In situ detection and genotyping of individual mRNA molecules

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Increasing knowledge about the heterogeneity of mRNA expression within cell populations highlights the need to study transcripts at the level of single cells. We present a method for detection and genotyping of individual transcripts based on padlock probes and *in situ* target-primed rolling-circle amplification. We detect a somatic point mutation, differentiate between members of a gene family and perform multiplex detection of transcripts in human and mouse cells and tissue.

In population-based assays that analyze the content of many cells, molecules in rare cells may escape detection. Furthermore, such assays provide no information concerning which of the molecules detected originate from which cells. Expression in single cells can vary substantially from the mean expression detected in a heterogeneous cell population[1,2]. Single-cell studies, preferentially with single-molecule sensitivity, are therefore essential for studying the fluctuation and sequence variation in expressed transcripts. Fluorescence *in situ* hybridization (FISH) has been used to detect single mRNA molecules *in situ*, using either probes labeled with multiple fluorophores[3] or multiple probes labeled with single fluorophores[4]. Although permitting determination of transcript copy numbers in individual cells, these techniques cannot resolve highly similar sequences, so they cannot be used to study, for example, allelic inactivation or splice variation and cannot distinguish among gene family members. The only option available for assigning transcript variants to a single cell in a given tissue involves PCR of laser-capture microdissected material[5,6], which is time consuming and error prone, and thus not suitable for diagnostics.

As an alternative to PCR- and hybridization-based methods, padlock probes[7] have for many years been used to analyze nucleic acids. These highly selective probes are converted into circular molecules by target-dependent ligation upon perfect hybridization to the target sequence. Circularized padlock probes can be amplified by rolling-circle amplification (RCA) *in situ*[8], providing information about the localization of target molecules at the single-cell level. We have previously published a robust protocol for *in situ* detection of mitochondrial DNA molecules in which the target DNA is used to prime the RCA reaction[9], causing the rolling circle product (RCP) to be anchored to the target molecule. RNA molecules can also serve as templates for the ligation of padlock probes[10], but RNA detection with padlock probes *in situ* has so far proven more difficult than DNA detection and is subject to limitations[11] (see **Supplementary Note 1**).

Here, we accomplish transcript detection *in situ* by first converting the mRNA into localized cDNA molecules that are detected with padlock probes and target-primed RCA (**Fig. 1a**). With cDNA the ligation of padlock probes is highly specific, enabling genotyping

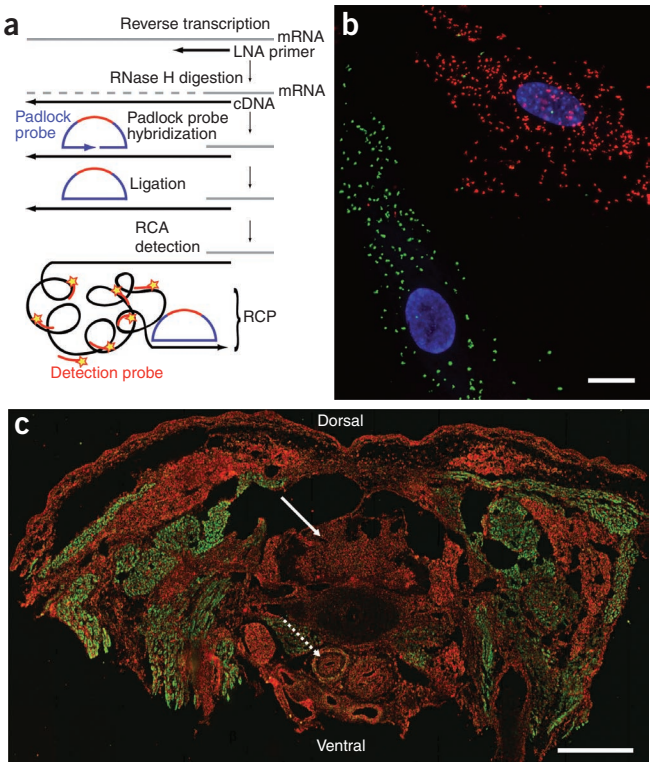

Figure 1 | Detection of individual transcripts *in situ* with padlock probes and target-primed RCA. (**a**) Schematic representation of the procedure. cDNA is created using locked nucleic acid (LNA)-modified primers and is probed after degradation of mRNA by RNase H. RCPs are identified through hybridization of fluorescent detection probes. (**b**) Detection of a single nucleotide difference in β-actin transcripts in cocultured human and mouse fibroblast cells. Green, human β-actin sequence variant; red, mouse β-actin sequence variant. Blue, cell nuclei. Scale bar, 20 μm. (**c**) Detection of α1- (green) and β-actin (red) transcripts in fresh frozen mouse embryonic tissue (E14.5). Solid arrow, spinal cord; dashed arrow, trachea. Scale bar, 500 μm.

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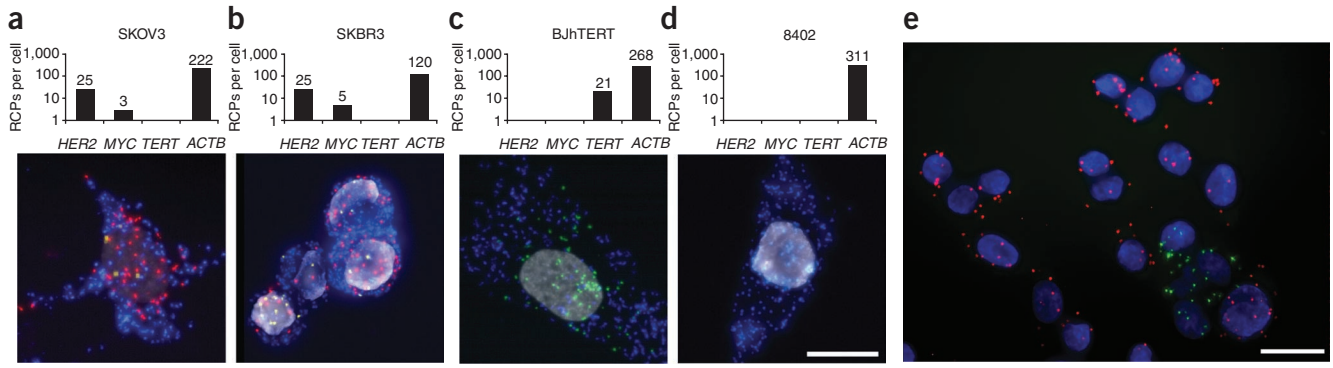

Figure 2 | Multiplex *in situ* detection of cancer-related transcripts in cancer and primary human cell lines. (**a**–**d**) Quantification of RCPs in the different cell lines is shown in the bar graph, with a representative micrograph of each cell type shown below. Detection of *HER2* (red), *MYC* (yellow), *TERT* (green) and *ACTB* (blue) transcripts in human ovarian carcinoma cells (SKOV3) (**a**), human breast carcinoma cells (SKBR3) (**b**), *TERT* immortalized human fibroblast cells (BJhTERT) (**c**) and primary human fibroblast culture GM08402 (**d**). Scale bar, 50 μm. (**e**) Genotyping of a *KRAS* point mutation in a coculture of human wild-type (ONCO-DG-1) and mutant (SW-480) cells, showing wild-type (green) and mutant (red) RCPs. Scale bar, 20 μm. Cell nuclei are shown in gray (**a**–**d**) or blue (**e**).

of individual transcript molecules. We describe important parameters for design of primers and probes in **Supplementary Notes 2** and **3** and **Supplementary Figures 1** and **2**.

We first used the method to detect β-actin (*ACTB*) transcripts in cultured human cells with two different padlock probes, targeting sequences in the first and last exon, respectively. We obtained abundant, bright, spot-like signals localized to the cytoplasm of cells, consistent with previous observations of this transcript[3] (**Supplementary Fig. 3**). The detection efficiency was similar for the two padlock probes, indicating that in this case detection was not highly dependent on target position along the transcript. In contrast, when reverse transcriptase was omitted from the cDNA synthesis reaction, no signals were detected, verifying that the signals were cDNA dependent. We estimated the overall *in situ* detection efficiency to be ~30% of available transcripts, on the basis of a comparison to quantitative PCR (qPCR) data for β-actin mRNA in the GM08402 cell line (2,000 copies per cell). There was considerable variation in the number of signals among cells (**Supplementary Fig. 4**), consistent with other reports of intercellular variation in β-actin mRNA expression[1].

To demonstrate high selectivity of detection, we arranged an assay for detection of single-nucleotide variants of transcripts *in situ*. Expressed polymorphisms are rare in β-actin, and we therefore used a single-base difference between the human and mouse β-actin sequences as genotyping target. We subjected cocultured human and mouse fibroblast cells to *in situ* genotyping of cDNA using padlock probes PLP-βhum (human) and PLP-βmus (*Mus musculus*) and target-primed RCA. There was a clear-cut distinction between the two subpopulations of cells in the coculture (**Fig. 1b**). The preference for perfectly matched padlock probes at the circularization step ensures distinction between the two targets by the ligase. (Ligase fidelity is discussed in **Supplementary Note 4**.)

To test the method in fixed tissue sections, we targeted the closely related skeletal muscle α1-actin (*Acta1*) and cytoplasmic β-actin (*Actb*) transcripts[12] in fresh frozen tissue from an E14.5 mouse embryo cross sectioned at the level of the neck. The two actin transcripts were successfully detected in the tissue using padlock probes designed with target sequences differing by a single base. The α1-actin signals were mainly distributed to skeletal muscles, whereas β-actin signals were widely distributed but showed slightly more signals in the non-muscular tissue (**Fig. 1c**). We further demonstrated the ability to distinguish three transcripts from the same gene family by including a probe specific for the cytoplasmic γ1-actin (*Actg1*) transcript (**Supplementary Fig. 5**).

To test the method's ability for multiplex detection of transcripts for expression profiling, we designed padlock probes for the three cancer-related transcripts *HER2* (also known as *ERBB2*), *cMyc* (also known as *MYC*) and *TERT*. Using β-actin as a reference transcript, we assayed these transcripts in four cell lines (a human ovarian carcinoma cell line, a human breast carcinoma cell line, a *TERT*-immortalized human foreskin fibroblast cell line and a primary fibroblast cell culture). The levels of expression of the cancer-related genes differed among the cell lines (**Fig. 2a–d**). The ovarian and breast carcinoma cell lines showed similar patterns of expression of the *HER2* and *cMyc* transcripts, whereas the *TERT*-immortalized fibroblast was the only cell type with a detectable level of the *TERT* transcript. All four cell lines expressed β-actin, and in the normal fibroblasts this was the only investigated transcript expressed at a detectable level. We compared these results to qPCR data and to available literature and found good correlation with the expected relative expression levels in the different cell lines and a notable consistency in detection efficiency among the different transcripts (**Supplementary Note 5**). We noticed large cell-to-cell variation in expression for all investigated transcripts (**Supplementary Fig. 6**), which is consistent with previous studies of expression in single cells in cultures[1,2,11].

We also used the technique to assess *HER2* transcript distribution in a fresh frozen HER2-positive human breast cancer tissue section (**Supplementary Fig. 7**). We found expression to vary widely among the cells, consistent with the expected presence of cancer cells and normal stroma in the tumor tissue.

Finally, we applied our method to genotype a *KRAS* point mutation in *KRAS* wild-type and mutant cells (**Fig. 2e** and **Supplementary Fig. 8**). The different cell types could be clearly distinguished on the basis of the color of their corresponding RCPs. Activating mutations of the *KRAS* oncogene are found in 17%–25% of all human tumors[13], and assays to monitor these mutations and other tumor cell–specific markers in tissue specimens *in situ* could be of great value for clinical pathology

investigations. We further investigated the potential for studies of allelic expression by analyzing 77 cells from a cell line heterozygous for a point mutation in *KRAS*. We observed an average allelic ratio of 48% wild-type transcripts, with considerable cell-to-cell variation (**Supplementary Figs. 4b** and **8d**), indicating a balanced allelic transcription. In this experiment, all heterozygous cells with more than seven RCPs showed signals from both alleles. For cells showing fewer than seven signals, it will be difficult to determine the potential for biallelic expression and extent of unbalanced allelic expression in single cells.

Our method for *in situ* analysis of mRNA has a detection resolution that may allow the study of differences in the relative expression of two allelic transcripts directly in tissue. The importance of these types of studies was recently highlighted in two papers describing large-scale analyses of allele-specific expression[14,15], revealing that many genes undergo this type of transcriptional regulation and that the allelic expression can differ among tissues. Furthermore, it has been shown that most human genes undergo alternative splicing[16], which could now also potentially be studied at the single-cell level using this method. Because to our knowledge no other *in situ* method exists today that can perform multiplex detection of expressed single nucleotide sequence variants, we believe that our method to visualize transcriptional variation directly in cells and tissues will be of value in both research and diagnostics, providing new insights about the human transcriptome.

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

C.L. and I.G. designed and performed experiments and analyzed data. M.N. supervised. All authors contributed to conceiving the project and writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

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ONLINE METHODS

Cell culture. The cell lines GM08402 (Coriell Cell Repositories) and BJhTERT were cultured in MEM without phenol red and L-glutamine (Gibco) supplemented with 10% FBS (Sigma), 1× nonessential amino acids (Gibco), 2 mM L-glutamine (Sigma) and 1× penicillin-streptomycin (PEST, Sigma). Mouse embryonic fibroblasts (MEF) were cultured in DMEM without phenol red and L-glutamine (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and 1× PEST. ONCO-DG-1, SW-480, A-427 (all three from DSMZ), SKOV3 and SKBR3 were cultured in RPMI culture medium (Sigma) supplemented with 10% FBS, 2 mM L-glutamine and 1× PEST.

Preparation of tissue sections. Fresh frozen 9-μm sections of E14.5 mouse embryos were placed on Superfrost Plus Gold slides (Thermo Scientific). Fully anonymized fresh frozen human tissue sections from a HER2-positive breast cancer were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish Biobank Legislation. Breast tissue sections of 4 μm thickness were placed on Starfrost microscope slides (Instrumedics).

Sample pretreatment for *in situ* experiments. Cells were seeded on Superfrost Plus slides (Thermo Scientific) and allowed to attach. When the cells reached the desired confluency they were fixed in 3% (w/v) paraformaldehyde (Sigma) in PBS for 30 min at room temperature (20–23 °C). After fixation, slides were washed twice in DEPC-treated PBS (DEPC-PBS) and dehydrated through a series of 70%, 85% and 99.5% ethanol for 3 min each. The molecular reactions were performed in Secure-seals (Grace Bio-Labs, 9 mm in diameter and 0.8 mm deep) attached to the slides. A 50-μl reaction volume was used for each sample. To make the RNA more readily available for cDNA synthesis, 0.1 M HCl was applied to the cells for 10 min at room temperature. This was followed by two brief washes in DEPC-PBS.

Tissues were treated similarly to cell lines, with a few exceptions. Tissue fixation was performed in 2% (w/v) paraformaldehyde in PBS. The tissue was then permeabilized with 0.01% pepsin (Sigma) in 0.1 M HCl at 37 °C for 2 min. Molecular reactions were carried out with a reaction volume of 100 μl in Secure-seals (13 mm in diameter, 0.8 mm deep; Grace Bio-Labs) mounted over the tissue. Reverse transcription was carried out overnight and incubation times for ligation, RCA and detection probe hybridization were doubled. For the mouse tissue, ligation was carried out with T4 DNA ligase.

Oligonucleotide sequences. Oligonucleotide sequences (**Supplementary Tables 1–3**) were designed using GenBank accession numbers NM_001101.3 (*ACTB*), NM_007393.3 (*Actb*), NM_198253.2 (*TERT*), NM_002467 (*MYC*), NM_001005862.1 (*ERBB2*), NM_009606 (*Acta1*), NM_009609 (*Actg1*) and NM_033360 (*KRAS*). All padlock probes were 5′-phosphorylated at a concentration of 2 μM with 0.2 U μl^{-1} T4 polynucleotide kinase (Fermentas) in the manufacturer's buffer A plus 1 mM ATP for 30 min at 37 °C, followed by 10 min at 65 °C. For β-actin transcript detection in cultured cells, primer P-βe1 was used for detection with padlock probe PLP-βe1, primer P-βe6 with padlock probe PLP-βe6, primer P-βhum with padlock probe PLP-βhum and primer P-βmus with padlock probe PLP-βmus unless otherwise indicated. *TERT* was detected with primer P-TERT and padlock probe PLP-TERT, *cMyc* with primer P-cMyc and padlock probe PLP-cMyc and *HER2* with primer P-HER2 and padlock probe PLP-HER2. For detection of transcripts in mouse tissue, primer P-α1βmus was used with padlock probe PLP2-βmus for β-actin and with padlock probe PLP-α1mus for α1-actin, and primer P-γ1mus was used together with padlock probe PLP-γ1mus for γ1-actin detection. For *KRAS* genotyping, primer P-KRAS was used in combination with the padlock probes PLP-KRAS-wtGGT, PLP-KRAS-mutGTT and PLP-KRAS-mutGAT.

***In situ* cDNA detection procedure.** Samples were preincubated in M-MuLV reaction buffer. Then 1 μM of cDNA primer was added to the slides with 20 U μl^{-1} of RevertAid H minus M-MuLV reverse transcriptase (Fermentas), 500 nM dNTPs (Fermentas), 0.2 μg μl^{-1} BSA (NEB) and 1 U μl^{-1} RiboLock RNase Inhibitor (Fermentas) in the M-MuLV reaction buffer. Slides were incubated for 3 h to overnight at 37 °C. After incubation, slides were washed briefly in PBS-T (DEPC-PBS with 0.05% Tween-20 (Sigma)), followed by a postfixation step in 3% (w/v) paraformaldehyde in DEPC-PBS for 30 min at room temperature. After postfixation, the samples were washed twice in PBS-T.

To make the target cDNA strands available for padlock probe hybridization, the RNA portion of the created RNA-DNA hybrids was degraded with ribonuclease H. This was performed in the same step as the padlock probe hybridization and ligation. For most reactions, Ampligase (Epicentre) was used for ligation. Samples were first preincubated in Ampligase buffer (20 mM Tris-HCl, pH 8.3, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD and 0.01% Triton X-100). Ligation was then carried out with 100 nM of each padlock probe in a mix of 0.5 U μl^{-1} Ampligase, 0.4 U μl^{-1} RNase H (Fermentas), 1 U μl^{-1} RiboLock RNase Inhibitor, Ampligase buffer, 50 mM KCl and 20% formamide. Incubation was performed first at 37 °C for 30 min, followed by 45 min at 45 °C. For detection of actin transcript isoforms in mouse embryonic tissue sections, ligation was instead carried out using T4 DNA ligase (Fermentas). Samples were then first preincubated in T4 DNA ligase buffer (Fermentas). Then 100 nM of each padlock probe was added with 0.1 U μl^{-1} T4 DNA ligase, 0.4 U μl^{-1} RNase H, 1 U μl^{-1} RiboLock RNase Inhibitor and 0.2 μg μl^{-1} BSA in T4 DNA ligase buffer supplemented with 0.5 mM ATP and 250 mM NaCl. Slides were then incubated at 37 °C for 30 min. After ligation with Ampligase or T4 DNA ligase, slides were washed in DEPC-treated 2× SSC with 0.05% Tween-20 at 37 °C for 5 min and rinsed in PBS-T.

Slides were preincubated briefly in Φ29 DNA polymerase buffer (Fermentas). RCA was then performed with 1 U μl^{-1} Φ29 DNA polymerase (Fermentas) in the supplied reaction buffer, 1 U μl^{-1} RiboLock RNase Inhibitor, 250 μM dNTPs, 0.2 μg μl^{-1} BSA and 5% glycerol. Incubation was carried out for 60 min at 37 °C. The incubation was followed by a wash in PBS-T.

RCPs were visualized using 100 nM of each corresponding detection probe in 2× SSC and 20% formamide at 37 °C for 30 min. Slides were then washed in PBS-T, the Secure-seals were removed and the slides were dehydrated using a series of 70%, 85% and 99.5% ethanol for 3 min each. The dry slides were mounted with Vectashield (Vector), containing 100 ng ml^{-1} DAPI to counterstain the cell nuclei. The protocol for counterstaining of cell membranes in **Supplementary Figure 3** is described in **Supplementary Note 6**.

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Image acquisition and analysis. Images of cultured cells were acquired using an AxioplanII epifluorescence microscope (Zeiss) equipped with a 100 W mercury lamp, a CCD camera (C4742-95, Hamamatsu), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, Cy3.5 and Cy5. A ×20 (Plan-Apocromat, Zeiss), ×40 (Plan-Neofluar, Zeiss) or ×63 (Plan-Neofluar, Zeiss) objective was used for capturing the images. Images were collected using the Axiovision software (release 4.3, Zeiss). Exposure times for cell images were 260–340 ms (at ×20 magnification), 10–80 ms (×40) or 220 ms (×63) for DAPI; 40 ms (×40) or 220 ms (×63) for FITC; 560–640 ms (×20), 110–160 ms (×40) or 200 ms (×63) for Cy3; 110 ms (×40) or 250 ms (×63) for Texas Red; and 6,350 ms (×20), 180 ms (×40) or 350 ms (×63) for Cy5. For SKBR3 and SKOV3 cells, images were collected as *z*-stacks to ensure that all RCPs were imaged. The tissue section in **Figure 1c** was imaged using a Mirax Midi slide scanner (3D Histech) equipped with a CCD camera (AxioCam MRm, Zeiss) and a ×20 Plan-Apochromat objective. Exposure times in the slide scanner were 45 ms for DAPI, 270 ms for Cy3, 340 ms for Texas Red and 3,200 ms for Cy5.

For quantification, the numbers of RCPs and cell nuclei in images were counted digitally using BlobFinder software[17] (version 3.0_beta). For cultured cells, the quantification was done on five 20× microscope images (approximately 20–30 cells for each sample). The total number of RCPs was divided by the number of nuclei for each image. The average for each sample was then calculated from the result of the five images and is reported as RCPs per cell. The procedure for single-cell quantification used in **Supplementary Figure 4** is described in **Supplementary Note 7**.

Images displayed for illustration in this publication were processed using image editing software for clarity in print. The threshold for different color channels was set using Photoshop CS3 (**Fig. 1** and **Supplementary Figs. 3** and **6** online; Adobe) or ImageJ 1.41o (**Fig. 2a–d** and **Supplementary Figs. 5** and **7** online; US National Institutes of Health). In **Figure 2a**,**b**, a maximum intensity projection was created in Axiovision using the collected *z*-stack images. For clearer visualization of signals in the cell images in **Figure 2** and **Supplementary Figure 8**, a maximum filter was also applied in all channels except the DAPI channel, using ImageJ. The image from the slide scanner displayed for illustration was prepared using the Mirax viewer software (**Fig. 1c**; version 1.11, 3D Histech).

qPCR for β-actin transcript quantification in cells. Two separate passages of the cell line GM08402 were collected after counting of cells, and total RNA was purified from the cells using the PARIS kit (Ambion) with the protocol for RNA isolation from total cell lysate. Traces of DNA were removed from the purified RNA using the DNA-free kit (Ambion). First-strand cDNA synthesis was carried out with 700 ng of template RNA in a mix containing 20 U RevertAid H minus M-MuLV reverse transcriptase (Fermentas) in the corresponding enzyme buffer, 0.5 μg oligo(dT) primer (20-mer), 1 mM dNTPs and 1 U μl^{-1} RiboLock RNase Inhibitor. Samples were incubated at 37 °C for 5 min, followed by 42 °C for 60 min. The reaction was stopped by heating to 70 °C for 10 min. A preparative PCR was carried out to synthesize template for standard curve creation. For this PCR, 1 μl of cDNA from one of the cell passages was amplified in a mix of 0.02 U μl^{-1} Platinum Taq DNA polymerase (Invitrogen), PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 200 nM ACTBfwd primer and 200 nM ACTBrev primer in a total volume of 50 μl. PCR was carried out with 2 min at 95 °C, followed by cycling 45 times (95 °C for 15 s, 50 °C for 15 s, and 72 °C for 1 min) and finishing with 72 °C for 5 min. The PCR product was purified using the Illustra GFX PCR and gel band purification kit (GE Healthcare) according to the protocol for purification of DNA from solution. The concentration of the purified PCR product was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and the number of molecules per microliter was calculated. qPCR was run with 2 μl of template cDNA, or diluted standard curve PCR product, with SYBR Green (Invitrogen), 0.02 U μl^{-1} Platinum Taq DNA polymerase, PCR buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 200 nM ACTBfwd primer and 200 nM ACTBrev primer in a total volume of 30 μl. The qPCR was run using the same program as for the preparative PCR. Standard curve samples were run in duplicates of the same sample and cDNA samples from the two passages of cells were run in triplicates.

Calculations of transcript copy numbers for the two cell passages were based on the number of counted cells at harvest. The average β-actin mRNA copy number for the cell line was then determined.

The protocol for efficiency estimation by qPCR for the *in situ* multiplex detection experiment is found in **Supplementary Note 8**.

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CORRIGENDUM: *In situ* detection and genotyping of individual mRNA molecules

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In the version of this article initially posted online, primer information for detection of mouse α- and β-actin in tissue was incorrect in Online Methods. The error has been corrected in the HTML and PDF versions of the article.