The PITCh dTAG vectors for rapid protein degradation of endogenous targets

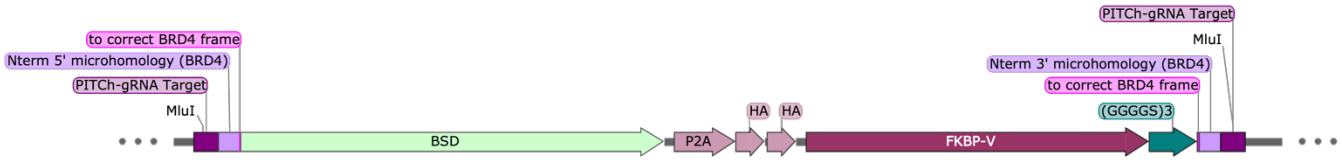
The dissection of complex biological systems requires target-specific control of protein function or abundance. Genetic perturbations have markedly advanced science but are variably limited by off-target effects, multi-component complexity and irreversibility. Most limiting to the study of fast biology is the requisite delay from modulation to experimental measurement. To enable the rapid and selective control of single protein abundance, our group created a chemical biology system that leverages the potency of cell-permeable heterobifunctional chemical degraders. Prior research demonstrated selective degradation of BET bromodomain transcriptional co-activators (BRD2, BRD3, BRD4) using heterobifunctional small molecules that bridge BET bromodomains to an E3 ubiquitin ligase, cereblon (Winter*, Buckley*, et al., Science 2015). Catalytic-like biochemical activity of these small molecules allows rapid and target-specific turnover without degradation of the ligand. Use of E3-binding heterobifunctional small molecules has now been validated for several other targets. Chemical probes established by this approach powerfully enable the study of target biology, but each requires the up-front identification of a target-selective ligand. We therefore created a single, generalizable approach to rapidly degrade allele-specific protein chimeras for biological investigation and early target validation (Nabet*, Roberts*, Buckley*, et al., Nat. Chem. Biol. 2017).

Our 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 generalizable strategy to study the immediate consequent biology of protein loss. In our study, we modified the PITCh vector system to tag the N-terminus of BRD4 using microhomology-mediated end joining (MMEJ)**. This protocol describes how to design and modify vectors in the PITCh dTAG arsenal to tag any target of interest.** Please refer to Sakuma T, et al., Nat. Protoc. 2016 for a description of the original PITCh vector system for MMEJ-based CRISPR knock-in.

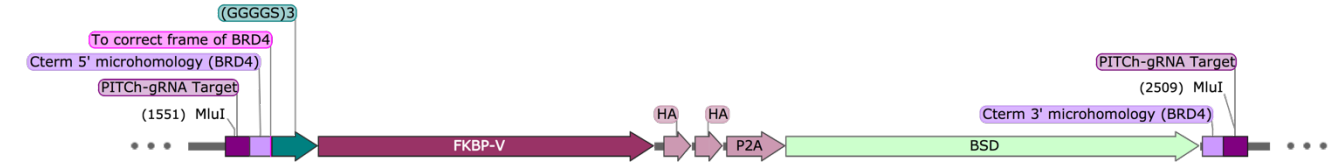
Step 1: Choosing a target and PITCh dTAG vector: After identifying the target of interest, determine which terminus can accommodate the $FKBP12^{F36V}$ tag without disrupting protein function. A literature search can help determine which terminus is favorable for tagging. If it is unclear which terminus 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 of interest, before investing in knock-in experiments. Lentiviral gateway cloning-compatible vectors for exogenous expression of tagged genes are available on Addgene: pLEX_305-N-dTAG (Addgene #91797) and pLEX_305-C-dTAG (Addgene #91798). After choosing a terminus to knock-in the tag, choose a PITCh dTAG vector with your preferred selectable marker (puromycin or blasticidin resistance).

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

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

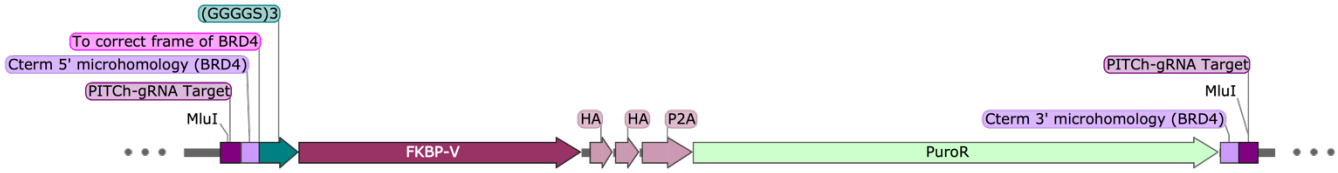


pCRIS-PITChv2-dTAG-BSD^{R} (BRD4) – Tags the C-terminus and contains a blasticidin resistance cassette (Addgene #91792)

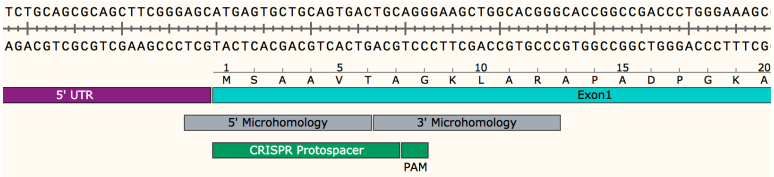


pCRIS-PITChv2-Puro^{R}-dTAG (BRD4) – Tags the N-terminus and contains a puromycin resistance cassette (Addgene #91796)

pCRIS-PITChv2-dTAG-Puro^{R} (BRD4) – Tags the C-terminus and contains a puromycin resistance cassette (Addgene #91793)



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. An example of an sgRNA targeting the first exon of the serine/threonine-protein kinase, PLK1, is shown below for N-terminal tagging:



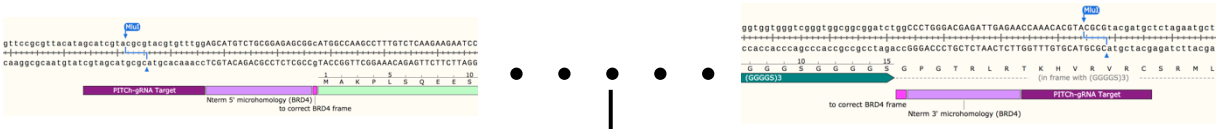
Step 3: Annotate your guide: For SpCas9, the cut site is 3bp upstream of the PAM. As such, the 5' microhomology and 3' microhomology regions will be the 20 bp immediately up and downstream of the cut site. Note that the cut site is 2 bp into the 6th codon and will need to be corrected when designing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

Step 4: Purify your backbone: From the constructs listed above, notice the presence of MluI sites flanking the dTAG cassette. These sites will be used to cut out the cassette to facilitate Gibson assembly later on. Digest vector (2 μg) with MluI, run the digest on a gel, and purify the backbone band with a DNA gel extraction kit.

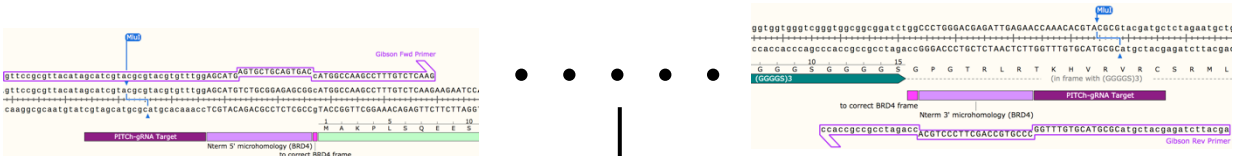
Step 5a: Design primers to incorporate new microhomologies: New microhomologies for your gene of interest were defined in the “annotate your guide” section. In the case of the PLK1 example below, the bases that were used for BRD4 to correct the frame (in hot pink) in the original vector will be kept, 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 outlined for PLK1 will allow replacement of microhomology sequences targeting your gene of interest. Remember to correct for frame differences if necessary.

5’ primer **3’ primer**

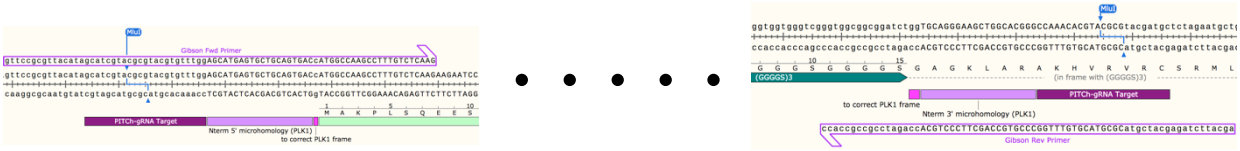
original dTAG cassette with BRD4 microhomologies



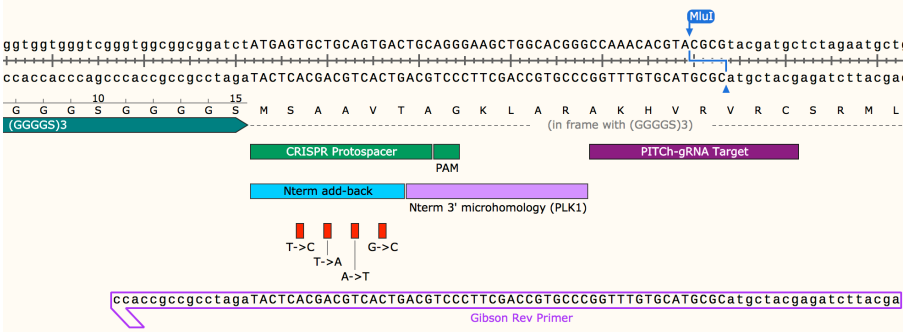
PLK1 primer including new microhomologies



New PLK1 knock-in dTAG cassette after PCR



Step 5b: Recommended method to addback bases in primer design: While MMEJ is a convenient way of doing CRISPR knock-ins, 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. The longer PCR reverse primer may make the PCR more difficult, but extending the primer sequence of the forward primer as well, 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, four silent mutations were made in the primer sequence to prevent binding of the sgRNA.

Step 6: Cassette PCR: Using primers designed in Step 6a or 6b, perform a PCR using the uncut PITCh dTAG vector, run the PCR 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 the Gibson assembly reaction (NEBuilder is recommended) using the purified PCR product and cut backbone. Transform appropriate bacteria with the reaction, grow up using Ampicillin selection, and purify your plasmid. Sequence the plasmid to confirm incorporation of new microhomologies:

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: pX330A-nBRD4/PITCh (Addgene, #91794) was cloned as previously described (Sakuma T, et al., Nat. Protoc. 2016; Ran, F.A. et al. Nat. Protoc. 2013). Briefly, N-terminal targeting guides for BRD4 were designed using http://crispr.mit.edu/. Oligonucleotides from IDT were annealed and inserted into the pX330A-1x2 (Addgene, #58766) plasmid after BbsI (NEB) digestion to generate pX330A-1x2-nBRD4. The PITCh sgRNA sequence from pX330S-2-PITCh (Addgene #63670) was inserted into pX330A-1x2-nBRD4 using Golden Gate assembly (NEB). The resulting plasmid pX330A-nBRD4/PITCh contained both the PITCh sgRNA, BRD4-specific sgRNA, and Cas9.

Step 9: Cell line construction: 293T cells were plated in 10 cm plates and co-transfected with 1 µg of pCRIS-PITChv2-BSD^{R}-dTAG (BRD4) and 2 µg of pX330A-nBRD4/PITCh using Lipofectamine 2000 (Thermo Fisher Scientific), according to manufacturer's instructions. After 6 hours of transfection, the media was aspirated and replaced with appropriate growth media and cells were allowed to incubate for and additional 48 hours. Subsequently, cells were selected with 10 µg/mL blasticidin, and single cell clones were isolated in 96-well format. To isolate genomic DNA, cells were transferred to a v-bottom PCR plate (VWR, #82006-636), washed twice with cold PBS, mixed with 60 µL of directPCR (tail) (Viagen Biotech, #102-T) and 0.4 mg/mL proteinase K (Life Technologies, #AM2548), and incubated overnight at 55 ºC. To inactivate proteinase K, cells were incubated at 85 ºC for 1.5 hours. Cell debris was spun down at 500 xg for 5 minutes. To genotype the cells and confirm knock-in, PCR reactions were performed using 1 µl of genomic DNA, 2x Q5 hot start master mix (NEB), and the following primers: TCCCTCTGGCCAACTTGGCTA (forward) and TGGTCTGCCTCTTGG-GCTTGTTA (reverse). Amplified DNA was purified by gel extraction (Qiagen) and sequenced using the PCR primers.

Alternatives to the PITCh approach: In addition to MMEJ-based CRISPR knock-in, in collaboration with Rick Young's lab, we also recently employed homology-directed repair and dual fluorescent screening (mCherry or BFP) to knock-in FKBP12^{F36V} at the YY1 locus (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 Weintraub et al.

Citation: Please reference the following publication for the use of this material:

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