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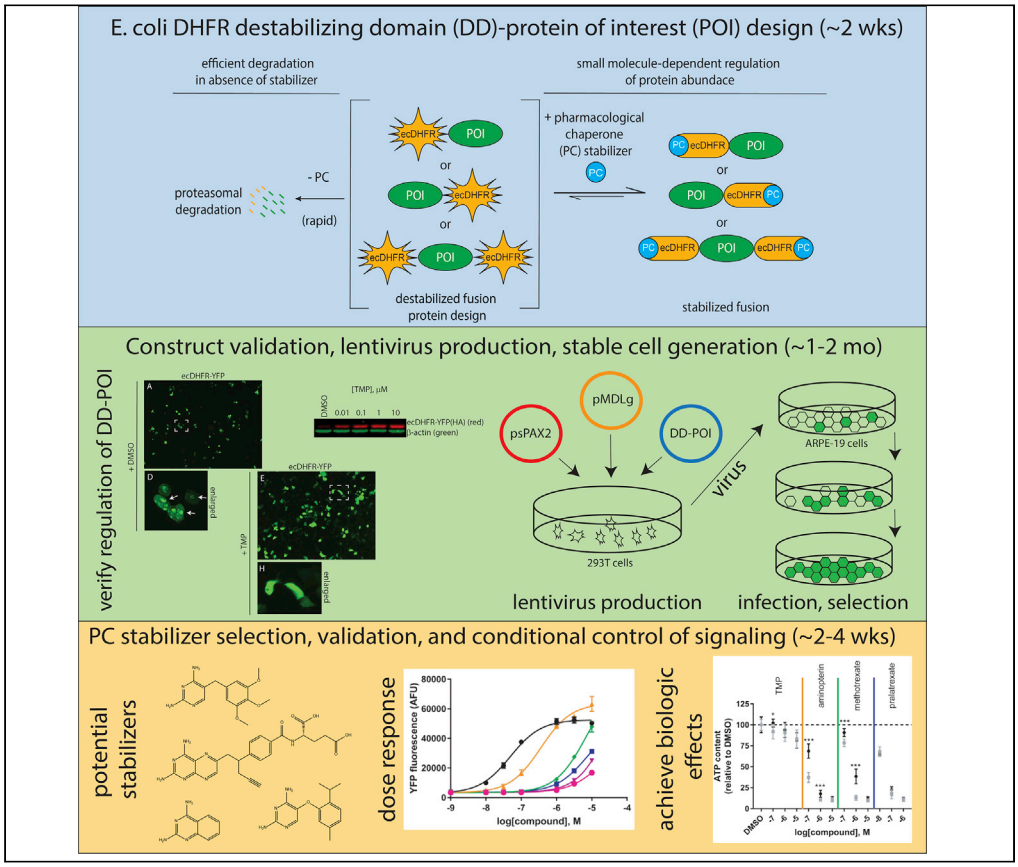
Protocol for Designing Small-Molecule-Regulated Destabilizing Domains for *In Vitro* Use



The use of destabilizing domains (DDs) to conditionally control the abundance of a protein of interest (POI) using a small-molecule stabilizer has gained increasing traction both *in vitro* and *in vivo*. Yet there are specific considerations for the development and accurate control of user-defined POIs via DDs, as well as the identification of novel (and potentially synergistic) small-molecule stabilizers. Here, we describe a platform for achieving these goals.

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HIGHLIGHTS

Conditional protein of interest (POI) regulation by destabilizing domain (DD) fusion

Focus on the utility of the *E. coli* dihydrofolate reductase (ecDHFR) DD

Dynamically define the levels of a POI through diverse ligands

Simultaneous DD stabilization and control of synergistic cellular signaling pathways

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Protocol for Designing Small-Molecule-Regulated Destabilizing Domains for *In Vitro* Use

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SUMMARY

The use of destabilizing domains (DDs) to conditionally control the abundance of a protein of interest (POI) through a small-molecule stabilizer has gained increasing traction both *in vitro* and *in vivo*. Yet there are specific considerations for the development and accurate control of user-defined POIs via DDs, as well as the identification of novel (and potentially synergistic) small-molecule stabilizers. Here, we describe a platform for achieving these goals.

For complete details on the use and execution of this protocol, please refer to Ramadurgum et al. (2020).

BEFORE YOU BEGIN

Experimental Design Considerations

A destabilizing domain (DD) is a relatively short protein sequence that is inherently unstable under physiological conditions and is efficiently targeted for protein degradation. When fused to a protein of interest (POI), the entire fusion product is rapidly degraded under untreated conditions. However, the DD-POI fusion protein can be stabilized upon the addition of a small molecule ligand which binds to and stabilizes the DD, allowing for quick, conditional regulation of the POI. Our primary goal in this *STAR Protocols* paper is to guide prospective users of this system on the development of a DD-POI system and validate it in cultured cells.

In theory, the DD-based strategy can control the abundance of a wide variety of proteins involved in diverse biologic processes including, but not limited to cytosolic, nuclear, and membrane proteins. While application of the DD system does come with some caveats, its inherent modular flexibility and utility make it a unique and effective tool for probing biology and potentially treating disease (Datta et al., 2019).

Design of a User-Defined ecDHFR-DD for *In Vitro* Use

1. Currently, there are a limited number of DDs that have been validated either *in vitro* or *in vivo*. These include a modified version of FK506-binding protein 12 (FKBP12, (Banaszynski et al., 2006)), *E.* coli dihydrofolate reductase (ecDHFR, (Iwamoto et al., 2010)), and the estrogen receptor (Miyazaki et al., 2012). While these are the most characterized DDs, in theory, one could generate a DD from a destabilized form of any protein that retains an ability to bind a stabilizing ligand, thereby promoting protein abundance. Nonetheless, due to its extensive use in the literature both *in vitro* (Shoulders et al., 2013a, Shoulders et al., 2013b) and *in vivo* (Datta et al., 2018,

Peng et al., 2019, Ramadurgum et al., 2020), we have focused on optimization and use of the ecDHFR-DD.

2. Determining the appropriate ecDHFR-DD variant to use based on the selected organism.
   a. One of the first considerations when starting to develop an ecDHFR-DD fused to a POI is to determine in which species it will be ultimately tested. If being tested in mammalian systems, or other systems that are conventionally grown/raised at 37°C, then standard ecDHFR-DDs (i.e., containing the R12Y/G67S/Y100I mutations [an N-terminal DD] or N18T/A19V/G67S mutations [a C-terminal DD]) as described in (Iwamoto et al 2010) can be used. However, as the stability of ecDHFR-DDs are temperature sensitive, use of the ecDHFR-DD system at lower temperatures (e.g., 25°C) in organisms such as Drosophila or *C. elegans*, requires a different series of destabilizing mutations (i.e., R12H/G67S/Y100I/D132G [an N-terminal DD] or R12H/N18D/I61T/G67S/G121V [a C-terminal DD]) to ensure lower levels of basal expression (Cho et al., 2013, Kogenaru and Isalan, 2018).
3. Determining whether to use a C-terminal versus N-terminal ecDHFR-DD (or both).
   a. When initially generating the ecDHFR-DD-POI fusion construct, we recommend designing both an N-terminal DD and a C-terminal DD. It is sometimes unpredictable if, or whether, fusion of the ecDHFR-DD will adversely affect POI function. An additional consideration is to fuse both an N-terminal and C-terminal ecDHFR-DD to obtain even better control over regulation of a POI (Maji et al., 2017, Kogenaru and Isalan, 2018). Investment of time to generate multiple ecDHFR-DD-POI constructs at the outset of a project will prevent the need to re-generate the construct later if found to be non-functional or suboptimal.
   b. Choice of N- versus C- terminal DD tags may also affect the dynamic range of stabilization and the levels required for full stabilization. While these characteristics may not be critical for *in vitro* studies, it may be important to consider for *in vivo* studies where stabilizer levels may not reach a desired tissue at a sufficient concentration. As an example of this observation, an N-terminal ecDHFR fused to yellow fluorescent protein (YFP) was stabilized fully at 1 μM of trimethoprim (TMP), the canonical ecDHFR-DD stabilizer, whereas the equivalent C-terminal DD fusion demonstrated a wider dynamic range of stabilization reaching maximal fluorescence at ~10 μM (Iwamoto et al., 2010).
   c. If the reporter protein or POI does not tolerate the fusion of the ecDHFR-DD to either termini, it is theoretically possible to insert the DD internally within a POI where it might be better tolerated, although to our knowledge, this has not been attempted or accomplished. It is quite possible that splicing the DD into the middle of the POI sequence interrupts native activity and folding, unless the domain architecture is well understood. Therefore, this method will likely require structural modelling of the POI and a high degree of time and effort to successfully accomplish.
4. Choosing a fusion POI to regulate using the ecDHFR-DD.
   a. To effectively use DDs, it is important to ensure very low basal levels of the DD-POI under untreated conditions and effective, dose-dependent stabilization upon treatment with the small molecule stabilizer or pharmacological chaperone. Therefore, the fusion protein must be accessible to the proteasomal degradation machinery (to ensure rapid degradation) and the small molecule stabilizer must be able to penetrate the cell to be present where stabilization is sought. Thus, the ecDHFR-DD system has been used most commonly for regulating cytoplasmic (Vu et al., 2017) or nuclear fusion proteins (Chen et al., 2014, Cooley et al., 2014) where shuttling to the proteasome can easily occur. While some studies suggest that targeting the ecDHFR-DD to organelles such as the endoplasmic reticulum (ER) or mitochondria result in poor regulation (Sellmyer et al., 2012), other groups have been able to sucessfully control transmembrane and secreted proteins (Iwamoto et al., 2010, Quintino et al., 2018). This discrepancy may arise if the DD is able to sufficiently destabilize the fusion protein prior to being translocated to the ER or mitochondria, and thus would be targeted quickly for proteasomal degradation. Nonetheless, if the user is working with a protein that is targeted to the ER (e.g., lysosomal, transmembrane or secreted) or the mitochondia, they will have to assess the basal degradation rate carefully.

b. While the potential number of regulatable POIs is vast, we and others have focused largely on generating DDs fused to transcription factors (TFs) since these proteins can regulate multiple beneficial cellular pathways. Due to the complexity of the DNA binding pocket, TFs have long remained untargettable by small molecules with the exception of ligand-binding TFs such as estrogen and androgen receptors (Hagenbuchner and Ausserlechner, 2016). Therefore, the fusing of an ecDHFR-DD to a TF provides the unique opportunity to use small molecule-regulated TFs. Herein, we describe optimization of TFs as an example of the considerations that should be taken into account to generate an effective DD system.

c. Users should consider the half-life of the TF (or POI) of interest. Fusion of the ecDHFR-DD to such a TF could inadvertently stabilize the TF, increasing steady-state levels. We have observed this anecdotally with TFs with half-lives < ~0.5 h.

5. Genetically modifying the POI to achieve a constitutively active TF or dominant negative TF.

a. The primary goal of the ecDHFR-DD system is to be able to regulate cellular signaling immediately upon addition of a small molecule stabilizer. In many cases, this requires modification of the POI such that when it is present (i.e., when the small molecule is added), signaling is manipulated without the need for additional cellular input or post-translational modification. Thus, to activate downstream signaling, it is common to generate a constitutively active (ca) TF.

i. The most common way to generate a caTF is to remove known negative regulatory sequences that may promote the degradation of the TF or prevent its nuclear translocation. Such sequences, if retained within the ecDHFR-DD-TF, could result in little or no activity when the fusion protein is stabilized. Examples of these negative regulatory elements include, for example, the Keap1 binding domain of the antioxidant transcription factor, Nrf2 which promotes its ubiquitin-mediated proteasomal degradation (Itoh et al., 2003), or nuclear export signals (NESs) such as those found in signal transducers and activators of transcription (STAT) proteins (McBride et al., 2000).

b. Conversely, it may be desirable to repress endogenous signaling as opposed to activating signaling. In this instance, a dominant negative (dn) TF (or POI) would be necessary.

i. Given their modular and well-defined nature, many ecDHFR-DD-dnTFs introduced into cells have the ability to act as repressors of endogenous signaling. In most instances, when a TF homodimerizes or heterodimerizes with other TFs, a dominant negative version can be simply generated by deleting the transactivation domain, but retaining the remainder of the TF, including the DNA binding domain. Such dnTFs will still bind their target DNA, but can no longer recruit RNA polymerase to induce transcription, as in the case of dnNrf2 (Alam et al., 1999).

6. Select an appropriate expression vector for cloning the ecDHFR-DD-POI.

a. After identifying a plan for constructing one's ecDHFR-DD-POI, the next steps will be validation of its basal levels *in vitro*, determining the fold induction of fusion protein after stabilizer addition, and validation of the activity and subcellular localization of the ecDHFR-DD-POI.

⚠ CRITICAL: Since basal abundance of the ecDHFR-DD-POI largely relies on effective proteasomal degradation, it is critical to appropriately control the expression level of the ecDHFR-DD-POI *in vitro* such that the proteasome (or targeting there to) does not become overwhelmed. Regulation of ecDHFR-DD-POI expression levels can be achieved through lowering promoter strength (Qin et al., 2010), or by lowering the amount of DNA transfected/infected transiently.

Note: We have found that strong promoters (e.g., cytomegalovirus [CMV]) used at levels that favor efficient transfection levels leads to high levels of basal expression of the ecDHFR-DD-POI. However, we have noted that similar constructs introduced *in vivo* fare far better than the corresponding *in vitro* experiments would suggest. We recommend introducing one's ecDHFR-DD-POI into a lentivirus vector initially so that one can generate stable cell

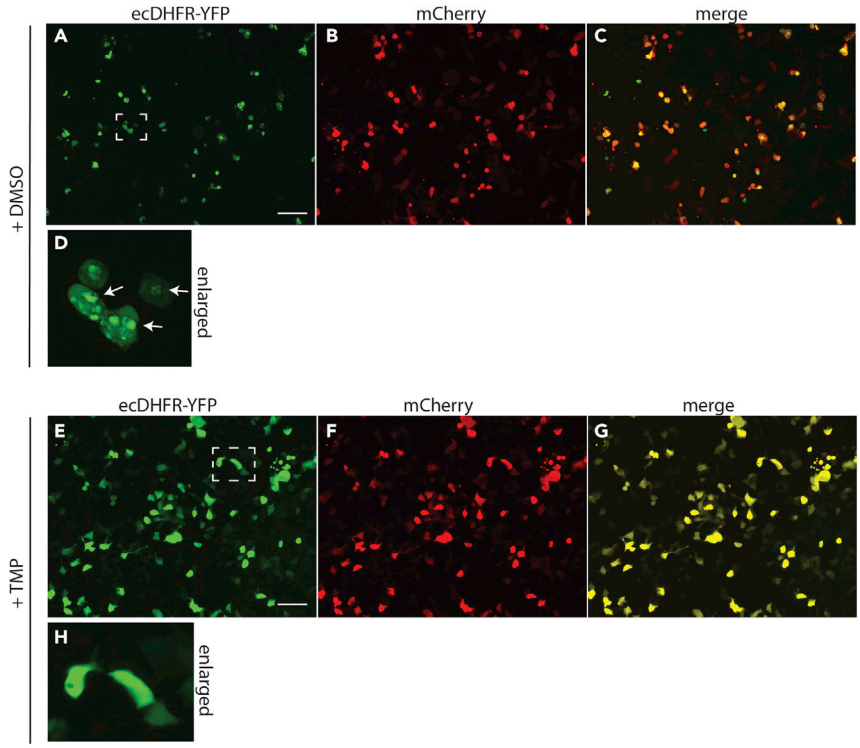

Figure 1. Fluorescence Microscopy of Transiently Transfected Cells with or without TMP Treatment

Example microscopy data from transient transfection of HEK-293-based cells with a construct encoding for ecDHFR-YFP IRES mCherry (500 ng/well of a 24 well using Lipofectamine 3000).

(A–D) Images of DMSO-treated cells. (C) is a merged image of (A) and (B). Note the punctate fluorescent signal originating from non-stabilized ecDHFR-YFP observable in (D). This is due to the elevated amount of DNA used for this particular transient transfection.

(E–H) Images of 1 μM (overnight, 14–18 h) TMP-treated cells. (G) is a merged image of (E) and (F). (H) is an enlarged image of the boxed area in (E). Note the diffuse fluorescent signal originating from TMP-stabilized ecDHFR-YFP. Images were acquired on the Zeiss Axio Observer D1 Fluorescent Microscope (Zeiss, Oberkochen, Germany). Scale bar, 100 μm.

populations that avoid very high levels of expression as typically seen in transient transfection experiments. (see Figures 1 and 2 for example data).

b. Looking ahead, for validation *in vivo*, researchers should design a plan for introducing their ecDHFR-DD-POI into vectors that are more suitable for long term, stable expression, such as the use of an adeno-associated virus (AAV) vectors containing a truncated chicken beta actin promoter (smCBA, (Ramadurgum et al., 2020)), or targeting vectors that could be used for the generation of knock-in mice at the ROSA26 or other loci. The user should also consider the size of their fusion protein, promoter, and reporter genes before choosing an expression vector. As a cautious reminder, AAV can only package a DNA insert that is less than ~4.7 kb inverted terminal repeat (ITR) to ITR, whereas lentivirus can accept DNA cargo up to ~9 kb.

c. Once an appropriate scheme has been developed to generate the user's ecDHFR-DD-POI construct(s), we recommend using conventional cloning techniques such as Gibson Assembly (Gibson et al., 2009) to assemble the final plasmid. This approach allows for the efficient modular assembly of up to 6 different components to generate a seamless plasmid containing said components in virtually any desired orientation.

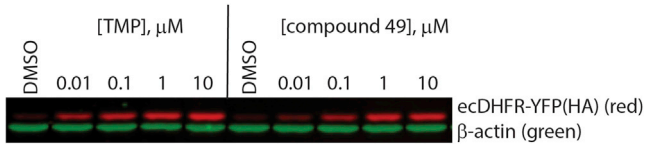

Figure 2. Stabilization of the ecDHFR-DD Is Dose Dependent

Example western blot of ecDHFR-YFP(HA) and β-actin originating from transiently transfected 293A cells (50 ng plasmid/well of a 48 well plate using Lipofectamine 3000) treated with 10 nM to 10 μM TMP or a different stabilizing compound termed "49." Note that TMP begins to stabilize below 10 nM (absent from this blot). Images were acquired using an Odyssey CLx imager (LI-COR, Lincoln, NE) with respective IRDye secondary antibodies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA Tag Mouse Monoclonal Antibody (2-2.2.14)	Invitrogen	Cat# 26183
β-Actin Rabbit Monoclonal Antibody	LI-COR	Cat# 926-42210
IRDye® 680RD Goat anti-Mouse IgG (H + L)	LI-COR	Cat# 926-68070
IRDye® 800CW Goat anti-Rabbit IgG (H + L)	LI-COR	Cat# 926-32211
Bacterial and Virus Strains		
Virus: pLenti CMV Puro R12Y/G67S/Y100I ecDHFR.YFP.HA	Ramadurgum et al, 2020	N/A
Bacteria: RN4220 wild-type S. aureus	Marrafini Laboratory, Rockefeller University	N/A
Bacteria: BW25113 wild-type DHFR *E. coli*	Toprak Laboratory, UT Southwestern	N/A
Chemicals, Peptides, and Recombinant Proteins		
Poly-D lysine	Sigma Aldrich	Cat# A-003-M
Benzonase Nuclease HC, Purity >99%	Sigma Aldrich	Cat# 71206
Halt Protease Inhibitor Cocktail, EDTA free	ThermoFisher Scientific	Cat# 78425
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	Cat# BP231-100
Tween™ 20	Fisher Scientific	Cat# BP337-100
Hanks buffered salt solution (HBSS)	Millipore Sigma	Cat# H6648
Ponceau S solution	Millipore Sigma	Cat# P7170
Trimethoprim (TMP)	Millipore Sigma	Cat# T7883
Gibco DMEM High Glucose	ThermoFisher Scientific	Cat# 11965118
Corning Basal Cell Culture Liquid Media – DMEM and Ham's F-12, 50/50 Mix with L-Glutamine and 15 mM HEPES	Fisher Scientific	Cat# MT10092CM
Fetal Bovine Serum, USDA Certified, Heat Inactivated	Omega Scientific	Cat# FB-02
Gibco Penicillin-Streptomycin-Glutamine (100X)	ThermoFisher Scientific	Cat# 10378-016
Gibco Trypsin-EDTA (0.25%), phenol red	ThermoFisher Scientific	Cat# 25200056
Gibco Opti-MEM	ThermoFisher Scientific	Cat# 31985088
Polybrene	Millipore Sigma	TR-1003-G

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientific	Cat# 23227
Human Dihydrofolate Reductase Assay Kit	Millipore Sigma	Cat# CS0340
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen	Cat# K210010
Maxi Prep Plus Kit	Qiagen	Cat# 12943
Lipofectamine™ 3000 Transfection Reagent	ThermoFisher Scientific	Cat# L3000015
Aurum Total RNA Mini kit	Bio-Rad	Cat# 7326820
qScript cDNA Synthesis Kit	QuantaBio	Cat# 101414
Experimental Models: Cell Lines		
Human: HEK-293T	American Type Culture Collection	Cat# CRL-3216
Human: ARPE-19	American Type Culture Collection	Cat# CRL-2302
ARPE-19 ecDHFR.YFP.HA	Ramadurgum et al, 2020	N/A
Oligonucleotides		
Primer: CMV Forward Sequencing Primer 5′ CCAAGTACGCCCCCTATTGA 3′	Millipore Sigma	N/A
Recombinant DNA		
pLenti CMV Puro R12Y/G67S/Y100I ecDHFR.YFP.HA	Ramadurgum et al, 2020	N/A
psPAX2	Addgene	Plasmid #: 12260
pMD2.G	Addgene	Plasmid #: 12259
pLenti CMV/TO Puro DEST (670-1)	Addgene	Plasmid #: 17293
Other		
Corning Vacuum Filter/Storage Bottle System, 0.22 µm Pore 33.2 cm^2 PES Membrane, Sterile	Corning	Cat# 431097
Corning Costar Flat Bottom 6well Cell Culture Plates	Fisher Scientific	Cat# 07-200-83
Corning Costar 3603 96-well Clear Bottom Black Polystyrene Microplate	Fisher Scientific	Cat# 07-200-565
BD Biocoat Disposable Syringe, Non-Sterile, Luer-Lok, 5 mL,	Cole-Parmer	Cat# EW-07944-06
GE Healthcare Whatman Puradisc 25 mm Syringe Filter: Sterile and Non-Pyrogenic 0.45 µm Polyethersulfone Membrane with Polypropylene Housing	Fisher Scientific	Cat# 6780-2504
15 mL conical tube	Corning	Cat# 430052
Zeiss Axio Observer D1 Fluorescent Microscope	Zeiss	N/A
Odyssey CLx Imager	LI-COR	N/A
Celigo Imaging Cytometer	Nexcelom	N/A
IN Cell Analyzer 6000	GE Healthcare	N/A
IVIS Spectrum	PerkinElmer	N/A
QuantStudio 6	Life Technologies	N/A
Software and Algorithms		
GraphPad Prism	GraphPad Software, Inc	https://www.graphpad.com/scientific-software/prism/
SnapGene	GSL Biotech	https://www.snapgene.com/

MATERIALS AND EQUIPMENT

HEK-293T Culture Media

Reagent	Final Concentration	Amount
DMEM, high glucose	N/A	500 mL
FBS	10%	50 mL
penicillin-streptomycin-glutamine (100X)	1X	5.5 mL

Filter this solution through a 0.22 μm PES membrane (Corning) under sterile conditions.

ARPE-19 Culture Media

Reagent	Final Concentration	Amount
DMEM and Ham's F-12, 50/50 Mix with L-Glutamine and 15 mM HEPES	N/A	500 mL
FBS	10%	50 mL
penicillin-streptomycin-glutamine (100X)	1X	5.5 mL

Filter this solution through a 0.22 μm PES membrane (Corning) under sterile conditions.

STEP-BY-STEP METHOD DETAILS

Lentivirus Production of the ecDHFR-DD-POI Construct

Timing: 3–4 days

Here we detail the steps required for the production of lentivirus encoding for the ecDHFR-DD-POI fusion protein. The virus produced can then be used to infect a cell line of interest and generate a stable cell line expressing the ecDHFR-DD fusion protein. While non-viral methods can be utilized, the use of a stable cell line is ideal for subsequent experiments to ensure reproducibility. While transducing cells with lentivirus often results in a heterogenous polyclonal population due to variations in the number of integration copies of the ecDHFR-DD-POI construct and the genomic context which influences their expression, we have found such cell lines to be sufficient for the purpose of validation experiments. It is up to the user whether they wish to generate single colony clones, which will take much more time.

We recommend the use of HEK-293T cells for their high transfection efficiency and resulting high levels of lentivirus titer. Cells should be low passage (< P10) and cultured with DMEM containing high glucose supplemented with 10% fetal bovine serum (FBS) and 1X penicillin/streptomycin/glutamine (PSQ). Lipofectamine 3000 provides excellent transfection efficiency in these cells.

1. In a 6-well plate, add 1 mL of Poly-D-Lysine and tilt plate to coat the well evenly. Allow the plate to sit at room temperature (20°C–23°C) for 5 min.
2. Trypsinize the HEK-293T cells using 0.25% trypsin-EDTA and perform a cell count using a hemocytometer.
3. Remove the Poly-D-Lysine and wash the well with 1 mL of media. Poly-D-Lysine can be toxic at high concentrations, so it is important to aspirate any that is remaining and unbound. Aspirate the media from the well.

4. Plate 1 × 10^6 HEK-293T cells in a total volume of 1.5 mL into each well. Allow cells to attach overnight (14–18 h). Use one well of a 6 well for each lentivirus construct wished to be produced.
5. For each reaction, combine 125 μL of Opti-MEM medium with 5 μL of P3000 reagent, 1.43 μg of viral packaging DNA (psPAX2 plasmid), and 476 ng of viral envelope DNA (VSV-G is recommended due to its broad tropism, this is included in the pMD2.G plasmid). Finally, add 595 ng of the pLenti plasmid DNA containing the ecDHFR-DD fusion gene (pLenti CMV Puro plasmid, for example, as the vector backbone). Vortex for 10 seconds and incubate at room temperature (20°C–23°C) for 2 min.

   Note: As a positive control, we suggest to use ecDHFR-DD fused to a fluorescent protein such as YFP [https://www.addgene.org/73188/] to gauge transfection/infection efficiency and to also estimate how well the ecDHFR-DD would be turned over/stabilized under idealistic conditions. Additionally, we prefer to use the pLenti CMV Puro backbone because of the rapidity with which stable cells can be generated under puromycin-selection conditions.

   Note: it is highly recommended to use high-quality DNA originating from endotoxin-free midi/maxi preps for each of the above listed DNAs.

   Note: The described transfection is utilizing second-generation lentivirus (three plasmid system). We find this to be a nice compromise between safety and sufficient titers. However, if the user so desires, third-generation lentivirus (four plasmid system) could also be utilized for increased safety and lower risks of replication-competent events.

6. For each reaction, in another tube combine 125 μL of Opti-MEM medium with 7.5 μL of L3000 reagent.
7. Combine the volumes from steps 5 and 6, vortex for 10 seconds, and incubate at room temperature (20°C–23°C) for 5–10 min. During this time, the transfection complex forms. This is your transfection reagent.
8. Gently pipette the 250 μL of transfection reagent into the existing media in the well containing the HEK-293T cells which were seeded the day before. Rock the plate gently to mix.
9. 6 h post transfection, aspirate the existing media and add 2 mL of fresh media. Continue incubation overnight (14–18 h).

   Note: at this point, we consider that the cells are producing lentivirus, so it is important to bleach (1%–2% final concentration, 20 min at room temperature (20°C–23°C)) all waste material and pipette tips that have encountered the media at this point. We routinely use Clorox wipes to disinfect biosafety cabinet working surfaces.

   Note: we also recommend using aerosol barrier tips to prevent contamination of pipettes with lentivirus.

10. 24 h post transfection, the media in the well will contain virus. Collect this media while utilizing the proper safety equipment and technique into a 15 mL conical tube. Store this media at 4°C and add 2 mL of fresh, warm media to the well.
11. 48 h post transfection, collect the media once again and add to the 15 mL conical tube described in step #10. Bleach and discard the 6-well plate.
12. Filter the collected media through a 0.45 μm PES filter and aliquot into single use screw top tubes. We recommend using up to 500 μL per vial. The lentivirus can be used immediately or frozen at −80°C for later use. This is an appropriate pause point, if necessary.

   Note: The collected virus is crude, and viral titer will vary from batch to batch. If one will be producing multiple viruses for comparison, it is best to perform the virus production simultaneously. Nonetheless, crude virus will suffice for the purposes of this protocol. If a better

handle on viral titer is desired, the user can also perform a p24 ELISA or functional titer (e.g., Crystal violet staining) to more accurately determine and normalize titers.

Note: The VSV-G envelope protein will not tolerate freeze-thaws. A >10-fold reduction in titer can be expected if the lentivirus is frozen and thawed. We highly recommend storing it as single use frozen aliquots.

Note: Using a filter pore size of 0.22 μm or less in step #12 will shear the virus and compromise its ability to infect cells.

Generate a Stable Cell Line Expressing the ecDHFR-DD-POI

Timing: 1–2 weeks, 1–2 months if selecting single colony clones

Here we describe the use of crude lentivirus extract to generate a stable cell line expressing the ecDHFR-DD-POI construct. The amount of crude lentivirus media used will vary depending on how easily the target cell line is infected.

13. Plate target cells (e.g., ARPE-19) to be infected with lentivirus at a density of 1×10^5 cells/well of a 12 well (or scale accordingly). Allow to attach overnight (14–18 h).

Note: while ARPE-19 cells tolerate (and even prefer) confluent conditions, one may have to adjust the seeding density according to their cell type of choice.

Note: always include an additional well that will serve as uninfected cells. These cells should die quickly after prolonged incubation with the selective antibiotic, whereas infected cell should survive.

14. Dilute polybrene into full DMEM media to reach a final concentration of 1–10 μg/mL. Make enough media to cover cells in their appropriate dish. For ARPE-19 cells, we use 1 μg/mL polybrene.

Note: the final concentration of polybrene used will depend on the cell line selected for stable cell generation. Prolonged polybrene use can kill cells or cause differentiation (when used on ARPE-19 cells). Other cells (293-based, for example) are remarkably resilient to polybrene.

15. Thaw a single lentivirus aliquot at room temperature (20°C–23°C). For each well to be infected, transfer 50–100 μL into the appropriate amount of polybrene-containing media.
16. Remove old media on cells, add polybrene/lentivirus containing media on top, incubate for 24 h. Alternatively, for suspension cells, spinoculation can be used.
17. After 24 h, change the media and replace with fresh media. Allow cells to recover.
18. After another 24 h (48 h post infection), add media containing the appropriate selective antibiotic. For puromycin selection, we use 1 μg/mL.
19. Replace the media every 2–3 days with new media containing the selective antibiotic. Continue to expand the cells during this period and monitor them under the microscope. Inclusion of selective antibiotic during the passaging can help accelerate the selection process. The resulting population is polyclonal so we recommend continued use of the selective reagent for at least 3–5 passages after initial selection to ensure stable integration. If the ecDHFR-DD-POI is suspected to be toxic, we recommend to continuously include selective antibiotic during all subsequent culturing and passaging, except for experiments.

Note: if there is no apparent cell death 1–2 weeks after antibiotic addition, verify that your cells did not initially contain a selective antibiotic cassette. For example, HEK-293T cells are

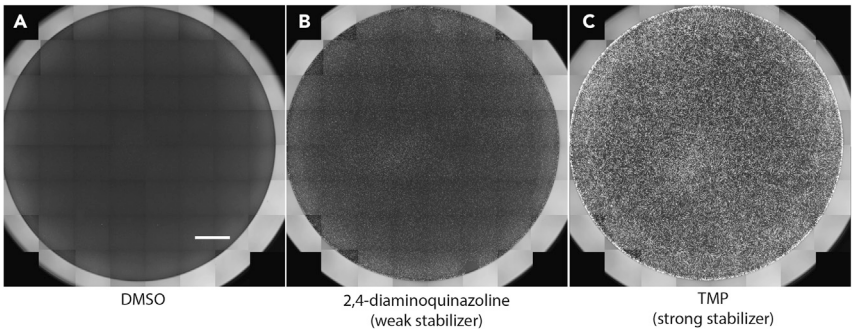

Figure 3. Whole-Well Fluorescence Analysis Provides a Convienent, Sensitive, and Accurate Quantitation Method for ecDHFR-DD Stabilization

Example whole-well microscopy obtained on a Celigo Imaging Cytometer (Nexcelom Bioscience, Lawrence, MA) of stable, lentivirus-infected ARPE-19 ecDHFR-YFP cells treated with either DMSO (A), 2,4-diaminoquinazoline (B), or TMP (C) for 24 h. Note the very low apparent basal fluorescence in (A) compared to Figure 1A. Scale bar, 2 mm.

inherently resistant to G418 after introduction of the large T-cell antigen. Thus, this antibiotic cannot be used for stable cell selection in HEK-293T cells. Alternative explanations for the lack of cell death could be a complete, 100% infection rate, or high-resistance to the selective antibiotic.

Evaluating ecDHFR-DD-POI Basal Abundance and Dynamic Range in Newly Generated Stable Cell Lines

Timing: 3–4 days

Once the stable cell line(s) is/are established, it is important to evaluate initial basal and stabilized expression of the ecDHFR-DD-POI transgene using western blotting and quantitative polymerase chain reaction (qPCR), regardless of the actual desired activity of the construct. This will allow one to evaluate basal expression in the absence of stabilizer and the dynamic range when stabilizer is added. Significant increases in ecDHFR-DD-POI should be observed at the protein level (e.g., western blot) after stabilization, but not at the mRNA (e.g., qPCR) level as the stabilizer works at the protein, not transcript level. Initially we describe how to accomplish this using the canonical ecDHFR ligand, TMP, but later describe the possibility to use alternative stabilizing molecules with potentially complementary functions to the ecDHFR-DD-POI. (see Figures 3 and 4 for data obtained using stable cell lines).

20. Trypsinize or detach the cells from the culture vessel, determine the cell count, and dilute to the preferred seeding density. For ARPE-19 cells, we use a concentration of 1×10^5 cells/well of a 12 well for western blotting and qPCR. Allow cells to attach overnight (14–18 h).

Note: If your ecDHFR-DD-POI has a reporter attached to it, such as a fluorescent protein or a luciferase, it is possible to perform higher-throughput assays (i.e., 96-well plates) after confirming that western blotting and the reporter assay are in accordance.

21. Dissolve TMP in DMSO to a final concentration of 100 mM. Make small aliquots of this stock and store at −20°C, avoid more than 5 freeze-thaw cycles.
22. Using serial dilution, prepare media with compound concentrations ranging from 100 μM down to 1 nM in 10-fold dilutions. This range should capture the full dynamic range of TMP. In addition, prepare a negative control with an equal volume of DMSO as the highest concentration dilution.
23. Treat cells for 24 h.

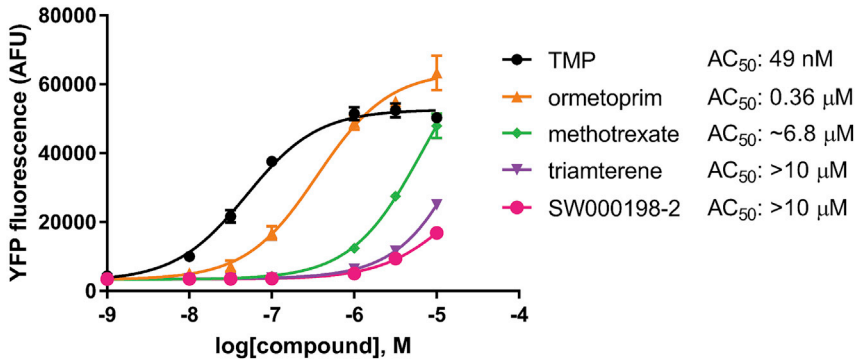

Figure 4. Newly Identified Stabilizers Demonstrate Unique Dose-Response Curves

Example dose-response curve obtained using high content imaging in stable ARPE-19 ecDHFR-YFP cells after treatment with stabilizers of varying potency. Images and fluorescence were measured on the IN Cell Analyzer 6000 (GE Healthcare, Chicago, IL). Data are represented as mean ± SD.

24. For western blotting, harvest cells by washing with Hank's buffered salt solution (HBSS) followed by in-well lysis using radio immunoprecipitation precipitation assay (RIPA) buffer supplemented with benzonase (0.05 - 0.1 U/μL final concentration) and protease inhibitor. Rock multiwell plate for 2 min at room temperature (20°C–23°C), then transfer cell lysate to a microcentrifuge tube. Spin the lysate at 21,100 × *g* at 4°C for 10 min. Transfer the supernatant to a new tube and freeze at −20°C until further use. This is an appropriate pause point, if necessary. When ready, normalize soluble protein content using a bicinchoninic acid (BCA) assay or similar kit. Proceed with conventional western blotting procedures. While antibodies used will depend on the ecDHFR-DD-POI, one can also consider using a polyclonal ecDHFR antibody that works very well for western blotting (custom-made by Pacific Immunology, provided by Prof. Eugene I. Shakhnovich, Harvard University). (see Figure 2 for example western blotting data originating from transiently transfected cells followed by stabilization)

The user should also note that the resulting dose-response curve is sigmoidal when graphed logarithmically, and while the raw values may fluctuate, the half-maximal activity value (AC_{50}) should remain consistent. The curve will eventually flatten at higher concentrations (usually between 1 μM and 10 μM), indicating maximal stabilization of the DD. Based on the individual construct, this may occur at differing concentrations, or it may even lay outside the range of assayed concentrations, indicating a wide dynamic range. A wide dynamic range can indicate efficient and rapid turnover of the DD at lower concentrations. Conversely, a more moderate dynamic range may indicate increased stability at lower concentrations or at basal levels. (see Figure 4 for example data demonstrating these phenomena)

25. For qPCR analysis, wash cells with HBSS followed by trypsinization for 3–5 min at 37°C, neutralize with full media, spin cells at 900 × *g* at 4°C for 5 min, wash with cold HBSS, spin again at 900 × *g* at 4°C for 5 min, aspirate, and freeze pellet at −80°C until further use. This is an appropriate pause point, if necessary. Extract RNA using any number of methods, we use the Aurum Total RNA Mini kit (BioRad), followed by cDNA synthesis using qScript SuperMix (Quantabio). As with western blotting, qPCR primers or TaqMan probes will be dependent on the ecDHFR-DD-POI that is used (see Figure 5 for example qPCR versus reporter signal data +/- TMP in mice).

It is important to confirm that if leaky levels of the ecDHFR-DD-POI are observed, that such levels are not sufficient to activate or repress signaling on their own, in the absence of the small molecule stabilizer.

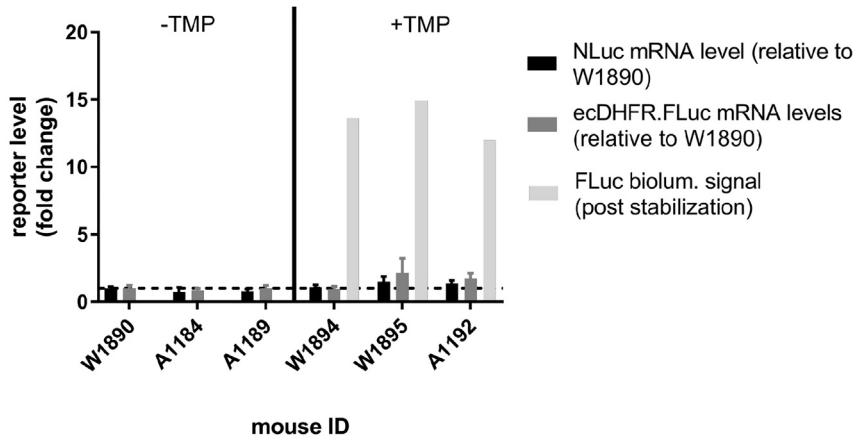

Figure 5. Confirmation of ecDHFR-DD Regulation at the Protein, Not Transcript Level

Example data demonstrating no changes at the mRNA level of NanoLuc (NLuc) or ecDHFR-firefly luciferase (FLuc) after TMP administration, but obvious high levels of luminescent signaling of the ecDHFR-FLuc reporter. Mice were intravitreally injected with adeno-associated virus encoding for a constitutively expressed NLuc and ecDHFR-FLuc. Next, mice were treated with TMP (1 mg via gavage) or remained untreated. Bioluminescence was measured in 3 mice before and after 6 h of TMP treatment on the IVIS Spectrum (PerkinElmer Inc., Waltham, MA). Neural retina were then harvested from all mice and processed for qPCR measuring NLuc and FLuc transcripts. qPCR was performed on a QuantStudio 6 (Life Technologies, Carlsbad, CA). Data are represented as mean ± SD. FLuc signal is a single value.

Evaluating ecDHFR-DD-POI Activity in Newly Generated Stable Cell Lines

Subsequent to evaluation that TMP predictably and effectively elevates ecDHFR-DD-POI abundance, the next step is to confirm that indeed the ecDHFR-DD-POI is active and functions as expected when stabilized. In many cases, one could use the cell lysates or cDNA that were obtained in the previous section to confirm this. Alternatively, one can culture or treat cells with the necessary pharmacologic or genetic perturbations to evaluate their expected activity. For example, previously, we fused ecDHFR-DD to IκBα, an inhibitor of NFκB, a proinflammatory transcription factor (Vu et al., 2017). To evaluate the ecDHFR-DD activity, we stabilized ecDHFR-DD-IκBα and then treated ARPE-19 cells with a pro-inflammatory cytokine, interleukin 1α (IL1α). Subsequently, we evaluated NFκB translocation to the nucleus (something that IκBα would prevent if active) and the expression of downstream cytokines, IL-1β and IL-6 by ELISA and qPCR.

Using an assay specific to the construct, basal levels of the ecDHFR-DD-POI should be assessed to verify that under untreated conditions, the abundance of the fusion protein is negligible. Such assays may include fluorescent or luminescent reporter assays, translocation assays, morphometric assays, ELISAs, western blots, or qPCR. Dose-response curves are recommended. The appropriate timing of stabilization will have to be determined experimentally and will depend on several factors including unknown repressive signaling pathways, xenobiotic removal pathways, and endogenous feedback loops.

Using Non-TMP ecDHFR-DD Pharmacological Stabilizers – the Potential for Synergistic Signaling Regulation

Timing: up to 4–5 days

While TMP is the canonical stabilizer for the ecDHFR-DD, we have found that non-antibiotic TMP derivatives (Peng et al., 2019) or other compounds containing the 2,4-diaminopyrimidine (or triazine) ring with an aryl group (Ramadurgum et al., 2020) can also promote ecDHFR-DD-POI abundance, and thus be used to conditionally control cellular signaling. Interestingly, the chemical space that can stabilize the ecDHFR-DD appears to be rather vast. This opens up the possibility of using a

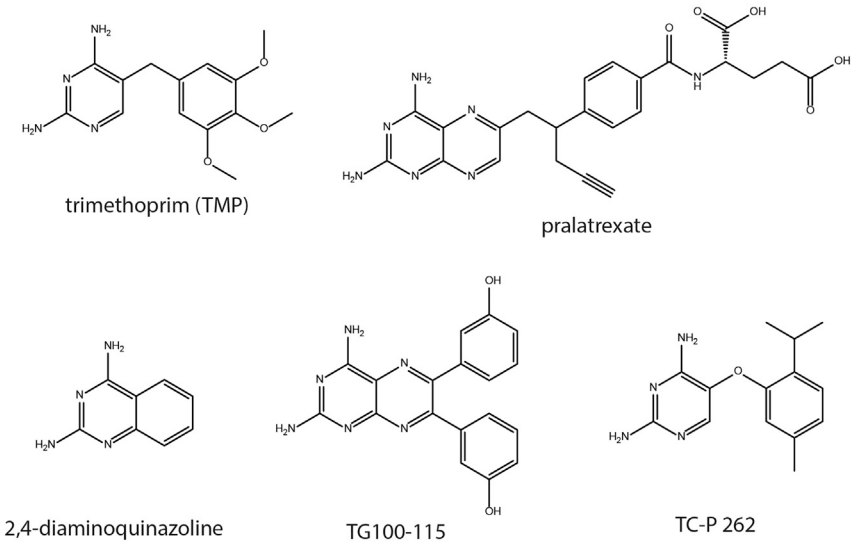

Figure 6. Examples of Validated Potential Stabilizers that Contain the Minimum 2,4-diaminopyrimidine/Aryl Structure for ecDHFR-DD Stabilization

non-TMP small molecule to simultaneously stabilize the ecDHFR-DD, but also act on another endogenous supporting pathway to achieve a potentially synergistic response. Based on our work thus far, such endogenous pathways include, but are not limited to cFMS kinase inhibition, PI3Kγ/δ inhibition, $P2X_3$ and $P2X_{2/3}$, ENaC, and pharmacological chaperoning of other proteins.

26. As a starting point, one can begin using already validated compounds described in (Ramadurgum et al., 2020), or they could source from the list of virtually identified compounds (Table S1 in Ramadurgum et al., 2020) most of which were not validated. (see Figure 6 for example stabilizers)

27. Proceed with performing dose-response curves and downstream evaluation as described above in "Evaluating ecDHFR-DD-POI Basal Abundance and Dynamic Range in Newly Generated Stable Cell Lines".

Note: Even in DMSO, some of the 2,4-diaminopyrimidine compounds are poorly soluble and must be warmed at 37°C to dissolve completely. Additionally, it is possible that DMSO solubility may max out at 500 μM, unlike TMP, which is soluble >100 mM in DMSO. Nonetheless, it is important to try minimize the final DMSO concentration and keep it below 0.5% to reduce off-target disturbances of the cells.

Note: TMP and its derivatives for the most part have limited water solubility (~0.2 mg/mL for TMP [~690 μM]). Nonetheless, this amount of soluble TMP is certainly sufficient for basic *in vitro* studies as well as for *in vivo* regulation as long as sufficient amounts of this solution is used/imbibed (Datta et al., 2018). Additional solubility can be achieved through pH reduction and generation of salt forms of 2,4-diaminopyrimidine-containing compounds or TMP. For example, TMP sulfate found in POLYTRIM® eye drops is soluble to at least 1 mg/mL at pH 4.0–6.2.

Note: The virtual screen that we performed searched for 2,4-diaminopyrimidine-containing molecules, but did not specifically screen for 2,4-diaminotriazine molecules, which would further expand the available set of molecules for ecDHFR DD stabilization.

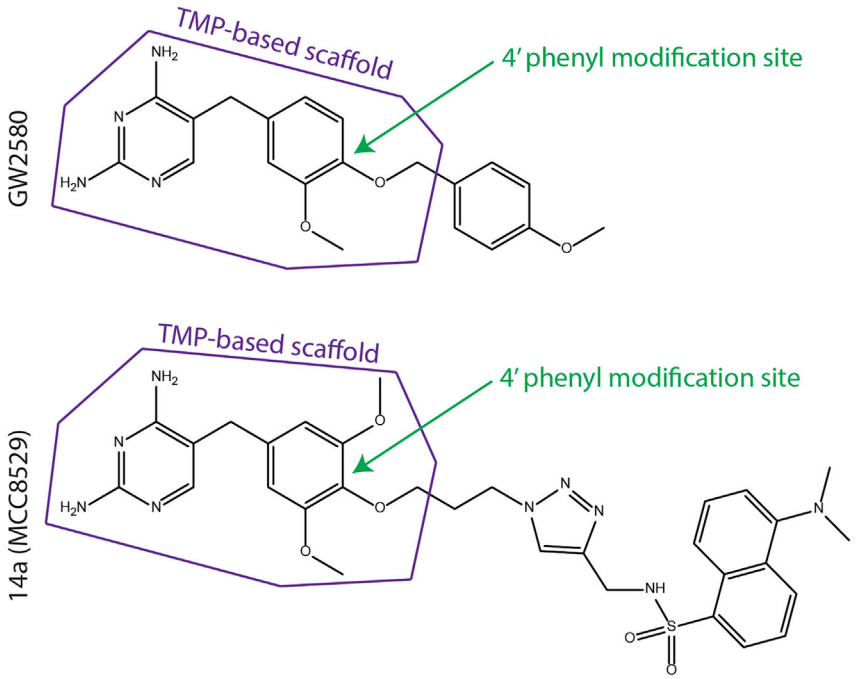

Figure 7. The 4′ Phenyl Position of the TMP Scaffold is a Well-Tolerated Modification Site for Custom ecDHFR-DD Stabilizers

Example validated ecDHFR-DD stabilizers with a TMP scaffold, but 4′ phenyl modifications that are apparently well tolerated in the ecDHFR structure, which also renders the new compounds inactive (or inefficient) antibiotics.

28. If the user is unable to find an existing molecule, they could rationally design and synthesize one. Theoretically, part of another molecule can be fused to TMP at the 4′ phenyl position without compromising its stabilizing activity to a high degree (see Figure 7 for example stabilizing compounds with modification at the 4′ phenyl site).

Evaluation and Reduction of Stabilizer Off-Target Activity

When using small molecule stabilizers (including TMP), it is important to note potential adverse side effects due to the small molecule. As a compound that binds tightly to ecDHFR, TMP is an effective antibiotic. Similarly, many TMP analogs are also antibiotics. If the desire is to use one's ecDHFR-DD-POI *in vivo*, we feel that it is sub-optimal to use a compound that could potentially disrupt the brain-gut axis (Carabotti et al., 2015) through disruption of gut microbiome. Furthermore, if thinking very long term, towards potential translation in humans, development of antibiotic resistance is also a significant concern. Surprisingly, though, subtle modifications of the base TMP structure can compromise its antibiotic properties while not appreciably changing its ability to regulate an ecDHFR-DD-POI.

Additionally, TMP is a highly specific compound with a >10,000 fold affinity for ecDHFR versus human DHFR (hsDHFR). Modifications to the TMP structure lead to the likelihood that the compound could become more promiscuous and target other DHFRs, which is undesirable. Specifically, hsDHFR is involved in *de novo* synthesis of nucleotides, meaning that hsDHFR inhibitors are likely to be toxic to highly proliferative cells that rely heavily on such pathways , such as immune cells or cancer cells (Villa et al., 2019). To test whether newly identified stabilizers inhibit hsDHFR, a simple commercially-available assay kit (Sigma) can be used.

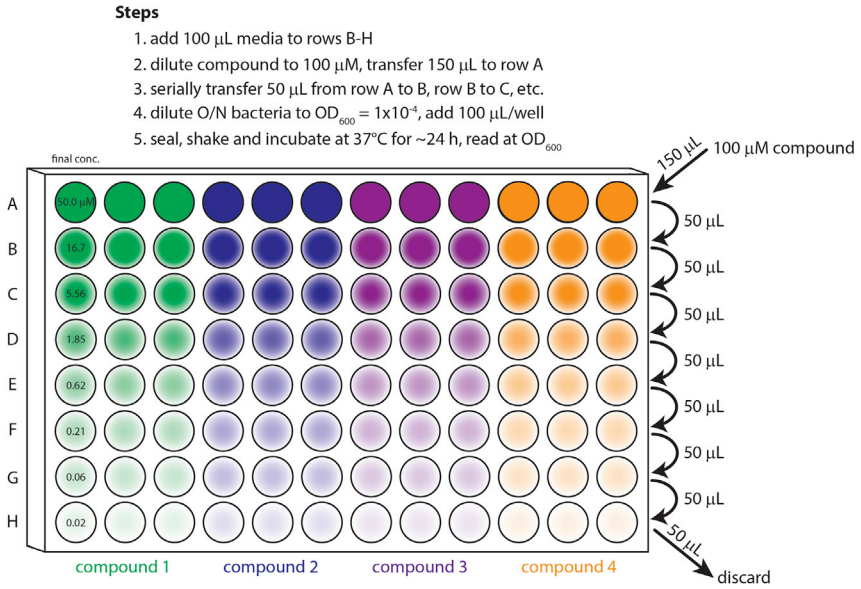

Figure 8. Layout and General Procedure for Performing 96 Well Bacterial Growth Assays

Bacterial Growth Assay

Timing: 48 h

To rule out antibiotic activity of a newly identified small molecule, we recommend testing both gram-positive (e.g., *S. aureus*) and gram-negative (e.g., *E. coli*) bacteria, incubating wild-type bacterial cultures with the small molecule compounds for 24 h. In the interest of preventing cross-contamination, the user should perform separate bacterial growth assays for the gram-positive and gram-negative strains. (see Figure 8 for an example plate layout and basic protocol).

29. Grow wild-type (WT) *E. coli* cultures (e.g., BW25113 or MG1655) overnight (14–18 h) in 25 mL M9 minimal media (supplemented with 0.4% [w/v] glucose and 0.2% [w/v] amicase) at 37°C. If using gram-positive bacteria, we recommend WT RN4220 *S. aureus* grown overnight (14–18 h) in Luria-Bertani (LB) broth at 37°C.

Note: We have found that growth inhibition curves of TMP in *S.* aureus in LB seem to be shifted towards less growth inhibition than expected, possibly because of the levels of thymidine in LB. To avoid this, one could alternatively use brain-heart-infusion (BHI) broth.

30. Measure the optical density (OD_{600}) of the overnight culture and dilute it to an OD = 1×10^{-4} in M9 minimal media or LB broth, depending on the culture.

Note: Do not dilute less than 20 µL into the corresponding media. Pipetting such small amounts of cultures can result in inconsistent dilutions and uneven growth.

31. To fully evaluate bacterial growth under all possible conditions, growth must be assessed with the positive control ligand TMP, the small molecule of interest, DMSO, and only media. In this respect, the user is able to confirm the antibiotic activity of TMP, potential antibiotic activity from the small molecule of interest, potential toxicity from DMSO, and growth under ideal conditions in plain media. Furthermore, prepare media without bacteria to function as a blank when comparing OD. To fully use the 96-well plate, we suggest testing the small molecule across 8

dilutions in triplicate (24 wells total). In this regard, the user can conveniently dilute vertically in serial dilutions. Four different compounds can be evaluated within a single 96 well plate (see Figure 8 for an example plate layout).

32. In the experimental wells, add 100 μL of minimal media or LB media to each row except the very first within the set of 8 dilutions. This is to prepare the wells for the serial dilutions described in step 34.
33. Prepare stock concentrations of compounds (10 mM or 100 mM in DMSO).
34. Prepare 600 μL of 100 μM of TMP and any other stabilizing compounds in M9 or LB media. Make an equivalent dilution of DMSO to serve as a vehicle control. Using a 12 well multichannel pipette, prepare dilutions by adding 150 μL of 100 μM compound to the first well (which was purposely left empty), mix, and take 50 μL to add to the next well (a 3-fold dilution). Mix the second well and continue to transfer 50 μL across the remaining wells in a similar pattern. Discard the last 50 μL originating from the last dilution well. Add 100 μL of media containing diluted bacteria to all wells (except for media only wells). The first well should have a concentration of 50 μM and the last should be 0.02 μM as the final concentration.

⚠ CRITICAL: DMSO is also serially diluted out in its own wells in a similar way to compound dilution. This is important to assess whether DMSO itself may have adverse effects on bacterial growth.

35. Seal plates with an acetate sticker or similar cover, incubate at 37°C at 200–250 rpm for 24 h.
36. After 24 h, measure the OD_{600}. We perform this assay as an endpoint reading. Alternatively, one can also perform a kinetic read, if desired, and if they have a plate reader with such capabilities.

⚠ CRITICAL: All handling and experimentation during the bacterial growth assay should be under sterile conditions using a flame.

Determining Dynamic Stabilization and Destabilization Effects of New Compounds

Timing: 48 h

Up to this point, we have mainly suggested the use of a stabilizer in a dose-response curve at a (somewhat) arbitrary timepoint of 24–48 h (since that is when maximal stabilization occurs). However, one unique aspect of the destabilizing domain is the ability to turn "off" the strategy simply by removing (and washing out) the small molecule stabilizer. Different stabilizing compounds will vary in terms of their kinetics of stabilization and destabilization once removed. For example, we found that TMP analogs ormetoprim and diaverdine were able to stabilize to similar degrees at 10 μM after 24 h, but once removed, ecDHFR-YFP fluorescence reduced more quickly to baseline (Ramadurgum et al., 2020) than with TMP. Thus, such a compound could turn "on" with similar kinetics, but once removed, would result in quicker "off" rates of signaling. Conversely, compounds such as methotrexate stabilized and maintained ecDHFR-DD abundance well after removal, allowing for sustained regulation of abundance (Ramadurgum et al., 2020). If new compounds are used for stabilization of the ecDHFR-DD-POI, we recommend testing their ability to dynamically stabilize and destabilize. The easiest method to evaluate the kinetics of a stabilizer is through a fluorescent or luminescent reporter gene. If this is not available to the user, we recommend performing an ELISA or possibly dot blot to directly measure fusion protein abundance at multiple concentrations and timepoints in a multiwell format.

37. Trypsinize or detach the stable cells from the culture vessel, determine the cell count, and dilute to the preferred seeding density.
38. Plate cells at 1×10^4 cells/well in a 96-well plate and allow to attach overnight (14–18 h).

39. The next day, dissolve the small molecule compound in the chosen solvent at a concentration of 100 mM. We recommend DMSO as it can dissolve a variety of compounds - both polar and nonpolar.

Note: Some 2,4-diaminopyrimidine-containing compounds must be warmed to dissolve completely. In addition, certain compounds may not dissolve at concentrations of 100 mM, 10 mM, or even 1 mM.

40. Using serial dilution, prepare media with compound concentrations ranging from 100 μM to 1 nM using 10-fold dilutions. Make sure to resuspend each dilution to achieve the most consistent results. This range can usually capture the dynamic range of a compound. In addition, prepare a negative control with an equal volume of DMSO as the highest concentration of dilution.

Note: We recommend performing dose-response in triplicate with at least 3–6 wells for the DMSO negative control.

41. Aspirate the old media from the wells and add 100 μL of compound-containing media to the wells. This timepoint is considered t = 0 h.
42. The user can harvest the cells at select timepoints, or if a fluorescent or non-lytic luminescent reporter is used, one can measure in well until a plateau has been reached (typically 24 h). We recommend 2, 4, 6, 8 and 24 h "on" timepoints.
43. Afterwards, remove the treated media, wash the cells with an isotonic buffer such as HBSS. We recommend either washing twice with 100 μL or washing once with a larger volume such as 200–300 μL.
44. At this point, the user can measure stabilization at various timepoints until the final timepoint (typically 48 h). Again, if possible, we recommend using 2, 4, 6, 8, 24, and 48 h "off" timepoints.

EXPECTED OUTCOMES

Figures 1, 2, 3, 4, and 5 provide expected outcomes of this protocol.

Transient transfections can be used initially to estimate the basal levels and activity of a newly generated DD-POI. In cases where too much DNA is used (Figures 1A and 1D), excessive background of the DD-POI can be observed as obvious punctate aggregates, which become stabilized and diffuse when stabilizer is added (Figures 1E and1H). Transiently transfected cells (this time transfected with much lower amounts of DNA) can also be used to visualize DD-POI stabilization using dose-response curves (Figure 2) and to test newly generated potential stabilizers.

Upon generation of a stable cell line, the cells may be imaged for fluorescence (Figures 3 and 4). The resulting values can be used to construct a dose-response curve in order to compare stabilizing molecules to each other and to the negative control (Figure 4). Validation experiments should be performed to demonstrate, for example, that DD-POI mRNA levels do not change with stabilizer addition, demonstrating that regulation is at the protein level (Figure 5).

TMP analogues such as the ones shown in Figure 6 may also prove useful to the user as either an additional control or an endogenously active small molecule. However, the user also has the option to modify TMP at the 4′ phenyl position to reduce antibiotic activity or modify its properties without compromising stabilizing ability (Figure 7).

LIMITATIONS

While broadly applicable, the ecDHFR-DD system is not going to work for absolutely every POI. Some proteins will not tolerate tagging at the N- or C- terminus. Other POIs may be just too small to be tagged with a 153 residue ecDHFR-DD. Yet others might be trafficked to the extracellular space or other proteasome-inaccessible subcellular contexts. Another limitation of the ecDHFR-DD system is that it relies on an active proteasome. Thus, under conditions where the proteasome is not active, or it compromised, there is the possibility that the ecDHFR-DD may accumulate at higher basal levels than desired, affecting the dynamic range of the system and basal signaling.

TROUBLESHOOTING

In the described experiments, issues may arise due to mishandling of materials or contamination of reagents. Please ensure that all reagents and materials are stored according to instructions, are handled properly, and are freshly aliquoted to minimize contamination. Use of calibrated pipettes is strongly recommended as is the use of aerosol barrier tips wherever possible.

Problem 1: Increased Basal Signal from the ecDHFR-DD Fusion Protein

Increased basal levels may be indicative of insufficient degradation by the proteasome (which could be cell type dependent), increased fusion protein stability due to the ecDHFR-DD, or too high levels of expression.

Potential Solution 1

Ways to overcome this problem include making knock-in cell lines that use an endogenous, typically less strong promoter, using lower levels of virus for infection, or re-engineering the position of the DD.

Potential Solution 2

Be sure to explore placing the ecDHFR-DD on different termini (or both) to identify an ideal construct.

Problem 2: Low Stabilized Signal from the ecDHFR-DD Fusion Protein

After evaluating ecDHFR-DD-POI levels post stabilizer treatment, the user may notice these values are too low, even with the canonical stabilizer, TMP. This could be indicative of poor initial transduction efficiency which may be a result of low viral titer. Low levels of the stabilized fusion protein may also suggest poor fusion protein stability, or alternative means of protein degradation (i.e., lysosomal).

Potential Solution 1

We recommend performing an ELISA or functional titer to establish viral titer. If the titer is low, the user will need to use larger quantities when producing the stable cell line, or re-perform the lentivirus production.

Potential Solution 2

We recommend utilizing a control fusion protein control to assess whether the fusion protein itself is the cause of the low signal. One such fusion protein is the ecDHFR-YFP utilized in (Ramadurgum et al., 2020) (pLenti CMV Puro R12Y/G67S/Y100I ecDHFR-YFP-HA).

Potential Solution 3

Inclusion of degradation inhibitors as additional controls would be warranted in this instance. Use of MG-132 should significantly increase ecDHFR-DD-POI levels. Additionally, use of lysosomal neutralizing agents (ammonium chloride) or autophagy inhibitors (3-methyladenine) could be used to narrow the potential mechanism of protein degradation if MG-132 or TMP do not elevate ecDHFR-DD-POI levels.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John D. Hulleman (john.hulleman@utsouthwestern.edu)

Materials Availability

Plasmids used in this study are either available from the Lead Contact with a completed Materials Transfer Agreement, or can be purchased through Addgene (details listed in the Key Resources Table).

Data and Code Availability

The original study by (Ramadurgum et al., 2020) generated a unique molecule dataset of potential DD stabilizing compounds, which are available in the Supplementary Information of the referenced publication.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.D.H.; Writing – Original Draft, P.R. and J.D.H.; Writing – Review & Editing, P.R. and J.D.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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