Takara Bio USA, Inc.

ProteoTuner™ Plasmid-Based Shield Systems User Manual

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I. Introduction

A. Summary

Analyzing protein function is a key focus in discovery-based cell biology research. ProteoTuner technology 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 must have access to proteasomes within the cell. Cell regions that lack such access (e.g., the ER lumen) will not allow DD-tagged protein degradation.

A variety of ProteoTuner Shield Systems are available:

Your choices include N- or C-terminal DD fusions; conventional plasmid or viral delivery; and systems with or without a Living Colors® Fluorescent Protein marker for transfection. One system contains a tag for ProLabel quantitation. ProteoTuner technology also plays an important role in the On-Demand Fluorescent Reporter Systems. This manual describes the **Plasmid-Based ProteoTuner Shield Systems**, which provide plasmid delivery of DD-fusion proteins (via transfection) to your target cells. You can learn about all of our ProteoTuner Shield Systems at **www.clontech.com**

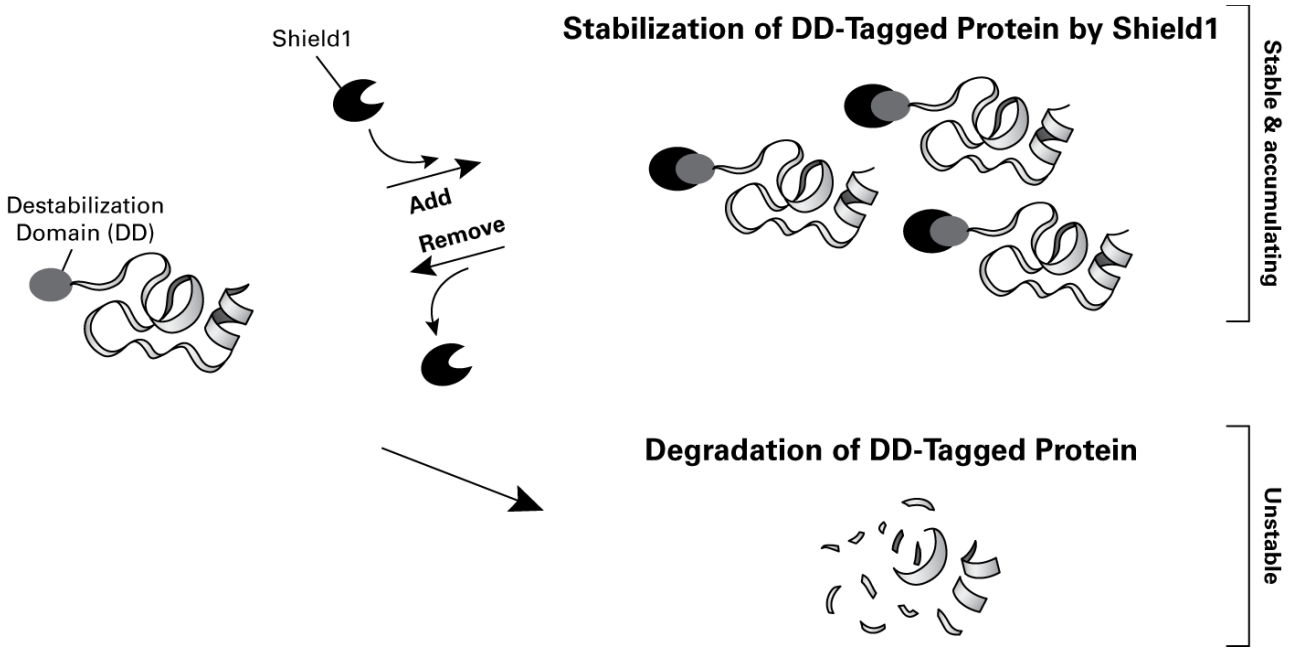

Figure 1. Ligand-dependent, targeted, and reversible protein stabilization. A small destabilization domain (DD; grey) is fused to a target protein of interest. The small membrane-permeable ligand Shield1 (black) binds to the DD and protects it from proteasomal degradation. Removal of Shield1, however,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

II. List of Components

Store all components at -20°C.

A. Expression Systems

ProteoTuner Shield System N (Cat. No. 632172)

- pPTuner Vector (20 µg) (Cat. No. 632170; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

ProteoTuner Shield System C (Cat. No. 631072)

- pPTunerC Vector (20 µg) (Cat. No. 631071; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

ProteoTuner Shield System N (w/ AcGFP1) (Cat. No. 632168)

- pPTuner IRES2 Vector (20 µg) (Cat. No. 631036; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

B. On Demand Reporter Systems

DD-AmCyan1 Reporter System (Cat. No. 632191)

- pDD-AmCyan1 Reporter (20 µg) (Cat. No. 632194; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

DD-tdTomato Reporter System (Cat. No. 632190)

- pDD-tdTomato Reporter (20 µg) (Cat. No. 632193; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

DD-ZsGreen1 Reporter System (Cat. No. 632192)

- pDD-ZsGreen1 Reporter (20 µg) (Cat. No. 632195; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

CRE DD Cyan Reporter System (Cat. No. 631089)

- pCRE-DD-AmCyan1 Reporter (10 µg) (Cat. No. 631090; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

CRE DD Green Reporter System (Cat. No. 631085)

- pCRE-DD-ZsGreen1 Reporter (10 µg) (Cat. No. 631086; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

CRE DD Red Reporter System (Cat. No. 631087)

- pCRE-DD-tdTomato Reporter (10 µg) (Cat. No. 631088; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

NFkappaB DD Cyan Reporter System (Cat. No. 631083)

- pNFkB-DD-AmCyan1 Reporter (10 µg) (Cat. No. 631084; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

NFkappaB DD Green Reporter System (Cat. No. 631079)

- pNFkB-DD-ZsGreen1 Reporter (10 µg) (Cat. No. 631080; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

NFkappaB DD Red Reporter System (Cat. No. 631081)

- pNFkB-DD-tdTomato Reporter (10 µg) (Cat. No. 631082; not sold separately)
- Shield1 (500 µl) (also sold separately as Cat. No. 632189)

III. Additional Materials Required

A. Shield1

Each ProteoTuner Shield System includes 500 µl of Shield1 (0.5 mM; see Section II). Additional Shield1 can also be purchased separately in the following sizes:

Cat. No.	Product Name	Size
632189	Shield1 (0.5 mM)	500 µl
632188	Shield1*	5 mg

*Designed for *in vivo* use; supplied in a dry-down format.

B. ProteoTuner Accessory Products

Cat. No.	Product Name	Size
631073	DD Monoclonal Antibody	50 µl
632196	Proteotuner Quantitation System	500 µl

C. Mammalian Cell Culture Supplies

- Culture medium, supplies, and additives specific for your cells
- Trypsin/EDTA (e.g., Sigma, Cat. No. T4049)
- 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. Nos. C6164 or C6039), for freezing ProteoTuner cell lines.
- Cloning cylinders or discs for isolating colonies of adherent cell lines (Sigma, Cat. No. C1059*)*
- 6-well, 12-well, and 24-well cell culture plates; 10 cm cell culture dishes

D. Antibiotics for Selecting Stable Cell Lines

Table 1. Recommended Antibiotic Concentrations for Selecting & Maintaining Stable Cell Lines

		Recommended Concentration (µg/ml)	
Cat. No.	**Antibiotic**	**Selecting Colonies[1]**	**Maintenance**
631308	G418 (5 g)	100–800	200
631307	G418 (1 g)		

[1] The appropriate dose must be determined empirically for your specific cell line.

E. Xfect™ Transfection Reagent

Xfect Transfection Reagent provides high transfection efficiency and low cytotoxicity for most commonly used cell types, including 293T cells.

Cat. No.	Transfection Reagent
631317	Xfect Transfection Reagent (100 rxns)
631318	Xfect Transfection Reagent (300 rxns)

F. In-Fusion® HD Cloning System

In-Fusion is a revolutionary technology that permits highly efficient, seamless, and directional cloning. For more information, visit www.clontech.com/infusion

Cat. No.	In-Fusion Cloning Kit
639645	In-Fusion HD Cloning System (10 rxns)
639646	In-Fusion HD Cloning System (50 rxns)
639647	In-Fusion HD Cloning System (100 rxns)

G. Stellar™ Competent Cells

Stellar Competent Cells are recommended by Clontech for cloning of lentiviral and retroviral vectors. Propagation of vectors containing repeat sequences such as viral LTRs using other strains of *E.coli* may result in plasmid rearrangements. Stellar Competent Cells are sold separately and provided with all In-Fusion HD Cloning Systems.

Cat. No.	Competent Cells
636763	Stellar Competent Cells (10 x 100 µl)
636766	Stellar Competent Cells (50 x 100 µl)

IV. ProteoTuner Assay Protocol Overview

1. Protein Stabilization

In order to stabilize your protein of interest, you need to 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 Shield1-based stabilization rate for your protein of interest as well as testing different Shield1 concentrations (50 nM–1,000 nM).
- 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.

2. Protein Destabilization

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

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

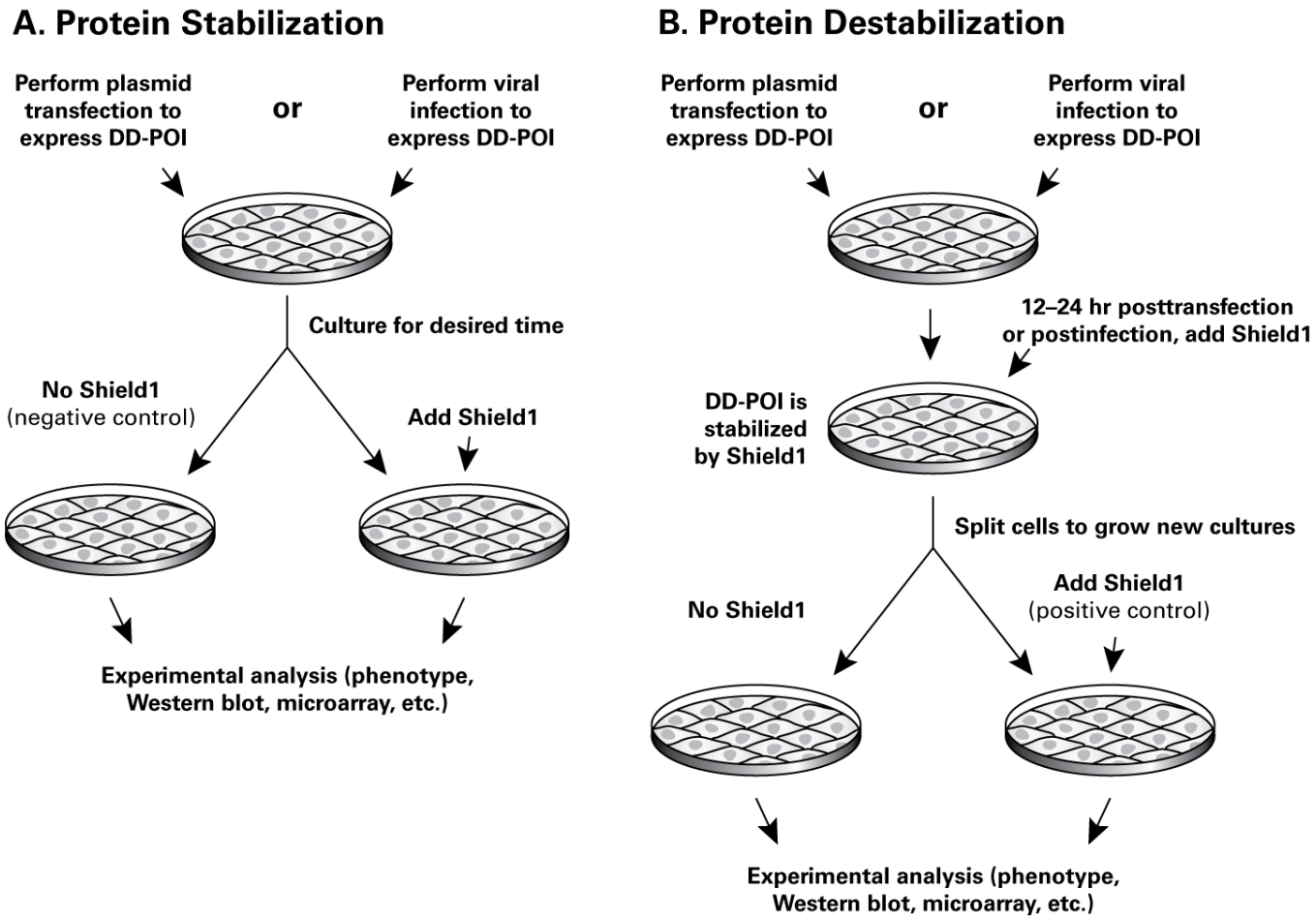

Figure 2. Overview of the ProteoTuner Shield Systems protein stabilization and destabilization protocols. Both protocols are based on Shield1's ability to reversibly stabilize DD-tagged fusion proteins (see Figure 1). **Panel A.** In order to observe the effects of stabilizing your protein of interest (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. Creating Vector Constructs Encoding DD-Tagged Proteins of Interest

A. Protocol: Creating ProteoTuner Vector Constructs using In-Fusion HD

The ProteoTuner Shield Systems N and C provide plasmid-based vectors for adding a DD-tag to your protein of interest. Each vector includes either a Living Colors Fluorescent Protein or an antibiotic selection marker for transfection, or both (Figures 4–7).

Cloning Guidelines

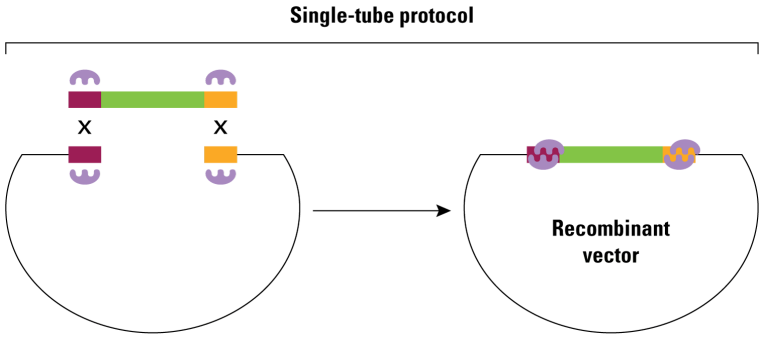

Figure 3. The In-Fusion HD Single-Tube Cloning Protocol.

1. To obtain a renewable source of plasmid DNA, transform the plasmid vector provided with your system into a suitable *E. coli* host strain, such as **Stellar Competent Cells** (Section III.G). These cells are recommended for use with our highly efficient, precise **In-Fusion HD Cloning Systems** (Section III.F) in cloning ProteoTuner constructs, and are provided with all In-Fusion HD Cloning Systems. In-Fusion HD technology (Figure 3) is described at **www.clontech.com/infusion**.
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. Insert your cDNA into the multiple cloning site (MCS) of the vector, in-frame with the DD domain. The gene of interest cloned in-frame with the N-terminal version of the DD tag should contain its own stop codon. The gene of interest cloned in-frame with the C-terminal version of the DD tag should contain no intervening stop codons.
4. We recommend using **Xfect Transfection Reagent** (Section III.E) to transfect ProteoTuner constructs into your target cells, as described in the Xfect Transfection Reagent Protocol-at-a-Glance, available at **www.clontech.com/manuals**.

Selecting Transfectants

- **Selection by fluorescent protein**: The ProteoTuner Shield System N (w/ AcGFP1) [Cat. No. 632168—see Figure 4] 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.

  NOTE: For optimal expression of a fluorescent protein downstream of the IRES in pPTuner IRES2 Vector (Figure 4), the DD-tagged gene of interest upstream of the IRES should be ≤ 2.5 kb.

- **Antibiotic selection**: If required, stable transfectants of all ProteoTuner plasmid-based Shield Systems can be selected using G418 (Section III.D).

VI. Protein Stabilization & Destabilization Using ProteoTuner Cell Lines

A. Protocol: Optimizing Shield1 Concentration and Incubation Time of Transfected Cells

Before you begin, transfect your DD construct of interest into your target cells (Section V.A, Step 4).

1. 12–24 hours posttransfection, split the transfected cells from Section V.A, Step 4 into different plates or separate wells of a 6-well plate, or your preferred plate format.

   To begin incubation of the transfected cells with Shield1 at predetermined time intervals and concentrations, replace the medium in the plates containing the transfected cells with medium containing the appropriate amount of Shield1, diluted as described below. Maintain at least one culture in medium containing no Shield1 as a negative control.

   NOTE: In the case of adherent cells, let the cells reattach after the split before removing the medium.

   a. Recommended Shield1 Concentrations and Time Points

   - Try Shield1 concentrations between 0.1 nM and 1,000 nM for different lengths of time (30 minutes to 12+ hours) to determine the best experimental conditions.

     NOTE: Your protein of interest may be detectable as early as 15–30 minutes after addition of the stabilizing ligand, Shield1 (Banaszynski et al., 2006).

   b. General Guidelines for Preparing Medium Containing Shield1

   - Dilute the supplied Shield1 stock solution (0.5 mM, supplied in ethanol) in tissue culture media to the final concentration(s) needed in your experiment.

     EXAMPLE: Preparation of 10 ml of medium containing 500 nM of Shield1: Dilute 10 µl of Shield1 stock solution (500 µM) in 10 ml of medium to yield a final concentration of 500 nM.

   - Working concentrations of Shield1 can be obtained by adding it directly from ethanol stocks, or by diluting it serially in culture medium just before use.
   - Dilute the Shield1 stock solution using one of the two following types of culture medium:
     1) **Culture medium that has already been used to culture the cells:** 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.
     2) **Fresh culture medium:** 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.
   - If you are making serial dilutions of Shield1 into culture medium, the highest concentration should not exceed 5 µM, to ensure complete solubility in the (aqueous) culture medium.
   - In either case, the final concentration of ethanol in the medium added to mammalian cells should be kept below 0.5% (a 200-fold dilution of a 100% ethanol solution) to prevent this solvent from having a detrimental effect on the cells.

2. After adding the medium containing Shield1 at the appropriate concentration and for the appropriate length of time, the effect of stabilizing your DD-tagged protein of interest can be analyzed with an assay that is appropriate for your experiment, e.g., Western blot.

B. Protocol: DD-Protein Stabilization of Transfected Cells

Before you begin, transfect your DD construct of interest into your target cells (Section V.A, Step 4) and determine the optimal Shield1 concentration and incubation time (see Section VI.A).

Stabilizing a protein of interest in attached cells

1. 12–24 hours posttransfection, split the cells into at least two parallel cultures (the number of plates depends on the number of samples you would like to collect).
2. Culture the cells (all plates) in medium without Shield1 until the cells are attached to each plate.

   NOTE: Shield1 does not interfere with the attachment process. Therefore, Shield1 can be added immediately after splitting if required for your experimental needs.

3. Dilute Shield1 to the optimal concentration determined in Section VI.A. We recommend final concentrations of ~50–1,000 nM Shield1 in the cell culture medium.
4. Remove the culture medium and replace it with warm medium with or without Shield1. Shield1 added to the experimental plate(s) will protect the DD-tagged protein of interest from proteasomal degradation, causing a rapid increase in its level in the cell.
5. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest.

Stabilizing a protein of interest in cells grown in suspension

1. 12–24 hours posttransfection, divide the cell suspension evenly into at least two tubes (the number of tubes depends on the number of samples you would like to collect).
2. Dilute Shield1 to the optimal concentration determined in Section VI.A. We recommend final concentrations of ~50–1,000 nM Shield1 in the cell culture medium.
3. Centrifuge the tubes (from Step 1) for 5 minutes at ≤1,000 rpm.
4. Remove the culture medium and replace with warm media with or without Shield1 (prepared in Step 2) as determined by your needs.

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

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

C. Protocol: DD-Protein Destabilization

Before you begin, transfect your DD construct of interest into your target cells (Section V.A, Step 4). Culture your cells in medium containing Shield1 at the optimal concentration determined in Section VI.A to stabilize your protein of interest.

Destabilizing a protein of interest in attached cells

Method A

Requires splitting cells (for quickest destabilization)

1. After stabilizing the protein of interest for the desired length of time via Shield1, remove the medium containing Shield1.
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.) and split them into at least two new cell culture plates (the number of plates depends on the number of samples you would like to collect).
4. Culture the cells in one plate in medium containing Shield1 (positive control) and culture the cells in the other plate(s) in medium without Shield1.

   NOTE: Growing the cells in the absence of Shield1 causes the fast degradation of the previously stabilized protein of interest.

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

Method B

No splitting required (for slower destabilization)

1. After stabilizing the protein of interest for the desired length of time via Shield1, remove the medium containing Shield1.
2. In order to destabilize the protein of interest, wash the cells in the plates by rinsing them three times with warm culture medium without Shield1.
3. Culture the cells in culture medium without Shield1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest.

Destabilizing a protein of interest in cells grown in suspension

1. After stabilizing the protein of interest for the desired length of time via Shield1, 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).
2. Centrifuge the tubes for 5 minutes at ≤1,000 rpm and remove the culture medium.
3. Resuspend one pellet in culture medium with Shield1 at the appropriate concentration (positive control) and resuspend the remaining pellet(s) in culture medium without Shield1.
4. Collect cells at specific time points (defined by your needs) in order to analyze and compare cells with and without the stabilized DD fusion protein of interest.

D. 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 (Section VI.B).
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 (Section VI.C).

VII. References

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VIII. Troubleshooting

Problem	Possible Explanation	Solution
The DD-tagged protein is already detectable in the absence of Shield1.	The expression level of the protein of interest fused to the DD domain is too high, especially in the case of a DD-tagged protein of interest localized to the plasma membrane.	Transfect cells with a lower amount of plasmid.
Addition of Shield1 does not result in any of the expected effect(s).	The Shield1 concentration is too low.	Increase the amount of Shield1 added.
	The monitoring assay is not sensitive enough.	Make sure to include a positive control when performing your assay.
	The volume of Shield1 used causes cells to die due to high solvent concentration.	Prepare a more concentrated stock solution.
	Poor target cell viability	• Optimize passage number of target cells. • Optimize culture conditions of target cells. • Optimize tissue culture plasticware

Appendix A: Creating Vector Constructs Encoding DD-Tagged Proteins of Interest

A. ProteoTuner Expression System Vector Maps

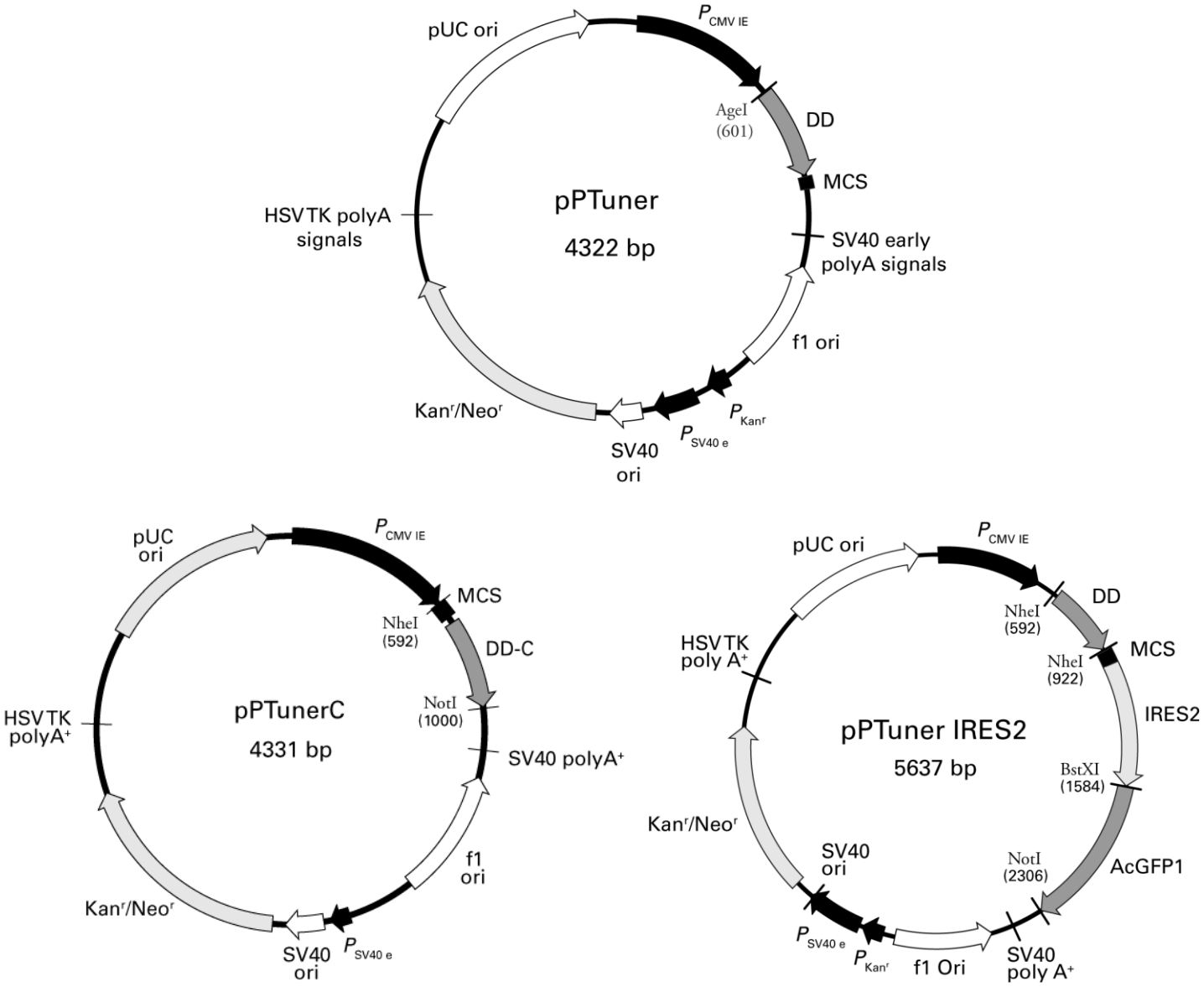

Figure 4. pPTuner, pPTunerC, and pPTuner IRES2 Vector maps.
For more detailed vector information, see **www.clontech.com**

B. ProteoTuner On Demand Reporter System Vector Maps

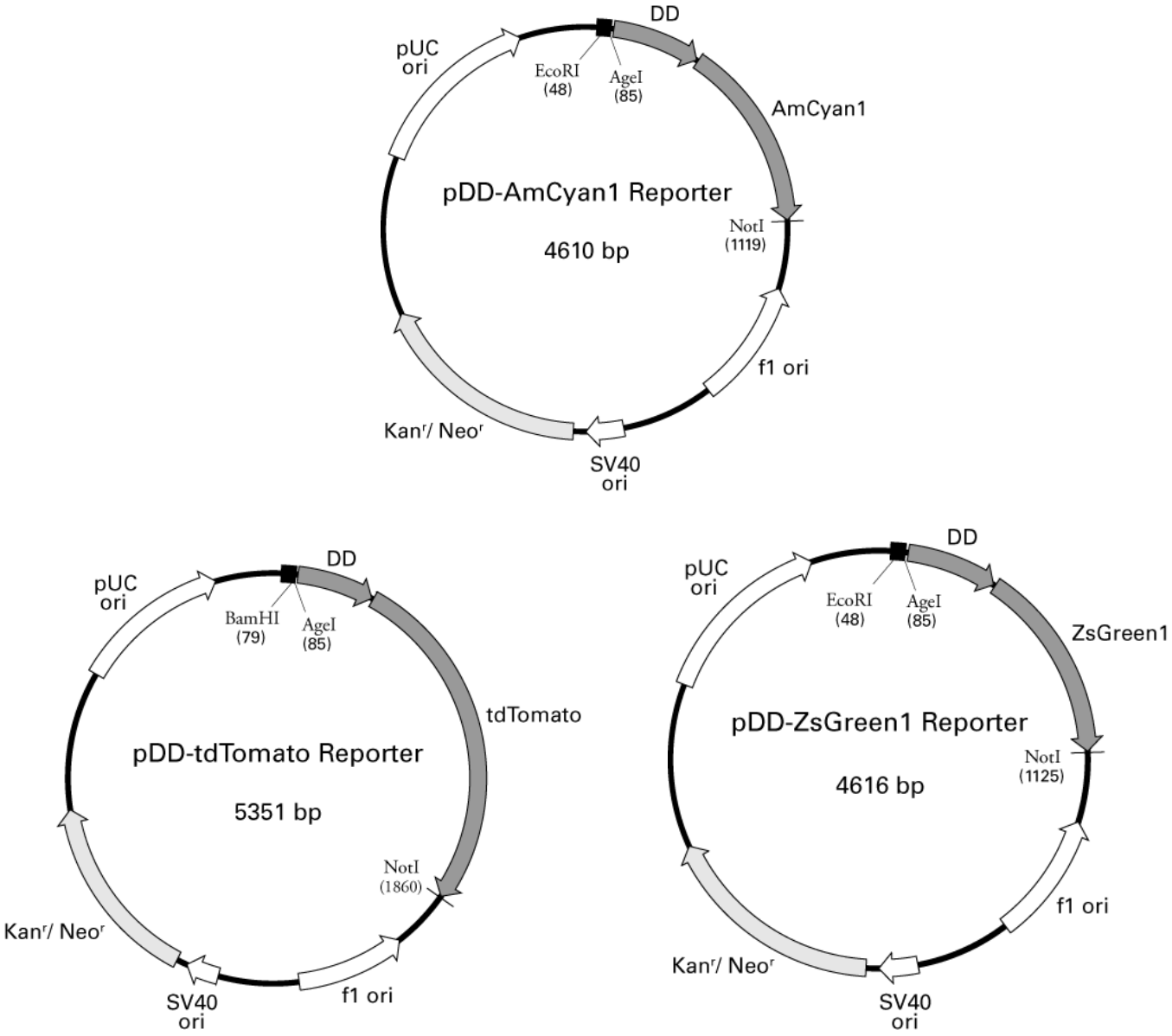

Figure 5. pDD-AmCyan1, pDD-tdTomato, and pDD-ZsGreen1 Reporter maps.
For more detailed vector information, see **www.clontech.com**

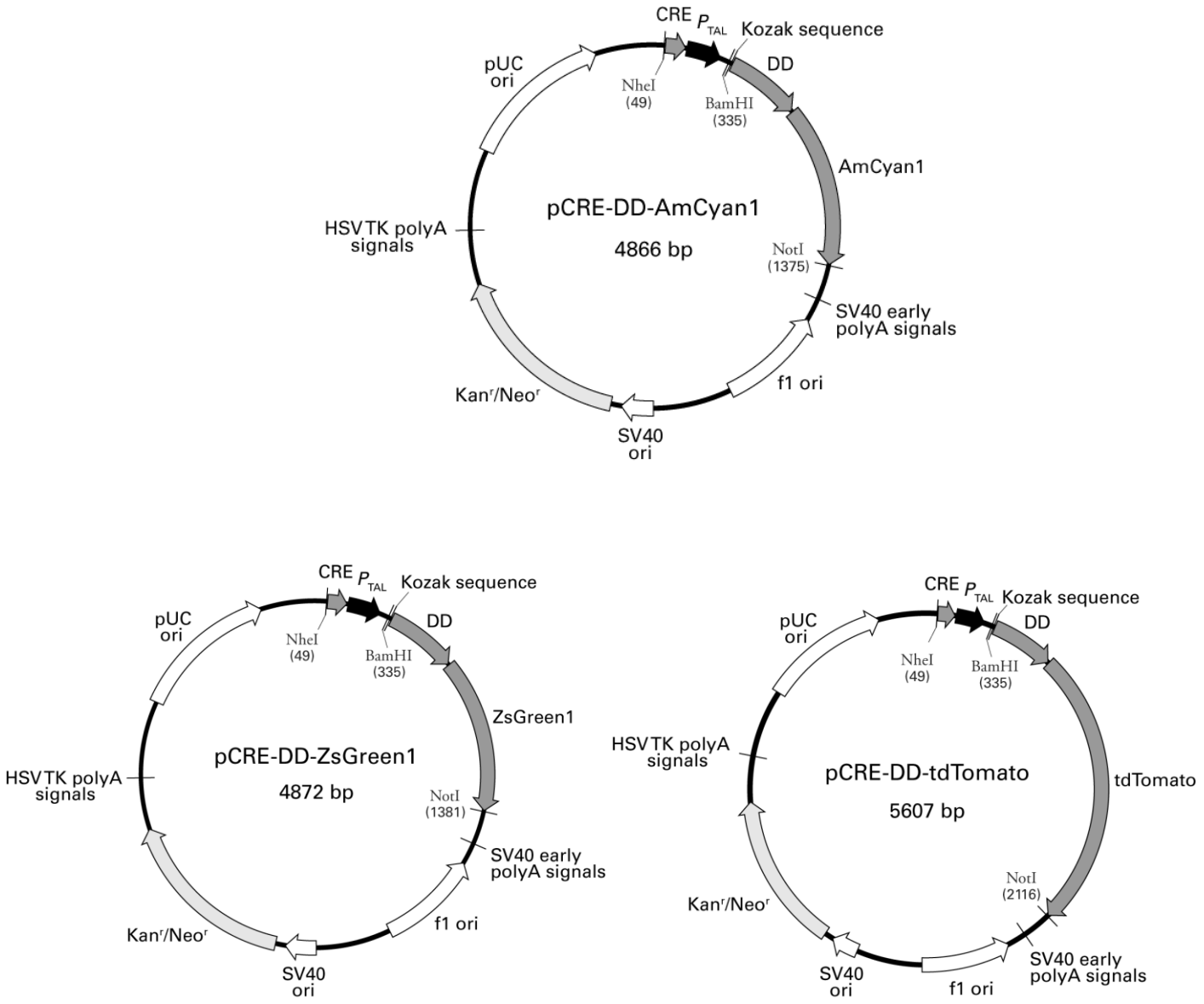

Figure 6. pCRE-DD-AmCyan1, pCRE-DD-ZsGreen1, and pCRE-DD-tdTomato Reporter maps.
For more detailed vector information, see **www.clontech.com**

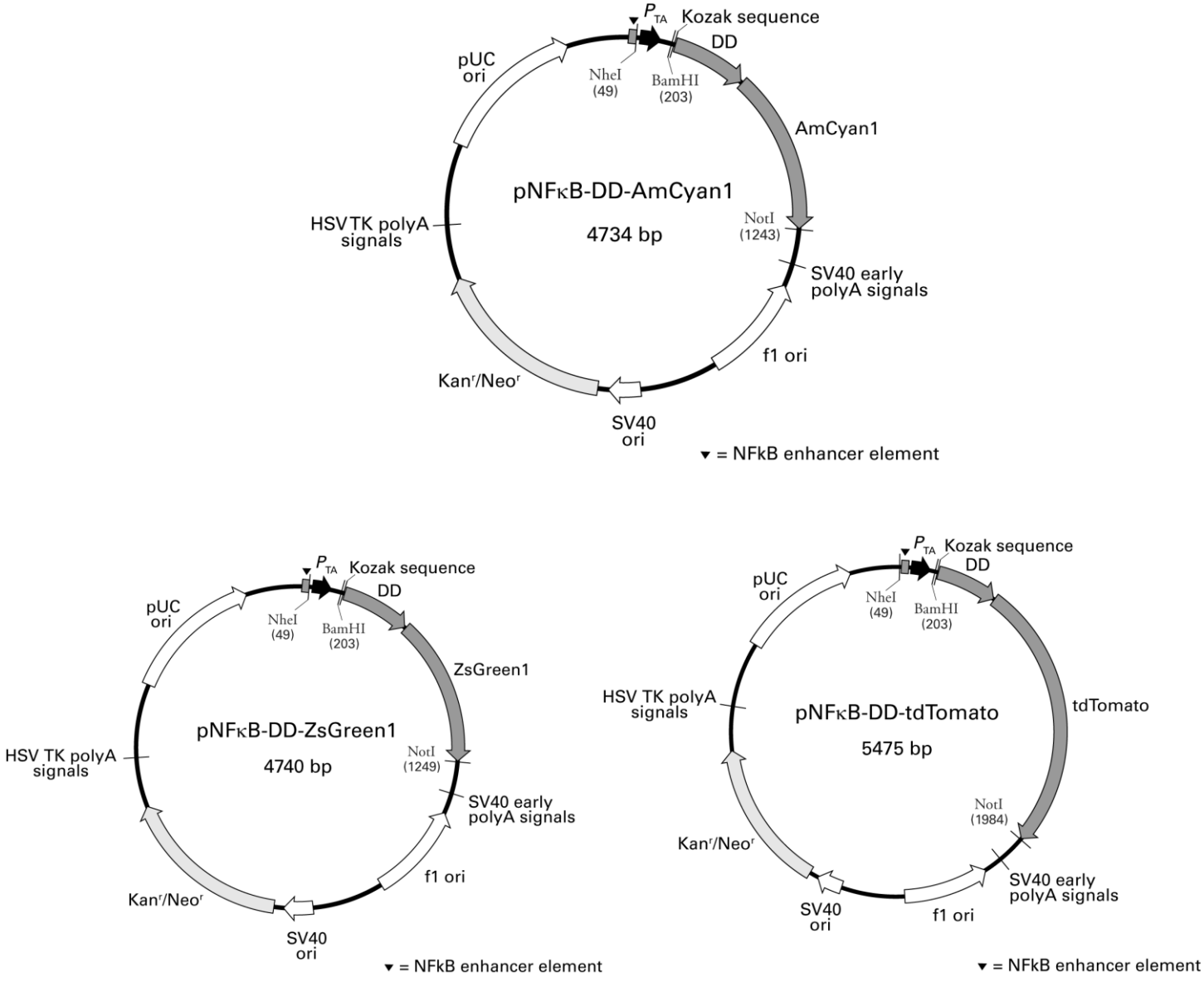

Figure 7. pNFkB-DD-AmCyan1, pNFkB-DD-ZsGreen1, and pNFkB-DD-tdTomato Reporter maps.
For more detailed vector information, see **www.clontech.com**

Appendix B: Preparing and Handling Cell Line Stocks

A. Protocol: Freezing Cell Line Stocks

Once you have created and tested your ProteoTuner cell line, you must prepare multiple frozen aliquots to ensure a renewable source of cells, according to the following protocol:

1. Expand your cells to multiple 10 cm dishes or T75 flasks.
2. Trypsinize and pool all of the cells, then count the cells using a hemocytometer.
3. Centrifuge the cells at 100 x g for 5 min. Aspirate the supernatant.
4. Resuspend the pellet at a density of at least 1–2 x 10^6 cells/ml in freezing medium. Freezing medium can be purchased from Sigma (Cat. Nos. C6164 & C6039), or use 70–90% FBS, 0–20% medium (without selective antibiotics), and 10% DMSO.
5. Dispense 1 ml aliquots into sterile cryovials and freeze slowly (1°C per min). For this purpose, you can place the vials in Nalgene cryo-containers (Nalgene, Cat. No. 5100-001) and freeze at –80°C overnight. Alternatively, place vials in a thick-walled styrofoam container at –20°C for 1–2 hr. Transfer to –80°C and freeze overnight.
6. The next day, remove the vials from the cryo-containers or styrofoam containers, and place in liquid nitrogen storage or an ultra-low temperature freezer (–150°C) for storage.
7. Two or more weeks later, plate a vial of frozen cells to confirm viability.

B. Protocol: Thawing Cell Line Frozen Stocks

To prevent osmotic shock and maximize cell survival, use the following procedure to start a new culture from frozen cells:

1. Thaw the vial of cells rapidly in a 37°C water bath with gentle agitation. Immediately upon thawing, wipe the outside of the vial with 70% ethanol. All of the operations from this point on should be carried out in a laminar flow tissue culture hood under strict aseptic conditions.
2. Unscrew the top of the vial slowly and, using a pipet, transfer the contents of the vial to a 15 ml conical centrifuge tube containing 1 ml of prewarmed medium (without selective antibiotics such as puromycin). Mix gently.
3. Slowly add an additional 4 ml of fresh, prewarmed medium to the tube and mix gently.
4. Add an additional 5 ml of prewarmed medium to the tube and mix gently.
5. Centrifuge at 100 x g for 5 min, carefully aspirate the supernatant, and GENTLY resuspend the cells in complete medium without selective antibiotics. (This method removes the cryopreservative and can be beneficial when resuspending in small volumes. However, be sure to treat the cells gently to prevent damaging fragile cell membranes.)

6. Mix the cell suspension thoroughly and add to a suitable culture vessel. Gently rock or swirl the dish/flask to distribute the cells evenly over the growth surface and place in a 37°C humidified incubator (5–10% CO_2 as appropriate) for 24 hr.

   NOTE: For some loosely adherent cells (e.g. HEK 293-based cell lines), we recommend using collagen-coated plates to aid attachment after thawing. For suspension cultures, suspend cells at a density of no less than 2 x 10^5 cells/ml.

7. The next day, examine the cells under a microscope. If the cells are well-attached and confluent, they can be passaged for use. If the majority of cells are not well-attached, continue culturing for another 24 hr.

   NOTE: Note: For some loosely adherent cell lines (e.g., HEK 293-based cell lines), complete attachment of newly thawed cultures may require up to 48 hr.

8. Expand the culture as needed. The appropriate selective antibiotic(s) should be added to the medium after 48–72 hr in culture. Maintain cell lines in complete culture medium containing a maintenance concentration of G418, as appropriate (Section III.D).

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