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Data Sheet

IDO1 Cell-Based Assay Kit
Catalog #72031

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. Indoleamine 2,3-dioxygenases (IDO1 & IDO2), two of the rate limiting enzymes in this pathway, are upregulated in many tumors, providing cancer cells with an avenue for immune evasion.

Description
The human *IDO1 Cell-Based Assay Kit* is designed to monitor the activity of exogenously expressed human IDO1 in cultured cells. The kit contains a transfection-ready expression vector for full length human IDO1 (hIDO1). Upon expression, hIDO1 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 hIDO1.

Application

- Screen for inhibitors of hIDO1 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
hIDO1 Expression Vector (Component A)	100 µl (50 ng DNA/µl)	-20°C
IDO1 Assay Medium Supplement 1 (Component B)	200 µl	4°C
IDO1 Assay Medium Supplement 2 (Component C)	200 µl	-20°C
IDO1 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.
- INCB024360 (BPS Cat. #27338), 10 mM stock in DMSO (optional reference inhibitor for IDO1 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 preparing transfection cocktail for multiple wells.

Sample protocol to determine the effect of reference inhibitor INCB024360 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 **hIDO1 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 **Assay Medium Supplement 1 (Component B)** and **Assay Medium Supplement 2 (Component C)** at 37°C. Vortex briefly before use.
*Note: There may be a small amount of precipitate in **Assay Medium Supplement 2 (Component C)**. This will not interfere with the assay.*

6. Prepare fresh *Assay Medium* by diluting **Assay Medium Supplement 1 (Component B)** 1:100 and **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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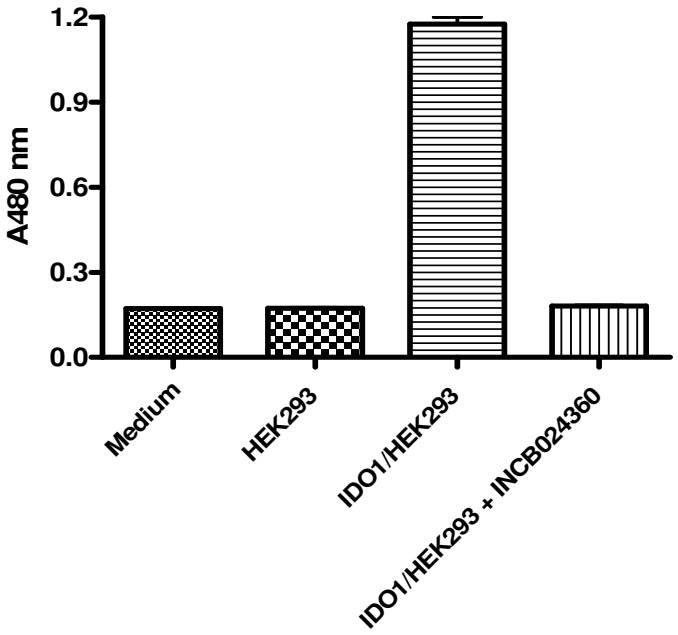
Figure 1. Inhibition of hIDO1 enzyme activity by known IDO1 inhibitor, INCB024360.



INCB024360 completely blocks hIDO1 enzyme activity at a concentration of 1 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, IDO1-transfected HEK293 plus all assay components, IDO1-transfected HEK293 plus all assay components and 1 µM INCB024360.

Sample protocol to determine the IC50 of the reference inhibitor INCB024360 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 IDO1 from HEK293 cells

3. After ~24 hours of transfection, thaw **Assay Medium Supplement 1 (Component B)** and **Assay Medium Supplement 2 (Component C)** at 37°C. Vortex briefly before use. Prepare fresh Assay Medium by diluting **Assay Medium Supplement 1 (Component B)** 1:100 and **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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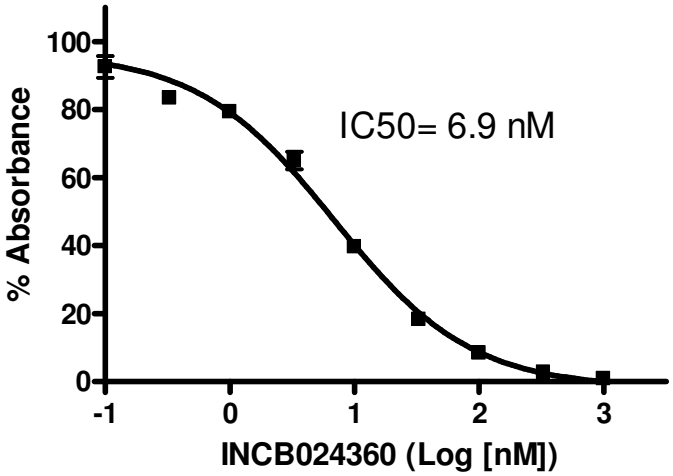
4. Remove culture medium and treat transfected cells with varying concentrations of the test inhibitor in 200 µl of *Assay Medium*. In this case, we used INCB024360. Add 200 µl of *Assay Medium* containing DMSO to cell-free control wells (for determining background absorbance) and vector-free HEK293 cell control wells (for negative control on any basal level of IDO1 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 hIDO1 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 hIDO1 activity to reference inhibitor INCB024360.



The results are shown as percentage of absorbance. The normalized absorbance for hIDO1 transfected cells without inhibitor treatment was set at 100%. The IC_{50} of INCB024360 is ~ 6.9 nM.

References

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