Generating a CRISPR BromoTag Brd4BD2 L387A Knock-in Cell Line

This protocol for creating CRISPR BromoTag knock-in cell line is intended as a guide only and is based on the methods described in (1) and (2). For full experimental details please read the original published papers.

Step 1: Design and Synthesis of Guide RNA and donor

There are 3 essential genetic components that need to be transfected into the cells for this CRISPR-Cas systems to work:

- A pair of offset **single-guide RNA (sgRNA),** each one contains 5' a target sequence that is complementary to opposite strands of the target site, as well as an invariant RNA scaffold backbone. Each sgRNA should be controlled by a U6 RNA polymerase III promoter.
- **Cas9^{D10A} expression cassette**
- **Donor DNA construct**

A fluorescence reporter and antibiotics, e.g. Puromycin (**Cat. No. 4089**), resistance genes should also be included to enable clonal selection and isolation. These components can also be cloned into various plasmid vectors with different combinations or produced by *de novo* synthesis, depending on available tools and the sequence.

Guide RNA: To minimize off-target activity, the double nicking strategy uses the **D10A Nickase** form of **Cas9** (PAM: 5' NGG immediately following the target sequence) with pairs of **sgRNA** chosen for increased specificity (4). Prior to designing the sgRNAs, identify the appropriate features of the target integration site such, start/stop codons and localization sequences, within the gene of interest for N-terminal or C-terminal integration, respectively. The pair of sgRNAs must be designed such that 5′ overhangs are generated upon nicking; and target loci for the sgRNA pairs must also be offset with an optimal gap of 0–20 bp to facilitate efficient double nicking. We recommend using an online CRISPR Design Tool to select target sites for homozygous or heterozygous knock-in and for designing sgRNA against genomic regions of interest to target. Different sgRNAs can result in different Cas9 cleavage efficiencies. We suggest designing multiple candidate sgRNA pairs to test the efficiency. The sgRNA can be prepared as single-stranded oligodeoxynucleotides (ssODNs), e.g., by PCR-generated U6-sgRNA expression cassettes or they can be cloned into a pSpCas9 plasmid. It has been reported that the latter resulted in higher cleavage efficiency (1).

> Note: The T7 endonuclease 1 (T7E1) mismatch detection assay can be used for validating different gRNA designs (5). Using a Cas9^{wt} single gRNA approach is feasible, if the reduced targeting efficiency of the double nicking strategy preclude its use in tagging.

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Donor DNA is designed to have a sequence 500 bp upstream and 500 bp downstream of the designated tag insertion site of the target protein, forming a pair of homology arms flanking the DNA sequence encoding for $Brd4^{BD2\ L387A}$. The sequence for a flexible linker (-Gly-Gly-) between $Brd4^{BD2\ L387A}$ and the target protein of interest should be added. As above identification of the start and stop codons is crucial for N-terminal or C-terminal integration with design of homology arms to ensure integration immediately downstream or upstream of the respective codons. We recommend that expected integration product is confirmed in silico to check that the sequences remain in frame.

> Note: Targeting efficiencies can vary widely depending on cell type, target locus, type of repair donor and location of modification relative to the double-strand break site. As a rule of thumb, single-base correction rates drop approximately 4-fold at 100 bp away from the target site, and beyond 200 bp away, selection markers may be required (1).

For clonal isolation of cell lines after transfection via Fluorescence Activated Cell Sorting (FACS), a reporter/marker can be added with the BromoTag to denote successful integration of the knock-in construct. The sequence of eGFP is provided in the appendix as an optional integration marker. 2A self-cleaving peptides (P2A) splice sequence can be added between the integration marker and the BromoTag to separate the tagged protein and the marker.

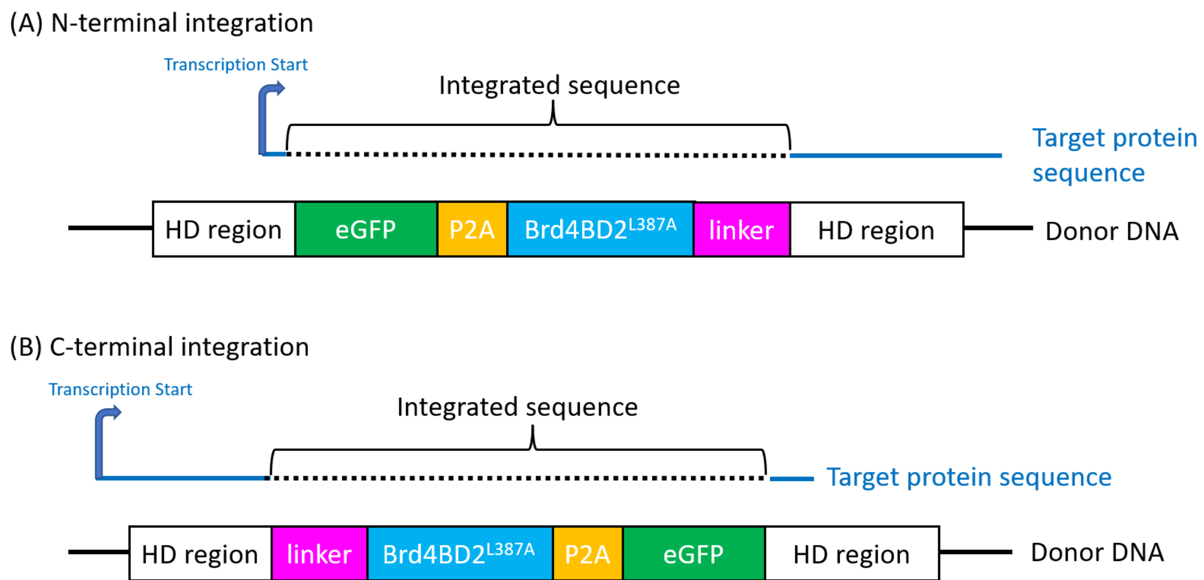

Figure 1. Suggested design of the knock-in construct of the donor DNA for N-terminal **(A)** or C-terminal **(B)** integration of the BromoTag. Please see the appendix for the sequences.

Step 2: Transfection

Cells are simultaneously transfected with the three genetic components in the plasmid vector or as ssODNs.

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- Mix at equimolar ratios and use no more than 500 ng of total DNA. Cells are optimal at 70–90% confluency. To increase the relative population of cells undergoing homologous recombination, transfection should be performed in the presence of 0.1 μM of the DNA ligase IV inhibitor SCR7 (Cat. No. 5342).
- The following day, wash cells before applying fresh medium containing SCR7 (0.1 μM) and appropriate antibiotics for selection.
- Repeat on the following day
- The following day, wash cells for the third time; add fresh medium without SCR7 or antibiotics to allow for recovery.
- On the fourth day, cells should be washed and then cultured with fresh medium containing the antibiotics and SCR7 (0.1 μM). Repeat this process for a further 2 days, then wash with PBS before recovery in media for a further 20 days.

Note: The CRISPR-Cas system has been used in a number of mammalian cell lines. Conditions may vary for each cell line. Fugene HD transfection reagent and lipofectamine 2000 have been used for transfecting the genetic components in HEK293 cells (1-3). Electroporation can be used for cell lines that are conventionally more challenging to transfect with lipofectamine.

Step 3: Clonal Isolation of Cell Lines

For generating clonal cell lines after transfection, FACS (selection by fluorescent marker), serial dilutions (selection by genotyping) or other isolation procedures can be used for isolating single cells followed by an expansion period to establish a new clonal cell line. It is worth noting that cell types can vary substantially in their responses to FACS or single-cell isolation, and literature specific to the cell type of interest should be consulted.

Note: Upon single cell sorting, it is crucial that cells should be spun down in their plates (~200-300 g) as this greatly increases their survivability after sorting. The use of conditioned media (contains filtered media from healthy growing cells) can also be used to enhance the survival and growth of any sorted cells.

Step 4: Knock-in Validation

Following clonal isolation, we recommend validating the Knock-In with at least two methods. Validation methods are listed below:
PCR Assay: Validate the insertion of Bromo-tag using PCR amplification with primers designed in the flanking homology arms and visualization by Gel Electrophoresis. Successful insertion of Bromo-Tag should yield a ~1160bp increase in fragment size.
RNA Validation: Confirm the expression of the Bromo-tagged transcript through RNA purification, cDNA generation with PCR-based knock-in confirmation.
Western Blot: Validate expression of the Bromo-Tagged protein of interest directly via Western Blot using the BromoTag Antibody (Novus, NBP3-17999)

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DNA Sequencing: Perform Sanger Sequencing of the insertion site to identify proper integration events, for this we recommend designing primers at least 200bp on either side of the sgRNA sites and subsequently optimizing them. Tools such as ICE can assist in the interpretation of this data. If desired, deep sequencing of the PCR products can be undertaken.

> Note: Sequencing based validation of integration is considered a Gold-standard. For PCR amplification alone or in combination with Sanger or NGS sequencing we recommend using Primer design software such as Primer3 and tools such as Primer-BLAST will assist in designing primers and consider off-target amplification. With optimal design the same PCR Primers can be used PCR validation and Sequencing.

References

1. **Ran** *et al* (2013) Genome engineering using the CRISPR-Cas9 system Nat.Protoc. ***8*** 2281. PMID: 24157548.
2. **Bond** *et al* (2021) Development of BromoTag: a "Bump-and-Hole"-PROTAC system to induce potent, rapid, and selective degradation of tagged target proteins J.Med.Chem. ***64*** 15477. PMID: 34652918.
3. **Tovell** *et al* (2019) Rapid and reversible knockdown of endogenously tagged endosomal proteins via an optimized HaloPROTAC degrader ACS.Chem.Biol. ***14*** 882. PMID: 30978004.
4. **Ran** *et al* (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity Cell ***154*** 1380. PMID: 23992846.
5. **Sentmanat** *et al* (2018) A survey of validation strategies for CRISPR-Cas9 editing. Sci.Rep. ***8*** 888. PMID: 29343825.

Custom gene engineering services are available from Bio-Techne.

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Appendix

Sequence Map and Feature annotation of Bromo-Tag Integration

Brd4$^{BD2\ L387A}$ (Bromo-Tag) Sequence
P2A Sequence
eGFP Sequence
Flexible Linker Sequence

N-terminal Bromo-Tag Integration

Homology Arm 1-
GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGA
CGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGC
TGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCA
CCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTT
CAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAA
CTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAA
GGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAG
CCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGC
CACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGC
GACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGAC
CCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTC
GGCATGGACGAGCTGTACAAGGCAACAAACTTCTCACTACTCAAACAAGCAGGTGACGTGGAGG
AGAATCCCGGCCTGTGAAGGACGTGCCCGACTCTCAGCAGCACCCAGCACCAGAGAAGAGCA
GCAAGGTCTCGGAGCAGCTCAAGTGCTGCAGCGGCATCCTCAAGGAGATGTTTGCCAAGAAGC
ACGCCGCCTACGCCTGGCCCTTCTACAAGCCTGTGGACGTGGAGGCACTGGGCGCCCACGACT
ACTGTGACATCATCAAGCACCCCATGGACATGAGCACAATCAAGTCTAAACTGGAGGCCCGTGA
GTACCGTGATGCTCAGGAGTTTGGTGCTGACGTCCGATTGATGTTCTCCAACTGCTATAAGTACA
ACCCTCCTGACCATGAGGTGGTGGCCATGGCCCGCAAGCTCCAGGATGTGTTCGAAATGCGCT
TTGCCAAGATGCCGGACGAGGGGGGG-Homology Arm 2

C-Terminal Bromo-Tag Integration

Homology Arm 1 -
GGGGGGGTGAAGGACGTGCCCGACTCTCAGCAGCACCCAGCACCAGAGAAGAGCAGCAAGGT
CTCGGAGCAGCTCAAGTGCTGCAGCGGCATCCTCAAGGAGATGTTTGCCAAGAAGCACGCCGC
CTACGCCTGGCCCTTCTACAAGCCTGTGGACGTGGAGGCACTGGGCGCCCACGACTACTGTGA
CATCATCAAGCACCCCATGGACATGAGCACAATCAAGTCTAAACTGGAGGCCCGTGAGTACCGT
GATGCTCAGGAGTTTGGTGCTGACGTCCGATTGATGTTCTCCAACTGCTATAAGTACAACCCTCC
TGACCATGAGGTGGTGGCCATGGCCCGCAAGCTCCAGGATGTGTTCGAAATGCGCTTTGCCAA
GATGCCGGACGAGGCAACAAACTTCTCACTACTCAAACAAGCAGGTGACGTGGAGGAGAATCC
CGGGCCTGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG
ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTAC
GGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTC

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GTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACG
ACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCG
AGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACT
ACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAA
GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCC
CATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAG
CAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGAT
CACTCTCGGCATGGACGAGCTGTACAAG-Homology Arm 2

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