USER GUIDE

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BLOCK-iT™ RNAi Target Screening System

A Gateway®-adapted destination vector for rapid screening of siRNA, Stealth RNAi®, or shRNA target sequences for RNAi analysis in mammalian cells

Catalog number V470-20

Revision date 2 March 2012
Publication Part number 25-0723

MAN0000453

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

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Contents

Kit Contents and Storage

Kit Components

This manual is supplied with the pSCREEN-iT™/lacZ-DEST Gateway® Vector Kit (Cat. no. V470-20). The following reagents are supplied with the kit.

Reagent	Composition	Amount
pSCREEN-iT™/lacZ-DEST	150 ng/µL in: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.	6 µg
pSCREEN-iT™/lacZ-GW/CDK2 Control Vector	0.5 µg/µL in: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 20 µL.	10 µg
Positive lacZ Stealth RNAi® Control	20 µM Stealth RNAi® in: 10 mM Tris-HCl, pH 8.0 20 mM NaCl 1 mM EDTA, pH 8.0	125 µL
Scrambled Negative Stealth RNAi® Control	20 µM Stealth RNAi® in: 10 mM Tris-HCl, pH 8.0 20 mM NaCl 1 mM EDTA, pH 8.0	125 µL
1X RNA Annealing/Dilution Buffer	10 mM Tris-HCl, pH 8.0 20 mM NaCl 1 mM EDTA, pH 8.0	1 mL

Shipping and Storage

The pSCREEN-iT™/lacZ-DEST Gateway® Vector Kit is shipped on dry ice. Upon receipt, **store all reagents at –20°C**.

Additional Reagents for Screening

The following products can be used for screening with the pSCREEN-iT™/lacZ-DEST Gateway® Vector Kit. For more detailed information about each product, including a description of the reagents, refer to the product manual. For ordering information, see page 44.

Product
FluoReporter® lacZ/Galactosidase Quantitation Kit
Lipofectamine® 2000 Reagent
Gateway® LR Clonase® II Enzyme Mix
pCR™8/GW/TOPO® TA Cloning Kit

Product Use

For research use only. Not intended for any human or animal therapeutic or diagnostic use.

Introduction

Product Overview

Introduction

The BLOCK-iT™ RNAi Target Screening System uses a *lacZ*-based reporter vector that is specifically designed to facilitate accurate and sensitive screening of RNAi molecules targeted towards a gene of interest in mammalian cells. The reporter vector is adapted with Gateway® Technology to allow easy generation of a screening construct containing your target gene or sequence of interest fused to the *lacZ* reporter gene. The screening construct is then cotransfected with the RNAi molecule into mammalian cells, and target gene knockdown assessed by measuring β-galactosidase reporter readout. The System is suitable for screening a variety of RNAi molecules including double-stranded RNA (dsRNA) oligomers (*i.e.* Stealth RNAi® or siRNA) or plasmids expressing short hairpin RNA (shRNA).

For more information about the Gateway® Technology, see page 2.

Advantages of the BLOCK-iT™ RNAi Target Screening System

Use of the BLOCK-iT™ RNAi Target Screening System to facilitate screening of RNAi molecules targeted towards a gene of interest provides the following advantages:

- Uses a reporter vector to provide a rapid and efficient way to screen and assess the effectiveness of a wide variety of RNAi molecules including siRNA, Stealth RNAi®, or shRNA-expressing plasmids targeted towards a gene of interest.
- The pSCREEN-iT™/lacZ-DEST reporter vector facilitates fusion of your target gene or sequence of interest to the *lacZ* reporter, allowing accurate and highly sensitive readout of target RNA knockdown without the need for antibodies to the target protein or prior knowledge of the knockdown phenotype.
- The System is sensitive enough to discriminate between highly active (*i.e.* induces > 85% target RNA knockdown) and moderately active (*i.e.* induces 60–85% target RNA knockdown) RNAi molecules.
- Target gene knockdown can be analyzed in common, easily transfected cell types, even those that do not express the target endogenously.
- The level of target RNA knockdown observed with an RNAi molecule in the screening system correlates with the level of endogenous mRNA knockdown attained as measured by qRT-PCR, thus eliminating the need for specialized equipment and validated primer sets.
- The pSCREEN-iT™/lacZ-DEST reporter vector is Gateway®-adapted for easy recombinational cloning of any target gene or sequence of interest from an entry clone, including Ultimate™ ORF Clones.
- Analysis can be carried out within 24 hours of transfection, allowing essential or toxic genes to be targeted over a short enough time period to permit cell survival.

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The Gateway® Technology

Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems. The reporter vector in the BLOCK-iT™ RNAi Target Screening System is adapted with Gateway® Technology to facilitate generation of a screening construct. To generate the screening construct, simply:

1. Clone your target gene or sequence of interest into pCR™8/GW/TOPO® or any other suitable Gateway® entry vector to create an entry clone. Alternatively, obtain an Ultimate™ ORF Clone containing your target gene of interest.
2. Perform an LR recombination reaction between the entry clone and the pSCREEN-iT™/lacZ-DEST reporter vector to generate the screening construct.
3. Proceed to your screening experiment.

For detailed information about the Gateway® Technology, refer to the Gateway® Technology with Clonase® II manual which is available from www.lifetechnologies.com/support or by contacting Technical Support (see page 45).

BLOCK-iT™ RNAi Products

A large selection of BLOCK-iT™ RNAi products is available for purchase to facilitate RNAi analysis in mammalian and invertebrate systems including those that:

- Facilitate production and expression of shRNA molecules in mammalian cells. These vector-based systems allow constitutive or regulated expression of shRNA molecules in mammalian cells.
- Facilitate expression of shRNA molecules in any mammalian cell type. Adenoviral and lentiviral vectors are available to allow transient or stable shRNA expression, respectively, in dividing or non-dividing mammalian cells.
- Facilitate production and delivery of synthetic short interfering RNA (siRNA), diced siRNA (d-siRNA), or double-stranded RNA (dsRNA) for RNAi analysis in mammalian cells or invertebrate organisms, as appropriate.

A variety of other products to aid your RNAi research are available. For more information about any of the BLOCK-iT™ RNAi products and other RNAi resources, see the RNAi Central application portal at www.lifetechnologies.com/rnai.

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Purpose of this Manual

This manual provides an overview of the BLOCK-iT™ RNAi Target Screening System and provides instructions and guidelines to:

1. Perform an LR recombination reaction between the pSCREEN-iT™/lacZ-DEST vector and a suitable entry clone containing the target gene or sequence of interest to generate a screening construct.
2. Co-transfect the pSCREEN-iT™/lacZ-DEST screening construct and an RNAi molecule targeting the gene of interest into mammalian cells.
3. At an appropriate time after transfection, harvest cells and assay for β-galactosidase activity to determine the efficacy of the RNAi molecule in inducing target gene knockdown.

For guidelines to generate a suitable entry clone, see page 11. For detailed instructions, refer to the manual for the entry vector you are using. If you are using the pCR™8/GW/TOPO® vector to generate an entry clone, refer to the pCR™8/GW/TOPO® TA Cloning Kit manual. This manual is available for downloading from www.lifetechnologies.com/support or by contacting Technical Support (see page 45).

Note

The LR Clonase® II Enzyme Mix and Lipofectamine® 2000 Reagent included in the BLOCK-iT™ RNAi Target Screening System are available separately for purchase and are supplied with individual documentation detailing general use of the product. **For instructions to use these products specifically with the BLOCK-iT™ RNAi Target Screening System, follow the recommended protocols in this manual.**

Important

The BLOCK-iT™ RNAi Target Screening System is designed to help you screen and identify effective RNAi molecules targeted to a particular gene of interest. Although the system has been designed to help you screen RNAi molecules in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the principles of gene silencing and the various types of RNAi effector molecules including siRNA, Stealth RNAi® duplexes, and short hairpin RNA (shRNA). We highly recommend that users possess a working knowledge of tissue culture techniques, lipid-mediated transfection, and the RNAi pathway. For more information about the following topics, refer to these published references:

- RNAi pathway: see Dykxhoorn *et al.*, 2003 and Hannon, 2002
- Stealth RNAi®: see www.lifetechnologies.com/rnai
- siRNA: see Caplen *et al.*, 2001; Elbashir *et al.*, 2001; Elbashir *et al.*, 2001
- DNA-based shRNA expression vectors: see Brummelkamp *et al.*, 2002; Czauderna *et al.*, 2003; Paddison *et al.*, 2002

The BLOCK-iT™ RNAi Target Screening System

Introduction

Many groups have demonstrated that specific RNAi molecules targeting different regions of a transcript can vary widely in their effectiveness at inducing gene silencing (Bohula *et al.*, 2003; Holen *et al.*, 2002; Kawasaki *et al.*, 2003; Vickers *et al.*, 2003). Although significant improvements have been made in the design rules used to select effective RNAi molecules (Schwarz *et al.*, 2003), testing the efficacy of each RNAi molecule is currently a tedious and time-consuming process. We have developed the BLOCK-iT™ RNAi Target Screening System to provide a means to easily and empirically compare RNAi molecules for their effectiveness at inducing target gene knockdown. This system is based on transfection and does not require prior knowledge of the cellular knockdown phenotype, antibodies to detect target protein, or specialized equipment as would be needed to perform other types of RNAi analysis.

Components of the System

The BLOCK-iT™ RNAi Target Screening System facilitates rapid and accurate screening of RNAi molecules targeted against a gene of interest for RNAi analysis. The System includes the following major components:

1. The pSCREEN-iT™/lacZ-DEST Gateway® destination vector into which the target gene or sequence of interest will be transferred via LR recombination reaction. The resulting screening construct allows expression of your gene of sequence of interest as a *lacZ* fusion transcript. For more information about the pSCREEN-iT™/lacZ-DEST vector, see page 8 and pages 41–42.
2. Positive and negative control Stealth RNAi® molecules that may be included in the screening experiment to verify the functionality of the system. For more information about the Stealth RNAi® molecules, see page 8.
3. 1X RNA Annealing/Dilution Buffer for dilution of your RNAi molecule stock solutions, as needed to obtain optimal transfection and screening results.

The following products can be used as part of the BLOCK-iT™ RNAi Target Screening System, and can be purchased separately (see page 44 for ordering):

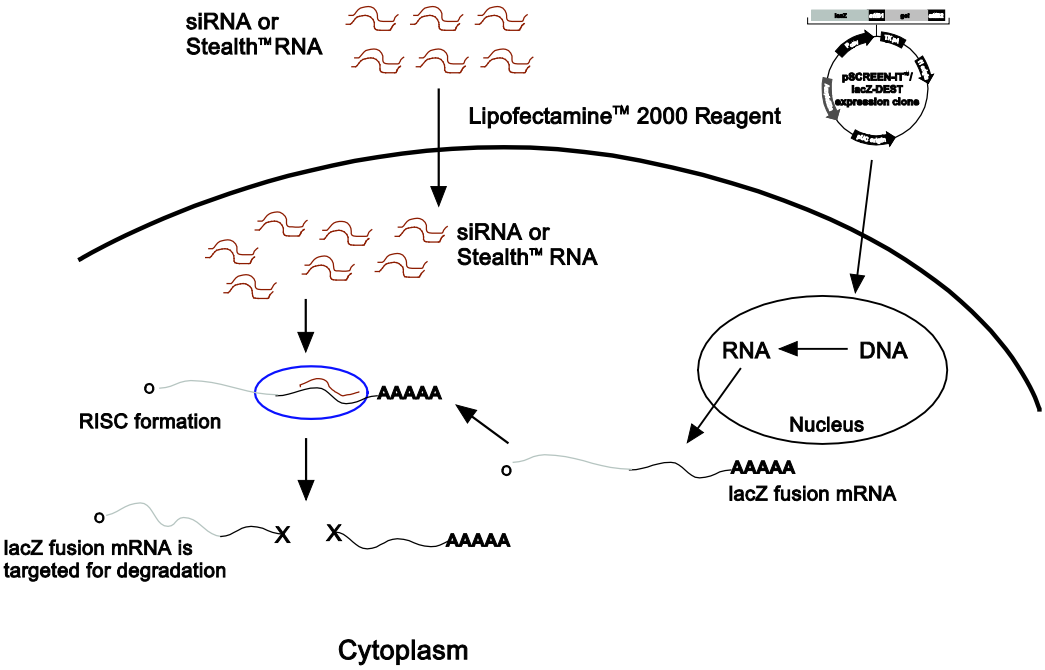
1. The pCR™8/GW/TOPO® TA Cloning Kit containing the pCR™8/GW/TOPO® vector for production of an entry clone. The vector is TOPO®- and Gateway®-adapted to allow rapid, 5-minute TOPO® Cloning of a *Taq* polymerase-amplified PCR product encoding your target gene or sequence of interest, and easy transfer of the target into the pSCREEN-iT™/lacZ-DEST reporter vector, respectively. For detailed information about the pCR™8/GW/TOPO® vector, refer to the pCR™8/GW/TOPO® TA Cloning Kit manual.
2. LR Clonase® II Enzyme Mix to facilitate transfer of the target gene of interest into pSCREEN-iT™/lacZ-DEST.
3. Lipofectamine® 2000 Reagent for highly efficient delivery of the screening construct and the corresponding RNAi molecule to mammalian cells.
4. FluoReporter® lacZ/Galactosidase Quantitation Kit containing an improved fluorogenic substrate for highly sensitive detection of β-galactosidase activity. For more information about the kit, see page 9.

Once you have generated your pSCREEN-iT™/lacZ-DEST screening construct, you will cotransfect the vector with an RNAi molecule of interest into mammalian cells and assay for target gene knockdown by measuring β-galactosidase readout. For more information about how the System works, see the next page.

Continued on next page

How the System Works

In the BLOCK-iT™ RNAi Target Screening System, you will clone your target gene or sequence of interest downstream of the *lacZ* gene to generate a screening construct. Transfection of the screening construct into mammalian cells allows expression of a fusion *lacZ* transcript. Simultaneous delivery of an active RNAi molecule to the cells induces cleavage of the *lacZ* fusion transcript, which is then measured by the resulting reduction in β-galactosidase reporter expression and activity (see figure below). The system utilizes the RNAi machinery in mammalian cells but does not require that the target gene be endogenously expressed. This provides the added benefit that target knockdown can be analyzed in common, easily transfected cell types. Finally, analysis is typically carried out within 24 to 48 hours following transfection, allowing essential or toxic genes to be targeted over a short enough time period to permit cell survival.



RNAi Molecules

The BLOCK-iT™ RNAi Target Screening System may be used to screen various types of RNAi molecules including:

- Stealth RNAi® duplexes
- siRNA
- Plasmids expressing short hairpin RNA (shRNA)

To design and synthesize Stealth RNAi® duplexes, siRNA, or shRNA oligos for use in RNAi analysis, use the online RNAi Designer tool available at www.lifetechnologies.com/rnai.

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Sensitivity of the System

When using the BLOCK-iT™ RNAi Target Screening System to screen a panel of RNAi molecules targeting a particular gene of interest, you may assess the potency of each RNAi molecule based on its effectiveness in inducing β-galactosidase knockdown. We generally categorize the efficacy of RNAi molecules as follows:

- Highly active RNAi molecule – induces > 85% target gene knockdown
- Moderately active RNAi molecule – induces 60–85% target gene knockdown
- Inactive RNAi molecule – induces < 60% target gene knockdown

The sensitivity of the System is such that within a certain class of RNAi molecules, one can identify those that are the most potent in inducing target gene knockdown. For example, among highly active RNAi molecules, this System can distinguish between those that induce 85%, 90%, or 95% target gene knockdown.

Target Sequence Options

When generating your pSCREEN-iT™/lacZ-DEST screening construct for use in screening RNAi molecules, you may fuse any target sequence to the *lacZ* reporter including:

- A sequence encoding the gene of interest (*i.e.* open reading frame (ORF)) **or**
- 5′ or 3′ untranslated region (UTR) of the target gene

If you are fusing an ORF to the *lacZ* gene, we recommend fusing the target sequence in frame with the reporter so that a β-galactosidase fusion protein will be expressed. See the discussion below and on page 7 for the reasons why we recommend this option.

Size of the Target Gene

You may fuse a target sequence of any size to the *lacZ* reporter in pSCREEN-iT™/lacZ-DEST; however, addition of amino acids from the target protein to the C-terminus of β-galactosidase can affect the expression levels and activity of the β-galactosidase fusion protein. How much so will depend on the nature and length of the target protein. In some cases, you may not observe any detectable β-galactosidase fusion protein expression from the pSCREEN-iT™/lacZ-DEST screening construct following transfection. If so, you may want to try fusing a shorter region of the target gene (*i.e.* 200 bp to 1 kb) to *lacZ* or placing a stop codon between *lacZ* and the target gene of interest to create an RNA-only fusion.

Note: There are a number of advantages and disadvantages associated with creating an RNA-only fusion (see discussion on the next page). When considering this option, carefully weigh the advantages and disadvantages associated with using this approach.

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Advantages to Creating an RNA-Only Fusion

In limited instances (*e.g.* no β-galactosidase fusion protein expressed when fusing your target gene in frame with the *lacZ* reporter in pSCREEN-iT™/lacZ-DEST), you may want to generate a screening construct that expresses an RNA-only fusion by placing a stop codon between *lacZ* and the target gene. Expression of a *lacZ* fusion transcript offers the following advantages over expression of a protein fusion:

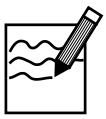
- β-galactosidase protein is more likely to be expressed since the only amino acids that are added to the C-terminus of β-galactosidase are those contributed by the *att*B1 site (see the diagram on page 14). Because the amount of β-galactosidase expressed depends in part on the stability of the fusion transcript, note that the amount of β-galactosidase protein expressed may still vary from screening construct to screening construct.
- Since no part of the target mRNA would be translated into protein, no pleiotropic effects due to overexpression of the target gene should be observed.
- Expression of an RNA-only fusion obviates the need to position the inserted gene or gene fragment of interest in frame with *lacZ*.

Important: While there are a number of advantages to expressing an RNA-only fusion, there is also a disadvantage associated with this option (see below).

Disadvantage to Creating an RNA-Only Fusion

While expression of a *lacZ*/target gene RNA-only fusion may be desirable for target screening in some cases, this approach also has a disadvantage. We have observed that the apparent knockdown achieved with a particular RNAi molecule can be negatively affected by the distance between the stop codon and the target site of the RNAi molecule. That is, the farther away the target site from the stop codon, the lower the percentage of β-galactosidase knockdown observed, even with RNAi molecules that are known to be highly active. This phenomenon could result in ranking of effective RNAi molecules as ineffective simply because the target site is distal to the *lacZ* stop codon. This trend is consistent with the hypothesis that mRNA transcripts cleaved by the RISC in the 3' UTR can produce functional protein while being slowly degraded by exonucleases. Under this model, as the distance from the stop codon increases, so does the time it takes for the degradation to reach the protein coding region. Note that many other factors can also affect fusion transcript stability. **Because of this disadvantage, we recommend fusing the target gene in frame with *lacZ* (to express the fusion protein) whenever possible.**

Note: The difference in apparent knockdown achieved with a particular RNAi molecule targeted against a *lacZ* RNA-only fusion transcript or a fusion protein expressed from the screening construct is minimal when the target sequence is ≤ 300 bp.



Note

Screening data obtained with RNAi molecules targeted to regions distal to the *lacZ* junction in a screening construct expressing a *lacZ* RNA-only fusion transcript does not correlate as well with qRT-PCR analysis (of the endogenous transcript) as does screening vector data obtained with the same RNAi molecules in a screening construct expressing a fusion protein.

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Features of the pSCREEN-iT™/lacZ-DEST Vector

The pSCREEN-iT™/lacZ-DEST vector contains the following features:

- Human CMV promoter for high-level, constitutive expression of the *lacZ*/target gene fusion
- *lacZ* gene that is fused to the target gene of interest and functions as a reporter for target gene knockdown following delivery of the screening construct and RNAi molecule to mammalian cells
- Two recombination sites, *att*R1 and *att*R2, downstream of the *lacZ* gene for recombinational cloning of the target gene of interest from an entry clone
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- The *ccd*B gene located between the *att*R sites for negative selection
- pUC origin for high-copy replication of the plasmid in *E. coli*
- Ampicillin resistance gene for selection in *E. coli*

Note

Note that the pSCREEN-iT™/lacZ-DEST vector does not contain a selectable marker. The screening construct containing your gene of interest can **only** be used in transient screening experiments, and not to generate stable cell lines.

Control Stealth RNAi® Duplexes

The BLOCK-iT™ RNAi Target Screening System includes the Positive lacZ Stealth RNAi® Control and the Scrambled Negative Stealth RNAi® Control for use as positive and negative controls for *lacZ* reporter gene knockdown in mammalian cells. The Positive lacZ Stealth RNAi® molecule is targeted to and downregulates *lacZ* mRNA while the Scrambled Negative Stealth RNAi® molecule does not target any human gene and induces minimal knockdown in mammalian cells. Because it is targeted to *lacZ*, the Positive lacZ Stealth RNAi® Control may be used as a positive control for β-galactosidase knockdown in every screening experiment irregardless of the target gene. For more information about Stealth RNAi®, see the next page.

Note: In GripTite™ 293 MSR cells, the Positive lacZ Stealth RNAi® Control is a moderately active RNAi molecule, inducing 70–80% knockdown of β-galactosidase.

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Stealth RNAi®

Stealth RNAi® is chemically modified dsRNA developed to overcome the limitations of traditional siRNA. Using Stealth RNAi® for RNAi analysis offers the following advantages:

- Produces effective target gene knockdown at levels that are equivalent to or greater than those achieved with traditional siRNA
- Reduces non-specific effects caused by induction of cellular stress response pathways
- Exhibits enhanced stability for greater flexibility in RNAi analysis

For more information about Stealth RNAi®, see the RNAi Central application portal at www.lifetechnologies.com/rnai or contact Technical Support (see page 45). To design and order Stealth RNAi® duplexes for your target genes, see the RNAi Designer at www.lifetechnologies.com/rnai.

FluoReporter® lacZ/Galactosidase Quantitation Kit

The FluoReporter® lacZ/Galactosidase Quantitation Kit can be used with the BLOCK-iT™ RNAi Target Screening System to facilitate highly sensitive measurement of β-galactosidase activity in solution or in cell extracts prepared from cells expressing the lacZ/target gene fusion from your pSCREEN-iT™/lacZ-DEST screening construct (see page 44 for ordering). The kit uses an improved fluorogenic substrate, 3-carboxy-umbelliferyl β-D-galactopyranoside (CUG) to allow higher aqueous solubility and increased fluorescence efficiency. This results in a lower threshold of β-galactosidase detection (*i.e.* 0.5 picograms) over that normally achieved with the more commonly used 4-methylumbelliferyl β-D-galactopyranoside (MUG) substrate.

How the FluoReporter® Kit Works

To use the FluoReporter® Kit, you will add the CUG substrate and an aliquot of cell extract to a well in a 96-well microtiter plate. The β-galactosidase catalyzes the enzymatic cleavage of the CUG substrate to 7-hydroxycoumarin-3-carboxylic acid, a highly fluorescent product (λ_{ex} = 386 nm, λ_{em} = 448 nm). The fluorescence of the sample can be quantitated in a fluorescence microplate reader equipped with an excitation filter centered at 390 nm and an emission filter centered at 460 nm.

Experimental Outline

Experimental Outline

The table below describes the general steps required to generate a pSCREEN-iT™/lacZ-DEST screening construct, and to use the screening construct to screen a set of RNAi molecules for target gene knockdown.

Step	Action	Pages
1	Generate or obtain a Gateway® entry clone containing your target gene or sequence of interest. **Note:** You may use an Ultimate™ ORF clone available for purchase or generate your own entry clone by cloning the gene of interest into pCR™8/GW/TOPO® or another suitable entry vector.	11–12
2	Perform an LR recombination reaction between pSCREEN-iT™/lacZ-DEST and the entry clone containing your target gene or sequence of interest to generate a screening construct.	13–17
3	Purify plasmid DNA from your pSCREEN-iT™/lacZ-DEST screening construct.	21
4	Cotransfect the pSCREEN-iT™/lacZ-DEST plasmid and your RNAi molecule into mammalian cells.	18–26
5	Harvest cells 24–48 hours following transfection and prepare a cell lysate.	27
6	Assay the cell lysate for β-galactosidase activity.	28–32

Methods

Generating an Entry Clone

Introduction

To recombine your gene of interest into pSCREEN-iT™/lacZ-DEST, you will first need to generate an entry clone containing the target gene or sequence of interest using one of the options discussed below. General guidelines are provided in this section to help you generate the entry clone.

Options to Generate an Entry Clone

A number of options exist to generate an entry clone containing your target gene or sequence of interest. Choose the option that best fits your needs.

Option	Procedure
1	Use an existing Gateway® entry clone containing your target gene of interest or one of the Ultimate™ ORF Clones. **Note:** Entry clones containing an RNAi cassette that is generated in the BLOCK-iT™ pENTR™/U6 or pENTR™/H1/TO vector **are not suitable** for use in this application. However, these shRNA-expressing plasmids may be used as RNAi knockdown reagents.
2	Use the pCR™8/GW/TOPO® TA Cloning Kit to generate the entry clone. The pCR™8/GW/TOPO® vector facilitates simple generation of an entry clone using a 5-minute TOPO® Cloning reaction with a *Taq* polymerase-amplified PCR product. **Note:** The pCR™8/GW/TOPO® TA Cloning Kit is supplied with Cat. no. K4916-00, but is also available separately for purchase (see page 44 for ordering information).
3	Use another suitable Gateway® entry vector to generate the entry clone. See www.lifetechnologies.com/support or contact Technical Support for more information about the many other entry vectors available for purchase.

Ultimate™ ORF Clones

If you wish to target a human or murine gene of interest, we recommend using an Ultimate™ Human ORF (hORF) or Mouse ORF (mORF) Clone, respectively, available for purchase. Each Ultimate™ ORF Clone is a fully-sequenced clone provided in a Gateway® entry vector that is ready-to-use in a Gateway® LR recombination reaction with pSCREEN-iT™/lacZ-DEST. For more information about the Ultimate™ ORF Clones available, see www.lifetechnologies.com/support or contact Technical Support (see page 45).

Note: If you use an Ultimate™ ORF Clone in an LR recombination reaction with pSCREEN-iT™/lacZ-DEST, the gene of interest will be cloned in frame with the *lacZ* reporter gene.

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Insert Requirements

For compatibility with the BLOCK-iT™ RNAi Target Screening System, keep the following in mind when generating an insert to clone into an appropriate entry vector:

- The gene of interest **should** be in frame with the N-terminal *lacZ* ORF after recombination with the pSCREEN-iT™/lacZ-DEST vector.

  Tip: If you are producing a PCR product to clone into an entry vector (*e.g.* pCR™8/GW/TOPO®), design the forward PCR primer such that the translation reading frame of the PCR product is in the same frame as the –AAA-AAA- triplets in the *att*L1 site of the entry vector. **Note that the first three base pairs of the PCR product should constitute a functional codon.** For more information, refer to the manual for the entry vector you are using.
- If you want to express an RNA-only fusion after recombination with the pSCREEN-iT™/lacZ-DEST vector, add a stop codon to the beginning of your insert.
- Although you will be fusing your protein to the N-terminal *lacZ* ORF after recombination with the pSCREEN-iT™/lacZ-DEST vector, you may include the ATG initiation codon for your protein in the insert. Inclusion of a Kozak consensus sequence is not necessary.
- Make sure that your gene of interest contains a stop codon for proper translation termination of the β-galactosidase fusion protein.

  Note: If you do not include a stop codon in your insert, note that stop codons in two reading frames are present in the pSCREEN-iT™/lacZ-DEST vector downstream of the *att*R2 site. Use of these stop codons will result in addition of amino acids to the end of your fusion protein.

Using pCR™8/GW/TOPO®

To generate an entry clone in pCR™8/GW/TOPO®, you will:

- Amplify your target gene or sequence of interest using *Taq* polymerase and the appropriate PCR primers
- TOPO® Clone the PCR product into pCR™8/GW/TOPO® in a 5-minute TOPO® Cloning reaction
- Transform the TOPO® reaction into competent *E. coli* and select for entry clones

For detailed instructions and guidelines to generate your entry clone using pCR™8/GW/TOPO®, refer to the pCR™8/GW/TOPO® TA Cloning Kit manual. This manual is available for downloading from www.lifetechnologies.com/support or by calling Technical Support (see page 45).

Using Other Entry Vectors

If you are using other Gateway® entry vectors, refer to the manual supplied with the entry vector you are using for detailed instructions and guidelines to generate an entry clone. All entry vector manuals are available for downloading from www.lifetechnologies.com/support or by contacting Technical Support.

Creating Expression Clones

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into the pSCREEN-iT™/lacZ-DEST vector to create an expression clone. To ensure that you obtain the best possible results, we recommend that you read this section and the section entitled **Performing the LR Recombination Reaction** (pages 15–17) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform an LR recombination reaction using the *att*L-containing entry clone (or any Ultimate™ ORF Clone) and the *att*R-containing pSCREEN-iT™/lacZ-DEST vector. **Note:** Both the entry clone and the destination vector can be supercoiled (see **Important Note** below).
2. Transform the reaction mixture into a suitable *E. coli* host (see page 15).
3. Select for expression clones (see the next page for a diagram of the recombination region of expression clones in pSCREEN-iT™/lacZ-DEST.

Important

The pSCREEN-iT™/lacZ-DEST vector is supplied as a supercoiled plasmid. Although the Gateway® Technology manual has previously recommended using a linearized destination vector for more efficient LR recombination, further testing has found that linearization of pSCREEN-iT™/lacZ-DEST is **not** required to obtain optimal results for any downstream application.

Propagating the Destination Vector

To propagate and maintain the pSCREEN-iT™/lacZ-DEST vector, use the supplied 150 ng/µL solution in TE, pH 8.0 to transform One Shot® *ccd*B Survival™ T1^R Chemically Competent *E. coli* (see page 44 for ordering). The *ccd*B Survival™ T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 100 µg/mL ampicillin and 15–30 µg/mL chloramphenicol.

Note: Do not use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects.

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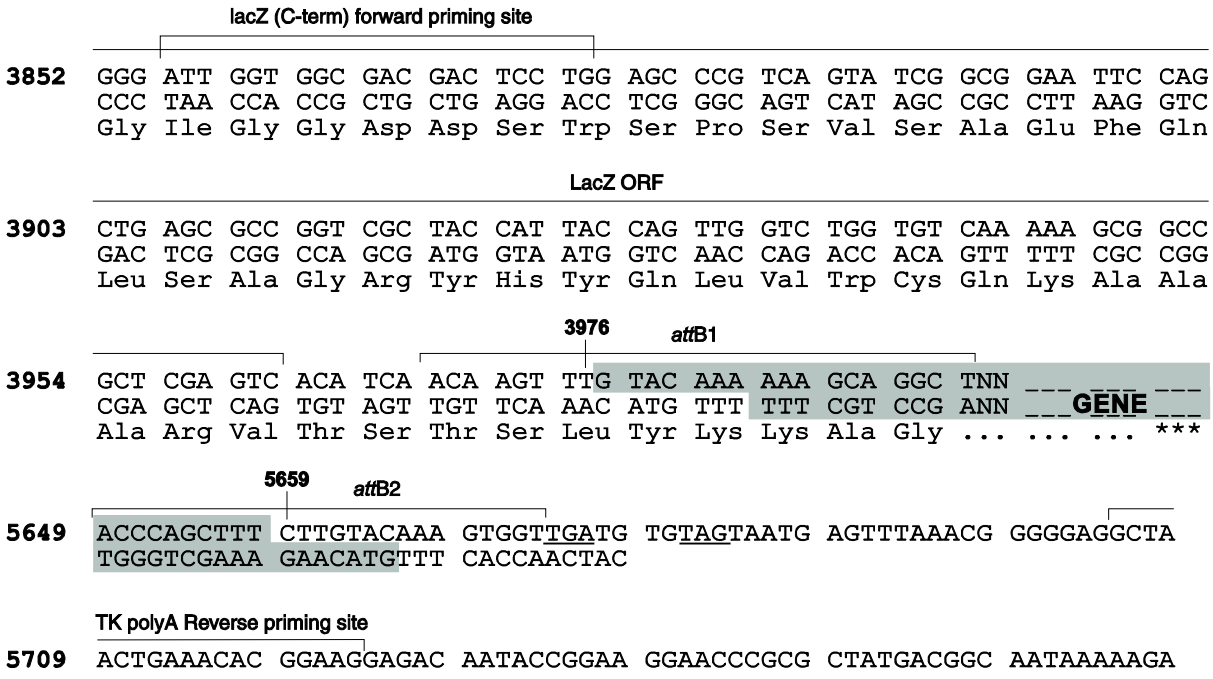
Recombination Region of pSCREEN-iT™/ lacZ-DEST

The recombination region of the expression clone resulting from pSCREEN-iT™/lacZ-DEST × entry clone is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the entry clone into the pSCREEN-iT™/lacZ-DEST vector by recombination. Non-shaded regions are derived from the pSCREEN-iT™/lacZ-DEST vector.
- Bases 3976 and 5659 of the pSCREEN-iT™/lacZ-DEST sequence are indicated.
- Potential stop codons that are located downstream of the *att*B2 site are underlined.



Performing the LR Recombination Reaction

Introduction

Follow the guidelines and instructions in this section to perform the LR recombination reaction using the entry clone and the pSCREEN-iT™/lacZ-DEST vector. We recommend including a negative control (no LR Clonase® II) in your experiment to help you evaluate your results.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including TOP10, Mach1™-T1^R, or DH5α™ for transformation (see page 44 for ordering information). **Do not** transform the LR recombination reaction into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

LR Clonase® II Enzyme Mix

LR Clonase® II enzyme mix is available for purchase to catalyze the LR recombination reaction (see page 44 for ordering). The LR Clonase® II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase® Reaction Buffer previously supplied as separate components in LR Clonase® enzyme mix into an optimized single-tube format for easier set-up of the LR recombination reaction. Use the protocol provided on page 16 to perform the LR recombination reaction using LR Clonase® II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase® enzyme mix, if desired. To use LR Clonase® enzyme mix, follow the protocol provided with the product. **Do not** use the protocol for LR Clonase® II enzyme mix provided in this manual.

Positive Control for LR Reaction

The pENTR™-gus plasmid is included with the LR Clonase® II enzyme mix for use as a positive control for the LR recombination reaction. You may use this entry clone in your LR recombination reaction to verify the efficiency of the LR reaction. The resulting expression clone may be used to express a *lacZ/gus* fusion, if desired. For a map of pENTR™-gus, see the LR Clonase® II manual.

Materials Needed

- Purified plasmid DNA of your entry clone (50–150 ng/µL in TE Buffer, pH 8.0)
- pSCREEN-iT™/lacZ-DEST vector (150 ng/µL)
- pENTR™-gus control (if desired, supplied with Cat. no. K4916-00, Box 4)
- LR Clonase® II enzyme mix (supplied with Cat. no. K4916-00, Box 4; store at -20°C until immediately before use)
- 2 µg/µL Proteinase K solution (supplied with Cat. no. K4916-00, Box 4; thaw and keep on ice until use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- Sterile 0.5 mL microcentrifuge tubes
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates containing 100 µg/mL ampicillin

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Setting Up the LR Recombination Reaction

Follow this procedure to perform the LR reaction between the entry clone and the pSCREEN-iT™/lacZ-DEST vector. If you want to include a negative control, set up a separate reaction but omit the LR Clonase® II enzyme mix.

1. Add the following components to 0.5 mL microcentrifuge tubes at room temperature and mix.

Component	Sample	Positive Control
Entry clone (50–150 ng/reaction)	1–7 µL	–
pENTR™-gus (50 ng/µL)	–	2 µL
pSCREEN-iT™/lacZ-DEST vector (150 ng/µL)	1 µL	1 µL
TE Buffer, pH 8.0	to 8 µL	5 µL

2. Remove the LR Clonase® II enzyme mix from –20°C and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase® II enzyme mix briefly twice (2 seconds each time).
4. To the sample above, add 2 µL of LR Clonase® II enzyme mix. Mix well by pipetting up and down.

   Reminder: Return LR Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate the reaction at 25°C for 1 hour.

   Note: Extending the incubation time to 18 hours typically yields more colonies.
6. Add 1 µL of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Transform 1 µL of the LR recombination reaction into a suitable competent *E. coli* host (follow the manufacturer's instructions) and select for expression clones.

   Note: You may store the LR reaction at –20°C for up to 1 week before transformation, if desired.

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^8 cfu/µg, the LR recombination reaction should result in greater than 5,000 colonies if the entire LR reaction is transformed and plated.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be chloramphenicol-sensitive **and** ampicillin-resistant. Transformants containing a plasmid with a mutated *ccdB* gene will be chloramphenicol- and ampicillin-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/mL chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Continued on next page

Sequencing

Sequencing the expression construct is not required as transfer of the target gene of interest from the entry vector into the pSCREEN-iT™/lacZ-DEST vector preserves the orientation and reading frame of the gene. However, if you wish to confirm that your gene of interest in pSCREEN-iT™/lacZ-DEST is in the correct orientation and in frame with the lacZ ORF, you may sequence your expression construct. We recommend using the following primers to help you sequence your expression construct. Refer to the diagram on page 14 for the location of the primer binding sites in the expression vector.

Primer	Sequence
lacZ (C-term) Forward	5′-ATTGGTGGCGACGACTCCTG-3′
TK polyA Reverse	5′-CTTCCGTGTTTCAGTTAGC-3′

Note: For your convenience, we offer a custom primer synthesis service. For more information, see www.lifetechnologies.com or call Technical Support (see page 45).

General Guidelines for Screening

Introduction

Once you have generated a pSCREEN-iT™/lacZ-DEST expression construct containing your target sequence fused to the *lacZ* reporter, you may use this screening construct to screen any type of RNAi molecule targeted towards your gene including:

- Stealth RNAi®
- siRNA
- shRNA-expressing plasmids

If you have multiple RNAi molecules, you may use the pSCREEN-iT™/lacZ-DEST screening construct to measure the effectiveness of each molecule in inducing target gene knockdown. To screen the RNAi molecules, you will cotransfect the pSCREEN-iT™/lacZ-DEST expression construct and the RNAi molecule into a dividing mammalian cell line and assay for knockdown of β-galactosidase reporter activity. This section provides general guidelines for transfection and discusses factors that can affect the success of your screening experiment. We recommend reading this section before beginning your screening experiment.

Factors Affecting Screening Success

A number of factors can influence the degree of success achieved with your screening experiment including:

- The mammalian cell line used for screening
- Method of transfection and the transfection reagent used
- Amount of RNAi molecule transfected
- Amount of pSCREEN-iT™/lacZ-DEST plasmid transfected
- Transfection format and number of transfections per RNAi molecule
- The size of the target gene
- The location of the sequence targeted by the RNAi molecule

Each of these factors is discussed in greater detail in this section.

Selecting a Cell Line

You may screen your RNAi molecules using any dividing mammalian cell line of choice, even one that does not endogenously express the target gene of interest. When choosing a cell line to use for your screening experiments, we recommend choosing one with the following characteristics:

- Transfects efficiently (*i.e.* easy-to-transfect)
- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18–25 hours
- Non-migratory

We generally use the GripTite™ 293 MSR cell line (see page 44 for ordering) for our screening experiments, but the parental HEK293 cell line or other 293 derivatives are also suitable.

Continued on next page

Culturing Cells

The health of your cells at the time of transfection can affect the success of the screening experiment. Use of "unhealthy" cells can negatively affect the transfection efficiency, resulting in variability and low-to-moderate target gene knockdown. For optimal results, follow the guidelines below to culture your mammalian cells before use in transfection:

- Make sure that cells are healthy and greater than 90% viable.
- Subculture and maintain cells as recommended by the supplier of your cell line. Do not allow cells to overgrow before passaging.
- Use cells that have been subcultured for less than 20 passages.

Methods of Transfection

For established cell lines (*e.g.* 293, A549), consult original references or the supplier of your cell line for the optimal method of transfection. Pay particular attention to media requirements, when to pass the cells, and at what dilution to split the cells.

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al.*, 1977), lipid-mediated (Felgner and Ringold, 1989), and electroporation (Chu *et al.*, 1987; Shigekawa and Dower, 1988). Choose the method and reagent that provides the highest efficiency transfection in your mammalian cell line. For a recommendation, see below.

If you are screening Stealth RNAi® molecules or siRNA, remember that you will be transfecting plasmid DNA (pSCREEN-iT™/lacZ-DEST construct) **and** double-stranded RNA (dsRNA). When choosing a transfection reagent, make sure that you select a transfection reagent that provides highly efficient delivery of both DNA **and** RNA to mammalian cells. For a recommendation, see below.

For high-efficiency transfection of DNA and dsRNA in a broad range of mammalian cell lines, we recommend using the cationic lipid-based Lipofectamine® 2000 Reagent available for purchase (Ciccarone *et al.*, 1999). Using Lipofectamine® 2000 for transfection offers the following advantages:

- Provides the highest transfection efficiency in many mammalian cell types.
- DNA- (and/or dsRNA)-Lipofectamine® 2000 complexes can be added directly to cells in culture medium in the presence of serum.
- Removal of complexes, medium change, or medium addition following transfection is not required, although complexes can be removed after 4-6 hours without loss of activity.

Lipofectamine® 2000 Reagent is available separately for purchase (see page 44 for ordering information).

Continued on next page

Opti-MEM® I

To facilitate optimal formation of DNA- (and dsRNA)-Lipofectamine® 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available for purchase (see page 44 for ordering information). For more information about Opti-MEM® I, see www.lifetechnologies.com or call Technical Support (see page 45).

Amount of DNA and RNAi Molecule to Use for Transfection

When performing the screening experiment, you will be measuring target gene knockdown using an artificial system rather than knockdown of the endogenous target transcript. Because you are simultaneously cotransfecting the pSCREEN-iT™/lacZ-DEST screening construct and the RNAi molecule into mammalian cells, and because you do not need to deliver the RNAi molecule to all cells to achieve an RNAi response, the level of sensitivity of target gene knockdown achieved with this system (as measured by β-galactosidase readout) is greater than that achieved with endogenous target gene knockdown. **Because of the sensitivity of the system, we have found that a lower amount of RNAi molecule is required to elicit an RNAi response.** Indeed, transfecting dsRNA or shRNA-containing plasmid DNA at amounts typically used in RNAi analysis (*e.g.* 50 pmoles of siRNA or 600 ng of shRNA plasmid in a 24-well format) in the context of this system can **swamp** the system, resulting in significant knockdown of β-galactosidase expression even from RNAi molecules with low to moderate activity. Consider the following when setting up your transfection:

- Use 2 to 20-fold less RNAi molecule (*i.e.* Stealth RNAi®, siRNA, or shRNA plasmid DNA) in the cotransfection with the screening construct. See page 26 for recommended reagent amounts to use for different tissue culture formats. Optimize as necessary for your mammalian cell line.
- To maximize transfection efficiency and prevent cell toxicity, make sure that the total amount of nucleic acid transfected (*i.e.* screening vector construct + RNAi molecule) does not exceed the amount recommended by the manufacturer of the transfection reagent you are using. If you are using Lipofectamine® 2000 for transfection, see page 26 for the recommended range of reagent amounts to use for different tissue culture formats.

pSCREEN-iT™/lacZ-GW/CDK2 Control

The pSCREEN-iT™/lacZ-GW/CDK2 plasmid is supplied with the kit for use as a positive control to help you optimize transfection conditions in your mammalian cell line. The pSCREEN-iT™/lacZ-GW/CDK2 plasmid expresses the human CDK2 gene as a C-terminal fusion with the *lacZ* gene. Transfecting the plasmid alone into the mammalian cell line of interest helps to establish a baseline measurement of the amount of β-galactosidase fusion protein expressed in your cells. For more information about the pSCREEN-iT™/lacZ-GW/CDK2 vector and the human CDK2 gene, see page 43.

Continued on next page

To facilitate optimization of transfection conditions for your mammalian cell line, we recommend using the BLOCK-iT™ Fluorescent Oligo available for purchase (see page 44 for ordering). The BLOCK-iT™ Fluorescent Oligo allows strong, easy fluorescence-based assessment of dsRNA oligomer uptake into mammalian cells, and is ideal for use as an indicator of transfection efficiency. For more information about the BLOCK-iT™ Fluorescent Oligo, see the RNAi Central application portal at www.lifetechnologies.com/rnai or call Technical Support (see page 45).

The effective concentration of RNAi molecule required to induce an RNAi response (assuming the RNAi molecule is active) depends in part on the transfection efficiency of the mammalian cell line and may vary from cell line to cell line. After you have optimized transfection conditions for your mammalian cell line and have determined an appropriate amount of RNAi molecule to transfect to obtain an RNAi response, make sure that you use this same amount when screening other RNAi molecules in the same cell line. That is, to accurately compare the effectiveness of an RNAi molecule relative to other RNAi molecules targeted to the same gene in a particular cell line, you should deliver the same amount of each RNAi molecule to the cells.

Transfection Format

You may perform the screening experiment in any tissue culture format. We generally do the following:

- Transfect cells in 24-well format
- For each sample, transfect cells in triplicate. This increases the accuracy of results obtained and accounts for variability associated with transfection.

Note: You may perform the screening experiment in 96-well format, but transfection in this format typically requires more optimization as results obtained are more sensitive to assay variability.

Plasmid Preparation

Once you have generated your pSCREEN-iT™/lacZ-DEST expression clone, you **must** isolate plasmid DNA for transfection. This also applies to shRNA-containing plasmids. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from contamination with phenol and sodium chloride. Contaminants will kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink® HQ Mini Plasmid Purification Kit (see page 44 for ordering) or CsCl gradient centrifugation.

Resuspend the purified plasmid DNA in sterile water or TE Buffer, pH 8.0 to a final concentration ranging from 0.1–3.0 µg/µL.

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Recommended Positive and Negative Controls

We recommend including the following positive and negative controls in **each** screening experiment to help you interpret your results. The screening vector construct is the pSCREEN-iT™/lacZ-DEST vector containing your target gene or sequence of interest.

1. **Mock transfection** (*i.e.* no screening vector, no RNAi molecule): Include this control to assess the effects of the transfection reagent on your mammalian cells.
2. **Screening vector construct only:** This control provides a baseline measurement of the amount of the β-galactosidase fusion protein expressed in mammalian cells after transfection.

   Reminder: If you are transfecting your mammalian cell line for the first time and want to optimize your transfection conditions, use the pSCREEN-iT™/lacZ-GW/CDK2 vector.
3. **Screening vector construct + positive control RNAi molecule:** The positive control RNAi molecule can be an active RNAi molecule targeted to your gene of interest or the Positive lacZ Stealth RNAi® Control supplied with the kit. Use of the Positive lacZ Stealth RNAi® Control effectively targets the *lacZ* reporter gene, resulting in > 70% knockdown of β-galactosidase expression.
4. **Screening vector construct + negative control RNAi molecule:** The negative control RNAi molecule can be an inactive RNAi molecule targeted to your gene of interest or the Scrambled Negative Stealth RNAi® Control supplied with the kit. Use of the Scrambled Negative Stealth RNAi® control does not target any human gene and should induce minimal knockdown of β-galactosidase expression when transfected into mammalian cells at concentrations less than 50 nM.

Transfecting Cells Using Lipofectamine® 2000

Introduction

This section provides a protocol to cotransfect your pSCREEN-iT™/lacZ-DEST screening construct and a corresponding RNAi molecule (*i.e.* Stealth RNAi®, siRNA, or shRNA plasmid) into mammalian cells using Lipofectamine® 2000 Reagent. Before beginning, read the previous section for guidelines and tips to help you design your screening experiment.

Experimental Outline

To perform a screening experiment, you will:

- Co-transfect the pSCREEN-iT™/lacZ-DEST screening construct and your RNAi molecule into mammalian cells using Lipofectamine® 2000.
- Harvest cells and prepare a cell lysate 24–48 hours after transfection.
- Assay the cell lysates for β-galactosidase activity.

Important

Note that the guidelines provided in this section regarding the time period in which to harvest cells are optimized for transfection with Lipofectamine® 2000. If you are using another transfection reagent, you will need to determine the optimal transfection conditions to use and when to harvest cells to obtain the best screening results.

Materials to Have on Hand

- Mammalian cell line cultured in the appropriate growth medium
- pSCREEN-iT™/lacZ-DEST screening construct (150 ng/µL in TE Buffer, pH 8.0)
- Stealth RNAi® or siRNA of interest (20 µM stock in 1X RNA Annealing/Dilution Buffer) or shRNA expression plasmids of interest (0.1-3.0 µg/µL in sterile water or TE Buffer, pH 8.0)
- 20 µM Positive lacZ Stealth RNAi® control (supplied with the kit)
- 20 µM Scrambled Negative Stealth RNAi® control (supplied with the kit)
- 1X RNA Annealing/Dilution Buffer (supplied with the kit)
- pSCREEN-iT™/lacZ-GW/CDK2 control plasmid (supplied with the kit; 0.5 µg/µL in TE)
- Lipofectamine® 2000 Reagent (see page 44 for ordering; store at 4°C and mix gently before use)
- Opti-MEM® I Reduced Serum Medium (pre-warmed; see page 20)
- Appropriate tissue culture plates and supplies
- Dulbecco's Phosphate-Buffered Saline (D-PBS; see page 44 for ordering)
- Cell Lysis Buffer (25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, pH 8.0, 10% glycerol, 0.1% Triton-X-100; see page 37 for a recipe)

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Transfecting Cells Using Lipofectamine® 2000, Continued

General Guidelines for Transfection

Follow these general guidelines when using Lipofectamine® 2000 to cotransfect your pSCREEN-iT™/lacZ-DEST screening construct and the RNAi molecule of interest into mammalian cells.

- Use low-passage cells, and make sure that cells are healthy and greater than 90% viable before transfection.
- **Transfect cells at 80–90% confluence.**
- **Do not add antibiotics to the medium during transfection** as this reduces transfection efficiency and causes cell death.
- For optimal results, use Opti-MEM® I Reduced Serum Medium to dilute Lipofectamine® 2000, DNA, and dsRNA oligomers prior to complex formation.
- Stealth RNAi® duplexes or siRNA are generally supplied as a 20 µM stock solution. If you are performing transfection in a format smaller than a 6-well dish (*e.g.* 24-well format), we recommend diluting the 20 µM stock solution 10- to 20-fold in 1X RNA Annealing/Dilution Buffer (supplied with the kit) to prepare a 1–2 µM stock solution, as appropriate. Use the 1–2 µM stock solution for transfection. Store the 2 µM stock solution at –20°C.

  Example: To prepare a 2 µM stock solution, dilute 2 µL of the 20 µM siRNA or Stealth RNAi® stock solution in 18 µL of 1X RNA Annealing/Dilution Buffer).
- To increase accuracy and reduce assay variability, we recommend performing **triplicate** transfections for each sample condition.

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Transfecting Cells Using Lipofectamine® 2000, Continued

Transfection Procedure

Use this procedure to cotransfect the pSCREEN-iT™/lacZ-DEST screening construct containing your target gene or sequence of interest and the RNAi molecule into mammalian cells using Lipofectamine® 2000. Refer to the table in **Suggested Reagent Amounts and Volumes**, page 26 for the appropriate reagent amounts and volumes to add for different tissue culture formats. Remember to include the proper positive and negative controls in your experiment (see page 22).

1. One day before transfection, plate cells in the appropriate amount of growth medium **without antibiotics** such that they will be 80–90% confluent at the time of transfection.
2. **For each transfection sample,** prepare DNA-RNAi molecule-Lipofectamine® 2000 complexes as follows.
   a. Dilute the DNA and RNAi molecule in the appropriate amount of Opti-MEM® I Medium without serum. Mix gently.
   b. Mix Lipofectamine® 2000 gently before use, then dilute the appropriate amount in Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA and RNAi molecule with the diluted Lipofectamine® 2000. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur. The solution may appear cloudy, but this will not impede the transfection.
3. Add the DNA-RNAi molecule-Lipofectamine® 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4. Incubate the cells at 37°C in a CO_2 incubator until you are ready to harvest cells and assay for β-galactosidase activity (see **Preparing Cell Lysates**, page 27). Removal of complexes or media change is not required; however, growth medium may be replaced after 4–6 hours without loss of transfection activity.

   Tip: We recommend harvesting cells 24–48 hours after transfection.

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Transfecting Cells Using Lipofectamine® 2000, Continued

Suggested Reagent Amounts and Volumes

The table below lists the range of recommended reagent amounts and volumes to use to transfect cells in various tissue culture formats. As a starting point, use an amount of pSCREEN-iT™/lacZ-DEST DNA (see column 4), dsRNA or shRNA plasmid DNA (see column 5), and Lipofectamine® 2000 (see column 7) that falls around the mid-point of the recommended range, then optimize conditions for your cell line by varying reagent amounts within the recommended range. If you wish to perform transfection in 96-well format, see the additional guidelines in **Guidelines for Transfection in 96-Well Format**, below.

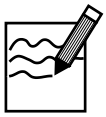
Example: We typically use 150 ng of screening vector DNA, 5 pmol of Stealth RNAi®, and 1 µL of Lipofectamine® 2000 to transfect GripTite™ 293 MSR cells in 24-well format.

Tip: 20 µM dsRNA (*i.e.* siRNA or Stealth RNAi®) = 20 pmol/µL.

Culture Vessel	Relative Surface Area (vs. 24-well)	Volume of Plating Medium	pSCREEN-iT™/ lacZ-DEST DNA (ng) Amt	dsRNA (pmol)/ shRNA DNA (ng) Amt[1]	DNA/RNA Dilution Volume (µL)[2]	Lipid (µL) and Dilution Volume (µL)
96-well	0.2	100 µL	10–100 ng	0.1–1 pmol/150–300 ng	25 µL	0.2–0.5 µL in 25 µL
48-well	0.4	200 µL	50–100 ng	0.5–5 pmol/150–300 ng	25 µL	0.3–0.8 µL in 25 µL
24-well	1	500 µL	100–200 ng	1–10 pmol/300–600 ng	50 µL	0.5–1.5 µL in 50 µL
6-well	5	2 mL	500–1000 ng	5–50 pmol/1.5–3 µg	250 µL	2.5–6 µL in 250 µL

[1]dsRNA = siRNA or Stealth RNAi®; shRNA DNA = shRNA-containing plasmid

[2]Dilute the pSCREEN-iT™/lacZ-DEST DNA **and** the dsRNA or shRNA DNA into this volume of Opti-MEM® I.



Note

Note that for highly potent RNAi molecules (*i.e.* RNAi molecules inducing > 90% target knockdown), the amount of dsRNA or shRNA DNA required to obtain effective knockdown may be less than the amounts specified in the table above (see column 5). This needs to be determined empirically for each cell line.

Guidelines for Transfection in 96-Well Format

You may perform the screening experiment in 96-well format, if desired. Note that in this format, the results obtained from the screening experiment are much more sensitive to well-to-well variability caused by differences in cell density, transfection efficiency, and reagent amounts used. If you are transfecting cells in 96-well format, significant optimization of transfection conditions may be required. Follow the guidelines below to cotransfect mammalian cells in 96-well format:

- To address potential problems caused by well-to-well variability, we recommend performing more replicates for each sample condition; **we generally transfect each sample into 6–7 individual wells**.
- When plating cells, make sure that cells are evenly distributed over the surface of each well. As with the other tissue culture formats, transfect cells at 80–90% confluence.
- Use the following range of recommended reagent amounts and volumes listed in the table above and optimize accordingly.
- We recommend harvesting cells and assaying for β-galactosidase activity 24 hours after transfection.

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Preparing Cell Lysates

Follow this procedure to prepare cell lysates from your untransfected and transfected cells. Use the amount of Cell Lysis Buffer recommended in column 2 of the table below as a starting point. Optimize your β-galactosidase assay by varying the amount of Cell Lysis Buffer used within the recommended range (see column 3).

1. Remove the growth medium from each well of the tissue culture dish and wash the cells once with D-PBS.
2. Add the appropriate amount of Cell Lysis Buffer (see recipe on page 37) to each well containing cells.

Tissue-Culture Format	Amt of Cell Lysis Buffer (µL)	Cell Lysis Buffer Range to Optimize (µL)
96-well	100 µL	25–100
48-well	250 µL	100–250
24-well	500 µL	125–500
6-well	2000 µL	600–2000

3. Transfer the plate containing cells and Cell Lysis Buffer to –80°C for at least 20 minutes until samples are frozen.

   Note: You may store samples for up to one month at this stage by wrapping the plate with parafilm or plastic wrap and storing at –80°C.

4. Proceed to assay for β-galactosidase activity (see the next page).

Guidelines to Perform the β-galactosidase Assay

Introduction

Once you have prepared cell lysates of your untransfected and transfected cells, you can assay each sample for β-galactosidase activity using the FluoReporter® lacZ/Galactosidase Quantitation Kit (see page 44 for ordering). The kit uses a fluorogenic substrate to allow highly sensitive measurement of β-galactosidase activity in cell extracts using a fluorescence microplate reader equipped with the proper filter set. Guidelines to perform the quantitation assay are provided in this section. For instructions to perform the assay, see the next section, page 30.

Note: The FluoReporter® lacZ/Galactosidase Quantitation Kit is also available separately for purchase (see page 44 for ordering). Other methods or commercial kits may also be used to assay for β-galactosidase activity. Refer to manufacturer's instructions to perform the assay.

Assay Format

We recommend performing the β-galactosidase assay in a 96-well format. This allows rapid analysis of multiple samples and minimizes the amount of cell lysate required for each assay. For other recommendations, see page 29.

Fluorescence Plate Readers and Filter Sets

You may use any fluorescence plate reader to detect the fluorescence signal after performing the β-galactosidase assay. Keep the following in mind:

- For optimal sensitivity, we recommend using a bottom-read fluorescence plate reader (*e.g.* Gemini-EM Fluorescence Microtiter Plate Reader, Molecular Devices, CytoFluor® 4000 Fluorescence Plate Reader, Applied Biosystems, or Safire Microplate Reader, Tecan). Top-read fluorescence plate readers (*e.g.* Gemini-XS Fluorescence Microtiter Plate Reader, Molecular Devices) can be used.
- To detect the blue fluorescence signal, use a fluorescence microplate reader equipped with an excitation filter centered at ~390 nm and an emission filter centered at ~460 nm.

  Note: Filter sets are included with some fluorescence plate readers, while others require that filters be obtained separately. If you need to obtain filters separately, we recommend using the following filter set from Chroma Technologies (Cat. no. 31047):

Excitation filter	D405/10x
Dichroic mirror	425DCLP
Emission filter	D460/50m

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Guidelines to Perform the β-galactosidase Assay, Continued

General Recommendations

Follow the general recommendations below to perform the β-galactosidase assay:

- We recommend performing the β-galactosidase assay in a black-walled, clear-bottom microtiter plate with low autofluorescence (Costar, Cat. nos. 3603 or 3631). Using a black-walled microtiter plate blocks any signal from adjoining wells during quantitation by the fluorescence microplate reader.
- Some plates/plate readers exhibit edge effects that may affect data. If edge effects are noticed, consider the plate layout when setting up the assay.
- Do not touch the bottom of the microtiter plate or allow dust to cover the tissue culture surface. Fingerprints and dust can autofluoresce, introducing well-to-well variability in replicate wells.
- Include the Reference Standard and the appropriate controls (mock transfection, screening construct only transfection) in your experiment.

Reference Standard

The Reference Standard (7-hydroxycoumarin-3-carboxylic acid) is provided in the FluoReporter® lacZ/Galactosidase Quantitation Kit to serve as an instrument-independent control, and can be used to normalize fluorescence. This allows a single standard curve to be used for assays performed at different times, even if performed on different instruments or with different instrument settings. The reference standard can also be used to convert the fluorescence signal into moles of product.

Generating a Standard Curve

When using the FluoReporter® lacZ/Galactosidase Quantitation Kit, we recommend generating a standard curve using purified β-galactosidase solutions of known concentration. Generating a standard curve will allow you to:

- Determine the linear detection range of β-galactosidase based on your reagents, buffers, and fluorescence microplate reader
- Convert the fluorescence readings for your samples into picograms of β-galactosidase

To generate a standard curve and for an example of a standard curve, see the **Appendix**, pages 39–40.

Performing the β-galactosidase Assay

Introduction

This section provides instructions to perform the β-galactosidase assay using the reagents supplied in the FluoReporter® lacZ/Galactosidase Quantitation Kit.

Experimental Outline

To assay your samples for β-galactosidase activity, you will:

1. Add an aliquot of cell extract and the CUG substrate to wells in a 96-well microtiter plate.

   Recommendation: For increased accuracy, we recommend performing the assay in triplicate.

2. Incubate the sample(s) at room temperature for 30 minutes.
3. Add a stop buffer to terminate the reaction.
4. Measure fluorescence signal using a fluorescence microplate reader equipped with the appropriate filter set (see page 28).

Amount of Cell Extract to Assay

The β-galactosidase assay is generally performed using 10 µL of cell extract. If your sample contains high levels of β-galactosidase activity, the fluorescence signal may exceed the linear range of detection. In this case, you may need to dilute the cell extracts in Cell Lysis Buffer (see page 37 for a recipe) prior to performing the assay.

Materials Needed

- Cell extracts of interest (in Cell Lysis Buffer from Step 3, page 27)
- 40 mM CUG Substrate Reagent (supplied with the kit, Box 2; see the next page for handling guidelines)
- 10 mM Reference Standard (optional; supplied with the kit, Box 2; see the next page for handling guidelines)
- Reaction Buffer (0.1 M sodium phosphate, pH 7.3, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol; see page 37 for a recipe)

  Note: You will need approximately 10 mL of Reaction Buffer for every 96-well plate. If you plan to generate a standard curve or use the Reference Standard, you will need additional Reaction Buffer to prepare the enzyme dilution buffer and dilute the Reference Standard.

- Stop Buffer (0.2 M Na_2CO_3; see page 38 for a recipe)

  Note: You will need approximately 5 mL of Stop Buffer for every 96-well plate.

- Enzyme Dilution Buffer (if generating a standard curve; Reaction Buffer containing 1 mg/mL BSA; see the Appendix, page 39 for guidelines)
- 1 µg/mL β-galactosidase solution in Enzyme Dilution Buffer (if generating a standard curve; see the Appendix, page 39 for guidelines)

Continued on next page

Performing the β-galactosidase Assay, Continued

Handling the Reagents

The CUG Substrate Reagent is supplied as a 40 mM stock solution in 100 mM sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, and 110 mM β-mercaptoethanol while the Reference Standard is supplied as a 10 mM stock solution in dimethylformamide. Follow the guidelines below when handling each stock solution.

- The CUG Substrate Reagent and the Reference Standard are light sensitive. Store the CUG Substrate Reagent at –20°C, protected from light. Store the Reference Standard at –20°C or 4°C. The stock solutions are stable for at least 6 months if stored properly.
- When using, thaw the CUG substrate stock solution at room temperature, protected from light. Thaw immediately before use. **Do not expose to room temperature for an extended period of time as spontaneous hydrolysis will occur.** After use, return stock solution to –20°C storage.

  Note: The Reference Standard does not freeze.
- The CUG Substrate Reagent stock solution may be frozen and thawed multiple times without loss of fluorescence signal if handled properly.

Before Starting

- Prepare a 1.1 mM working solution of the CUG Substrate Reagent by diluting 275 µL of the 40 mM stock solution with 9.73 mL of Reaction Buffer. You will need approximately 10 mL of CUG working solution for each 96-well microtiter plate. Scale up the volume needed accordingly. **Do not** leave the CUG Substrate Reagent at room temperature for an extended period of time (see handling instructions above).

  Note: Store the working solution at –20°C for at least six months.
- If you are using the Reference Standard, dilute the 10 mM Reference Standard 100-fold into 200 µL of Reaction Buffer to prepare a 0.1 mM working solution (*i.e.* add 5 µL of 10 mM Reference Standard to 495 µL of Reaction Buffer).

Continued on next page

Performing the β-galactosidase Assay, Continued

β-Galactosidase Assay Procedure

Follow this procedure to assay your samples for β-galactosidase activity.

1. Remove the plate containing cell lysates (Step 3, page 27) from the freezer and thaw the cell lysates at room temperature for 30–45 minutes.
2. Rock the plate gently to mix the solution, then pipette 10 µL of cell lysate into individual wells of a black-walled, 96-well microtiter plate. Take the clear solution; **do not** pipette any insoluble material into the 96-well plate. Wrap the plate containing unused cell lysate with parafilm or plastic wrap and store at –80°C.

   Tip: For more accurate results, we recommend assaying each sample in triplicate.
3. Pipet 10 µL of Reaction Buffer into a well to serve as a blank.
4. Add 100 µL of the 1.1 mM CUG substrate working solution to each well containing 10 µL of cell lysate.
5. Optional: Pipet 100 µL of the 0.1 mM Reference Standard into an empty well.
6. Incubate the samples at room temperature for 30 minutes.

   Important: If you are comparing results to a previously generated standard curve, incubation time is critical. Always use the same incubation time and temperature to ensure accurate quantitation.
7. Add 50 µL of Stop Buffer to each well to terminate the reaction. In addition to terminating the reaction, the Stop Buffer causes an increase in the fluorescence of the product.
8. Measure the fluorescence signal in each well using a fluorescence microplate reader equipped with the appropriate filter set (see page 28).

   Important: Measure fluorescence signal within 15 minutes of adding the Stop Buffer. If comparing results to a previously generated standard curve, use the same time interval between stopping the reaction and reading the fluorescence signal.
9. Analyze results (see below).

Analyzing Results

Analyze the fluorescence of the samples by subtracting the fluorescence of the blank from that of each sample. If the Reference Standard is used, divide the corrected fluorescence by the background-subtracted fluorescence of the Reference Standard. Use the standard curve to determine the amount of β-galactosidase in each well, if desired.

Example of Expected Results

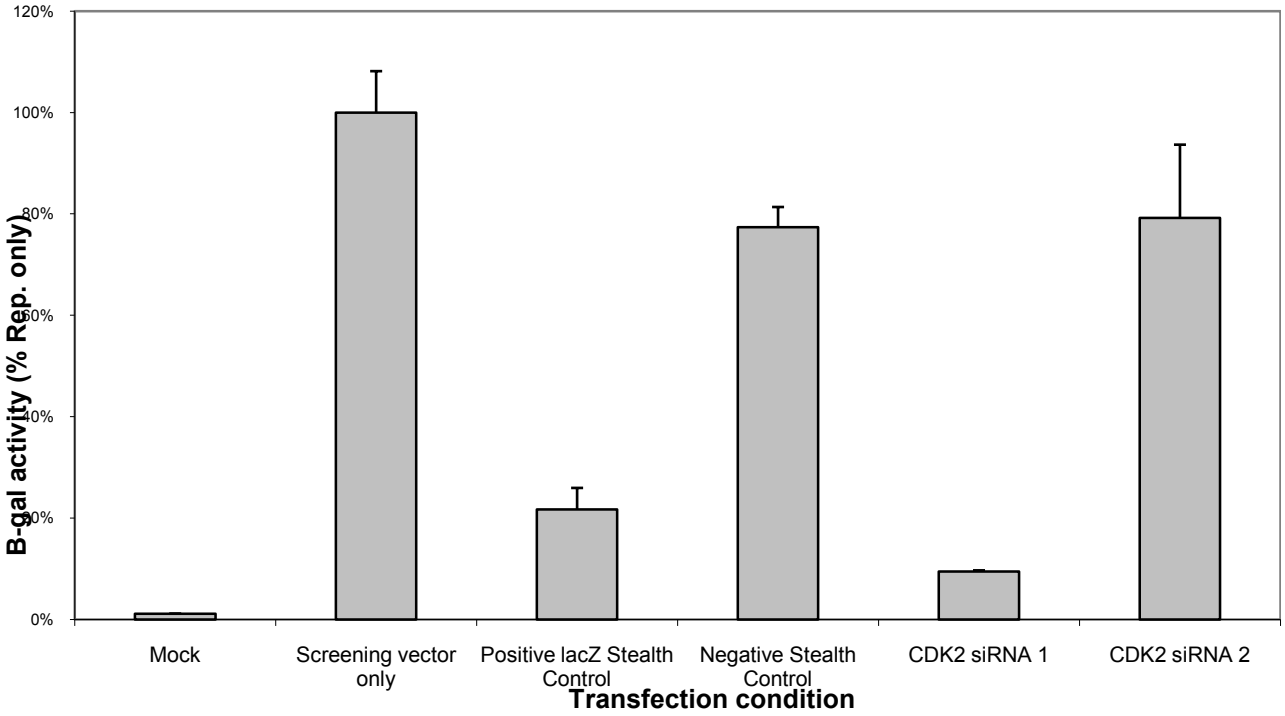
Example 1: Screening siRNA Targeting the Human CDK2 Gene

In this experiment, we wish to screen several synthetic siRNA targeting the human CDK2 gene (*i.e.* CDK2 siRNA 1 and CDK2 siRNA 2). An Ultimate™ hORF entry clone containing the human CDK2 gene (ORF no. IOH21140) was transferred into pSCREEN-iT™/lacZ-DEST using the LR recombination reaction to generate the pSCREEN-iT™/lacZ-GW/CDK2 screening construct.

GripTite™ 293 MSR cells (see page 44 for ordering) plated in a 24-well plate were transfected using Lipofectamine® 2000 with either the pSCREEN-iT™/lacZ-GW/CDK2 screening vector alone or together with a Stealth RNAi® control or one of the CDK2 siRNA. Twenty-four hours after transfection, cell lysates were prepared and assayed in triplicate for β-galactosidase activity using the FluoReporter® lacZ/Galactosidase Quantitation Kit reagents and the procedure on page 32. The β-galactosidase activity reported is normalized to the % activity obtained from the screening vector (*i.e.* reporter) alone.

Results:

- The results indicate that CDK2 siRNA 1 is a highly active siRNA for human CDK2 as measured by > 85% knockdown of *lacZ* reporter activity. In contrast, CDK2 siRNA 2 is not an active siRNA, with only 20% knockdown of *lacZ* reporter activity achieved.
- The results obtained from the screening experiment correlate with real-time quantitative RT-PCR (qRT-PCR) analysis of the endogenous CDK2 transcript.



Troubleshooting

Introduction

Review the information in this section for help to troubleshoot your recombination and screening experiments.

LR Reaction and Transformation

The table below lists some potential problems and possible solutions that may help you troubleshoot the LR recombination and transformation procedures.

Problem	Reason	Solution
Few or no colonies obtained after transformation of LR reaction	LR recombination reaction not treated with proteinase K	Treat reaction with proteinase K before transformation.
	Did not use the suggested amount of LR Clonase® II enzyme mix or LR Clonase® II enzyme mix was inactive	• Make sure to store the LR Clonase® II enzyme mix at –20°C. • Do not freeze/thaw the LR Clonase® II enzyme mix more than 10 times. • Use the recommended amount of LR Clonase® II enzyme mix (see page 16). • Test another aliquot of the LR Clonase® II enzyme mix.
	Not enough LR reaction transformed	Transform 2–3 µL of the LR reaction into a suitable chemically competent *E. coli* strain.
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating.
	Too much entry clone DNA used in the LR reaction	Use 50–150 ng of the entry clone in the LR reaction.
	Used low efficiency competent cells	Use competent *E. coli* with a transformation efficiency ≥ 1×10^8 cfu/µg.

Screening Experiment

The table below lists some potential problems and possible solutions that may help you troubleshoot the co-transfection and β-galactosidase reporter activity assay procedures.

Problem	Reason	Solution
Knockdown observed when cotransfecting screening construct and negative control (*i.e.* inactive) RNAi molecule	Too much RNAi molecule transfected	Reduce the amount of RNAi molecule transfected.

Continued on next page

Screening Experiment, Continued

Problem	Reason	Solution
Low levels of β-galactosidase activity obtained when screening construct alone is transfected **Note:** Assumes that Lipofectamine® 2000 used for transfection	Low transfection efficiency: • Used poor quality pSCREEN-iT™/lacZ-DEST screening construct plasmid DNA (*e.g.* DNA contaminated with phenol) • Transfected unhealthy mammalian cells; cells exhibit low viability • Cells transfected in media containing antibiotics (*e.g.* penicillin/streptomycin) • Did not transfect enough screening construct plasmid DNA • Mammalian cells plated too sparsely • Used a cell line that does not transfect efficiently • Plasmid DNA:transfection reagent ratio used not optimal	• Use the PureLink® HQ Mini Plasmid Purification Kit (see page 44 for ordering) or CsCl gradient centrifugation to prepare DNA. • Use healthy mammalian cells under passage 20. Do not overgrow; make sure cells are > 90% viable before transfection. • **Do not** add antibiotics to media during transfection; this reduces transfection efficiency and causes cell death. • Use an amount of plasmid DNA that falls within the range recommended on page 26. • Plate cells such that they are 80–90% confluent at the time of transfection. • Use a different mammalian cell line for transfection (*e.g.* GripTite™ 293 MSR). • Use an amount of plasmid DNA and lipid that falls within the range recommended on page 26.
	C-terminal fusion of your target gene to *lacZ* interferes with β-galactosidase activity or expression	• Reduce the amount of Cell Lysis Buffer used to lyse cells (see procedure on page 27). • Test pSCREEN-iT™/lacZ-GW/CDK2 for β-galactosidase fusion protein expression. • Reduce the size of the target sequence fused to *lacZ*; use a DNA fragment ranging from 200 bp to 1 kb. • Place a stop codon before the beginning of your target sequence.
	Lipofectamine® 2000 Reagent handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion before use. **Do not vortex.**

Continued on next page

Screening Experiment, Continued

Problem	Reason	Solution
Poor knockdown or no knockdown observed when cotransfecting screening construct and positive control (*i.e.* highly potent) RNAi molecule	Insufficient amount of RNAi molecule transfected	• Increase the amount of RNAi molecule transfected. • Optimize cotransfection conditions for your cell line by varying screening construct plasmid DNA, RNAi molecule, and lipid amounts used.
	Cell lysate assayed contained too much β-galactosidase	Dilute the cell lysate in Cell Lysis Buffer and repeat the β-galactosidase detection assay. Make sure that the amount of β-galactosidase in the sample is within the linear range of detection.
Significant cytotoxicity observed **Note:** Assumes that Lipofectamine® 2000 used for transfection	Too much Lipofectamine® 2000 used	Reduce the amount of Lipofectamine® 2000 used (see page 26 for the recommended amounts of lipid to use).
	Mammalian cells plated too sparsely	Plate cells such that they are 80–90% confluent at the time of transfection.
	Too much nucleic acid (*i.e.* screening construct DNA + RNAi molecule) transfected	Reduce the total amount of nucleic acid transfected (see page 26 for the recommended amounts of screening construct DNA and RNAi molecule to use).
No fluorescence signal (*i.e.* no β-galactosidase activity in all samples)	CUG substrate stock solution exposed to light during storage	Store the CUG substrate stock solution at -20°C, protected from light.
	Used the incorrect filter set	Measure fluorescence using a fluorescence microplate reader equipped with an excitation filter centered at 390 nm and an emission filter centered at 460 nm.
CUG substrate exhibits fluorescence signal in the absence of β-galactosidase	CUG substrate has spontaneously hydrolyzed	• Do not leave the CUG substrate stock solution at room temperature for extended periods of time. • Store the CUG substrate stock solution at –20°C, protected from light.
Observe well-to-well variability in replicate wells (most notable when using top-read fluorescence plate readers)	Bubbles are present in the cell lysates	Carefully transfer cell lysates to a new tissue culture plate, taking care not to introduce bubbles. Read fluorescence signal.
	Touched the bottom of the microtiter plate	Do not touch the bottom of the microtiter plate as fingerprints can autofluoresce.
	Microtiter plate covered with dust or lint	Dust can autofluoresce. Keep the bottom and top surface of the microtiter plate free of dust.

Appendix

Recipes

Cell Lysis Buffer

25 mM Tris-HCl, pH 8.0
0.1 mM EDTA, pH 8.0
10% glycerol
0.1% Triton X-100

1. In a sterile beaker, combine the following:

1 M Tris-HCl, pH 8.0	12.5 mL
0.5 mM EDTA, pH 8.0	100 mL
Glycerol	50 mL
Triton X-100	5 mL
Sterile deionized water	332.5 mL
Total volume	500 mL

2. Stir to mix thoroughly.
3. Filter-sterilize and store at 4°C.

Reaction Buffer

Follow this procedure to prepare 10 mL of Reaction Buffer for use with the reagents supplied in the FluoReporter® lacZ/Galactosidase Quantitation Kit. To prepare a larger volume of Reaction Buffer, scale up the amounts of each reagent accordingly. For a recipe to prepare 1 M Sodium Phosphate, pH 7.3, see the next page.

Composition:

0.1 M Sodium Phosphate, pH 7.3
1 mM $MgCl_2$
45 mM β-mercaptoethanol

Recipe:

1. In a 15 mL sterile, conical tube, combine the following:

1 M Sodium Phosphate, pH 7.3	1 mL
1 M $MgCl_2$	10 µL
β-mercaptoethanol	31.5 µL
Sterile deionized water	8.96 mL
Total volume	10 mL

2. Mix thoroughly.
3. Store at room temperature until use.

Continued on next page

1 M Sodium Phosphate, pH 7.3

Materials Needed:

Sodium phosphate monobasic monohydrate ($H_2NaPO_4 \cdot H_2O$; Sigma, Cat. no. S-9638)
Sodium phosphate dibasic (HNa_2PO_4; Sigma, Cat. no. S-7907)

Recipe:

1. Prepare 2 M stock solutions of each reagent:
   a. 2 M $H_2NaPO_4 \cdot H_2O$: Dissolve 55.2 g in 200 mL sterile deionized water.
   b. 2 M HNa_2PO_4: Dissolve 56.8 g in 200 mL sterile deionized water.
2. In a beaker, combine the following:

2 M $H_2NaPO_4 \cdot H_2O$	23 mL
2 M HNa_2PO_4	77 mL
Sterile deionized water	100 mL
Total volume	200 mL

3. Stir to mix thoroughly. This is the 1 M Sodium Phosphate, pH 7.3 solution.
4. Filter-sterilize and store at room temperature.

Stop Buffer

Stop Buffer = 0.2 M Na_2CO_3 (Sigma, Cat. no. 71350)

1. To prepare a 2 M stock solution of Na_2CO_3, add 10.6 g of Na_2CO_3 to 45 mL of sterile deionized water. Stir to mix and bring the volume up to 50 mL with sterile deionized water. Filter-sterilize.
2. Dilute an aliquot of the 2 M Na_2CO_3 stock solution 10-fold in sterile deionized water (*e.g.* add 1 mL of 2 M Na_2CO_3 to 9 mL of sterile deionized water) to prepare a 0.2 M working solution.
3. Store at room temperature until use.

Generating a β-Galactosidase Standard Curve

Introduction

Follow the guidelines provided in this section to generate a standard curve using purified β-galactosidase solutions and reagents supplied in the FluoReporter® lacZ/Galactosidase Quantitation Kit.

Materials Needed

- Bovine Serum Albumin (BSA; see page 44 for ordering)
- 1 µg/mL β-galactosidase (Sigma, Cat. no. G4155) in Enzyme Dilution Buffer (see below)
- 1.1 mM working solution of CUG Substrate Reagent (see page 31)
- Reaction Buffer (see page 37 for a recipe)
- 0.1 mM working solution of Reference Standard (see page 31)
- 96-well black-walled, microtiter plate

Before Starting

1. Prepare Enzyme Dilution Buffer by adding BSA to a final concentration of 1 mg/mL in 1 mL of Reaction Buffer.
2. Prepare a fresh 1 µg/mL solution of β-galactosidase in Enzyme Dilution Buffer. Keep at room temperature until use.
3. Prepare 10-fold serial dilutions of the β-galactosidase solution ranging from 10^{-1} to 10^{-4} in Enzyme Dilution Buffer. For each dilution, dilute the β-galactosidase solution into Enzyme Dilution Buffer to a final volume of 100 µL (*i.e.* dilute 10 µL of β-galactosidase solution into 90 µL of Enzyme Dilution Buffer). Keep at room temperature until use.
4. If you are using the Reference Standard, dilute the 10 mM Reference Standard 100-fold into 200 µL of Reaction Buffer to prepare a 0.1 mM working solution (*i.e.* add 5 µL of 10 mM Reference Standard to 495 µL of Reaction Buffer).

Performing the β-galactosidase Assay

Follow the procedure below to perform the β-galactosidase assay.

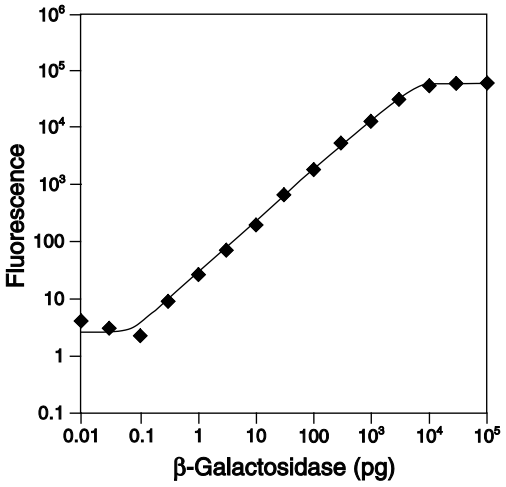
1. Into individual wells in a 96-well black-walled microtiter plate, pipet 10 µL of each of the purified β-galactosidase dilutions (10^{0} to 10^{-4} dilutions), yielding 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg standards. For more accurate results, assay each sample in triplicate.
2. Pipet 10 µL of Reaction Buffer into a well to serve as a blank.
3. Pipet 100 µL of the 0.1 mM Reference Standard into an empty well (if desired).
4. Add 100 µL of the 1.1 mM CUG substrate working solution to each well containing β-galactosidase.
5. Follow Steps 6–8 of the **β-galactosidase Assay Procedure**, page 32.

Continued on next page

Generating a β-Galactosidase Standard Curve, Continued

Generating the Standard Curve

To generate a standard curve, first subtract the fluorescence of the blank from that of each of the samples containing the purified β-galactosidase solutions. If the standard curve will be used for comparison with assays performed at a later date, divide the background-subtracted fluorescence of the β-galactosidase standards by the background-subtracted fluorescence of the reference standard. Plot the resulting corrected fluorescence intensities versus enzyme amount on a log-log scale. Adjust the values for enzyme amount to compensate for the purity of the enzyme preparation. Alternatively, plot fluorescence versus units of β-galactosidase activity. A standard curve (without reference standard normalization) should resemble the sample curve shown below.

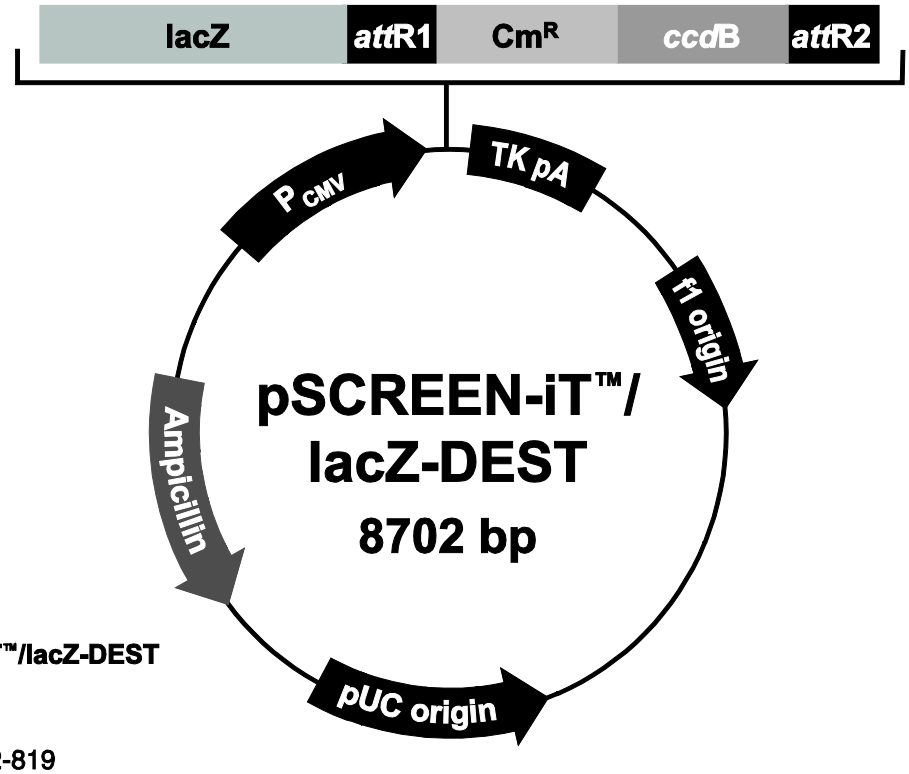


Note that the assay has a linear detection range of about 0.5 to over 1000 pg β-galactosidase, and that fluorescence units ranging from about 10 to 10^5 fall within the linear range of the assay. The lower detection limit corresponds to about ten *lacZ*-positive NIH 3T3 cells per well.

Map and Features of pSCREEN-iT™/lacZ-DEST

Map of pSCREEN-iT™/lacZ-DEST

The map below shows the elements of pSCREEN-iT™/lacZ-DEST. DNA from the entry clone replaces the region between the *att*R sites at bases 3976 and 5659. The vector sequence for pSCREEN-iT™/lacZ-DEST is available at www.lifetechnologies.com or by contacting Technical Support (see page 45).



Features of pSCREEN-iT™/lacZ-DEST
8702 nucleotides

CMV promoter: bases 232-819
LacZ ORF: bases 891-3962
lacZ (C-term) forward priming site: bases 3855-3874
*att*R1 recombination site: bases 3969-4093
Chloramphenicol resistance gene: bases 4202-4861
*ccd*B gene: bases 5203-5508
*att*R2 recombination site: bases 5549-5673
TK polyadenylation sequence: bases 5698-5969
TK polyA reverse priming site: bases 5705-5723
f1 origin: bases 6005-6433
pUC origin: bases 6888-7561 (C)
bla promoter: bases 8567-8665 (C)
Ampicillin resistance (*bla*) gene: bases 7706-8566 (C)

(C) = complementary strand

Continued on next page

Map and Features of pSCREEN-iT™/lacZ-DEST, Continued

Features of the Vector

The pSCREEN-iT™/lacZ-DEST vector (8702 bp) contains the following elements. All features have been functionally tested and the vector fully sequenced.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows high-level expression of the *lacZ* fusion transcript in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
LacZ ORF	Used to create an N-terminal fusion of lacZ to your target gene, and functions as a reporter for knockdown of the target transcript.
lacZ (C-term) forward priming site	Allows sequencing of the insert.
*att*R1 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the target gene or sequence of interest from a Gateway® entry clone (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Allows negative selection of the plasmid.
TK polyA reverse priming site	Allows sequencing of the insert.
Herpes Simplex Virus Thymidine Kinase (TK) polyadenylation sequence	Allows efficient transcription termination and polyadenylation of mRNA (Cole and Stacy, 1985).
f1 origin	Allows rescue of single-stranded DNA.
pUC origin	Allows high-copy replication and maintenance of the plasmid in *E. coli*.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.

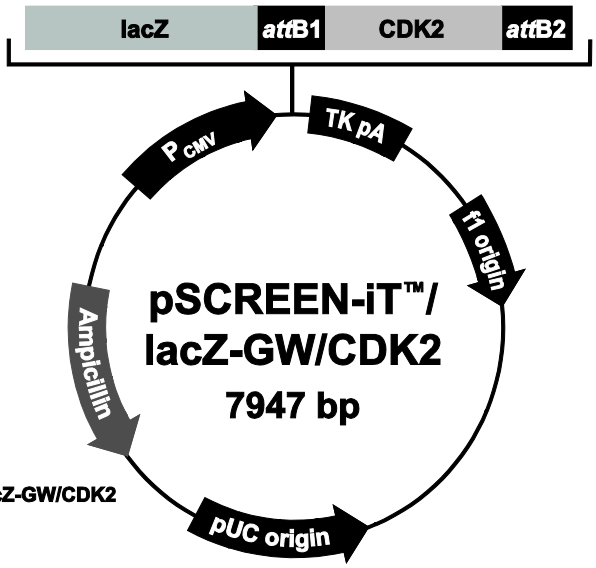
Map of pSCREEN-iT™/lacZ-GW/CDK2

Description

pSCREEN-iT™/lacZ-GW/CDK2 is a 7947 bp control vector containing the human CDK2 gene (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.*, 1991; Tsai *et al.*, 1991) fused to the *lacZ* reporter gene, and was generated by performing an LR recombination with the pSCREEN-iT™/lacZ-DEST vector and an Ultimate™ hORF Clone containing the human CDK2 gene (Invitrogen Clone ID No. IOH21140; Genbank Accession No. NM_001798).

Map of pSCREEN-iT™/lacZ-GW/CDK2

The map below shows the elements of pSCREEN-iT™/lacZ-GW/CDK2. The nucleotide sequence of the vector is available from www.lifetechnologies.com or by calling Technical Support (see page 45).



Features of pSCREEN-iT™/lacZ-GW/CDK2
7947 nucleotides

CMV promoter: bases 232-819
LacZ ORF: bases 891-3962
lacZ (C-term) forward priming site: bases 3855-3874
*att*B1 recombination site: bases 3969-3993
CDK2 ORF: bases 3996-4892
*att*B2 recombination site: bases 4894-4918
TK polyadenylation sequence: bases 4943-5214
TK polyA reverse priming site: bases 4950-4968
f1 origin: bases 5250-5678
pUC origin: bases 6133-6806 (C)
bla promoter: bases 7812-7910 (C)
Ampicillin resistance (*bla*) gene: bases 6951-7811 (C)

(C) = complementary strand

CDK2

CDK2 is a member of the serine/threonine protein kinase family, and is a catalytic subunit of the cyclin-dependent protein kinase complex whose activity is restricted to the G1-S phase and essential for cell cycle G1/S phase transition. The protein associates with and is regulated by the regulatory subunits of the complex including cyclin A or E, CDK inhibitor p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B). Its activity is also regulated by its protein phosphorylation.

Accessory Products

Introduction

The products listed in this section may be used with the BLOCK-iT™ RNAi Target Screening Kits. For more information, refer to www.lifetechnologies.com or call Technical Support (see page 45).

Accessory Products

Many of the reagents supplied in the BLOCK-iT™ RNAi Target Screening System as well as other products suitable for use with the kit are available separately for purchase. Ordering information is provided below.

Product	Amount	Cat. no.
pCR™8/GW/TOPO® TA Cloning Kit		
with One Shot® TOP10 Chemically Competent E. coli	20 reactions	K2500-20
with One Shot® Mach1™-T1^R Chemically Competent E. coli	20 reactions	K2520-20
Gateway® LR Clonase® II Enzyme Mix	20 reactions 100 reactions	11791-020 11791-100
One Shot® TOP10 Chemically Competent *E. coli*	20 × 50 µL	C4040-03
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 × 50 µL	C8620-03
One Shot® *ccd*B Survival™ T1^R Chemically Competent *E. coli*	10 reactions	C7510-03
Lipofectamine® 2000 Reagent	0.75 mL 1.5 mL	11668-027 11668-019
Opti-MEM® I Reduced Serum Medium	100 mL 500 mL	31985-062 31985-070
Dulbecco's Phosphate-Buffered Saline (D-PBS)	500 mL 1 L	14190-144 14190-136
BLOCK-iT™ Fluorescent Oligo	2 × 125 µL (20 µM) 75 µL (1 mM)	2013 13750-062
FluoReporter® lacZ/Galactosidase Quantitation Kit	1000 reactions	F-2905
Blasticidin	50 mg	R210-01
PureLink® HQ Mini Plasmid Purification Kit	100 reactions	K2100-01
GripTite™ 293 MSR Cell Line	3 × 10^6 cells × 2 vials	R795-07
Bovine Serum Albumin (BSA)	150 mg	15561-020

Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com/support.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Limited Use Label License ULB ccdB Selection Technology

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Life Technologies' policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Life Technologies' commercially available Gateway® Technology.

Gateway® Entry Clones

Life Technologies understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

Gateway® Expression Clones

Life Technologies also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Life Technologies. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Life Technologies.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase® from Life Technologies is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Life Technologies' Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license, and questions concerning such commercial use should be directed to Life Technologies' licensing department at 760-603-7200.

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989). Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229.

Bohula, E. A., Salisbury, A. J., Sohail, M., Playford, M. P., Riedemann, J., Southern, E. M., and Macaulay, V. M. (2003). The Efficacy of Small Interfering RNAs Targeted to the Type 1 Insulin-Like Growth Factor Receptor (IGF1R) is Influenced by Secondary Structure in the IGF1R Transcript. J. Biol. Chem. *278*, 15991-15997.

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A System for Stable Expression of Short Interfering RNAs in Mammalian Cells. Science *296*, 550-553.

Caplen, N. J., Parrish, S., Imani, F., Fire, A., and Morgan, R. A. (2001). Specific Inhibition of Gene Expression by Small Double-Stranded RNAs in Invertebrates and Vertebrate Systems. Proc. Natl. Acad. Sci. USA *98*, 9746-9747.

Chen, C., and Okayama, H. (1987). High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. Mol. Cell. Biol. *7*, 2745-2752.

Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the Efficient Transfection of Mammalian Cells with DNA. Nucleic Acids Res. *15*, 1311-1326.

Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999). Lipofectamine™ 2000 Reagent for Rapid, Efficient Transfection of Eukaryotic Cells. Focus *21*, 54-55.

Cole, C. N., and Stacy, T. P. (1985). Identification of Sequences in the Herpes Simplex Virus Thymidine Kinase Gene Required for Efficient Processing and Polyadenylation. Mol. Cell. Biol. *5*, 2104-2113.

Czauderna, F., Santel, A., Hinz, M., Fechtner, M., Durieux, B., Fisch, G., Leenders, F., Arnold, W., Giese, K., Klippel, A., and Kaufmann, J. (2003). Inducible shRNA Expression for Application in a Prostate Cancer Mouse Model. Nuc. Acids Res. *31*, e127.

Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003). Killing the Messenger: Short RNAs that Silence Gene Expression. Nat. Rev. Mol. Cell Biol. *4*, 457-467.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-Nucleotide RNAs Mediate RNA Interference in Cultured Mammalian Cells. Nature *411*, 494-498.

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional Anatomy of siRNA for Mediating Efficient RNAi in *Drosophila melanogaster* Embryo Lysate. EMBO J. *20*, 6877-6888.

Elledge, S. J., and Spottswood, M. R. (1991). A New Human p34 Protein Kinase, CDK2, Identified by Complementation of a cdc28 Mutation in *Saccharomyces cerevisiae*, is a Homolog of Xenopus Eg1. EMBO J. *10*, 2653-2659.

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References, Continued

Felgner, P. L. a., and Ringold, G. M. (1989). Cationic Liposome-Mediated Transfection. Nature *337*, 387-388.

Hannon, G. J. (2002). RNA Interference. Nature *418*, 244-251.

Holen, T., Amarzguioui, M., Wiiger, M., Babaie, E., and Prydz, H. (2002). Positional Effects of Short Interfering RNAs Targeting the Human Coagulation Trigger Tissue Factor. Nuc. Acids Res. *30*, 1757-1766.

Kawasaki, H., Suyama, E., Iyo, M., and Taira, K. (2003). siRNAs Generated by Recombinant Human Dicer Induce Specific and Significant But Target Site-Independent Gene Silencing in Human Cells. Nuc. Acids Res. *31*, 981-987.

Kertbundit, S., Greve, H. d., Deboeck, F., Montagu, M. V., and Hernalsteens, J. P. (1991). *In vivo* Random β-glucuronidase Gene Fusions in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *88*, 5212-5216.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949.

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129.

Ninomiya-Tsuji, J., Nomoto, S., Yasuda, H., Reed, S. I., and Matsumoto, K. (1991). Cloning of a Human cDNA Encoding a CDC2-Related Kinase by Complementation of a Budding Yeast cdc28 Mutation. Proc. Natl. Acad. Sci. USA *88*, 9006-9010.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002). Short Hairpin RNAs (shRNAs) Induce Sequence-Specific Silencing in Mammalian Cells. Genes Dev. *16*, 948-958.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. Cell *115*, 199-208.

Shigekawa, K., and Dower, W. J. (1988). Electroporation of Eukaryotes and Prokaryotes: A General Approach to the Introduction of Macromolecules into Cells. BioTechniques *6*, 742-751.

Tsai, L. H., Harlow, E., and Meyerson, M. (1991). Isolation of the Human cdk2 Gene that Encodes the Cyclin A- and Adenovirus E1A-Associated p33 Kinase. Nature *353*, 174-177.

Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003). Efficient Reduction of Target RNAs by Small Interfering RNA and RNase H-Dependent Antisense Agents: A Comparative Analysis. J. Biol. Chem. *278*, 7108-7118.

Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Cheng, Y.-C., and Axel, R. (1977). Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells. Cell *11*, 223-232.

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