Human Tumor Protein p53 (p53) Reporter Assay System

2x 48 Assays in 96-well Format
Product # IB25001-48

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Technical Manual
(version 7.4i)

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2x48 Assays each in 96-well Format

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I. Description

▪ Background ▪

Tumor protein P53, also known as p53, acquired its reputation as "guardian of the genome" through its ability to sense and respond to cellular stress, preventing accumulation of DNA damage and subsequent formation of malignancies. p53 is a transcription factor and tumor suppressor that responds to cellular stress by regulating genes involved in a diverse array of cellular responses, including but not limited to, cell cycle arrest, DNA repair, apoptosis, senescence, and autophagy, thus minimizing the negative consequences of genetic mutation.[1] p53 is expressed at low levels in cells in the absence of stress, regulated by various factors including MDM2 (also known as HDM2), which acts as a negative regulator through its E3 ubiquitin ligase activity, and WIP1/PPM1D, which also acts as a negative regulator of p53 through its ability to dephosphorylate specific residues on both p53 and MDM2, leading to the destabilization of p53 and the stabilization of HDM2, respectively. p53 in turn directly influences the expression of these two negative regulators, as both MDM2 and WIP1/PPM1D are target genes of p53.[2]

p53 is the most commonly mutated gene in human cancer formation, and is therefore of interest in cancer research and therapeutic development.[1] Potential therapeutics may target the p53 pathway in several ways. These include restoration of mutated p53 to its wild-type conformation and targeting regulators of p53 activity, for example, by inhibiting MDM2. The small molecule PRIMA-1, as well as derivates of the thiosemicarbazone family, have been shown to restore wild-type p53 activity, and several classes of MDM2 inhibitors, including nutlins, have been developed and studied.[3] Since the causes, results, and regulation of p53 activation are diverse and context-dependent, therapeutics targeting the p53 pathway are necessarily diverse as well.

▪ The Assay System ▪

INDIGO's **p53 Reporter Cells** include the luciferase reporter gene functionally linked to a p53-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in human p53 activity. Accordingly, the principal application of this reporter assay is in the screening of test compounds to quantify any functional activity that they may exert against human p53.

Human p53 Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate preparatory steps such as the spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

INDIGO's Reporter Assays are all-inclusive cell-based assay systems. In addition to p53 Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting test samples, the reference p53 activator Doxorubicin (hydrochloride), Luciferase Detection Reagent, and two cell culture-ready assay plates.

▪ The Assay Chemistry ▪

INDIGO's 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).

INDIGO'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 at least 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 wells. Immediately prior to setting up an assay, the master stocks of test samples are serially diluted using one of two alternative strategies:

1.) As described in *Step 7*, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

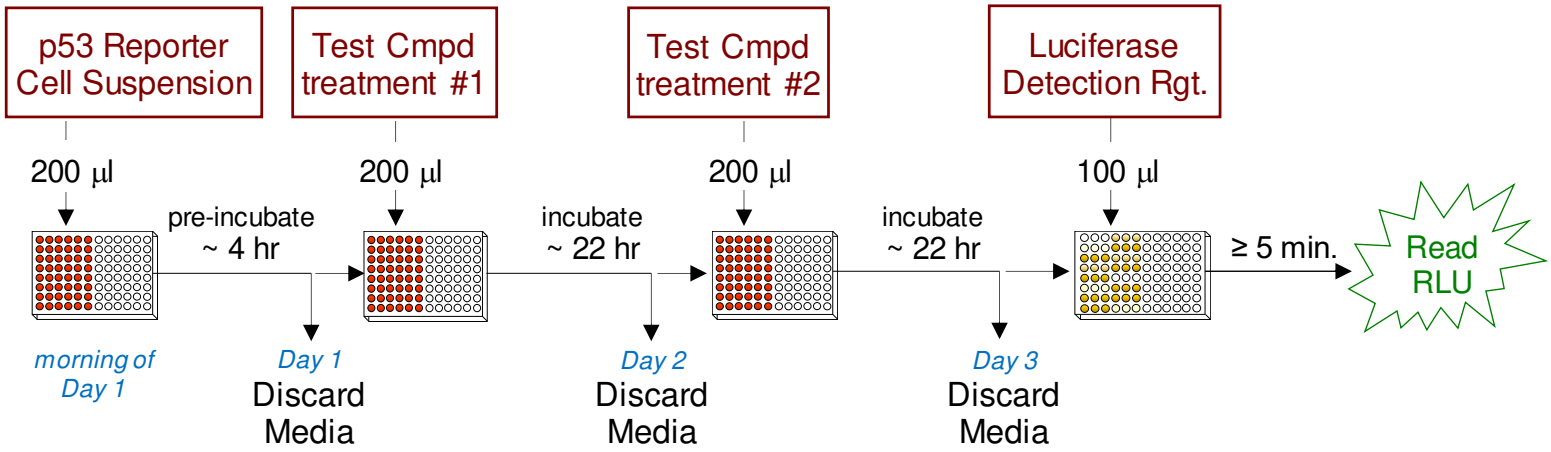
Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared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

Regardless of the dilution method used, the final concentration of total DMSO (or any organic solvent) carried over into assay wells should not 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 then considered to be 'single-use' reagents.

▪ Assay Scheme ▪

Figure 1. Assay workflows for p53 activation assays. In brief, 200 µl / well of Reporter Cells are dispensed into the assay plate, which is then pre-incubated for 4 - 6 hours. After the pre-incubation period the culture media are discarded and the prepared treatment media are added (200 µl / well). Following a first 22 - 24 hours treatment period the media are discarded and freshly prepared treatment media are added (200 µl / well). Following a second 22 - 24 hour treatment period the media are again discarded and Luciferase Detection Reagent is added (100 µl / well). After a brief plate-rest period, the intensity of light emission (in terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



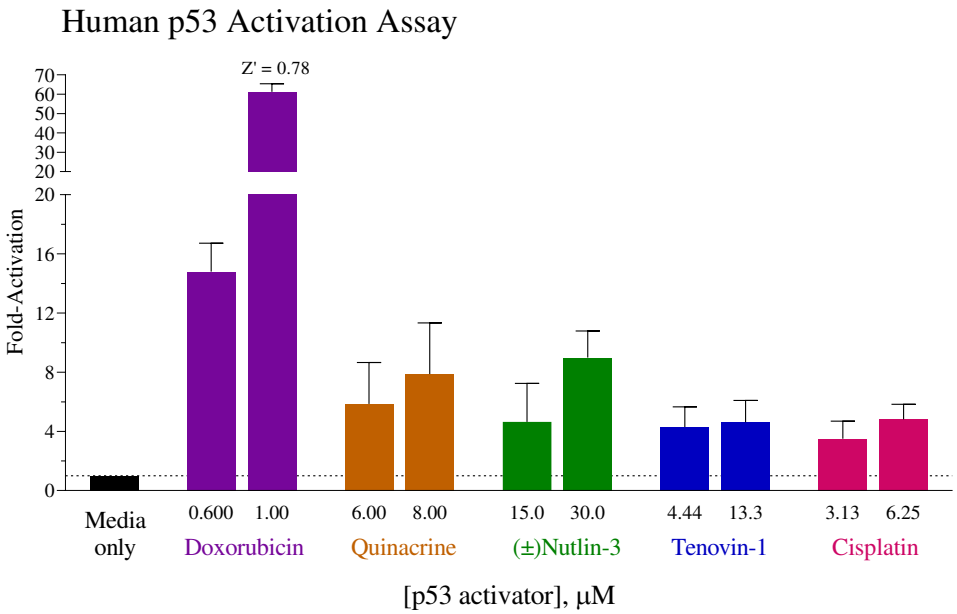


Figure 2. Activation of p53.
Activation of p53 is demonstrated by treating reporter cells with Doxorubicin hydrochloride (provided), Quinacrine hydrochloride hydrate, (±)-Nutlin-3, Tenovin-1 (all from Cayman Chemical), and Cisplatin (Tocris). Reporter Cells were treated following the '~24 hour + ~24 hour' dosing regimen described in this Technical Manual, and depicted in Figure 1. Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 3). Fold-activation and Z' values were calculated as described by Zhang, *et al*. (1999)[4]. All graphical manipulations were performed using GraphPad Prism software. The high Z' score confirms the robust performance of this assay[4].

II. Product Components & Storage Conditions

This Human p53 Assay kit contains materials sufficient to perform two independent assays of 48-wells each, using 96-well format assay plates.

Reporter cells are temperature sensitive! To ensure maximal viability the tubes 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 tubes of reporter cells in dry ice.

The two aliquots of p53 Reporter Cells are each provided as single-use reagents. 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.*
▪ p53 Reporter Cells	2 x 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 21 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 90 mL	-20°C
▪ Doxorubicin (hydrochloride)*, 1.0 mM (in DMSO) (reference for p53 activation)	1 x 80 µL	-20°C
▪ Luciferase Detection Reagent (LDR)	2 x 6.0 mL	**-80°C**
▪ 96-well, assay plate (white, sterile, cell-culture ready)	2	ambient

*Doxorubicin (hydrochloride) (CAS No. 25316-40-9) is a potent activator of the p53 pathway[5].

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, cell culture treated, for viewing cells on *Day 3.*

DAY 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 (or similar vessel)

DAY 3
- ▪ plate-reading luminometer.

IV. Assay Protocol

Please review the entire assay protocol before starting. Completing the assay requires ***two*** overnight incubations. *Steps 1-11* are performed on ***Day 1***, requiring less than 2 hours of bench work and a 4-hour pre-incubation step to complete. *Steps 12-16* are performed on ***Day 2*** and require less than 2 hours of bench work. *Steps 17 -22* are performed on ***Day 3*** and require less than 1 hour to complete.

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

1.) Remove the **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 tube of **CRM** from the 37°C water bath and sanitize its outside surface with a 70% ethanol swab.

Second, retrieve a tube of **p53 Reporter Cells** from -80°C storage and place it directly into dry ice for transport to the laminar flow hood. Use 1 tube of reporter cells if setting up 48 assay wells in one 96-well format plate, or 2 tubes of cells if intending to set up 96 assay wells using one (or both) assay plates. When ready, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml of 37°C CRM directly into a single tube of frozen cells. Repeat with the second tube of cells if thawing both at the same time. Place the tube(s) of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **10.5 ml** *per tube*.

3.) Retrieve the tube(s) 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 sterile media basin. If both tubes of reporter cells were thawed combine the cell suspensions in the single media basin. Using an 8-chanel pipette, dispense 200 µl/well of cell suspension into the assay plate; 48 assay wells per tube of reporter cells thawed.

> *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). Performing cytotoxicity analyses is recommended for the p53 assay.
>
> *NOTE 4.2:* 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 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.
>
> *NOTE 4.3:* 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.

5.) Pre-incubate reporter cells. Place the assay plate(s) into a mammalian cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) 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 the CSM from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound(s) and Reference Compound treatment media at the desired final assay concentrations. 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 **90 ml** total of CSM.

NOTE: Total DMSO (or any organic solvent) carried over into assay reactions should not exceed 0.4%.

***Activation*-mode assays**. This p53 Assay kit includes a 1.0 mM stock solution of Doxorubicin hydrochloride, a potent activator of the p53 pathway over a narrow concentration window. A 1.0 µM treatment concentration will provide significant activation of the p53 pathway. Note that higher concentrations run the risk of inducing cytotoxicity. Treatment concentrations below 0.2 uM provoke only weak activation of p53. Always include 'no treatment' ('media only') control wells.

8.) At the end of the pre-incubation period discard the culture media. The preferred method is to use a 'wrist flick' to manually eject media into an appropriate waste collection 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 appropriate wells of 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.) Return remaining CRM and CSM to a refrigerator (+4°C) for storage up to 1 week. For longer-term storage refreeze these media.

DAY 2 Assay Protocol: All steps must be performed using aseptic technique.

12.) Remove the CSM from refrigerator storage and warm in a 37°C water bath.

13.) Prepare the Test Compound(s) and Reference Compound treatment media at the desired final assay concentrations, as before in *Step 7*.

14.) At the end of the 22 - 24 hour treatment incubation period, discard the treated culture media into a biohazardous waste container, as described previously in *Step 8*.

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

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

DAY 3 Assay Protocol: Subsequent manipulations do not require special regard for aspectic technique and may be performed on a bench top.

17.) Near the end of the second treatment period, retrieve a tube (or both tubes, if both tubes of reporter cells were used) of **Luciferase Detection Reagent (LDR)** from freezer storage and place it in a low-light area so that the reagent may thaw and equilibrate to room temperature. Do not actively warm the LDR above room temperature; if needed, a room temperature water bath may be used to expedite thawing.

18.) Turn on the plate-reader and set the instrument to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set the read-time per assay well to 0.5 second (500 mSec), *or less*.

19.) Transfer the thawed **LDR** into a media basin.

20.) Following the treatment period, discard the media contents by manually ejecting it into an appropriate container for biohazardous waste. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

21.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5-10 minutes following the addition of LDR. Do not shake the plate during this period.

22.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human p53 Assay Kit	
IB25001-48	2x 48 p53 assays in 96-well assay plates
Bulk 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 performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Citations

[1] Kastenhuber, E, Lowe, S. (2017) Putting p53 in context. Cell. 170(6), 1062-1078.

[2] Ladds, M, et al. (2019) Small molecular activators of the p53 response. J. Mol. Cell Biol. 11(3), 245-254.

[3] Hientz, K, et al. (2017) The role of p53 in cancer drug resistance and targeted chemotheraphy. Oncotarget. 8(5), 8921-8946.

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

$$Z' = 1 - [3*(SD^{Ref\ EC100} + SD^{Untreated}) / (RLU^{Ref\ EC100} - RLU^{Untreated})]$$

[5] Lorenzo, E, et al. (2002) Doxorubicin induces apoptosis and CD95 gene expression in human primary endothelial cells through a p53-dependent mechanism. J. Biol. Chem. 277(13), 10883-10892.

VII. Limited Use Disclosures

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