Review

Improved applications of the tetracycline-regulated gene depletion system

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Summary **Tightly controlled expression of transgenes in mammalian cells is an important tool for biological research, drug discovery, and future genetic therapies. The tetracycline-regulated gene depletion (Tet-Off) system has been widely used to control gene activities in mammalian cells, because it allows strict regulation of transgenes but no pleiotropic effects of prokaryotic regulatory proteins. However, the Tet-Off system is not compatible with every cell type and this is the main remaining obstacle left for this system. Recently, we overcame this problem by inserting an internal ribosome entry site (IRES) to drive a selectable marker from the same tetracycline-responsive promoter for the transgene. We also employed a CMV immediate early enhancer/β-actin (CAG) promoter to express a Tet-controlled transactivator. Indeed, the Tet-Off system with these technical modifications was applied successfully to the human pre-B Nalm-6 cell line in which conventional Tet-Off systems had not worked efficiently. These methodological improvements should be applicable for many other mammalian proliferating cells. In this review we give an overview and introduce a new method for the improved application of the Tet-Off system.**

Keywords: Tetracycline-regulated gene depletion system, Tet-Off system, gene targeting

1. Introduction

The tetracycline (Tet)-regulated gene depletion (Tet-Off) system, originally proposed by Gossen and Bujard (*1*), has been widely applied to control gene activity in eukaryotes. The system functions in cultured cells from mammals, plants, amphibians, and insects, as well as, in organisms including: yeast, *Drosophila*, plants, mice, and rats (*http://www.zmbh.uni-heidelberg.de/Bujard/homepage.html*). In order to set up a functional Tet-Off system, a stable host cell line containing two different expression units needs to be isolated, as shown in Figure 1. The first unit is a plasmid to express a regulatory protein termed Tet-controlled transactivator (tTA), which is a fusion protein of an *Escherichia coli* (*E. coli*) Tet repressor (TetR) and the transcriptional transactivation domain of herpes simplex virus protein 16 (VP16AD). The second unit is a response plasmid that expresses a gene of interest (GOI) under the control of a Tet-response element (TRE), which is a minimal RNA polymerase II promoter sequence fused downstream from the multiple Tet resistance operator in *Tn10* of *E. coli*. In the doubly stable cell harboring the tTA expression vector and the response vector, the tTA can bind to the TRE to stimulate the onset of transcription of *GOI* if the cells are cultured in the

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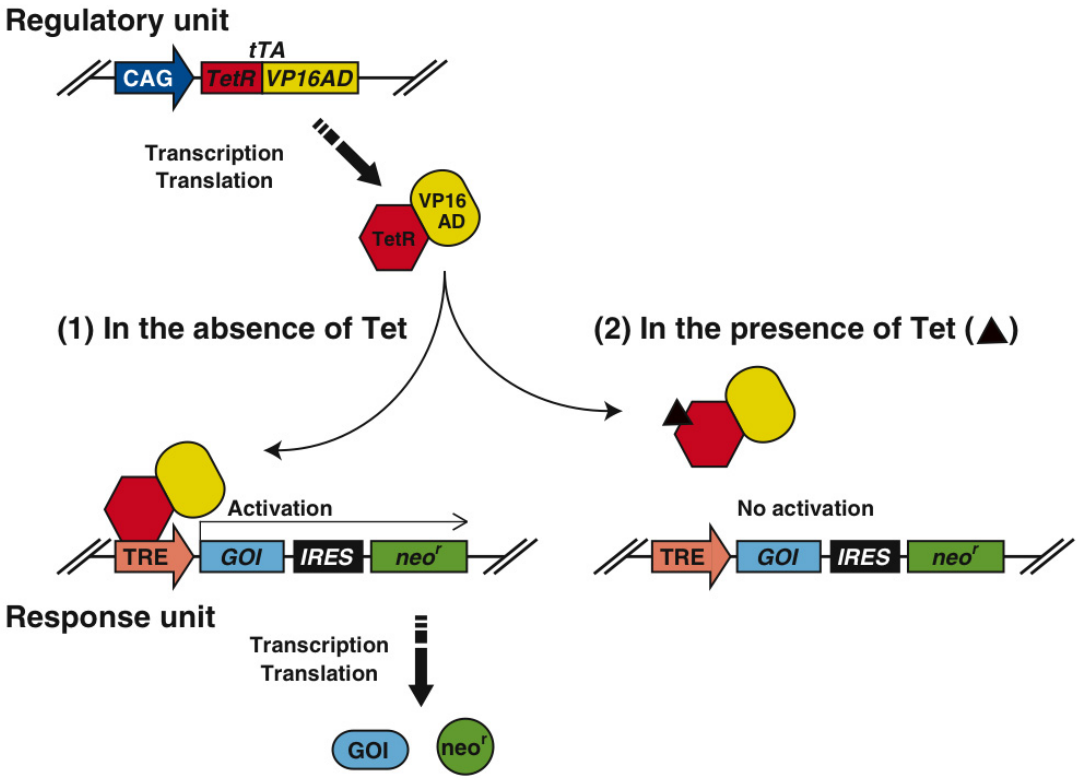

Figure 1. The principles and the strategy of the improved Tet-Off System. To set up a Tet-Off system, two expression units are required to be integrated into the genome of the host cells. The first is a regulatory unit to express tTA, a fusion protein of TetR and VP16AD). The second is a response unit in which *GOI* is placed downstream of TRE to express *GOI* in a tTA/TRE-dependent manner. **(1)** In the absence of Tet, tTA binds to TRE and activates TRE-derived transcription of *GOI*. **(2)** In the presence of Tet (closed triangle), TRE-derived expression of *GOI* is shut off promptly since Tet-bound tTA cannot access TRE. In our improved Tet-Off system, CAG promoter is used to express tTA in place of CMV promoter. In addition, IRES was inserted between *GOI* and drug-resistant gene (*neo*^{r}) in the response unit. The cell clones that express optimal amounts of TRE-derived transgenes can be isolated efficiently as drug-resistant clones.

absence of Tet. In contrast, the tTA is prevented from binding to the TRE and consequently transcription of *GOI* is abolished if the cells are cultured in the presence of Tet, even at concentrations far below cytotoxic levels for living cells.

Among a number of inducible gene expression systems so far developed (*1-5*), the Tet-Off system has been widely used, because it has been improved to overcome the initial technical problems, including leaky expression, loss of regulation, and slow suppression (see reviews in *6,7*). The main problem left for the Tet-Off system was that it does not work effectively in every cell type, as described (a protocol issued by Clontech 13 September 2005; *8-10*). However, this problem was overcome using the following technical modifications: switching of the promoter to express tTA from the human cytomegalovirus IE (CMV) to CMV immediate early enhancer/β-actin (CAG) (*11*) in the tTA expression vector; insertion of an internal ribosome entry site (IRES) followed by a drug-resistant gene in the response vector. These improvements enabled us to obtain rare clones that express robust amounts of tetracycline-regulated transgenes, in the human Nalm-6 pre-B cell line. This is a cell line that is not always compatible with the conventional Tet-Off system. As the Nalm-6 is a rare cell line that is efficient for gene targeting by homologous recombination, the successful application of the Tet-Off system allowed us to generate a 'tetracycline-inducible conditional gene deficient' Nalm-6 cell clone in which the expression of *GOI* can be depleted with the addition of Tet on a knockout background (*12,13*).

As the CAG promoter and the IRES sequence work effectively in most vertebrate cells, we expect that the improved Tet-Off system will be applicable to many other proliferating cells in which the conventional Tet-Off system would not work. In this review, the methodological improvements introduced in the Tet-Off system is summarized and a protocol for its application to the Nalm-6 cell line is introduced as a compelling example.

2. The choice of promoter to express tTA

The promoter to express tTA should be selected dependent on what types of the cells are used. For example, the CAG promoter (*11*) is a better choice than the CMV promoter for the human Nalm-6 cell line. Figure 2 is a transient Luc assay in which a Luc reporter vector was introduced into the Nalm-6 cell

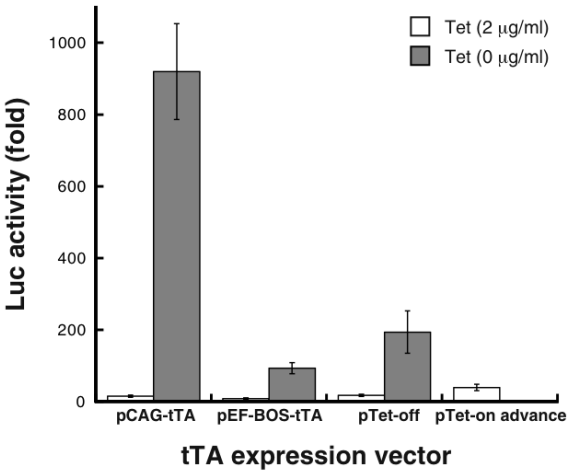

Figure 2. The efficacy of the promoter to express tTA. The histogram represents the fold increase in the induction of luciferase (Luc) expression in the transient Luc assay, in which pUHC13-3 (pTRE-Luc) was introduced alone or with pCAG-tTA (CMV enhancer and chicken β-actin promoter), pEF-BOS-tTA (SV40 enhancer and human EF-1α promoter), pTet-off or pTet-On advance (CMV promoter IE) into Nalm-6 cells. Twelve hours after transfection, the cells were separated into two aliquots, one was used for the depletion by Tet and the other for no depletion. After incubation for 36 h, cell extracts were prepared to measure the Luc activities as relative light units (RLUs). The Luc activity was normalized by the RLUs of the extract from the cells transfected with pTRE-Luc alone, and used to calculate the relative Luc activity (y-axis). Each independent experiment was performed in triplicate, and the results are expressed as the mean fold induction compared with the negative control (pTRE-Luc alone) ± standard error of mean (SEM).

line to monitor TRE-derived Luc expression. It was demonstrated that the Nalm-6 cell lines in which tTA was expressed under the control of CAG promoter (pCAG-tTA) displayed higher levels of Luc activities (on average about four-fold higher) than the Nalm-6 cell lines in which tTA was expressed under the control of CMV promoter (pTet-Off; BD Biosciences, San Jose, CA, USA). As the CAG promoter insures a stable expression of transgene in a wide rage of mammalian cells, it may be the first choice for general applications. The map of the pCAG-tTA we constructed is illustrated in Figure 3A.

3. Insertion of the IRES-drug resistance gene in the response vector

The IRES is a virus-derived nucleotide sequence in which a cap-independent translation is allowed to commence even in the middle of mRNA. Therefore, when the IRES sequence is located between the first *GOI* and the second drug resistance gene in the response vector, it permits the expression of the second gene used to monitor the expression of the first gene (*14,15*). Taking advantage of this property of the IRES, a few cell clones that express a TRE-derived transgene can be isolated efficiently by seeking drug resistance clones, instead of examining the expression of the transgene in each clone by Western blotting.

The conventional protocol for the Tet-Off system recommends a sequential transfection of the tTA expression vector and then the response vector. However, the screening system using the IRES-drug-resistant gene allowed us to transfect these two vectors simultaneously to obtain cell clones with optimal levels of TRE-derived transgene expressions, saving time by approximately a month or two. Importantly, the system worked effectively in other cell lines than the Nalm-6. The map of the typical response vector containing IRES-drug-resistance gene is shown in Figures 3B-D.

4. Materials

4.1. *Web resources*

1. Human genome database: *http://genome.ucsc.edu/cgi-bin/hgGateway*
2. NCBI BLAST home: *http://blast.ncbi.nlm.nih.gov/Blast.cgi*
3. Kazusa DNA Research Institute for full-length cDNAs of KIAA clones: *http://www.kazusa.or.jp/j/resources/clone.html*
4. Invitrogen for full-length cDNAs of IMAGE clones: *http://clones.invitrogen.com/index.php*

4.2. *Cell cultures*

1. Medium: Dulbecco's Modified Eagles Medium and Roswell Park Memorial Institute-1640 medium were used for adherent and suspension cells, respectively. The medium was supplemented with 50 mL (10%) fetal bovine serum (Kohjin-Bio, Saitama, Japan; *Note*: tetracycline-negative!), 5 mL (1%) penicillin-streptomycin solution (Sigma-Aldrich, St Louis, MO, USA), 5 mL (2 mM) glutamine (Sigma-Aldrich), and 50 μM 2-mercaptoethanol.
2. Petri dish for adherent culture (9.4 cm × 1.6 cm; Greiner Japan, Tokyo, Japan).
3. Petri dish for suspension culture (9.4 cm × 2 cm; Greiner Japan).
4. Cell banker for frozen stocks of cells (Nihon Zenyaku, Fukushima, Japan).

4.3. *Preparation of cDNA*

1. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA).
2. SuperScript™ First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen).
3. PrimeSTAR™ HS DNA polymerase (Takara Bio, Otsu, Japan).
4. Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA).
5. PCR-Script™ Amp Cloning Kit (Stratagene, La Jolla, CA, USA).

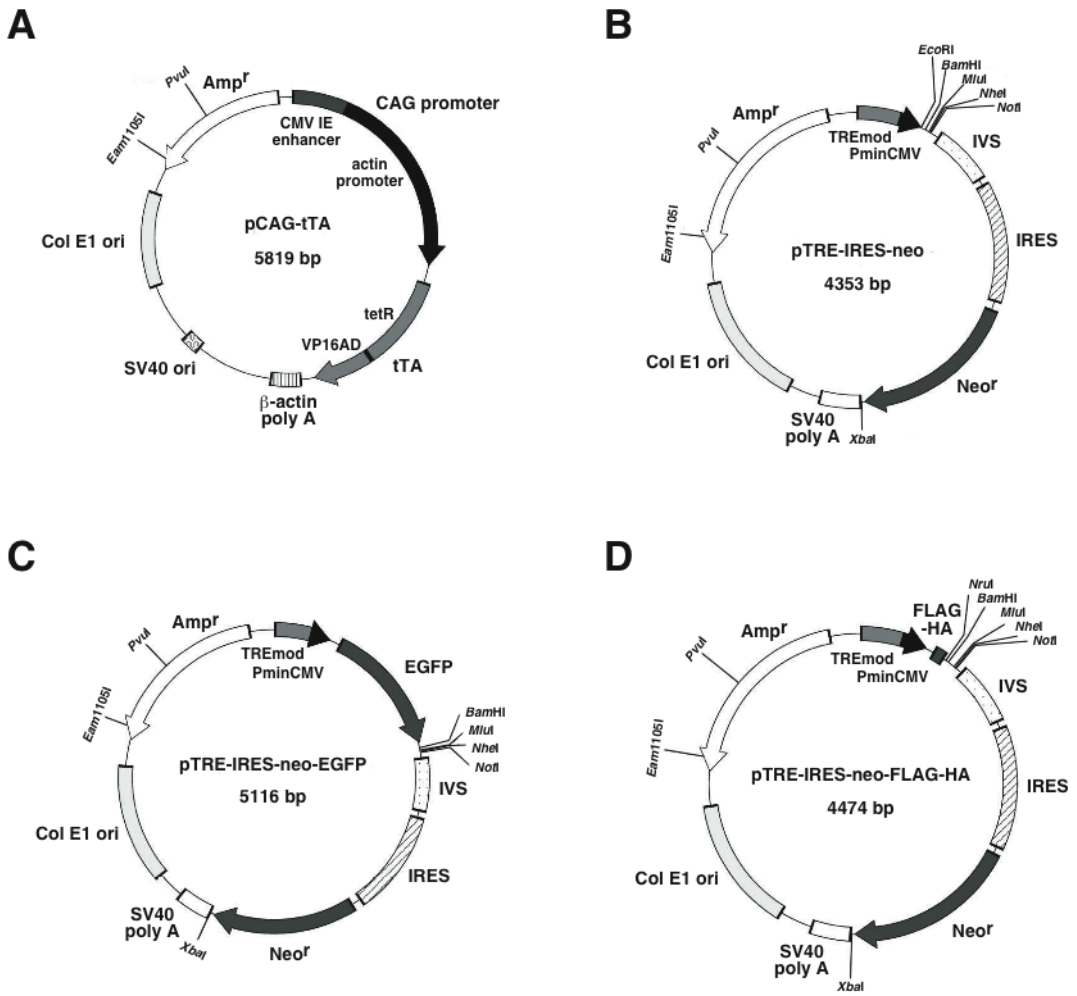

Figure 3. Schematic diagrams of tTA expression vector and response vectors. (A) Expression of tTA is driven by the CAG promoter in the pCAG-tTA. The tTA expression vector harboring neomycin resistant gene (pCAG-tTA-neo) for the sequential transfection procedure is also available. **(B-D)** A series of response plasmids were constructed from pTRE-tight vector. The response vector containing the hygromycin or puromycin resistant gene is available. Detailed information of these vectors is available upon request.

6. GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA).
7. Chemically competent DH5α or XL10-Gold cells.

4.4. *Transfection and Drug Screening*

1. Transfection: Nucleofector I (Amaxa Inc., Gaithersburg, MD, USA), Nucleofector Kit T (Amaxa Inc.).
2. Drug screening: For the case of Nalm-6 cell lines, 1.0 mg/mL of L-histidinol (Sigma-Aldrich), 0.2 μg/mL of puromycin (BD Biosciences), 1.0 mg/mL of G418 (GIBCO, Grand Island, NY, USA), or 0.35 μg/mL of hygromycin B (Clontech, Mountain View, CA, USA). *Note*: Optimal concentrations for drug screening should be checked beforehand for each cell line!
3. 96-well, 24-well, and 6-well flat-bottom plates for suspension culture (Sumitomo Bakelite Co. LTD., Tokyo, Japan).
4. 8-channel and 12-channel pipettes (Thermo Scientific Inc., Rockford, IL, USA).
5. Gel-loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water.

4.5. *Luciferase (Luc) assay*

1. Luciferase Assay System (Promega). *Note*: Reconstituted Luc assay reagent should not be thawed above 25°C and also avoid multiple freeze-thaw cycles.
2. Luminat LB9507 (Berthold, Wildbad, Germany).

4.6. *Western blot analysis*

1. 2× Sodium Dodecyl Sulfate (SDS) sample buffer: 100 mM Tris-HCl (pH 6.8), 4.0% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol.
2. Phosphate-buffered saline: 2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4.
3. SuperSignal WestFemto Maximum Sensitivity Substrate (Thermo Scientific Inc.).

5. Protocols

5.1. *Preparation of cDNA*

A full-length cDNA is prepared by RT-PCR with the total RNA obtained from the cells or the organisms of interest, but some full-length cDNAs or ESTs can be obtained (IMAGE clones, KIAA clones, and so on: see *4.1*. Materials). The sequence of the obtained cDNA should be confirmed carefully to discriminate among alternative splicing products, polymorphisms, and amplification errors.

5.1.1. *Preparation of total RNA from cultured cells*

Total RNA is prepared using TRIzol Reagent. Roughly, 1×10^7 cells are required to prepare > 10 μg total RNA which is sufficient for the subsequent RT-PCR reactions.

5.1.2. *Preparation of cDNA*

1st strand synthesis – A typical reaction of RT-PCR with oligo (dT) primer is shown as follows, although the RT-PCR with specific primers is acceptable.

1. Prepare a 10 μL reaction mixture (I) as follows:

Prepared total RNA	to make 5.0 μg
dNTP (10 mM)	1.0 μL
Oligo(dT) primer (50 μM)	1.0 μL
DEPC-treated MilliQ water	to make 10 μL

2. Incubate at 65°C for 5 min and then put it on ice for 1 min.
3. Prepare a 9 μL reaction mixture (II) as follows:

10× RT buffer	2.0 μL
25 mM $MgCl_2$	4.0 μL
0.1 M DTT	2.0 μL
RNase inhibitor	1.0 μL

4. Mix 10.0 μL of the reaction Mixture (I) and 9.0 μL of the reaction Mixture (II).
5. Add 1.0 μL of SuperScript II reverse transcriptase, incubate at 50°C for 50 min, incubate at 85°C for 5 min, and then put it on ice.
6. Add 1.0 μL of RNase H, and incubate at 37°C for 20 min.
7. Resulting 1st strand cDNA was kept on ice or stored at -20°C until the next procedures.

PCR amplification – Primers used for PCR are designed for the subsequent construction of the TRE-responsive expression vectors, especially when an epitope-tag fused protein is expressed. For the amplification of full-length cDNA, proofreading polymerase (with a 3′-5′ exonuclease activity) should be used. A typical PCR reaction with PrimeSTAR HS DNA polymerase is shown below.

1. Prepare a 50 μL reaction mixture (III) as follows:

5× PCR buffer	10.0 μL
cDNA	1.0~2.0 μL
5′ primer (10 μM)	1.25 μL (final 250 nM)
3′ primer (10 μM)	1.25 μL (final 250 nM)
PrimeSTAR HS DNA polymerase	0.5 μL
dNTP (2.5 mM)	4.0 μL (final 200 μM each)
Sterile MilliQ water	to make 50 μL

2. PCR program:

98°C	3 min	
98°C	10 sec	┐
55°C	5 sec	├ 30 cycles
72°C	1 min/kb	┘
72°C	10 min	
4°C	∞	

3. Purify the PCR product using Wizard® SV Gel and PCR Clean-Up System.
4. Subclone the PCR product into pPCR-Script using PCR-ScriptTM Amp Cloning Kit.
5. Confirm the sequence.

5.1.3. *Construction of the Tet-responsive expression vector*

To obtain a Tet-responsive expression vector, the cDNA prepared to express a full-length or a partial, a wild-type or a mutated, or an epitope-tagged or a non-tagged protein, is integrated at the given multiple cloning sites of the pTRE-IRES-neo vector (Figures 3B-D). Expression of a tagged-fusion protein is advantageous because it enables easy confirmation of the Tet-responsive expression of the transgenes by Western blot analysis using anti-tag antibody. Expression of green fluorescence proteins (GFP)-fusion also makes it possible to analyze localization and dynamics of the expressed proteins by microscopic observation even when the specific antibodies against the expressed proteins are not available. The pTRE-IRES-series vectors for FLAG-HA- or GFP-fusion can be obtained upon request (Figures 3C and 3D).

5.2. *Transfection and screening of drug-resistant colonies*

In the following protocol, the tTA expression vector and the TRE-responsive vector are simultaneously introduced into the cells to save time, although the protocol issued by Clontech recommends a sequential transfection of the two vectors (*www.clontech.com*; Cat. No. 630921, published in September 13, 2005). The method to deliver the vector into the cells is dependent on the cell type. Lipofection is effective for adherent cells, while electroporation works better for suspension cells. Nucleofection is a transfection method that enables efficient transfer of nucleic acids

into cells considered difficult or even impossible to transfect. Nucleofection uses a combination of optimized electrical parameters, generated by a special device called Nucleofector (Amaxa Inc.), with cell-type specific reagents (Amaxa Inc.). As standard electroporation is not efficient in some suspension cells, nucleofection is recommended for those cells. In this section, a protocol to introduce DNA into human pre-B Nalm-6 cells using Nucleofector is shown as an example.

Day 0:

1. Prepare healthy growing Nalm-6 cells.
2. Linearize the pCAG-tTA vector (Figure 3A) and pTRE-IRES-neo vector prepared in section *5.1.3.*, using a single cutting enzyme.

Day 1:

1. Perform phenol-chloroform extraction, ethanol precipitation and then rinse with ice-cold 70% ethanol.
2. Dry the DNA on a clean bench and suspend the DNA pellet in sterile MilliQ water.
3. Check the concentration of the DNA before transfection.
4. Pre-warm the complete culture medium (0.5 mL per sample) at 37°C.
5. Pre-warm Nucleofector Solution T at room temperature (RT).
6. Perform a cell viability check for Nalm-6 cells. *Note*: Cells for transfection should be healthy and 2 × 10^6 cells for each transfection are required.
7. Harvest the cells by centrifugation at 90 g for 5 min at 22°C and completely aspirate the supernatant (Be careful because cell pellets are less-tightly packed in this step!).
8. Suspend the cells with 100 µL of Nucleofector Solution T.
9. Add < 5 µg digested plasmid DNAs to the suspended cells (The molar ratio of the pCAG-tTA and pTRE-IRES-neo expression vector is roughly 5 to 1, and the total amount of DNA is limited to less than 5 µg for the Amaxa system).
10. Transfer the cell/DNA mixture into an Amaxa certified cuvette.
11. Perform transfection with Nucleofector I using the C-05 program of the machine.
12. Quickly add 500 µL pre-warmed culture medium into the cuvette and mix it well but slowly.
13. Transfer the cell/DNA mixture to a 6-well plate with a pipet.
14. Culture the cells in a humidified 5% CO_2 incubator at 37°C for 24 h.

Day 2:

1. Prepare the screening medium containing a selection drug at the appropriate concentration. *Note*: The optimal concentration of the drug should be determined whenever new lots of the drug are used!
2. Count the cell number to check the viability of the transfected Nalm-6 cells. *Note*: Around 80% cell viability is preferred.
3. Seed the cells at around 2,000~5,000 viable cells per well in the screening medium on 96-well flat-bottom plate.

Day 14-21

1. After 2~3 weeks of culturing, drug-resistant colonies were selected and transferred to new 96-well flat-bottom plates.
2. These cell colonies were grown for the preparation of SDS-PAGE samples.

5.3. *Confirmation of the TRE-responsive expression*

1. When there are colonies in the 96-well flat-bottom plate, the cell clones are transferred to a 6-well plate or 10-cm dish to allow cells to grow until there are more than 1 × 10^6 cells.
2. Count the number of the cells, harvest more than 1 × 10^6 cells in a 15-mL tube by centrifugation at 190 g for 3 min at 4°C, and carefully discard the supernatant.
3. Suspend the cell pellet in 1 mL PBS (-), transfer it to a 1.5-mL tube, centrifuge at 1,000 rpm (190 g) for 3 min at 4°C, and carefully discard the supernatant.
4. Suspend the cell pellet in 50 µL of 2× SDS sample buffer per 1 × 10^6 cells.
5. Incubate the cells at 95°C for 3 min. The samples are not very viscous at this concentration, but they can be sonicated in a water bath sonicator, if necessary.
6. Load 10 µL of heat-denatured sample into each lane (*Note*: Whole cell extracts derived from around 1~2 × 10^5 cells are appropriate amounts for 1 mm-thick SDS-PAGE and western blot analysis).
7. SDS-PAGE analysis and western blot analysis are performed as described (Molecular Cloning, 3rd edition).

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