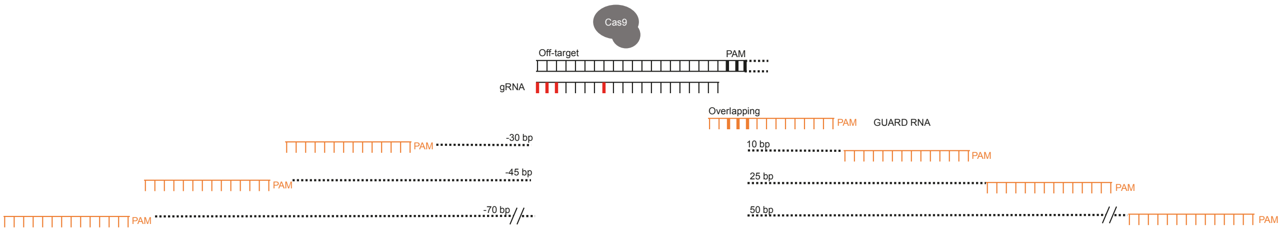
CRISPR GUARD protects off-target sites from Cas9 nuclease activity using short guide RNAs

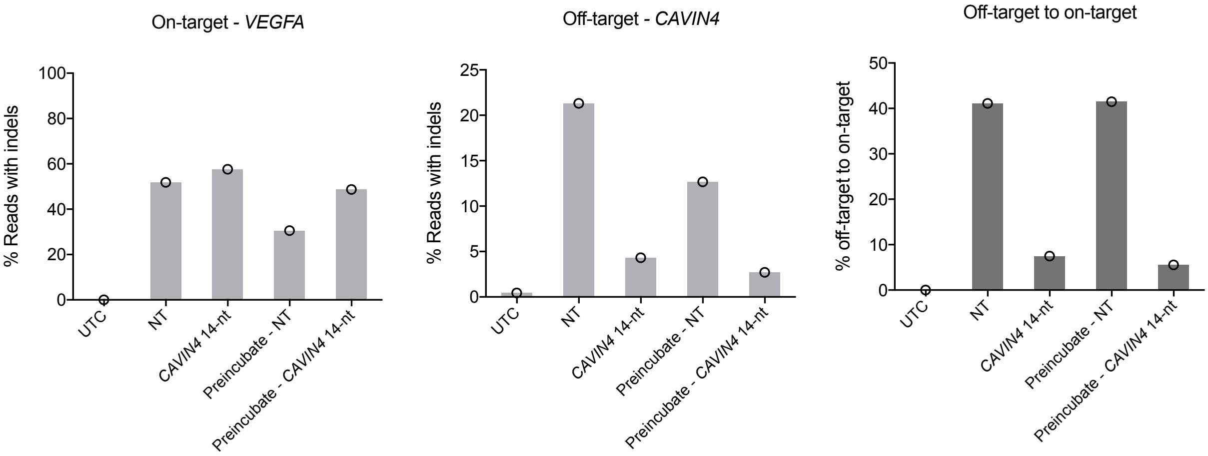
Coelho et al.

Supplementary Figure 1



Schematic of the experiment described in Fig. 2e.

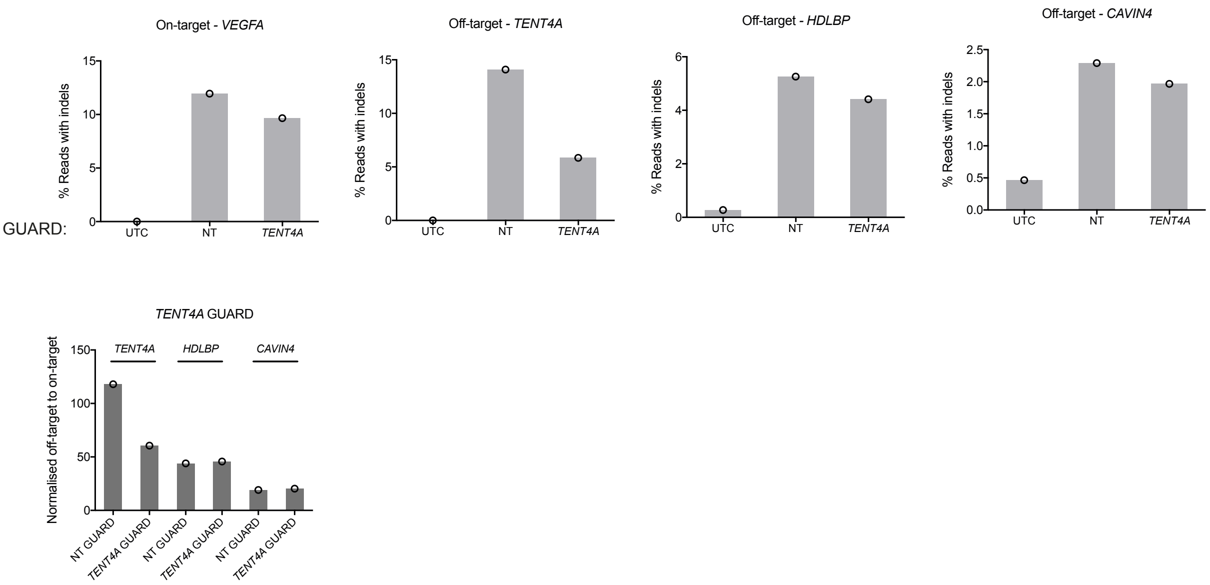
Supplementary Figure 2



Pre-incubation of GUARD RNAs in cells does not provide additional protection of off-target sites. Indel rates from amplicon sequencing of Cas9-expressing HEK293 cells transfected with *VEGFA* on-target gRNA and *CAVIN4* proximal off-target GUARD RNA at ratio of 25:25 nM. Transfection was performed concomitantly, or by first transfecting with GUARD RNA and 18 h later, transfecting with the on-target gRNA ('pre-incubate' condition). The ratio of off-target to on-target is represented in the right-hand panel.

Source data are provided as a Source Data file.

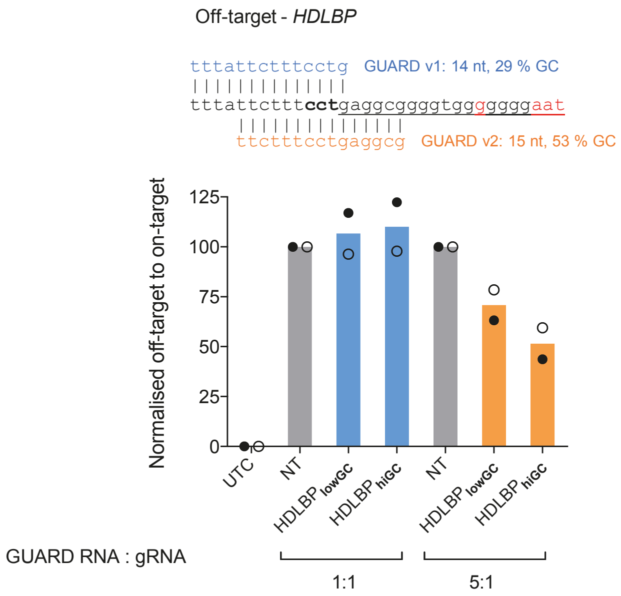
Supplementary Figure 3



CRISPR GUARD protection of one off-target site does not lead to increased off-target mutagenesis at other sites. Amplicon sequencing analysis of HEK293 cells electroporated with wild-type Cas9 protein complexed with *VEGFA* on-target gRNA, and dCas9 protein complexed with *TENT4A* GUARD RNA. On-target cutting (*VEGFA*), and *TENT4A, HDLBP* and *CAVIN4* proximal off-target sites were analysed for indel formation. The ratio of off-target to on-target is represented in the lower panel.

Source data are provided as a Source Data file.

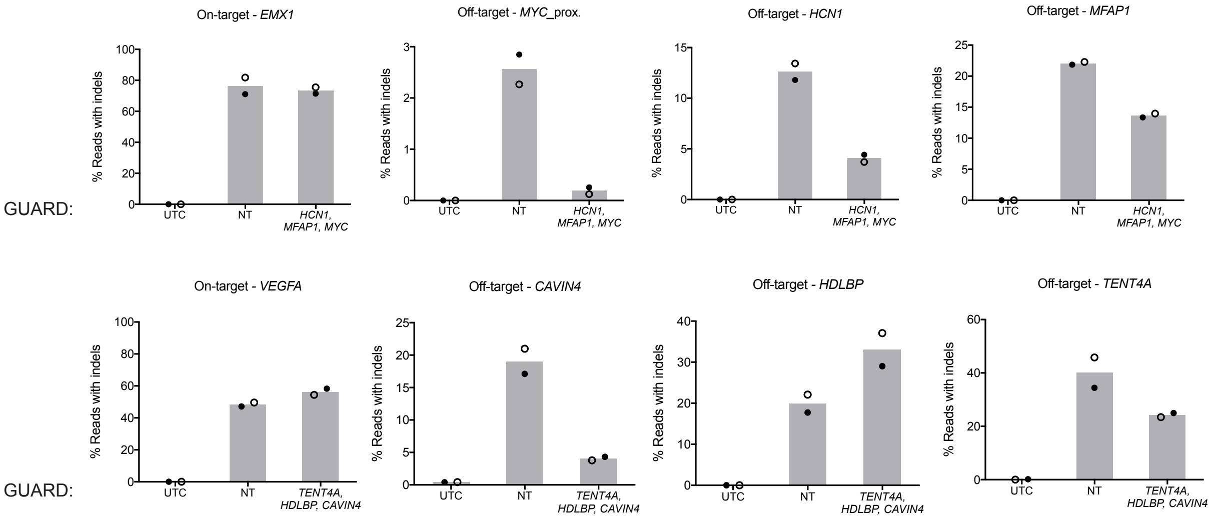
Supplementary Figure 4



Rational redesign of poorly performing GUARD RNAs by increasing GC-content. Shown is the *VEGFA* gRNA off-target region *HDBLP*, with the mismatched gRNA and two distinct GUARD RNA designs. Indel rates for 25:25 nM and 125:25 nM GUARD RNA to gRNA ratios from NGS of amplicons from Cas9-expressing HEK293 cells are expressed as an off-target to on-target ratio. Mean of two independent experiments with symbols representing each replicate.

Source data are provided as a Source Data file.

Supplementary Figure 5

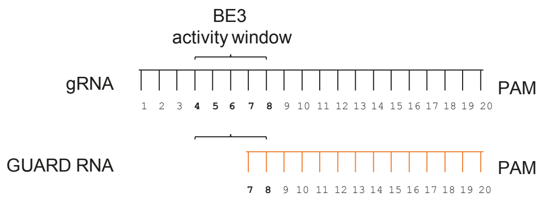


Multiplexed delivery of GUARD RNAs provides protection against multiple Cas9 off-targets simultaneously. Indel rates from amplicon sequencing of Cas9-expressing HEK293 cells transfected with *EMX1* or *VEGFA* on-target gRNAs, and three GUARD RNAs (25:25:25:25 nM ratio). Mean of two independent experiments with symbols representing each replicate.

Source data are provided as a Source Data file.

Supplementary Figure 6

a

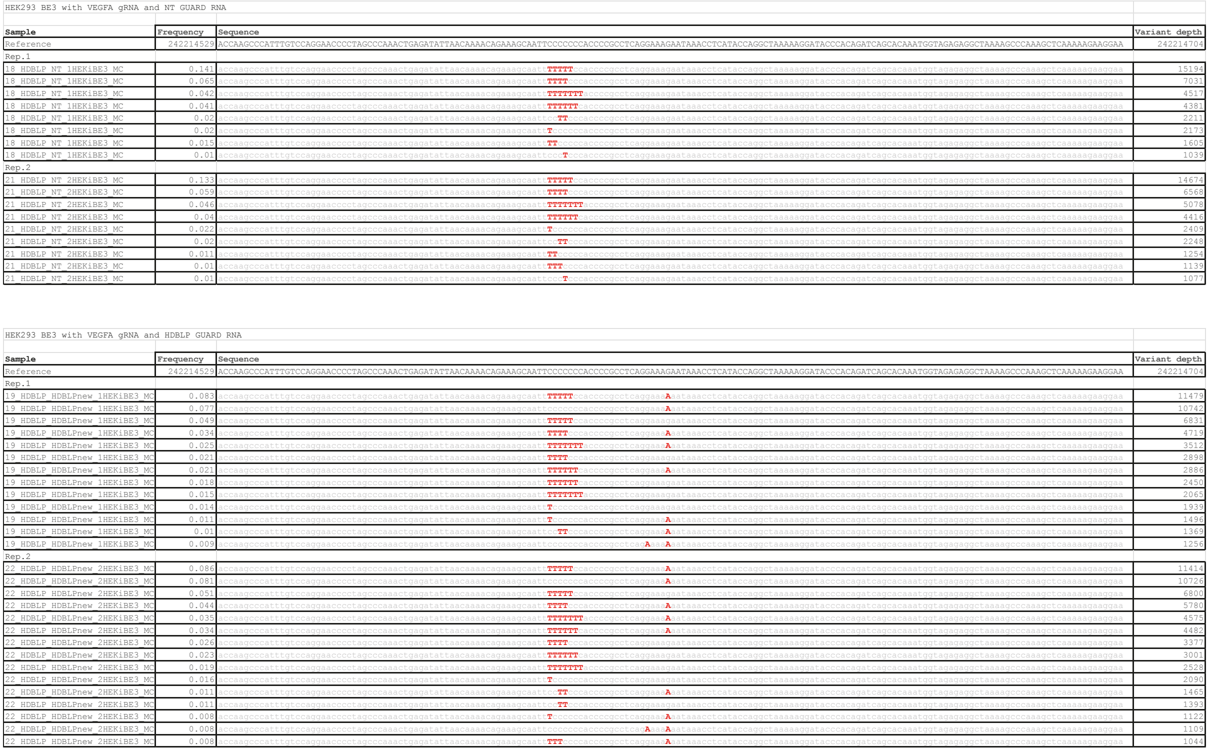


b

On-target EMX1	5′ GAGTCCGAGCAGAAGAAGAA**GGG** 3′
OFF MFAP1	5′ GAGTCTAAGCAGAAGAAGAA**GAG** 3′
GUARD1	5′ TAAGCAGAAGAAGAA**GAG** 3′
OFF MYC proximal	5′ GAGTCCTAGCAGGAGAAGAA**GAG** 3′
GUARD2	3′ **GGT**CGTACTGGACTCAG 5′

On-target VEGFA	5′ GACCCCCTCCACCCCGCCTCCGG 3′
OFF HDLBP	5′ ATTCCCCCCCACCCCGCCTC**AGG** 3′
GUARD1	3′ **GGG**GCGGAGTCCTTTCTT 5′
OFF CAVIN4	5′ ACACCCCCCCACCCCGCCTC**AGG** 3′
GUARD2	5′TCAGGACGTCCAAC**AGG** 3′

c



HEK293 BE3 with VEGFA gRNA and NT GUARD RNA

Sample	Frequency	Sequence	Variant depth
Reference	242214529	ACCAAGCCCATTTGTCCAGGAACCCCTAGCCCAAACTGAGATATTAACAAAACAGAAAGCAATTCCCCCCCACCCCGCCTCAGGAAAGAATAAACCTCATACCAGGCTAAAAAGGATACCCACAGATCAGCACAAATGGTAGAGAGGCTAAAAGCCCAAAGCTCAAAAAGAAGGAA	242214704
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HEK293 BE3 with VEGFA gRNA and HDBLP GUARD RNA

Sample	Frequency	Sequence	Variant depth
Reference	242214529	ACCAAGCCCATTTGTCCAGGAACCCCTAGCCCAAACTGAGATATTAACAAAACAGAAAGCAATTCCCCCCCACCCCGCCTCAGGAAAGAATAAACCTCATACCAGGCTAAAAAGGATACCCACAGATCAGCACAAATGGTAGAGAGGCTAAAAGCCCAAAGCTCAAAAAGAAGGAA	242214704
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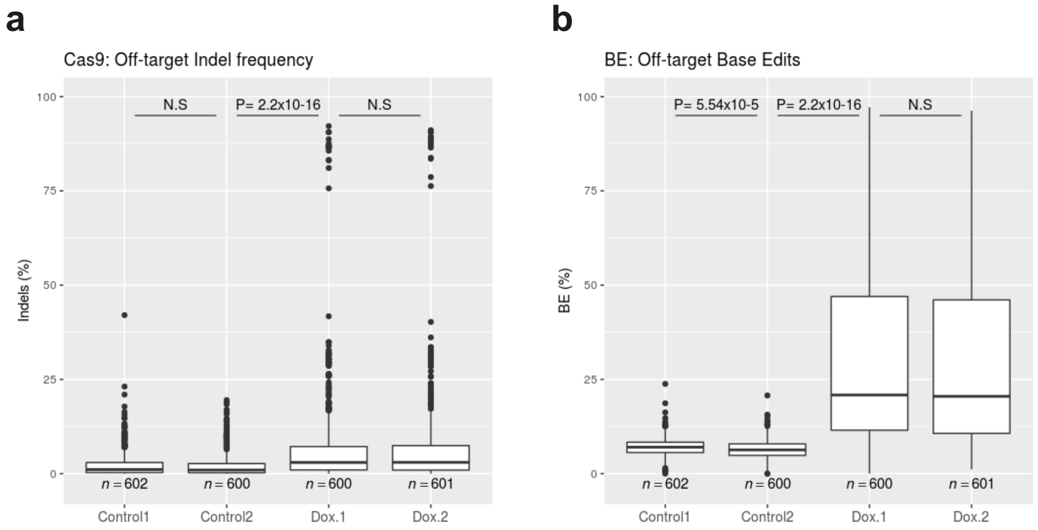
(a) Schematic representation of the predicted base editing activity window for full-length, 20-nt gRNAs and short GUARD RNAs.

(b) Sequence and position of the gRNA on-target, off-target sites and GUARD RNAs used for protection, annotated as in Figure 1.

(c) Linked and unlinked base editing events caused by on-target *VEGFA* gRNA binding and *HDLBP* GUARD RNA binding. Base editing rates from amplicon sequencing of BE3-expressing HEK293 cells transfected with *VEGFA* on-target gRNA (25 nM) and the indicated GUARD RNA (125 nM). NGS reads for the off-target region are shown with edited nucleotides highlighted in red.

Source data are provided as a Source Data file.

Supplementary Figure 7

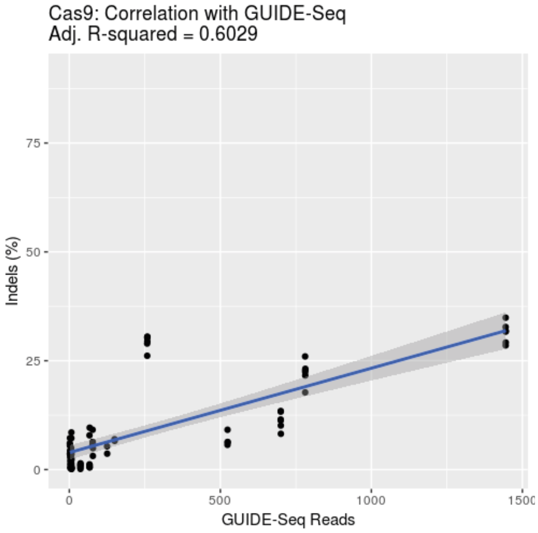


(a) Box and whiskers plot of off-target indel frequency for each replicate of the Cas9 screen.

(b) Box and whiskers plot of off-target SNP frequency for each replicate of the BE3 screen.

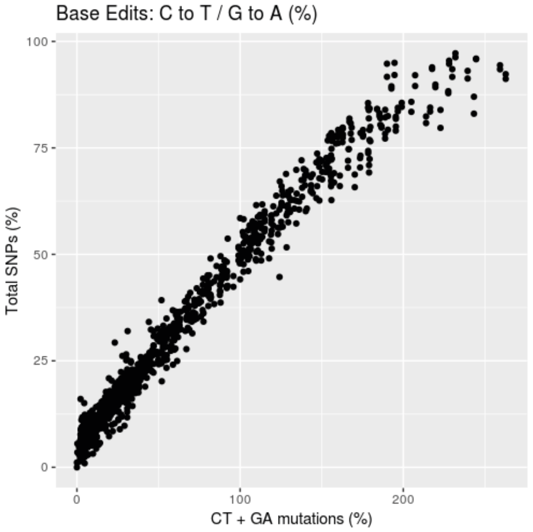
Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, outliers. Unpaired, two-tailed student's *t*-test.

Supplementary Figure 8



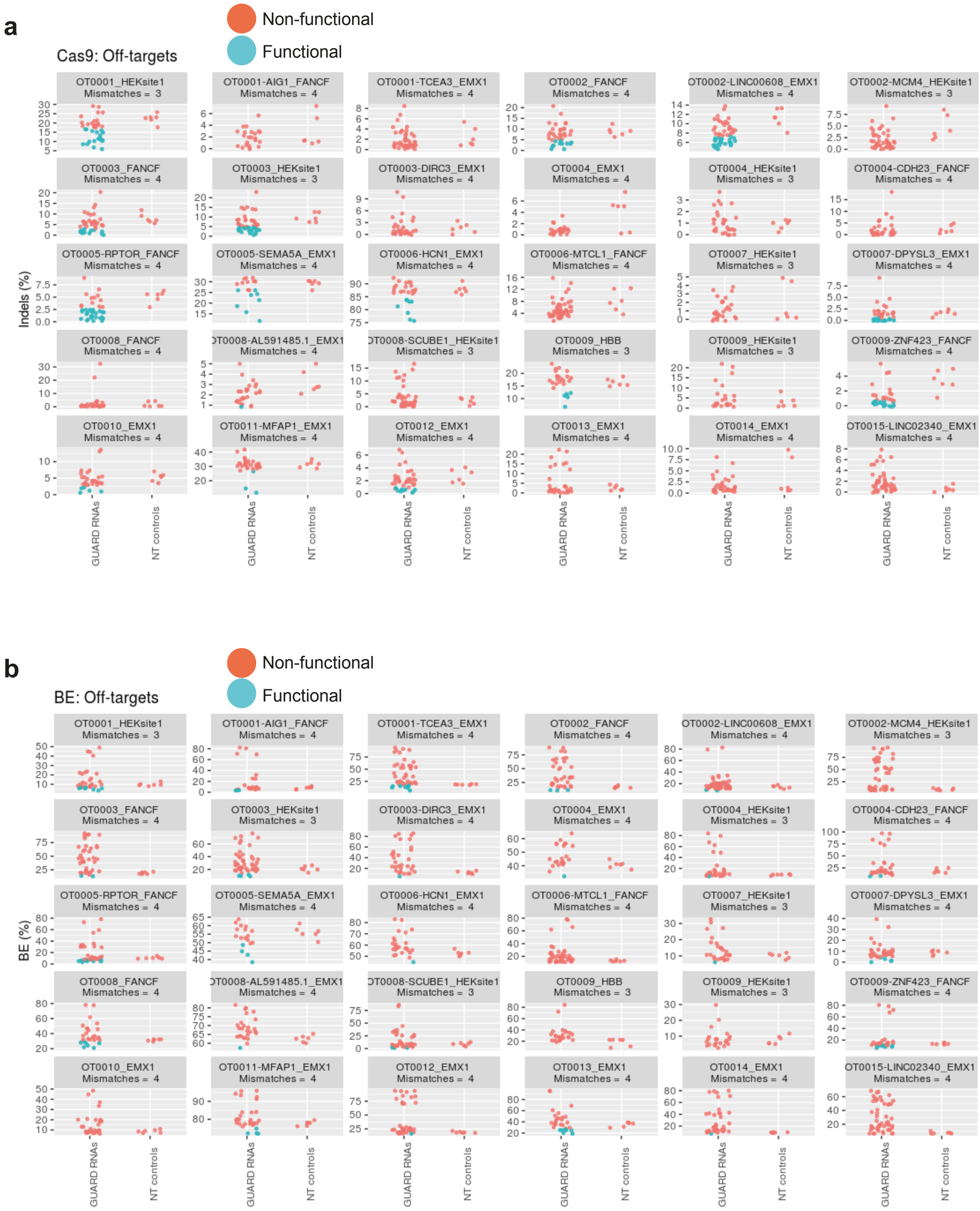
Dot plot showing the correlation of non-targeting GUARD RNA editing rates (% reads with indels) and publicly available GUIDE-Seq read counts for off-targets used in the Cas9 screen. The shaded area represents the 95 % confidence interval.

Supplementary Figure 9



For the BE3 GUARD RNA screen, correlation between total SNPs (% of reads) and C->T and G->A (sum % of reads). As some reads contained both C->T and G->A mutations, the % exceeds 100 % in some cases. Data represent all GUARD RNAs from two independent replicates of doxycycline-treated cells.

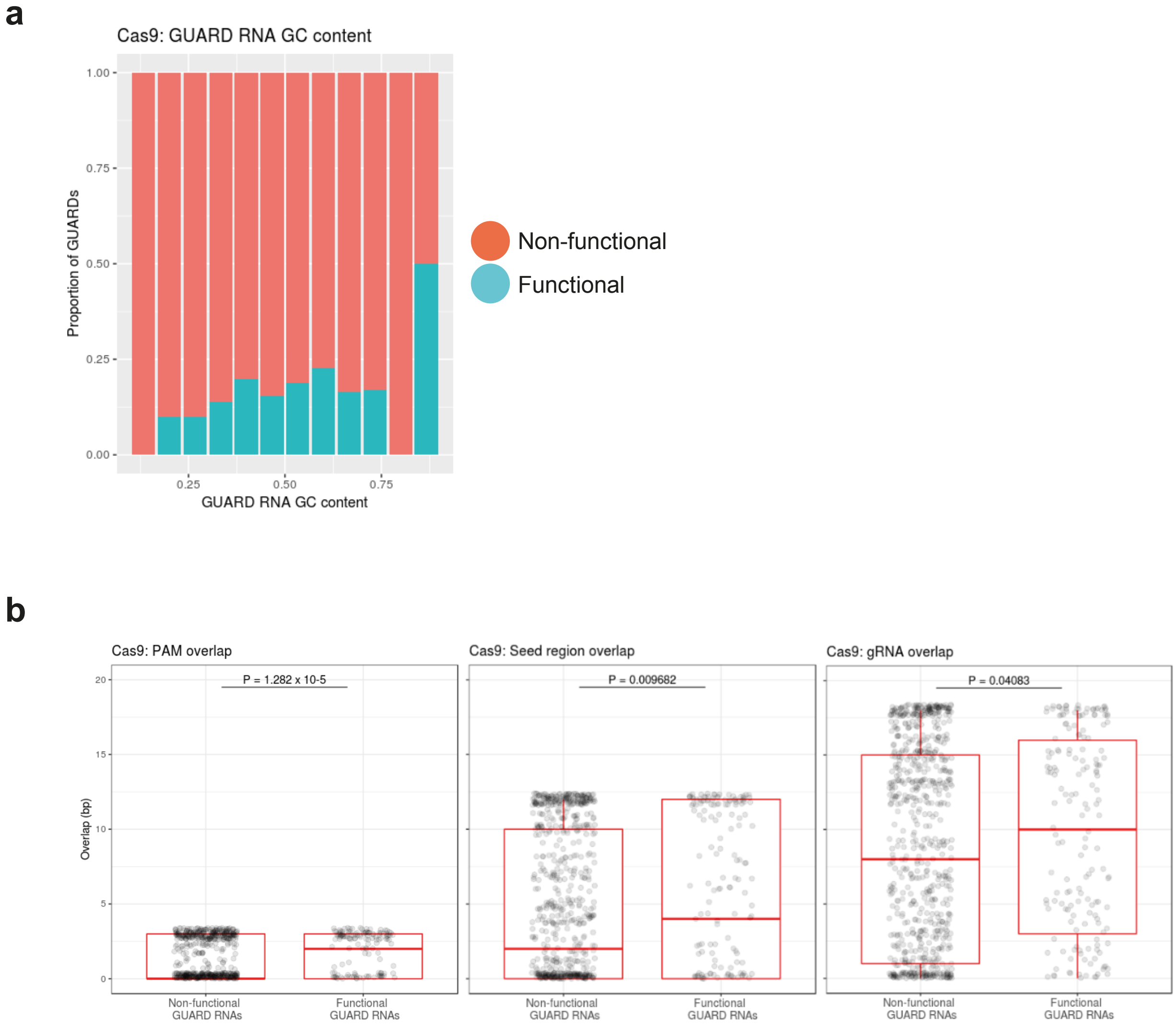
Supplementary Figure 10



(a) GUARD RNA performance for Cas9 and,

(b) BE3. Indel rates (% of NGS reads with indels) and BE rates (% of NGS reads with SNPs) for a 30 off-target sites. Off-targets are labelled with an identifier, gene name (if genic), gRNA name, and the number of mismatches between the gRNA and the off-target site. Data are pooled from two independent experiments from cells treated with doxycycline. Each point represents a GUARD RNA and are compared to three non-targeting (NT) control RNAs. GUARD RNAs are classed as “functional” if they reduce off-target editing by at least two standard deviations from the mean of the NT controls.

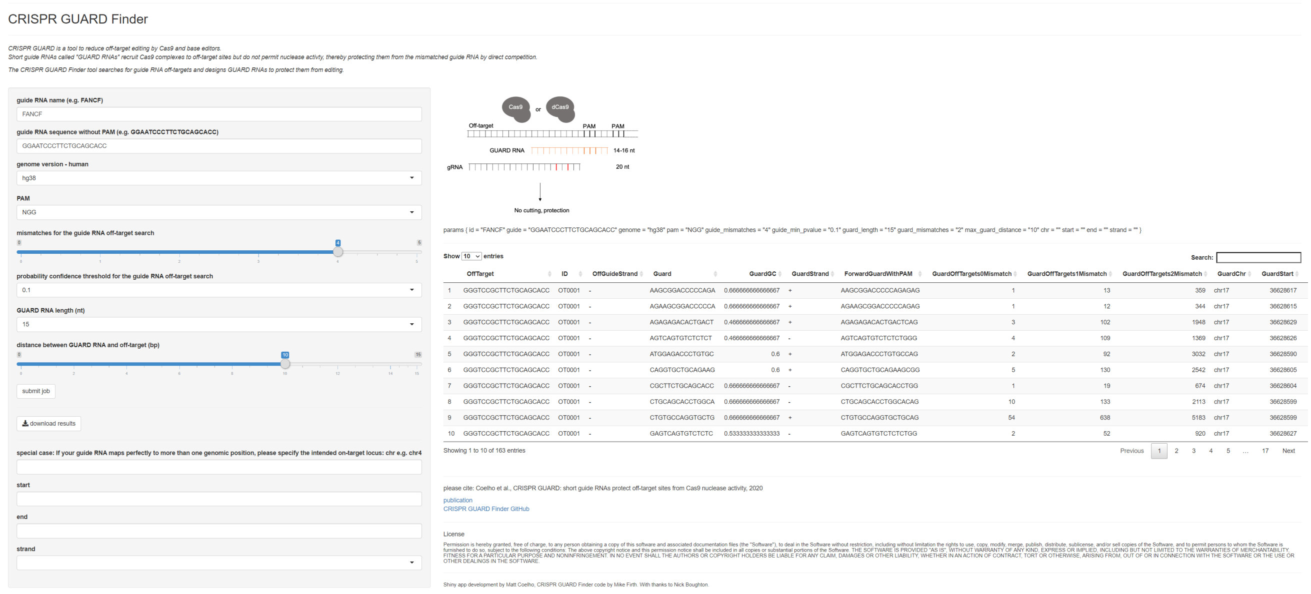
Supplementary Figure 11



(a) Distribution of functional GUARD RNAs found in the Cas9 screen with increasing GUARD RNA GC-content (%GC).

(b) Functional GUARD RNAs have more overlap with the off-target gRNA binding region. Overlap with the gRNA PAM, seed region including the PAM and entire protospacer sequence including the PAM (bp) was compared for non-functional and functional GUARD RNAs from the Cas9 screen using an unpaired, two-tailed student's *t*-test. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range. Unpaired, two-tailed student's *t*-test.

Supplementary Figure 12



CRISPR GUARD Finder

CRISPR GUARD is a tool to reduce off-target editing by Cas9 and base editors.
Short guide RNAs called "GUARD RNAs" recruit Cas9 complexes to off-target sites but do not permit nuclease activty, thereby protecting them from the mismatched guide RNA by direct competition.

The CRISPR GUARD Finder tool searches for guide RNA off-targets and designs GUARD RNAs to protect them from editing.

guide RNA name (e.g. FANCF)
FANCF

guide RNA sequence without PAM (e.g. GGAATCCCTTCTGCAGCACC)
GGAATCCCTTCTGCAGCACC

genome version - human
hg38

PAM
NGG

mismatches for the guide RNA off-target search
4

probability confidence threshold for the guide RNA off-target search
0.1

GUARD RNA length (nt)
15

distance between GUARD RNA and off-target (bp)
10

special case: If your guide RNA maps perfectly to more than one genomic position, please specify the intended on-target locus: chr e.g. chr4

start

end

strand

params { id = "FANCF" guide = "GGAATCCCTTCTGCAGCACC" genome = "hg38" pam = "NGG" guide_mismatches = "4" guide_min_pvalue = "0.1" guard_length = "15" guard_mismatches = "2" max_guard_distance = "10" chr = "" start = "" end = "" strand = "" }

	OffTarget	ID	OffGuideStrand	Guard	GuardGC	GuardStrand	ForwardGuardWithPAM	GuardOffTargets0Mismatch	GuardOffTargets1Mismatch	GuardOffTargets2Mismatch	GuardChr	GuardStart
1	GGGTCCGCTTCTGCAGCACC	OT0001	-	AAGCGGACCCCCAGA	0.666666666666667	+	AAGCGGACCCCCAGAGAG	1	13	359	chr17	36628617
2	GGGTCCGCTTCTGCAGCACC	OT0001	-	AGAAGCGGACCCCCA	0.666666666666667	+	AGAAGCGGACCCCCAGAG	1	12	344	chr17	36628615
3	GGGTCCGCTTCTGCAGCACC	OT0001	-	AGAGAGACACTGACT	0.466666666666667	+	AGAGAGACACTGACTCAG	3	102	1948	chr17	36628629
4	GGGTCCGCTTCTGCAGCACC	OT0001	-	AGTCAGTGTCTCTCT	0.466666666666667	-	AGTCAGTGTCTCTCTGGG	4	109	1369	chr17	36628626
5	GGGTCCGCTTCTGCAGCACC	OT0001	-	ATGGAGACCCTGTGC	0.6	+	ATGGAGACCCTGTGCCAG	2	92	3032	chr17	36628590
6	GGGTCCGCTTCTGCAGCACC	OT0001	-	CAGGTGCTGCAGAAG	0.6	+	CAGGTGCTGCAGAAGCGG	5	130	2542	chr17	36628605
7	GGGTCCGCTTCTGCAGCACC	OT0001	-	CGCTTCTGCAGCACC	0.666666666666667	-	CGCTTCTGCAGCACCTGG	1	19	674	chr17	36628604
8	GGGTCCGCTTCTGCAGCACC	OT0001	-	CTGCAGCACCTGGCA	0.666666666666667	-	CTGCAGCACCTGGCACAG	10	133	2113	chr17	36628599
9	GGGTCCGCTTCTGCAGCACC	OT0001	-	CTGTGCCAGGTGCTG	0.666666666666667	+	CTGTGCCAGGTGCTGCAG	54	638	5183	chr17	36628599
10	GGGTCCGCTTCTGCAGCACC	OT0001	-	GAGTCAGTGTCTCTC	0.533333333333333	-	GAGTCAGTGTCTCTCTGG	2	52	920	chr17	36628627

Showing 1 to 10 of 163 entries

please cite: Coelho et al., CRISPR GUARD: short guide RNAs protect off-target sites from Cas9 nuclease activity, 2020

License

Shiny app development by Matt Coelho, CRISPR GUARD Finder code by Mike Firth. With thanks to Nick Boughton.

An example workflow of running the “CRISPR GUARD Finder” tool. The user defines parameters such as PAM, guide RNA sequence, search genome, off-target probability, mismatches and GUARD RNA length. The output contains predicted off-targets, possible GUARD RNAs and their relevant sequence features.

Supplementary Table 1

No detectable Cas9-induced indels caused by CRISPR GUARD

Sample	Amplicon	Total reads	% Reads with indel
EMX1 Untransfected	*EMX1*	812998	0.00
EMX1 gRNA only - Positive control	*EMX1*	983231	81.98
CAVIN4 Untransfected	*CAVIN4*	512079	0.34
CAVIN4 14 nt GUARD	*CAVIN4*	1007520	0.33
CAVIN4 15 nt GUARD	*CAVIN4*	1076240	0.35
HCN1 Untransfected	*HCN1*	1674461	0.00
HCN1 14 nt GUARD	*HCN1*	1465594	0.00
MYC prox. Untransfected	*Myc* prox.	1718187	0.10
MYC prox. 14 nt GUARD	*Myc* prox.	1589391	0.10
TENT4A Untransfected	*TENT4A*	1293639	0.00
TENT4A 14 nt GUARD	*TENT4A*	1334905	0.00

Indel rates from NGS of amplicons from Cas9-expressing HEK293 cells transfected with *EMX1* on-target gRNA (full-length, 20-nt guide RNA as a control), or an array of different functional GUARD RNAs at 125 nM.

Supplementary Table 2

List of guide RNAs and GUARD RNAs

RNA molecules	**Sequence**
EMX1 gRNA	GAGTCCGAGCAGAAGAAGAA**GGG**
EMX1 on-target GUARD	CGAGCAGAAGAAGAA**GGG**
EMX1 OT1 HCN1 GUARD	AAGGCATGGAGTAA**AGG**
EMX1 OT2 MFAP1 GUARD	TAAGCAGAAGAAGAA**GAG**
EMX1 OT3 MYC proximal GUARD	GACTCAGGTCATGC**TGG**
VEGFA gRNA	GACCCCCTCCACCCCGCCTC**CGG**
VEGFA on-target GUARD	CCCGCGCCCGGAGG**CGG**
VEGFA OT1 TENT4A GUARD	CCTCCGGGCAAGTG**AGG**
VEGFA OT2 HDLBP GUARD 1	TTTATTCTTTCCTG**AGG**
VEGFA OT2 HDLBP GUARD 2	TTCTTTCCTGAGGCG**GGG**
VEGFA OT3 CAVIN4 proximal GUARD	TCAGGACGTCCAAC**AGG**
VEGFA OT3 CAVIN4 competitive 15 nt GUARD	CCCCCACCCCGCCTC**CGG**
VEGFA OT3 CAVIN4 proximal 15 nt GUARD	CTCAGGACGTCCAAC**AGG**
VEGFA OT3 CAVIN4 proximal with spacer GUARD	AATTACTCAGGACGTCCAAC**AGG**
HBB gRNA	CTTGCCCCACAGGGCAGTAA**CGG**
FANCF gRNA	GGAATCCCTTCTGCAGCACC**TGG**
HEKsite1 gRNA	GGGAAAGACCCAGCATCCGT**GGG**
Non-targeting gRNA (NT) IDT	GCCCCGCCGCCCTCCCCTCC
Non-targeting (NT) GUARD 1 screen (Avana NO_CURRENT_1)	AAGAGGAATAGTAGC
Non-targeting (NT) GUARD 2 screen (Avana NO_CURRENT_2)	GAAACGTGACTAAAG
Non-targeting (NT) GUARD 3 screen (Avana NO_CURRENT_3)	TTAGGCACGGTTACT

PAMs are indicated in bold.

Supplementary Table 3

List of primers

Primer name	Sequence
qPCR EMX1 For.	5′-TCCAGAACCGGAGGACAAAG-3′
qPCR EMX1 Rev.	5′-CACCGGTTGATGTGATGGGA-3′
qPCR EMX1 OT1 HCN1 For.	5′-TTCTGAGGGCTGCTACCTGT-3′
qPCR EMX1 OT1 HCN1 Rev.	5′-GAGTGGCCAGAGTCCAGCTTGG-3′
qPCR EMX1 OT2 MFAP1 For.	5′-CACGGCCTTTGCAAATAGAG-3′
qPCR EMX1 OT2 MFAP1 Rev.	5′-GGCTTTCACAAGGATGCAGT-3′
qPCR EMX1 OT3 MYC prox. For.	5′-CCAGACTCAGTAAAGCCTGGA-3′
qPCR EMX1 OT3 MYC prox. Rev.	5′-TGGCCCCAGTCTCTCTTCTA-3′
qPCR VEGFA For.	5′-CTGACCAGTCGCGCTGACGG-3′
qPCR VEGFA Rev.	5′-CAGAAGTTGGACGAAAAGTTTCAGTGCG-3′
qPCR VEGFA OT1 TENT4A For.	5′-TCCTACAAGTAACAGTCCAAGAA-3′
qPCR VEGFA OT1 TENT4A Rev.	5′-TTCTGCAACTTAACTTACGTGAAA-3′
qPCR VEGFA OT2 HDLBP For.	5′-ACCAAGCCCATTTGTCCAGG-3′
qPCR VEGFA OT2 HDLBP Rev.	5′-TCCTTCTTTTTGAGCTTTGGGC-3′
qPCR VEGFA OT3 CAVIN4 For.	5′-TCCATACCAGCAGCAGTTCC-3′
qPCR VEGFA OT3 CAVIN4 Rev.	5′-CTCACCTCAGCTCCTGCAC-3′
Amplicon Seq. EMX1 For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** TGA GTT TCT CAT CTG TGC CCC-3′
Amplicon Seq. EMX1 Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GT TTG TGG TTG CCC ACC CTA-3′
Amplicon Seq. EMX1 OT1 HCN1 For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** TTC TGA GGG CTG CTA CCT GT-3′
Amplicon Seq. EMX1 OT1 HCN1 Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GC CCA ATC ATT GAT GCT TTT-3′
Amplicon Seq. EMX1 OT2 MFAP1 For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CAC GGC CTT TGC AAA TAG AG-3′
Amplicon Seq. EMX1 OT2 MFAP1 Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GG CTT TCA CAA GGA TGC AGT-3′
Amplicon Seq. EMX1 OT3 MYC prox. For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** CCA GAC TCA GTA AAG CCT GGA-3′
Amplicon Seq. EMX1 OT3 MYC prox. Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**TG GCC CCA GTC TCT CTT CTA-3′
Amplicon Seq. VEGFA For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** GGA CAG ACA GAC AGA CAC CG-3′

Amplicon Seq. VEGFA Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA** **G**GC CCG AGC TAG CAC TTC TC-3′
Amplicon Seq. OT1 VEGFA TENT4A For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** TCC TAC AAG TAA CAG TCC AAG AA-3′
Amplicon Seq. OT1 VEGFA TENT4A Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA** **G**TT CTG CAA CTT AAC TTA CGT GAA A-3′
Amplicon Seq. OT2 VEGFA HDLBP For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** ACC AAG CCC ATT TGT CCA GG-3′
Amplicon Seq. OT2 VEGFA HDLBP Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA** **G**TC CTT CTT TTT GAG CTT TGG GC-3′
Amplicon Seq. OT3 VEGFA CAVIN4 For.	5′-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG** TCC ATA CCA GCA GCA GTT CC-3′
Amplicon Seq. OT3 VEGFA CAVIN4 Rev.	5′-**GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA** **G**CT CAC CTC AGC TCC TGC AC-3′

In bold are adapter sequences used for the addition of next-generation indexing primers.