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Data Sheet

TDO Cell-Based Assay Kit
Catalog #72033

Background
L-tryptophan (L-Trp) is an essential amino acid necessary for protein synthesis in mammalian cells and the L-Trp to kynurenine (Kyn) pathway is firmly established as a key regulator of innate and adaptive immunity. Catabolism of L-Trp to Kyn maintains an immunosuppressive microenvironment by starving immune cells of L-Trp and releasing degradation products of L-Trp that have immunosuppressive functions. Tryptophan 2,3 dioxygenase (TDO), one of the rate limiting enzymes in this pathway, is upregulated in many tumors, providing cancer cells with an avenue for immune evasion.

Description
The human *TDO Cell-Based Assay Kit* is designed to monitor the activity of exogenously expressed human TDO in cultured cells. The kit contains a transfection-ready expression vector for full length human TDO (hTDO). Upon expression, hTDO can catalyze L-Trp conversion to Kyn, which gets released and is easily detected in the assay medium. The kit also includes specialized assay medium and all components necessary to fully activate hTDO.

Application

- Screen for inhibitors of hTDO enzyme in a cellular context.
  Note: The expression vector is designed for use in transient transfection protocols. It is NOT SUITABLE for transformation and amplification in bacteria.

Components

Component	Amount	Storage
hTDO Expression Vector (Component A)	100 µl (50 ng DNA/µl)	-20°C
TDO Assay Medium Supplement 1 (Component B)	2 x 200 µl	4°C
TDO Assay Medium Supplement 2 (Component C)	200 µl	-20°C
Detection Reagent (Component D)	200 mg	Room temperature

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Materials Required but Not Supplied

- HEK293 cells (or other appropriate mammalian cell line) and corresponding cell culture medium
- 96-well tissue culture plate
- 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)
- 6.1 N Trichloroacetic acid (Sigma #T0699)
- Acetic acid (Sigma #320099)
- Transparent 96-well plate
- Spectrophotometer capable of measuring absorbance at λ=470 - 490 nm.
- 680C91 (Tocris #4392), 100 mM stock in DMSO (optional reference inhibitor for TDO inhibition)

Generalized Transfection and Assay Protocols

The following procedure is designed to transfect the expression vector into HEK293 cells using Lipofectamine 2000 in a 96-well format. To transfect different cell lines and/or 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 recommended transfection protocol. Transfection conditions 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.
Note: we recommend setting up each condition in at least triplicate, and prepare transfection cocktail for multiple wells.

Sample protocol to determine the effect of reference inhibitor 680C91 on exogenously expressed hIDO1 in HEK293 cells:

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

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

   a. Dilute 1 µl **hTDO Expression Vector (Component A)** in 15 µl of Opti-MEM I medium (antibiotic-free). Mix gently.

   b. In a separate tube, 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 both tubes for 5 minutes at room temperature.
   Note: Prepare this dilution cocktail in volumes sufficient for the whole experiment.

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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. Add 30 µl of DNA/Lipofectamine complex to each well containing cells and medium. Mix gently by tapping the plate.

4. Incubate cells at 37°C in a CO_2 incubator overnight.

5. After ~24 hours of transfection, thaw **TDO Assay Medium Supplement 1 (Component B)** and **TDO Assay Medium Supplement 2 (Component C)** at 37°C. Vortex briefly before use.
*Note: There may be a small amount of precipitate in **TDO Assay Medium Supplement 2 (Component C)**. This will not interfere with the assay.*

6. Prepare fresh *Assay Medium* by diluting **TDO Assay Medium Supplement 1 (Component B)** 1:50 and **TDO Assay Medium Supplement 2 (Component C)** 1:100 into cell culture medium. Dilute the test compound to the desired concentration in fresh *Assay Medium*. Remove the cell culture medium from transfected cells and replace with 200 µl of Assay Medium containing the test compound. Incubate cells overnight at 37°C in a CO_2 incubator.
Note: The final concentration of DMSO in the cell culture should not exceed 0.3%.

7. The next day, remove 140 µl of medium from each well of the cell culture and transfer into a fresh 96-well plate. Add 10 µl of 6.1 N trichloroacetic acid to each well. Incubate the plate at 50°C for 30 min. Centrifuge the plate at 2500 rpm for 10 minutes to remove any sediment. If a plate centrifuge in not available, the liquid can be transferred to a microcentrifuge tube and spun briefly to pellet any solids.

8. While the plate is incubating, prepare *Detection Reagent Solution* by dissolving **Detection Reagent (Component D)** at a 50-fold dilution in acetic acid, e.g. 200 mg in 10 ml undiluted acetic acid. Prepare only enough reagent required for the assay.

9. Transfer 100 µl of supernatant to a transparent 96-well plate and mix with 100 µl of fresh *Detection Reagent Solution*. Incubate the plate at room temperature for 10 minutes, then measure absorbance at 480 nm using a microplate reader.

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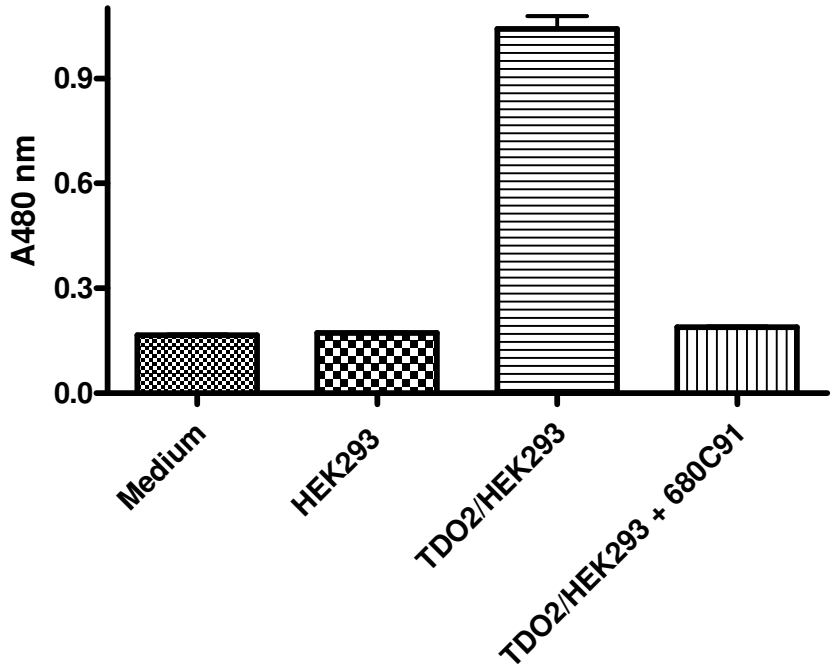
Figure 1. Inhibition of hTDO enzyme activity by known TDO inhibitor, 680C91.



680C91 completely blocks hTDO enzyme activity at a concentration of 100 µM. The results are shown as raw absorbance data at 480 nm. Conditions from left to right: medium only (no cells), untransfected HEK293 cells plus all assay components, TDO-transfected HEK293 plus all assay components, TDO-transfected HEK293 plus all assay components and 100 µM 680C91.

Sample protocol to determine the IC50 of the reference inhibitor 680C91 on exogenously expressed hIDO1 in HEK293 cells:

1. One day before transfection, seed HEK293 cells at a density of 30,000 cells in 100 µl of growth medium into each well of a tissue culture-treated 96-well plate. Incubate cells at 37°C in a CO_2 incubator overnight.

2. On the next day, transfect cells following the procedure described in *Generalized Transfection and Assay Protocol,* steps 1 through 4. Keep a few wells of untransfected HEK293 cells as a negative control for any basal level of TDO from HEK293 cells

3. After ~24 hours of transfection, thaw **TDO Assay Medium Supplement 1 (Component B)** and **TDO Assay Medium Supplement 2 (Component C)** at 37°C. Vortex briefly before use. Prepare fresh *Assay Medium* by diluting **TDO Assay Medium Supplement 1 (Component B)** 1:50 and **TDO Assay Medium Supplement 2 (Component C)** 1:100 into cell culture medium. *Note: There may be a small amount of precipitate in **Assay Medium Supplement 2 (Component C)**. This will not interfere with the assay.*

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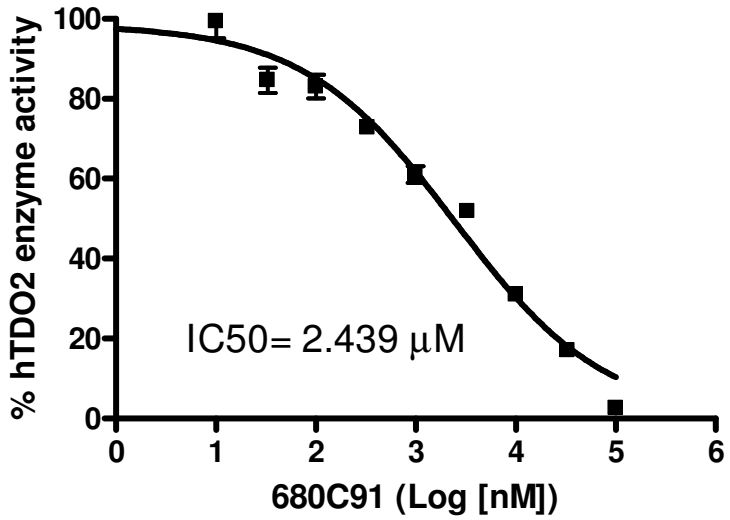
4. Remove culture medium and treat transfected cells with the test inhibitor, in this case, we used 10 µM 680C91 in 200 µl of fresh *Assay Medium*. Add 200 µl of *Assay Medium* containing DMSO to cell-free control wells (for determining background absorbance) and vector-free HEK293 cell control wells (for negative control on any basal level of TDO from HEK293 cells). Set up each treatment in at least triplicate. Incubate cells overnight at 37°C in a CO_2 incubator. *Note: The final DMSO concentration should not exceed 0.3%.*

5. On the next day, detect product in assay medium following the procedure in *Generalized Transfection and Assay Protocols*, steps 7-9.

6. Data analysis: in the absence of the reference inhibitor and presence of hTDO expression vector, the absorbance (At) in each should be set to 100%. The absorbance of cell-free control wells (Ab) in each data set should be defined as 0%. The percent absorbance in the presence of reference inhibitor compound is calculated according to the following equation: % Absorbance = (A-Ab)/(At-Ab), where A= the absorbance in the presence of the compound and expression vector.

Figure 2. Dose response of hTDO activity to reference inhibitor 680C91.



The results are shown as percentage of absorbance. The normalized absorbance for hTDO transfected cells without inhibitor treatment was set at 100%. The IC_{50} of 680C91 is ~ 2.439 µM.

References

1. Yue, E., *et al.*, *J. Med. Chem.* 2009; **52:** 7364–7367.
2. Liu, X., *et al., Blood.* 2010; **115(17):** 3520-3530.

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