ProteoTuner™ Systems User Manual

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I. Introduction

Analyzing protein function is a key focus in discovery-based cell biology research. The ProteoTuner technology, developed by Wandless and colleagues (Banaszynski *et al.,* 2006), allows you to directly investigate the function of a specific protein of interest—by directly manipulating the level of the protein itself. This fast regulation occurs directly at the protein level, rather than at the mRNA or promoter induction level, and enables you to control the quantity of a specific protein in the cell, in as little as 15 to 30 minutes.

This revolutionary method takes advantage of ligand-dependent, tunable stabilization/destabilization of the protein of interest. It is based on a 12 kDa mutant of the FKBP protein (the destabilization domain, or DD) that can be expressed as a tag on your protein of interest. In the presence of the small (750 Da), membrane-permeant, stabilizing ligand Shield1, the DD-tagged protein of interest is stabilized (protected from proteasomal degradation) and accumulates inside the cell (Figure 1). Ligand-dependent stabilization occurs very quickly: DD fusion proteins have been shown to accumulate to detectable levels just 15–30 minutes after the addition of Shield1 (Banaszynski *et al.*, 2006).

The ProteoTuner method is not restricted to protein *stabilization*—it can also be used to *destabilize* the DD-tagged protein when you culture your cells in medium without Shield1, allowing proteasomal degradation of the DD-protein (Figure 1). This makes it possible to "tune" the amount of stabilized DD-tagged protein present in the cell by titrating the amount of Shield1 in the culture medium, and to repeatedly stabilize and destabilize the protein of interest using the same set of cells.

NOTE: To be degraded effectively, the DD fusion protein needs to have access to the proteasomes within the cell. Regions of the cell that do not have access to the proteasomes (e.g., the lumen of the ER) will not allow DD-tagged protein degradation.

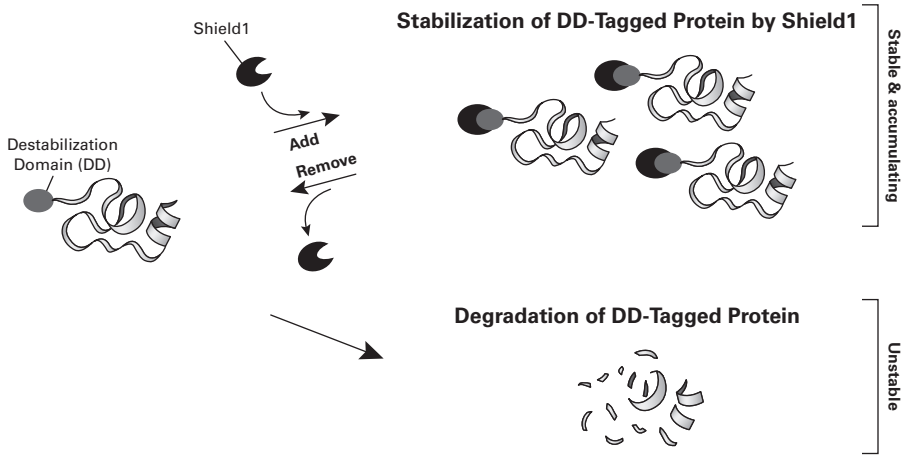

Figure 1. Ligand-dependent, targeted, and reversible protein stabilization. A small destabilization domain (DD; grey) is fused to a target protein of interest. The small membrane-permeable ligand Shield1 (black) binds to the DD and protects it from proteasomal degradation. Removal of Shield1, however, causes rapid degradation of the entire fusion protein. The default pathway for the ProteoTuner systems is degradation of the DD-tagged protein, unless Shield1 is present to stabilize it.

I. Introduction continued

This technology has been used successfully in a variety of applications and organisms (Table I).

Table I: ProteoTuner Technology is Applicable to a Wide Variety of Cell Types & Organisms

Protein	Organism/Cell Type	Reference
SIK1 (salt-inducible kinase)	Mouse myocytes	Berdeaux *et al.,* 2007
Rab11a (a small GTP binding protein; dominant negative version)	*Toxoplasma gondii*	Herm-Götz *et al.,* 2007
Falcipain-2	*Plasmodium falciparum*	Armstrong *et al.,* 2007
IL2	HCT116 colon cancer line (xenografted into mouse)	Banaszynski *et al.,* 2008
TNF-α	Mouse	
RhoA; Cdc42	NIH3T3, HEK 293T, HeLa, and COS-1 cells	Banaszynski *et al.,* 2006
Membrane protein CD8α	NIH 3T3 cells	
Cyclin B1	HeLa	Chu *et al.* 2008
TRPV5 (membrane ion channel)	HEK 293	Schoeber *et al.*, 2008
GPI-anchored protein		
MHC class I		
	Xenopus	
	Zebrafish	

Several systems are available: with either conventional plasmid or viral vectors, and with or without a Living Colors® Fluorescent Protein marker for transfection. Some systems also include a fluorescent protein reporter, or a tag for ProLabel™ quantitation (Table II).

Table II: Characteristics of the ProteoTuner Systems

System	Conventional Plasmid Delivery	Viral Delivery	Antibiotic Resistance*	Fluorescent Protein
ProteoTuner™ System	Yes	No	G418	No
ProteoTuner™ IRES2 System	Yes	No	G418	(IRES2) AcGFP1 marker
Retro-X™ ProteoTuner™ System	Yes	Retroviral	(IRES) Puromycin	No
Retro-X™ ProteoTuner™ IRES System	Yes	Retroviral	No	(IRES) ZsGreen1 marker
Lenti-X™ ProteoTuner™ System	Yes	Lentiviral	(IRES) Puromycin	No
Lenti-X™ ProteoTuner™ Green System	Yes	Lentiviral	No	(IRES) ZsGreen1 marker
DD-tdTomato Reporter System	Yes	No	G418	tdTomato reporter
DD-AmCyan1 Reporter System	Yes	No	G418	AmCyan1 reporter
DD-ZsGreen1 Reporter System	Yes	No	G418	ZsGreen1 reporter
ProteoTuner™ Quantitation System	Yes	No	G418	No

* In mammalian cells.

II. List of Components

Store Shield1 and all plasmids at –20°C.

ProteoTuner System (Cat. No. 632172)
- 20 µg pPTuner Vector (Cat. No. 632170; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

ProteoTuner IRES2 System (Cat. No. 632168)
- 20 µg pPTuner IRES2 Vector (Cat. No. 631036; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

Retro-X ProteoTuner System (Cat. No. 632171)
- 20 µg pRetroX-PTuner Vector (Cat. No. 632169; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

Retro-X ProteoTuner IRES System (Cat. No. 632167)
- 20 µg pRetroX-PTuner IRES Vector (Cat. No. 631035; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

Lenti-X ProteoTuner System (Cat. No. 632173)
- 20 µg pLVX-PTuner Vector (Cat. No. 632174; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

Lenti-X ProteoTuner System Green (Cat. No. 632175)
- 20 µg pLVX-PTuner Green Vector (Cat. No. 632176; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

DD-tdTomato Reporter System (Cat. No. 632190)
- 20 µg pDD-tdTomato Reporter (Cat. No. 632193; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

DD-AmCyan1 Reporter System (Cat. No. 632191)
- 20 µg pDD-AmCyan1 Reporter (Cat. No. 632194; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

DD-ZsGreen1 Reporter System (Cat. No. 632192)
- 20 µg pDD-ZsGreen1 Reporter (Cat. No. 632195; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)

ProteoTuner Quantitation System (Cat. No. 632196)
- 20 µg pPTuner Q Vector (Cat. No. 632197; not sold separately)
- 60 µl Shield1 (60 µl; Cat. No. 631037)
- 1 each ProLabel Detection Kit II (Cat. No. 631629)

Shield1
- 60 µl Shield1 (Cat. No. 631037)
- 200 µl Shield1 (Cat. No. 631038)
- 500 µl Shield1 (Cat. No. 632189)

III. Additional Materials Required

A. Mammalian Cell Culture Supplies

- Tissue culture plates or flasks
- Cell culture media
- Trypsin-EDTA (Trypsin; Sigma, Cat. No. T3924)
- Dulbecco's phosphate buffered saline (DPBS; VWR, Cat. No. 82020-066 or Sigma, Cat. No. D8662)
- Cell Freezing Medium, with or without DMSO (Sigma, Cat. No. C6164 or C6039)
- Tet System Approved Fetal Bovine Serum (FBS; Clontech, Cat. Nos. 631101, 631105, 631106 & 631107)

B. Antibiotics for Clonal Selection

Prior to using antibiotics to select transduced cells, determine the optimal selection concentration for each cell type.

- **G418** is available from Clontech (Cat. No. 631307).
  Note that the effective weight is about 0.7 g per gram of powder. Make a 10 mg/ml stock solution by dissolving 1 g of powder in approximately 70 ml of DMEM or α-MEM (without supplements). Filter sterilize and store at 4°C.
  - Recommended working concentration range: 50–800 µg/ml
  - Maintenance of stable cell lines: 100 µg/ml
  - Selection (e.g., HEK 293, HeLa or CHO cells): 400–500 µg/ml
- **Puromycin** is available from Clontech (Cat. Nos. 631305 & 631306)
  - Recommended working concentration range for viral systems: 1–2 µg/ml

C. Additional Materials for Retro-X™ ProteoTuner Systems

- Retro-X Universal Packaging System (Cat. No. 631530)

  NOTE: Please refer to the Retroviral Gene Transfer and Expression User Manual (PT3132-1, available at **http://www.clontech.com/support/manuals.asp**) for a complete list of materials required.

- Retro-X qRT-PCR Titration Kit (Cat. No. 631451)

  NOTE: Please refer to the Retro-X qRT-PCR Titration Kit User Manual (PT3952-1, available at **http://www.clontech.com/support/manuals.asp**) for a complete list of materials required.

D. Additional Materials for Lenti-X™ ProteoTuner Systems

- Lenti-X HT Packaging System (Cat. Nos. 632160 & 632161)

  NOTE: Please refer to the Lenti-X Lentiviral Expression Systems User Manual (PT3983-1, available at **http://www.clontech.com/support/manuals.asp**) for a complete list of materials required.

- Lenti-X 293T Cell Line (Cat. No. 632180)
  Alternatively, you can use the HEK 293T cell line; American Type Culture Collection HEK 293T/17 (ATCC No. CRL-11268™)
- Tet System Approved Fetal Bovine Serum (Cat. Nos. 631101, 631105, 631106 & 631107)
- Lenti-X qRT-PCR Titration Kit (Cat. No. 632165)

  NOTE: Please refer to the Lenti-X qRT-PCR Titration Kit User Manual (PT4006-1, available at **http://www.clontech.com/support/manuals.asp**) for a complete list of materials required.

IV. ProteoTuner Assay Protocol Overview

Protein Stabilization

In order to stabilize your protein of interest, you can add the stabilizing ligand, Shield1, to one of two parallel cell cultures which were previously untreated with Shield1 (Figure 2, Panel A). The other culture will be continuously cultured in the absence of Shield1 as a negative control.

The added Shield1 will protect your DD-tagged protein of interest from proteasomal degradation, causing a dramatic increase in its level in the cell. Stabilization has been reported in as little as 15–30 minutes (Banaszynski *et al.*, 2006) but we recommend performing a time course experiment in order to determine the stabilization rate after addition of Shield1 for your protein of interest.

At different time points, analyze the treated and control cells using your method of choice (e.g., Western blot or phenotypic analysis), depending on your experimental goals.

Protein Destabilization

The default pathway of the ProteoTuner Systems is rapid destabilization and degradation of the DD-tagged protein (Figure 1). In order to destabilize/degrade your protein of interest that has previously been stabilized, split cells which were previously treated with Shield1 into two parallel cultures (Figure 2, Panel B). One culture will continue to be cultured in the presence of Shield1 as a positive control, and the second (experimental) culture will be cultured without the stabilizing ligand, Shield1.

In the absence of Shield1, the DD-tagged protein of interest will be degraded rapidly. Degradation half lives of one to two hours have been reported (Banaszynski *et al.*, 2006), but we recommend performing a time course experiment in order to assess the rate of degradation of your protein of interest.

At different time points, analyze the treated and control cells using your method of choice (e.g., Western blot or phenotypic analysis), depending on your experimental goals.

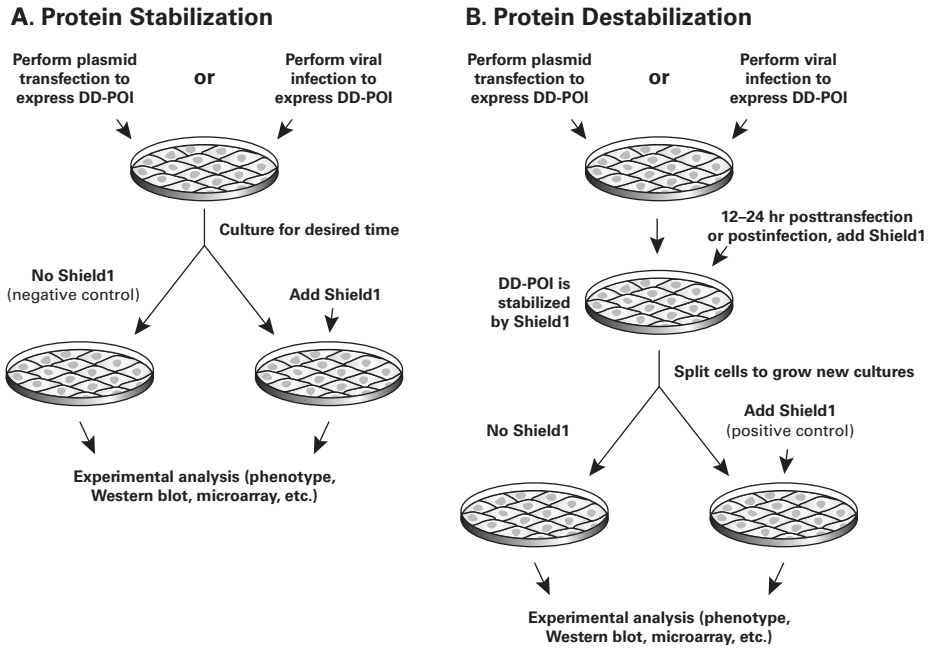

Figure 2. Overview of the ProteoTuner protein stabilization and destabilization protocols. Both protocols are based on Shield1's ability to reversibly stabilize DD-tagged fusion proteins (see Figure 1). **Panel A.** In order to observe the effects of stabilizing your protein of interest (POI), begin with cells cultured in medium that does not contain Shield 1. Then add Shield1, and as your DD-protein of interest is stabilized, perform your experimental analysis at defined time points in order to determine the protein's effects. **Panel B.** To observe the effects of the loss of your protein of interest, begin with cells cultured in medium that contains Shield1, and then split the cells into medium without Shield1 to destabilize your DD-protein of interest. Then perform your experimental analysis at defined time points in order to determine the effects of the loss of your protein of interest.

V. ProteoTuner System Protocols

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

Protocol

A. Protocol: Propagating Plasmid ProteoTuner Vectors

The ProteoTuner IRES2 System and ProteoTuner System include plasmid-based vectors. Each vector includes either a Living Colors® Fluorescent Protein or an antibiotic selection marker for transfection and transduction (Table II).

Selection by fluorescent protein: The **ProteoTuner IRES2 System** contains an internal ribosome entry sequence (IRES) following the DD-tagged gene of interest. This allows AcGFP1 to be translated independently of the DD-tagged protein. AcGFP1 expression is *not* regulated by the stabilizing ligand, Shield1. Therefore, detection of green fluorescence in a cell by either fluorescence microscopy or flow cytometry analysis indicates that the cell has been transfected and is expressing your DD-tagged protein of interest.

Antibiotic selection: If required, stable transfectants can be selected using G418 (Cat. Nos. 631305 & 631306). Please see Part III.B, Additional Materials Required, for more information about antibiotic selection.

1. To ensure that you have a renewable source of plasmid DNA, transform the plasmid vector provided with your system into a suitable *E. coli* host strain (e.g., DH5α). See the enclosed Vector Information Packet for further DNA propagation details.
2. To generate plasmid DNA for cloning purposes, use a suitable **NucleoBond®** or **NucleoSpin Kit®**. NucleoBond Xtra Kits provide the fastest and most convenient means available to achieve high yields of transfection-quality plasmid DNA. See **www.clontech.com** for available kits and options.
3. Using standard cloning techniques, insert your cDNA into the multiple cloning site (MCS) of the vector, in-frame with the DD domain. Your gene of interest must contain its own stop codon. The resulting construct can than be transfected into the cells of interest using your method of choice. We recommend using In-Fusion™ PCR Cloning Kits to perform your cloning.

Website

NOTE: For more information on Living Colors Fluorescent Proteins, please refer to our website, **http://www.clontech.com/colors**

V. ProteoTuner System Protocols continued

Protocol

B. Protocol: Propagating Viral ProteoTuner Vectors

We offer both retroviral and lentiviral delivery of ProteoTuner technology. Each vector includes either a Living Colors® Fluorescent Protein or an antibiotic selection marker for transfection and transduction (Table II).

Selection by fluorescent protein: The **Retro-X ProteoTuner IRES System** and the **Lenti-X ProteoTuner Green System** each include a vector which contains an IRES sequence following the DD-tagged gene of interest. This allows ZsGreen1 to be translated independently of the DD-tagged protein. ZsGreen1 expression is not regulated by the stabilizing ligand, Shield1. The detection of green fluorescence in a cell by either fluorescence microscopy or flow cytometry analysis indicates that the cell has been transduced and is expressing your DD-tagged protein of interest.

Website

NOTE: For information on Living Colors Fluorescent Proteins, please visit **http://www.clontech.com/colors**

Antibiotic selection: The **Retro-X ProteoTuner System** and the **Lenti-X ProteoTuner System** each include a vector which contains an IRES sequence following the DD-tagged gene of interest. This allows the antibiotic resistance gene for puromycin to be translated independently of the DD-tagged protein. This can be used to establish stable cell lines by selection with G418 (Cat. Nos. 631305 & 631306). Please see Part III.B, Additional Materials Required, for more information about antibiotic selection.

1. To ensure that you have a renewable source of plasmid DNA, transform the vector provided with your system into a suitable *E. coli* host strain which can prevent recombination between homologous LTRs (e. g., Supercharge EZ10 Electrocompetent Cells, Cat. No. 636756). See the enclosed Vector Information Packet for further DNA propagation details.
2. To generate the construct of interest, insert your gene of interest into the multiple cloning site (MCS) in-frame with the DD domain, using standard cloning techniques. Your gene of interest must contain its own stop codon but no poly A site. We recommend using In-Fusion PCR Cloning Kits to perform your cloning.
3. Produce your retrovirus or lentivirus:
   - **To generate retrovirus** from your pRetro-X-PTuner construct, use the Retro-X Universal Packaging System (Cat. No. 631530) according to the Retroviral Gene Transfer and Expression User Manual (PT3132-1). See the Vector Information Packet included with your pRetro-X-PTuner vector for further DNA propagation details. You can perform this initial transfection by your method of choice. We recommend using the CalPhos™ Mammalian Transfection Kit (Cat. No. 631312).

     Website

     NOTE: The Retroviral Gene Transfer and Expression User Manual (PT3132-1) is available at **http://www.clontech.com/support/manuals.asp**
   - **To generate lentivirus** from your pLVX-PTuner construct, use the Lenti-X HT Packaging System (Cat. Nos. 632160 & 632161) to cotransfect your construct into Lenti-X 293T Cells (Cat. No. 632180) according to the Lenti-X Lentiviral Expression Systems User Manual (PT3983-1). See the Vector Information Packet included with your pLVX-PTuner vector for further DNA propagation details.

     Website

     NOTE: The Lenti-X Lentiviral Expression Systems User Manual (PT3983-1) is available at **http://www.clontech.com/support/manuals.asp**
4. Collect the viral supernatant 48–72 hours posttransfection. At this point you must determine your viral titer and infect your desired target cells.
   - **For retroviruses**, we recommend using the Retro-X qRT-PCR Titration Kit (Cat. No. 631451) to determine your viral titer.
   - **For lentiviruses**, we recommend using the Lenti-X qRT-PCR Titration Kit (Cat. No. 632165) to determine your viral titer.

V. ProteoTuner System Protocols continued

Protocol

C. Protocol: Optimizing Shield1 Concentration and Incubation Time

Before you begin, transfect (or infect) your DD construct of interest into your cells of interest, using your method of choice (See Protocols A–B, above).

1. 12–24 hours posttransfection (for plasmid vectors) or postinfection (for viral vectors), split the cells into at least two parallel cultures (a plate treated with Shield1 and an untreated negative control).

Dilute the amount of Shield1 needed for your experiment.

Shield1 is provided as a 0.5 mM stock solution. It needs to be diluted to the final concentration in warm (37°C) culture medium, and mixed well. We recommend beginning with a range between 50–1,000 nM for the final concentration of Shield1.

You can dilute the Shield1 stock solution with culture medium that has already been used to culture the cells, or with fresh culture medium:

- Collect the media supernatant from your cell culture into a clean and sterile container and add the appropriate amount of Shield1 to reach the appropriate final concentration. After mixing, add the medium containing Shield1 back into the plate, or
- Warm up the appropriate volume of fresh culture media needed for your experiment to ~37°C. Then add the appropriate volume of Shield1 stock solution, to obtain the final concentration of Shield1 to be used in the experiment.

Example: Preparing 10 ml of culture media containing 500 nM Shield1.

10 ml	Warm culture medium (37°C; either collected from your tissue culture plate, or fresh media) in a sterile tube.
10 µl	0.5 mM stock solution
10 ml	500 nM Shield1 (final concentration)

2. Optimize the Shield1 concentration:

   a. The final concentration of Shield1 used in the experiment may vary, depending on the type of cells used as well as your particular DD-tagged protein of interest. Because the system is tunable, you can regulate or "tune" the amount of stabilized DD-fusion protein of interest in your cells by adding either more or less of the stabilizing ligand Shield1 to the culture media.

   b. We suggest testing various Shield1 concentrations within the recommended range (50–1,000 nM Shield1 in your culture medium) in order to determine the concentration that best fits your experimental needs.

4. Optimize the Shield1 incubation time.

Your protein of interest may be detectable as early as 15–30 minutes after addition of the stabilizing ligand, Shield1 (Banaszynski *et al.,* 2006). We recommend performing a time course experiment in order to determine the optimal incubation time with Shield1.

V. ProteoTuner System Protocols continued

D. Protocol: DD-Protein Stabilization

Before you begin, transfect (or infect) your DD construct of interest into your cells of interest, using your method of choice (See Protocols A–B, above).

1. 12–24 hours posttransfection (for plasmid vectors) or postinfection (for viral vectors), split the cells into at least two parallel cultures (an experimental plate which will be treated with Shield1, and an untreated negative control).

   For cells growing in suspension, collect your cells and distribute the cell suspension evenly into at least two tubes (the number of tubes depends on the number of samples you would like to collect). Centrifuge the cells for 5 minutes at ≤1,000 RPM, and remove the media.

2. Dilute the Shield1 to the optimal concentration determined in Protocol C. We recommended final concentrations of ~50–1,000 nM Shield1 in the cell culture medium.

3. Once the cells are attached to each plate or resuspended in each tube, remove the culture medium and replace with warm media with or without Shield1, respectively.

   The added Shield1 will protect your DD-tagged protein of interest from proteasomal degradation, causing a rapid increase in its level in the cell.

4. Collect cells at specific time points after treating with Shield1 (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest.

E. Protocol: DD-Protein Destabilization

Before you begin, transfect (or infect) your DD construct of interest into your cells of interest, using your method of choice (See Protocols A–B, above). Culture your cells in medium containing Shield1 at the optimal concentration determined in Protocol C, to stabilize your protein of interest.

1. 12–24 hours posttransfection (for plasmid vectors) or postinfection (for viral vectors), split the cells into at least two parallel cultures (an experimental plate treated with Shield1 and an untreated negative control).

2. Rinse the cells with warm Dulbecco's Phosphate Buffered Saline (TC grade).

3. Detach the cells by your method of choice (trypsin, cell dissociation buffer, etc.).

   For cells growing in suspension, collect your cells and distribute the cell suspension evenly in at least two tubes (the number of tubes depends on the number of samples you would like to collect). Centrifuge the cells for 5 minutes at ≤1,000 RPM and remove the media. Then resuspend the cell pellet in media with and without the appropriate concentration of Shield1 and culture for the appropriate amount of time determined by your needs. Proceed to step 6.

4. Split the cells into at least two new cell culture plates.

5. Culture the cells in one plate in the presence of Shield1 in the medium (positive control) and culture the second plate without Shield1.

6. Collect cells at specific time points (defined by your needs) after splitting and growing them in media with and without Shield1 in order to analyze and compare cells with and without the stabilized DD fusion protein of interest.

NOTE: To remove Shield1 without splitting the cell culture, wash cells three times with culture medium (without Shield1).

V. ProteoTuner System Protocols continued

Protocol

F. Protocol: Working with Stable Cell Lines Expressing a DD-Tagged Protein of Interest

1. After establishing a stable cell line, you can culture your cells either in the absence or the presence of Shield1, depending on your experimental needs.
2. If you grow your cells in the absence of Shield1, your protein of interest will be destabilized and expressed only at a very low level in your stable cell line. Then Shield1 can be added to rapidly increase the amount of your protein of interest (Protocol D).
3. Maintenance in, or addition of Shield1 to a stable cell line will stabilize your protein of interest and quickly increase its level in the cell (Protocol E).

VI. References

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