User Manual

SPRINTer™ Endogenous Protein Turnover Biosensor Cell Lines

For Detection of Drug-Mediated Modulation of Endogenous Protein Levels

Document Number 70-425 Revision 0

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Important: Please read this entire user manual before proceeding with the assay.

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Overview

SPRINTer Endogenous Protein Turnover Biosensor Cell Lines are stable clonal cell lines used for monitoring drug-induced protein turnover of a specific endogenous target. Genome engineering or editing techniques, such as CRISPR/Cas9, are used to introduce a small tag into the endogenous locus of interest. This allows for the quantitation of changes in target protein levels when cells are treated with therapeutic agents such as targeted protein degraders.

Assay Principle

The SPRINTer assay utilizes the established Enzyme Fragment Complementation (EFC) technology. EFC uses a split β-galactosidase (β-gal) enzyme, which consists of the Enzyme Donor (ED) and the Enzyme Acceptor (EA) fragments. Independently, these fragments have no β-gal enzymatic activity. However, when forced to complement, e.g. as a result of protein-protein interaction, they form an active β-gal enzyme that hydrolyzes a substrate to produce a chemiluminescent signal However, when the 2 fragments are brought in close proximity, e.g. as a result of protein-protein interaction, they are forced to complement and form an active β-gal enzyme that can then hydrolyze a chemiluminescent substrate to produce a light signal. This is represented/illustrated in Figure 1.A below.

The SPRINTer Endogenous Protein Turnover Biosensor Cell Lines have been engineered to introduce the small 42 amino acid fragment of β-gal, ED into the endogenous locus of the desired target gene (e.g. BRD4, cMyc, etc.) in an appropriate cell background (e.g. the colorectal carcinoma model HCT-116 line or the chronic myeloid leukemia model K-562 line). Expression of the target gene from its native promoter results in the production of an ED-tagged target protein, as shown in Figure 1.B below. Addition of the exogenous Enzyme Acceptor (EA) and buffer lyses the cell and forces complementation of the ED and EA enzyme fragments. This results in the formation of a functional β-gal enzyme that hydrolyzes substrate to generate a chemiluminescent signal, allowing quantitation of drug-induced changes in ED-target protein levels. Treatment of these engineered biosensor cells with therapeutics that promote turnover of the target protein (such as PROTACs-mediated protein degradation) results in a decrease in the chemiluminescent signal, as illustrated in Figure 1.C below.

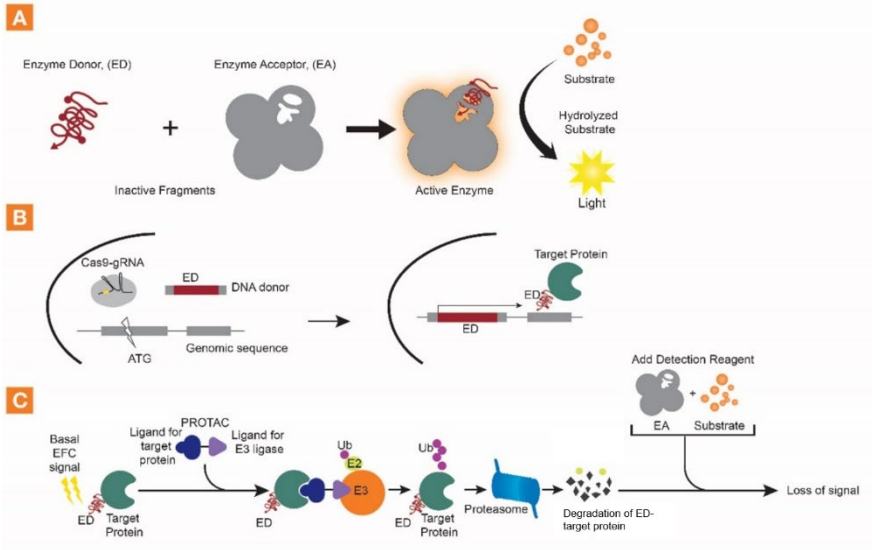

Figure 1. Principle of SPRINTer Biosensor Cell Lines: A. Illustration of Enzyme Fragment Complementation technology. **B.** A schematic illustration of CRISPR-mediated knock-in of ED tag into a target locus to create an ED-fusion protein for EFC assay. Cas9, gRNA and a donor DNA encoding the ED fragment are delivered into cells. Cell clones with robust EFC signal are isolated and optimized for protein turnover assay. **C.** Example of an application for the SPRINTer Endogenous Protein Turnover Biosensor Cell Line: measurement of PROTAC-mediated endogenous protein turnover. The heterobifunctional PROTAC molecule facilitates ubiquitination of the ED-tagged protein by bringing it into proximity of a specific E3 ligase. As a result, the ED-tagged protein undergoes degradation. A loss of the chemiluminescent signal is observed when the complementary enzyme fragment, EA, is added with the detection reagents.

Materials Provided

Components	
2 vials of cells	Refer to cell line-specific datasheet for shipped cell density

Storage Conditions

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

- Short-term (less than 24 hours): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (longer than 24 hours): Vials should ONLY be stored in liquid nitrogen (vapor phase ONLY) or in ultra-low temperature (-150°C) freezers.

Additional Materials Required

Refer to the cell line-specific datasheet to determine the appropriate media and reagents required for the specific cell line used in the assay.

Material	Ordering Information
Control molecule	Refer to the cell line-specific datasheet
AssayComplete™ Cell Culture Kit	Refer to the cell line-specific datasheet
AssayComplete Cell Plating (CP) Reagent*	Refer to the cell line-specific datasheet
AssayComplete Cell Detachment Reagent	92-0009 (for adherent cells)
AssayComplete Thawing Reagent	Refer to the cell line-specific datasheet
AssayComplete Freezing Reagent	Refer to the cell line-specific datasheet
PathHunter® PL/PK Detection Kit	93-0812
96-well White, Flat-Bottom TC-Treated, Sterile Plates with Lid, 10 plates/pack	92-0027
384-well White, Flat-Bottom, TC-Treated, Sterile Plates with Lid, 10 plates/pack (if applicable)	92-0015
96-Well Green, V-bottom, Untreated, Non-sterile Dilution Plates	92-0011
Dulbecco's Phosphate-Buffered Saline (PBS)	Corning, Cat. No. 21-031-CM or similar
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Multimode or luminescence reader	Refer to discoverx.com/instrument-compatibility
DMSO (for compound reconstitution and intermediate dilutions)**	
Single and multichannel micropipettes and pipette tips	
50 mL and 15 mL polypropylene tubes	

1.5 mL microtubes
Tissue culture disposable pipettes (1 mL-25 mL) and tissue culture flasks (T25 and T75 flasks, etc.)
Cryovials for freezing cells
Hemocytometer
Humidified tissue culture incubator (37°C and 5% CO_2)

* It is not recommended to substitute the Cell Plating (CP) Reagent that has been specified in the datasheet for the cell line.

** Refer to the ligand datasheet for the recommended dilution buffer.

Unpacking Cell Cryovials

Two cryovials are shipped on dry ice, and contain cells in 1 mL of AssayComplete™ Freezing Reagent (refer to the cell line-specific datasheet for the number of cells provided in each vial). The following procedures are for safe storage, handling and removal of cryovials from the vapor phase of liquid nitrogen storage.

 Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

1. Cells must arrive in a frozen state on dry ice.

    Contact technical support immediately, if cells received were already thawed.

2. Frozen cell cryovials must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, cryovials must be stored in the vapor phase of liquid nitrogen.

    Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

3. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container.

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4. Wait for at least one minute for any liquid nitrogen that may be present inside the vial to evaporate, before handling the vials.

Proceed with the thawing and propagation protocol in the following section. Refer to the cell line-specific datasheet for a list of the appropriate AssayComplete products mentioned in the following protocols.

Cell Culture Protocol – Adherent cells

The following procedures are for thawing adherent cells from cryovials, seeding and expanding the cells, and freezing the cells down once they have been propagated.

Cell Thawing

The following is a protocol for thawing cells in a T75 flask.

1. Pre-warm the AssayComplete™ Thawing Reagent in a 37°C water bath for 15 minutes, as indicated on the cell line-specific datasheet.

2. Add 15 mL of the AssayComplete Thawing Reagent into a T75 flask inside a sterile tissue culture hood. Set aside for Step 6. DO NOT add selection antibiotics to the thawing reagent.

3. Remove the cryovials from -80°C or liquid nitrogen vapor storage and immediately place them in dry ice prior to thawing.

Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid nitrogen vapor storage, use tongs to place them immediately in dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time, to avoid inadvertent thawing of the cells.

4. Place the cryovials containing the cells briefly (30 seconds to 1 minute) in a 37°C water bath, until only small ice crystals remain and the cell pellet is almost completely thawed. DO NOT centrifuge or vortex freshly thawed cells.

Do not leave the frozen cell vials in the water bath for longer than 1 minute. Prolonged thawing at 37ºC may result in cell death.

5. Decontaminate the external surface of the vials by spraying and wiping them with 70% ethanol. Transfer them to a tissue culture hood.

6. Using a sterile pipette, gently transfer the thawed cells to the pre-filled T75 flask. Incubate the flask at 37°C and 5% CO_2.

7. Maintain the cells in culture until they are 70-80% confluent. Then, proceed to Cell Recovery instructions. Do not split if cells are below this confluency, or growth issues may occur.

Cell Recovery

The following is a protocol for ensuring maximal cell recovery once they become 70-80% confluent in a T75 flask. The initial thawing and the first passage are conducted using the thawing reagent without any selection antibiotics.

1. Pre-warm the AssayComplete Thawing Reagent in a 37°C water bath for 15 minutes. DO NOT add selection antibiotics to the Thawing Reagent.

2. Remove the T75 flask from the tissue culture incubator and place it in a sterile tissue culture hood.

3. Gently aspirate the media from the T75 flask.

4. Add 5 mL of PBS into the T75 flask, and gently tip the flask side to side to ensure that the cells are rinsed.

5. Gently aspirate PBS from the flask.

6. Add 1 mL of 0.25% Trypsin-EDTA to the flask. Gently rock the flask back and forth to ensure that the inner surface of the flask is uniformly covered with trypsin.

7. Incubate the flask at 37°C and 5% CO_2 for 2 to 3 minutes, or until the cells have detached.

8. Remove the flask from the incubator and observe the cells under a microscope to confirm that the cells have detached. Gently tap the edge of the flask to detach cells from the surface, if necessary. If the cells do not detach easily, then return the flask to the incubator for an additional 1 to 2 minutes and repeat this step until almost all cells are in suspension. This typically does not exceed 5 minutes.

9. Add 4 mL of the AssayComplete™ Thawing Reagent to the T75 flask. Using a pipette, gently rinse the cells with the reagent.

10. Gently pipet the cell suspension up and down several times to generate a single cell suspension without any clumps.

11. Split the cells conservatively for the first passage after thawing, using the AssayComplete Thawing Reagent. Refer to the cell line-specific datasheet for the recommended split ratio.

12. Add 5 mL of AssayComplete Thawing Reagent to a new T75 or T225 flask, followed by addition of the cell suspension (volume determined in Step 11).

13. Add an additional volume of the thawing reagent to reach a final volume of 15 mL for a T75 flask, or 45 mL for a T225 flask.

14. Transfer the flask to a tissue culture incubator and incubate the cells at 37°C and 5% CO_2.

Cell Propagation

The cells can be propagated after a successful recovery, which can be determined by monitoring them under a microscope. Healthy cells should adhere uniformly to the surface of the flask, with only a few cells remaining in suspension.

To maintain logarithmic growth of the cells, cultures should be maintained in a sub-confluent monolayer.

1. If the cells appear healthy:

    a. Exchange the AssayComplete™ Thawing Reagent with 15 mL of the recommended AssayComplete Cell Culture Reagent for a T75 flask (45 mL for T225 flasks), supplemented with the recommended concentration of selection antibiotic.

    Refer to the cell line-specific datasheet to determine the correct Cell Culture Kit, recommended antibiotic, and antibiotic concentration for the cell line.

Cell culture media is prepared by mixing the components provided in the cell line-specific AssayComplete™ Cell Culture Kit, and appropriate selection antibiotics. Refer to the Cell Culture Kit's datasheet for instructions on using its components.

b. Return the flask to a tissue culture incubator, then proceed to Step 3.

2. If the cells do not appear to be healthy, or if confluency is <25%,

   a. Incubate the flask for additional 24 to 48 hours to allow for cell recovery before adding AssayComplete Cell Culture Reagent.

3. Once the cells have reached 70-80% confluency, split the cells every 2 to 3 days, based on the doubling time of the cell line.

15. Use the AssayComplete Cell Culture Reagent supplemented with the proper selection antibiotics (as indicated on the cell line-specific datasheet) to split the cells. Refer to the cell line-specific datasheet for the recommended split ratio.

Cell Cryopreservation

The following procedure is for freezing cells that have been propagated in T75 or T225 flasks. This protocol assumes that the cells have reached 70-80% confluency in a sufficient number of flasks to fill the desired number of cryovials. The cells will be cryopreserved in aliquots of 1 mL/vial, at the desired number of cells per vial (e.g. 1 x 10^6 per vial).

1. Remove T75 (or T225) flasks from the incubator and place them in a sterile tissue culture hood.


Care should be taken while handling flasks to avoid contamination.

2. Slowly aspirate the media from the flasks.

3. Add 10 mL of PBS into each T75 flask (or 15 mL for a T225 flask), and swirl it to rinse the cells.

4. Gently aspirate PBS from the flask.

5. Add 1 mL of 0.25% Trypsin-EDTA to T75 flasks (or 3 mL to T225 flasks).

6. Gently rock the flask back and forth to ensure that the inner surface of the flask is uniformly covered with Trypsin-EDTA.

7. Incubate the flasks at 37°C and 5% CO_2 for 2 to 3 minutes, or until the cells have detached.

8. Remove the flask from the incubator and view under a microscope to confirm that the cells have detached. If necessary, gently tap the edge of the flask to detach cells from the surface.

9. Add 5 mL of the cell culture media to each T75 flask (or 15 mL to each T225 flask).

10. Using a pipette, slowly rinse the cells from the surface of the flask with the added media. Slowly pipette up and down several times to achieve a single cell suspension with no cell clumps.

11. Transfer the cell suspension from the T75 flask into a 15 mL conical centrifuge tube. If using a T225 flask, transfer cells to a 50 mL conical centrifuge tube. If necessary, add an additional volume of the cell culture media to the flasks (e.g. 3 mL for T75 flasks or 5 mL for T225 flasks).

12. Rinse the flask to collect the remaining cells, then transfer the cell suspension into the conical tube. Slowly pipette up and down several times to ensure that a single cell suspension is formed.

13. To determine the concentration of cells in the suspension:

    13.1. Set aside a small fraction (0.5 mL or less) of the suspended cells in a separate tube.

    13.2. Transfer an appropriate portion of this fraction to a hemocytometer (typically 10 µL of cell suspension) or another cell counting device.

    13.3. Count viable cells, calculate the concentration of cells in the suspension, and then calculate the total number of cells in the 15 mL (or 50 mL) centrifuge tube.

14. Centrifuge the cell suspension prepared in Step 12 at 300 x g for 4 minutes.

15. After centrifugation, carefully discard the supernatant, without disturbing the cell pellet.

16. Based on the total cell number calculated in Step 13, resuspend the cells to the desired concentration (e.g. 1-2 x 10^6 cells/mL) with ice-cold AssayComplete™ Freezing Reagent (as defined in the cell line-specific datasheet).

17. Make aliquots by transferring 1 mL of cell suspension into the labeled 2 mL cryogenic tubes. Seal the tubes tightly.

18. Freeze cells in a -80°C freezer at a controlled rate of -1°C/minute overnight in a dedicated cell freezer or commercially-available freezing chamber (pre-chilled to 4°C). For short-term storage, vials can be stored in the -80°C freezer for a maximum of two weeks.

19. Transfer the vials into vapor phase of liquid nitrogen for long-term storage.

Keep cells on ice during this process to maintain cell viability.

Cell Culture Protocol – Suspension Cells

The following procedures are for thawing suspension cells from cryovials, seeding and expanding the cells, and freezing them down once they have been propagated. Refer to the cell line specific-datasheet for the specific AssayComplete™ products listed in the protocol below.

Cell Thawing

1. Add 9 mL of the AssayComplete™ Thawing Reagent in a T25 flask and place it in a humidified 37°C and 5% CO_2 incubator for 15 minutes to equilibrate pH and temperature of the reagent.

2. Remove the cell cryovials from -80°C or liquid nitrogen vapor phase storage and immediately place them in dry ice.

    Safety Warning: A face shield, gloves, and a lab coat should be worn at all times when handling frozen vials. When removing cryovials from liquid nitrogen storage, use tongs and immediately place the vials on dry ice in a covered container. Wait at least 1 minute for any liquid nitrogen inside the vial to evaporate. Do not touch the bottom of the vials at any time, to avoid inadvertent thawing of the cells.

3. Place the cryovials containing the cells briefly (30 seconds to 1 minute) in a 37°C water bath, under sterile conditions, until only small ice crystals remain and the cell pellets are almost thawed. DO NOT centrifuge or vortex freshly thawed cells.

    Prolonged thawing at 37°C may result in cell death.

4. Decontaminate the surface of the vials by spraying and wiping them with 70% ethanol. Transfer them to a tissue culture hood.

5. Gently transfer the thawed cells into the pre-filled T25 flask using a pipette, and incubate at 37°C and 5% CO_2 for 48 hours before proceeding with cell propagation.

Cell Propagation

1. Passage the suspension cells in the T25 flask when they have reached a density of 1 x 10^6 cells/mL as determined by taking an aliquot of cells to determine density. Do not let the cells exceed 3 x 10^6 cells/mL.

   1.1. Remove cells from the flask and transfer them to a conical tube (if necessary, add 5 mL of cell culture media to the flask, and rinse the flask to collect the remaining cells. Transfer this additional volume to the conical tube).

   1.2. Centrifuge at 180 x g for 3 minutes at room temperature to pellet cells.

   1.3. Decant the supernatant or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.

2. For routine passaging, resuspend the cells at a concentration of 1-2 x 10^5 cell/mL by adding the appropriate volume of pre-warmed cell culture media, supplemented with selection antibiotics for the given cell line. Refer to the recommendations in the table below for final culture volumes.

3. Note: If passaged every other day, we recommend seeding at a density of 2 x 10^5 cells/mL. If the interval between passages is longer than two days, we recommend seeding at a density of 1 x 10^5 cells/mL.

Flask Size	T25	T75	10 cm Dish
Final Culture Volume	10 mL	20 mL	10 mL

Cell Cryopreservation

Note: The following procedure is for freezing cells from T75 flasks. If smaller flasks are used, adjust the volumes accordingly. This protocol assumes that cells have reached the desired cell density in a sufficient number of flasks to fill the desired number of cryovials. The cells will be cryopreserved in aliquots of 1 mL/vial, at the desired number of cells per vial (e.g. 2 x 10^6 per vial).

1. It is recommended to freeze the cells at a low passage number (2 to 3 passages). For optimal assay performance, ensure that the cells are in logarithmic growth phase at the time of freeze down.

2. Remove the cell suspension from the flask and transfer it to a conical tube (if necessary, add an additional 5 mL of cell culture media to the flask and rinse it to collect the remaining cells. Transfer this additional volume to the conical tube).

3. Take an aliquot of the cells to determine the cell number.

4. Centrifuge the tube at 180 x g for 3 minutes at room temperature to pellet the cells.

5. Decant the supernatant, or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.

6. Immediately resuspend the cells in ice-cold AssayComplete™ Freezing Reagent to a concentration of 2 x 10^6 cells/mL.

7. Make aliquots by transferring 1 mL of cell suspension into the labeled 2 mL cryogenic tubes. Seal the tubes tightly.

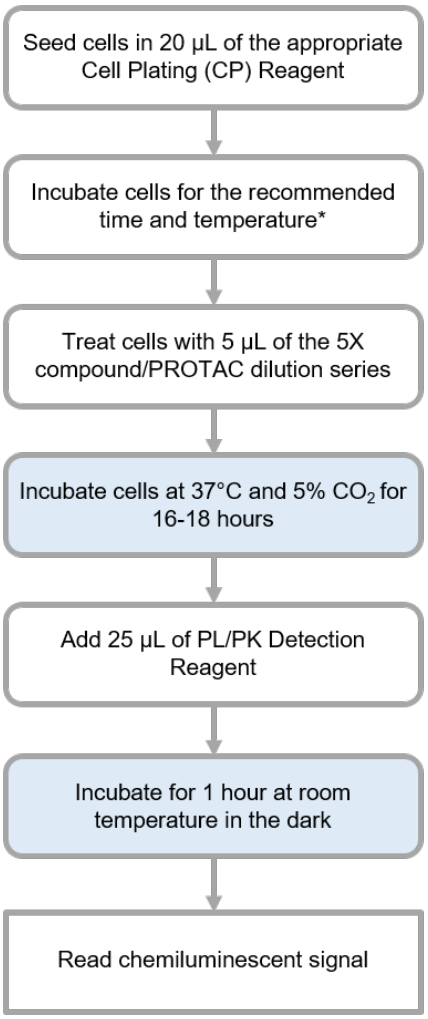
8. Freeze cells in a -80°C freezer at a controlled rate (-1°C/minute) overnight in a dedicated cell freezer or commercially available freezing chamber (pre-chilled to 4°C). For short-term storage, vials can be stored in the -80°C freezer for a maximum of two days.

9. Transfer the vials into the liquid nitrogen vapor phase for long-term storage.

Keep cells on ice during this time to maintain cell viability.

Protocol Schematic

Quick-Start Procedure: The flowchart below summarizes the assay protocol to be run in a 384-well plate.



*Refer to cell line-specific datasheet for specific recommendations.

‡Room temperature refers to a range of 23-25°C.

Detailed Assay Protocol: Small Molecules or Targeted Protein Degraders

The following protocol is designed for evaluating the ability of small molecules or targeted protein degraders (e.g. PROTACs) to promote target protein turnover, in a 384-well format.

For assays to be run in a 96-well plate, refer to the table below for assay reagent volumes. For experiments in a 96-well plate, the sample dilution series should be prepared at 10X the final desired concentration.

Assay Reagents (Volume per Well)	96-Well Plate**	384-Well Plate
Cell Plating Volume (µL)*	90	20
Ligand (µL)	10	5
Working Detection Solution (µL)	100	25
Total Assay Volume (µL)	200	50

*Refer to the cell line-specific datasheet for the recommended cell density.
**For an assay in a 96-well plate, a recommended cell number per well would be 2X the recommended cell number per well for a 384-well plate. Additional optimization of the cell number may be required.

1. Preparation and Plating: Adherent Cells

The following protocol provides steps for harvesting and preparing adherent cells for plating in an assay plate. This protocol assumes that cells have reached a 70-80% confluency and have been cultured in the recommended cell culture media. For cell line-specific information on the appropriate AssayComplete™ Cell Plating (CP) Reagent, Cell Culture Kit, control ligand, incubation times and temperature, refer to the cell line-specific datasheet.

1.1. Ensure that the cells are in the logarithmic growth phase prior to use in the assay.

1.2. Warm the CP Reagent and cell culture media in a clean 37°C water bath for 15 minutes. Refer to the cell line-specific datasheet for the recommended CP Reagent and cell culture media.

1.3. Dissociate the cells and resuspend them in fresh media.

1.3.1. Aspirate the media from the T75 flasks. Add 10 mL of room temperature PBS into each flask, and gently swirl it to rinse the cells. Aspirate PBS from the flask.

Use calcium- and magnesium-free PBS for rinsing cells prior to detachment.

1.3.2. Dissociate the cells by adding 1.5 mL of Assay Complete Cell Detachment Reagent. Gently rock the flask back and forth to ensure that the surface of the flask is uniformly covered.

Do not use trypsin for this step; especially with assays interrogating membrane-anchored receptors (e.g. GPCRs). Use of trypsin can negatively affect assay results.

1.3.3. Incubate the flask at 37°C and 5% CO_2 in a humidified incubator for 5 minutes, or until the cells have detached.

1.3.4. Remove the flask from the incubator and confirm that the cells have detached by viewing under a microscope. Gently tap the edge of the flask to detach cells from the inner surface, if necessary.

1.3.5. Add 8-10 mL of the cell culture media to each flask and pipette up and down a few times to dissociate cells.

1.4. Remove an aliquot of the cells from the flask and determine the cell density (e.g. using a hemocytometer or automated cell counter).

1.5. Transfer an appropriate volume of the cell suspension to a conical tube. The appropriate volume of cell suspension is based on the number of cells required for the number of samples being run in the assay.

1.6. Centrifuge the conical tube at 300 x g for 4 minutes at room temperature to pellet cells.

1.7. Decant the supernatant or carefully remove the media with a 10 mL pipette, without disturbing the cell pellet.

1.8. Resuspend the cell pellet in an appropriate volume of the recommended AssayComplete™ Cell Plating Reagent to achieve the required concentration of cells. The volume of CP reagent used is based on the number of cells in the pellet from Step 1.4 (e.g. to achieve 5,000 cells/well in 20 µL, resuspend cells at 2.5 x 10^5 cells/mL). Refer to the cell line-specific datasheet for the recommended concentration of cells.

1.9. Pour the cell suspension into a sterile reagent reservoir. Transfer 20 µL of the cell suspension into each well of a 384-well assay plate using a multichannel pipette

1.10. Incubate the assay plate at 37°C and 5% CO_2 before proceeding with ligand preparation. Refer to the cell line-specific datasheet for recommended cell incubation time.

2. Preparation and Plating: Suspension Cells

The following protocol is for harvesting cells from a T75 flask (with cell density of 1 x 10^6 cells/mL) and preparing them for plating in an assay plate at the desired concentration (optimal cell density). The protocol assumes that cells have been cultured in their specified cell culture media. The cell culture media comprises the cell line-specific AssayComplete Cell Culture Reagent mixed with its associated components provided in the Cell Culture Kit and supplemented with appropriate selection antibiotics.

2.1. Warm the CP Reagent in a clean 37°C water bath for 15 minutes.

2.2. Take an aliquot of cells (in suspension) from the flask, and determine the cell density by first counting cells, and then calculating the cell concentration per mL. Use the calculated concentration to determine the total number of cells in the tissue culture flask.

2.3. Remove the cells from the flask and transfer them into a conical tube (if necessary, add an additional 5 mL of media to the flask and rinse to collect the remaining cells. Transfer the additional volume to the conical tube).

2.4. Centrifuge at 180 x g for 3 minutes at room temperature to pellet cells.

2.5. Decant the supernatant or carefully remove the media with a 10 mL pipette without disturbing the cell pellet.

2.6. Resuspend the cell pellet to the required concentration (e.g. to achieve 5,000 cells/well in 20 µL, resuspend cells at 2.5 x 10^5 cells/mL) in pre-warmed CP Reagent. Refer to the cell line-specific datasheet for the recommended concentration of cells and CP Reagent.

2.7. Using a multichannel pipette and reagent reservoir, seed 20 µL of the cell suspension into one 384-well assay plate.

2.8. Incubate the assay plate at 37°C and 5% CO_2. Refer to the cell line specific-datasheet for the recommended cell incubation time. Proceed to the Preparation: Reference Compound or PROTAC section.

3. Preparation: Reference Compound or a PROTAC

When optimizing assay conditions, it is recommended to include a standard curve of a reference compound to verify that the cell line is working optimally. For the SPRINTer BRD4 and c-Myc Biosensor cell lines, we recommend the PROTAC MZ-1 as reference compound that is available from Tocris; Cat. No. 6154/5. MZ1 is also used as an example in the protocol below.

Follow the steps below to prepare serial dilutions of the reference compound or sample in a 96-well master dilution plate by making 4-fold, 11-point serial dilutions in the appropriate AssayComplete™ Cell Plating (CP) Reagent containing 5% DMSO, or the appropriate ligand dilution buffer (as specified on the ligand datasheet).

Note: The reference compound or sample should be prepared at 5X the final screening concentration. In order to maintain constant DMSO concentrations for all dilutions, we recommend that serial dilutions be prepared in an appropriate dilution buffer supplemented with 5% DMSO.

3.1. Add 45 µL of appropriate AssayComplete Plating Reagent containing 5% DMSO to Wells A2 to A12 of each row in the master dilution plate to contain sample (e.g. Row A will contain serial dilutions of the reference compound, while additional rows will contain test samples). This represents sufficient volume to aliquot the reference compound or test sample into 2 rows of the 384-well assay plate (quadruplicates per dose). The dilution volume may be adjusted according to the number of replicate wells required for the experiment.

3.2. Prepare the top concentration of the reference compound in CP Reagent (without 5% DMSO) at 5X the desired top dose in the assay.

3.2.1. For MZ1; top dose in the assay is 10 µM (5X is 50 µM). Add 5 µL of 1 mM MZ1 stock (in 100% DMSO) to 95 µL of appropriate AssayComplete Plating Reagent.

3.2.2. Refer to the cell line-specific datasheet for the recommended top screening concentration of the reference compound for the biosensor cell line being used.

3.3. Add 60 µL of the 50 µM solution of the reference compound (prepared in Step 3.2.1) to Well A1 of the master dilution plate.

3.4. Using a clean pipette tip, transfer 15 µL of solution from Well A1 into Well A2, and mix thoroughly by pipetting up and down several times.

3.5. Replace the pipette tip and transfer 15 µL of solution from Well A2 into Well A3, and mix well. Repeat this process until Well A11, resulting in an eleven-point, 1:4 dilution series. No sample is transferred to Well A12 as this is the negative control well.

3.6. Set up serial dilutions for test samples in a similar manner, using the ligand diluent recommended for the cell line on the cell line-specific datasheet. Ensure that the final DMSO concentration is comparable for all doses.

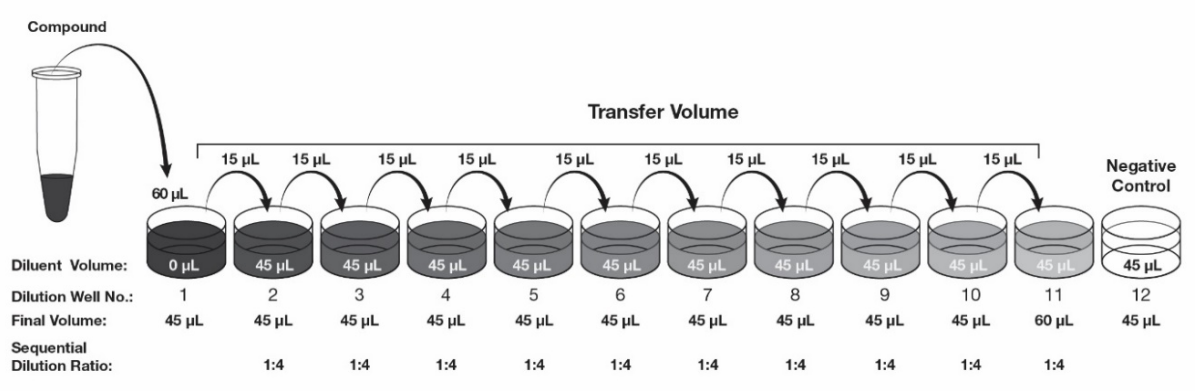


Figure 2. Reference Compound Serial Dilutions: Make eleven 4-fold serial dilutions of the reference compound in a master dilution plate.

4. Addition: Reference Compound or PROTAC

4.1. Using a 12-channel multichannel pipette, transfer 5 µL of the 5X reference compound serial dilution (prepared in Step 3) from Row A of the 96-well master dilution plate into Row A of the 384-well assay plate (2 transfer steps; 1 transfer step adds sample to every other well in the first row of the 384-well assay plate). This represents two replicate wells per dose.

4.2. For quadruplicate wells per dose, transfer 5 µL of the 5X reference compound serial dilution (prepared in Step 3) from Row A of the 96-well master dilution plate into Row B of the 384-well assay plate (2 transfer steps; 1 transfer step adds sample to every other well in the 384-well assay plate).

4.3. Add additional prepared samples to appropriate rows of the 384-well assay plate. The recommended format of sample addition is shown in the Representative Assay Plate Map.

4.4. Cover the plate with a lid and incubate for 16–18 hours at 37°C and 5% CO_2.

Representative Assay Plate Map

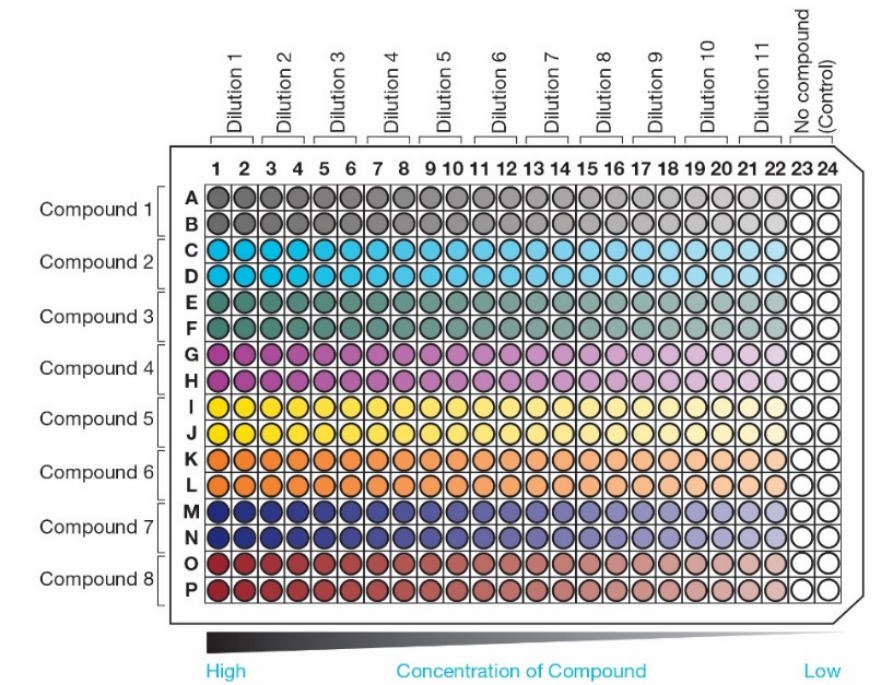


Figure 3. Assay Plate Map: This plate map shows an 11-point dose curve, with 4 data points at each concentration for one reference and seven test samples per plate with a 1:4 serial dilution scheme.

5. Detection

5.1. Prepare the working stock solution of the PL/PK detection reagent in a 15 mL polypropylene tube or a reagent reservoir. The detection kit includes EA Reagent, Lysis Buffer and Substrate Reagent. Mix the kit components in the ratio indicated in the table below:

Working Detection Solution (PL/PK) for a 384-Well Format		
Components	Volume Ratio	Volume per Plate (mL)
EA Reagent	1	2
Lysis Buffer	1	2
Substrate Reagent	4	8
Total Volume		**12**

5.2. Transfer 25 µL (or a volume equivalent to the assay volume) of the working detection solution to each well of the assay plate.
Optional: Place the plate on an orbital shaker at 350 rpm for 1 minute to achieve uniform mixing.

5.3. Incubate the assay plate for 1 hour at room temperature in the dark.

Detection Reagents are light sensitive, hence incubation in the dark is necessary.

6. Reading: Assay Plate

Read samples on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube readers or 5 to 10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.

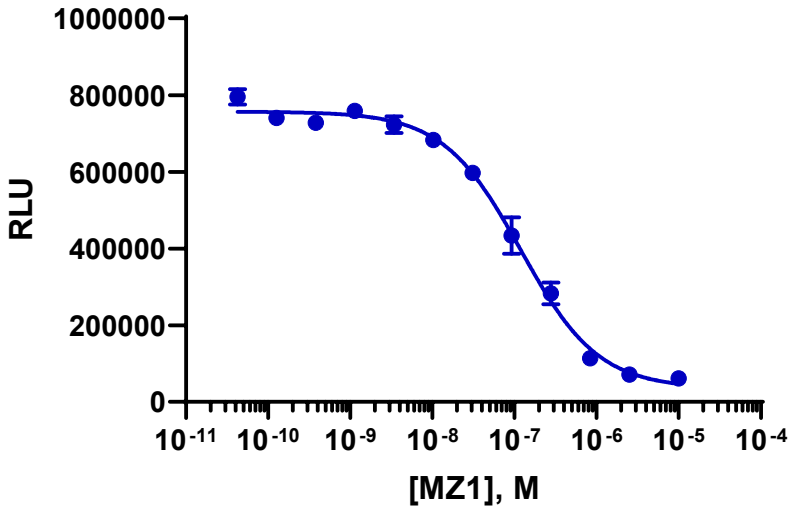
Data analysis can be performed using any statistical analysis software, such as GraphPad Prism, Softmax Pro, Gen5, Microsoft Excel, etc.

Typical Results

The following graph is an example of a typical dose-response curve using a c-Myc biosensor line, for measuring MZ1-mediated turnover of the endogenous c-Myc protein. MZ1 promotes degradation of BRD4, a transcriptional regulator of c-Myc. Degradation of BRD4 leads to a reduction in c-Myc transcription and subsequent c-Myc protein production. The data shows a potent, dose-dependent decrease in ED-c-Myc protein levels in the SPRINTer HCT-116 c-Myc Biosensor cell line after a 16 hour incubation with MZ1.

The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism 8.3.1.

A.



B.

EC_{50} (nM)	119.3
S/B	12.5

Figure 4. Typical Results: Representative **A**, dose-response curve and **B**, the EC_{50} and assay window observed for the decrease in c-Myc protein production when BRD4 is treated with MZ1.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Sub-optimal cell growth conditions	Refer to the datasheet for cell culture conditions.
	High DMSO/solvent concentration	Maintain DMSO/solvent at low concentrations (<1%) in serial dilutions of samples.
	Incorrect agonist used or incorrect agonist incubation time	Refer to the datasheet for recommended agonist and assay conditions.
	Incorrect preparation of agonist	Refer to the specific datasheet to ensure proper handling, dilution, and storage of agonist.
	Sub-optimal time course for induction	Optimize time course of induction with control agonist.
Decreased response	Higher passages give reduced performance	PathHunter cells are stable for at least 10 passages. Use low passage cells whenever possible.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should be prepared just prior to use.
	Problem with cell growth, cell viability, or cell density	Refer to the Cell Culture Protocol sections of this user manual for cell culture conditions.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 1 second/well.
Experimental S/B does not match datasheet value	Incorrect incubation temperature	Check and repeat the assay at the correct incubation temperature, as indicated on the cell line-specific datasheet
	Sub-optimal incubation time	Refer to the cell line-specific datasheet and optimize incubation time with control agonist.
	Slow-growing cells	Use functionally-validated and optimized AssayComplete™ media and reagents to improve assay performance.
EC_{50} is right-shifted	Ligand not stored properly	Follow directions for ligand storage as indicated in the ligand datasheet. Store in small aliquots at -20°C. Do not freeze/thaw more than twice.

	Incorrect top dose for the dose-response curve	Consult the dose-response curve on the cell line-specific datasheet for appropriate top concentration of ligand.
	Quality of ligand used in the assay	Differences in ligand vendors can also affect assay performance - be sure to use DiscoverX supplied ligand as a positive control to ensure that the assay works well in your hands before testing ligands or molecules from other sources.
	Problems with sample stability	Hydrophobic samples should be tested for solubility and may be diluted in buffer containing 0.1% BSA.
	Problems with plate type	Non-binding surface plates may be necessary for hydrophobic samples.
	Trypsin used for cell harvesting/plating	Use only AssayComplete™ Cell Detachment reagent to harvest cells for the assay.

For questions on using this product, please contact Technical Support at 1.866.448.4864 or DRX_SupportUS@eurofinsUS.com

Document Revision History

Revision Number	Date Released	Revision Details
0	March 2021	• New document

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