Journal of
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MDPI

Contents

1. Supplementary Figures

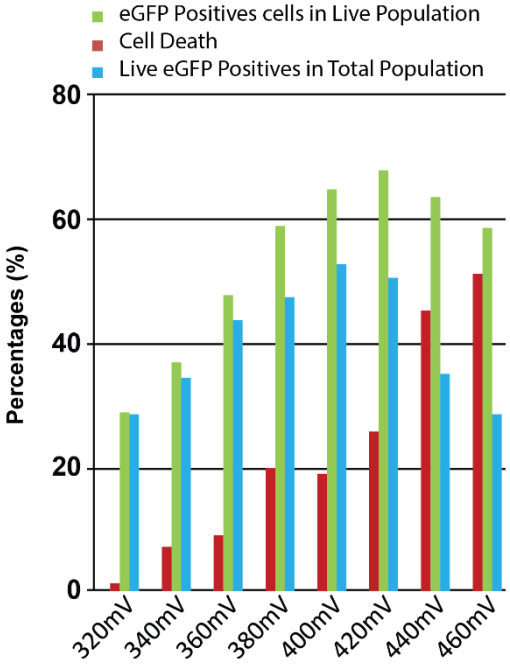

Figure S1. Flow cytometry of electroporated MEL cell line. Optimization of plasmid electroporation conditions in MEL cells. MEL cells were analyzed by flow cytometry 48 h after delivering the GFP reporter plasmid by electroporation, using a range of voltages and constant 1050 μF capacitance. Cell death (% SYTOX Red positives) was background-corrected for that of the non-electroporated negative control (≈20%, not shown). Optimal electroporation conditions (highlighted in yellow) were those with the highest percentage of live GFP positives in the total population (400 mV and 1050 μF).

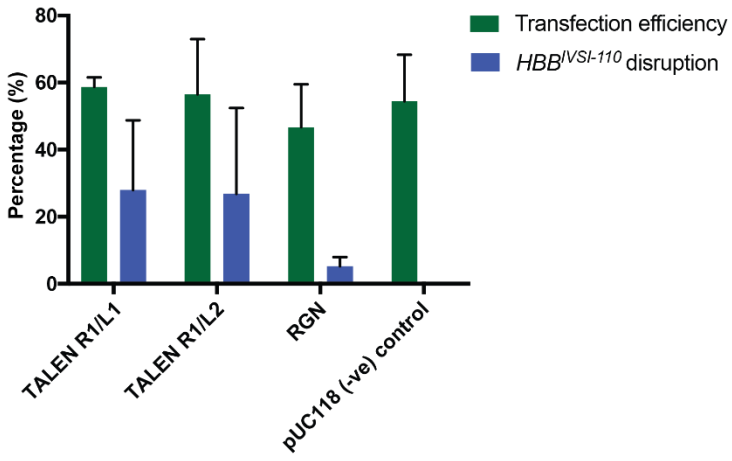

Figure S2. Transfection and targeted disruption efficiencies in MEL *HBB*^{IVS} bulk cells. Transfection efficiency is shown as the average percentage of GFP positives measured by flow cytometry 48 h post-electroporation (green bars), and percentages of *HBB*$^{IVSI\text{-}110(G>A)}$-targeted disruption on day 5 post-electroporation of (recovered) MEL *HBB*^{IVS} bulk populations as measured by the T7E1 assay (blue bars). All displayed data comprised the average values of biological triplicates (n = 3; ±SD).

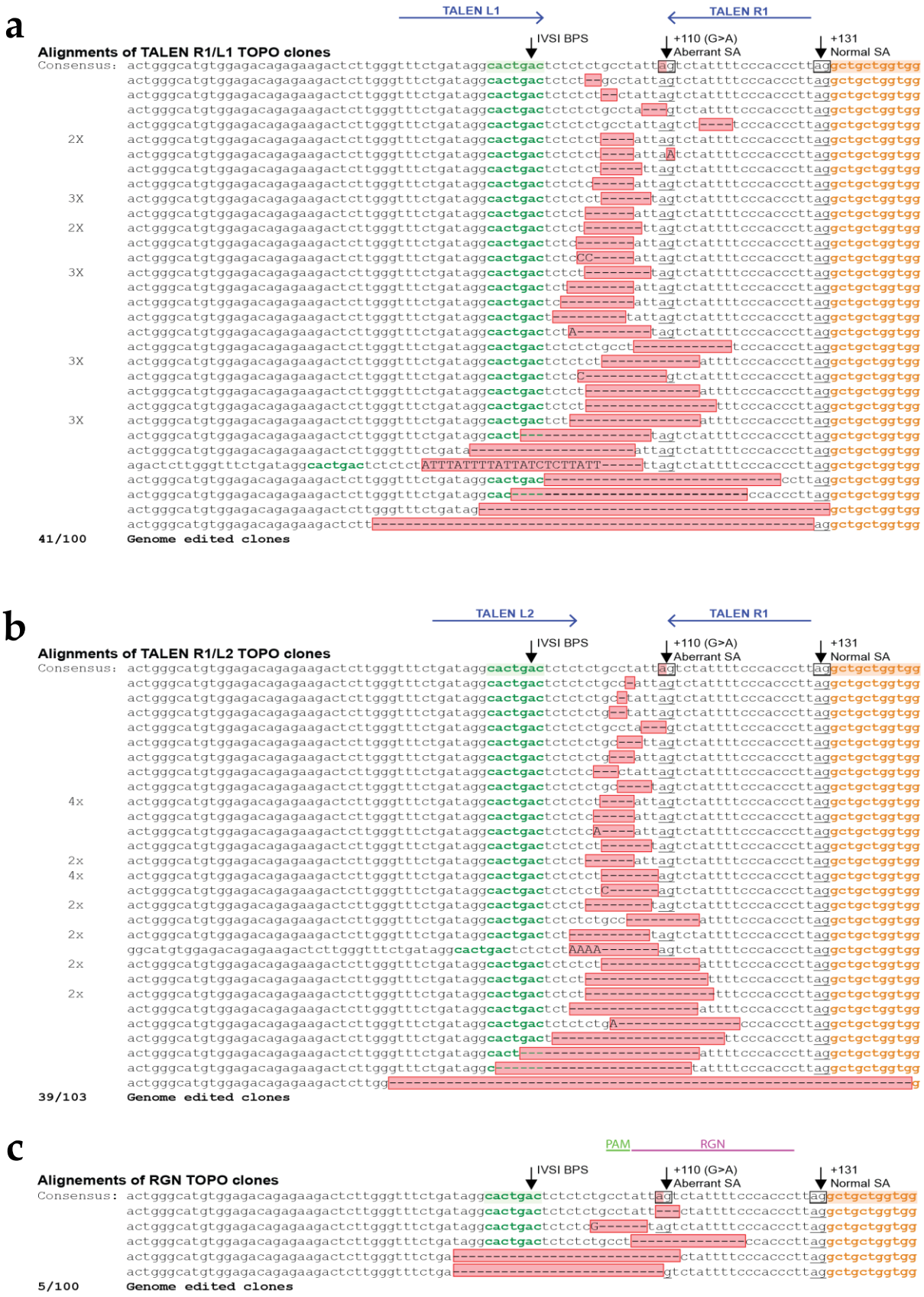

Figure S3. Characterization of TALEN- and RGN-induced indels in MEL-*HBB*^{IVS} as bacterial clones. Alignments of TOPO clones holding disrupted *HBB*$^{IVSI\text{-}110(G>A)}$ amplicons derived from HBB TALEN R1/L1 (**a**), TALEN R1/L2 (**b**) and RGN (**c**) genome-modified bulk MEL-*HBB*^{IVS} populations. TOPO clones were aligned primarily based on the size of the indel and secondarily based on indel proximity

to the normal splice acceptor site (+131 Normal SA). Intron 1 is shown unshaded, the intron-1 branchpoint site (IVSI BPS) in green, exon 2 in orange, the *HBB*$^{IVSI\text{-}110(G>A)}$ mutation in red, and the NHEJ-induced indels in pink. Aberrant (+110 (G>A) Aberrant SA) and normal (+131 Normal SA) splice acceptor sites are underlined (ag) sequences on the consensus sequence. Combined editing events of insertions (upper case) and deletions are shown. Binding sites of TALEN monomers are shown as blue arrows (A and B) and RGN gRNA and PAM sequence as purple and green lines (C) above each consensus sequence.

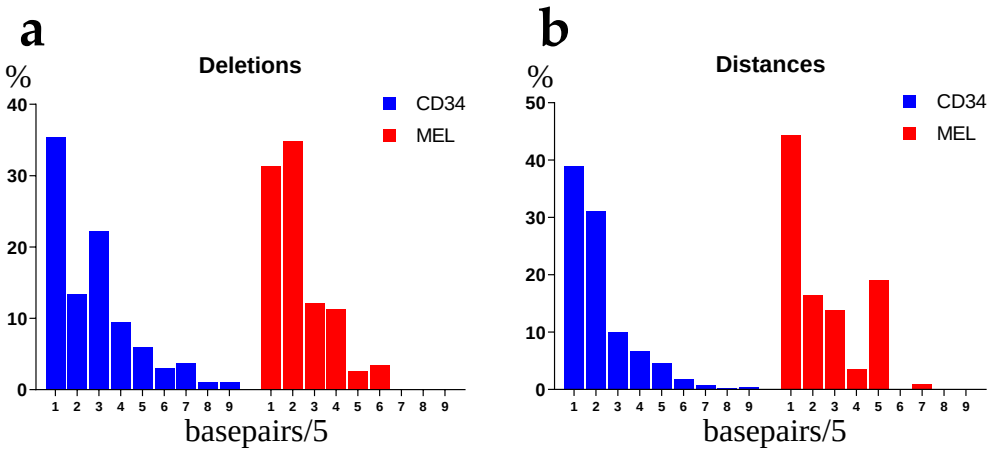

Figure S4. Comparison of TALEN data in CD34+ and MEL cells. Frequencies (**a**) of deletion sizes of the original *HBB*$^{IVSI\text{-}110(G>A)}$ on-target sequence and (**b**) the greatest distance of deletions from the predicted cleavage site are compared for *HBB*$^{IVSI\text{-}110(G>A)}$–homozygous CD34^{+} cells [1] and MEL-HBBIVS cells (this study) after categorization in bin sizes of five base pairs. The Spearman constant for the correlation of the categorized data is 0.865 (p value of correlation: 0.007) for (**a**) and 0.848 (p value of correlation: 0.005) for (**b**). The Spearman constant for the underlying uncategorized data is 0.786 (p value of correlation: 3.196×10^{-12}) for (**a**) and 0.745 (P value of correlation: 1.580×10^{-10}) for (**b**).

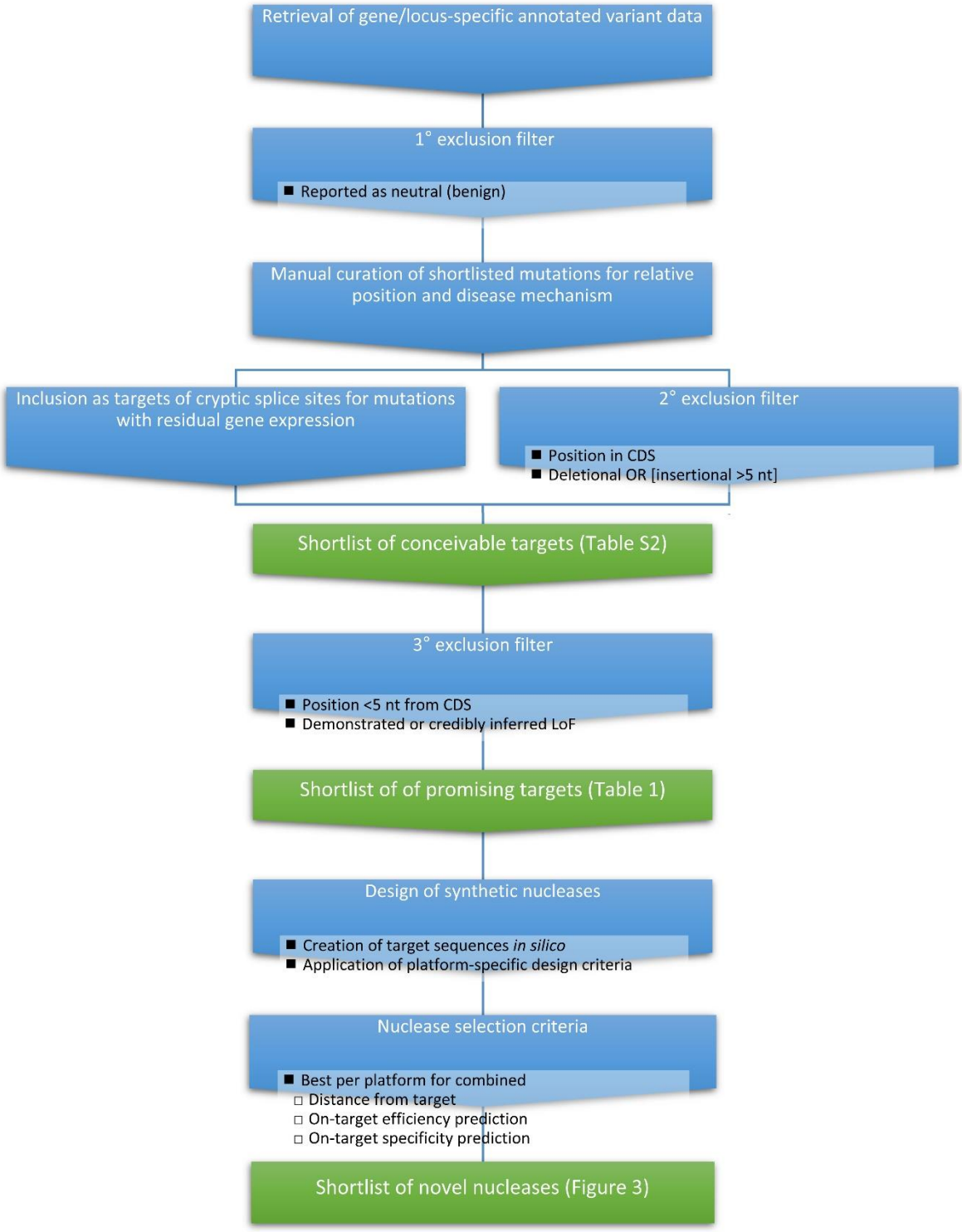

Figure S5. Schematic workflow of the DARE target and nuclease compilation for new target loci. 2° and 3° exclusion filters can be combined, where a list of conceivable targets is not of interest. CDS—coding sequence; LoF—loss of function; nt—nucleotide.

2. Supplementary Tables

Table S1. Known β-thalassemia mutations passing initial filter criteria for analysis.

IthaID[1]	Common Name	HGVS Name	Type of mutation	Region	Exon→[2]	References
3081[3]	-223 T>C	HBB:c.-273T>C	Likely RE[4] LoF[5]	Upstream promoter	273 nt[6]	[2]
1	-190 G>A	HBB:c.-240G>A	Likely RE LoF	Upstream promoter	240 nt	[3]
2	-102 C>A	HBB:c.-152C>A	RE LoF	CACCC box, distal	152 nt	[4]
3	-101 C>T	HBB:c.-151C>T	RE LoF	CACCC box, distal	151 nt	[5]
4	-101 C>G	HBB:c.-151C>G	RE LoF	CACCC box, distal	151 nt	[6]
3059	-98 T>A	HBB:c.-148T>A	Possible RE LoF	near CACCC boxes	148 nt	[7]
5	-93 C>G	HBB:c.-143C>G	RE LoF	near CACCC boxes	143 nt	ITHANET[7]
6	-92 C>T	HBB:c.-142C>T	RE LoF	near CACCC boxes	142 nt	[8]
7	-90 C>T	HBB:c.-140C>T	RE LoF	CACCC box, proximal	140 nt	[7]
3224	-90 C>G	HBB:c.-140C>G	RE LoF	CACCC box, proximal	140 nt	[9]
8	-88 C>T	HBB:c.-138C>T	RE LoF	CACCC box, proximal	138 nt	[7]
9	-88 C>A	HBB:c.-138C>A	RE LoF	CACCC box, proximal	138 nt	[10]
2178	-88 C>G	HBB:c.-138C>G	RE LoF	CACCC box, proximal	138 nt	[11]
10	-87 C>G	HBB:c.-137C>G	RE LoF	CACCC box, proximal	137 nt	[12]
11	-87 C>T	HBB:c.-137C>T	RE LoF	CACCC box, proximal	137 nt	[13]
12	-87 C>A	HBB:c.-137C>A	RE LoF	CACCC box, proximal	137 nt	[14]
13	-86 C>G	HBB:c.-136C>G	RE LoF	CACCC box, proximal	136 nt	[15]
14	-86 C>A	HBB:c.-136C>A	RE LoF	CACCC box, proximal	136 nt	[13]
3077	-83 G>A	HBB:c.-133G>A	Possible RE LoF	near CACCC & CCAAT	133 nt	[16]
3069	-77 G>C	HBB:c.-127G>C	Possible RE LoF	near CACCC & CCAAT	127 nt	[17]
3386	-76 C>A	HBB:c.-126C>A	RE LoF	CCAAT box	126 nt	[18]
15	-73 A>T	HBB:c.-123A>T	RE LoF	CCAAT box	123 nt	[19]
2997	-72 T>A	HBB:c.-122T>A	RE LoF	CCAAT box	122 nt	[20]
2171	-71 C>T	HBB:c.-121C>T	Likely RE LoF	DRE[8]	121 nt	[21]
3043	-71 C>T	HBB:c.-121C>T	Likely RE LoF	DRE	121 nt	[11]
16	-56 G>C	HBB:c.-106G>C	Likely RE LoF	DRE	106 nt	[3]
17	-50 G>A	HBB:c.-100G>A	Likely RE LoF	DRE	100 nt	[22]
3060	-42 C>G	HBB:c.-92C>G	Likely RE LoF	DRE	92 nt	[7]
2172	-41 A>T	HBB:c.-91A>C	Likely RE LoF	DRE	91 nt	[23]
18	-32 C>A	HBB:c.-82C>A	Likely RE LoF	DRE	82 nt	[24]
19	-32 C>T	HBB:c.-82C>T	Likely RE LoF	DRE	82 nt	[25]
20	-31 A>G	HBB:c.-81A>G	RE LoF	TATA (ATAAA) box	81 nt	[26]
21	-31 A>C	HBB:c.-81A>C	RE LoF	TATA (ATAAA) box	81 nt	[27]
22	-30 T>A	HBB:c.-80T>A	RE LoF	TATA (ATAAA) box	80 nt	[28]
23	-30 T>C	HBB:c.-80T>C	RE LoF	TATA (ATAAA) box	80 nt	[29]
2179	-30 T>G	HBB:c.-80T>G	RE LoF	TATA (ATAAA) box	80 nt	[11]
25	-29 A>G	HBB:c.-79A>G	RE LoF	TATA (ATAAA) box	79 nt	[30]
26	-29 A>C	HBB:c.-79A>C	RE LoF	TATA (ATAAA) box	79 nt	[31]
28	-28 A>C	HBB:c.-78A>C	RE LoF	TATA (ATAAA) box	78 nt	[32]
29	-28 A>G	HBB:c.-78A>G	RE LoF	TATA (ATAAA) box	78 nt	[33]
30	-27 A>T	HBB:c.-77A>T	Likely RE LoF	near TATA (ATAAA)	77 nt	[34]
2175	-26 A>C	HBB:c.-76A>C	Likely RE LoF	near TATA (ATAAA)	76 nt	[23]
32	-25 G>C	HBB:c.-75G>C	Likely RE LoF	near TATA (ATAAA)	75 nt	[25]
2565	-25 G>T	HBB:c.-75G>T	Likely RE LoF	near TATA (ATAAA)	75 nt	[35]
34	CAP +1 A>C	HBB:c.-50A>C	LoF	5′ UTR	50 nt	[36]
3464	CAP +3 A>T	HBB:c.-48A>T	LoF	CAP initiator element	48 nt	[37]
35	CAP +8 C>T	HBB:c.-43C>T	Likely LoF	5′ UTR	43 nt	[38]
36	CAP +10 -T	HBB:c.-41delT	Mild LoF	5′ UTR	41 nt	[39]
2494	CAP +16 A>G	HBB:c.-35A>G	Mild LoF	5′ UTR	35 nt	[40]
3345	CAP +22 G>T	HBB:c.-29G>T	Mild LoF	5′ UTR	29 nt	[41]
38	CAP +22 G>A	HBB:c.-29G>A	Mild LoF	5′ UTR	29 nt	[42]
2536	CAP +30 T>A	HBB:c.-21T>A	Mild LoF	5′ UTR	21 nt	[43]
39	CAP +33 C>G	HBB:c.-18C>G	Mild LoF	5′ UTR	18 nt	[44]
2176	CAP +39 C>T	HBB:c.-12C>T	Mild LoF	5′ UTR	12 nt	[45]
40	CAP +40 to +43 (-AAAC)	HBB:c.-11_-8delAAAC	LoF	5′ UTR	8 nt	[46]
41	CAP +45 (G>C)	HBB:c.-6G>C	Mild LoF	5′ UTR, Kozak sequence	6 nt	[47]
107	IVS I-5 G>A	HBB:c.92+5G>C	Activation of cSD[9]; partial SD LoF	SD[10]-proximal	5 nt	[12,48]

111	IVS I-6 T>C	HBB:c.92+6T>C	Activation of cSD; partial SD LoF	SD-proximal	6 nt	[49]
112	IVS I-7 A>T	HBB:c.92+7A>T	Unknown	SD-proximal	7 nt	ITHANET[7]
3276	IVS I-7 A>G	HBB:c.92+7A>G	Unknown	SD-proximal	7 nt	ITHANET[7]
3445	IVSI-13G	HBB:c.92+13	Potential target	cSD activated by IthaID	13 nt	[12]
113	IVS I-110 G>A	HBB:c.93-21G>A	**Confirmed target; GG>GA (aSA)**[11]	aSA	21 nt	[1,50]
3008	IVS I-115 A>T	HBB:c.93-16A>T	AT>TT (effect unclear)	Intronic	16 nt	[51]
114	IVS I-116 T>G	HBB:c.93-15T>G	TT>GT (potential aSD)[12]	Intronic	15 nt	[52]
115	IVS I-128 T>G	HBB:c.93-3T>G	SS LoF	3' pyrimidine run	3 nt	[53]
116	IVS I-129 A>C	HBB:c.93-2A>C	SS LoF	SA[13]	2 nt	[54]
117	IVS I-129 A>G	HBB:c.93-2A>G	SS LoF	SA	2 nt	[55]
118	IVS I-130 G>C	HBB:c.93-1G>C	SS LoF	SA	1 nt	[56]
119/120	IVS I-130 G>A	HBB:c.93-1G>A	SS LoF	SA	1 nt	[7,57–59]
200	IVS II-1 G>A	HBB:c.315+1G>A	SS LoF	SD	1 nt	[60,61]
201	IVS II-1 G>C	HBB:c.315+1G>C	SS LoF	SD	1 nt	[62]
202	IVS II-1 G>T	HBB:c.315+1G>T	SS LoF	SD	1 nt	ITHANET[7]
203	IVS II-2 T>C	HBB:c.315+2T>C	SS LoF	SD	2 nt	[63]
204	IVS II-2 T>A	HBB:c.315+2T>A	SS LoF	SD	2 nt	ITHANET[7]
3226	IVS II-2 T>G	HBB:c.315+2T>G	SS LoF	SD	2 nt	[9]
208	IVS II-5 G>C	HBB:c.315+5G>C	Activation of cSD; partial LoF	Intronic	5 nt	[64,65]
3446	IVS II-579G	HBB:c.316-272	cSA[14] activated by IthaID 214	Intron	270 nt	[12]
210	IVS II-613 C>T	HBB:c.316-238C>T	Activation of cryptic splice site	Intron	238 nt	[66]
211	IVS II-654 C>T	HBB:c.316-197C>T	**Confirmed target; GC>GT (aSD)**	Intron	197 nt	[50,67]
212	IVS II-705 T>G	HBB:c.316-146T>G	Activation of cryptic splice site	Intron	146 nt	[68,69]
213	IVS II-726 A>G	HBB:c.316-125A>G	Likely block of RNA processing	Intron	125 nt	[70]
214	IVS II-745 C>G	HBB:c.316-106C>G	aSD, activating cSA IthaID3446	Intron	106 nt	[12]
215	IVS II-761 A>G	HBB:c.316-90A>G	AT>GT (potential aSD	Intron	90 nt	ITHANET[7]
2183	IVS II-781 C>G	HBB:c.316-70C>G	CT>GT (potential aSD)	Intron	70 nt	[11]
216	IVS II-815 C>T	HBB:c.316-36C>T	CT>GT (potential aSD)	Intron	36 nt	[71]
217	IVS II-837 T>G	HBB:c.316-14T>G	AT>AG (potential aSA)	Intron	14 nt	[72]
218	IVS II-843 T>G	HBB:c.316-8T>G	LoF	3' pyrimidine run	8 nt	[73]
219	IVS II-844 C>A	HBB:c.316-7C>A	Presumed LoF, very mild	3' pyrimidine run	7 nt	[74]
220	IVS II-844 C>G	HBB:c.316-7C>G	Presumed LoF, very mild	3' pyrimidine run	7 nt	[75,76]
221	IVS II-848 C>A	HBB:c.316-3C>A	LoF	3' pyrimidine run	3 nt	[53,77]
222	IVS II-848 C>G	HBB:c.316-3C>G	LoF	3' pyrimidine run	3 nt	[78]
3045	IVS II-848 C>T	HBB: c.316-3C>T	Presumed LoF	3' pyrimidine run	3 nt	[11]
267	Terminal CD +6 C>G [CAP +1480]	HBB:c.*6C>G	Mild LoF	3′ UTR	6 nt	[79]
2177	Terminal CD +32	HBB:*32A>C	Presumed LoF	3′ UTR	32 nt	[45]
268	Terminal CD +47	HBB:c.*47C>G	Presumed LoF	3′ UTR	47 nt	ITHANET[7]
3443	Cap +1570 (T>C)	HBB:c*96T>C	Presumed LoF, very mild	3′ UTR	96 nt	[80]
278	Poly A (-AATAA) AATAAA> -----	HBB:c.*108_*112delAATAA	LoF	poly(A) signal	108 nt	[81]
270	Poly A (A>C) AATAAA>CATAAA	HBB:c.*108A>C	Mild LoF	poly(A) signal	108 nt	[82]
271	Poly A (A>G) AATAAA>GAT	HBB:c.*108A>G	LoF	poly(A) signal	108 nt	[83]
277	Poly A -AT	HBB:c.*109_*110delAT | HBB:c.*110_*111delTA	LoF	poly(A) signal	109 nt	[84]
272	Poly A (T>C) AATAAA>AACAAA	HBB:c.*110T>C	Mild LoF	poly(A) signal	110 nt	[85]
273	Poly A (T>A) AATAAA>AAAAAA	HBB:c.*110A>C	Mild LoF	poly(A) signal	110 nt	[86]
3046	Poly(A) AATAAA>AAT--A	HBB:c.*111_*112delAA	Mild LoF	poly(A) signal	111 nt	[87]
274	Poly A (A>G) AATAAA>AATGAA	HBB:c.*111A>G	Mild LoF	poly(A) signal	111 nt	[88]

2198	Poly A (A>T) AATAAA>AATATA	HBB:c.*112A>T	Mild LoF	poly(A) signal	112 nt	[89]
275	Poly A (A>G) AATAAA>AATAGA	HBB:c.*112A>G	Mild LoF	poly(A) signal	112 nt	[88]
276	Poly A (A>G) AATAAA>AATAAG	HBB:c.*113A>G	Mild LoF	poly(A) signal	113 nt	[90]
2564	3'UTR +1592	HBB:c.*118A>G	Very mild or benign	Conserved +1592 nt in 3'	1592 nt	[91]
2463	3'UTR +101 G>C	HBB:c.*233G>C	Very mild or benign	3' UTR-adjacent	101 nt	[92]

Fill color for "*Type of mutation*" indicates likely suitability for DARE, based on *Region*, severity and level of characterization. Absence of fill color – —unsuitable for DARE; orange fill color—likely unsuitable for DARE; yellow fill color—possibly suitable for DARE; green fill color—likely or proven suitable for DARE and included in Table 1 of the manuscript.

[1] Nucleotide-specific target ID from ITHANET (www.ithanet.eu); [2] Distance of the target from the nearest exon; [3] IthaID3083 was the only *HBB* mutation detected in heterozygosity in a β-thalassemic patient. The second mutation is presumed to have escaped detection. IthaID3083 would be a potential target if it turned out to be dominant after all; [4] RE—response element; [5] LoF—loss of function; [6] nt—nucleotide; [7] Unpublished data retrieved from the ITHANET Portal [93]; [8] DRE—direct repeat element; [9] cSD—cryptic splice donor; [10] SD—splice donor; [11] aSA—aberrant splice acceptor; [12] aSD—aberrant splice donor; [13] SA—splice acceptor; [14] cSA—cryptic splice acceptor

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