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Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA

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Targeted genomic manipulation by Cas9 can efficiently generate knockout cells and organisms via error-prone nonhomologous end joining (NHEJ), but the efficiency of precise sequence replacement by homology-directed repair (HDR) is substantially lower[1,2]. Here we investigate the interaction of Cas9 with target DNA and use our findings to improve HDR efficiency. We show that dissociation of Cas9 from double-stranded DNA (dsDNA) substrates is slow (lifetime ~6 h) but that, before complete dissociation, Cas9 asymmetrically releases the 3′ end of the cleaved DNA strand that is not complementary to the sgRNA (nontarget strand). By rationally designing single-stranded DNA (ssDNA) donors of the optimal length complementary to the strand that is released first, we increase the rate of HDR in human cells when using Cas9 or nickase variants to up to 60%. We also demonstrate HDR rates of up to 0.7% using a catalytically inactive Cas9 mutant (dCas9), which binds DNA without cleaving it.

The type II CRISPR endonuclease Cas9 and an engineered single-guide RNA (sgRNA) form a ribonucleoprotein (RNP) complex that can be readily targeted to desired DNA sequences. This targeting activity is driven by 20 nucleotides of RNA-DNA base-pairing between the sgRNA and the target DNA strand protospacer and through interactions between the nontarget DNA strand's protospacer-adjacent motif (PAM; 5′-NGG-3′ for *Streptococcus pyogenes* Cas9) and Cas9's PAM-interaction motif (PIM) (**Fig. 1a**)[3,4]. After binding, the HNH and RuvC nuclease domains within Cas9 respectively cleave the target and nontarget strands of substrate DNA. Inactivating these domains with D10A (HNH) and/or H840A (RuvC) mutations yields Cas9 variants that nick a single strand of duplex DNA or are catalytically inactive (dCas9)[5].

Because Cas9 may behave differently from other genome targeting reagents, such as zinc finger nucleases (ZFNs) or TAL effector nucleases (TALENs)[6,7], a molecular understanding of the steps between DNA cleavage and the appearance of genomic edits is desirable. *In vitro* fluorescence measurements show that Cas9 associates tightly with both ends of cut DNA duplex, maintaining contact with its target site for at least 45 min (the quench time of the fluorophore), and releases the PAM-proximal side of the break in the presence of high concentrations of denaturant[8]. Understanding the nature and duration of Cas9-DNA interactions under physiological conditions could suggest strategies to increase Cas9's effectiveness as a genome-editing tool, because protection of cut DNA by Cas9 may obscure DNA lesions from cellular surveillance and repair pathways.

We used Bio-Layer Interferometry (BLI) to determine the *in vitro* kinetics of Cas9 RNP interaction with substrate DNA under native conditions[9]. A biotinylated 55-base-pair (bp) substrate DNA (λ1, described in ref. 8 and **Supplementary Note 1**) was immobilized to a streptavidin-coated BLI probe, and the binding and dissociation of Cas9 or catalytically inactive dCas9 was measured (**Supplementary Fig. 1a**). Despite the ability of Cas9 to cleave the target DNA (**Supplementary Fig. 1b**), we observed that both proteins have identical affinities for DNA (K_d 1.2 ± 0.1 nM, **Fig. 1b** and **Supplementary Fig. 1c**) as well as identical off-rates of ~$5.0 \pm 0.3 \times 10^{-5}$ s^{-1}, which equates to a lifetime of 5.5 h. The tight interaction of both RNP variants with substrate DNA was sgRNA dependent and required a PAM (**Supplementary Fig. 1d**).

To resolve whether Cas9 preferentially dissociates from one end of cut duplex DNA under physiological conditions, we labeled substrate DNA on each side of the nuclease cut site with a distinct fluorophore and monitored the dissociation of cut fragments using an electrophoretic mobility shift assay (EMSA). By chasing with unlabeled duplex competitor DNA, we determined the off-rate of the PAM-distal side of the cut to be $1 \pm 0.2 \times 10^{-5}$ s^{-1} and the off-rate of the PAM-proximal side of the cut to be $6 \pm 0.8 \times 10^{-6}$ s^{-1} (**Fig. 1c**; quantified in **Supplementary Fig. 1e**), which are very similar to values measured by BLI. Cas9's lifetime on DNA is therefore approximately sevenfold longer than the lower bound previously established by fluorescence means[8], and under native conditions Cas9 symmetrically dissociates from the target duplex. We note that exponentially growing mammalian cells require only 1 h to repair 90% of the double-strand breaks caused by ionizing radiation[10] but 15 h to resolve 90% of Cas9 lesions[11],

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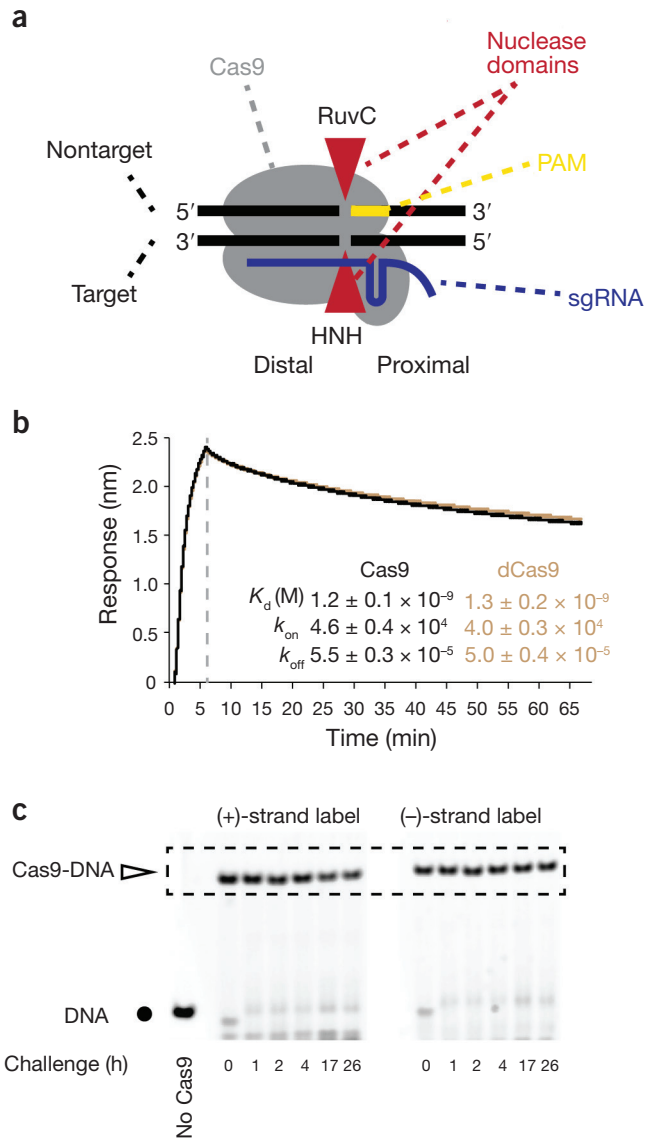

Figure 1 Cas9 interacts stably with substrate DNA. (**a**) Schematic of Cas9's interaction with substrate DNA. Cas9 (gray) complexed with sgRNA (dark blue) binds to DNA (black) comprising target and nontarget strands. Cas9-PAM interactions occur on the nontarget strand; sgRNA-DNA annealing occurs on the target strand. RuvC (His840) and HNH (Asp10) nuclease domains cut the nontarget and target strands, respectively (red triangles). (**b**) BLI measurements of association (left of dotted line) and dissociation (right of dotted line) of Cas9 (black trace) or dCas9 (brown trace) with λ1 dsDNA. Mean ± s.d. kinetic values calculated from $n = 2$ experiments are inset. See **Supplementary Figure 1** for data fitting. (**c**) Electrophoretic mobility shift assay (EMSA) measuring dissociation of Cas9 from substrate dsDNA. Cas9 RNP was equilibrated with S1 dsDNA for 16 h, after which unlabeled challenge dsDNA was added for the indicated time and reaction products were visualized on a native polyacrylamide gel. Black circle, labeled substrate DNA (no Cas9); open triangle, Cas9-DNA complex; dashed box, region of interest box. Data shown are representative of $n = 2$ experiments (quantified in **Fig. 2d**). Subsequent figures highlight the region of interest corresponding to the dashed lines.

suggesting that the long lifetime of the Cas9-DNA complex could be a limiting factor in genome editing. Direct comparisons between our *in vitro* data and the timing of DNA repair in cells could be complicated by many factors, but cellular single-molecule measurements of Cas9 binding also indicate an unusually long residence time at on-target sites[12], consistent with a model in which Cas9 stably binds to substrate DNA, concealing the underlying double-strand break and preventing recognition by genome surveillance factors.

To identify intermediate species that form during Cas9-mediated DNA cleavage, we challenged the Cas9-DNA complex with unlabeled DNA of various compositions (**Fig. 2a** and **Supplementary Fig. 2**). Notably, incubation of Cas9-DNA with an excess of ssDNA identical to the target strand caused the fluorophore attached to the 5′ end of the nontarget strand to be lost from the complex, whereas a fluorophore attached to the 5′ end of the target strand was not affected by either double- or single-stranded challenge DNA (**Fig. 2b**, lanes 2–4). By systematically labeling the 5′ or 3′ termini of either strand of the substrate DNA, we found that target-strand challenge DNA removes the PAM-distal nontarget strand from the Cas9-DNA complex without affecting the other three strands (**Supplementary Fig. 3a**). This strand-removal activity required sequence complementarity between Cas9-bound DNA and challenge DNA outside the protospacer sequence (**Fig. 2b**, lanes 8–10), but the PAM sequence of the challenge DNA was dispensable (**Fig. 2b**, lanes 5–7). These results, and the appearance of a new, fluorescently labeled product whose size is consistent with cleaved nontarget strand annealed to challenge DNA (**Supplementary Note 2**), indicate that single-stranded challenge DNA does not compete for Cas9 binding, but instead anneals to the nontarget strand. Strand removal was dependent on the concentration of this challenge DNA as well as on nuclease activity (**Supplementary Fig. 3b**). By recruiting two RNP complexes to a single target DNA in a 'PAM-inward' orientation, such as is used in paired-nick editing experiments[13], we found that we could remove the PAM-distal nontarget strand from each complex with the appropriate challenge ssDNA (**Fig. 2c**). Hence, although Cas9 globally dissociates from duplex DNA in a symmetric fashion (**Fig. 1c**), it appears that the enzyme locally releases the PAM-distal nontarget strand after cleavage but before dissociation. This strand is furthermore available for annealing to complementary challenge DNA and can be extruded from the Cas9-DNA complex by branch migration (**Supplementary Fig. 4**).

To determine whether Cas9 asymmetrically releases DNA during *in vivo* gene targeting in human cells, we used a terminal transferase end-labeling assay to measure the accessibility of the 3′-hydroxyls on either side of a Cas9-induced double-stranded break immediately after cleavage. We found that the nontarget strand is PAM-distally end-labeled sixfold more effectively than the target strand, and this preferential labeling follows the orientation of the Cas9 complex, such that sgRNAs targeting opposite strands induce labeling of opposite hydroxyls (**Supplementary Fig. 5**). Preferential accessibility of a single strand at the site of a Cas9 break raises the possibility that single-strand break recognition may play an unanticipated role in Cas9-mediated genome editing, but more work will be required to explore this hypothesis.

Having shown that Cas9 releases the PAM-distal nontarget strand but tightly engages the other three strands, we explored the nature of complexes formed when strand extrusion is prevented, either by catalytic inactivation of nuclease domains or by topological prevention of branch migration. Combining substrate DNA with Cas9 or the Cas9H840A mutant, which both cut the nontarget strand, preserved the challenge-dependent removal of the nontarget strand. However, the Cas9D10A mutant and dCas9, which leave the nontarget strand intact, instead exhibited a supershifted product when provided with target-strand challenge. This is consistent with stable annealing of the ssDNA challenge to the nontarget strand and formation of a Cas9-sgRNA-dsDNA-ssDNA complex (**Fig. 2d** and **Supplementary Fig. 3c**). This model predicts that loading two RNPs onto a single substrate DNA in a PAM-out orientation should prevent strand removal by branch

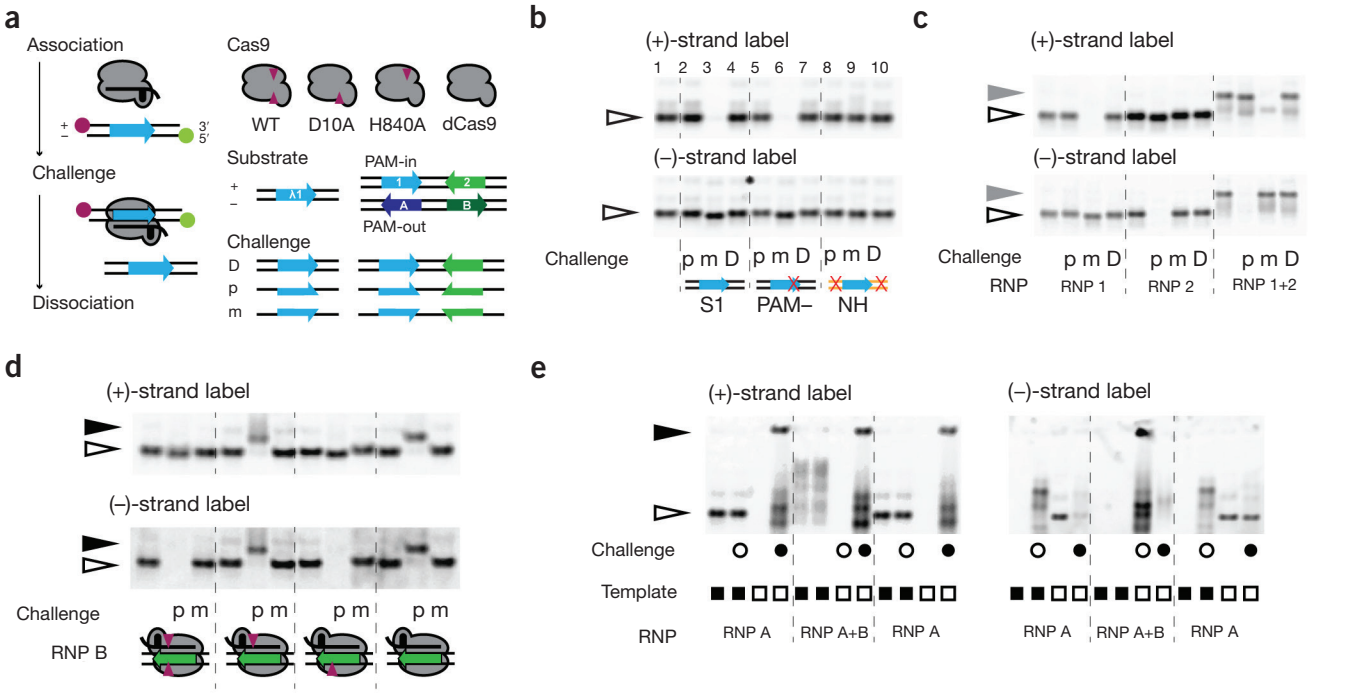

Figure 2 Complementary DNA anneals to the nontarget strand of the RNP-dsDNA complex. (**a**) Schematic for EMSA assays. Association: RNP is equilibrated for 10 min with fluorescently labeled substrate DNA (magenta, Cy5; green, Cy3) containing protospacer-PAM sequences (blue and green arrows). Challenge and Dissociation: reactions are incubated with or without unlabeled challenge DNA for 10 min and products are resolved on a native polyacrylamide gel. Nuclease variants (top right inset) are wild type (WT, with two magenta arrows representing catalytically active nuclease domains), the Cas9D10A and Cas9H840A nickase variants (with single magenta arrows) and the catalytically dead D10A H840A dCas9 variant (with no arrows). DNA substrates (middle right inset) contain one or two protospacer-PAM sequences (arrows). Dual binding of RNP to substrate DNA was investigated using sgRNA pairs that targeted protospacer-PAM sites in PAM-in (RNPs 1 and 2) or PAM-out (RNPs A and B) orientations. Unlabeled challenge DNA (lower right inset) was provided as double-stranded (D) or single-stranded (plus strand, p; minus strand, m) species. Data presented are representative of $n = 2$ biological replicates and cropped to highlight the region of interest. Uncropped gels from all experiments are presented in **Supplementary Note 2**. The Cas9, sgRNA and substrate DNA for each EMSA experiment is schematically presented in **Supplementary Figure 2**. Nuclease activity was verified using denaturing gels (**Supplementary Fig. 1b**). (**b**) Challenging a stable Cas9-DNA complex with ssDNA complementary to the PAM-distal nontarget strand leads to removal of this strand from the complex. Challenge DNAs were identical to substrate DNA (S1 substrate challenge; lanes 2–4), identical to substrate DNA with PAM disrupted (PAM$^-$ challenge; lanes 5–7), or disrupted the complementarity of the sequence flanking the protospacer-PAM (no-homology (NH) challenge; lanes 8–10). Lane 1, no challenge DNA. Open triangle, RNP-DNA complex. (**c**) Loading multiple Cas9 molecules in a PAM-in orientation allows displacement of either PAM-distal nontarget strand. One or two Cas9 molecules were loaded onto D1 substrate DNA, then challenged with the indicated challenge DNA species. Open triangle, RNP-DNA; solid gray triangle, 2×Cas9-DNA product. (**d**) Challenge DNA anneals to the uncut nontarget strand when Cas9 nuclease domains are inactivated. EMSA performed as described in **a**. Cas9, Cas9D10A, Cas9H840A and dCas9 nuclease variants were used as diagrammed below (following the visual scheme in **a**). Open triangle, RNP-DNA; solid black triangle, supershifted product. (**e**) Challenge DNA anneals to the nontarget strand when strand displacement is prevented by adjacent Cas9-DNA interactions in a PAM-out orientation. EMSA performed as described in **a** except that the fluorophore location was varied. Cas9 and dCas9 nuclease variants were used as diagrammed. D1 substrate dsDNA was labeled with Cy5 on the plus strand (solid square) or Cy3 on the minus strand (open square). Challenge ssDNAs were labeled with Cy5 on the plus strand (solid circle) or Cy3 on the minus strand (open circle). Open triangle, RNP-DNA; solid black arrow, well-shifted products.

migration because the stable protein-DNA interaction on the PAM-proximal side of each RNP would topologically block branch migration from the other complex (**Supplementary Fig. 2**). This may be similar to the situation encountered during genomic targeting with even a single Cas9 nuclease, in which chromatin factors such as nucleosomes should prevent branch migration.

We investigated strand-annealing activity in PAM-out complexes to directly monitor the incorporation of fluorescently labeled challenge DNA into supershifted products. For PAM-out RNPs bound to either strand of the substrate, addition of target-strand challenge DNA resulted in retention of the fluorescent challenge rather than in strand removal (**Fig. 2e**). This is in contrast to the PAM-in configuration, which does not present a topological barrier and allows strand removal (**Fig. 2c**). Taken together, these observations demonstrate that the nontarget strand is accessible for annealing to complementary ssDNA even when branch migration is prevented. Cas9 binding therefore not only melts the nontarget DNA strand from the target strand, but also renders the nontarget strand accessible for annealing to exogenous nucleic acid.

Short ssDNA donors containing a mutation of interest have been used to stimulate HDR events, the frequency of which can be increased by administering cell cycle or DNA-damage repair inhibitors[11,14–16]. We asked whether designing an ssDNA donor to optimize annealing to the exposed nontarget strand could boost the frequency of HDR events in the absence of chemical intervention. To explore this possibility, we generated ssDNA donor molecules that have varying sequence overlap on the 5′ and 3′ side of the break and are complementary to either the nontarget or the target strand, and measured their ability to support Cas9-mediated conversion of a stably integrated BFP reporter to GFP via a three-nucleotide mutation (**Fig. 3a**,**b**). Nucleofection of Cas9 RNPs with a donor DNA complementary to the nontarget strand stimulated

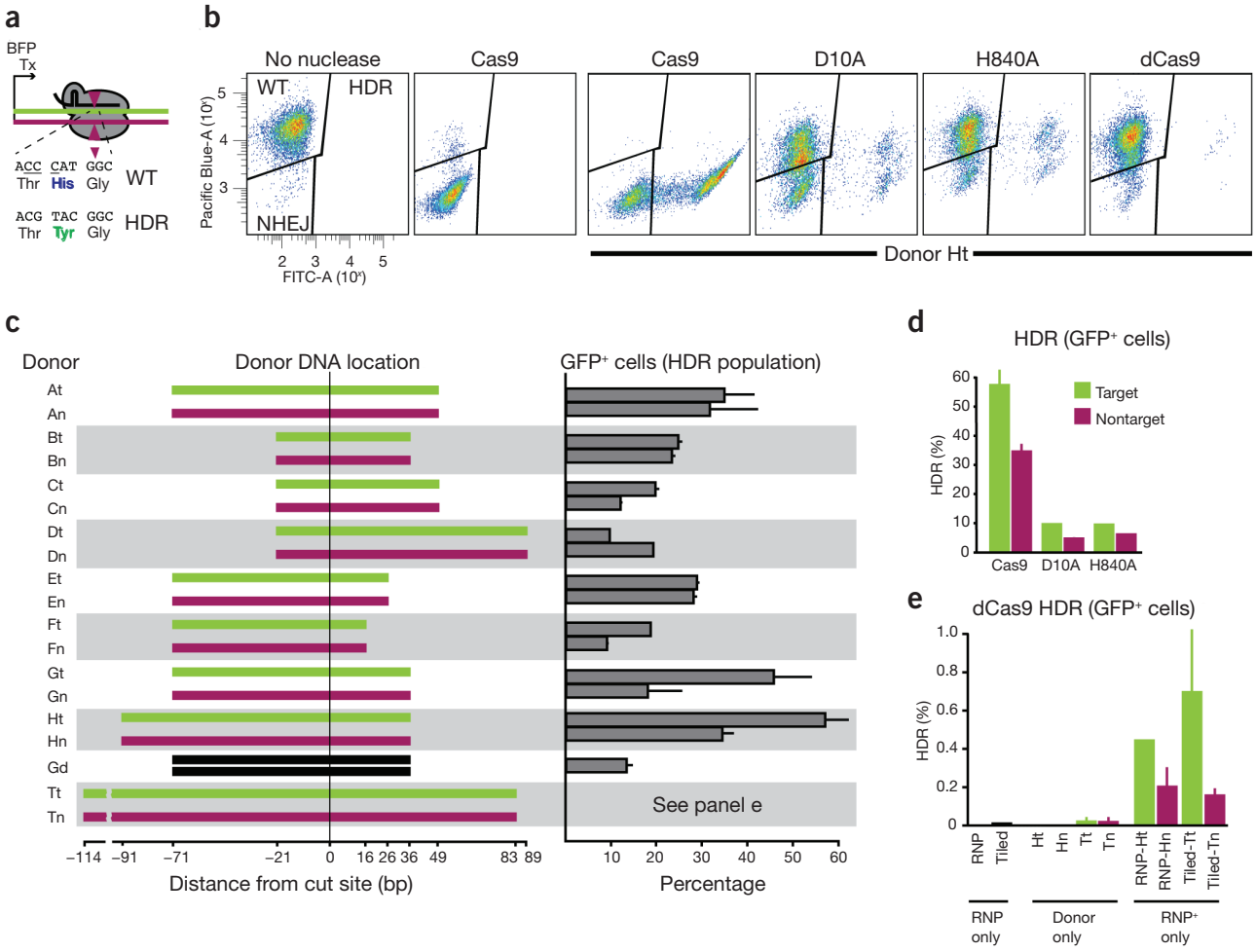

Figure 3 Delivery of ssDNA donors complementary to the nontarget strand drives efficient HDR using Cas9, nickases and dCas9. (**a**) Schematic for HDR at a BFP reporter locus. Target strand (green) or nontarget strand (magenta) donor ssDNAs were generated with the indicated overlaps on either side of the Cas9 cut site in the BFP reporter. The sequences of the unedited (WT, BFP) and edited loci (HDR, GFP) are presented inset (PAM reverse complement, underlined; cut site, magenta arrow). (**b**) HDR, NHEJ and unedited populations can be measured using flow cytometry. BFP-GFP flow cytometry scatter plots for BFP reporter cells (leftmost panel), BFP reporter cells edited with Cas9 (Cas9), or BFP reporter cells edited with the indicated nuclease and Donor Ht. Data shown is representative of $n = 2$ experiments, and all flow cytometry plots are shown in **Supplementary Note 3**. Gated populations are WT, BFP$^+$ cells; NHEJ, BFP$^-$ GFP$^-$ cells; and HDR, GFP$^+$ cells. (**c**) Optimized donor DNA is complementary to the nontarget strand and has a characteristic size. HDR frequencies for editing with target (t), nontarget (n) or double-stranded (d) donor DNAs are presented at right as mean ± s.d. for $n \geq 2$ independent experiments. All flow cytometry plots are presented in **Supplementary Note 3**. (**d**) Target-strand donor stimulates greater levels of HDR for all Cas9 variants. HDR frequencies are quantified from editing experiments using the indicated nuclease and donor Ht (target strand, green) or Donor Hn (nontarget strand, magenta). Data presented as mean ± s.d. from $n \geq 2$ independent experiments. (**e**) Single or tiled-dCas9 molecules support HDR. HDR frequencies from dCas9 editing experiments as presented in **d** except that control (RNP only, Donor only) reactions are shown alongside editing reactions. RNP, single dCas9; tiled, equimolar amounts of dCas9 targeting four distinct sites on the coding strand of the BFP reporter.

HDR frequencies up to 2.6-fold greater than those obtained with a donor DNA complementary to the target strand (**Fig. 3c**, Gt versus Gn) and 4-fold greater than those obtained with a double strand donor DNA of the same length (**Fig. 3c**, Gt versus Gd).

Our observation that donor DNA complementary to the nontarget strand is more effective than donor complementary to the target strand is consistent with previous studies using symmetric single-stranded donor oligonucleotides (ssODNs) to introduce mutations at the *EMX1* (ref. 15) and *AAVS1/PPP1R12C* (ref. 17) loci in human cell lines. Notably, strand bias was not observed when AAVS-1 breaks were generated using ZFNs[18], suggesting that donor-strand bias may be specific to Cas9 breaks. In contrast to results from previous studies, which focused on donor DNA symmetric around the break[15,17,18], we observed that asymmetric donor DNA optimized for annealing by overlapping the Cas9 cut site with 36 bp on the PAM-distal side, and with a 91-bp extension on the PAM-proximal side of the break, supported HDR frequencies of 57 ± 5%. This HDR frequency, obtained through simple rules of ssDNA donor design, is greater than rates obtained using symmetric donors and chemical or genetic intervention, such as cell cycle arrest or knockdown of NHEJ repair[14,16], and invites attempts at combinatorial treatment that could boost frequencies further. Shorter or longer overlaps with the nontarget strand compromised editing efficiency, possibly by reducing the stability of annealing to the nontarget strand or requiring extensive invasion into the duplex region farther from the Cas9 complex. Increases in HDR frequency obtained by exploiting the strand bias and asymmetry effects were consistent when measured in multiple cell types (HEK293 and K562) and at multiple genomic loci (HEK293 with integrated BFP, *CXCR4*, *CCR5* and

EMX1; K562 with *CXCR4*, *CCR5*; **Supplementary Figs. 6–8**). Notably, the geometric design principles used for increased HDR remained consistent with respect to Cas9's physical engagement of each locus rather than its genomic orientation. Thus, HDR enhancement by precise donor–nontarget strand complementarity appears robust with respect to the choice of transcript template or coding strand.

Because we observed that *in vitro* strand annealing was independent of nuclease activity (**Fig. 2**), we investigated the potential of optimized ssDNA donors to edit the BFP reporter when paired with Cas9 variants in which one or both nuclease domains were disrupted. Cas9D10A stimulates 2–3% HDR when delivered via plasmid in a "paired nick" configuration[13,19], but efficient HDR with a single nickase has not been reported[20]. Using RNP electroporation, we observed that Cas9D10A (nicking the target strand) and Cas9H840A (nicking the nontarget strand) each stimulated HDR frequencies of ~10% when provided with the optimized asymmetric target-strand donor DNA, but also silenced the BFP reporter, presumably by inducing error-prone NHEJ (**Fig. 3b**,**d** and **Supplementary Fig. 9a**). This latter observation raises concerns about the use of paired nickases for editing, because off-target cuts associated with each single nickase could be mutagenic. The high efficiency of editing with RNP delivery relative to plasmid delivery may reveal these unappreciated NHEJ events, because indels caused by individual plasmid-expressed nickases have previously been reported at the low end of the detection level for T7E1 or Surveyor assays[4,13,19]. We also observed a small but measurable (0.4 ± 0.0%) frequency of HDR when catalytically inactive dCas9 was used in editing experiments (**Fig. 3b**,**e**). dCas9 was less effective at stimulating HDR than Cas9, Cas9D10A or Cas9H840A but should edit without introducing breaks in genomic DNA (**Supplementary Fig. 9b**). For all nuclease variants tested, HDR occurred at approximately a twofold higher frequency when donor DNA complementary to the nontarget strand was provided relative to donor DNA complementary to the target strand (**Fig. 3d**), suggesting that strand annealing affects HDR in the absence of nuclease activity, consistent with the ability to stably form Cas9-dsDNA-ssDNA complexes *in vitro* (**Fig. 2d**,**e**, and **Supplementary Fig. 3c**).

As the annealing step is thought to be rate limiting in oligonucleotide-mediated repair, we speculated that binding multiple dCas9 molecules to the same strand would displace large portions of genomic DNA for annealing to an ssDNA donor and might increase the frequency of dCas9-mediated HDR. Tiling three dCas9 molecules on the same strand resulted in a twofold increase (to 0.7 ± 0.3%) in HDR when paired with an ssDNA donor that can anneal to the nontarget strand, but a twofold decrease (0.2 ± 0.04%) when paired with a donor that can anneal to the opposite strand (**Fig. 3e** and **Supplementary Fig. 9b**). The rate of dCas9-mediated HDR, although low compared to that of wild-type Cas9-mediated mutation, is substantially greater than that obtained with oligonucleotide alone (0.02 ± 0.03%; **Fig. 3e**). We anticipate that dCas9-mediated mutation could be useful in certain therapeutic applications, similar to X-SCID[21] and tyrosinemia[22], where edited cells have a major fitness advantage and/or NHEJ events at on- or off-target sites would be a significant hazard.

We have found that although Cas9 binds stably to DNA substrates, it makes one strand upstream of the PAM, and identical in sequence to the RNA protospacer, accessible both *in vitro* and *in vivo*. Cas9-mediated interrogation of potential CRISPR targets has recently been shown to play a role in protospacer acquisition by Cas1, Cas2 and Csn2 (ref. 23), and it is tempting to speculate that Cas9's ability to render the nontarget strand available for annealing to exogenous factors could potentiate this process. The ability of Cas9 to release one PAM-distal strand of DNA is consistent with recently published structural data showing that the target DNA strand is buried from solvent and wrapped around the sgRNA, whereas the RuvC active site lines a wide, solvent-exposed basic cleft that appears poised to channel the PAM-distal region of the nontarget strand (**Supplementary Fig. 10**). This structural asymmetry also explains an early observation that Cas9 cleaves the target strand in one precise location 3 bp from the PAM, whereas the nontarget strand is cut in variable locations[12], likely because that strand is free to breathe in and out of the nuclease domain. Collectively, the biochemical and structural asymmetry of Cas9's interaction with substrate DNA indicates that Cas9 globally remains bound to both sides of the cut while locally releasing the PAM-distal side of the nontarget strand. Cas9's monomeric asymmetry is conceptually distinct from other targeted nucleases such as ZFNs or TALENS in which asymmetric targeting domains are bridged by weak dimeric FokI-FokI interactions[24]. ZFNs and TALENs also release cut DNA under native conditions *in vitro*[25], whereas denaturing conditions must be used for Cas9 (**Supplementary Fig. 1b**). Thus, strategies for Cas9-mediated editing may differ from those used with other gene-editing tools and may even differ for engineered dCas9-FokI editing[26].

Most optimization of genome editing has focused on biasing repair outcomes toward HDR and away from NHEJ by chemically or genetically inactivating components of the NHEJ pathway, chemically activating HDR or manipulating the cell cycle[14–16,27]. These *trans* interventions, although highly useful in some contexts, may be undesirable during therapeutic gene editing because they alter the cellular capacity to respond to damage at other sites in the genome. We have found that Cas9-mediated HDR frequencies can be increased by rationally designing the orientation, polarity and length of the donor ssDNA to match the properties of the Cas9-DNA complex. We also found that these donor designs, when paired with tiled catalytically inactive dCas9 molecules, can stimulate HDR to approximately 1%, almost 50-fold greater than donor alone. The exact mechanism by which donor design increases Cas9-mediated HDR remains to be investigated. Evidence that the repair of DSBs using ssODNs in budding yeast occurs through the single-strand annealing (SSA) pathway[28] suggests a model in which optimal donors anneal to the strand released from Cas9 or dCas9 and either bias the choice of repair pathway toward SSA or increase the local concentration of the donor. The high frequency of HDR seen with ssODN-mediated Cas9 editing would thus be consistent with the emerging view that microhomology-mediated end joining and/or SSA play a substantial physiological role in metazoan cells[29,30]. We anticipate that simple strategies to increase HDR frequency that take advantage of detailed knowledge of the mechanism of Cas9 action will be valuable for basic research and therapeutic gene editing.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Plasmid sequences are available at Addgene with accession numbers 69090, 71820, 71821, 71822, 71823, 71824 and 78125.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.D.R. and J.E.C. designed experiments; C.D.R., G.J.R. and G.L.C. performed experiments; M.A.D. designed and constructed the BFP reporter cell line; C.D.R. and J.E.C. analyzed data; C.D.R. and J.E.C. wrote the manuscript with contributions from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cas9 and RNA preparation. Cas9 (pCR1002, Addgene 71820), dCas9 (pCR1003, Addgene 71821), Cas9-2NLS (pCR1053, Addgene 69090), D10ACas9-2NLS (pCR1054, Addgene 71822), H840ACas9-2NLS (pCR1055, Addgene 71823), and dCas9-2NLS (pCR1056, Addgene 71824) were purified by a combination of affinity, ion exchange, and size exclusion chromatographic steps as previously described[31], except protein was eluted at 40 μM in 20 mM HEPES KOH, pH 7.5, 5% glycerol, 150 mM KCl, 1 mM DTT. **Supplementary Note 4** contains expressed sequences for each of these vectors (Addgene 69090 and 71820–71824).

sgRNAs were generated by HiScribe (NEB E2050S) T7 *in vitro* transcription using PCR-generated DNA as a template[31,32]. Complete sequences for all sgRNA templates can be found in **Supplementary Note 1**.

Bio-Layer interferometry. The Octet RED384 Bio-Layer Interferometry machine and Streptavidin (SA) Biosensors are available from ForteBio (Menlo Park, CA). All steps were performed in Reaction Buffer (20 mM Tris, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.01% Tween, 50 μg/mL Heparin) at 37 °C. Biosensors were incubated with 300 nM double-stranded DNA (Single RNP Substrate) biotinylated on the 5′ terminus of the nontarget strand for 180 s and free DNA was washed away. 200 nM Cas9 (expressed from pCR1002) or dCas9 (expressed from pCR1003) was mixed with a 20% molar excess of sgRNA and incubated for 10 min to form RNP. Biosensor tips conjugated to substrate DNA were incubated with RNP for 300 s to load RNP. Biosensor-dsDNA-RNP complexes were allowed to dissociate in Reaction Buffer for 3,600 s. Response curves for each biosensor were normalized against biosensors conjugated to DNA but without RNP (buffer-only control). Normalized response curves were processed using Octet software version 7 to obtain reported kinetic values.

EMSA assays. Nuclease and sgRNA were incubated in Reaction Buffer for 30 min to form RNP. Substrate DNA was added and RNP loading was allowed to take place for a defined interval (16 h, equilibrium experiments; 10 min, strand displacement/annealing experiments). Assembled RNP-dsDNA complexes were incubated with challenge DNA for the reported amount of time. Standard reactions conditions were: Substrate DNA, 100 nM; Cas9, 500 nM; sgRNA, 500 nM; challenge DNA, 1,500 nM. All reactions were performed at 37 °C. Sequences for all substrate and challenge oligonucleotides (IDT) can be found in **Supplementary Note 1**.

Cell lines. HEK293 cells were obtained from ATCC and verified mycoplasma-free (Lonza Mycoalert LT-07). All cells were maintained in DMEM supplemented with 10% FBS and 100 μg/mL Penicillin-Streptomycin.

Reporter strain construction. HEK293 cells were transduced with lentivirus expressing a BFP reporter construct under the EF1alpha promoter (Addgene 71825; **Supplementary Note 1** for expressed sequence of plasmid). Dilution cloning was used to isolate clonal populations with robust expression of BFP (as measured by flow cytometry). Cell populations were periodically sorted on a BD FACSJAZZ to maintain BFP expression levels.

Nucleofection editing experiments. 100 pmol of Cas9-2NLS (or variants) was diluted to a final volume of 5 μL with Cas9 buffer (20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol and 1 mM TCEP) and mixed slowly into 5 μL of Cas9 buffer containing 120 pmol of L2 sgRNA. The resulting mixture was incubated for 10 min at RT to allow RNP formation. 2×10^{-5} HEK293 cells were harvested, washed once in PBS, and resuspended in 20 μL of SF nucleofection buffer (Lonza, Basel, Switzerland). 10 μL of RNP mixture, 100 pmol of donor DNA, and cell suspension were combined in a Lonza 4d strip nucleocuvette. Reaction mixtures were electroporated using setting DS150, incubated in the nucleocuvette at RT for 10 min, and transferred to culture dishes containing pre-warmed media[32]. Editing outcomes were measured 4 and 7 days post-nucleofection by flow cytometry. Seven-day results are presented in the figures.

Analysis of editing rates by digestion. Editing was monitored by T7 Endonuclease I (T7E1) or restriction enzyme digestion essentially as described[15]. Briefly, edited cells were harvested 2 days after nucleofection and genomic DNA was extracted using a Qiagen DNeasy kit (Qiagen, Venlo, Netherlands). 200 ng of genomic DNA was used as a template in a PCR reaction using KAPA high GC buffer (KAPA Biosystems, Wilmington, MA) and standard PCR conditions (95 °C for 5 min, 35 cycles of 98 °C for 20 s, 62 °C for 15 s and 72 °C for 30 s, and one cycle of 72 °C for 1 min). 200-ng aliquots of purified PCR DNA were subjected to T7EI or PciI digestion (NEB, Ipswich, MA) and resolved on a 2% agarose gel. Gel images were quantified using ImageJ.

Terminal transferase–qPCR assay. 667 pmol of Cas9-2NLS (or variants) was diluted to a final volume of 20 μL with Cas9 buffer and mixed slowly into 20 μL of Cas9 buffer containing 800 pmol of sgRNA. The resulting mixture was incubated for 10 min at RT to allow RNP formation. 4×10^6 cells were harvested, washed once in PBS, and resuspended in 100 μL of SF nucleofection buffer (Lonza V4XC-2032). 50 μL of RNP mixture, 100 pmol of donor DNA, and cell suspension were combined in a Lonza 4d strip nucleocuvette. Reaction mixtures were electroporated using setting DS150, incubated in the nucleocuvette at RT for 10 min, and transferred to culture dishes containing pre-warmed media. Cells were allowed to recover for three hours, then fixed in 2% formaldehyde for 15 min at 4 °C and permeabilized overnight in 70% ethanol at –20 °C. 10^{-6} cells were rehydrated in terminal transferase (TdT) buffer (Roche 03333574001) for 10 min at 37 °C and incubated with 800 U of TdT and 2 nmol of Biotin-16-dUTP (Roche 11093070910) for 30 min at 37 °C. Labeled cells were resuspended in Lysis Buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT) and disrupted by sonication in a Covaris S220. Cross-links were reversed by incubation for 4 h at 65 °C and cell slurry was cleared by centrifugation at maximum speed for 10 min.

Biotinylated DNA in the supernatant was bound to streptavidin MyOne C1 Dynabeads (Life Technologies 65002) and washed 3× in Wash Buffer (5 mM Tris, 0.5 mM EDTA, 1 M NaCl). Nonbiotinylated strands were dissociated by incubation in 20 mM NaOH for 10 min. The washed beads were resuspended to a final concentration of 10 μg/μL in dH_2O. Two microliters of bead slurry was used as template for qPCR.

A total of three independent cultures were analyzed for each sgRNA on an Eppendorf Nexus X1 qPCR machine using primers listed in **Supplementary Note 1**. Each reaction was performed using DyNAmo HS SYBR Green qPCR kit (Fisher Scientific F-410L) in a total volume of 20 μL with primers at a final concentration of 300 nM. Annealing was performed at 62 °C. Fold enrichment of the assayed DNA segments over the unlabeled ACT1B locus was calculated using the $2^{-\Delta\Delta Ct}$ method essentially as described[33].

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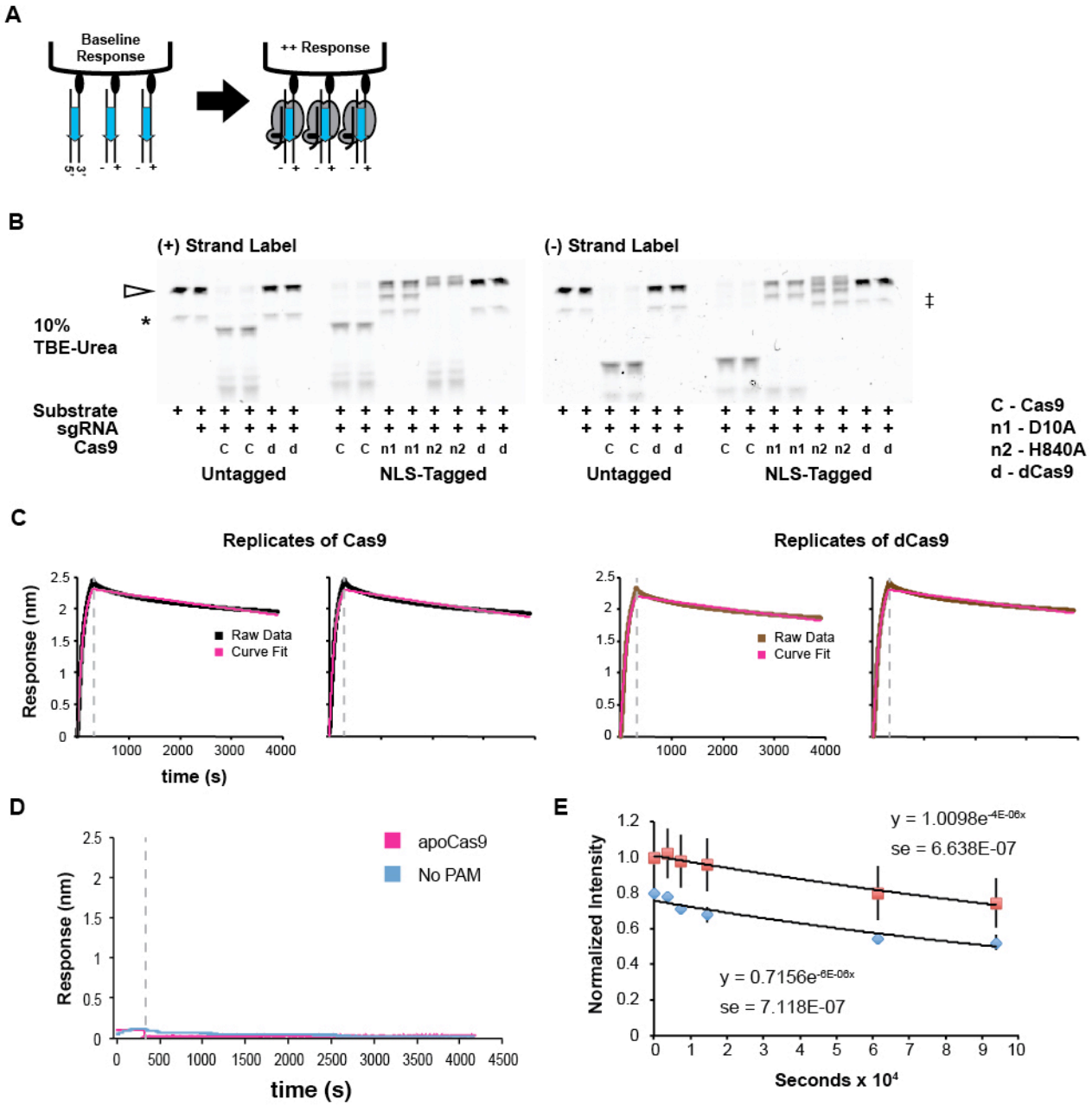
doi:10.1038/nbt.3481

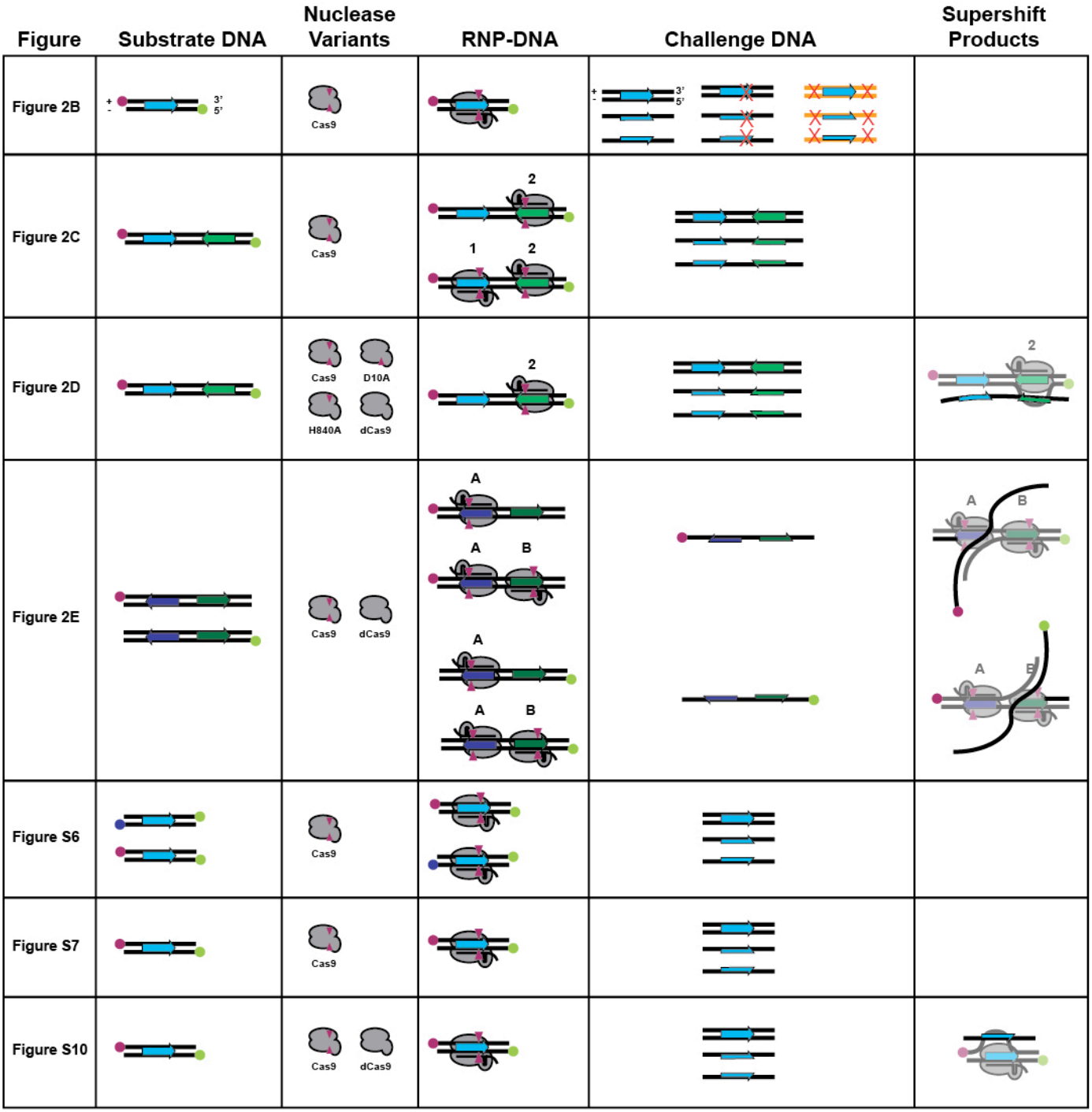


Supplementary Figure 1

Supporting data for Figure 1

(A) Schematic of BLI assay used to measure dissociation. 5' monobiotinylated substrate DNA (identical to λ1, Figure 2) is associated with streptavidin-coated sensor tips (black oval) and baseline signal is established (left panel). Association phase (right panel) loads Cas9 onto substrate dsDNA and measures response. Dissociation phase (not shown) transfers the tip into buffer and monitors dissociation of Cas9. (B) Cas9, Cas9D10A, and Cas9H840A cleave DNA while dCas9 does not. Cas9 nucleases were incubated with or without sgRNA for 30 minutes and associated with λ1 substrate DNA (Figure 2A) for ten minutes. Untagged (pCR1002 and pCR1003, Document S3) and NLS-tagged (pCR1053-pCR1056, Document S3) Cas9 variants were tested and found to have equivalent activity. -Reaction products were resolved on a 10% TBE-Urea gel. Open arrow, uncut substrate DNA; *, excess Cy5 labeled ssDNA; ‡, excess Cy3 labeled ssDNA. Data presented is representative of n=2 experiments. (C) Fit between BLI data (thick trace) and calculated kinetic values (maroon trace) for Cas9 (black) and dCas9 (brown). Replicate data is shown. (D) Cas9 interacts specifically with substrate dsDNA. BLI traces show no interaction of apoCas9 (no sgRNA) with substrate dsDNA (maroon trace) or Cas9 with

substrate dsDNA lacking a PAM (blue trace). (n=2). (E) Gel densitometry of Figure 1B. Mean ± SD normalized intensity of + strand (blue) and – strand (red) shifted products were plotted as a function of time. The indicated regression lines were used to calculate koff. Equations and standard errors of the regression coefficient (se) are presented for each trace.



Supplementary Figure 2

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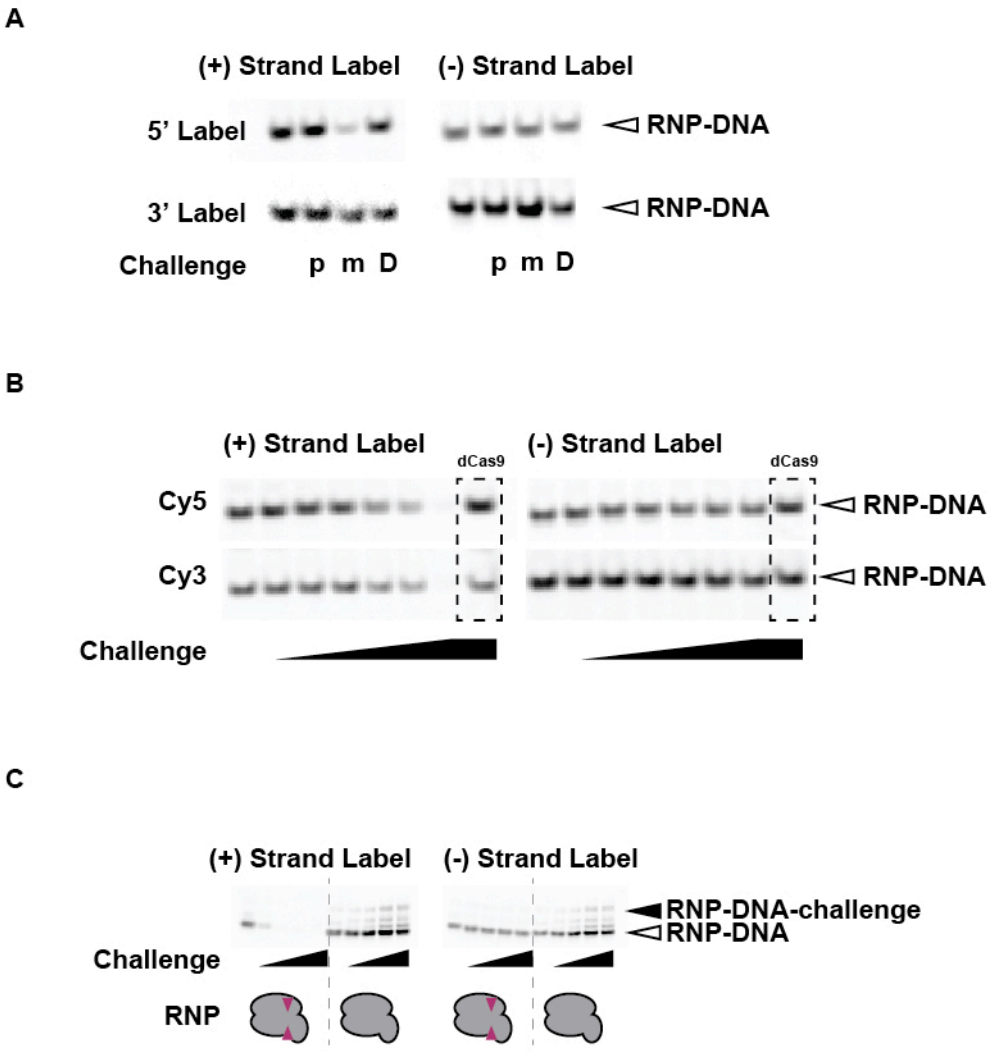
Schematic of reagents and experimental design for EMSA experiments.

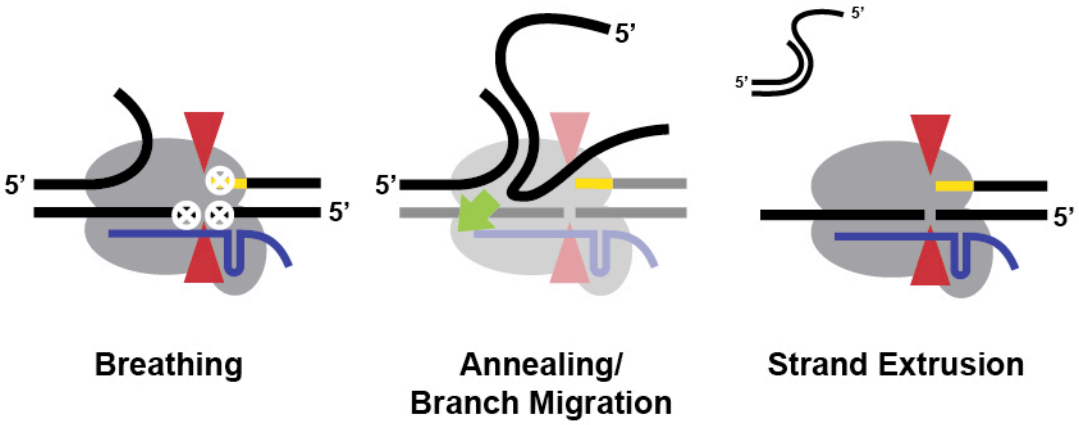
Potential supershift products are presented where appropriate.



Supplementary Figure 3

Supporting data for Figure 2

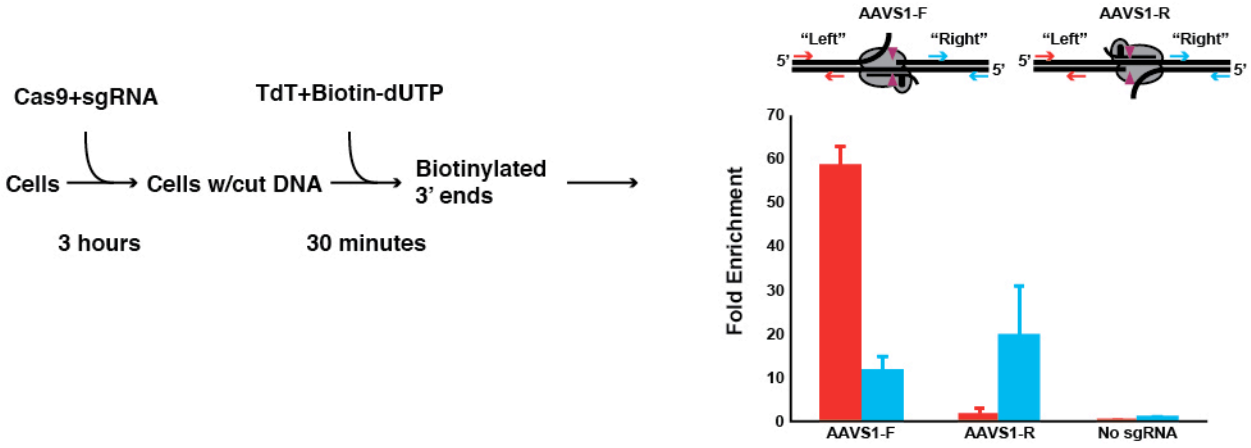
(A) The non-target strand is released on the PAM-distal side of the cut. One Cas9 molecule was loaded onto substrate DNA fluorescently labeled at the 5' or the 3' terminus of each strand (Supplementary Figure 3). Only the 5' non-target strand can be removed from the complex by a challenge DNA. Open arrow, RNP-DNA complex. (B) Removal of the non-target strand depends upon the concentration of the challenge DNA but is independent of the labeling fluorophore. Single RNP EMSA was conducted as described in Figure 2A, except challenge concentration was varied from 0-1500nM (0, 30, 75, 150, 300, 600,1500nM). Catalytically inactive dCas9 was used in lane 8 (dashed box) to demonstrate that nuclease activity is required for strand extrusion activity. Substrate DNA fluorescently labeled at the 5' termini with Cy5 or Cy3 as indicated. Open arrow, RNP-DNA complex. (C) Strand annealing occurs in single-RNP substrates when the non-target strand is left intact. Cas9 or dCas9 variants were loaded onto substrate DNA as indicated and as described in Supplementary Figure 3. Challenge concentration was varied from 0-5uM (0, 500, 1500, 2500, 5000nM). Open arrow, RNP-DNA complex; solid black arrow, supershifted products.



Supplementary Figure 4

Model for challenge-mediated non-target strand removal activity.

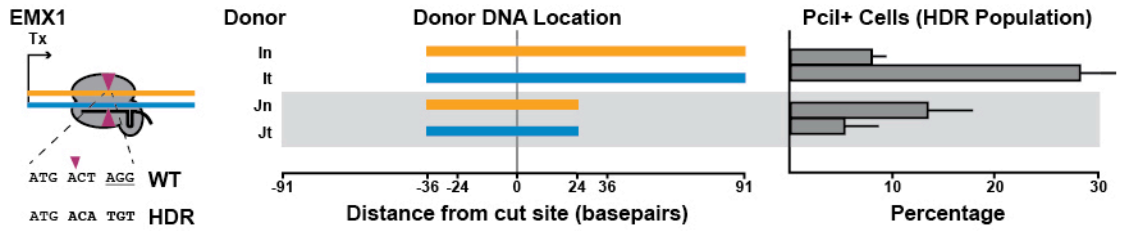
1) After duplex cleavage, Cas9 holds onto three ends of the target DNA (white crossed circles), but the PAM-distal non-target strand is released from the Cas9-DNA complex. 2) Complementary DNA anneals to released strand. 3) Branch migration results in extrusion from the Cas9-DNA complex.



Supplementary Figure 5

The non-target strand is available for enzymatic modification in cells.

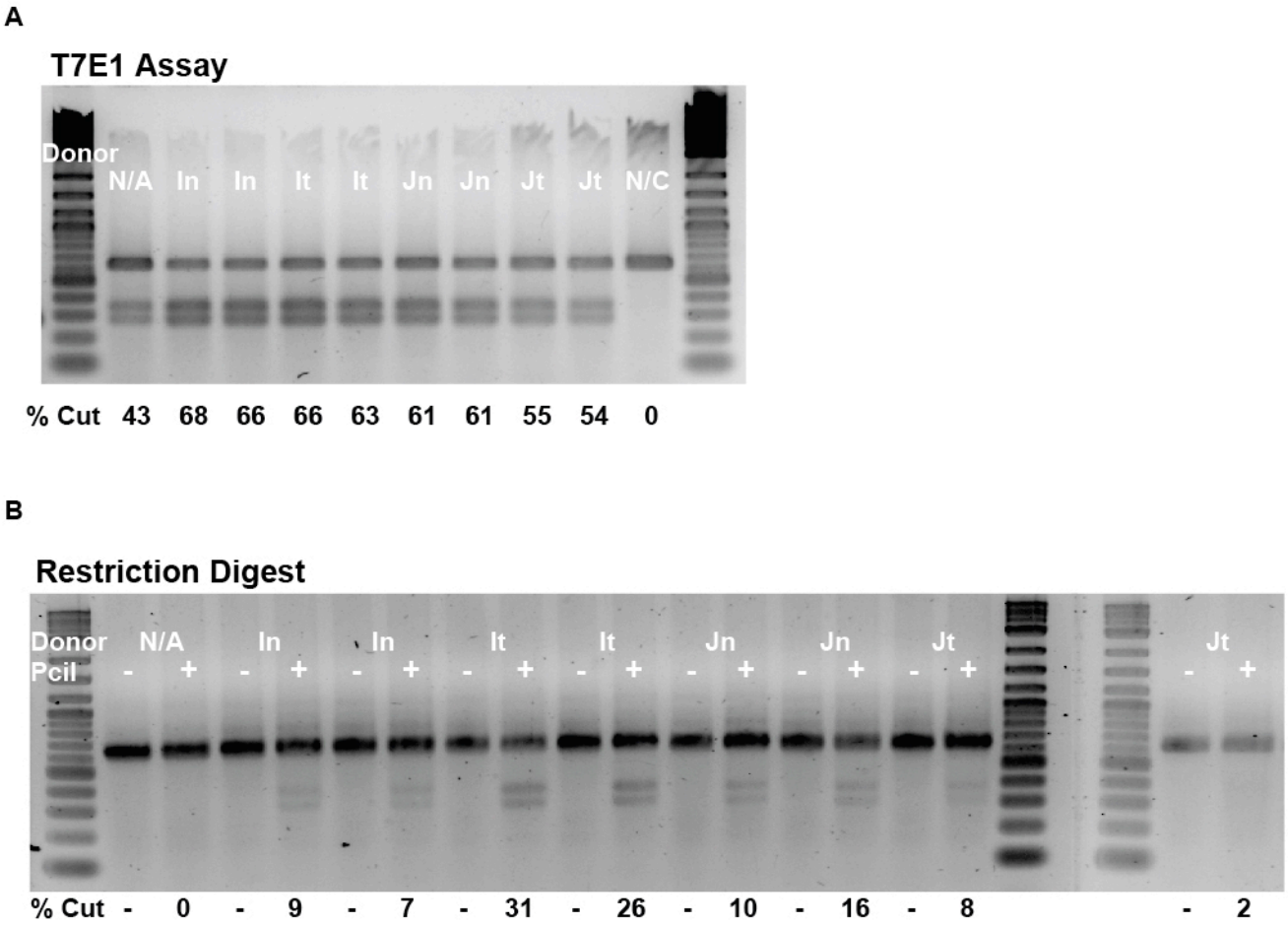
Cas9 was targeted to either strand of the AAVS1 locus (AAVS1-F or AAVS1-R) and terminal transferase was introduced to 3' end-label cut DNA with biotin. After streptavidin immunoprecipitation, end-labeling on either side of the break was determined by the ability to qPCR amplify sequences using the indicated primer pairs (Left and Right). Results are presented as the mean +/- SD fold enrichment (n=3) of labeled DNA over uncut control DNA (ACT1).



Supplementary Figure 6

Strand-bias for optimized donor DNA is independent of genomic locus and gene transcription.

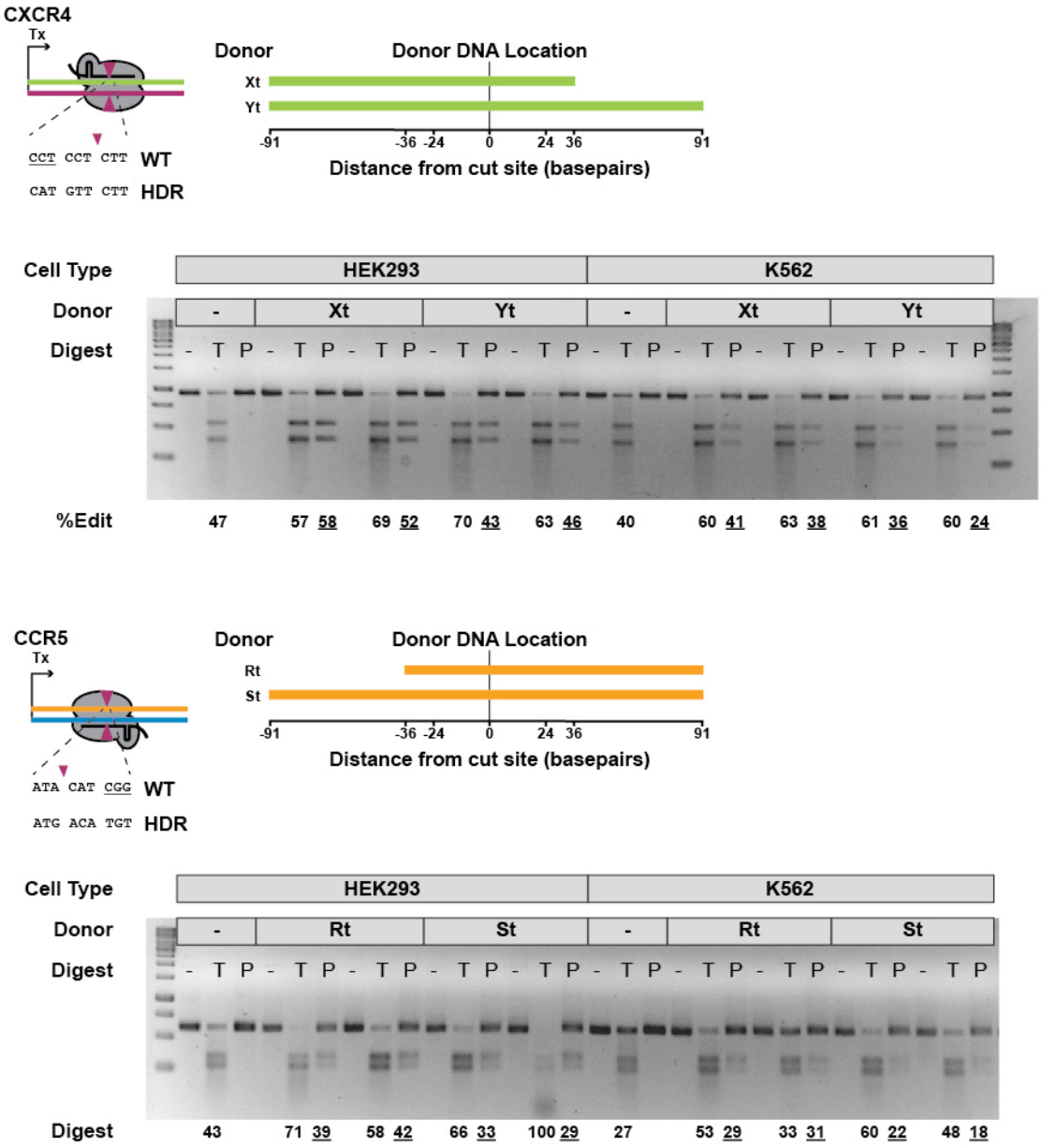
Cas9 targets the template strand of the EMX1 locus as diagrammed at left. Target strand (blue) or non-target strand (orange) donor ssDNAs were generated with the indicated overlaps on either side of the Cas9 cut site at EMX1. The sequences of the unedited and edited loci are presented inset (PAM sequence, underlined; cut site, magenta arrow; PciI site, bold font). HDR frequencies for editing with each donor are presented at right as mean +/- SD for n≥2 two independent experiments



Supplementary Figure 7

Editing the EMX1 locus with and without donor DNA.

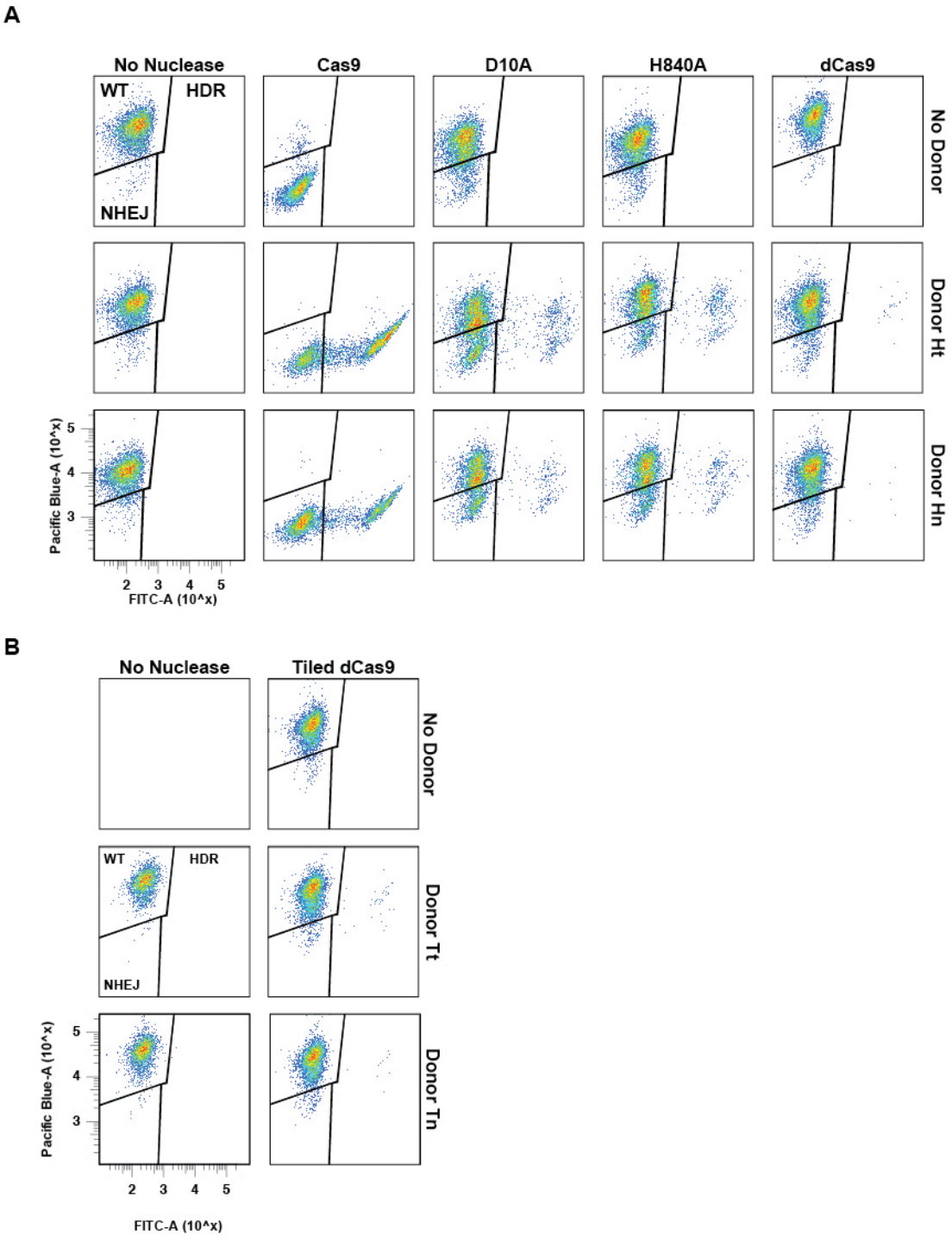
(A) The EMX1 locus is not cut as efficiently as the BFP locus. PCR amplification and T7E1 digestion were performed on cells edited using the indicated donor DNA (N/A – no donor, N/C – no Cas9). %Cut was quantified by gel densitometry. Compare to 95% total editing seen at the BFP locus (Supplementary Figure 9A). (B) HDR incorporation of a PciI site into the EMX1 locus shows donor strand-bias. PCR amplification (-) or PCR amplification and PciI digestion (+) was performed on cells edited using the indicated donor DNA (N/A – no donor). %Cut was quantified by gel densitometry and used to generate bar graphs in Supplementary Figure 6. Each nucleofection was performed in biological duplicate



Supplementary Figure 8

Asymmetric donors stimulate HDR at the CXCR4 and CCR5 loci in HEK293 and K562 cells.

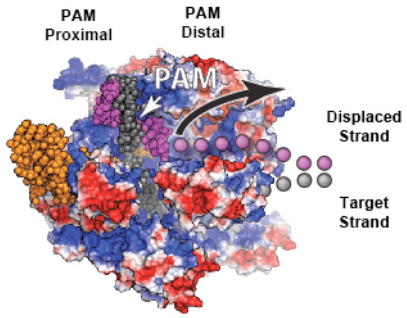
Cas9 was targeted to the CXCR4 or CCR5 loci in HEK293 and K562 cells. Target strand donors with the diagrammed overlaps were generated for each locus. The sequences of the unedited and edited loci are presented inset (PAM sequence, underlined; cut site, magenta arrow). T7 editing (T) and HDR frequencies (P; underlined) are presented at the bottom of each gel. Each nucleofection was performed in biological duplicate.



Supplementary Figure 9

Sample flow cytometry plots.

(A) Representative flow cytometry data used to create bar graphs shown in Figure 3B. (B) Representative flow cytometry data used to create bar graphs shown in Figure 3C.



Supplementary Figure 10

Structural data is consistent with asymmetric release of substrate by Cas9.

A surface electrostatic view of Cas9, sgRNA (orange), and non-target (purple) or target (grey) DNA strands7. PAM-Cas9 interaction, white arrow; putative path of non-target strand, purple dots; presumed direction of non-target strand extrusion, black arrow.

Supplementary Note 1: Sequence of Editing Reagents

sgRNA template DNA (protospacer annealing in CAPS)

λ1
ccagtgaattctaatacgactcactatagACGCATAAAGATGAGACGCGt
tttagagctatgctgttttggaaacaaaacagcatagcaagttaaaataa
ggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttt
tg

A
ccagtgaattctaatacgactcactataggCCATATCTGTGAGGGAGCCA
gttttagagctatgctgttttggaaacaaaacagcatagcaagttaaaat
aaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttt
tttg

1
ccagtgaattctaatacgactcactataggAGATTCTAAATCCTGCTCCT
gttttagagctatgctgttttggaaacaaaacagcatagcaagttaaaat
aaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgctttt
tttg

B
ccagtgaattctaatacgactcactataGGTCTCGTAGTCCGGTGAGCgt
tttagagctatgctgttttggaaacaaaacagcatagcaagttaaaataa
ggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttt
tg

2
ccagtgaattctaatacgactcactataGGGGAGAGTGACCGGCTCACgt
tttagagctatgctgttttggaaacaaaacagcatagcaagttaaaataa
ggctagtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttt
tg

AAVS1-F
ggatcctaatacgactcactatagTGTCCCTAGTGGCCCCACTGgtttta
gagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaa
aagtggcaccgagtcggtgctttttt

AAVS1-R
ggatcctaatacgactcactatagACAGTGGGGCCACTAGGGACgtttta
gagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaa
aagtggcaccgagtcggtgctttttt

BFP-L2
ggatcctaatacgactcactatagGCTGAAGCACTGCACGCCATgtttta
gagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaa
aagtggcaccgagtcggtgctttttt

EMX1-F
ggatcctaatacgactcactatagCGATGTCACCTCCAATGACTgtttta
gagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaa

aagtggcaccgagtcggtgctttttt

Substrate DNA (protospacer, bold; PAM, bold underline)

S1 AGCAGAAATCTCTGCT**GACGCATAAAGATGAGACGCTGG**AGTACAAACGT
CAGCT

PAM- AGCAGAAATCTCTGCT**GACGCATAAAGATGAGACGC**TcGAGTACAAACGT
CAGCT

NH tatgagatgactctga**GACGCATAAAGATGAGACGCTGG**gtgacctaacg
taaga

D1 GGGATGGGAGGTGTG**AAGATTCTAAATCCTGCTCCTTGGCTCCCTCACAG
ATATGG**CCCAGAAAGGCCGC**GGTCTCGTAGTCCGGTGAGCCGGTCACTCT
CCCC**GAGGTCCCACACTCCC

BFP ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGT
CGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG
GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACC
ACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGA**CCCA
TGGCGTGCAGTGCTTCAGC**CGCTACCCCGACCACATGAAGCAGCACGACT
TCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTC
TTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGG
CGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAAC
GTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAA
GATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCAC
TACCTGAGCACCCAGTCCAAGCTGAGCAAAGACCCCAACGAGAAGCGCGA
TCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCA
TGGACGAGCTGTACAAG

EMX1 AATGGGGAGGACAT**CGATGTCACCTCCAATGACTAGG**GTGGGCAACCACA

AAVS1 TCTGTCACCAAT**CCTGTCCCTAGTGGCCCCACTGTGG**GGTGGAGGGGACA

CXCR4 TGTCAGTGGCCGA**CCTCCTCTTTGTCATCACGCTTC**CCTTCTGGGC

CCR5 TCAAGTCCAATCTA**TGACATCAATTATTATACATCGG**AGCCCTGCCA

Donor

At (CR236)	CCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC TCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGAC CACATGAAGCAGCACGACTT
An (CR237)	AAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCC GTACGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGG TGGTGCAGATGAACTTCAGG
Bt (CR238)	CCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCC GACCACATGA
Bn (CR239)	TCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTG GTCACGAGGG
Ct (CR254)	CCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCC GACCACATGAAGCAGCACGACTT
Cn (CR255)	AAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCC GTACGTCAGGGTGGTCACGAGGG
Dt (CR256)	CCCTCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCC GACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTA CGTCCAGGAGCGC
Dn (CR257)	GCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCT GCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGG GTGGTCACGAGGG
Et (CR278)	CCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC TCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCC
En (CR279)	GGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTGGTCACGAGGG TGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGG
Ft (CR280)	CCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC TCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAG
Fn (CR281)	CTGAAGCACTGCACGCCGTACGTCAGGGTGGTCACGAGGGTGGGCCAGGG CACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGG

Gt (CR252)	CCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCC TCGTGACCACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGAC CACATGA
Gn (CR253)	TCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTG GTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAA CTTCAGG
Ht (CR282)	GCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCT GCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACGTACGGCGTGCAGT GCTTCAGCCGCTACCCCGACCACATGA
Hn (CR283)	TCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTG GTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAA CTTCAGGGTCAGCTTGCCGTAGGTGGC
Tt (CR276)	TGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACGCTGAAG TTCATCTGCACCACCGGCAAGCTGCCGGTGCCCTGGCCCACCCTCGTGAC CACCCTGACGTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACGACATGA AGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAG
Tn (CR277)	CTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCA TGTCGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTACGTCAGGGTGGTC ACGAGGGTGGGCCAGGGCACCGGCAGCTTGCCGGTGGTGCAGATGAACTT CAGCGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACA
In (CR287)	AGGCCAATGGGGAGGACATCGATGTCACCTCCAATGacatgtGTGGGCAACC ACAAACCCACGAGGGCAGAGTGCTGCTTGCTGCTGGCCAGGCCCCTGCGTGG GCCCAAGCTGGACTCTGGCCACT
It (CR288)	AGTGGCCAGAGTCCAGCTTGGGCCCACGCAGGGGCCTGGCCAGCAGCAAGCA GCACTCTGCCCTCGTGGGTTTGTGGTTGCCCACacatgtCATTGGAGGTGAC ATCGATGTCCTCCCCATTGGCCT
Jn (CR289)	AGGCCAATGGGGAGGACATCGATGTCACCTCCAATGacatgtGTGGGCAACC ACAAACCC
Jt (CR290)	GGGTTTGTGGTTGCCCACacatgtCATTGGAGGTGACATCGATGTCCTCCCC ATTGGCCT

Xt (oCR408)	ATGGATTGGTCATCCTGGTCATGGGTTACCAGAAGAAACTGAGAAGCATGAC GGACAAGTACAGGCTGCACCTGTCAGTGGCCGACaTgtTCTTTGTCATCACG CTTCCCTTCTGGGCAGTTGATGC
Yt (oCR409)	ATGGATTGGTCATCCTGGTCATGGGTTACCAGAAGAAACTGAGAAGCATGAC GGACAAGTACAGGCTGCACCTGTCAGTGGCCGACaTgtTCTTTGTCATCACG CTTCCCTTCTGGGCAGTTGATGCCGTGGCAAACTGGTACTTTGGGAACTTCC TATGCAAGGCAGTCCATGTCATCTAC
Rt (oCR411)	ACAAAACCAAAGATGAACACCAGTGAGTAGAGCGGAGGCAGGAGGCGGGCTG CGATTTGCTTCACATTGATTTTTTGGCAGGGCTCacATGTATAATAATTGAT GTCATAGATTGGACTTGACACTT
St (oCR412)	ACAAAACCAAAGATGAACACCAGTGAGTAGAGCGGAGGCAGGAGGCGGGCTG CGATTTGCTTCACATTGATTTTTTGGCAGGGCTCacATGTATAATAATTGAT GTCATAGATTGGACTTGACACTTGATAATCCATcttgttccaccctgtgcat aaataaaaagtgatctttataaagt

qPCR Primers

ACT1B-Fwd	CTGGAACGGTGAAGGTGACA
ACT1B-Rev	AAGGGACTTCCTGTAACAACGC
AAVS1L-Fwd	CAGAAAAGCCCCATCCTTAGGC
AAVS1L-Rev	CGGGCAGGTCACGCATC
AAVS1R-Fwd	GCAGCAAACATGCTGTCCTG
AAVS1R-Rev	TTGCTTTCTTTGCCTGGACAC

PCR amplification primers

EMX1-F	GCCATCCCCTTCTGTGAATGTTAGAC
EMX1-R	GGAGATTGGAGACACGGAGAGCAG
CXCR4-F	AGAGGAGTTAGCCAAGATGTGACTTTGAAACC
CXCR4-R	GGACAGGATGACAATACCAGGCAGGATAAGGCC
CCR5-F	CTCCATGGTGCTATAGAGCA
CCR5-R	GCCCTGTCAAGAGTTGACAC

Supplementary Note 2: Full length (uncropped) gels used in main figures. Dashed boxes indicate region of interest presented in main figures.

Figure 1

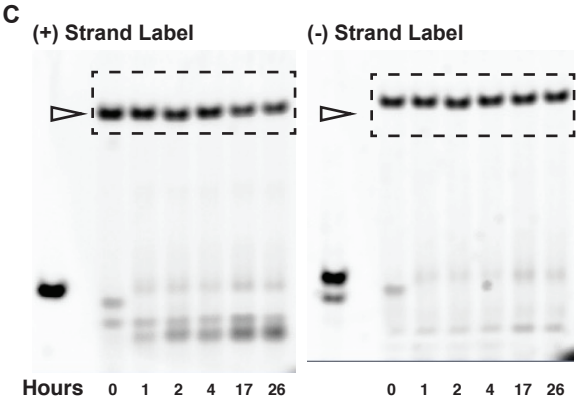


Figure 2

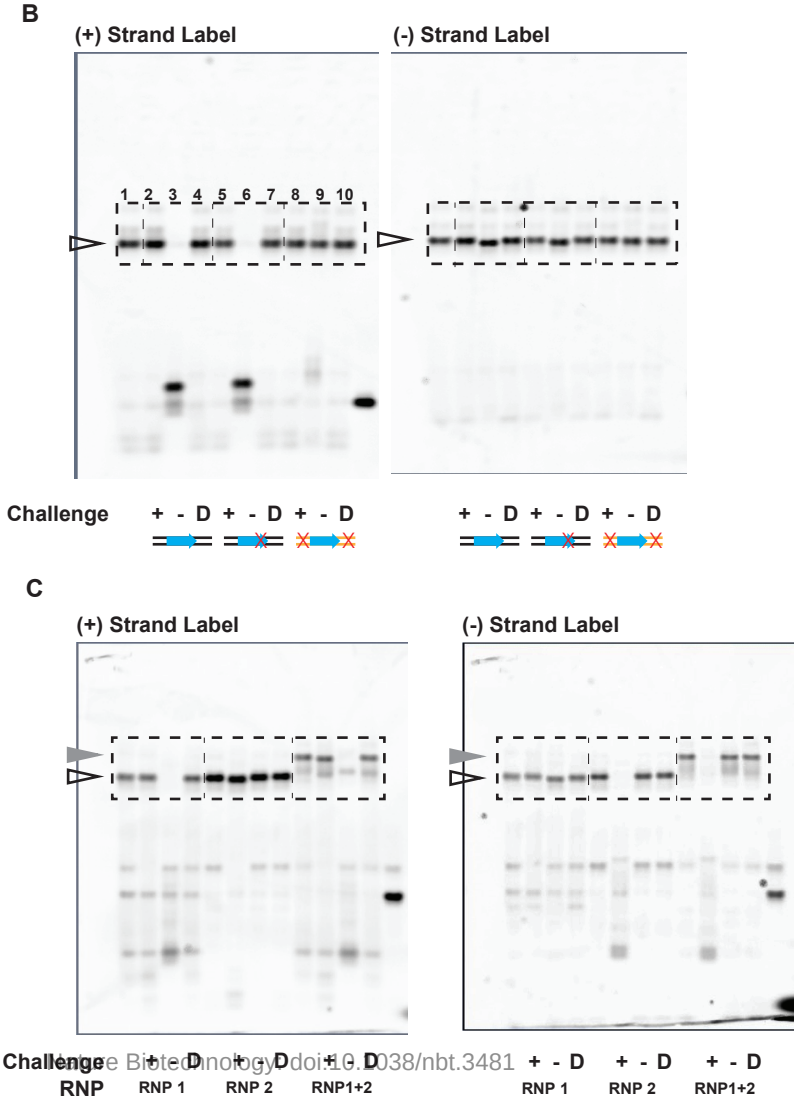
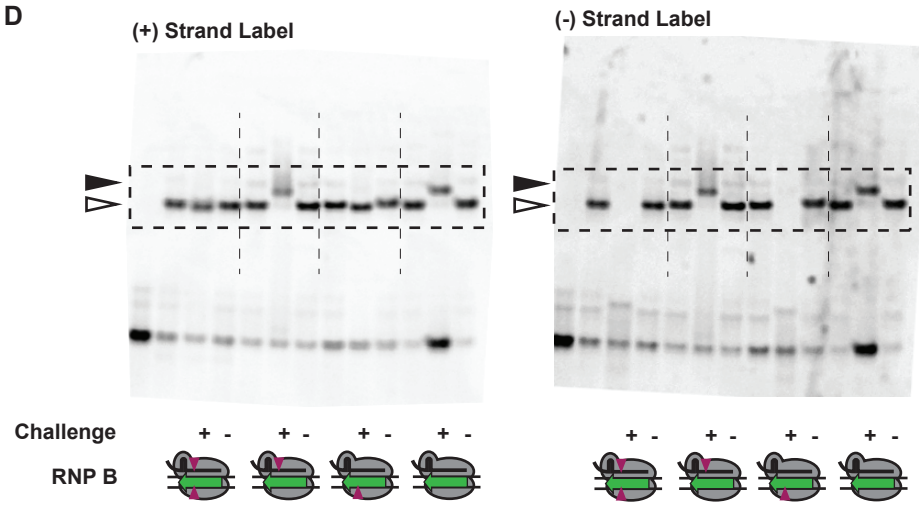
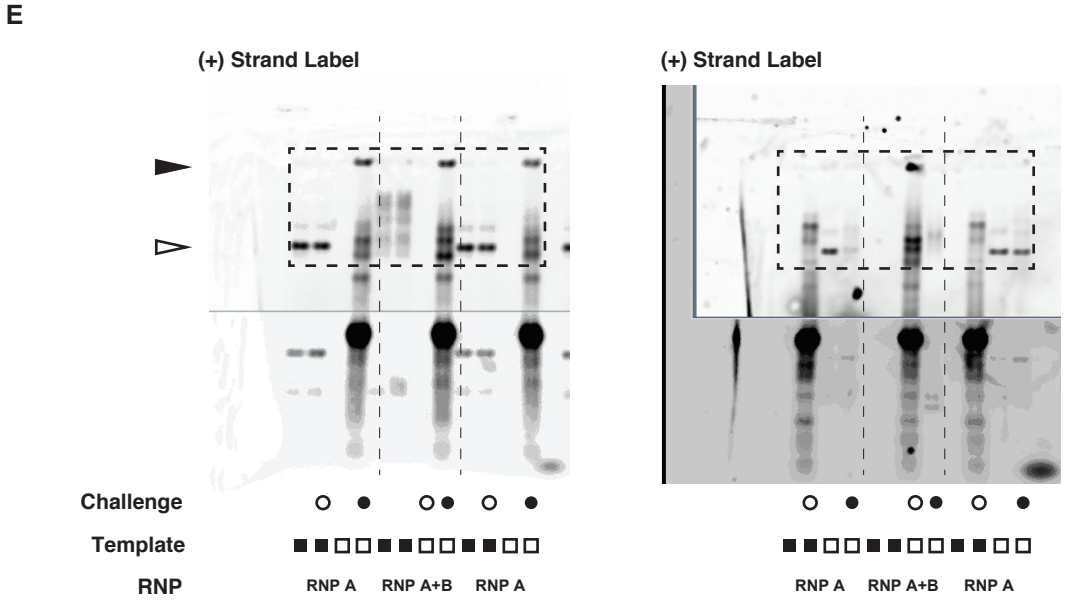


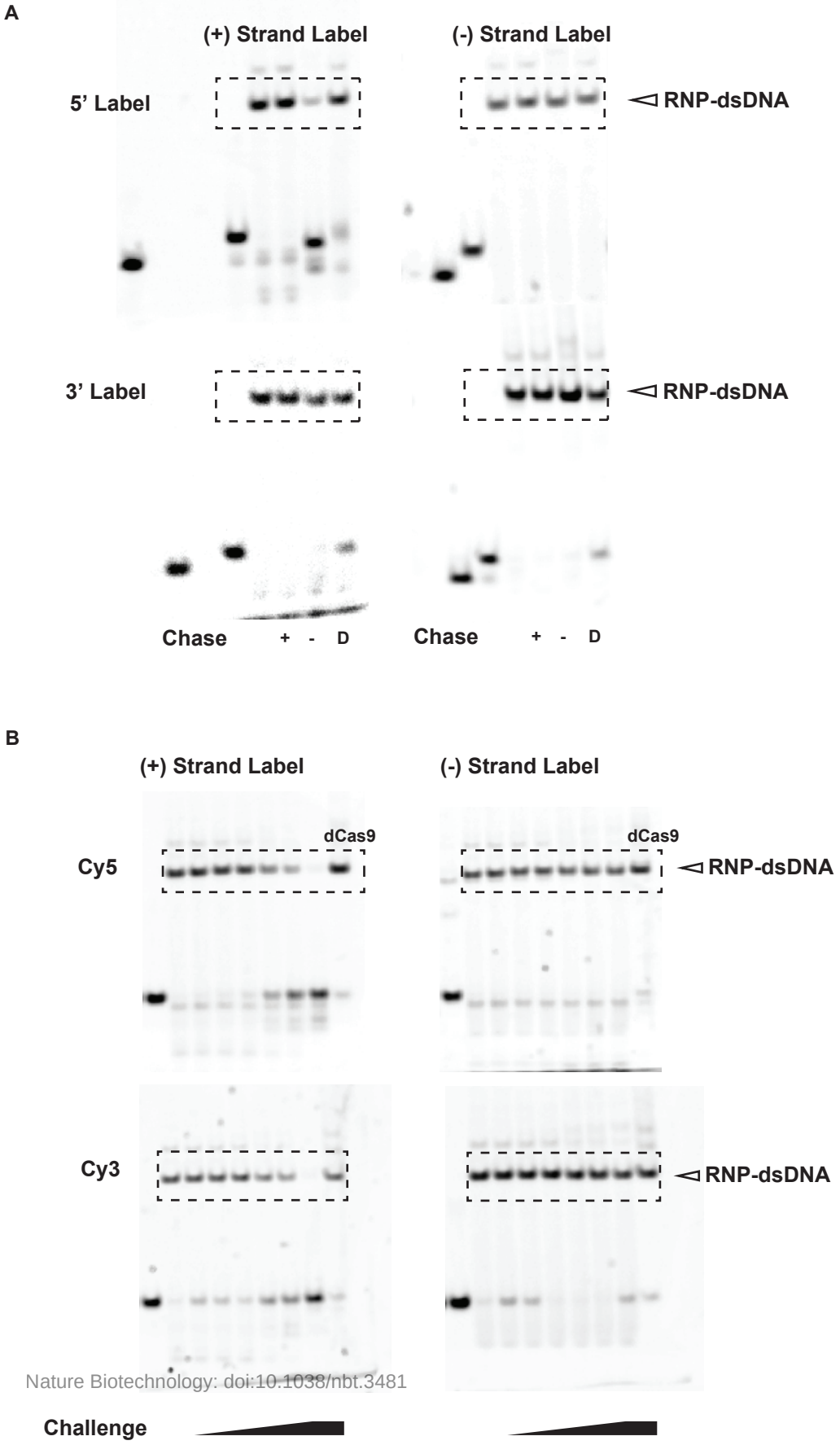
Figure 2



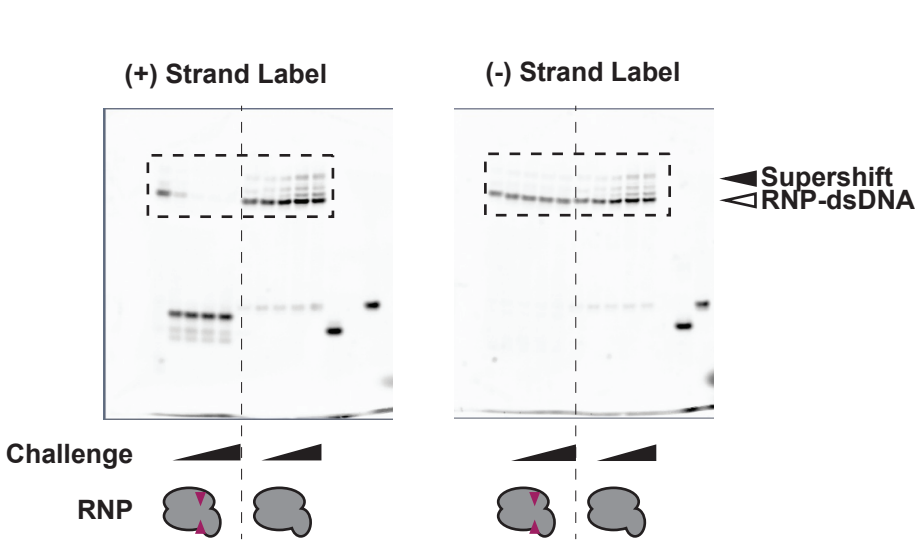


Two different exposures merged to form this image.

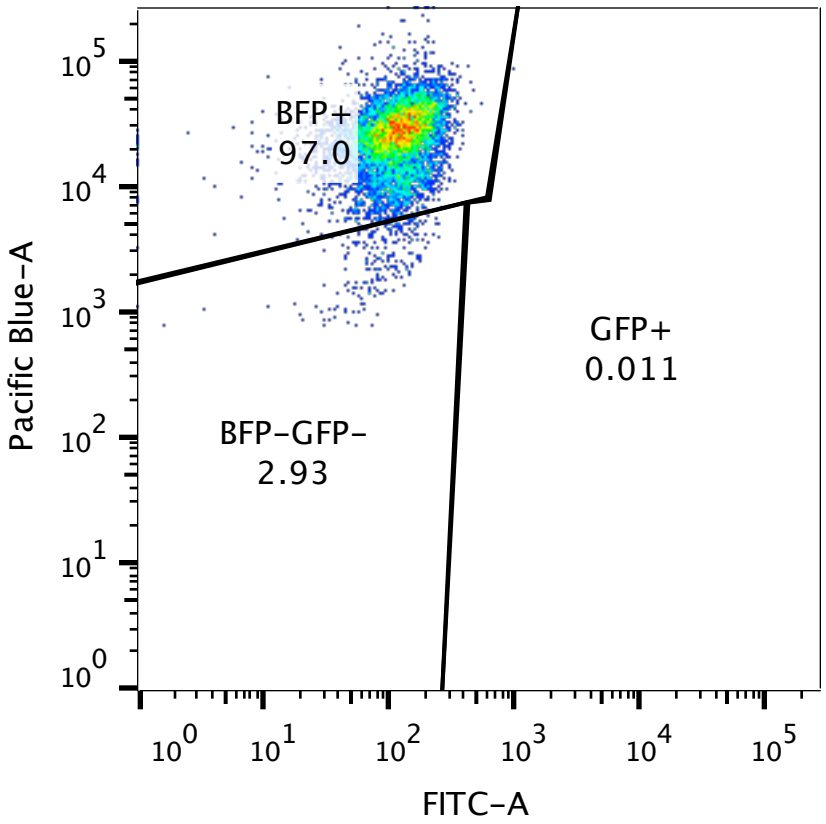
Supplementary Figure 3



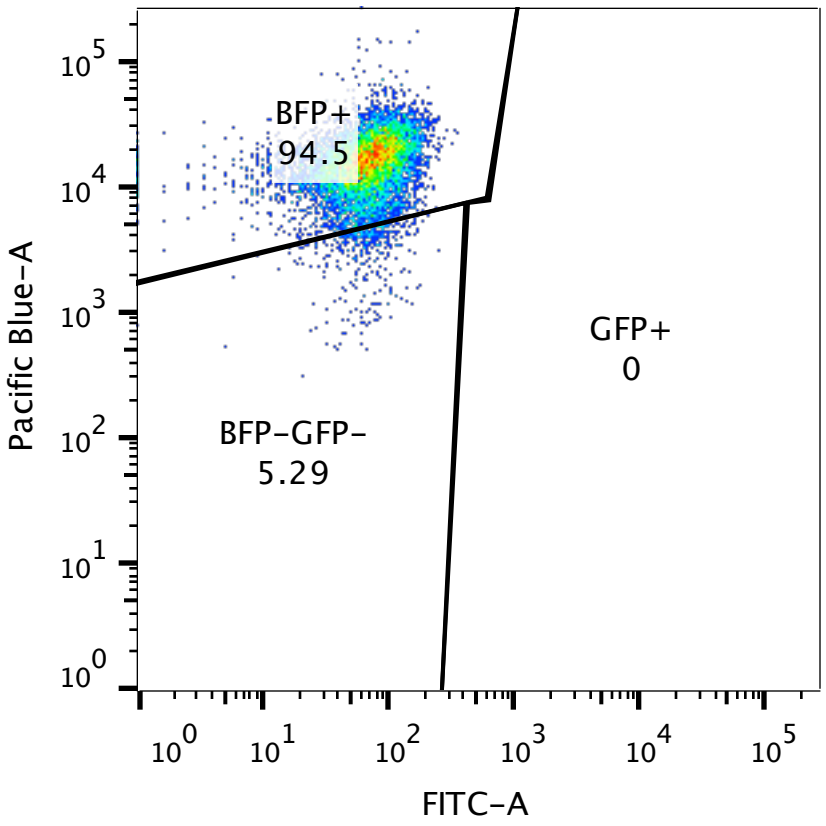
Supplementary Figure 3



Supplementary Note 3: All flow cytometry plots.

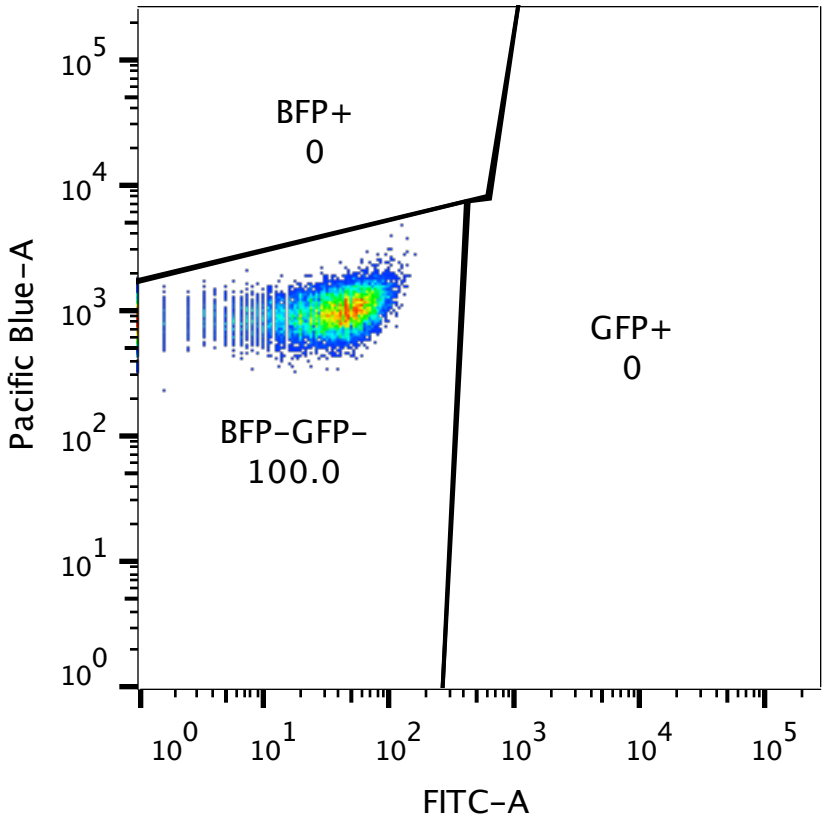


BFP Cell Line 1



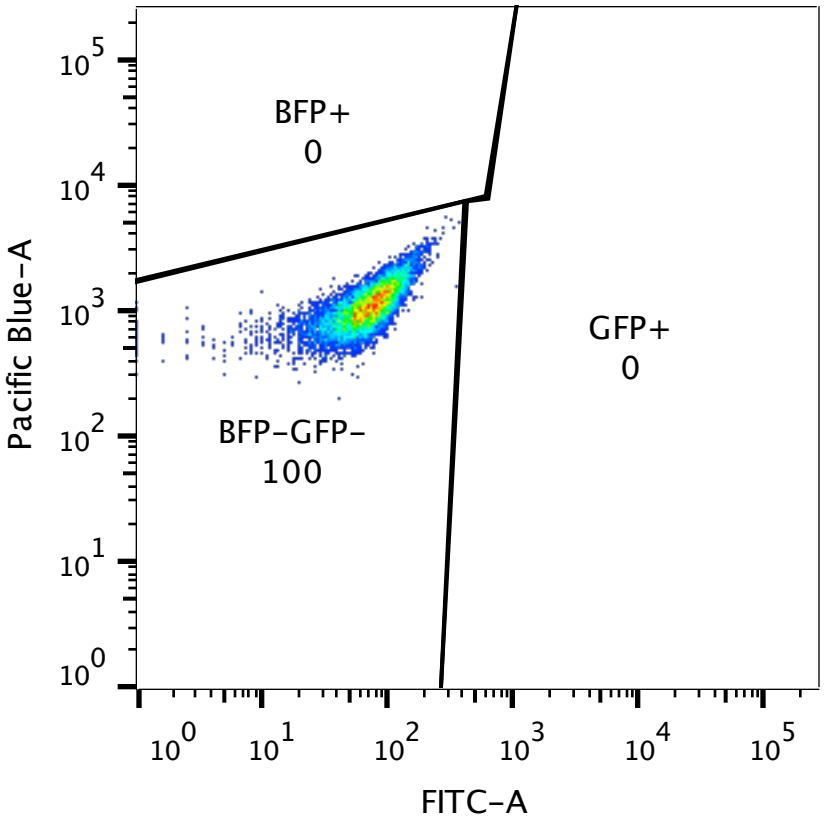
BFP Cell Line 2

Nature Biotechnology: doi:10.1038/nbt.3481



Parental (HEK293) Cell Line 1

Nature Biotechnology: doi:10.1038/nbt.3481



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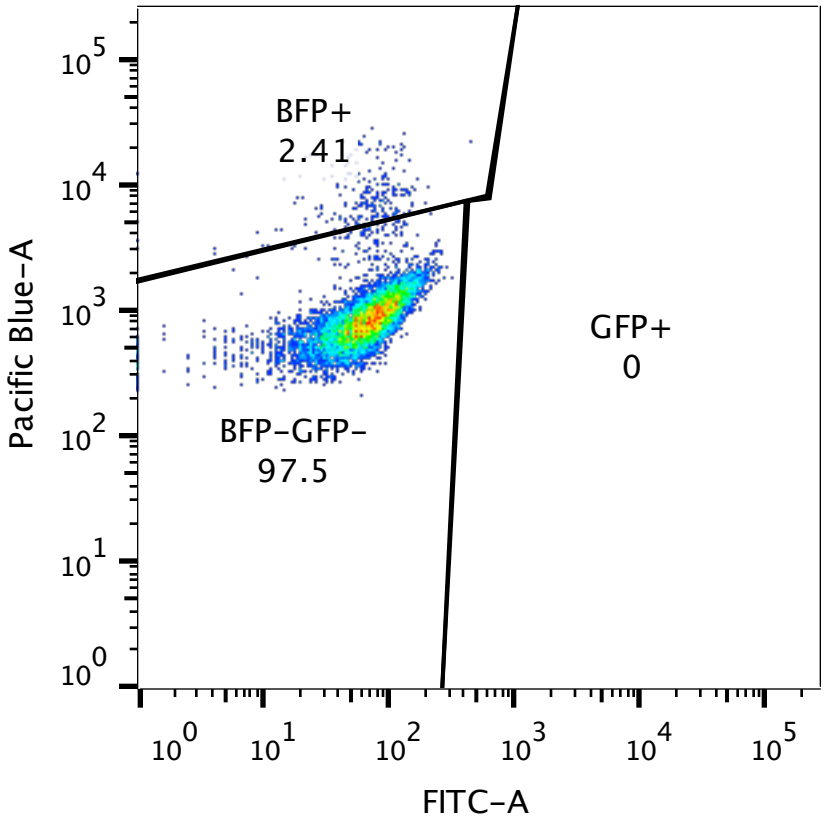
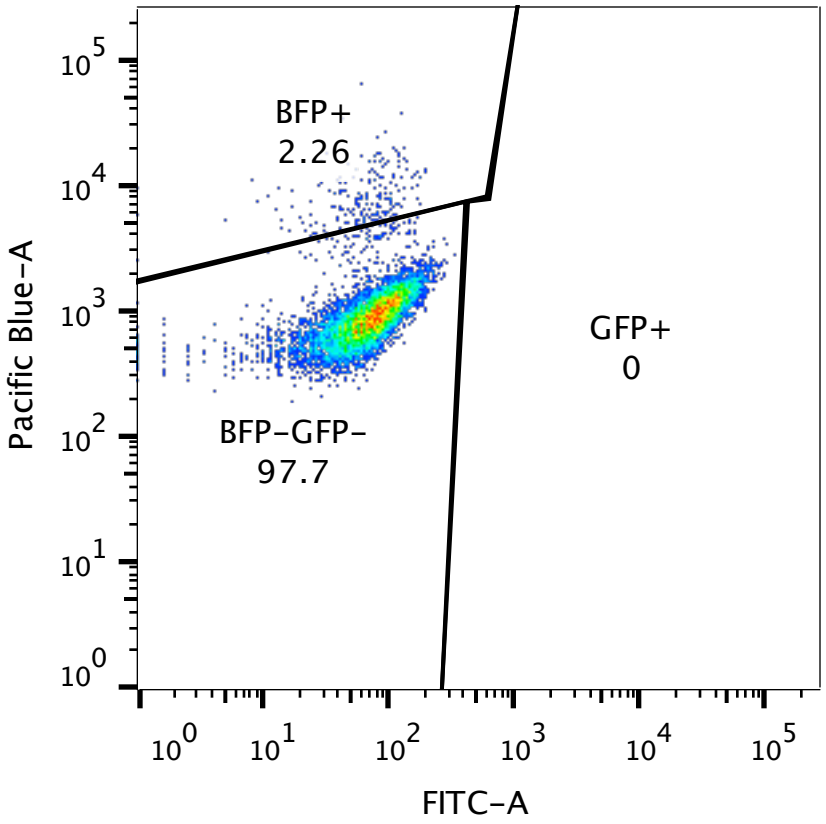
Nature Biotechnology: doi:10.1038/nbt.3481



Cas9 – No Donor 1

Nature Biotechnology: doi:10.1038/nbt.3481



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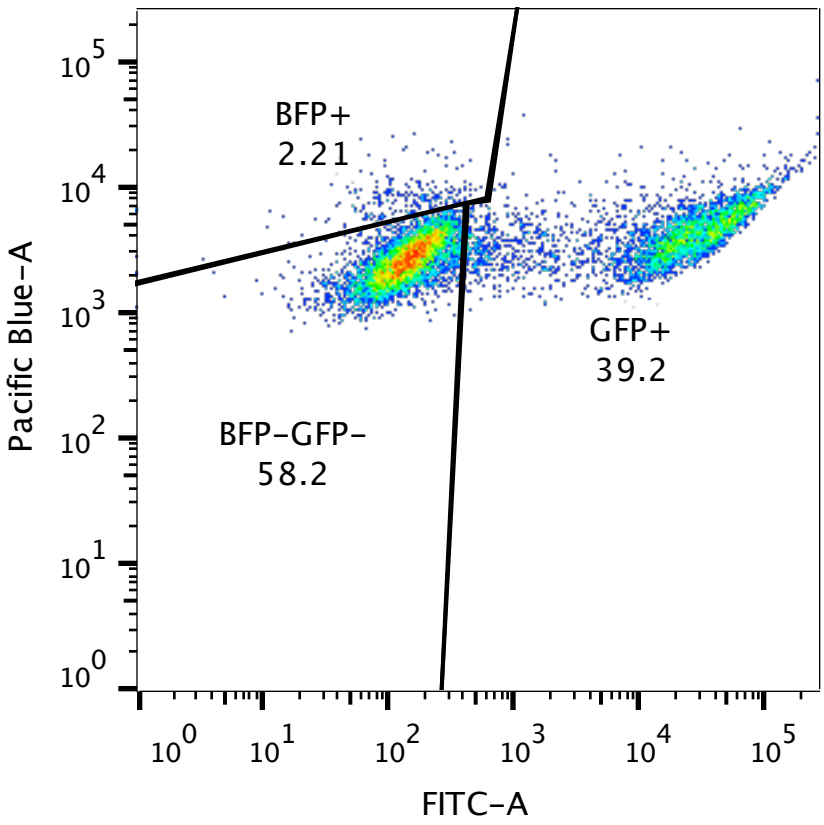
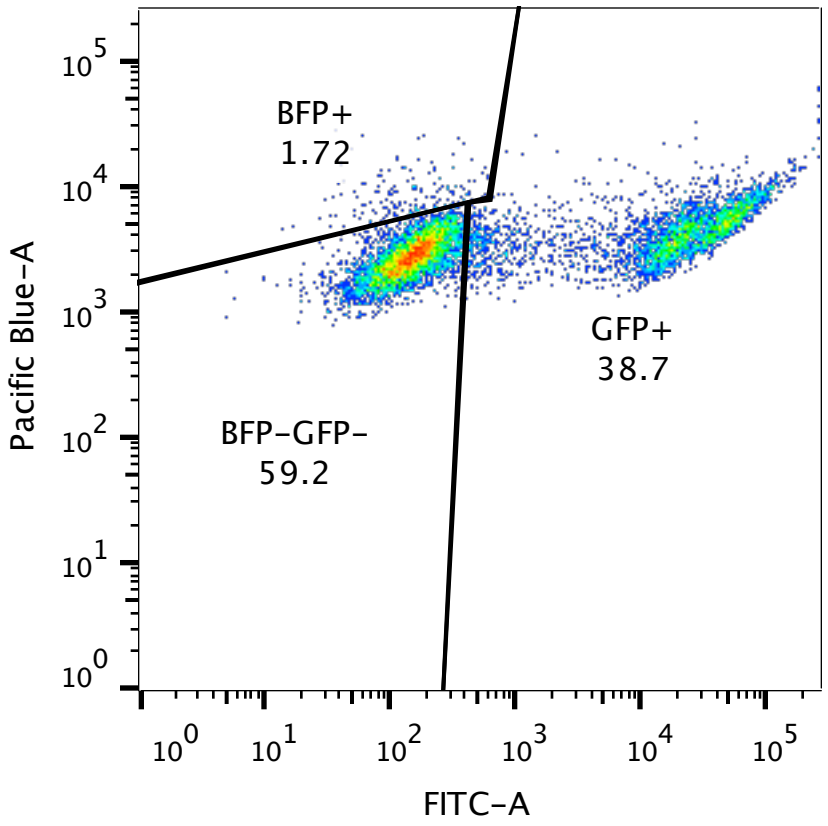
Nature Biotechnology: doi:10.1038/nbt.3481



Cas9 – Donor At 1



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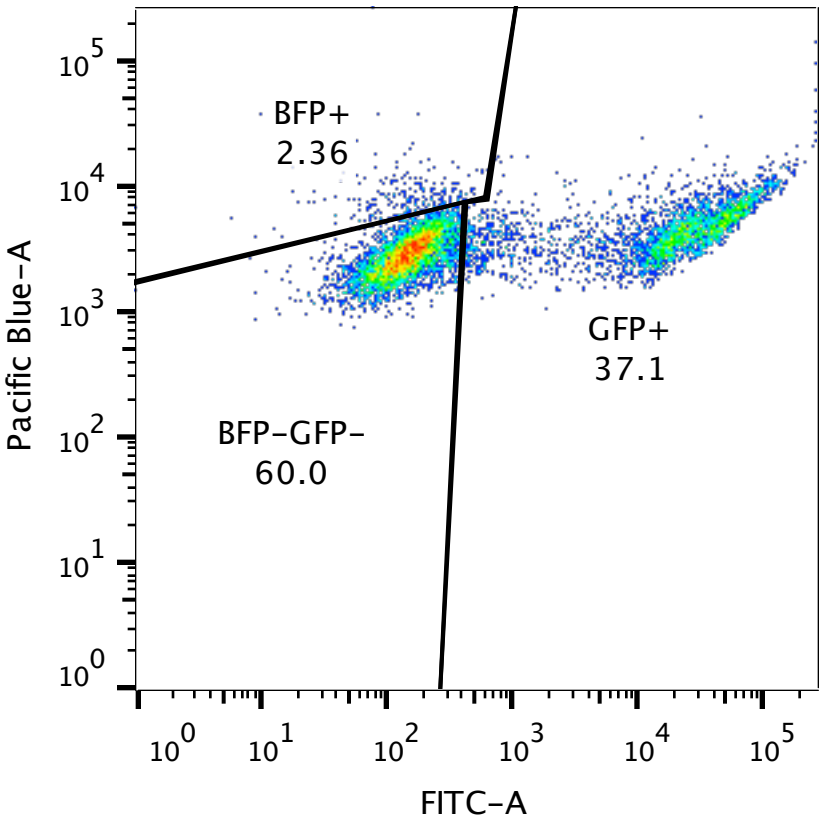
Nature Biotechnology: doi:10.1038/nbt.3481



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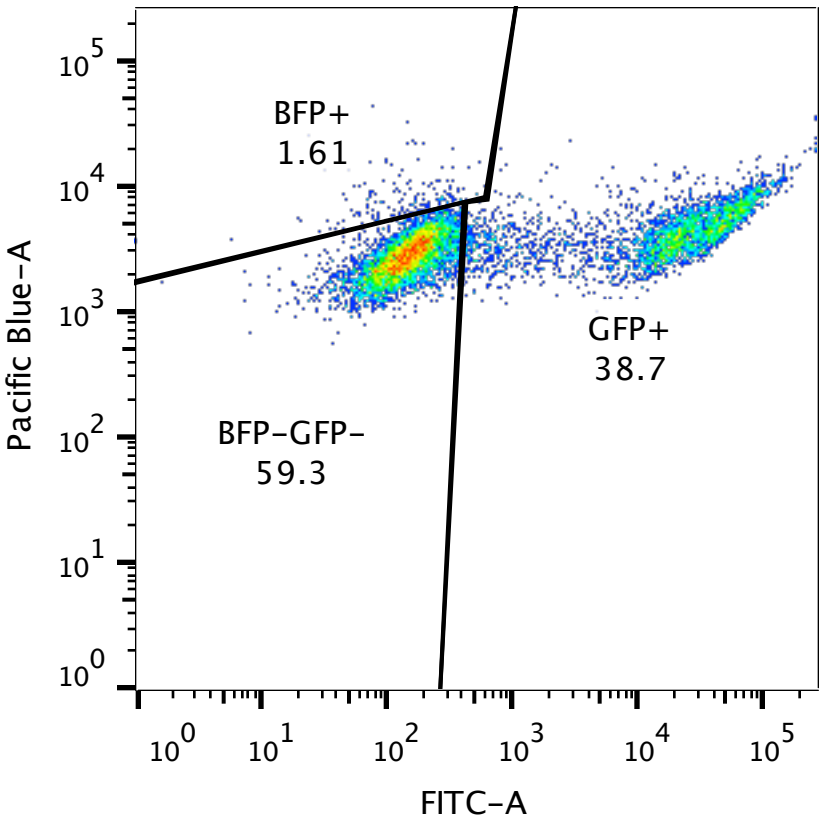
Nature Biotechnology: doi:10.1038/nbt.3481



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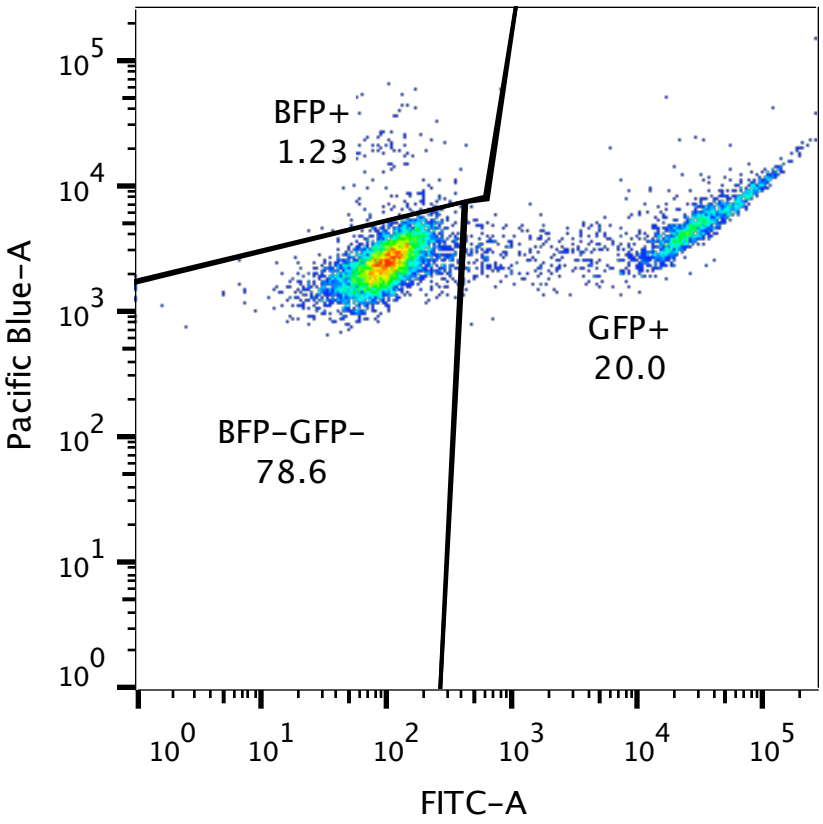
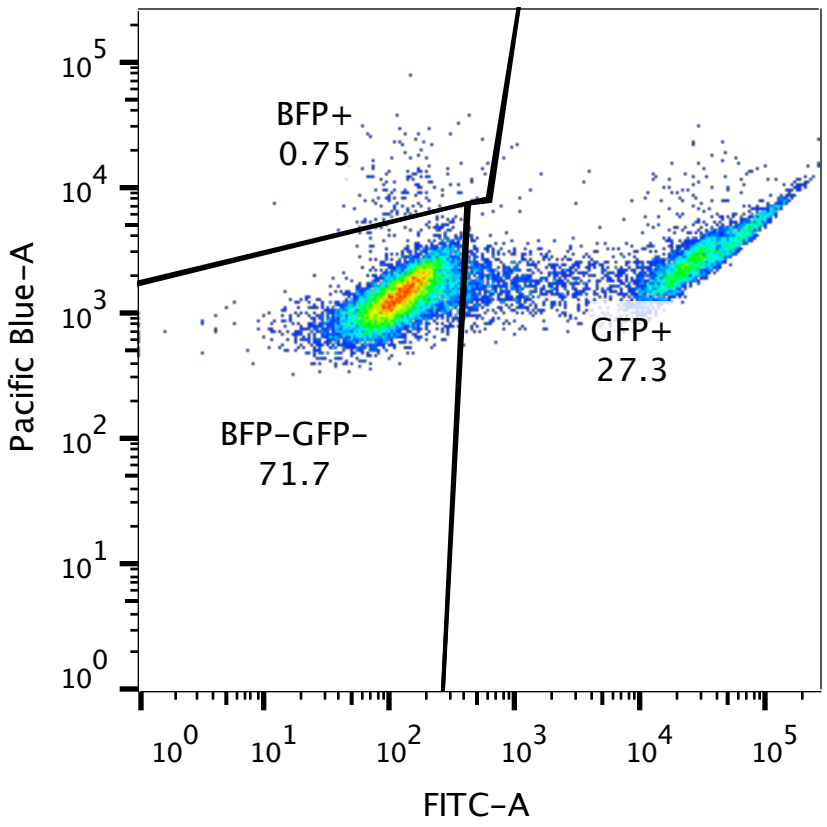


Cas9 – Donor Bt 1

Nature Biotechnology: doi:10.1038/nbt.3481



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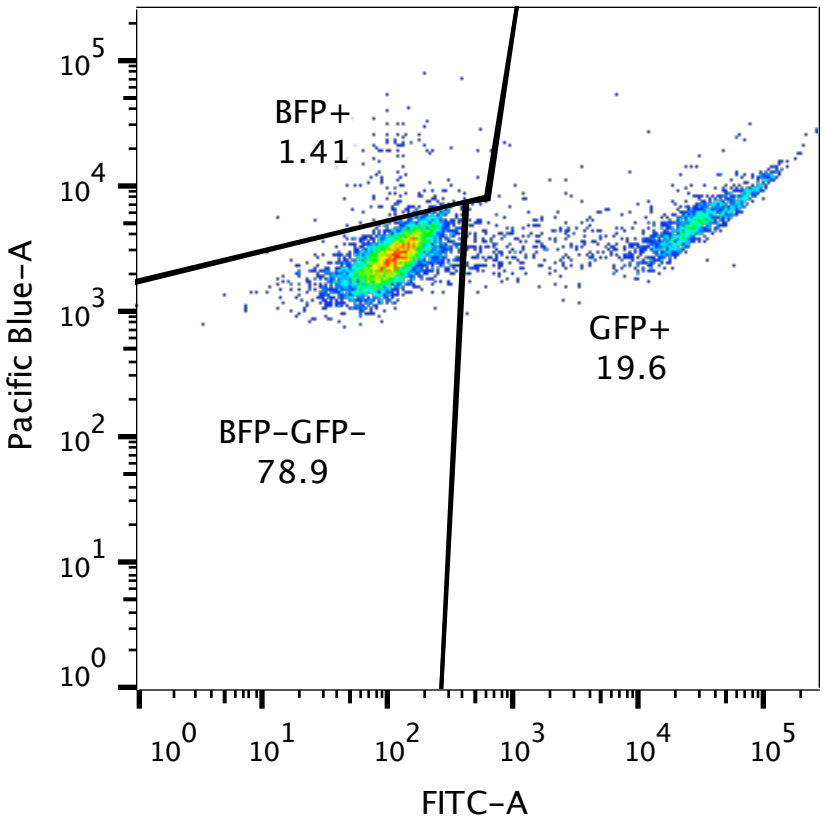

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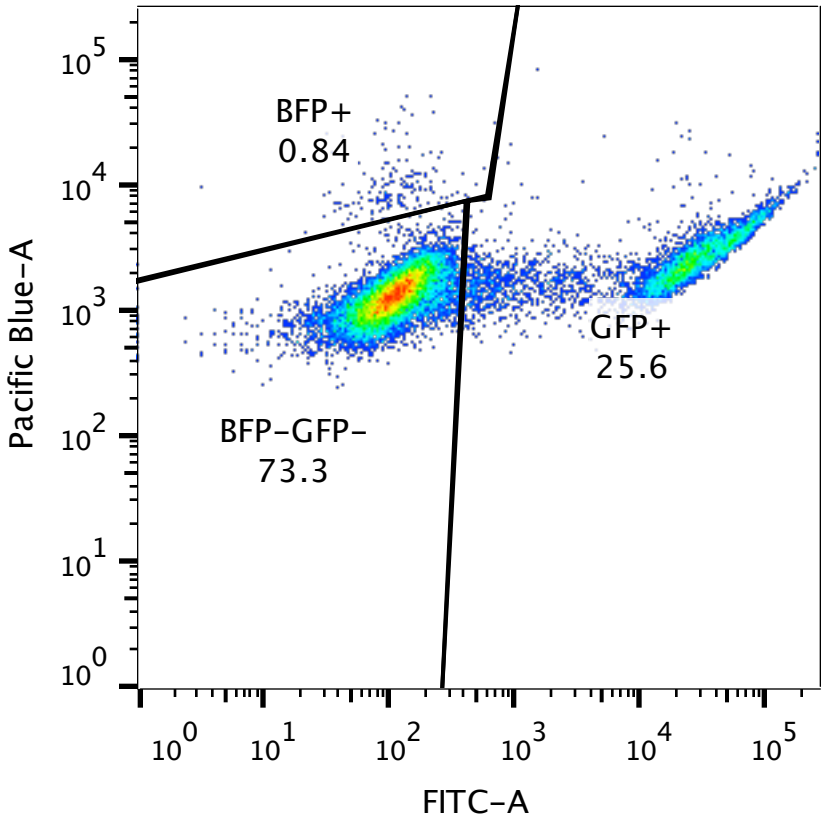
Nature Biotechnology: doi:10.1038/nbt.3481



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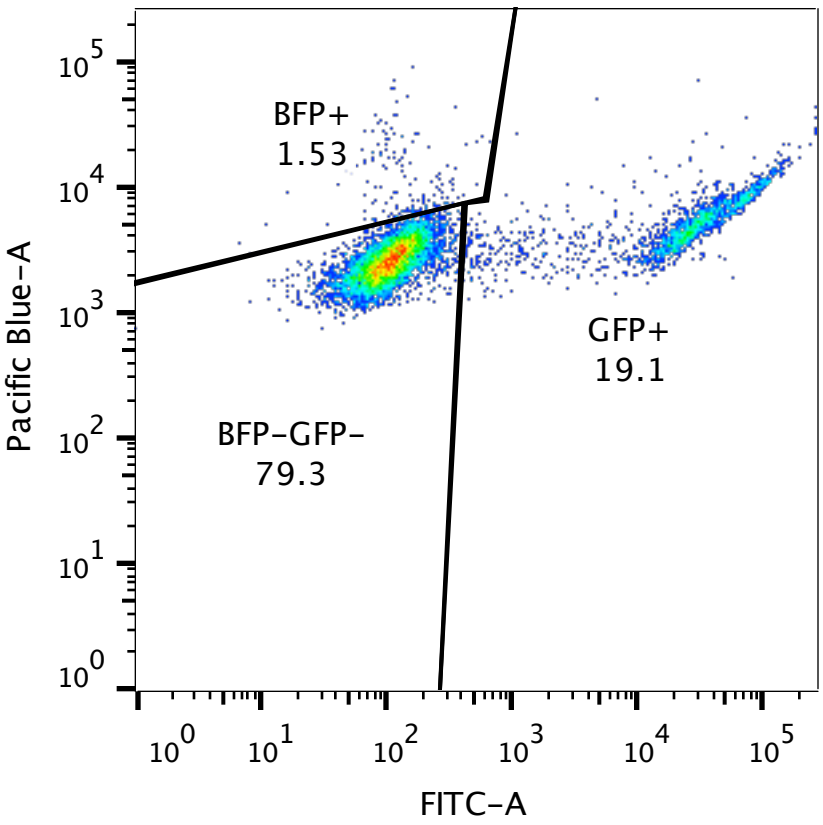

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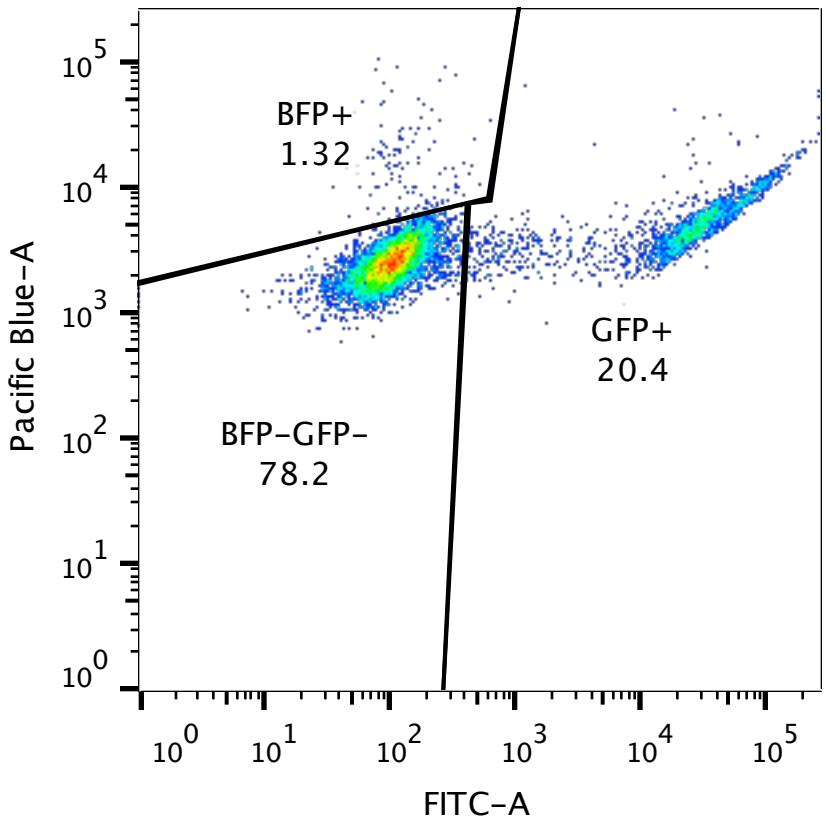

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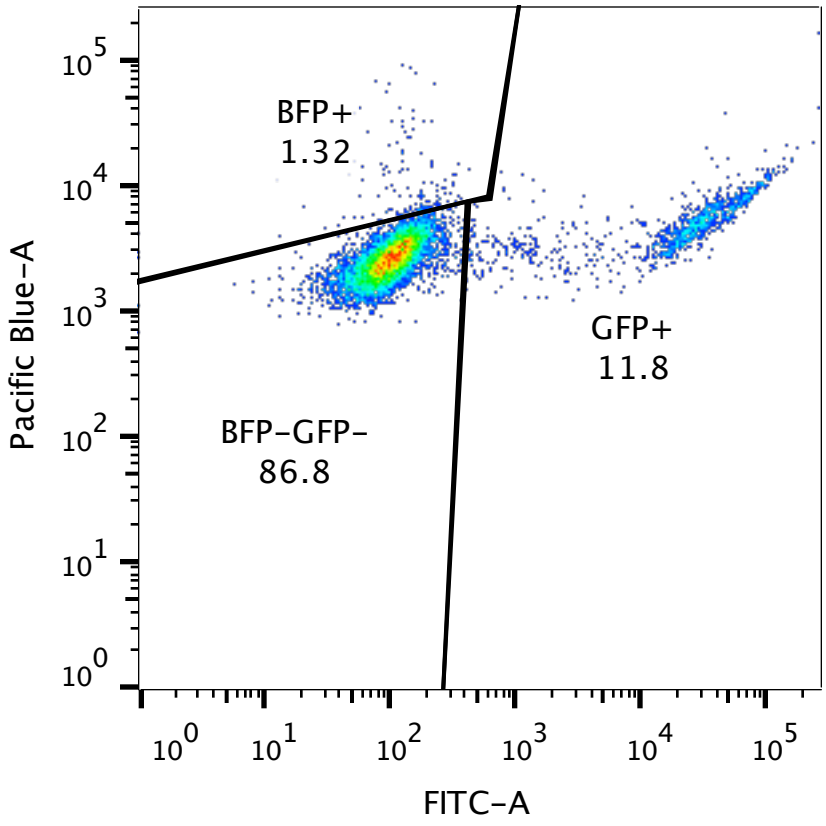
Nature Biotechnology: doi:10.1038/nbt.3481



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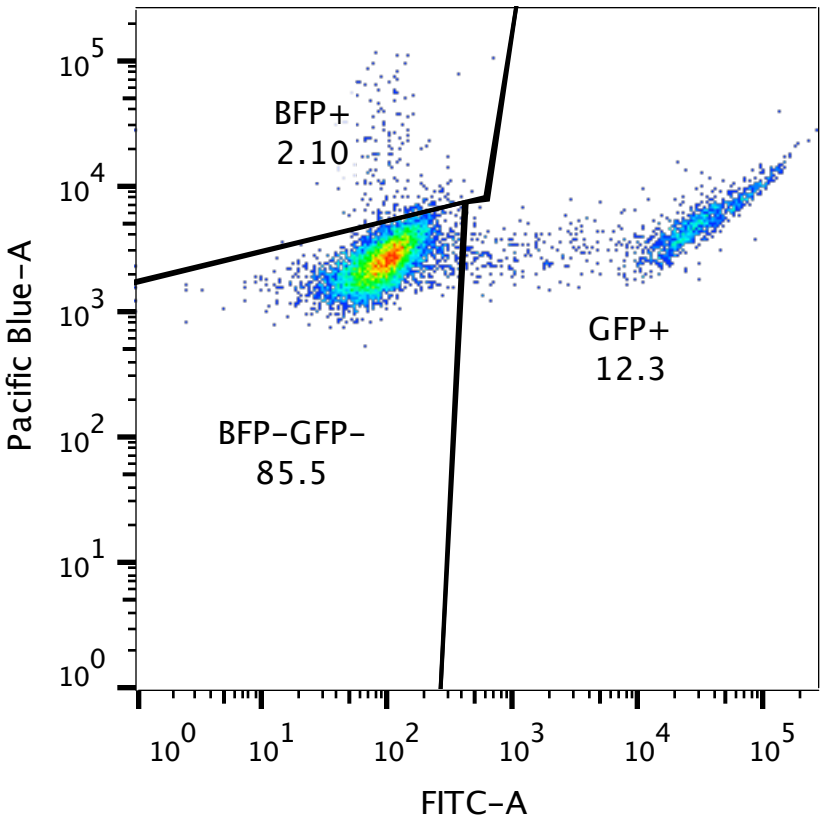

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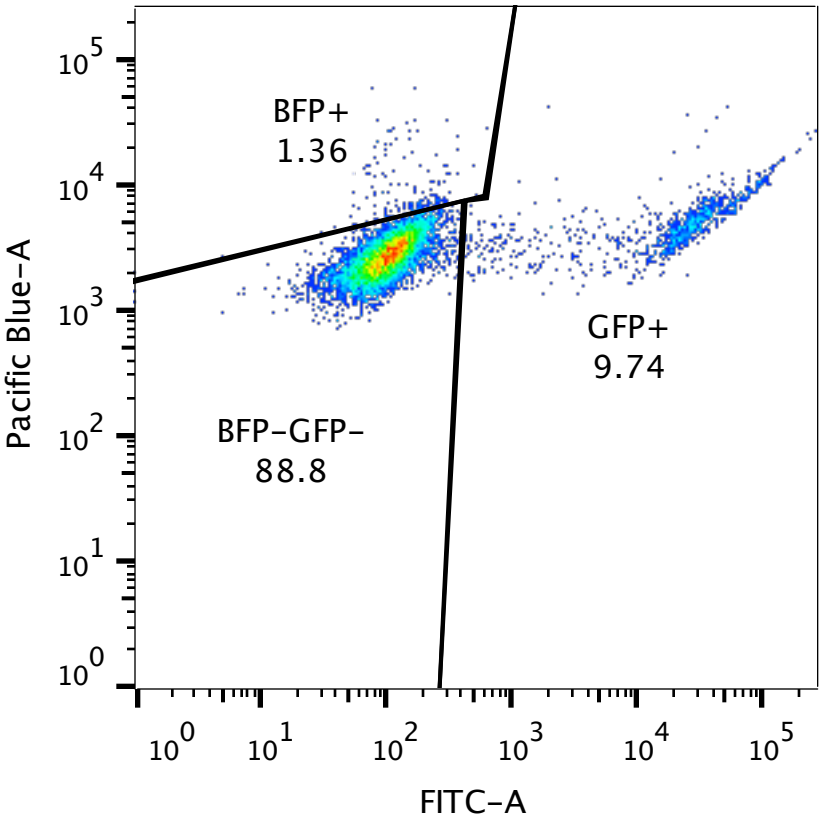

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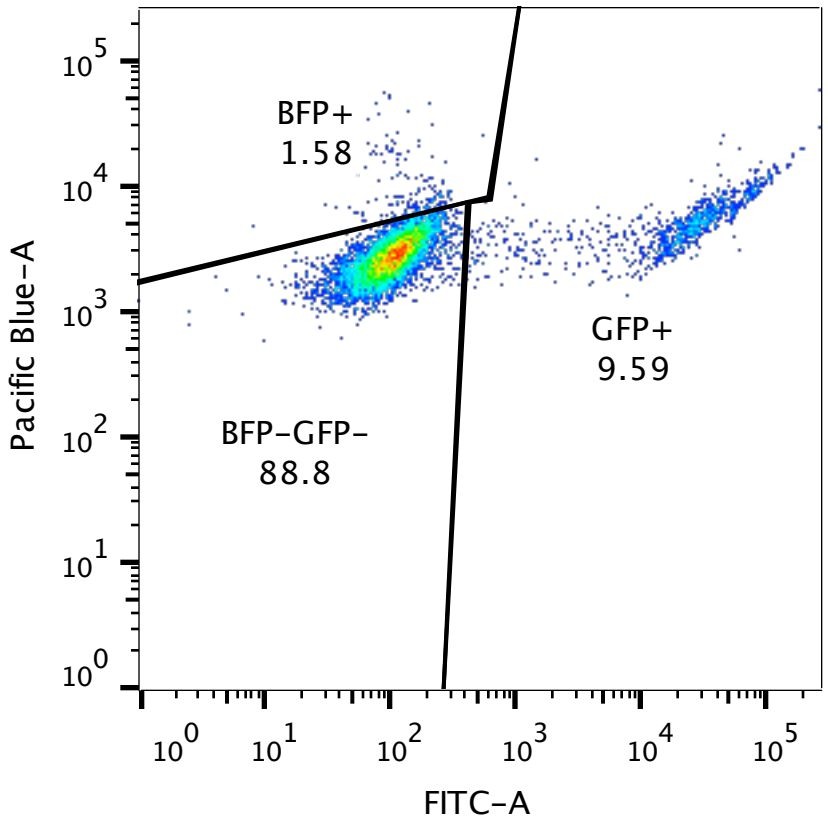

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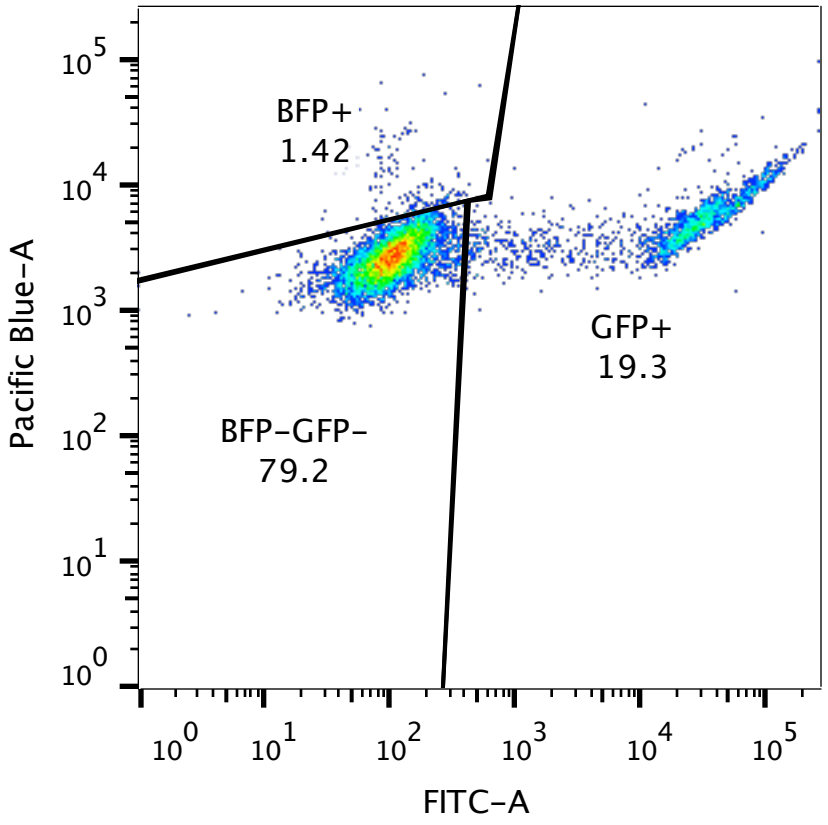

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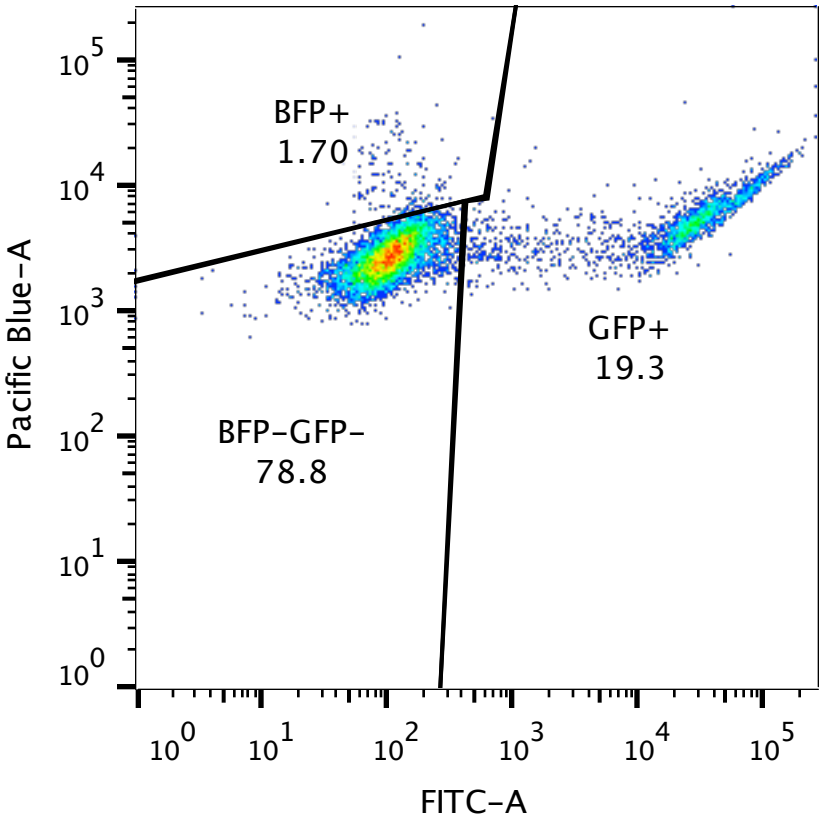

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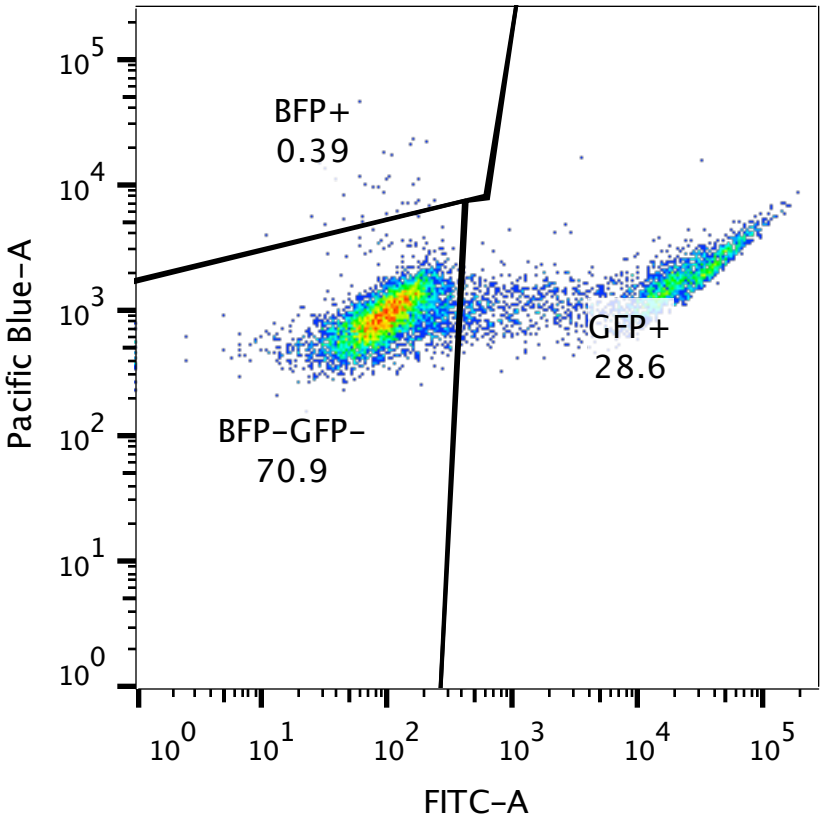

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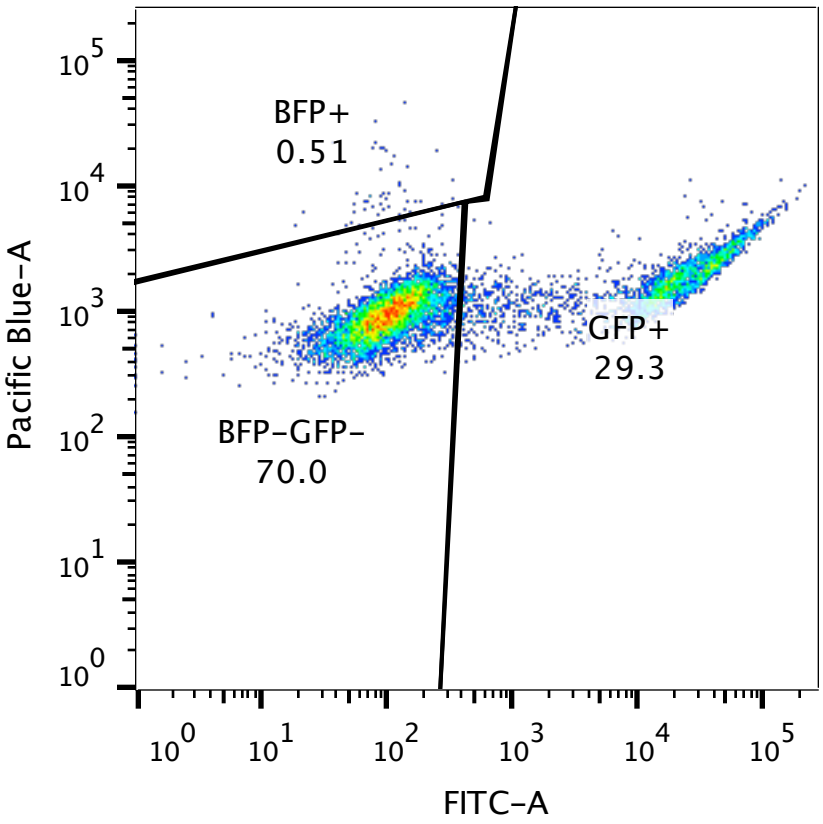

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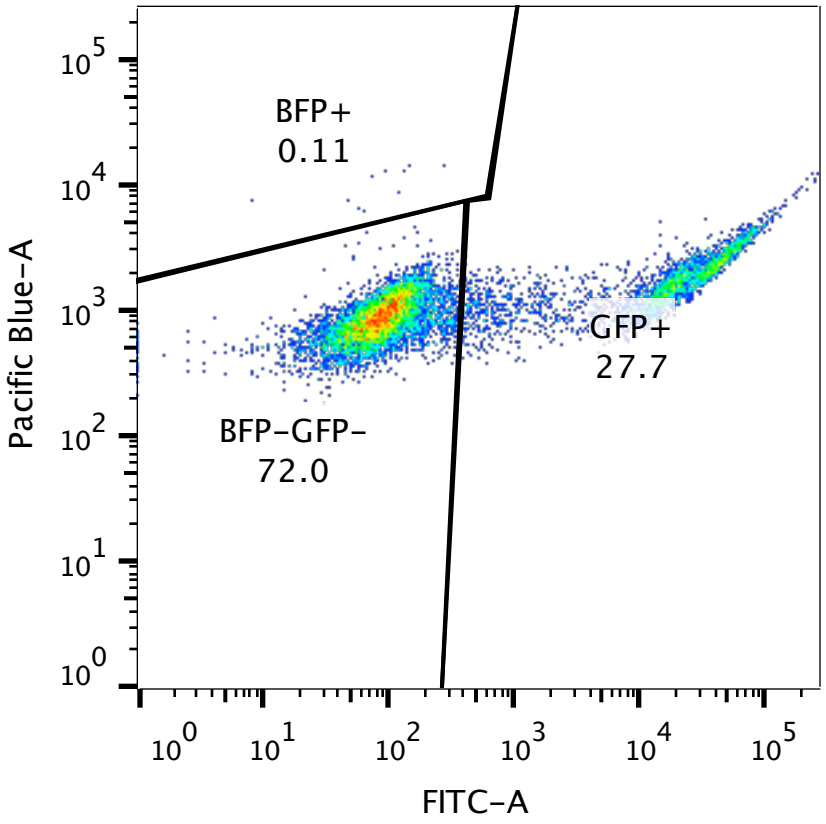

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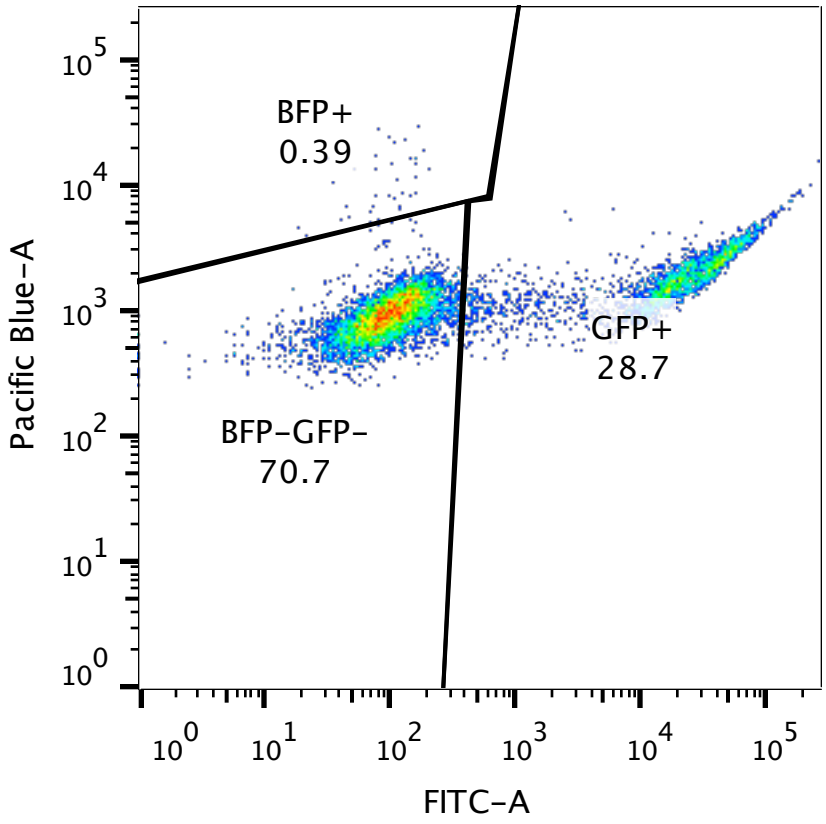

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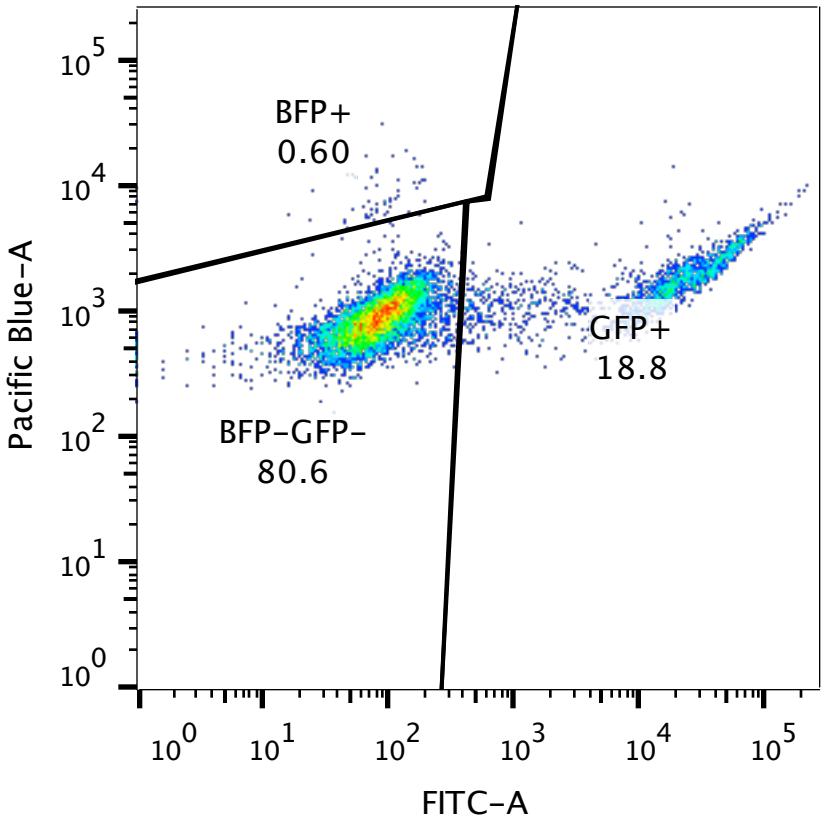

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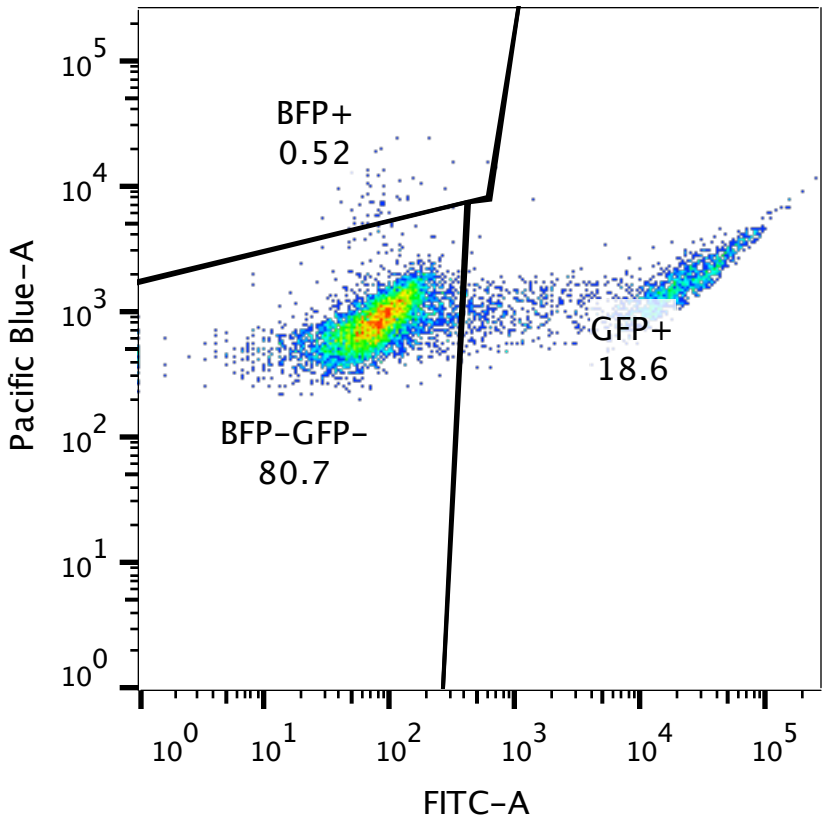

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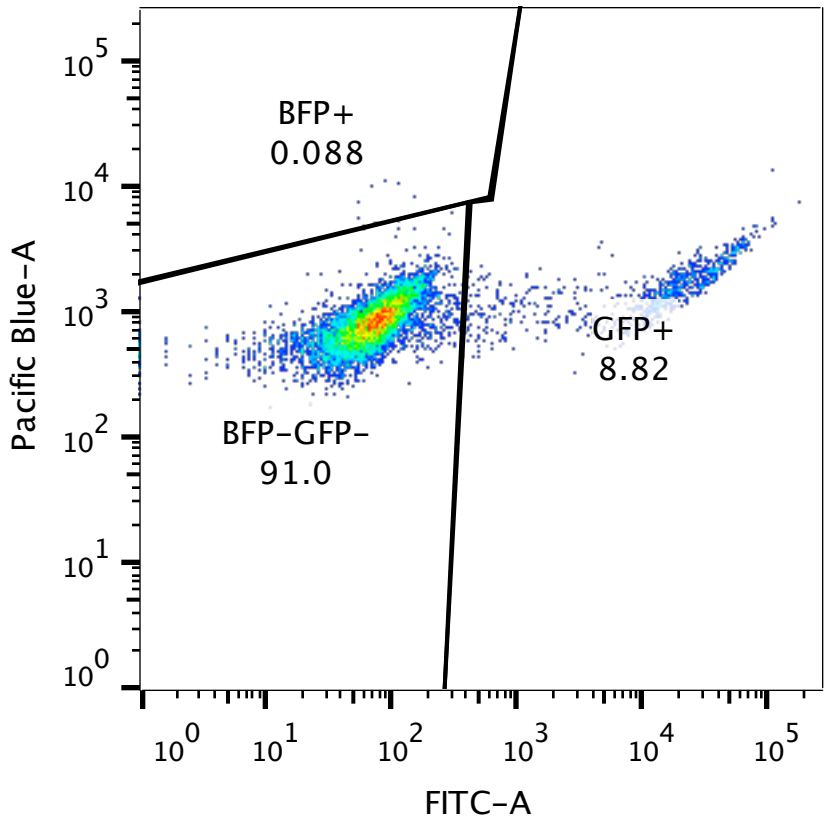

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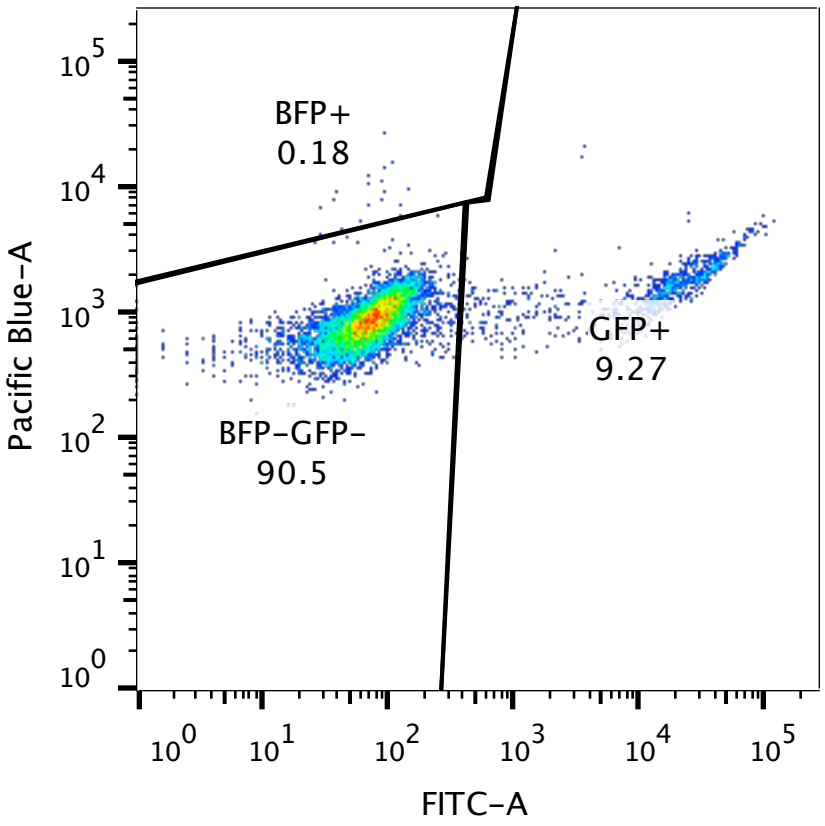

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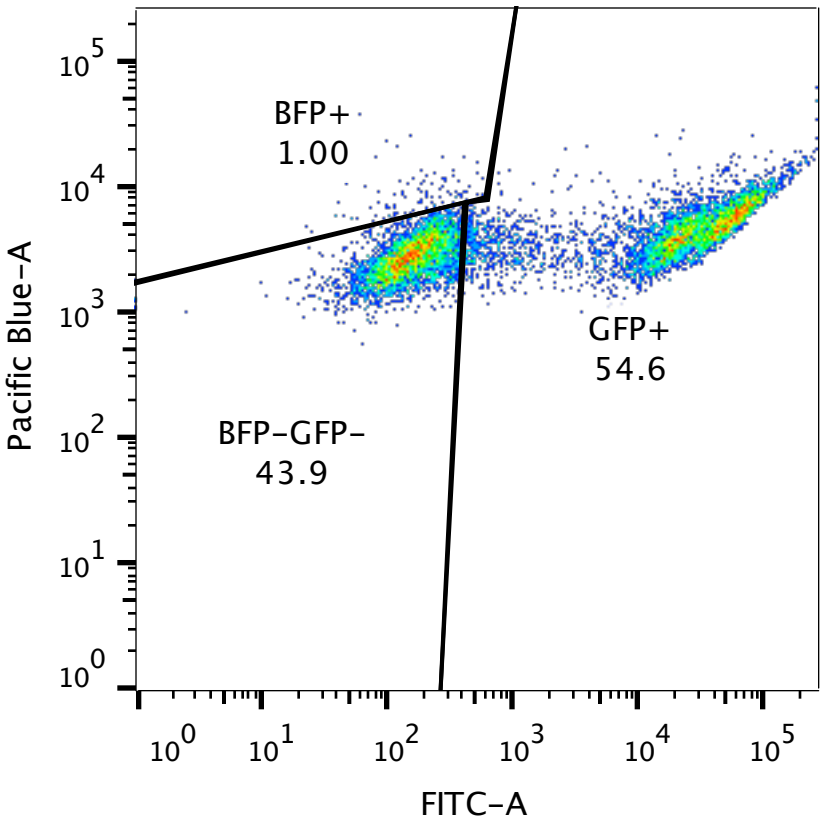
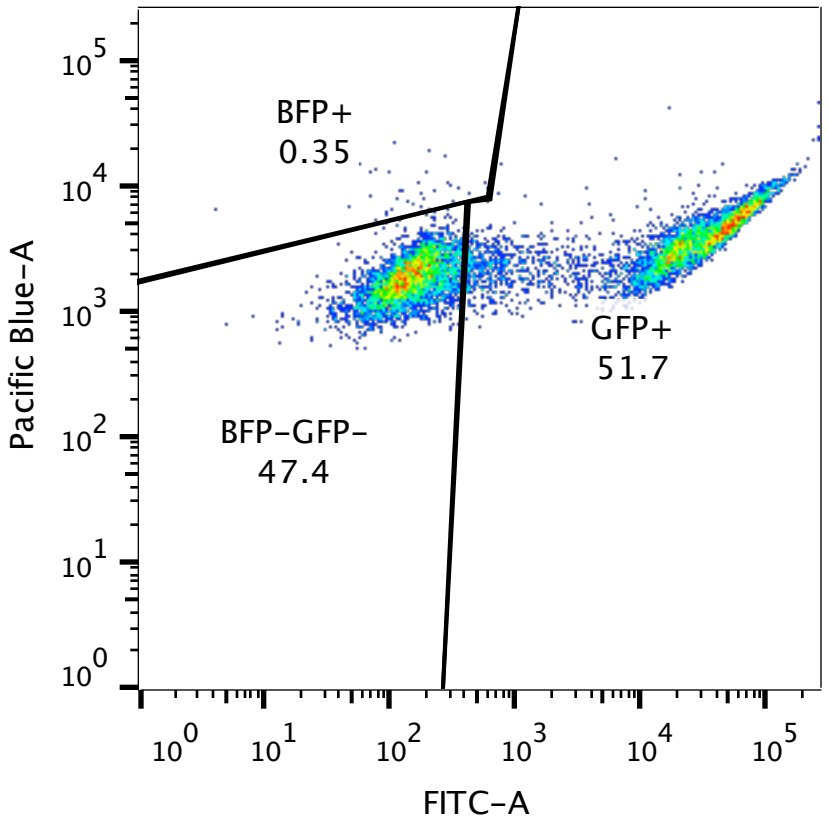


Cas9 – Donor Gt 1

Nature Biotechnology: doi:10.1038/nbt.3481



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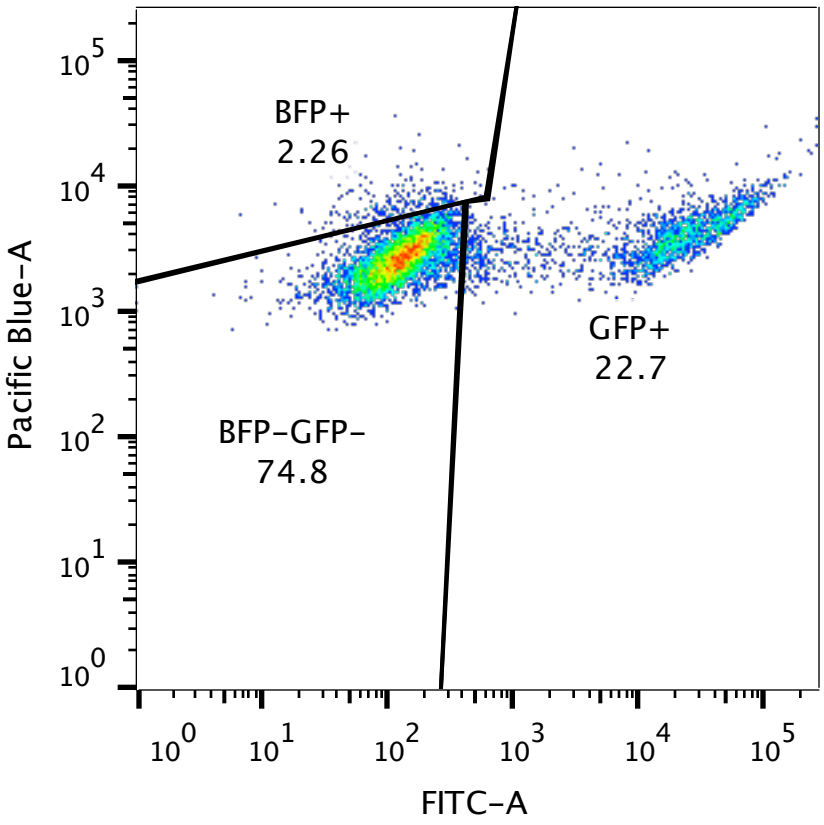
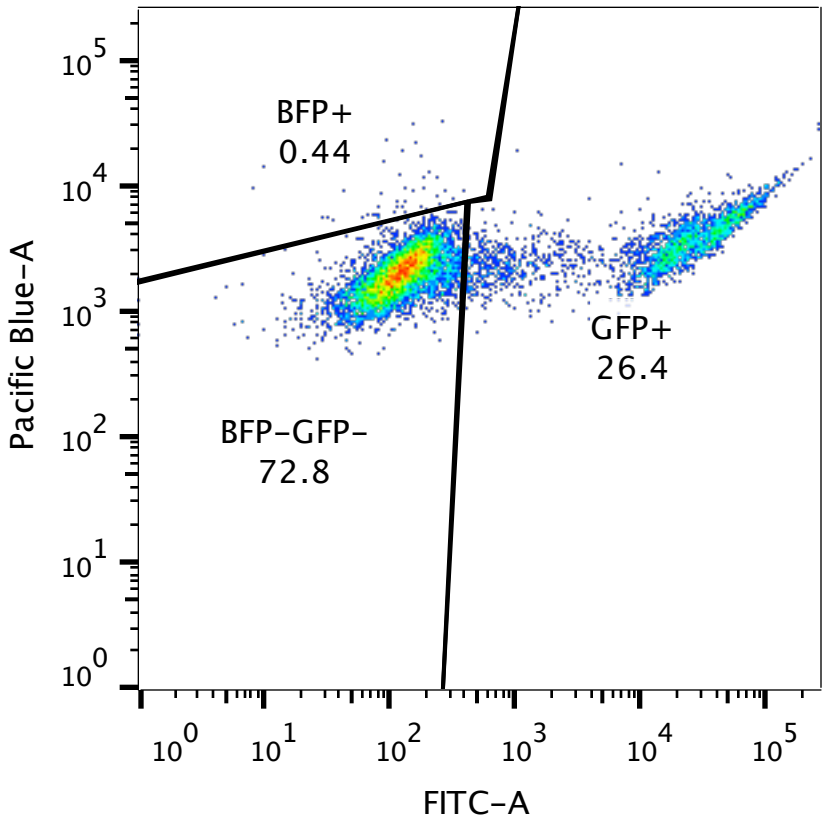
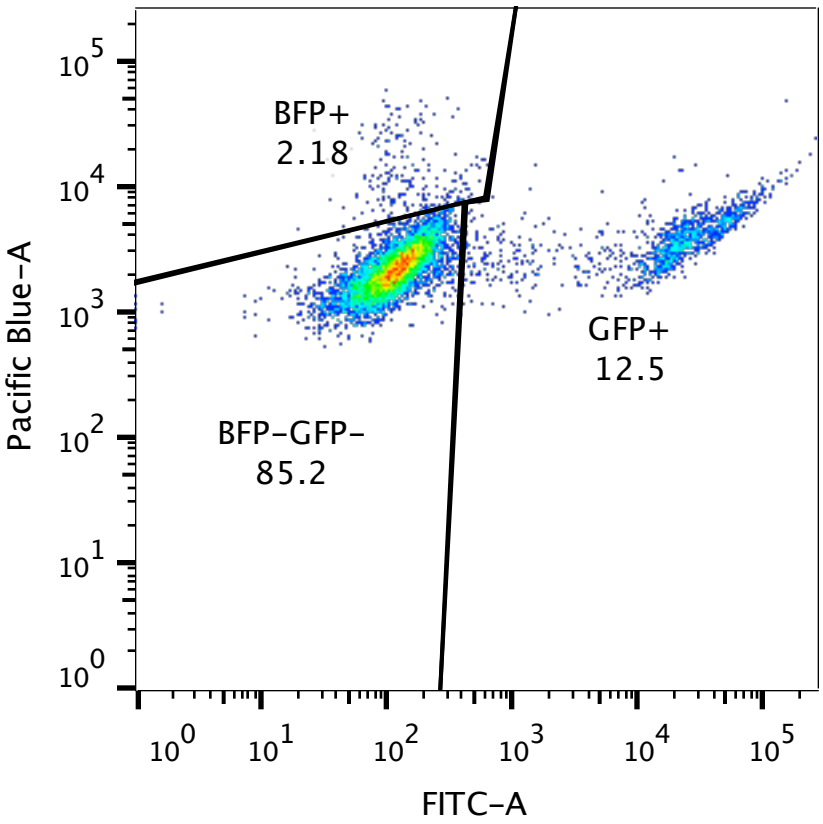


Cas9 – Donor Gn 1



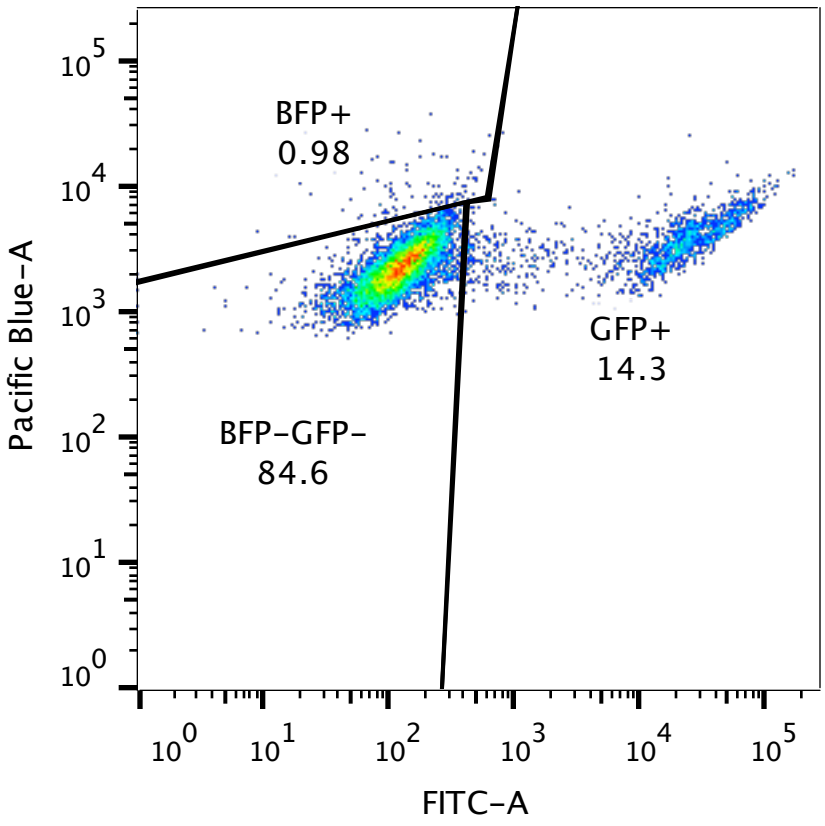
Cas9 – Donor Gn 2

Nature Biotechnology: doi:10.1038/nbt.3481



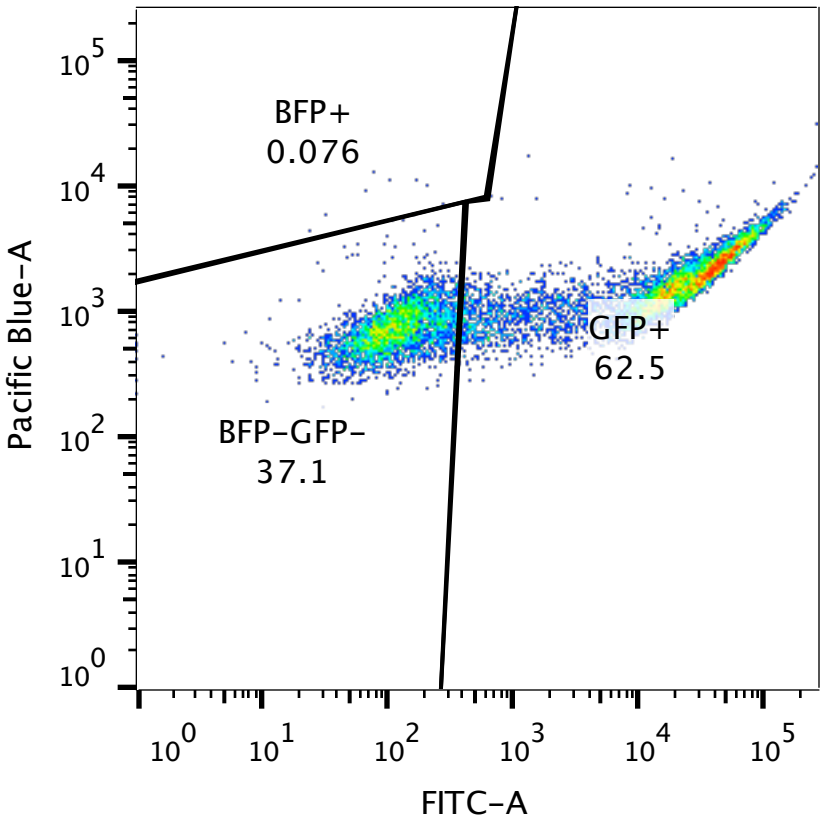
Cas9 – Donor Gt/Gn 1
(Duplex)

Nature Biotechnology: doi:10.1038/nbt.3481



Cas9 – Donor Gt/Gn 2
(Duplex)

Nature Biotechnology: doi:10.1038/nbt.3481



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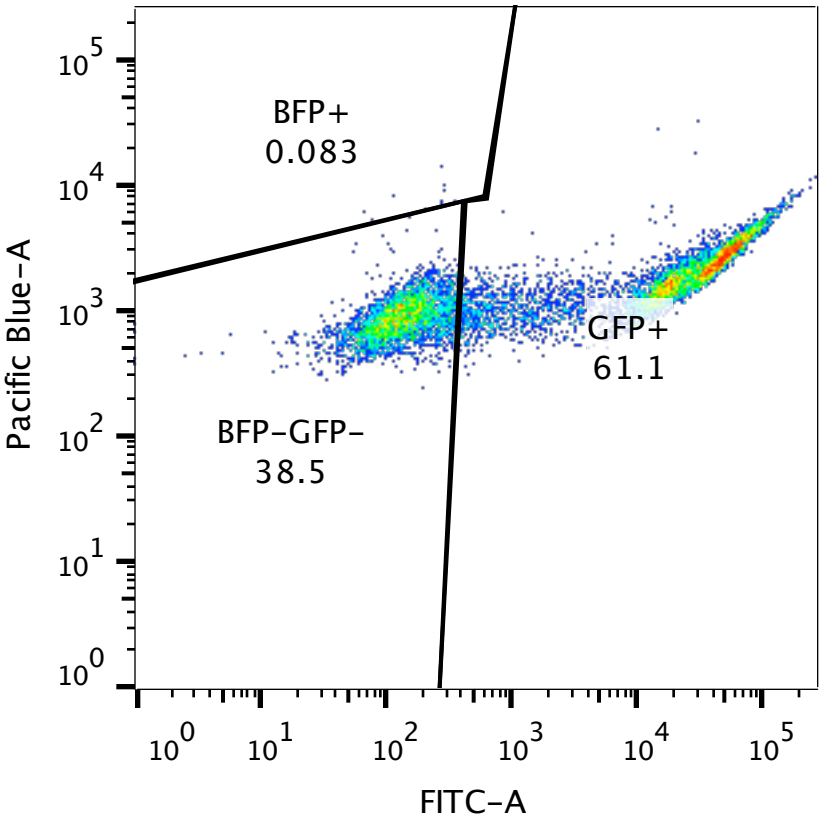
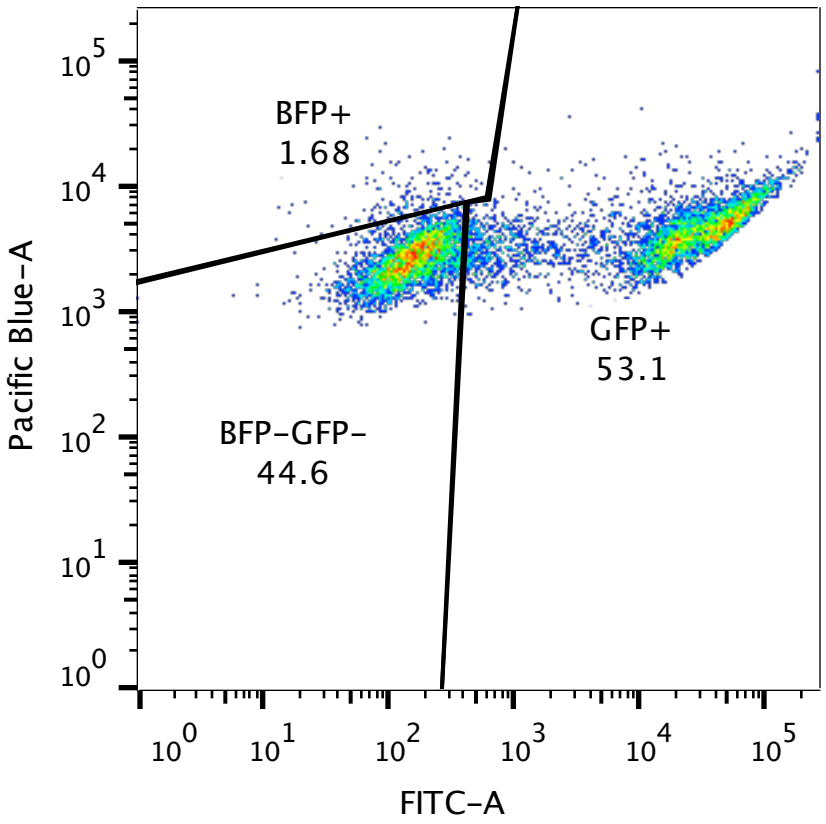


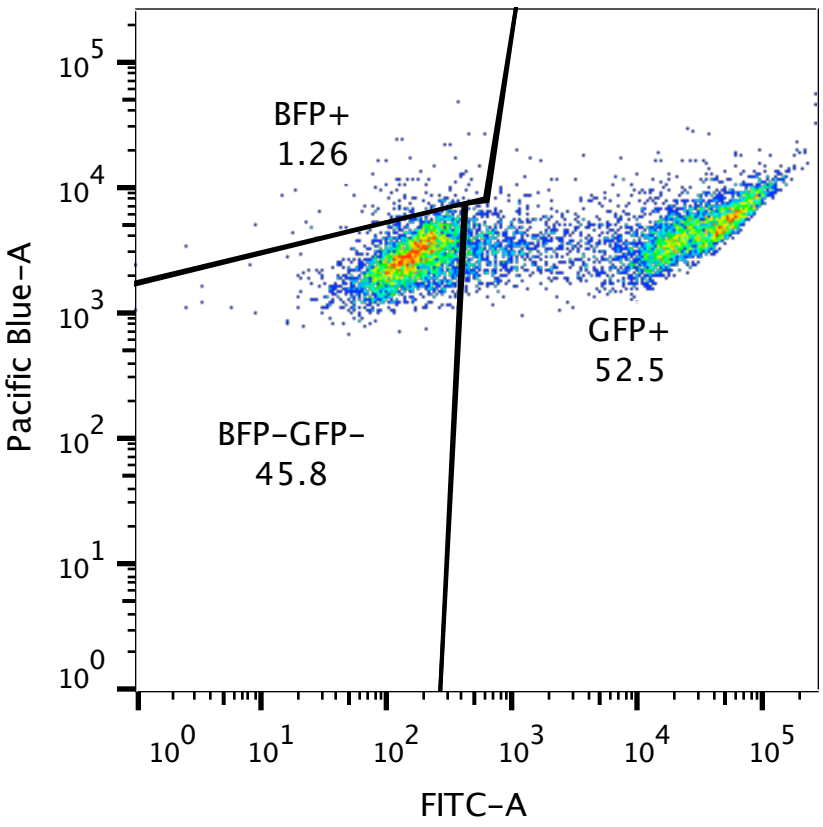
Cas9 - Donor Ht 2

Nature Biotechnology: doi:10.1038/nbt.3481



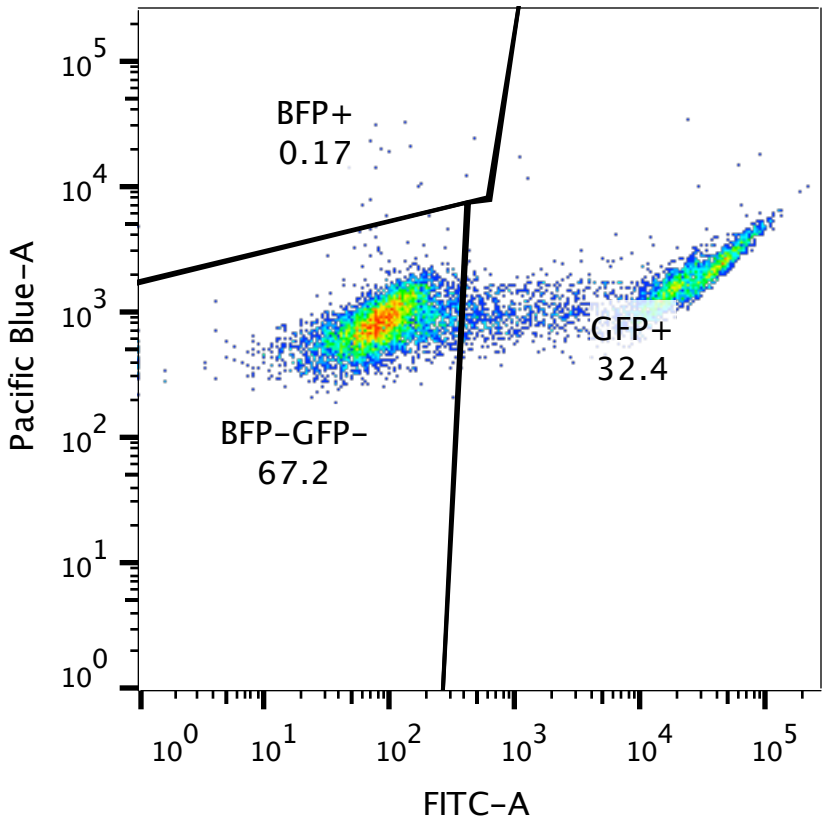
Cas9 – Donor Ht 3

Nature Biotechnology: doi:10.1038/nbt.3481

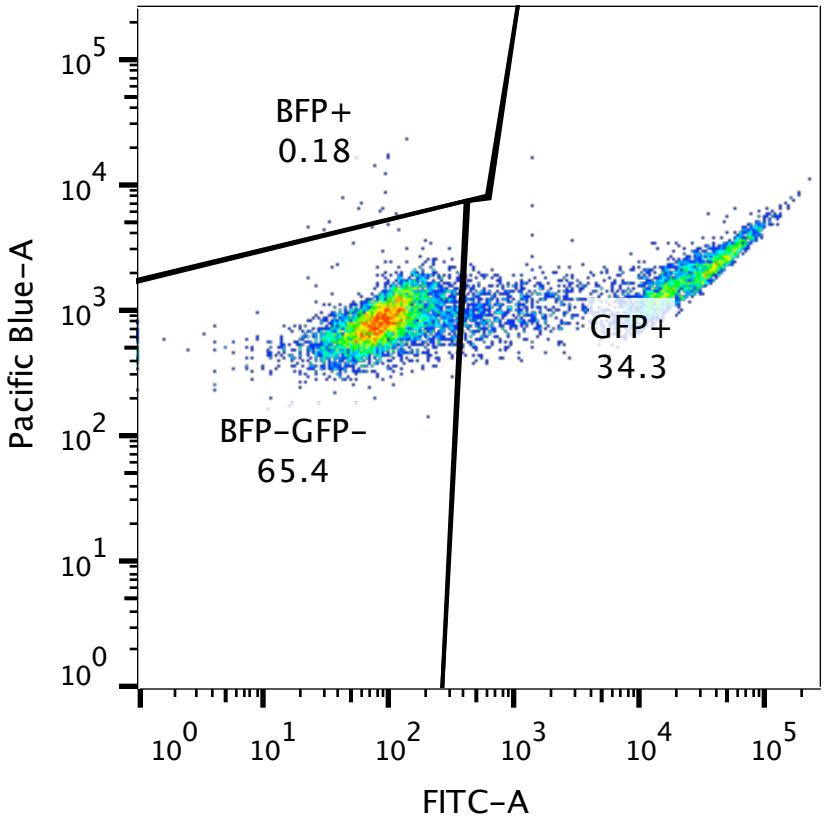


Cas9 - Donor Ht 4

Nature Biotechnology: doi:10.1038/nbt.3481

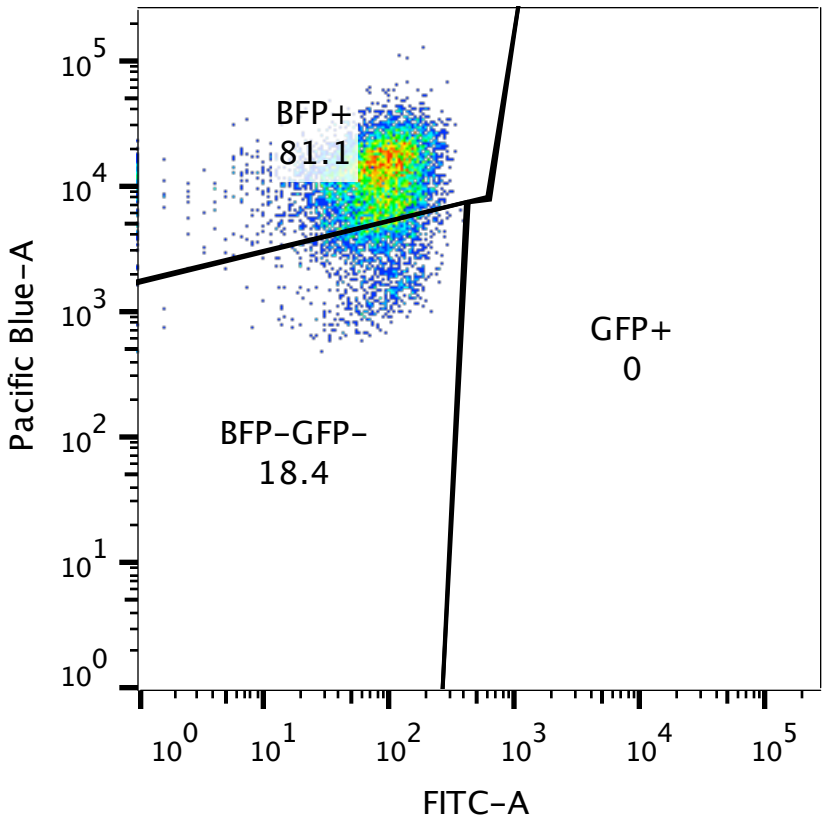


Cas9 – Donor Hn 1



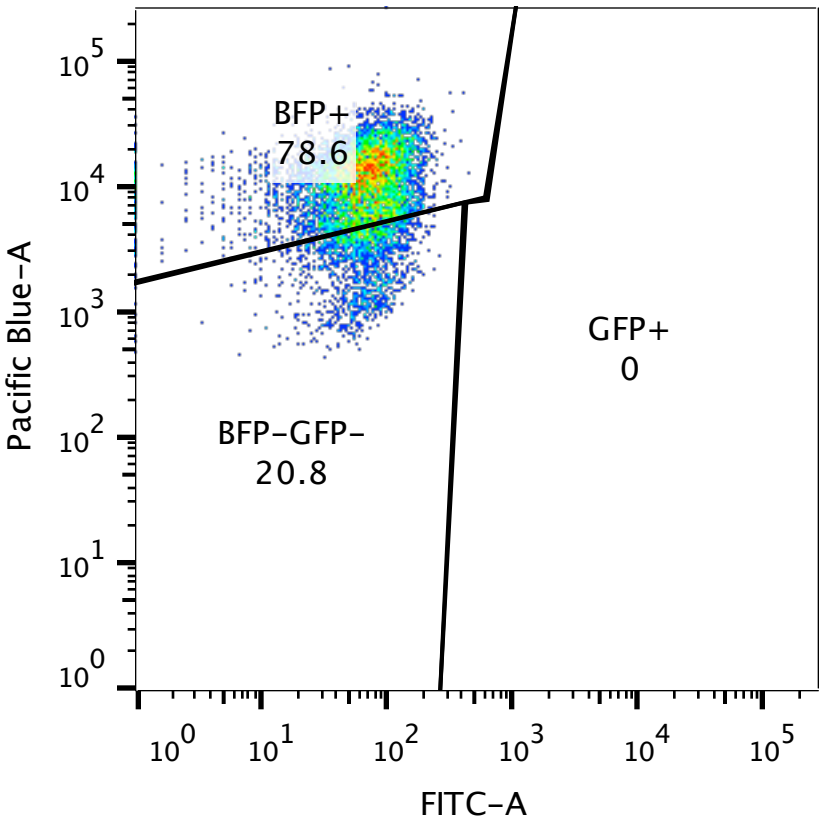
Cas9 - Donor Hn 2

Nature Biotechnology: doi:10.1038/nbt.3481



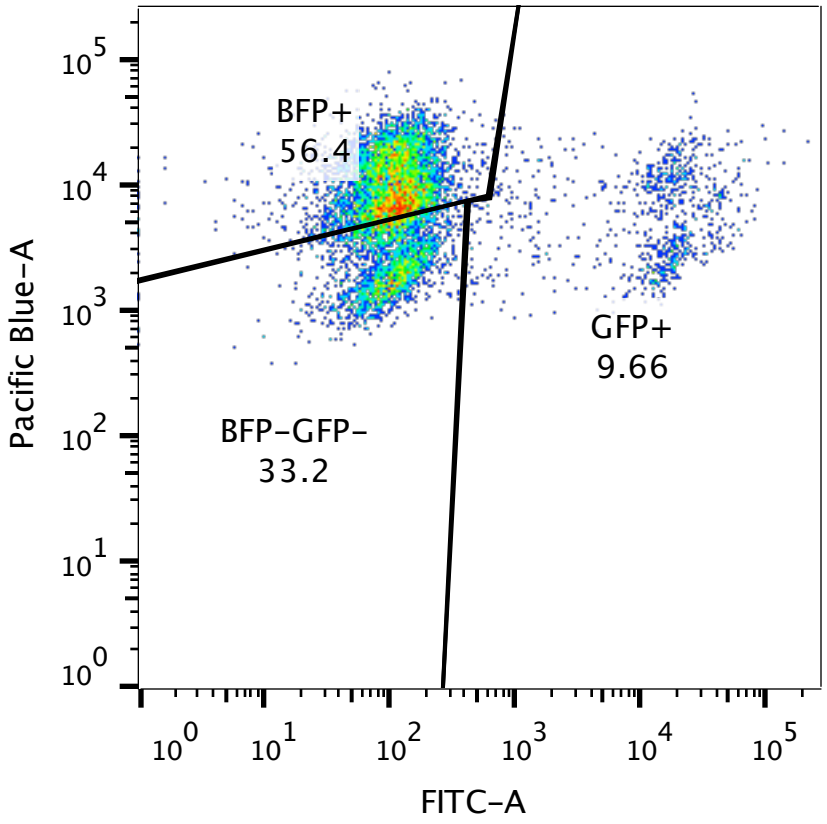
nCas9(D10A) – No Donor 1

Nature Biotechnology: doi:10.1038/nbt.3481



nCas9(D10A) - No Donor 2

Nature Biotechnology: doi:10.1038/nbt.3481



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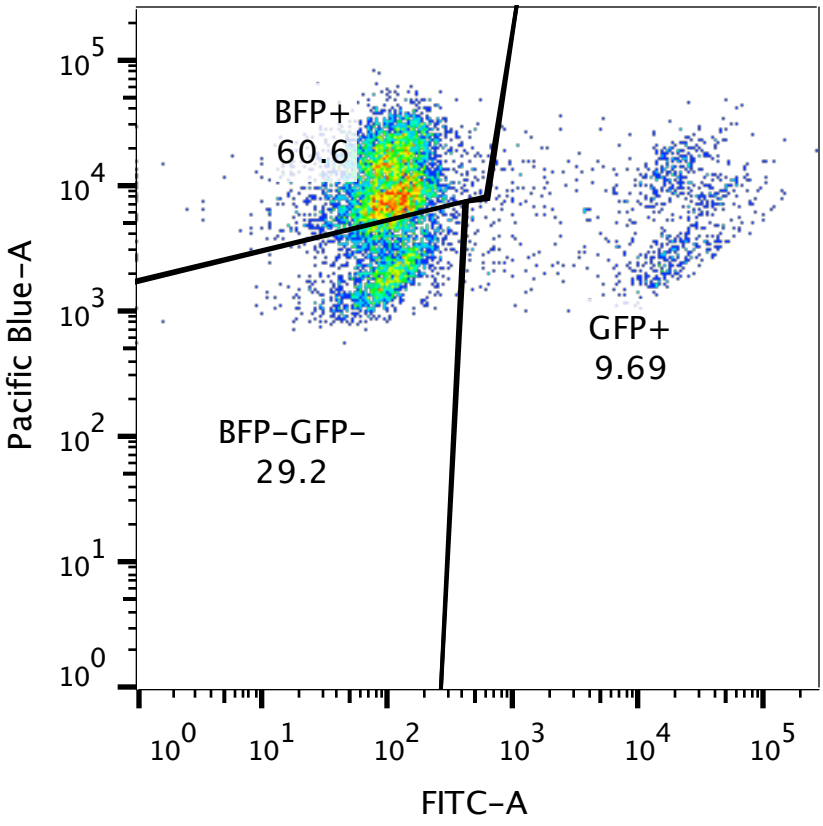
Nature Biotechnology: doi:10.1038/nbt.3481



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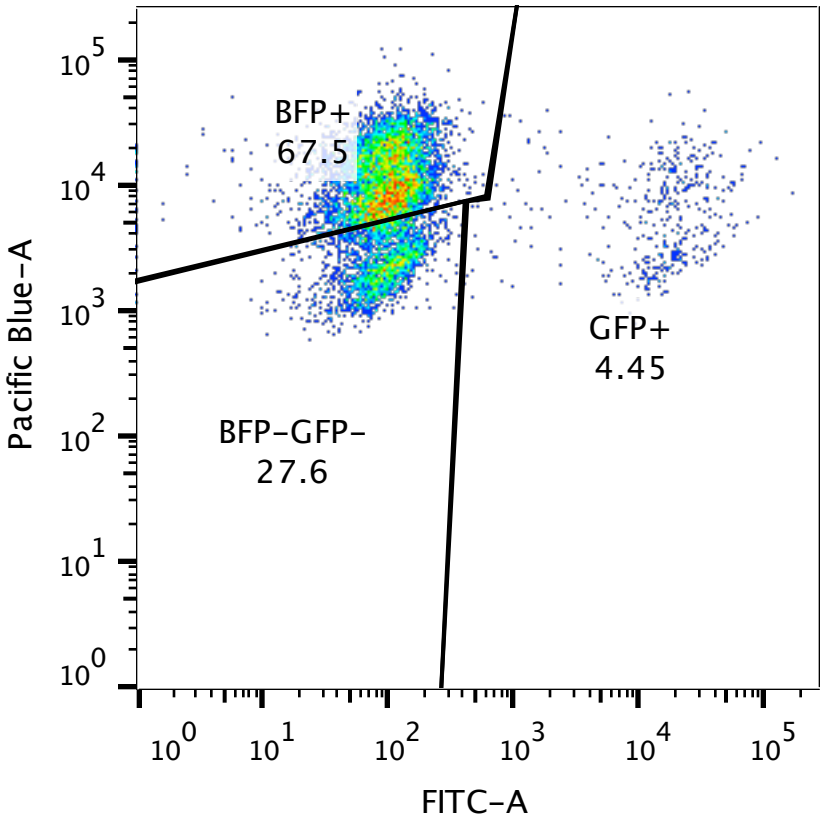

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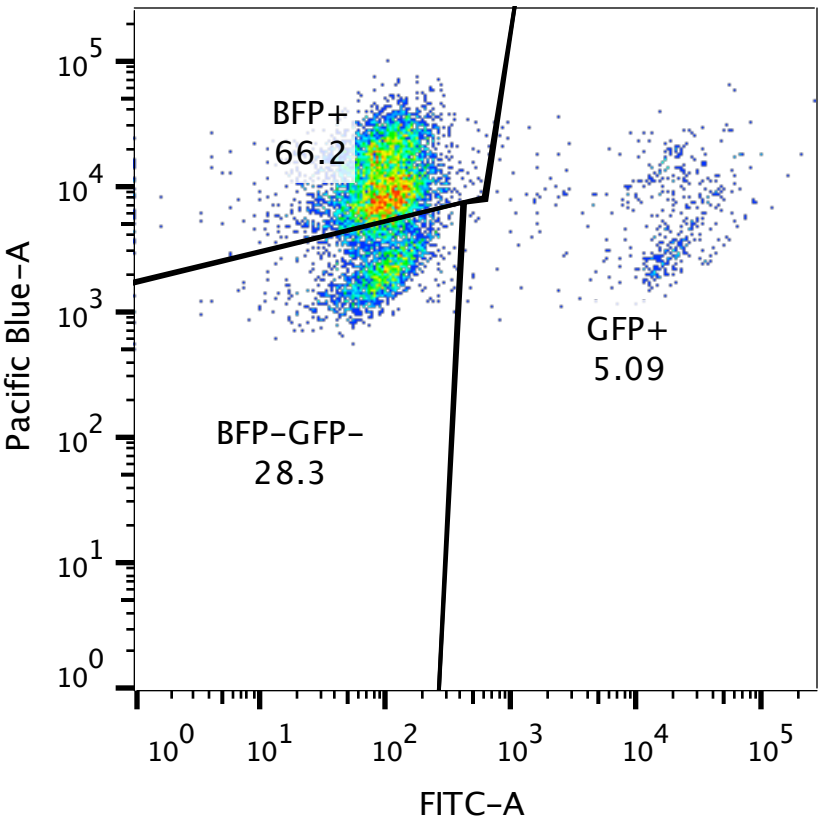
Nature Biotechnology: doi:10.1038/nbt.3481



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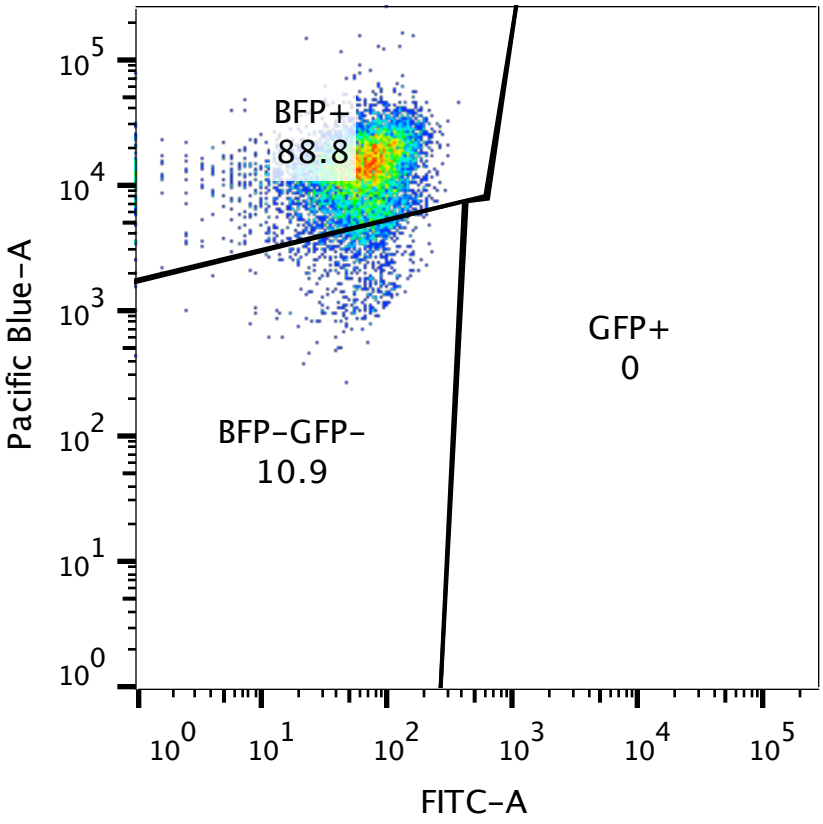

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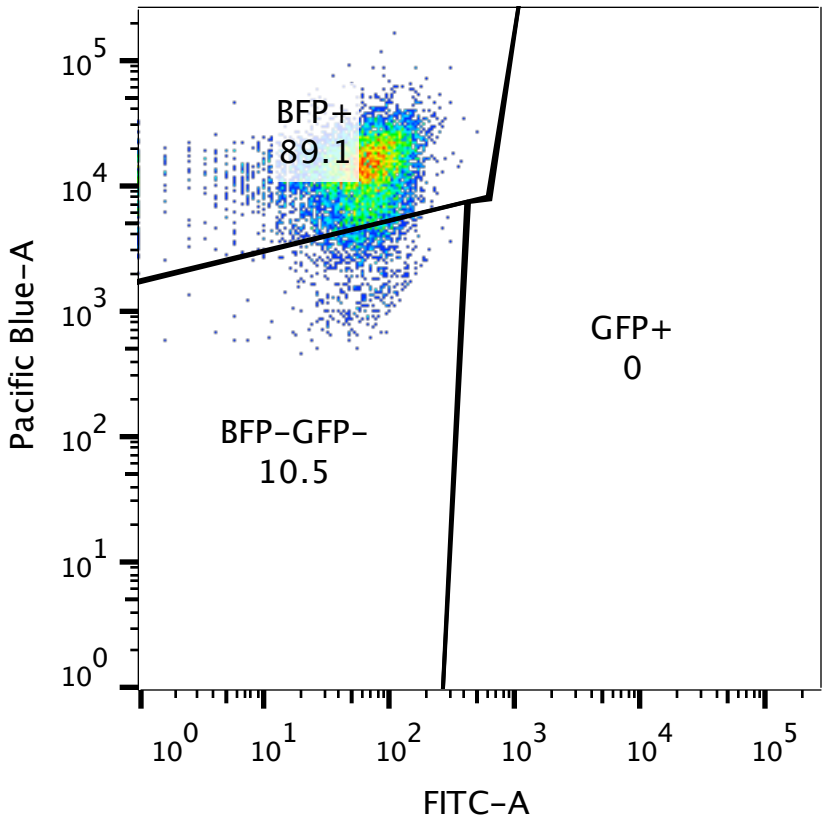

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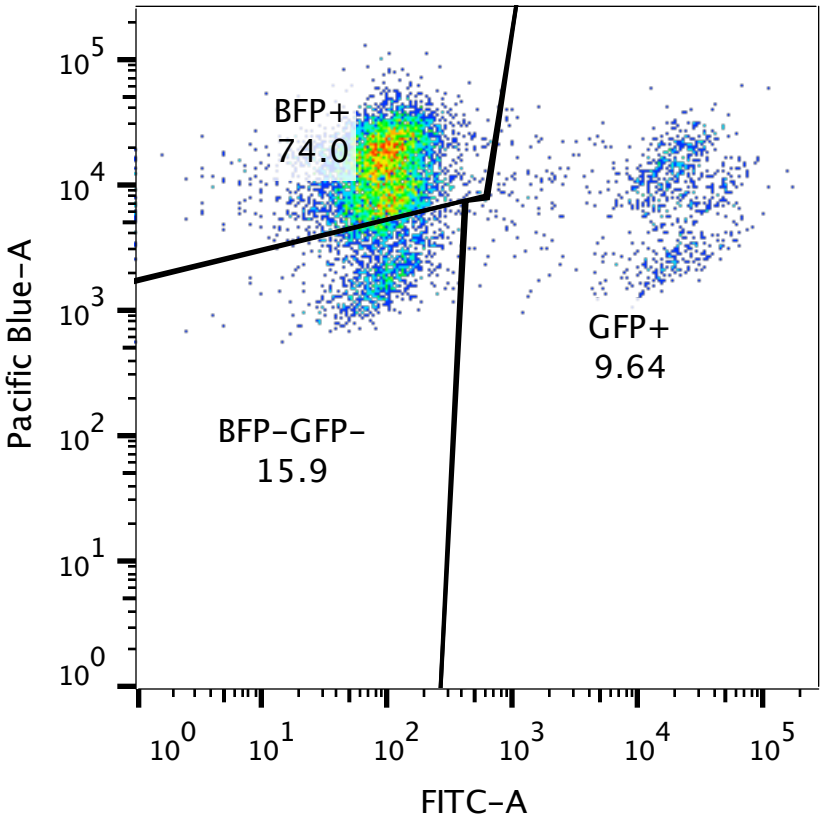

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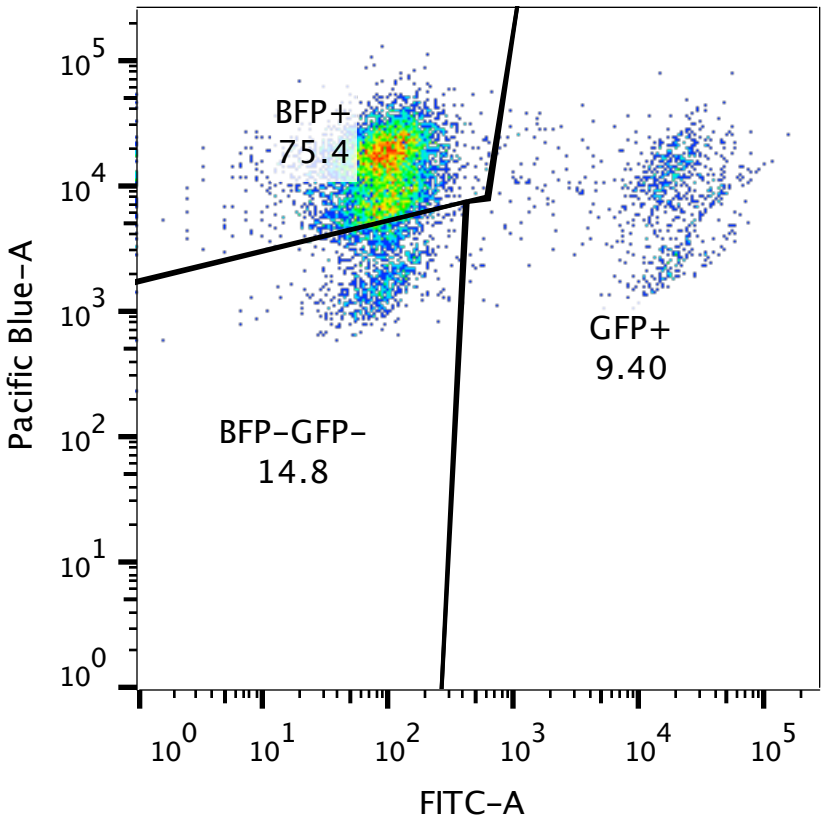
Nature Biotechnology: doi:10.1038/nbt.3481



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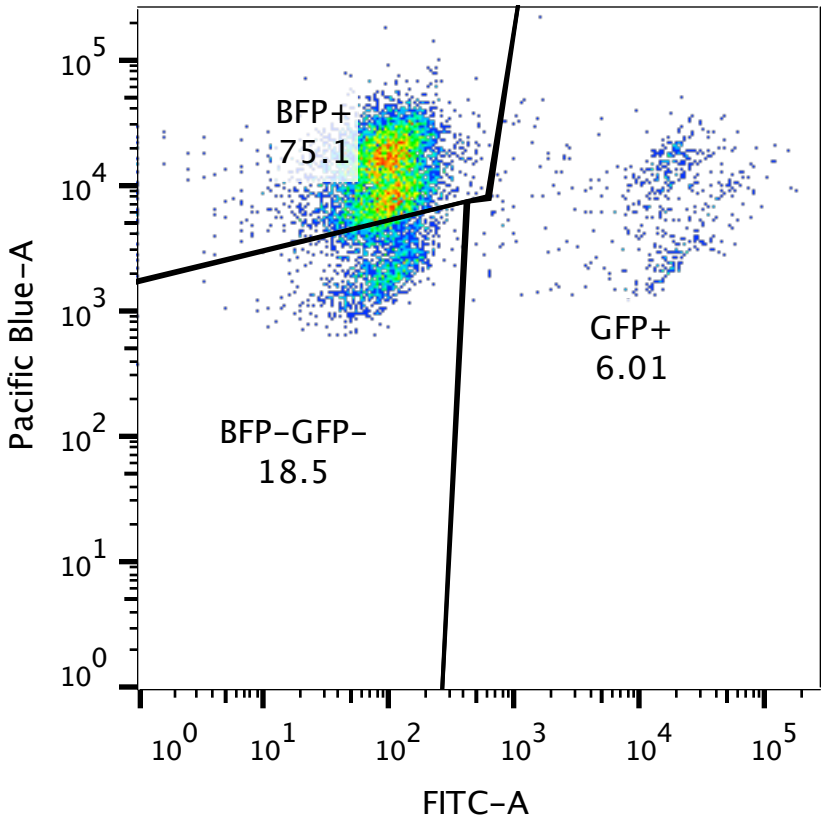
Nature Biotechnology: doi:10.1038/nbt.3481



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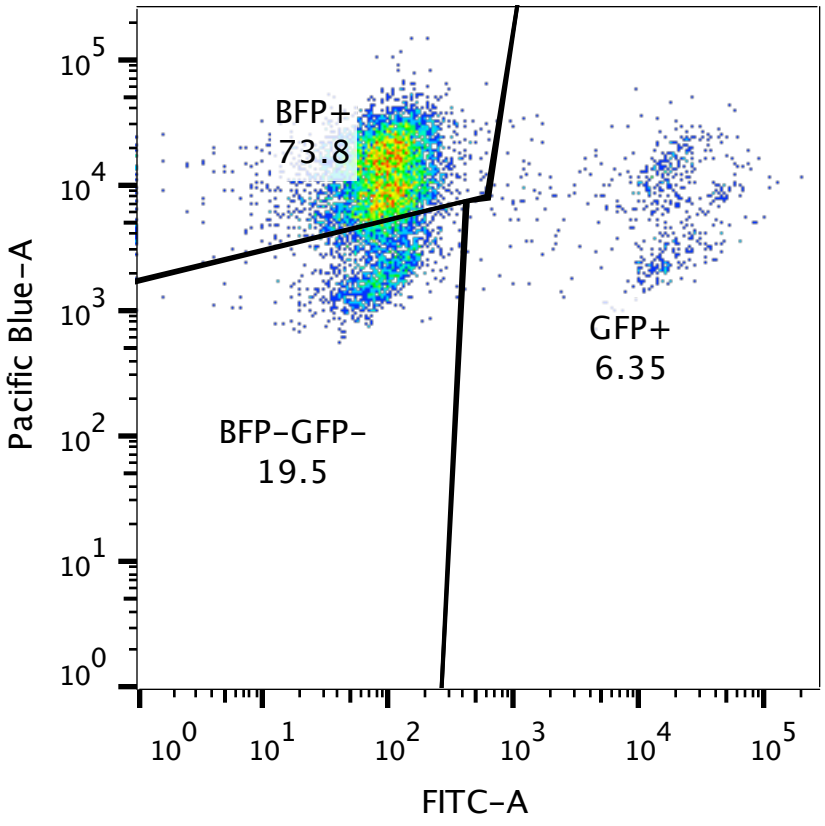
Nature Biotechnology: doi:10.1038/nbt.3481



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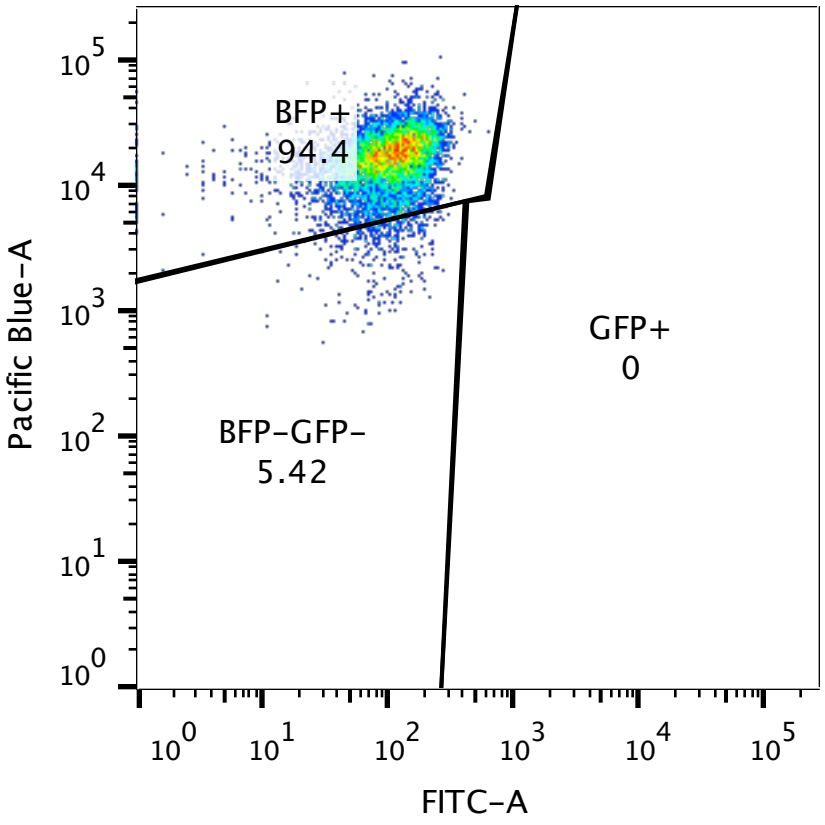
Nature Biotechnology: doi:10.1038/nbt.3481



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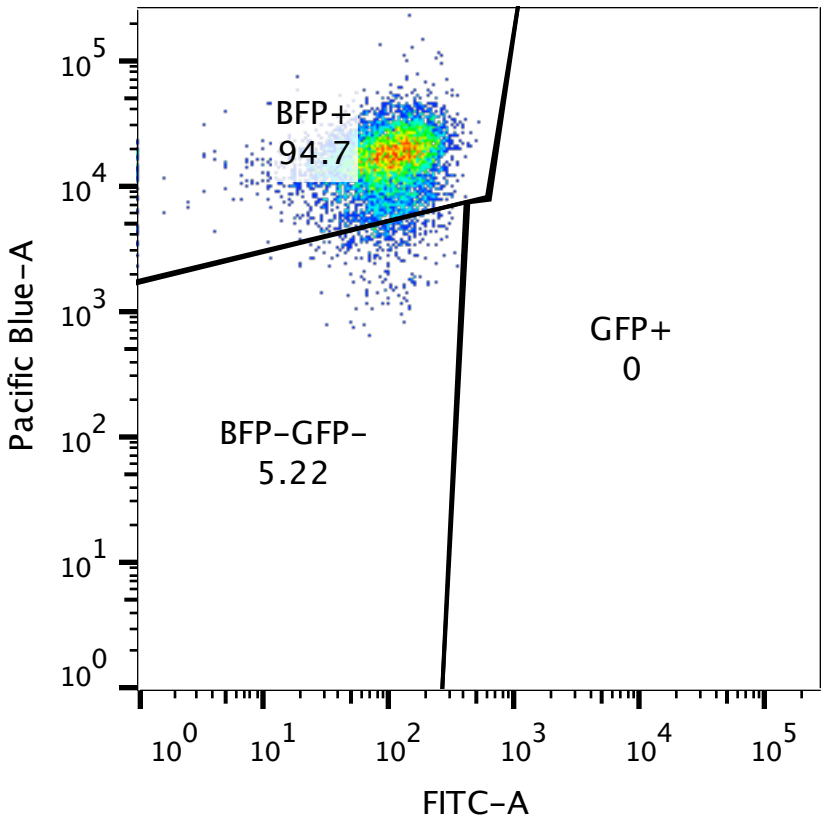

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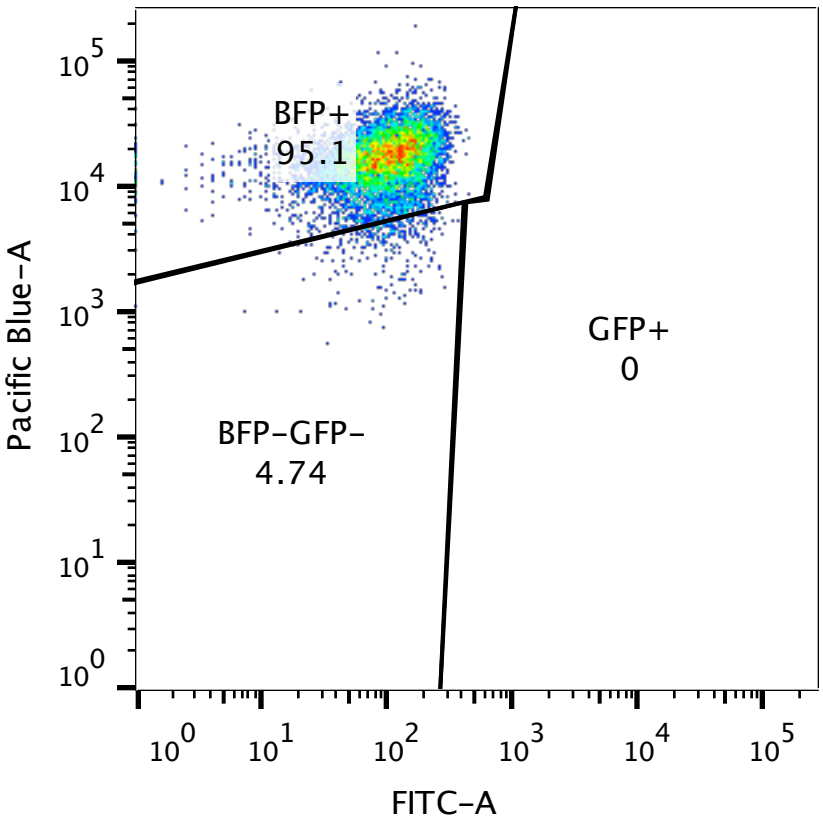

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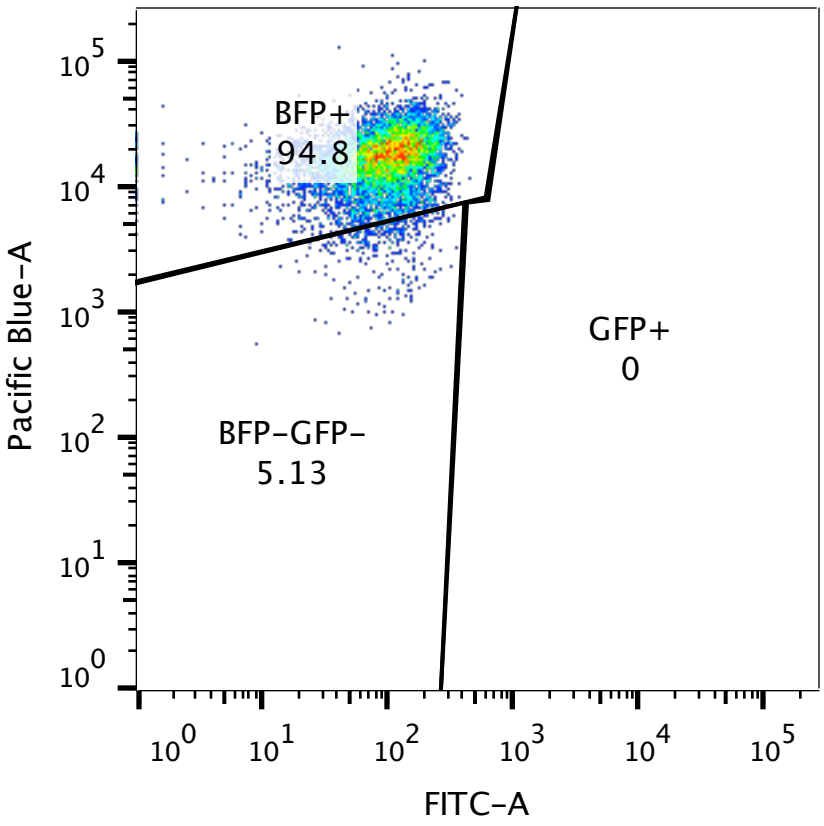

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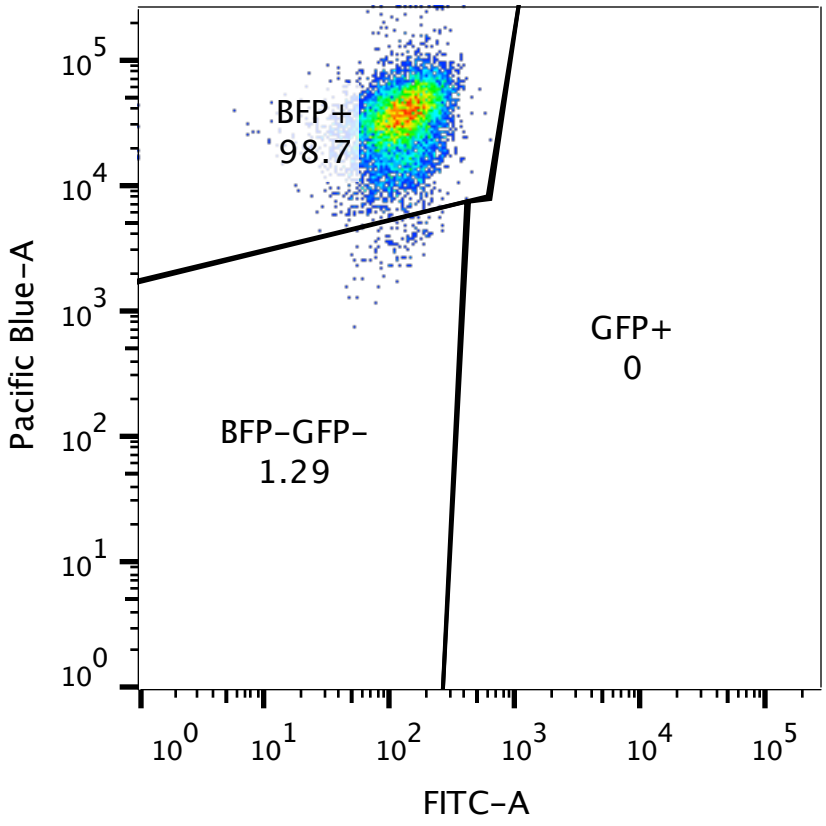
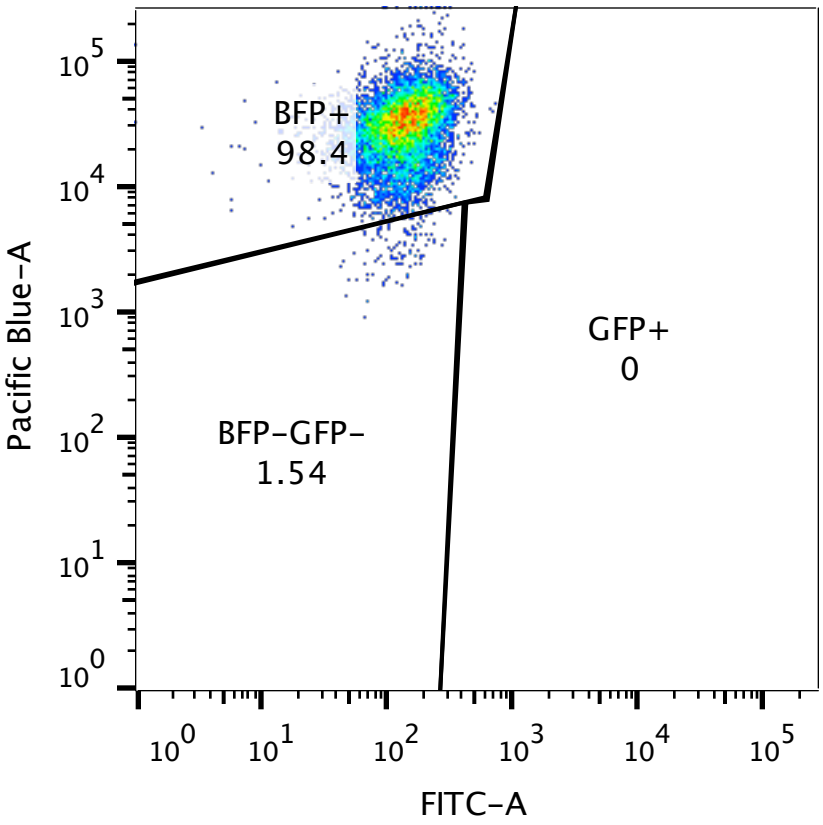


dCas9 - No Donor 1

Nature Biotechnology: doi:10.1038/nbt.3481



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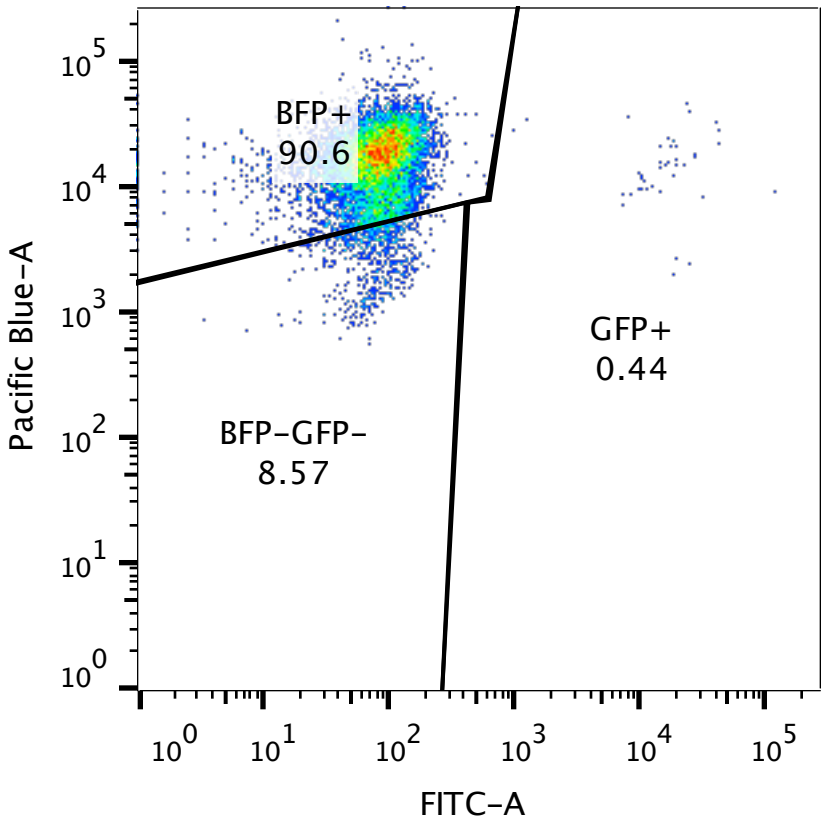
Nature Biotechnology: doi:10.1038/nbt.3481



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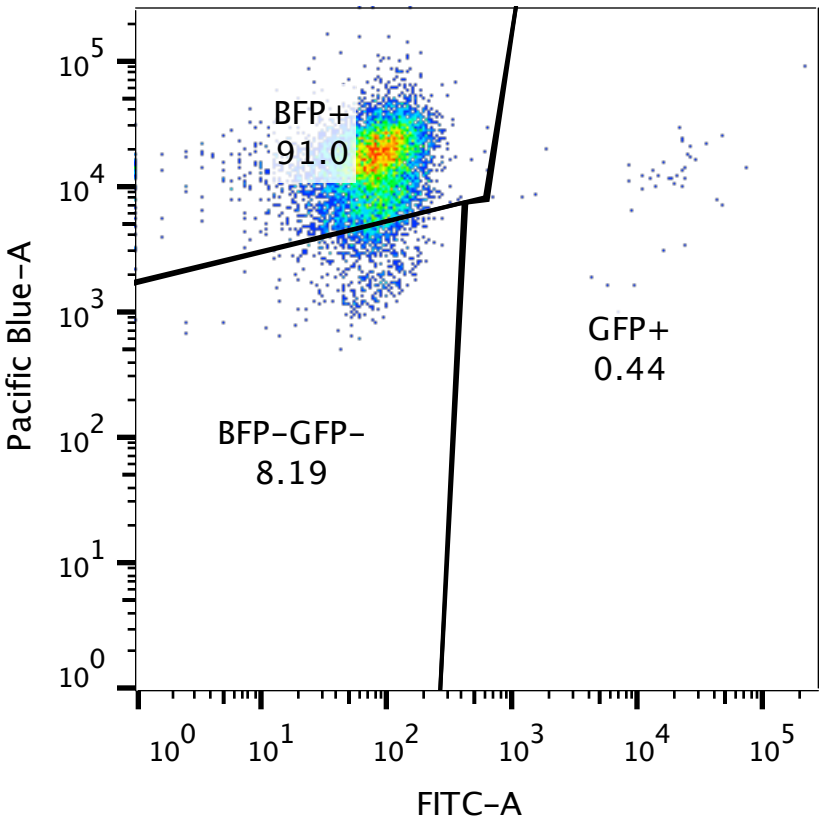

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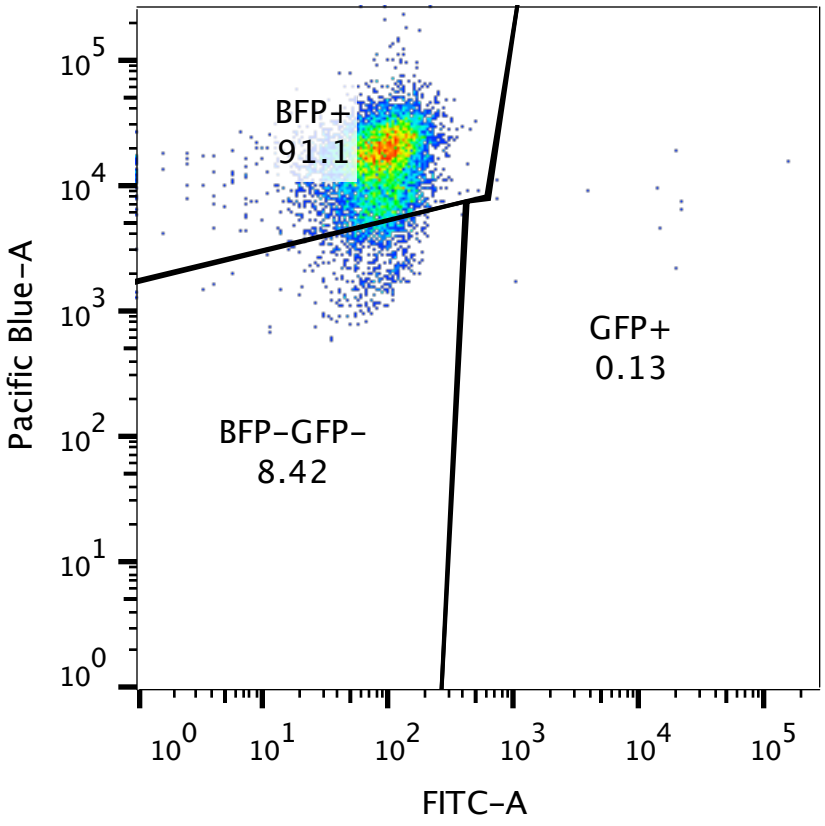

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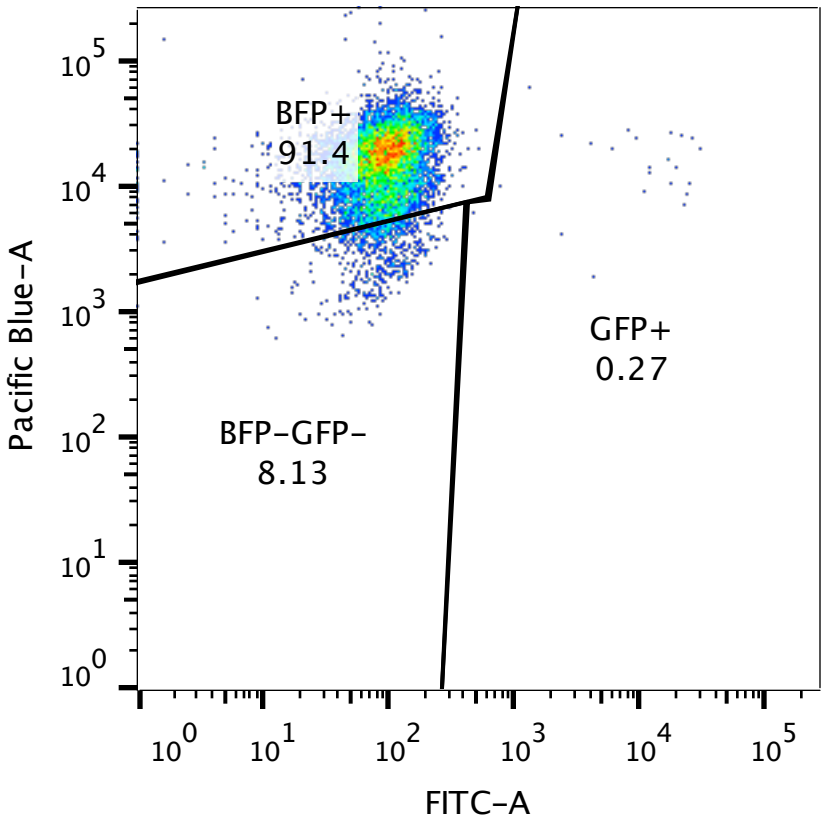

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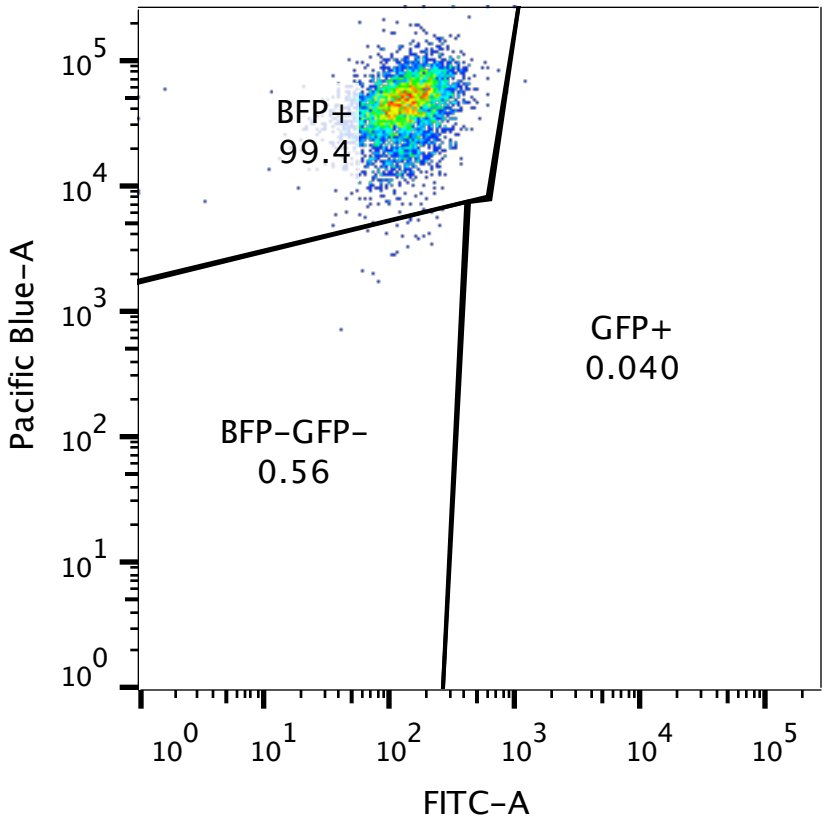
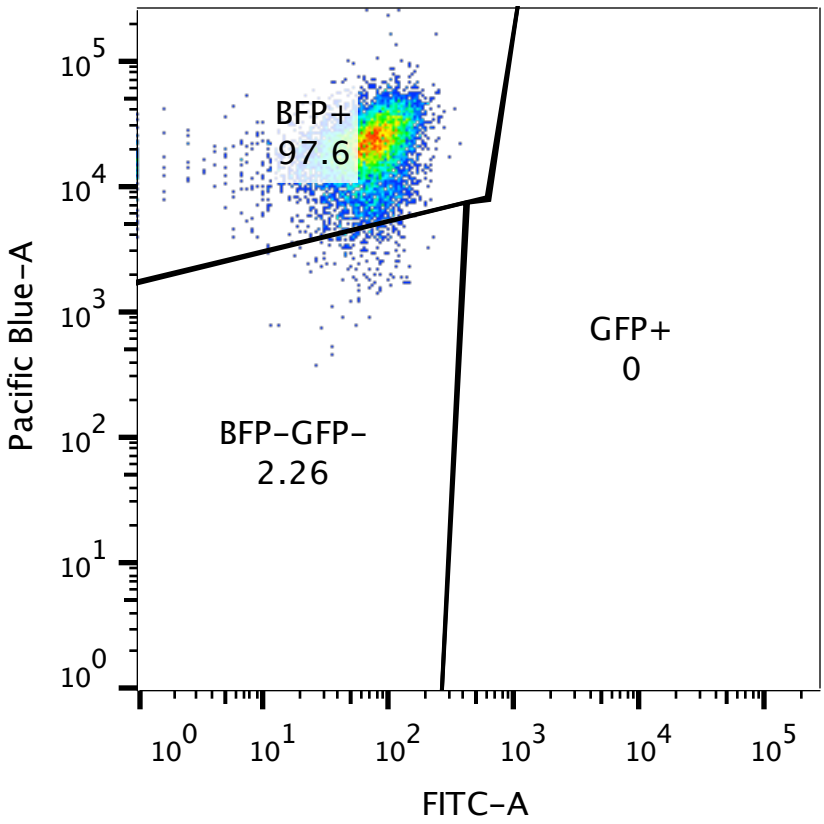


No Nuclease - Donor Tt 1

Nature Biotechnology: doi:10.1038/nbt.3481



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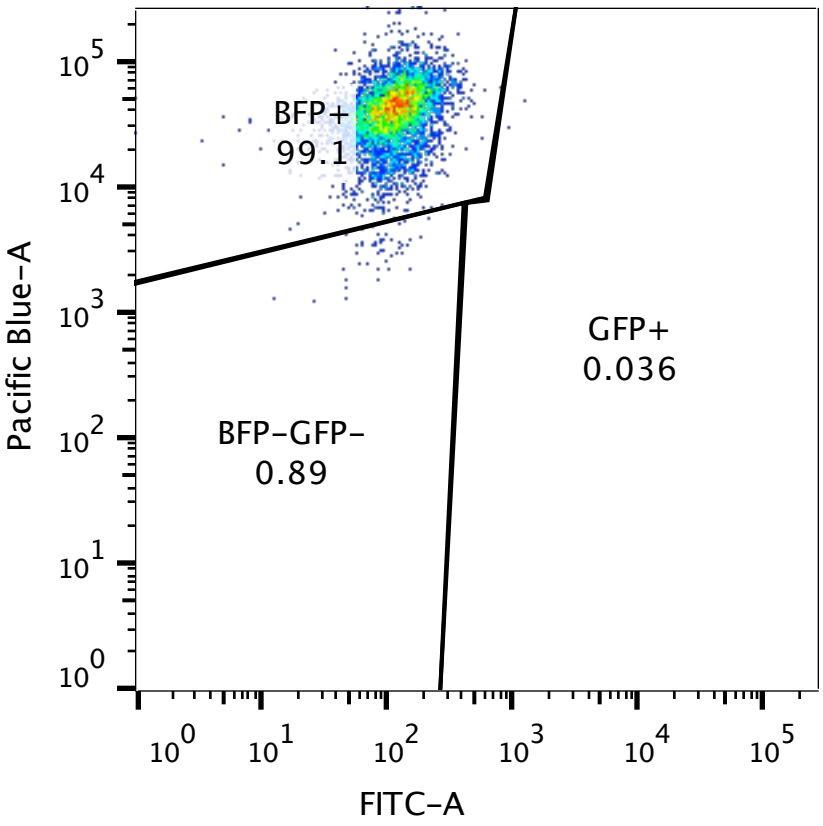

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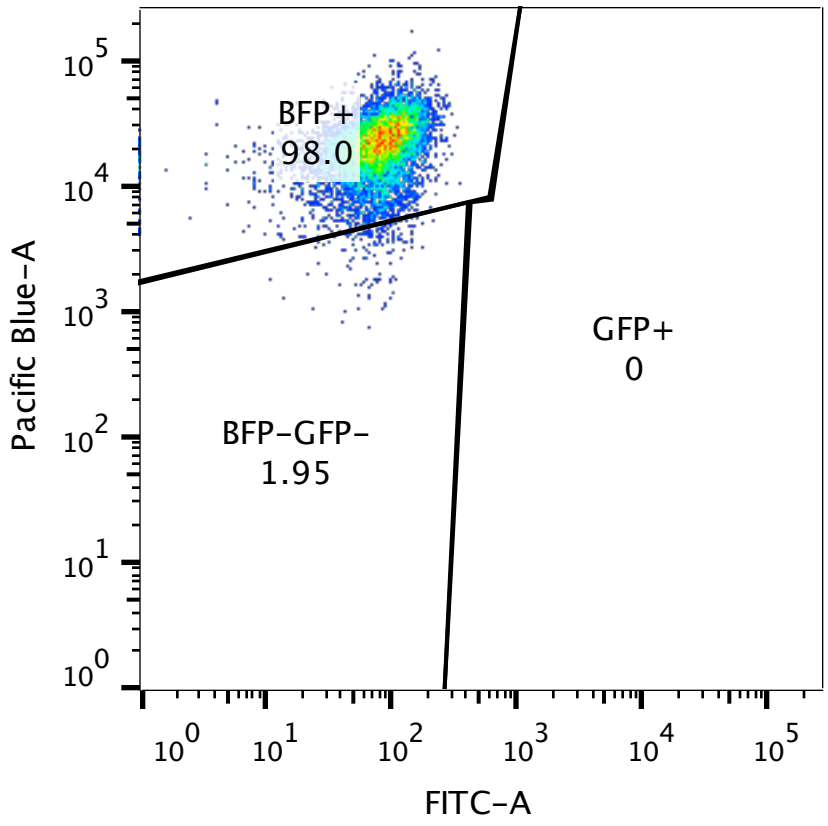

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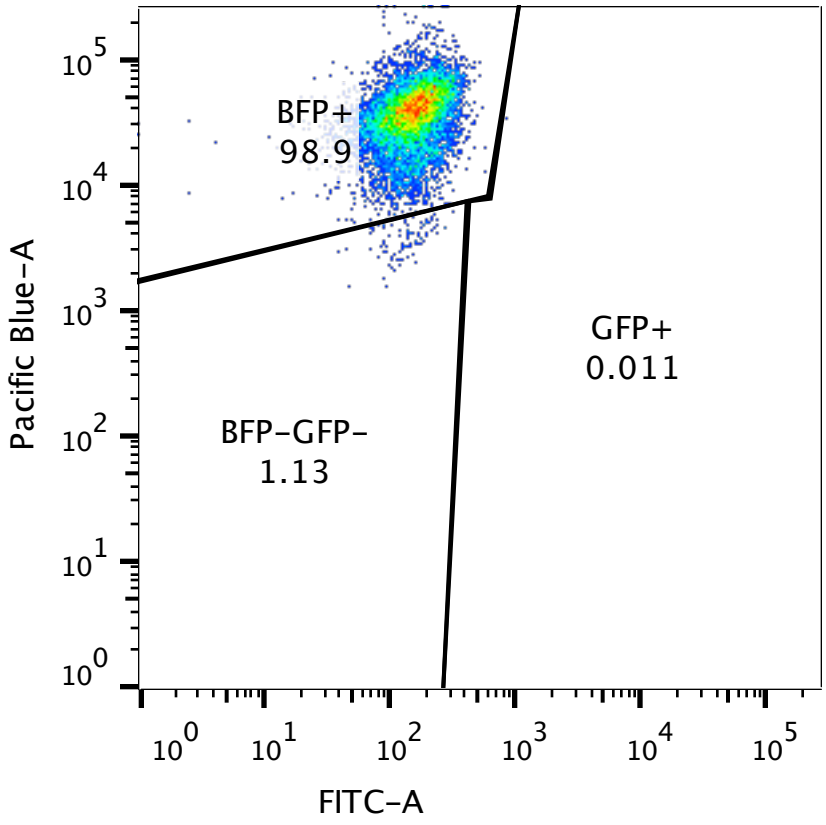
Nature Biotechnology: doi:10.1038/nbt.3481



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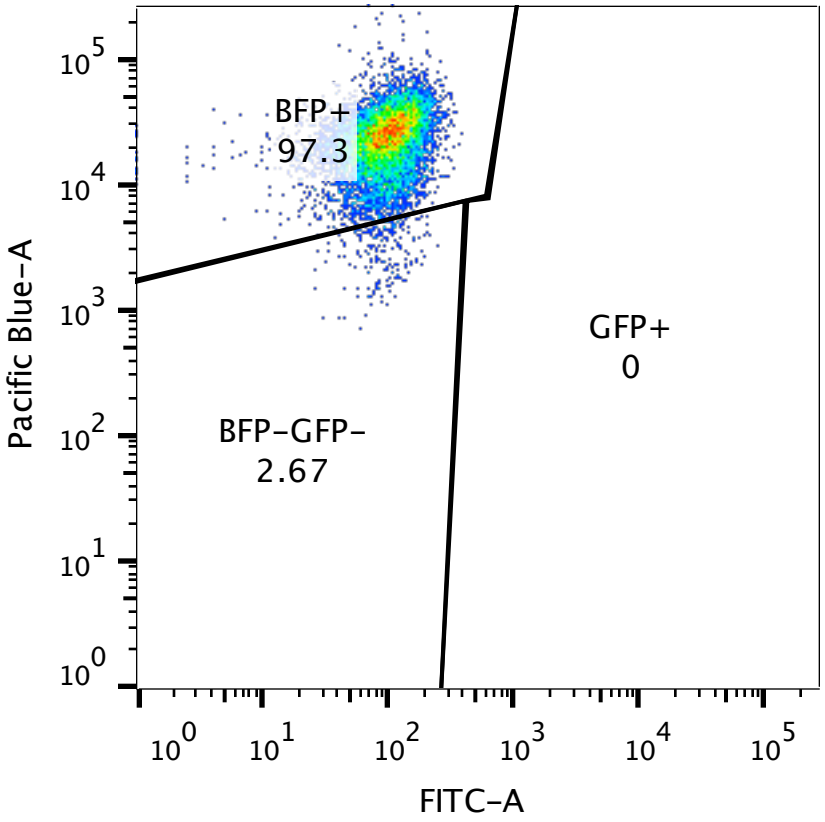

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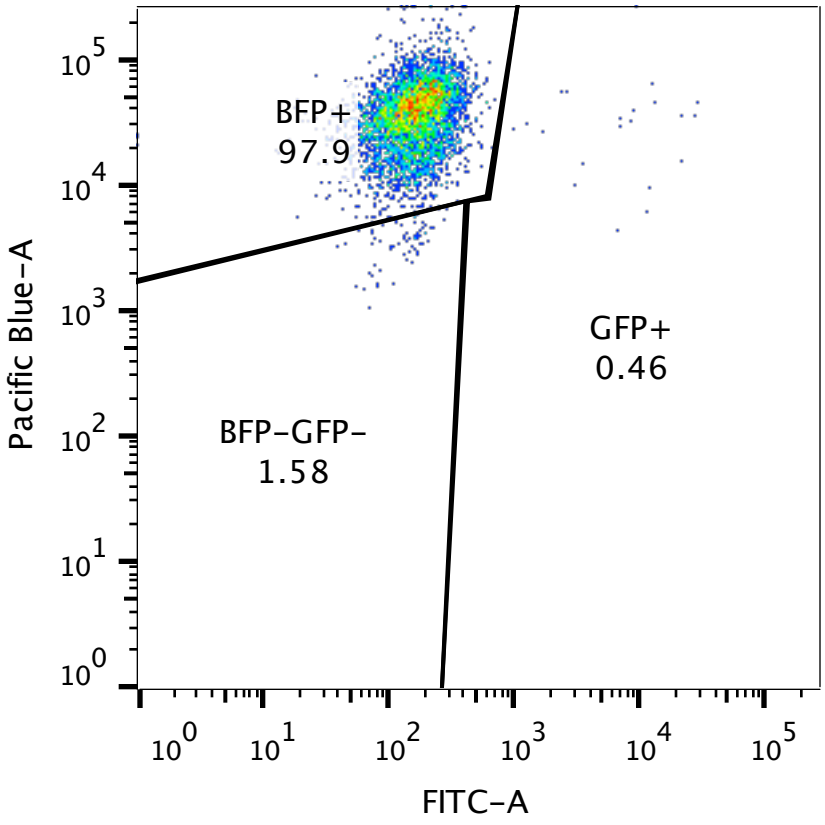

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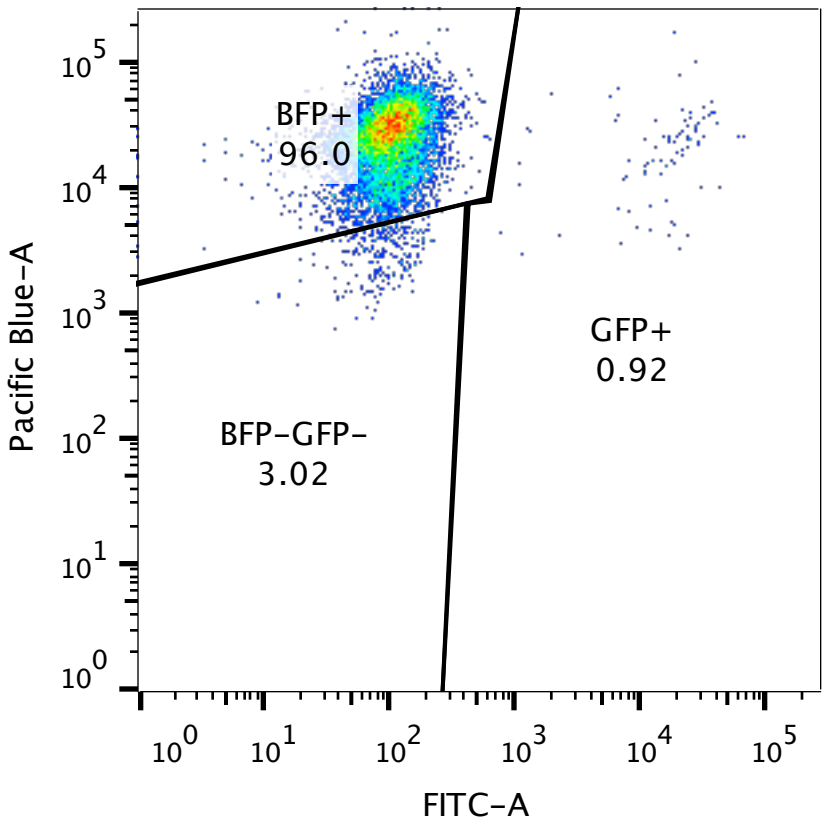

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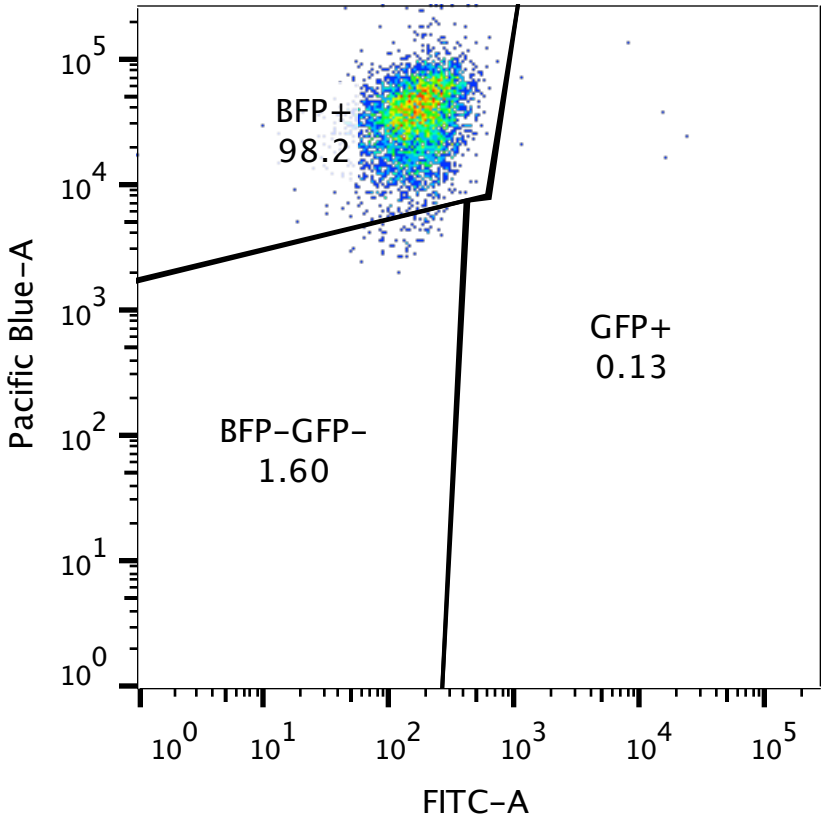
Nature Biotechnology: doi:10.1038/nbt.3481



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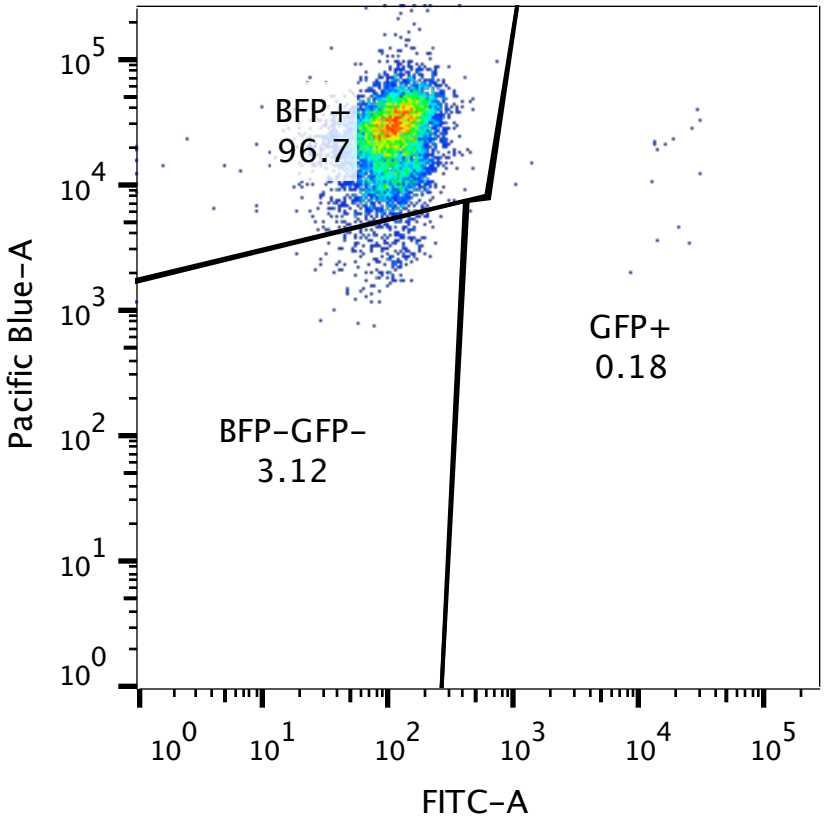
Nature Biotechnology: doi:10.1038/nbt.3481



Tiled dCas9 - Donor Tn 2

Nature Biotechnology: doi:10.1038/nbt.3481

Supplementary Note 4 –Vectors

pCR1002 Sequence T7promoter-His10-MBP-TEV-Cas9
(T7 Promoter to T7 Terminator)

ATGAAATCTTCTCACCATCACCATCACCATCACCATCACCATGGTTCTTCTATGAAAAT
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CCAATACGTGAACAAGCAGAAAATATTATTCATTTATTTACGTTGACGAATCTTGGAGC
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pCR1003 Sequence T7promoter-His10-MBP-TEV-dCas9
(T7 Promoter to T7 Terminator)

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pCR1053 Sequence T7promoter-His10-MBP-TEV-Cas9-2NLS
(T7 Promoter to T7 Terminator)
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pCR1054 Sequence T7promoter-His10-MBP-TEV-D10ACas9-2NLS
(T7 Promoter to T7 Terminator)
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pCR1055 Sequence T7promoter-His10-MBP-TEV-H840ACas9-2NLS
(T7 Promoter to T7 Terminator)
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pCR1056 Sequence T7promoter-His10-MBP-TEV-dCas9-2NLS
(T7 Promoter to T7 Terminator)
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BFP Destination Clone (EF1alpha promoter to terminator) BFP reporter in caps, H66 codon in bold, protospacer+PAM underlined.

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