Human TEAD4/YAP
(Hippo Pathway)
Reporter Assay System

96-well Format Assays
Product # IB16001

▪

Technical Manual
(version 7.2)

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I. Description

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▪ Background ▪

The Hippo tumor suppressor pathway coordinates cellular signals that modulate cell proliferation, tissue homeostasis and organ size. This signaling primarily regulates the ability of YAP, or its paralog TAZ, to bind and co-activate the TEAD family of transcription factors (TEAD1-4).[1]

YAP confers traits that sustain cell proliferation, inhibit apoptosis, promote angiogenesis, and develop resistance to therapies. The TEAD/YAP complex is therefore considered an oncogene regulator, as the dysregulation of YAP is strongly associated with the onset and progression of several cancers, such as prostate and pancreatic cancer.[2] As such, the Hippo pathway is a premier target for the development of novel, specific, small molecule inhibitors.

As depicted in **Figure 1**, the evolutionarily conserved Hippo pathway comprises a complex activation cascade of protein kinases. Activated MST1/2 forms a complex with SAV1 to phosphorylate and activate LATS1/2 kinases. Activated LATS in complex with MOB1 phosphorylate YAP and/or TAZ, leading to cytoplasmic retention *via* association with 14-3-3 σ. Alternatively, phosphorylated YAP may be ubiquitinated and targeted for proteasomal degradation. Under these conditions the expression of genes associated with pro-proliferative cell function are reduced. The inhibition, or dysregulation, of this kinase cascade leads to reduced phosphorylation of YAP, their translocation to the nucleus and association with TEAD transcription factors. The assembly of an active TEAD/YAP (or TEAD/TAZ) transcription complex induces expression of oncogenic target genes involved in mediating cell proliferation, migration, and survival.[1-4]

▪ The INDIGO Hippo Pathway Assay System ▪

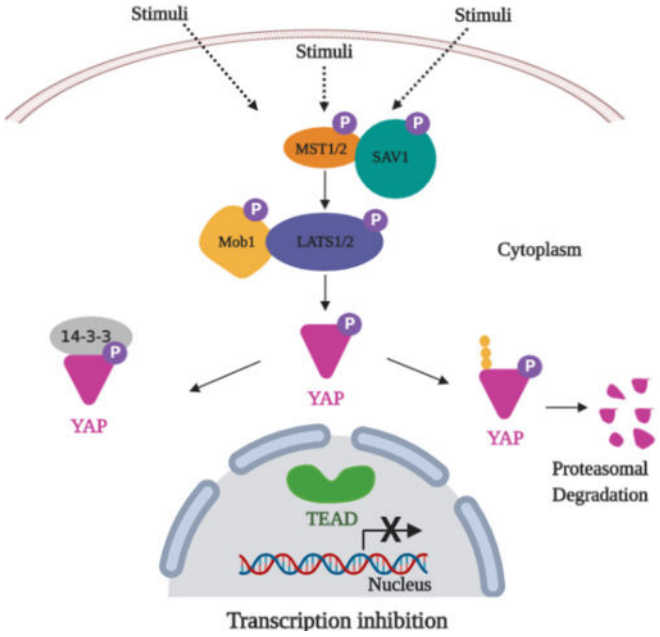
This Hippo pathway assay system utilizes proprietary human cells engineered to provide high-level expression of a hybrid form of the **Human Transcriptional Enhanced Associate Domain 4 Protein (TEAD4)**, whereby the DNA binding domain (DBD) of the native TEAD4 has been substituted with that of the yeast Gal4-DBD. Additionally, these reporter cells express the requisite transcriptional co-activator **Human Yes-Associated Protein 1 (YAP)** as well as the luciferase reporter gene functionally linked to tandem Gal4 Upstream Activation Sequence (UAS) genetic response elements. The use of a hybrid Gal4(DBD)-TEAD4 is a well-used strategy [4], and ensures that any drug-induced changes in reporter gene expression are the direct result of changes in Gal4(DBD)-TEAD4 / YAP transcriptional activity.

To reiterate, the reporter cells provided in this kit present the "Hippo Off", or oncogenic pathway. Specifically, the Gal4(DBD)-TEAD4 transcription factor and the YAP co-activator are over-expressed to produce a constitutively active transcription complex that delivers high-level expression of the luciferase reporter gene. The primary application of this assay system is to screen test materials for *inhibitory* activities against the active TEAD4/YAP transcription complex.

INDIGO's Reporter Cells are transiently transfected and prepared as frozen stocks using a proprietary **CryoMite™** process. This cryo-preservation method allows for the immediate dispensing of healthy, division-competent reporter cells into assay plates. There is no need for intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup.

INDIGO's assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to TEAD4/YAP Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, the reference inhibitor of TEAD Flufenamic acid [5], Luciferase Detection Reagents, and a cell culture-ready assay plate.

A.) Hippo '*On*' Pathway: TEAD inactive → target gene expression "off"



B.) Hippo '*Off*' Pathway: YAP/TEAD active → target gene expression "on"

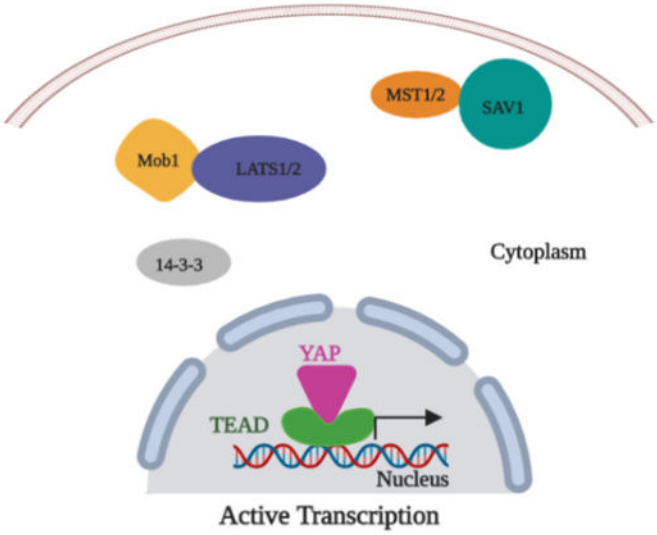


Figure 1. General overview of the TEAD/YAP (Hippo) signaling pathway.

A.) Hippo-*ON* homeostasis is maintained by a variety of upstream stimuli (external and internal) that maintain a state of phosphorylation of MST1/2 and LATS1/2 kinases. The ensuing phosphorylation of YAP leads to cytoplasmic retention and eventual degradation, either through its association with 14-3-3 or E3 ligase-dependent ubiquitination and proteasomal degradation. In the absence of its co-activator partner, the transcription factor TEAD is inactive.

B.) When the Hippo pathway is *OFF* non-phosphorylated YAP translocates into the nucleus and associates with TEAD to form a functional transcription complex that drives the expression of YAP/TEAD-dependent target genes.

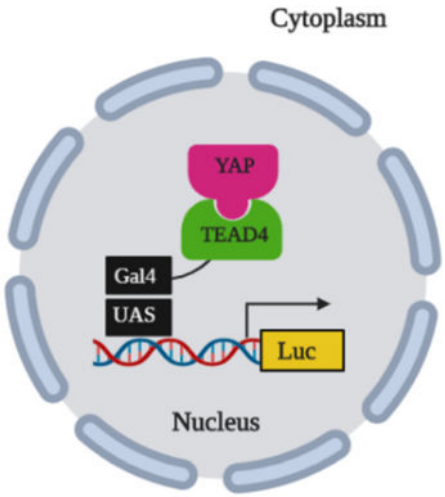


Figure 2. Overview of the GAL4(DBD)-TEAD4/YAP (Hippo) signaling pathway.
INDIGO's reporter cells express *i.)* a GAL4 DNA Binding Domain (DBD) hybrid version of TEAD4, *ii.)* YAP, and *iii.)* the luciferase reporter gene functionally linked to tandem GAL4 Upstream Activation Sequences (UAS), as well as a minimal promoter. As demonstrated in *Figure 3*, these reporter cells deliver constitutive expression of the Luc reporter gene that is exclusive to, and dependent on, Gal4(DBD)-TEAD4/YAP association. As such, this assay kit presents the "Hippo-*Off*" pathway.

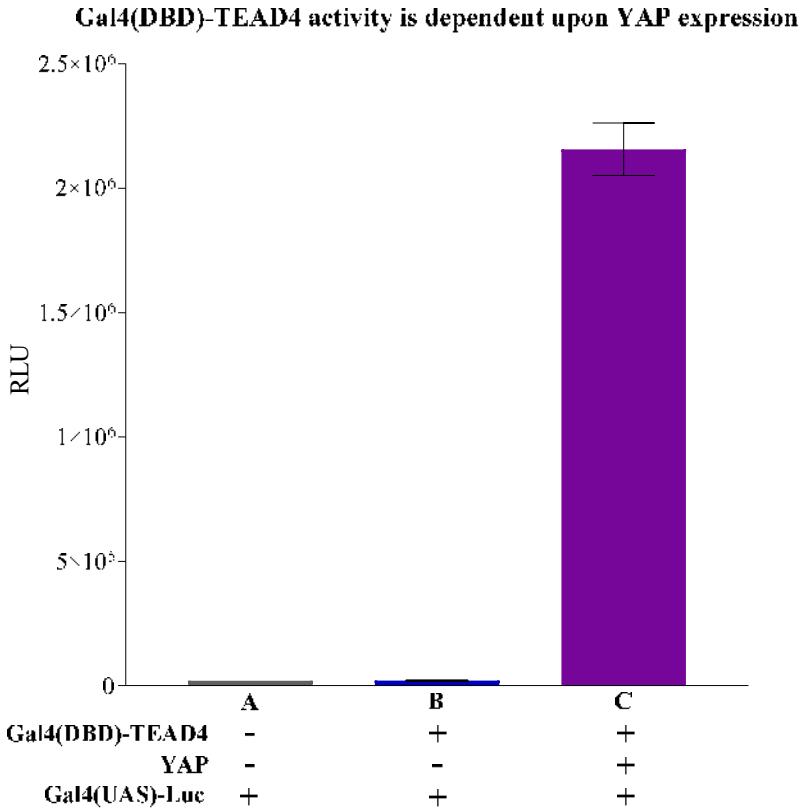


Figure 3. TEAD4 transcriptional activity is dependent upon YAP expression.
HEK293 cells were transfected with the Gal4(UAS)-Luc +/- YAP co-activator and Gal4(DBD)-TEAD4, as indicated. Following 24 hr incubation cells were processed to quantify luciferase activity per well (n = 4). Average relative light units (RLU) are plotted. Error-bars depict standard deviation (SD). Cell variation "C" represents the Reporter Cells provided in this kit, delivering constitutive expression of Luc that is dependent on the functional association between YAP and Gal4(DBD)-TEAD4. As such, INDIGO's reporter cells express the 'Hippo-*Off* 'pathway (as depicted in *Figure 2*).

▪ The Assay Chemistry ▪

INDIGO's Reporter assay kits capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO_2, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

Assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5-minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Preparation of Test Compounds ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

As described in *Step 7* of the assay protocol, **Compound Screening Medium (CSM)** may be used to make serial dilutions of test compounds to achieve the desired concentrations of treatment media.

Alternatively, if test compound solubility is expected to be problematic, DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should *never* exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

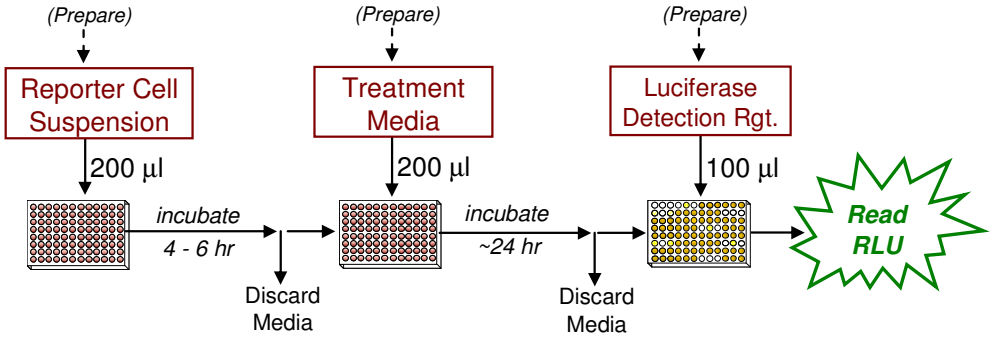
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 3. Assay workflow. *In brief*, 200 µl of reporter cell suspension is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared 1x-concentration treatment media are added. Following ~24 hr incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Validation and Performance ▪

INDIGO's TEAD4/YAP Reporter cells have been validated using compounds described to inhibit, by a variety of mechanism, TEAD/YAP dependent transcriptional activity. **Figure 4** demonstrates that INDIGO's assay is sufficiently sensitive to measure significant drug-induced reductions in TEAD4/YAP transcription at sub-toxic treatment concentrations, even for verteporfin (see NOTE, below). Nonetheless, including an independent assessment of compound-induced cytotoxicity is strongly advised. INDIGO's Live Cell Multiplex (LCM) Assay is specifically optimized for this application.

NOTE: Caution is advised when interpreting data from loss-of-activity assays when even moderate concentrations of the compound being tested is cytotoxic. As an example, verteporfin, a commonly cited FDA-approved drug that interacts with YAP, induces high-level toxicity in cultured cells. This is likely due to its ability to oligomerize proteins involved in critical cellular processes, such as autophagy and cytoskeletal maintenance [6-7]. The occurrence of dose-dependent compound-induced cytotoxicity will always complicate the proper interpretation of results from assays that quantify an inhibition of function. Performing coincident cytotoxicity analyses of compound treated cells is recommended.

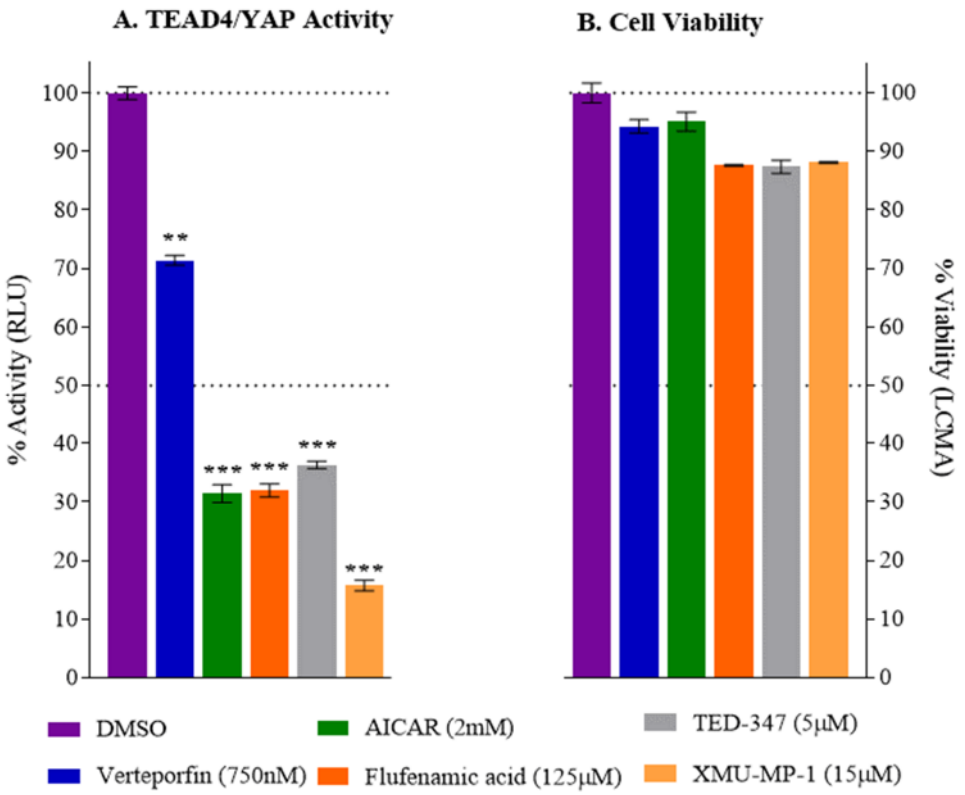


Figure 4. TEAD4/YAP (Hippo) Reporter Assay Validation.

The TEAD4/YAP Reporter Cell suspension was dispensed into a 96-well assay plate, which then underwent a preliminary 4-hour incubation. The pre-incubation media were discarded, and the following preparations of treatment media were added to the assay wells (n = 4 / treatment):

- DMSO ('vehicle only' Control treatment)
- Flufenamic Acid (binds to TEAD to inhibit TEAD/YAP transcription[5])
- Verteporfin (promotes YAP degradation[6-7])
- AICAR (promotes phosphorylation of YAP *via* AMPK[3] → degradation)
- XMU-MP-1 (cell-type specific interaction with YAP[8-9])
- TED-347 (Binds to TEAD to inhibit TEAD/YAP transcription activity[1])

After 22 hr incubation (44 hr incubation for TED-347) treatment media were discarded, Luciferase Detection Reagent was added, and RLU/well were quantified. Average RLU values are normalized as % Activity relative to the respective DMSO control treatment. INDIGO's Live Cell Multiplex (LCM) Assay was used to quantify cell viability, which is normalized as % Live Cells relative to the DMSO control. Values of ≥ 85% Live Cells at the assay endpoint indicate non-significant cytotoxicity. For both assays, error-bars depict coefficient of variation (%CV). ** and *** = p value ≤ 0.001 and ≤ 0.0001, respectively, as determined by two-way T-test. Reference compounds were obtained from Cayman Chemical, except for TED-347, which was obtained from SelleckChem.

II. Product Components & Storage Conditions

This Human TEAD4/YAP (Hippo) Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	*Amount*	*Storage Temp.*
▪ TEAD4/YAP Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ Flufenamic acid 125 mM (in DMSO) (inhibitor of TEAD4/YAP association)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

▪ dry ice bucket (*Step 2*)
▪ cell culture-rated laminar flow hood.
▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
▪ 37°C water bath.
▪ 70% alcohol wipes
▪ 8-channel electronic, repeat-dispensing pipettes & sterile tips
▪ disposable media basins, sterile.
▪ sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
▪ *Optional:* clear 96-well assay plate, sterile, *collagen-coated*, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring a 4-6 hr pre-incubation step. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

DAY 1 Assay Protocol: All steps must be performed using aseptic technique.

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

> *NOTE 4.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).
>
> *NOTE 4.2:* Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.
>
> *NOTE 4.3:* Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 4 - 6 hours.

6.) ***Near the end of the pre-incubation period:*** Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations. Use **CSM** to prepare the desired treatment concentrations of the reference and test compound stocks. In *Step 9*, the prepared treatment media will be dispensed at **200 µl / well** into the strip-wells. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

> NOTE: Total organic solvent carried over into assay reactions should never exceed 0.4%.

This Assay kit includes a 125 mM stock solution of Flufenamic Acid, an inhibitor of TEAD4/YAP complex formation. A treatment concentration of 125 µM provides significant inhibition of the TEAD4/YAP transcription complex without inducing significant cytotoxicity (cell viability ≥ 85%). Always include 'no treatment' (or 'vehicle only') control wells.

8.) At the end of the 4-6 hr pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

9.) Dispense **200 µl / well** of each prepared treatment media into the assay plate.

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

11.) For greater convenience on *Day 2*, retrieve **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on a bench top.

12.) Approximately 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

13.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

14.) *Immediately before proceeding to Step 15*, gently invert the tubes of Detection Substrate and Detection Buffer several times to ensure homogenous solutions, then transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

16.) Add 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Citations

[1] Bum-Erdene. K, *et. al.* (2019) Small-Molecule Covalent Modification of Conserved Cysteine Leads to Allosteric Inhibition of the TEAD-Yap Protein-Protein Interaction, Cell Chemical Biology: **26**, 378-389.

[2] Nouri. K, *et. al.* (2019) Identification of Celastrol as a Novel YAP-TEAD Inhibitor for Cancer Therapy by High Throughput Screening with Ultrasensitive YAP/TAZ-TEAD Biosensors, Cancers: **11**, 1596-1614.

[3] DeRan. M, *et. al.* (2014) Energy Stress Regulates Hippo-YAP Signaling Involving AMPK-Mediated Regulation of Angiomotin-like 1 Protein, Cell Reports: **9**, 495-503.
[4] Zhao. B, *et. al.* (2008) TEAD mediates YAP-dependent gene induction and growth control, Genes & Development: **22**, 1962-1971

[5] Pobbati. A, *et. al.* (2015) Targeting the Central Pocket in Human Transcription Factor TEAD as a Potential Cancer Therapeutic Strategy, Structure: **23**, 2076-2086.

[6] YA-Wen. M, *et. al.* (2016) Verteporfin induces apoptosis and eliminates cancer stem-like cells in uveal melanoma in the absence of light activation, Am J Cancer Res: **12**, 2816-2830.

[7] Calses. PC, *et. al.* (2019) Hippo Pathway in Cancer: Aberrant Regulation and Therapeutic Opportunities, Trends in Cancer: **5**, 297-307.

[8] Mitchell. E, *et. al.* (2020), XMU-MP-1 induces growth arrest in a model human mini-organ and antagonizes cell-cycle dependent paclitaxel cytotoxicity, Cell Division: **15:11**.

[9] Kastan, N, *et. al.* (2020) Small-Molecule inhibition of Lats kinases promotes Yap-dependent proliferation in postmitotic mammalian tissues, bioRxiv 10.1101/2020.02.11.944157.

VI. Related Products

Product No.	*Product Descriptions*
Human TEAD4/YAP (Hippo) Assay Products	
IB16001-32	Human TEAD4/YAP (Hippo) Assay System 3x 32 assays in 96-well format
IB16001	Human TEAD4/YAP (Hippo) Assay System 1x 96-well format assay
IB16002	Human TEAD4/YAP (Hippo) Assay System 1x 384-well format assays
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

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Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.