Cleanup Module, following the manufacturer's instructions, and then eluting with 30 μl EB buffer or cDNA eluting buffer, respectively.
■ **PAUSE POINT** cDNAs can be stored at −20 or −80 °C.

Examination of the quality of the final cDNA product

55| It is important to examine the quality of the final cDNA product. Choose some of the Step-54 products at random (e.g., 8 out of 20 samples) and subject them to a non-labeling IVT reaction on a small scale (e.g., a 5-μl scale): prepare non-labeling IVT reaction mixture in a 0.2 ml thin-walled PCR tube with a cap using the MEGA script T7 kit, at room temperature:

Number of reactions	×1
10× reaction buffer	0.5 μl
ATP solution	0.5 μl
CTP solution	0.5 μl
GTP solution	0.5 μl
UTP solution	0.5 μl
Nuclease-free water	1.0 μl
cDNA (Step-54 product)	1.0 μl
Enzyme mix	0.5 μl
Total volume	5.0 μl

56| Incubate at 37 °C for 2–16 h and purify with the RNeasy kit following the manufacturer's instructions, eluting with 50 µl Rnase-free water.
▲ CRITICAL STEP Set the incubation time according to the IVT labeling protocol for the microarray experiments.

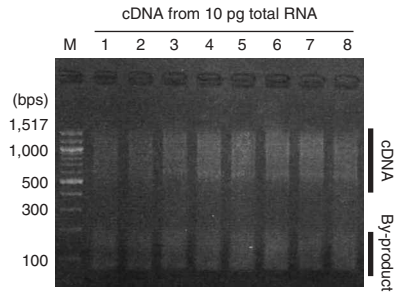

Figure 3 | A typical result of 2% agarose gel electrophoresis of the cDNAs amplified from 10 pg ES cell total RNA. We subjected 0.4 µl diluted ES cell total RNA (25 pg μl^{-1}) to eight independent amplifications. The initial amplification was performed with 24 cycles instead of 20 cycles in order to obtain a quantity of DNA sufficient to be visualized with ethidium bromide (EtBr). A 5 µl portion out of 50 µl of total cDNAs was loaded (lanes 1–8), and 100 ng of the 100 bp DNA ladder (NEB; N3231S) was loaded in lane M as a molecular weight marker. By-products are supposed to be the result of amplification of primer V1$(dT)_{24}$ that is left unreacted at Steps 15 and 20, tailed with poly(dA) in Step 24[13]. The cDNAs range in size from 500 to 2,000 bps, whereas by-products are less than 200 bps.

57| Measure the amount of RNA by UV260 and analyze the RNA with 2% agarose gel electrophoresis.
▲ CRITICAL STEP Confirm that the majority of the RNA smear is distributed across >300 bps by agarose gel electrophoresis (see ANTICIPATED RESULTS).
▲ CRITICAL STEP Confirm that the amount of product RNA is 4–25 µg.

58| Subject 1–2 µg of the Step-56 product to RT using SuperScript III, and purify with the Qiaquick PCR purification kit, following the manufacturer's instructions, eluting with 50 µl EB buffer.

59| Compare the relative abundance of gene expressions in the Step-58 (IVT-RT product) and Step 54 products (final PCR product) with Q-PCR. Prepare templates for Q-PCR by diluting the IVT-RT and PCR products with H_2O at ratios of 1:5 and 1:40, respectively. Use 2 µl template DNA for a 10 µl per well-scale Q-PCR.
▲ CRITICAL STEP Confirm that the IVT reaction did not distort the representation of the PCR product (see also ANTICIPATED RESULTS).

Application to IVT labeling procedures

60| Subject the Step-54 product to the standard IVT labeling protocol supported by a commercial oligonucleotide microarray system according to the manufacturer's instructions. Typically, we apply 3–8 µl to the Affymetrix Eukaryotic Target Preparation protocol, starting from "Synthesis of Biotin-Labeled cRNA for Both the One-Cycle and Two-Cycle Target Labeling Assays" step.
▲ CRITICAL STEP The optimal amount of cDNAs subjected to IVT labeling should be determined depending on the microarray system, the quality of the purified cDNA and/or the original single cells used for the analysis.

● TIMING
Day 1: Steps 1–2, 1 h
Steps 3–10, 1.5 h
Steps 11–33, 5 h
Step 34, 2.5 h
Day 2: Steps 35–36: Time depends on sample numbers. We usually perform 20 amplifications per operator, and it takes 1.5 h
Step 37 (optional), 3.5 h
Steps 38–42, 1 h

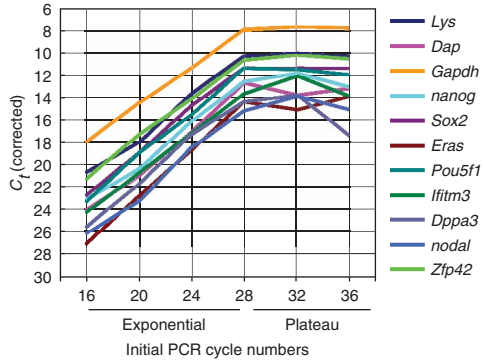

Figure 4 | Amplification curves of genes in cDNAs from 10 pg ES cell total RNA during PCR amplification. For the initial PCR, we performed two independent amplifications with each of 16, 20, 24, 28, 32 and 36 cycles. cDNAs amplified by 16–24 cycles were then diluted with H_2O by 1:10, whereas those amplified by 28–36 cycles were diluted by 1:100. Then, 2 µl of diluted cDNA was applied to the 10 µl per well-scale Q-PCR. Q-PCR was performed on two spike RNAs (*Lys* and *Dap*) and nine endogenous genes [glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)[27], *nanog*[28,29], SRY (sex-determining region Y)-box 2 (*Sox2*)[30], ES cell-expressed Ras (*Eras*)[31], POU domain class 5 transcription factor 1 (*Pou5f1*, also called Oct3/4)[32], interferon-induced transmembrane protein 3 (*Ifitm3*, also called *fragilis/mil-1*)[23], developmental pluripotency-associated 3 (*Dppa3*, also called *stella/PGC7*)[23,33], *nodal*[34] and zinc-finger protein 42 (*Zfp42*, also called *Rex1*)[35].] The threshold cycle (C_t) values of Q-PCR are plotted against the cycle numbers of the initial PCR amplifications [the threshold cycle is defined as the cycle at which the fluorescence emission exceeds the fixed threshold. The threshold was set as ΔRn (normalized reporter fluorescence) of 0.2]. The C_t values for samples amplified by 28–36 cycles are corrected by subtracting $\log_2 10$ (=3.322), so that they are comparable to those amplified by 16–24 cycles. The exponential (16–24 cycles) and plateau phases (24–32 cycles) are indicated. Note that the exponential amplification is represented linearly in the initial PCR cycle versus C_t plot.

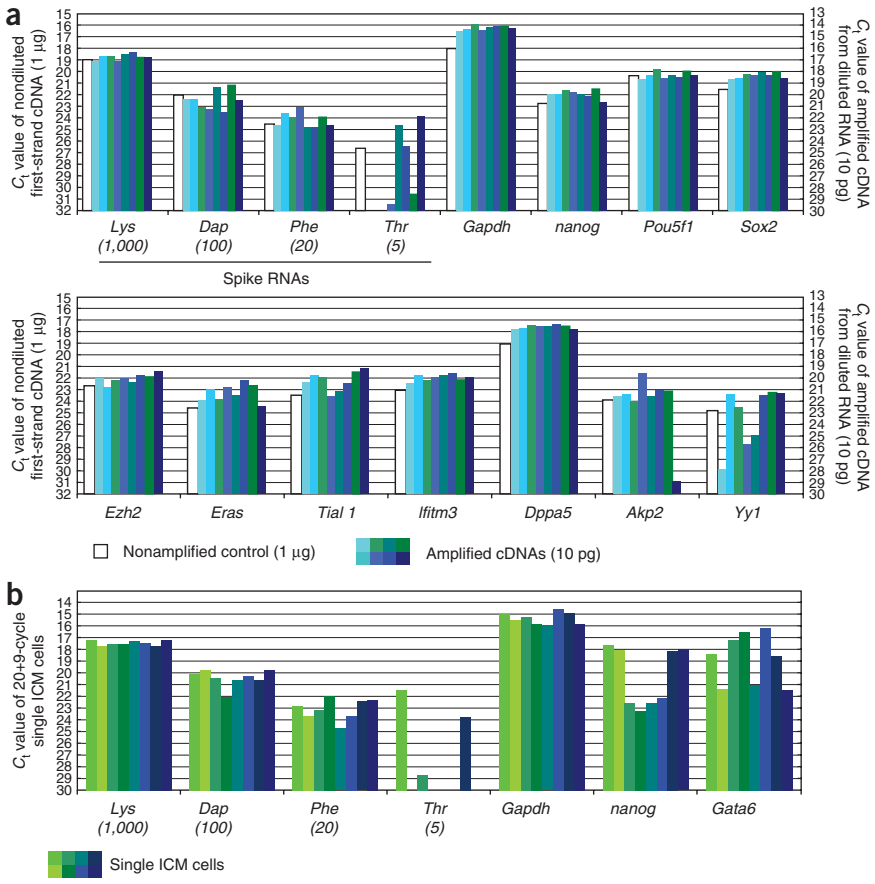

Figure 5 | Anticipated representation of gene expressions after cDNA amplifications. (**a**) Preservation of transcript abundance through the initial 20-cycle PCR amplification. We performed eight independent amplifications from the 10 pg ES cell total RNA, and also prepared first-strand cDNA from 1 μg, non-diluted ES cell total RNA as a non-amplified control. The amplified samples and non-amplified controls were purified and dissolved in 50 μl buffer EB. Each of them was diluted with H_2O by 1:10, 2 μl of which was applied to the 10 μl per well-scale Q-PCR as a template. The open bars represent gene expressions in the non-amplified control (left axis). The blue- or green-filled bars represent gene expressions in the independently amplified cDNAs (right axis). (**b**) Gene expressions of eight single ICM cells after 20+9-cycle amplification (each represented by differently colored bar). We cut out the embryonic fragment that contained the ICM under a dissection microscope and dissociated the cells by trypsin (**Fig. 1a**). The cDNA products were diluted with H_2O by 1:40, 2 μl of which was applied to a 10 μl per well-scale Q-PCR. The C_t values of Q-PCR are shown. The gene nomenclatures are as follows: enhancer of zeste homolog 2 (*Ezh2*)[36], Tia1 cytotoxic granule-associated RNA binding protein-like 1 (*Tial1*, also called *Tiar*)[37], developmental pluripotency associated 5 (Dppa5, also called *Esg1*)[38], alkaline phosphatase 2 liver (*Akp2*, also called *Tnap*)[39], YY1 transcription factor (*Yy1*)[40] and GATA binding protein 6 (*Gata6*)[41].

Step 43, 2 h
Step 44: Time depends on samples numbers. For example, it takes ~1 h for ten samples
Day 3: Step 45 (optional): Time depends on sample numbers. For example, it takes 2 h to prepare 192 reactions (i.e., 12 cells × 8 genes × 2 duplicates) and 2 h for 35-cycle real-time PCR
Day 4: Steps 46–47: Time depends on sample numbers. For example, it takes ~3 h for eight samples (i.e., electrophoresis twice using 110 × 60 mm agarose gel)
Steps 48–52, 2 h
Step 53, 30 min
Step 54: Time depends on sample numbers. For example, it takes 1 h for eight samples
Day 5: Step 55, 1 h
Step 56, 2–16 h
Day 6: Step 57: 1 h
Step 58: 5 h
Step 59: 3 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Loss of embryonic fragments during transfer	Embryonic fragments stick to the bottom of the dish or to the inside of the micropipette	Skip PBS-EDTA treatment and prolong the trypsin-EDTA treatment (e.g., to 10 min)
Incomplete dissociation of single cells	Insufficient trypsin treatment	Prolong the trypsin-EDTA treatment and pipette the embryonic fragments gently and thoroughly using mouth pipettes with larger diameters first and then those with smaller diameters (e.g., ~100 μm and ~50, respectively)

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
No and/or low efficiency of control amplifications	Degradation of diluted RNA during preparation	Compare amplified cDNAs with two independently diluted RNA samples by Q-PCR (e.g., $N = 6$ for each dilution lot). RNA degradation should cause the difference between dilution lots Check the C_t values of RT products from non-diluted total RNAs
	Degradation of RNA during the procedure	Ensure that the tubes containing the cell lysis buffer are well chilled on ice
	Contamination during handling	Use distilled DNase/RNase-free pre-packed pipette tips Keep the operator's breath away from the reaction tubes (e.g., wear a dust mask) Keep dust away from the bench Float the tubes on water baths very gently to prevent water from touching the tube caps
	Contamination from DDW	Aliquot the bulk water into 1.5-ml tubes and store at −20 °C. Thaw only the amount to be used for the procedure If necessary, use other DDW products
	Improper reaction temperatures	Check the thermal cycler settings and measure the temperature of the water baths. Smaller water baths are often problematic
	Decay of enzymatic activity by shearing forces	Tap the reaction tubes gently to mix the reagents (typically less than ten times). Never knock the tube roughly or use Voltex mixer
	Decay of enzymatic activity by thermal agitation	Ensure that the mastermixes are well chilled on ice before adding enzymes
	Degradation of RT products by exonuclease I	Properly inactivate exonuclease I. The remaining active exonuclease I may degrade the cDNA products Perform heat inactivation at 80 °C or at a higher temperature. Inactivation at lower temperature increases the risk of having a considerable amount of active enzyme
	Insufficient degradation of the unreacted initial primer	Run the small amount of the initial amplified product by agarose gel electrophoresis (see ANTICIPATED RESULTS). If exonuclease I fails to degrade the unreacted primers, the PCR product results in one continuous smear from 100 to 1,500 bps, rather than in two separate DNA smears, as shown in **Figure 3** To determine whether or not exonuclease I works, check a negative control (i.e., water as the starting material). If the unreacted primer is not eliminated by exonuclease I, DNA smear derived from poly-dA-tailed primers will appear with the absence of mRNA as the starting material
	Inappropriate choice of genes for Q-PCR check	Choose genes of appropriate expression level carefully (the safer limit is >20 copies per cell) based on the results of RT products from the non-diluted total RNA.
	Degradation of reagents and/or enzymes	Refresh the reagents. dATP, dNTP and/or DDT may require frequent refreshing. Aliquot the bulks into 1.5-ml tubes and store at −20 °C Although we tend to suspect the enzymes for insufficient amplifications, in our cases, they were the least problematic factor
No and/or low efficiency of initial amplification	Cells were not picked into the tubes	Ensure that the cells are picked by the micropipette and placed into tubes
	mRNA degradation	Proceed with the cell isolation process as quickly as possible, preferably within 1 h
	Insufficient cDNA synthesis and/or PCR amplification	Check the amplification procedure (Steps 1–36) using diluted total RNA

TABLE 1 | Troubleshooting table (continued).

Problem	Possible reason	Solution
No and/or low efficiency of spike RNA amplification	Degradation of spike RNA	Prepare a high-concentration spike RNA mixture first, and then dilute it to an appropriate level. Do not dilute individual spike RNAs first Do not use Voltex or tap tubes to mix the spike RNA solutions, so as to prevent the contents of the tubes from touching the caps The final spike RNA concentration is extremely low. The spike RNA solution is much more difficult to prepare than the single-cell-level diluted total RNA. Establish the amplification procedure using the endogenous genes before working with spike RNAs
	Insufficient cDNA synthesis and/or PCR amplification	Check the amplification procedure (Steps 1–36) using diluted total RNA
Low efficiency of gel purification	Large amount of gel applied to the purification	The efficiency of gel purification depends on the amount of the gel, as described in the manufacturer's instructions (Qiaquick gel extraction kit). Make the gel as thin as possible
	Insufficient amount of cDNA applied to the purification	The efficiency of gel purification also depends on DNA amounts. If the cDNA amount is so small that the smear is not visually detected in the gel excising process (e.g., cDNAs of small cells such as from flies), try to increase the amount of DNA for gel purification

ANTICIPATED RESULTS

Control amplification

We consider it essential to perform control amplifications using single-cell-level RNA (10 pg) before working with real single cells. **Figure 3** shows a typical result of gel electrophoresis of an initial amplification product. In this instance, we performed 24 cycles instead of 20 at Step 34 to visualize the PCR products. The initial amplification progressed exponentially when less than 28 PCR cycles were performed, retaining relative abundance of the individual cDNAs (**Fig. 4**). When there were more than 28 PCR cycles, however, the amplification rapidly reached a plateau. As the plateau could be the result of dNTP depletion during PCR, we believe that an initial PCR cycle number of less than 28 (e.g., 20–24) is safe to retain the representation of gene expression.

To examine whether or not the amplification was representative and reproducible, we compared the levels of 15 gene expressions (11 endogenous genes and four spike RNAs) among eight independently amplified samples by Q-PCR (**Fig. 5a**). In **Figure 5**, we provide the raw C_t values of the genes we examined by Q-PCR to present a practical measure to see if the single-cell PCR was successful. This shows that the majority of the genes we examined were reproducibly amplified in the eight independent amplifications, although the precise measurement of expression levels requires normalization and correction by the efficiency of Q-PCR primers (see refs. 13,25). The proportion of each gene expression was also essentially retained after the 20-cycle PCR.

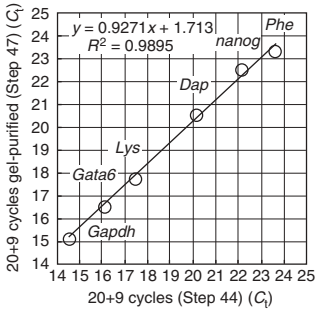

Figure 6 | Preservation of transcript abundance through the gel purification step. We performed 20+9 cycles (Step 44) and gel purification (Step 47) for one of the single-ICM-cell cDNAs, and compared gene representation between these two steps. Q-PCR was performed on three spike RNAs (*Lys*, *Dap* and *Phe*) and endogenous genes (*Gapdh*, *nanog* and *Gata6*), and the C_t values were plotted. The inset equations show the regression line and squared regression coefficient (R^2) of the plots.

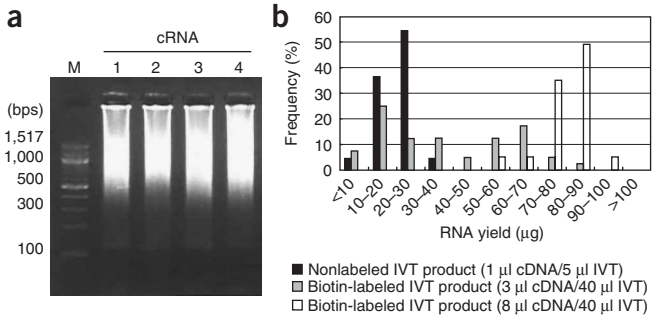

Figure 7 | Typical results of IVT. (**a**) Typical result of 2% agarose gel electrophoresis for non-labeled IVT product (Step 56) from the final cDNAs (Step 54). cRNAs (0.5 μl) were loaded and stained with EtBr (lanes 1–4). The 100-bp DNA ladder was loaded in lane M as a molecular weight marker. (**b**) Histogram of IVT yields. Black, gray and white bars represent frequencies of non-labeled IVT product (5-μl scale), biotin-labeled IVT product (3 μl cDNA in 40-μl scale reaction) and biotin-labeled IVT product (8 μl cDNA in 40-μl scale reaction), respectively.

Amplification of cDNA from single ICM cells

We also performed amplification from single ICM cells in mouse blastocysts[13] using the initial 20-cycle PCR and a subsequent nine-cycle PCR (Steps 38–44), as described (**Fig. 5b**). The C_t values of the housekeeping gene *Gapdh* and the spike RNAs were comparable with those of the control amplification from diluted, single-cell-level ES total RNA (**Fig. 5**). The endogenous gene expressions show that the amplified cDNAs from single ICM cells have a complexity reflecting the nature of the original cells (**Fig. 5b** and see also ref. 13).

Next, we subjected 20 randomly chosen single ICM cells to microarray analysis[13]. After nine-cycle amplification, we performed gel purification (Steps 46 and 47). The representation of the cDNAs was well conserved through the gel purification step (**Fig. 6**). The purified cDNAs were then subjected to the final one-cycle amplification (Steps 48–54) and non-labeling IVT for the cDNA quality test (Steps 55–59), for which we randomly chose eight of the 20 samples. A typical electrophoresis result and the distribution of the IVT yield of the non-labeling IVT are shown in **Figure 7**. The RNA products were then converted to single-stranded cDNA by RT and subjected to Q-PCR analysis to analyze the gene representation of the IVT products (**Fig. 8**). **Figure 8** shows that the IVT-RT process brought essentially no distortion in gene representation. We have observed that when the final one-cycle PCR was omitted, the IVT yield significantly decreased and gene representation was distorted (not shown), although we have not identified the cause. The final cycle therefore should not be omitted.

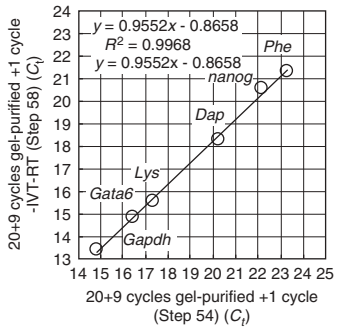

Figure 8 | Preservation of transcript abundance through the IVT reaction. An anticipated result of Step 59 is shown. We performed the IVT template preparation throughout (Step 54) for one of the single-ICM-cell cDNAs. We also performed the non-labeled IVT reaction using the same cDNA sample and reverse-transcribed the IVT product (Step 58). We compared gene representation between these two steps by plotting the C_t values of Q-PCR, as in **Figure 6**.

The IVT biotin-labeling procedure supported by the Affymetrix system can be performed using 3–8 µl volumes of the final cDNA (Step 60). The distribution of the labeled RNA yield was shown for IVT labeling using both 3 and 8 µl volumes (**Fig. 7b**). The 8 µl volume provided better, more stable yields of labeled cRNAs.

ACKNOWLEDGMENTS We thank all the members of our laboratory for their discussion of this study. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by a PRESTO project grant from the Japan Science and Technology Agency.

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

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Lockhart, D.J. *et al.* Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* **14**, 1675–1680 (1996).
2. Adams, M.D. *et al.* Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**, 1651–1656 (1991).
3. Boguski, M.S., Lowe, T.M. & Tolstoshev, C.M. dbEST—database for "expressed sequence tags". *Nat. Genet.* **4**, 332–333 (1993).
4. Velculescu, V.E., Zhang, L., Vogelstein, B. & Kinzler, K.W. Serial analysis of gene expression. *Science* **270**, 484–487 (1995).
5. Brady, G., Barbara, M. & Iscove, N. Representative *in vitro* cDNA amplification from individual hemopoietic cells and colonies. *Methods Mol. Cell. Biol.* **2**, 17–25 (1990).
6. Brady, G. & Iscove, N.N. Construction of cDNA libraries from single cells. *Methods Enzymol.* **225**, 611–623 (1993).
7. Van Gelder, R.N. *et al.* Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* **87**, 1663–1667 (1990).
8. Eberwine, J. *et al.* Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. USA* **89**, 3010–3014 (1992).
9. Saito, H., Kubota, M., Roberts, R.W., Chi, Q. & Matsunami, H. RTP family members induce functional expression of mammalian odorant receptors. *Cell* **119**, 679–691 (2004).
10. Klur, S., Toy, K., Williams, M.P. & Certa, U. Evaluation of procedures for amplification of small-size samples for hybridization on microarrays. *Genomics* **83**, 508–517 (2004).
11. Ji, W., Zhou, W., Gregg, K., Lindpaintner, K. & Davis, S. A method for gene expression analysis by oligonucleotide arrays from minute biological materials. *Anal. Biochem.* **331**, 329–339 (2004).
12. Iscove, N.N. *et al.* Representation is faithfully preserved in global cDNA amplified exponentially from sub-picogram quantities of mRNA. *Nat. Biotechnol.* **20**, 940–943 (2002).
13. Kurimoto, K. *et al.* An improved single-cell cDNA amplification method for efficient high-density oligonucleotide microarray analysis. *Nucleic Acids Res.* **34**, e42 (2006).
14. Tietjen, I. *et al.* Single-cell transcriptional analysis of neuronal progenitors. *Neuron* **38**, 161–175 (2003).
15. Chazaud, C., Yamanaka, Y., Pawson, T. & Rossant, J. Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2–MAPK pathway. *Dev. Cell* **10**, 615–624 (2006).
16. Baugh, L.R., Hill, A.A., Slonim, D.K., Brown, E.L. & Hunter, C.P. Composition and dynamics of the *Caenorhabditis elegans* early embryonic transcriptome. *Development* **130**, 889–900 (2003).
17. Adams, M.D. *et al.* The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185–2195 (2000).
18. Church, R.B. & Robertson, F.W. Biochemical analysis of genetic differences in the growth of *Drosophila*. *Genet. Res.* **7**, 383–407 (1966).
19. Rahner, C., Fukuhara, M., Peng, S., Kojima, S. & Rizzolo, L.J. The apical and basal environments of the retinal pigment epithelium regulate the maturation of tight junctions during development. *J. Cell Sci.* **117**, 3307–3318 (2004).
20. Jost, J.P., Ohno, T., Panyim, S. & Schuerch, A.R. Appearance of vitellogenin mRNA sequences and rate of vitellogenin synthesis in chicken liver following primary and secondary stimulation by 17 beta-estradiol. *Eur. J. Biochem.* **84**, 355–361 (1978).
21. Brown, D.D. & Gurdon, J.B. Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA* **51**, 139–146 (1964).
22. Dulac, C. & Axel, R. A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83**, 195–206 (1995).
23. Saitou, M., Barton, S.C. & Surani, M.A. A molecular programme for the specification of germ cell fate in mice. *Nature* **418**, 293–300 (2002).
24. Chiang, M.K. & Melton, D.A. Single-cell transcript analysis of pancreas development. *Dev. Cell* **4**, 383–393 (2003).
25. Yabuta, Y., Kurimoto, K., Ohinata, Y., Seki, Y. & Saitou, M. Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biol. Reprod.* **75**, 705–716 (2006).

26. Kamme, F. *et al.* Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. *J. Neurosci.* **23**, 3607–3615 (2003).
27. Seipp, S. & Buselmaier, W. Isolation of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) cDNA from the distal half of mouse chromosome 16: further indication of a link between Alzheimer's disease and glycolysis. *Neurosci. Lett.* **182**, 91–94 (1994).
28. Chambers, I. *et al.* Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655 (2003).
29. Mitsui, K. *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631–642 (2003).
30. Avilion, A.A. *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* **17**, 126–140 (2003).
31. Takahashi, K., Mitsui, K. & Yamanaka, S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. *Nature* **423**, 541–545 (2003).
32. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391 (1998).
33. Sato, M. *et al.* Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. *Mech. Dev.* **113**, 91–94 (2002).
34. Brennan, J. *et al.* Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* **411**, 965–969 (2001).
35. Rogers, M.B., Hosler, B.A. & Gudas, L.J. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development* **113**, 815–824 (1991).
36. Laible, G. *et al.* Mammalian homologues of the Polycomb-group gene Enhancer of zeste mediate gene silencing in *Drosophila* heterochromatin and at *S. cerevisiae* telomeres. *EMBO J.* **16**, 3219–3232 (1997).
37. Kawakami, A. *et al.* Identification and functional characterization of a TIA-1-related nucleolysin. *Proc. Natl. Acad. Sci. USA* **89**, 8681–8685 (1992).
38. Astigiano, S. *et al.* Changes in gene expression following exposure of nulli-SCCl murine embryonal carcinoma cells to inducers of differentiation: characterization of a down-regulated mRNA. *Differentiation* **46**, 61–67 (1991).
39. MacGregor, G.R., Zambrowicz, B.P. & Soriano, P. Tissue non-specific alkaline phosphatase is expressed in both embryonic and extraembryonic lineages during mouse embryogenesis but is not required for migration of primordial germ cells. *Development* **121**, 1487–1496 (1995).
40. Safrany, G. & Perry, R.P. Characterization of the mouse gene that encodes the delta/YY1/NF-E1/UCRBP transcription factor. *Proc. Natl. Acad. Sci. USA* **90**, 5559–5563 (1993).
41. Morrisey, E.E., Ip, H.S., Lu, M.M. & Parmacek, M.S. GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. *Dev. Biol.* **177**, 309–322 (1996).