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Quantitative analysis of gene expression in a single cell by qPCR

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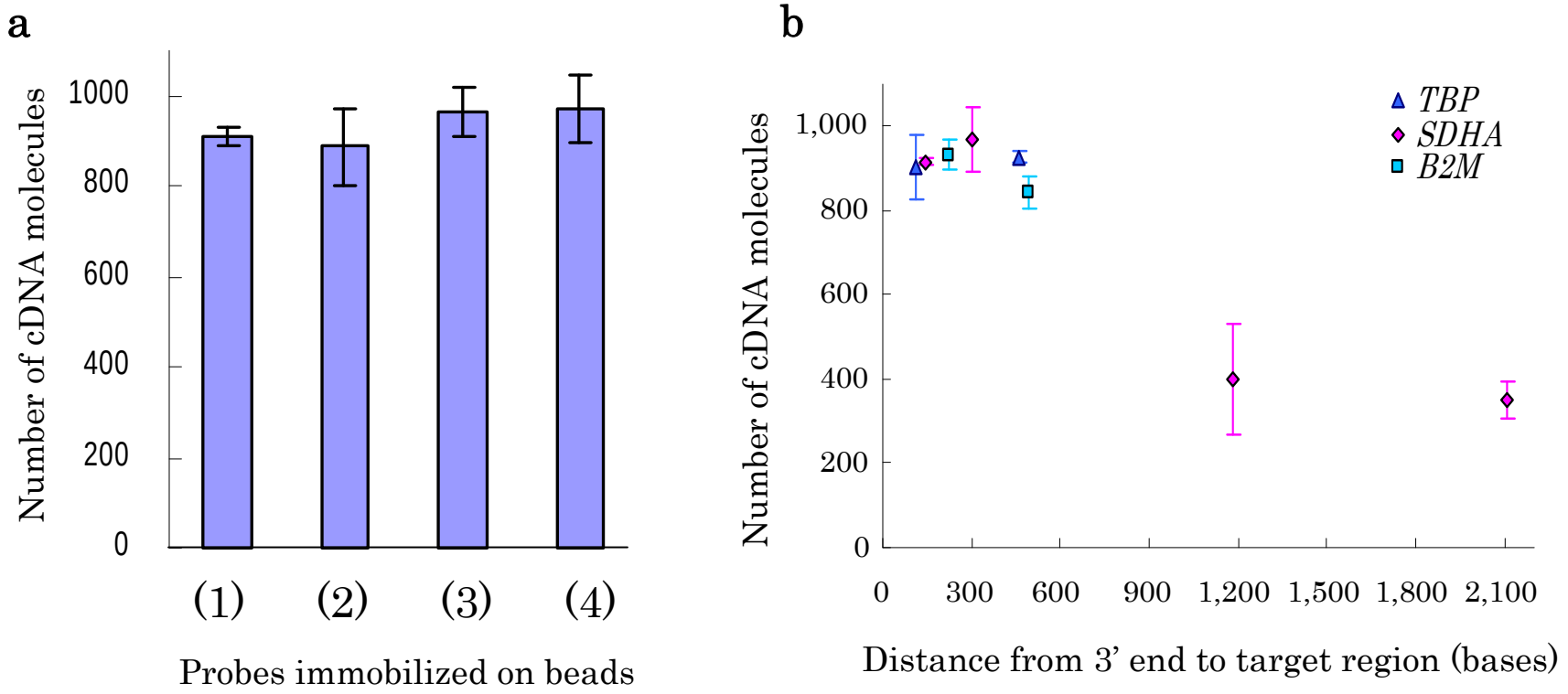
Supplementary Figure 1. RT efficiencies of four probes and 3' bias in cDNA synthesis



(a) RT efficiencies with four types of probes (1) oligo$(dT)_{30}$, (2) gene-specific, (3) oligo$(dT)_{25}$VN, and (4) LNA for a model RNA (SDHA, 10^3 molecules) were roughly the same (mean± s.d., n=3). (b) With 10^3 molecules of model RNA (*TBP*, *SDHA*, and *B2M*), the 3' bias was evaluated by carrying out qPCR at different target region of the cDNA species (mean±s.d., n=3). The estimated number of copies decreased with the distance between the PCR portions and 3' termini in a region over 500 bases from the termini.

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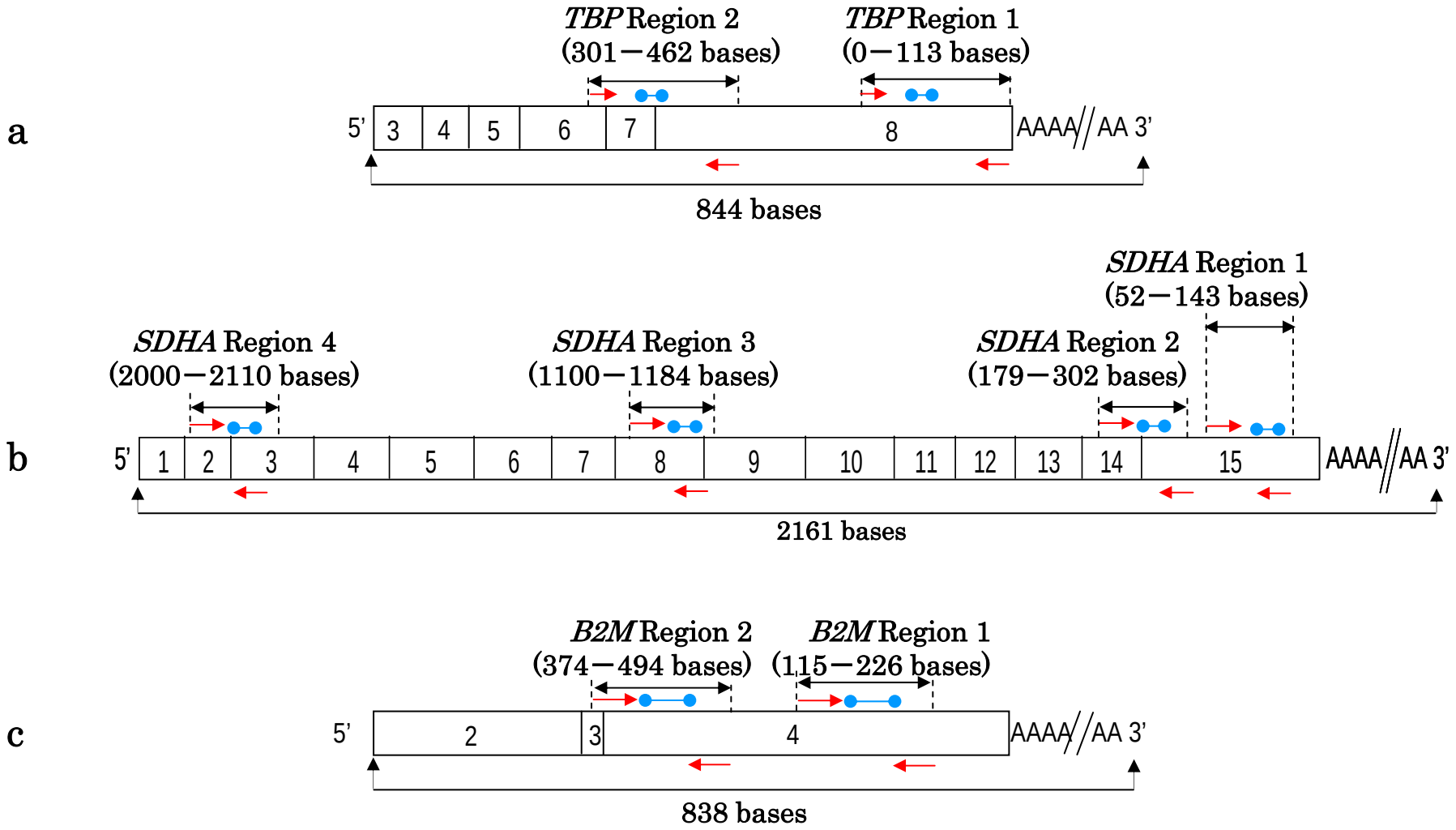
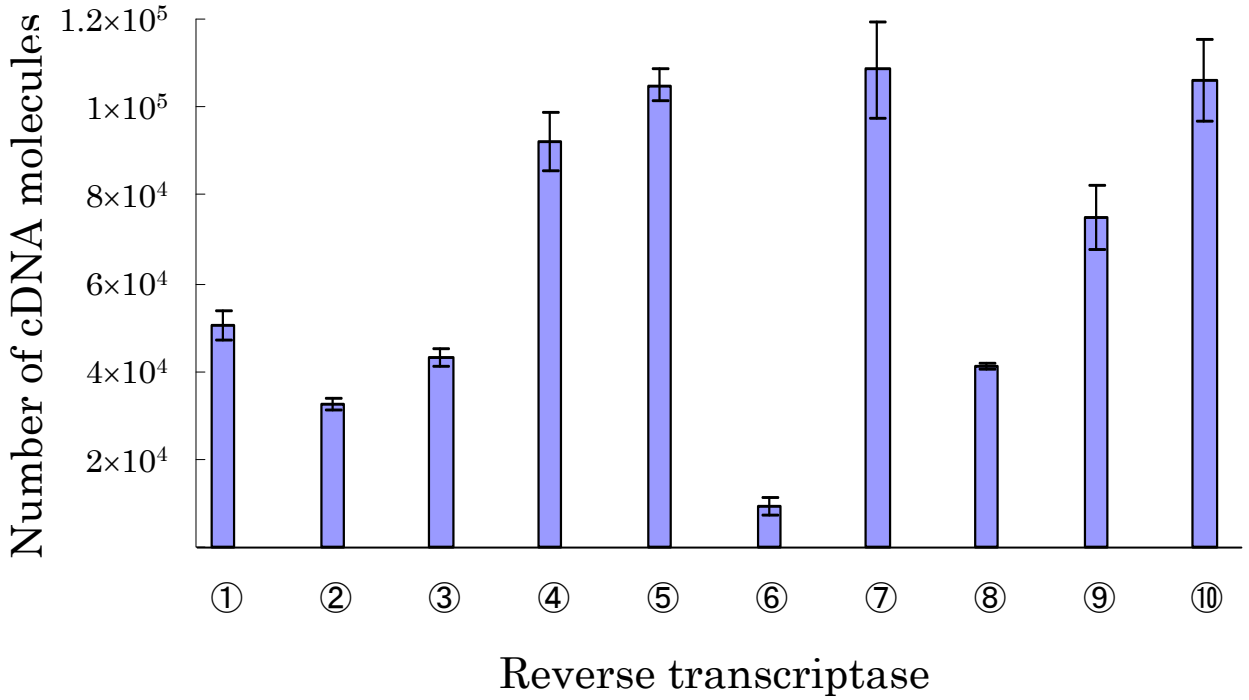
Supplementary Figure 2. Structure of model RNA and target region for qPCR



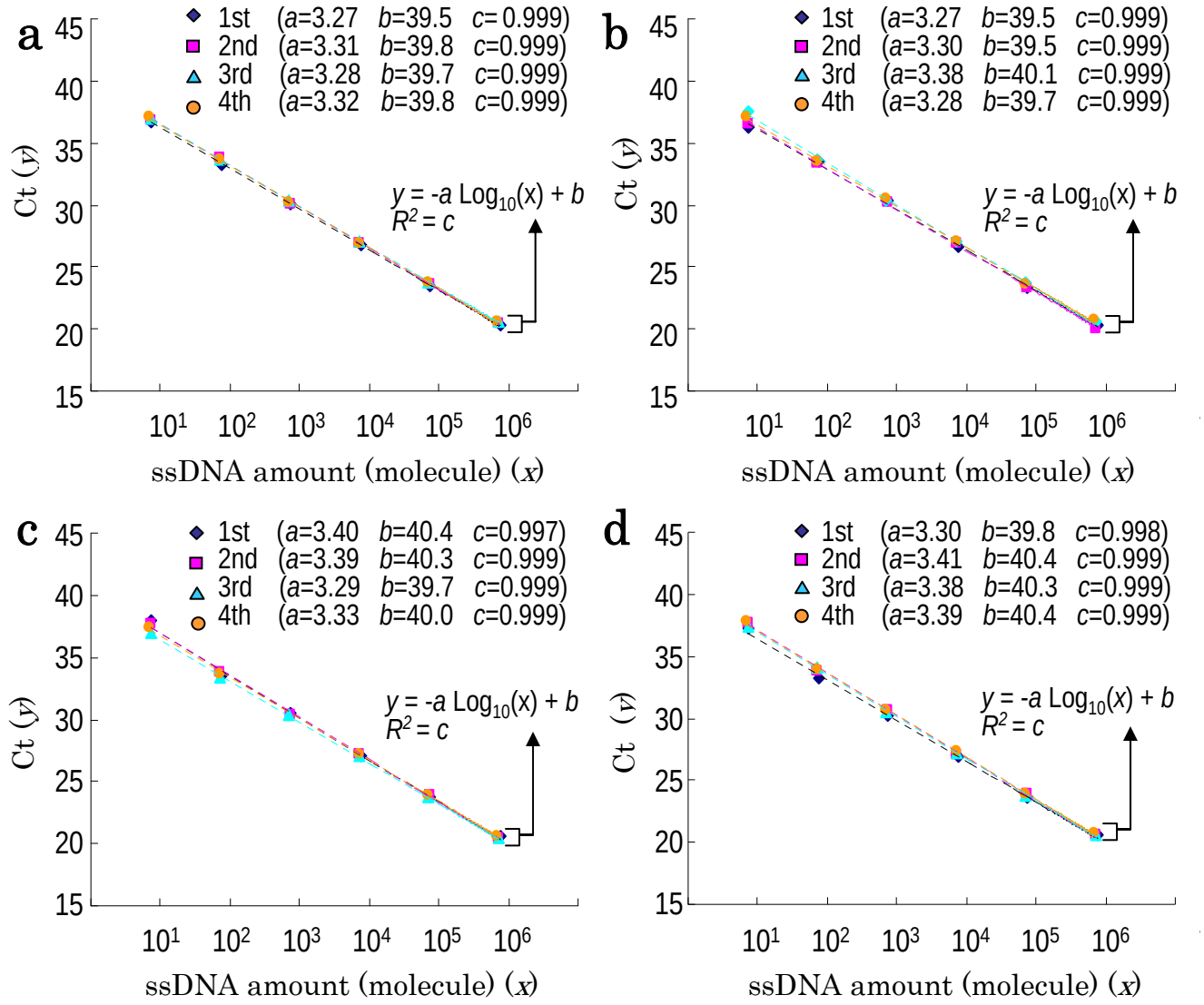
Structure of model RNA (a: *TBP*, b: *SDHA*, and c: *B2M*) and q-PCR primers (red arrows) and probes (blue bars) at different distances from 3' end. Number in each box is number of exons.

Supplementary Figure 3. Selection of optimum reverse transcriptase



Number of cDNA molecules synthesized with model RNA (10^5 molecules, *SDHA*) and ten reverse transcriptases (mean±s.d, n=2). ①TaKaRa One step RNA PCR Kit (TaKaRa), ②Bca BEST RNA PCR Kit (TaKaRa), ③cDNA Synthesis Kit (TaKaRa), ④Super Script III (Invitrogen), ⑤ThermoScript RT (Invitrogen), ⑥MiScript RT (QIAGEN), ⑦Advantage RT (Clontech), ⑧PrimeScript RT (Clontech), ⑨ReverTra Ace-α (TOYOBO), ⑩Transcriptor cDNA Synthesis Kit (Roche). Four of them (④, ⑤, ⑦, and ⑩) gave high RT efficiency. However, repetition qPCR-analysis with RT ⑤ was impossible due to significant nonspecific adsorption of the magnetic beads on the surface. RTs ⑦ and ⑩ were not applicable to our method due to bead aggregation.

Supplementary Figure 4. Evaluation of reproducibility in reuse of standard ssDNA templates



As there were four different target genes, four sets of repeated qPCR were carried out by changing the analysis order of the target genes. The orders were *TBP→SDHA→B2M→EEF1G, SDHA→B2M→EEF1G→TBP, B2M→EEF1G→TBP→SDHA, and EEF1G→TBP→SDHA→B2M.* Four standard curves of the same gene were over-plotted: (a) *TBP*,(b) *SDHA*, (c) *B2M*, and (d) *EEF1G*.

Supplementary Table 1. Primer sequences

	Gene	Region	Primer	Seq. (5'→3')	modification	Product size(bp)
Standard DNA	*TBP*	1 and 2	Forward	GAGCTGTGATGTGAAGTTTCC		548
			Reverse	CTCCCTCAAACCAACTTGTC	5' Dual biotin	
		2	Forward	GAGCTGTGATGTGAAGTTTCC		265
			Reverse	GGAGGCAAGGGTACATGAG	5' Dual biotin	
	SDHA	1, 2, 3, and 4	Forward	CGAGGTTTTCACTTCACTGTT		2131
			Reverse	GAAGCAAGGGACAAAGGTAA	5' Dual biotin	
		2	Forward	AGCACTGGAGGAAGCACAC		342
			Reverse	GAAGCAAGGGACAAAGGTAA	5' Dual biotin	
	B2M	1 and 2	Forward	CCGTGTGAACCATGTGACTT		566
			Reverse	AACCACAACCATGCCTTAC	5' Dual biotin	
		2	Forward	CCGTGTGAACCATGTGACTT		264
			Reverse	CAACCTGCTCAGATACATCAAA	5' Dual biotin	
	EEF1G	1	Forward	AGCTGCAATCTCATCACTGG		319
			Reverse	TGATGGCAAGAGATGTTCACT	5' Dual biotin	
qPCR	*TBP*	1	Forward	ACCAGGTGATGCCCTTCT		113
			Reverse	ATAGCAGCACGGTATGAGC		
			TBP_MGB 1	CGCAGCGTGACTGTGAGTT	5' FAM / 3' NFQ, MGB	
		2	Forward	ACCCACCAACAATTTAGTAGTTA		131
			Reverse	GCTCTGACTTTAGCACCTGTTA		
			TBP_MGB 2	AGCCAGAGTTATTTCCTGG	5' FAM / 3' NFQ, MGB	
	SDHA	1	Forward	TGTCCATGTCATAACTGTCTTCA		91
			Reverse	AAGCTCCCAGCCACTAGGT		
			SDHA_MGB 1	AAGAAGGAGTACATTGAAG	5' FAM / 3' NFQ, MGB	
		2	Forward	CACTGGGAAGGTCACTCTG		123
			Reverse	TTCTGTCATCACCACATCTTG		
			SDHA_MGB 2	CCATTCGCTCCTACTGAT	5' FAM / 3' NFQ, MGB	
		3	Forward	TAGAGATGTGGTGTCTCGGTC		84
			Reverse	TGCAGGTAGACGTGATCTTTC		
			SDHA_MGB 3	AGATCCGAGAAGGAAGAG	5' FAM / 3' NFQ, MGB	
		4	Forward	CGAGGTTTTCACTTCACTGTT		110
			Reverse	ACCACCACTGCATCAAATTC		
			SDHA_MGB 4	TCTGCTCAGTATCCAGTAGT	5' FAM / 3' NFQ, MGB	
	B2M	1	Forward	CAACATCTTGGTCAGATTTGAA		111
			Reverse	CCCAAATTCTAAGCAGAGTATG		
			B2M_MGB 1	CTTGCACTCAAAGCTTGT	5' FAM / 3' NFQ, MGB	
		2	Forward	GCATCATGGAGGTTTGAAG		120
			Reverse	TATAACCCTACATTTTGTGCAT		
			B2M_MGB 2	CGCATTTGGATTGGATGA	5' FAM / 3' NFQ, MGB	
	EEF1G	1	Forward	TTTCCGCTGAGTCCAGATT		149
			Reverse	CCCTGATTGAAGGCTTTG		
			MGB Probe 1	TGGACTACGAGTCATACACA	5' FAM / 3' NFQ, MGB	

	Gene	Primer	Seq. (5'→3')	Product size(bp)
model RNA	*TBP*	Forward	TAATACGACTCACTATAGGGCTTCGGAGAGTTCTGGGATT	844
		Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATAGCAGCACGGTATGAGCA	
	SDHA	Forward	TAATACGACTCACTATAGGGCCGAGGTTTTCACTTCACTG	2161
		Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAGCAAGGGACAAAGGTAA	
	B2M	Forward	TAATACGACTCACTATAGGGACTCCAAAGATTCAGGTTTACTC	838
		Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCATGCCTTACTTTATCAAATG	
	EEF1G	Forward	TAATACGACTCACTATAGGGATGCACCACAACAAACAGG	1020
		Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGATGGCAAGAGATGTTCACT	

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Supplementary Table 2. RT reaction conditions

No.	Reverse Transcriptase	Reaction condition (Total volume; 20μL)				Beads Aggregation
		Step 1: Hybridization		Step 2: Reverse Transcription		
		Components	Incubation	Additional Components	Incubation	
1	TaKaRa One step RNA PCR Kit (AMV) (TaKaRa)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (11 μL)	70°C 5 min ↓ 4°C 1 min	10× One-step RNA PCR Buffer (2 μL) $MgCl_2$ (25 mM) (2 μL) RNase Inhibitor (1 μL) AMV RTase XL (1 μL)	50°C 60 min	○
2	Bca BEST RNA PCR Kit Ver. 1.1 (TaKaRa)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (1.5 μL) 2× Bca 1st Buffer (10 μL)		$MgSO_4$ (25 mM) (4 μL) RNase Inhibitor (0.5 μL) Bca Best polymerase (1 μL)	30°C 5 min ↓ 65°C 60 min	○
3	cDNA Synthesis Kit (M-MLV Version) (TaKaRa)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (11 μL)		5× Buffer (4 μL) RNase Inhibitor (1 μL) M-MLV RT (1 μL)	50°C 60 min	×
4	Super Script III (Invitrogen)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads(10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (11 μL)		5× RT Buffer (4 μL) 0.1-M DTT (1 μL) RNase OUT (1 μL) Super Script III (1 μL)	50°C 60 min	○
5	ThermoScript RT (Invitrogen)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (10 μL)		5× RT Buffer (4 μL) 0.1-M DTT (1 μL) RNase OUT (1 μL) ThermoScript RT (1 μL)	55°C 60 min	○
6	MiScript Reverse Transcription Kit (QIAGEN)	RNA (10^5 molecules/μL) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (13 μL)		miScript RT Buffer (4 μL) miScript RT (1 μL)	37°C 60 min	○
7	advantage RT- for -PCR Kit (Clontech)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (11.5 μL)		5× Buffer (4 μL) RNase Inhibitor (0.5 μL) MMLV RT (1 μL)	42°C 60 min	×
8	PrimeScript Reverse Transcriptase (Clontech)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (11.5 μL)		RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (11.5 μL)	42°C 60 min	×
9	ReverTra Ace-α (TOYOBO)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (10 μL)		5× Buffer (4 μL) RNase Inhibitor (2 μL) ReverTra Ace (1 μL)	42°C 60 min	○
10	Transcriptor First Strand cDNA Synthesis kit (Roche)	RNA (10^5 molecules/μL) (1 μL) dNTP Mix (10 mM) (1 μL) Oligo$(dT)_{30}$ beads (10^7/μL) (1 μL) 0.1% Tween20 (10-mM Tris) (12 μL)		5× Buffer (4 μL) RNase Inhibitor (0.5 μL) ReverTra Ace (0.5 μL)	55°C 60 min	×

Supplementary Table 3. Immobilization efficiency of dsDNA on beads

Gene	dsPCR product		Immobilization efficiency (%)	
	Size (bp)	GC (%)	a) one PCR product	b) four PCR products
TBP	265	69.2	95.6	98.5
SDHA	342	50.6	92.4	96.7
B2M	264	37.9	95.5	95.5
EEF1G	319	53.3	98.0	96.1

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Supplementary Table 4. Standard deviation in qPCR.

(a) Standard ssDNA templates immobilized on beads (n=10) were reused in qPCR to examine mean of DNA (no. of molecules), standard deviation (no. of molecules), and coefficient of variation(%). (b) The qPCR results for standard ssDNA template solutions (n=10, no beads) are summarized.

a

ssDNA immobilized on beads	1st *TBP*			2nd *SDHA*			3rd *B2M*			4th *EEF1G*		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
7.5	9.3	3.8	40.5	8.8	4.4	50.6	10.1	6.4	63.5	8.6	3.5	41.4
7.5×10^1	66.9	11.0	16.4	71.8	14.3	19.9	71.2	9.4	13.1	66.2	16.2	24.5
7.5×10^2	714.9	90.7	12.7	793.7	73.0	9.2	702.8	72.5	10.3	838.8	72.0	8.6
7.5×10^3	7567.4	374.7	5.0	7420.1	414.9	5.6	7729.9	430.0	5.6	8006.8	856.0	10.7
7.5×10^4	79136.7	3775.0	4.8	78379.7	6310.9	8.1	77701.2	3126.8	4.0	72947.8	9808.0	13.4
7.5×10^5	751641	34254.8	4.6	726477	41181.0	5.7	752771	35121.7	4.7	736044	42059.4	5.7

b

ssDNA in solution	*EEF1G*		
	Mean	SD	CV
7.5	8.7	5.2	59.7
7.5×10^1	77.9	17.9	23.0
7.5×10^2	719.5	93.8	13.0
7.5×10^3	6918.5	572.5	8.3
7.5×10^4	76671.0	2934.7	3.8
7.5×10^5	790691	62538.7	7.9

Supplementary Table 5. Gene expression levels of four genes in single cells

Cell no.	cDNA molecules per cell			
	TBP	*SDHA*	*B2M*	*EEF1G*
1	34	181	197	1117
2	21	268	343	1665
3	13	166	239	1464
4	8	254	185	2126
5	11	103	280	1527
6	10	29	569	1225
7	11	68	104	1367
8	13	280	351	1957
9	22	75	108	686
10	12	111	97	1304
11	3	334	136	1189
12	17	214	183	1159
13	7	89	280	1416
14	9	448	156	1291

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Supplementary Table 6. Measured amounts of cDNA and standard deviations

	Sample	Gene	Avg. no. of cDNA molecules	SD	CV(%)
Actual cell	1 cell	*TBP*	13.7	7.89	57.6
		SDHA	187	119.1	63.7
		B2M	230.5	128.7	55.8
		EEF1G	1391.6	358.1	25.7
	10 cells	*TBP*	56.9	9.66	17.0
		SDHA	1336.6	398.7	29.8
		B2M	1637	205.2	12.5
		EEF1G	12288.9	1718.5	14.0
	100 cells	*TBP*	1218.3	132	10.8
		SDHA	19384.6	5186.5	26.8
		B2M	25777.5	5175.4	20.1
		EEF1G	148287.3	19100.3	12.9
	1000 cells	*TBP*	12150.4	652.5	5.4
		SDHA	113702.4	14952	13.2
		B2M	202913.9	26219.3	12.9
		EEF1G	748693.4	113796	15.2
Diluted mRNA	2 pg (1 cell alike)	*TBP*	16.5	2.6	15.9
		SDHA	146.5	14.7	10.0
		B2M	402.3	55.2	13.7
		EEF1G	2314.9	223.3	9.6
	20 pg (10 cells alike)	*TBP*	161.9	12.1	7.5
		SDHA	1869.3	248.3	13.3
		B2M	4087.8	540.3	13.2
		EEF1G	22960.7	2880.7	12.5
	200 pg (100 cells alike)	*TBP*	1643.7	106.1	6.5
		SDHA	11993.3	1528.6	12.7
		B2M	32180.9	3954.4	12.3
		EEF1G	192172.6	18602.1	9.7
	2 ng (1,000 cells alike)	*TBP*	14082.3	873.9	6.2
		SDHA	96998.4	12494.8	12.9
		B2M	298326	23859.9	8.0
		EEF1G	1234030.9	137034	11.1

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Supplementary Protocol

The steps in our proposed method are illustrated in **Figure A**.

Selection of target sequences in genes and primer design

1. Obtain information on the target gene sequences and exon/intron boundaries from database (Ensembl and NCBI Entrez Gene).
2. Design PCR primers and MGB fluorescent probes using OLIGO (TaKaRa Bio) and Primer Express (Applied Biosystems). The PCR primers should be free of duplexes and hairpins. All qPCR primers should be designed no more than 500 bases away from the polyT tail to avoid 3' bias. Forward primers for qPCR should hybridize to the last exon-exon junction to prevent amplification of genomic DNA.
3. Use BLAST to confirm that the primer sequences do not have a high degree of homology to human genomic DNA sequences so as to avoid nonspecific amplification.

Cell culture and single-cell sampling

1. Culture HCT 116 cells (ATCC, 48 hours) in a 25-cm^2 flask containing 5 ml of DMEM medium (Invitrogen) supplemented with 10% FBS (Invitrogen) in 5% CO^2 at 37ºC.
2. Rinse cells once with PBS, add 0.5 ml of trypsin (0.25% trypsin, 1 mM EDTA·4Na, Invitrogen) and keep at 37°C for 1 minute.
3. Add 1 ml of the medium to the solution and centrifuge it at 1000 rpm for 3 min at 4°C.
4. Remove supernatant and resuspend cell pellet in 3 ml of PBS.
5. Dilute cell suspension with PBS, and take 100 μl PBS containing 50–100 cells and place it on the lid of a 96-well plate (Falcon).
6. Under a microscope, use a capillary tip (ϕ = 190 μm; Drummond Scientific) to manually pick up a single cell in 1 μl of PBS and transfer it to a nonstick PCR tube (Axygen Scientific, dip-coated with 1% PMB80 (AI BIO CHIPS) beforehand to prevent nonspecific adsorption of mRNA) containing 1 μl of PBS and cooled it on ice.

   Critical Step: The interval between cell sampling and the end of cell lysis should be as short as possible to prevent the change in gene expression levels during the handling. We completed the process within 30 min.

Preparation of cDNA libraries from a single-cell

1. Add 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.
2. Incubate it at 75°C for 10 minutes in a thermal cycler (Applied Biosystems) to lyse the cell and place it on ice.
3. Add 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) to it and mix the solution by pipetting.
4. Incubate the solution at room temperature for 5 minutes to digest genomic DNA.

5. Add 1.2 µl of EDTA (2.5 mM, pH 8.0) and incubate the solution at 70°C for 5 minutes in a thermal cycler to deactivate DNase. Then place it on ice.
6. Add 17.6 µl of a bead suspension (10^7 oligo$(dT)_{30}$-immobilized beads, 568 µM dNTP mix and 0.089% Tween20, 8.9 mM Tris-HCl (pH 8.0)) to the solution. (It mixes naturally because the added amount is large.)
7. Incubate the solution at 70°C for 5 minutes in a thermal cycler, and cool it down to 4°C gradually (1.5~3.0°C/sec) to hybridize mRNA to the oligo$(dT)_{30}$ probes.
8. Add 9 µl of RT solution (50 mM Tris-HCl (pH 8.3), 75 M KCl, 3 mM $MgCl_2$, 11 mM DTT, 40U RNase OUT, 200U Super Script III RT, Invitrogen) and mixed them gently by pipetting.
9. Shake the tube at 750 rpm at 50°C for 50 minutes in a microincubator (Taitec, M-36).
10. Incubate it at 85°C for 1.5 minutes in a thermal cycler to deactivate RT enzyme and then place the tube on ice.
11. Add 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) into the tube and mix the solution.
12. Shake the tube at 750 rpm at 37°C for 30 minutes in a microincubator (Taitec, M-36) to digest the mRNA.
13. Remove the supernatant from the tube over keeping the beads with an NdFeB magnet (Hitachi Metals) and wash the beads once with 50 µl of washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)).
14. Add 3.6 µl of resuspension buffer (1% PMB80, 10 mM Tris-HCl (pH 8.0)) to the tube and disperse the cDNA-immobilized beads and then spin them down. (The beads absorbed on the tube inner surface can be easily recovered through the resuspension process.)

Critical Step: Before using the oligo$(dT)_{30}$-immobilized beads (10^7 beads /µl), wash them thoroughly with equivalent amount of washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)) to remove completely nonspecific oligo$(dT)_{30}$ adsorbed on the beads surface.

Preparation of standard ssDNA templates immobilized on beads

1. Amplify DNA fragments including the target region for qPCR (region2: *TBP, SDHA*, *B2M;* region1: *EEF1G*) with cDNA prepared from the HCT116 cells and primers listed in **Supplementary Table 1** online.
2. Remove the excess primers in each sample with a QIAquick PCR Purification Kit (QIAGEN).
3. Determine the concentrations of the dual-biotinated PCR products by UV absorption.
4. Diluted the dual-biotinated PCR products for the four genes with the binding and washing buffer (20 mM Tris-HCl (pH 8.0), 0.5-mM EDTA, 1 M NaCl) and mixed to make a 50 µl of PCR solution containing 10^6 /µl of each of the product molecules.
5. Wash streptavidin-coated beads (5×10^8 beads, φ = 1 µm, Dynal)with 50 µl of binding and washing buffer three times.
6. Suspend the beads in 50 µl of the binding and washing buffer.

7. Add 50 µl of the PCR solution to the same volume of streptavidin-coated beads.
8. Mix them at 750 rpm at room temperature for an hour.
9. Wash the beads twice with 100 µl of the washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)) and suspend them in 50 µl of RT-PCR grade water (Ambion) (each 9.5×10^5 molecules per 10^7 beads)).
10. Wash them twice with 50 µl of 95°C washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)) to denature the dsDNA for preparing ssDNA template fabrication.
11. Resuspend the beads 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) and hold them at 95°C for 10 seconds followed by 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds with a thermal cycler to remove completely the nonspecific DNA adsorbed on the beads. (By this procedure, the nonspecific DNA adsorbed on beads was completely removed.)
12. Remove the supernatant from the tube, and resuspend the beads in 50 µl of washing buffer (0.1% Tween20, 10 mM Tris-HCl (pH 8.0)).
13. Produce a ten-fold dilution series 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

1. Dispense 16.4 µl solution (1×Premix Ex Taq, 1 µM of each *TBP* primer pair, 0.25 µM *TBP* MGB fluorogenic probe, and 5% formamide) into each well of a 384-well-microplate (Applied Biosystems). Then, add 3.6µl of cDNA library sample (10^7 beads) to each well and mix gently.
2. Dispense 19 µl solution (1×Premix Ex Taq, 1 µM of each *TBP* primer pair, 0.25 µM *TBP* MGB fluorogenic probe, 0.18% PMB80, 1.8 mM Tris-HCl and 5% formamide) into each well of a 384-well-microplate (Applied Biosystems). Then, add 1 µl of standard ss template sample (10^7 beads) to each well and mix the solution gently.
3. Perform a qPCR analysis under condition 3 (95°C for 10 seconds followed by 3 cycles of 95°C for 5 seconds and 55°C for 30 seconds, and 37 cycles of 85°C for 5 seconds and 55°C for 30 seconds).
4. Make the standard curve by plotting the Ct values on y axis and amount of DNA molecules on x axis with data of standard ssDNA templates.
5. Estimate the numbers of target molecules in the cDNA library from the standard curve.
6. Transfer the standard ssDNA templates as well as the cDNA library samples to new nonstick tubes. To recover the beads adsorbed on the well walls of the 384-well plate, wash the wells with 20 µl of the washing buffer (0.1% Tween20, 10 mM Tris-HCl) and add the buffer to the samples in the nonstick tubes.
7. Remove supernatant from the beads, and resuspend the beads in 3.6 µl of suspension liquid (1% PMB80, 10 mM Tris-HCl (pH 8.0)).
8. Perform a qPCR analyses of the other three target genes sequentially (*SDHA*→*B2M*→*EEF1G*) using the same standard ssDNA temples and cDNA libraries. The reaction conditions for all the analyses are the same as those for the first *TBP* analysis described above.

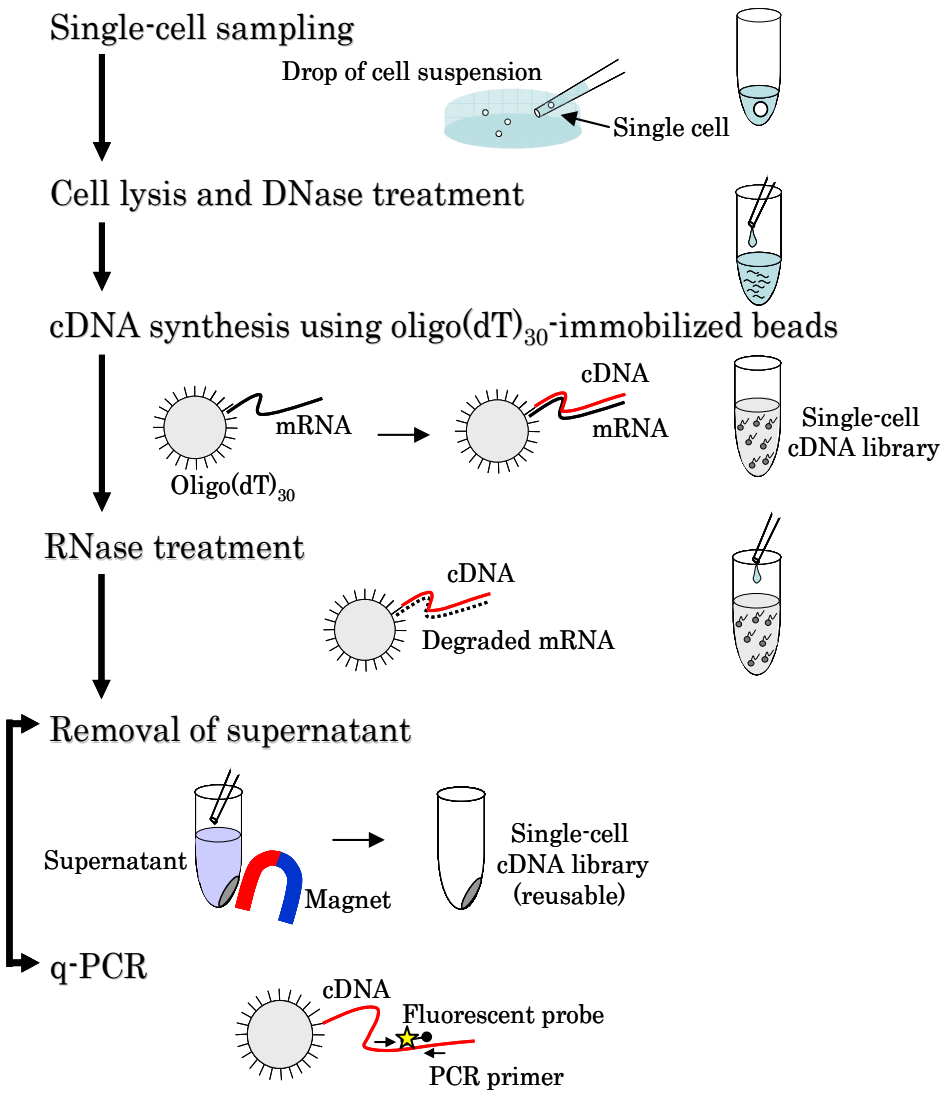

Figure A Outline of the methods.

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Supplementary Methods

Estimation of efficiency of DNA immobilization on beads

The efficiency of the DNA immobilization on the beads was estimated by measuring the amounts of DNA in five solutions: A—PCR solution before immobilization, B—supernatant after immobilization, C—supernatant after first wash, D—supernatant after second wash, and E—supernatant after third wash. The last three solutions might have included DNA desorbed from the beads during the washing processes. The washing was carried out with the binding and washing buffer described above. Four DNA fragments corresponding to the target genes were prepared by qPCR. One (Case A) or four kinds (Case B) of PCR products (each containing 10^6 molecules and dual–biotinated) were immobilized on 10^7 streptavidin-coated beads, and the numbers of DNA molecules in the solutions were quantitatively analyzed by qPCR with the same primers and MGB fluorescent probe (region2: *TBP, SDHA*, *B2M,* region1: *EEF1G*) listed on **Supplementary Table 1.** The efficiency of dsDNA immobilization on the beads was estimated using

$$\left\{\frac{[SolutionA]-2\times([SolutionB]+[SolutionC]+[SolutionD]+[SolutionE])}{[SolutionA]}\right\}\times 100\ .$$

The results are listed in **Supplementary Table 3**.

Evaluation of reproducibility of standard curves in reuse of standard ssDNA templates

The reproducibility of the standard curves was investigated by performing qPCR repeatedly with the same standard ssDNA templates (10^7 beads per μl) containing from 7.5 molecules to 7.5×10^5 molecules per 10^7 beads. As there were four different target genes, four sets of repeated qPCR (sets a, b, c, and d) were carried out by changing the analysis order of the target genes.

The analysis order was *TBP*→*SDHA*→*B2M*→*EEF1G* in set a, *SDHA*→*B2M*→*EEF1G*→*TBP* in set b, *B2M*→*EEF1G*→*TBP*→*SDHA* in set c, and *EEF1G*→*TBP*→*SDHA*→*B2M* in set d. Each 20 μl reaction mixture contained 1× Premix Ex Taq, 1 μM each of forward and reverse primers, 0.25 μM MGB fluorogenic probe, 10^7 beads with immobilized DNA, 0.18% PMB80, 5% formamide, and 1.8 mM Tris-HCl. The PCR cycle profile was 95°C for 10 seconds followed by 3 cycles of 95°C for 5 seconds and 55°C for 30 seconds, and 37 cycles of 85°C for 5 seconds and 55°C for 30 seconds. After quantitative analysis for the first target genes, the standard ssDNA templates were transferred to nonstick tubes. To recover the beads (of the standard ssDNA templates) adsorbed on the well walls of the 384-well plate, we washed the wells with 20 μl of the washing buffer (0.1% Tween20, 10 mM Tris-HCl) and added the buffer to the samples in nonstick tubes. After the supernatant was removed, the beads were resuspended in 3.6 μl of suspension liquid (1% PMB80, 10 mM Tris-HCl (pH 8.0)). The quantitative analyses of the other three targets were performed by reusing the standard ssDNA templates. The standard curves, obtained for the four sets of measurements are

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summarized in **Supplementary Figs. 4a–d**. The number of DNA molecules was corrected using the desorption rate (2.8%) obtained in the experiments in which ssDNA reusability was evaluated using the third thermal cycle profile.

Selection of reverse transcription probes

The efficiencies of capturing mRNA and RT were evaluated using four types of oligo-probes immobilized on beads.

The cDNA libraries for the evaluation were produced by RT reaction using 1000 molecules of the model RNA (*SDHA*) together with 10^7 magnetic beads immobilizing oligo$(dT)_{30}$ probes, gene–specific probes, oligo$(dT)_{25}$VN probes, or LNA probes (EXIQON) (n=3 each). The gene-specific probes (SDHA Reverse primer for standard DNA, **Supplementary Table 1**, Tm=62°C,) had the complementary sequence next to the poly(A) tail. The oligo$(dT)_{25}$VN probes had two additional bases, V and N, at the 3' terminus and an oligo$(dT)_{25}$ sequence: V=(A,G,C mixture) and N=(A,G,C,T mixture). The LNA$(dT)_{30}$ probes had an oligo$(dT)_{30}$ sequence including ten artificial nucleotides placed at every three bases to increase Tm by 10°C or more. The total number of probes immobilized on 10^7 beads was about 1.5×10^{12}.

To prepare standard samples for qPCR, PCR was carried out with about 5 ng cDNA (obtained from HCT116 cells) in order to produce a 2131-bp product labeled with double-biotin. The sequence of the primer set (*SDHA*, Region 1-4) was listed on **Supplementary Table 1**. We created standard sample with dsDNA concentrations ranging from 9.5 dsDNA templates to 9.5×10^5 templates per 10^7 beads as described in preparation of standard dsDNA templates immobilized on beads. The cDNA libraries produced from the model RNA and the standard samples were analyzed simultaneously by qPCR. The result is shown in **Supplementary Fig 1a**.

Selection of target regions free from 3' bias

The cDNA samples (n=3 each) produced from 1000 molecules of the model RNA (*TBP, SDHA, and B2M*) were analyzed by qPCR for different target regions as shown in **Supplementary Fig. 2** (TBP: region 1 and 2, SDHA: region 1, 2, 3. and 4, and B2M: region 1 and 2). The qPCR primer and prove sequences are listed on **Supplementary Table 1.** To prepare standard samples for qPCR, PCR product was produced with cDNA obtained from HCT116 cells and PCR primers listed on **Supplementary Table 1.** Using these PCR products, we prepared the standard dsDNA at concentrations ranging from 9.5 molecules to 9.5×10^5 molecules per 10^7 beads. The cDNA samples (n=3, each) produced from the model RNA and the standard samples were analyzed simultaneously by qPCR for different PCR regions. Quantitative analysis was carried out using Premix Ex Taq (TaKaRa Bio) by measuring the fluorescence intensities during thermal cycling (95°C for 10 seconds followed by 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds). The results are shown in **Supplementary Fig. 1b**.

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Selection of reverse transcriptase for creating single-cell cDNA library.

Because the selection of the reverse transcriptase to use is very important for producing a cDNA library efficiently, we evaluated ten reverse transcriptases by measuring the number of cDNAs they produces from 10^5 molecules of a model RNA for *SDHA* (2161 bases).

The RT reaction conditions are listed in **Supplementary Table 2**. All the reactions were carried out in single tubes in accordance with the conditions recommended by the suppliers. The model RNA (10^5 molecules) was hybridized with oligo$(dT)_{30}$ immobilized on 10^7 magnetic beads. qPCR was carried out using primers and MGB fluorogenic probe (*SDHA,* region 2) listed in **Supplementary Table 1**.The quantification results are shown in **Supplementary Fig. 3**.

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