Oddělení funkční genomiky a proteomiky
Přírodovědecká fakulta Masarykovy university

SYNTETICKÉ OLIGONUKLEOTIDY

Hana Konečná

CENTRÁLNÍ LABORATOŘ
Masarykovy Univerzity v Brně

ODDĚLENÍ FUNKČNÍ GENOMIKY A PROTEOMIKY

Laboratoř molekulární fyziologie rostlin

Centrální laboratoř
CL

Laboratoř analýzy biologicky významných komplexů

Laboratory of Plant Molecular Physiology
L M F R
MASARYK UNIVERSITY

LDMC • FGP-ABIMC

CENTRÁLNÍ LABORATOŘ

- přístup k pokročilým technologiím na bázi sdílené instrumentace a její kvalifikované obsluhy

→ proteomické techniky
→ genomické techniky

- výuka - přednášky a praktické kurzy
- členství v ABRF
  (Association of Biomolecular Resource Facilities)

TECHNIKY V CENTRÁLNÍ LABORATOŘI

- sekvenování a fragmentační analýza DNA
- syntéza a purifikace oligonukleotidů

- kapalinová chromatografie
- gelová elektroforéza
- analýza obrazu
- digesce proteinů
- hmotnostní spektrometrie

- minisklad reagencií pro molekulární biologii

SYNTETICKÉ OLIGONUKLEOTIDY

- definice
- aplikace
- modifikace
- syntéza
- purifikace
- kontrola kvality

- design sekvence
- zásady navrhování
- software OLIGO 7
- praktická ukázka

oligonukleotid

orientace! polymeráza!

- krátká jednořetězcová struktura
- DNA nebo RNA (event. PNA)
- **hydroxyl** na obou koncích (normálně na 5´- konci fosfát)

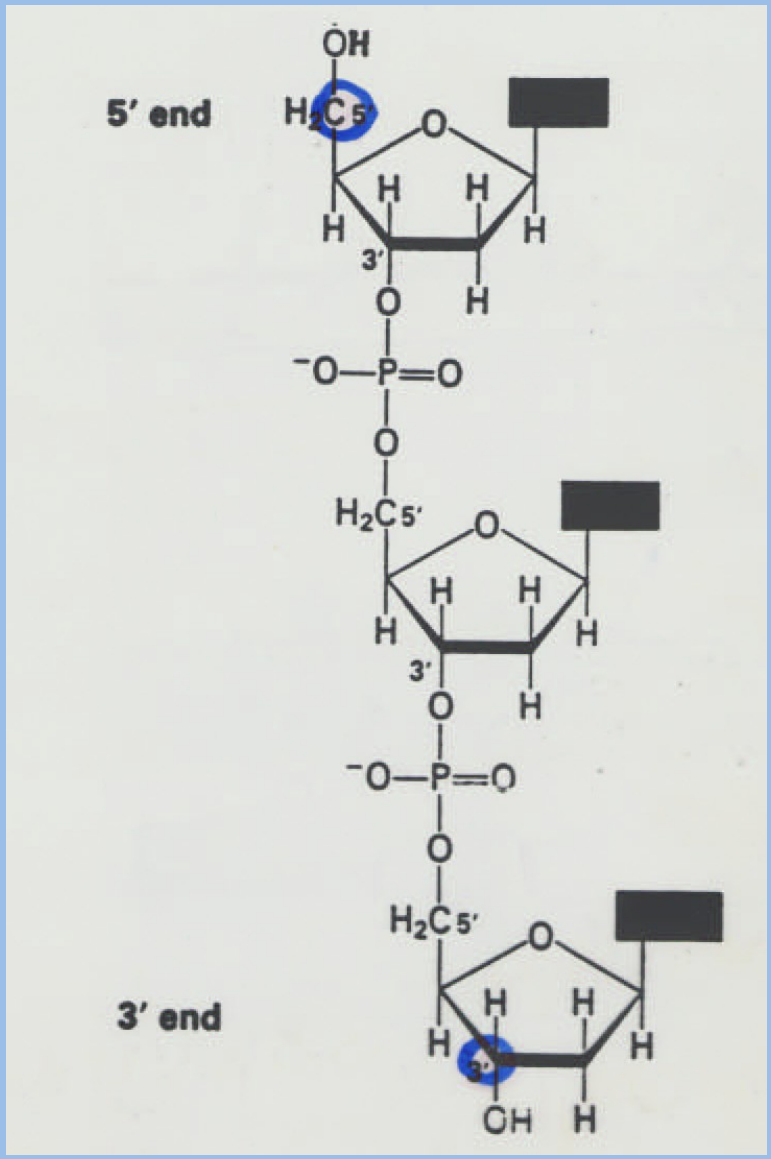
oligonukleotid

↓

syntetický oligonukleotid

↓

primer

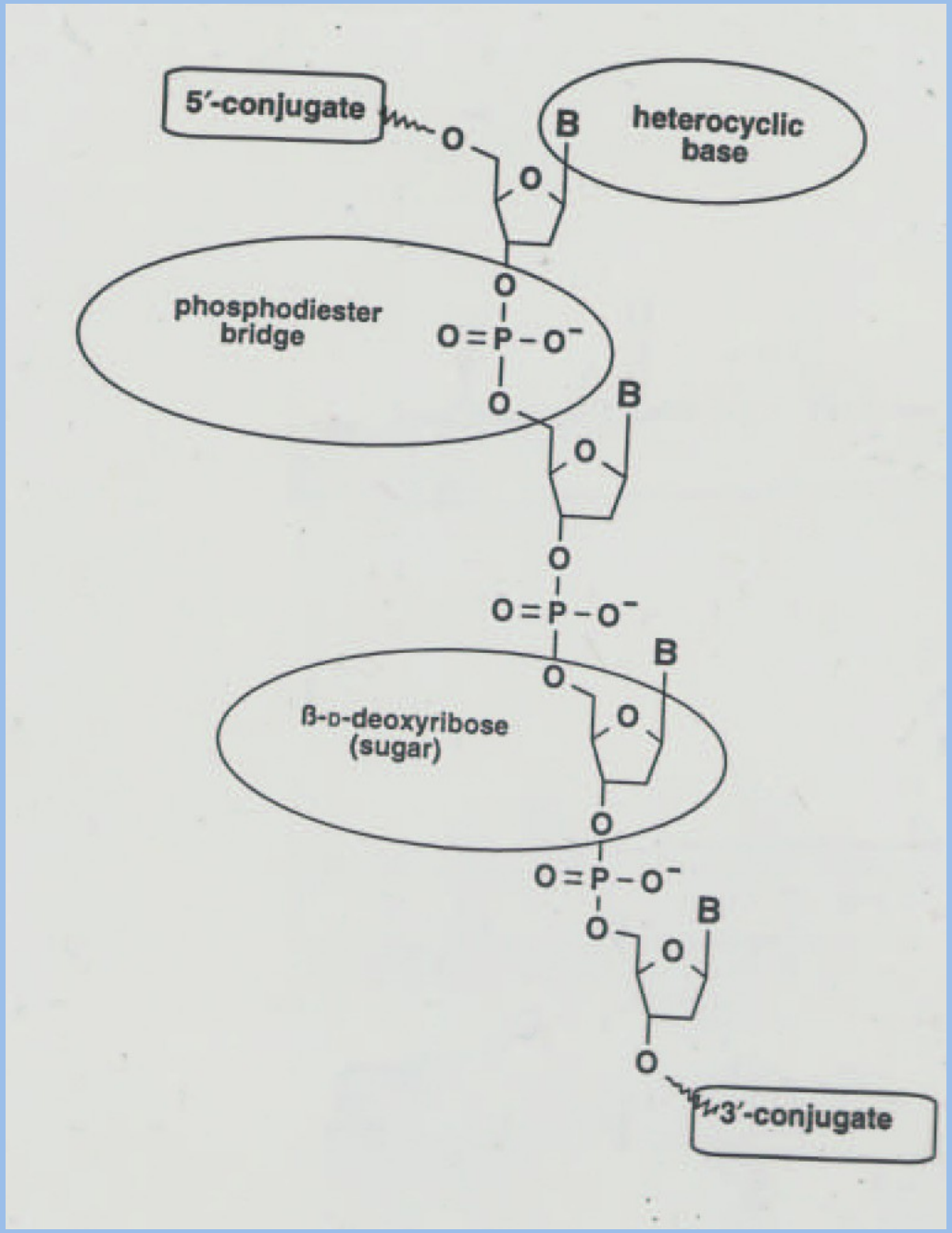


Aplikace syntetických oligonukleotidů

- konstrukce duplexů
- primery pro syntézu komplementární DNA
  PCR, Real-Time PCR
- hybridizační sondy pro klonování
- místně cílená mutageneza
- strukturální rentgenová analýza NA
- NMR studia interakcí DNA-protein
- potenciální léčiva
- gene arrays

Modifikace

- degenerace
- konce řetězce
- báze
- fosfát
- cukr
- PNA



Degenerované oligonukleotidy

2-deoxyinosin ?

univerzální báze:
3-nitropyrrol ?
5-nitroindol ?

M	A or C
R	A or G
W	A or T
S	C or G
Y	C or T
K	G or T
V	A or C or G
H	A or C or T
D	A or G or T
B	C or G or T
N	G or A or T or C
X	G or A or T or C

www.glenres.com

Degenerované oligonukleotidy

Příklady:

ACG TAC GTA CGT ACG TAC	nedegenerovaný
ACG TA**M** GTA CGT ACG TAC	M = A/C
ACG TAC GTA C**D**T ACG TAC	D = A/G/T
ACG TAC GTA CGT ACG **N**AC	N = A/C/G/T

Modifikace na 5´- konci

postsyntetické modifikace

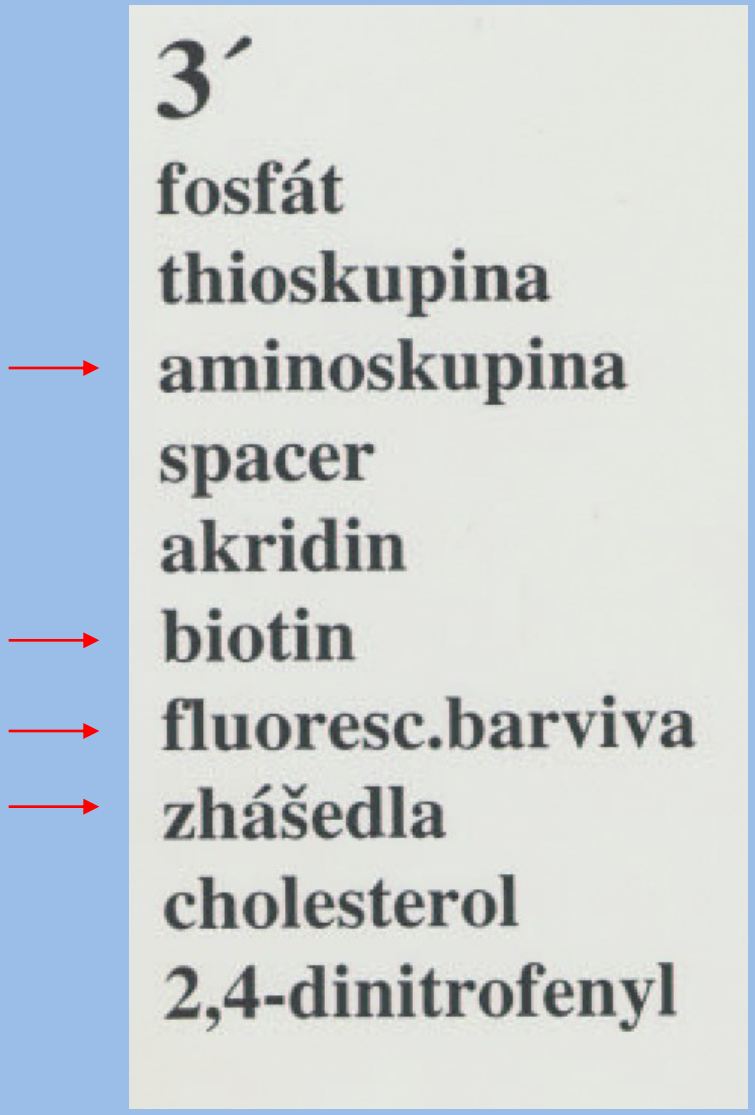
sekvenování
fragmentační analýza
gene arrays
Real-Time PCR

5´

fosforylace
aminoskupina
thioskupina
digoxigenin
biotin
enzymy
psoralen
akridin
cholesterol
fluoresc. barviva
zhášedla
2,4-dinitrofenyl
TBR-chelát
spacer
větvení
blokáda

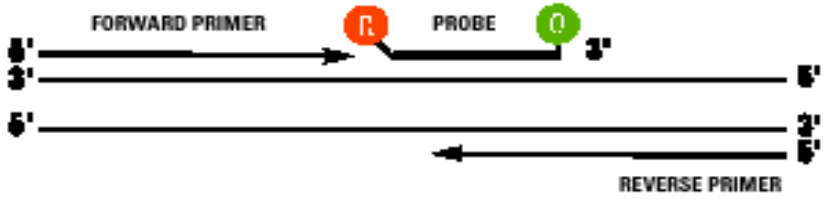
Modifikace na 3´- konci

derivatizovaná matrice

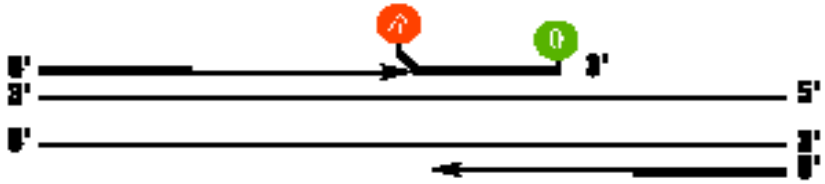


Real-Time PCR

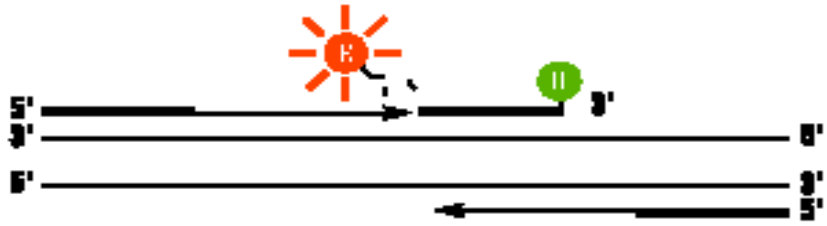
- 2x značená sonda
- REPORTER
- QUENCHER



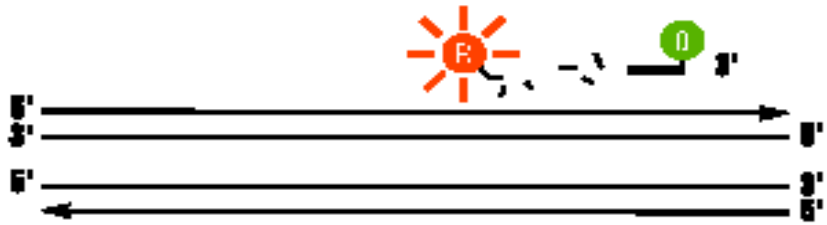
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



3. **Cleavage:** During each extension cycle, the DNA polymerase cleaves the reporter dye from the probe.



4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



Další modifikace

fosforothioáty
fosforodithioáty
H-fosfonáty
metylfosfonáty

← páteř

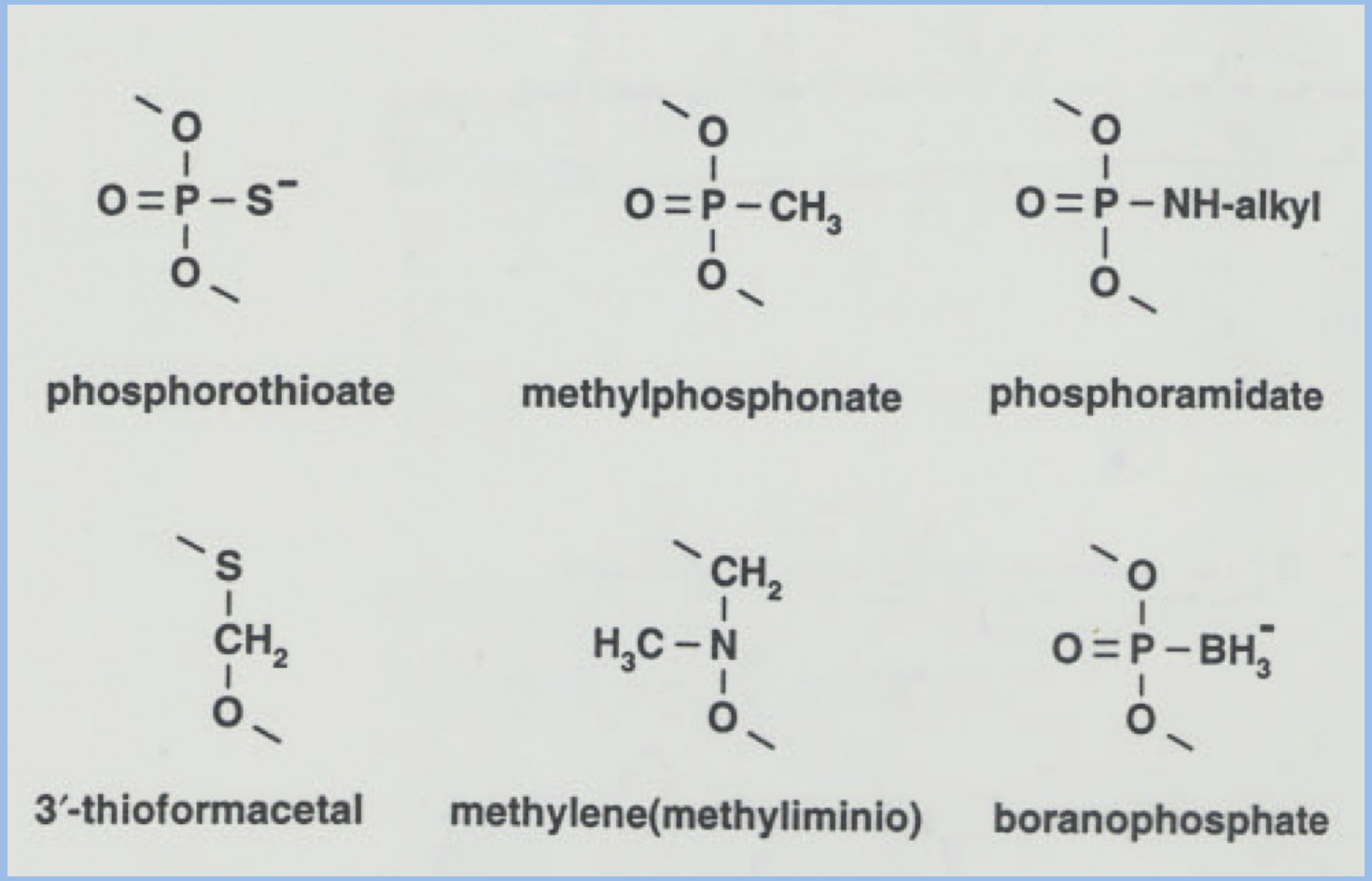
cukr →

modifikace v 2´pozici
modifikace ribózové jednotky

Terapeutika

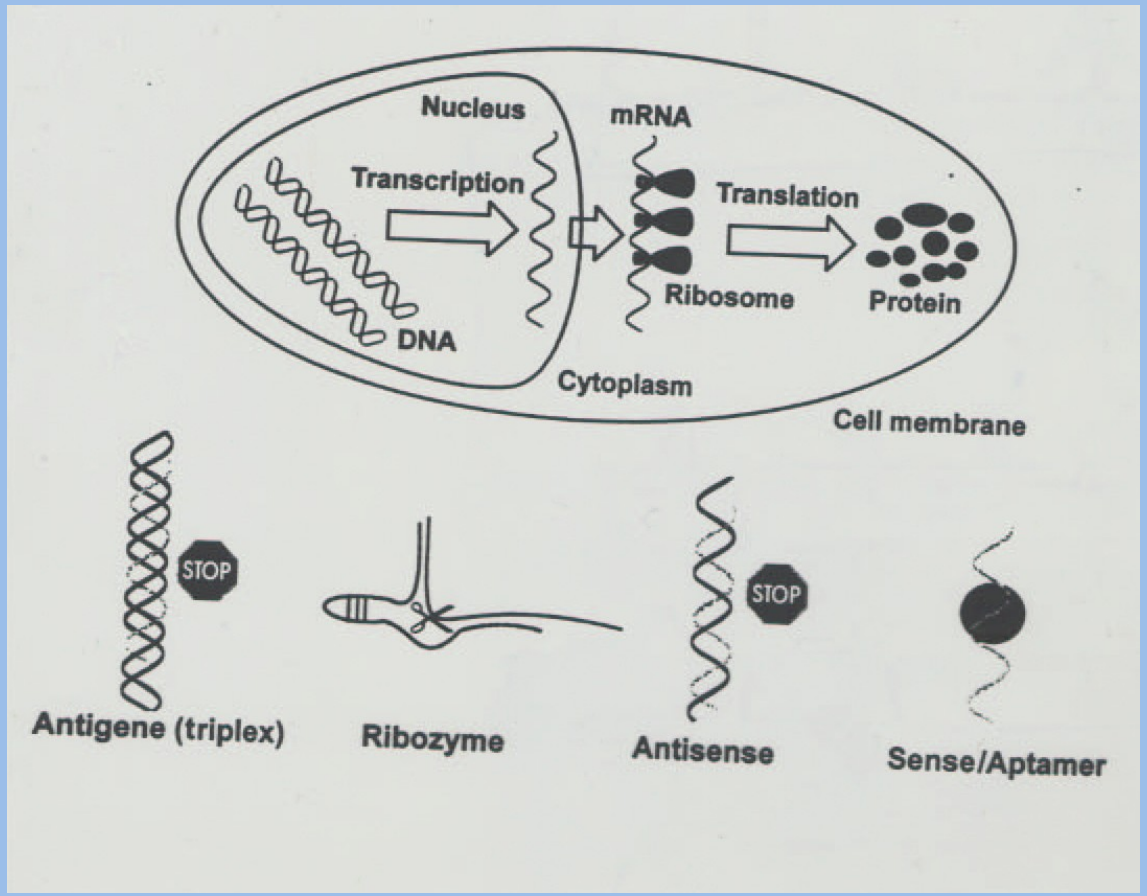
nedegradována nukleázami!

→ modifikace fosfodiesterové vazby



ANTISENSE oligonukleotid

- oligonukleotid nebo analog
- komplementární k segmentu RNA nebo DNA
- vazbou inhibuje jejich normální funkci

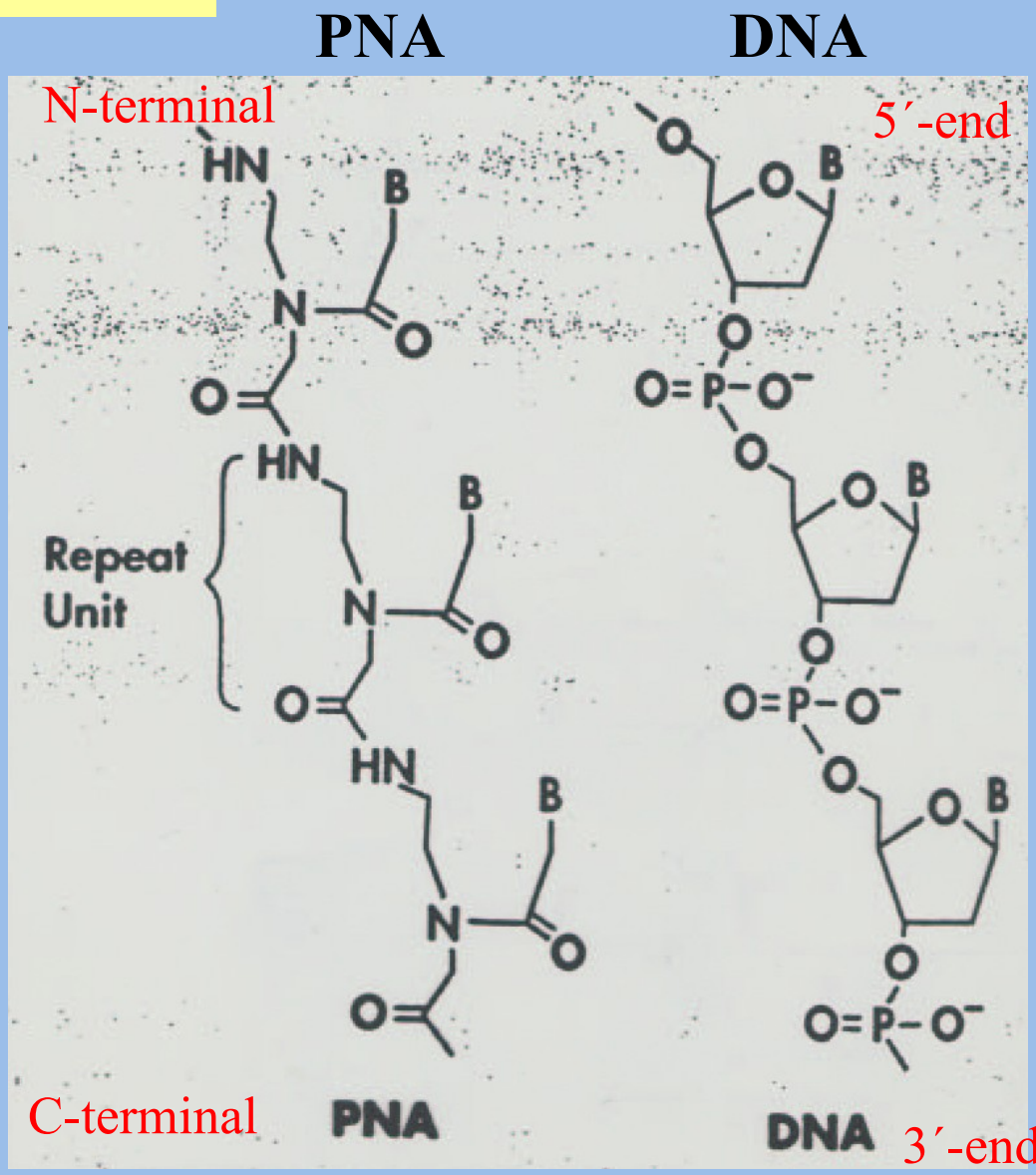


Peptidonukleová kyselina

PNA

- nenabitá molekula
- vazba k DNA/RNA

N-(2-aminoethyl)-glycin →



Peptidonukleová kyselina

PNA

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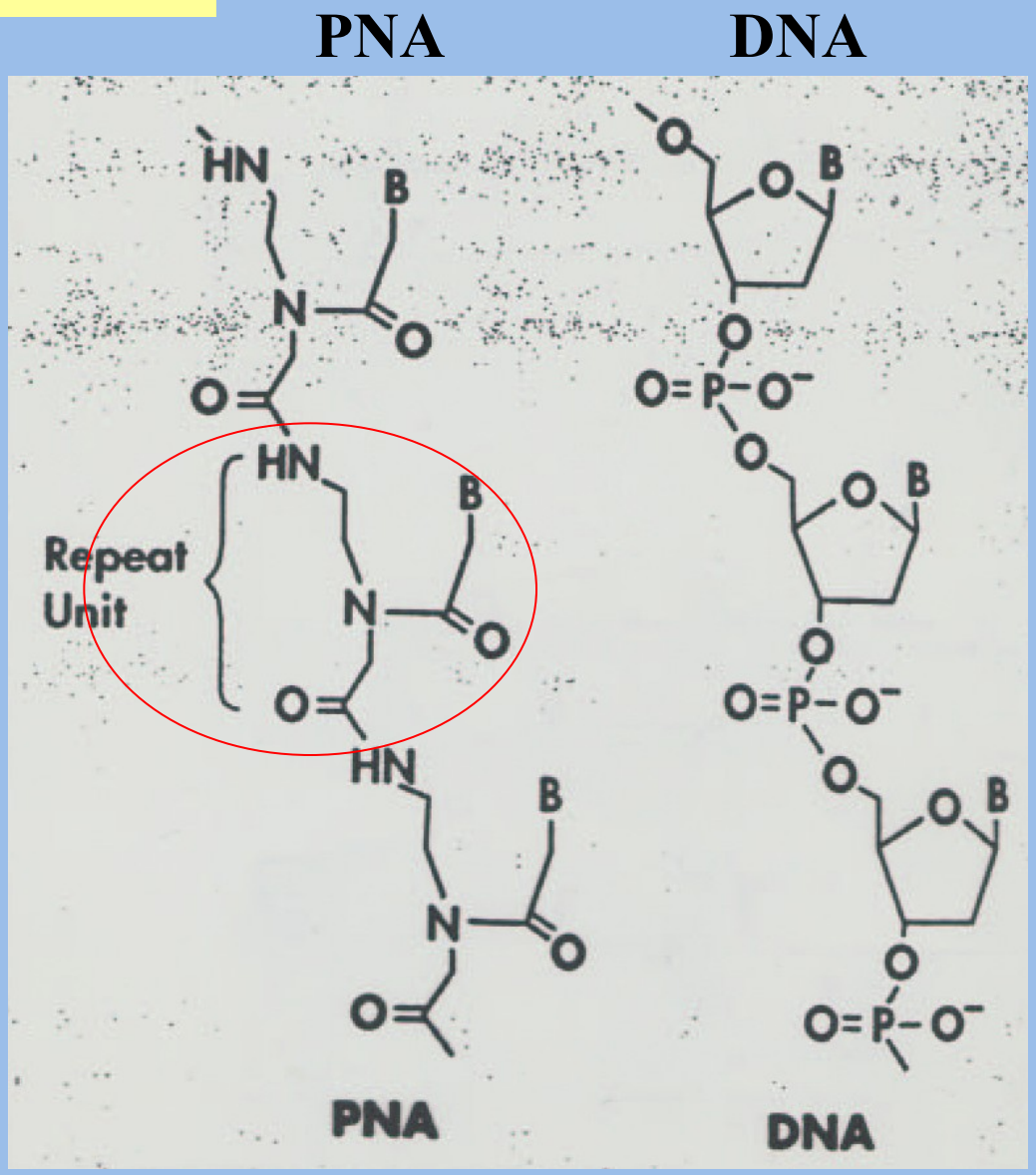
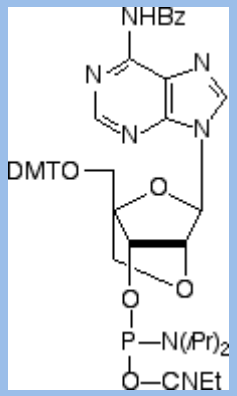


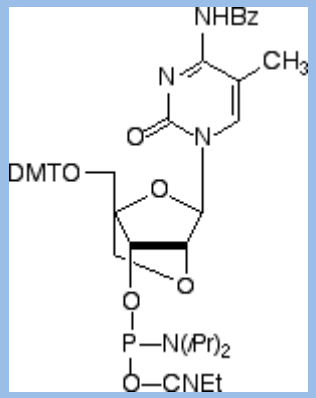
Vlastnosti PNA

- vysoká termostabilita
- T_m nezávisí na obsahu solí
- vyšší specificita
- vyšší afinita
- rezistentní k enzymům...

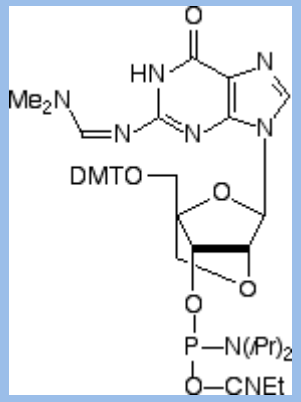
LNA



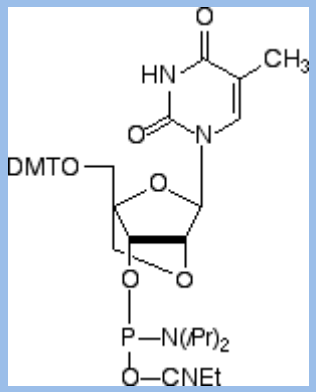
Bz-A-LNA



5-Me-Bz-C-LNA



dmf-G-LNA



T-LNA

Locked Nucleic Acid

2'-O, 4'-C methylenový můstek

potlačená flexibilita ribofuranózového kruhu

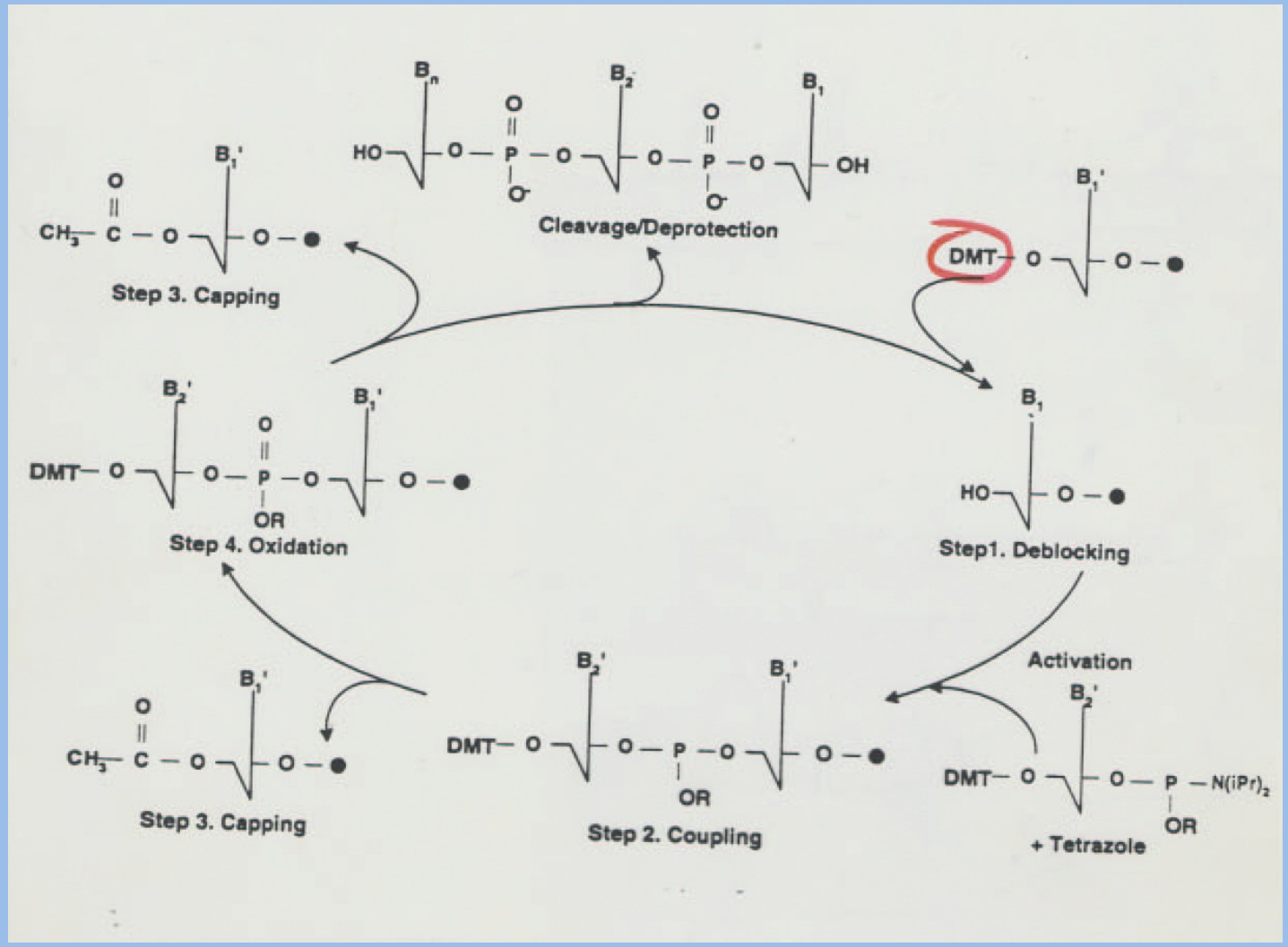
struktura je zamčena do rigidní C3-endo konformace

zlepšená hybridizace

výjímečná biostabilita

Syntéza oligonukleotidu

- organická syntéza na pevné fázi
- od 3´-konce k 5´-konci
- bezvodé prostředí



OLIGONUKLEOTIDY

design

syntéza

purifikace

EXPEDITE 8909

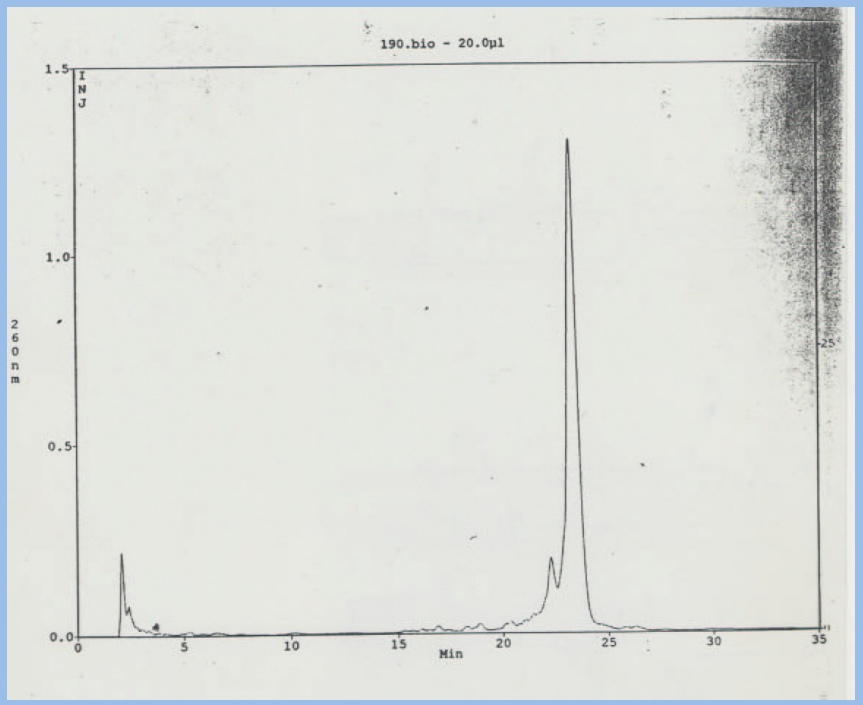
EXPEDITE 8909

- rychlost
- nízká spotřeba reagencií
- několik koncentračních rozsahů
- dvě paralelní syntézy
- protokoly pro DNA, RNA, PNA, fosforothioáty
- Workstation: možnost editace základních protokolů - syntéza modifikací (značení biotinem, fluroscenčními značkami, degenerované oligonukleotidy, užití inosinu, aminoderiváty aj.)

Kontrola kvality

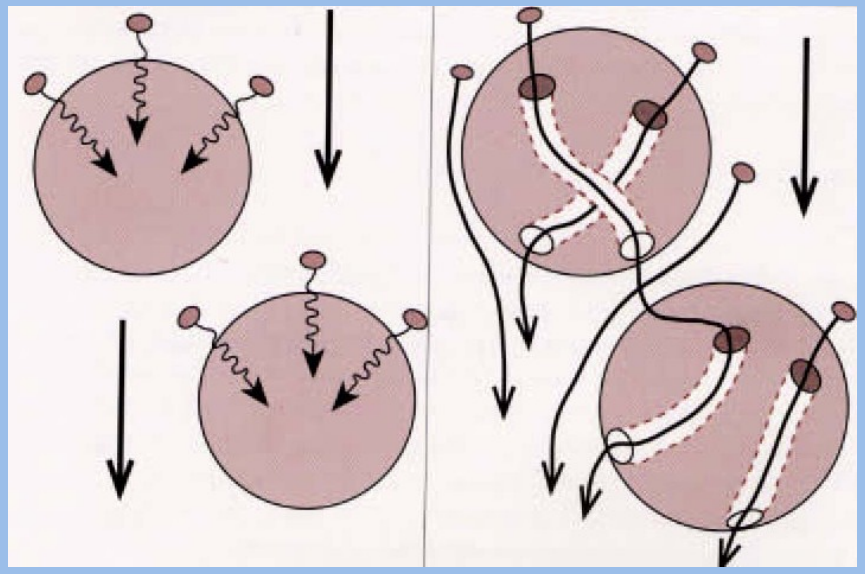
HPLC
Perfúzní chromatografie

- anex
- RP

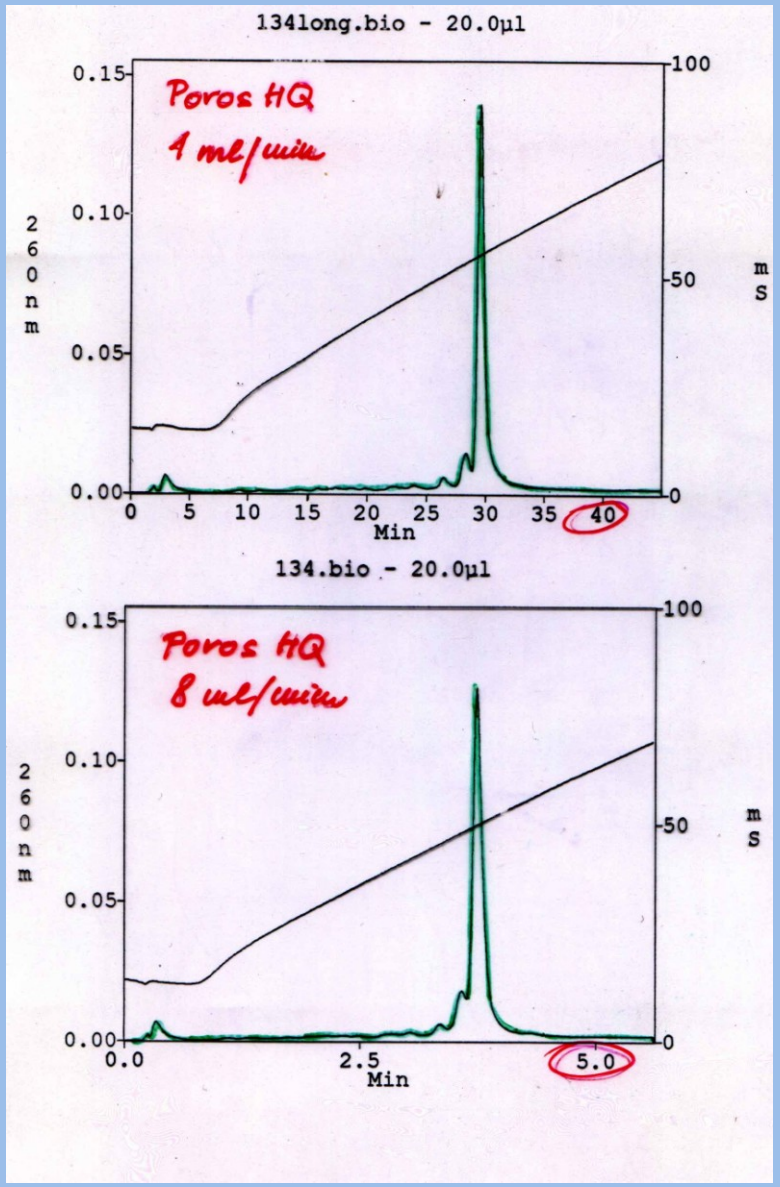


Perfúzní chromatografie

POROS

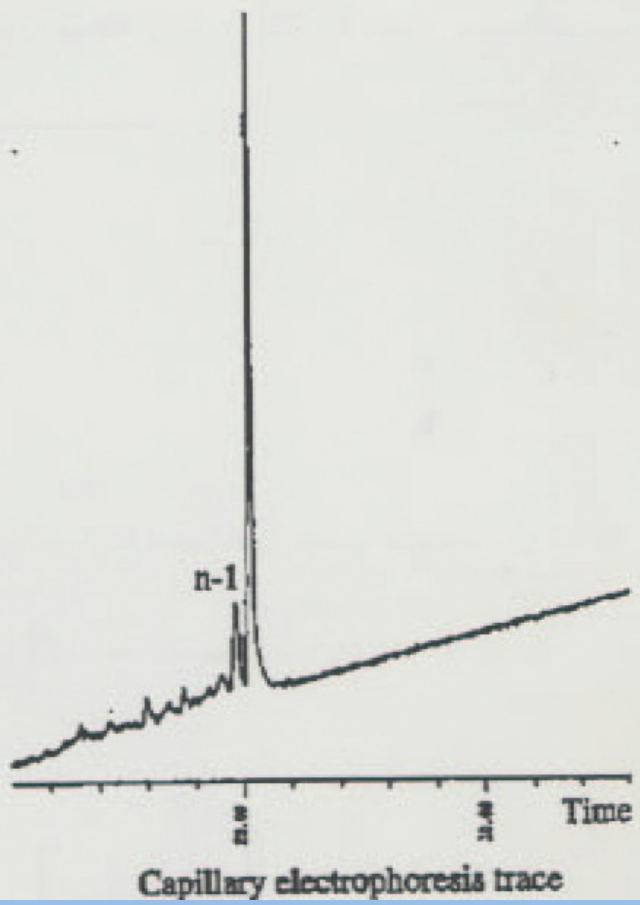


klasický sorbent POROS

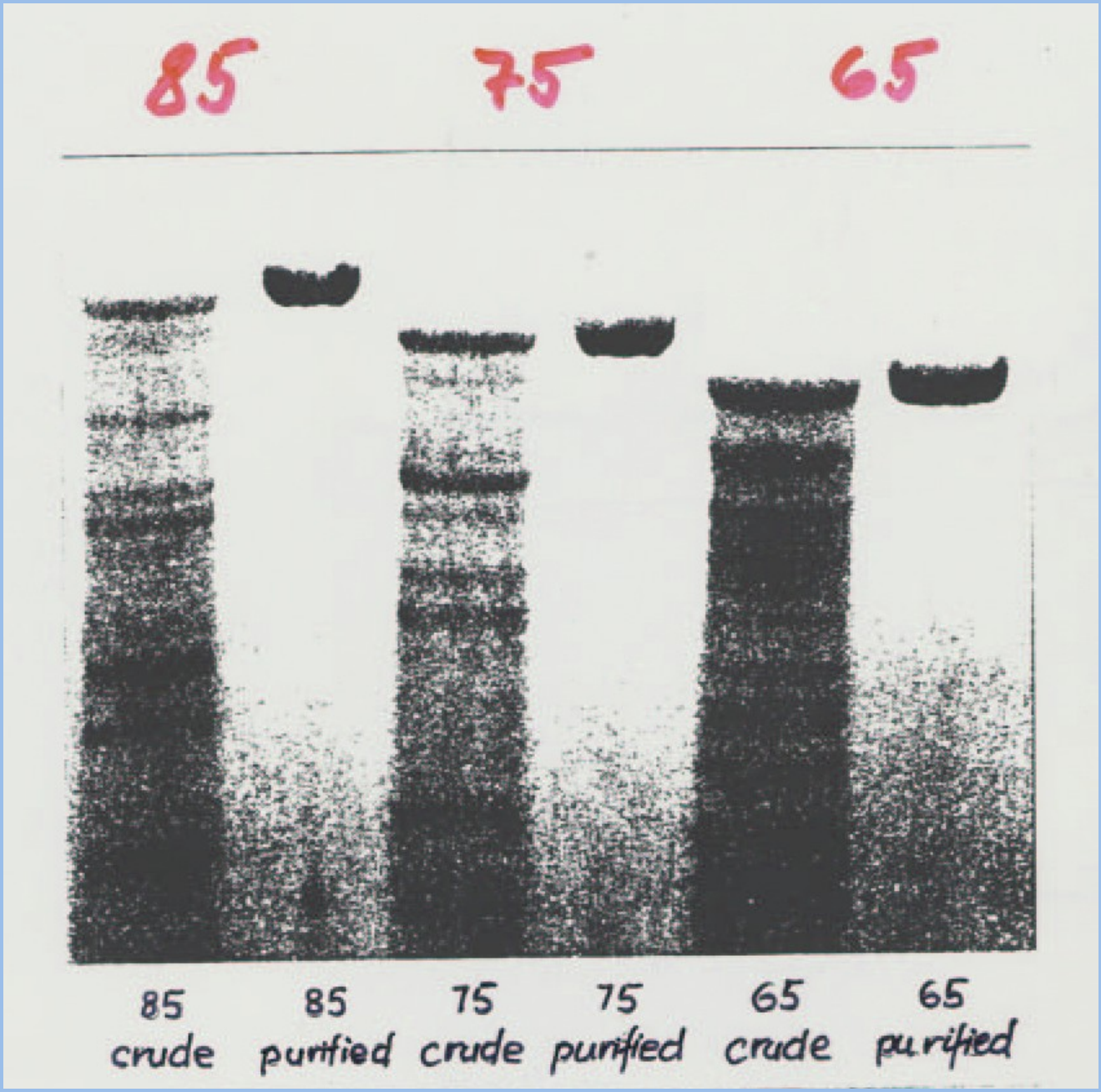


Maldi-Tof MS

CE

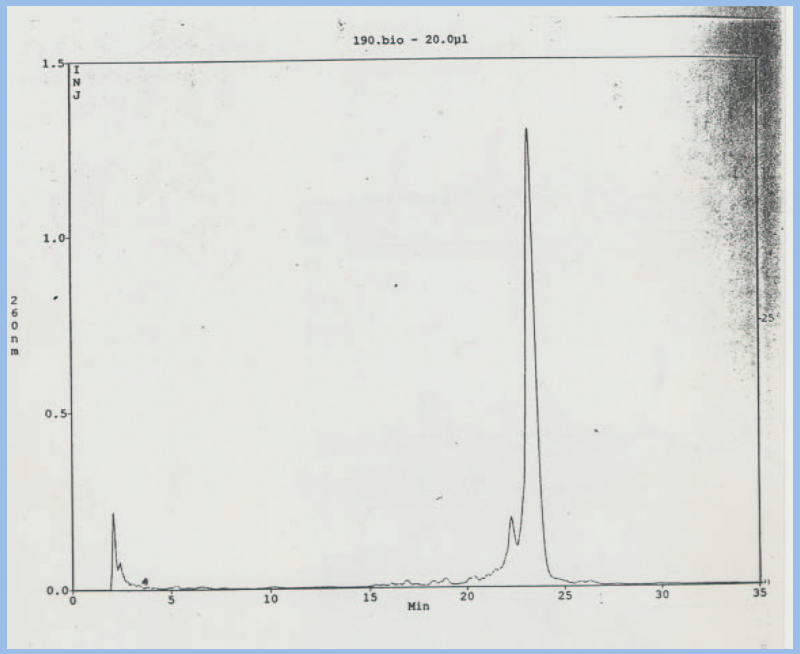


PAGE

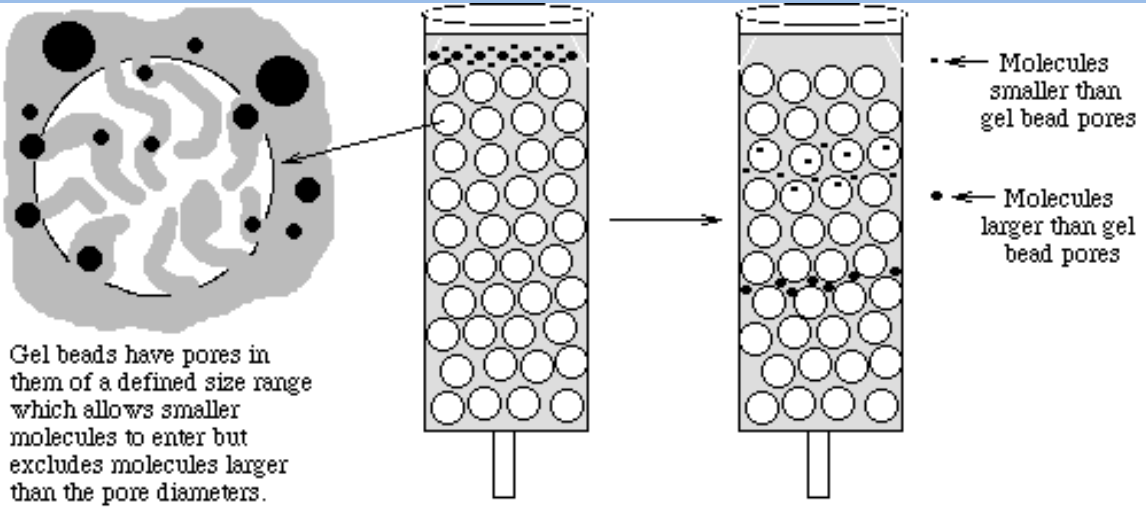

85
75
65
85 crude
85 purified
75 crude
75 purified
65 crude
65 purified

PURIFIKACE

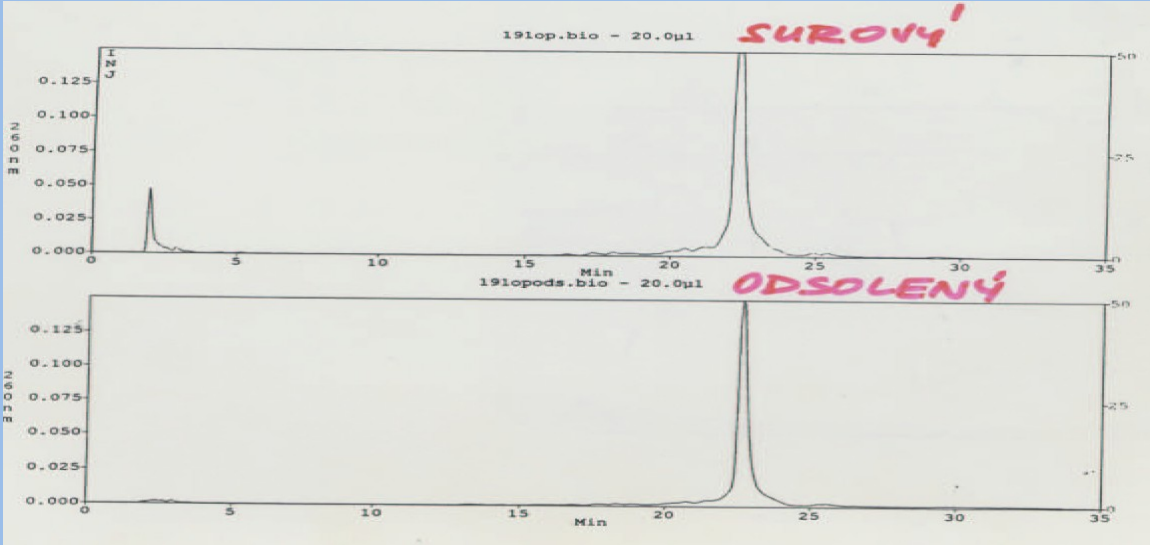
Sephadex
RP cartridge
HPLC



Odsolování



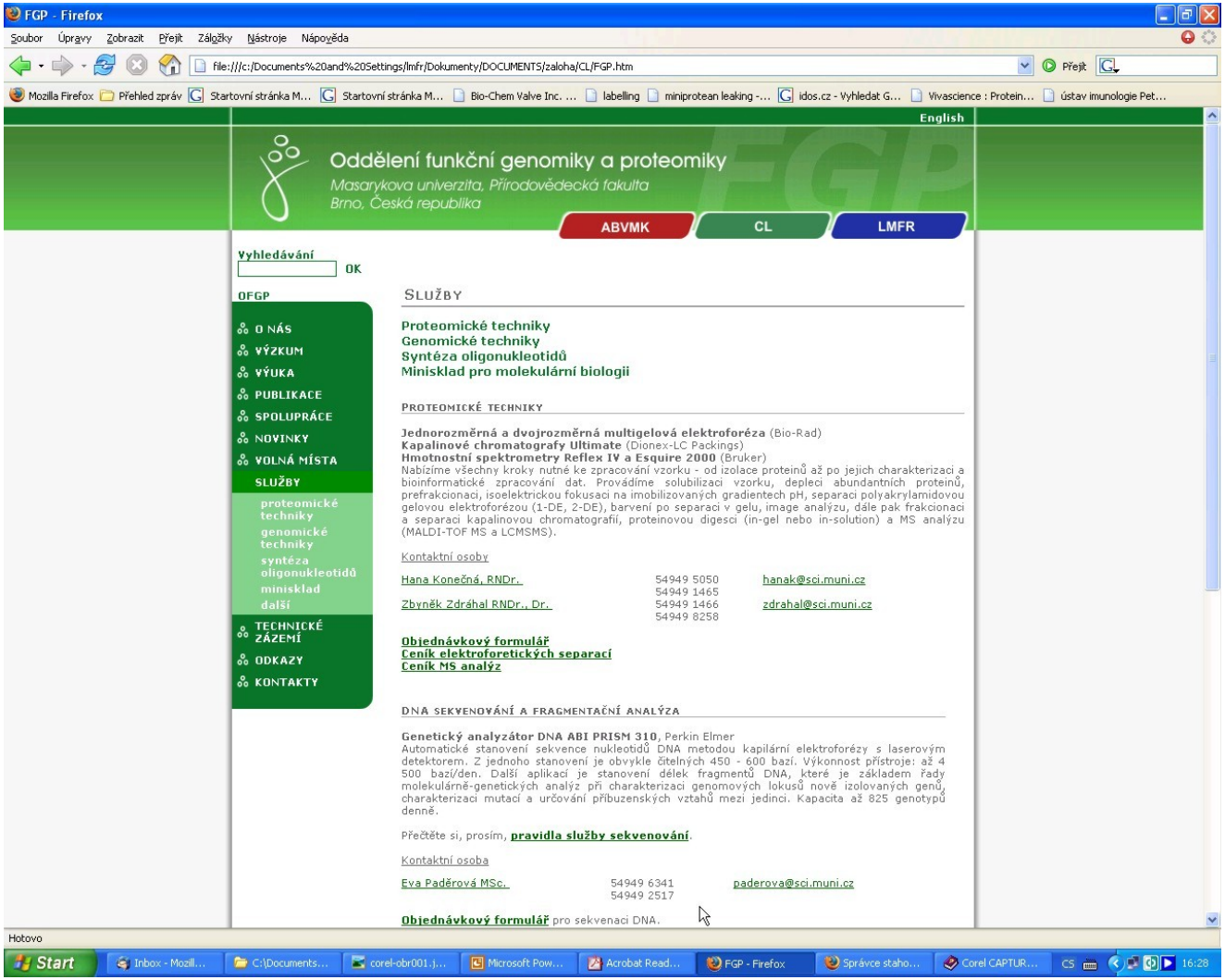
- soli
- oligonukleotid



OBJEDNÁVKY

www.sci.muni.cz/FGP

- on-line
- e-mail
- písemně



DESIGN OLIGONUKLEOTIDU

- manuální
- počítačový

www.protocol-online.org/prot/Research_Tools/Online_Tools/Oligo_Design/index.html

Hlavní kritéria pro sekvenci PCR primeru

- vysoce specifické
- netvoří dimery a vlásenky
- stabilní duplexy s aktivní sekvencí
- nepříliš stabilní 3´-konec

OLIGO 6

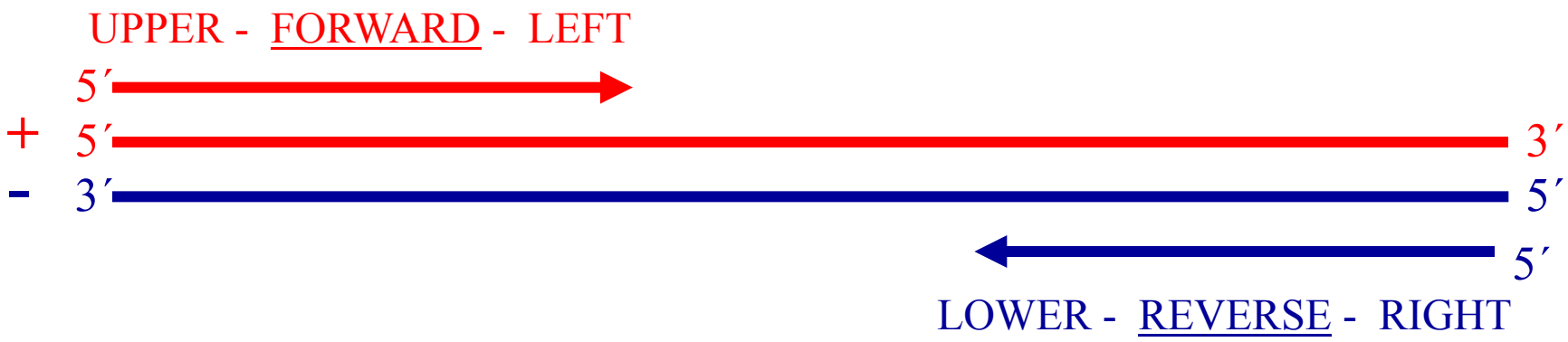
- PCR primery,
- hybridizační sondy
- sekvenační primery

OLIGO 7 (od roku 2008)

- TaqMan sondy
- primery pro *nested PCR*
- *molecular beacons*
- siRNA

Terminologie PCR primerů

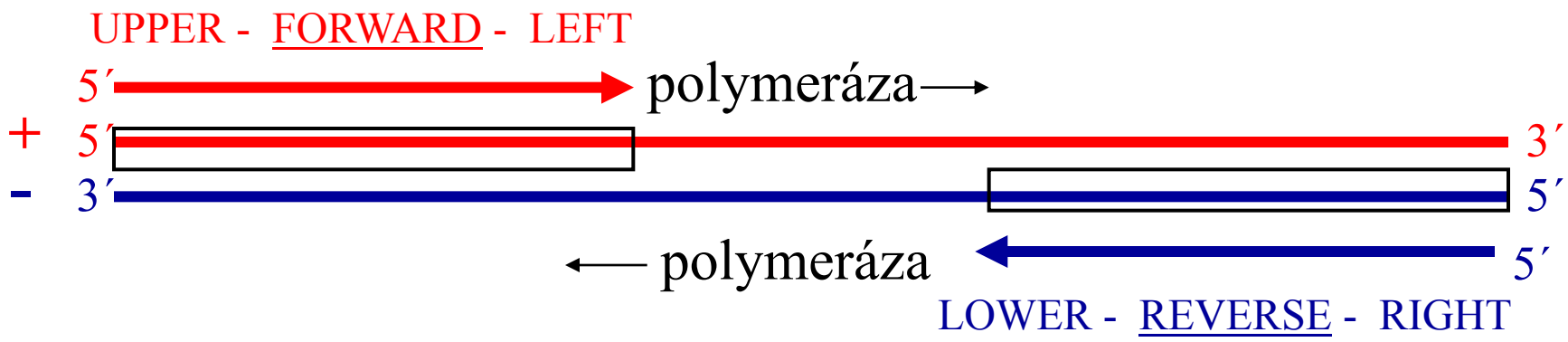
forward primer… část sekvence + vlákna
reverse primer… část sekvence - vlákna



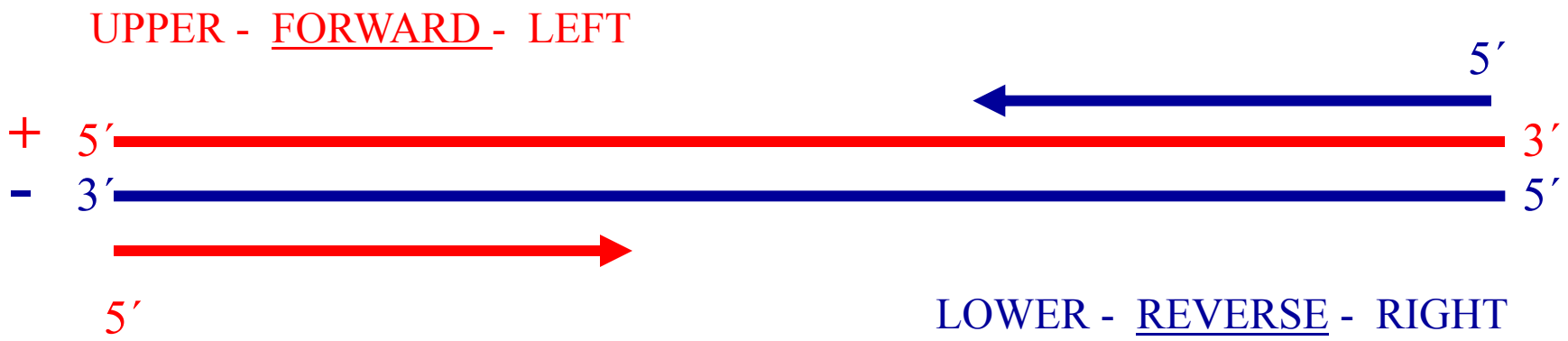
Terminologie

forward primer… část sekvence + vlákna

reverse primer… část sekvence - vlákna



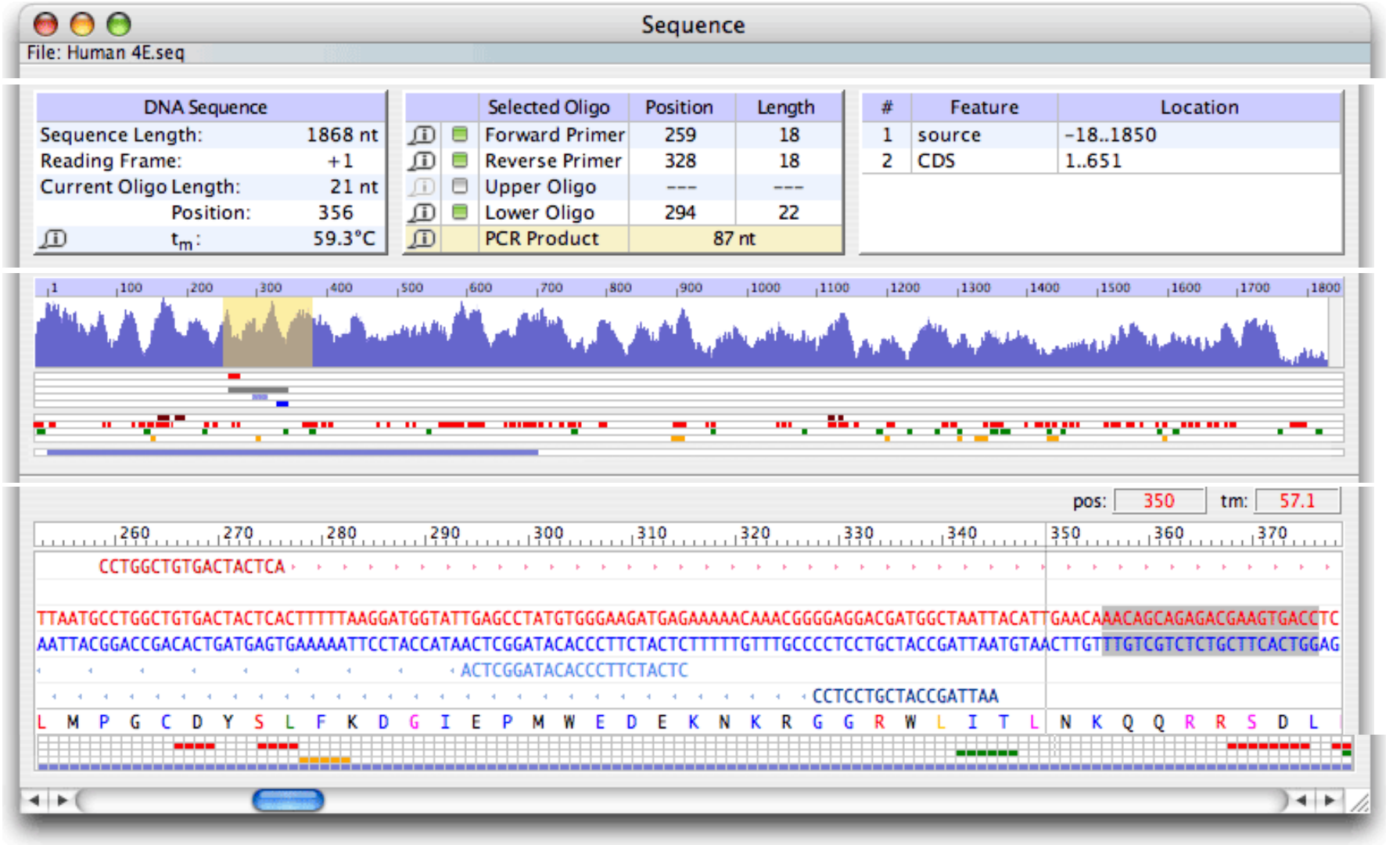
Nasedání PCR primerů

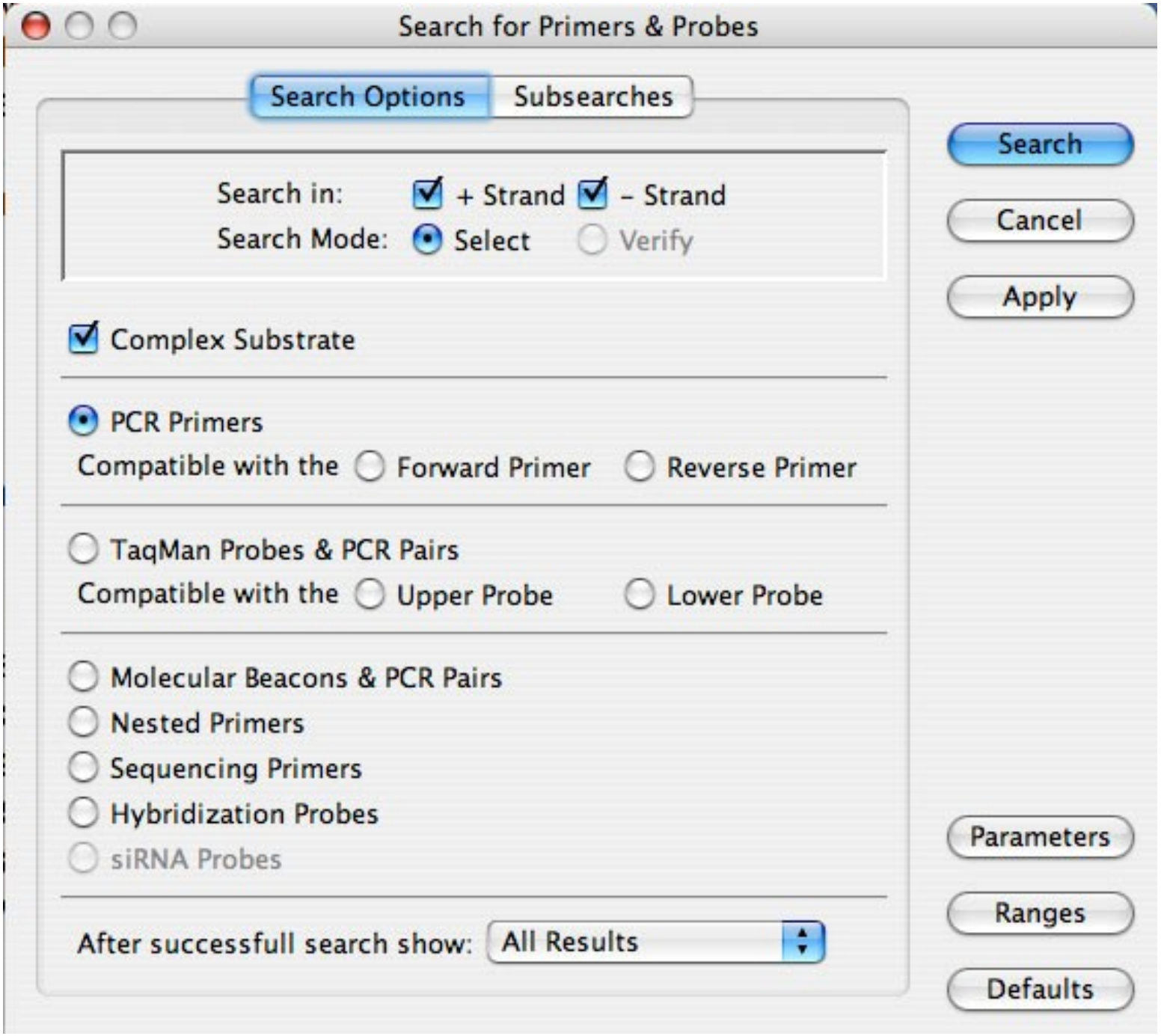


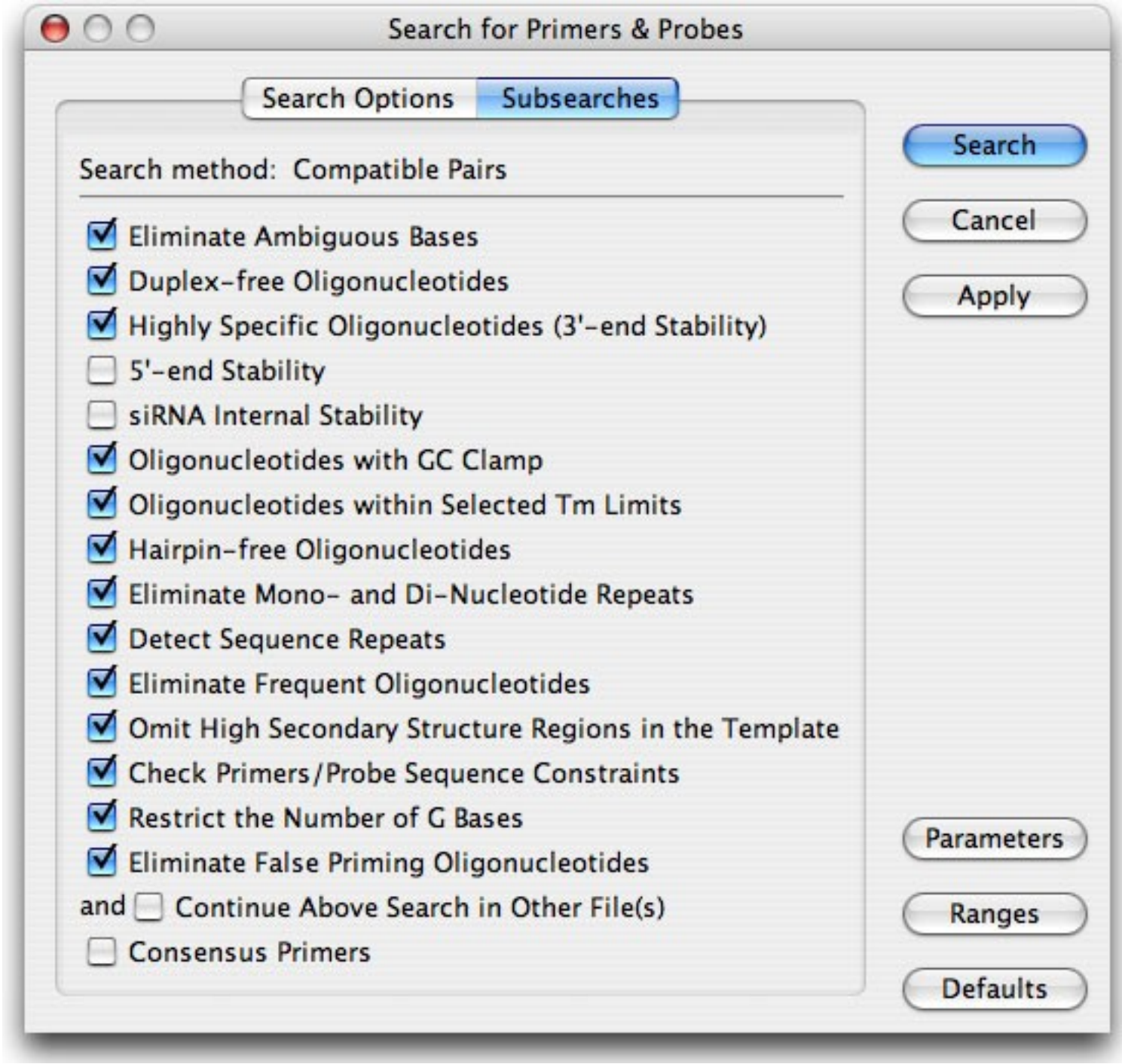
5´ CTT CTG CTC AAT CTT TCT AC 3´ **FORWARD**

5´
1 ATGGCTTCTG CTCAATCTTT CTACAACCAA AGCTCTGTCT TGAAAATCAA
51 TGTCATGGTT GTGGACGATG ATCATGTTTT CCTTGATATC ATGTCACGCA
101 TGCTTCAACA CTCCAAATAC AGAGGTAATT AAATATTATT ATCATATTAT
151 ATATAATATG TTATTGATTT TTTGTTTGTG ATTTCATTTA GATTTTTATT
201 TCTATGATTT CTTAGCATGA AATACAATTT TTGGAGAAAC AACTAGCAGT
251 TTTAAAAACA AAACTTGAAT TTTGAGAAAT TCAAAGATGT TATATATATA
301 TGTCAAAATT TAACAATTAT TCTTCTAAAT CATCCGGATT CCGTTTACAT
351 GTACACATCT ACAATTTTCA ATTGAGGTAT TCTTGTTTTG ATGCCTTTGA
401 GACGAATAGT TTGATTGATA AAAAAAATTC TAACCAATAT GATATATAAA
451 GTTTATTTTC TTTTTGTCAA ACCATACTTT ATACTATGTA ACTTTTTTAA
501 GAGATTATTG AAAATAGTTT ATTTATAAAA TAGTAACCTA TTGTTGAATT
551 AAAAAAAAAA AAAAAATTGT AAATCGTGTT TGCAAACGAC ATGTGATTTA
601 TCTTAGTTTA AAACTAGCTG ATATTCTTCA AATCGACTGT TCTTATAAGT
651 AATCAACCAA TTAGCATCAA TCACAATAAA TTGTAAACAC TTCAATGAAA
701 ATGGTGATTT TAAAGAATAT GTTTTACTTA TGTTATGAAC TATCTCAAAT
751 TTGTGAAATA TTTCATAACT AATGTGGAAA ACTATATAAC CCCTCCATAC
801 AAAACGTAAG TAAAATTTAT GAAATCCTAT CATTTTTAAA GGTTAAACCA
851 ATCAAAAAGT AATAATTCTT GGTACTTGCA ATATTTTTGT CATTATATTT
901 TAGTTTATTA ATTTTATTTT GATTAAATGG TTTTAGATCC ATCAGTTATG
951 GAGATCGCAG TTATAGCTGT AGACGATCCG AAGAAAGCAT TATCTACTCT
1001 AAAAATTCAA CGAGACAATA TAGATCTCAT AATCACAGAT TATTATATGC
1051 CTGGTATGAA CGGTTTACAA CTCAAAAAAC AAATCACTCA GGAATTTGGA
1101 AATTTACCGG TCTTAGGTAA CATTTTTTGT TCTTTACAAC TTAAATTAAA
3´

- 5´ TGA AGA ATA TCA GCT AGT TT 3´ **REVERSE**

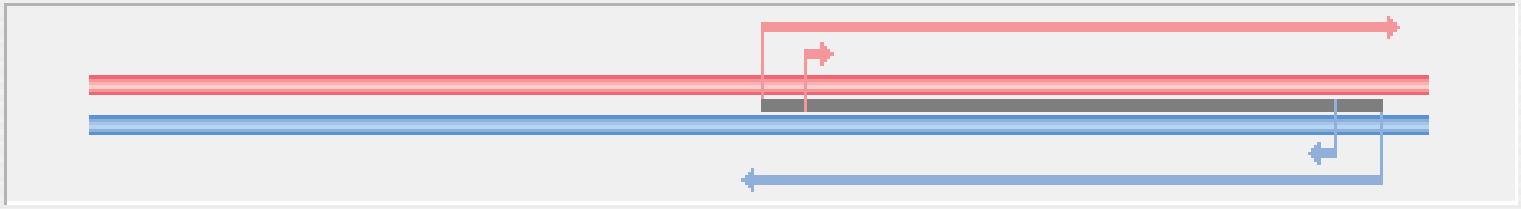

Sequence
File: Human 4E.seq
DNA Sequence
Sequence Length: 1868 nt
Reading Frame: +1
Current Oligo Length: 21 nt
Position: 356
t_m: 59.3°C
Selected Oligo | Position | Length
Forward Primer | 259 | 18
Reverse Primer | 328 | 18
Upper Oligo | --- | ---
Lower Oligo | 294 | 22
PCR Product | 87 nt
| Feature | Location
1 | source | -18..1850
2 | CDS | 1..651
pos: 350
tm: 57.1
CCTGGCTGTGACTACTCA
TTAATGCCTGGCTGTGACTACTCACTTTTTAAGGATGGTATTGAGCCTATGTGGGAAGATGAGAAAAACAAACGGGGAGGACGATGGCTAATTACATTGAACAAACAGCAGAGACGAAGTGACCTC
AATTACGGACCGACACTGATGAGTGAAAAATTCCTACCATAACTCGGATACACCCTTCTACTCTTTTTGTTTGCCCCTCCTGCTACCGATTAATGTAACTTGTTTGTCGTCTCTGCTTCACTGGAG
ACTCGGATACACCCTTCTACTC
CCTCCTGCTACCGATTAA
L M P G C D Y S L F K D G I E P M W E D E K N K R G G R W L I T L N K Q Q R R S D L


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
Molecular Beacons & PCR Pairs
Nested Primers
Sequencing Primers
Hybridization Probes
siRNA Probes
After successfull search show:
All Results
Search
Cancel
Apply
Parameters
Ranges
Defaults


Search for Primers & Probes
Search Options
Subsearches
Search method: Compatible Pairs
Eliminate Ambiguous Bases
Duplex-free Oligonucleotides
Highly Specific Oligonucleotides (3'-end Stability)
5'-end Stability
siRNA Internal Stability
Oligonucleotides with GC Clamp
Oligonucleotides within Selected Tm Limits
Hairpin-free Oligonucleotides
Eliminate Mono- and Di-Nucleotide Repeats
Detect Sequence Repeats
Eliminate Frequent Oligonucleotides
Omit High Secondary Structure Regions in the Template
Check Primers/Probe Sequence Constraints
Restrict the Number of G Bases
Eliminate False Priming Oligonucleotides
and Continue Above Search in Other File(s)
Consensus Primers
Search
Cancel
Apply
Parameters
Ranges
Defaults

PCR

File: Human 4E.seq



Optimal Annealing Temperature: 50.8 °C (Max: 66.3 °C)

	Position and Length		T_m [°C]	GC [%]	P.E.#	Score
Product	862		78.9	29.6	n/a	697
Forward Primer	918	22	56.9	45.5	471 / 471	840
Reverse Primer	1753	27	55.3	29.6	489 / 489	834
Upper Oligo	979	24	56.5	33.3	479 / 479	917
Lower Oligo	1694	23	55.4	39.1	457 / 457	841

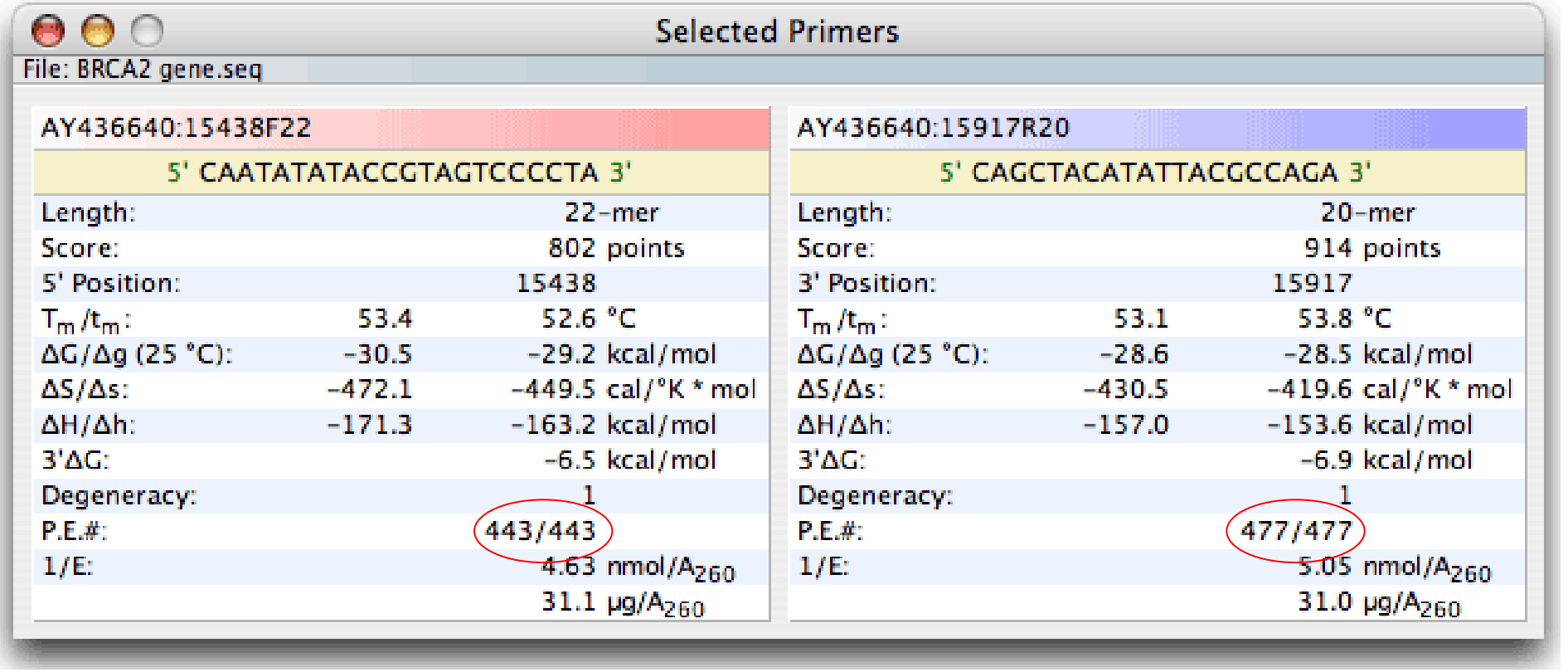
Product T_m – Reverse Primer T_m: 23.6 °C

Primers T_m difference: 1.6 °C

Comments:

	Concentration	
Forward Primer	200.0	nM
Reverse Primer	200.0	nM
Upper Oligo	200.0	nM
Lower Oligo	200.0	nM
Monovalent Cation	50.0	mM
Free Mg[2+]	0.7	mM

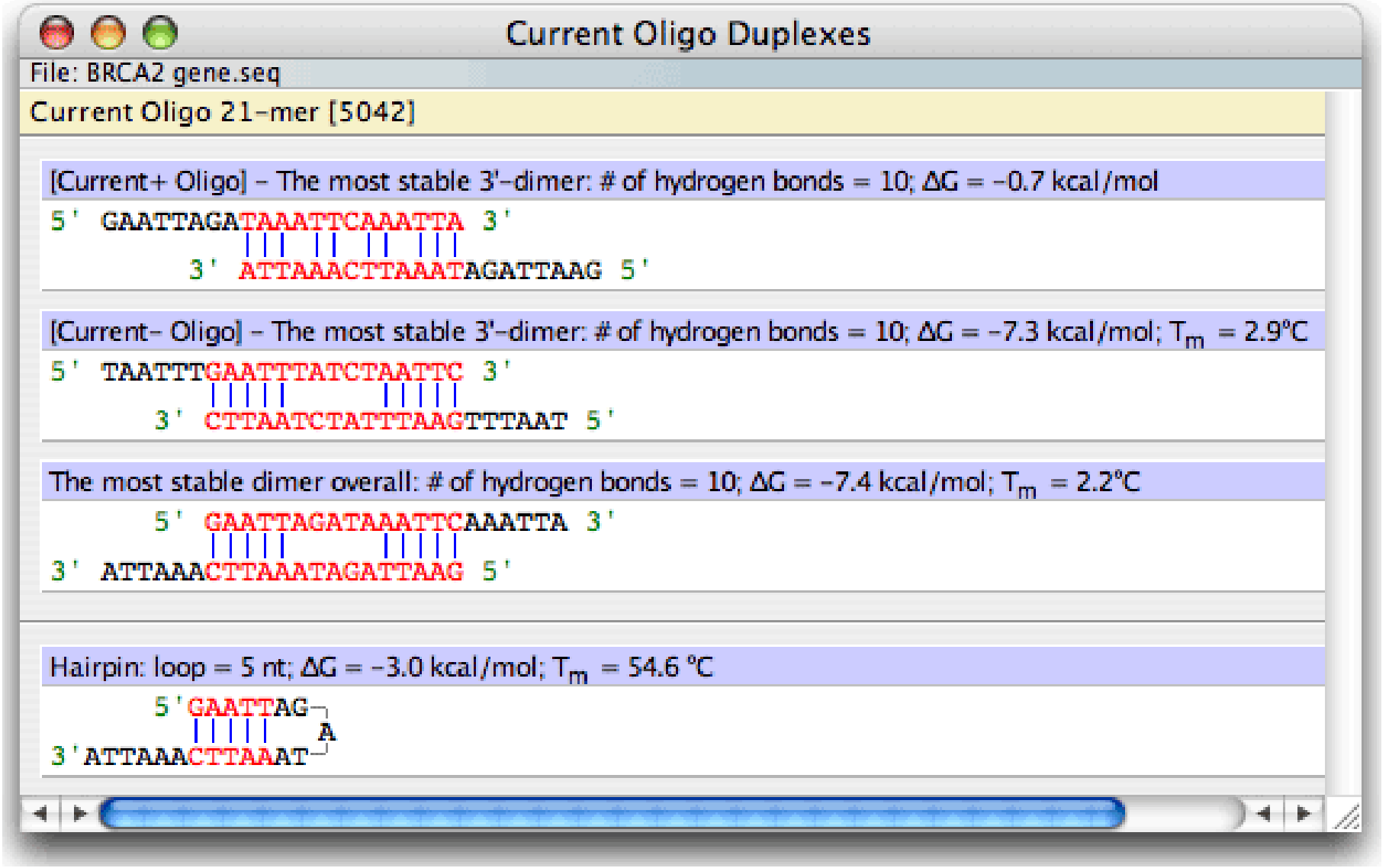
Total Na[+] Equivalent: 155.8 mM

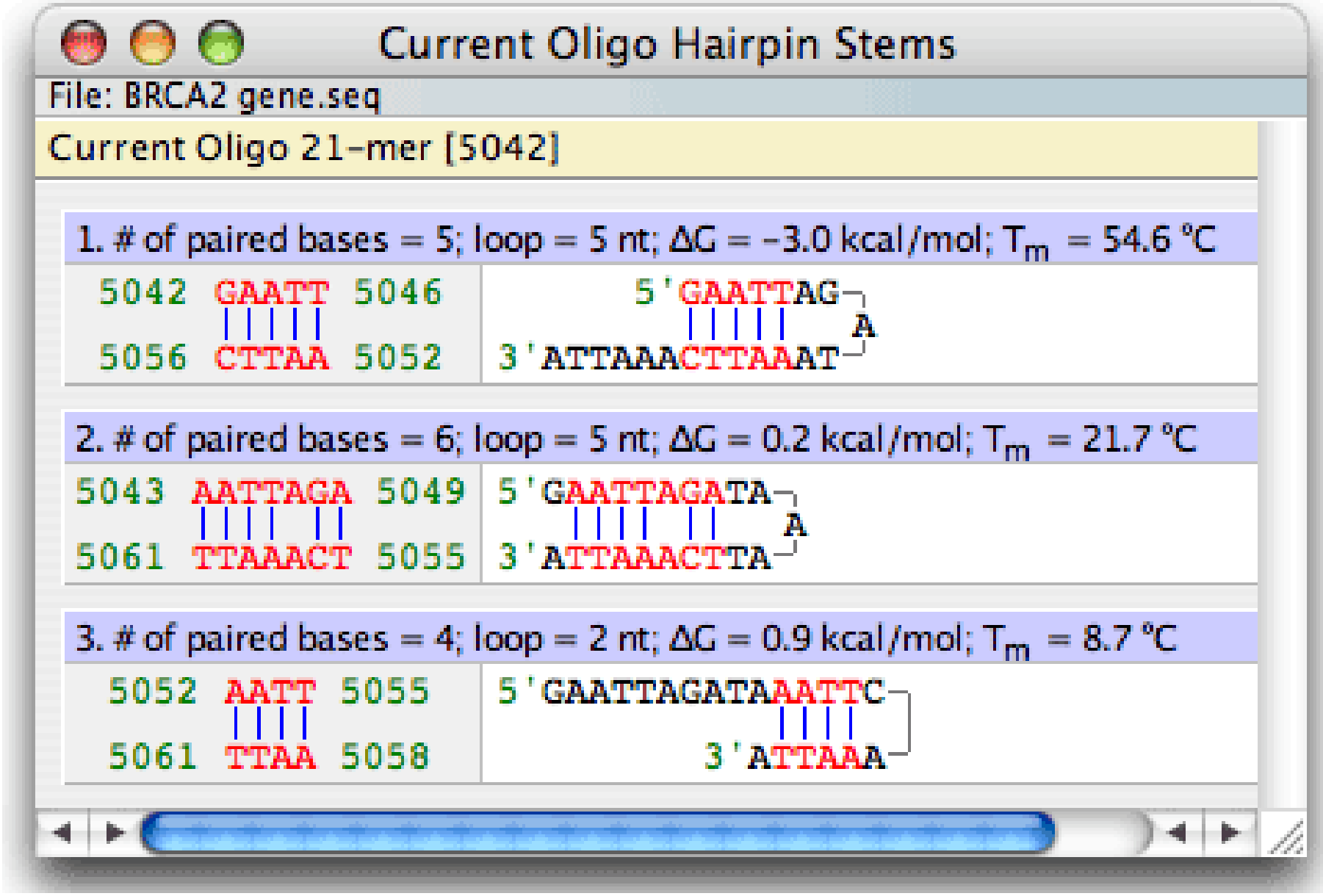


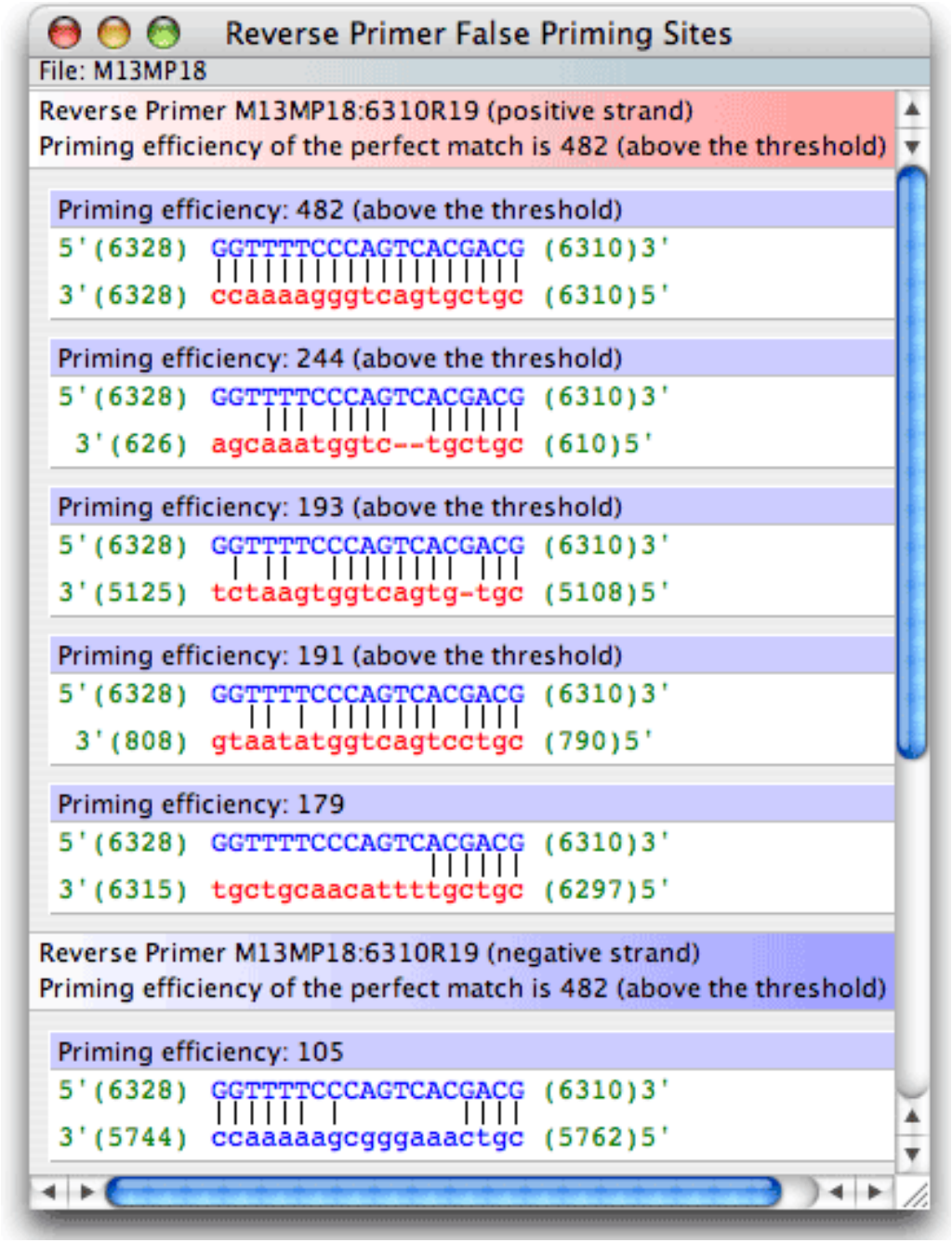
Priming Efficiency PE

DUPLEXY

- DIMER intermolekulární
- HAIRPIN intramolekulární




Current Oligo Hairpin Stems
File: BRCA2 gene.seq
Current Oligo 21-mer [5042]
1. # of paired bases = 5; loop = 5 nt; ΔG = -3.0 kcal/mol; Tm = 54.6 °C
5042 GAATT 5046
5056 CTTAA 5052
5'GAATTAG
A
3'ATTAAACTTAAAT
2. # of paired bases = 6; loop = 5 nt; ΔG = 0.2 kcal/mol; Tm = 21.7 °C
5043 AATTAGA 5049
5061 TTAAACT 5055
5'GAATTAGATA
A
3'ATTAAACTTA
3. # of paired bases = 4; loop = 2 nt; ΔG = 0.9 kcal/mol; Tm = 8.7 °C
5052 AATT 5055
5061 TTAA 5058
5'GAATTAGATAAATTC
3'ATTAAA


Reverse Primer False Priming Sites
File: M13MP18
Reverse Primer M13MP18:6310R19 (positive strand)
Priming efficiency of the perfect match is 482 (above the threshold)
Priming efficiency: 482 (above the threshold)
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(6328) ccaaaagggtcagtgctgc (6310)5'
Priming efficiency: 244 (above the threshold)
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(626) agcaaatggtc--tgctgc (610)5'
Priming efficiency: 193 (above the threshold)
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(5125) tctaagtggtcagtg-tgc (5108)5'
Priming efficiency: 191 (above the threshold)
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(808) gtaatatggtcagtcctgc (790)5'
Priming efficiency: 179
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(6315) tgctgcaacattttgctgc (6297)5'
Reverse Primer M13MP18:6310R19 (negative strand)
Priming efficiency of the perfect match is 482 (above the threshold)
Priming efficiency: 105
5'(6328) GGTTTTCCCAGTCACGACG (6310)3'
3'(5744) ccaaaaagcgggaaactgc (5762)5'

Forward Primer Composition

File: BRCA2 gene.seq

Forward Primer AY436640:6275F19

T_d	64.2°	[nearest neighbor method]
T_m	56.5°	[nearest neighbor method]
T_m	70.8°	[%GC method]
T_m	56°	[2(A+T)° + 4(G+C)° method]
T_m (RNA)[1M Na]	81°	[%GC method]
T_m (DNA:RNA)[1M Na]	74.7°	[%GC method]
A_{260}/A_{280}	1.59	[single strand]
Molecular Weight	5.8K	[one strand]
Molecular Weight	11.7K	[two strands]
µg/OD	47.4	[dsDNA]

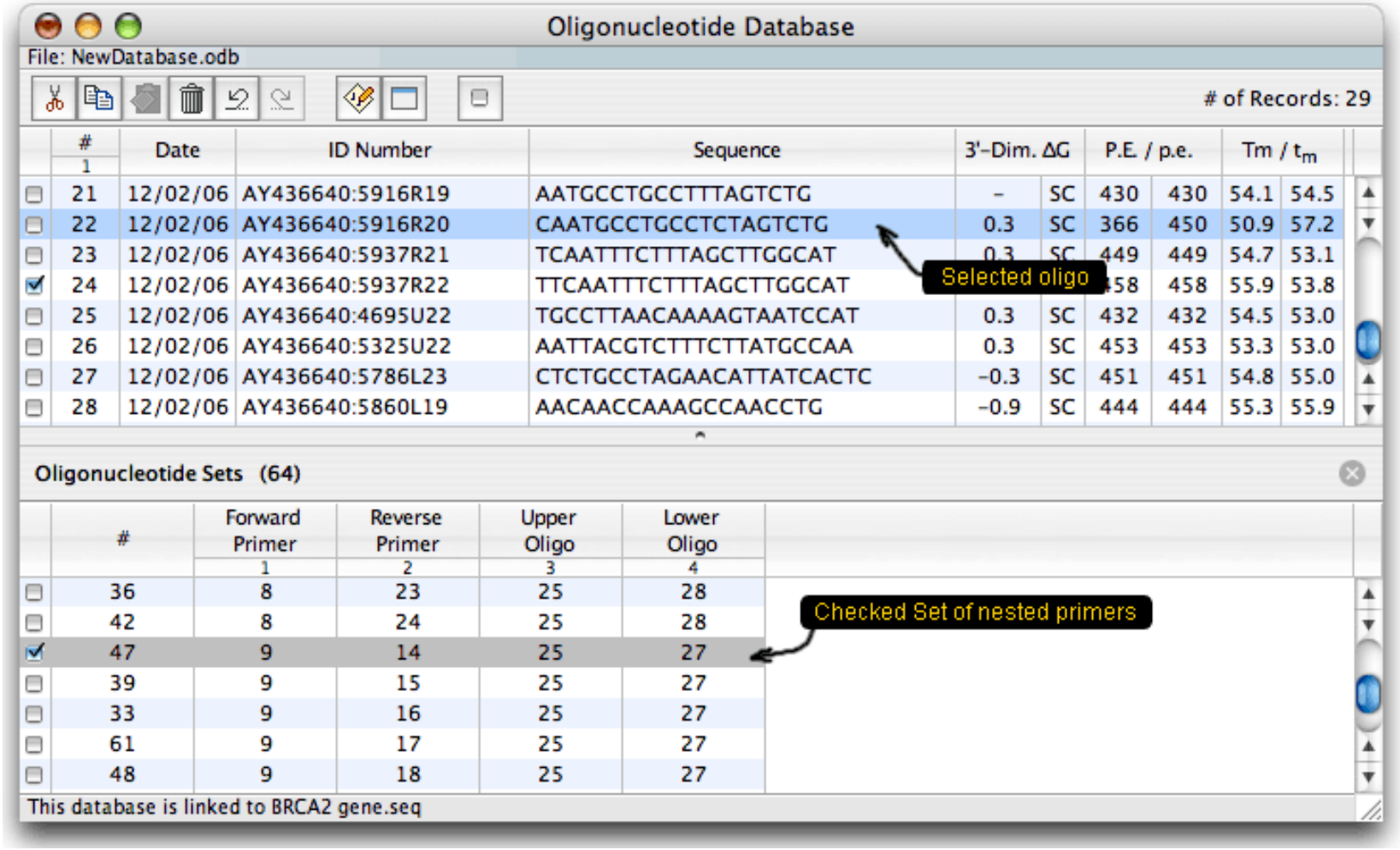
Base	Number & %	
A	2	[10.5%]
C	5	[26.3%]
G	4	[21.1%]
T	8	[42.1%]
A + T	10	[52.6%]
G + C	9	[47.4%]

DNA Melting Temperature in Various Salt and Formamide Concentrations [°C]

[mM]	xSSC	0%	10%	50%
1	0.006	24.8	18.3	-7.7
10	0.06	41.4	34.9	8.9
50	0.3	52.8	46.3	20.3
165	1	60.8	54.3	28.3
330	2	65.1	58.6	32.6
500	3	67.4	60.9	34.9
1000	6	70.8	64.3	38.3

Approximate t_m of the mismatched oligo
Mismatch $t_m = T_d - 1.2(\%\ \text{mismatch})°$

mism. #	t_m	mism. #	t_m
0	64.2	3	45.3
1	57.9	4	39.0
2	51.6	5	32.7

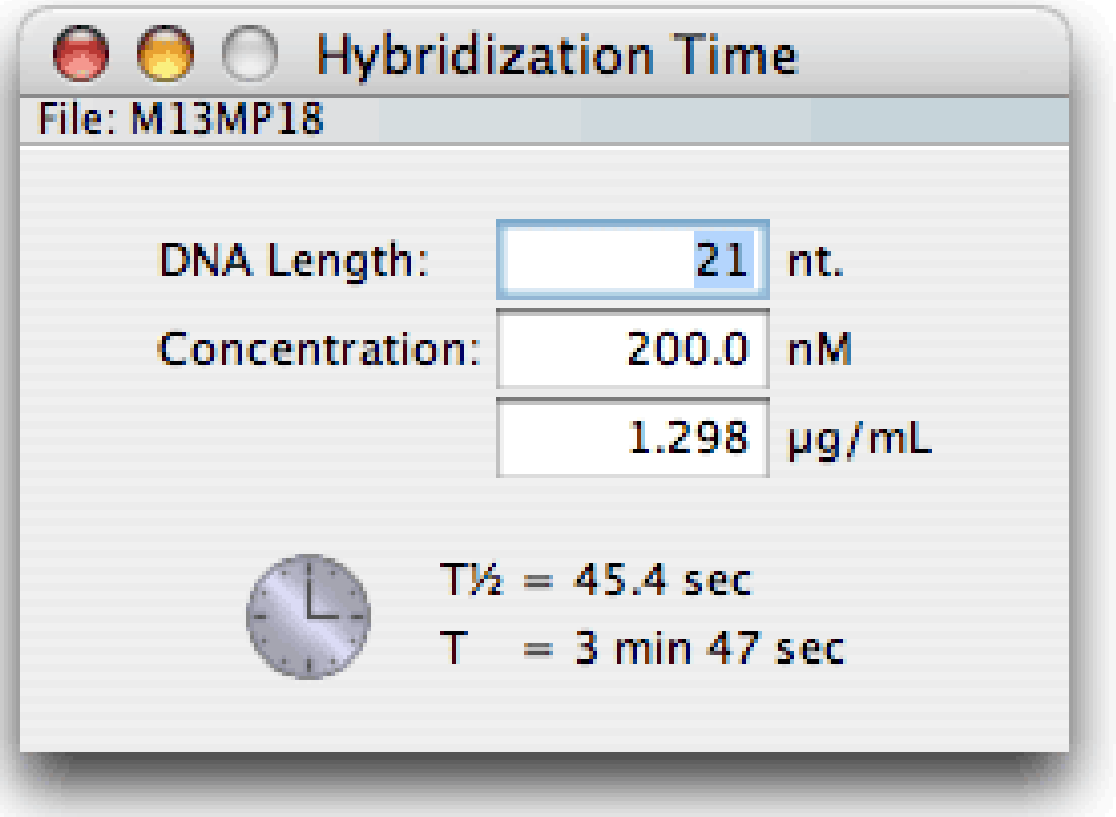

Oligonucleotide Database
File: NewDatabase.odb
# of Records: 29
1
Date
ID Number
Sequence
3'-Dim. ΔG
P.E. / p.e.
Tm / t_m
21 12/02/06 AY436640:5916R19 AATGCCTGCCTTTAGTCTG – SC 430 430 54.1 54.5
22 12/02/06 AY436640:5916R20 CAATGCCTGCCTCTAGTCTG 0.3 SC 366 450 50.9 57.2
23 12/02/06 AY436640:5937R21 TCAATTTCTTTAGCTTGGCAT 0.3 SC 449 449 54.7 53.1
24 12/02/06 AY436640:5937R22 TTCAATTTCTTTAGCTTGGCAT 458 458 55.9 53.8
25 12/02/06 AY436640:4695U22 TGCCTTAACAAAAGTAATCCAT 0.3 SC 432 432 54.5 53.0
26 12/02/06 AY436640:5325U22 AATTACGTCTTTCTTATGCCAA 0.3 SC 453 453 53.3 53.0
27 12/02/06 AY436640:5786L23 CTCTGCCTAGAACATTATCACTC -0.3 SC 451 451 54.8 55.0
28 12/02/06 AY436640:5860L19 AACAACCAAAGCCAACCTG -0.9 SC 444 444 55.3 55.9
Selected oligo
Oligonucleotide Sets (64)
1
Forward Primer 2
Reverse Primer 3
Upper Oligo 4
Lower Oligo
36 8 23 25 28
42 8 24 25 28
47 9 14 25 27
39 9 15 25 27
33 9 16 25 27
61 9 17 25 27
48 9 18 25 27
Checked Set of nested primers
This database is linked to BRCA2 gene.seq

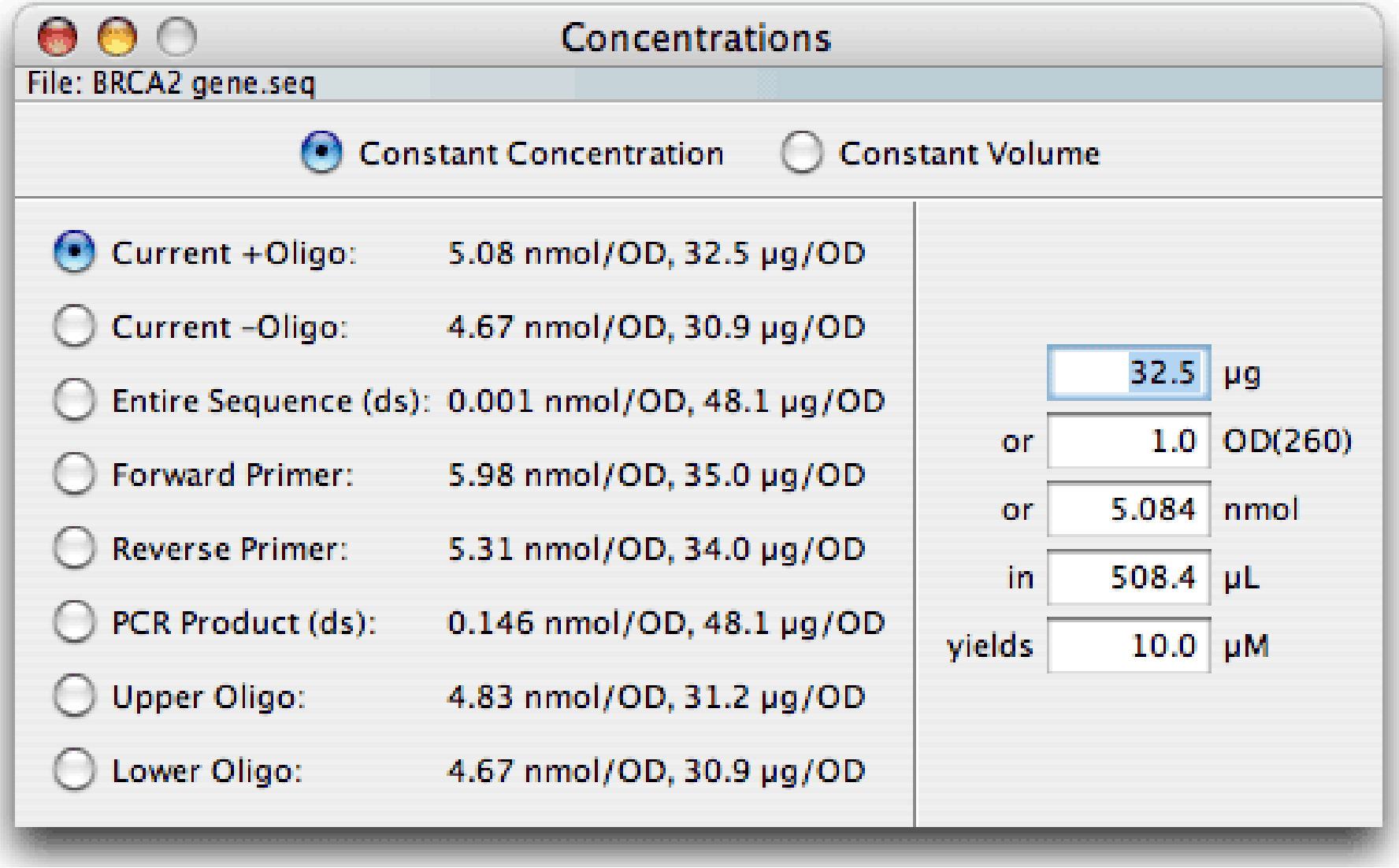
Restriction Enzyme Sites in Protein

File: BRCA2 gene.seq

#	Enzyme	Site	# Cuts	Positions & Fragment Sizes
41	KpnI	GT2VpzY6	8	-21253 23654 68 23722 52 23774 237 24011 585 24596 162 24758
				629 25387 1219 26606 22851
42	MluI	TR1RVyA7	5	-22233 22674 2824 25498 576 26074 106 26180 244 26424 23033
43	MunI	QL3NawI5	10	-21287 23620 355 23975 351 24326 282 24608 242 24850 72 24922
				351 25273 714 25987 187 26174 420 26594 22863
44	NaeI	AG2PAxR6	7	-21823 23084 597 23681 1286 24967 86 25053 573 25626 149 25775
				623 26398 23059
45	NarI	GA2APzR6	1	-20043 24864 24593
46	NcoI	PW3HGwM5	4	-22361 22546 336 22882 887 23769 531 24300 25157
47	NdeI	HM2IawY5	2	-20366 24541 1211 25752 23705
48	NheI	AS2LAx-6	16	-22276 22631 322 22953 185 23138 88 23226 27 23253 461 23714
				369 24083 312 24395 288 24683 151 24834 273 25107 536 25643
				402 26045 30 26075 210 26285 372 26657 22800

Search: 22454 to 27004 End Cut Type: Blunt, Odd, 3'-overhang, 5'-overhang


Hybridization Time
File: M13MP18
DNA Length:
21
nt.
Concentration:
200.0
nM
1.298
µg/mL
T½ = 45.4 sec
T = 3 min 47 sec


Concentrations
File: BRCA2 gene.seq
Constant Concentration
Constant Volume
Current +Oligo: 5.08 nmol/OD, 32.5 µg/OD
Current -Oligo: 4.67 nmol/OD, 30.9 µg/OD
Entire Sequence (ds): 0.001 nmol/OD, 48.1 µg/OD
Forward Primer: 5.98 nmol/OD, 35.0 µg/OD
Reverse Primer: 5.31 nmol/OD, 34.0 µg/OD
PCR Product (ds): 0.146 nmol/OD, 48.1 µg/OD
Upper Oligo: 4.83 nmol/OD, 31.2 µg/OD
Lower Oligo: 4.67 nmol/OD, 30.9 µg/OD
32.5 µg
or 1.0 OD(260)
or 5.084 nmol
in 508.4 µL
yields 10.0 µM

…nejlepším učitelem je praxe…