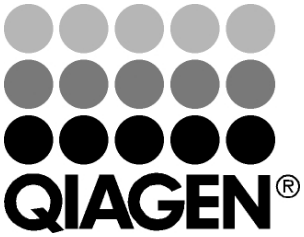
January 2011

Cignal Reporter Assay Handbook

For cell-based pathway activity assays



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Product Use Limitations

Cignal Reporter Assay Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

CONTENTS

I. Introduction

The Cignal Reporter Assays are designed for accurate, sensitive and quantitative assessment of the activation of signal transduction pathways. SABiosciences has developed a series of inducible reporter constructs that encode a reporter gene under the control of a basal promoter element (TATA box) joined to tandem repeats of specific transcriptional response elements (TRE; Figures 1A and 1B). Transcription factor activities can act as readouts for the intracellular status of many signal transduction pathways. Our constructs are specifically engineered for measuring changes in activity (both increases and decreases) of these signaling pathways. Each of the Cignal reporter assays is available in a dual-luciferase format. In addition, six of the Cignal reporter assays are also available as GFP reporter constructs. These include the CRE, SRE, AP-1, NFkB, SMAD, and TCF/LEF Cignal Reporter Assays. The Cignal reporter assays are valuable tools for deciphering gene function, as well as determining the mechanism of action of proteins, peptides, ligands, and small molecule compounds.

Each of the dual-luciferase formatted reporters encodes for the mammalian codon-optimized, non-secreted form of the firefly luciferase gene, carrying a protein-destabilizing sequence. Cells rapidly degrade the destabilized form of the firefly luciferase protein and hence the background luciferase activity (noise level) is greatly reduced. Due to low background activity, the magnitude of the response that can be measured (signal to noise ratio) as well as the speed of measuring changes in transcription are enhanced. The Cignal dual-luciferase reporter assays provide outstanding reproducibility, sensitivity, specificity, and signal to noise ratio. They are extremely useful for carrying out endpoint pathway regulation assays.

The Cignal GFP reporter assays enable you to monitor the dynamics of pathway activation on living cells with single cell resolution. The Cignal GFP reporter constructs utilize the Monster Green™ Fluorescent Protein reporter gene. Monster GFP is encoded by an improved synthetic version of the green fluorescent protein gene. This GFP expression cassette has been codon optimized to maximize mammalian cell expression and also utilizes an optimized Kozak sequence to increase translation efficiency. The synthetic GFP is an ideal fluorescent reporter, providing high-level fluorescence and minimal cytotoxicity. Moreover, the synthetic GFP gene is resistant to photobleaching. In addition, most consensus sequences for transcription factor binding have been removed from the synthetic GFP gene in order to minimize aberrant transcription and improve the reliability of the GFP as an accurate reporter. The spectral properties of the synthetic GFP are slightly red-shifted compared to other commercially available GFPs. Peak excitation occurs at 505nm, with a shoulder at 480nm; peak emission occurs at 515nm.

Benefits of Cignal Reporter Assays

- **PERFORMANCE**: Both the dual-luciferase and GFP reporter systems provide exceptional sensitivity, reproducibility, specificity, and signal to noise ratio

- **VERSATILITY**: Can monitor signal transduction pathway activity utilizing the dual-luciferase reporter system in an endpoint format assay, or measure pathway activation dynamics on live cells using the GFP reporter system

- **CONVENIENCE**: Transfection-ready constructs, including positive and negative controls, coupled with a transient reporter system, enable rapid analysis of signal transduction pathway regulation.

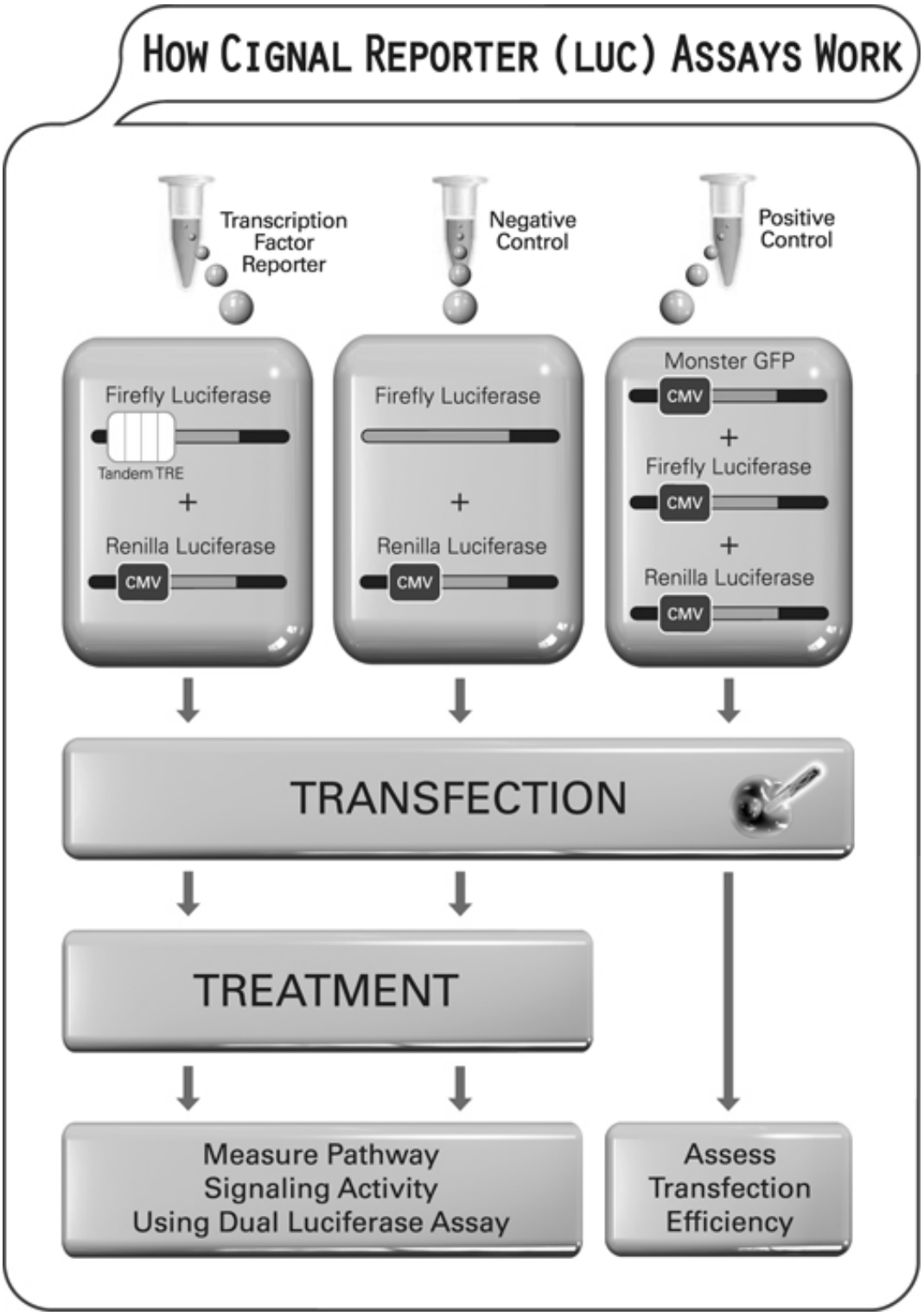


Figure 1A: Overview of Cignal Dual-Luciferase Reporter Assays Process.

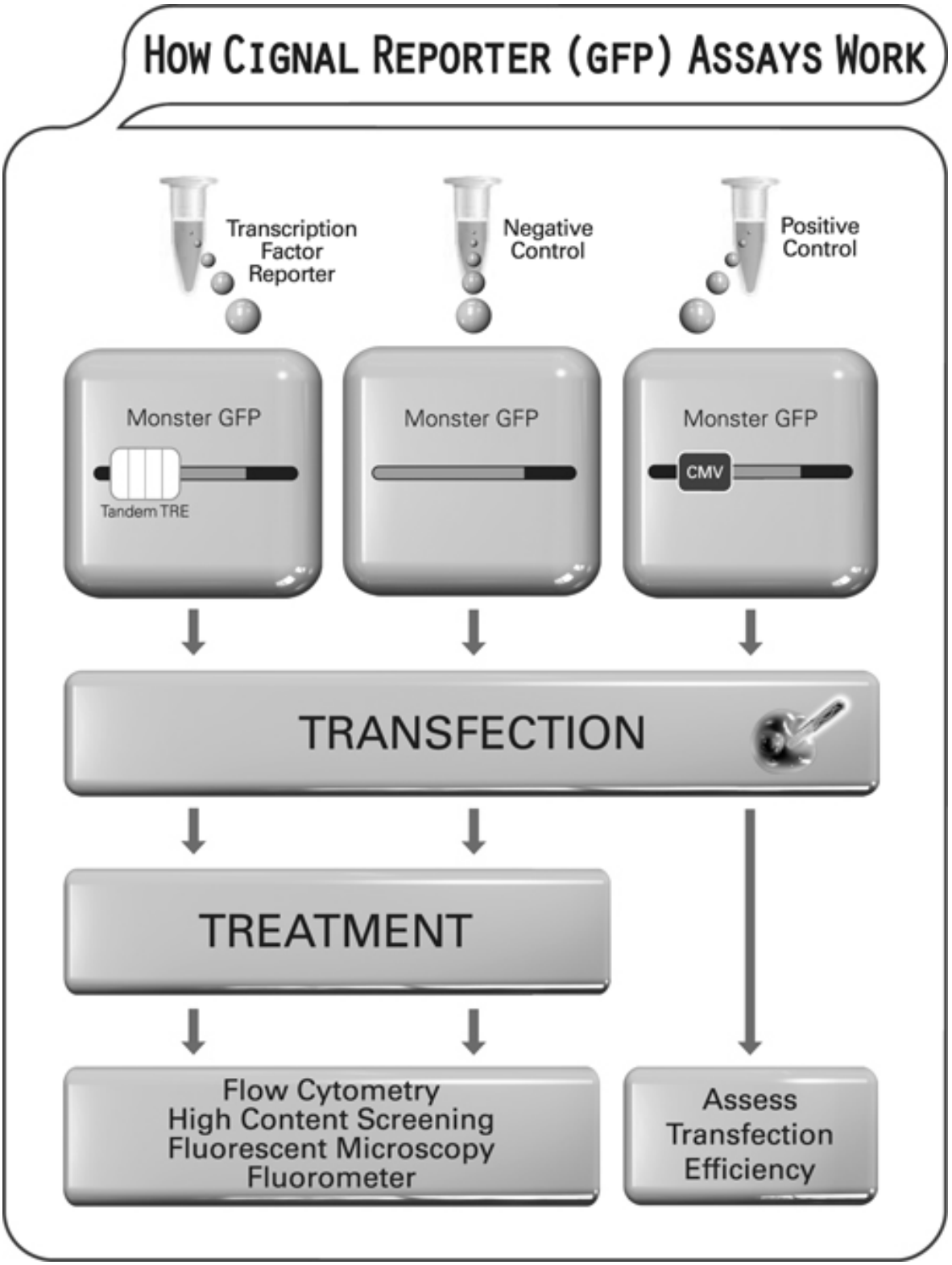


Figure 1B: Overview of Cignal GFP Reporter Assays Process.

II. Product Contents and Descriptions

A. Dual-Luciferase Reporter Assay Kits:

1. Kit Contents

Table 1: Cignal Reporter Assay Kit Specifications

Component	Specification	Concentration and total volume
Reporter	A mixture of an inducible transcription factor responsive firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	(100 ng/μl; 500 µl)
Negative control	A mixture of non-inducible firefly luciferase reporter and constitutively expressing *Renilla* construct (40:1).	(100 ng/μl; 500 µl)
Positive control	A mixture of a constitutively expressing GFP construct, constitutively expressing firefly luciferase construct, and constitutively expressing *Renilla* luciferase construct (40:1:1).	(100 ng/μl; 250 µl)

NOTE: *These constructs are **transfection-grade** and are ready for transient transfection. These constructs are specifically designed to inhibit transformation and are NOT MEANT for introduction and amplification in bacteria.*

2. Description:

Each Cignal Reporter Assay Kit includes the following components:

1. **Reporter:** The reporter is a mixture of inducible transcription factor responsive construct and constitutively expressing *Renilla* luciferase construct (40:1). The inducible transcription factor-responsive construct encodes the firefly luciferase reporter gene under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific Transcriptional Response Element (TRE; Figure 2A). This construct monitors both increases and decreases in the activity of a key transcription factor, which is a downstream target of a specific signaling pathway. The constitutively expressing *Renilla* construct encodes the *Renilla* luciferase reporter gene under the control of a CMV immediate early enhancer/promoter

(Figure 2B) and acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. It is also useful to confirm transfection and to verify active luciferase in the transfected culture.

2. **Negative control:** The negative control is a mixture of non-inducible reporter construct and constitutively expressing *Renilla* luciferase construct (40:1). The non-inducible reporter construct encodes firefly luciferase under the control of a basal promoter element (TATA box), without any additional transcriptional response elements (Figure 2C). The negative control is critical to identifying specific effects and determining background reporter activity.

3. **Positive control:** The positive control is a constitutively expressing GFP construct (Figure 2D), pre-mixed with a constitutively expressing firefly luciferase construct (Figure 2E), and a constitutively expressing *Renilla* luciferase construct (Figure 2B) (40:1:1). The positive control is necessary for visual confirmation of transfection. It is also useful for transfection optimization studies. The expression of the GFP from the positive control construct can be monitored by fluorescence microscopy using an excitation filter of 470 ± 20 nm (470 / 40 nm) and an emission filter of 515 nm (long pass).

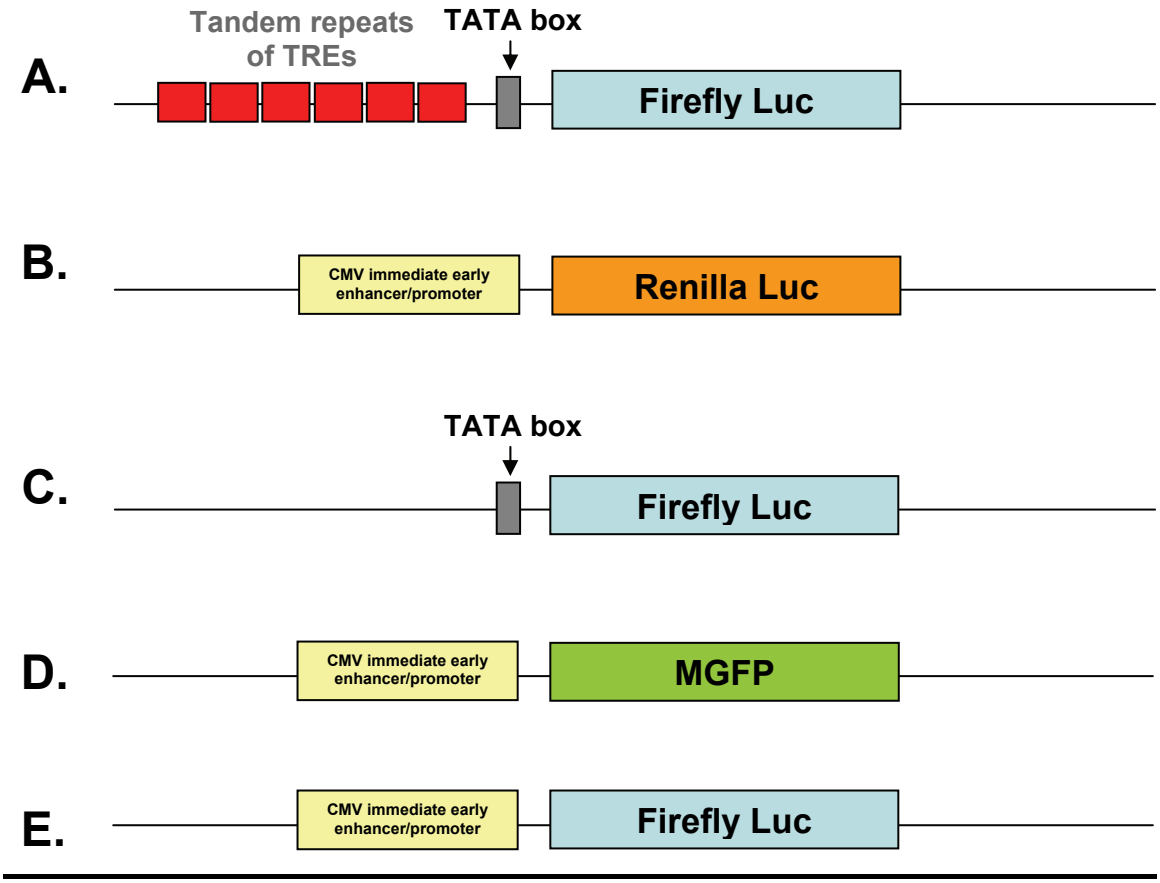


Figure 2: Schematic representation of constructs involved in the Cignal Reporter Assay. (A) The inducible transcription factor-responsive construct expressing firefly luciferase, (B) The constitutively expressing *Renilla* luciferase construct, (C) The non-inducible firefly luciferase reporter construct, (D) The constitutively expressing GFP construct, and (E) The constitutively expressing firefly luciferase construct.

B. GFP Reporter Assay Kits:

1. Kit Contents

Table 2: Cignal GFP Reporter Assay Kit Specifications

Component	Specification	Concentration and total volume
Reporter	An inducible transcription factor responsive GFP reporter	(100 ng/μl; 500 µl)
Negative control	A GFP reporter construct in which GFP expression is controlled by a minimal promoter	(100 ng/μl; 500 µl)
Positive control	A constitutively expressing GFP construct	(100 ng/μl; 250 µl)

NOTE: *These constructs are **transfection-grade** and are ready for transient transfection. These constructs are specifically designed to inhibit transformation and are NOT MEANT for introduction and amplification in bacteria.*

2. Description:

Each Cignal GFP Reporter Assay Kit includes the following components:

1. **Reporter:** The inducible transcription factor-responsive GFP reporter encodes the green fluorescent protein gene under the control of a basal promoter element (TATA box) joined to tandem repeats of a specific Transcriptional Response Element (TRE; Figure 3A). This construct monitors both increases and decreases in the activity of a key transcription factor, which is a downstream target of a specific signaling pathway

2. **Negative control:** The negative control is a GFP reporter that encodes the green fluorescent protein under the control of a basal promoter element (TATA box), without any additional transcriptional response elements (Figure 3B). The negative

control is critical to identifying pathway-specific effects and determining background reporter activity.

3. **Positive control:** The positive control is a constitutively expressing GFP construct (Figure 3C). The positive control is necessary for visual confirmation of transfection. It is also useful for transfection optimization studies.

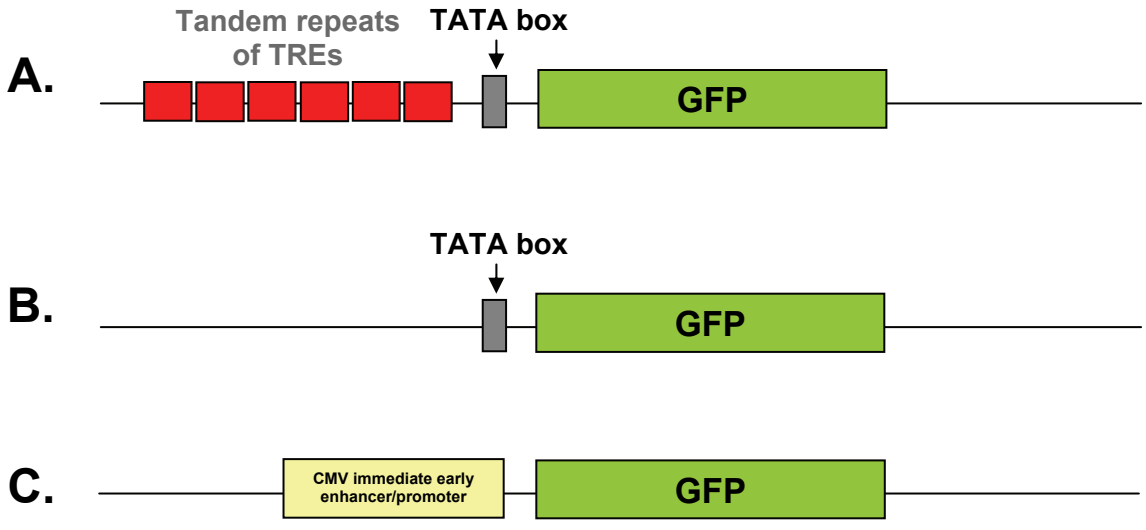


Figure 3: Schematic representation of constructs involved in Cignal GFP Reporter Assay. (A) The inducible transcription factor-responsive reporter expressing GFP, (B) The GFP reporter controlled by a minimal promoter (negative control), (C) The constitutively expressing GFP construct (positive control).

IMPORTANT NOTE: There are a few reports in the literature of the CMV regulatory element being activated by certain stimuli (see below). We recommend that you confirm that the stimulus used in each Cignal reporter assay does not induce the CMV regulatory element, in order to confirm that the CMV-Renilla construct is the appropriate normalization construct for your experiment. This can be done empirically by testing the impact of your stimulus on the Cignal positive control reporters, which are each under the control of the CMV enhancer/promoter cassette. If your stimulus is one of the very few reported activators of the CMV regulatory element, we advise using an alternative reporter as an internal control.

- W. Bruening, B. Giasson, W. Mushynski, and H. D. Durham. 1998. Nucleic Acids Research 26(2):486-489. **Activation of stress-activated MAP protein kinases up-regulates expression of transgenes driven by the cytomegalovirus immediate/early promoter**

- Madhu S. Malo, Moushumi Mozumder, Alexander Chen, Golam Mostafa, Xiao Bo Zhang, Richard A. Hodin. 2006. Analytical Biochemistry 350:307-309. **pFRL7: An ideal vector for eukaryotic promoter analysis**

III. Additional Materials Required:

- Mammalian cell line cultured in the appropriate growth medium
- Cell culture medium and standard cell culture supplies
- 96-well tissue culture plates
- Multi-channel pipettor and pipettor reservoirs
- Transfection reagent [We recommend Attractene Transfection Reagent (QIAGEN, cat. no. 301005), however, other transfection reagents work equally well]
- Polystyrene test tubes (BD FALCON, Cat # 352099)
- Opti-MEM® I Reduced Serum Medium (Invitrogen, Cat. No. 31985-062)
- Fetal bovine serum (FBS)
- Non-essential amino acids (NEAA) (Invitrogen, Cat. No. 11140-050)
- Penicillin/Streptomycin
- Hemacytometer
- Dual-Luciferase® Assay System
    - Dual-Luciferase® Reporter Assay System (Promega, Cat. No. E1910)
      This system requires cell lysis, and is well-suited for the rapid quantitation of both luciferase reporters when using luminometers with reagent auto-injectors.
    - Dual-Glo® Luciferase Assay System (Promega, Cat. No. E2920)
      This system is used to assay for both luciferase reporters on intact cells in growth medium. This system can be used with any luminometer, including those without reagent auto-injectors.
- 96-well white opaque flat bottom microtiter plate

- Luminometer
- FACS, flow cytometer, fluorescent microscope, or fluorometer

IV. Protocol:

A. Before you begin:

1. Cell line selection: The Cignal Reporter Assay may be used with various mammalian cell lines. Cell lines show a great deal of variation in the levels of signaling proteins. The transcriptional activator activities in the cell line used will determine the sensitivity of the assay. A cell line should be selected based on the functionality of the signal transduction pathway under investigation, as well as for the "transfectability" of the cell line (see below).

2. Transfection reagent selection: We recommend the use of Attractene Transfection Reagent (cat. no. 301005) as a transfection reagent. The Cignal Reporter Assay, however, also performs equally well with other transfection reagents such as Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027), or FuGENE 6 (Roche, Cat. No. 1815091). When using alternative transfection reagents, please refer to the manufacturer's instructions on the use of those reagents.

3. Optimization of transfection conditions: The sensitivity of the Cignal Reporter Assay depends on the transfection efficiency. The transfection efficiency, in turn, primarily depends upon cell line used. Therefore, it is very important to optimize the transfection conditions for each cell type under study. Variables to consider, when optimizing the transfection conditions include cell density, cell viability, amount of DNA, ratio of DNA to transfection reagent, transfection complex formation time, and transfection incubation time (see the detailed protocols for our recommendations). The positive control construct included with each Cignal Reporter Assay can be used for determining the optimal transfection conditions.

4. Optimization of assay condition: The response rate in the Cignal Reporter Assay depends on the assay conditions (conditions of the experimental treatment). To obtain maximum response given by any stimulus, perform dosing and time-course studies. The optimal amount of stimulus and the time of treatment must be obtained empirically for each experiment (see different protocols for our recommendations).

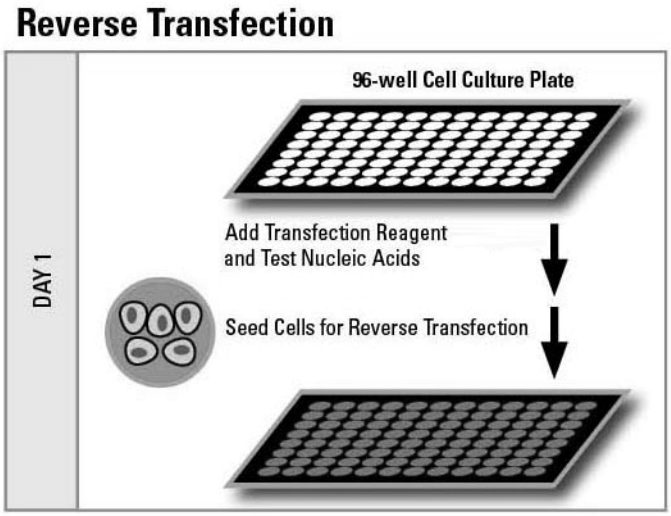
5. Important recommendations for best results:
    A. Perform all transfections in **triplicate** to minimize variability among treatment groups.
    B. Include positive and negative controls in each experiment to obtain reliable results.

C. Use low-passage cells that are actively growing and are greater than 90% viable, for maximal transfection efficiencies.
D. Do not add antibiotics to media during transfection, as this may cause cell death.
E. Take care to always seed the same number of cells in each well, in order to maximize the reproducibility of your experiment.
F. Serum induces various signaling pathways, leading to cross-talk and high background. Therefore, use reduced amounts of serum (0.5%) in the assay medium during the experimental treatment to minimize these serum effects.

B. Generalized Transfection Protocols:

We recommend using reverse transfection protocols with the Attractene Transfection Reagent throughout the Cignal Reporter Assays User Manual. This is due to the time savings and improved reproducibility of using this method, compared to traditional forward transfection methods. However, Cignal Reporter Assays also work well with traditional forward transfection methods and transfection reagents from other vendors. Below are general protocol overviews for the Cignal Reporter Assays, using either reverse or forward transfection approaches.

1. Reverse Transfection Protocol Overview (1 DAY PROCEDURE)



DAY 1

- Prepare nucleic acid mixtures in appropriate ratios. This may include any of the following combinations, depending upon the experimental design (we recommend carrying out each transfection condition in triplicate):

Experimental transfection

i. Cignal Reporter + test nucleic acid (expression plasmids, shRNA plasmids, or siRNAs)

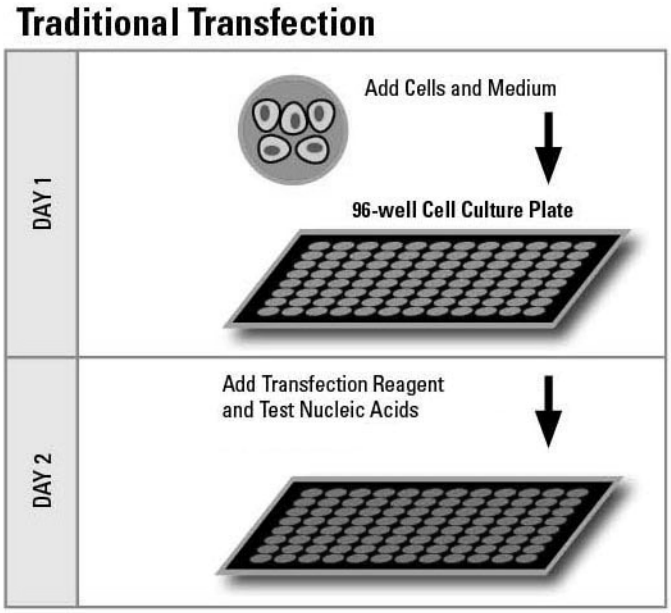
Control transfections

ii. Cignal Reporter + negative control for test nucleic acid
iii. Cignal Negative Control + test nucleic acid (expression plasmids, shRNA plasmids, or siRNAs)
iv. Cignal Negative Control + negative control for test nucleic acid
v. Cignal Positive Control

- Dilute Attractene into Opti-MEM
- Add diluted Attractene to nucleic acid mixtures, incubate at room temperature for 20 minutes
- Trypsinize (if necessary), count, and suspend cells to appropriate density
- Aliquot transfection complexes into wells
- Immediately seed cells to each well *

*** For detailed information on the transfection conditions, and treatment of cultures post-transfection, refer to the application-specific protocols within this user manual.**

2. Traditional Transfection Protocol Overview (2 DAY PROCEDURE)



DAY 1

- Trypsinize (if necessary), count, and suspend cells to appropriate density
- Seed cells into multiwell plate(s)

DAY 2

- Prepare nucleic acid mixtures in appropriate ratios. This may include any of the following combinations, depending upon the experimental design (we recommend carrying out each transfection condition in triplicate):

Experimental transfection

i. Cignal Reporter + test nucleic acid (expression plasmids, shRNA plasmids, or siRNAs)

Control transfections

ii. Cignal Reporter + negative control for test nucleic acid
iii. Cignal Negative Control + test nucleic acid (expression plasmids, shRNA plasmids, or siRNAs)
iv. Cignal Negative Control + negative control for test nucleic acid
v. Cignal Positive Control

- Dilute Attractene Transfection Reagent into appropriate medium (**If you are using a transfection reagent other than Attractene Transfection Reagent follow their manufacturer's protocol for transfection**)
- Add diluted transfection reagent to nucleic acid mixtures, incubate at room temperature for 20 minutes
- Aliquot transfection complexes into wells containing overnight cell cultures

C. Co-transfection Protocol for siRNA + Reporter Assay

The following protocol is designed to reverse transfect adherent cell line, HEK-293H, using Attractene Transfection Reagent (cat. no. 301005) in a 96-well plate format. The Cignal Reporter Assay works well with transfection reagents from other vendors. *If you are using a transfection reagent other than Attractene Transfection Reagent follow their manufacturer's protocol for optimizing transfection*. The Cignal Reporter Assay also works well using traditional forward transfection protocols. Moreover, if you are using plates or wells of different size, adjust the components in proportion to the surface area (see section IV.H). **This is just a general guideline; the optimal conditions/amounts should be optimized according to the cell type and the study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death.

Table 2: Guidelines for setting up co-transfections of siRNA and Cignal Reporter Assays. Table 2 represents the total components needed, on a per well basis, for each condition to be tested. Note that individual components must be added sequentially, as instructed in the protocol.

#	Cignal Reporter (per well)	Cignal Negative Control (per well)	Cignal Positive Control (per well)	Specific siRNA (per well)	Negative Control siRNA (per well)	Opti-MEM Nucleic Acid Diluent (per well)	Attractene (per well)	Opti-MEM Attractene Diluent (per well)	Time of transfection (hours)
1	100 ng (1.0 µl)				2 pmol	25 µl	0.6 µl	25 µl	48 h or 72 h
2	100 ng (1.0 µl)			2 pmol		25 µl	0.6 µl	25 µl	
3		100 ng (1.0 µl)			2 pmol	25 µl	0.6 µl	25 µl	
4		100 ng (1.0 µl)		2 pmol		25 µl	0.6 µl	25 µl	
5			100 ng (1.0 µl)			25 µl	0.6 µl	25 µl	

1. The recommended experimental setup, on a **per well basis**, follows. Please note that we recommend setting up multiple replicates for each condition, and preparing transfection cocktail volumes sufficient for transfecting multiple wells. In addition, we advise always taking 5-10% extra amounts of nucleic acid, Opti-MEM® serum-free culture medium, and Attractene to compensate for pipettor error when setting up transfection cocktails (steps 1 through 4).

Add 25 µl of Opti-MEM® to each of 5 polystyrene tubes, along with the following:

Experimental transfection

- 1 µl (100 ng) Cignal reporter + 2 pmol sequence-specific siRNA

Control transfections

- 1 µl (100 ng) Cignal reporter + 2 pmol negative control siRNA
- 1 µl (100 ng) Cignal negative control + 2 pmol sequence-specific siRNA
- 1 µl (100 ng) Cignal negative control + 2 pmol negative control siRNA
- 1 µl (100 ng) Cignal positive control

Mix each transfection cocktail gently.

2. Prepare an Attractene dilution for 5 tubes (mentioned in step 1) by dispensing 3 µl of Attractene into 125 µl of Opti-MEM® serum-free culture medium (for every well dilute 0.6 µl of Attractene in 25 µl of Opti-MEM® serum-free culture medium) in a polystyrene test tube. Mix gently and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 25 µl of diluted Attractene into each of the five tubes containing 25 µl of the diluted nucleic acids (1:1 ratio) as detailed in Table 2.

4. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells* in a culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37ºC in a

humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 4 x 10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA**. To ensure reproducible transfection results, it is important to accurately measure the cell density with a hemacytometer or an automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, aliquot 50 µl of specific constructs-siRNA-Attractene complexes into the appropriate wells.

7. Add 100 µl of prepared cell suspension (4 x 10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum) to each well containing constructs-siRNA-Attractene complexes. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16-24 hours.

9. After 16-24 hours of transfection, change the medium to complete growth medium (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin).

10. To study the effect of knockdown, we recommend harvesting cells 48 or 72 hours after transfection to perform dual-luciferase assay.

12. The **luciferase assay** can be developed by using Dual-Luciferase Reporter Assay System from Promega (Cat. No. 1910). Follow the manufacturer's protocol for developing the assay.
Expression of the **Monster GFP** reporter can be monitored via FACS, flow cytometry, fluorescent microscopy, or standard fluorometry. The spectral properties of the Monster Green Fluorescent Protein are slightly red-shifted compared to other commercially available GFP reporters. We recommend using the standard FACS settings of an argon laser (488 nm excitation) and filters of 530±15 nm (530/30 nm) for emission. When analyzing GFP expression via fluorescent microscopy or standard fluorometry, we recommend using standard fluoroisothiocyanate (FITC) filters [excitation of 470±20 nm and an emission filter of 515 nm (long pass)].

*Cells that have been passed 1:3 or 1:4 the day before are generally more easily transfected than cells that have reached a confluent state at the time of use.

** In most cases, cells grow well in Opti-MEM® serum-reduced growth medium with 3-5% FBS due to extra growth factors and nutrients supplied in Opti-MEM®. Cell should reach ~50-90% confluence once attached to the wells, otherwise increase the cell numbers.

D. Co-transfection Protocol for shRNA + Reporter Assay

The following protocol is designed to reverse transfect adherent cell line, HEK-293H, using Attractene Transfection Reagent (cat. no. 301005) in a 96-well plate format. The Cignal Reporter Assay works well with transfection reagent from other vendors. *If you are using transfection reagent other than Attractene Transfection Reagent follow their manufacturer's protocol for transfection*. The Cignal Reporter Assay also works well using traditional forward transfection protocols. Moreover, if you are using plates or wells of different size, adjust the components in proportion to the surface area (see section IV.H). **This is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death.

Table 3: Guidelines for setting up co-transfections of a shRNA vector and Cignal Reporter Assay. Table 3 represents the total components needed, on a per well basis, for each condition to be tested. Note that individual components must be added sequentially, as instructed in the protocol.

#	Cignal Reporter (per well)	Cignal Negative Control (per well)	Cignal Positive Control (per well)	Specific shRNA (per well)	Negative Control shRNA (per well)	Opti-MEM Nucleic Acid Diluent (per well)	Attractene (per well)	Opti-MEM Attractene Diluent (per well)	Time of transfection (hours)
1	100 ng (1.0 µl)				200 ng	25 µl	0.6 µl	25 µl	48 h or 72 h
2	100 ng (1.0 µl)			200 ng		25 µl	0.6 µl	25 µl	
3		100 ng (1.0 µl)			200 ng	25 µl	0.6 µl	25 µl	
4		100 ng (1.0 µl)		200 ng		25 µl	0.6 µl	25 µl	
5			100 ng (1.0 µl)			25 µl	0.6 µl	25 µl	

1. The recommended experimental setup, on a **per well basis**, follows. Please note that we recommend setting up multiple replicates for each condition, and preparing transfection cocktail volumes sufficient for transfecting multiple wells. In addition, we advise always taking 5-10% extra amounts of nucleic acid, Opti-MEM® serum-free culture medium, and Attractene to compensate for pipettor error when setting up transfection cocktails (steps 1 through 4).
Add 25 µl of Opti-MEM® to each of 5 polystyrene tubes, along with the following:

Experimental transfection

- 1 µl (100 ng) Cignal reporter + 200 ng sequence-specific shRNA

Control transfections

- 1 µl (100 ng) Cignal reporter + 200 ng negative control shRNA

- 1 µl (100 ng) Cignal negative control + 200 ng sequence-specific shRNA
- 1 µl (100 ng) Cignal negative control + 200 ng negative control shRNA
- 1 µl (100 ng) Cignal positive control

Mix each transfection cocktail gently.

2. Prepare an Attractene dilution for 5 tubes (mentioned in step 1) by dispensing 3 µl of Attractene into 125 µl of Opti-MEM® serum-free culture medium (for every well dilute 0.6 µl of Attractene in 25 µl of Opti-MEM® serum-free culture medium) in a polystyrene test tube. Mix gently and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 25 µl of diluted Attractene into each of the five tubes containing 25 µl of diluted constructs (1:1 ratio) as detailed in Table 3.

4. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells* in a culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37ºC in a humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 4 $\times 10^5$ cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA**. To ensure reproducible transfection results, it is important to accurately measure the cell density with a hemacytometer or automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, aliquot 50 µl of specific constructs-shRNA-Attractene complexes into the appropriate wells.

7. Add 100 µl of prepared cell suspension (4 $\times 10^5$ cells/ml in Opti-MEM® containing 5% of fetal bovine serum) to each well containing construct-shRNA-Attractene complexes. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16-24 hours.

9. After 16-24 hours of transfection, change the medium to complete growth medium (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin).

10. To study the effect of knockdown, we recommend harvesting cells 48 or 72 hours after transfection to perform dual-luciferase assay.

11. The **luciferase assay** can be developed by using Dual-Luciferase Reporter Assay System from Promega (Cat. No. 1910). Follow the manufacturer's protocol for developing the assay.

Expression of the **Monster GFP®** reporter can be monitored via FACS, flow cytometry, fluorescent microscopy, or standard fluorometry. The spectral properties of the Monster Green Fluorescent Protein are slightly red-shifted compared to other commercially

available GFP reporters. We recommend using the standard FACS settings of an argon laser (488 nm excitation) and filters of 530±15 nm (530/30 nm) for emission. When analyzing GFP expression via fluorescent microscopy or standard fluorometry, we recommend using standard fluoroisothiocyanate (FITC) filters [excitation of 470±20 nm and an emission filter of 515 nm (long pass)].

*Cells that have been passed 1:3 or 1:4 the day before are generally more easily transfected than cells that have reached a confluent state at the time of use.

**In most cases, cells grow well in Opti-MEM® serum reduced growth medium with 3%-5% FBS due to extra growth factors and nutrients supplied in Opti-MEM®. Cell should reach ~50-90% confluence once attached to the wells, otherwise increase the cell numbers.

E. Co-transfection Protocol for Expression Vector + Reporter Assay

The following protocol is designed to reverse transfect adherent cell line, HEK-293H, using Attractene Transfection Reagent (cat. no. 301005) in a 96-well plate format. The Cignal Reporter Assay works well with transfection reagent from other vendors. *If you are using transfection reagent other than Attractene Transfection Reagent follow their manufacturer's protocol for transfection*. The Cignal Reporter Assay also works well using traditional forward transfection protocols. Moreover, if you are using plates or wells of different size, adjust the component in proportion to the surface area (see section IV.H). **This is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and the study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death.

Table 4: Guidelines for setting up co-transfections of an expression vector and Cignal Reporter Assay. Table 4 represents the total components needed, on a per well basis, for each condition to be tested. Note that individual components must be added sequentially, as instructed in the protocol.

#	Cignal Reporter (per well)	Cignal Negative Control (per well)	Cignal Positive Control (per well)	Experimental Vector with Gene of Interest (per well)	Experimental Vector Without Insert (per well)	Carrier DNA[a]	Opti-MEM Nucleic Acid Diluent (per well)	Attractene (per well)	Opti-MEM Attractene Diluent (per well)	Time of transfection (hours)
1	100 ng (1.0 µl)				100 ng	100 ng	25 µl	0.6 µl	25 µl	32 h - 48 h
2	100 ng (1.0 µl)				200 ng		25 µl	0.6 µl	25 µl	
3	100 ng (1.0 µl)			100 ng		100 ng	25 µl	0.6 µl	25 µl	
4	100 ng (1.0 µl)			200 ng			25 µl	0.6 µl	25 µl	
5		100 ng (1.0 µl)			100 ng	100 ng	25 µl	0.6 µl	25 µl	
6		100 ng (1.0 µl)			200 ng		25 µl	0.6 µl	25 µl	
7		100 ng (1.0 µl)		100 ng		100 ng	25 µl	0.6 µl	25 µl	
8		100 ng (1.0 µl)		200 ng			25 µl	0.6 µl	25 µl	
9			100 ng (1.0 µl)				25 µl	0.6 µl	25 µl	

[a] Carrier DNA means any empty plasmid, such as a pUC or a pBR plasmid.

1. The recommended experimental setup, on a **per well basis**, follows. Please note that we recommend setting up multiple replicates for each condition, and preparing transfection cocktail volumes sufficient for transfecting multiple wells. In addition, we advise always taking 5-10% extra amounts of nucleic acid, Opti-MEM® serum-free

culture medium, and Attractene to compensate for pipettor error when setting up transfection cocktails (steps 1 through 4).

Add 25 µl of Opti-MEM® to each of 9 polystyrene tubes, along with the following:

Experimental transfections

- 1 µl (100 ng) Cignal reporter + 100 ng experimental vector expressing gene of interest + 100 ng carrier DNA
- 1 µl (100 ng) Cignal reporter + 200 ng experimental vector expressing gene of interest

Control transfections

- 1 µl (100 ng) Cignal reporter + 100 ng negative control expression vector + 100 ng carrier DNA
- 1 µl (100 ng) Cignal reporter + 200 ng negative control expression vector
- 1 µl (100 ng) Cignal negative control + 100 ng experimental vector expressing gene of interest + 100 ng carrier DNA
- 1 µl (100 ng) Cignal negative control + 200 ng experimental vector expressing gene of interest
- 1 µl (100 ng) Cignal negative control + 100 ng negative control expression vector + 100 ng carrier DNA
- 1 µl (100 ng) Cignal negative control + 200 ng negative control expression vector
- 1 µl (100 ng) Cignal positive control

Mix each transfection cocktail gently.

2. Prepare an Attractene dilution for 9 tubes (mentioned in step 1) by dispensing 5.4 µl of Attractene into 225 µl of Opti-MEM® serum-free culture medium (for every well dilute 0.6 µl of Attractene in 25 µl of Opti-MEM® serum-free culture medium) in a polystyrene test tube. Mix gently and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 25 µl of diluted Attractene into each of the nine tubes containing 25 µl of diluted constructs (1:1 ratio) as detailed in Table 4.
4. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells* in a culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37ºC in a humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 4×10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA**. To ensure reproducible transfection results, it is important to accurately measure the cell density with a hemacytometer or automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, aliquot 50 µl of specific complexes into the appropriate wells.

7. Add 100 µl of prepared cell suspension (4 ×10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum) to each well containing construct-vector-Attractene complexes. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16-24 hours.

9. After 16-24 hours of transfection, change the medium to complete growth medium (DMEM with 10% FBS, 0.1mM NEAA, 1mM Sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin).

11. To study the effect of the gene product, we recommend harvesting cells 32 hours or 48 hours after transfection to perform the dual-luciferase assay.

12. The **luciferase assay** can be developed by using Dual-Luciferase Reporter Assay System from Promega (Cat. No. 1910). Follow the manufacturer's protocol for developing the assay.
Expression of the **Monster GFP** reporter can be monitored via FACS, flow cytometry, fluorescent microscopy, or standard fluorometry. The spectral properties of the Monster Green Fluorescent Protein are slightly red-shifted compared to other commercially available GFP reporters. We recommend using the standard FACS settings of an argon laser (488 nm excitation) and filters of 530±15 nm (530/30nm) for emission. When analyzing GFP expression via fluorescent microscopy or standard fluorometry, we recommend using standard fluoroisothiocyanate (FITC) filters [excitation of 470±20 nm and an emission filter of 515nm (long pass)].

*Cells that have been passed 1:3 or 1:4 the day before are generally more easily transfected than cells that have reached a confluent state at the time of use.

**In most cases, cells grow well in Opti-MEM® serum reduced growth medium with 3%-5% FBS due to extra growth factors and nutrients supplied in Opti-MEM®. Cell should reach ~50-90% confluence once attached to the wells, otherwise increase the cell numbers.

F. Transfection and Treatment Protocol for Reporter Assay + Small Molecules/Organic Compounds

The following protocol is designed to reverse transfect adherent cell line, HEK-293H, using Attractene Transfection Reagent (cat. no. 301005) as a transfection reagent in 96-well plate format. The Cignal reporter assay works well with transfection reagent from other vendors. *If you are using transfection reagent other than Attractene follow their manufacturer's protocol for transfection*. The Cignal Reporter Assay also works well using traditional forward transfection protocols. Moreover, if you are using plates or wells of different size, adjust the component in proportion to the surface area (see section IV.H). **This is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and the study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death.

Table 5: Guidelines for studying the effect of small molecules/organic compounds. Table 5 represents the total components needed, on a per well basis, for each condition to be tested. Note that individual components must be added sequentially, as instructed in the protocol.

#	**Cignal Reporter** (per well)	**Cignal Negative Control** (per well)	**Cignal Positive Control Construct** (per well)	**Small Molecule/ Organic Compound** (per well)	**Opti-MEM DNA diluent** (per well)	**Attractene** (per well)	**Opti-MEM Attractene diluent** (per well)	**Time of Transfection** (hours)
1	100 ng (1.0 µl)				25 µl	0.6 µl	25 µl	30 h or 42 h
2	100 ng (1.0 µl)			1X[a]	25 µl	0.6 µl	25 µl	
3	100 ng (1.0 µl)			10X	25 µl	0.6 µl	25 µl	
4	100 ng (1.0 µl)			100X	25 µl	0.6 µl	25 µl	
5		100 ng (1.0 µl)			25 µl	0.6 µl	25 µl	
6		100 ng (1.0 µl)		1X	25 µl	0.6 µl	25 µl	
7		100 ng (1.0 µl)		10X	25 µl	0.6 µl	25 µl	
8		100 ng (1.0 µl)		100X	25 µl	0.6 µl	25 µl	
9			100 ng (1.0 µl)		25 µl	0.6 µl	25 µl	

[a] 1X is a smallest appropriate amount of small molecule or organic compound expected to modulate signaling pathway.

1. The recommended experimental setup, on a **per well basis**, follows. Please note that we recommend setting up multiple replicates for each condition, and preparing transfection cocktail volumes sufficient for transfecting multiple wells. In addition, we advise always taking 5-10% extra amounts of nucleic acid, Opti-MEM® serum-free

culture medium, and Attractene to compensate for pipettor error when setting up transfection cocktails (steps 1 through 4).

Set up three polystyrene tubes, as follows

Experimental transfections

Tubes 1 - 4: 100 µl Opti-MEM® + 4 µl (400 ng) Cignal reporter (**4 volumes for conditions 1 to 4 of Table 5**; for every well dilute 1 µl (100 ng) of Cignal reporter in 25 µl of Opti-MEM® serum-free culture medium)

Control transfections

Tubes 5 - 8: 100 µl Opti-MEM® + 4 µl (400 ng) Cignal negative control (**4 volumes for conditions 5 to 8 of Table 5**; for every well dilute 1 µl (100 ng) of Cignal reporter in 25 µl of Opti-MEM® serum-free culture medium)

Tube 9: 25 µl Opti-MEM® + 1µl (100 ng) Cignal positive control

Mix each transfection cocktail gently.

2. Prepare an Attractene dilution for 9 tubes (mentioned in step 1) by dispensing 5.4 µl of Attractene into 225 µl of Opti-MEM® serum-free culture medium (for every well dilute 0.6 µl of Attractene in 25 µl of Opti-MEM® serum-free culture medium) in a polystyrene test tube. Mix gently and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 100 µl of diluted Attractene to the two tubes (mentioned in step 1) containing equal volume (100 µl) of diluted Cignal reporter, and add 25 µl of diluted Attractene into the positive control tube containing 25 µl of diluted constructs (1:1 ratio) as detailed in Table 5.

4. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells* in culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37 ºC in a humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 4 $\times 10^5$ cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA**. To ensure reproducible transfection results, it is important to accurately determine the cell density with a hemacytometer or an automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, aliquot 50 µl of specific complexes into the appropriate wells.

7. Add 100 µl of prepared cell suspension (4 $\times 10^5$ cells/ml in Opti-MEM® containing 5% of fetal bovine serum) to each well containing constructs- Attractene complexes. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16 hours.

9. After 16 hours of transfection, change medium to assay medium (Opti-MEM® containing 0.5% of fetal bovine serum, 1% NEAA, 100 U/ml Penicillin and 100 µg/ml Streptomycin).
10. After 24 hours of transfection, treat the cells, as described in Table 5, with 1×, 10× and 100× amount of small molecule or organic compound (1× is the lowest appropriate amount of small molecule or organic compound expected to modulate the signaling pathway).

11. To study the effect of small molecule or organic compound, we recommend harvesting cells 6 hours or 18 hours after treatment to perform dual-luciferase assay.

12. The **luciferase assay** can be developed by using Dual-Luciferase Reporter Assay System from Promega (Cat. No. 1910). Follow the manufacturer's protocol for developing the assay.
Expression of the **Monster GFP** reporter can be monitored via FACS, flow cytometry, fluorescent microscopy, or standard fluorometry. The spectral properties of the Monster Green Fluorescent Protein are slightly red-shifted compared to other commercially available GFP reporters. We recommend using the standard FACS settings of an argon laser (488 nm excitation) and filters of 530±15 nm (530/30 nm) for emission. When analyzing GFP expression via fluorescent microscopy or standard fluorometry, we recommend using standard fluoroisothiocyanate (FITC) filters [excitation of 470±20 nm and an emission filter of 515 nm (long pass)].

*Cells that had been passed 1:3 or 1:4 the day before are generally more easily transfected than cells that have reached a confluent state at the time of use.

**In most cases, cells grow well in Opti-MEM® serum reduced growth medium with 3%-5% FBS due to extra growth factors and nutrients supplied in Opti-MEM®. Cell should reach ~50-90% confluence once attached to the wells, otherwise increase the cell numbers.

G. Transfection and Treatment Protocol for Reporter Assay + Peptide/Recombinant Protein

The following protocol is designed to reverse transfect adherent cell line, HEK-293H, using Attractene Transfection Reagent (cat. no. 301005) in a 96-well plate format. The Cignal reporter assay works well with transfection reagent from other vendors. *If you are using transfection reagent other than Attractene follow their manufacturer's protocol for transfection*. The Cignal Reporter Assay also works well using traditional forward transfection protocols. Moreover, if you are using plates or wells of different size, adjust the component in proportion to the surface area (see section IV.F). **This is just a general guideline; the optimal conditions/amounts should be adjusted according to the cell type and the study requirements.** Read the protocol completely before starting the experiment.

IMPORTANT: (1) Do not add antibiotics to media during transfection as this causes cell death.

Table 6: Guidelines for studying the effect of a peptide or recombinant protein.

Table 6 represents the total components needed, on a per well basis, for each condition to be tested. Note that individual components must be added sequentially, as instructed in the protocol.

#	**Cignal Reporter** (per well)	**Cignal Negative Control** (per well)	**Cignal Positive Control** (per well)	**Peptide or Recombinant Protein** (per well)	**Opti-MEM DNA Diluent** (per well)	**Attractene** (per well)	**Opti-MEM Attractene diluent** (per well)	**Time of Transfection** (hours)
1	100 ng (1.0 µl)				25 µl	0.6 µl	25 µl	30 h or 42 h
2	100 ng (1.0 µl)			1×[a]	25 µl	0.6 µl	25 µl	
3	100 ng (1.0 µl)			10×	25 µl	0.6 µl	25 µl	
4	100 ng (1.0 µl)			100×	25 µl	0.6 µl	25 µl	
5		100 ng (1.0 µl)			25 µl	0.6 µl	25 µl	
6		100 ng (1.0 µl)		1×	25 µl	0.6 µl	25 µl	
7		100 ng (1.0 µl)		10×	25 µl	0.6 µl	25 µl	
8		100 ng (1.0 µl)		100×	25 µl	0.6 µl	25 µl	
9			100 ng (1.0 µl)		25 µl	0.6 µl	25 µl	

[a] 1X is a smallest appropriate amount of interfering peptide/recombinant protein/growth factor expected to modulate signaling pathway.

1. The recommended experimental setup, on a **per well basis**, follows. Please note that we recommend setting up multiple replicates for each condition, and preparing transfection cocktail volumes sufficient for transfecting multiple wells. In addition, we advise always taking 5-10% extra amounts of nucleic acid, Opti-MEM® serum-free

culture medium, and Attractene to compensate for pipettor error, when setting up transfection cocktails (steps 1 through 4).

Set up three polystyrene tubes, as follows:

Experimental transfections

Tubes 1 – 4: 100 µl Opti-MEM® + 4 µl (400 ng) Cignal reporter (**4 volumes for conditions 1 to 4 of Table 6**; for every well dilute 1 µl (100 ng) of Cignal reporter in 25 µl of Opti-MEM® serum-free culture medium)

Control transfections

Tubes 5-8: 100 µl Opti-MEM® + 4 µl (400 ng) Cignal negative control (**4 volumes for conditions 5 to 8 of Table 6**; for every well dilute 1 µl (100 ng) of Cignal reporter in 25 µl of Opti-MEM® serum-free culture medium)

Tube 9: 25 µl Opti-MEM® + 1µl (100 ng) Cignal positive control

Mix each transfection cocktail gently.

2. Prepare an Attractene dilution for 9 tubes (mentioned in step 1) by dispensing 5.4 µl of Attractene into 225 µl of Opti-MEM® serum-free culture medium (for every well dilute 0.6 µl of Attractene in 25 µl of Opti-MEM® serum-free culture medium) in a polystyrene test tube. Mix gently and set the tube at room temperature for 5 minutes.

3. After the 5 minute incubation, add 100 µl of diluted Attractene to the two tubes (mentioned in step 1) containing equal volume (100 µl) of diluted Cignal reporter, and add 25 µl of diluted Attractene into the positive control tube containing 25 µl of diluted constructs (1:1 ratio) as detailed in Table 6.

4. Mix gently and incubate for 20 minutes at room temperature to allow complex formation to occur.

5. Meanwhile, wash cells* in culture dish once with Dulbecco's PBS **without calcium and magnesium**, and treat with 1-3 ml trypsin-EDTA for 2-5 minutes at 37 ºC in a humidified atmosphere containing 5% CO_2. Suspend the cells in 7-9 ml of Opti-MEM® containing 5% of fetal bovine serum, then centrifuge the cells down, remove the supernatant, and resuspend the cells to 4 ×10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum and 1% NEAA**. To ensure reproducible transfection results, it is important to accurately determine the cell density with a hemacytometer or an automated cytometry device.

6. After the 20 minute incubation for complex formation is completed, aliquot 50 µl of specific complexes into the appropriate wells.

7. Add 100 µl of prepared cell suspension (4 ×10^5 cells/ml in Opti-MEM® containing 5% of fetal bovine serum) to each well containing constructs-Attractene complexes. This gives a final volume of 150 µl. Mix gently by rocking the plate back and forth.

8. Incubate cells at 37°C in a 5% CO_2 incubator for 16 hours.

9. After 16 hours of transfection, change medium to assay medium (Opti-MEM® containing 0.5% of fetal bovine serum, 1% NEAA, 100 U/ml Penicillin and 100 µg/ml Streptomycin).

10. After 24 hours of transfection, treat the cells, as described in Table 12, with 1x, 10x and 100x amount interfering peptide/recombinant protein/growth factor (1x is an smallest appropriate amount of small molecule or organic compound expected to modulate signaling pathway).

11. To study the effect of interfering peptide/recombinant protein/growth factor, we recommend harvesting cells 6 hours or 18 hours after treatment to develop luciferase assay.

12. The **luciferase assay** can be developed by using Dual-Luciferase® Reporter Assay System from Promega (Cat. No. 1910). Follow the manufacturer's protocol for developing the assay.
Expression of the **Monster GFP** reporter can be monitored via FACS, flow cytometry, fluorescent microscopy, or standard fluorometry. The spectral properties of the Monster Green Fluorescent Protein are slightly red-shifted compared to other commercially available GFP reporters. We recommend using the standard FACS settings of an argon laser (488 nm excitation) and filters of 530±15 nm (530/30nm) for emission. When analyzing GFP expression via fluorescent microscopy or standard fluorometry, we recommend using standard fluoroisothiocyanate (FITC) filters [excitation of 470±20 nm and an emission filter of 515 nm (long pass)].

*Cells that had been passed 1:3 or 1:4 the day before are generally more easily transfected than cells that have reached a confluent state at the time of use.

** In most cases, cells grow well in Opti-MEM® serum reduced growth medium with 3%-5% FBS due to extra growth factors and nutrients supplied in Opti-MEM®. Cell should reach ~50-90% confluence once attached to the wells, otherwise increase the cell numbers.

H. Scaling up transfection experiments:

To transfect cells in different tissue culture formats, vary the amounts of constructs, number of cells, and volume of Attractene and medium used in proportion to the surface area, as shown in the Table 7. The parameters shown in Table 7 are standardized for HEK-293H cells. **Use these parameters as a starting point to optimize transfections for your cell line of interest.**

Table 7. Reagent amounts for transfecting cells in different size culture vessels

Type of Plate	Surface Area (cm^2 per well)	Starting amount of construct (ng / well)	Starting Volume of Attractene (µl / well)	Starting Volume of Attractene (µl / well)	Volume of Cell Suspension (µl / well)	Starting No. of Adherent Cells (per Well)	Volume of Opti-MEM Medium (µl)	siRNA / shRNA Vector or Gene Expression Vector (per Well)
96-well	0.3	100	0.6	0.3	100	40,000	2 X 25[a]	2 pmol / 200 ng
48-well	0.95	150	1.8	0.8	250	130,000	2 X 50	5 pmol / 500 ng
24-well	1.9	250	3.2	1.6	500	250,000	2 X 50	10 pmol / 750 ng
12-well	3.8	500	6.4	3.2	1000	500,000	2 X 100	20 pmol / 1.5 µg
6-well	9.4	1000	16.0	8.0	2500	1500,000	2 X 250	50 pmol / 4.0 µg
35 mm	8.0	1000	16.0	8.0	2500	1500,000	2 X 250	50 pmol / 4.0 µg
60 mm	21	2000	36.0	18.0	5000	3.0×10^6	2 X 500	100 pmol / 8.0 µg
100 mm	55	5000	90.0	45.0	15000 (15 ml)	9.0×10^6	2 X 1500	300 pmol / 25 µg

[a] 2X means one volume of Opti-MEM® medium for diluting constructs and another volume of Opti-MEM® medium for diluting Attractene.

For any other troubleshooting or technical questions about the Cignal Reporter Assay, please call one of our Technical Support representatives at 1-888-503-3187 or 301-682-9200 or email at support@SABiosciences.com.

Appendix:

Cignal Reporter Assay Kits

Pathway	Transcription Factor	Dual luciferase Cat. No.	MGFP Cat. No.
Amino Acid Deprivation	ATF4/ATF3/ATF2	CCS-8034L	
Androgen	Androgen Receptor	CCS-1019L	
Antioxidant Response	Nrf2 & Nrf1	CCS-5020L	
ATF6	ATF6	CCS-9031L	
C/EBP	C/EBP	CCS-001L	CCS-1001G
cAMP/PKA	CREB	CCS-002L	CCS-002G
Cell Cycle	E2F/DP1	CCS-003L	
DNA Damage	p53	CCS-004L	
Early Growth Response	EGR1	CCS-8021L	CCS-3021G
ER Stress	CBF/NF-Y/YY1	CCS-2032L	
Estrogen Receptor	Estrogen Receptor (ER)	CCS-005L	
GATA	GATA	CCS-1035L	
Glucocorticoid Receptor	Glucocorticoid Receptor (GR)	CCS-006L	
Heat Shock Response	HSF	CCS-4023L	
Heavy Metal Response	MTF1	CCS-5033L	CCS-0033G
Hedgehog	Gli	CCS-6030L	
Hepatocyte Nuclear Factor 4	HNF4	CCS-3039L	
Hypoxia	HIF-1	CCS-007L	
Interferon Regulation	IRF-1	CCS-7040L	
Interferon Type I	STAT1/STAT2	CCS-008L	CCS-008G
Interferon Gamma	STAT1/STAT1	CCS-009L	
KLF4	KLF4	CCS-4036L	CCS-9036G
Liver X Receptor	LXRa	CCS-0041L	

MAPK/ERK	Elk-1/SRF	CCS-010L	CCS-010G
MAPK/JNK	AP-1	CCS-011L	CCS-011G
MEF2	MEF2	CCS-7024L	
c-Myc	Myc/Max	CCS-012L	
Nanog	Nanog	CCS-7037L	
NFκB	NFκB	CCS-013L	CCS-013G
Notch	RBP-Jκ	CCS-014L	CCS-1014G
Oct4	Oct4	CCS-0025L	
Pax6	Pax6	CCS-3042L	
PI3K/AKT	FOXO	CCS-1022L	CCS-6022G
PKC/Ca^{++}	NFAT	CCS-015L	
PPAR	PPAR	CCS-3026L	
Progesterone	Progesterone Receptor	CCS-6043L	
Retinoic Acid Receptor	RAR	CCS-016L	
Retinoid X Receptor	RXR	CCS-9044L	
Sox2	Sox2	CCS-0038L	
SP1	SP1	CCS-6027L	CCS-1027G
STAT3	STAT3	CCS-9028	
TGFβ	SMAD2/SMAD3/SMAD4	CCS-017L	CCS-017G
Vitamin D	VDR	CCS-2029L	
Wnt	TCF/LEF	CCS-018L	CCS-018G
Xenobiotic	AhR	CCS-2045L	CCS-7045G

Cignal Reporter Assay Controls

Control Construct	Components	Concentration And Volume	Catalog Number
Cignal Negative Control (LUC)	A mixture of non-inducible firefly luciferase reporter construct and constitutively expressing *Renilla* luciferase construct (40:1).	100 ng/µl; 500 µl	CCS-NCL
Cignal Positive Control (LUC)	A mixture of a constitutively expressing GFP construct, constitutively expressing firefly luciferase construct, and constitutively expressing *Renilla* luciferase element (40:1:1).	100 ng/µl; 250 µl	CCS-PCL
Cignal Negative Control (GFP)	A GFP reporter construct in which GFP expression is controlled by a minimal promoter	100 ng/µl; 500 µl	CCS-NCG
Cignal Positive Control (GFP)	A constitutively expressing GFP construct	100 ng/µl; 250 µl	CCS-PCG

Ordering Information

Product	Contents	Cat. no.
Cignal Reporter Assay Kits	Assays in dual-luciferase or GFP format	Varies

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

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Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

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Finland ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

France ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

Germany ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Hong Kong ■ Orders 800 933 965 ■ Fax 800 930 439 ■ Technical 800 930 425

Ireland ■ Orders 1800 555 049 ■ Fax 1800 555 048 ■ Technical 1800 555 061

Italy ■ Orders 800-789-544 ■ Fax 02-334304-826 ■ Technical 800-787980

Japan ■ Telephone 03-6890-7300 ■ Fax 03-5547-0818 ■ Technical 03-6890-7300

Korea (South) ■ Orders 080-000-7146 ■ Fax 02-2626-5703 ■ Technical 080-000-7145

Luxembourg ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

Mexico ■ Orders 01-800-7742-639 ■ Fax 01-800-1122-330 ■ Technical 01-800-7742-436

The Netherlands ■ Orders 0800-0229592 ■ Fax 0800-0229593 ■ Technical 0800-0229602

Norway ■ Orders 800-18859 ■ Fax 800-18817 ■ Technical 800-18712

Singapore ■ Orders 1800-742-4362 ■ Fax 65-6854-8184 ■ Technical 1800-742-4368

Spain ■ Orders 91-630-7050 ■ Fax 91-630-5145 ■ Technical 91-630-7050

Sweden ■ Orders 020-790282 ■ Fax 020-790582 ■ Technical 020-798328

Switzerland ■ Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

UK ■ Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

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