Human
Transforming Growth Factor Beta Receptors I/II
Reporter Assay System

(TGFβR)

96-well Format Assays
Product # IB12001

▪

Technical Manual
(version 7.2b)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human TGFβR Reporter Assay System
96-well Format Assays

I. Description

▪ The Assay System ▪

This TGFβR assay utilizes proprietary human cells that provide constitutive expression of the **Human type I** and **type II Transforming Growth Factor beta Receptors (TGFβR I/II),** both of which are transmembrane serine/threonine kinase receptors. Receptor activation results when TGF-β binds to RII, which then phosphorylates and forms a heterodimer complex with RI. The mechanisms of TGFβ–RI/RII activation and ensuing signal transduction cascade have been well studied and involve the phosphorylation and interplay of a variety of Smad regulatory proteins and transcription factors.[1] Additionally, cross-talk between the activated TGFβR and NF-κB signal transduction pathways have been characterized.[2]

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to tandem TGF-β Response Sequences (TRS) derived from the human plasminogen activator inhibitor-1 (PAI-1) promoter, a well characterized TGF-β responsive target gene[3]. The TRS sequences are readily bound by activated dimeric $Smad3^{PP}$/Smad4 to initiate formation of a transcription complex. Quantifying changes in luciferase activity in drug treated cells *vs.* untreated (or vehicle treated) reporter cells provides a sensitive surrogate measure of drug-induced changes in TGFβR activity.

Considering its role in cell proliferation and the progression of many types of cancers, the TGFβ Receptors continues to command much interest as targets for the development of novel, specific, and predominantly inhibitory drugs and antibodies. Accordingly, the primary application of this assay is in the screening of drug candidates to quantify any functional activity, either agonistic or inhibitory, that they may exert against the human TGFβR complex.

Reporter Cells are transiently transfected and prepared as frozen stocks using INDIGO's 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 cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations or cell titer adjustments prior to assay setup.

INDIGO's TGFβR Assay kit provides the convenience of an all-inclusive cell-based assay system. In addition to TGFβR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples. Also included is the reference agonist TGF-β1, Luciferase Detection Reagents, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor 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.

[1]Targeting TGF-β signaling. Pennison M and Pasche B (2007) Curr Opin Oncol:19, 579 (PMID 17906455).

[2]TGF-β and NF-κB signal pathway crosstalk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. Freudisperger C, *et. al.* (2013) Oncogene:32, 1549 (PMID 22641218).

[3]Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. Dennler S, et. al. (1998) EMBO:17, 3091 (PMID 9606191).

▪ Preparation of Test Compounds ▪

Small molecule 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.

It is recommended that test materials that are protein ligands or antibodies be solvated in aqueous buffered solutions with carrier protein (*e.g.,* PBS + 0.1% BSA) at concentrations *no less* than 10x-concentrated relative to the highest desired treatment concentration.

Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*. Note that the final concentration of DMSO carried over into assay wells should *never* exceed 0.4%.

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

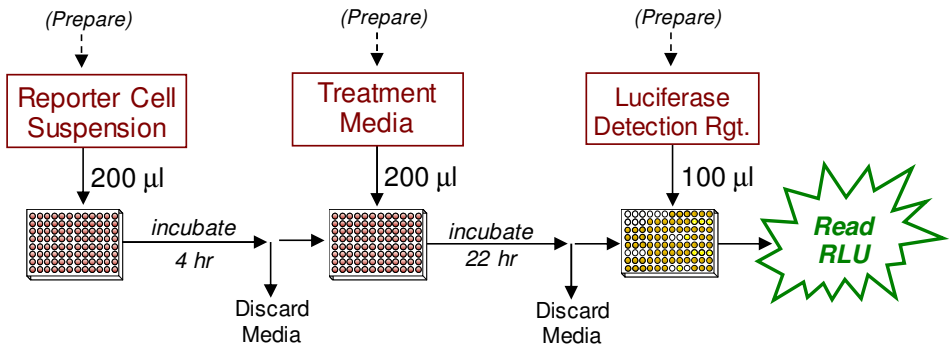
▪ Considerations for Automated Dispensing ▪

When using an automated dispensing instrument to process a small number of assay plates, first carefully consider the dead volume requirement of your 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 1. Assay workflow. *In brief*, 200 µl of Reporter Cells are dispensed into wells of the assay plate and incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared treatment media are added. Following 22-24 hr incubation discard treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



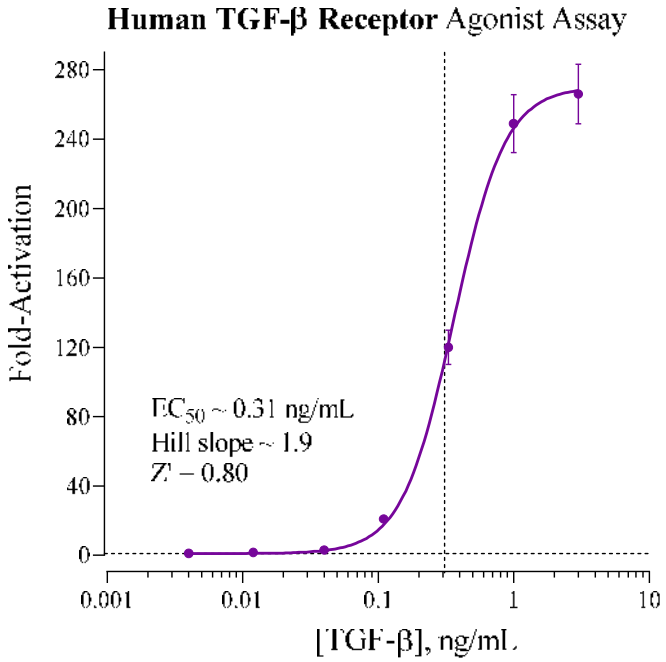


Figure 2a. Agonist dose-response of the TGFβR.
Dose-response analyses of TGFβR Reporter Cells were performed according to the protocol provided in this Technical Manual. TGFβR Reporter Cells were treated with TGF-β1 using a range of 7 concentrations (n = 4/conc.) generated in 3-fold decrements: 3.0, 1.0, .33, .11, .037, .012 and 0.0041 ng/mL, and including 'untreated' (*i.e.*, 'vehicle only') control wells, as described in **Appendix 1**. Luminescence/well was quantified and the average relative light units (RLU) and corresponding standard deviation (SD), percent coefficient of variation (%CV) and Fold-Activation values were determined for each treatment concentration. Z' values were calculated as described by Zhang, *et al*. (1999)[4]. Non-linear regression analyses and IC_{50} calculations were performed using GraphPad Prism software.

These data confirm the robust performance of this TGFβR Assay and demonstrate its suitability for use in HTS applications.

[4] Zhang JH, *et. al.* (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4**(2), 67-73.

$$Z' = 1 - [3*(SD^{Reference} + SD^{Vehicle\ Bkg}) / (RLU^{Reference} - RLU^{Vehicle\ Bkg})]$$

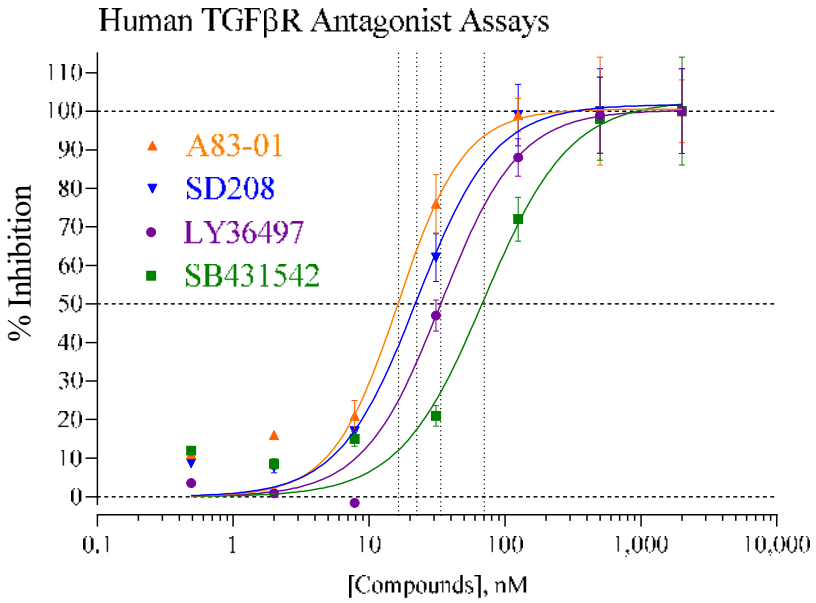


Figure 2b. Antagonist dose-response of the TGFβR.
200 µl / well of TGFβR Reporter Cell suspension was dispensed into the 96-well assay plate, which was then placed in a cell culture incubator for four hours. Approximately 30 minutes before the end of the 4 hr pre-culture period, treatment media were prepared by first supplementing CSM with TGF-β1 to a final concentration of 0.6 ng / ml (an approximate EC_{80} concentration), then using that medium to prepare serial dilutions of the reference antagonists A83-01, SD208, LY36497 and SB431542 (all from Tocris). For each drug, seven treatment concentrations were prepared in 4-fold decrements: 2000, 500, 125, 31.3, 7.81, 1.95 and 0.488 nM. At the end of the pre-culture period media were discarded from the assay wells and 200 µl /well of respective treatment media were dispensed (n = 4/conc.), including 'vehicle only' control wells. Residual DMSO was ≤ 0.1% per well in the assay plate. Following a 22 hr incubation period media were discarded, Luciferase Detection Reagent was added, and Luminescence per well was quantified. Values of average relative light units (RLU) and corresponding standard deviation (SD), percent coefficient of variation (%CV), Fold-Inhibition, and % Inhibition were determined for each treatment concentration. Depicted is a plot of % Inhibition of TGFβR *vs.* Log_{10}[nM] concentration of the various drugs. Non-linear regression analyses and IC_{50} calculations were performed using GraphPad Prism software.

II. Product Components & Storage Conditions

This Human TGFβR 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 Reporter 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 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.*
▪ TGFβR 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
▪ TGF-β1, 3.0 µg/mL (in PBS/0.1%BSA) (reference agonist for TGFβR)	1 x 20 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

▪ container of dry ice (see *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* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
▪ *Optional:* clear 96-well assay plate, cell culture treated, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

The Day 1 Assay protocol begins on the next page. Please review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring less than 2 hours of bench work and a 4 hr incubation step to complete. *Steps 12-17* are performed on **Day 2** and require less than 1 hour to complete.

▪ A word about Inhibition-mode assay setups ▪

Receptor inhibition assays expose the Reporter Cells to a fixed, sub-maximal concentration (typically between EC_{50} – EC_{85}) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for chemical inhibition or neutralizing antibody activities. This TGFβR Assay kit includes a 3.0 µg/mL stock solution of **TGF-β1**, the physiological agonist of TGFβR, that may be used to setup inhibition-mode assays. 0.6 ng/mL TGF-β1 typically approximates EC_{80} in this assay. Hence, it presents a reasonable concentration of agonist to use when screening test compounds for inhibitory activity.

Add TGF-β1 to a bulk volume of **CSM**, as described above. This agonist-supplemented medium is then used to prepare serial dilutions of test material stocks to achieve the desired respective final assay concentrations. This is an efficient and precise method of setting up TGFβR inhibition assays, and it is the method presented in *Step 7b* of this protocol.

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 **TGFβR Reporter Cells** from -80°C storage, place it directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, 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 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 gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an electronic, repeat-dispensing 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 'cell blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of plate-specific 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, preferably collagen-coated, 96-well assay plate. Continue to process this plate in identical manner to the white assay plate.

5.) Pre-culture reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 -6 hours.

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

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

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

NOTE: Total DMSO carried over into assay reactions should never exceed 0.4%.

***a. Agonist*-mode assays**. This TGFβR Assay kit includes a concentrated stock of TGF-β1, 3.0 µg/mL prepared in PBS/0.1%BSA. The following 7-point treatment series, with concentrations generated using serial 3-fold dilutions, provides a complete dose-response: 3.0, 1.0, .33, .11, .037, .012 and 0.0041 ng/mL. **APPENDIX 1** provides guidance for generating such a dilution series. Always include 'no treatment' (or 'vehicle') controls.

~ or ~

***b. Antagonist*-mode assays**. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist **TGF-β1** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired series of treatment concentrations.

8.) At the end of the 4-6 hr pre-culture period, discard the media by 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.

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 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 85%) 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.) 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. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

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*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of room temperature **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, discard the media by 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 the prepared **LDR** into all wells 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. Related Products

Human TGFβR Assay Products	
Product No.	***Product Descriptions***
IB12001-32	Human TGFβR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB12001	Human TGFβR Reporter Assay System 1x 96-well format assay
IB12002	Human TGFβR Reporter Assay System 1x 384-well format assays
Bulk volumes of TGFβR Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
Product No.	***Product Descriptions***
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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VI. Limited Use Disclosures

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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 current version available.

APPENDIX 1

Example scheme for the serial dilution of the reference agonist TGF-β1, and the setup of a TGFβR dose-response assay.