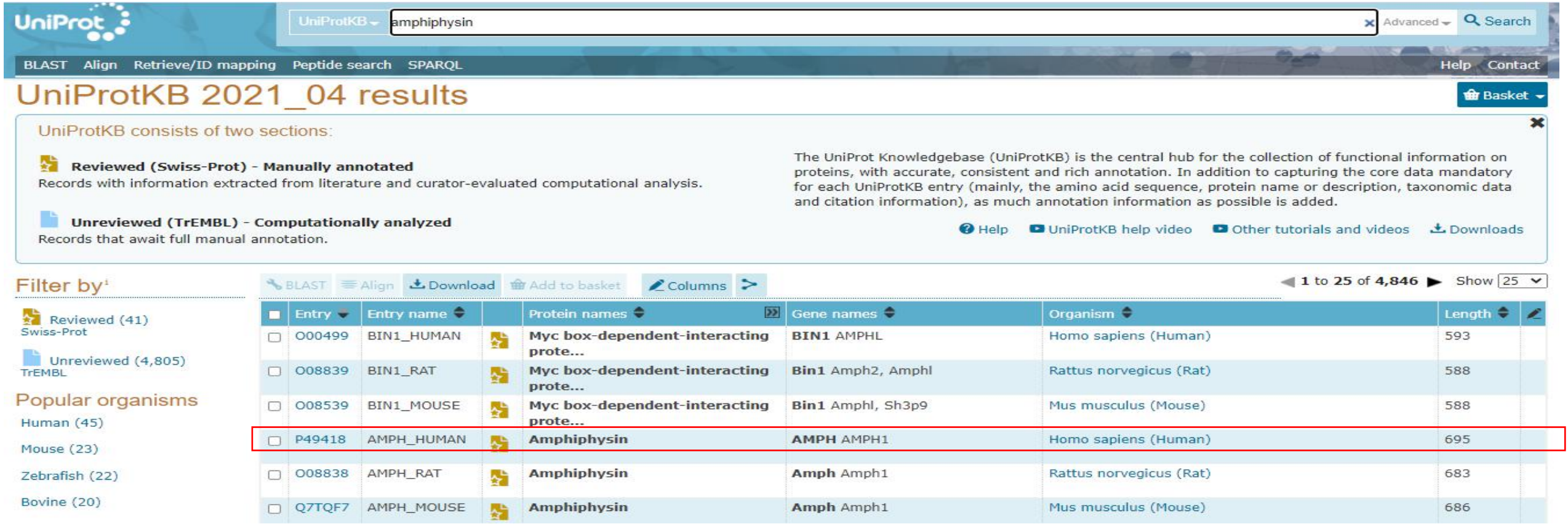
载体构建与细胞转染
Western Blot技术介绍

范俊婉

20220303

CDS序列

https://www.uniprot.org/



Sequence databases

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	X81438 mRNA Translation: CAA57197.1
(•) EMBL[i]	AF034996 mRNA Translation: AAC02977.1
( ) GenBank[i]	AC011309 Genomic DNA Translation: AAS07391.1
( ) DDBJ[i]	AC012490 Genomic DNA Translation: AAS07563.1
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	CCDS5456.1 [P49418-1]
PIR[i]	S62400
RefSeq[i]	NP_001626.1, NM_001635.3 [P49418-1]
	NP_647477.1, NM_139316.2 [P49418-2]

Genome annotation databases

Ensembl[i]	ENST00000325590; ENSP00000317441; ENSG00000078053 [P49418-2]
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GeneID[i]	273
KEGG[i]	hsa:273
UCSC[i]	uc003tgu.4, human [P49418-1]

Keywords - Coding sequence diversity[i]

Alternative splicing


NCBI Resources How To
Sign in to NCBI
Nucleotide
Nucleotide
amphiphysin
Search
Create alert Advanced
Help
COVID-19 Information
Public health information (CDC) | Research information (NIH) | SARS-CoV-2 data (NCBI) | Prevention and treatment information (HHS) | Español
Species
Animals (3,523)
Plants (4)
Fungi (372)
Protists (2)
Viruses (5)
Customize ...
Molecule types
genomic DNA/RNA (1,305)
mRNA (2,097)
Customize ...
Source databases
Summary 20 per page Sort by Default order
Send to:
Items: 1 to 20 of 3913
<< First < Prev Page 1 of 196 Next > Last >>
Danio rerio strain Tuebingen chromosome 2, GRCz11 Primary Assembly
1. 59,640,629 bp linear DNA
Accession: NC_007113.7 GI: 1196813952
Assembly BioProject BioSample Protein PubMed Taxonomy
GenBank FASTA Graphics
Filters: Manage Filters
Results by taxon
Top Organisms [Tree]
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Homo sapiens (269)
Mus musculus (105)
Saccharomyces cerevisiae (56)
Danio rerio (37)
All other taxa (3062)
More...

4. **Homo sapiens amphiphysin**, mRNA (cDNA clone MGC:34636 IMAGE:5181100), complete cds
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GenBank FASTA Graphics

5. **Homo sapiens amphiphysin** (AMPH), transcript variant 1, mRNA
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Accession: NM_001635.4 GI: 1519314873
Protein PubMed Taxonomy
GenBank FASTA Graphics

6. **Homo sapiens amphiphysin** (AMPH), transcript variant 2, mRNA
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Accession: NM_139316.3 GI: 1890270254
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GenBank FASTA Graphics

7. **Homo sapiens amphiphysin** II mRNA, complete cds
1,998 bp linear mRNA
Accession: U87558.1 GI: 2160718
Protein PubMed Taxonomy
GenBank FASTA Graphics

amphiphysin (3913) Nucleotide

Homo sapiens amphiphysin (AMPH), transcript variant 1, mRNA Nucleotide

Aifm2, a NADH oxidase, supports robust glycolysis and is required for cold- and ...

Isolation and Differentiation of Stromal Vascular Cells to Beige/Brite Cells

See more...

Homo sapiens amphiphysin (AMPH), transcript variant 1, mRNA

NCBI Reference Sequence: NM_001635.4

FASTA Graphics

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/note= major polyA site

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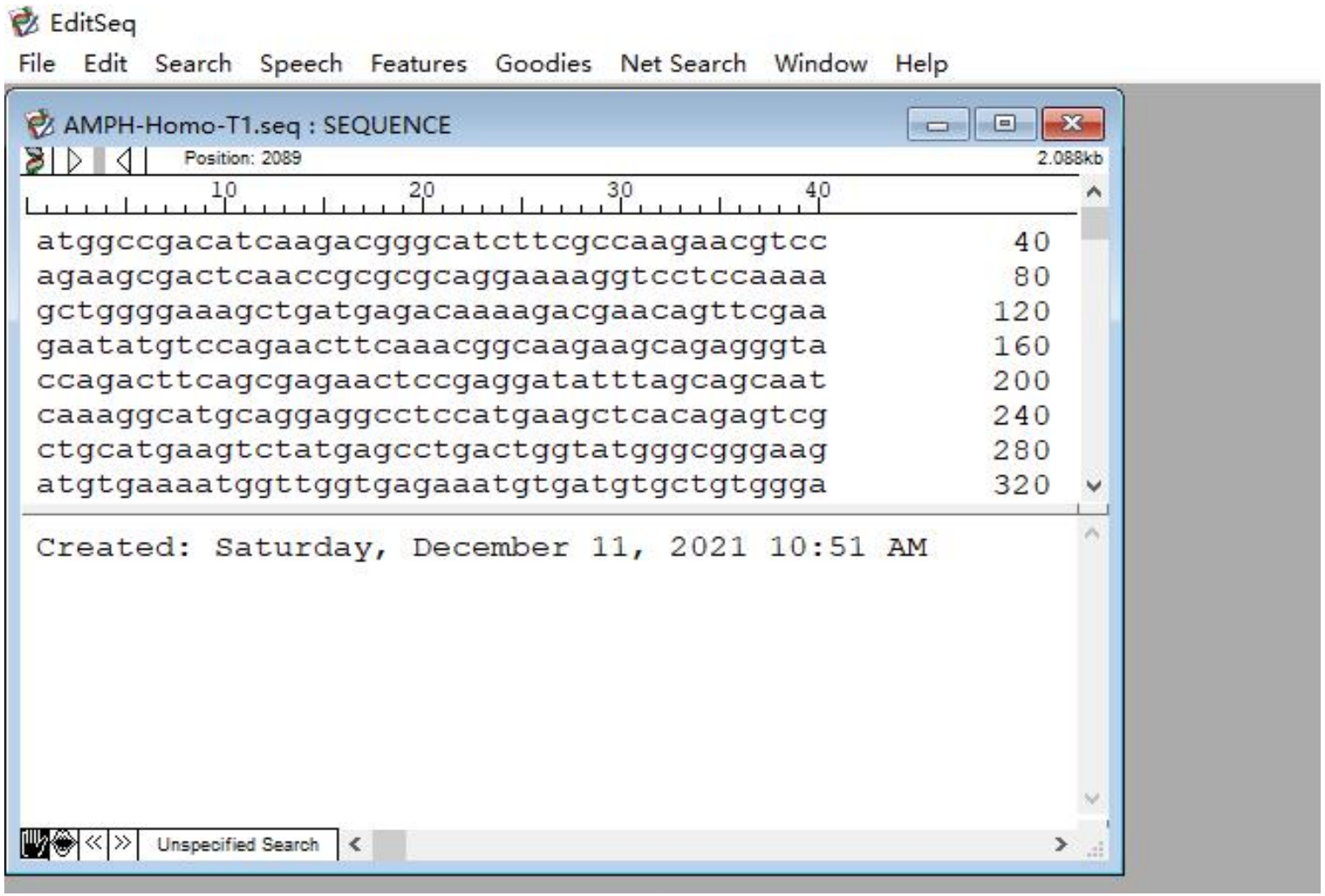
DNASTAR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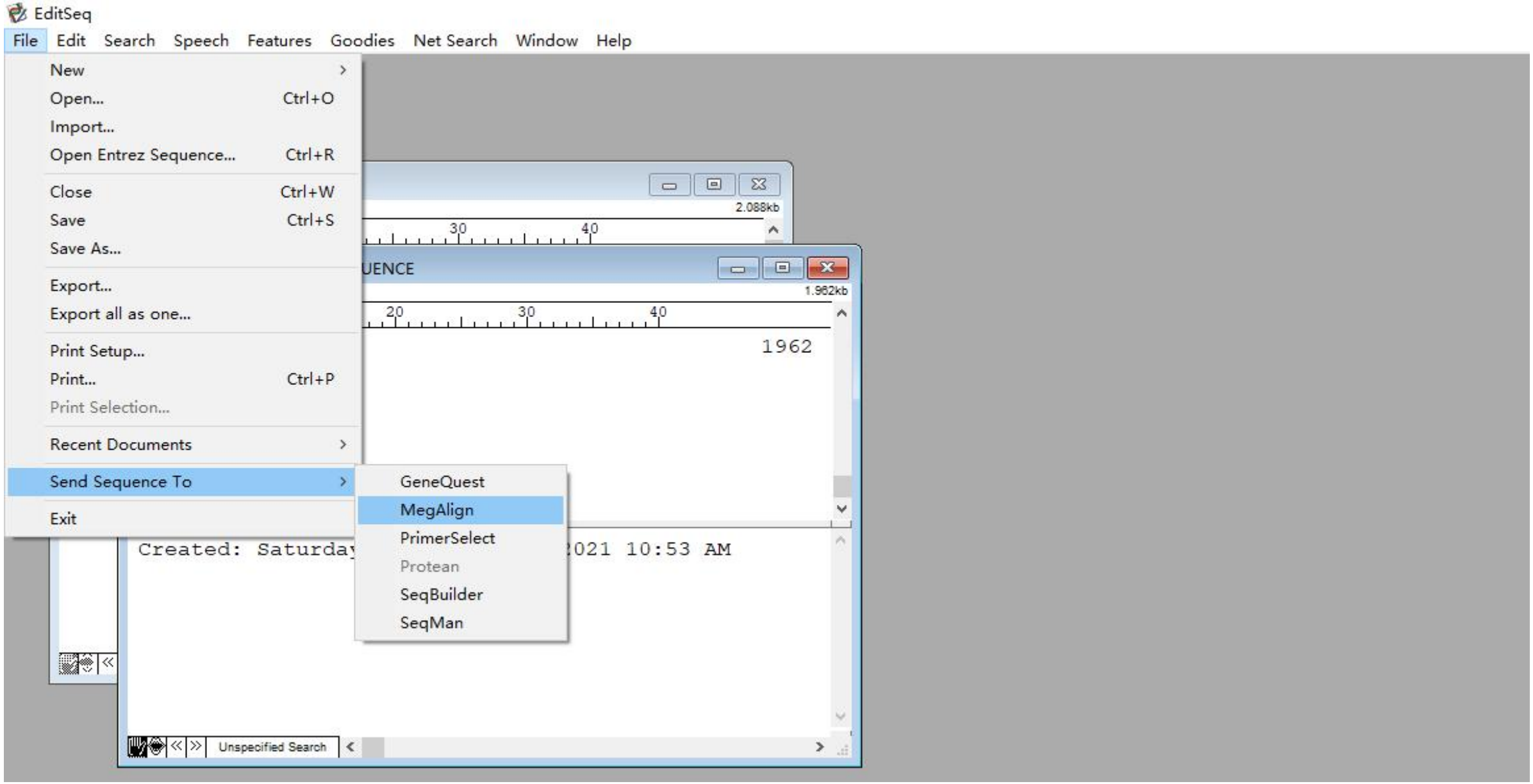
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Import...
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Save Ctrl+S
Save As...
Export...
Export all as one...
Print Setup...
Print... Ctrl+P
Print Selection...
Recent Documents
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Exit
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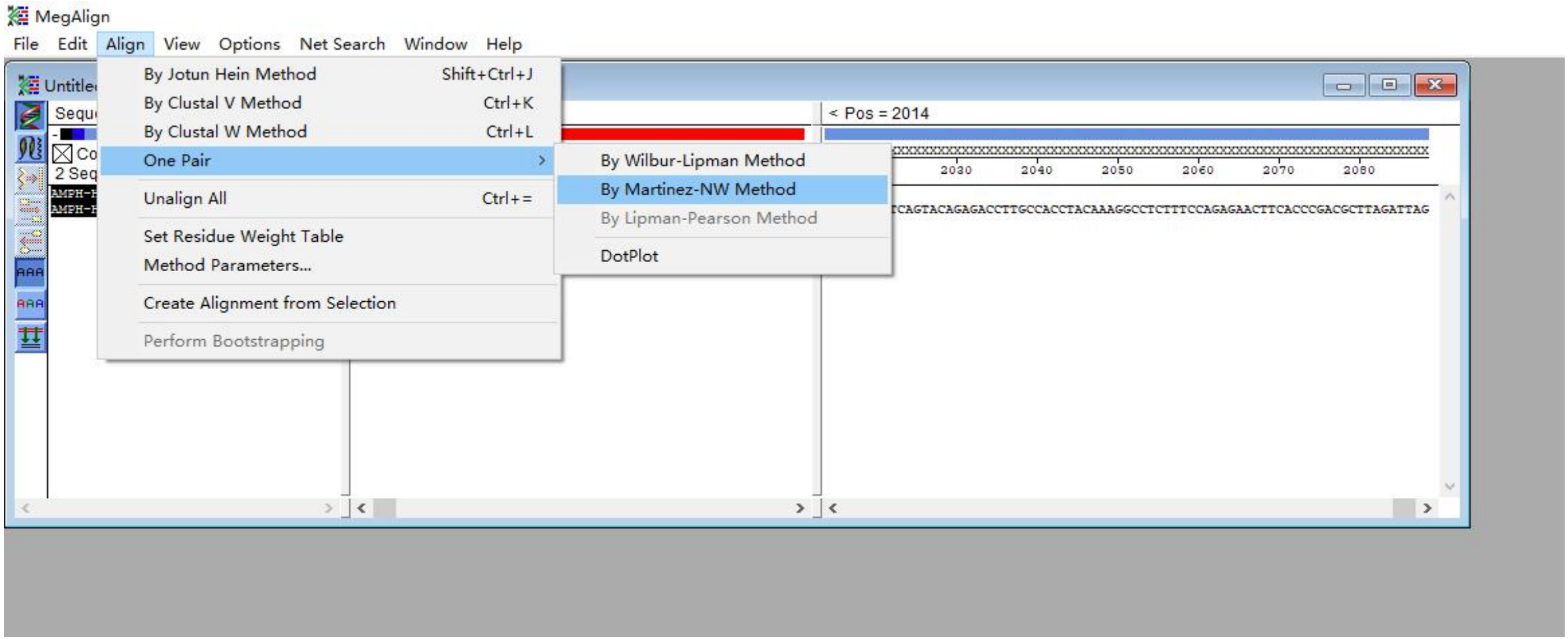
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Window
Help
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By Clustal V Method Ctrl+K
By Clustal W Method Ctrl+L
One Pair
Unalign All Ctrl+=
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Method Parameters...
Create Alignment from Selection
Perform Bootstrapping
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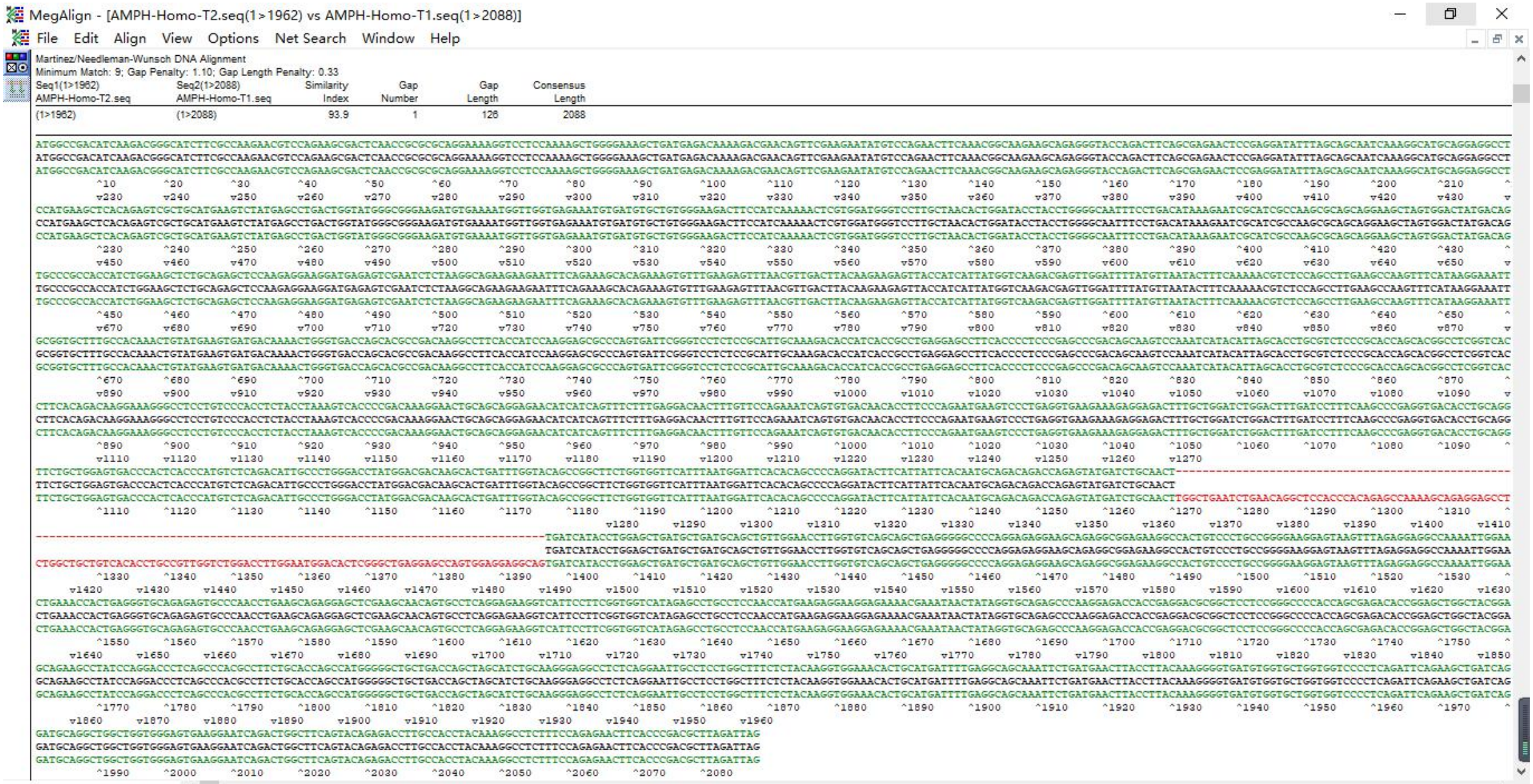
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Seq1(1>1962) Seq2(1>2088) Similarity Gap Gap Consensus
AMPH-Homo-T2.seq AMPH-Homo-T1.seq Index Number Length Length
(1>1962) (1>2088) 93.9 1 126 2088

目的片段的扩增

没有现成质粒：

1．通过查文献，看看这个基因在哪些组织中高表达，取组织，提RNA，反转成cDNA，以cDNA为模板，设计引物扩增目的片段。

2．如果组织取不到，也可以用对应种属的细胞系，提RNA，反转成cDNA，以cDNA为模板扩增。

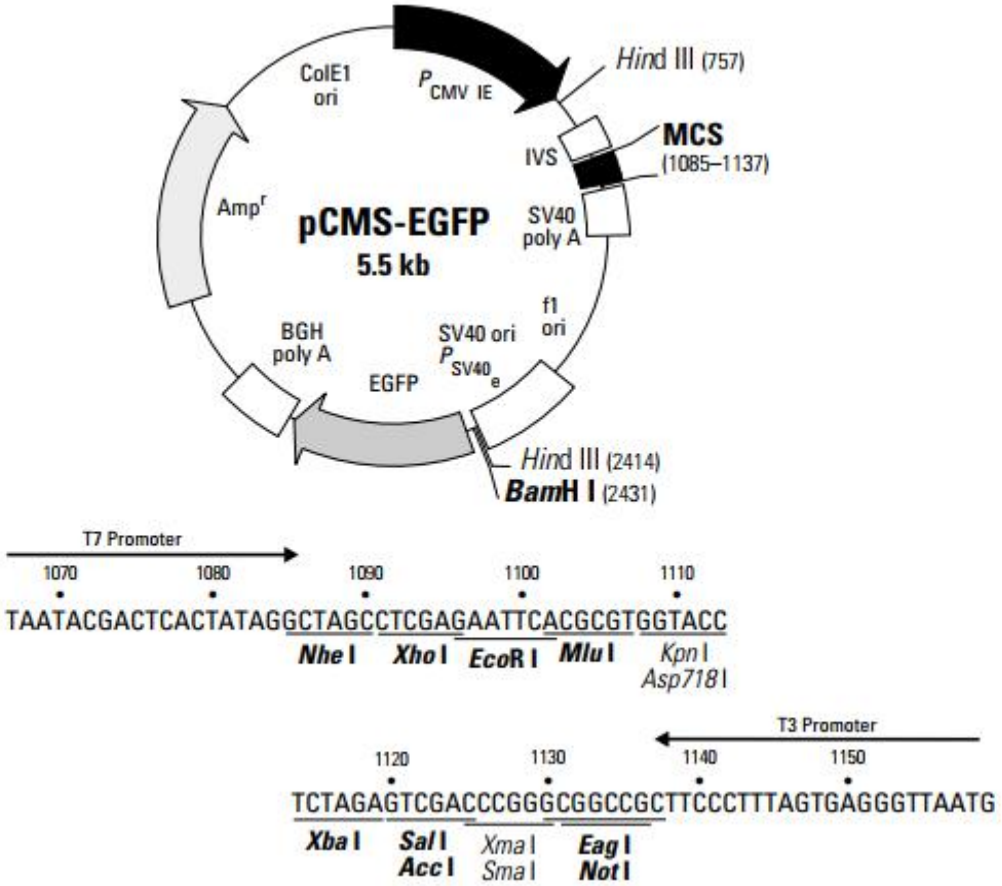
3．　　公司合成目的序列。

有现成质粒：

1．测序确定质粒的序列信息；
2．根据确定的序列信息，设计引物扩增目的片段。

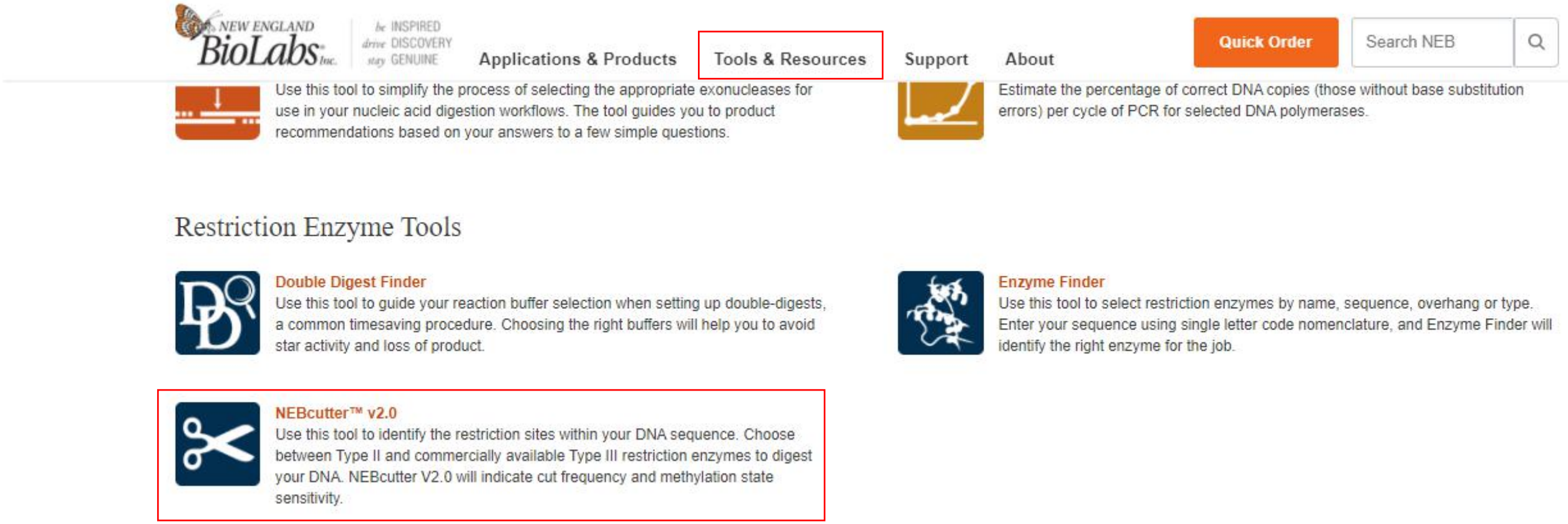
设计引物

1．首先确定目的片段连入的载体信息



2．确定目的片段连入载体的酶切位点

https://www.neb.com/



NEW ENGLAND BioLabs Inc.
[NEB homepage]

NEBcutter V2.0

Program Guide | Help | Comments

This tool will take a DNA sequence and find the large, non-overlapping open reading frames using the E.coli genetic code and the sites for all Type II and commercially available Type III restriction enzymes that cut the sequence just once. By default, only enzymes available from NEB are used, but other sets may be chosen. Just enter your sequence and "submit". Further options will appear with the output. **The maximum size of the input file is 1 MByte, and the maximum sequence length is 300 KBases.**

What's new in V2.0 Citing NEBcutter

Local sequence file: 选择文件 未选择任何文件

GenBank number: [Browse GenBank]

Standard sequences:
Plasmid vectors
Viral + phage

or paste in your DNA sequence: *(plain or FASTA format)*

```
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agccttgaag
ccaagtttcataaggaaattgcggtgctttgccacaaactgtatgaagtgatgacaaaactgggtgacca
gcacgccgac
aaggccttcaccatccaaggagcgcccagtgattcgggtcctctccgcattgcaaagacaccatcaccgc
ctgaggagcc
ttcacccctcccgagcccgacagcaagtccaaatcatacattagcacctgcgtctcccgcaccagcacgg
```

Submit

More options

Set colors

The sequence is: Linear / Circular

Enzymes to use:
- NEB enzymes
- All commercially available specificities
- All specificities
- All + defined oligonucleotide sequences
- Only defined oligonucleotide sequences

[define oligos]

NEW ENGLAND BioLabs Inc.
NEBcutter

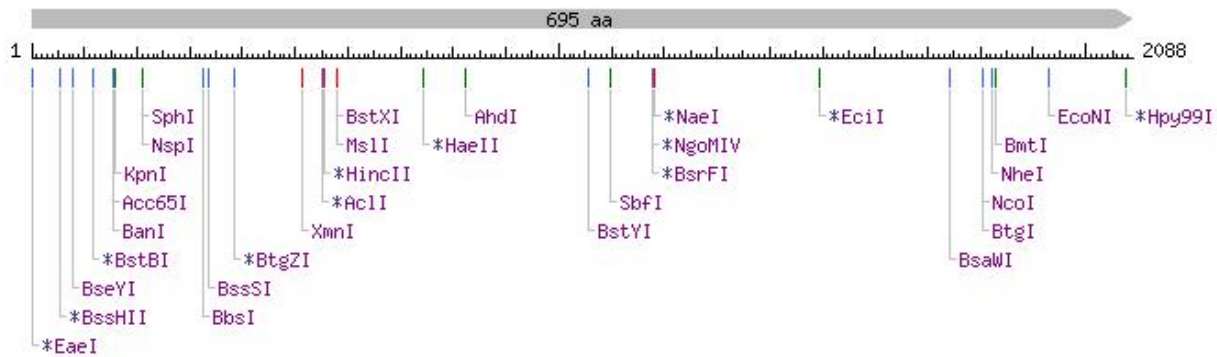
Linear Sequence: *unnamed sequence*

Help | Comments

Display: - NEB single cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs

GC=52%, AT=48%

Cleavage code	Enzyme name code
▼ | blunt end cut	Available from NEB
▼ | 5′ extension	Has other supplier
▼ | 3′ extension	Not commercially available
▼ | cuts 1 strand	*: cleavage affected by CpG meth.
	#: cleavage affected by other meth.
	(enz.name): ambiguous site



Main options	Availability	Display	Zoom	List
New DNA	All commercial	2 cutters	Zoom in	0 cutters
Custom digest	All	3 cutters	More...	1 cutters
View sequence				All sites
ORF summary				Save all sites
Save project				Flanking enzymes
Print				

Minimum ORF length to display: 100 aa. OK

Enzymes that don't cut

unnamed sequence

Number of cuts = 0 OK Save as text file

#	Enzyme	Specificity
1	AatII	G▲ACGT▼C
2	AccI	GT▼MK▲AC
3	AfeI	AGC▼GCT
4	AflII	C▼TTAA▲G
5	AflIII	A▼CRYG▲T
6	AgeI	A▼CCGG▲T
7	AleI	CACNN▼NNGTG
8	ApaLI	G▼TGCA▲C
9	AscI	GG▼CGCG▲CC
10	AseI	AT▼TA▲AT
11	AsiSI	GCG▲AT▼CGC
12	AvrII	C▼CTAG▲G
13	BaeI	▲(N)5▼(N)10ACNNNNGTAYC(N)7▲(N)5▼
14	BamHI	G▼GATC▲C
15	BcgI	▲NN▼(N)10CGA(N)6TGC(N)10▲NN▼
16	BglII	A▼GATC▲T
17	BlpI	GC▼TNA▲GC
18	BmgBI	CAC▼GTC

根据这个表格，与载体上多克隆位点的酶切位点进行匹配，筛选出两个常用的内切酶

TAATACGACTCACTATAGGCTAGCATGGATTACAAGGATGACGACGATAAGGAG CTC CAT ATG ACT AGT CTC G

Nhe1 flag Sac1 Nde1 Spe1 Xho1

AG AAT TCA CGC GTG GTA CCT CTA GAG TCG ACC CGG GCG GCC GCT TCC CTT TAG TGA GGG TTA ATG

EcoR1 Mlu1 Kpn1 Xba1 Sal1 Xma1 Eag1

Acc1 Sma1 Not1

选酶切位点时，一定要考虑移码的问题

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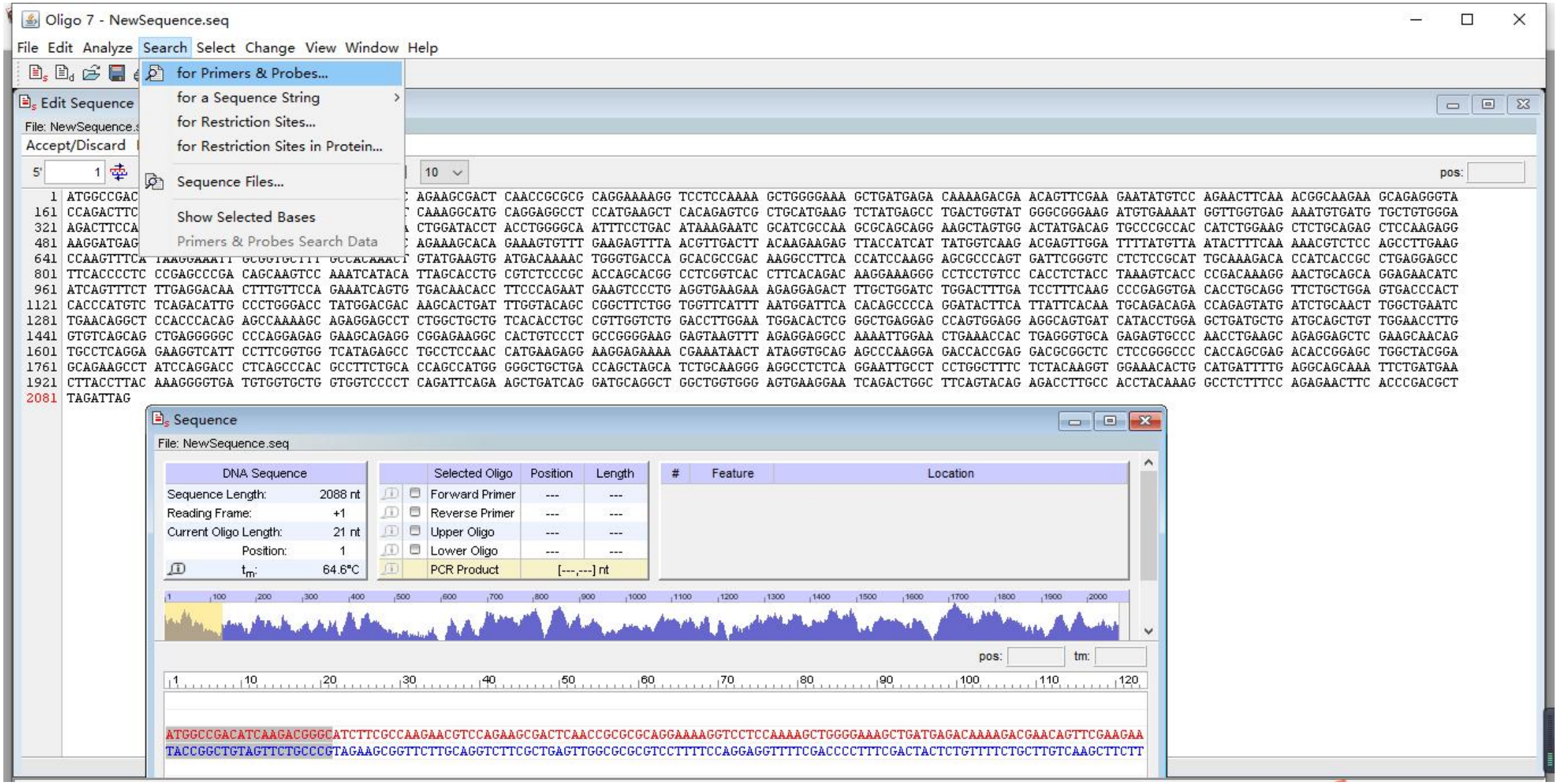
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Show Selected Bases
Primers & Probes Search Data
Edit Sequence
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Accept/Discard
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Lower Oligo --- ---
PCR Product [---,---] nt
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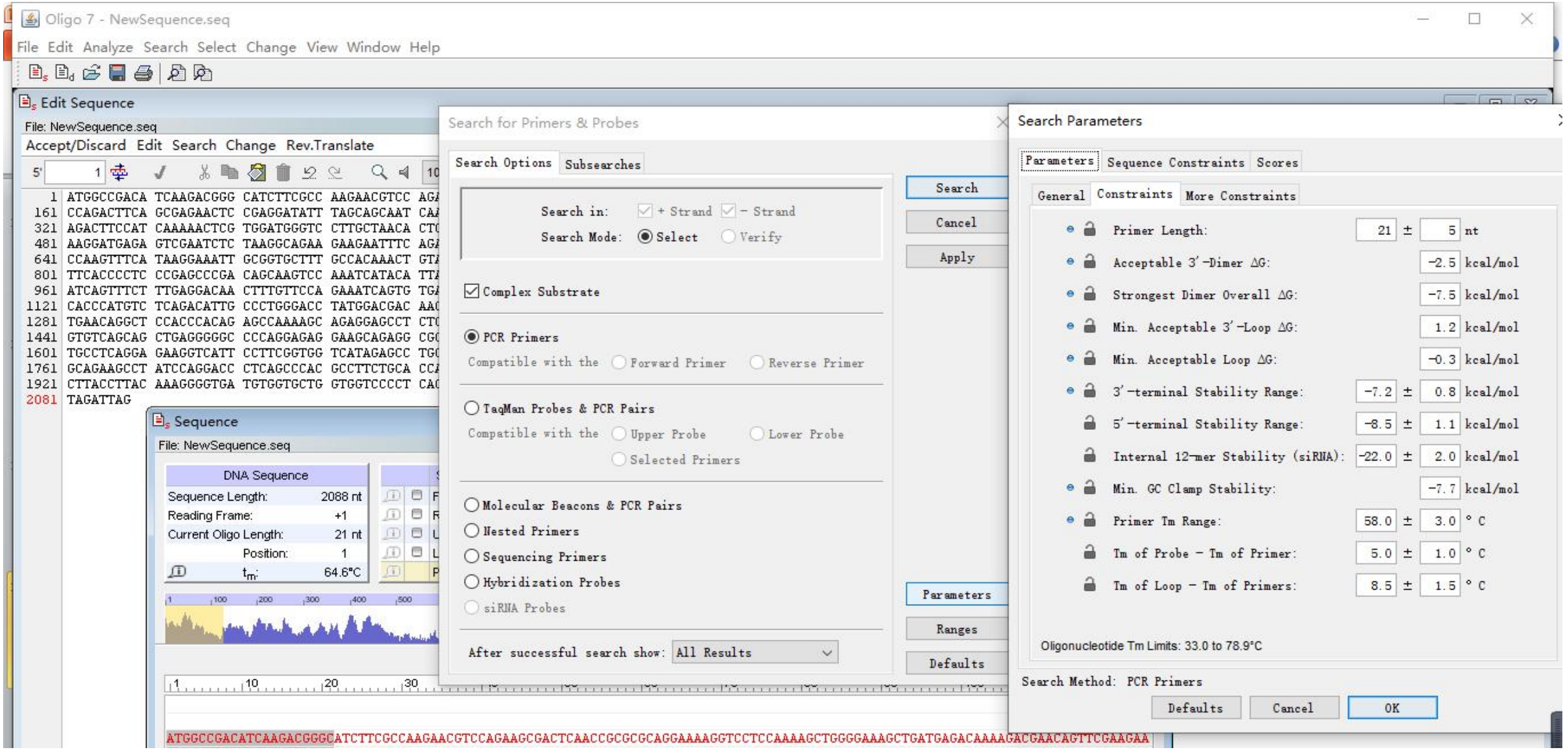
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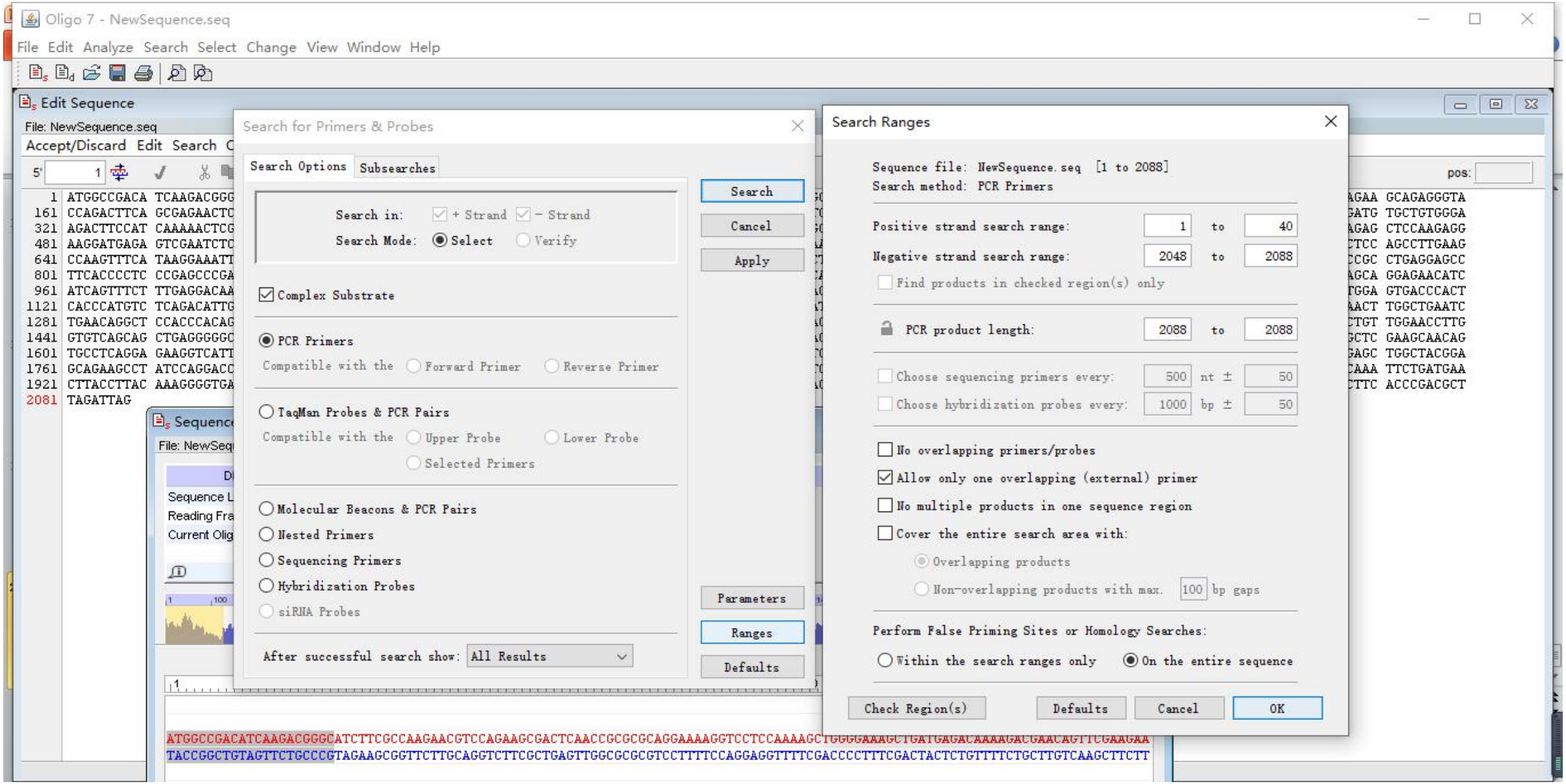
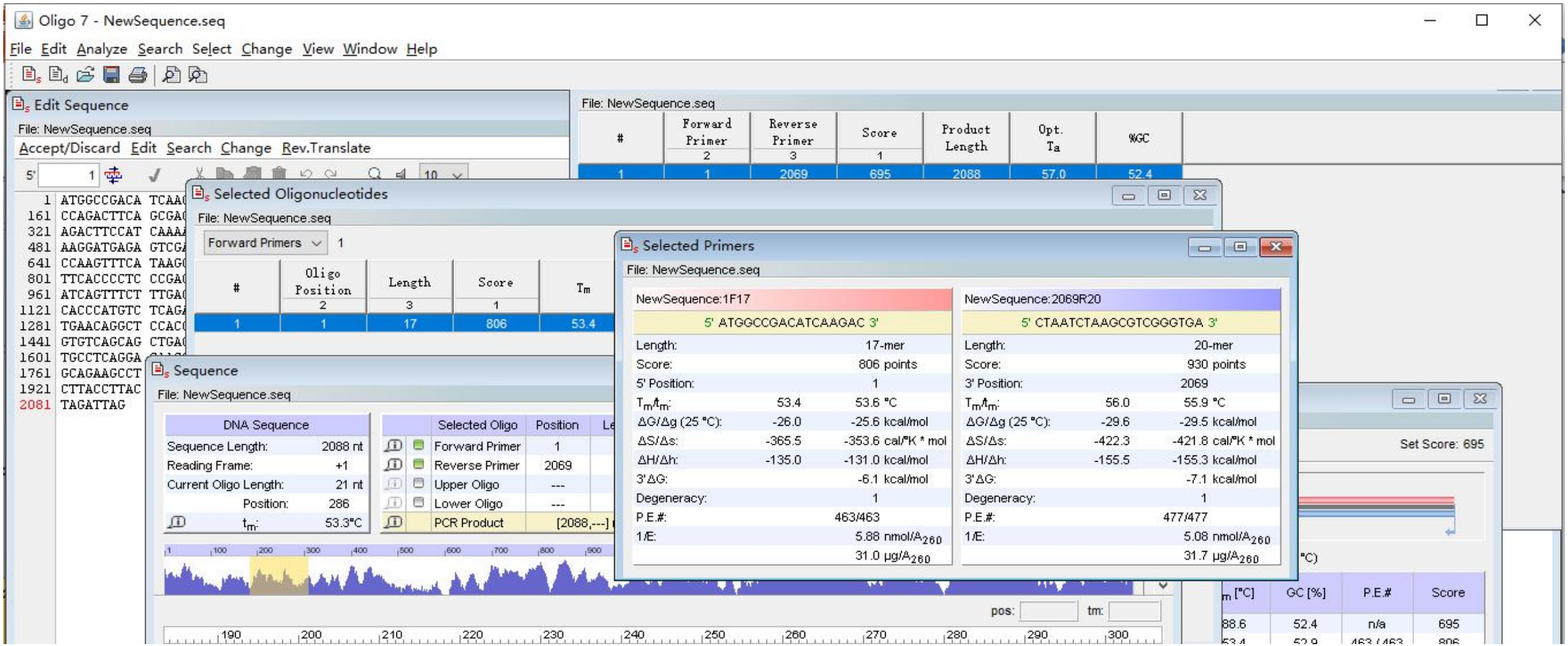
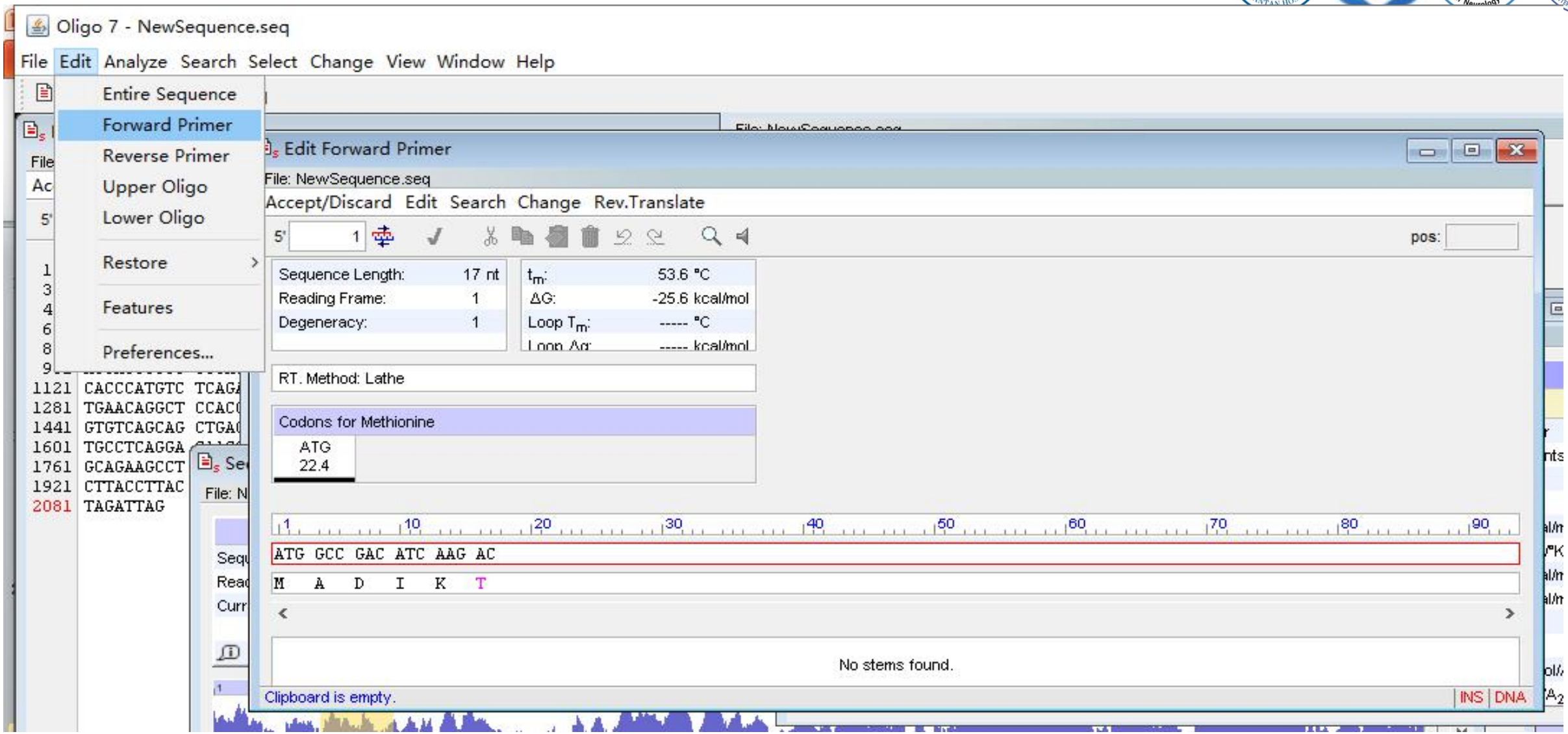
Position: 1
t_m: 64.6°C
ATGGCCGACATCAAGACGGGCATCTTCGCCAAGAACGTCCAGAAGCGACTCAACCGCGCGCAGGAAAAGGTCCTCCAAAAGCTGGGGAAAGCTGATGAGACAAAAGACGAACAGTTCGAAGAA
Search for Primers & Probes
Search Options
Subsearches
Search in: + Strand − Strand
Search Mode: Select Verify
Complex Substrate
PCR Primers
Compatible with the Forward Primer Reverse Primer
TaqMan Probes & PCR Pairs
Compatible with the Upper Probe Lower Probe
Selected Primers
Molecular Beacons & PCR Pairs
Nested Primers
Sequencing Primers
Hybridization Probes
siRNA Probes
After successful search show: All Results
Search
Cancel
Apply
Parameters
Ranges
Defaults
Search Parameters
Parameters
Sequence Constraints
Scores
General
Constraints
More Constraints
Primer Length: 21 ± 5 nt
Acceptable 3′-Dimer ΔG: −2.5 kcal/mol
Strongest Dimer Overall ΔG: −7.5 kcal/mol
Min. Acceptable 3′-Loop ΔG: 1.2 kcal/mol
Min. Acceptable Loop ΔG: −0.3 kcal/mol
3′-terminal Stability Range: −7.2 ± 0.8 kcal/mol
5′-terminal Stability Range: −8.5 ± 1.1 kcal/mol
Internal 12-mer Stability (siRNA): −22.0 ± 2.0 kcal/mol
Min. GC Clamp Stability: −7.7 kcal/mol
Primer Tm Range: 58.0 ± 3.0 °C
Tm of Probe − Tm of Primer: 5.0 ± 1.0 °C
Tm of Loop − Tm of Primers: 8.5 ± 1.5 °C
Oligonucleotide Tm Limits: 33.0 to 78.9°C
Search Method: PCR Primers
Defaults
Cancel
OK


Oligo 7 - NewSequence.seq
File Edit Analyze Search Select Change View Window Help
Edit Sequence
File: NewSequence.seq
Accept/Discard Edit Search
Search for Primers & Probes
Search Options
Subsearches
Search in: + Strand - Strand
Search Mode: Select Verify
Complex Substrate
PCR Primers
Compatible with the Forward Primer Reverse Primer
TaqMan Probes & PCR Pairs
Compatible with the Upper Probe Lower Probe
Selected Primers
Molecular Beacons & PCR Pairs
Nested Primers
Sequencing Primers
Hybridization Probes
siRNA Probes
After successful search show: All Results
Search
Cancel
Apply
Parameters
Ranges
Defaults
Search Ranges
Sequence file: NewSequence.seq [1 to 2088]
Search method: PCR Primers
Positive strand search range: 1 to 40
Negative strand search range: 2048 to 2088
Find products in checked region(s) only
PCR product length: 2088 to 2088
Choose sequencing primers every: 500 nt ± 50
Choose hybridization probes every: 1000 bp ± 50
No overlapping primers/probes
Allow only one overlapping (external) primer
No multiple products in one sequence region
Cover the entire search area with:
Overlapping products
Non-overlapping products with max. 100 bp gaps
Perform False Priming Sites or Homology Searches:
Within the search ranges only
On the entire sequence
Check Region(s)
Defaults
Cancel
OK


Oligo 7 - NewSequence.seq
File Edit Analyze Search Select Change View Window Help
Edit Sequence
File: NewSequence.seq
Accept/Discard Edit Search Change Rev.Translate
File: NewSequence.seq
| Forward Primer 2 | Reverse Primer 3 | Score 1 | Product Length | Opt. Ta | %GC
1 | 1 | 2069 | 695 | 2088 | 57.0 | 52.4
Selected Oligonucleotides
File: NewSequence.seq
Forward Primers 1
| Oligo Position 2 | Length 3 | Score 1 | Tm
1 | 1 | 17 | 806 | 53.4
Selected Primers
File: NewSequence.seq
NewSequence:1F17
5' ATGGCCGACATCAAGAC 3'
Length: 17-mer
Score: 806 points
5' Position: 1
Tm/tm: 53.4 53.6 °C
ΔG/Δg (25 °C): -26.0 -25.6 kcal/mol
ΔS/Δs: -365.5 -353.6 cal/°K * mol
ΔH/Δh: -135.0 -131.0 kcal/mol
3'ΔG: -6.1 kcal/mol
Degeneracy: 1
P.E.#: 463/463
1/E: 5.88 nmol/A260
31.0 µg/A260
NewSequence:2069R20
5' CTAATCTAAGCGTCGGGTGA 3'
Length: 20-mer
Score: 930 points
3' Position: 2069
Tm/tm: 56.0 55.9 °C
ΔG/Δg (25 °C): -29.6 -29.5 kcal/mol
ΔS/Δs: -422.3 -421.8 cal/°K * mol
ΔH/Δh: -155.5 -155.3 kcal/mol
3'ΔG: -7.1 kcal/mol
Degeneracy: 1
P.E.#: 477/477
1/E: 5.08 nmol/A260
31.7 µg/A260
Sequence
File: NewSequence.seq
DNA Sequence
Sequence Length: 2088 nt
Reading Frame: +1
Current Oligo Length: 21 nt
Position: 286
tm: 53.3°C
Selected Oligo | Position
Forward Primer 1
Reverse Primer 2069
Upper Oligo ---
Lower Oligo ---
PCR Product [2088,---]
Set Score: 695
m [°C] | GC [%] | P.E.# | Score
88.6 | 52.4 | n/a | 695
pos:
tm:


Oligo 7 - NewSequence.seq
File Edit Analyze Search Select Change View Window Help
Entire Sequence
Forward Primer
Reverse Primer
Upper Oligo
Lower Oligo
Restore
Features
Preferences...
Edit Forward Primer
File: NewSequence.seq
Accept/Discard Edit Search Change Rev.Translate
5' 1
pos:
Sequence Length: 17 nt
Reading Frame: 1
Degeneracy: 1
t_m: 53.6 °C
ΔG: -25.6 kcal/mol
Loop T_m: ----- °C
Loop Δg: ----- kcal/mol
RT. Method: Lathe
Codons for Methionine
ATG
22.4
1 10 20 30 40 50 60 70 80 90
ATG GCC GAC ATC AAG AC
M A D I K T
No stems found.
Clipboard is empty.
INS DNA
1121 CACCCATGTC TCAGA
1281 TGAACAGGCT CCAC
1441 GTGTCAGCAG CTGA
1601 TGCCTCAGGA
1761 GCAGAAGCCT
1921 CTTACCTTAC
2081 TAGATTAG

F：CCG CTCGAG ATGGCCGACATCAAGAC
R：GC TCTAGA CTAATCTAAGCGTCGGGTGA

NEB 保护碱基表（根据固定的序列）

我的经验：其实，对于常用的一些很好使的内切酶，如 HindIII、BamHI、SacI 等，保护碱基[size=1em]可以任意选择。此时，可以考虑使用与模板上对应序列配对的一个或者几个碱基作为保护碱基。如果两条引物的 GC 含量和 Tm 值相差较大，还可以通过调整保护碱基来使之接近。

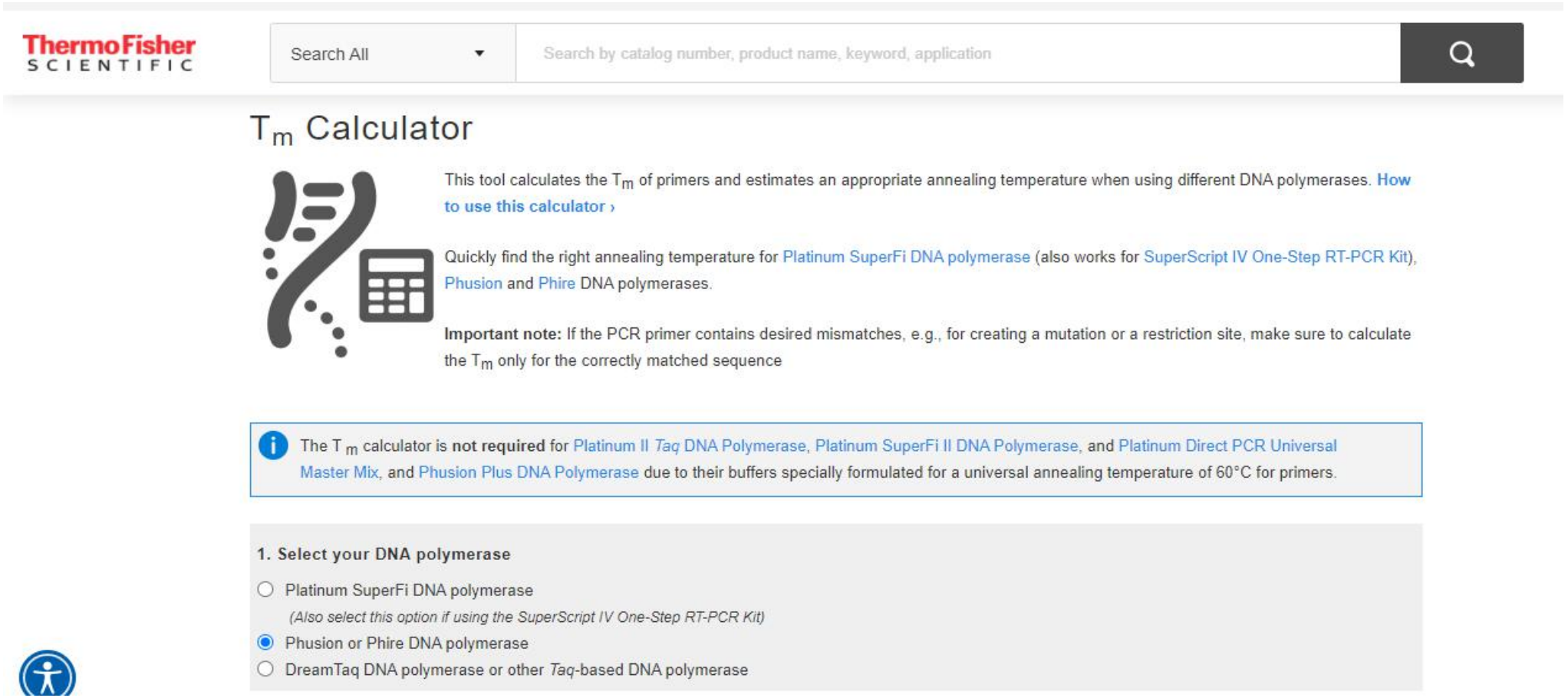
下面是 NEB 公司网站提供的保护碱基参考。

Enzyme	Oligo Sequence	Chain Length	% Cleavage 2 hr	% Cleavage 20 hr
	GGTCGACC	8	0	0
AccI	CGGTCGACCG	10	0	0
	CCGGTCGACCGG	12	0	0
	CACATGTG	8	0	0
AflIII	CCACATGTGG	10	>90	>90
	CCCACATGTGGG	12	>90	>90
	GGCGCGCC	8	>90	>90
AscI	AGGCGCGCCT	10	>90	>90
	TTGGCGCGCCAA	12	>90	>90
	CCCCGGGG	8	50	>90
AvaI	CCCCCGGGGG	10	>90	>90
	TCCCCCGGGGGA	12	>90	>90

目的片段扩增

PCR由变性--退火--延伸三个基本反应步骤构成：

- 确定退火温度；
- 延伸时间：30s/1kb



2. Select input method

- (●) Single pair
- ( ) Batch

3. Type or paste your sequence

Primer#1: 5'- ATGGCAAGCTTATCAAGAC 19 nt: A=7.0 T=4.0 C=4.0 G=4.0 CG=42.11%

Primer#2: 5'- TTAGCCAGAGAGGGCTTC 18 nt: A=4.0 T=4.0 C=4.0 G=8.0 CG=55.56%

Clear

4. PCR conditions

Primer conc. 0.5 µM

Results

Export table data into Excel

ID #1	Sequence #1	Molecular weight g/mol	Extinction coefficient l/(mol˙cm)	Tm °C	ID #2	Sequence #2	Molecular weight g/mol	Extinction coefficient l/(mol˙cm)	Tm °C	Annealing Temperature °C
Primer#1	ATGGCAAGCTTATCAAGAC	5820.9	191200.0	58.5	Primer#2	TTAGCCAGAGAGGGCTTC	5539.7	174100.0	61.3	58.5

酶切

- 目的片段的PCR产物酶切；
- 载体酶切

Restriction Enzyme Single/Double Digestion

Transition to new BSA-free NEBuffer™: View Announcement.

XhoI

XbaI

✖ clear 2nd selection

Digest in rCutSmart Buffer Show Detailed Protocol

Name	Cat #	Temp °C	Supplied Buffer	Add SAM	% Activity in NEBuffer™			
					r1.1	r2.1	r3.1	rCutSmart
XhoI	R0146	37	rCutSmart Buffer	No	75	100	100	100
XbaI	R0145	37	rCutSmart Buffer	No	10	100	75	100

Name	Time-Saver ™	Heat Inactivation (°C)	Methylation Sensitivity
XhoI	Yes	65	cpg (Impaired)
XbaI	Yes	65	dam (Blocked by Overlapping)

Notes:

1. Digest in rCutSmart Buffer (or NEBuffer 4 + rAlbumin) at 37 °C.

酶连

外源DNA片段与质粒载体的连接　　（10ul）

DNA片段	3ul
载体	1ul
T4 DNA Ligase	1ul
Buffer T4 DNA Ligase	1ul
水	4ul

反应条件：22℃水浴3-4h或者16℃水浴过夜。

一般目的片段：载体摩尔比约为3:1，但是通常也可以按体积比。

连接产物转化

将10ul连接产物加入到不少于5-7倍体积感受态细胞溶液中，冰浴30min，42℃热激30s后，冰上静置2min；加入500ul LB培养基，37℃ 200rmp恒温培养1h；4000rmp离心1min弃上清，同时保留100ul混匀菌体沉淀后均匀涂布抗性平板上；37℃恒温箱培养过夜。

质粒转化

取质粒1ul或50-100ng加入10ul感受态细胞溶液中，步骤同上，但是37℃ 200rmp恒温培养1h，一般不用离心，直接取20-30ul涂板就可以。

注：
1 一定要区分质粒和连接产物转化的不同。
2 根据实验要求选择所需要的感受态。
3 在选择抗性平板的时候要根据所选载体来确定。

挑单克隆，摇菌，提质粒

一般挑2-3个单克隆，加入10ml对应抗性的培养基；摇过夜，提质粒。

细胞转染---脂质体转染

利用带正电的阳离子脂质体，与带负电的核酸磷酸骨架形成复合物，经过内吞被导入细胞。

Lip3000

Lipofectamine™ 3000 Reagent

USER GUIDE

invitrogen

Document Part No. 100022234 Publication No. MAN0009872 Rev C.0

Package Contents

Catalog Numbers	Size:
L3000001	0.1 mL
L3000008	0.75 mL
L3000015	1.5 mL
L3000075	5 × 1.5 mL
L3000150	15 mL

Storage Conditions

- Store at 4°C (do not freeze).

Required Materials

- Plasmid DNA (0.5–5 µg/µL stock)
- Opti-MEM™ Reduced Serum Medium
- Microcentrifuge tubes

Timing

Preparation: 10 minutes
Incubation: 10–15 minutes
Final Incubation: 1–3 days

Selection Guide

Lipofectamine™ Reagents
Go online to view related products.

Product Description

- Lipofectamine™ 3000 Reagent is a proprietary formulation for transfecting nucleic acids into a wide range of eukaryotic cells and especially designed for hard to transfect cells

Important Guidelines

- Make DNA-Lipofectamine™ 3000 complexes in serum-free medium such as Opti-MEM™ Reduced Serum Medium and add directly to cells in culture medium, in the presence or absence of serum/antibiotic.
- It is not necessary to remove complexes or change/add medium after transfection.
- The amount of Lipofectamine™ 3000 Reagent for successful transfection varies. Start any new transfection by testing the recommended two concentrations of Lipofectamine™ 3000 Reagent to determine an optimum amount.

Lipofectamine™ 3000 Reagent Protocol

Protocol Outline

A. Plate cells so they will be 70–90% confluent at the time of transfection.
B. Prepare plasmid DNA-lipid complexes (recommend 2 doses of lipid).
C. Add DNA-lipid complexes to cells.

Transfection Amounts

Component	96-well	24-well	6-well
DNA per well	100 ng	500 ng	2500 ng
P3000™ Reagent per well	0.2 µL	1 µL	5 µL
Lipofectamine™ 3000 Reagent per well	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL

Transfection of siRNA

To transfect cells with siRNA, follow the protocol as described for DNA but **do not** add P3000™ Reagent when diluting the siRNA (step 3).

Limited Product Warranty

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Timeline			Steps
Day 0	1		Seed cells to be 70–90% confluent at transfection
Day 1	2		Dilute Lipofectamine™ 3000 Reagent in Opti-MEM™ Medium (2 tubes) – Mix well
	3		Prepare master mix of DNA by diluting DNA in Opti-MEM™ Medium, then add P3000™ Reagent – Mix well
	4		Add Diluted DNA to each tube of Diluted Lipofectamine™ 3000 Reagent (1:1 ratio)
	5		Incubate
	6		Add DNA-lipid complex to cells
Day 2–4	7		Visualize/analyze transfected cells

Procedure Details (Two Reaction Optimization)

Component	96-well	24-well	6-well
Adherent cells	1–4 × 10^4	0.5–2 × 10^5	0.25–1 × 10^6
Opti-MEM™ Medium	5 µL × 2	25 µL × 2	125 µL × 2
Lipofectamine™ 3000 Reagent	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL
Opti-MEM™ Medium	10 µL	50 µL	250 µL
DNA (0.5–5 µg/µL)	0.2 µg	1 µg	5 µg
P3000™ Reagent (2 µL/µg DNA)	0.4 µL	2 µL	10 µL
Diluted DNA (with P3000™ Reagent)	5 µL	25 µL	125 µL
Diluted Lipofectamine™ 3000 Reagent	5 µL	25 µL	125 µL

Incubate for 10–15 minutes at room temperature.

Component (per well)	96-well	24-well	6-well
DNA-lipid complex	10 µL	50 µL	250 µL
DNA amount	100 ng	500 ng	2500 ng
P3000™ Reagent	0.2 µL	1 µL	5 µL
Lipofectamine™ 3000 Reagent used	0.15 and 0.3 µL	0.75 and 1.5 µL	3.75 and 7.5 µL

Incubate cells for 2–4 days at 37°C. Then, analyze transfected cells.

Vigofect

操作方法：

准备培养细胞：

1) 转染前24小时，接种适量细胞（接种数量可参考附表）。至转染时细胞密度以40~60%为宜（80~90%亦可）。

2) 转染前1小时，更换新鲜的完全培养液（体积可参考附表），置37℃，5% CO_2 培养。

配制转染工作液：（6孔板或35 mm平皿，2 ml培养液）

3) 取5~8 μg DNA（起始用量5 μg），加入稀释液中至总体积为100 μl，轻轻混匀，室温放置。

4) 取VigoFect 1~4μl（起始用量2 μl），加入稀释液中至总体积为100 μl，轻轻混匀，室温放置5分钟。

5) 将稀释的VigoFect逐滴加入稀释的DNA溶液中，轻轻混匀，所得的转染工作液在室温放置15分钟。

6) 将转染工作液轻轻混匀，逐滴加入2 ml培养液中，轻轻混匀培养液，置37℃，5% CO_2 培养。

细胞后续处理：

7) 24~48小时后，观察或收取细胞。

8) 稳定转染时，于转染后24~48小时消化细胞分至3~5个培养皿中，加适当浓度的相应抗生素（如G418）筛选。

表：建议的起始转染条件。

培养容器	转染前一天接种细胞数	转染时培养液体积	DNA用量与稀释后体积		VigoFect用量与稀释后体积	
	万	ml	μg	μl	μl	μl
96孔板	1-1.5	0.1	0.25	5	0.1	5
24孔板	5-10	0.5	1.25	25	0.5	25
6孔板	20-40	2	5	100	2	100
35mm平皿	20-40	2	5	100	2	100
60mm平皿	40-60	4	10	200	4	200

提高转染效率的方法：

1) 使用高质量、高纯度的质粒DNA。
2) 转染时细胞应处在生长旺盛状态。
3) 从起始用量开始，调整配制转染液中DNA和VigoFect的用量（保持转染工作液总体积不变），以确定不同细胞的最佳转染条件。一般固定DNA用量（5 μg），与系列含量的VigoFect混合，选取VigoFect的最佳用量；也可固定VigoFect用量（2 μl），与系列含量的DNA混合，选取DNA的最佳用量。
4) 进一步减少转染时培养液体积（转染后6~16小时再补加足量培养液）。

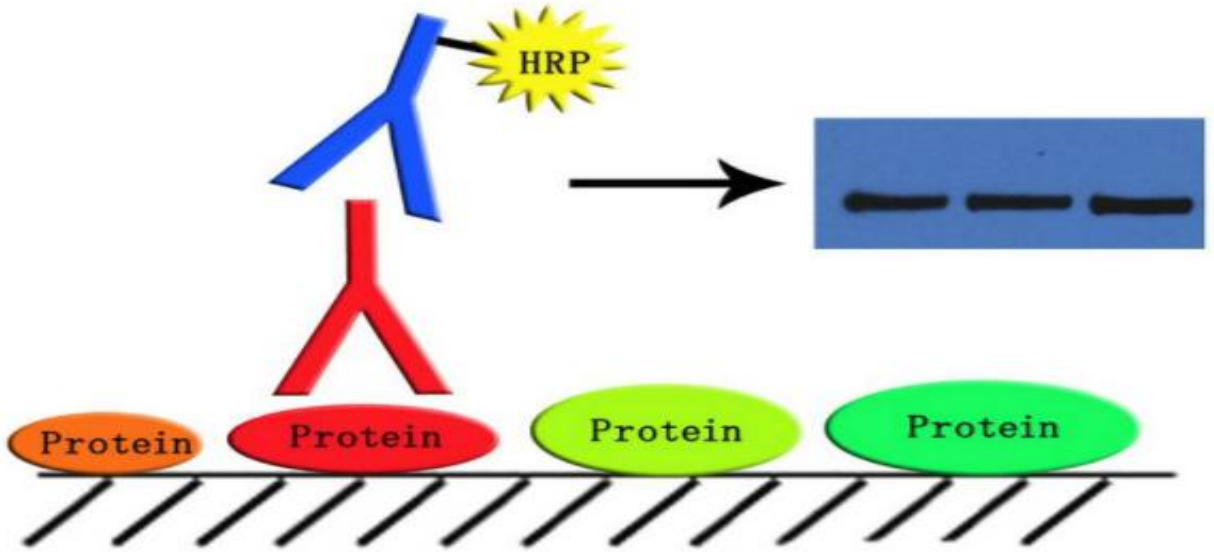
Western blot

为什么要做?

- 研究一些体外的蛋白质分子，寻找目的蛋白是否存在于样品当中。
- 蛋白质的表达情况，上调或下调表达，在不同的样品中，如疾病与正常的样品之间的表达差异性。

Western blot实验原理

Western blot 是利用已知的特异性抗体与抗原（即组织细胞中的目的蛋白）能特异性结合的原理，通过化学反应使标记于结合后的特异性抗体上的显示剂显示一定的颜色或发光来对组织细胞内的目的蛋白定性及半定量的研究。



Western blot流程

样本制备

SDS-PAGE电泳

转印

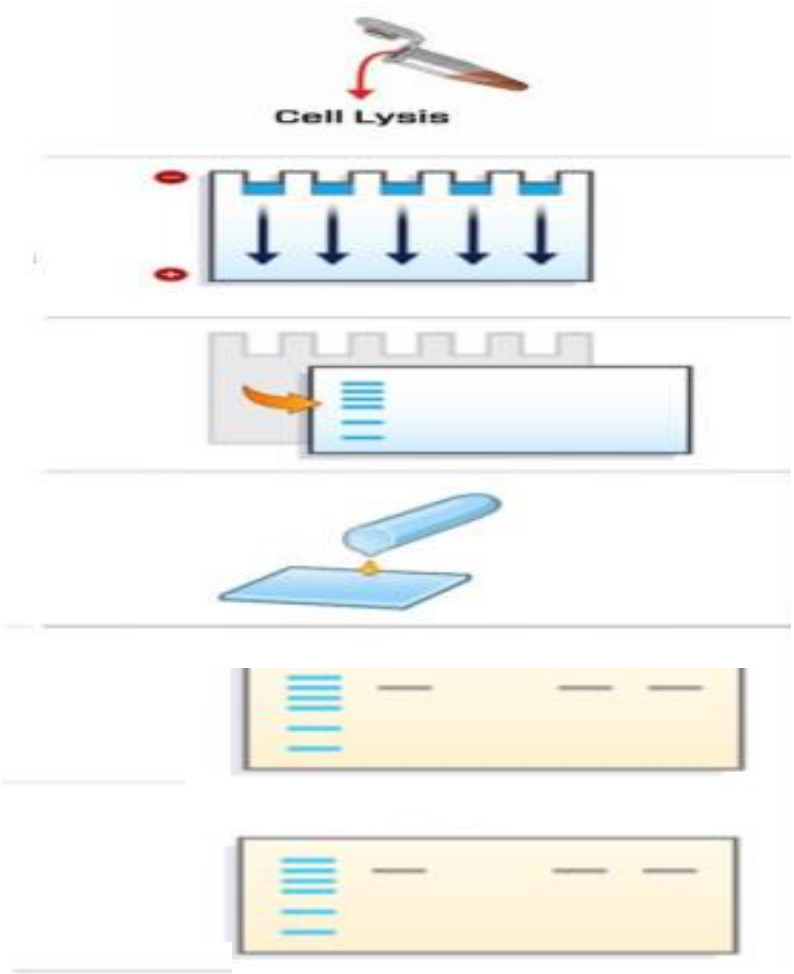
封闭

孵育一抗

孵育二抗

化学发光检测

近红外荧光检测



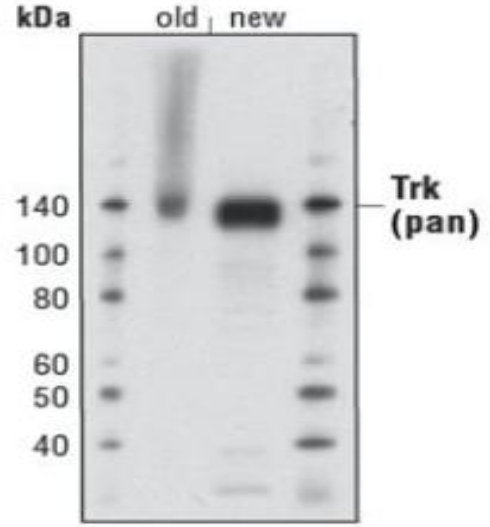
蛋白样品制备

确保样本充分裂解，预防蛋白降解

- 样本制备时建议采用细胞裂解/组织匀浆和超声破碎相结合的方法
- 尽可能地使用新鲜样本

组织匀浆器

超声细胞粉碎机



SDS-PAGE电泳

- SDS是一种很强的阴离子表面活性剂，它可以断开分子内和分子间的氢键，破坏蛋白质分子的二级和三级结构。
- 强还原剂巯基乙醇可以断开二硫键，破坏蛋白质的四级结构，使蛋白质分子被解聚成肽链形成单链分子。解聚后的侧链与SDS充分结合形成带负电荷的蛋白质-SDS复合物。
- 蛋白质-SDS复合物在凝胶电泳时，不再受蛋白质电荷和形状的影响，而只取决于蛋白质分子量的大小。

	作用	缓冲液PH	凝胶浓度
浓缩胶	使蛋白样品浓缩	pH6.8Tris-Cl	低，2-5%
分离胶	使蛋白样品分离	pH8.8Tris-Cl	高，根据蛋白大小

转膜

常用的转膜方法有湿转和半干转

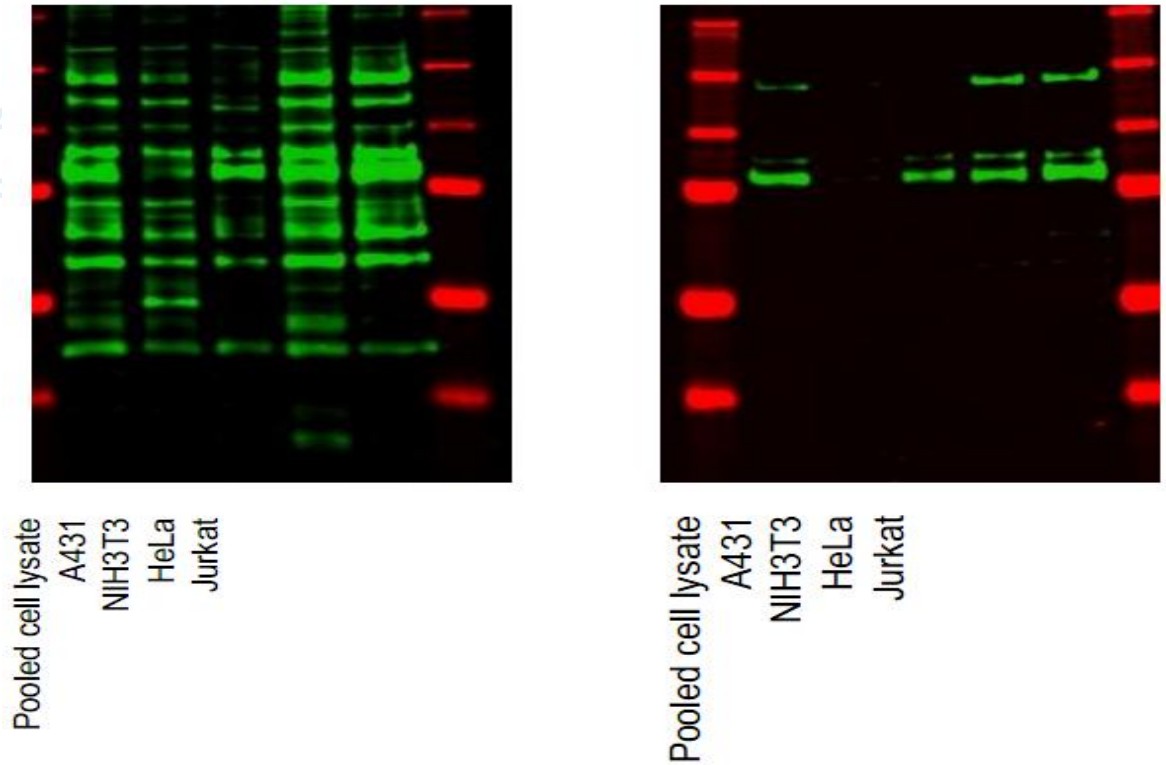
区别：

湿转：速度比较慢，转移效率高，适合分子量大的蛋白；

半干转：速度比较快，转移效率稍低，适合分子量较小的蛋白。

封闭

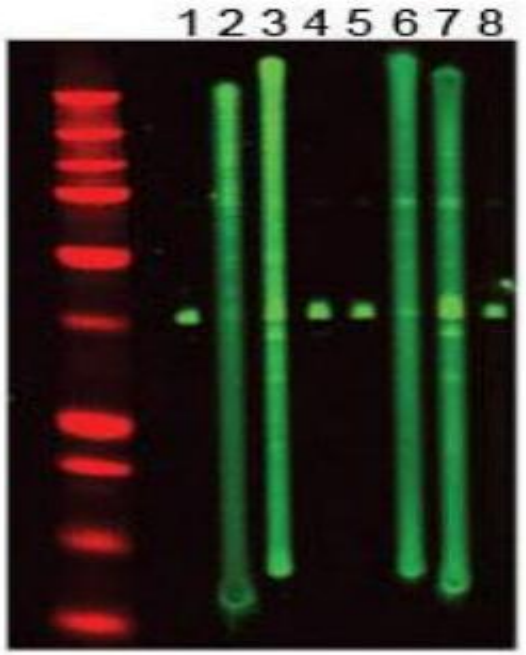
- 为避免作为检测试剂的特异性第一抗体与膜发生非特异性结合，使非特异性背景提高，需对膜上的潜在结合位点进行封闭处理。
- 5%脱脂奶粉或BSA（室温孵育1h ）。



一抗、二抗孵育

抗体孵育：Western blot成败的关键

抗体孵育是Western blot实验的关键步骤，抗体效价和抗体的正确使用对Western blot结果起决定性影响作用。



	Antibody	Host	Manufacturer	Part #
1	α-GAPDH	Mouse	Ambion	4300
2	GAPDH	Sheep	AbCam	ab35348
3	GAPDH	Rabbit	Rockland	600-401-A33
4	GAPDH	Mouse	AbCam	ab8245
5	GAPDH	Chicken	ProSci Inc.	XW-7214
6	GAPDH (N-14)	Goat	Santa Cruz Bio	sc-20356
7	GAPDH (V-18)	Goat	Santa Cruz Bio	sc-20357
8	α-GAPDH	Mouse	Sigma	G8795

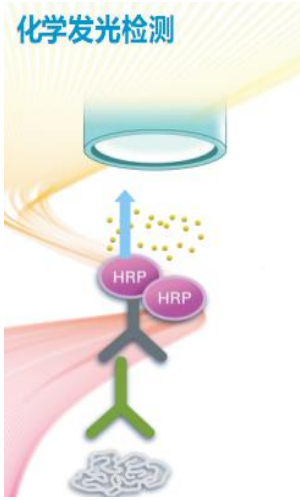
如图所示为loading Hela细胞裂解液后，用8种不同的GAPDH一抗进行检测。不同厂家的一抗，检测效果差异很大。1、4、5、8通道对应厂家的一抗较好。

显色

原理：偶联了HRP/ALP的二抗在加入发光底物后发生酶促反应，通过暗室压片或CCD化学发光成像仪进行检测。

实现方式：暗室压片、CCD化学发光成像仪

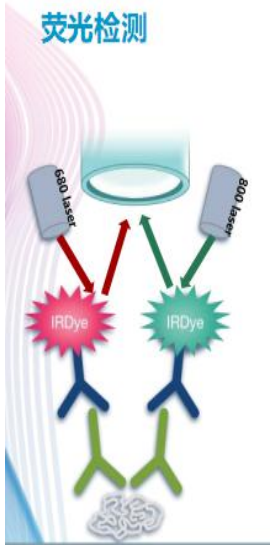
优点：化学发光检测较经典，灵敏度高



原理：二抗上结合的荧光染料被特定波长的光源激发，产生的荧光信号被收集并转换成电信号，通过灰度值的分析来检测蛋白的表达水平以及蛋白修饰水平如磷酸化修饰等

实现方式： 可见荧光成像仪，近红外荧光成像仪

优点：近红外荧光背景更低，动态范围更广、可实现准确定量双色成像；应用可拓展性强；



Western Blot常见问题分析

SDS-PAGE电泳

- 凝胶漏液?
- 凝胶时间不对?
- 纹理和脱尾现象?
- 微笑条带?
- 倒微笑条带?

- 两块玻璃板底部要对齐
- 凝胶太慢可能是AP、TEMED剂量不足或失效；
  凝胶太快可能是AP、TEMED用量过多
- 样品溶解不佳，加样前离心
- 凝胶不均匀冷却
- 凝胶和玻璃板组成“三明治”底部有气泡

转膜

- 凝胶肿胀或卷曲?
- 条带歪斜或漂移?
- 单个或多个白点?

- 转膜之前将凝胶在转膜缓冲液中浸泡5-10 min
- 转膜仪长期使用导致海绵变薄，“三明治”结构不紧凑导致，可在两块海绵之间垫上少许普通的草纸
- 确保膜和凝胶之间没有气泡

转膜

- 转移到膜上的蛋白很少?
- 转膜缓冲液过热?

- 当蛋白分子量小于10kD时，减少转膜时间；使用小孔径的膜；提高分离胶的浓度；当蛋白分子量较大时，则相反
- 缓冲液中离子浓度太低，电流或电压太高，转膜过程中注意降温

Western blot结果

- 背景太高?
- 杂交信号弱?
- 出现非特异性条带?

- 封闭不充分；抗体与封闭剂出现交叉反应；抗体浓度过高
- 抗体保存不当；抗原不充足；膜漂洗过度
- 一抗不是唯一特异性的；二抗出现非特异性结合

谢谢