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

Coelho et al.



Schematic of the experiment described in Fig. 2e.



Pre-incubation of GUARD RNAs in cells does not provide additional protection of offtarget 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.



CRISPR GUARD protection of one off-target site does not lead to increased offtarget 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.



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.



Multiplexed delivery of GUARD RNAs provides protection against multiple Cas9 offtargets 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.

BE3 activity window				
gRNA		Π	PAM	
GUARD RNA	1 2 3 <b>4 5 6</b>	/ * 	PAM	
		78	<b>8</b> 9 10 11 12 13 14 15 16 17 18 19 20	

b

а

On-target EMX1	5' GAGTCCGAGCAGAAGAAGAAGAAGAAGAA
OFF MFAP1	5' GAGTCTAAGCAGAAGAAGAAGAAGAAGA 3'
GUARD1	5' TAAGCAGAAGAAGAAGAAGAAGA 3'
OFF MYC proximal	5' GAGTCCTAGCAGGAGAAGAA <b>GAG</b> 3'
GUARD2	3' <b>GGT</b> CGTACTGGACTCAG 5'
On-target VEGFA	5' GACCCCTCCACCCGCCTCCGG 3'
OFFHDLBP	5' ATTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
GUARD1	3' <b>GGG</b> GCGGAGTCCTTTCTT 5'
OFF CAVIN4	5' ACACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
GUARD2	5' TCAGGACGTCCAACAGG 3'

С

HEK293 BE3 with VEGFA gRNA an	d NT GUARD F	22A	
Sample	Frequency	Sequence	Variant depth
Reference	242214529		242214704
Rep.1			
18 HDBLP NT 1HEKIBE3 MC	0.141	cosequent to the carge acceptage cost agree at take as as a carge and cast the concept carge agree a take of the carge take agree as a take of t	15194
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18 HDBLP NT 1HEKIBE3 MC	0.042	ccease.ccttttttccease.ccctage.ccctage.ccease.ctgage.actgage.actgage.gcast.tttttttt	4517
18 HDBLP NT 1HEKIBE3 MC	0.041	consecuting to aggest according to the second s	4381
18 HDBLP NT 1HEKIBE3 MC	0.02	i ceaego cratti gi ceaggaa cooti ago ceaa actgaga ta ti aceaaa ageaa ti com ceaego teaggaaa gaataaa en to to to ceaego taa aaggata ceee caaga teago acaa atggtagagaggetaaa ago ceaaaaggata	2211
18 HDBLP NT 1HEKIBE3 MC	0.02	consecuting to segment of the second	2173
18 HDBLP NT 1HEKIBE3 MC	0.015	l ceasge ceatting ceasge accest age ceasacing against maceasaa eagaa age an time cee cease age and a a accest exact on the ceasge and a a age ceasa and a general and a general age	1605
18 HDBLP NT 1HEKIBE3 MC	0.01	$\  \  \  \  \  \  \  \  \  \  \  \  \  $	1039
Rep.2			
21 HDBLP NT 2HEKIBE3 MC	0.133	icca ago coattig coaggaa cootago coa a cigaga ta tiacaaaa cagaaag caati minin cooco cooto aggaaagaataaa coto cata coaggota aaaggata cooca caga toago coaaaatggtaga gagaggota aaag coasaatggtaga gagaggota aaag coasaa gaaggaa	14674
21_HDBLP_NT_2HEKiBE3_MC	0.059	consigned at the transmission of the second	6568
21 HDBLP NT 2HEKIBE3 MC	0.046	iccaageeeatttgtocaggaaceeetageceaaactgagatattaacaaaageaat <sup>a</sup> in	5078
21 HDBLP NT 2HEKIBE3 MC	0.04	$\  \  \  \  \  \  \  \  \  \  \  \  \  $	4416
21_HDBLP_NT_2HEKiBE3_MC	0.022	locaagoocat t t gt ocaggaa cooct agoocaa act gaga ta t taacaaaa agoaa t Tococco coocgoot caggaa agaa taa oo toata coaggot aaaaggat accoccagga toagoocaa a t gt agaga ggot aaaagoocaa ago coa aa agoocaa a t gt agaga ggot aaaagoocaa ago coa a agoocaa ago coa a agoocaa a t gt agaga ggot aaaagoocaa ago coa a agoocaa ago coa agoocaa ago coa agoocaa agoocaa agoocaa agoocaa agoocaa agoocaa agoocaa agoocaa agoocaa agoo	2409
21 HDBLP NT 2HEKIBE3 MC	0.02	uccaagcocattgt.ccaggaaccoctagcocaaactgagatattaacaaaacagaaagcaattoc 🎞 o caccoogoot caggaaagaataaacot o ataccaggat aaaaaggat accaacagat cagaacaaatggtagaaggot aaaaggot caaaaggaggot aaaaggag	2248
21 HDBLP NT 2HEKIBE3 MC	0.011	$\  \  \  \  \  \  \  \  \  \  \  \  \  $	1254
21_HDBLP_NT_2HEKiBE3_MC	0.01	l con ago contitig to agga a contra go con a a transmission and a second de la contra transmission a second de	1139
O.S. service as a company in an O. service	0.04		4.022

#### HEK293 BE3 with VEGFA gRNA and HDBLP GUARD RNA

Sample	Frequency	Sequence	Variant depth
Reference	242214529		242214704
Rep.1			
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.083	crease cost tig transported agric case tig against take as a caga a grant titter can cogort cagga a A intancost of take agget as a agget grant agget grant agget as a ggt again ggt	11479
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.077	sceaego catititi de casega e contago casa et que ta tacasa e casa e e que a case e que ta secanda e que ta e case e ta concerce e ta que a que ta e case e ta concerce e ta que a que ta e case e ta concerce e ta que a que ta e case e ta e c	10742
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.049	Creating of the approximation of the second se	6831
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.034	coca agree at tig to aggaa cooct agree as a tig aga ta ti accasa a cagaa agrea ti TTTT cocaccorgoot ca ggaa <b>A</b> sa taa cot cata coaggot a sa aggat accooct aggaa agat agga ggot a sa agree da a agget a sa agree da agget agget agget agget agget ggot a sa agree da agget	4719
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.025	consisting to aggesere of agreement agreement and a sease capability of the sease o	3512
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.021	$\label{eq:construction} to cappe access act gaget at tasces a cape a geast transformation concept cappe access agat cape a concept cape a c$	2898
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.021	l ccaagcocatttgt ccaggaaccectagcecaaactgagatattaacaaaacagaaagcaatt <b>TTTTT</b> . acccogoot caggaaa <b>a</b> aagaaagaa	2886
19 HDBLP HDBLFnew 1HEKiBE3 MC	0.018	considered that a considered and a second	2450
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.015	$\label{eq:construction} construction and the second cons$	2065
19 HDBLF HDBLFnew 1HEKiBE3 MC	0.014	$bccasgcccatttgtccaggaacccctagcccaaactgagatattaccagaaagccaatt\mathbf{T}ccccccaccccccccctcaggaaagaataaacctcataccaggctaaaaggatacccacaggatcagcaaaatggtagagaggggtaaaagccaatggtagagagggtaaaagccaatggtagagagggtaaaagccaatggtaggagaggaagaagaaggaataaacctcataccaggctaaaaggatacccccaggctaggagagggggaaggaa$	1939
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.011		1496
19 HDBLF HDBLFnew 1HEKiBE3 MC	0.01	bccaagcccatttgtccaggaacccctagcccaaactgagatattacagaaagcaattccrcccccccccc	1369
19 HDBLP HDBLPnew 1HEKiBE3 MC	0.009		1256
Rep.2			
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.086		11414
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.081	$ccaageccatttgtccaggaaccectagcccaaactgggatattaacaaaaggcaattcccecccaccecegcctcaggaaa \\ A aataaacctcataccaggataccectagcacaaatggtagagagggtaaaagccaatatcccaaagatccaaaatggtagagagggtaaaaagccaaaatggtagagagggtaaaagccaaaatggtagagagggaaagcaaatccaccaagatccaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaaaatggtagagagggaaagcaattccaccaccecegctcaggaaagcaaatggtagaggaagcaaatccaccaccaccacaaatggtagagaggaaagcaaaatggtagagaggaaagcaaatggtagagaggaaagcaaatccaccacaaatggtagagaggaagcaaaatggtagagaggaaaaaatggtagagaggaagcaaaatggtagagaagcaaatccaccacacaca$	10726
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.051	accasgoccatttgtocaggaaccoctagoccaaactgagatattaacaaaacagaaagcaat intrr caccocgoctcaggaaagaataaacctcataccaggataccaggaacagaataggtagagagggtagagaggggtagagaggggtagagagggtagagagggtagagggggg	6800
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.044		5780
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.035	$ccaagcccatttgtccaggaacccctagcccaaactgggatattaacaaaacagaaagcaatt{\tt minimi} accccgcctcaggaaaAaataaacctcataccaggatacccctagcacaaatggtagagagggtaaaaagccaaaatggtagagaggctaaaagcccaaaatggtagagaggctaaaagcccaaaatggtagagaggaa$	4575
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.034	$ccasgcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaatt\tau \tau \tau \tau$	4482
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.026	$ccaageccatttgtccaggaaccectagcccaaactgggatattaacaaaacagaaageaat i {\tt TTTT} cccacecegectcaggaaagaataaacctcataccaggataccectagcacaaatggtagagagggtaaaaageataaaccectagcacaaatggtagagagggtaaaageataaaageataaaccectagcacaaatggtagagaggagag$	3377
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.023	$ccaagcccatttgtccaggaacccctagcccaaactgggatattaacaaaacagaaagccaatt{\tt mmm}cacccqcctcaggaaagaataaacctcataccaggatacccctagcacaaatggtagagagggtaaaagccaaaatggtagagagggtaaaagccaaaatggtagagagggaaagaataaacctcataccaggctaaaaaggatacccctagcacaaatggtagagaggggaaagaataaacctcataccaggctaaaaaggatacccctagcacaaatggtagagaggggaaagcaattggtagagaggaagaataaacctcataccaggctaaaaaggatacccctagcccaaatggtagagaggggaaagcaattggtagagaggaagaataaaccccctagcctaaaaggatacccctagcccaaatggtagagaggggaaagaataaacccccacaggataccccaaaatggtagagaggggaaacccctagcccaaatggtagagaggaagcaattggtagagaggaagcaattggtagagaggaagaagaagaagaagaagaagaataaaccccacaggatacccccacaggatacccccaaatggtagagaggggaagcaattggtagagaggaagaagaagaagaagaagaagaagaa$	3001
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.019	$ccasgcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaatt\tau arr rr rr$	2528
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.016	ccaagcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaattTcccccacccgcctcaggaaagaataaacctcataccaggctaaaaaggatacccacagatcagcacaaatggtagagggtaaaagcccaaagctcaaaaggaag	2090
22 HDBLF HDBLFnew 2HEKiBE3 MC	0.011	$ccaageccatttgtccaggaaccectageccaaactgggatattaacaaaagecaattecr\mathbf{T}cccacecegectcaggaaaAaataaaectcataccaggetcaaaaggataccectageccaaastggtagagaggggtaaaageccaaastggtagagagggtaaaageccaaaatggtagagagggtaaaageccaaaatggtagagagggaaagecaaaatggtagagagggaaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagaggggaaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagagggaagecaaaatggtagagaggaagecaaaatggtagagaggaagecaaaatggtagagaggaagecaaaatggtagagaggetaaaaagecaaaatggtagagaggaagecaaaatggtagagaggaagecaaaatggtagagagecaaaatggtagagaggaagecaaaatggtagagaggaagecaaaatggtagagaggaagecaaaatggtagagagecaaaatggtagagaggaagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagagecaaaatggtagagageaaaatggtagagagecaaaatggtagagagegaatagecaaaatggtagagagegaaaatggtagagaggaaggaagga$	1465
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.011	ccasgcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaattccTT.ccaccccgcctcaggaaagaataaacctcataccaggctaaaaaggatacccacagatcagcacaaatggtagaggggtaaaagcccaaaggctaaaaggatgaa	1393
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.008	ccaagcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaattTcccccacccgcctcaggaaaAaataaccaggctaaaaaggataccaacagatcagcacaaatggtagaggggtaaaaggccaaaaggtcgaaaaggaag	1122
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.008	ccaagcccatttgtccaggaacccctagcccaaactgagatattaacaaaacagaaagcaattccccccaccccgcctcagAnaaAaataaccaggctaaaaaggatacccacagatcagcacaaatggtagaggggtaaaaggccaaaaggtcgaaaaggaag	1109
22 HDBLP HDBLPnew 2HEKiBE3 MC	0.008		1044

- (a) Schematic representation of the predicted base editing activity window for fulllength, 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 offtarget region are shown with edited nucleotides highlighted in red.



- (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.



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.



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.





# GUARD RNAS Ē

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- (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.



b



- (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.

CRISPR GUARD Finder									
CHISPR QUARD is a tool to reace of keeper wating by Card and base waters. Short puide River called "QUARD River" noout Card compares to of keaper sites but do not permit nuclease exting, thereby protecting them from the mismit The CRISPR QUARD River tool services for guide RIVA of kergets and designs GUARD RIVAs to protect them from eating.	tched guide RNA by direct competition.								
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15 · · ·	3 GGGTCCGCTTCTGCAGCACC OT0001 -	AGAGAGAGACACTGACT 0.46866	00000000007 +	AGAGAGAGACACTGACTCAG	3	102	1948	chr17	35628629
	4 GGGTCCGCTTCTGCAGCACC OT0001 -	AGTCAGTGTCTCTCT 0.468868	00000000007 -	AGTCAGTGTCTCTCTGGG	4	109	1369	chr17	35628626
distance between GUARD RNA and off-arget (bp)	5 GGGTCCGCTTCTGCAGCACC OT0001 -	ATGGAGACCCTGTGC	0.6 +	ATGGAGACCCTGTGCCAG	2	92	3032	chr17	36628590
	6 GGGTCCGCTTCTGCAGCACC OT0001 -	CAGGTGCTGCAGAAG	0.6 +	CAGGTOCTGCAGAAGCGG	5	150	2542	chr17	35628605
schrift inh	7 GEGTCCGCTTCTGCAGCACC OT0001 -	OGCTTCTGCAGCACC 0.66666	00000000000 -	COCTTCTGCAGCACCTOG	1	19	674	chr17	35628604
u u u u u u u u u u u u u u u u u u u	8 GGGTCCGCTTCTGCAGCACC OT0001 -	CTGCAGCACCTGGCA 0.66666	. 0000000000000000000000000000000000000	CTGCAGCACCTGGCACAG	10	133	2113	chr17	35628599
A download samile	9 GGGTCCGCTTCTGCAGCACC OT0001 -	CTGTGCCAGGTGCTG 0.66666	+ 0000000000000000000000000000000000000	CTGTGCCAGGTGCTGCAG	54	638	5183	chr17	35628599
	10 GGGTCCGCTTCTGCAGCACC OT0001 -	GAGTCAGTGTCTCTC 0.53333	10333333333 -	GAGTCAGTGTCTCTCTGG	2	52	920	chr17	35628627
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	Showing 1 to 10 of 163 entities						Previous 1 2 3 4	5	17 Next
start	please cite: Coelho et al., CRISPR GUARD: short quide RNAs protect off-	target sites from Cas9 nuclease act/vity,	. 2020						
	publication								
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strand v	Permission is hereby granted, free of charge, to any person obtaining a copy of this so familihed to do so, subject to the following conditions. The above copyright notice and PTINESS FOR A PARTICULTURE PURPOSE AND INCIMINIFICACEMENT. IN NO EVEN OTHER DEALINGS IN THE SOFTIMMRE.	hware and associated documentation files (the this permission notice shall be included in all I SHALL THE AUTHORS OR COPYRIGHT HI	e "Software"), to deal in the Software wit copies or substantial portions of the Soft IOLDERS BE LIABLE FOR ANY CLAIM,	hout restriction, including without limits www. THE SOFTWARE IS PROVIDED DAMAGES OR OTHER LIABILITY, W	fion the rights to use, copy, modify, merg PAG ISP, WITHOUT WARRANTY OF AP HETHER IN AN ACTION OF CONTRAC	e, publish, distribute, sublicense, and/or s N KIND, EXPRESS OR IMPLIED, INCLU , TORT OR OTHERWISE, ARISING FRO	el copies of the Software, and to permit po DING BUT NOT LIMITED TO THE WARE M, OUT OF OR IN CONNECTION WITH 1	NOTES OF MERCI HE SOFTWARE C	2 Software is SHANTABLITY OR THE USE OR
	Shiny app development by Matt Coelho, CRISPR GUARD Finder code by Mike Firth. 1	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 offtargets, possible GUARD RNAs and their relevant sequence features.

#### Supplementary Table 1

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	<i>Myc</i> prox.	1718187	0.10
MYC prox. 14 nt GUARD	<i>Myc</i> prox.	1589391	0.10
TENT4A Untransfected	TENT4A	1293639	0.00
TENT4A 14 nt GUARD	TENT4A	1334905	0.00

No detectable Cas9-induced indels caused by CRISPR GUARD

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 <b>GGG</b>
EMX1 on-target GUARD	CGAGCAGAAGAAGAA <b>GGG</b>
EMX1 OT1 HCN1 GUARD	AAGGCATGGAGTAA <b>AGG</b>
EMX1 OT2 MFAP1 GUARD	TAAGCAGAAGAAGAA <b>GAG</b>
EMX1 OT3 MYC proximal GUARD	GACTCAGGTCATGC <b>TGG</b>
VEGFA gRNA	GACCCCCTCCACCCGCCTC <b>CGG</b>
VEGFA on-target GUARD	CCCGCGCCCGGAGG <b>CGG</b>
VEGFA OT1 TENT4A GUARD	CCTCCGGGCAAGTG <b>AGG</b>
VEGFA OT2 HDLBP GUARD 1	TTTATTCTTTCCTG <b>AGG</b>
VEGFA OT2 HDLBP GUARD 2	TTCTTTCCTGAGGCG <b>GGG</b>
VEGFA OT3 CAVIN4 proximal GUARD	TCAGGACGTCCAAC <b>AGG</b>
VEGFA OT3 CAVIN4 competitive 15 nt GUARD	CCCCCACCCGCCTC <b>CGG</b>
VEGFA OT3 CAVIN4 proximal 15 nt GUARD	CTCAGGACGTCCAAC <b>AGG</b>
VEGFA OT3 CAVIN4 proximal with spacer GUARD	AATTACTCAGGACGTCCAAC <b>AGG</b>
HBB gRNA	CTTGCCCCACAGGGCAGTAA <b>CGG</b>
FANCF gRNA	GGAATCCCTTCTGCAGCACC <b>TGG</b>
HEKsite1 gRNA	GGGAAAGACCCAGCATCCGT <b>GGG</b>
Non-targeting gRNA (NT) IDT	GCCCCGCCGCCTCCCCTCC
Non-targeting (NT) GUARD 1 screen	AAGAGGAATAGTAGC
(Avana NO_CURRENT_1)	
Non-targeting (NT) GUARD 2 screen	GAAACGTGACTAAAG
(Avana NO_CURRENT_2)	
(Avana NO, CURRENT, 3)	TTAGGCACGGTTACT
(Avana NO_CURRENT_3)	

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'-TGGCCCCAGTCTCTTCTA-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'-TCCTTCTTTTGAGCTTTGGGC-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 Sea. EMX1 Rev.	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA
Amplicon Sog. EMX1 OT1	GGT TTG TGG TTG CCC ACC CTA-3
HCN1 For.	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG TTC TGA GGG CTG CTA CCT GT-3'
Amplicon Seg. EMX1 OT1	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA
HCN1 Rev.	GCC CCA ATC ATT GAT GCT TTT-3'
Amplicon Seg. EMX1 OT2	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG
MFAP1 For.	CAC GGC CTT TGC AAA TAG AG-3'
Amplicon Seq. EMX1 OT2	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA
MFAP1 Rev.	GGG CTT TCA CAA GGA TGC AGT-3'
Amplicon Seq. EMX1 OT3 MYC	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG
prox. For.	CCA GAC TCA GTA AAG CCT GGA-3'
Amplicon Seq. EMX1 OT3 MYC	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA
prox. Rev.	GTG GCC CCA GTC TCT CTT CTA-3'
Amplicon Seg VEGEA For	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG
Amplicon Seq. VEGEA FUI.	GGA CAG ACA GAC AGA CAC CG-3'

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

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

primers.