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Data Sheet

The Transfection Collection™ – GAL4 Transient Pack
Glucocorticoid Receptor Pathway
Catalog #: 79265

Background
The glucocorticoid signaling pathway plays an important role in development, fluid homeostasis, cognition, immune response and metabolism. Glucocorticoids are a class of steroid hormones that bind to the glucocorticoid receptor, causing it to translocate to the nucleus. Upon translocation, the receptor can regulate the transcription of a large number of genes, including those that regulate glucose metabolism and inflammatory responses.

Description
The GAL4 *Transient Pack* is designed to provide the tools necessary for transiently transfecting and monitoring the activity of the glucocorticoid signaling pathway in cultured HEK293 cells. The kit contains transfection-ready vectors containing firefly luciferase as a glucocorticoid pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the TWO-Step Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the GAL4 Transient Pack is the expression vector for the glucocorticoid receptor ligand binding domain that is fused to the DNA binding domain (DBD) of GAL4 (GAL4 DBD-GR). This fusion construct activates firefly luciferase expression under the control of a multimerized GAL4 upstream activation sequence (UAS). This allows for specific detection of glucocorticoid-induced activation of the glucocorticoid receptor without the need for individual transcriptional targets and with low cross-reactivity for other nuclear receptor pathways. The GAL4/UAS reporter is premixed with constitutively expressing *Renilla* (sea pansy) luciferase vector, which serves as an internal positive control for transfection efficiency.

The pack also includes a non-inducible firefly luciferase vector premixed with constitutively-expressing *Renilla* luciferase vector as a negative control. The non-inducible luciferase vector contains a firefly luciferase gene under the control of a minimal promoter, but without any additional response elements. The negative control is critical for determining pathway-specific effects and background luciferase activity.

Additionally, the pack includes cell culture medium (BPS Medium 1) that has been optimized for use with HEK293 cells*. BPS Medium 1 includes MEM medium, 10% fetal bovine serum, 1% non-essential amino acids, sodium pyruvate, and 1% Pen/Strep. Finally, the pack provides the TWO-Step Luciferase (Firefly & Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The

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TWO-step luciferase reagents can be used directly in cells in growth medium, and can be detected with any luminometer; automated injectors are not required.

*Note: the kit may be used with other cell lines than HEK293, but an alternate cell culture medium may be required for optimal cell growth.

Application

- Monitor glucocorticoid signaling pathway activity.
- Screen activators or inhibitors of the glucocorticoid signaling pathway.

Components

Component	Amount	Storage
Reporter (Component A) GAL4/UAS luciferase reporter vector + constitutively expressing *Renilla* luciferase vector	500 µl (60 ng DNA/µl)	-20°C
Negative Control Reporter (Component B) Non-inducible luciferase vector + constitutively expressing *Renilla* luciferase vector	500 µl (60 ng DNA/µl)	-20°C
GAL4 DBD-GR (Component C) Expression vector for ligand binding domain of the glucocorticoid receptor + GAL4 DNA binding domain	250 µl (100 ng DNA/µl)	-20°C
Negative Control Expression vector (Component D) Expression vector with GAL4 DNA binding domain only	250 µl (100 ng DNA/µl)	-20°C
Firefly Luciferase Reagent Buffer	10 ml	-20°C
Firefly Luciferase Reagent Substrate (100x)	100 µl	-20°C *Protect from light*
Renilla Luciferase Reagent Buffer	10 ml	Room Temp.
Renilla Luciferase Reagent substrate (100x)	100 µl	-20°C *Protect from light*
BPS Medium 1	100 ml	+4°C

Note: These vectors are ready for transient transfection. They are NOT SUITABLE for transformation and amplification in bacteria.

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Materials Required but Not Supplied

- HEK293 cells. Other mammalian cell lines may also be used, but an alternate cell culture medium may be required for optimal cell growth.
- 96-well tissue culture plate or 96-well tissue culture treated white clear-bottom assay plate (Corning, #3610)
- Transfection reagent for mammalian cell line. [We use Lipofectamine™ 2000 (Life Technologies #11668027), however, other transfection reagents should work equally well.]
- Opti-MEM I Reduced Serum Medium (Life Technologies, #31985-062)
- Luminometer

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the reporter into HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfect cells in different tissue culture formats, adjust the amounts of reagents and cell number in proportion to the relative surface area. If using a transfection reagent other than Lipofectamine 2000, follow the manufacturer's transfection protocol. Transfection condition should be optimized according to the cell type and study requirements.
All amounts and volumes in the following setup are provided on a per well basis.

1. One day before transfection, seed cells at a density of ~30,000 cells per well in 100 µl of BPS Medium 1 so that cells will be 90% confluent at the time of transfection.

2. The next day, for each well, prepare complexes as follows:

   a. Dilute DNA mixtures in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently. Depending upon the experimental design, the DNA mixtures may be any of the following combinations:
      - 1 µl of **Reporter** (component A); in this experiment, the control transfection is 1 µl of **Negative Control Reporter** (component B).
      - 1 µl of **Reporter** (component A) + **experimental vector** expressing gene of interest (such as component C); in this experiment, the control transfections are: 1 µl of **Reporter** (component A) + **negative control expression vector** (such as component D), 1 µl of **Negative Control Reporter** (component B) + **experimental vector** expressing gene of interest, and 1 µl of **Reporter** (component A) + **negative control expression vector** (component D).
      - 1 µl of **Reporter** (component A) + **specific siRNA**; in this experiment, the control transfections are: 1 µl of **Reporte**r (component A) + **negative control siRNA**, 1 µl of **Negative Control Reporter** (component B) + **specific siRNA**, and 1 µl of **Negative Control Reporter** (component B) + **negative control siRNA**.

Note: we recommend setting up each condition in at least triplicate, and preparing transfection cocktail for multiple wells to minimize pipetting errors.

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b. Mix Lipofectamine 2000 gently before use, then dilute 0.35 µl of Lipofectamine 2000 in 15 µl of Opti-MEM I medium (antibiotic-free). Incubate for 5 minutes at room temperature. Note: Prepare this dilution cocktail in volumes sufficient for the whole experiment.

c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine 2000. Mix gently and incubate for 25 minutes at room temperature.

3. Carefully remove and discard 30 µl of media from each of the wells of cell culture, taking care not to disturb the cells or touch the bottom of the well with the pipet tip. Add the 30 µl of complexes to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a 5% CO_2 incubator. After ~24 hours of transfection, change medium to fresh growth medium. ~48 hours after transfection, perform the TWO-Step Luciferase Assay System (below).

TWO-Step Luciferase Assay Procedure

1. Thaw **Firefly Luciferase Reagent Buffer** by placing the reagent in a room temperature water bath. Equilibrate the buffer to room temperature and mix well before use. Note: It is important that the **Firefly Luciferase Reagent Buffer** be at room temperature before use.

2. Calculate the amount of Firefly Luciferase Assay Working solution needed for the experiment (**Firefly Luciferase Reagent Buffer** + **Firefly Luciferase Reagent Substrate**). Immediately prior to performing the experiment, prepare the Firefly Luciferase Assay Working Solution by diluting **Firefly Luciferase Reagent Substrate** into **Firefly Luciferase Reagent Buffer** at a 1:100 ratio and mix well. Avoid exposing to excessive light. Only use enough of each component for the experiment, remaining **Firefly Luciferase Reagent Buffer** and **Firefly Luciferase Reagent Substrate** should be stored separately at -20°C.

3. Remove multi-well plate containing mammalian cells from incubator. Note: plates must be compatible with luminescence measurement by luminometer being used.

4. Add equal volume of Firefly Luciferase Assay Working Solution (step 2) to the culture medium in each well. Example: 96-well plate with 100 µl of culture medium requires 100 µl of Firefly Luciferase Assay Working Solution per well.

Gently rock the plates for at least 15 minutes at room temperature. Measure firefly luminescence using a luminometer. The signal under these conditions will be stable for more than 2 hours at room temperature. For maximal light intensity, measure samples within 1 hour of reagent addition.

5. Calculate the amount of Renilla Luciferase Assay Working Solution needed for the experiment (**Renilla Luciferase Reagent Buffer** + **Renilla Luciferase Reagent Substrate**). Prepare the Renilla Luciferase Assay Working Solution by diluting **Renilla Luciferase Reagent**

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Substrate into **Renilla Luciferase Reagent Buffer** at a 1:100 ratio and mix well. Avoid exposing to excessive heat or light. Only use enough of each component for the experiment.

6. Add equal volume of Renilla Luciferase Assay Working Solution (step 5) to each well. Example: 96-well plate with 100 µl of culture medium + 100 µl Firefly Luciferase Reagent requires 100 µl of Renilla Luciferase Assay Working Solution per well.

7. Gently rock the plates for ~1 minute at room temperature. Measure Renilla luminescence using a luminometer.

8. Data analysis: subtract background (wells with medium and luciferase reagent only) from all the readings. To obtain the normalized luciferase activity for the ISRE reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from ISRE reporter to Renilla luminescence from the control Renilla luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

To study the effect of activators/inhibitors on the glucocorticoid pathway, treat the cells with tested activator/inhibitor after ~6 hours or 24 hours of transfection. Perform TWO-step luciferase assay ~48 hours after transfection.

Sample protocol to determine the effect of Dexamethasone on GAL4 DBD-GR combined with the GAL4/UAS reporter in HEK293 cells

Additional materials required in this experimental setup

- Dexamethasone (Sigma, D1756), stock solution in DMSO
- HEK293 cells
- 96-well tissue culture treated white clear-bottom assay plate (Corning, #3610)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells per well into white clear-bottom 96-well plate in 100 µl of BPS Medium 1. Incubate cells at 37°C in a CO_2 incubator overnight.

2. The next day, transfect 1 µl of GAL4/UAS luciferase reporter (component A) or 1 µl negative control reporter (component B) with 0.5 µl of GAL4 DBD-GR (component C) or negative control expression vector (component D) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~24 hours of transfection, treat transfected cells with 3 µM or various concentrations of Dexamethasone (if determining response curve) in 50 µl of fresh BPS Medium 1. Add 50 µl of BPS Medium 1 to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate. Incubate cells at 37°C in a CO_2 incubator for ~24 hours.

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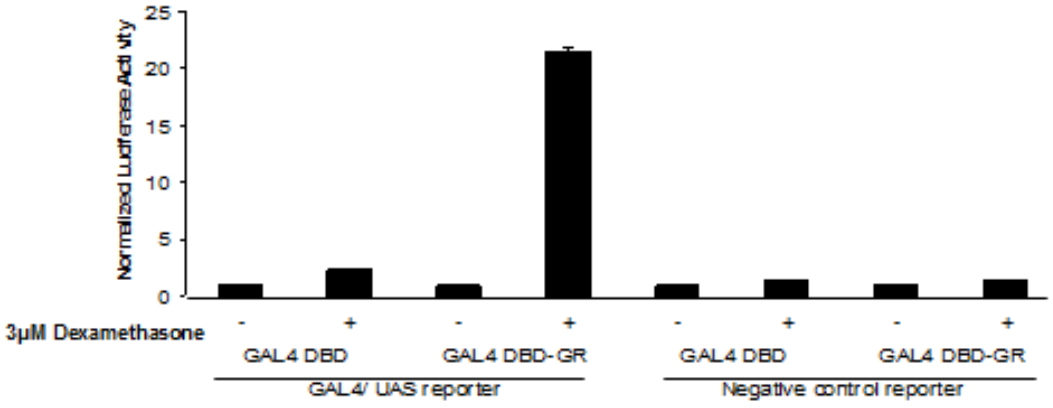
4. After ~48 hours of transfection, perform TWO-step luciferase assay as described above **in TWO-Step Luciferase Assay Procedure**. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer . Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

5. To obtain the normalized luciferase activity for GAL4/UAS reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from GAL4/UAS reporter to *Renilla* luminescence from the control *Renilla* luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

Figure 1. (A) Dexamethasone-induced activation of the GAL4/UAS reporter with the GAL4 DBD-GR expression vector. The results are shown as normalized GAL4/UAS luciferase reporter activity.



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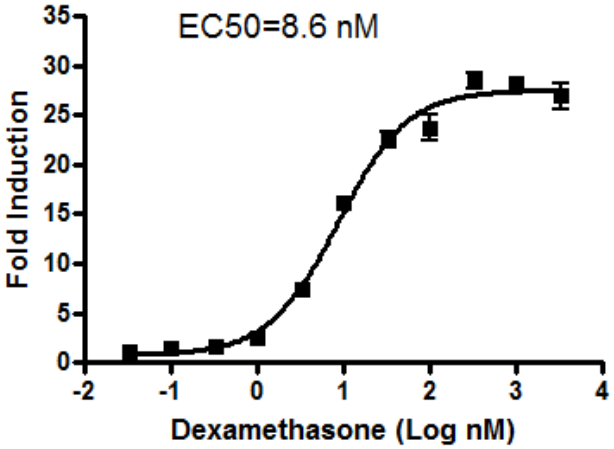
(B) Dose response of GAL4 DBD-GR expression vector with GAL4/UAS reporter activity to Dexamethasone. The results are shown as fold induction of normalized GAL4/UAS luciferase reporter activity. Fold induction was determined by comparing values against the mean value for control cells without Dexamethasone treatment.



Sample protocol to determine the effect of antagonists of the Glucocorticoid signaling pathway on Dexamethasone-induced GAL4 DBD-GR with GAL4/UAS reporter activity in HEK293 cells:

Additional materials required in this experiment setup

- Dexamethasone (Sigma-Aldrich, D1756), stock solution in DMSO
- Mifepristone/ RU-486 (Sigma, #M8046)
- 96-well tissue culture treated white clear-bottom assay plate (Corning #3610)

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells in 100 µl of BPS Medium 1 into each well of a white clear-bottom 96-well plate. Incubate cells at 37°C in a 5% CO_2 incubator overnight.

2. The next day, transfect 1 µl of GAL4/UAS luciferase reporter (component A) with 0.5 µl of GAL4 DBD-GR (component C) or negative control expression vector (component D) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~24 hours of transfection, treat transfected cells with 100 nM (final concentration) of the glucocorticoid pathway antagonist Mifepristone in 45 µl of fresh BPS Medium 1. Set up each treatment in at least triplicate. Include a subset of untreated, control wells that do not receive Mifepristone.

4. Incubate cells at 37°C in a CO_2 incubator for ~1 hour.

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5. Add Dexamethasone (final concentration 10 nM) in 5 µl of BPS Medium 1 to stimulated wells (cells treated with Dexamethasone, with or without Mifepristone); add 5 µl of BPS Medium 1 to the unstimulated control wells (for determining the basal activity); add 50 µl of BPS Medium 1 to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate.

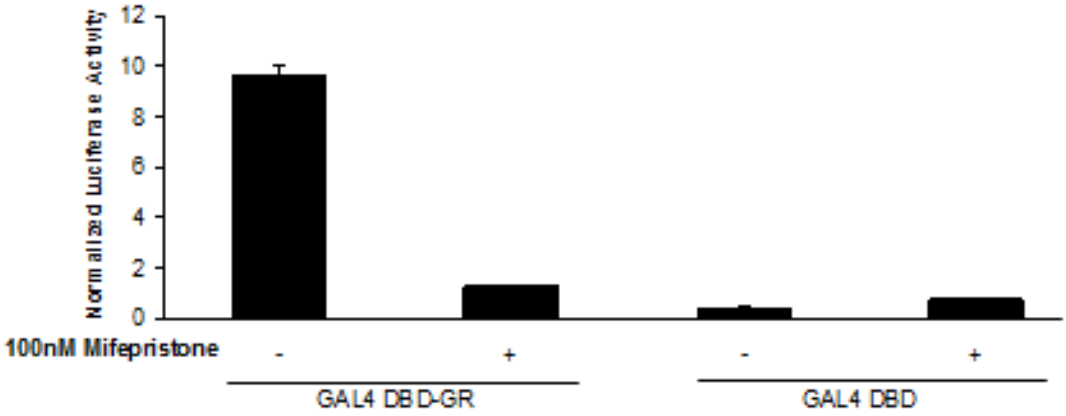
6. Incubate cells at 37°C in a CO_2 incubator for ~24 hours.

7. Perform TWO-step luciferase assay as described above in TWO-Step Luciferase Assay Procedure. Briefly, dilute 100x Firefly Luciferase Reagent Substrate into Firefly Luciferase Reagent Buffer that has been equilibrated to room temperature. Add 50 µl of Firefly Luciferase reagent per well and rock at room temperature for ~15 minutes, then measure firefly luminescence using a luminometer. Dilute 100x Renilla Luciferase Reagent Substrate into Renilla Luciferase Reagent Buffer. Add 50 µl of Renilla Luciferase reagent per well, rock at room temperature for ~1 minute and measure Renilla luminescence.

8. To obtain the normalized luciferase activity for GAL4/UAS reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from GAL4/UAS reporter to *Renilla* luminescence from the control *Renilla* luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

Figure 2. Inhibition of Dexamethasone-induced GAL4 DBD-GR with GAL4/UAS reporter activity by the Glucocorticoid antagonist, Mifepristone.

(A) Mifepristone completely blocks Dexamethasone-induced GAL4 DBD-GR with GAL4/UAS reporter activity. The results are shown as normalized GAL4/UAS luciferase reporter activity.



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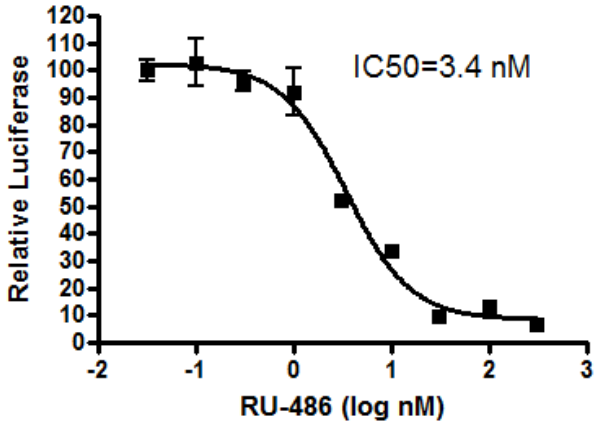
(B) Dose response of inhibition of Dexamethasone-induced GAL4 DBD-GR with GAL/UAS reporter activity to Mifepristone. The results are shown as percentage of GAL4/UAS reporter activity. The normalized luciferase activity for GAL4 DBD-GR transfected cells treated with 10 nM Dexamethasone without Mifepristone treatment was set at 100%.



Reference

Paguio A, *et al*. (2010) Improved TWO-Step-Luciferase Reporter Assays for Nuclear Receptors. *Curr Chem Genomics*. **4:** 43-49.

Refills

Product	Cat. #	Size
GAL4 Reporter Kit	60522	500 rxns.
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-1	10 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-2	100 mL
TWO-Step Luciferase (Firefly & Renilla) Assay System	60683-3	1 L
BPS Medium 1	79259	100 ml

Related Products

Product	Cat. #	Size
GAL4 Reporter (Luc)-HEK293 Recombinant Cell Line	60656	2 vials
GR-GAL4 Reporter (Luc)-HEK293 Recombinant Cell Line	60655	2 vials
AP-1 Reporter Kit	60612	500 rxns.
NF-κB Reporter Kit	60614	500 rxns.
ARE Reporter Kit	60514	500 rxns.
Myc Reporter Kit	60519	500 rxns.
Notch1/CSL Reporter Kit	60509	500 rxns.
AP-1 Reporter – HEK293 Cell Line	60405	2 vials
NF-κB Reporter – HEK293 Cell Line	60650	2 vials
ARE Reporter – HepG2 Cell Line	60513	2 vials
Notch1/CSL Reporter - HEK293 Cell Line	60652	2 vials