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# Data Sheet

# The Transfection Collection™ – Mouse Notch1/CSL Transient Pack
## (Notch Signaling Pathway)
### Catalog #79285

**Background**

The Notch signaling pathway controls cell fate decisions in vertebrate and invertebrate tissues. NOTCH signaling is triggered through the binding of a transmembrane ligand to Notch transmembrane receptor (NOTCH1/ NOTCH2/NOTCH3/NOTCH4) on a neighboring cell. This results in proteolytic cleavage of the NOTCH receptor, releasing the constitutively active intracellular domain of NOTCH (NICD). NICD translocates to the nucleus and associates with transcription factors CSL (CBF1/RBPJκ/Suppressor of Hairless/Lag-1) and coactivator Mastermind to turn on transcription of Notch-responsive genes.

**Description**

The Notch1/CSL Transient Packis designed to provide the tools necessary for transiently transfecting and monitoring the activity of the Notch signaling pathway in HEK293 cultured cells. The kit contains transfection-ready vectors containing firefly luciferase as a Notch pathway-responsive reporter and constitutively expressing Renilla luciferase as a transfection control. It also includes the Dual Luciferase detection reagents to detect both luciferase activities and specialized medium for growing and assaying HEK293 cells.

The key to the Notch1/CSL Transient Pack is the expression vector for NOTCH1 that has a deletion of the entire extracellular domain (Notch1ΔE). Inside the cells, the NOTCH1 ΔE can be cleaved by γ-secretase and active NOTCH1 NICD is released into the nucleus. The kit also contains CSL (CBF1/RBP-Jκ) luciferase reporter vector, which is a Notch pathway-responsive reporter. This reporter contains the firefly luciferase gene under the control of multimerized CSL responsive elements upstream of a minimal promoter. The CSL (CBF1/RBP-Jκ) 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.

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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 Dual Luciferase (Firefly-Renilla) Assay System. These luciferase reagents provide highly sensitive, stable detection of firefly luciferase activity and Renilla luciferase activity. The dual 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 Notch signaling pathway activity.
- Screen activators or inhibitors of Notch signaling pathway.
- Study effects of RNAi or gene overexpression on the activity of Notch pathway.

**Components**

| Component | Amount | Storage |
|---|---|---|
| **Reporter (Component A)** AP1 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 |
| **Notch1ΔE (Component C)** Expression vector for intracellular domain of Notch1 | 250 µl (100 ng DNA/µl) | -20°C |
| **Negative Control Expression vector (Component D)** Empty expression vector without Notch1 | 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 SUITBLE for transformation and amplification in bacteria.*

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**Materials Required but Not Supplied**

- HEK293 cells. Other mammalian cell lines can 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 (Invitrogen, #11668027). However, other transfection reagents should work equally well.]
- Opti-MEM I Reduced Serum Medium (Invitrogen, #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. 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 **Reporte**r (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 **Negative Control Reporter** (component B) + **negative control expression vector**.
      - 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

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**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 prepare transfection cocktail for multiple wells.

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 $CO_2$ incubator. After ~24 hours of transfection, change medium to fresh BPS Medium 1. ~48 hours after transfection, perform the Dual Luciferase Assay System (below).

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

**Dual 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 with 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.

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Gently rock the plates for ≥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 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 Notch reporter, subtract the background luminescence, then calculate the ratio of firefly luminescence from Notch reporter to Renilla luminescence from the control Renilla luciferase vector. To determine theµ fold induction, divide the value for the treated cells by the value for the untreated cells.

**Sample protocol to determine the effect of NOTCH 1 on the CSL (CBF1/RBP-Jκ) reporter in HEK293 cells**

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 µµl of BPS Medium 1. Incubate cells at 37°C in a $CO_2$ incubator overnight.

2. Next day, transfect 1 µl of CSL (CBF1/RBP-Jκ) luciferase reporter (component A) with 0.5 µl of Notch1ΔE (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, change medium to 50 µl of fresh BPS Medium 1. Add 50 µl of BPS Medium 1 to cell-free control wells (for determining background luminescence).

4. After ~48 hours of transfection, perform dual luciferase assay as described above in Dual 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.

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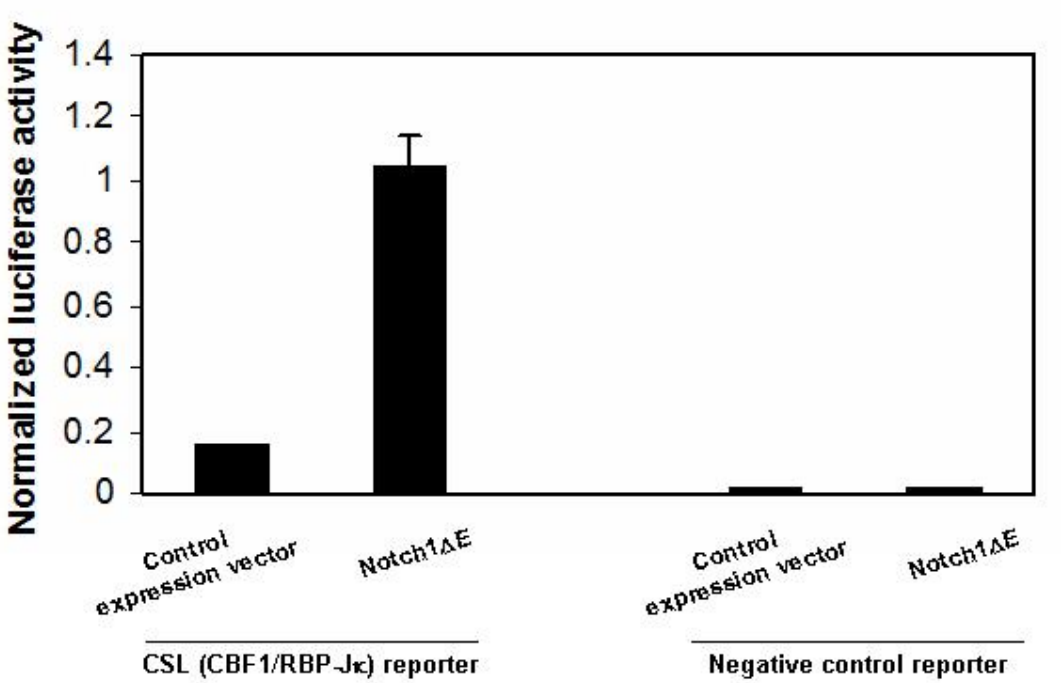
5. To obtain the normalized luciferase activity for CSL (CBF1/RBP-Jκ) reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from CSL (CBF1/RBP-Jκ) reporter to *Renilla* luminescence from the control *Renilla* luciferase vector. To determine the fold induction, divide the value for the treated cells by the value for the untreated cells.

**Figure 1. Notch1ΔE induced the expression of CSL (CBF1/RBP-Jκ) reporter.** The results are shown as normalized CSL (CBF1/RBP-Jκ) luciferase reporter activity.




**Sample protocol to determine the effect of antagonists of Notch signaling pathway on Notch1ΔE-induced CSL (CBF1/RBP-Jκ) reporter activity in HEK293 cells:**

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 $CO_2$ incubator overnight.

2. Next day, transfect 1 µl of CSL (CBF1/RBP-Jκ) luciferase reporter (component A) with 0.5 µl of Notch1ΔE (component C) into cells following the procedure in **Generalized Transfection and Assay Protocols.**

3. After ~24 hours of transfection, treat transfected cells with the Notch pathway inhibitor DAPT (γ-secretase inhibitor) in 50 µl of fresh BPS Medium 1. Add 50 µl of BPS Medium 1

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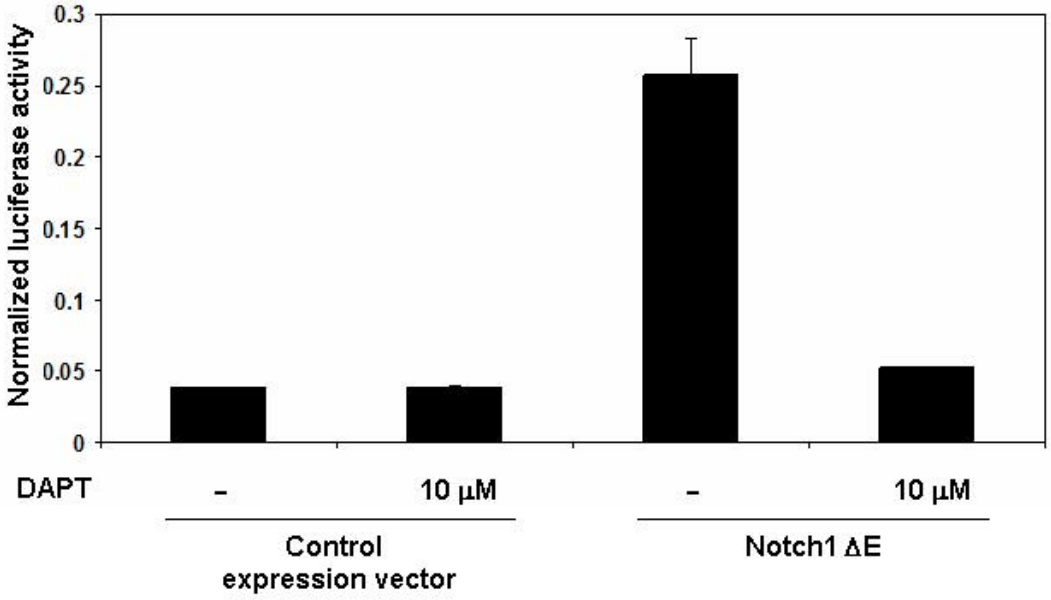
to cell-free control wells (for determining background luminescence). Set up each treatment in at least triplicate. Incubate cells at 37°C in a $CO_2$ incubator for ~24 hours.

4. After ~48 hours of transfection, perform dual luciferase assay as described above in **Dual 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 CSL (CBF1/RBP-Jκ) reporter, subtract background luminescence, then calculate the ratio of firefly luminescence from CSL (CBF1/RBP-Jκ) 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 Notch1ΔE-induced CSL (CBF1/RBP-Jκ) reporter activity by Notch pathway inhibitor, DAPT (γ-secretase inhibitor).**



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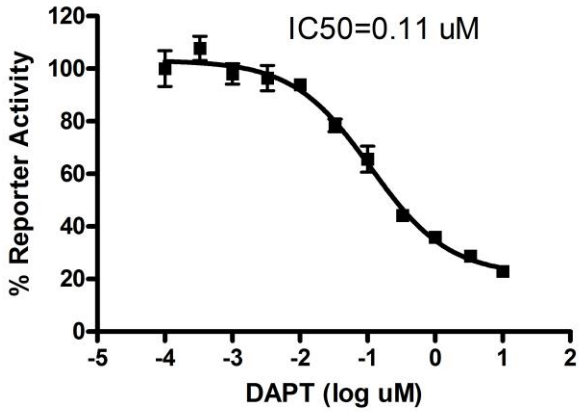
**2a**. DAPT completely blocks Notch1ΔE-induced CSL (CBF1/RBP-Jκ) reporter activity. The results are shown as normalized CSL (CBF1/RBP-Jκ) luciferase reporter activity.




**2b.** Dose response of Notch1ΔE-induced CSL (CBF1/RBP-Jκ) reporter activity to DAPT. The results are shown as percentage of CSL (CBF1/RBP-Jκ) reporter activity. The normalized luciferase activity for Notch1ΔE transfected cells without DAPT treatment was set at 100%. The $IC_{50}$ of DAPT is ~ 0.11 µM.

## References

1. Lu FM *et al*. (1996) Constitutively active human Notch1 binds to the transcription factor CBF1 and stimulates transcription through a promoter containing a CBF1-responsive element. *Proc. Natl. Acad. Sci. USA* **93(11):** 5663-5667.
2. Kanungo, *et al.* (2008) The Notch signaling inhibitor DAPT down-regulates cdk5 activity and modulates the distribution of neuronal cytoskeletal proteins*. J. Neurochem*. **106:** 2236.

## Refills

| Product Name | Catalog # | Size |
|---|---|---|
| Notch1/CSL Reporter Kit (Notch Signaling Pathway) | 60509 | 500 rxns. |
| BPS Medium 1 | 79259 | 100 ml |
| Dual Luciferase (Firefly-Renilla) Assay System | 60683-1 | 10 mL |
| Dual Luciferase (Firefly-Renilla) Assay System | 60683-2 | 100 mL |
| Dual Luciferase (Firefly-Renilla) Assay System | 60683-3 | 1 L |

## Related Products

| Product Name | Catalog # | Size |
|---|---|---|
| Notch1/CSL Reporter - HEK293 Cell Line | 60652 | 2 vials |
| TW-37 | 27775-1 | 10 mg |
| TW-37 | 27775-2 | 50 mg |