# Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells

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**Human embryonic stem (ES) cells are pluripotent cell lines that have been derived from the inner cell mass (ICM) of blastocyst stage embryos [1–3]. They are characterized by their ability to be propagated indefinitely in culture as undifferentiated cells with a normal karyotype and can be induced to differentiate in vitro into various cell types [1, 2, 4–6]. Thus, human ES cells promise to serve as an unlimited cell source for transplantation. However, these unique cell lines tend to spontaneously differentiate in culture and therefore are difficult to maintain. Furthermore, colonies may contain several cell types and may be composed of cells other than pluripotent cells [1, 2, 6]. In order to overcome these difficulties and establish lines of cells with an undifferentiated phenotype, we have introduced a reporter gene that is regulated by a promoter of an ES cell-enriched gene into the cells. For the introduction of DNA into human ES cells, we have established a specific transfection protocol that is different from the one used for murine ES cells. Human ES cells were transfected with enhanced green fluorescence protein (EGFP), under the control of murine *Rex1* promoter. The transfected cells show high levels of GFP expression when in an undifferentiated state. As the cells differentiate, this expression is dramatically reduced in monolayer cultures as well as in the primitive endoderm of early stage (simple) embryoid bodies (EBs) and in mature EBs. The undifferentiated cells expressing GFP can be analyzed and sorted by using a Fluorescence Activated Cell Sorter (FACS). Thus, we have established lines of human ES cells in which only undifferentiated cells are fluorescent, and these cells can be followed and selected for in culture. We also propose that the pluripotent nature of the culture is made evident by the ability of the homogeneous cell population to form EBs. The ability to efficiently transfect human ES cells will provide the means to study and manipulate these cells for the purpose of basic and applied research.**

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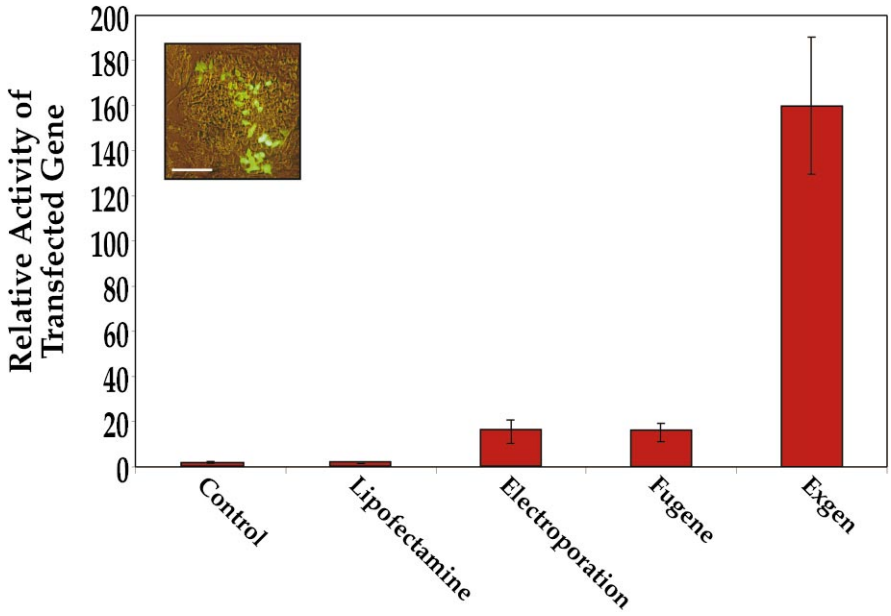
## Results and discussion

The objective of this study was to obtain pure clones of human ES cells that are genetically modified so that their undifferentiated phenotype can be followed and selected for in vitro. Thus, we aimed at introducing the EGFP reporter gene under the control of a promoter of an ES cell-enriched gene into human ES cells. By tagging the undifferentiated cells with GFP, we wished to monitor the differentiation status of the cells in culture during growth and propagation as well as following spontaneous and induced differentiation. For this purpose, we chose to use the well-characterized promoter sequence of the murine *Rex1* gene [7]. *Rex1* is a retinoic acid-regulated zinc finger protein that is expressed in preimplantation mouse embryos (including the inner cell mass), trophoblast, and spermatocytes as well as in undifferentiated murine ES cells and some embryonic carcinoma (EC) cell lines [7, 8]. This gene is rapidly downregulated upon differentiation of the embryonic cells. Hence, by introducing *Rex1*-regulated gene markers (*Rex1*-EGFP) into human ES cells, we should be able to express these markers in pluripotent cells, allowing the determination of the differentiation status of these cells in culture.

In order to introduce *Rex1*-EGFP fusion gene into human ES cells, we had to establish a method to transfect the human embryonic cells with DNA. Although ES-like cell lines are now available from a large array of mammalian species (for a review, see Prelle et al. 1999 [9]), there are no published protocols for DNA transfection in any of the species, other than mice. In the mouse, electropora-

**Figure 1**



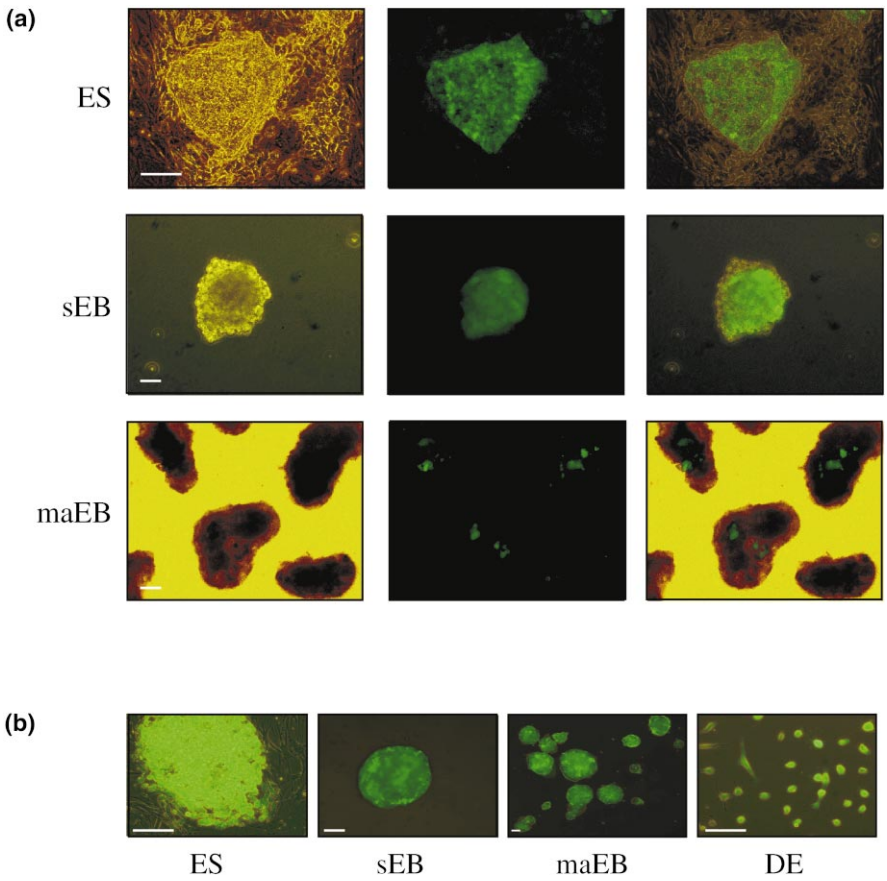

Transfection of DNA into human ES cells. DNA was introduced into human ES cells either by electroporation or by using several commercial reagents such as LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas). To determine the efficiency of DNA introduction by each of the methods, the cells were transfected with a construct of firefly Rennila protein under the control of a TK promoter. The cells were harvested 48 hr after transfection, and luminosity of the Rennila protein was monitored using a luminometer. Results are given in the histogram as the relative activity of the transfected gene (luminosity units per mg of total protein), following the subtraction of the values obtained from samples of the appropriate MEF-only controls. Each experiment was repeated three times, and the mean with standard error is shown. Inset: human ES cells transiently transfected with EGFP under the control of the housekeeping gene E1F (elongation factor I) promoter. Note the green fluorescent ES cells that incorporated the foreign DNA. The scale bar indicates 100 μm.

tion was found to be the method of choice for introducing foreign DNA into ES cells [10]. However, human ES cells do not survive electroporation well. Therefore, we compared the efficiencies of several chemical-based methods for the transfection of H9 human ES cells [1] (passage 40–50). Initially, an expression construct of EGFP under the control of the housekeeping gene elongation factor I (E1F) was introduced into human ES cells by several different reagents. Transient expression of the GFP was observed in no more than 10% of the cells, mainly by the human ES cells, and not by the feeder layer of mouse embryonic fibroblasts (MEF) (over 80% of the fluorescent cells had ES cell morphology and resided within the colony boundaries) (Figure 1, inset). To allow quantification and comparison of transfection efficiencies between protocols, a TK-firefly Renilla luciferase reporter gene (Dual Luc Reporter Assay Kit, Promega) was introduced into growing colonies of human ES cells, either by LipofectAMINE Plus (Life Technologies), FuGENE (Boehringer Mannheim), or ExGen 500 (Fermentas) (performed according to the manufacturer's protocols). Cell samples were lysed (using the passive lysis buffer of the assay kit) and evaluated for the efficiency of transient transfection by measuring the relative activity of luciferase in respect to protein concentration (as determined by the Bradford method [BIO-RAD Protein Assay]) 48 hours after transfection. A clear difference between ExGen 500, FuGENE, LipofectAMINE Plus, and electroporation was apparent. Transfection with ExGen 500 seems to deliver DNA into human ES cells in an order of magnitude more efficiently than other reagents that we have examined (Figure 1).

Using the transfection protocol of ExGen 500, a *Rex1*-EGFP expression vector, which includes the neo selectable marker, was delivered into human ES cells. The following day, cells were trypsinized and replated on a feeder of inactivated MEF that was resistant to neomycin ($MEF^{Neo+}$), allowing the clonal propagation of transfected cells by G418 selection. At 14 days in culture, neomycin-resistant fluorescent colonies were isolated and propagated for several passages while maintaining their level of fluorescence (up to 13 passages), allowing the establishment of individual cell lines. In our experience, stable clones were derived in an efficiency of $\sim 10^{-5}$ of the transfected cells.

Of the various neo resistant colonies, we have established 10 cell lines, 4 of which were examined under different culture conditions (Figure 2). When grown on feeder cells in the presence of leukemia inhibitory factor (LIF) (to support undifferentiated growth), high expression of GFP was detected in the small and densely packed cells of the undifferentiated colony. The fluorescent emission overlaps well with the discrete margins of the colony and is absent in the periphery, where spontaneous differentiation takes place (Figure 2a). When the transfected human ES cells were induced to differentiate by growing as cell aggregates in suspension culture, fluorescence gradually declines, initially, in the outer surface of 4 day old simple

**Figure 2**

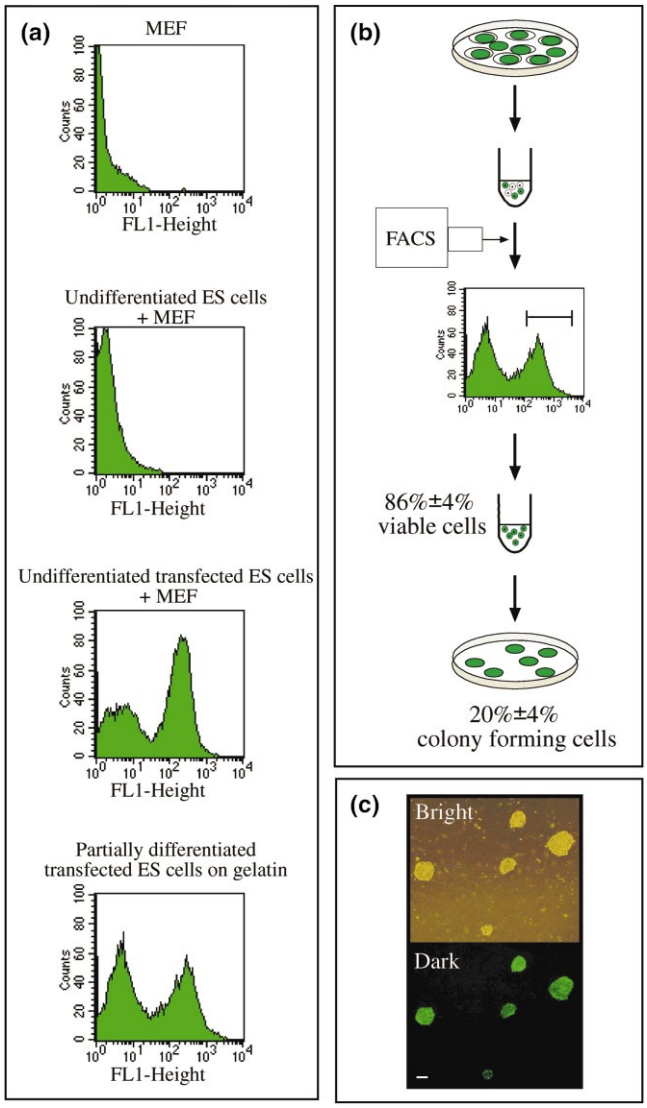



Isolation of human ES clones transfected with a marker for undifferentiated cells. **(a)** Human ES cells underwent stable transfection with EGFP fused to the murine *Rex1* minimal promoter sequence. The transfected ES cells and their differentiated cell derivatives are shown: simple embryoid body (sEB), and mature embryoid bodies (maEBs). The left and middle columns are photos of bright and dark fields, respectively. The right column is the overlay of the two photos. Note that only the undifferentiated cells are fluorescent. The fluorescent ES colony is surrounded by differentiated nonfluorescent cells. The simple EB is labeled only in the middle and not in the peripheral primitive endodermal cells [11]. Mature EBs are generally not fluorescent, and only very distinct areas in them are still fluorescent (probably residual undifferentiated cells). The scale bar indicates 100 μm. **(b)** The stable transfection of human ES cells with a constitutively expressed EGFP construct, driven by the mouse *PGK* promoter. Overlay photos of the dark on bright field of the transfected ES cells (ES) and their differentiated cell derivatives are shown: simple embryoid bodies (sEB), mature embryoid bodies (maEB), and differentiating embryonic cells derived from dissociated embryoid bodies (DE). Note that GFP is expressed by all cells, differentiated and undifferentiated, in the proliferating ES colony as well as by all cells of simple and mature EBs (including those in the outer layer of the sEB, where differentiation of primitive endoderm is taking place in the mouse EBs [11]). The scale bar indicates 100 μm.

EBs, where a layer of primitive endoderm was demonstrated in the mouse [11]. Later, if maintained to form mature EBs (20 days in suspension), the fluorescence practically ceases, apart from a few cores of undifferentiated cells (Figure 2a). This is in contrast to transfections of constructs driven by constitutively expressed promoters (*PGK*, phosphoglycerate kinase 1; and *CMV*, cytomegalovirus), in which expression of GFP was observed in both undifferentiated and differentiated cells of the colony (Figure 2b).

In an attempt to distinguish between populations of undifferentiated and differentiated human ES cells, we have analyzed the *Rex1*-EGFP transfected cell lines by FACS (Figure 3). Cell samples of MEF, undifferentiated human ES cells, and a mixture of undifferentiated and differentiated transfected cell lines were characterized according to their fluorescent emission. As expected, a clear difference in fluorescent intensity exists between the undifferentiated cultures of untransfected and transfected cell lines. In addition, when comparing EGFP-transfected human ES cells to their differentiated derivatives, a reduction in emission intensity is observed (Figure 3a). This shift in fluorescence emission represents a transition, from undifferentiated to differentiated, in the state of the cells. To allow the collection and selective propagation of the most fluorescent cells in the culture, three different GFP-expressing human ES cell lines (3–4 cell sample replicates per clone) were sorted by FACS (Figure 3b). Cell sorting was performed according to the background level of fluorescence that had been obtained by the analysis of untransfected human ES cells. The different cell samples were individually sorted for collection into tubes containing 25,000–50,000 cells each. By comparing the total cell count and the number of viable cells prior and following cell sorting (determined by trypan blue staining), we could show that the FACS procedure had no detrimental effect on cell viability, as 86% of the sorted cells were viable. Moreover, by plating the isolated cells on $MEF^{Neo+}$ 10 $cm^2$ culture dishes and allowing their propagation in vitro, we demonstrated their ability to develop into undifferentiated fluorescent-labeled human ES colonies, with an efficiency of 20% ± 4% (n = 11) (ranging from 2% to 41%) (Figure 3b). In our procedure, many sorted cells were grown in the same culture dish, potentially allowing

**Figure 3**




FACS analysis and cell sorting of the transfected human ES cells. **(a)** Human ES cells transfected with *Rex1*-EGFP construct were analyzed by FACS according to the intensity of green fluorescence emission (FL1 height). Cell samples of MEF and undifferentiated human ES cells were used as controls. Fluorescent intensity between undifferentiated human ES cells, transfected human ES cells, and their differentiated cell culture derivatives (obtained by growth on gelatin-coated plates in the absence of LIF and bFGF) was then compared. The high-fluorescent intensity peak represents GFP positive cells, while the low-intensity peak represents background levels that may result either from autofluorescence or residual promoter activity. **(b)** Cell sorting of three GFP-expressing cell lines was performed by FACS. Following trypsin digestion, cell samples (3–4 replicates per clone) were evaluated for percentage of cell viability (84%) and sorted according to the intensity of green fluorescent emission. The collected cell samples (25,000–50,000) were redetermined for cell viability (86%) and replated on $MEF^{Neo+}$ culture dishes (2,500–8,000 cells per dish). Following growth in vitro, cell culture dishes were inspected and recorded for total number of proliferating human ES colonies (20% $\pm$ 4% [n = 11]). **(c)** Photos of fluorescent-labeled proliferating human ES colonies (top, bright field; and bottom, dark field) obtained 4 days after cell sorting by FACS.

mutual support of growth and relatively high plating efficiency. This differs from the single cell dilution procedure by which the clonality of human ES cells was conferred [6]. After FACS sorting, the cells have a morphology indistinguishable from that seen before, but we have not yet tested them for pluripotency.

In our research, we have developed a stem cell selection approach in an attempt to facilitate maintenance of human ES cells in vitro. Currently, the available methods applied for this purpose involve the identification and isolation of single colonies under a dissecting microscope; however, these procedures are time consuming and labor intensive. As an alternative, we suggest a method for purifying undifferentiated cells by cell sorting the fluorescent-labeled cells from a mixed population. Similar selection of undifferentiated clones may be achieved by introducing into the cells a gene that enables drug selection, such as neo resistance gene, under the regulation of an ES-specific promoter [12]. By generating pure populations of undifferentiated cells, as described above, we should be able to avoid the loss of human ES cultures due to their spontaneous differentiation in vitro. Our system of introducing a cell-specific selectable marker into the genome of undifferentiated human ES cells provides a model for isolating specific cell types for transplantation from heterogeneous cell cultures obtained by induced differentiation. Similarly, such methods may be considered for eliminating human ES cells by negative selection prior to transplantation of differentiated cells, avoiding the risk of tumor induction.

The expression of *Rex1*-regulated reporter gene by the cells in the growing colony illustrates that these cells maintained their undifferentiated phenotype. In addition, the transfected cells can develop into undifferentiated colonies that maintain their ability to form EBs in vitro. These results support previous work that demonstrated the clonality of human ES cells [6] and the capacity of these homogenous cultures to differentiate into the three germ layers.

Finally, we report the first isolation of genetically engineered human ES cell lines and describe an efficient protocol for transfecting these cells. By introducing genetic modifications into their genome, we should be able to manipulate them in vitro and use them as vectors in cell-based therapies as well as for other biomedical and research purposes.

## Materials and methods

*Cell culture*

Human ES cells (H9 [1], passage 40–50) were cultured on a Mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder layer (obtained from 13.5 day embryos) in 80% KnockOut DMEM medium (GIBCO-BRL), supplemented with 20% KnockOut SR (a serum-free formulation) (GIBCO-BRL), 1 mM glutamine (GIBCO-BRL), 0.1 mM $\beta$-mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (50 units/ml), Streptomycin (50 $\mu$g/ml), and 4 ng/ml basic fibroblast

growth factor (bFGF). The cells were grown in the presence of LIF ($10^3$ units/ml, GIBCO-BRL), although its necessity for supporting undifferentiated growth in human ES cells is currently unclear [1, 2]. The undifferentiated cell cultures were induced to differentiate in vitro into EBs by omitting LIF and bFGF from the growth media and allowing aggregation in petri dishes [3]. Following the formation of simple EBs by a 5 day cell aggregation step, cell masses were either trypsin dissociated and left to grow as a monolayer on fibronectin-creating cultures of differentiated embryonic (DE) cells [5] or further expanded in suspension and allowed to develop into 20 day old mature EBs (maEBs) (yielding cavitated and cystic EBs). In addition, we allowed some undifferentiated cells to undergo spontaneous differentiation as a monolayer by growing them on 0.1% gelatin-coated plates (Merck) in the absence of LIF and bFGF.

*Plasmid construction*

*Rex1*-EGFP and *PGK*-EGFP expression vectors were constructed by the deletion of the *CMV* promoter sequence from pEGFP-N1 (Clontech) and the insertion of either the mouse *Rex1* promoter sequence (700 bp) into the HindIII restriction site or the mouse *PGK* (phosphoglycerate kinase 1) promoter (515 bp) into the EcoRI and BamHI restriction sites. These constructs contained an SV40-driven neo selectable marker. The use of SV40 promoter in our system was sufficient to confer G418 resistance by driving the neo gene, although it was somewhat inefficient in mouse ES cells.

*Transfection and establishment of transgenic cell lines*

Fully expanded and undifferentiated human ES cells underwent stable transfection with *Rex1*-EGFP, *CMV*-EGFP, or *PGK*-EGFP plasmid DNA by the ExGen 500 transfection system (Fermentas). Transfection of human ES cells was carried out in 6-well trays on MEF, two days after plating, and was performed as described by the manufacturer's protocol. Specifically, 2 μg of plasmid DNA plus 10 μl of the transfecting agent ExGen 500 were added to $\sim 3\times10^5$ cells in a final volume of 1 ml media per well. The cells were centrifuged at 280 × g for 5 min and incubated at 37°C in a moist chamber for an additional 45 min. Residuals of the transfecting agent were removed by washing the cells twice with PBS. The following day, the cells were trypsinized and $\sim 5\times10^5$ were replated on each 10 cm culture dish containing inactivated $MEF^{Neo+}$. Two days following replating, G418 (200 ng/ml) was administered to the growth medium, allowing the selective propagation of transfected cells in culture. By day 14, neo resistant fluorescent-labeled colonies were identified by a fluorescent microscope (up to 10 colonies per plate). Using our constructs, over 80% of the neo resistant colonies were also GFP positive. Single transgenic colonies were picked by a micropipette, dissociated into small clumps of cells, and transferred into a 24-well culture dish on a fresh feeder of $MEF^{Neo+}$. The cells continuously proliferated in the presence of G418 and formed a large number of expanding undifferentiated colonies.

*FACS analysis and cell sorting*

FACS analysis of *Rex1*-EGFP-expressing cells was performed on a FACSCalibur system (Becton-Dickenson), according to their green fluorescent emission. Undifferentiated human ES cells were used to set the background level of fluorescence. Transfected cells, either undifferentiated (grown on MEF cells in the presence of LIF) or partially differentiated (obtained by growth on gelatin in the absence of LIF and bFGF) were analyzed for fluorescence intensity and compared to control cells.

GFP-expressing cell lines were sorted by FACS according to their fluorescence emission. Following trypsin digestion and centrifugation, cell pellets ($\sim 2–5\times10^6$ cells from each cell line) were resuspended in PBS, filtered by a 70 μM cell strainer (Falcon), and divided into four different tubes, which were kept on ice under sterile conditions. Total cell counts and percent of viable cells were determined for each sample by 0.5% trypan blue staining (1:1 volume) prior to analysis and sorting by FACS. Cell samples were sorted for the collection of 25,000–50,000 cells in 50 ml conical tubes (Falcon) precoated with BSA (4% in PBS). Following centrifugation (5 min, 1000 rpm), the cells were resuspended in 0.5 ml PBS, analyzed for cell viability as described above, and plated on $MEF^{Neo+}$ 10 cm culture dishes. Following 4 days in culture in the presence of G418 (200 ng/ml), the cell cultures were inspected under the microscope, and the total number of colonies per plate was recorded.

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