Quantitative analysis of gene expression in a single cell by qPCR

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We developed a quantitative PCR method featuring a reusable single-cell cDNA library immobilized on beads for measuring the expression of multiple genes in a single cell. We used this method to analyze multiple cDNA targets (from several copies to several hundred thousand copies) with an experimental error of 15.9% or less. This method is sufficiently accurate to investigate the heterogeneity of single cells.

Molecular biology in general and the human genome project in particular have provided a massive amount of information about genomes and proteins, and there is now much interest in understanding living systems at the molecular level. Because the smallest metabolically functional unit of a living organism is a single cell, many people are trying to analyze the molecular components in single cells. But information from typical ensemble measurements of mRNA and proteins is averaged from millions of cells. The individual cells from the same tissue may actually differ from each other and have different roles[1]. There have recently been several attempts to investigate the heterogeneity of gene expression in individual embryo cells, neurons, immunocytes and cancer cells[2–9]. These single-cell approaches have attracted much attention[10–12], and efforts are underway to develop tools for analyzing the components in single cells[13–15].

Our ultimate goal is to establish a method for quantifying all the mRNA in a single cell. This requires developing technologies to efficiently and reproducibly extract the mRNA needed to produce cDNA from a single cell and quantify the cDNA for each target gene. Previous work in this area has primarily used whole RNA or cDNA amplification coupled with DNA chips or quantitative PCR (qPCR). More recently, a single-cell mRNA analysis method using whole cDNA amplification coupled with digital counting of amplified cDNA with an ultra-high-throughput DNA sequencer was reported[16]. It was effective at finding many splicing variants, but in terms of quantitative analysis, it seems to have ambiguity owing to the amplification processes. As qPCR is considered to be the most accurate quantitative method at the present time, the best way to achieve accurate quantitative analysis is to use direct qPCR from a cDNA pool without pre-amplification. Although there have been several reports of qPCR being used to quantify expression of a few genes in a single cell, the analysis of multiple genes in a single cell is rather difficult because it requires dividing the sample, which reduces the sample size for measurement and therefore the detection sensitivity.

Although the use of oligo(dT)-immobilized beads for creating a cDNA library from multiple cells has been reported[17–19], in most cases the library has been used for reverse transcriptase PCR followed by agarose-gel electrophoresis analysis. The combination of qPCR and a bead-supported single-cell cDNA library is attractive for quantifying the expression of multiple genes by permitting repeated use of the library. However, it is difficult to reuse a cDNA library because of adsorption of PCR products on the beads and tube surfaces and desorption of immobilized cDNA from the beads during thermal cycling.

We overcame these difficulties by adding surfactant to the reaction mixture and by lowering the temperature during qPCR to reduce the thermal damage to the bead surfaces, which causes cDNA desorption. This method allowed accurate qPCR-mediated quantification of as few as several copies of mRNA from multiple genes in a single cell (**Supplementary Protocol**).

The important factors for producing single-cell cDNA libraries and using them for qPCR are the number of oligo(dT)$_{30}$-immobilized beads (capture beads), the 3′ bias in the cDNA production and the selection of transcriptase.

The number of oligo(dT)$_{30}$ probes on a capture bead was about 1.5×10^5. We estimated the number of mRNA molecules in a single cell to be 10^5–10^6. To optimize the number of capture beads, we estimated the mRNA capture rate together with the reverse transcription rate. As the number of mRNA molecules varied between cells, we used a model RNA (10^1–10^9 molecules, *EEF1G*) for the estimation. We created model cDNA libraries with various numbers of capture beads. For all of them, the number of cDNA molecules increased with the number of capture beads up to 10^7 capture beads (cDNA production efficiency was almost 100% for model RNA with less than 10^6 copies) (**Fig. 1a**). The reverse transcription efficiency with 10^8 capture beads was much lower for model RNA with over 10^6 copies. Therefore, the number of capture beads should be kept below 10^8. We used 10^7 capture beads. The capture beads included about 1.5×10^{12} oligo(dT)$_{30}$ probes.

The cDNA production efficiencies with oligo(dT)$_{30}$ probes for four model RNA (*TBP*, *SDHA*, *B2M* and *EEF1G*; 10^3 molecules each) were almost the same (**Fig. 1b**). Moreover, the production efficiencies with four types of probes (oligo(dT)$_{30}$, gene-specific, oligo(dT)$_{25}$VN (where V is an A, G and C mixture, and N is an A, G, C and T mixture) and locked nucleic acid (LNA)) for a model RNA (*SDHA*; 10^3 molecules) were the same (**Supplementary Fig. 1a** and **Supplementary Methods**).

It has been reported that a cDNA library produced with oligo(dT) probes has a 3′ bias, so cDNA lengths are not uniform

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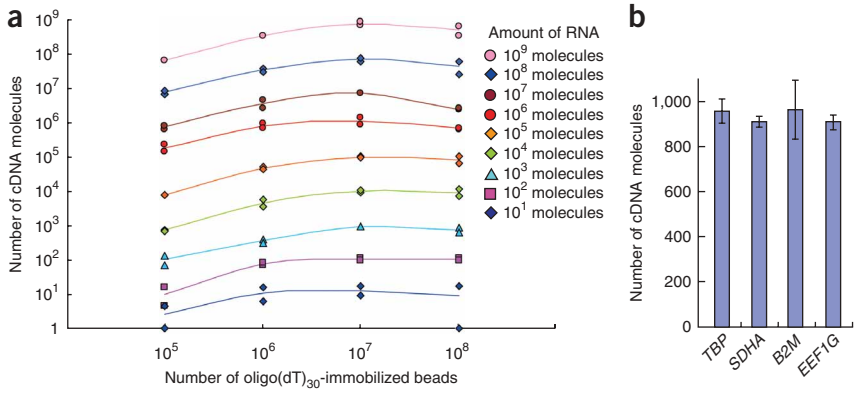

Figure 1 | Efficiency of cDNA production. (**a**) Total number of cDNA molecules produced on beads with various amounts of model RNA (*EEF1G*) versus number of oligo(dT)$_{30}$-immobilized beads ($n = 2$). (**b**) Reverse transcription efficiencies of four target model RNA on capture beads (1,000 molecules each) (mean ± s.d., $n = 3$).

even for the same gene species. Although this is a serious problem for quantitative gene expression analysis by qPCR, it can be overcome by selecting a region as close as possible (that is, no more than 500 bases away) to the poly(T) termini (corresponding to the 3′ end of the mRNA) for the analysis (**Supplementary Fig. 1b**, **Supplementary Fig. 2** and **Supplementary Table 1**). It is also important to design the forward primer for qPCR so that it hybridizes to the last exon-exon junction of the target mRNA to avoid amplifying the residual genomic DNA fragments.

The selection of reverse transcriptase is important for obtaining uniform cDNA libraries. We compared ten commercially available reverse transcriptases and found that SuperScript III (Invitrogen) best suited our requirements. It was particularly attractive because of its enzyme handling ease and high reverse transcription efficiency (**Supplementary Fig. 3** and **Supplementary Table 2**).

Usually qPCR is carried out without beads. In our experiments, during the first several PCR cycles, immobilized cDNA fragments were used as templates for the PCR while the beads were dispersed in the solution. The beads then gradually sank to the bottom. We evaluated the effect on qPCR of co-existing beads for four kinds of dsDNA samples (free DNA as well as dsDNA immobilized on 10^6, 10^7 and 10^8 beads) by plotting the relationship between the amount of DNA and the number of threshold cycles (Ct). We prepared dsDNA-immobilized beads by streptavidin–dual biotin bonding (**Supplementary Table 3** and **Supplementary Methods**) and determined that the standard curves had a strong linear correlation (**Fig. 2a**), indicating that quantitative analysis is possible.

The coefficients of determination (R^2) for the standard curves were greater than 0.998, so an accurate quantitative analysis could be carried out in all cases. As long as we used less than 10^7 beads, the standard curve coincided with that of the free DNA sample. Therefore, we used 10^7 DNA-immobilized beads for quantitative analysis. This was also the optimum number of beads for cDNA production.

The desorption of cDNA from beads during thermal cycling limits the reusability and therefore the number of analyzable gene species. This limitation can be overcome by lowering the operating temperature. We evaluated the amounts of ssDNA (four DNAs: *EEF1G*, *B2M*, *SDHA* and *TBP*) immobilized on beads after repeated qPCR under three different temperature conditions (**Fig. 2b**). Under condition 1 (DNA polymerase from Applied Biosystems), the PCR profile was 95 °C for 10 min and then 50 cycles of 95 °C for 15 s and 60 °C for 60 s during which there was substantial cDNA desorption. Under condition 2 (DNA polymerase from TaKaRa Bio), the PCR profile was 95 °C for 10 s and then 45 cycles of 95 °C for 5 s and 60 °C for 30 s, during which DNA desorption was substantially lower; however, the reduction was not sufficient. We determined the amounts of DNA remaining on the beads after 10 qPCR operations under condition 2 at five different denaturation temperatures (**Fig. 2c**). These results suggest that the

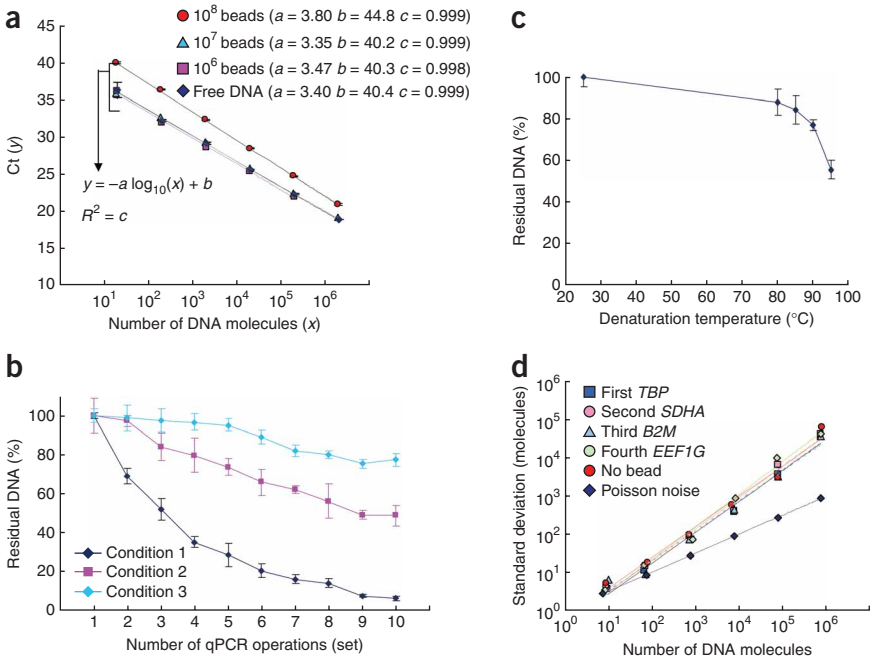

Figure 2 | Characteristics of qPCR with beads. (**a**) Number of threshold cycles for various numbers of DNA molecules with beads was investigated. qPCR (with *EEF1G* as the target gene) was carried out with a free-DNA sample and three different amounts of dsDNA immobilized on beads (10^6, 10^7 and 10^8 beads in 20 µl of reaction solution; mean ± s.d., $n = 3$). (**b**) Amount of DNA (*EEF1G*) remaining on beads after an initial thermal operation (condition 2) was defined as 100%. After a subsequent thermal operation under condition 1, 2 or 3, the percentage of residual DNA was estimated (mean ± s.d., $n = 3$). (**c**). Desorption after ten qPCR operations at five denaturation temperatures under condition 2 was estimated by qPCR (mean ± s.d., $n = 3$). Amount of DNA on beads without qPCR was defined as 100%. (**d**) Standard deviation for qPCR reusing DNA-immobilized beads ($n = 10$).

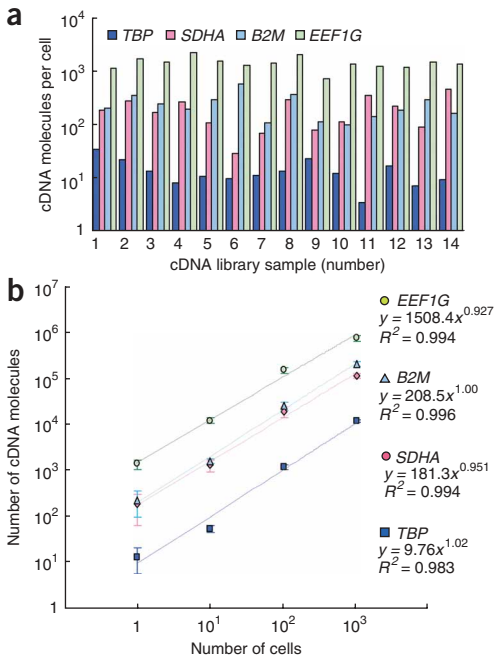

Figure 3 | Number of cDNA molecules measured in single cells and cell pools. (**a**) Cell-to-cell variation in gene expression among single cells. (**b**) Average gene expression for single cells as well as cell pools (single cell: mean ± s.d., $n = 14$; 10–1,000 cells: mean ± s.d., $n = 5$).

desorption can be greatly reduced by lowering the temperature to 80–85 °C. As lowering the denaturation temperature in qPCR requires decreasing the melting temperature of the PCR products, we added formamide to the samples to allow low-temperature PCR without affecting the amplification rate. The optimum formamide concentration was 5%, and the best PCR profile (condition 3) was 95 °C for 10 s, 3 cycles of 95 °C for 5 s and 55 °C for 30 s, and 37 cycles of 85 °C for 5 s and 55 °C for 30 s.

The average desorption rate under condition 3 was only 2.8%, which is sufficient for analyzing expression from 10–20 genes with one cDNA library. As the desorption rates of standard DNA and the target cDNA were almost the same, the changes in the Ct values resulting from desorption of the target cDNA were compensated for by changing the Ct values of the standard DNA to obtain an accurate quantitative result even after repeated use of a cDNA library.

To test the reusability of the cDNA library in qPCR, we quantitatively analyzed the standard ssDNA templates corresponding to the four genes immobilized on beads under condition 3, changing the order in which we measured them to obtain standard curves for the four genes. All standard curves for repeated analysis coincided with each other and were quite reproducible (**Supplementary Fig. 4**), indicating that DNA-immobilized beads are reusable and that the measurement order did not affect the results. The standard deviations were roughly equal to those obtained with DNA solution (no beads) (**Fig. 2d** and **Supplementary Table 4**) and did not increase with the number of reuses. This suggests that the major errors came from the pipetting and PCR processes, not from the desorption or bead-recovery process.

We quantitatively analyzed gene expression using four housekeeping genes with 14 single-cell cDNA libraries (**Supplementary Table 5**), together with pooled-cell cDNA libraries (**Fig. 3** and **Supplementary Table 6**). Although the relative expression of the four genes was similar, the absolute amounts differed from cell to cell. The average numbers of cDNA molecules per cell were 13.7 ± 7.9 copies for *TBP*, 187 ± 119 copies for *SDHA*, 231 ± 129 copies for *B2M* and 1,392 ± 358 copies for *EEF1G* (mean ± s.d., $n = 14$) and were proportional to the number of cells (**Fig. 3b**), indicating that the number of cDNA molecules for a single-cell sample was reasonable. As indicated by the error bars in the data shown in **Figure 3b**, the s.d. in the number of cDNA molecules was very large for the single cells. It was much larger than that for qPCR with sample reuse (**Fig. 2d**). Even though the cDNA was produced from diluted mRNA so as to include all the process errors, the s.d. for samples obtained with a small number of cells were very large (**Fig. 4**). The s.d. obtained for diluted-mRNA were less than 15.9% of the average amounts for cDNA ($n = 5$; **Supplementary Table 6**). This is comparable to the experimental error for the whole process.

As shown by the reusability of standard ssDNA templates in qPCR, for more than 750 molecules of ssDNA template, the s.d. were 4.0–13.4% for the average amounts of standard DNA (**Fig. 2d** and **Supplementary Table 4**). This is comparable to that for the Poisson noise for less than about 20 ssDNA molecules. It is thus possible to detect 7.5 ssDNA molecules on average with this method. When we used a model cDNA library produced from diluted mRNA, the noise was 6.2–15.9% of the average amount for cDNA. This is because the errors include those from reverse transcription and qPCR processes with sample reuse (**Fig. 4** and **Supplementary Table 6**). As the differences in the errors between

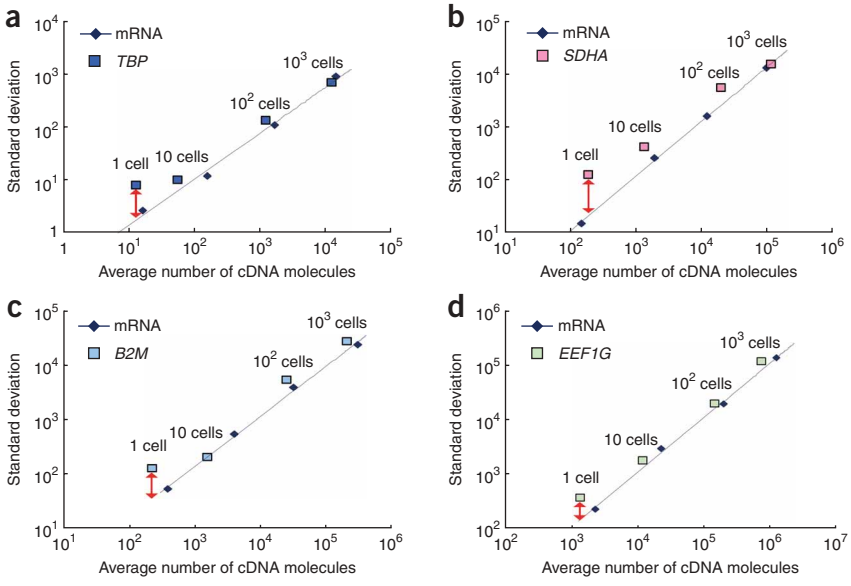

Figure 4 | Change in standard deviation of the number of target cDNA molecules. (**a**–**d**) Standard deviation for qPCR analysis of cDNA synthesized with diluted mRNA as well as cells ($n = 5$) for *TBP* (**a**), *SDHA* (**b**), *B2M* (**c**) and *EEF1G* (**d**). Arrows show heterogeneity of single cells.

these two cases (qPCR without and with reverse transcription processes) were not substantial, the experimental error in the reverse transcription process was negligible.

The errors discussed above are experimental errors related to using this method. As shown in **Figure 4**, the s.d. of the mRNA measurements for single cells were much larger than the experimental errors. Although the expression fluctuated greatly from cell to cell, the average expression amounts correlated with those obtained with pooled samples. The high noise for single-cell measurements might reflect fluctuations in gene expression among cells even under controlled conditions. As the experimental errors related to using this method were much smaller than the fluctuations, these fluctuations must have originated in the cells themselves. This means that the quantitative analysis for multiple gene expression is accurate enough for various applications and should bring new perspectives to the understanding of complex biological processes.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

K.T. helped design the experiments, performed all the experiments, analyzed the data and helped prepare the manuscript. T.K. invented the method used for sample reuse and discussed the experimental result. H.K. helped design the experiments, analyzed the data and helped prepare the manuscript.

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ONLINE METHODS

Primer design and selection of target sequences for qPCR. OLIGO (version 6; TaKaRa Bio) and Primer Express (version 1.5; Applied Biosystems) were used to design the PCR primers and minor groove–binding (MGB) probes so as to avoid duplex and hairpin formations. Using a BLAST program, we confirmed that the primer sequences did not have a high degree of homology to human genomic DNA sequences, thereby avoiding nonspecific amplification.

Cell culturing and single-cell sampling. Human colon carcinoma cells (HCT 116, Amercian Type Culture Collection) were cultured in 5 ml of advanced Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) under 5% CO_2 at 37 °C for 24 h. After the cells were rinsed once with PBS, they were kept at 37 °C for 1 min with 0.5 ml of trypsin (0.25% trypsin, 1 mM EDTA, Invitrogen). After 1 ml of the medium was added, the cell suspension was centrifuged at 1,000 r.p.m. (208*g*) for 3 min at 4 °C. The supernatant was removed, and the cell pellet was resuspended in PBS. The 100 µl PBS solution, containing 50–100 cells, was placed on the lid of a 96-well plate (Falcon). Under a microscope (Olympus CK40), a single cell together with 1 µl of PBS was picked up manually with a capillary tip (diameter (ϕ) = 190 µm; Drummond Scientific). It was transferred into a nonstick PCR tube (Axygen Scientific) containing 1 µl of PBS and cooled on ice. To prevent nonspecific adsorption of mRNA into the inner wall, the tube was dip-coated with 1% PMB80 (AI BIO CHIPS) beforehand.

Preparation of cDNA libraries from single cell. All the processes were carried out in one tube to minimize sample loss. We added 1.1 µl of cell-lysis solution (mixture of 1 µl resuspension buffer and 0.1 µl Lysis Enhancer; Invitrogen) to a PCR tube containing a single cell suspended in 2 µl PBS. The cell was lysed at 75 °C for 10 min. After the tube was cooled to 4 °C, 0.86 µl of DNase solution (0.5 U DNase I in 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl) was added and the solution was mixed. The solution was kept at room temperature (20–25 °C) for 5 min to enable digestion of the genomic DNA. The DNase was deactivated by adding 1.2 µl of EDTA (2.5 mM, pH 8.0) and heating the solution at 70 °C for 5 min. After the solution had cooled to 4 °C, 17.6 µl of a bead suspension (10^7 oligo$(dT)_{30}$-immobilized beads, 568 µM dNTP mix and 0.089% Tween-20, 8.9 mM Tris-HCl (pH 8.0)) was added, and the solution was mixed. The immobilized beads were produced by mixing streptavidin-coated beads (ϕ = 1 µm, Dynal, C1) with dual-biotinated oligo$(dT)_{30}$ (Integrated DNA Technologies). Each bead had 1.5×10^5 oligo$(dT)_{30}$ probes on its surface. After the sample solution was heated at 70 °C for 5 min, it was allowed to cool to 4 °C, which hybridized the mRNA molecules to the oligo$(dT)_{30}$ probes. The reverse transcription (RT) reaction was carried out by adding 9 µl of RT solution (50 mM Tris-HCl (pH 8.3), 75 M KCl, 3 mM $MgCl_2$, 11 mM DTT, 40 U RNase OUT, 200 U Super Script III RT; Invitrogen) and shaking the tube at 750 r.p.m. at 50 °C for 50 min in a microincubator (Taitec M-36). The sample was then heated at 85 °C for 1.5 min to deactivate the RT enzyme. After the solution had cooled to 4 °C, 1 µl of RNase solution (1 U RNase H (Invitrogen) in 30 mM Tris-HCl, 0.07 mM DTT, 50 mM KCl, 5 mM $MgCl_2$ and 0.02% Tween20) was added to the PCR tube, and the tube was shaken at 750 r.p.m. at 37 °C for 30 min. The supernatant was removed with an NdFeB magnet (Hitachi Metals). The cDNA-immobilized beads were then washed once with 50 µl of washing buffer (0.1% Tween-20, 10 mM Tris-HCl (pH 8.0)). After the washing buffer was removed, the cDNA-immobilized beads were dispersed in 3.6 µl of resuspension buffer (1% PMB80, 10 mM Tris-HCl (pH 8.0)).

Preparation of standard dsDNA templates immobilized on beads. The DNA fragments (region2: *TBP, SDHA*, *B2M;* region1: *EEF1G*) were amplified by PCR with cDNA prepared from the HCT116 cells and primers listed in **Supplementary Table 1**. The fragments contained a target region for qPCR. The excess primers in each sample were removed using a QIAquick PCR Purification kit (Qiagen). The concentrations of the dual-biotinated PCR products were determined by UV-light absorption.

Streptavidin-coated beads (5×10^8 beads, ϕ = 1 µm; Dynal) were suspended in 50 µl of the binding and washing buffer (20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 1 M NaCl) after being washed with 50 µl of that buffer three times. The dual-biotinated PCR products for the four genes were diluted with the binding and washing buffer and mixed to make a solution containing 10^6 μl^{-1} of each of the product molecules. The PCR products were immobilized on beads by adding 50 µl of the PCR solution to the same volume of streptavidin-coated beads and then mixing them at 750 r.p.m. at room temperature for 1 h. We measured the amounts of DNA in the solution before and after the immobilization by qPCR and estimated the immobilization efficiency to be 95%. The DNA-immobilized beads were washed twice with 100 µl of the washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)). After this buffer was removed, the beads were suspended in 50 µl of RT-PCR grade water (Ambion). The amount of each gene of dsDNA immobilized on the beads was estimated to be 9.5×10^5 molecules per 10^7 beads. A tenfold dilution series was produced by repeatedly diluting the sample with the washed intact beads. We prepared bead solutions containing the same number of beads (10^7 beads per µl) with different amounts of the standard dsDNA templates (immobilized on the beads) at concentrations ranging from 9.5 molecules to 9.5×10^5 molecules per 10^7 beads.

Preparation of standard ssDNA templates immobilized on beads. To evaluate the reusability of cDNA libraries and to estimate the number of cDNA multiple genes in the library, we needed standard ssDNA templates for qPCR. To make these templates, we needed to denature the dsDNA and prevent any DNA from adsorbing in the beads. The dsDNA-immobilized beads (50 µl, each 9.5×10^5 molecules per 10^7 beads) were prepared as for standard dsDNA templates. The beads were washed twice with 50 µl of 95 °C washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)) to denature the dsDNA in preparation for ssDNA template fabrication. After the beads were resuspended in 950 µl of q-PCR buffer (1× Premix Ex Taq (TaKaRa Bio), 0.013% Tween20, 1.3 mM Tris-HCl (pH 8.0) and 5% formamide), they were held at 95 °C for 10 s and then subjected to 45 cycles of 95 °C for 5 s and 60 °C for 30 s. Although about 80% of the ssDNA initially captured remained on the beads after this procedure, the nonspecific DNA that had adsorbed in the beads was completely removed. After the supernatant was removed, the beads were resuspended in 50 µl of RT-PCR grade water.

The amount of each ssDNA immobilized on the beads was estimated by qPCR to be about 7.5×10^5 molecules per 10^7 beads. A tenfold dilution series was produced by repeatedly diluting the sample with washed intact beads. This produced standard ssDNA template solutions containing four different immobilized ssDNA fragments at concentrations ranging from 7.5 molecules to 7.5×10^5 molecules per 10^7 beads.

Quantitative analysis of cDNA in single-cell cDNA libraries. The expression levels of the four target genes were analyzed sequentially (in the order *TBP*, *SDHA*, *B2M* and *EEF1G*) with a qPCR sequence detection system (Applied Biosystems, ABI PRISM 7900, version 2.1). The qPCR analysis was carried out with a 20 µl solution containing 1 × Premix Ex Taq, 1 µM of each *TBP* primer pair, 0.25 µM *TBP* MGB fluorogenic probe, a cDNA library (10^7 beads), 0.18% PMB80, 5% formamide and 1.8 mM Tris-HCl. The standard ssDNA templates and a single-cell cDNA library were analyzed simultaneously by measuring fluorescence during thermal cycling (95 °C for 10 s followed by 3 cycles of 95 °C for 5 s and 55 °C for 30 s, and 37 cycles of 85 °C for 5 s and 55 °C for 30 s) to produce amplification plots. The threshold number of cycles (Ct, $\Delta Rn = 0.2$) was the number of cycles at which the products reached predetermined amounts selected automatically. The relationships between the number of DNA molecules and Ct were obtained using the standard ssDNA templates and plotted as standard curves. The number of target molecules in the cDNA library was estimated from these curves.

After the first *TBP* analysis, the standard ssDNA templates as well as the cDNA library samples were transferred to new nonstick tubes for the subsequent *SDHA* analysis. To recover the beads adsorbed on the well walls of the 384-well plate, we washed the wells with 20 µl of the washing buffer (0.1% Tween-20, 10 mM Tris-HCl) and added the buffer to the samples in the nonstick tubes. After the supernatant was removed, the beads were suspended in 3.6 µl of suspension liquid (1% PMB80, 10 mM Tris-HCl (pH 8.0)). qPCR analyses of the other three target genes were performed sequentially (*SDHA*, *B2M*, *EEF1G*) using the same standard ssDNA temples and cDNA libraries. The reaction conditions for all the analyses were the same as those for the first analysis described above. The sequences of PCR primers and MGB fluorogenic probes (region 2, *TBP*, *SDHA* and *B2M*; region 1, *EEF1G*) and the sizes of the products are listed on **Supplementary Table 1**. The results for 14 single cells and cell pools are summarized in **Supplementary Tables 5** and **6**.

Evaluation of DNA desorption from beads. The ssDNA-immobilized beads (50 µl, each 7.5×10^5 molecules per 10^7 beads) were prepared as for standard ssDNA templates. Using this sample, we evaluated the desorption of the immobilized DNA using the three different thermal cycle profiles described in the main text. The PCR buffer for the first contained 1× TaqMan Universal PCR Master mix (Applied Biosystems), 0.015% Tween-20, and 1.5-mM Tris-HCl (pH 8.0), that for the second contained 1× Premix Ex Taq, 0.013% Tween-20, and 1.3 mM Tris-HCl (pH 8.0), and that for the third contained 1× Premix Ex Taq (TaKaRa Bio), 0.013% Tween-20, 5% formamide and 1.3 mM Tris-HCl (pH 8.0). Two hundred microliters of ssDNA template solution (10^7 per µl) were diluted with 3,800 µl of buffer1 or buffer2 or buffer3. After the thermal cycles, the PCR buffer was removed, and the beads were resuspended in 200 µl of RT-PCR–grade water. Twenty µl of each suspension was removed for evaluating the rates of DNA desorption during the first set of thermal cycles, and 180 µl of the remaining solution was again diluted with 3,600 µl of each buffer (20× dilution) and used to make a series of diluted samples. The thermal cycles and the partial sampling were repeated ten times to get a series of heat-treated samples (each 20 µl, 10^7 beads per µl). After the heat treatment, the number of DNA molecules still immobilized on the 10^7 beads was measured by qPCR for the *EEF1G* gene fragments with the same primer and MGB fluorogenic probe listed in **Supplementary Table 1**.

Preparation of diluted mRNA and evaluation of measurement errors. Total RNA (14 µg) was extracted from 10^6 cells using an RNeasy mini kit (Qiagen). After DNase treatment, the mRNA was purified using an Oligotex-dT30 kit (TaKaRa Bio), and phenol-chloroform extraction was performed. After ethanol precipitation, the mRNA concentrations were estimated by measuring the UV-light absorption. A series of diluted mRNA mixtures (2 pg μl^{-1}, 20 pg μl^{-1}, 200 pg μl^{-1} and 2 ng μl^{-1}) were prepared, and the numbers of copies of the target genes were estimated using qPCR. The numbers corresponded to those for 1, 10, 100 and 1,000 cells, respectively.

The measurement errors were estimated using the diluted-RNA series ($n = 5$). Almost the same conditions and processes used for actual cells were used for these diluted mixtures. A solution of 6.6 µl containing 2 µl PBS, 1.1 µl cell-lysis solution (mixture of 1 µl resuspension buffer and 0.1 µl of cell-lysis Enhancer; Invitrogen), 0.86 µl of DNase solution (0.5 U DNase I in 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 50 mM KCl), 1.2 µl EDTA (2.5 mM) and 1 µl of each mRNA mixture was added to the nonstick tubes. To prevent adsorption of mRNA into the inner wall, the tube was dip-coated with 1% PMB80 beforehand. After the solution was heated at 70 °C for 5 min and then allowed to cool to 4 °C, 17.6 µl of bead-containing suspension (10^7 oligo$(dT)_{30}$-immobilized beads) was added. The tubes were again heated at 70 °C for 5 min and then allowed to cool to 4 °C to enable the mRNA molecules to hybridize to the oligo$(dT)_{30}$ probes on the beads. RT reactions were initiated by adding 9 µl of RT solution to the tubes and then incubating the tubes at 50 °C for 50 min while shaking them at 750 r.p.m. The tubes were then heated at 85 °C for 1.5 min. After the tubes had cooled to 4 °C, 1 µl of RNase solution (1 U RNase H (Invitrogen) in 30 mM Tris-HCl, 0.07 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, and 0.02% Tween-20) was added, and the tubes were again shaken at 750 r.p.m. at 37 °C for 30 min. After the supernatant was removed, the beads were washed once with 50 µl of washing buffer and then resuspended in 3.6 µl of suspension liquid. qPCR analysis for the model cDNA samples was carried out in the same way as for the actual samples from single cells. The measured amounts of cDNA together with the s.d. are listed on **Supplementary Table 6**.

Preparation of model RNA samples. Four kinds of PCR products (*TBP*, *SDHA*, *B2M* and *EEF1G*) were amplified with a forward primer anchored to a T7 promoter sequence and a reverse primer anchored to an oligo$(dT)_{30}$ sequence. The sequences of PCR primers and the sizes of the products are listed in **Supplementary Table 1**.

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After examination of the products with a bioanalyzer (Agilent, 2100), the excess primers were removed from the products using the QIAquick PCR Purification Kit. After ethanol precipitation, the concentrations of DNA were measured by UV absorption.

RNA was synthesized by incubating 500 ng of each PCR product at 37 °C for 1 h in a 10 µl reaction mixture containing 90 nmol of dATP, dCTP, dGTP, and dUTP, 10 nmol of DTT, 1 µl of AmpliScribe T7-Flash Enzyme (Epicentre Biotechnologies), and 1 × AmpliScribe buffer. The RNA samples were purified by DNase and protease-K treatments, after which phenol-chloroform extraction was done twice in accordance with the common procedure. The residual dNTP in the purified RNA samples was then removed with an Oligotex-dT30 kit, and the phenol-chloroform extraction was performed once again. After ethanol precipitation, the pellets were resuspended in 100 µl of RT-PCR–grade water and the RNA concentrations were measured by UV absorption.

Protocol for investigating optimum number of oligo(dT)$_{30}$-immobilized magnetic beads for cDNA libraries. We performed reverse transcriptions using various amounts of oligo(dT)$_{30}$-immobilized beads and model RNA (*EEF1G*). A solution containing 2 µl PBS, 1.1 µl cell-lysis solution, 0.86 µl DNase solution, and 1.2 µl EDTA (2.5 mM) was placed in 20 nonstick tubes that had been dip-coated with 1% PMB80 before the experiments. The solutions and process were the same as those used for the actual samples. After 1 µl of model RNA samples (10^1–10^9 molecules) was added to tubes, they were heated at 70 °C for 5 min and then allowed to cool to 4 °C. After 17.6 µl of bead solution (10^5–10^8 oligo(dT)$_{30}$-immobilized beads) was added to each tube, the tubes were heated at 70 °C for 5 min and then allowed to cooled to 4 °C to enable the RNA molecules to hybridize to the oligo(dT)$_{30}$ probes. The model cDNAs were produced by RT reactions in which 9 µl of RT solution was added to tubes that were then incubated at 50 °C for 50 min while being shaken at 750 r.p.m. The products were heated at 85 °C for 1.5 min to deactivate the RT enzymes. After the tubes cooled to 4 °C, the supernatant-containing residual reagents were removed. The model samples were washed once with 50 µl of the washing buffer (0.1% Tween20, 10-mM Tris-HCl (pH 8.0)) and then resuspended in 3.6 µl of suspension liquid (1% PMB80, 10 mM Tris-HCl (pH 8.0)).

qPCR for these model samples were carried out using *EEF1G* primer set and MGB fluorogenic probe listed in **Supplementary Table 1** by measuring the fluorescence intensities during thermal cycling (95 °C for 10 s followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s) to produce amplification plots.