

Получение генно- модифицированных кроликов с использованием новых геномных технологий

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– ВИЖ имени академика Л.К. Эрнста»**

Часть 1. Стратегия использования CRISPR/Cas9 технологии в получении кролика-биомодели - продуцента биологически активного белка с молоком.

Применение технологии CRISPR/Cas9 – эффективный метод изменения состава молока животных:

- гуманизация молока - замещение генов эндогенных молочных белков у с/х животных генами белков молока человека;
- исключение из состава молока какого-либо белка (аллергена);
- модификация молочных белков с целью повышения питательной ценности и усвояемости, переработки их в разные молочные продукты, для получения из них биологически активных пептидов в качестве вакцин
- замена эндогенного белка белком фармакологического или диагностического назначения;

Стратегия модификации гена WAP кролика с использованием технологии CRISPR/Cas9:

Цель:

- нокаут гена WAP кролика
- сайт-специфическая интеграция гена маркерного белка для оценки эффективности работы CRISPR/Cas9 компонентов в экспериментах *in vitro*.

Способы достижения цели:

- Создание конструкции, содержащей плечи гомологии к соответствующим областям гена WAP кролика и фрагмент *cmvEGFP*.
- Поиск таргетных последовательностей в гене WAP кролика: получение компонентов системы CRISPR/Cas9

Анализ возможных генетических модификаций эмбрионов, возникших в результате работы микроинъектированных компонентов системы CRISPR/Cas9 и ГИК (NHEJ, HDR).

Анализ гена WAP:

1. Общая информация

Gene: WAP ENSOCUG00000022314

(*Ensembl Genome Browser* – открытый геномный браузер)

Ensembl BLAST/BLAT VEP Tools BioMart Downloads Help & Docs Blog

Rabbit (OryCus2.0) Search Rabbit

Location: 10,45,322,775-40,324,485 Gene: WAP Transcript: WAP-201

Transcript-based displays

- Summary
- Sequence
 - Exons
 - cDNA
 - Protein
- Protein information
 - Protein summary
 - Domains & features
 - Variants
 - 3D Protein model
- Genetic Variation
 - Variant table
 - Variant image
 - Population comparison
 - Comparison image
- External References
 - General identifiers
 - Oligo probes
- Supporting evidence
- ID History
 - Transcript history
 - Protein history

Configure this page Custom tracks Export data Share this page Bookmark this page

Transcript: WAP-201 ENSOCUT00000022305.1

Description: whey acidic protein (Swiss-PROT gene:Act 1000002251.4)

Location: [Chromosome 10: 45,322,775-40,324,485](#) reverse strand

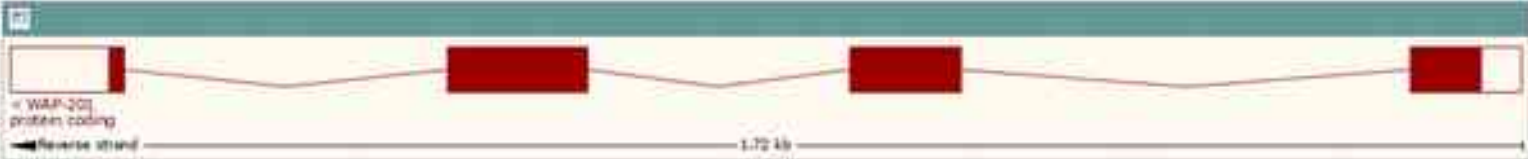
About this transcript: This transcript has 4 exons and is annotated with 8 domains and features.

Gene: This transcript is a product of gene [ENSOCUG00000022314](#) [Hide transcript table](#)

Show/hide columns (1 hidden)

Name	Transcript ID	bp	Protein	Biotype	UniProt	RefSeq	Flags
WAP-201	ENSOCUT00000022305.1	543	127aa	Protein coding	P09412	NM_001052390 RP_001075050	

Summary



Statistics: Exons: 4, Coding exons: 4, Transcript length: 543 bps, Translation length: 127 residues

UniProt: This transcript corresponds to the following UniProt identifiers: [P09412](#)

Version: [ENSOCUT00000022305.1](#)

Type: Protein coding

Annotation Method: Annotation produced by the Ensembl [genebuild](#)

Ensembl release 95 - January 2019 © [EMBL](#) [EBI](#)

[Permanent link](#) - [View in action site](#)

Подробнее:

Show/hide columns		Filter					
No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence						ctcctcctcctgtgtccaaacctttaaatgcatcccgaggccccaagaacacc
1	ENSOCUE00000254032	45,324,495	45,324,371	-	1	126	ATCCACACACTGGCTGCTGCGGACACAGCCTACACACCTGACCATGCTCTCTCAT CAGCCTGGCCCTCGCCCTGCTCGCCCTGGAGGCGCCCTCGCTCTGGCCGCCAAATTGAT CGCTCCAG
	Intron 1-2	45,324,370	45,323,859			512	gtaggccacagctgtgcttctcctcctc.....cttgtgtgtcctgtcccggtgtgag
2	ENSOCUE00000247312	45,323,858	45,323,733	1	1	126	TGCAGTTCATGTGCTCCGAGCCACGCTCTCCGAGGAGACGCTCTGGCTCAATGACAAAG ACTGTCTCGGCGAGCACCTGTGTGTGTCCAGCCCGCCGCGCGCTCTGACAGACCCCCA TCATCG
	Intron 2-3	45,323,732	45,323,433			300	gtatcgtagccacactgaaggctc.....ccagaaaaggccctgtctctccccag
3	ENSOCUE00000263449	45,323,432	45,323,274	1	1	159	TCCCTACCCCCAAGGCTGGCCGCTGCCCTGGGTCCAGGCGCCAAATGCTGTCCAGTTGT GTGAGGAGCTGAGCGACTGTGCCAAGGACATCGAGTCCAGGGGGGAGACAGAAGTGCTGCT TCAGCCGCTGGCCATGCGCTATCTGGAACCCATGCTAG
	Intron 3-4	45,323,273	45,322,905			369	gtatgtgtcctgagccctcccgagg.....tttgtccccaactgtgtctcctgag
4	ENSOCUE00000254385	45,322,904	45,322,775	1	-	130	AGAGCACTCCCGAGTGAGCCGCTATCCAGAGGAGTCTGGCTGCCAGGAGTTGGGCT GATTCGCTCTCTGACCCAGAGAGCTTGTGACGCTCTCTGCTGCTCTAATAAGACTA CTCAGCTGA
	3' downstream sequence						tggtctgtgtgtgtgtgtccatctgccctgggagcttgggaaaccagtgac.....

Аминокислотная последовательность кислого сывороточного протеина:

MRCLISLALG¹⁰ LLALEAALAL²⁰ APKFIAPVQV³⁰ MCPEPSSEE⁴⁰ TLCLSDNDCL⁵⁰
GSTVCCPSAA⁶⁰ GGSCRTPIIV⁷⁰ PTPKAGRCPW⁸⁰ VQAPMLSQLC⁹⁰ EELSDCANDI¹⁰⁰
ECRGDKKCCF¹¹⁰ SRCAMRYLEP¹²⁰ ILESTPQ¹²⁷

В молоке кролика – около 15 г/л WAP

NCBI National Center for Biotechnological Information, USA.

(информация о базах данных белковых доменов, ДНК (GenBank) и РНК, базах данных статей научной литературы (PubMed) и др..)

Gene:

Full Report

WAP whey acidic protein [*Oryctolagus cuniculus* (rabbit)]

Gene ID: 1000002018, updated on 27-Jan-2018

Summary

Gene symbol: WAP
Gene description: whey acidic protein
See related: [Ensembl:ENSPCUC000000000011](#)
Gene type: protein coding
RefSeq status: PROVISIONAL
Organism: [Oryctolagus cuniculus](#)
Lineage: Eukaryota, Metazoa, Chordata, Craniata, Vertebrata, Eumetazoa, Mammalia, Eutheria, Euarchontoglires, Glires, Lagomorpha, Leporidae, Oryctolagus

Genomic context

Location: chromosome 10 [See WAP in Genome Data Viewer](#)
Exon count: 4

Annotation release	Status	Assembly	Chr	Location
102	current	OryCun2.9 (GCF_000003625.3)	10	NC_013678.1 (45322770..45324498, complement)

Chromosome 10 - NC_013678.1

Genomic regions, transcripts, and products

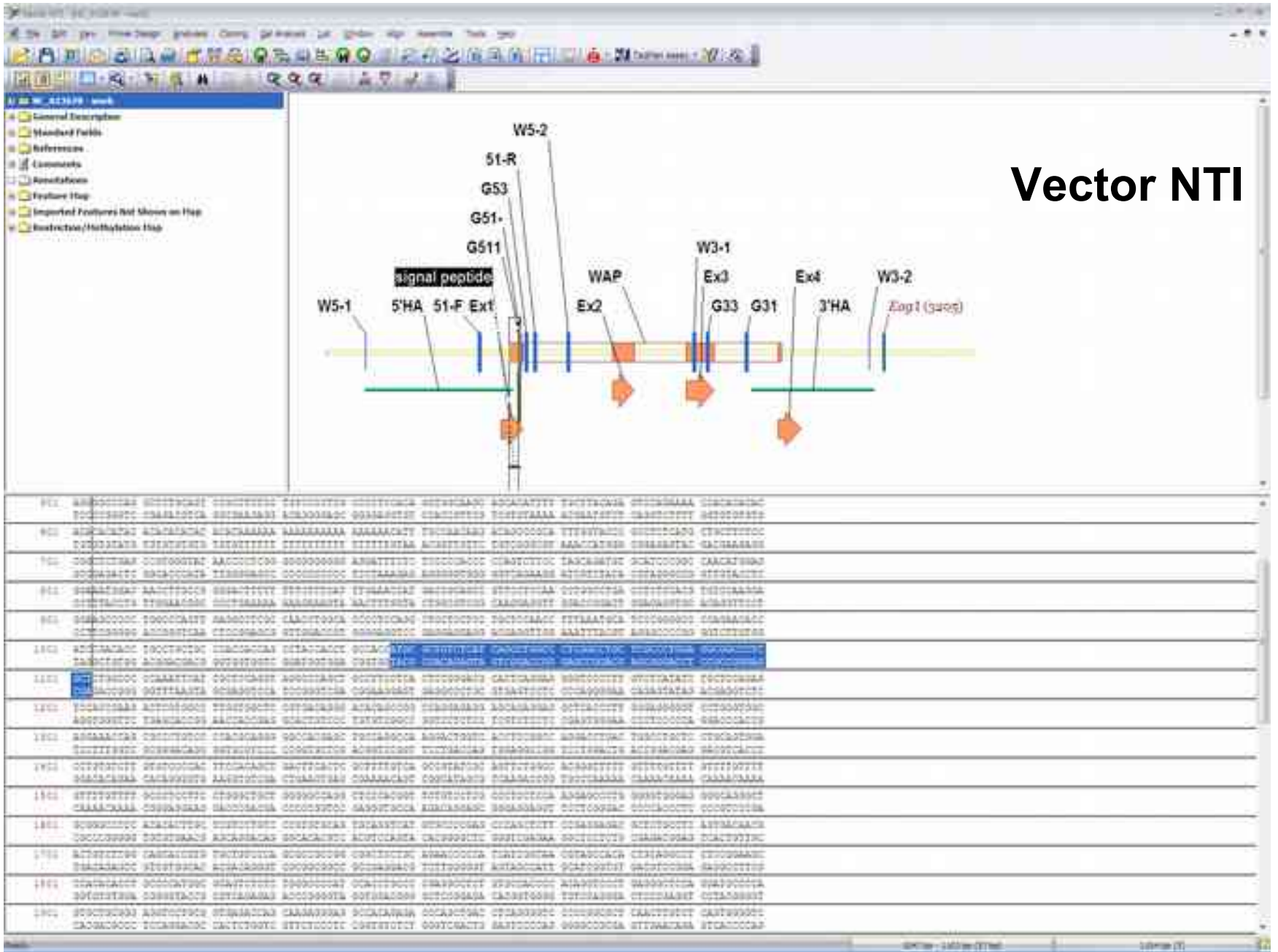
Genomic Sequence: NC_013678.1 Chromosome 10 Reference OryCun2.9 Primary Assembly

[Go to \[nucleotide\]](#) | [CDS](#) | [Exons](#) | [Intron](#) | [Gene](#)

Sequence, Loading...
Genes, NCBI Oryctolagus cuniculus Annotation Release 102, 2018-09-13, Loading...
Genes, Ensembl release 99, Loading...
RNA-seq read coverage, aggregate (filtered), NCBI Oryctolagus cuniculus Annotation Release 102, Loading...
RNA-seq transcript coverage, aggregate (filtered), NCBI Oryctolagus cuniculus Annotation Release 102, Loading...
RNA-seq exon coverage, aggregate (filtered), NCBI Oryctolagus cuniculus Annotation Release 102, Loading...

Находим нужную последовательность с подробным описанием, сохраняем в формате *.gb для работы в программе **Vector NTI**

The screenshot shows the NCBI GenBank entry for Oryctolagus cuniculus breed Thorbecke inbred chromosome 10, C genome shotgun sequence. The accession number is F069876.1. The page includes a summary of the sequence, its history, and links to related resources. A red circle highlights the "Download" button in the top right corner.

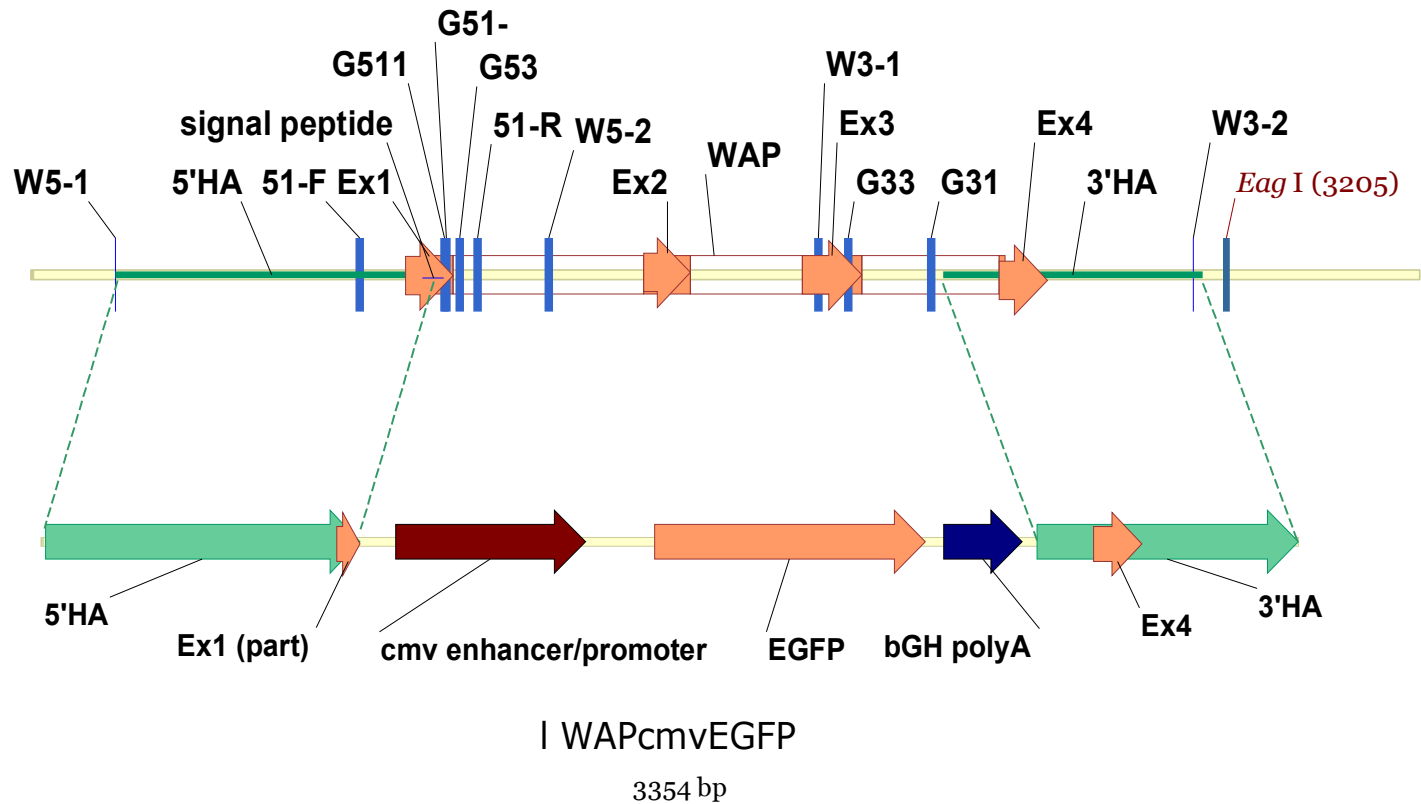


Vector NTI

Так будет происходить гомологичная рекомбинация (HDR):

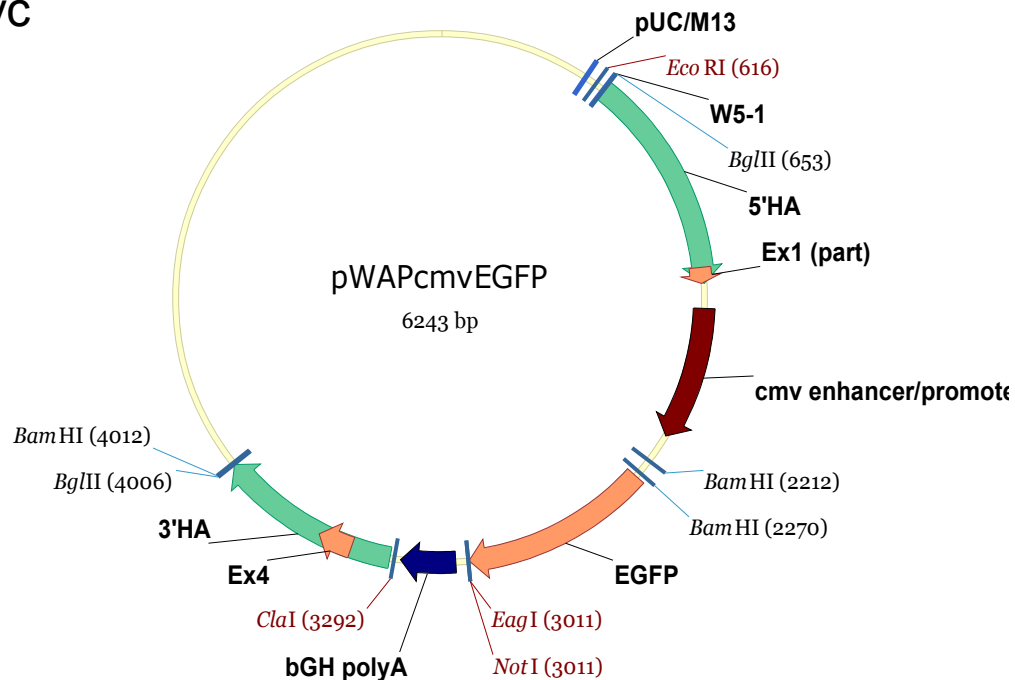
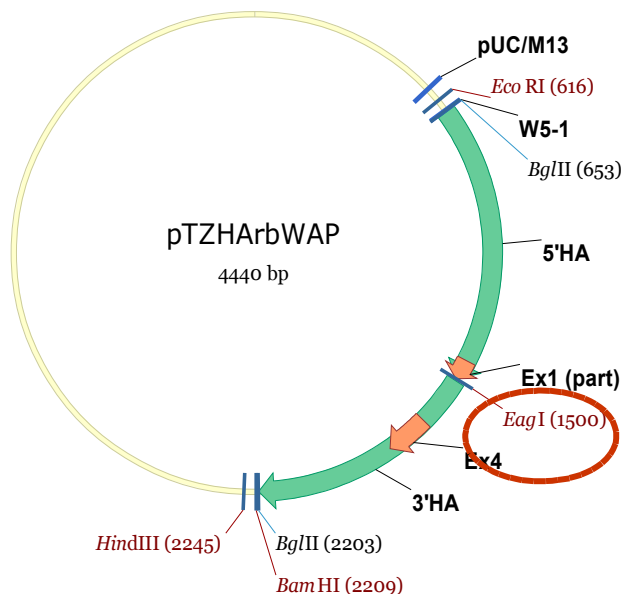
Whey Acidic Protein

Location [transcript](#) - [chromosome 10: 45,322,775-45,324,498](#)



Генная конструкция для HDR knock-in

Плазмида ***pTZHArbWAP*** – кассета для интеграции трансгена в локус гена кислого сывороточного протеина кролика



pWAPcmvEGFP – по сайту *Eag*I встроен фрагмент *cmvEGFPbGHpolyA*

Подбор мишеней для образования ДЦР ДНК

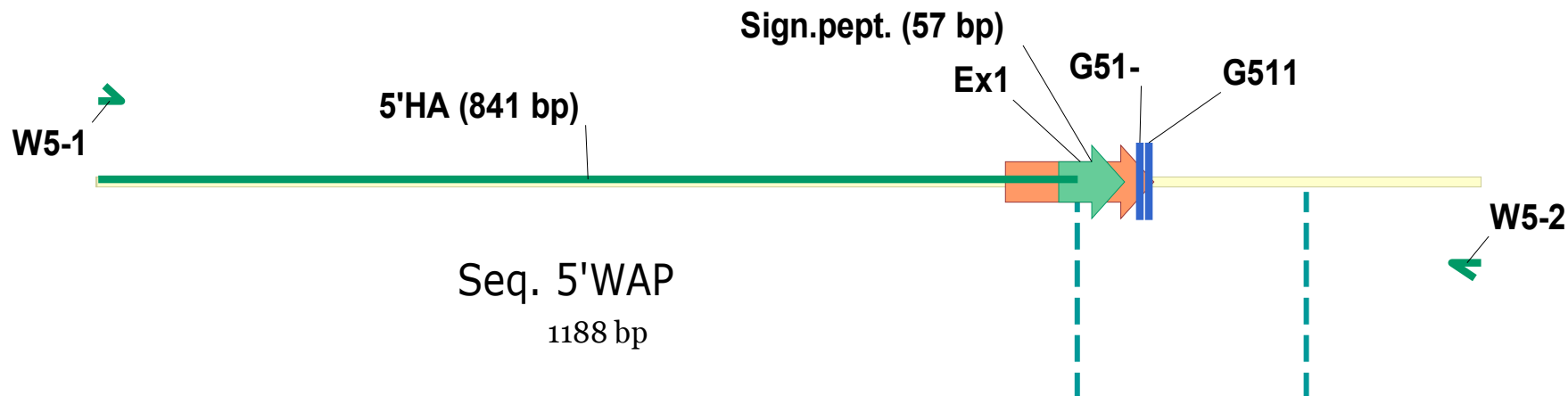
Подбор гРНК в программах:

CRISPR direct <http://crispr.dbcls.jp/>

CRISPOR v.4.8. <http://crispor.tefor.net/>

CHOPCHOP <http://chopchop.cbu.uib.no/>

Поиск мишеней в 5'-области:



Последовательность для поиска мишеней гРНК
(выбрали 200 п.н.):

ATGCGCTGTCTCATCAGCCTGGCCCTCGGCCTGCTCGCCCTGG

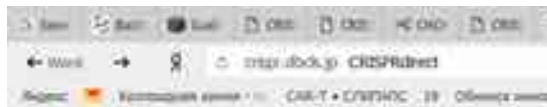
AGGCGGCCCTCGCTCTGGCCC **CCAAT** | **TCATCGC** |

TCCAGgtaggcccagctgccttcctcactccgggacgcactcaggaggggtccccttgtctc
atatctgctccagagtccacccaagactcgtggccttggtggctccgtgacagggacacag

CRISPR direct

<http://crispr.dbcls.jp/>

Naito Y, Hino K, Bono H, Ui-Tei K. (2015) CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics*, 31, 1120-1123.



Enter an accession number (e.g. NM_006299) or genome location (e.g. hg19:chr7:900000-901000): ?

retrieve sequence

or Paste a nucleotide sequence: ?

```
>sample sequence
atgcctgtgtctcatcagcctggcctcctggcctgtctgcctggagcggcctcctgtctgtgcccctcaaatcctcgtcctcaggttaggecca
gtgccttctcactccgggacgcactcaggaggggtcccttctgtctcctatctgtctcagagtcaccccaagactcgtgccttctgttgc
tcctgtgacagggaacatag
```

or upload sequence file: ? Выберите файл Файл не выбран

PAM sequence requirement: NGG (e.g. NGG, NRG) ?

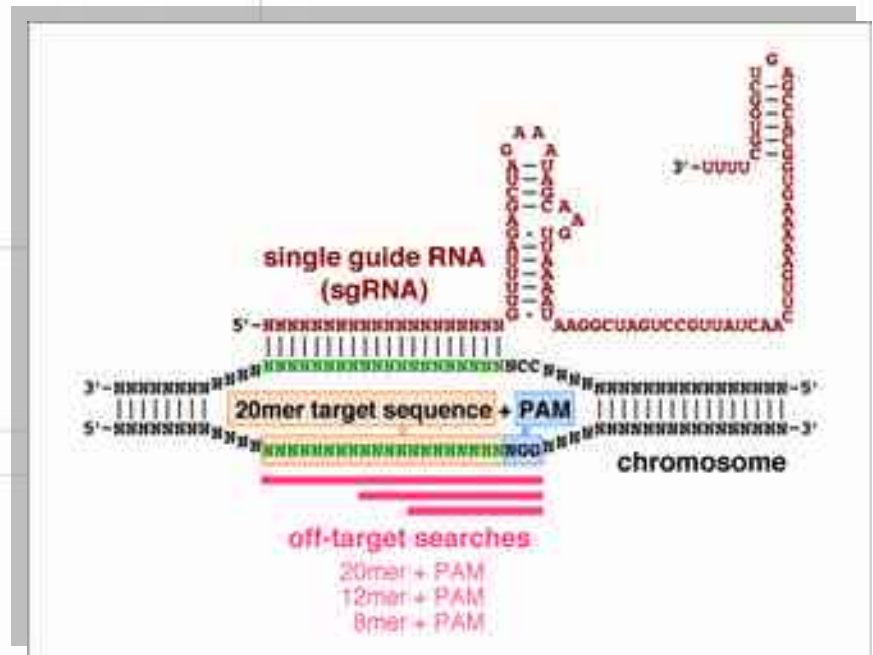
Specificity check:

Rabbit (*Oryctolagus cuniculus*) genome, Broad/oryCun2 (Apr, 2009)

design

Whats' new:

- 2018-05-07 Added 12 genomes - [List](#)
- 2017-12-11 Added Carrot genome v2.0 from Phytozome.
- 2017-11-30 Added 326 genomes from CyanoBase - [List](#)



gRNA sequence calculator

Input:

Target sequence + PAM:

octaacctggagcgaatgaatttgg

sgRNA 3' sequence:

GGTGGAGAC GAGGAGGAC AGGAGAGAG AGGAGAGAG GAGGAGAGC
GGGAGAGAG GAGGAGAGC GAGGAGAGC

Output:

sgRNA sequence (read only):

octaacctggagcgaatgaatttgg
GAGGAGAGC GAGGAGAGC GAGGAGAGC

☒ RNA → DNA (U → T)

show highly specific target only

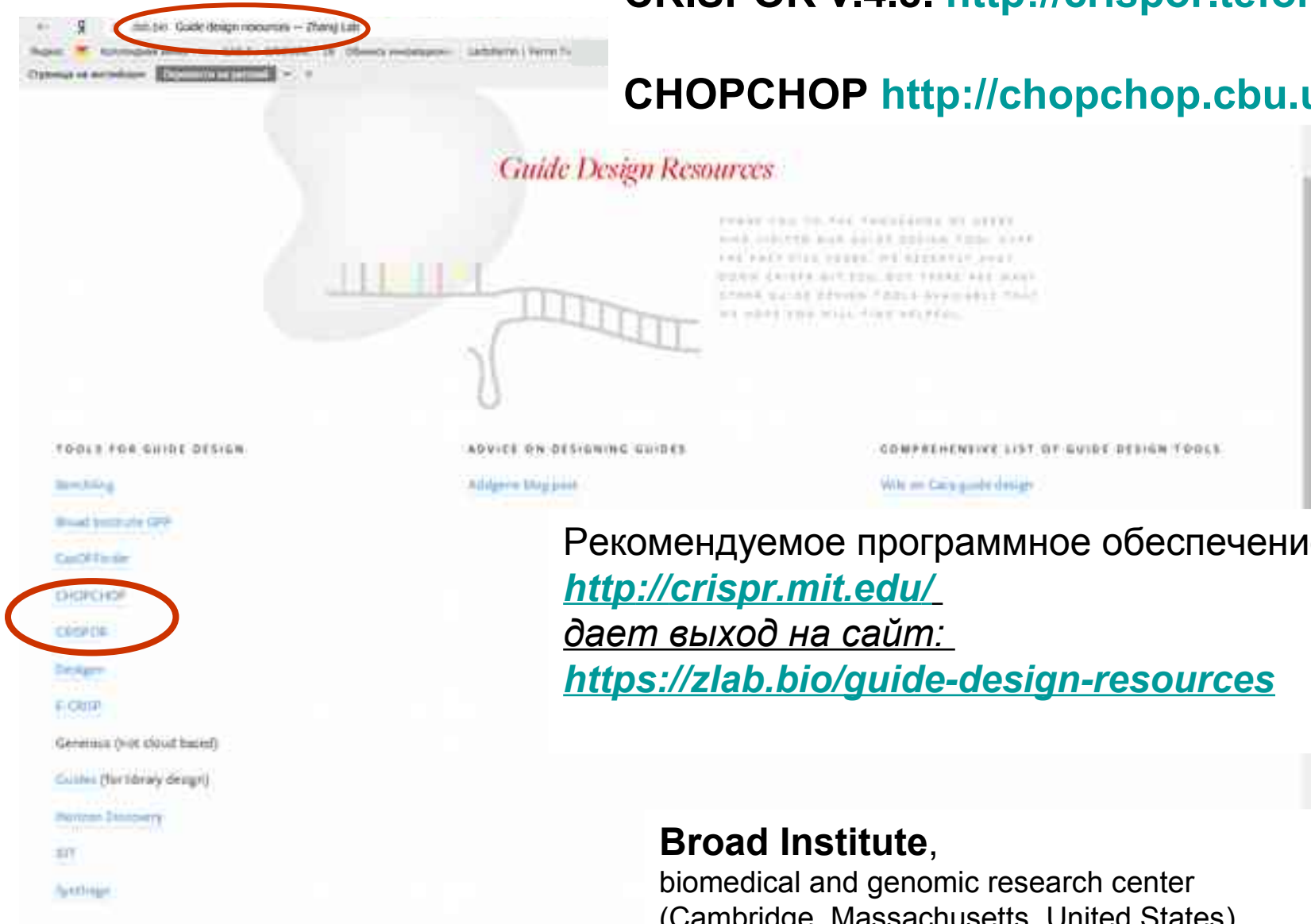
Show 50 entries

Search:

position	target sequence	sequence information	number of target
start end	20mer+PAM (total 23mer)	GC% of 20mer Tm of 20mer TTTT in 20mer restriction sites	20mer +PAM 12mer +PAM
7 - 29	+ tgtctcatcagcctggccct [gRNA]	60.00 % 79.24 °C	1 [detail] 234 [det]
18 - 40	- cctcggcctcggcctgctggcc [gRNA]	85.00 % 89.85 °C	1 [detail] 56 [det]
21 - 43	+ ggcctcggcctgctggccct [gRNA]	85.00 % 89.85 °C	1 [detail] 33 [det]
23 - 45	- cctcggcctgctggccctggag [gRNA]	75.00 % 85.01 °C	1 [detail] 6 [det]
24 - 46	+ actcggcctgctggccctggag [gRNA]	88.00 % 86.94 °C	1 [detail] 21 [det]
24 - 46	- cctcggcctgctggccctggag [gRNA]	80.00 % 86.94 °C	1 [detail] 21 [det]
27 - 49	+ cgcctgctcgcctggagcggg [gRNA]	80.00 % 86.94 °C	1 [detail] 20 [det]
30 - 52	- cctcggcctgctggagcggcc [gRNA]	85.00 % 89.85 °C	1 [detail] 34 [det]
38 - 60	- cctcggagcgcctcgcctcgt [gRNA]	75.00 % 85.01 °C	1 [detail] 30 [det]
39 - 61	+ cctcggagcgcctcgcctcgt [gRNA]	80.00 % 86.76 °C	1 [detail] 19 [det]
39 - 61	- cctcggagcgcctcgcctcgt [gRNA]	80.00 % 86.76 °C	1 [detail] 47 [detail]
50 - 72	- cctcggcctgctggccccaattc [gRNA]	60.00 % 78.25 °C	1 [detail] 28 [detail] 2860 [detail]
61 - 83	+ cctcggcctgctggccccaattc [gRNA]	60.00 % 78.32 °C	1 [detail] 62 [detail] 3982 [detail]
61 - 83	+ ggcctcggcctgctggccccaattc [gRNA]	60.00 % 77.25 °C	1 [detail] 2 [detail] 754 [detail]
62 - 84	- cctcggcctgctggccccaattc [gRNA]	50.00 % 72.30 °C	1 [detail] 4 [detail] 7150 [detail]
63 - 85	- cctcggcctgctggccccaattc [gRNA]	45.00 % 69.29 °C	1 [detail] 5 [detail] 8660 [detail]
64 - 86	- cctcggcctgctggccccaattc [gRNA]	45.00 % 69.67 °C	1 [detail] 3 [detail] 10072 [detail]
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65 - 87	- cctcggcctgctggccccaattc [gRNA]	50.00 % 72.67 °C	1 [detail] 3 [detail] 4055 [detail]
79 - 101	- cctcggcctgctggccccaattc [gRNA]	70.00 % 83.80 °C	1 [detail] 20 [detail] 1792 [detail]
88 - 110	+ cctcggcctgctggccccaattc [gRNA]	65.00 % 79.43 °C	1 [detail] 45 [detail] 6287 [detail]
88 - 110	- cctcggcctgctggccccaattc [gRNA]	65.00 % 80.53 °C	1 [detail] 96 [detail] 13261 [detail]
89 - 111	+ cctcggcctgctggccccaattc [gRNA]	65.00 % 79.43 °C	1 [detail] 65 [detail] 8748 [detail]
89 - 111	- cctcggcctgctggccccaattc [gRNA]	70.00 % 81.82 °C	1 [detail] 162 [detail] 15831 [detail]
96 - 118	- cctcggcctgctggccccaattc [gRNA]	65.00 % 80.46 °C	1 [detail] 19 [detail] 10885 [detail]
100 - 122	+ cctcggcctgctggccccaattc [gRNA]	70.00 % 80.40 °C	1 [detail] 3 [detail] 1212 [detail]
100 - 122	- cctcggcctgctggccccaattc [gRNA]	70.00 % 80.49 °C	1 [detail] 5 [detail] 2527 [detail]
103 - 125	+ cctcggcctgctggccccaattc [gRNA]	70.00 % 80.49 °C	1 [detail] 8 [detail] 5745 [detail]
104 - 126	+ cctcggcctgctggccccaattc [gRNA]	65.00 % 80.40 °C	1 [detail] 6 [detail] 6129 [detail]
105 - 127	+ cctcggcctgctggccccaattc [gRNA]	70.00 % 80.38 °C	1 [detail] 13 [detail] 8883 [detail]
107 - 129	+ cctcggcctgctggccccaattc [gRNA]	70.00 % 81.08 °C	1 [detail] 10 [detail] 1893 [detail]
129 - 151	- cctcggcctgctggccccaattc [gRNA]	45.00 % 69.15 °C	1 [detail] 16 [detail] 6033 [detail]
130 - 152	- cctcggcctgctggccccaattc [gRNA]	45.00 % 69.15 °C	1 [detail] 33 [detail] 7197 [detail]
130 - 152	- cctcggcctgctggccccaattc [gRNA]	70.00 % 80.40 °C	1 [detail] 20 [detail] 5065 [detail]

CRISPOR v.4.8. <http://crispor.tefor.net/>

CHOPCHOP <http://chopchop.cbu.uib.no/>



Рекомендуемое программное обеспечение
<http://crispr.mit.edu/>
дает выход на сайт:
<https://zlab.bio/guide-design-resources>

Broad Institute,
biomedical and genomic research center
(Cambridge, Massachusetts, United States).
Feng Zhang laboratory

CRISPOR v.4.8. <http://crispor.tefor.net/>

Your input sequence is 200 bp long. It contains 44 possible guide sequences. Shown below are their PAM sites and the expected cleavage position located ~3bp 5' of the PAM site. Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the

[illegible]

Ranked by default from highest to lowest specificity score (Pine et al., *Nat Genet* 2011). Click on a column title to rank by a score. If you use this website, please cite our [CRISPR paper in Gen. Exp.](#) 2016. Too much information? Look at the [CRISPR manual](#).

Yeast Strain	Guide Sequence + PAM + Restriction Enzymes Only G- Only GC- Only A-	MFE Specificity Score	CFD Spec. Score	Predicted Efficiency	Outcome	Off-targets for 3-1-2-3-4 re-matches + next to PAM	Genome browser links to matches sorted by CFD off-target score exon only chr chr chr chr	
				Depth - 1000 Max-Maps	On-Off-Frame Log2			
65 / 100	CTACCTGCGCGGCTGATTTT TTT Enzymes: BamHI, MluI, MspI, NcoI, PstI, XbaI Cloning / PCR primers	93	97	18	41	68	None 0-0-2-0-44 0-0-0-0-0 46 off-targets	www.ncbi.nlm.nih.gov/ncbi2281 www.ncbi.nlm.nih.gov/ncbi2281 Off-target primers
81 / 100	GGCGCGGATTTTATGCTCTC AAG Enzymes: SfiI, DdeI, BspI, XbaI Cloning / PCR primers	92	98	51	28	71	None 0-0-1-6-82 0-0-0-0-0 89 off-targets	www.ncbi.nlm.nih.gov/ncbi2281 Off-target primers
85 / 100	CTGATTTTCTCTGCGGCTT AAG Enzymes: BamHI, SfiI, DdeI, PstI, XbaI Cloning / PCR primers	90	95	61	38	73	None 0-0-0-3-51 0-0-0-0-0 34 off-targets	Off-target primers
100 / 100	CTGCTTATGCGGATTTT TTT Enzymes: MspI, BspI, SfiI, PstI Cloning / PCR primers	90	96	39	55	64	None 0-0-0-6-38 0-0-0-0-0 34 off-targets	www.ncbi.nlm.nih.gov/ncbi2281 www.ncbi.nlm.nih.gov/ncbi2281 Off-target primers

If you use this website, please cite our **CRRPDR paper in Gen Biol 2016**. Too much information? Look at the **CRRPDR manual**.

Download as Excel tables: [Guides](#) / [Off-targets](#) / [Substituting multi-gene's constant](#)

**Программа дает
перечень офф-таргетов
с демонстрацией
ошибок...**

...предлагает последовательности олигонуклеотидов для вставки в одну из подходящих плазмид, например:

To clone the guide into

pX330-U6-Chimeric_BB-CBh-hSpCas9 (Zhang lab) + derivatives,

use these primers:

Name	Primer Sequence
guideRNA65rvU6sensepX330	CACCG <u>C</u> CTACCTGGAGCGATGAATT
guideRNA65rvU6antisensepX330	AAACAATTCATCGCTCCAGGTAGG <u>C</u>

Summary of main cloning/expression primers

guideRna65rvT7sense	TAGGCCTACCTGGAGCGATGAATT
guideRna65rvT7antisense	AAACAATTCATCGCTCCAGGTAGG
guideRNA65rvT7crTarget	GAAATTAATACGACTCACTATAGCCTACCTGGAGCGATGAATTGT TTTAGAGCTAGAAATAGCAAG
guideRNAallT7common	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTA GCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
guideRNA65rvU6sensepX330	CACCG <u>C</u> CTACCTGGAGCGATGAATT
guideRNA65rvU6antisensepX330	AAACAATTCATCGCTCCAGGTAGG <u>C</u>



...праймеры для ПЦР-амплификации фрагментов, содержащих мишени...

PCR to amplify the on-target site

Use these primers to amplify a genomic fragment around the on-target site:

OntargetGuideRna65rvLeft AGTCTTGGGTGGACTCTGGA Tm 59.809

OntargetGuideRna65rvRight GACCTCTCCACGTGTCCAAG Tm 60.038

Genome fragment with validation primers (underlined) and guide sequence (yellow)

Maximum amplicon length: 400 bp - for >= 250bp paired reads ▼ Primer Tm: 60 deg. ▼

Genomic sequence chr10:45324365-45324388 including primers, genomic forward strand:

AGTCTTGGGTGGACTCTGGAGCAGATATGAGACAAGGGGACCCCTCCTGAGTGCGTCCCGAGTGAGGAAGGCAGCTGGG
CCTACCTGGAGCGATGAATTTGGGGGCCAGAGCGAGGGCCGCCCTCCAGGGCGAGCAGGCCGAGGGCCAGGCTGATGAGAC
AGCGCATGGTGGCAGGTGGTAGGCTGGTGGTGGGCAGCAGGCAGGTGTCCGATGGTGTCTGGGGCCCCGGGATGCATTT
AAAGGTTGGAGCAGGAGGAGGAGCCTGGAGGGGTGCCAGGTTGGCCAGGCCTCAACTGGGCCAGSGGGCTTCCTCCTTGG
ACACGTGGAGAGGTC

AGTCTTGGGTGGACTCTGGAGCAGATATGAGACAAGGGGACCCCTCCTGAGTGCGTCCCGAGTGAGGAAGGCAGCTGGG
CCTACCTGGAGCGATGAATTTGGGGGCCAGAGCGAGGGCCGCCCTCCAGGGCGAGCAGGCCGAGGGCCAGGCTGATGAGAC
AGCGCATGGTGGCAGGTGGTAGGCTGGTGGTGGGCAGCAGGCAGGTGTCCGATGGTGTCTGGGGCCCCGGGATGCATTT
AAAGGTTGGAGCAGGAGGAGGAGCCTGGAGGGGTGCCAGGTTGGCCAGGCCTCAACTGGGCCAGSGGGCTTCCTCCTTGG
ACACGTGGAGAGGTC

Sequence length: 334

Method: Primer3.2 with default settings, target length: 250-400 bp.



AGTCTTGGCTGGACTCTGGAGCAGATATGAGACACGGGGGACCCCTCCTGAGTGGCTCCCGAGAGTGAGGAGAGGCACCTGAG
CCTACCTGGAGGCCATGCAATTTGGGGGCGAGAGCGAGGGGCGCCCTCCGGGGCGAGCAGGGGCGAGGGGCGAGGGCTGATGAGAC

**...перечень
рестриктаз для
анализа ПЦР-
продукта...**

DNA break repair outcome predictions

Guide: CCTACCTGGAGCGATGAATT TGG

The following table lists possible deletions. Each sequence below represents the context around the guide's target, with deleted nucleotides shown as "-". Sequences are ranked by micro-homology score. This score is correlated to the likelihood of finding a particular deletion, as predicted by the method of [Bao et al. 2014](#).

mh-Score	Sequence	Effect
	GAGTGAGGAAAGCAGCTGGGCTACCTGGAGCGATGAATTTGGGGGCCAGAGCGAGGGCCGGCTCCAGGGGAGCAGGGCC	(Wild-type sequence)
350	GGCAGCTGGGCTACCTGGAGCGA-----GGGCGGCTCCAGGG	21 bp deleted - no frameshift
273	GGCAGCTGGGCT-----AGAGCGAGGGCCGGCTCCAGGG	26 bp deleted - frameshift
248	GGCAGCTGGGCTACCTGG-----GGGCCAGAGCGAGGGCCGGCTCCAGGG	14 bp deleted - frameshift
222	GGCAGCTGGGCTACCTGGAGCGATG-----GGGGCCAGAGCGAGGGCCGGCTCCAGGG	6 bp deleted - no frameshift
210	GGCAGCTGG-----GGCCAGAGCGAGGGCCGGCTCCAGGG	24 bp deleted - no frameshift
188	GGCAGCTGGGCTACCTGGAGC-----CAGAGCGAGGGCCGGCTCCAGGG	15 bp deleted - no frameshift
188	GGCAGCTGGGCTACCTGG-----GGCCAGAGCGAGGGCCGGCTCCAGGG	15 bp deleted - no frameshift
179	GGCAGCTGGGCTACCTGG-----GCCAGAGCGAGGGCCGGCTCCAGGG	16 bp deleted - frameshift
172	GGCAGCTGG-----GCCAGAGCGAGGGCCGGCTCCAGGG	25 bp deleted - frameshift
170	GGCAGCTGGGCTACCTGG-----CCAGAGCGAGGGCCGGCTCCAGGG	17 bp deleted - frameshift
150	GGCAGCTGGGCT-----GGCTCCAGGG	38 bp deleted - frameshift
149	GGCAGCTGGGCTACCTGGAGCGATGA-----GCCAGGGCCGGCTCCAGGG	14 bp deleted - frameshift
143	GGCAGCTGGGCTACCTGGAG-----GGCCGGCTCCAGGG	25 bp deleted - frameshift
133	GGCAGCTGGGCTACC-----AGAGCGAGGGCCGGCTCCAGGG	22 bp deleted - frameshift
128	GGCAGCTGGGCTACCTGGAGCGA-----GCCAGGGCCGGCTCCAGGG	17 bp deleted - frameshift
126	GGCAGCTGG-----GGGCCAGAGCGAGGGCCGGCTCCAGGG	23 bp deleted - frameshift
122	GGCAGCTGGGCTACCTGGAGCGATGA-----GGCCGGCTCCAGGG	18 bp deleted - no frameshift
116	GGCAGCTGGGCTACCTGGAG-----AGCGAGGGCCGGCTCCAGGG	19 bp deleted - frameshift

...показывает варианты делеций в таргетной области

CHOPCHOP



Target:
RefSeq/NCBI/Ensembl gene name or genomic coordinates

W:
Reference species

Using:
Change default PAM and guide length in Options

For:
Please specify antibody in Clonify

sgRNA length without PAM:

PAM 3':
☒ NGG ☐ NAG ☐ NGA ☐ NRG (R = A or G) ☐ NNAAGAAW (W = A or T) ☐ NNNNGMTT (M = A or C) ☐ NNNERTT (R = A or G)
☐ Custom PAM:

Method for determining off-targets in the genome:
☒ Off-targets with up to mismatches in protospacer ([Pruett et al., 2012](#))
☐ Off-targets may have no more than mismatches in the protospacer seed region ([Cong et al., 2013](#))

Efficiency score:
☐ Doench et al. 2014 - only for NGG-PAM
☒ Doench et al. 2016 - only for NGG-PAM
☐ Chan et al. 2015 - only NGG and TGAAGAAW PAMs in sg13 and non10
☐ Xu et al. 2015 - only for NGG-PAM, but can be used with other PAMs
☐ Moreno-Mateos et al. 2015 - only for NGG-PAM
☐ G00

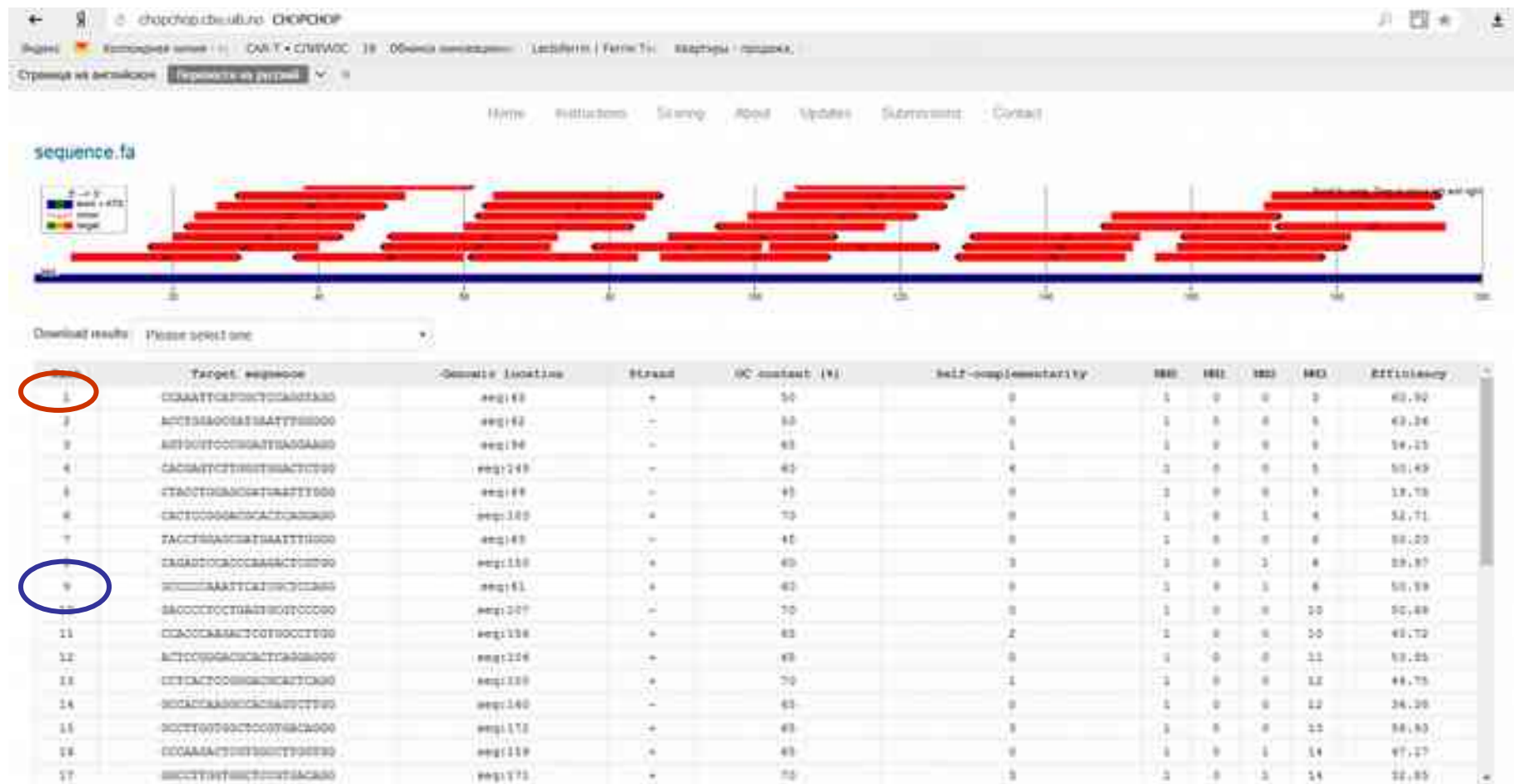
Repair profile prediction (Shen et al. 2016):
☒ iRE3C (recommended when you don't know which cell type)
☐ U2OS
☐ HEK293
☐ HCT116
☐ K562
☐ Don't calculate (saves time)

5' requirements for sgRNA:
☐ GW or TG
☐ GG

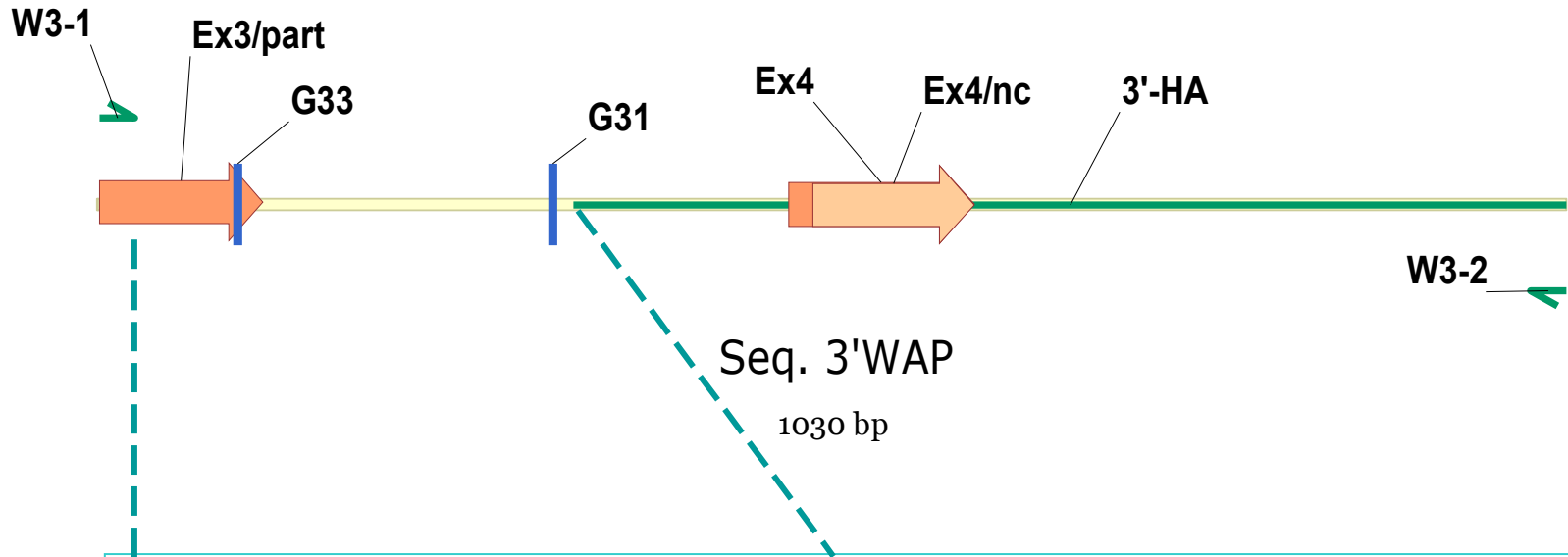
CHOPCHOP

<http://chopchop.cbu.uib.no/>

Возможности и интерфейс программы SNORCHOP



Поиск мишеней в 3'-области:



Последовательность для поиска мишеней гРНК
(выбрали 280 п.н. перед плечом гомологии):

**TGCAGGGGCGACAAGAAGTGCTGCTTCAGCCGCTGCGCCATGCGC
T|**

ATCTGGAACCCATCCTAGgtatgtgtcctgagccctcccaggcagggctgtcccttcagc
agggcccagggctcaggagtggatgtgggtgagtgaagggcactcggggacgcagggtggcaggc
gggacttggccctgggtggctcacaggccagcctgtacctttgccactgatctgagagggagtgagc
acagctccaggtatcggaggagtcgaag|gttaggagcctgggggtgtgtccaccagct

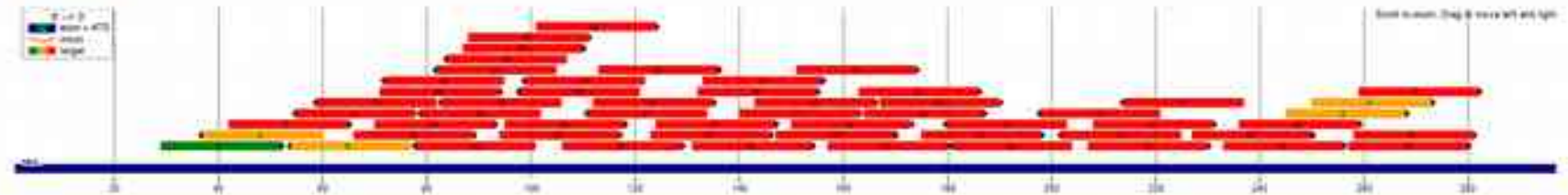
Ranked by default from highest to lowest specificity score (Hsu et al., [Nat Biomed 2013](#)). Click on a column title to rank by a score.

If you use this website, please cite our [CRISPR paper in Gen Biol 2016](#). Too much information? Look at the [CRISPR manual](#).

Download as Excel table: [Guides](#) / [Off-targets](#) / [Safeguarding malignancy assistant](#)

Position Strand	Guide Sequence + PAM + Restriction Enzymes ☑ Only G ☑ Only GG ☑ Only A ☑	MIT Specificity Score	CFD Spec. Score	Predicted Efficiency ☑ 50-100 ☑ Mon-Metases	Outscore ☑ Out-of-Frame ☑ Under	Off-targets for 0-1-2-3-4 mismatches + next to PAM ☑	Genome Browser links to matches sorted by CFD off-target score ☑ ☑ exons only ☐ chosen 10 only
50 / fw	GGCTGCGCAGGGGCTATC TGG Enzymes: XbaI, NdeI, Hpy188M Cloning / PCR primers	97	97	22	57	70 89	0-0-0-0-40 0-0-0-0-0 40 off-targets 4 exons: C1orf122 4 exons: ENSG00000222242
38 / rev	GATGGTTCTGGAAGAGGCGCA TGG Enzymes: HpaII, XbaI, NdeI, MspI Cloning / PCR primers	95	95	59	40	67 82	0-0-0-2-64 0-0-0-0-0 60 off-targets 4 exons: DNMT1
246 / rev	CTTGTGACTGCCCGATATC TGG ☑ Inefficient Enzymes: AclB1, StyD6, BspI, BstNI, Bst Cloning / PCR primers	94	96	47	15	70 77	0-0-0-4-52 0-0-0-0-0 56 off-targets 4 exons: C1orf10 4 exons: MCT1 4 exons: FMN2 4 exons: FMN2 4 exons: SLC39A1 4 exons: FMN2 4 exons: L10NA1 4 exons: NTSCC
971 / fw	TATCGAGAGATTCAGGTT AGG Enzymes: MspI Cloning / PCR primers	93	97	43	54	78 85	0-0-0-1-26 0-0-0-0-1 27 off-targets 4 exons: ZNF79
266 / fw	CGAGGTATCGAGAGATCGCA AGG Enzymes: HpaII, PstI, BspI, PstI Cloning / PCR primers	90	91	55	64	61 75	0-0-0-2-23 0-0-0-0-0 4 exons: ENSG00000222242 4 exons: C1orf122 4 exons: SLC39A1 4 exons: SLC39A1

sequence.fa

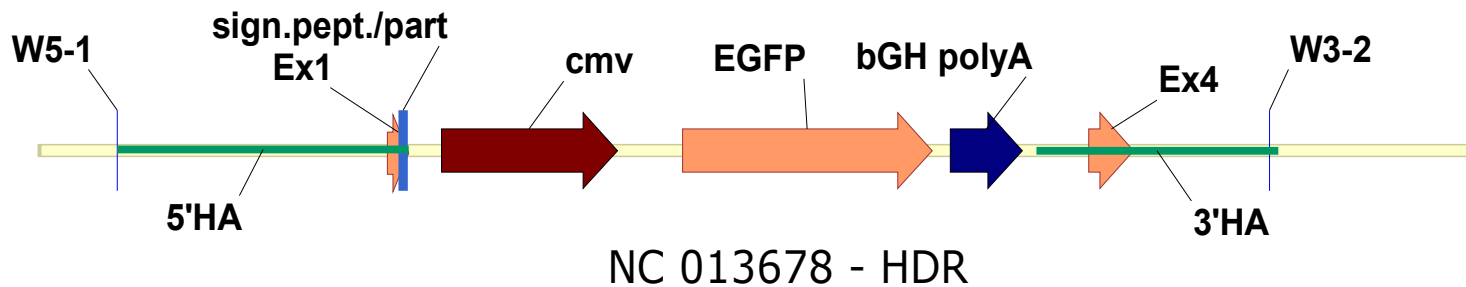


Download results: [Download](#)

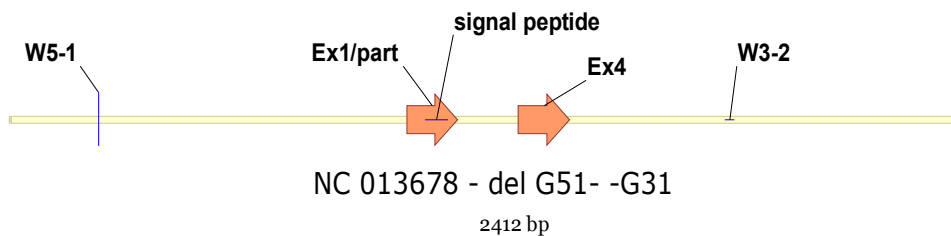
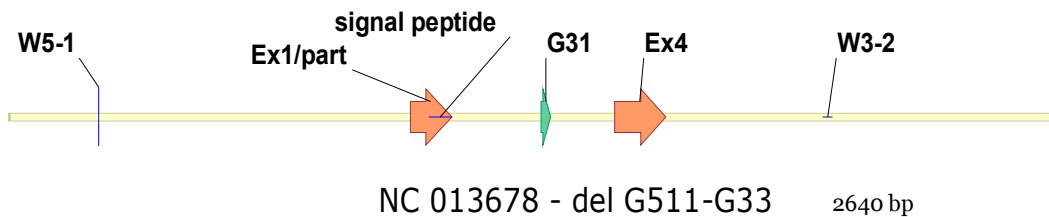
Rank	Target sequence	Seed position	Strand	GC content (%)	Self-complementarity	MS1	MS2	MS3	MS4	Efficiency
1	CGGCTGAGGATGAGGATGAGG	seq:10	+	75	2	1	0	0	0	21.31
2	CGGCTGAGGATGAGGATGAGG	seq:144	+	80	1	1	0	0	1	25.41
3	TATGAGGATGAGGATGAGG	seq:155	+	50	1	1	0	0	1	42.79
4	GATGAGGATGAGGATGAGG	seq:16	+	35	1	1	0	0	2	38.31
5	CGGCTGAGGATGAGGATGAGG	seq:15	-	49	2	1	0	0	2	39.32
6	GATGAGGATGAGGATGAGG	seq:159	+	35	0	1	0	0	3	52.99
7	CGGCTGAGGATGAGGATGAGG	seq:13	+	35	1	1	0	0	4	45.72
8	GATGAGGATGAGGATGAGG	seq:140	+	30	0	1	0	0	7	49.70
9	CGGCTGAGGATGAGGATGAGG	seq:16	-	35	2	1	0	0	7	43.24
10	CGGCTGAGGATGAGGATGAGG	seq:173	-	30	0	1	0	0	9	47.44
11	CGGCTGAGGATGAGGATGAGG	seq:141	+	70	0	1	0	0	11	44.10
12	GATGAGGATGAGGATGAGG	seq:100	+	35	0	1	0	0	5	48.54
13	GATGAGGATGAGGATGAGG	seq:139	+	85	2	1	0	1	12	41.91
14	CGGCTGAGGATGAGGATGAGG	seq:123	-	30	0	1	0	1	10	41.61
15	CGGCTGAGGATGAGGATGAGG	seq:109	-	35	0	1	0	1	14	41.73
16	GATGAGGATGAGGATGAGG	seq:128	+	80	0	1	0	2	13	42.88
17	GATGAGGATGAGGATGAGG	seq:10	-	30	1	1	0	1	10	42.88

Варианты модификаций гена rbWAP

1. Knock-in cmvEGFP (HDR)



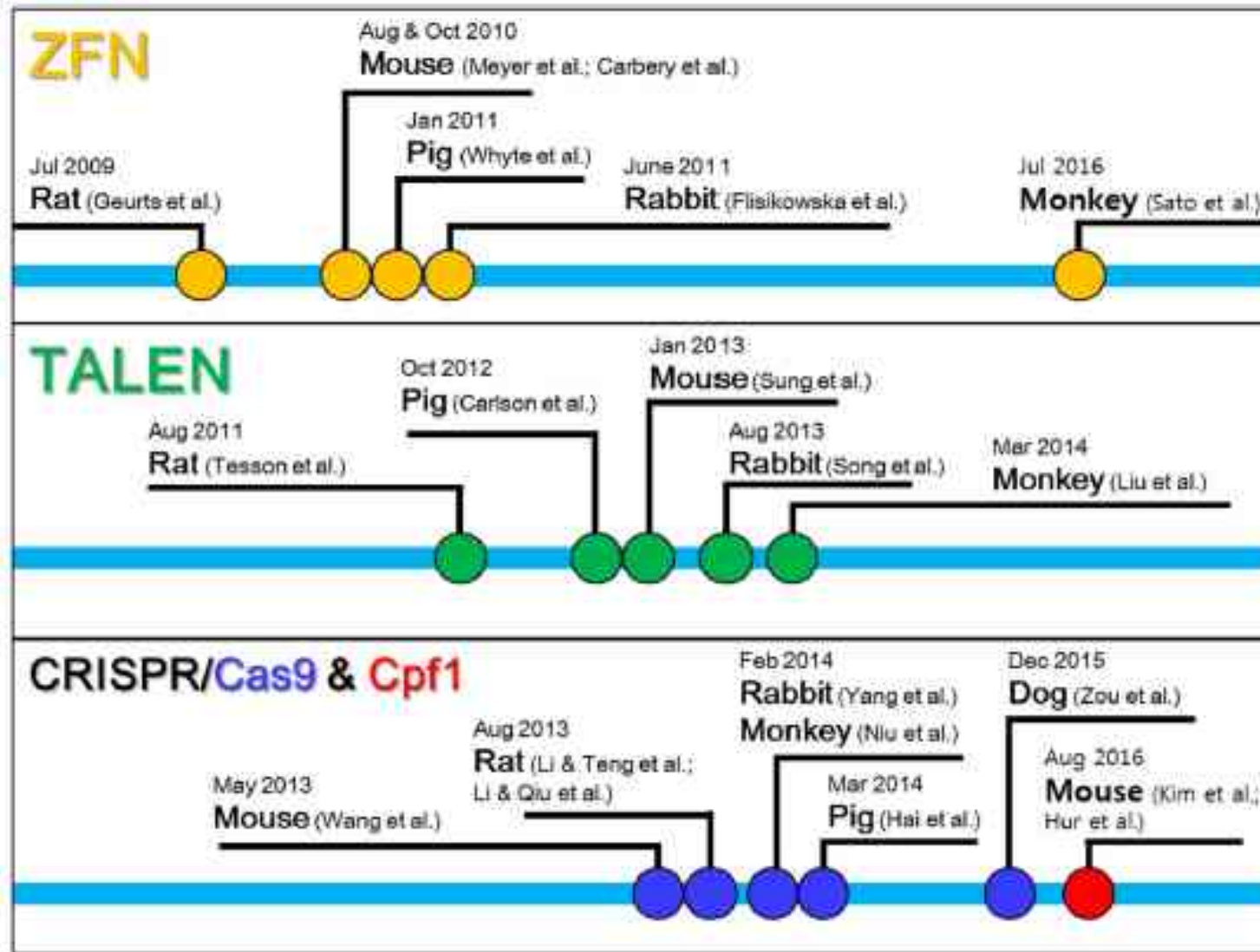
2. Возможны крупные делеции (варианты NHEJ)



3. Возможны мелкие делеции с нокаутом и без нокаута гена (варианты NHEJ) ...

Часть 2. Кролики-биомодели для медицинских и биологических исследований, полученные с использованием новых геномных технологий (обзор).

Хронология применения нуклеазных технологий на лабораторных животных. (Lee JG et al., 2018)



Кролики, нокаутные по одному гену

Ген-мишень	Функция гена, модель	Конц. НК, нг/мкл mRNA Cas9/ sgRNA/ кол-во sgRNA	Живые крольчата/ всего крольчат/ подсажено эмбр./ МИ эмбрионов	Биаллельны/ ГМ крольч./ всего крольчат	Ссылки
APOE	липидный обмен, атеросклероз	150 /6	12/?/94/?	10/10/12	Yang D, et al., 2014
CD36		150 /6	13/?/54/?	4/11/13	
LDLR		150 /6	28/?/93/?	0/9/28	
RyR2	аритмия сердца	150 /6	15/?/60/?	1/8/15	
		Итого:	68/?/301/?	15/38/68	
LDLR (Ex7)	Рецептор ЛПНП	49/13 sgRNA1	3/3 /12/14	3/3/3	Lu R et al., 2018
		49/13 sgRNA2	4/10/79/82	10/10/10	
GJA8 (Ex1)	катаракта	180/40/2	11/11/110/?	0/11/11	Yuan L, et al., 2016
CRYAA (Ex2)	αА-кристаллин	180/40/2	19/?/85/?	15/19/19	Yuan L, et al., 2017
TYR (Ex 1)	тирозиназа	pX330 - 5	9/?/67/77	1/2/9	Honda A, et al., 2015
TYR (Ex3, Δ105)	окраска шерсти	100/25/2	17/?/169/ ?	1(Δ105)/15/17	Song Y, et al., 2016
TYR (3'utr, Δ330)		100/25/2	4/?/84/92	3KO/4	Song Y, et al., 2017

Кролики, нокаутные по одному гену (продолжение)

Ген-мишень	Функция гена, модель	Конц. НК, нг/мкл mRNA Cas9/ sgRNA/ кол-во sgRNA	Живые крольчата/ всего крольчат/ подсажено эмбр./ МИ эмбрионов	Биаллельны/ ГМ крольч./ всего крольчат	Ссылки
MSTN (Ex1)	миостатин	180/40/2	20/?/158/?	6/16/20	Lv Q, et al., 2016
MSTN (Ex1-Ex3, Δ≈4600)	гипермышечный фенотип	200/20+20/2	3/5/56/80	24/34 (19-gRNA1, 18-gRNA2, 13-gRNA1+ gRNA2; 2-Δ4600b)	Guo et al., 2016
		100/20+20/2	29/32/194/218		
DMD (Ex51)	мышеч. дистрофия Дюшенна	200/50/2	33/?/128/?	22/26/33	Sui T, et al., 2018
FBN1 (Ex 65)	фибриллин-1, синдром Марфана	200/30/ 2	28/?/197/?	26/28/28	Chen M et al., 2018
PHEX (Ex1)	X-связанная гипофосфатемия, рахит	200/40/2	26/?/131/?	11/19/26	Sui T, et al., 2016 Ch
LMNA (Ex3)	синдром преждевр. старения	200/50/2	32/?/126/? (3 рец.)	29/30/32	Sui T, et al., 2019
Il2rg	Рецептор IL2	200/20	8/?/66/?	3/8/8	Yan Q, et al., 2014
Tiki1	Один из белков эмб-за и дифф-ки	200/20	5/?/30/?	3/5/5	
FOXN1	Иммунодефицит: развитие и функции лимфоцитов	150/6	21/?/76/? (3 реципиентки, подсаживали смесь эмбрионов)	?/4/20 эмбр	Song J, et al., 2017
RAG2		150/6		?/3/18 эмбр	
IL2RG		150/6		?/10/20 эмбр	
PRKDC		150/6		?/3/18 эмбр	
PAX4 (Ex3-	сахарный диабет	180/40/2	22/?/210/?	15/19/22	Xu Y. et al., 2018

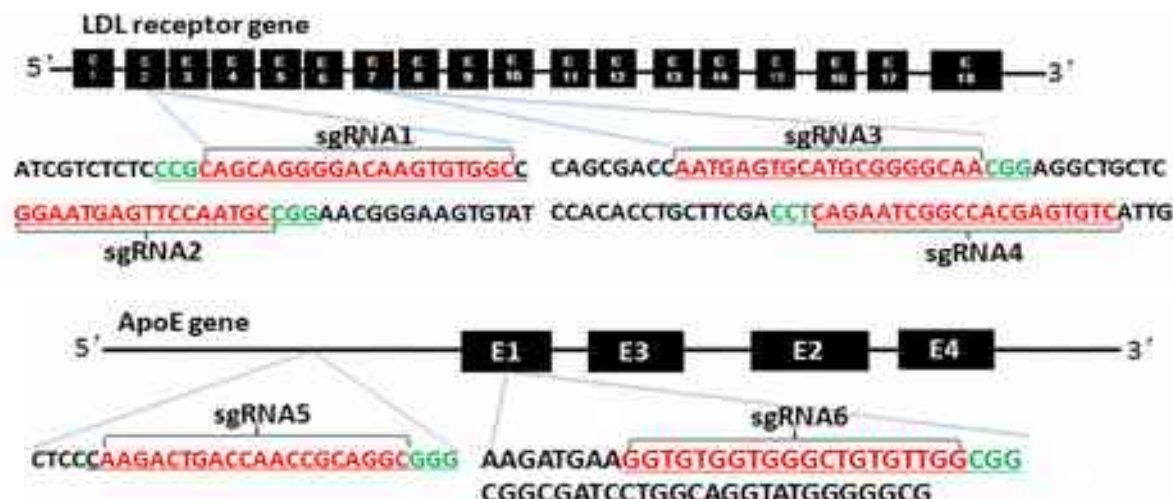
Кролики, нокаутные по нескольким генам одновременно

Гены-мишени	Функция гена, модель	Конц. НК, нг/мкл mRNA Cas9/ sgRNA/ кол-во sgRNA	Живые крольчата/ всего крольчат/ подсажено эмбр./ МИ эмбрионов	ГМ крольчата (пояснение)	Ссылки
<i>Il2rg</i>+<i>RAG1</i>	Иммунодефицит	200/20+20	5/?/67/?	2 <i>IL2RG^b</i> ; 5 <i>RAG1^b</i>	Yan Q, et al., 2014
<i>FOXN1</i>(Ex3)+ <i>PRKDC</i>(Ex1)+ <i>IL2RG</i>(Ex1) + <i>RAG1</i>(Ex1) + <i>RAG2</i>(Ex1)	Иммунодефицит	150/6	5/?/45/? (2 рецип.)	1 (<i>FOXN1^s</i> , <i>PRKDC^s</i> , <i>RAG1^s</i>); 1(<i>IL2RG^s</i> , <i>RAG1^s</i>); 1(<i>IL2RG^s</i>); 1 (<i>PRKDC^s</i> , <i>RAG1^s</i>); 1 (<i>FOXN1^s</i> , <i>PRKDC^s</i> , <i>IL2RG^b</i> , <i>RAG1^b</i>)	Song J, et al., 2017*
<i>RAG1</i>(Ex1) + <i>RAG2</i>(Ex1)		150 / 6	3/15 (1 рец.)	1 (<i>RAG1^b/RAG2^b</i>)/3KO	
<i>FUT1</i>+ <i>FUT2</i> + <i>SEC1</i> G1, G2 – общие	Фукозилтрансферазы	200/50	31/?/181/?	8/31 (<i>FUT1</i> -4, <i>FUT2</i> -4, <i>SEC1</i> -6: 1- по трем генам)	Liu H, et al., 2018
<i>LDLR</i> (Ex2(G1, G2) - Ex7(G3, G4) Δ≈2800 <i>ApoE</i> (5'utr (G5), Ex1 (G6))	Рецептор ЛПНП, аполипопротеин: гиперхолестеремия, атеросклероз	40/13 (G2+G4+G5)	4/10/96/?	1(Δ≈2800, б/а)/3 KO <i>LDLR</i> /4	Yuan T et al., 2019 **
		40/13 (G1+G3+G6)	2/3/109/?	0/2 KO (<i>LDLR</i> -Ex2 б/а; apoE-Ex1 б/а)/2	
		40/13 (G1+G2+G3+G4)	1/5/140/?	1(Δ≈2800, б/а)	



NuSRG – кролик ($FOXN1^s$, $PRKDC^s$, $IL2RG^b$, $RAG1^b$) и его ровесники дикого типа (WT)

* Song J, et al., 2017 *Production of immunodeficient rabbits by multiplex embryo transfer and multiplex gene targeting*. Sci Rep. 2017 Sep 22;7(1):12202. doi: 10.1038/s41598-017-12201-0.

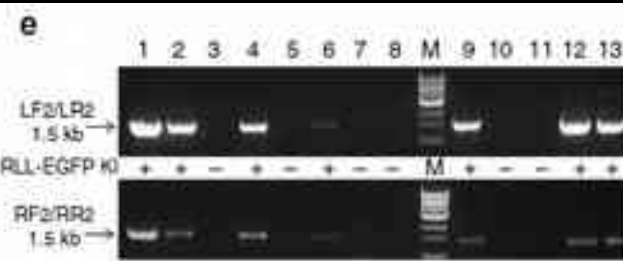
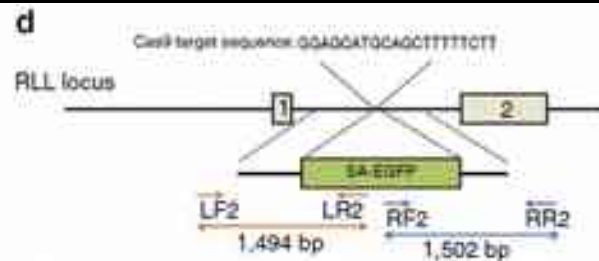


**** Yuan T, et al. Generation of hyperlipidemic rabbit models using multiple sgRNAs targeted CRISPR/Cas9 gene editing system. *Lipids Health Dis.* 2019 Mar 18;18(1):69. doi: 10.1186/s12944-019-1013-8. China Japan**

Microinjected sgRNA	sgRNA 2,4,5	sgRNA 1,3,6	sgRNA 1,2,3,4	Total
No. of embryos collected	113	137	179	429
No. of embryos injected	96	109	140	345
No. of recipients	5	5	6	16
No. of gestations	3	3	4	10
No. of pups born	10	3	5	18
No. of live pups	4	2	1	7
No. of mutant pups	3	2	1	6
No. of pups with large fragment deletion	1	0	1	2
Rate of pregnancy	60.0%(3/5)	60.0%(3/5)	6.7%(4/6)	62.5%(10/16)
Rate of mutations	75.0%(3/4)	100.0%(2/2)	100.0%(1/1)	85.7%(6/7)

Получения Knock-in (KI) кроликов

Ген-мишень	Конц. НК, нг/мкл mRNA Cas9/ sgRNA/ дополн. компонент	ДНК-вставка, нг/мкл	МИ	KI крольчата /крольчата с инделями/ всего крольчат/ транспл.эмбрион ов	Ссылка
<i>ROSA26-like locus (RLL)</i> (<i>Int1</i>)	100/6	0.5-kb 5'HA, 0.7-kb 3'HA: 2-kb EGFP expression cassette/ 100	CP	3/29/43/373	* Song J, et al., 2016
	100/6 / 7,5(RS-1)			10/18/38/146	
<i>ROSA26</i> (<i>Int1</i>)	150/6 / 7,5(RS-1)	0.5-kb 5'HA, 0.7-kb 3'HA: Cre reporter knock in - tdTomato, iEGFP/100	CP	7/?/20/100	Yang D, Song J, et al., 2016
<i>ATP7B</i> (Ex8) Модель гепатоцеребральной дистрофии (болезнь Вильсона)	20 / 10/ 2	ssODNs: 46-b 5'HA, 46-b 3'HA - точечные мутации с заменой 2 AK/ 50mM	МИ CP: через 19 ч	2/7 KO /14/62	Jiang W, et al., 2018
			через 14 ч после ХГч обработки доноров	9/5 KO /17/56	



* Song J, et al. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nat Commun 2016; 7: 10548.

Спасибо за внимание!

