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[32] Assaying the *Drosophila* Negative Feedback Loop with RNA Interference in S2 Cells

By PIPAT NAWATHEAN, JEROME S. MENET, and MICHAEL ROSBASH

Abstract

Transcriptional negative feedback loops play a critical role in the molecular oscillations of circadian genes and contribute to robust behavioral rhythms. In one key *Drosophila* loop, CLOCK and CYCLE (CLK/CYC) positively regulate transcription of *period* (*per*). The *period* protein (PER) then represses this transcriptional activation, giving rise to the molecular oscillations of *per* RNA and protein. There is evidence that links molecular oscillations with behavioral rhythms, suggesting that PER also regulates the expression of downstream genes, ultimately resulting in proper behavior rhythmicity. Phosphorylation of PER has also been shown to be critical for rhythms. DOUBLETIME (DBT) and casein kinase II (CKII) have been implicated in the phosphorylation of PER, which affects its stability as well as nuclear localization. We investigated the role of these kinases on PER transcriptional repression using the *Drosophila* S2 cell line in combination with RNA interference (RNAi) to knock down specific gene expression. This article describes the methods used to study PER repression activity in the S2 cell system as well as to exploit RNAi in this system. We also include protocols for immunocytochemistry and the application of leptomycin to differentiate direct effects on repression from indirect effects on subcellular localization. Finally, we discuss the generation of stable cell lines in the S2 cell system; these will be useful for experiments requiring homogeneous cell populations.

Introduction

Many eukaryotic and some prokaryotic organisms regulate their metabolism, physiology, and behavior with a circadian (~24-h) period. These

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rhythms have been shown to depend on complex feedback circuits involving transcriptional/translational regulation and occur in a diverse set of organisms, including cyanobacteria, fungi, plants, insects, and higher mammals such as mice and humans (Allada *et al.*, 2001; Panda *et al.*, 2002). The current model for the circadian pacemaker of *Drosophila melanogaster* posits two basic helix–loop–helix (bHLH) transcription factors, CLOCK (CLK) and CYCLE (CYC), which bind to upstream E boxes and activate the transcription of the *period* (*per*) and *timeless* (*tim*) genes as well as other direct target genes. The PERIOD and TIMELESS proteins (PER and TIM) are synthesized, associate in the cytoplasm, and the heterodimer is imported into the nucleus. The PER–TIM heterodimer then acts on CLK–CYC to inhibit *per* and *tim* transcription (Allada *et al.*, 2001; Panda *et al.*, 2002). A second feedback loop involves the two *Clk*-dependent transcription factors VRILLE and PDP1, and this loop drives *Clk* mRNA oscillations (Cyran *et al.*, 2003; Glossop *et al.*, 2003).

The molecular mechanisms through which PER and TIM repress the transcriptional activation of CLK/CYC are not yet well understood, although some evidence points to the importance of posttranslational modification of clock proteins (for review, see Allada *et al.*, 2001). There are two kinases in particular, DOUBLETIME (DBT) and casein kinase II (CKII), that make important contributions to circadian rhythmicity (Kloss *et al.*, 1998, 2001; Lin *et al.*, 2002; Martinek *et al.*, 2001; Price *et al.*, 1998). These kinases are imagined to function by phosphorylating clock proteins in a time-dependent manner and affecting protein stability as well as temporally gated nuclear localization of both PER and TIM (Curtin *et al.*, 1995; Dembinska *et al.*, 1997; Kim *et al.*, 2002; Shafer *et al.*, 2002; So and Rosbash, 1997). To decipher the precise role of these two kinases in the repression of CLK/CYC transcriptional activation, we took a reductionist approach and studied PER phosphorylation, nuclear localization, and transcriptional repression in a noncycling system, namely in cultured S2 cells. *cyc*, *ckII*, and *dbt* are expressed endogenously in S2 cells, whereas *Clk*, *per*, and *tim* are not (Darlington *et al.*, 1998).

Several S2 cell studies previously addressed CLK/CYC transcriptional activation and repression by PER/TIM (see later). They used expression vectors containing *Clk*, *per*, and/or *tim* genes together with a luciferase-expressing reporter gene. The reporter was preceded by a *per* or *tim* promoter or preceded by a set of artificial E boxes (Darlington *et al.*, 1998; McDonald *et al.*, 2001). CLK synthesis from an expression vector combines with endogenous CYC to drive transcription from the reporter gene, and then PER and/or TIM is assayed for its effect on transcriptional activity.

We also used this reporter system and focused on examining the effects of DBT and CKII on PER repression activity. In parallel, we also studied

the subcellular localization of PER. This is because the kinases have been proposed to affect subcellular localization, and this might be upstream of repression activity. We knocked down mRNA levels of these kinases by RNA interference (RNAi) and observed the effects on repression as well as nuclear localization with immunocytochemistry (ICC). To complement possible effects of RNAi on nuclear localization, we also utilized the nuclear export inhibitor leptomycin (LMB).

RNAi is a class of RNA-mediated gene silencing. In animals, double-stranded RNA (dsRNA) is detected, and a ribonuclease III (*Dicer*) cleaves the dsRNA into short pieces of 21–25 nucleotides called siRNAs (short interfering RNAs) (for review, see Agrawal *et al.*, 2003; Hannon, 2002; Novina and Sharp, 2004). siRNAs are recognized by the RNA-induced silencing complex (RISC). This ribonuclease complex unwinds the siRNA and helps anneal it to target mRNA, leading to its degradation. This mechanism is involved in viral defense mechanisms—viral dsRNA is detected and used to attack viral mRNA—as well as in development. Because RNAi exerts its effect on individual mRNAs, it has been used for the downregulation of specific gene expression. This is done by introducing siRNAs into mammalian cells with complementarity to an mRNA of interest. dsRNAs cannot be used in mammalian cells because they induce an interferon response (for review, see Mittal, 2004). This does not occur in S2 cells, so downregulation of gene expression can be achieved more simply, namely by just adding dsRNA to the cells; this was first demonstrated by Clemens *et al.* (2000). dsRNA enters S2 cells without any need for carrier or transfection agent; the effect is specific to the gene of interest and lasts for several days. This provides a convenient, effective, and long-lasting method of inhibiting gene expression.

Leptomycin was first isolated as an antifungal agent (Hamamoto *et al.*, 1983) and was later shown to be a nuclear export inhibitor (Wolff *et al.*, 1997). It has specificity for the CRM1–nuclear export receptor pathway (Ossareh-Nazari *et al.*, 1997). LMB has also been used successfully to inhibit protein export in *Drosophila* S2 cells (Abu-Shaar *et al.*, 1999), and the *Drosophila* homolog of *crm1* has been identified (Collier *et al.*, 2000; Fasken *et al.*, 2000). In the mammalian circadian system, mPER2 has been shown to be exported out of the nucleus via an LMB-sensitive pathway (Yagita *et al.*, 2002). *Drosophila* PER and TIM were subsequently shown to employ the same mechanism (Ashmore *et al.*, 2003; Nawathean and Rosbash, 2004).

As mentioned earlier, phosphorylation of PER by DBT and CKII has been proposed to be involved in PER degradation (by DBT) and nuclear localization (by both); however, the role of these kinases in regulating PER repression activity had not been investigated. We therefore made use of the

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Methodology

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S2 cell system to study CLK/CYC/PER transcriptional regulation and added the specific downregulation of these kinases by RNAi. We also examined the effects of the specific nuclear export inhibitor LMB on PER repression as well as subcellular localization. This article uses both transient transfection and stable transfection strategies in the S2 cell system to study these problems.

Methodology

Repression Assay in S2 Cells

All experiments were performed on *Drosophila* Schneider 2 (S2) cells. This S2 cell line was derived from a primary culture of late-stage (20–24 h old) *D. melanogaster* embryos. Many characteristics of the S2 cell line suggest that it is derived from a macrophage-like lineage (Schneider, 1972). S2 cells are grown at 25° without additional CO_2 on tissue culture flasks (75 cm^2) in an insect cell culture medium (HyQ SFX-Insect, Hyclone) containing 10% fetal bovine serum (FBS, GIBCO) and 1% antibiotic–antimycotic solution (GIBCO).

Vectors

Three promoters have been used to study CLK/CYC/PER transcription regulation: *per* and *tim* promoters (Darlington *et al.*, 1998) and the minimal sequence (CRS)-containing E-box promoter (Hao *et al.*, 1999; So *et al.*, 2000); all promoters were fused to *luciferase*. In general, these promoters behave similarly, i.e., they can be upregulated by CLK/CYC and downregulated by PER/TIM. There is, however, a smaller effect of PER/TIM in the case of the *per* or *tim* promoters, probably due to the presence of binding sites for other DNA-binding proteins within these natural promoters. In other words, the effect of PER is more pronounced on a promoter consisting of three repeats of the CRS sequence (69 nucleotides) of the *per* promoter and called p3 × 69-*luc* (So *et al.*, 2000); there is probably less of an effect from other transcription factors on this artificial promoter. *Clk* and *per* cDNAs were cloned into pAc V5/His6 (Invitrogen). They were in-frame with V5/His6 so that PER and CLK could be detected using the antiV5 antibody. These genes are driven by an *actin* promoter, which is constitutively active.

Choice of Transiently Transfected S2 Cells or Stable S2 Cell Lines

Most of the experiments were initially performed on transiently transfected S2 cells (Nawathean and Rosbash, 2004). Indeed, transient transfections allow an easy characterization of the PER repression parameters,

i.e., the relative amounts of the different plamids. Moreover, the use of dsRNA is particularly well suited for transient transfection. In contrast, stable cell lines are particularly useful when large amounts of material are desirable. For example, PER corepressors might be detectable by coimmunoprecipitation, and this is better done with stable lines.

Double-Stranded RNA

Double-stranded RNA was generated by *in vitro* transcription of both sense and antisense strands. The dsRNA is then incubated with S2 cells long enough to ensure efficient mRNA degradation and (hopefully) turnover of the encoded protein. In preparing dsRNA, care should be taken to avoid RNase contamination by wearing gloves and using DEPC-treated water. The dsRNA can be prepared in large scale and stored in aliquots at −20°.

Templates. Templates were amplified from either cDNA or genomic DNA. In the latter case, introns were avoided. Both forward and reverse primers contain a sequence complementary to the gene of interest as well as a T7 promoter sequence at the 5′ end of the primers for subsequent *in vitro* transcription by T7 polymerase (5′ TTAATACGACTCACTATAGGGAGA 3′). Polymerase chain reaction (PCR) products should be generally about 500–1000 bp in length (Worby *et al.*, 2001). Before continuing to the next step, we purify the PCR product with a Qiagen PCR purification kit and adjust the concentration to 125 ng/μl.

dsRNA Synthesis. The DNA template with a T7 promoter on both sense and antisense strands is used for *in vitro* transcription using an Ambion Megascript kit.

1. In a 20-μl reaction, mix 1 μg of the template (8 μl), 2 μl each of NTPs, 2 μl of reaction buffer (10X), and 2 μl of enzyme mix (containing T7 polymerase).
2. Mix gently and spin.
3. Incubate at 37° for 4–6 h or overnight.
4. Precipitate reaction at −20° for at least 30 min with 3 *M* sodium acetate to a final concentration of 10% and 2.5 volumes of 100% ethanol.
5. Centrifuge at 14,000 rpm for 15 min at 4°.
6. Remove the supernatant, and let the RNA pellet air dry for 10–15 min. Take care not to over dry, as it will be difficult to dissolve/resuspend the pellet.
7. Resuspend in 40 μl of DEPC water.
8. To anneal the sense and antisense RNA strands, denature by heating at 65° for 30 min and cool slowly by turning off the heat and

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letting the heating unit cool to room temperature. Measure the concentration at 260 nm (1 A_{260} = 45 μg/μl). Run a gel and check for RNA integrity and size (use ~1 μg for the gel).

9. Make a 3-μg/μl stock, aliquot, and store at −20°.

The normal yield of the *in vitro* transcription is approximately 150 μg per 20-μl reaction. The reaction volume can be scaled up.

Transient Transfection

Transfection of S2 cells can be performed using different reagents. We only used Cellfectin reagent (Invitrogen) in these studies, which is a liposome formulation. Transfection is generally performed when the S2 cells are seeded at 80% of confluence. In general, we also cotransfected the DNA with pCopia *renilla luciferase*, which serves as an internal control for transfection efficiency. Copia is a constitutively active promoter, and renilla luciferase requires a different substrate than firefly luciferase. Renilla luciferase activity can therefore be used for normalization to reduce variability of the results due to other factors.

Transfection

The following protocol is designed for one 35-mm well containing S2 cells.

1. In two 1.5-ml Eppendorfs prepare one tube with 100 μl of SFM (serum-free medium) + 10 μl Cellfectin (Invitrogen) and one tube with 100 μl of SFM + DNA.
2. Mix the two solutions gently in a 1.5-ml Eppendorf and wait 30 min at room temperature.
3. Add 800 μl of SFM.
4. Put this 1-ml solution in a well of a six-well plate pre-prepared with S2 cells. (Remove the culture medium in the well beforehand.)
5. Four hours later, add 1 ml of 20% FBS and 2% antibiotic–antimycotic medium. Incubate at 25° for 2–4 days.

Application of dsRNA

1. Plate the cells in SFM.
2. Add dsRNA 15 μg/2 ml of media in a six-well plate format, i.e., 5 μl RNA/well; mix well.
3. Incubate for 2–3 days (or more) for an efficient mRNA knockdown.

Usually, dsRNA is added 2 days before transfection, and cells are lysed 2 days after transfection. In our experience, 4 days of dsRNA is long

enough for an efficient RNA and protein knockdown. In general, we leave the cells in SFM during the incubation with dsRNA. Alternatively, 1 ml of SFM with the 15 μg of dsRNA can be used, to which 1 ml of 20% FBS-containing media is added 4 h later. This is preferable for longer incubation times with dsRNA, i.e., more than 3 days.

Use of Leptomycin

Leptomycin (Sigma) has been used successfully in a range of 10–400 n*M*. We generally use it at 20–100 n*M* with comparable results. To treat cells with LMB, we first remove the medium and then add new medium with an appropriate concentration of LMB and incubate for 8 h before proceeding to the next step, which is either a luciferase measurement or ICC.

Luciferase Bioluminescence Measurement in a Transient S2 Cell Culture

Both luciferase activities (firefly luciferase and *renilla* luciferase, which serves as an internal control; see earlier discussion) were quantified with the Dual-Luciferase reporter assay system (Promega) using a luminometer (#TD-20/20) from Turner Designs.

Protocol

1. Remove media.
2. Add 250 μl of lysis buffer per well (Promega). Shake for 10 min at room temperature.
3. Take liquid and centrifuge at 14,000 rpm for 10 min at 4°.
4. Take the supernatant and keep on ice.
5. In counting tubes, add 100 μl of luciferase substrate to 1–20 μl of the supernatant.
6. Quantify activity.
7. Add preprepared 100 μl Stop and Glo reagent (stock is 50×, dilute in Stop and Glo buffer).
8. Quantify the two luciferase activities.

Comparisons with Other Papers. The S2 cell system has been widely used to study circadian rhythm-relevant transcriptional regulation (e.g., see Ceriani *et al.*, 1999; Chang and Reppert, 2003; Darlington *et al.*, 1998; Nawathean and Rosbash, 2004; Rothenfluh *et al.*, 2000). Although results from different laboratories are largely consistent, there are some differences. For example, the role of TIM in modulating PER nuclear localization and PER repression activity is controversial and has been discussed elsewhere (Nawathean and Rosbash, 2004). Another quantitative difference between different studies is the strength of PER repression activity. For example, CLK:CYC transcriptional activity was reduced from 100% to 75% in one study (Darlington *et al.*, 1998) and reduced to less than 10% (10-fold)

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Transient S2 Cell Culture

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in another (Chang and Reppert, 2003). We believe that these differences are due to a number of factors. First, some reports used a fix amount of PER, which was probably suboptimal in those cases that reported weak repression activity. Second, a number of different reporter genes have been used. As described earlier, *per* and *tim* promoters probably contain other transcription factor-binding sequences, which increase background signal due to the activity of other S2 cell transcription factors. This has the effect of reducing potential PER repression activity. The artificial CRS (3 × 69) promoter sequence in contrast contains mainly, if not exclusively, CLK:CYC target E boxes. Third, experimental protocols have not been identical across different studies. For example, the amounts of the pAc*Clk* plasmid used range from 0.5 to 5 ng per well and, in some cases, the amount of plasmid was not even mentioned. Moreover, the amount of DNA needed for optimal transfection depends on the transfection reagent: 2 μg with Cellfectin and 20 μg with calcium phosphate per 1-ml reaction in our experience. Optimal conditions here are defined as the concentration of pAc*Clk* that gives maximal PER repression activity. In our experience, using a higher concentration of pAc*Clk* gives rise to lower PER repression activity. As a result, we suspect that some of the papers with lower PER activity simply used too high an amount of pAc*Clk*. This argument is just empirical (or a mix of empirical and guesswork), as the mechanism by which PER represses CLK/CYC transcription activation is not known. Therefore, it is not known what an optimal relationship between PER and CLK should be.

Immunocytochemistry Protocol

ICC is used to observe the subcellular localization of the protein of interest, in this case PER. We first seeded S2 cells in a six-well plate onto coverslips, which allows the convenient final mounting of the cells onto a slide. These cells can then be used for treatment with dsRNA, transfection, and/or incubation with LMB. Once ready for immunostaining, cells are fixed, blocked, and incubated with primary antibody. The subsequent secondary antibody is normally conjugated with fluorescence dye (for example, FITC). We use mounting medium containing DAPI, which will stain DNA, for ease of nuclear detection.

1. Seed S2 cells overnight or at least 2 h in serum-free media in six-well plates containing coverslips. This plate can then be used for the transfection and other treatments.
2. All the staining processes are also done in the six-well plate. Once the cells are ready for ICC, remove the medium and wash once briefly with 1 ml of cold phosphate-buffered saline (PBS).
3. Fix the cell with 1 ml 4% paraformaldehyde in PBS for 5 min at room temperature.

4. Wash three times for 10 min with cold PBS.
5. Remove the PBS and add 1 ml blocking solution (10% normal goat serum, 0.2% Triton X-100 in PBS). Place the plate on rocker at room temperature for 30 min.
6. Remove the blocking solution and add primary antibody (mouse antiV5—Invitrogen 1:500 in blocking solution). Incubate on the rocker at room temperature for 1–2 h.
7. Remove the primary antibody and wash the cell five times, each for 2 min, with cold PBS.
8. Add secondary antibody (goat antimouse FITC—Jackson research laboratory—1:200 in blocking solution; FITC excitation and emission wavelength—492/520 nm) and wrap the plate with aluminum foil to protect from light. Incubate on the rocker at room temperature for 1 h.
9. Remove the secondary antibody and wash 2 × 5 min with cold PBS, with aluminum foil wrapped around the plate. Mount onto a glass slide with mounting agent plus DAPI (Vector).

Generating Stable Cell Lines

Generality

To generate stable S2 cell lines, the plasmid(s) of interest first needs to be cotransfected with a selection vector, which confers drug resistance. Cells that have stably integrated the DNA are selected by applying the drug to the culture medium 4 days after the transfection. Duration of the selection depends on the selection vector used. We have used two different selection vectors shown to be appropriate for use with the *Drosophila* Expression system (DES, Invitrogen): pBSPuro (gift from M. Wilm, Heidelberg, Germany; Benting *et al.*, 2000) and pCoBlast (Invitrogen), which confer resistance to puromycin and blasticidin, respectively. A selection vector conferring hygromycin resistance (pCoHygro; Invitrogen) can also be used, but selection usually takes longer in this case (4 weeks after cotransfection, according to Invitrogen and McDonald *et al.*, 2001). With puromycin and blasticidin, only 2 weeks of selection are usually required to obtain a stable cell line. However, it may take somewhat longer depending on the transfection efficiency and cell density. When the selection is complete, the drug concentration in the medium can be decreased by a factor of two. Removing the drug completely should be avoided. This is because transgene silencing of multicopy repeats (what is usually the case in this kind of selection) can occur by a phenomenon still not well understood. In this case, drug reapplication after some weeks without treatment can induce death of almost all the cells.

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Using stable cell lines to study the repression of PER on CLK/CYC transcriptional activation requires some care. In our limited experience, this is because the amount of PER repression is highly dependent on the characteristics of the cell line. For this reason, we recommend two consecutive selections: (1) with pAc*Clk* and the reporter p3×69*luc*, to maximize CLK/CYC transcriptional activation of the reporter, and (2) with variable amounts of pA*cper*, to optimize repression. The decrease in luciferase activity after the second selection can then be directly correlated to the repression of PER on CLK/CYC transcriptional activation. The following protocol can be used for both types of selection (pBSPuro and pCoBlast).

Selection Protocol

Preparation of Plate

1. Culture S2 cells in 10% FBS and 1% antibiotic-antimycotic medium (call FBS medium) in a 75-cm^2 flask.
2. Remove the old medium and add 10 ml of fresh FBS medium.
3. Scrape the cells using a cell scraper (Corning).
4. Aspirate the 10 ml of culture medium containing the cells, count the cells using a hematocymeter, and dilute to 1×10^6 cells/ml with FBS medium.
5. Plate the cells in a six-well culture plate (Corning). Two milliliters containing 1×10^6 cells/ml is usually plated in one well.

Transfection. All transfections are performed using Cellfectin reagent (Invitrogen), which is well suited for insect cells. S2 cells are transfected with the plasmid(s) of interest and the selection vector. In the case of pBSPuro, we recommend a 5:1 ratio between reporter:selection vector. In the case of pCoBlast, the manufacturer recommendation ratio of 19:1 is fine. Decreasing the ratio generally causes an increase in the number of clones but a decrease in the incorporation of reporter into each clone, and increasing the ratio does the reverse. We recommend doing the transfection in a few wells (two to three) to increase the number of selected cells; two identical wells are usually sufficient.

Transfection is performed as described previously for transient transfection.

1. For one 35-mm well, mix 12 μl of Cellfectin and 88 μl of serum-free medium (SFM; Cellfectin at a final concentration of 12%) in a sterile 1.5-ml microcentrifuge tube or a culture tube.
2. In another tube, mix DNA (reporter and selection vector) in 100 μl of SFM.
3. Wait 10 min and then mix the two solutions gently.
4. Wait 30 min at room temperature.

5. Add 800 μl of SFM.
6. Remove the FBS medium from the wells.
7. Apply the 1 ml medium containing DNA and Cellfectin in a 35-mm well.
8. Four hours later, add 1 ml of 20% FBS, 2% antibiotic-antimycotic medium. Incubate at 25° for 3 days.

Selection. This step consists of killing S2 cells that have not stably integrated the selection vector by adding drug to the culture medium. Drug concentrations are 10 μg/ml for puromycin (http://www.mann.embl_heidelberg.de/GroupPages/PageLink/activities/iTAP/Experimental/StableCellLine.html) and 25 μg/ml for blasticidin (Invitrogen). Both drugs are dissolved in sterile distilled water at a concentration of 10 mg/ml for puromycin and 25 mg/ml for blasticidin. The stock solutions are then sterilized by filtration through a 0.22-μm pore, aliquoted in sterile tubes, and frozen at −20° until use. Stock solutions are not subject to multiple freeze/thaw cycles. After the stable lines are selected, drug concentrations are decreased by half (5 μg/ml for puromycin and 12.5 μg/ml for blasticidin).

1. Three to 4 days after transfection, replace the old FBS medium with 1 ml of fresh FBS medium containing puromycin or blasticidin at a concentration of 10 μg/ml for puromycin and 25 μg/ml for blasticidin.
2. Scrape the cells and transfer them into a 1.5-ml microcentrifuge tube.
3. Harvest the cells by spinning at 3000 rpm for 5 min at room temperature.
4. Remove the supernatant and add for a wash 1 ml of 10% FBS + same drug concentration.
5. Harvest the cells a second time at 3000 rpm for 5 min at room temperature.
6. Remove the supernatant and resuspend the cells in 1.5 ml of 10% FBS + drug.
7. Transfer the cells to a 25-cc flask.
8. Change the medium + antibiotic every 3–4 days for 2 weeks.

Application of dsRNA to Stable Cell Lines. As described earlier for transient transfections, dsRNA can just be added to the culture medium to efficiently silence a target gene. Nonetheless, we observed that silencing efficiency is generally improved if the dsRNA is transfected like the DNA, i.e., accompanied by transfection reagent.

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[33] Role
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By GABRIEI

Abstract

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Introduction

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