dTAG plasmids for rapid target protein degradation

Probing complex biological systems requires target-specific control of protein function or abundance. To enable the rapid and selective control of single protein abundance, we created a universally applicable system that uses cell-permeable small molecule degraders to induce proteasomal degradation of allele-specific protein chimeras within minutes (Nabet et al., *Nature Chemical Biology* 2018). The degradation tag (dTAG) system pairs a novel allele-specific degrader of FKBP12^{F36V} with expression of FKBP12^{F36V} in-frame with a target protein of interest. By transgene expression or CRISPR-mediated locus-specific knock-in, the dTAG system is a highly selective and generalizable strategy to study the immediate consequences of target protein degradation.

In this protocol, we describe how to modify vectors in the dTAG arsenal to N- or C-terminally tag any target of interest in order to enable exogenous protein expression using our lentiviral expression platform or endogenous CRISPR-mediated knock-in using a modified PITCh vector system. Please refer to Sakuma et al., *Nature Protocols* 2016 for a detailed description of the original PITCh vector system, which employs microhomology-mediated end joining (MMEJ)-based CRISPR knock-in.

Note on dTAG molecules: Please address your requests for dTAG molecules to Nathanael S. Gray (nathanael_gray@dfci.harvard.edu) or James E. Bradner (james.bradner@novartis.com). We will post an update with the vendor and catalog number once dTAG molecules become commercially available.

Step 1a: Exogenous lentiviral expression.

If the dTAG system has not been evaluated for your target of interest or cell line of interest, we recommend starting with the lentiviral expression platform. For each target of interest, it is important to determine which terminus can accommodate the FKBP12^{F36V} tag without disrupting protein function. A literature search can help determine which terminus is favorable for tagging. However, if it is unclear which terminus should be tagged, the dTAG lentiviral plasmids can serve as a rapid means of comparing functionality of N- and C-terminal tagged fusions and efficiency of degradation in the cell line of interest.

Lentiviral gateway cloning-compatible vectors for exogenous expression of tagged targets are available: pLEX_305-N-dTAG (Addgene #91797) and pLEX_305-C-dTAG (Addgene #91798). Each plasmid includes two tandem HA-tags for immunodetection of gene products and a puromycin selectable marker. Please refer to the Methods section of Nabet et al., *Nature Chemical Biology* 2018 for details on cloning of targets into these plasmids. Note that when designing plasmids for Gateway recombination cloning into pLEX_305-N-dTAG, a stop codon must be included at the end of the gene to prevent read-through into the recombination sequences.

In addition, CRISPR/Cas9 gene-editing can be employed to inactivate the endogenous gene in cell lines expressing the FKBP12^{F36V} tagged target, which can complement knock-in studies. Please refer to the Methods section of Erb et al. *Nature* 2017 and Huang et al. *eLife* 2017 for examples of sgRNA design and protocols employed to inactivate endogenous *ENL* or *MELK* in cell lines expressing FKBP12^{F36V}-tagged ENL or MELK, respectively.

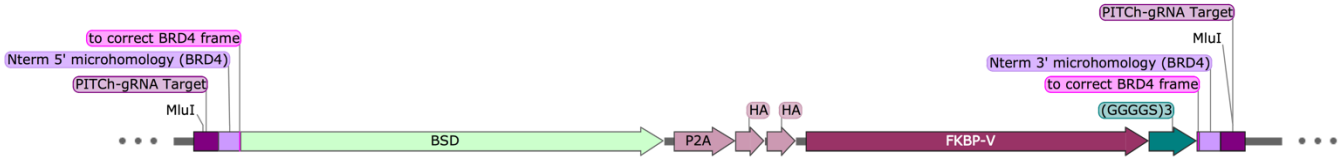
Step 1b: Selecting a PITCh dTAG plasmid for CRISPR-mediated knock-in.

The rest of the protocol will focus on employing CRISPR-mediated knock-in approaches to tag an endogenous locus. After choosing a terminus to knock-in the tag (**Step 1a**), choose a PITCh dTAG vector with your preferred selectable marker (puromycin or blasticidin resistance). While we have not evaluated

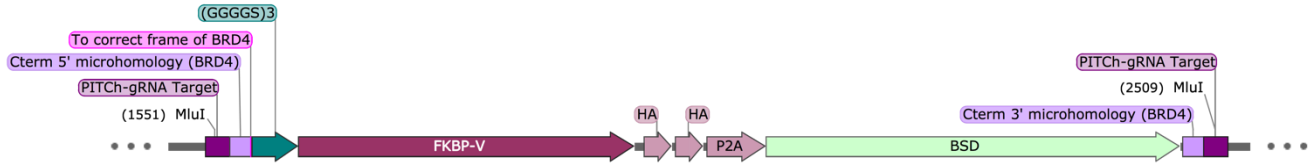
co-transfection of puromycin and blasticidin vectors together, using a dual selection strategy may increase the efficiency of identification of homozygous tagged clones.

Annotated GenBank files are available for each plasmid as noted below:

pCRIS-PITChv2-BSD-dTAG (BRD4) – Tags the N-terminus and contains a blasticidin resistance cassette (Addgene #91792)

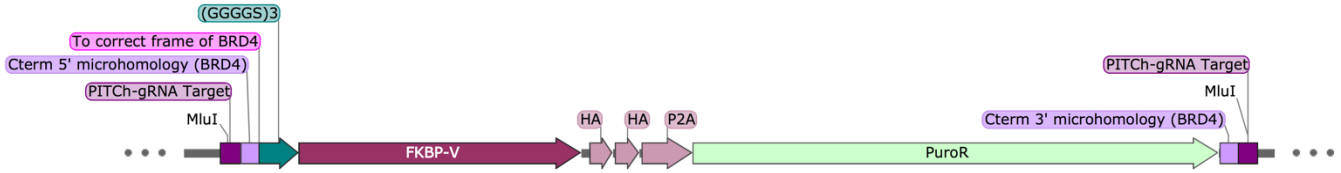


pCRIS-PITChv2-dTAG-BSD^{R} (BRD4) – Tags the C-terminus and contains a blasticidin resistance cassette (Addgene #91795)



pCRIS-PITChv2-Puro-dTAG (BRD4) – Tags the N-terminus and contains a puromycin resistance cassette (Addgene #91793)

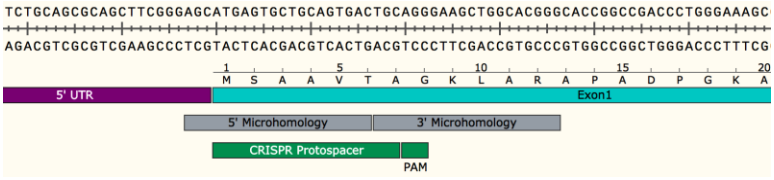
pCRIS-PITChv2-dTAG-Puro^{R} (BRD4) – Tags the C-terminus and contains a puromycin resistance cassette (Addgene #91796)



Step 2: Choosing a target sgRNA.

Design an sgRNA to target the N-terminus (5' UTR + first exon) or the C-terminus (last exon + 3' UTR) using computational methods or online servers (eg: crispr.mit.edu). Select an sgRNA that is as close as possible to the start (for N-terminal tags) or stop (for C-terminal tags) codons and has limited off-target effects. Cutting efficiency of selected sgRNAs can be evaluated prior to attempting knock-in experiments using surveyor nuclease or TIDE assays.

In this protocol, we will use tagging of a serine/threonine-protein kinase, PLK1, as an example. While we have not evaluated knock-in of FKBP12^{F36V} at the *PLK1* locus, an example of an sgRNA targeting the first exon for N-terminal tagging of PLK1 is shown below:



Step 3: Annotate your guide.

For SpCas9, the cut site is 3 bp upstream of the PAM motif. As such, the 5' microhomology and 3' microhomology regions will be the 20 bp immediately up and downstream of the cut site. Note that the cut site is 2 bp into the 6th codon and will need to be corrected when designing PCR primers for the dTAG cassette to prevent a frame shift in your tagged protein product.

Step 4: Digest and purify the backbone.

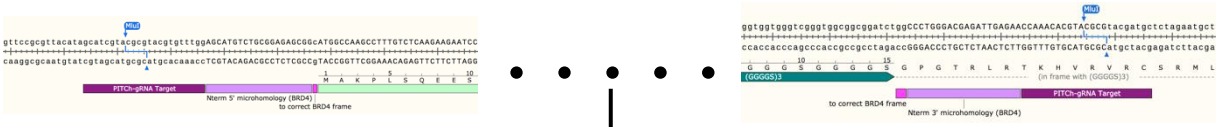
In the constructs listed above, MluI sites flank the dTAG cassette. These MluI sites will be used to cut out the cassette to facilitate Gibson assembly in **Step 7**. Digest the appropriate vector (2 μg) with MluI, run the digest on a gel, and purify the backbone band with a DNA gel extraction kit.

Step 5a: Design primers to incorporate new microhomology sequences.

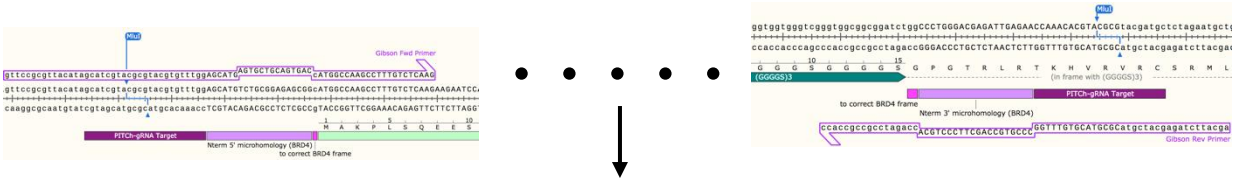
New microhomology sequences for your gene of interest were defined in **Step 3**. In the PLK1 example below, the bases that were used for BRD4 to correct the frame (in hot pink) in the original vector will be kept, since the sgRNA for PLK1 also cuts 2 bp into a codon, rather than between codons. Note that in order to clone into the MluI digested backbone using Gibson assembly, overlapping regions will need to extend ~20 bp beyond the MluI sites. Also, since Gibson assembly uses 5' exonucleases, sticky ends cannot be included as part of the Gibson assembly overhangs, as they will be cut back by the exonuclease. Employing primer designs such as those outlined for PLK1 will allow replacement of microhomology sequences targeting your gene of interest. Remember to correct for frame differences if necessary and refer to **Step 5b** to overcome a limitation with MMEJ.

5’ primer **3’ primer**

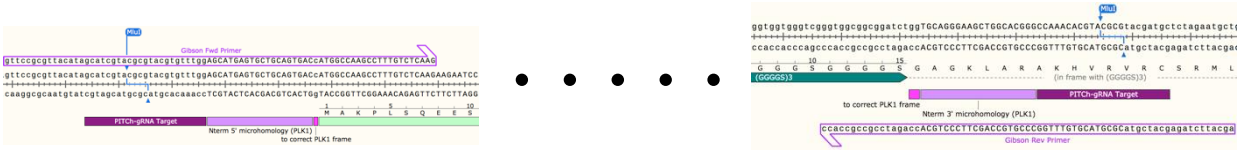
Original dTAG cassette with BRD4 microhomology sequences



PLK1 primer including new microhomology sequences

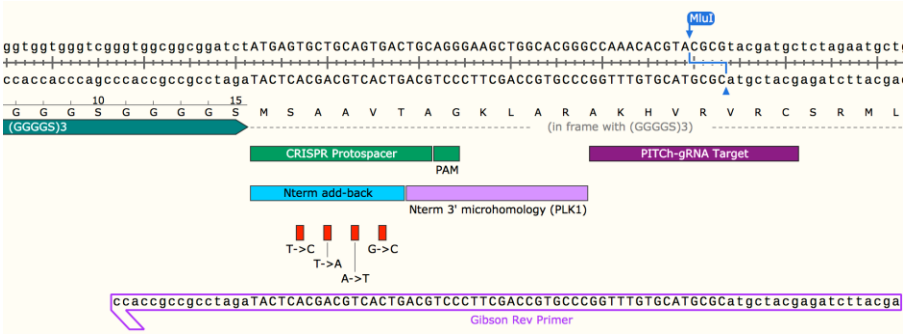


New PLK1 knock-in dTAG cassette after PCR



Step 5b: Recommended method to addback bases in primer design.

While MMEJ is a convenient way to perform CRISPR knock-in, a limitation is that a few bases of the target sequence can be removed based on the sgRNA cut site, potentially truncating the ends the tagged fusion protein. Steps that can be taken in cassette design to overcome this limitation. See below for the PLK1 example:



In the PLK1 example, we need to addback codons 1-6, which were separated from the rest of the gene due to the location of the sgRNA cut site. A longer reverse primer can be designed, which now includes these bases. While the longer PCR reverse primer may make the PCR more difficult, extending the sequence of the forward primer may help improve the PCR reaction.

Note that when adding back the sequence, the sgRNA binding site is likely recreated, and needs to be modified to avoid cutting of the cassette. The most efficient way to overcome this issue is to incorporate a silent mutation into the PAM so that the sgRNA no longer binds. In the PLK1 example, there are no possible substitutions that disrupt the PAM motif and retain glycine in position 8. In this case, two designs can be employed, and we would recommend testing both. As illustrated above, four silent mutations were made in the primer sequence to prevent binding of the sgRNA. An alternative would also be to remove the start codon (ATG), rather than make the indicated silent mutations.

Step 6: Cassette PCR.

Using primers designed in **Step 5a** or **5b** (**Step 5b** is recommended), perform a PCR reaction using the uncut PITCh dTAG vector. To purify the PCR product, run the PCR product on a gel, confirm PCR product size, and purify the PCR product with either a spin column or DNA gel extraction kit.

Step 7: Gibson Assembly.

Perform a Gibson assembly reaction (NEBuilder is recommended) using the purified PCR product and cut backbone from **Step 4**. Transform the assembly reaction using appropriate bacteria (STBL3 are recommended), grow up using Ampicillin selection, and purify the plasmid. Sequence the plasmid to confirm incorporation of new microhomology sequences:

Forward primer: TCGCCCTTAATTGTGAGCGGA
Reverse primer: GAAAGGACAGTGGGAGTGGCA

*Alternatively, traditional restriction enzyme cloning using MluI can be used, if preferred over Gibson assembly.

Step 8: Cas9/sgRNA vector construction.

Sakuma et al., *Nature Protocols* 2016 provides a detailed description on how to construct a plasmid containing the PITCh sgRNA, target-specific sgRNA, and Cas9 using pX330A-1x2 (Addgene #58766) and pX330S-2-PITCh (Addgene #63670). Please refer to the Methods section of Nabet et al., *Nature Chemical Biology* 2018 for details on generation of pX330A-nBRD4/PITCh (Addgene #91794), which contains the PITCh sgRNA, BRD4-specific sgRNA, and Cas9.

Step 9: Cell line construction.

Please refer to the Methods section of Nabet et al., *Nature Chemical Biology* 2018 for details on generation of BRD4 knock-in cell lines.

Alternative to the PITCh approach for achieving locus-specific knock-in.

In addition to MMEJ-based CRISPR knock-in, we recently employed homology-directed repair and dual fluorescent screening (mCherry and BFP) to knock-in $FKBP12^{F36V}$ at the *YY1* locus in collaboration with Richard Young's lab at the Whitehead Institute (Weintraub et al., *Cell* 2017). C-terminal targeting plasmids pAW62.YY1.FKBP.knock-in.mCherry (Addgene #104370) and pAW63.YY1.FKBP.knock-in.BFP (Addgene #104371) can be modified and employed for this approach, as outlined in the Methods sections of Weintraub et al., *Cell* 2017**.**

Citation: Please reference the following publication for the use of the materials described here:

The dTAG system for immediate and target-specific protein degradation. Nabet B, Roberts JM, Buckley DL, Paulk J, Dastjerdi S, Yang A, Leggett AL, Erb MA, Lawlor MA, Souza A, Scott TG, Vittori S, Perry JA, Qi J, Winter GE, Wong KK, Gray NS & Bradner JE. *Nature Chemical Biology (2018)* 10.1038/s41589-018-0021-8