

Supplemental Tables

Supplemental Table 1. gRNA target sequences.

gRNA	Sequence (5' to 3')	PAM sequence (5' to 3')	Position (hg19)	Strand	Compatible ABEs
gRNA1	TTAGTCTATTTCCACCCCT	TAG	chr11:5248033-5248052 (<i>HBB</i>)	-	SpRY-ABE8e, NG-ABE8e, ABE8e
gRNA2	ATTAGTCTATTTCCACCC	TTA	chr11:5248034-5248053 (<i>HBB</i>)	-	SpRY-ABE8e
gRNA3	TATTAGTCTATTTCCACC	CTT	chr11:5248035-5248054 (<i>HBB</i>)	-	SpRY-ABE8e
gRNA4	CTATTAGTCTATTTCCAC	CCT	chr11:5248036-5248055 (<i>HBB</i>)	-	SpRY-ABE8e
gRNA5	CCTATTAGTCTATTTCCCA	CCC	chr11:5248037-5248056 (<i>HBB</i>)	-	SpRY-ABE8e
gRNA6	GCCTATTAGTCTATTTCCC	ACC	chr11:5248038-5248057 (<i>HBB</i>)	-	SpRY-ABE8e
gRNA1_HD	TTGGTCTATTTCCACCCCT	TAG	chr11:5248033-5248052 (<i>HBB</i>)	-	SpRY-ABE8e, NG-ABE8e, ABE8e

PAM, Protospacer Adjacent Motif.

Supplemental Table 2. *In silico* predicted off targets.

Off-target	Sequence (5' to 3')	Mismatches	Position (hg19)	Strand	Score	Type
OT1	GTAGTCTATTTCCACCCCT	1	Chr10:36827935-36827954	+	0.12	Intergenic
OT2	CTAGCCTATTTCCACCCCT	2	Chr1:104536228-104536247	-	0.31	Intergenic
OT3	TCAGTCCATTTCCACCCCT	2	ChrX:136953484-136953503	+	0.36	Intergenic
OT4	TTGGTCGATTTCCACCCCT	2	Chr8:129251336-129251355	+	0.38	Intergenic
OT5	TTAGGTTATTTCCACCCCT	2	Chr3:65005077-65005096	-	0.40	Intergenic
OT6	TTTGTCTCTTTCCACCCCT	2	Chr12:40609156-40609175	+	0.42	Intergenic
OT7	TTTGTCTCTTTCCACCCCT	2	Chr8:89004815-89004834	-	0.42	Intergenic
OT8	TTAGTAAATTTCCACCCCT	2	Chr12:11337215-11337234	+	0.44	Intronic – <i>PRB4</i>
OT9	TTAGCCTAGTTCCACCCCT	2	ChrX:142880716-142880735	+	0.54	Intergenic
OT10	TTAGCTATTTCCACCCCT	0 (Del 16)	Chr4:59423629-59423647	-	0.70	Intergenic

OT, Off-target; Del, Deletion.

Supplemental Table 3. Primers used for targeted NGS.

Amplified region	F/R	Sequence (5' to 3')
On target	F	GCAGCGTCAGATGTGTATAAGAGACAGCTGGGCATGTGGAGACAGAG
	R	TGGGCTCGGAGATGTGTATAAGAGACAGTTGCCATGAGCCTTCACCTT
OT1	F	GCAGCGTCAGATGTGTATAAGAGACAGGCTCAGAAACAGGATGTCATCATTTCCTC
	R	TGGGCTCGGAGATGTGTATAAGAGACAGCTTCCCTAAATCAGCAACCCTTGG
OT2	F	GCAGCGTCAGATGTGTATAAGAGACAGGGTCATATTACAGGCCAAAAATAGAAAATCCTTGTGG
	R	TGGGCTCGGAGATGTGTATAAGAGACAGAAAAGTGCAACTTGGTGGCAGAATCTAATTTGAATC
OT3	F	GCAGCGTCAGATGTGTATAAGAGACAGTGTGGATTCTTGCAGGCACGG
	R	TGGGCTCGGAGATGTGTATAAGAGACAGGGATACAGAGAGAGGATGGAGAG
OT4	F	GCAGCGTCAGATGTGTATAAGAGACAGGATGCTAGGATCTGACTTCTGTGC

	R	TGGGCTCGGAGATGTGTATAAGAGACAGCCAAGAGCACTAGCCCTTCAC
OT5	F	GCAGCGTCAGATGTGTATAAGAGACAGCCCTCGGGCTTTTAACACATGC
	R	TGGGCTCGGAGATGTGTATAAGAGACAGCAGCTTCCACATGAGATGATTCCC
OT6	F	GCAGCGTCAGATGTGTATAAGAGACAGCTGGATCCCTTCAATAGCCTCCT
	R	TGGGCTCGGAGATGTGTATAAGAGACAGGTGGCCACTGCAAAGTCTTCC
OT7	F	GCAGCGTCAGATGTGTATAAGAGACAGCATCTTCAGTAAGTCAGTGTAGATGTACTTTCC
	R	TGGGCTCGGAGATGTGTATAAGAGACAGTTTTTTCCCTCTTCTGCCTCCTCC
OT8	F	GCAGCGTCAGATGTGTATAAGAGACAGCAGCTTCTGTACAGTGTTTAATCTATGCCATC
	R	TGGGCTCGGAGATGTGTATAAGAGACAGGACAAGCATATGTATACATAGTGCTGCTTTGAC
OT9	F	GCAGCGTCAGATGTGTATAAGAGACAGTGTCTCAGGCTCCCAAGCTTC
	R	TGGGCTCGGAGATGTGTATAAGAGACAGCAGACTTGGTGGATTTTACTAAGACCCG
OT10	F	GCAGCGTCAGATGTGTATAAGAGACAGTGGGCCATTCAATCTTTACTTTTG
	R	TGGGCTCGGAGATGTGTATAAGAGACAGGGAGTGGTTAACTGCCCCATT

F, forward primer; R, reverse primer.

Supplemental figure legends

Supplemental Figure 1. Impact of bystander edits on *HBB* expression.

A. Frequency of alleles carrying bystander 1 (C) at position 6 and bystander 2 (A) at position 8 in edited healthy donor (HD) and β -thalassemia (BT) samples (n=3; 3 β -thalassemia patients and 2 HD). Data are expressed as mean \pm SEM. **B.** RT-qPCR detecting β -globin mRNAs in erythroid cells derived from edited HD cells carrying the bystander edits (HD-IVS1-110). As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE). Data from erythroid cells derived from HD (n=2 donors) and control β -thalassemic HSPCs were included (BT-TE; 3 β -thalassemia patients). β -globin expression was normalized to α -globin. Data are expressed as mean \pm SEM (n=3). **** p \leq 0.0001 (One-way Anova, HD-BE vs other conditions). **C.** Expression of β -, γ^G - and γ^A - globin chains measured by RP-HPLC in HD RBCs. β -like-globin expression was normalized to α -globin. The α -non- α globin ratio is reported on top of the graph. RBCs were obtained from edited HD cells carrying the bystander edits (HD-IVS1-110). As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE). Data from erythroid cells derived from HD (n=2 donors) and β -thalassemic HSPCs were included (BT-TE; 3 β -thalassemia patients). Data are expressed as mean \pm SEM (n=3). Dotted lines indicate maximum and minimum values observed for β -globin in HD-TE conditions. **D.** Analysis of HbF, HbA and HbA₂ by CE-HPLC in HD RBCs. We calculated the percentage of each Hb type over the total Hb tetramers. RBCs were obtained from edited HD cells carrying the bystander edits (HD-IVS1-110). As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE). Data from erythroid cells derived from HD (n=2 donors) and β -thalassemic HSPCs were included (BT-TE; 3 β -thalassemia patients). Data are expressed as mean \pm SEM (n=3). **E.** Frequency of GPA⁺, CD71⁺ and CD36⁺ cells at day 19 of erythroid differentiation, as measured by flow cytometry analysis. RBCs were obtained from edited HD cells carrying the bystander edits (HD-IVS1-110). As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE) (n=2 donors). Data are expressed as mean \pm SEM (n=3). **F.** Frequency of enucleated cells at day 13, 16 and 19 of erythroid differentiation, as measured by flow cytometry analysis of cells stained with the DRAQ5 nuclear dye. RBCs were obtained from edited HD cells carrying the bystander edits (HD-IVS1-110). As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE) (n=2 donors). rep = replicate. Data are expressed as mean \pm SEM (n=3). **G.** Flow cytometry histograms showing the frequency of apoptotic cells (AnnexinV⁺-cells) in the 7AAD⁻ cell population of HD samples carrying the bystander edits (HD-IVS1-110) at day 13 of erythroid differentiation. As controls, we used erythroid cells derived from HD HSPCs transfected only with TE (HD-TE) or SpRY-ABE8e mRNA (HD-BE) (n=2 donors). Uns = unstained, rep = replicate (n=3).

Supplemental Figure 2. On and off-target editing in β -thalassemic cells.

A. Experimental protocol used for base editing experiments in β -thalassemic HSPCs. SpRY-ABE8e mRNA and synthetic gRNA1 were co-transfected in β -thalassemic HSPCs. Cells were plated in a methylcellulose-based medium under conditions supporting erythroid (BFU-E) and granulomonocytic (CFU-GM) differentiation, or

differentiated into mature RBCs using a three-phase erythroid differentiation protocol. **B.** Percent composition of Sanger sequencing traces measured to be significantly different from noise (in red), as assessed by EditR following Sanger sequencing in β -thalassemic CD34⁺ cells transfected with gRNA1/SpRY-ABE8e (cor) or with TE buffer (TE). Target base position is highlighted with a red box and the bystander edits with black boxes (3 donors). **C.** Frequency of base editing (left panel) and InDels (right panel) at the *HBB* gene and at the top-10 predicted off-targets (OTs) in control (BT-TE) and edited (BT-cor) β -thalassemic samples, as measured by targeted NGS sequencing (3 β -thalassemia patients). **** $p \leq 0.0001$ (Two-way ANOVA). On-target editing for the compound heterozygous BT2 was calculated considering all the reads harboring a G at the target site as “edited”. **D.** Frequency and sequence of modified and unmodified alleles in corrected β -thalassemic samples, as measured by targeted NGS sequencing. Target base position is highlighted with a red box. Bystander edits are present at positions 6 and 8 (black boxes), and at positions -1 and -2 although at low frequency. **E.** Product purity of SpRY-ABE8e, as indicated by the type of substitution measured by targeted NGS sequencing.

Supplemental Figure 3. CFC assay from β -thalassemic HSPCs.

A. CFC frequency in control (TE or BE) and corrected β -thalassemic HSPCs (cor). (2 β -thalassemia patients, BT0 data were not plotted as we obtained only few colonies even in control samples, i.e. ≤ 10). **B.** Frequency of corrected alleles (left panel) and InDels (right panel), as evaluated by Sanger sequencing in single colonies derived from corrected and control β -thalassemic HSPCs (BT1 and BT2 β -thalassemia patients). In the right panel, the dotted line represents the maximum background noise of InDels calculated by TIDE.

Supplemental Figure 4. RP-HPLC chromatographs of *in vitro* differentiated RBCs.

RP-HPLC chromatographs from *in vitro* differentiated RBCs derived from corrected β -thalassemic HSPCs (BT0-2#cor). As controls, we used HD and β -thalassemic HSPCs transfected only with SpRY-ABE8e mRNA (representative HD0#BE and BT0-2#BE chromatographs).

Supplemental Figure 5. Analysis of RBC parameters by quantitative phase microscopy

A-E. Quantitative phase microscopy analysis of individual RBCs obtained from corrected β -thalassemic HSPCs (BT-cor, red curve) at day 19 of differentiation. As controls, we used RBCs derived from β -thalassemic HSPCs transfected only with SpRY-ABE8e (BT-BE, black curve) or HD HSPCs transfected only with SpRY-ABE8e (HD-BE, gray curve) (3 β -thalassemia patients and 2 HDs). We reported the relative number of RBCs with different perimeter (A), surface (B), optical volume (C), dry mass (D) and surface density (E) values. Data were reported as overlaid histograms.

Supplemental Figure 6. Bystander editing efficiency in repopulating HSCs.

Frequency of edited alleles at on-target, bystander 1 and bystander 2 positions, as evaluated by EditR in input HSPCs and in the bone marrow 16 weeks after xenotransplantation. The frequency of base editing in the input was calculated in cells cultured in the HSPC medium (\blacktriangle), in liquid erythroid cultures (\blacktriangledown), BFU-E (\blacksquare) and CFU-GM (\blacklozenge) colonies. For editing at 16 weeks, each data point represents an

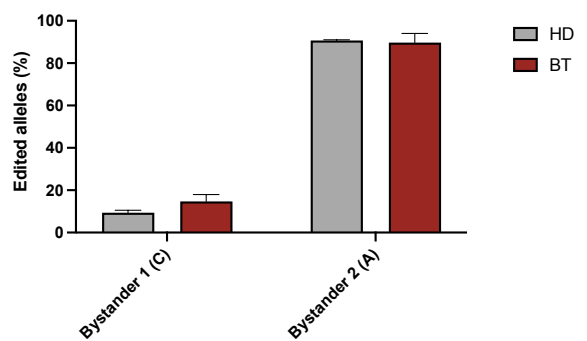
individual mouse transplanted with corrected HSPCs (n=5). Data are expressed as mean±SEM.

Supplemental references

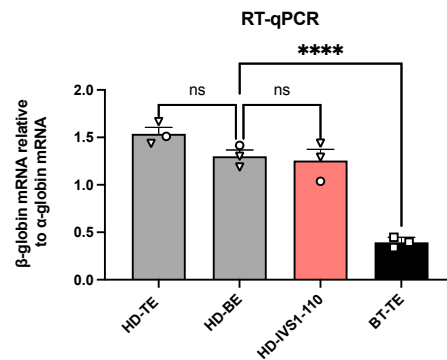
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Supplemental Figure 1

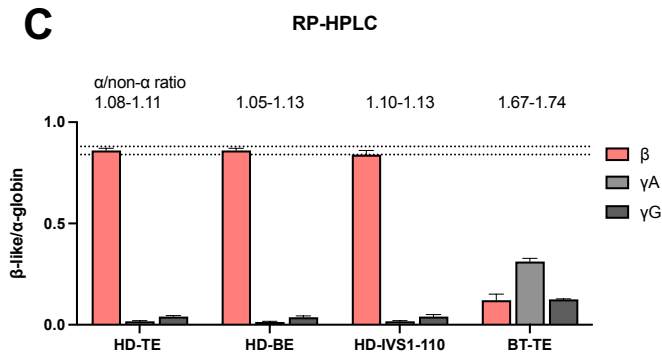
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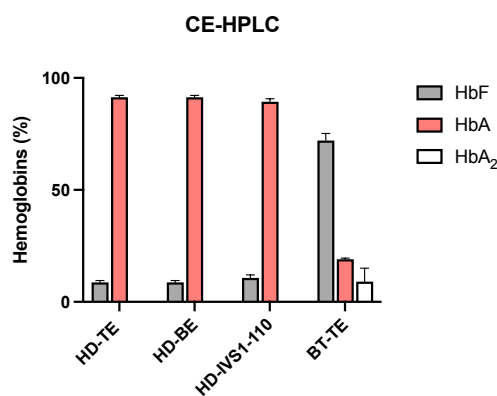
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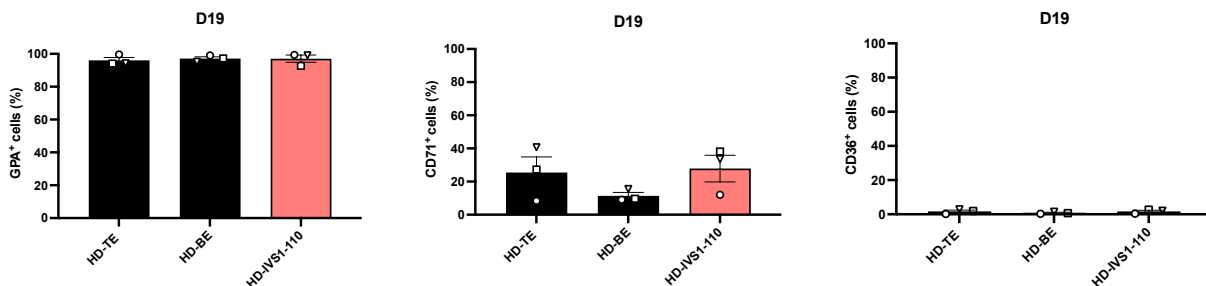
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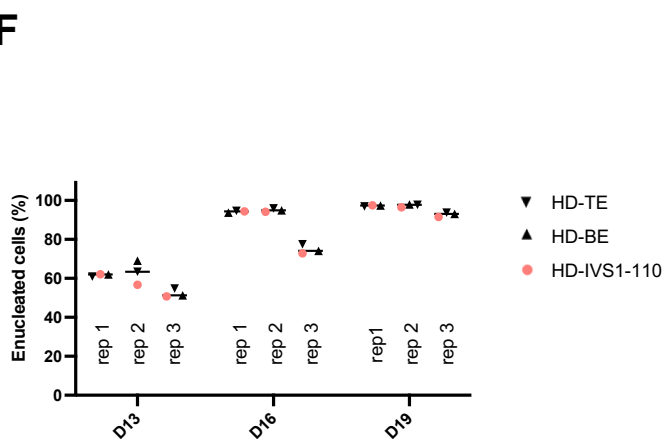
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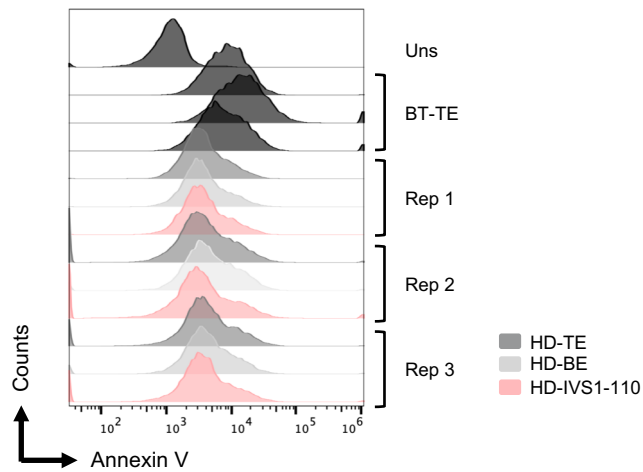
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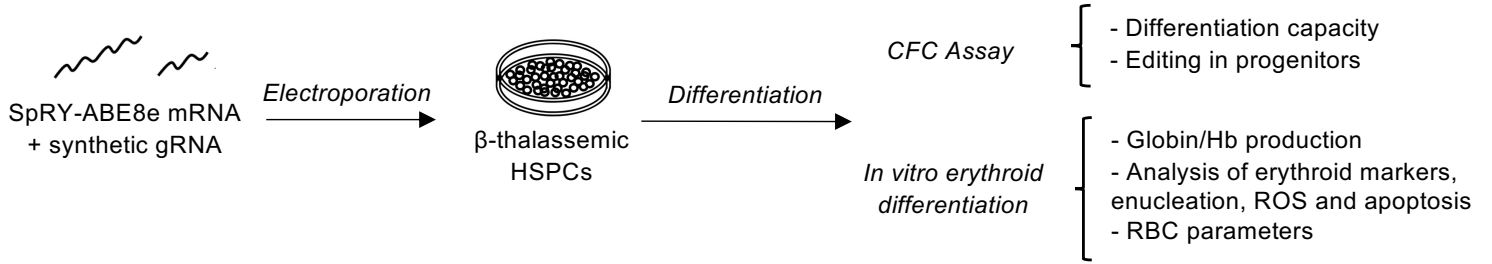


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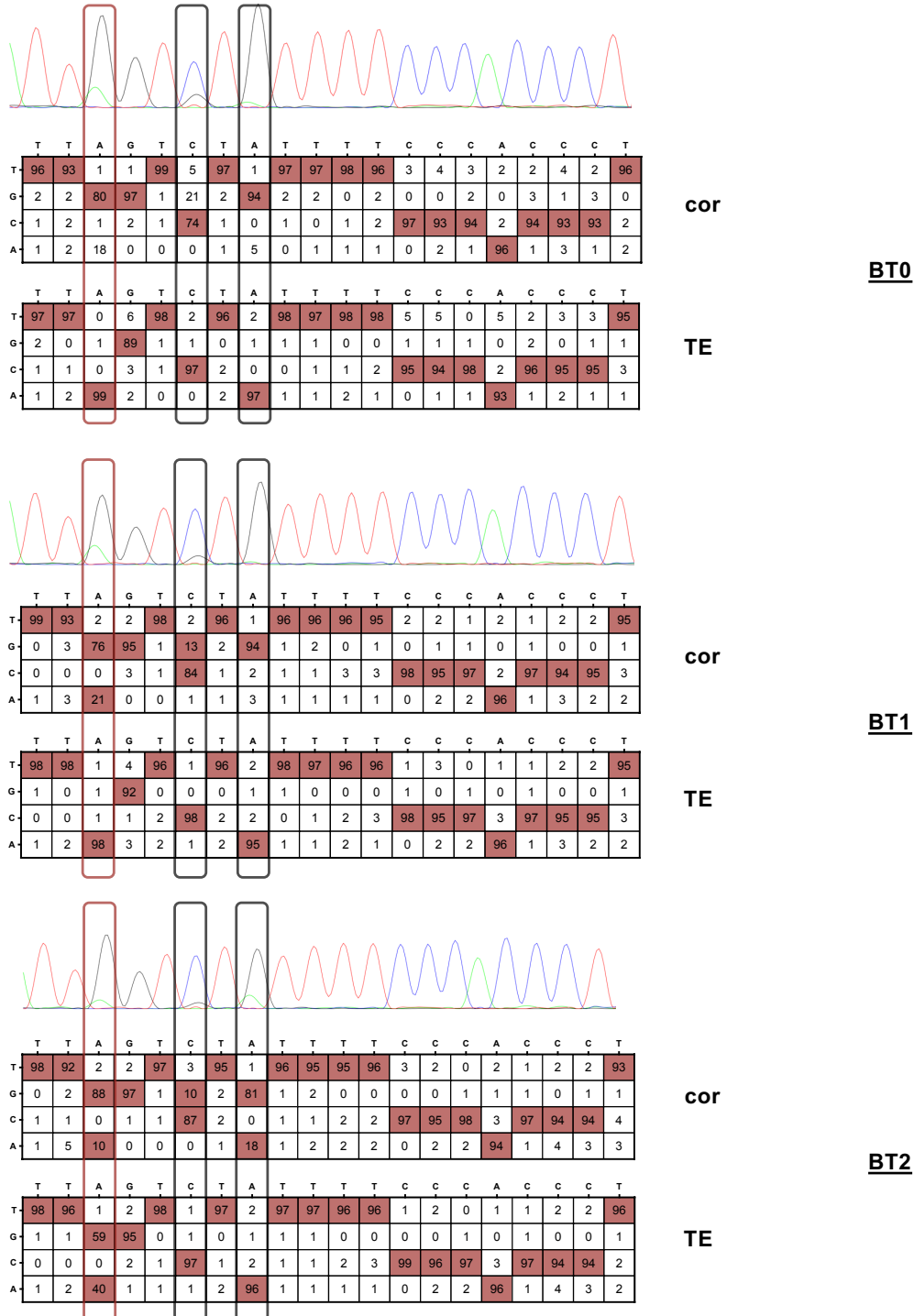


Supplemental Figure 2

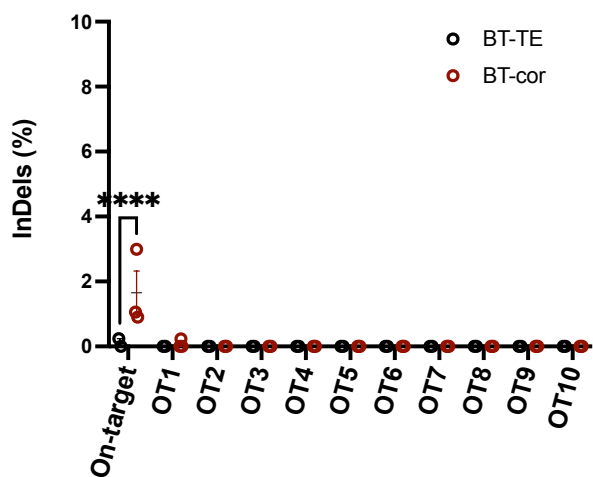
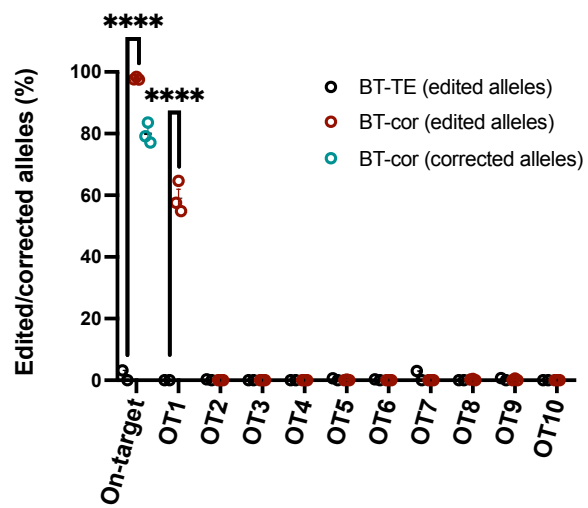
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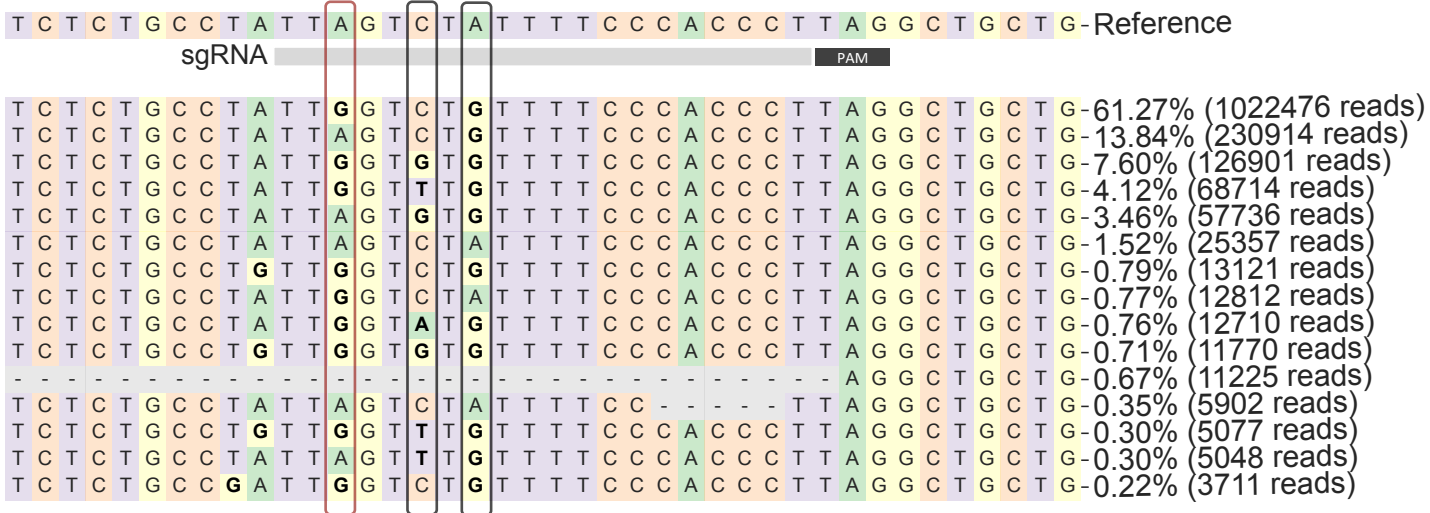


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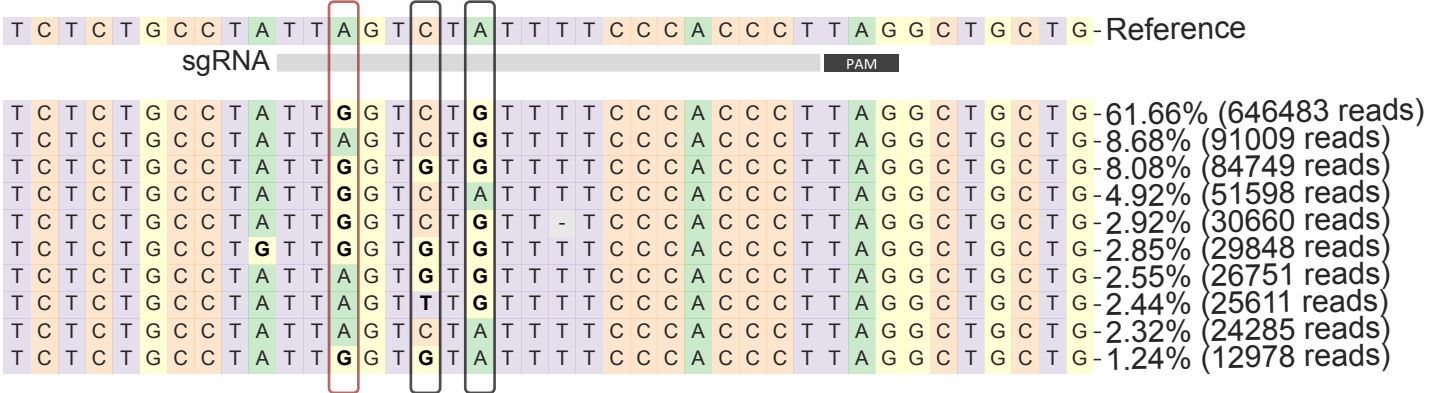


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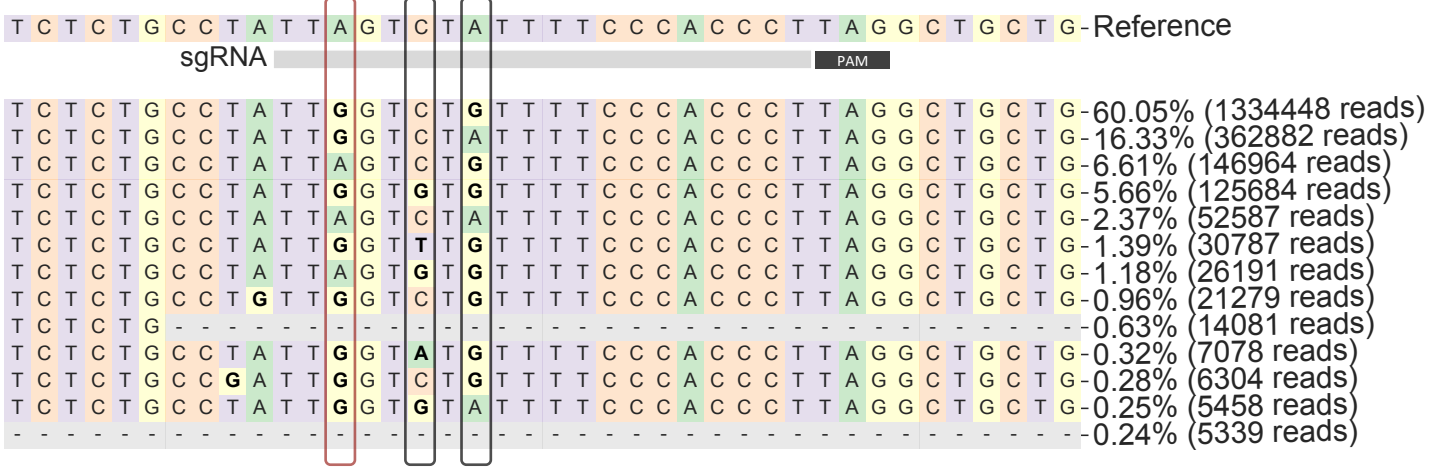
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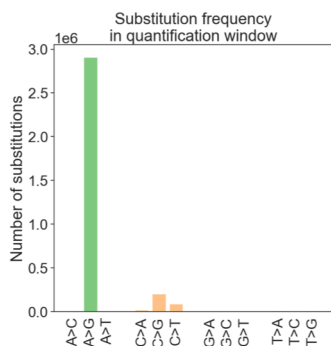
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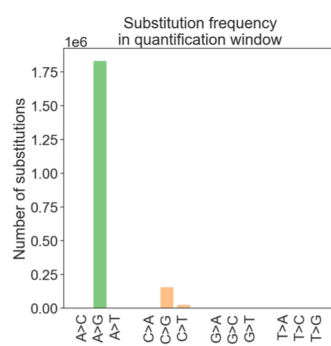
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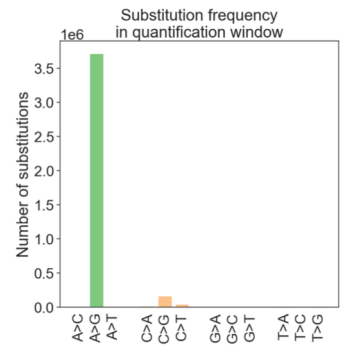
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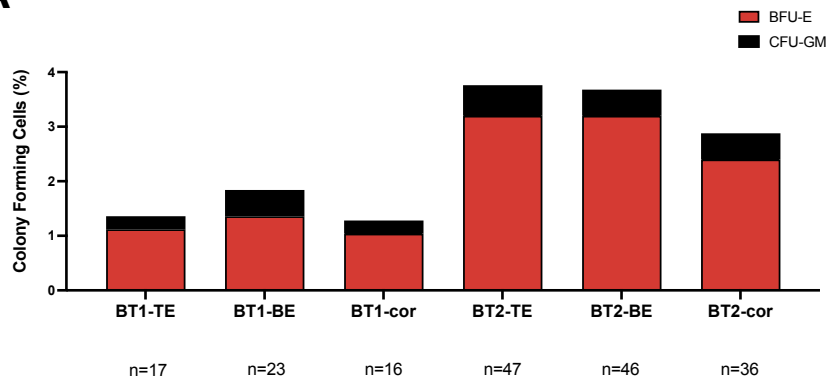


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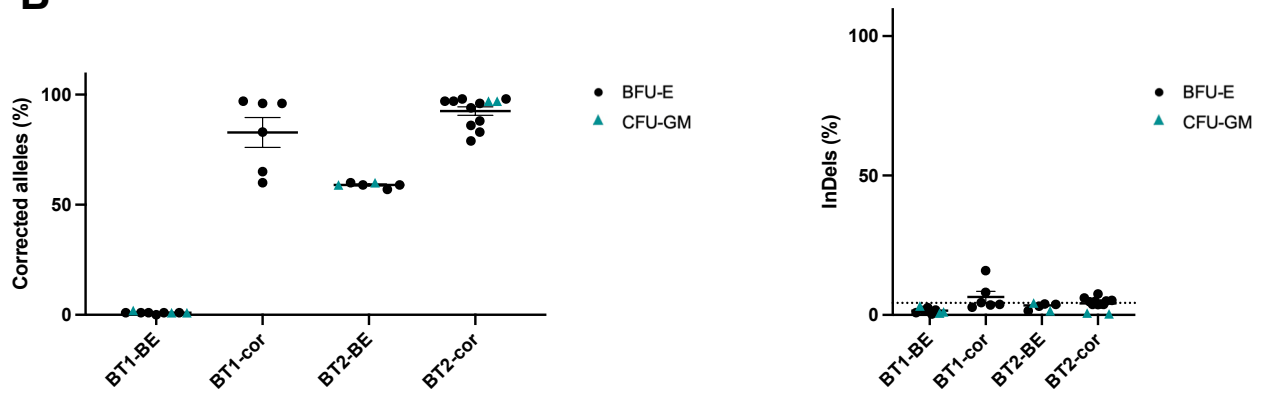


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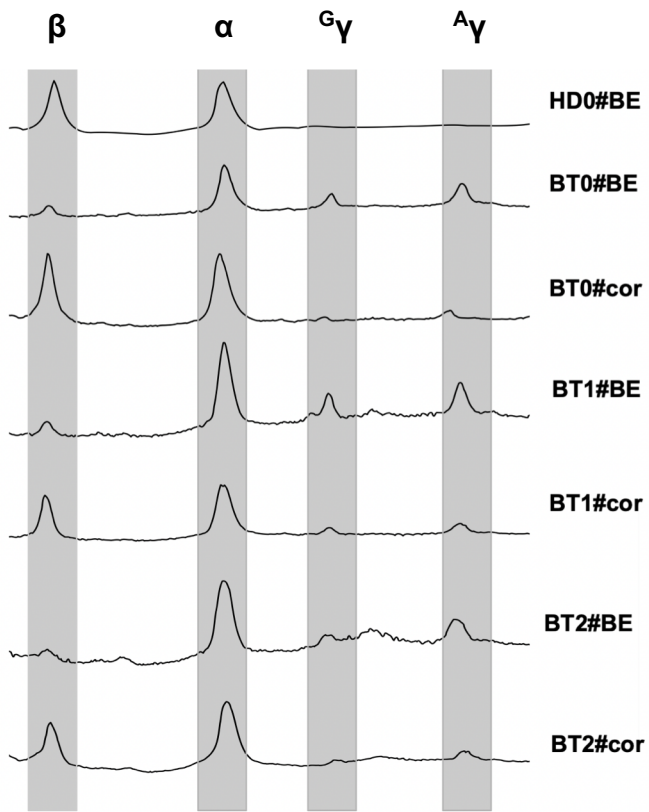
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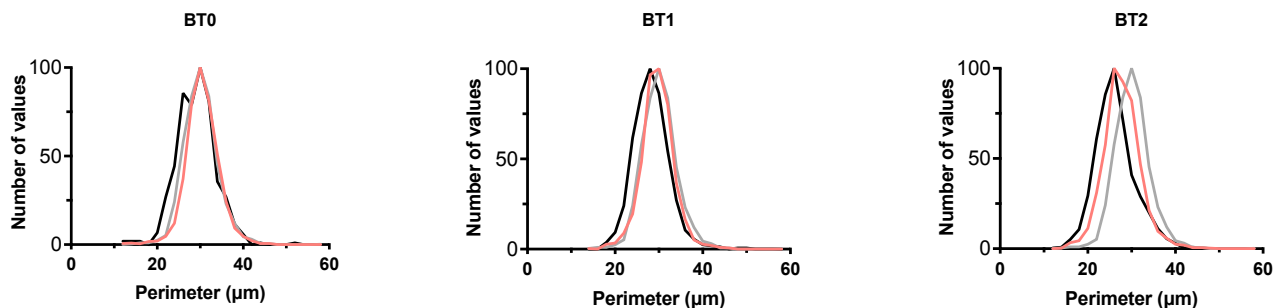
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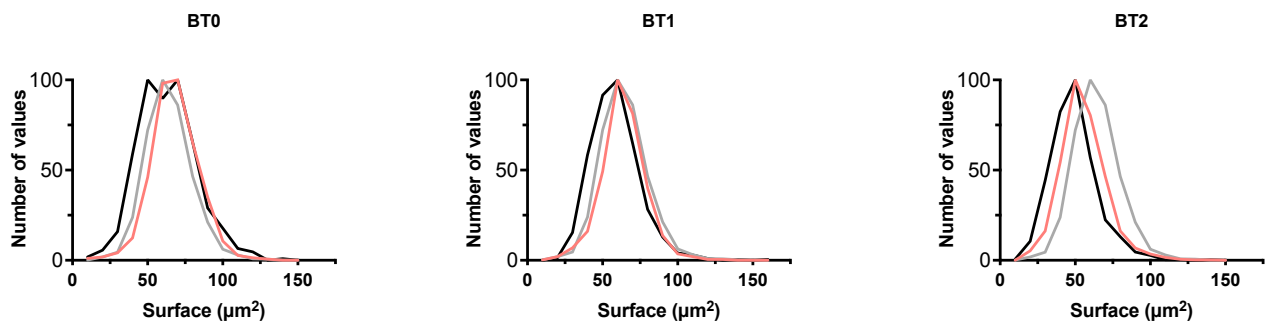
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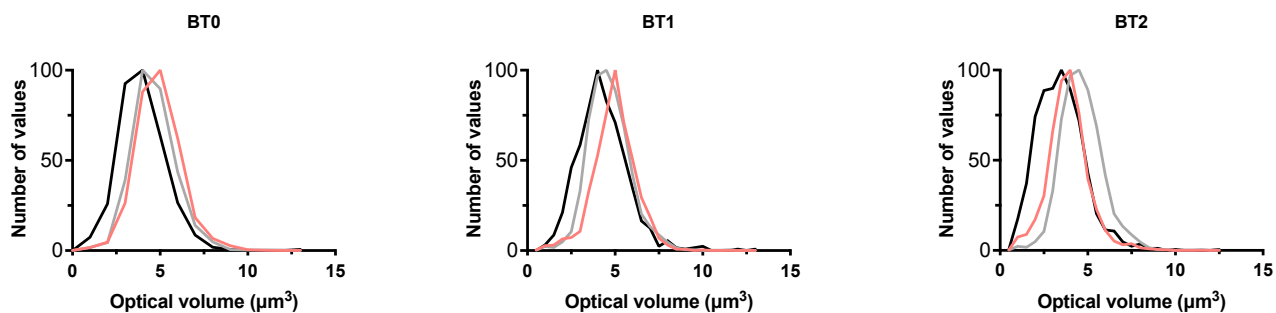
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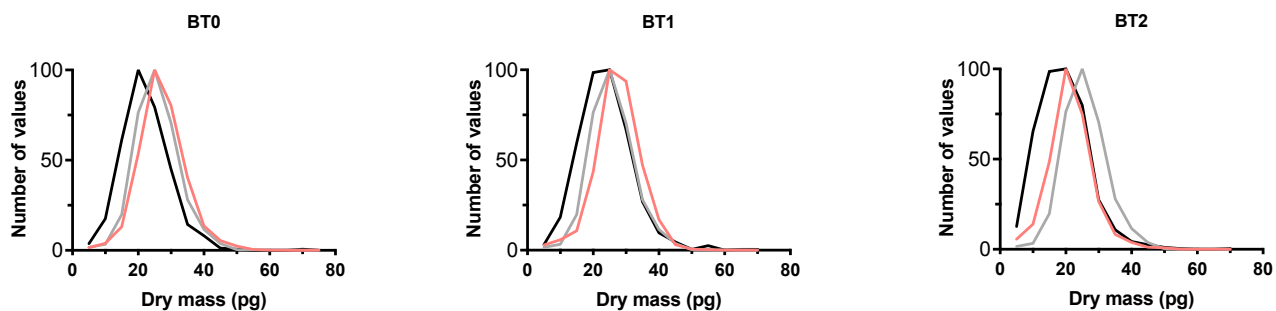
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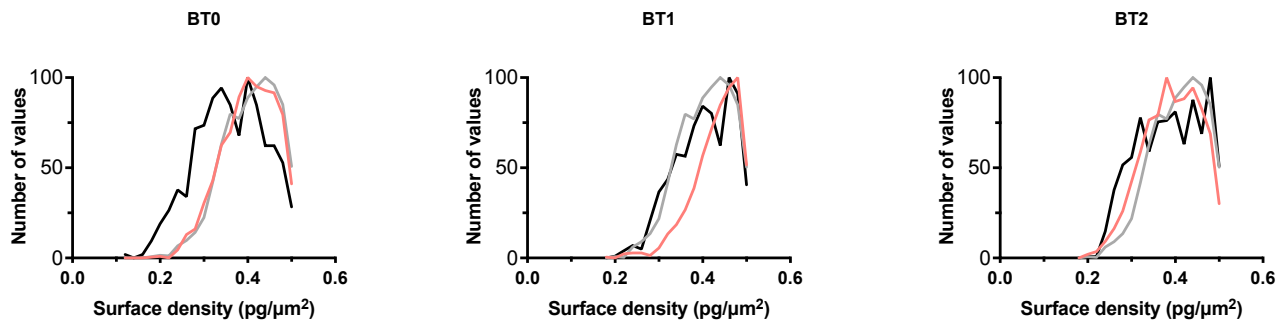
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D



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— HD-BE
 — BT-BE
 — BT-cor

Supplemental Figure 6

