školní rok 2016/2017, kurz Bi6405

# Metody molekulární biologie - cvičení

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Ústav experimentální biologie
Přírodovědecká fakulta MU

# Cíl cvičení

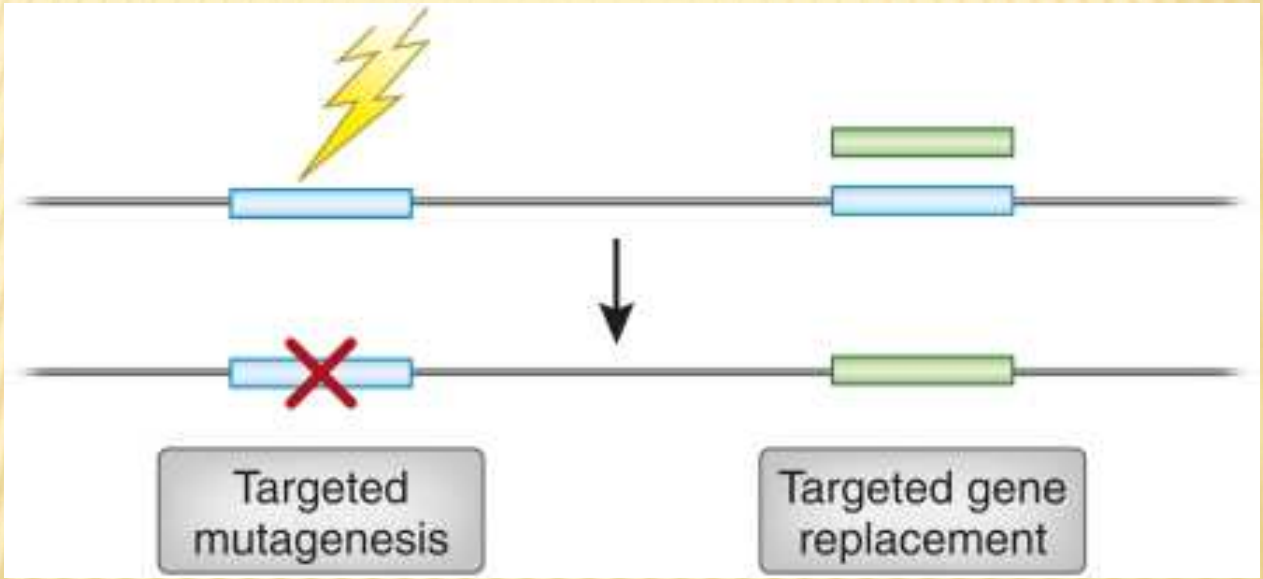
Potlačení/umlčení exprese vybraného genu

a) mutageneze v genomu pomocí metody CRISPR/Cas9

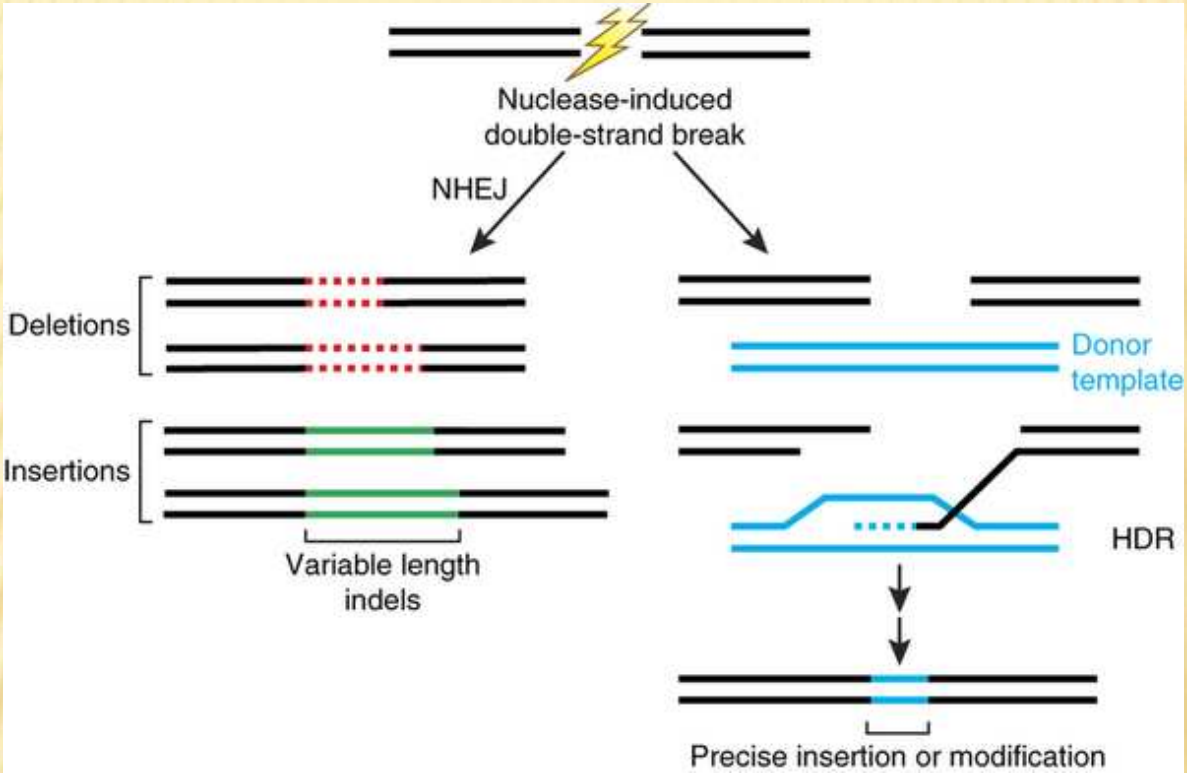
a) post-transkripčím umlčením exprese pomocí shRNA

→ funkce genu

# Cílená mutageneze přímo v genomech



# Cílená mutageneze přímo v genomech




od 80. let - četnost homologické rekombinace je zvýšena indukcí dsDNA zlomů

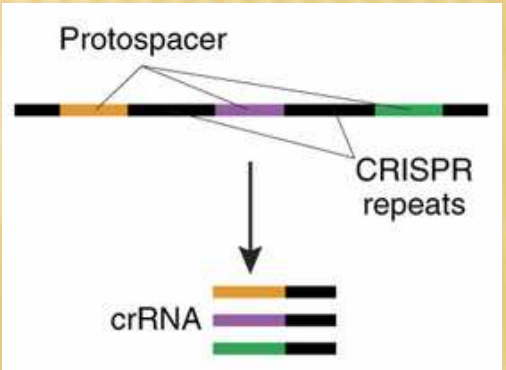
- snaha o vývoj systému pro sekvenčně specifickou tvorbu dsDNA zlomů

# CRISPR/Cas9

- CRISPR-associated protein 9 – nukleáza ze *Streptococcus pyogenes*
- adaptivní imunita bakterií proti virům (obecně cizorodé DNA)
- RGN – RNA-guided nuclease
- sekvenční specifita je dána interakcí DNA-RNA

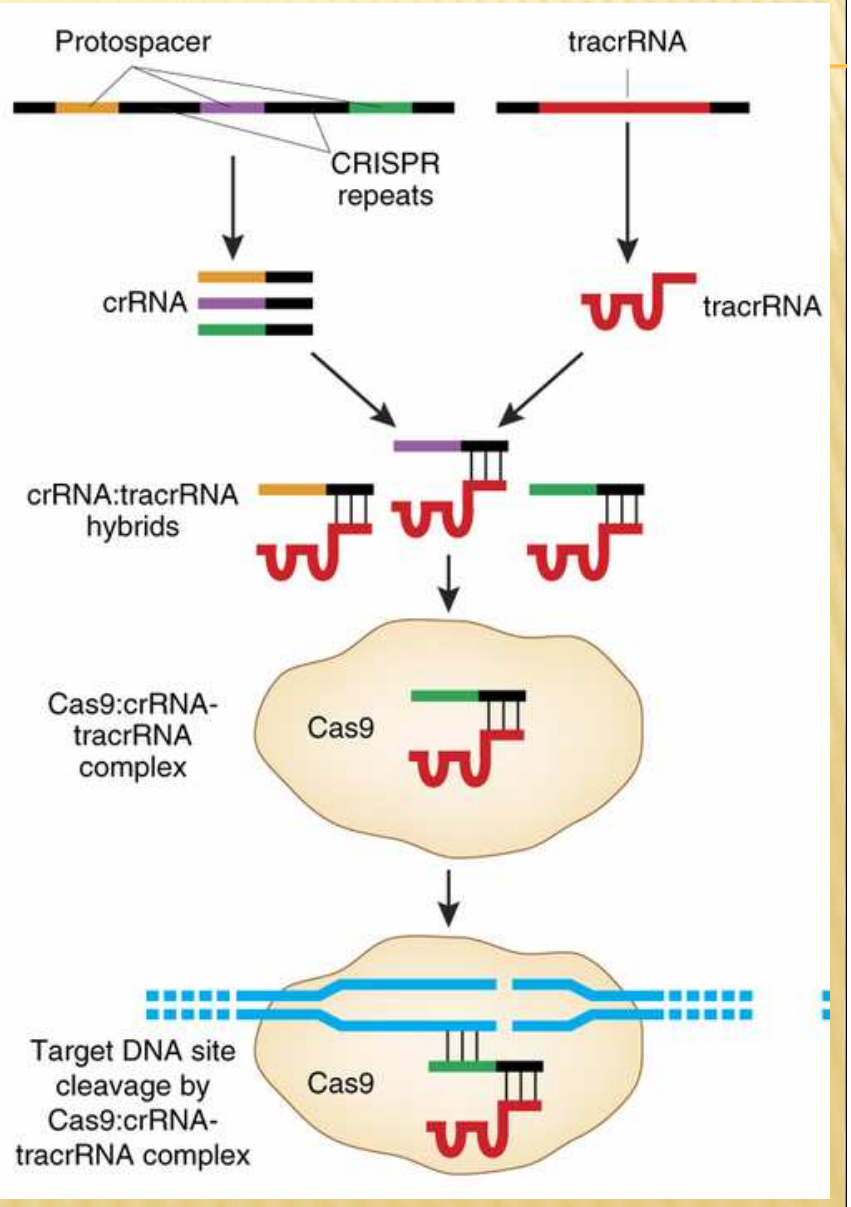
Bakterie – inkorporace cizorodé DNA do CRISPR repetic v genomu
- tyto následně přepsány do RNA (crRNA)

crRNA – photospacer – fragment cizorodé DNA
- CRISPR repetici



# CRISPR/Cas9

- crRNA poté hybridizuje s transaktivující CRISPR RNA (tracrRNA)
- tento komplex RNA interaguje s Cas9 nukleázou
- photospacer RNA navede celý komplex k cizorodé DNA (komplementarita sekvencí)
- výsledný ribonukleoproteinový komplex štěpí cizorodou komplementární DNA



# CRISPR/Cas9

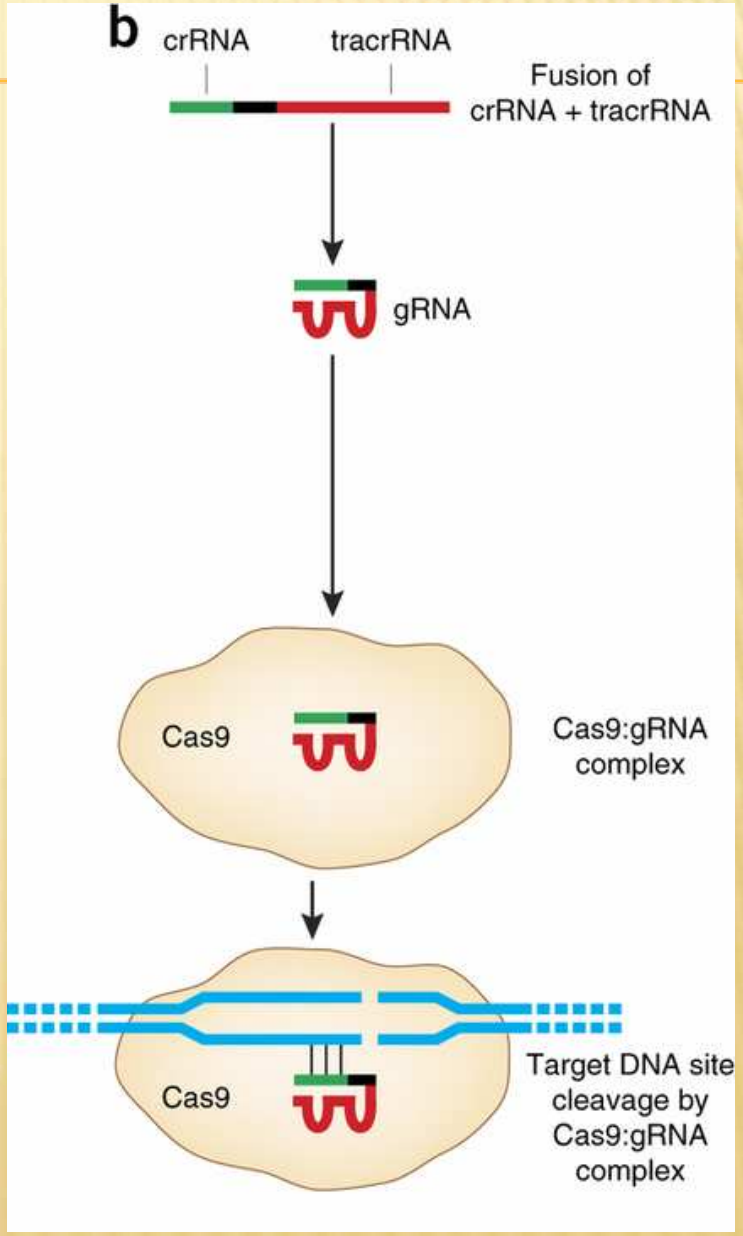
- celý systém modifikován pro cílenou mutagenezi

Vektor:

– guideRNA (gRNA) = crRNA + tracr RNA

- součástí gRNA i 20nt komplementární úsek k cílovému místu v genomové DNA

+ koexprese Cas9 nukleázy

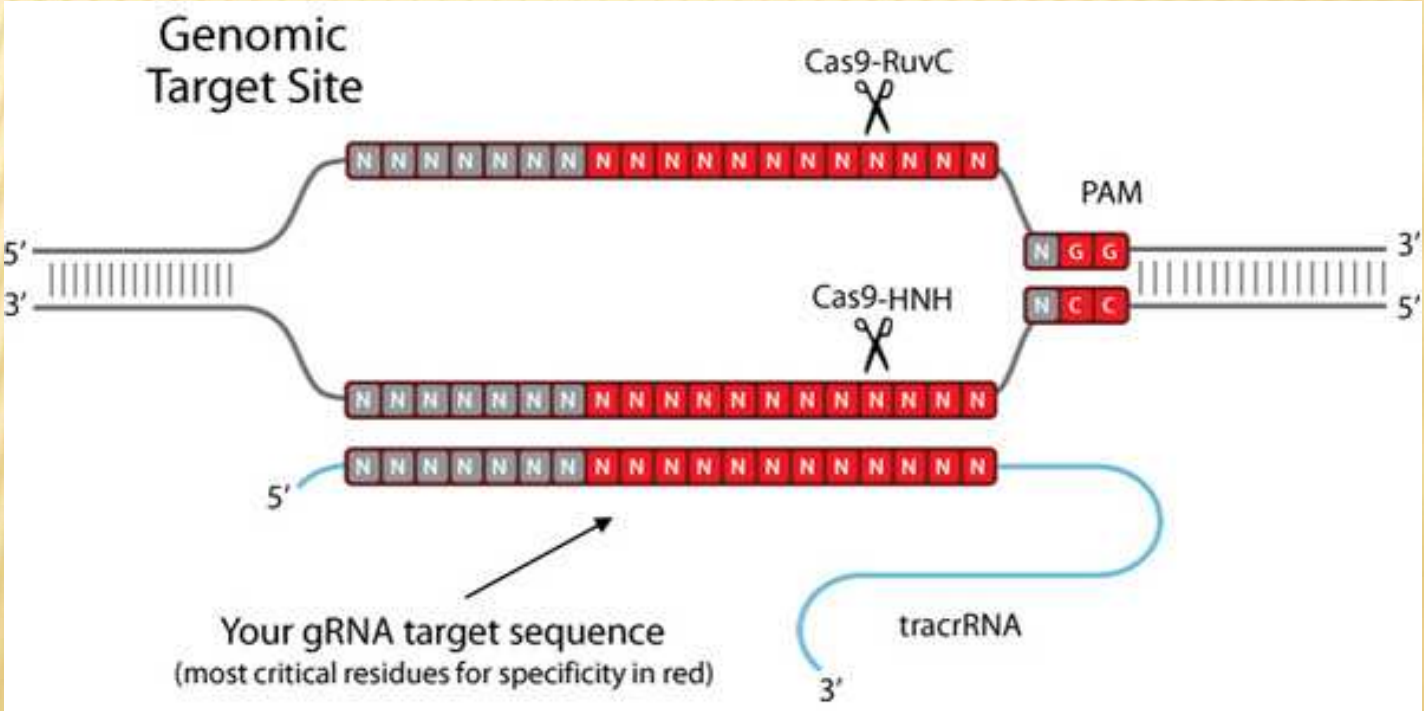


# CRISPR/Cas9

PAM – photospacer adjacent motif

- sekvence v těsném sousedství s gDNA
- nutná pro účinné štěpení Cas9 nukleázou

- původní systém „NGG" (ale vývoj systémů s jinými sekvencemi)
- dle systému cílová sekvence musí být ve formátu $N_{20}GG$



# CRISPR/Cas9 – zvýšení specifity

Pouze 20 nt naváděcí sekvence

→ off-target efekty

Cas9 – 2 nukleázové domény (RuvC, HNH)

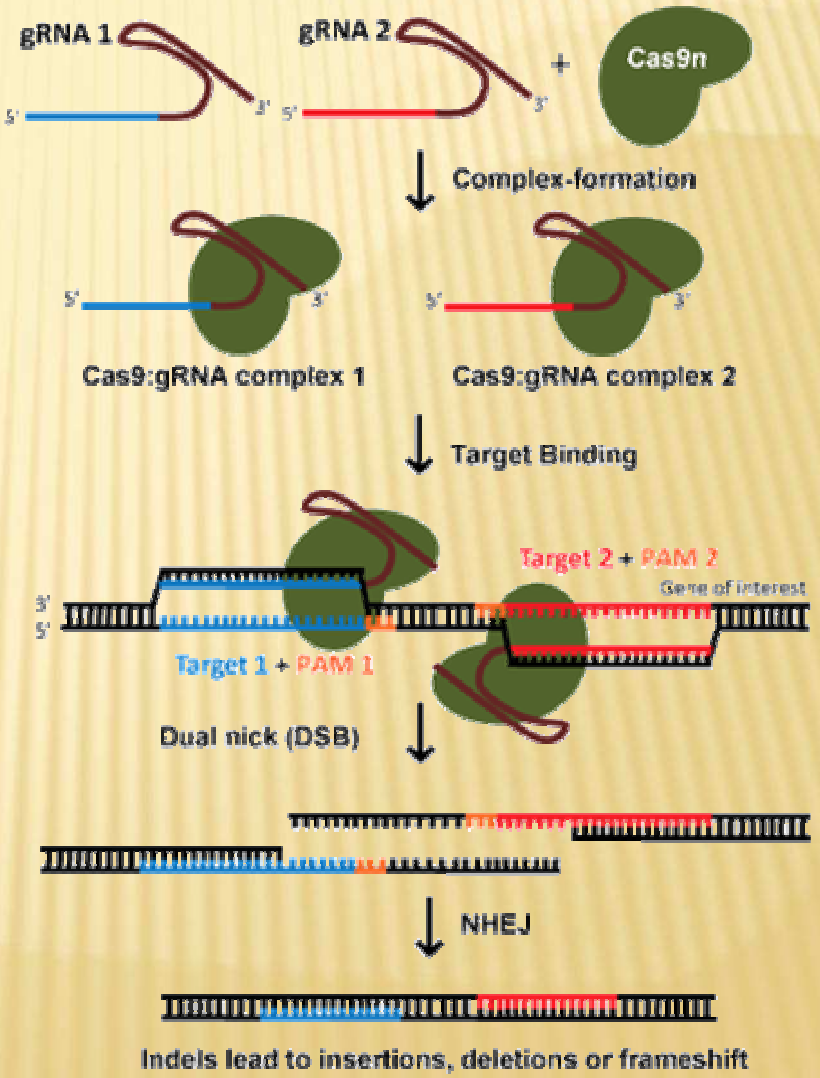
Cas9 nikázy – mutanti v jedné doméně
- indukce cílených SSB

-design – 2x SSB blízko sebe
- a na opačných řetězcích

↓

DSB

vyší specifita



# CRISPR/Cas9

http://genome-engineering.org/gecko/

http://www.addgene.org/crispr/



MIT

- dostupné vektory
- knihovny gDNA pro různé organismy
- validované gRNA
- …

- různé firmy – různé platformy (modifikace původního systému)

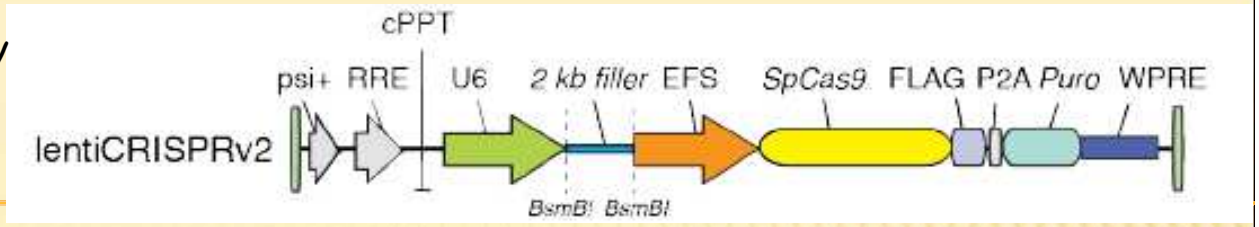
- pro design lze využít dostupný software

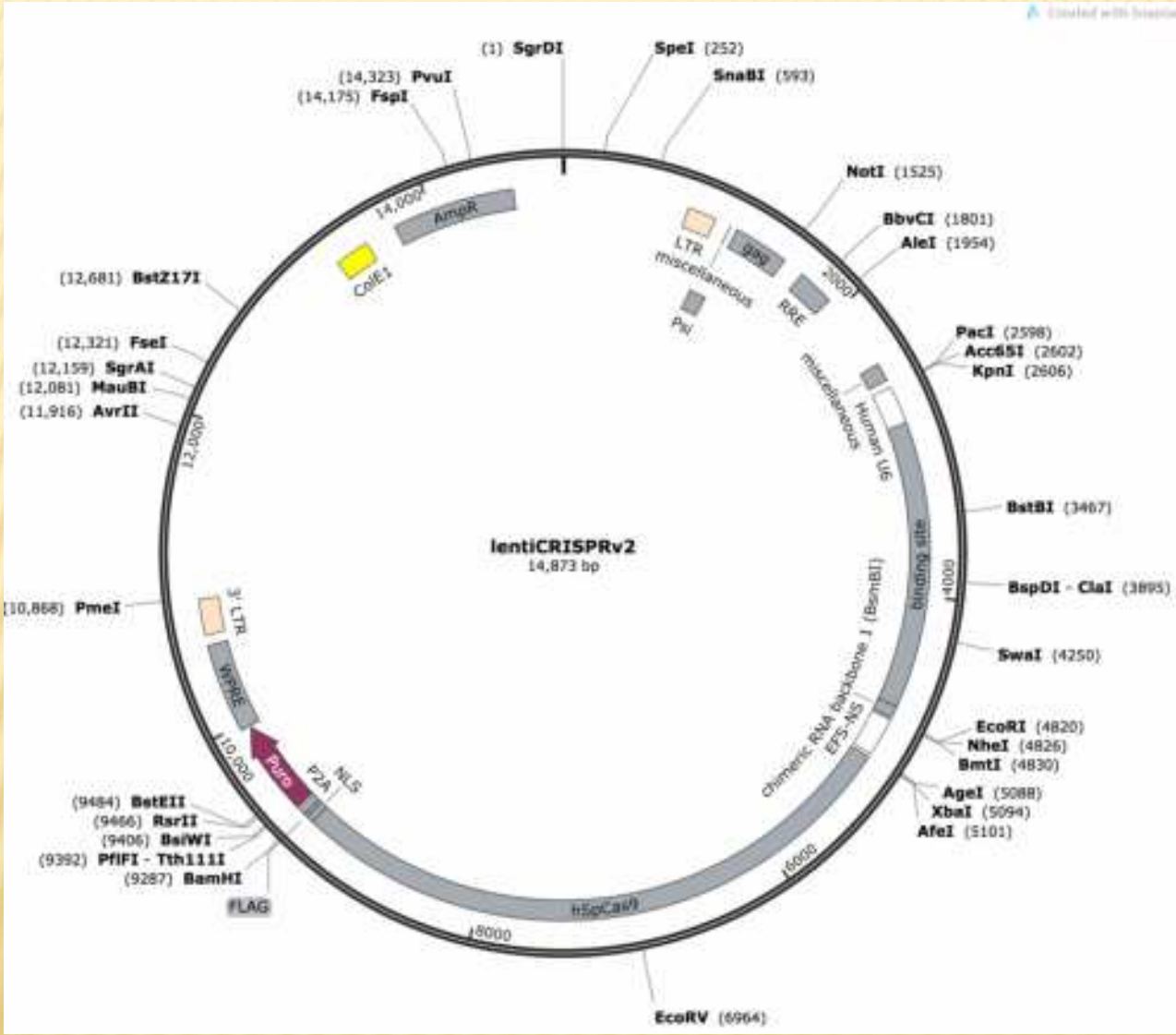
- http://crispr.mit.edu/
- http://www.e-crisp.org/E-CRISP/
- https://omictools.com/crispr-cas9-category

+ všechny větší i menší Lifetech firmy

http://www.addgene.org/52961/

# lentiCRISPRv2

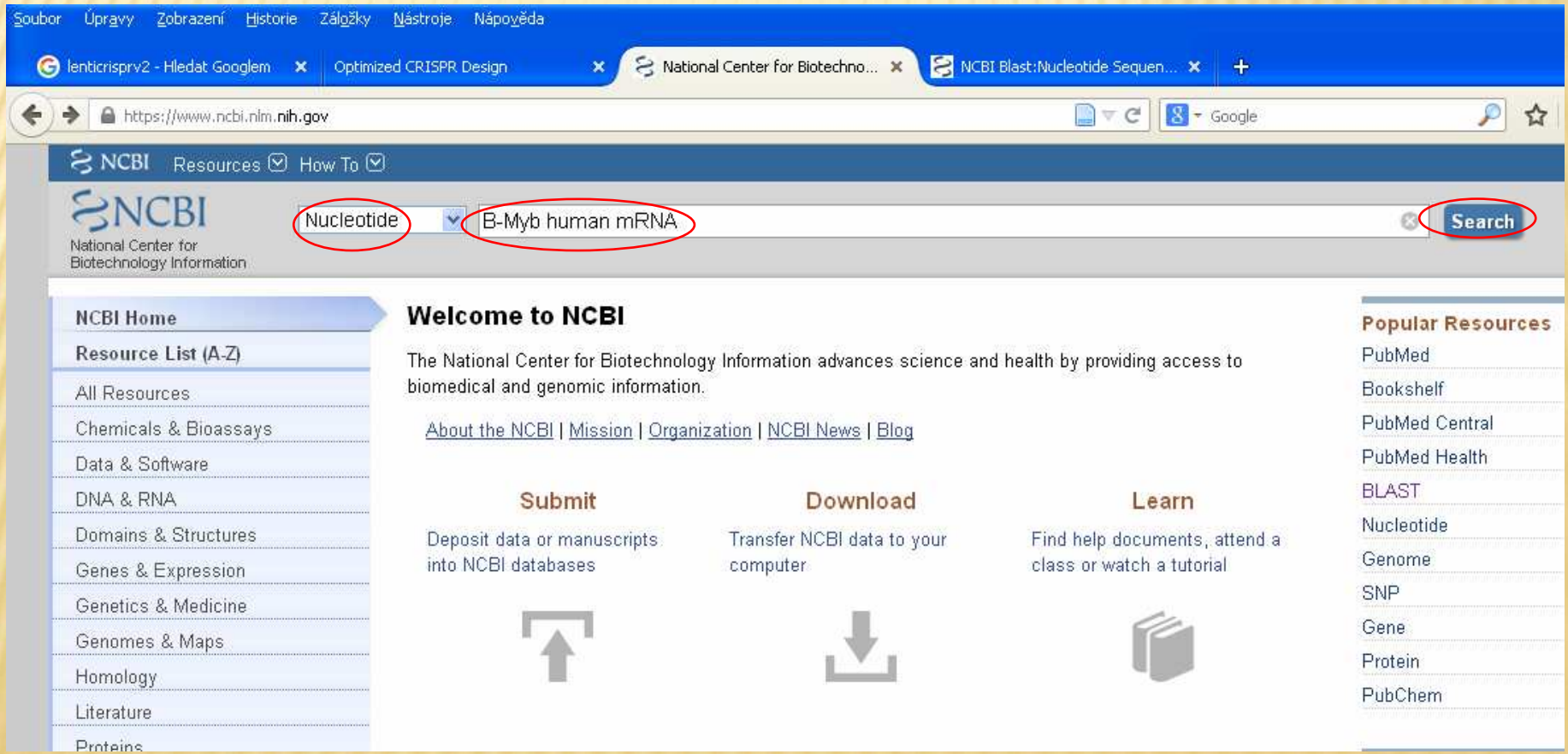


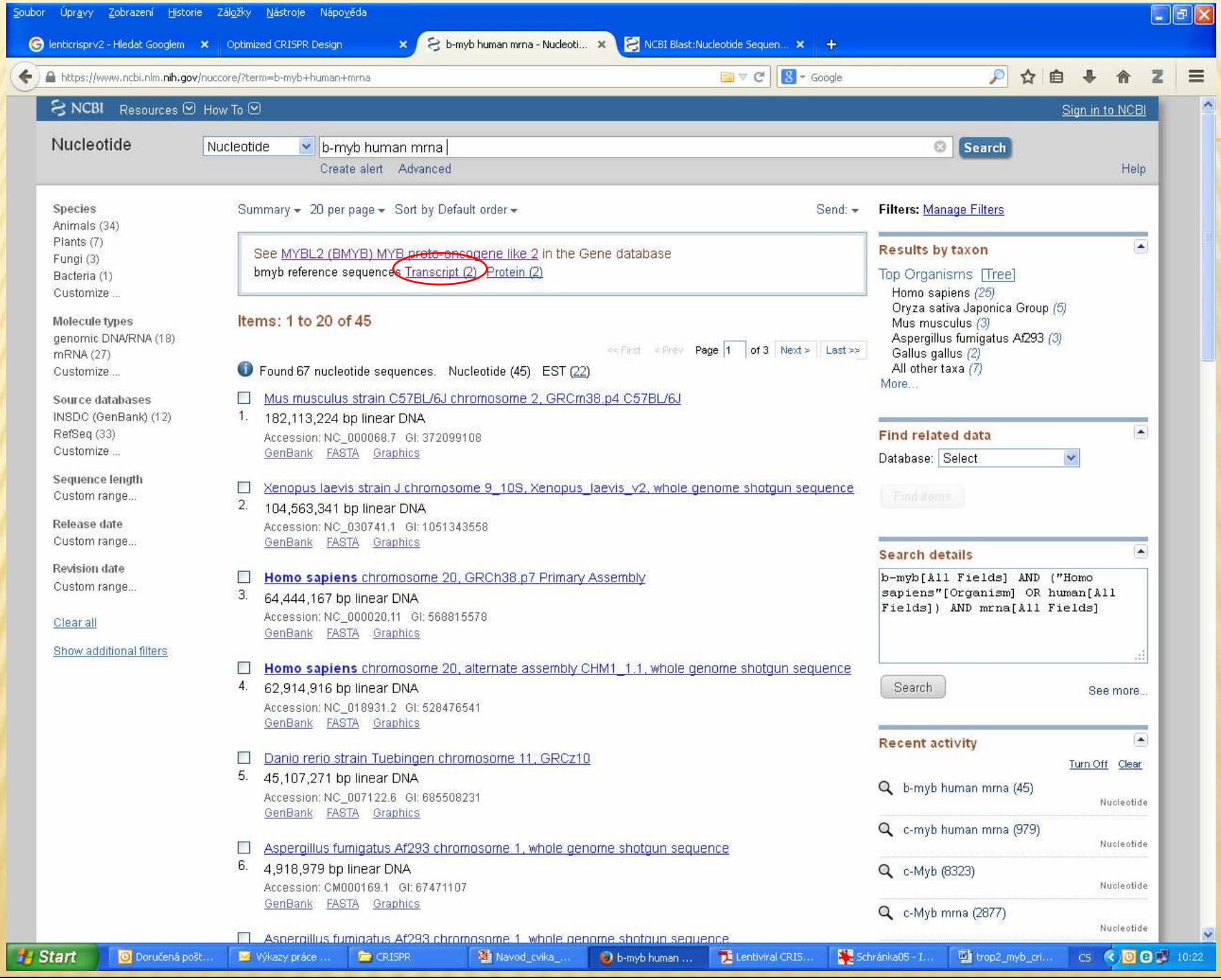



# design CRISPR/Cas9

Sekvence genu včetně znalosti kde začínají a končí exony

https://www.ncbi.nlm.nih.gov/

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Items: 1 to 20 of 45
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GenBank FASTA Graphics
2. Xenopus laevis strain J chromosome 9_10S, Xenopus_laevis_v2, whole genome shotgun sequence
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GenBank FASTA Graphics
5. Danio rerio strain Tuebingen chromosome 11, GRCz10
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6. Aspergillus fumigatus Af293 chromosome 1, whole genome shotgun sequence
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Aspergillus fumigatus Af293 chromosome 1, whole genome shotgun sequence
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Mus musculus (3)
Aspergillus fumigatus Af293 (3)
Gallus gallus (2)
All other taxa (7)
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Find items
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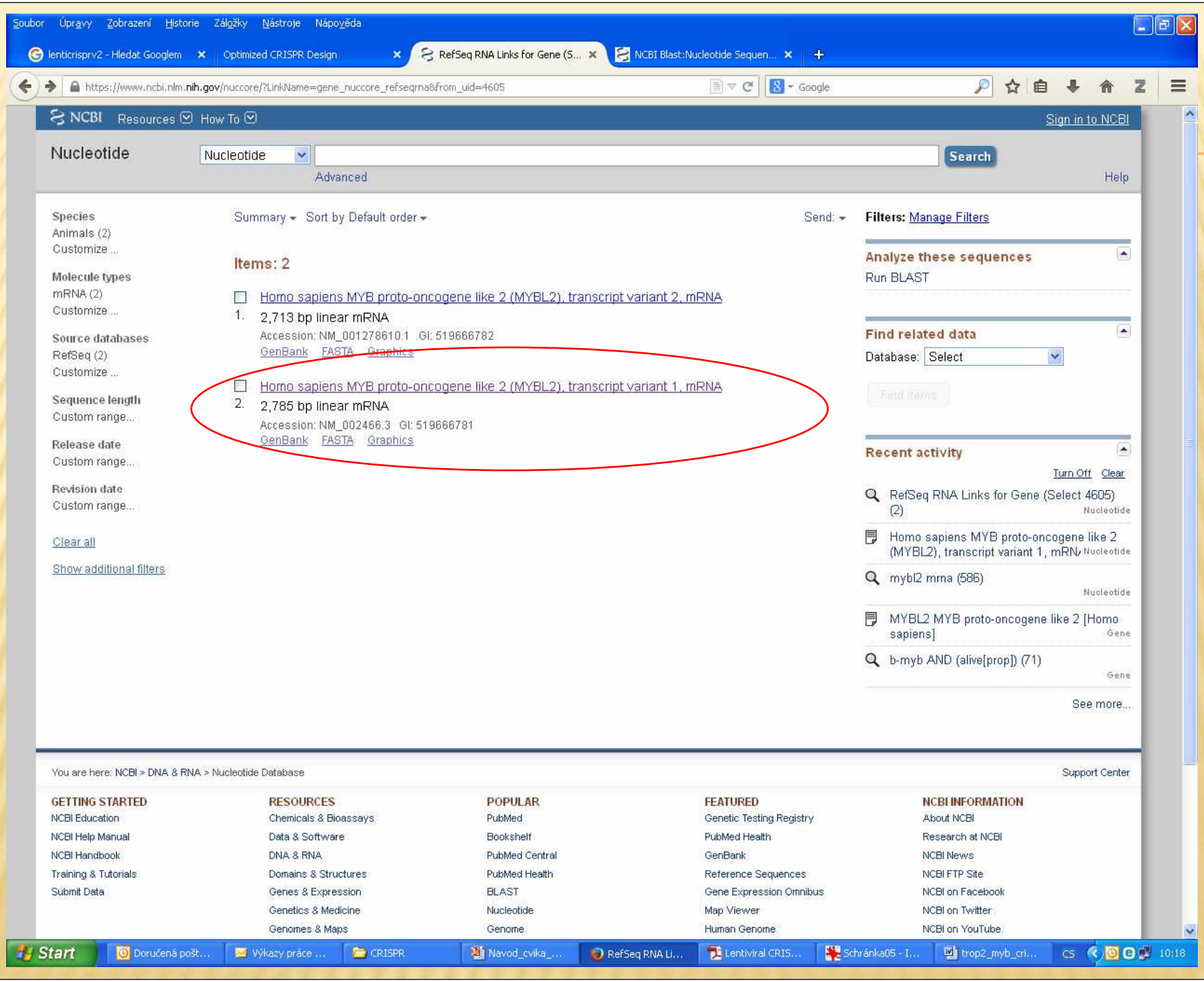
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Support Center
GETTING STARTED
NCBI Education
NCBI Help Manual
NCBI Handbook
Training & Tutorials
Submit Data
RESOURCES
Chemicals & Bioassays
Data & Software
DNA & RNA
Domains & Structures
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REFERENCE 1 (bases 1 to 2785)
AUTHORS Krakstad C, Tangen IL, Hoivik EA, Halle MK, Berg A, Werner HM, Raeder MB, Kusonmano K, Zou JX, Oyan AM, Stefansson I, Trovik J, Kalland KH, Chen HW and Salvesen HB.
TITLE ATAD2 overexpression links to enrichment of B-MYB-translational signatures and development of aggressive endometrial carcinoma
JOURNAL Oncotarget 6 (29), 28440-28452 (2015)
PUBMED 26308378
REMARK GeneRIF: Data indicate that gene expression alterations in endometrial carcinoma samples with high ATAD2 expression showed upregulation of several cancer-related genes including B-MYB gene.
REFERENCE 2 (bases 1 to 2785)
AUTHORS Tao D, Pan Y, Jiang G, Lu H, Zheng S, Lin H and Cao F.
TITLE B-Myb regulates snail expression to promote epithelial-to-mesenchymal transition and invasion of breast cancer cell
JOURNAL Med. Oncol. 32 (1), 412 (2015)
PUBMED 25502082
REMARK GeneRIF: We found that B-Myb upregulated expression of the key epithelial-to-mesenchymal transition regulator snail and that it mediated epithelial-to-mesenchymal transition activation and cell invasion by B-Myb.
REFERENCE 3 (bases 1 to 2785)
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Articles about the MYBL2 gene
A human interactome in three quantitative dimensions organized by stoichiomet [Cell. 2015]
ATAD2 overexpression links to enrichment of B-MYB-translational signature [Oncotarget. 2015]
The BioPlex Network: A Systematic Exploration of the Human Interactome. [Cell. 2015]
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TFAP2A acts as a transcriptional repressor during retinoic acid induced cell differentiation
Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors
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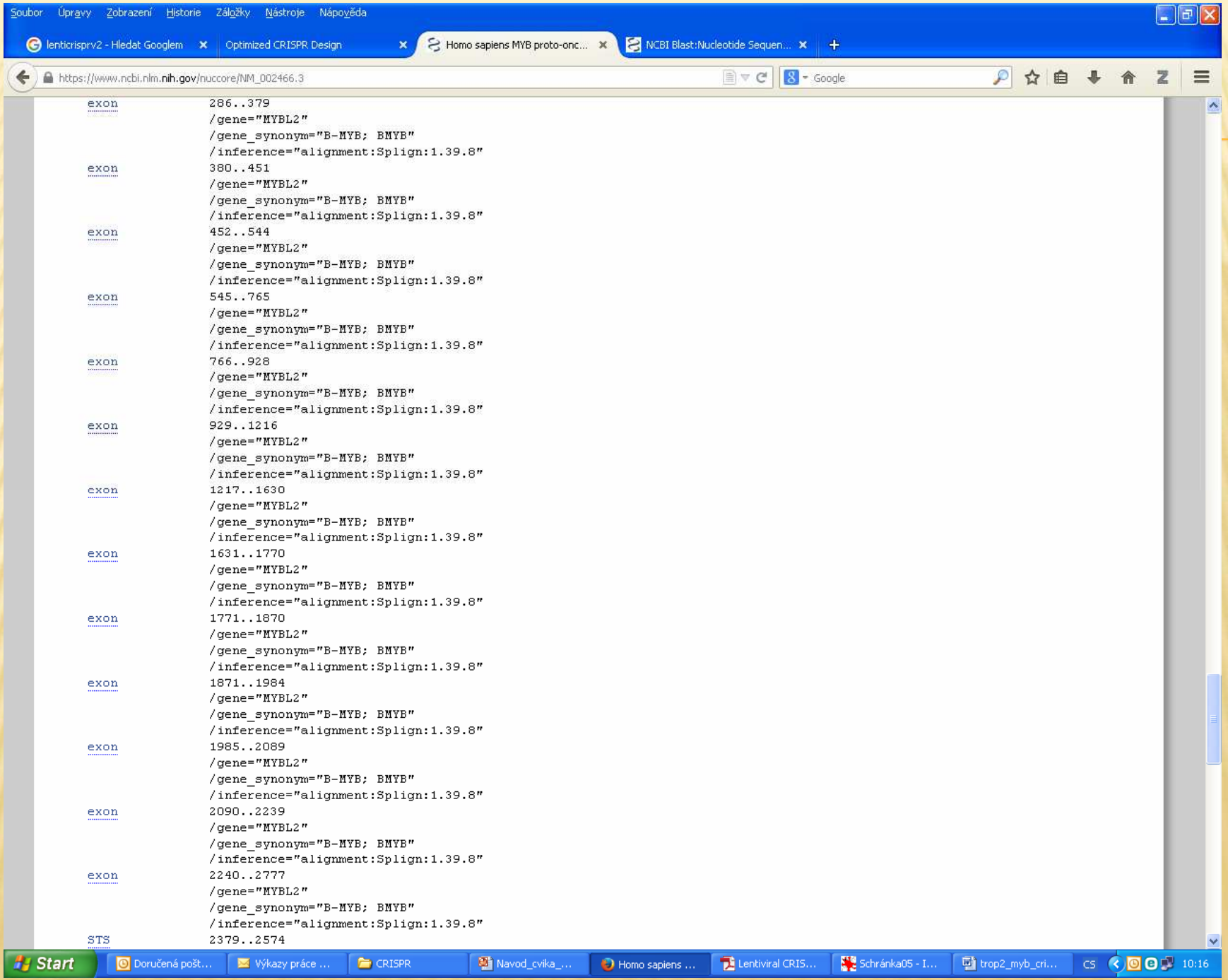
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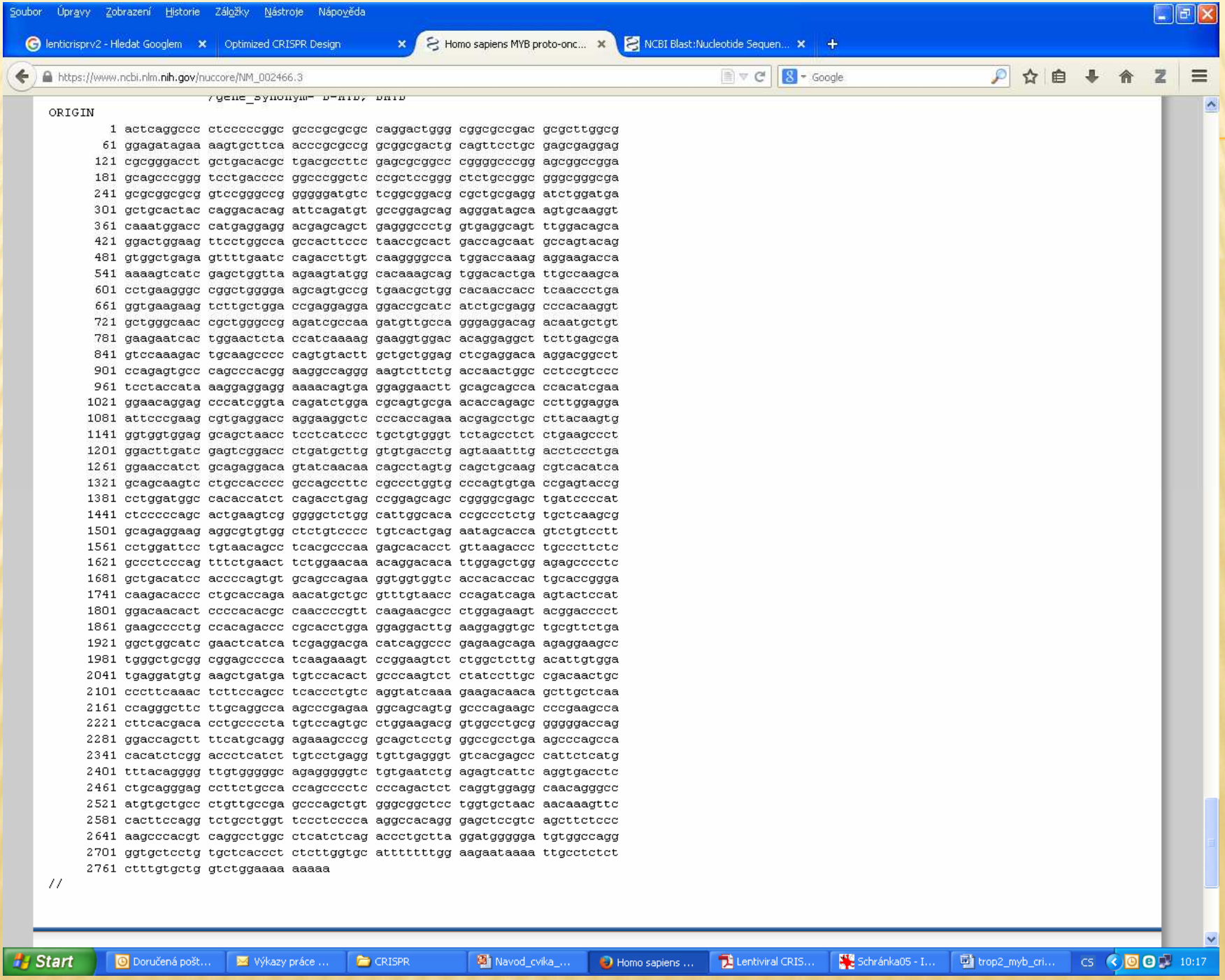
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1021 ggaacaggag cccatcggta cagatctgga cgcagtgcga acaccagagc ccttggagga
1081 attcccgaag cgtgaggacc aggaaggctc cccaccagaa acgagcctgc cttacaagtg
1141 ggtggtggag gcagctaacc tcctcatccc tgctgtgggt tctagcctct ctgaagccct
1201 ggacttgatc gagtcggacc ctgatgcttg gtgtgacctg agtaaatttg acctccctga
1261 ggaaccatct gcagaggaca gtatcaacaa cagcctagtg cagctgcaag cgtcacatca
1321 gcagcaagtc ctgccacccc gccagccttc cgccctggtg cccagtgtga ccgagtaccg
1381 cctggatggc cacaccatct cagacctgag ccggagcagc cggggcgagc tgatccccat
1441 ctcccccagc actgaagtcg ggggctctgg cattggcaca ccgccctctg tgctcaagcg
1501 gcagaggaag aggcgtgtgg ctctgtcccc tgtcactgag aatagcacca gtctgtcctt
1561 cctggattcc tgtaacagcc tcacgcccaa gagcacacct gttaagaccc tgcccttctc
1621 gccctcccag tttctgaact tctggaacaa acaggacaca ttggagctgg agagcccctc
1681 gctgacatcc accccagtgt gcagccagaa ggtggtggtc accacaccac tgcaccggga
1741 caagacaccc ctgcaccaga aacatgctgc gtttgtaacc ccagatcaga agtactccat
1801 ggacaacact ccccacacgc caaccccgtt caagaacgcc ctggagaagt acggacccct
1861 gaagcccctg ccacagaccc cgcacctgga ggaggacttg aaggaggtgc tgcgttctga
1921 ggctggcatc gaactcatca tcgaggacga catcaggccc gagaagcaga agaggaagcc
1981 tgggctgcgg cggagcccca tcaagaaagt ccggaagtct ctggctcttg acattgtgga
2041 tgaggatgtg aagctgatga tgtccacact gcccaagtct ctatccttgc cgacaactgc
2101 cccttcaaac tcttccagcc tcaccctgtc aggtatcaaa gaagacaaca gcttgctcaa
2161 ccagggcttc ttgcaggcca agcccgagaa ggcagcagtg gcccagaagc cccgaagcca
2221 cttcacgaca cctgccccta tgtccagtgc ctggaagacg gtggcctgcg gggggaccag
2281 ggaccagctt ttcatgcagg agaaagcccg gcagctcctg ggccgcctga agcccagcca
2341 cacatctccg accctcatct tgtcctgagg tgttgagggt gtcacgagcc cattctcatg
2401 tttacagggg ttgtgggggc agaggggtc tgtgaatctg agagtcattc aggtgacctc
2461 ctgcagggag cctttctgcca ccagcccctc cccagactct caggtggagg caacagggcc
2521 atgtgctgcc ctgttgccga gcccagctgt gggcggctcc tggtgctaac aacaaagttc
2581 cacttccagg tctgcctggt tccctcccca aggccacagg gagctccgtc agcttctccc
2641 aagcccacgt caggcctggc ctcatctcag accctgctta ggatgggggа tgtggccagg
2701 ggtgctcctg tgctcaccct ctcttggtgc attttttttgg aagaataaaa ttgcctctct
2761 ctttgtgctg gtctggaaaa aaaaa
//
Start
Doručená pošt...
Výkazy práce ...
CRISPR
Navod_cvika_...
Homo sapiens ...
Lentiviral CRIS...
Schránka05 - I...
trop2_myb_cri...
CS
10:17

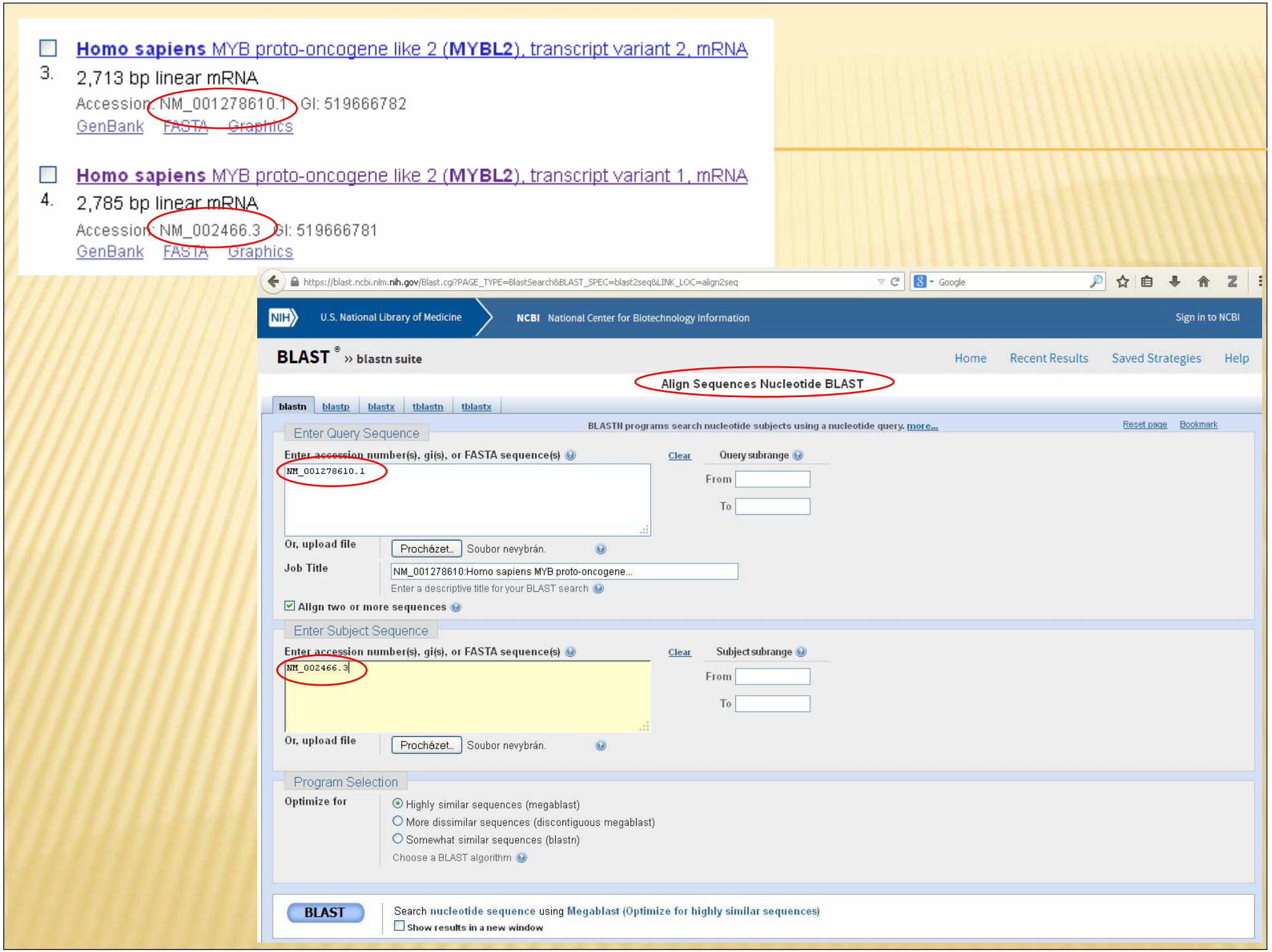
**human B-Myb**

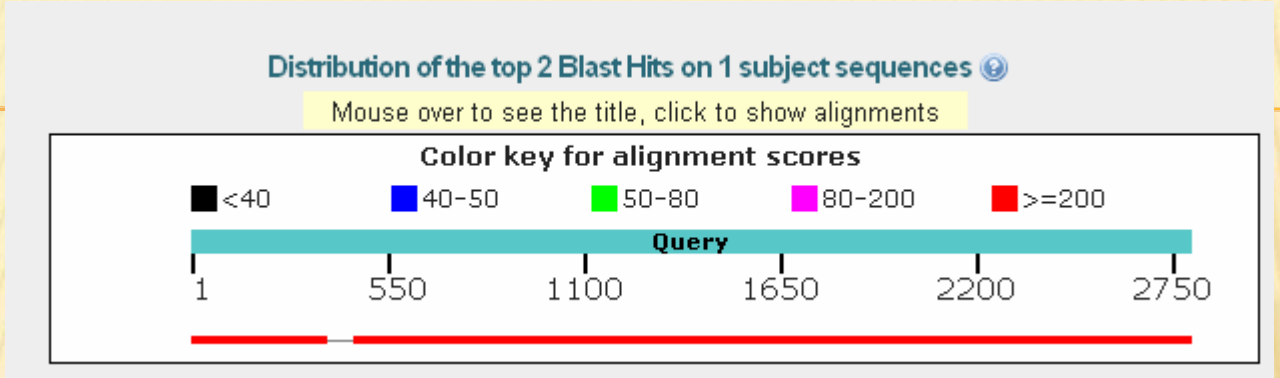
266

atgtctcggcggacgcgctgcgaggatctggatgagctgcactaccaggacacagattcagatgtgccggagcag
agggatagcaagtgcaaggtcaaatggacccatgaggaggacgagcagctgagggccctggtgaggcagtttgga
cagcaggactggaagttcctggccagccacttccctaaccgcactgaccagcaatgccagtacaggtggctgaga
gttttgaatccagaccttgtcaaggggccatggaccaaagaggaagaccaaaaagtcatcgagctggttaagaag

Kódující sekvence genu s vyznačenými exony

- max 250 nt dlouhá
- http://crispr.mit.edu/

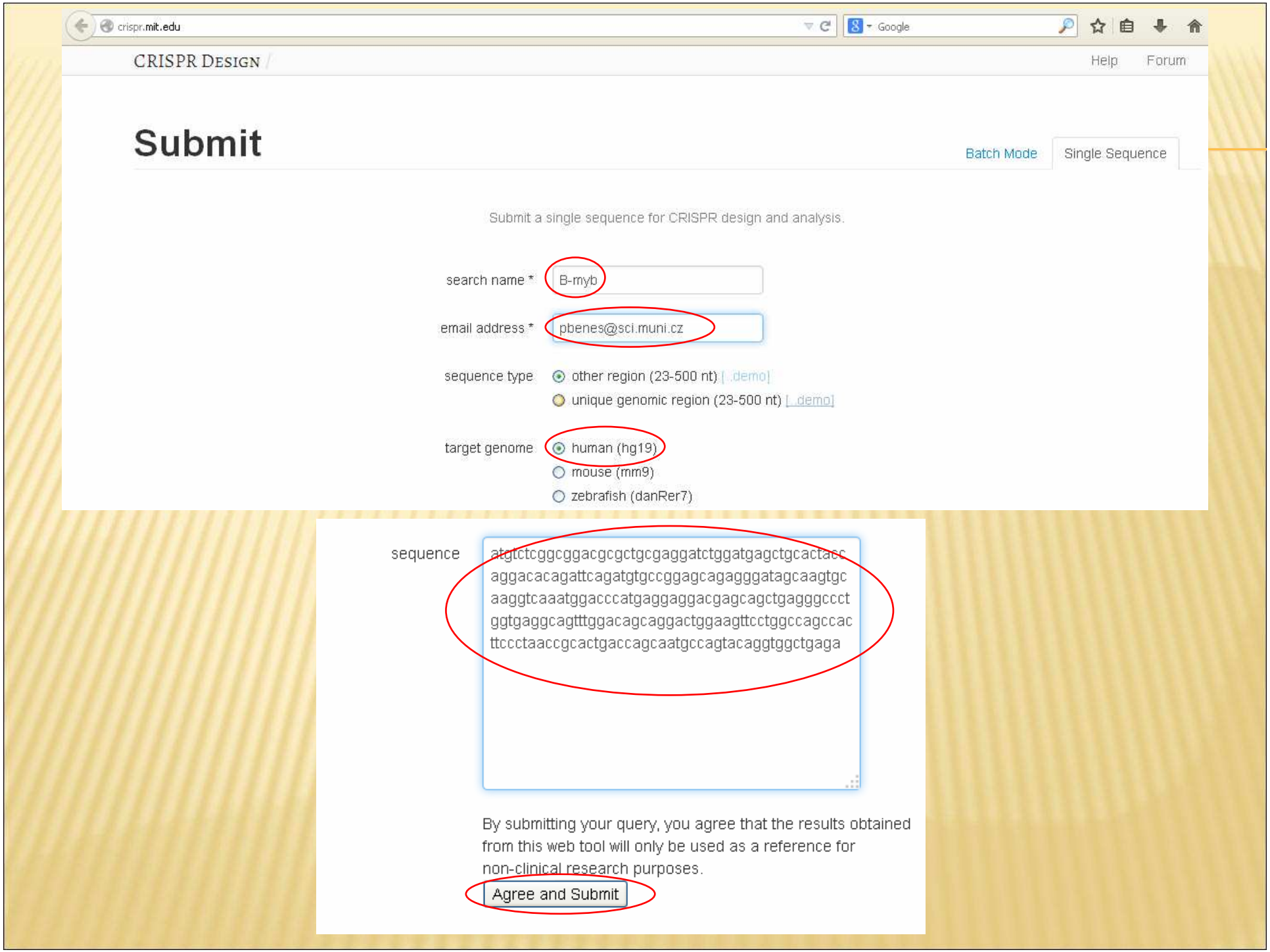

Homo sapiens MYB proto-oncogene like 2 (MYBL2), transcript variant 2, mRNA
3.
2,713 bp linear mRNA
Accession: NM_001278610.1 GI: 519666782
GenBank FASTA Graphics
Homo sapiens MYB proto-oncogene like 2 (MYBL2), transcript variant 1, mRNA
4.
2,785 bp linear mRNA
Accession: NM_002466.3 GI: 519666781
GenBank FASTA Graphics
https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq
U.S. National Library of Medicine
NCBI National Center for Biotechnology Information
Sign in to NCBI
BLAST » blastn suite
Home
Recent Results
Saved Strategies
Help
Align Sequences Nucleotide BLAST
blastn
blastp
blastx
tblastn
tblastx
BLASTN programs search nucleotide subjects using a nucleotide query. more...
Reset page
Bookmark
Enter Query Sequence
Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Query subrange
From
To
NM_001278610.1
Or, upload file
Procházet...
Soubor nevybrán.
Job Title
NM_001278610:Homo sapiens MYB proto-oncogene...
Enter a descriptive title for your BLAST search
Align two or more sequences
Enter Subject Sequence
Enter accession number(s), gi(s), or FASTA sequence(s)
Clear
Subject subrange
From
To
NM_002466.3
Or, upload file
Procházet...
Soubor nevybrán.
Program Selection
Optimize for
Highly similar sequences (megablast)
More dissimilar sequences (discontiguous megablast)
Somewhat similar sequences (blastn)
Choose a BLAST algorithm
BLAST
Search nucleotide sequence using Megablast (Optimize for highly similar sequences)
Show results in a new window




266

atgtctcggcggacgcgctgcgaggatctggatgagctgcactaccaggacacagattcagatgtgccggagcag
agggatagcaagtgcaaggtcaaatggacccatgaggaggacgagcagctgagggccctggtgaggcagtttgga
cagcaggactggaagttcctggccagccacttccctaaccgcactgaccagcaatgccagtacaggtggctgaga
gttttgaatccagaccttgtcaaggggccatggaccaaagaggaagaccaaaaa

- u transkriptu 2 chybí 3. exon (zelený)


crispr.mit.edu
Google
CRISPR Design
Help
Forum
Submit
Batch Mode
Single Sequence
Submit a single sequence for CRISPR design and analysis.
search name *
B-myb
email address *
pbenes@sci.muni.cz
sequence type
other region (23-500 nt) [ demo]
unique genomic region (23-500 nt) [ demo]
target genome
human (hg19)
mouse (mm9)
zebrafish (danRer7)
sequence
atgtctcggcggacgcgctgcgaggatctggatgagctgcactacc
aggacacagattcagatgtgccggagcagagggatagcaagtgc
aaggtcaaatggacccatgaggaggacgagcagctgagggccct
ggtgaggcagtttggacagcaggactggaagttcctggccagccac
ttccctaaccgcactgaccagcaatgccagtacaggtggctgaga
By submitting your query, you agree that the results obtained from this web tool will only be used as a reference for non-clinical research purposes.
Agree and Submit

Soubor Úpravy Zobrazení Historie Záložky Nástroje Nápověda

lenticrisprv2 - Hledat Googlem | Optimized CRISPR Design | Homo sapiens MYB proto-on... | NCBI Blast:Nucleotide Sequ... | Vyhledávání osob-Kontakty-... | Tereza Dohnalová Danhofer...

crispr.mit.edu/job/716734579046491

CRISPR Design / Job "B-myb" Help Forum

# CRISPR Job Submission "B-myb"

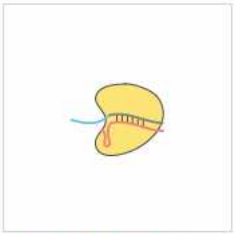
**Status:** Job is complete.

Downloads Job Info Results

## Results

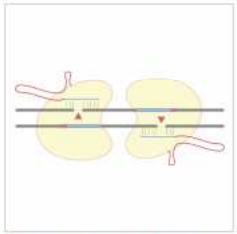
View online results for B-myb.

**Guides & offtargets**



Download as genbank

**Nickase analysis**

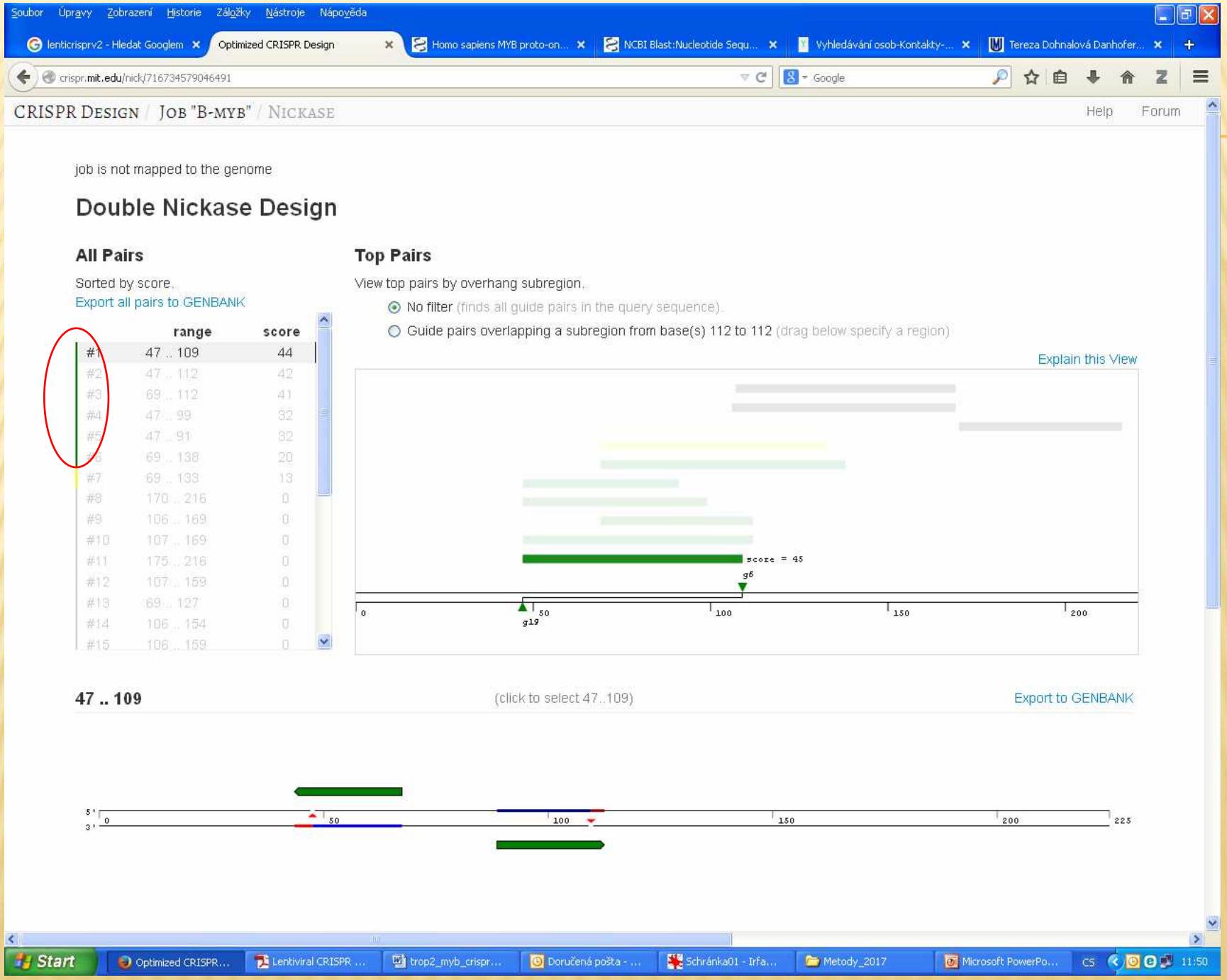


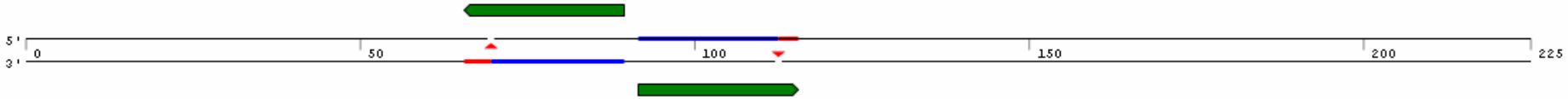
Download as genbank

The "zipper-like" annealing mechanics may explain why mismatches between the target sequence in the 3′ seed sequence completely abolish target cleavage, whereas mismatches toward the 5′ end are permissive for target cleavage.

Zhang Lab, MIT 2015

Start | Optimized CRISPR De... | Lentiviral CRISPR tool... | trop2_myb_crispr_no... | Doručená pošta - Mic... | CS | 11:45


Soubor Úpravy Zobrazení Historie Záložky Nástroje Nápověda
lenticrisprv2 - Hledat Googlem
Optimized CRISPR Design
Homo sapiens MYB proto-on...
NCBI Blast:Nucleotide Sequ...
Vyhledávání osob-Kontakty-...
Tereza Dohnalová Danhofer...
crispr.mit.edu/nick/716734579046491
CRISPR Design / Job "B-myb" / Nickase
Help Forum
job is not mapped to the genome
Double Nickase Design
All Pairs
Sorted by score.
Export all pairs to GENBANK
range score
#1 47 .. 109 44
#2 47 .. 112 42
#3 69 .. 112 41
#4 47 .. 99 32
#5 47 .. 91 32
#6 69 .. 138 20
#7 69 .. 133 13
#8 170 .. 216 0
#9 106 .. 169 0
#10 107 .. 169 0
#11 175 .. 216 0
#12 107 .. 159 0
#13 69 .. 127 0
#14 106 .. 154 0
#15 106 .. 159 0
Top Pairs
View top pairs by overhang subregion.
No filter (finds all guide pairs in the query sequence)
Guide pairs overlapping a subregion from base(s) 112 to 112 (drag below specify a region)
Explain this View
score = 45
g6
g19
0
50
100
150
200
47 .. 109
(click to select 47..109)
Export to GENBANK
5'
3'
0
50
100
150
200
225
Start
Optimized CRISPR...
Lentiviral CRISPR ...
trop2_myb_crispr...
Doručená pošta - ...
Schránka01 - Irfa...
Metody_2017
Microsoft PowerPo...
CS
11:50




**Overhang region**

ATGTCTCGGCGGACGCGCTGCGAGGATCTGGATGAGCTGCACTACCAGGACACA[illegible]AGATGTGCCGGAGCAGAGGGATAGCAAGTGCAAGGTCAAATGGACCCATGAGGAGGACGAGCAC[illegible]GGCCCTG

**Guide A** 59

**quality**
high
**cuts after position**
69 in query
**sequence**
CACTTGCTATCCCTCTGCTCCGG
**# offtargets**
237
**# genic offtargets**
33
Export off-targets to .csv

**Guide B** 71

**quality**
high
**cuts after position**
112 in query
**sequence**
GGTCAAATGGACCCATGAGGAGG
**# offtargets**
151
**# genic offtargets**
16
Export off-targets to .csv

**Pair score, A & B** 42

**quality**
high
**# offtarget pairs of A & B**
0
**# genic OT pairs of A & B**
0

- obě sekvence lze vklonovat do jediného vektoru s mutantní formou Cas9 (nikázou)

Soubor Úpravy Zobrazení Historie Záložky Nástroje Nápověda

lenticrisprv2 - Hledat Googlem | Optimized CRISPR Design | Homo sapiens MYB proto-on... | NCBI Blast:Nucleotide Sequ... | Vyhledávání osob-Kontakty-... | Tereza Dohnalová Danhofer...

crispr.mit.edu/job/716734579046491

CRISPR DESIGN / JOB "B-MYB" Help Forum

# CRISPR Job Submission "B-myb"

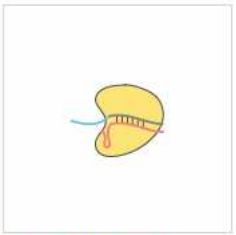
**Status:** Job is complete.

Downloads Job Info Results

## Results

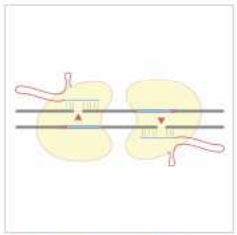
View online results for B-myb.

### Guides & offtargets



Download as genbank

### Nickase analysis



Download as genbank

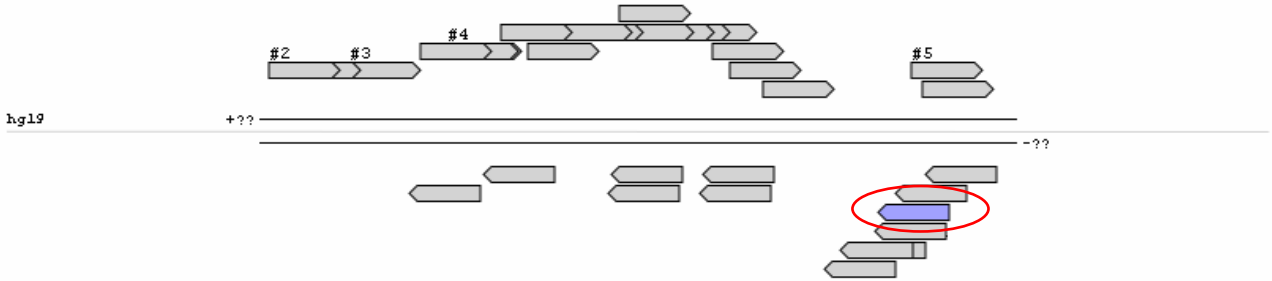
Zhang Lab, MIT 2015

Start | Optimized CRISPR De... | Lentiviral CRISPR tool... | trop2_myb_crispr_no... | Doručená pošta - Mic... | CS | 11:45

# "B-myb"

Spacers

Interactive results: mouse over a guide or explore below for details




## all guides

scored by inverse likelihood of offtarget binding

mouse over for details ... show legend

| | score | sequence | |
|---|---|---|---|
| Guide #1 | 86 | CATTGCTGGTCAGTGCGGTT | AGG |
| Guide #2 | 85 | GTCTCGGCGGACGCGCTGCG | AGG |
| Guide #3 | 82 | TCTGGATGAGCTGCACTACC | AGG |
| Guide #4 | 80 | ATTCAGATGTGCCGGAGCAG | AGG |
| Guide #5 | 80 | CTGACCAGCAATGCCAGTAC | AGG |
| Guide #6 | 75 | CAAGGTCAAATGGACCCATG | AGG |
| Guide #7 | 73 | GCCACCTGTACTGGCATTGC | TGG |
| Guide #8 | 71 | GGTCAAATGGACCCATGAGG | AGG |
| Guide #9 | 70 | ACCAGCAATGCCAGTACAGG | TGG |
| Guide #10 | 70 | GCGGACGCGCTGCGAGGATC | TGG |
| Guide #11 | 70 | GGTCAGTGCGGTTAGGGAAG | TGG |
| Guide #12 | 70 | TTCAGATGTGCCGGAGCAGA | GGG |
| Guide #13 | 67 | GCTGTCCAAACTGCCTCACC | AGG |
| Guide #14 | 65 | ATTGCTGGTCAGTGCGGTTA | GGG |
| Guide #15 | 62 | GGTGAGGCAGTTTGGACAGC | AGG |
| Guide #16 | 61 | GGACACAGATTCAGATGTGC | CGG |

## guide #1 quality score: 86

**guide sequence:** CATTGCTGGTCAGTGCGGTT AGG

**on-target locus:** unknown

**number of offtarget sites:** 100 (11 are in genes)

top 20 genome-wide off-target sites ☐ show all exonic

| sequence | score | mismatches | UCSC gene | locus |
|---|---|---|---|---|
| GATTTCTGTACAGTGCGGTTCAG | 0.8 | 4MMs [1:5:9:10] | | chr3:+128839272 |
| TACTGCTAGTCGGTGCGGTTCAG | 0.7 | 4MMs [1:3:8:12] | | chr3:+193871562 |
| TAGTGCTGGGCAGTGCTGTTAGG | 0.6 | 4MMs [1:3:10:17] | | chrX:+64899769 |
| CATGGTTTCTCAGTGCGGTTGAG | 0.5 | 4MMs [4:6:8:9] | | chr9:+126101702 |
| CTTTGCTCGTCAGGGCGGTTGGG | 0.4 | 3MMs [2:8:14] | NR_027018 | chr11:-3829338 |
| CATTGCTGGCCAGTGCTGTAAGG | 0.4 | 3MMs [10:17:20] | NM_201551 | chr11:+20949992 |
| GAGTGCTGGTGTGTGCGGTTCAG | 0.4 | 4MMs [1:3:11:12] | | chr12:+110541575 |
| CGGTGCTGCTCAGTGCAGTTCAG | 0.4 | 4MMs [2:3:9:17] | NM_013402 | chr11:-61579938 |
| CCTTGCTGGTGAGTGCGCTTAGG | 0.4 | 3MMs [2:11:18] | | chr11:+68869605 |
| CACTACTGATCAGTGCTGTTAAG | 0.4 | 4MMs [3:5:9:17] | | chr1:+76254707 |
| CTCTGCTTGTCAGTGCGATTGGG | 0.3 | 4MMs [2:3:8:18] | | chr12:+123202933 |
| CTCTGCTTGTCAGTGCGATTGGG | 0.3 | 4MMs [2:3:8:18] | | chr12:+123189486 |
| CATTTGTGGACAATGCGGTTAAG | 0.3 | 4MMs [5:6:10:13] | | chrY:+17926685 |
| GATGGCTGGCCAGTGCGTTTGGG | 0.3 | 4MMs [1:4:10:18] | | chr20:-62085902 |

266

atgtctcggcggacgcgctgcgaggatctggatgagctgcactaccaggacacagattcagatgtgccggagcag
agggatagcaagtgcaaggtcaaatggacccatgaggaggacgagcagctgagggccctggtgaggcagtttgga
cagcaggactggaagttcctggccagccacttccctaaccgcactgaccagcaatgccagtacaggtggctgaga
gttttgaatccagaccttgtcaaggggccatggaccaaagaggaagaccaaaaagtcatcgagctggttaagaag

CATTGCTGGTCAGTGCGGTT ☹
GTCTCGGCGGACGCGCTGCG ☹
TCTGGATGAGCTGCACTACC ok

CACCG TCTGGATGAGCTGCACTACC
AAAC GGTAGTGCAGCTCATCCAGA C

## Target Guide Sequence Cloning Protocol

In order to clone the target sequence into the lentiCRISPRv2 or lentiGuide-Puro backbone, synthesize two oligos of the following form. **All plasmids have the same overhangs** after *BsmBI* digestion and the same oligos can be used for cloning into lentiCRISPRv2, lentiGuide-Puro or lentiCRISPRv1.

Target Sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 NGG PAM

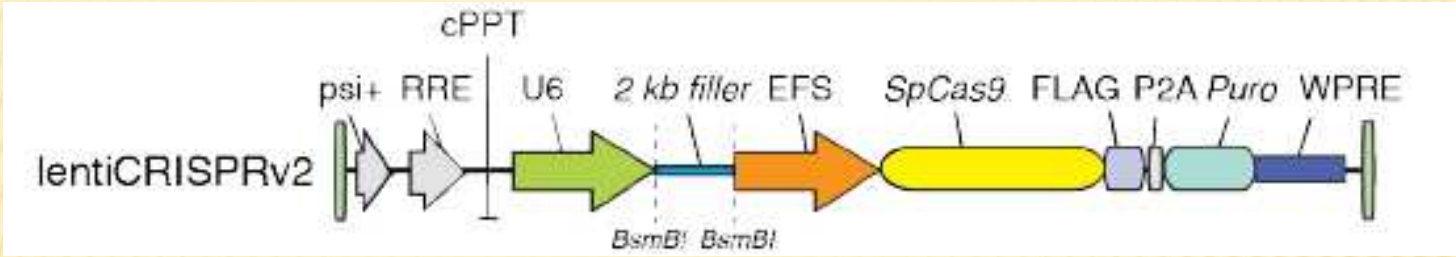
Oligo 1 → 5′ – CACCGNNNNNNNNNNNNNNNNNNN – 3′
3′ – CNNNNNNNNNNNNNNNNNNNCAAA – 5′ ← Oligo 2

*Example oligo design*: Note that the NGG PAM is **not** included in the designed oligos.

Target Sequence: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 NGG PAM

Genomic Sequence
5′ –...GACCA**CAGTCTGATCAGTTTTCCTT**GGGCTGCAA...– 3′
3′ –...CTGGT**GTCAGACTAGTCAAAAGGAA**CCCGACGTT...– 5′

Oligo 1 → 5′ – CACCG**CAGTCTGATCAGTTTTCCTT** – 3′
3′ – C**GTCAGACTAGTCAAAAGGAA**CAAA – 5′ ← Oligo 2

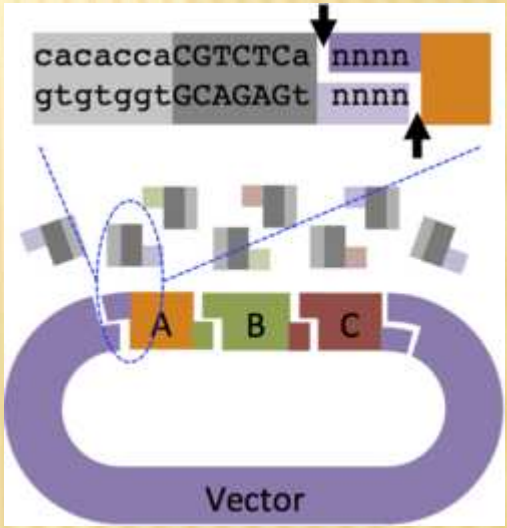



CACCGTCTGGATGAGCTGCACTACC

AAACGGTAGTGCAGCTCATCCAGAC

```
CACCGTCTGGATGAGCTGCACTACC
     CAGACCTACTCGACGTGATGGCAAA
```

RE BsmBI (ESp3I)




ligace, transformace E. coli ligační směsí, expanze klonů, izolace plazmidové DNA, sekvenace

# další postup …

- přenos plazmidu do eukaryotických buněk (transfekce)
- selekce a expanze puromycin-rezistentních buněk
- příprava jednotlivých klonů metodou limitního ředění
- analýza exprese cílového genu na úrovni proteinu (westernův přenos)
- izolace genomové DNA klonu(ů) s nulovou expresí cílového proteinu
- amplifikace cíleného úseku genomové DNA (200-400 nt)

Genomová DNA:

caataaaaataagtgaagcagaagtgcctatgtcttaaaaagaagaaaaagtaagagcctctctcccccagacct
ttccctagggtgggctggccgctgcctacacgttctccattatgtaaaacatatggacacaccatccttgaccct
tggcctgctttccccatagcgaggatctggatgagctgcactaccaggacacagattcagatgtgccggagcaga
gggatagcaagtgcaaggtcaaatggacccatgaggaggtgagtgccatggggaagagagggttgatggcagctg
ggggctgtccagaggaactgagcctggagtgtatttgatgtctcatcagatgggatgaagaggaggagccggtga
aggaagggatgggtcctctgattgtcctcatgcctcttctcagagcattctgggggattctcaattttgaggact
cagtgggtgggaaggggatgtgtgtcctagacctcagggcccattgtgtctcctgagcagctgccatgttctatg
tcatggacattgattgtgcttttgccttttgactgctaggaggtttggccaaatctgtaactcccccaccctt

*XhoI*

GAGC CTCGAG AAGTGAAGCAGAAGTGCCTATG

GAGC AAGCTT ACCTCCTAGCAGTCAAAAGGC

*HindIII*

# další postup …

- klonování zacíleného úseku genomové DNA do plazmidu vhodného pro sekvenaci (např. pGL3)

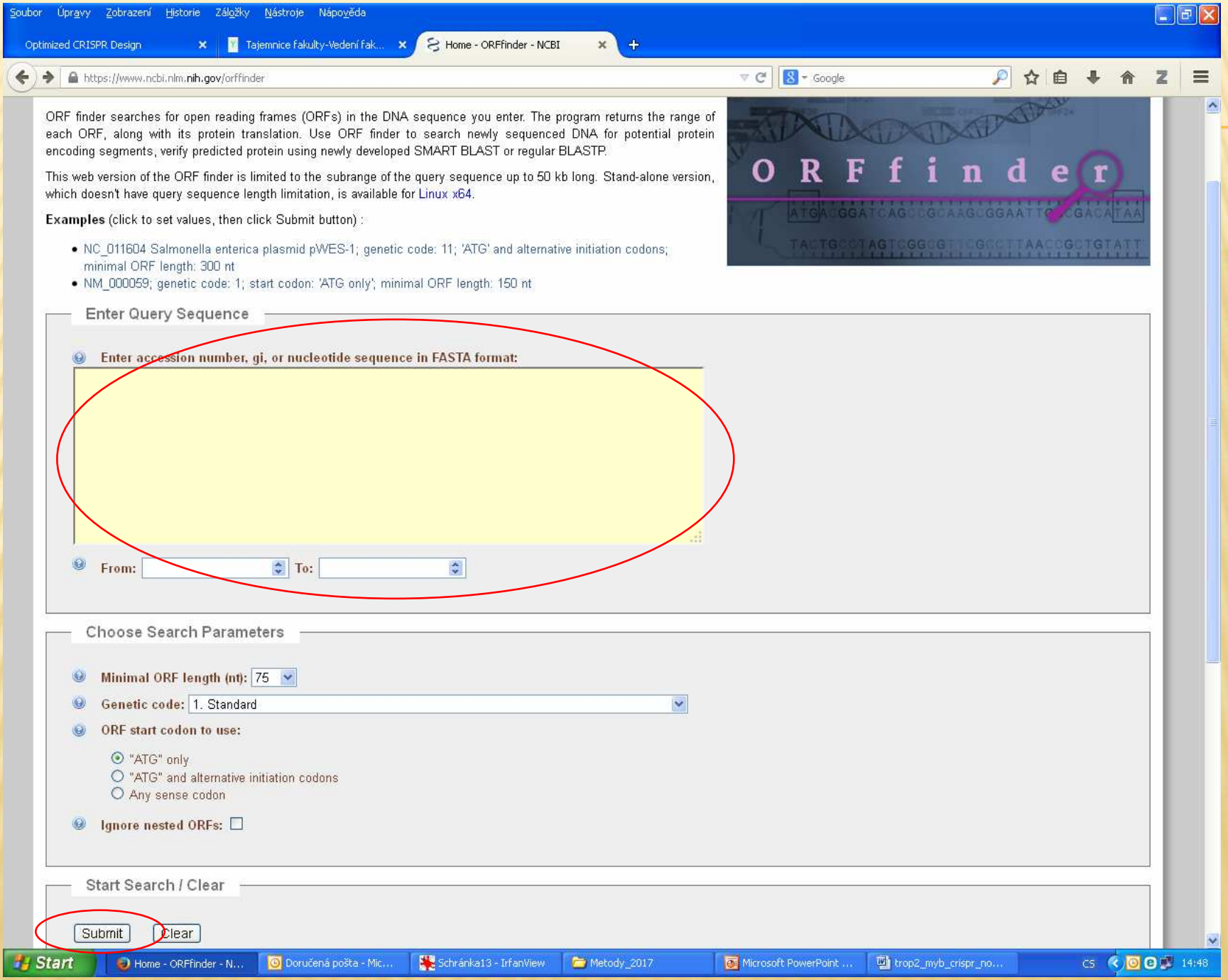
- sekvenace určitého počtu klonů (2 alely genu – min. 4 klony)
- analýza sekvenačních výstupů, identifikace mutací a jejích významu (ORF)

## Jaké mutace lze očekávat?

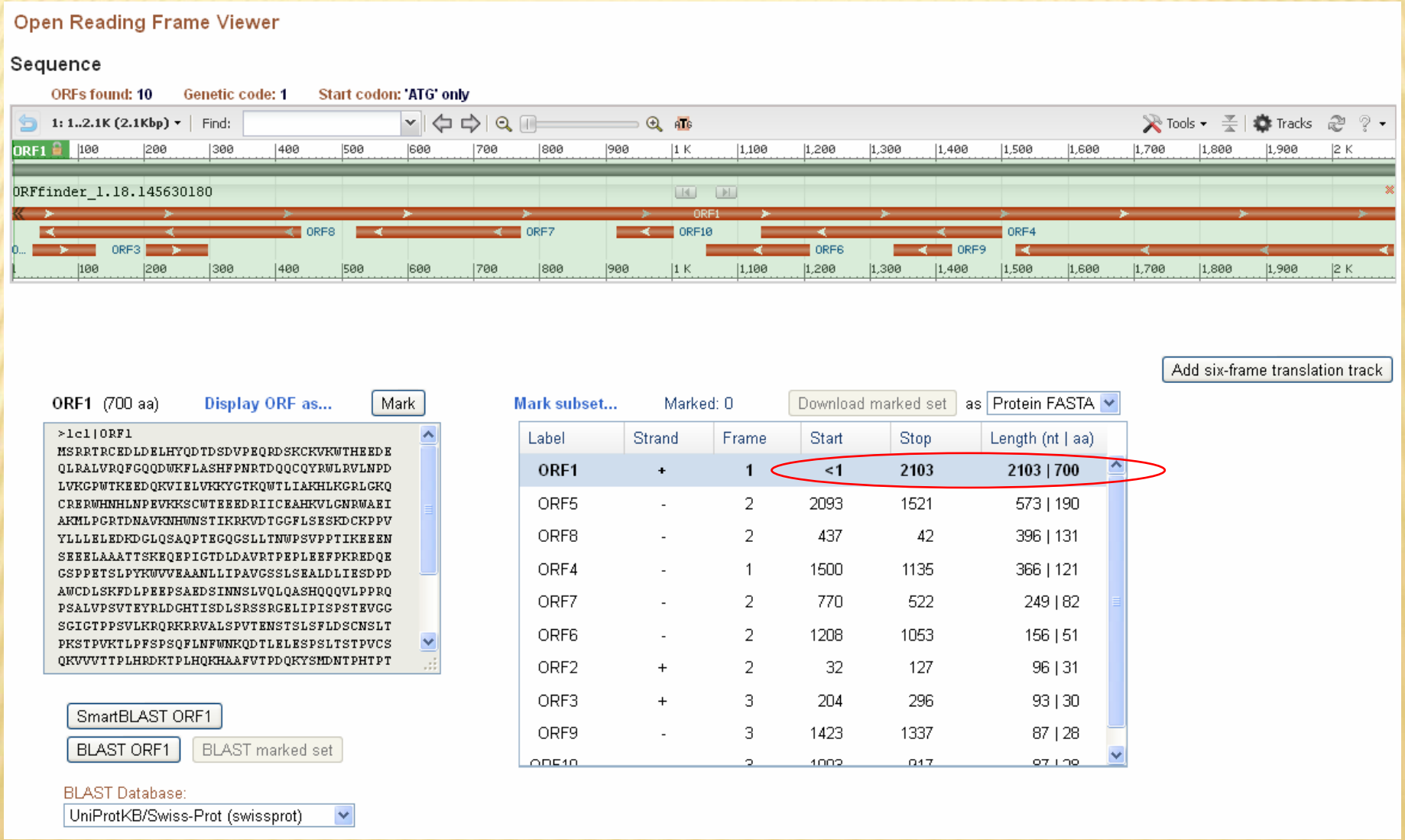
- jakékoli (nejčastěji malé inzerce/delece)
- Cas9 štěpí DNA 3-4 nukleotidy upstream od PAM sekvence
- ideální jsou malé posunové mutace, které mění čtecí rámec

→ předčasný stop kodon

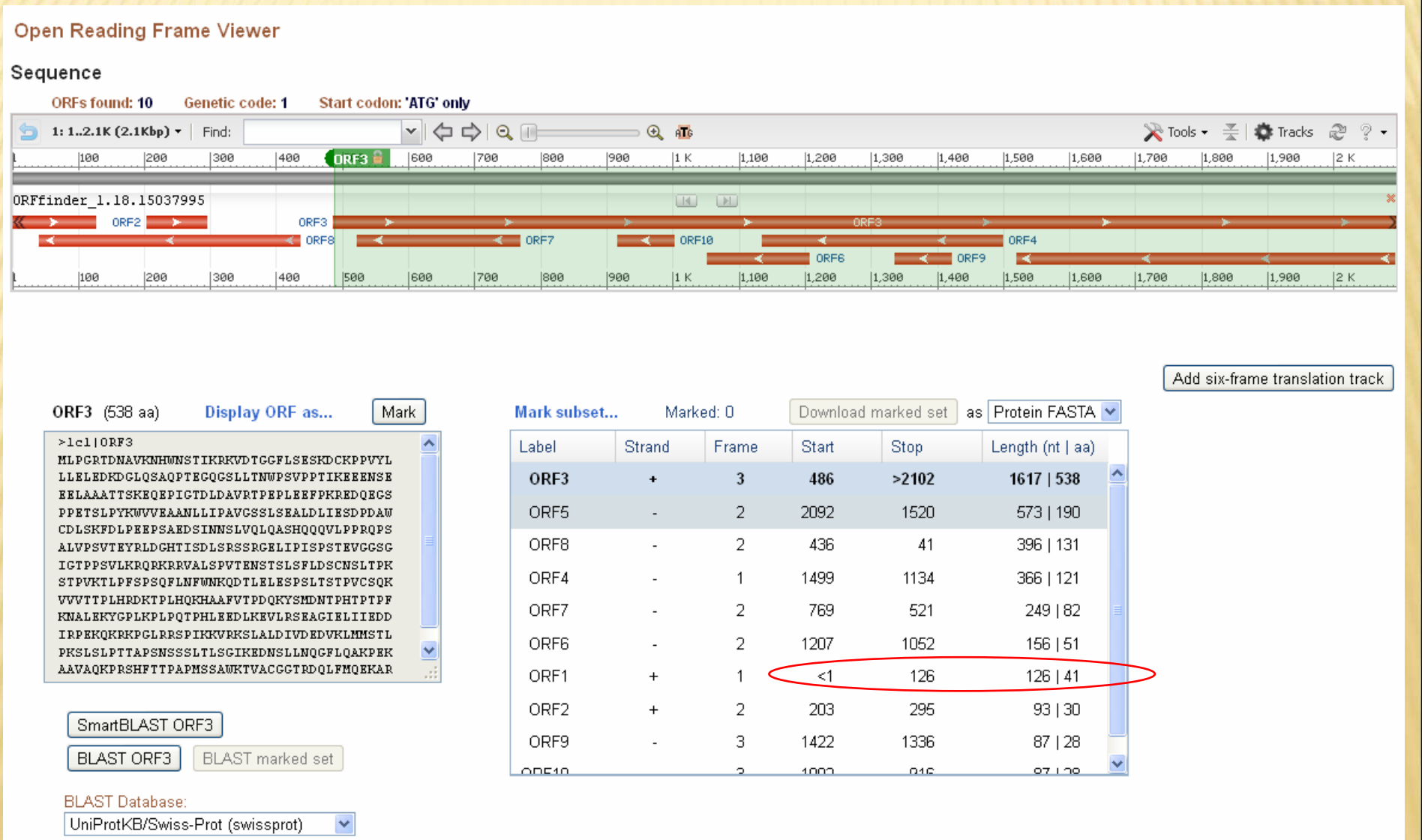
https://www.ncbi.nlm.nih.gov/orffinder


Soubor Úpravy Zobrazení Historie Záložky Nástroje Nápověda
Optimized CRISPR Design
Tajemnice fakulty-Vedení fak...
Home - ORFfinder - NCBI
https://www.ncbi.nlm.nih.gov/orffinder
Google
ORF finder searches for open reading frames (ORFs) in the DNA sequence you enter. The program returns the range of each ORF, along with its protein translation. Use ORF finder to search newly sequenced DNA for potential protein encoding segments, verify predicted protein using newly developed SMART BLAST or regular BLASTP.
This web version of the ORF finder is limited to the subrange of the query sequence up to 50 kb long. Stand-alone version, which doesn't have query sequence length limitation, is available for Linux x64.
Examples (click to set values, then click Submit button) :
NC_011604 Salmonella enterica plasmid pWES-1; genetic code: 11; 'ATG' and alternative initiation codons; minimal ORF length: 300 nt
NM_000059; genetic code: 1; start codon: 'ATG only'; minimal ORF length: 150 nt
ORFfinder
Enter Query Sequence
Enter accession number, gi, or nucleotide sequence in FASTA format:
From:
To:
Choose Search Parameters
Minimal ORF length (nt): 75
Genetic code: 1. Standard
ORF start codon to use:
"ATG" only
"ATG" and alternative initiation codons
Any sense codon
Ignore nested ORFs:
Start Search / Clear
Submit
Clear
Start
Home - ORFfinder - N...
Doručená pošta - Mic...
Schránka13 - IrfanView
Metody_2017
Microsoft PowerPoint ...
trop2_myb_crispr_no...
CS
14:48

# B-Myb


Open Reading Frame Viewer
Sequence
ORFs found: 10
Genetic code: 1
Start codon: 'ATG' only
1: 1..2.1K (2.1Kbp)
Find:
Tools
Tracks
ORF1
ORFfinder_1.18.145630180
ORF8
ORF7
ORF10
ORF4
ORF3
ORF6
ORF9
Add six-frame translation track
ORF1 (700 aa)
Display ORF as...
Mark
>lcl|ORF1
MSRRTRCEDLDELHYQDTDSDVPEQRDSKCKVKWTHEEDE
QLRALVRQFGQQDWKFLASHFPNRTDQQCQYRWLRVLNPD
LVKGPWTKEEDQKVIELVKKYGTKQWTLIAKHLKGRLGKQ
CRERWHNHLNPEVKKSCWTEEEDRIICEAHKVLGNRWAEI
AKMLPGRTDNAVKNHWNSTIKRKVDTGGFLSESKDCKPPV
YLLLELEDKDGLQSAQPTEGQGSLLTNWPSVPPTIKEEEN
SEEELAAATTSKEQEPIGTDLDAVRTPEPLEEFPKREDQE
GSPPETSLPYKWVVEAANLLIPAVGSSLSEALDLIESDPD
AWCDLSKFDLPEEPSAEDSINNSLVQLQASHQQQVLPPRQ
PSALVPSVTEYRLDGHTISDLSRSSRGELIPISPSTEVGG
SGIGTPPSVLKRQRKRRVALSPVTENSTSLSFLDSCNSLT
PKSTPVKTLPFSPSQFLNFWNKQDTLELESPSLTSTPVCS
QKVVVTTPLHRDKTPLHQKHAAFVTPDQKYSMDNTPHTPT
Mark subset...
Marked: 0
Download marked set
as
Protein FASTA
Label
Strand
Frame
Start
Stop
Length (nt | aa)
ORF1 + 1 <1 2103 2103 | 700
ORF5 - 2 2093 1521 573 | 190
ORF8 - 2 437 42 396 | 131
ORF4 - 1 1500 1135 366 | 121
ORF7 - 2 770 522 249 | 82
ORF6 - 2 1208 1053 156 | 51
ORF2 + 2 32 127 96 | 31
ORF3 + 3 204 296 93 | 30
ORF9 - 3 1423 1337 87 | 28
SmartBLAST ORF1
BLAST ORF1
BLAST marked set
BLAST Database:
UniProtKB/Swiss-Prot (swissprot)

# B-Myb - mutovaný



# Organizace cvičení (CRISPR)

- skupiny po 12 lidech (2x 6 hod praktických cvičení)

## Každá skupina

## … před praktickou částí cvičení …

- přídělen jeden cílový gen
- návrh strategie cílené mutageneze tohoto genu metodou CRISPR (miniprojekt)
- zaslání nejpozději v poslední neděli před začátkem cvik na pbenes@sci.muni.cz

## … po praktické části cvičení …

- výsledky získané na cvičeních budou zpracovány do tohoto miniprojektu
- včetně analýzy sekvenačních výstupů
- včetně vyhodnocení významu vzniklých mutací pro translaci cílového genu

# Miniprojekt (.doc)

- stručný úvod + cíl
- detailní popis návrhu gRNA sekvencí pro vybraný gen

---

- stručný popis výsledků dílčích kroků cílené mutageneze z praktických cvičení
- analýza výstupů ze sekvenace genomové DNA získaných klonů
- závěr

# RNA interference

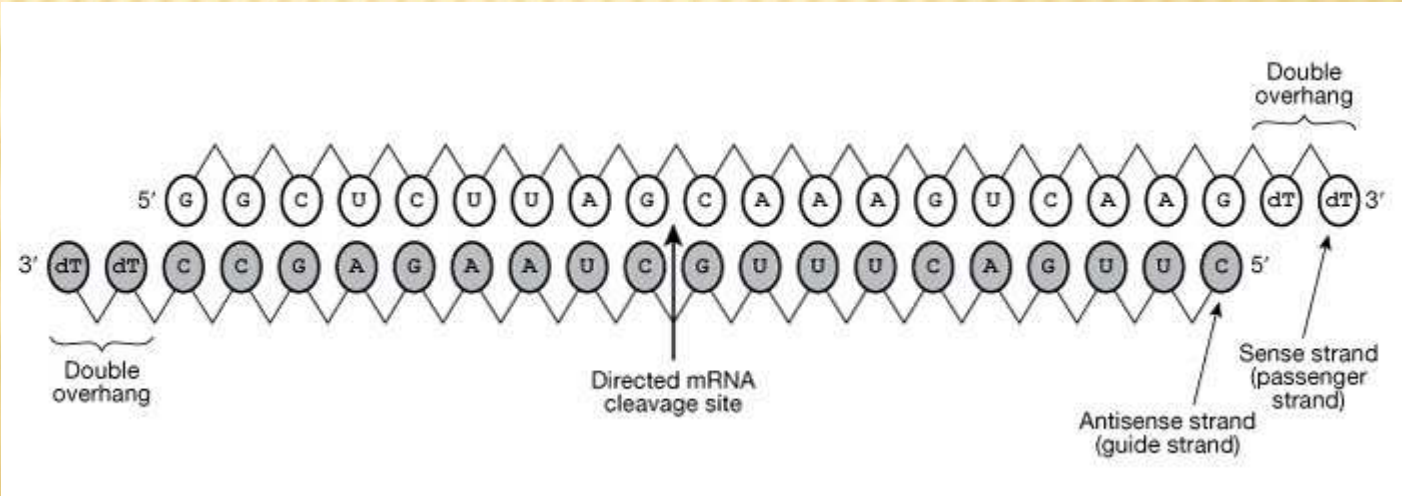
- http://www.nature.com/nrg/multimedia/rnai/animation/index.html
- mechanismus post-transkripčního umlčování genů
- využívá dvouřetězcové RNA pro interferenci s genovou expresí

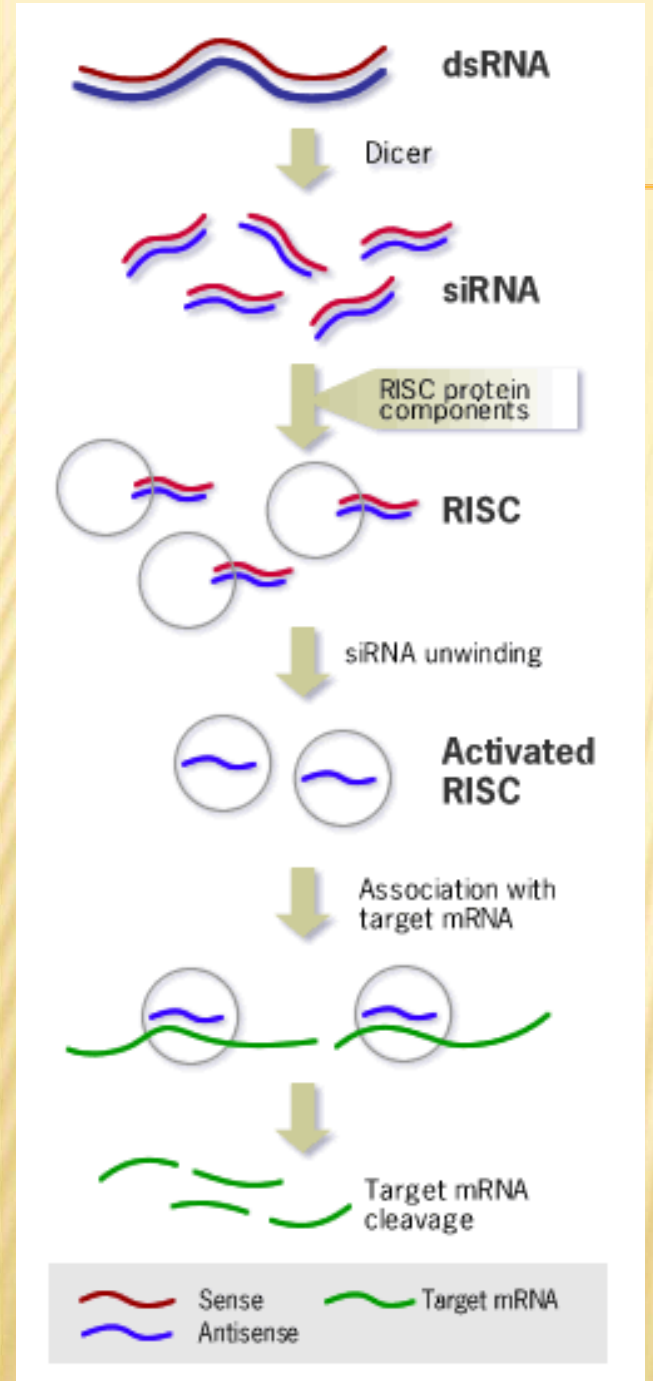
  (dsRNA efektivnější než ssRNA)

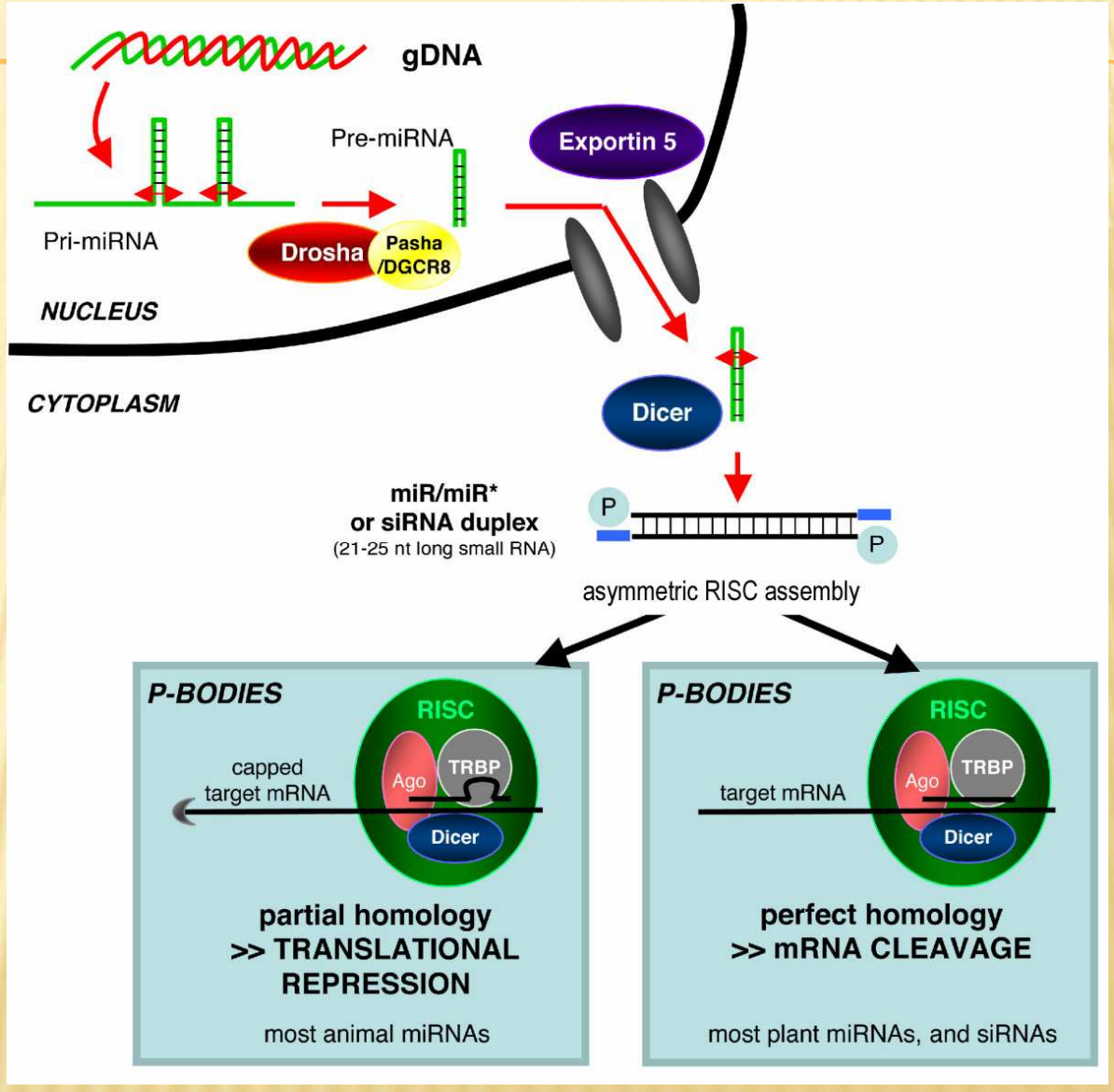
- podstatou je enzymová degradace nebo zastavení translace specifické mRNA
- miRNA vs siRNA

# siRNA

- malé nekódující dsRNA o velikosti 21-25 nukleotidů
- negativní regulátory genové exprese, které obvykle zprostředkovávají degradaci cílové mRNA (úplná homologie)
- exogenní původ (viry, syntetické)
- v cytoplazmě procesovány proteinem Dicer
- siRNA se váže k RISC, jedno vlákno se degraduje a druhé zprostředkovává degradaci nebo inhibici translace příslušné mRNA




dsRNA
Dicer
siRNA
RISC protein components
RISC
siRNA unwinding
Activated RISC
Association with target mRNA
Target mRNA cleavage
Sense
Antisense
Target mRNA


gDNA
Pre-miRNA
Exportin 5
Pri-miRNA
Drosha
Pasha /DGCR8
NUCLEUS
CYTOPLASM
Dicer
miR/miR*
or siRNA duplex
(21-25 nt long small RNA)
P
P
asymmetric RISC assembly
P-BODIES
RISC
capped target mRNA
Ago
TRBP
Dicer
partial homology
>> TRANSLATIONAL REPRESSION
most animal miRNAs
P-BODIES
RISC
target mRNA
Ago
TRBP
Dicer
perfect homology
>> mRNA CLEAVAGE
most plant miRNAs, and siRNAs

# miRNA versus siRNA

- obě RNA negativně regulují translaci
- miRNA je endogenní, siRNA je exogenní
- miRNA může, ale obvykle není úplně komplementární určitému transkriptu, proto jedna miRNA může blokovat translaci několika/mnoha transkriptů (desítky až stovky)
- siRNA je obvykle zcela komplementární – obvykle štěpení jediné cílové mRNA

# siRNA design

- validované siRNA dnes dodává řada firem (Dharmacon, Thermofisher, …)
- různý algoritmus, délka
- chemické modifikace (zvýšení stability, redukce off-target efektů)…
- směsi siRNA
- Kontroly !!!!!!!!!!!!!!!!

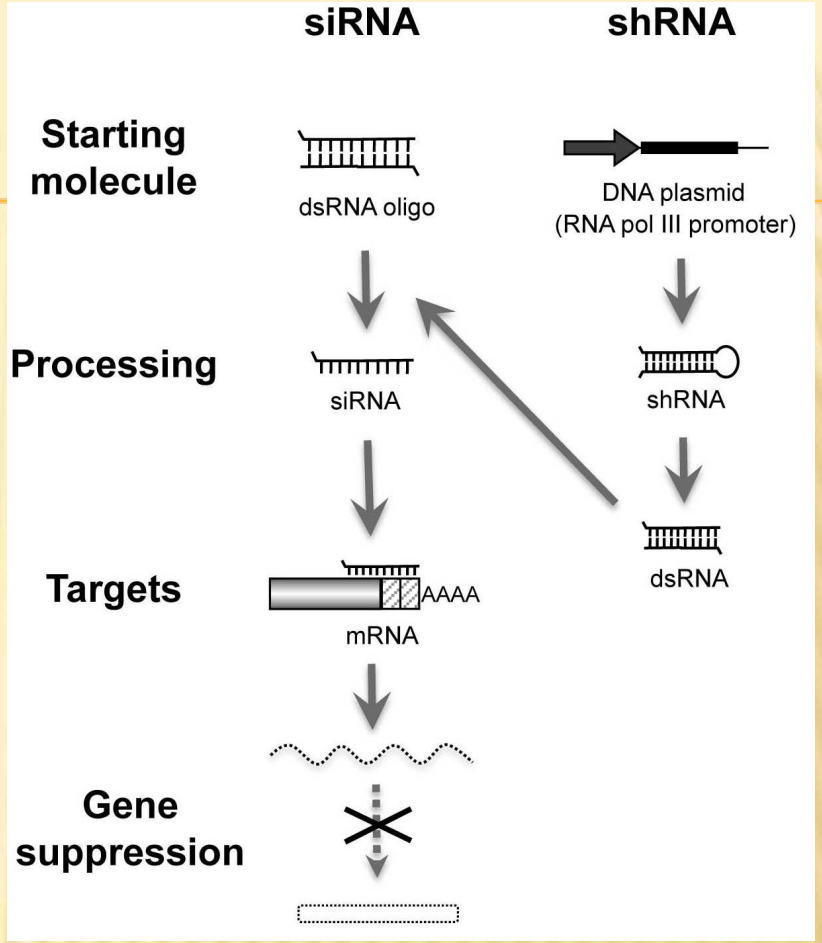
# siRNA design

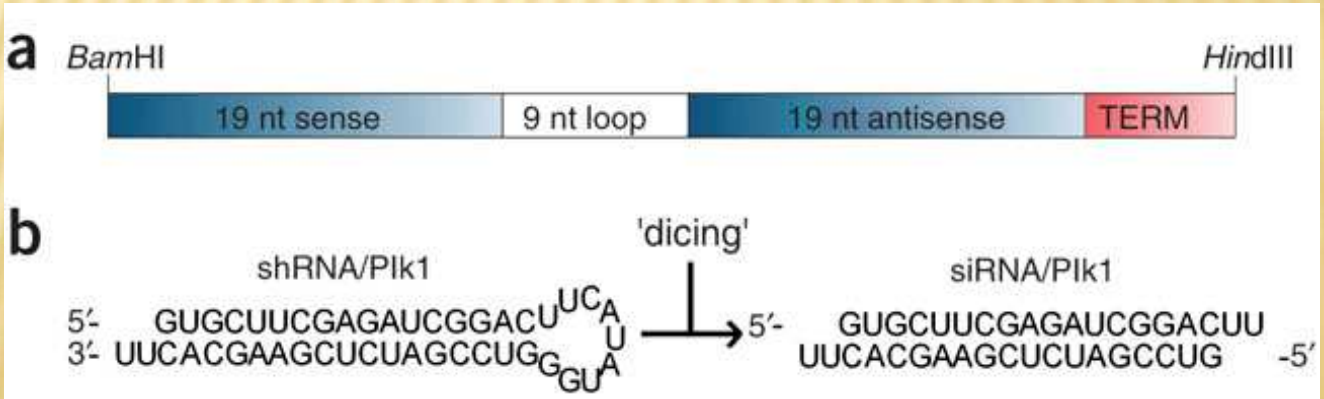
- 21-25 nt
- 2 nukleotidy přesah na 3´ koncích (obvykle UU)
- 30-60% *GC*
- ne cílené proti intronům, UTR a blízko *ATG* a terminačního kodónu
- homologie pouze k cílové mRNA

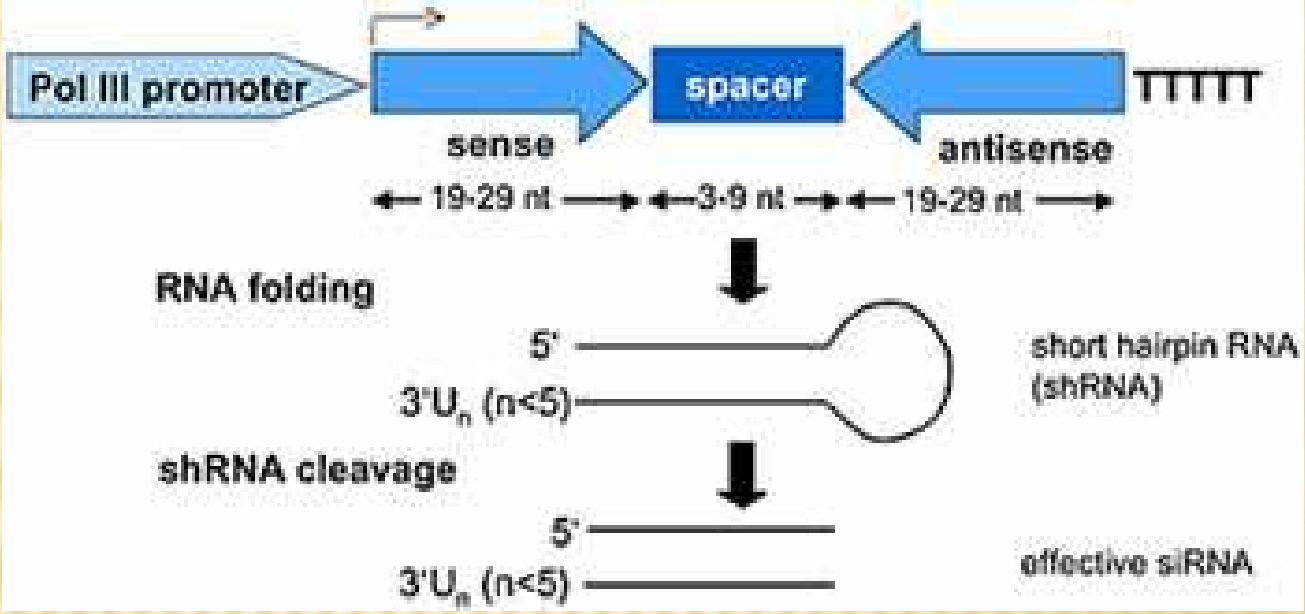
https://rnaidesigner.lifetechnologies.com/rnaiexpress/
http://dharmacon.gelifesciences.com/design-center/
http://www.sirnawizard.com/design.php

# shRNA

- produkovány z exogenního vektoru
- promotor polymerázy III
- syntéza a zpracování v buňce obdobné jako i mikroRNA




Pol III promoter
spacer
TTTTT
sense
antisense
19-29 nt
3-9 nt
19-29 nt
RNA folding
5'
3'U$_n$ (n<5)
short hairpin RNA
(shRNA)
shRNA cleavage
5'
3'U$_n$ (n<5)
effective siRNA

atgtctcggcggacgcgctgcgaggatctggatgagctgcactaccaggacacagattcagatgtgccggagcag
agggatagcaagtgcaaggtcaaatggacccatgaggaggacgagcagctgagggccctggtgaggcagtttgga
cagcaggactggaagttcctggccagccacttccctaaccgcactgaccagcaatgccagtacaggtggctgaga
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aaggtgctgggcaaccgctgggccgagatcgccaagatgttgccagggaggacagacaatgctgtgaagaatcac
tggaactctaccatcaaaaggaaggtggacacaggaggcttcttgagcgagtccaaagactgcaagcccccagtg
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accaactggccctccgtccctcctaccataaaggaggaggaaaacagtgaggaggaacttgcagcagccaccaca
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agtaaatttgacctccctgaggaaccatctgcagaggacagtatcaacaacagcctagtgcagctgcaagcgtca
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gctgcgtttgtaaccccagatcagaagtactccatggacaacactccccacacgccaaccccgttcaagaacgcc
ctggagaagtacggacccctgaagcccctgccacagaccccgcacctggaggaggacttgaaggaggtgctgcgt
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cggagccccatcaagaaagtccggaagtctctggctcttgacattgtggatgaggatgtgaagctgatgatgtcc
acactgcccaagtctctatccttgccgacaactgccccttcaaactcttccagcctcaccctgtcaggtatcaaa
gaagacaacagcttgctcaaccagggcttcttgcaggccaagcccgagaaggcagcagtggcccagaagccccga
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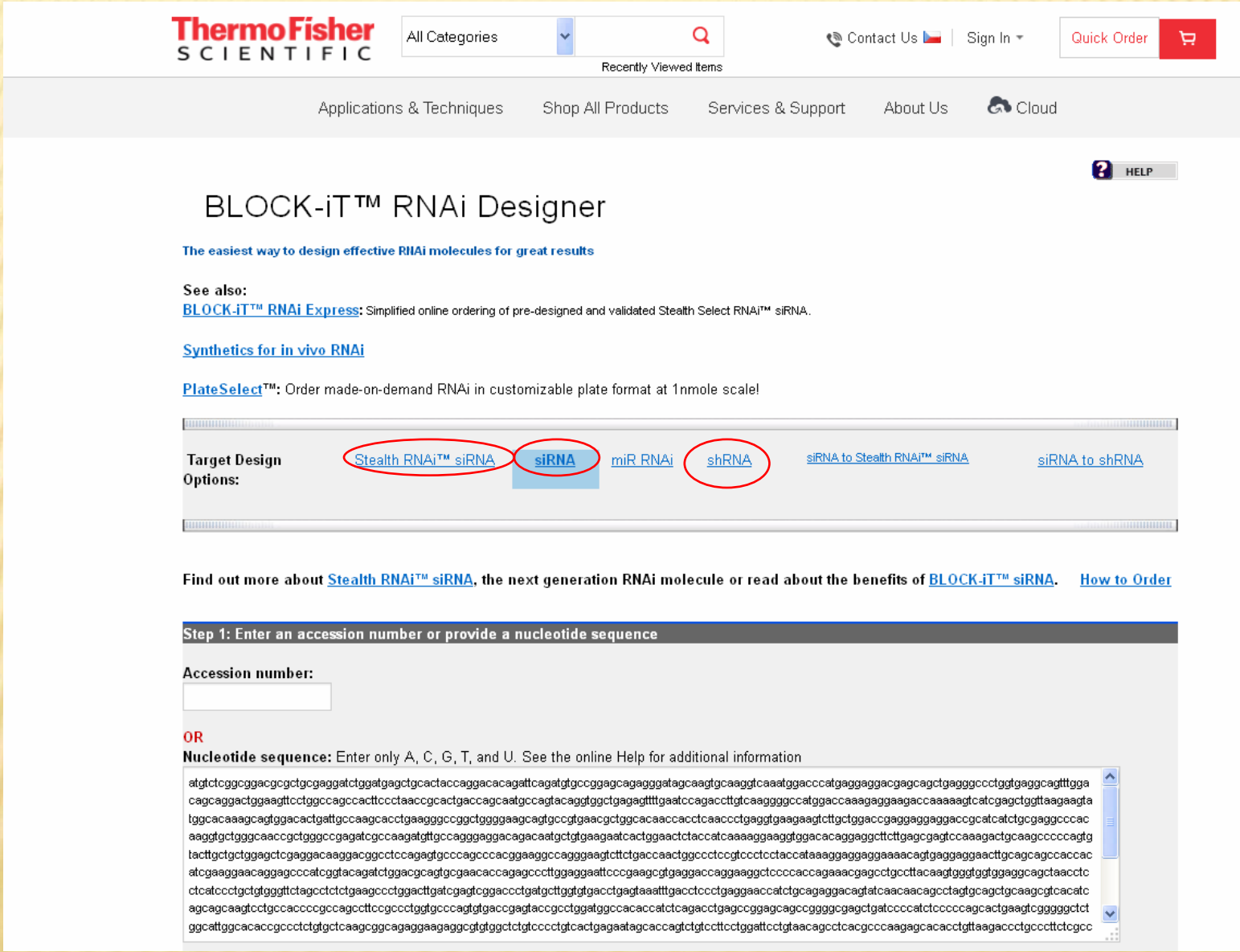
tga

https://rnaidesigner.thermofisher.com/rnaiexpress/



**Step 2: If you entered an accession number in Step 1, select regions for target design**

☑ Open reading frame (ORF) ☐ 5' UTR ☐ 3' UTR

**Step 3: Choose database for Blast**

Human - Homo sapiens

**NOTE:** BLAST is used to compare input sequence with sequences in the database to find unique regions against which to design RNAi targets. The databases contain representative gene sequences for that species. Blast databases were updated on May 01, 2008 and the design output reflects the most up-to-date designs.

**Step 4: Choose minimum and maximum G/C percentage**

Minimum G/C percentage: 35% Maximum G/C percentage: 55%

**Step 5: Select siRNA design options and click "RNAi Design" to design siRNA.**

◉ Default motif pattern: P: Proprietary (Recommended)

○ Tuschl's motif pattern: ☐ A: AA(N19)TT ☐ B: NA(N19)NN ☐ C: NA(RN17Y)NN ☐ D: NA(N18Y)NN

RNAi Design Reset Form

**Guarantee:** The BLOCK-iT™ RNAi Designer is such an effective tool for the design of siRNAs that if you order the three best siRNA sequences designed by the BLOCK-iT™ RNAi Designer, we guarantee that two of them will give greater than 70% knockdown of mRNA, given that the transfection efficiency in your experiment is at least 80%. If two or more fail to knock down your target RNA by at least 70% under these conditions, Invitrogen will design and ship a fourth siRNA to your target for free*.

*Please contact Invitrogen Technical Services to take advantage of this offer (800-955-6288 ext 2). Please be prepared to fax or email your order reference number, oligo sequences and data showing your transfection efficiency and knockdown. This offer is good for one free duplex per target.

**How to Order:** *You must have an account with Invitrogen to order custom oligos. After your oligos have been designed and added to the order form, you must enter a valid customer number that is keyed to your account. If you do not already have an account, contact your local representative to set up an account.*

**If you already know your RNAi oligo sequence:** Order oligos online at Order Custom Primers or by e-mail/Fax.

**Recommended siRNA**

| | | | | | |
|---|---|---|---|---|---|
| **Gene Name:** | Unknown | **Accession:** | Unknown | **GI:** | Unknown |
| **Organism:** | Unknown | **Length:** | 2103 | **ORF Region:** | Unknown |
| **Defintion:** | Unknown | | | | |

Continue | View Blast Results | Sort By: Start

**10 siRNA Sequences** (Up to 10 top scoring siRNA sequences are reported, sorted by the Start position and ranked as ★★★★★ to ★★★☆☆ to indicate knockdown probability). Select the sequence to order and click "Continue".

| Select | No. | Start | Sequence(DNA) | Region | GC% | Tuschl's pattern match* | Rank[1] |
|---|---|---|---|---|---|---|---|
| ☐ | 1 | 252 | GCCATGGACCAAAGAGGAA | | 52.64 | | ★★★★★ |
| ☐ | 2 | 391 | CCTGAGGTGAAGAAGTCTT | | 47.37 | | ★★★★½ |
| ☐ | 3 | 689 | CCGTCCCTCCTACCATAAA | | 52.64 | | ★★★★★ |
| ☐ | 4 | 912 | GGGTTCTAGCCTCTCTGAA | | 52.64 | | ★★★★½ |
| ☐ | 5 | 965 | GGTGTGACCTGAGTAAATT | | 42.11 | | ★★★★☆ |
| ☐ | 6 | 1292 | CCTTCCTGGATTCCTGTAA | | 47.37 | | ★★★★½ |
| ☐ | 7 | 1322 | CCAAGAGCACACCTGTTAA | | 47.37 | | ★★★★★ |
| ☐ | 8 | 1358 | CCTCCCAGTTTCTGAACTT | | 47.37 | | ★★★★★ |
| ☐ | 9 | 1379 | GGAACAAACAGGACACATT | | 42.11 | | ★★★★☆ |
| ☐ | 10 | 1491 | CCAGAAACATGCTGCGTTT | | 47.37 | BD | ★★★★★ |

Continue

Tuschl's pattern*: A=AA(N19)TT, B=NA(N19)NN, C=NAR(N17)YNN, D=NAN(17)YNN; Nucleotides: A = Adenine; T = Thymine; R = Adenine or Guanine (Purines); Y = Thymine or Cytosine (Pyrimidines); N = Any.

[1]**Note:** As a result of the recent update to the BLOCK-iT™ RNAi Designer, the "Star Scoring System" has also been updated to reflect our more highly refined RNAi duplex designs. Only the duplexes with the highest probability of success are provided which means that out of a possible five stars, no duplex has less than a three-star ranking. While each individual RNAi duplex is designed to achieve the highest quality results, we recommend selecting the top three star-ranked designs for your target of interest to guarantee your knockdown success.

# BLOCK-iT™ RNAi Designer

**The easiest way to design effective RNAi molecules for great results**

**See also:**
BLOCK-iT™ RNAi Express: Simplified online ordering of pre-designed and validated Stealth Select RNAi™ siRNA.

Synthetics for in vivo RNAi

PlateSelect™: Order made-on-demand RNAi in customizable plate format at 1nmole scale!

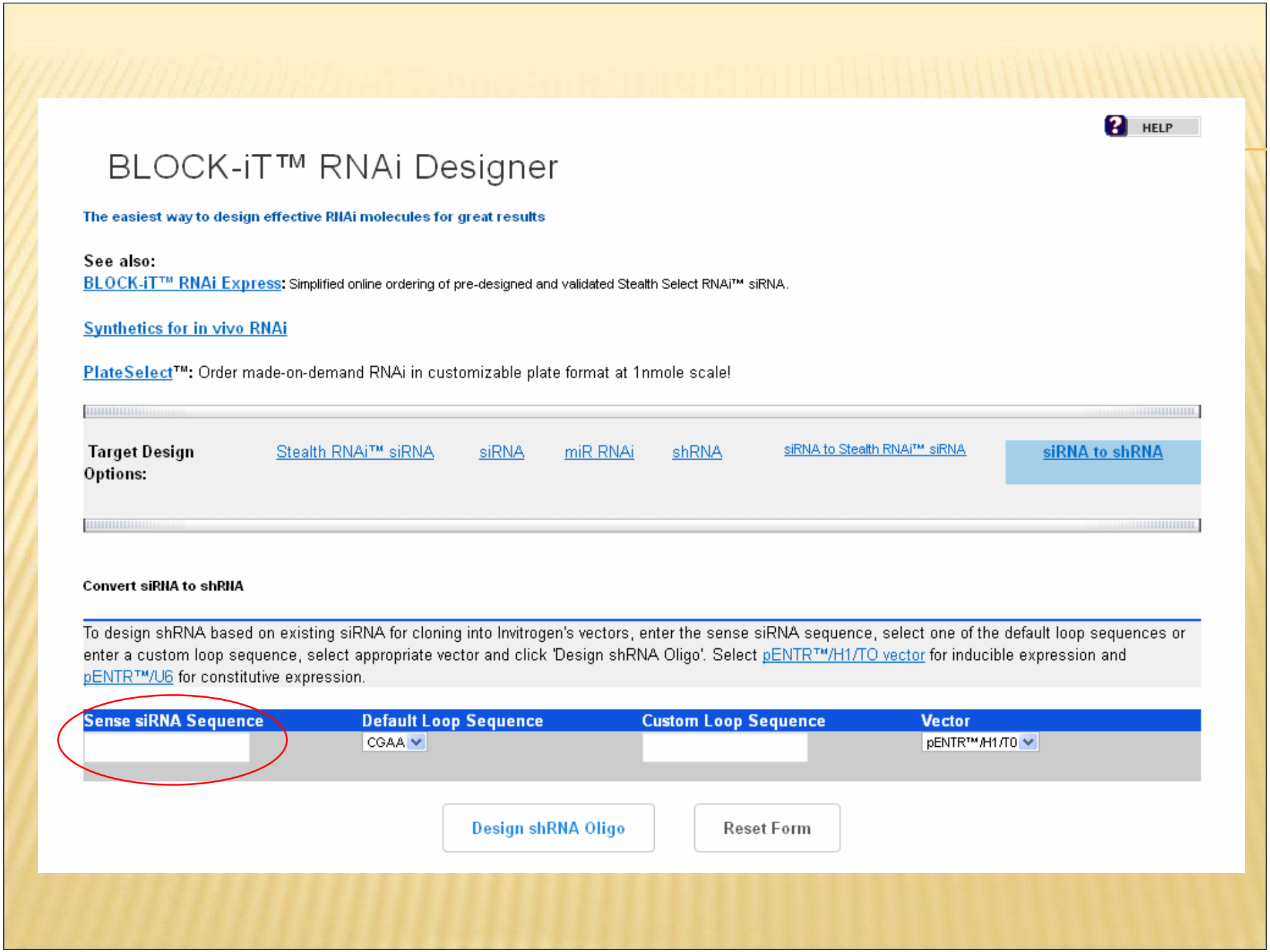
**Target Design Options:** Stealth RNAi™ siRNA | **siRNA** | miR RNAi | shRNA | siRNA to Stealth RNAi™ siRNA | siRNA to shRNA

**Selected siRNA with negative control sequences**

**The negative control sequence for each selected siRNA is displayed. Select control sequences to order and click 'Order Online'**

**Select order format first before selecting products to order*:** ○ Tube ○ Plate  Order Online  Email/Save Order

| Select | No. | Name | Start | Sequence(DNA) | Region | GC% | Rank |
|---|---|---|---|---|---|---|---|
| ☑ | 1 | siRNA_252 | 252 | GCCATGGACCAAAGAGGAA | | 52.64 | ★★★★★ |
| ☐ | | siRNA_control_252 | | GCCCAGGAAACGAGTAGAA | | 52.64 | |
| ☑ | 2 | siRNA_689 | 689 | CCGTCCCTCCTACCATAAA | | 52.64 | ★★★★★ |
| ☐ | | siRNA_control_689 | | CCGCTCCCATCTACCTAAA | | 52.64 | |
| ☑ | 3 | siRNA_1322 | 1322 | CCAAGAGCACACCTGTTAA | | 47.37 | ★★★★★ |
| ☐ | | siRNA_control_1322 | | CCAGAACACCCGTTAGTAA | | 47.37 | |
| ☑ | 4 | siRNA_1358 | 1358 | CCTCCCAGTTTCTGAACTT | | 47.37 | ★★★★★ |
| ☐ | | siRNA_control_1358 | | CCTTGACTCTTAAGCCCTT | | 47.37 | |
| ☑ | 5 | siRNA_1491 | 1491 | CCAGAAACATGCTGCGTTT | | 47.37 | ★★★★★ |
| ☐ | | siRNA_control_1491 | | CCAACAATCGTGCGAGTTT | | 47.37 | |


HELP
BLOCK-iT™ RNAi Designer
The easiest way to design effective RNAi molecules for great results
See also:
BLOCK-iT™ RNAi Express: Simplified online ordering of pre-designed and validated Stealth Select RNAi™ siRNA.
Synthetics for in vivo RNAi
PlateSelect™: Order made-on-demand RNAi in customizable plate format at 1nmole scale!
Target Design Options:
Stealth RNAi™ siRNA
siRNA
miR RNAi
shRNA
siRNA to Stealth RNAi™ siRNA
siRNA to shRNA
Convert siRNA to shRNA
To design shRNA based on existing siRNA for cloning into Invitrogen's vectors, enter the sense siRNA sequence, select one of the default loop sequences or enter a custom loop sequence, select appropriate vector and click 'Design shRNA Oligo'. Select pENTR™/H1/TO vector for inducible expression and pENTR™/U6 for constitutive expression.
Sense siRNA Sequence
Default Loop Sequence
CGAA
Custom Loop Sequence
Vector
pENTR™/H1/TO
Design shRNA Oligo
Reset Form

**The easiest way to design effective RNAi molecules for great results**

**See also:**

BLOCK-iT™ RNAi Express: Simplified online ordering of pre-designed and validated Stealth Select RNAi™ siRNA.

Synthetics for in vivo RNAi

PlateSelect™: Order made-on-demand RNAi in customizable plate format at 1nmole scale!

**Target Design Options:** Stealth RNAi™ siRNA | siRNA | miR RNAi | shRNA | siRNA to Stealth RNAi™ siRNA | **siRNA to shRNA**

**Order shRNA Oligo**

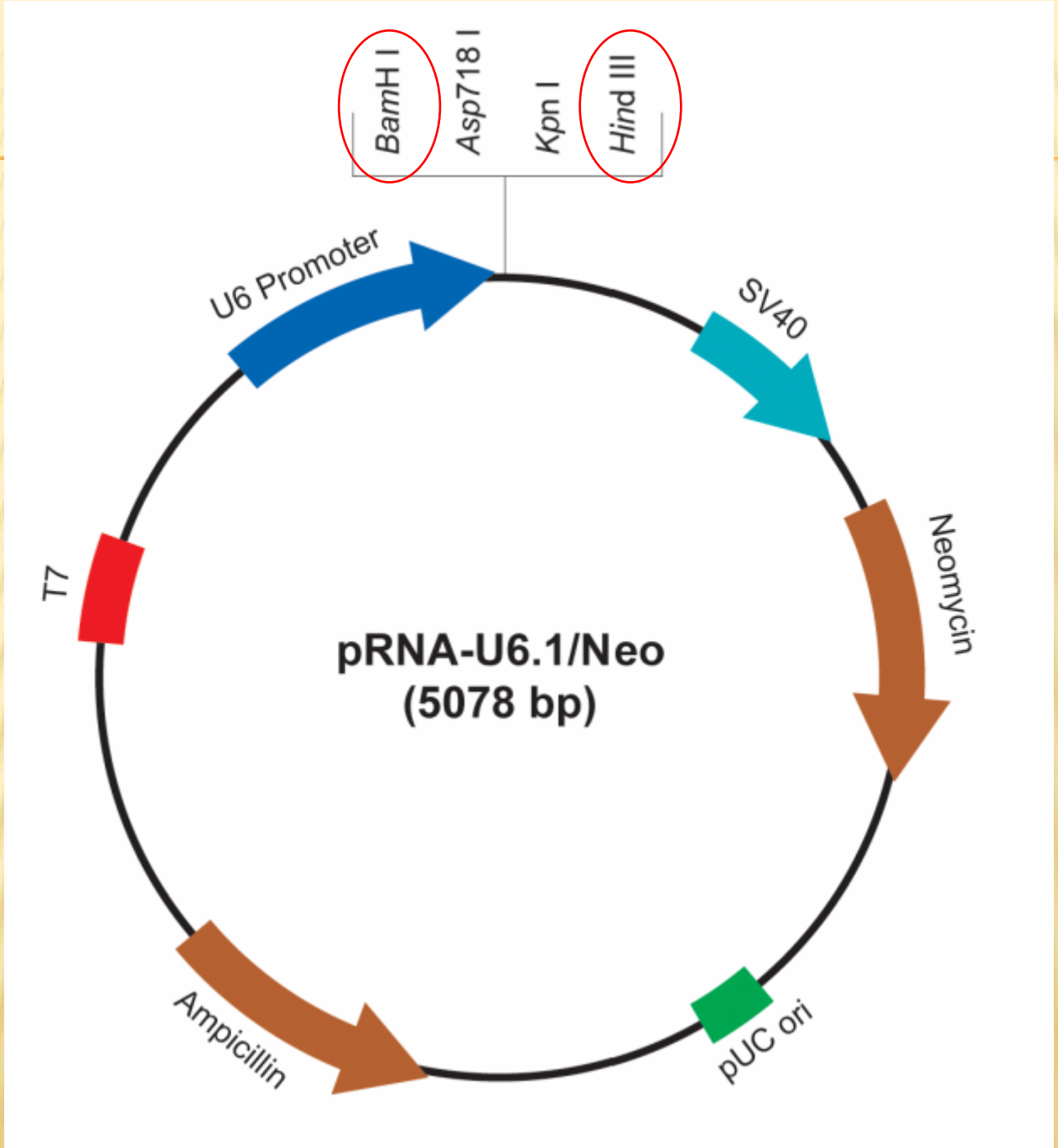
**shRNA Oligo Features:**

Linker | Sense Sequence | Loop Sequence | Antisense Sequence

| | |
|---|---|
| Top Strand | 5'-CACCGCCATGGACCAAAGAGGAACGAATTCCTCTTTGGTCCATGGC-3' |
| Bottom Strand | 5'-AAAAGCCATGGACCAAAGAGGAATTCGTTCCTCTTTGGTCCATGGC-3' |
| ds Oligo | 5'-CACCGCCATGGACCAAAGAGGAACGAATTCCTCTTTGGTCCATGGC-3'<br>\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|\|<br>3'-CGGTACCTGGTTTCTCCTTGCTTAAGGAGAAACCAGGTACCGAAAA-5' |

Note: You have to order both the top and bottom DNA strands (Minimum scale of synthesis = 50 nmole; 5' modifications = None; 3' Modifications = None; Purity = Desalted). Anneal the top and bottom strand oligos (shRNA Annealing Protocol (PDF)) to generate a double-stranded oligo with 4-nucleotide overhangs suitable for directional cloning into Invitrogen's BLOCK-iT™ Inducible H1 RNAi Entry Vector (pENTR™/H1/TO).

Order Online | Email/Save Order


BamH I
Asp718 I
Kpn I
Hind III
U6 Promoter
SV40
Neomycin
T7
pRNA-U6.1/Neo
(5078 bp)
Ampicillin
pUC ori

# B-Myb shRNA

*BamH I*

5’ GATCCGGCCATGGACCAAAGAGGAATTCAAGAGATTCCTCTTTGGTCCATGGCTTTTTT GG AAA3’

| Sense | Loop | Antisense | Termination Signal

5’AGCTTTTCCAAAAAAGCCATGGACCAAAGAGGAA*TCTCTTGAA*TTCCTCTTTGGTCCATGGCCG 3‘

*Hind III*

*BamH I*

GATCCGGCCATGGACCAAAGAGGAATTCAAGAGATTCCTCTTTGGTCCATGGCTTTTTT GGAAA

GCCGGTACCTGGTTTCTCCTTAAGTTCTCTAAGGAGAAACCAGGTACCGAAAAAACCTTTTCGA

*Hind III*

ligace, transformace E. coli ligační směsí, expanze klonů, izolace plazmidové DNA, sekvenace

# další postup …

- přenos plazmidu do eukaryotických buněk (přechodná transfekce)
- analýza exprese cílového genu na úrovni proteinu (westernův přenos)

# Organizace cvičení (shRNA)

- skupiny po 12 lidech (2x 6 hod praktických cvičení)

## Každá skupina

## … před praktickou částí cvičení …

- přídělen jeden cílový gen (stejný jako pro CRISPR)
- návrh siRNA/shRNA sekvence (miniprojekt)
- zaslání nejpozději v poslední neděli před začátkem cvik na pbenes@sci.muni.cz

## … po praktické části cvičení …

- výsledky získané na cvičeních budou zpracovány do tohoto miniprojektu

# Miniprojekt (.doc)

- stručný úvod + cíl
- detailní popis návrhu gRNA a siRNA/shRNA sekvencí pro vybraný gen

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- stručný popis výsledků z praktických cvičení:

a) dílčích kroků mutageneze cílového genu
b) dílčích kroků posttranskripčního umlčování cílového genu

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- analýza výstupů ze sekvenace genomové DNA získaných klonů
- závěr

# Cílové geny

Mus musculus

- Tacstd2 (Gene ID: 56753) – 30. března
- Myb (Gene ID: 17863) – 6. dubna
- Mybl2 (Gene ID: 17865) – 20. dubna
- Cxcl1 (Gene ID: 14825) – 27. dubna
- Icam1 (Gene ID: 15894) – 4. května
- Tnfrsf9 (Gene ID: 21942) – 11. května