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# Messenger RNA quantification after fluorescence activated cell sorting using intracellular antigens

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ABSTRACT

Recent studies using stem cells or cancer stem cells have revealed the importance of detecting minor populations of cells in blood or tissue and analyzing their biological characteristics. The only possible method for carrying out such procedures is fluorescence activated cell sorting (FACS). However, FACS has the following limitations. First, cells without an appropriate cell surface marker cannot be sorted. Second, the cells have to be kept alive during the sorting process in order to analyze their biological characteristics. If an intracellular antigen that was specific to a particular cell type could be stained with a florescent dye and then the cells can be sorted without causing RNA degradation, a more simple and universal method for sorting and analyzing cells with a specific gene expression pattern could be established since the biological characteristics of the sorted cells could then be determined by analyzing their gene expression profile. In this study, we established a basic protocol for messenger RNA quantification after FACS (FACS-mQ) targeting intracellular antigens. This method can be used for the detection and analysis of stem cells or cancer stem cells in various tissues.



## 1. Introduction

Stem cells have the potential to indefinitely self renew and differentiate into the various cell types of a particular tissue [1]. Their differentiation potential and capacity for tissue renewal and damage repair make stem cells valuable for regenerative medicine, tissue engineering, and biotechnology applications [2]. On the other hand, a series of mutations affecting the differentiation potential of stem cell and their unlimited growth potential may generate cancer stem cells (CSCs) [3–5]. There is increasing evidence that CSCs play an important role in the biological behavior of tumors, and they may even determine patient prognosis [6–10].

Stem cells and CSCs usually exist as minor populations of cells in a tissue [11]. They can be separated from other cells by fluorescent activated cell sorting (FACS) using antibodies that specifically bind to cell surface marker proteins or by the transfection of a plasmid carrying a cell type specific promoter and a reporter gene [6–12]. However, the majority of stem cells and CSCs do not possess such antigens or do not show specific promoter activity and so cannot be sorted in this manner. Furthermore, cell samples must be treated under sterilized conditions and kept alive throughout the whole procedure since the sorted cells need to be cultured in order to have their biological characteristics determined. In addition, transfection of a foreign reporter gene may result in the modification of the properties of the cell.

Many reports have described the sorting of various types of cells using intracellular protein markers and FACS [13–15]. However, few studies have then subjected the sorting cells to molecular analysis since the fixation and permeabilization required for the staining of intracellular antigens, especially nuclear antigens, often cause RNA degradation [16–19].

Thus, a simpler method for sorting and analyzing stem cells needs to be established. If cellular RNA could be preserved during the staining of intracellular antigenes, then the stained cells could be sorted by FACS, and their biological characteristics could be analyzed by obtaining their gene expression profile. In this study, we developed an in-tube fluorescence immunocytochemistry method for FACS that minimizes the degradation of cellular RNA and established a basic protocol for messenger RNA quantification after FACS (FACS-mQ) targeting intracellular antigens.

## 2. Materials and methods

### 2.1. Cell culture

Three cell lines were used in this study: the rat thyroid cell line FRTL-5, which expresses thyroglobulin (TG) and thyroid transcriptional factor 1 (TTF-1); the human lung carcinoma cell line PC3, which expresses TTF-1; and the human anaplastic thyroid

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carcinoma cell line 8305C, which does not express either of these genes. The FRTL-5 cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and the PC3 and 8305C cells were provided by the Human Science Research Resource Bank (Osaka, Japan).

The FRTL-5 cells were cultured in Ham's F-12 medium (Gibco, Paisley, Scotland, UK), supplemented with 5% newborn calf serum (Gibco); 2.5 mg/ml sodium bicarbonate ($NaHCO_3$) (Wako, Osaka, Japan); 100 U/ml penicillin (Meiji, Tokyo, Japan); 100 μg/ml streptomycin (Meiji); 250 ng/ml fungizone (Gibco); and a six-hormone preparation consisting of 100 μU/ml TSH (Sigma, St. Louis, MO, USA), 10 μg/ml bovine insulin (Sigma), 10 ng/ml somatostatin (Sigma), 360 pg/ml hydrocortisone (Sigma), 5 μg/ml transferrin (Gibco), and 100 ng/ml glycyl-L-histidyl-L-lysine acetate (Sigma). The PC3 and 8305C cells were grown in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 2 mg/ml $NaHCO_3$, 100 U/ml penicillin, 75 μg/ml streptomycin, and 250 ng/ml fungizone. All cells were maintained in a 5% $CO_2$–95% air atmosphere at 37 °C, and the medium was changed every third day until the cells were subconfluent.

### 2.2. In-tube immunocytochemistry for FACS

Ten ml tubes (Azone, Osaka, Japan) were used in all procedures. All media and buffers except the antibodies were mixed with 0.1% diethylpyrocarbonate (DEPC) (Sigma), stored overnight at room temperature, and then autoclaved. The FRTL-5, PC3, and 8305C cells were fixed with methanol (Wako) supplemented with 10% polyethylene glycols (molecular weight 300 kD, Wako) (UM-Fix) for 15 min at 4 °C. UM-Fix was replaced by phosphate-buffered saline (PBS) (Wako) supplemented with 0.1% Tween 20 (PBS-T) (Sigma) and kept in PBS containing 10% dimethyl sulfoxide (DMSO) (Sigma) at −80 °C until use.

On the day of immunocytochemistry, $1 \times 10^6$ cells were thawed rapidly in a water bath at 37 °C and then permeabilized with 0.1% TritonX-100 (Sigma) in PBS for 30 min at 4 °C. The cells were washed with PBS-T and then blocked with 0.5% blocking reagent (Roche Diagnostics) in PBS-T (blocking solution).

The blocked cells were then incubated in 100 μl of fluorescein-conjugated mouse anti-thyroglobulin antibody (No. 53905, Ana-Spec, San Jose, CA, USA) and R-phycoerythrin (R-PE)-conjugated TTF-1 antibody (DAKO, Carpinteria, CA Clone AG7G3/1) diluted 200 times in the blocking solution overnight at 4 °C. The antibodies were labeled with fluorescein and R-PE using the Fluorescein and R-PE Labeling Kit-$NH_2$ (Dojindo Molecular Technologies, Kumamoto, Japan), respectively. After being washed twice with PBS-T, the cells were analyzed using a flow cytometer.

### 2.3. Extraction of RNA from the cells and reverse transcription (RT)

Total RNA were extracted from the cells as described previously [20]. The recovered total RNA was reverse transcribed in an RT mixture, which contained 4 μl of 5× first strand buffer (Invitrogen, Tokyo, Japan), 10 mmol/l dithiothreitol (Invitrogen), 0.5 mmol/l deoxynucleotide triphosphates (dNTP) (Takara, Shiga, Japan), 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen), 2 U/μl RNase inhibitor (Takara), and 2.5 μmol/l random hexamer (Takara) in a total volume of 20 μl. The RT reaction was carried out for 10 min at 25 °C, 50 min at 42 °C, and 15 min at 70 °C.

### 2.4. Real-time quantitative polymerase chain reaction

The real-time quantitative polymerase chain reactions (PCR) for rat β-actin (*ACTB*), human *ACTB*, rat *TG*, and human pulmonary-associated surfactant B protein (*SFTPB*) mRNA were performed using SYBR® Green Master Mix and the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City CA, USA) according to the manufacturer's recommendations. One microliter of cDNA was used in the subsequent assays. The two primers used for the quantification of rat *ACTB* cDNA were as follows:

rACTBF (0.5 μM): 5′-CTGACAGGATGCAGAAGGA-3′;
rACTBR (0.5 μM): 5′-TGATCCACATCTGCTGGAA-3′.
Those used for human *ACTB* cDNA were as follows:
hACTBF (0.5 μM): 5′-TGGACATCCGCAAAGACCTG-3′;
rACTBR (0.5 μM): 5′-CCGATCCACACGGAGTACTT-3′.
Those used for rat *TG* cDNA were as follows:
rTGF (0.5 μM): 5′-ATGGCCAGTACCTACGTGAA-3′;
rTGR (0.5 μM): 5′-CCTTTGCCCTGTTGATAAGCC-3′.
Those used for human *SFTPB* cDNA were as follows:
hSFTBPF (0.5 μM): 5′-AGCTTTCTTCCTCGAGATG-3′;
hSFTPBR (0.5 μM): 5′-CACAGCAGAAATAGAATCACC-3′.

All primers were purchased from Operon Biotechnologies (Tokyo, Japan). The conditions for PCR were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A recombinant pGEM T-vector (Promega, Tokyo, Japan) containing a partial cDNA was constructed by PCR-cloning with the same primer sets as used in the PCR and was used as a standard sample.

### 2.5. Flow cytometry and data analysis

Flow cytometric analysis and sorting were performed using a FACS Vantage SE from Becton Dickinson (Mountain View, CA, USA) equipped with a 488-nm argon ion laser and emission filters for fluorescein and R-PE. For each sample, at least $10^4$ events were collected and analyzed. Data were analyzed using the DiVa software (Becton Dickinson). $10^4$ cells with strong fluorescent signals were sorted into TG positive, and TTF-1 positive and TG negative fractions. The sorted cells were stored in RNA later (Applied Biosystems).

## 3. Results

### 3.1. Cell preservation at −80 °C after fixation

After fixation, the cells were stored at −80 °C in PBS supplemented with 10% DMSO. The copy number of human *ACTB* mRNA was measured in $10^6$ 8305C cells stored for a period ranging from 1 day to 2 weeks. No significant decrease in the copy number was observed in the cells stored for 2 weeks (Fig. 1). In addition, no change in cell morphology was observed (data not shown).

### 3.2. The copy number of ACTB mRNA before and after immunocytochemistry

We measured the copy number of human *ACTB* mRNA in $10^6$ 8305C cells before and after immunocytochemistry. No significant decrease was observed after the immunocytochemistry procedure (Fig. 2).

### 3.3. Quantification of mRNA after FACS

After immunocytochemistry, the FRTL-5, PC3, and 8305C cells were analyzed and sorted by flow cytometry (Fig. 3). When double stained with fluorescein-labeled TG antibody and R-PE-labeled TTF-1 antibody, the FRTL-5 cells, which are known to express TG and TTF-1, showed a clear shift to the right and small shift to the upper. The PC3 cells, which are known to express TTF-1 but not TG, showed a clear upwards shift.