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Design of PCR Primers

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CEITEC Central European Institute of Technology
NCBR National Centre for Biomolecular Research

CG920 Genomics
Lecture 12

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OLIGONUCLEOTIDES

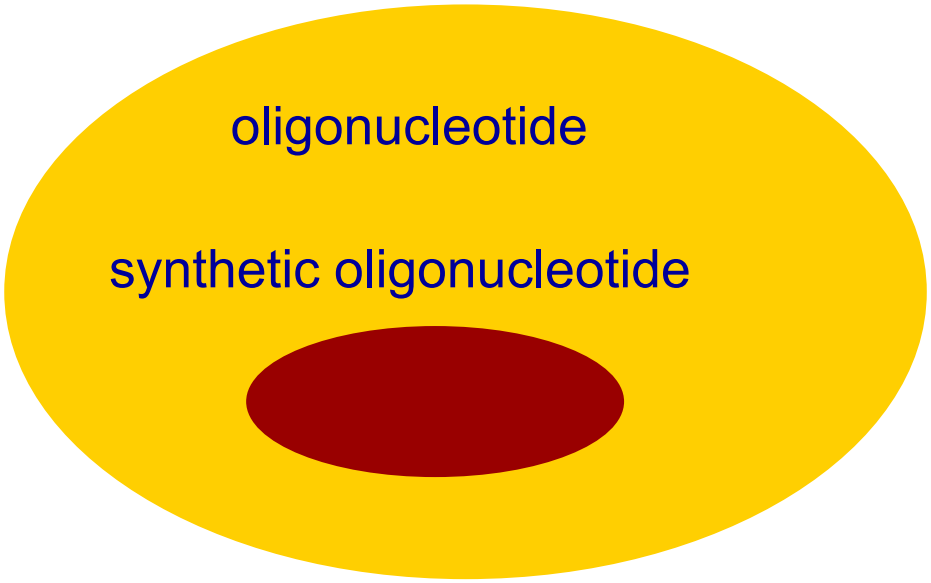
- definition
- aplications
- modifications
- synthesis
- purification
- quality control

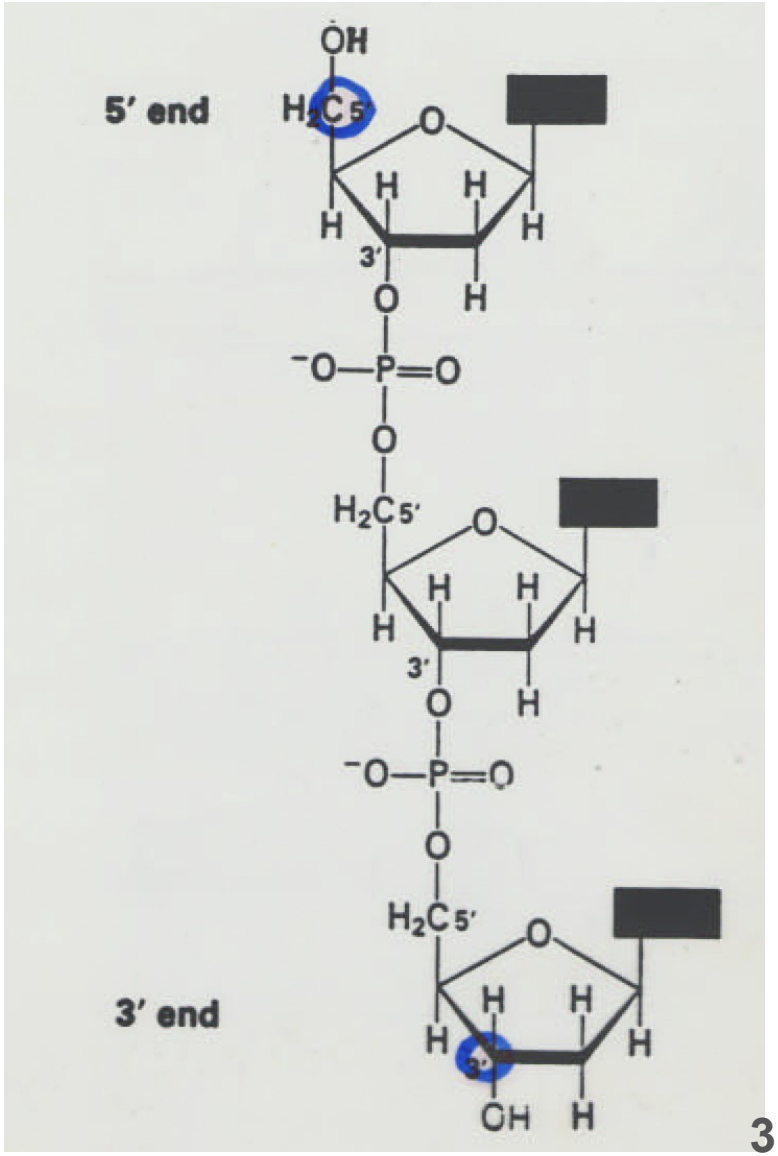
- design of sequence
- rules
- software OLIGO 7
- example

orientation! polymerase!

oligonucleotide

- short single stranded structure
- DNA or RNA (also PNA)
- **hydroxyl** on both ends
  (no phosphate on 5-end as usual)





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Aplications of synthetic oligonucleotides

- primers for synthesis of complementary DNA
  PCR, Real-Time PCR
- gene synthesis and recombinant proteins
- hybridisation probes for cloning
- site directed mutagenesis
- sequencing ang genetic profiling
- diagnostics – tests and biosensors
- gene arrays
- blocation of gene expression *antisense oligo*
- prospective therapeutics and DNA vaccines
- NMR monitoring of DNA – protein interactions
- structural X-ray analysis of NA

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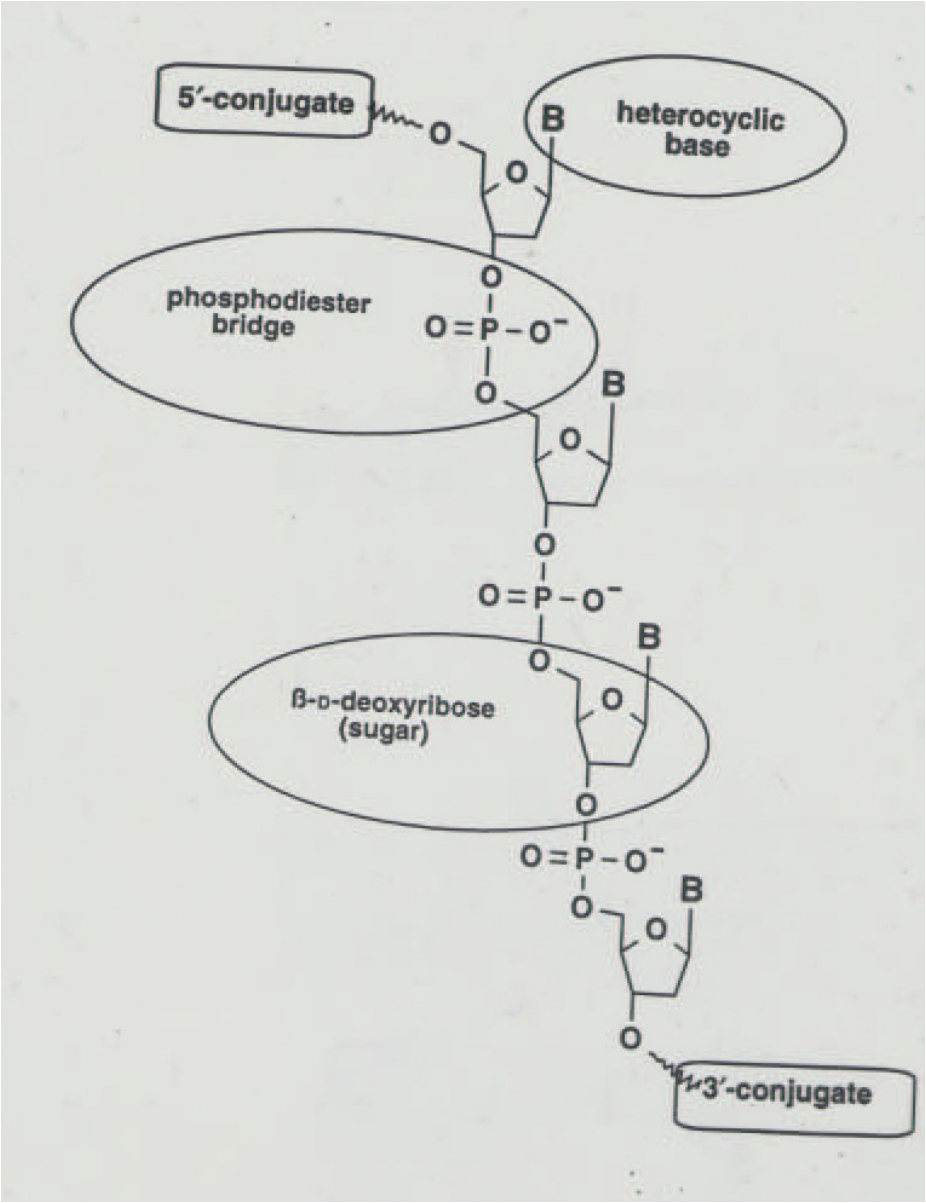
Modifications

- degeneration
- end of sequence
- bases
- phosphate
- carbohydrate
- PNA



Degenerated oligonucleotides

Examples:

ACG TAC GTA CGT ACG TAC	non-degenerated
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

Degenerated oligonucleotides

2-deoxyinosin

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

Modification on 5´- end

postsynthetic modifications

sequencing
fragmenation analysis
gene arrays
Real-Time PCR

- Phosphorylation
- Amino group
- Thio group
- Digoxigenin
- Biotin
- Enzymes
- Psoralen
- Acridine
- Cholesterole
- Fluorescent dyes
- Quenchers
- 2,4- dinitrophenyl
- Spacer
- Branching
- Blocation

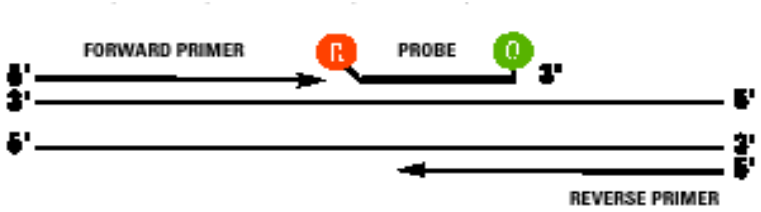
Modifications on 3´- end

derivatized matrix

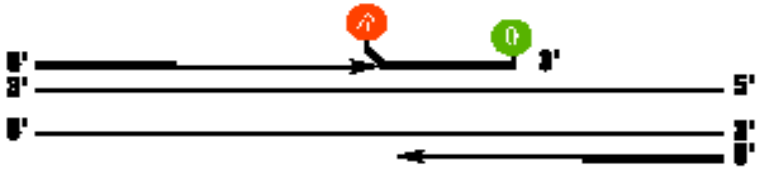
- Phosphate
- Thio group
- → Amino group
- Spacer
- Acridine
- → Biotin
- → Fluorescent dyes
- → Quenchers
- Cholesterole
- 2,4 dinitrophenyl

Real-Time PCR

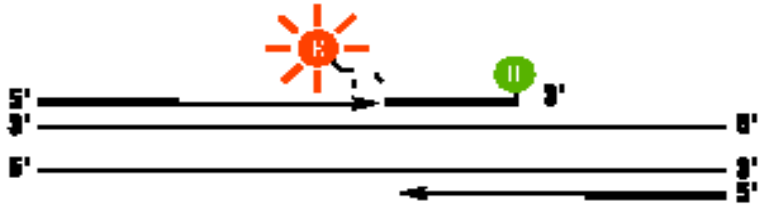
- 2x labeled probe
- REPORTER
- QUENCHER



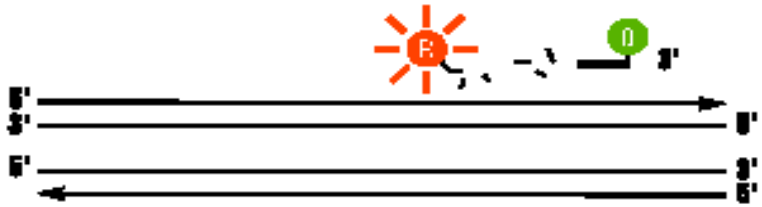
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



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.



Other modifications

Phosphorothioates
Phosphorodithioates
H-phosphonates
Methylphosphonates

← backbone

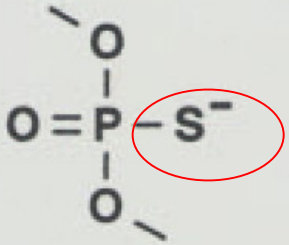
carbohydrate →

Modifications in 2´- position
Ribose modification

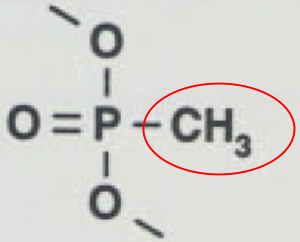
Therapeutics

→ Nondegradable by nucleases!

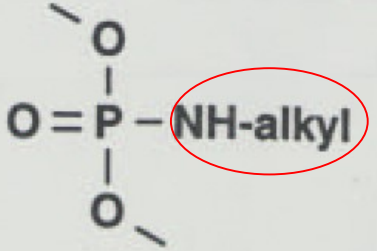
Modification of phosphodiester



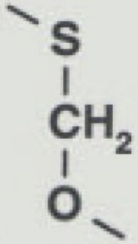
phosphorothioate



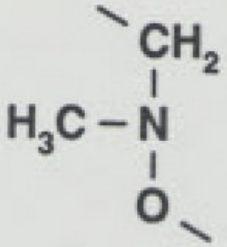
methylphosphonate



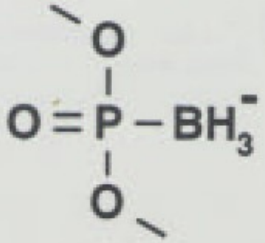
phosphoramidate



3′-thioformacetal



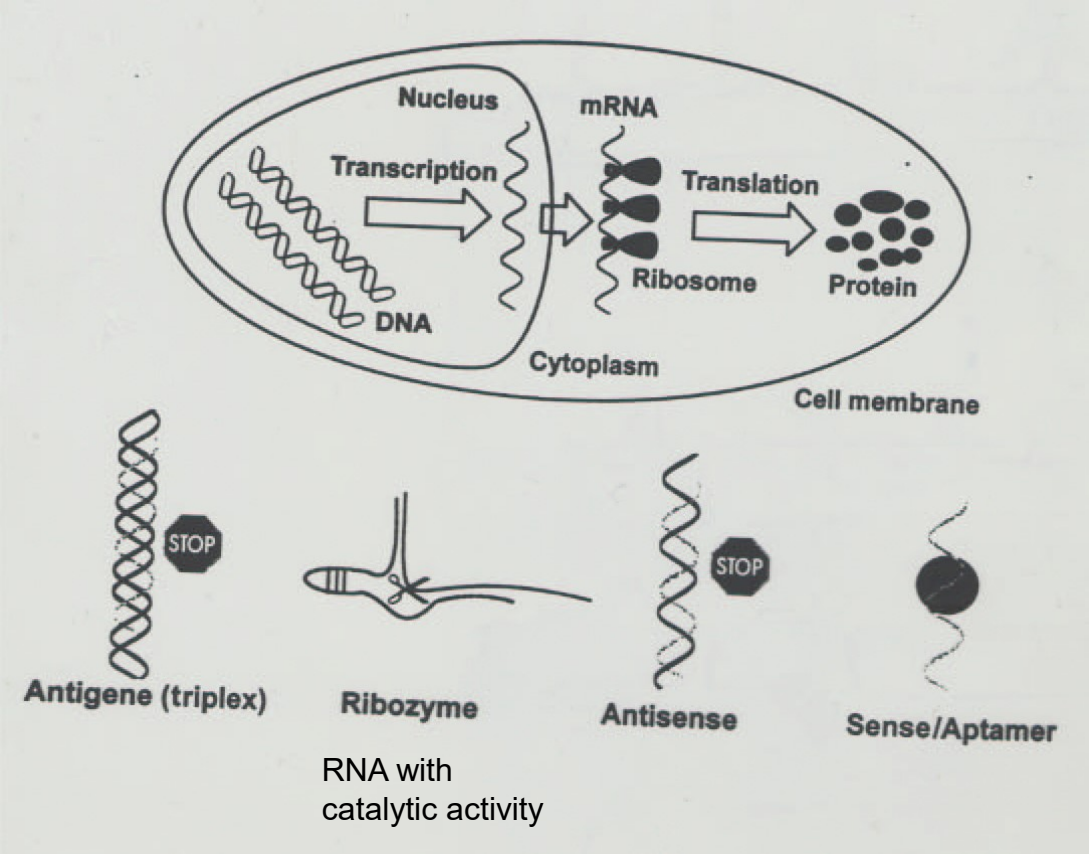
methylene(methyliminio)



boranophosphate

ANTISENSE oligonucleotide

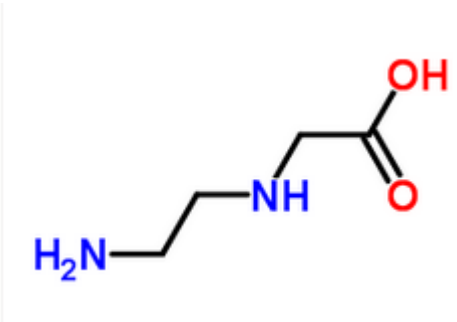
- oligonucleotide or analogue
- complementary to segment of RNA or DNA
- inhibition of normal function due to coupling

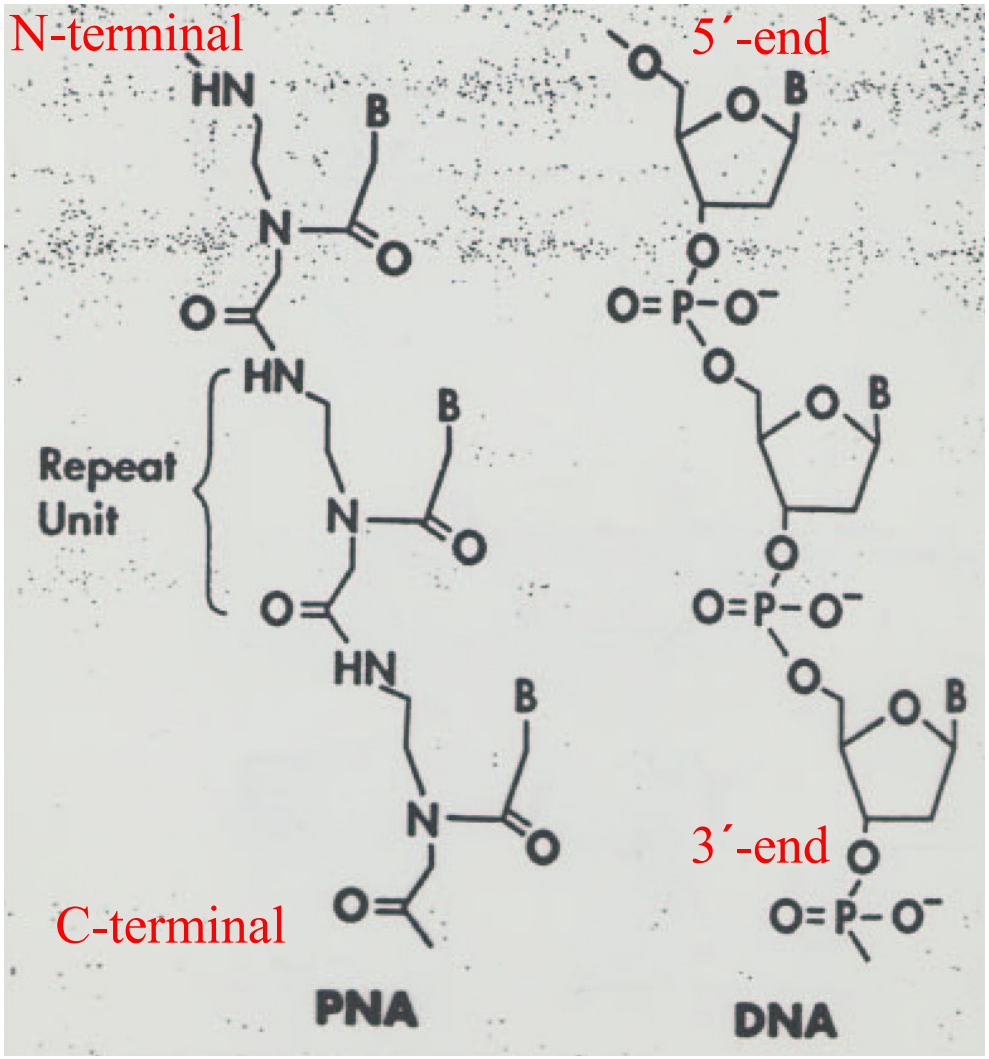


Peptidonucleic acid

- uncharged molecule
- binding with DNA/RNA

N-(2-aminoethyl)-glycine →





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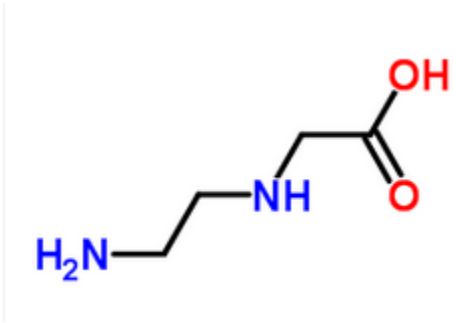

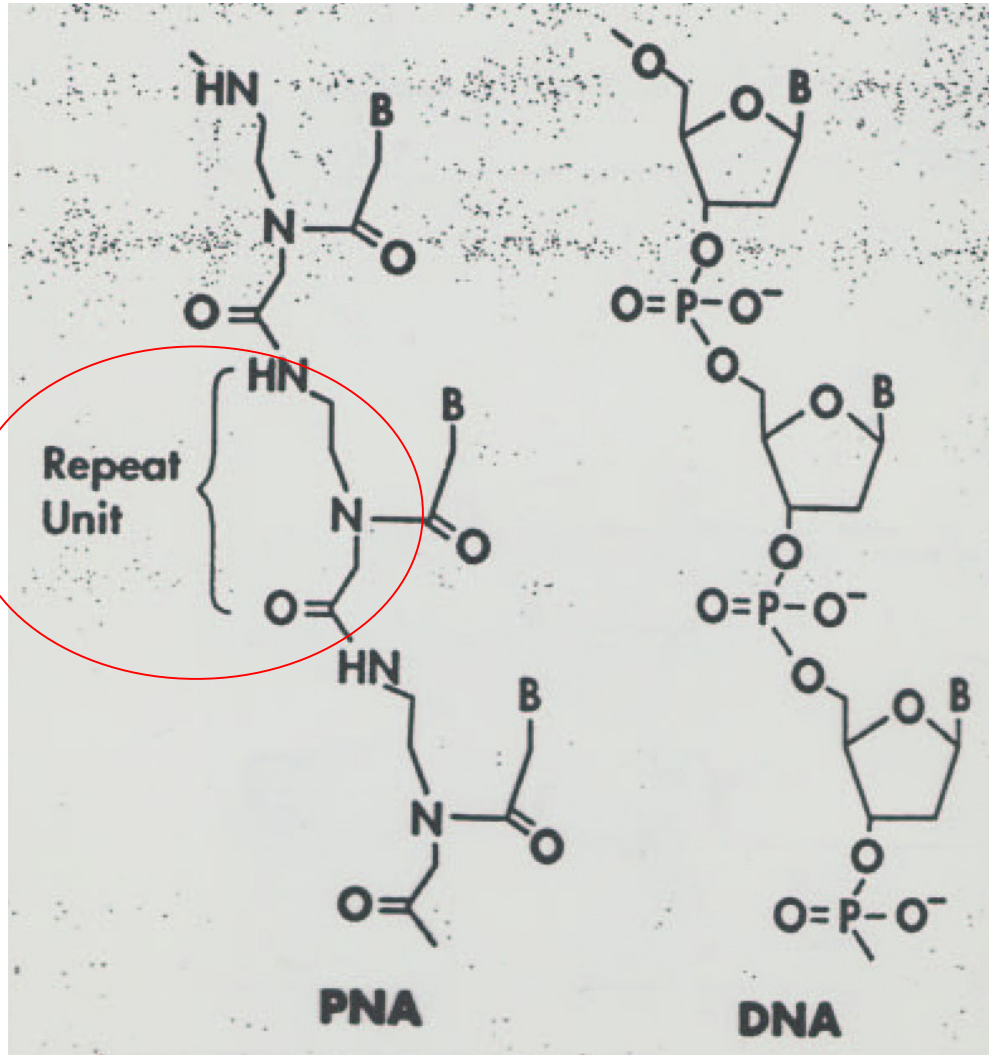

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Why PNA

- thermostable
- Tm not depending on salts
- high specificity
- high affinity
- resistant towards enzymes

Gambari R. *Expert Opin Ther Pat.* 2014, 24(3):267-94.
Peptide nucleic acids: a review on recent patents and technology transfer.

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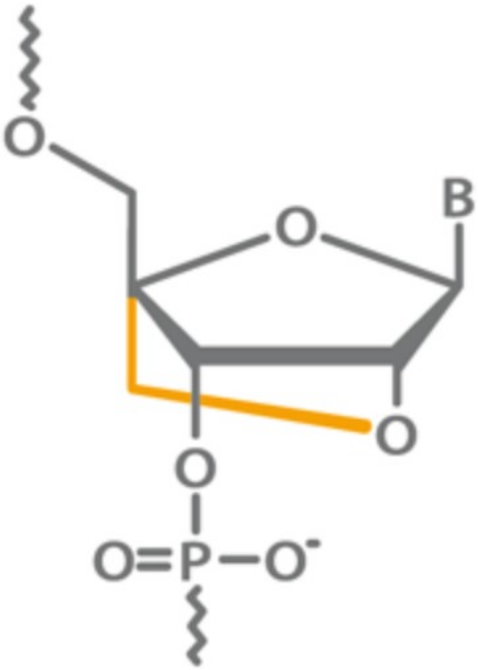
LNA



Locked Nucleic Acid

- 2'-O, 4'-C methylene bridge
- supressed flexibility of ribofuranose ring
- structure is locked into rigid C3-endo conformation
- enhanced hybridisation
- outstanding biostability

Molecular Therapy (2012); **20** 8, 1590–1598.
LNA-based Oligonucleotide Electrotransfer for miRNA Inhibition

OLIGONUCLEOTIDES

design

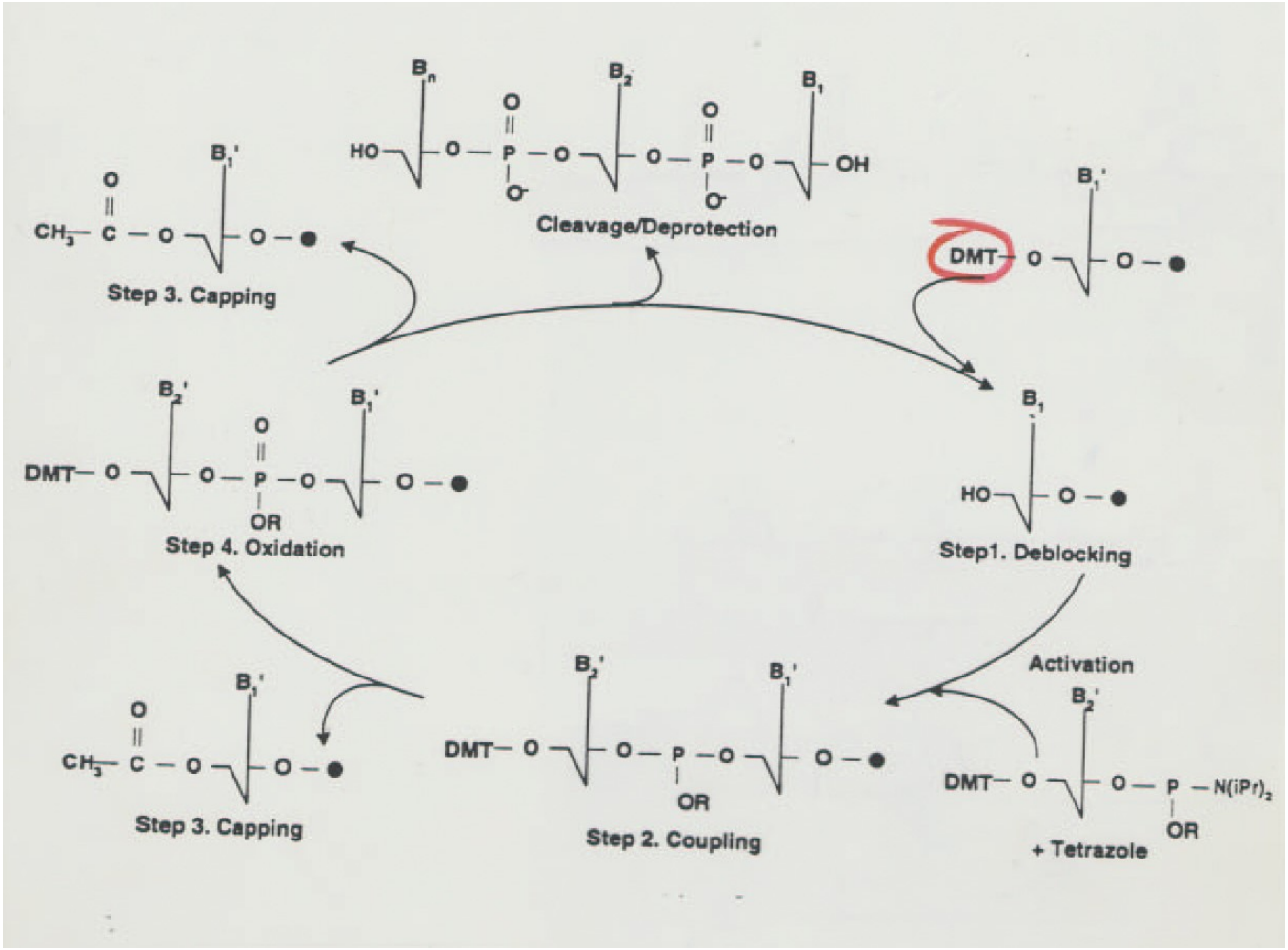
synthesis

purification

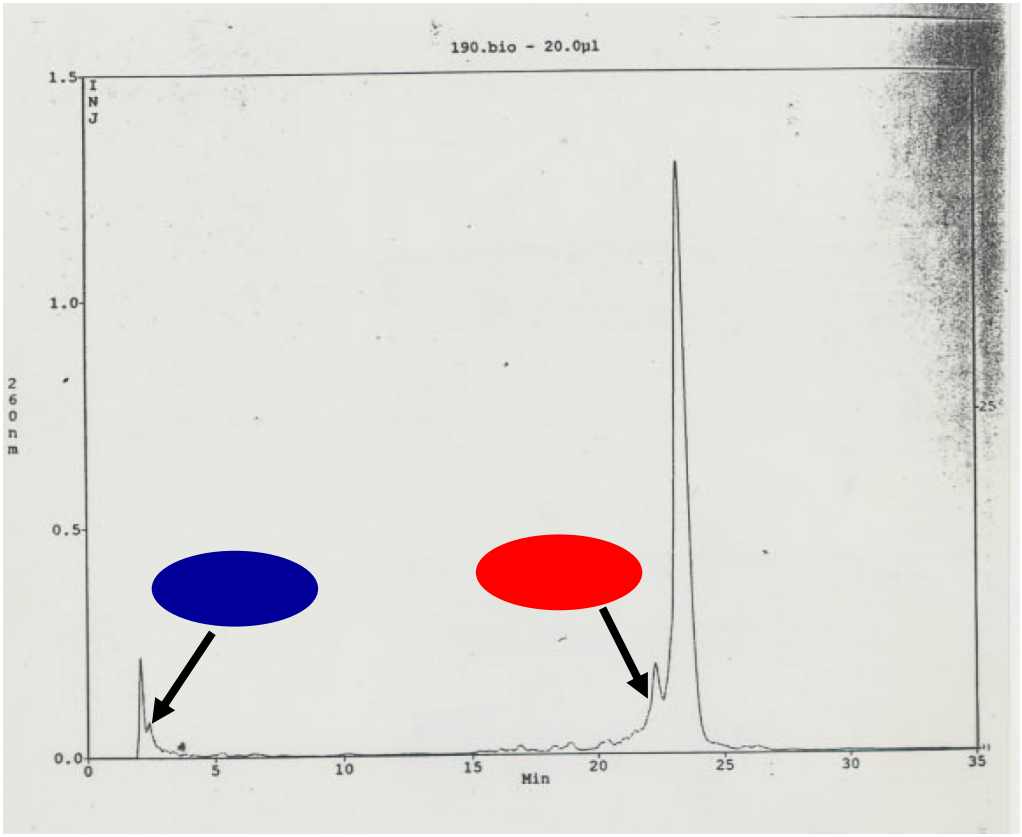
EXPEDITE 8909

Oligonucleotide Synthesis

- synthesis on solid matrix
- from 3´-end to 5´-end
- anhydrous environment



Quality Control

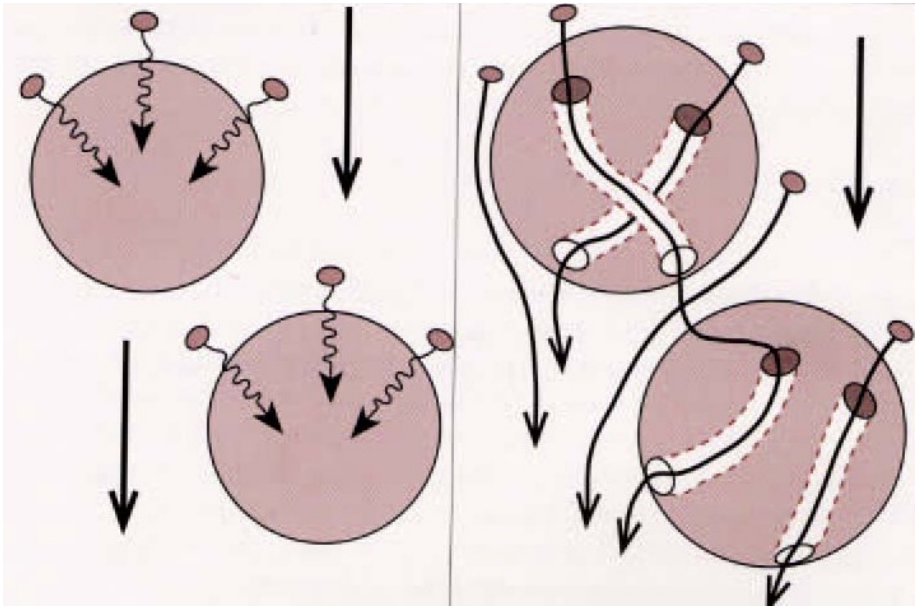


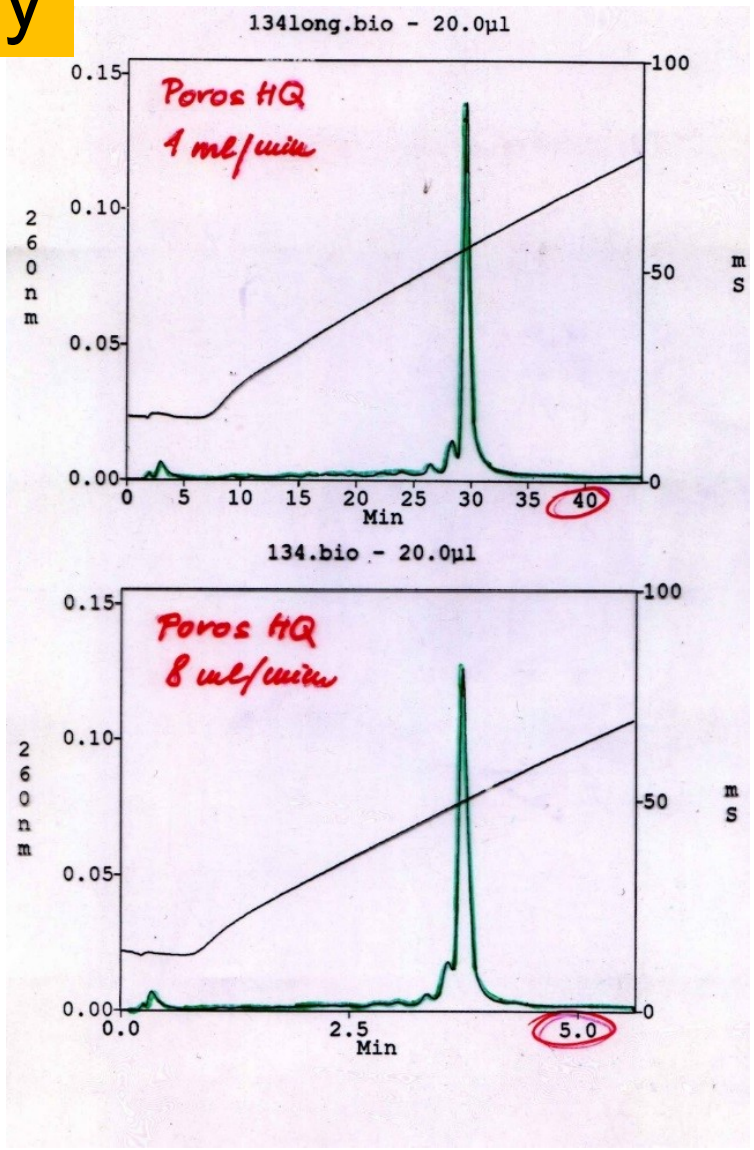
- HPLC
- perfusion chromatography

- anex
- RP

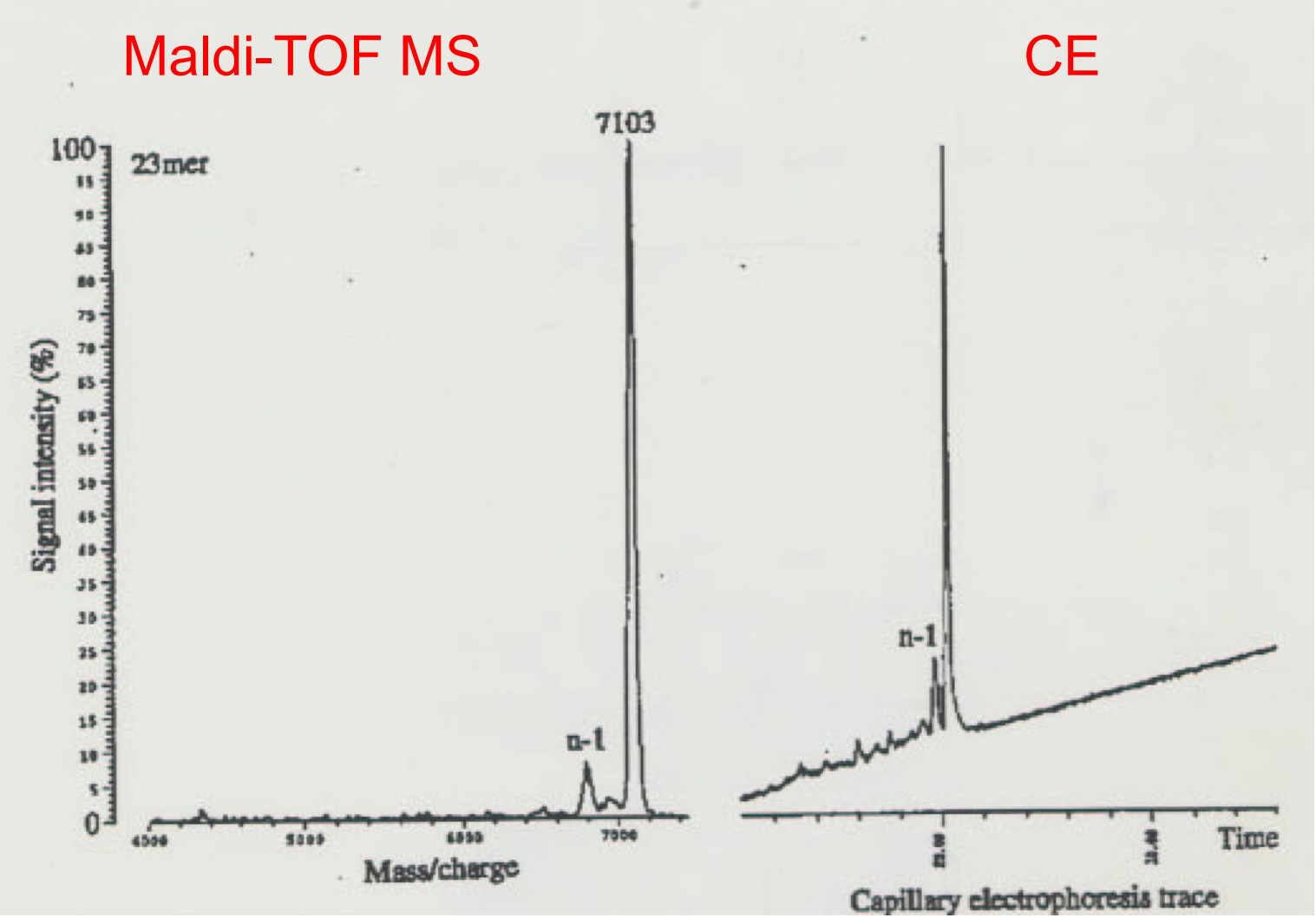
Perfusion chromatography

classical sorbent POROS





Quality Control



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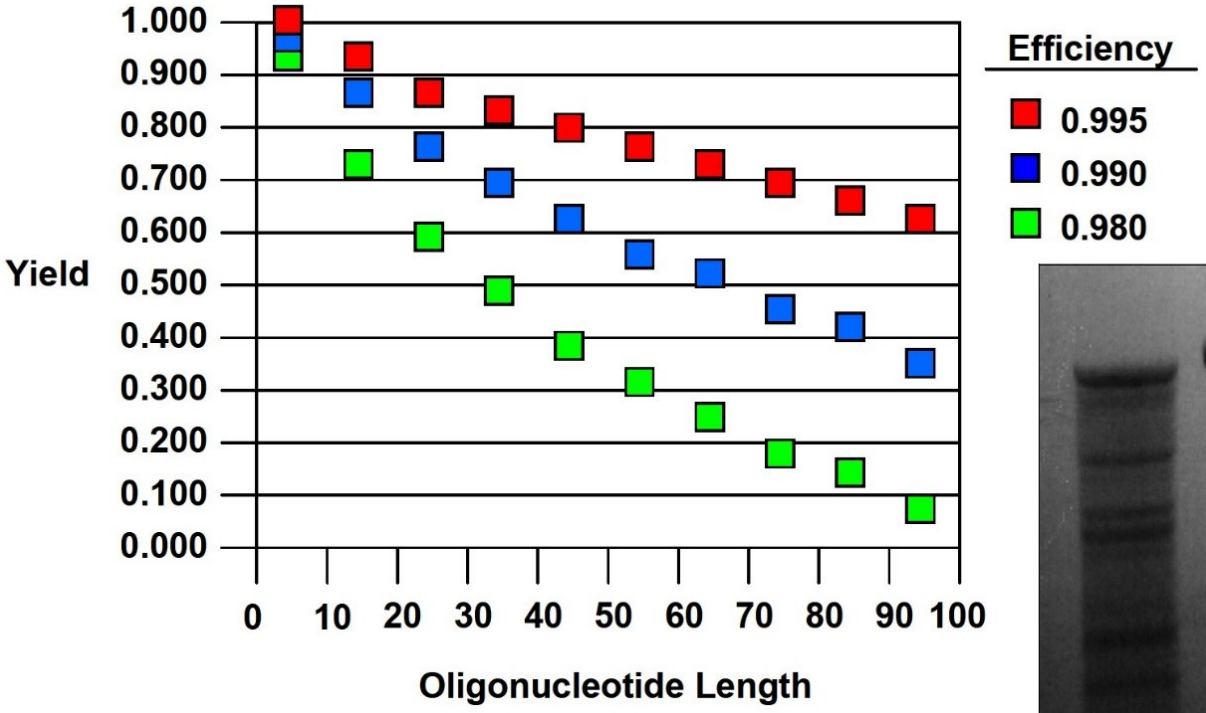
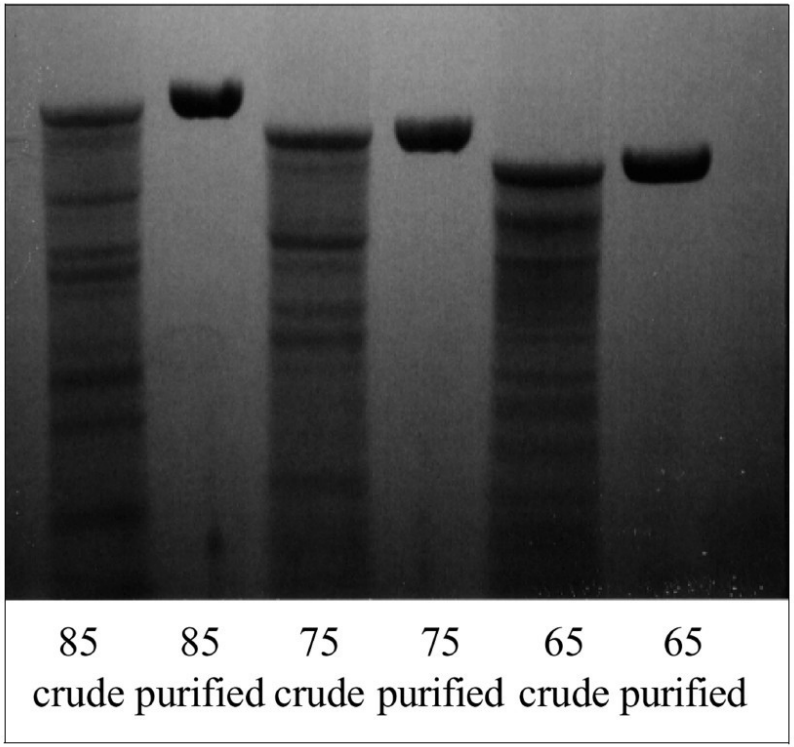
YIELD



PAGE of long-mers



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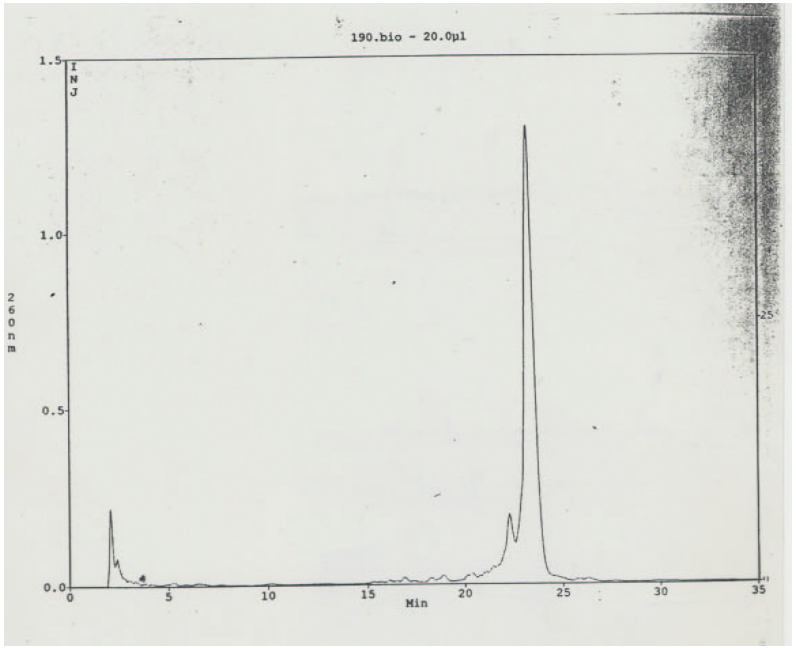
PURIFICATION

- Sephadex
- RP cartridge
- HPLC



OLIGONUCLEOTIDE DESIGN

- manual
- computer assisted

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

Main features of good PCR primer sequence

- highly specific
- no dimers and hairpins
- stable duplexes with active sequence
- lightly unstable 3´-end

OLIGO 6

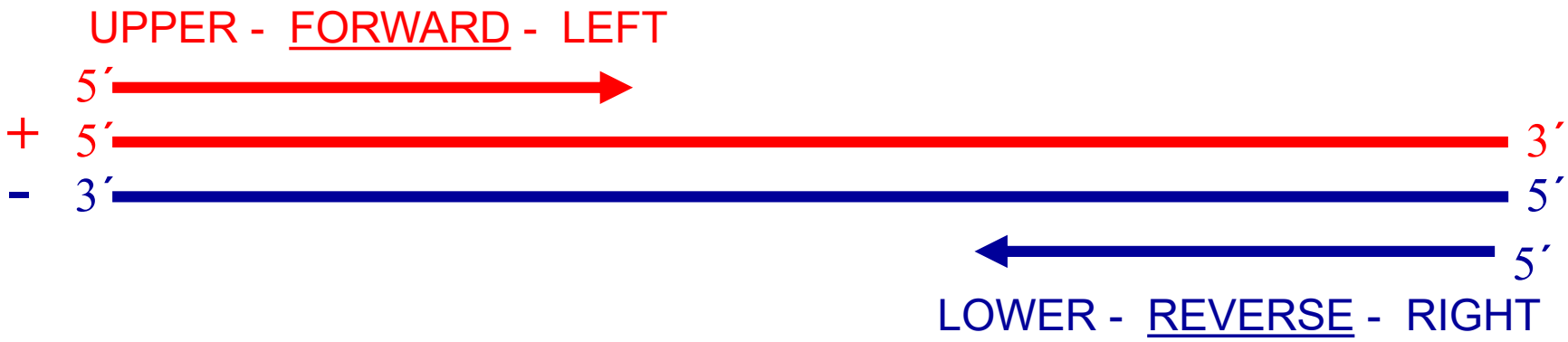
- PCR primers
- hybridisation probes
- sequencing primers

OLIGO 7 (from 2008)

- TaqMan probes
- primers for *nested PCR*
- *molecular beacons*
- siRNA

Terminology of PCR primers

forward primer… part of the + string
reverse primer… part of the - string



Terminology

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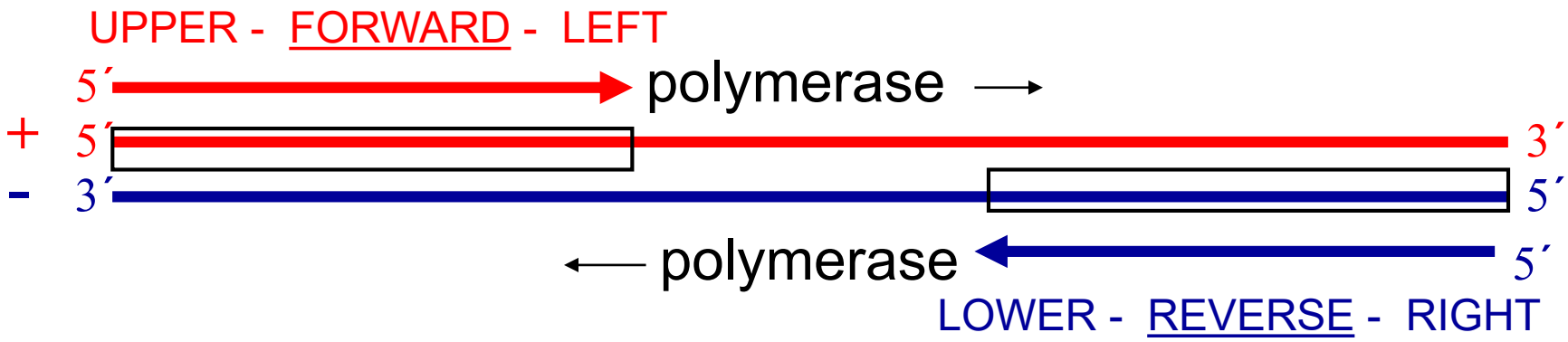
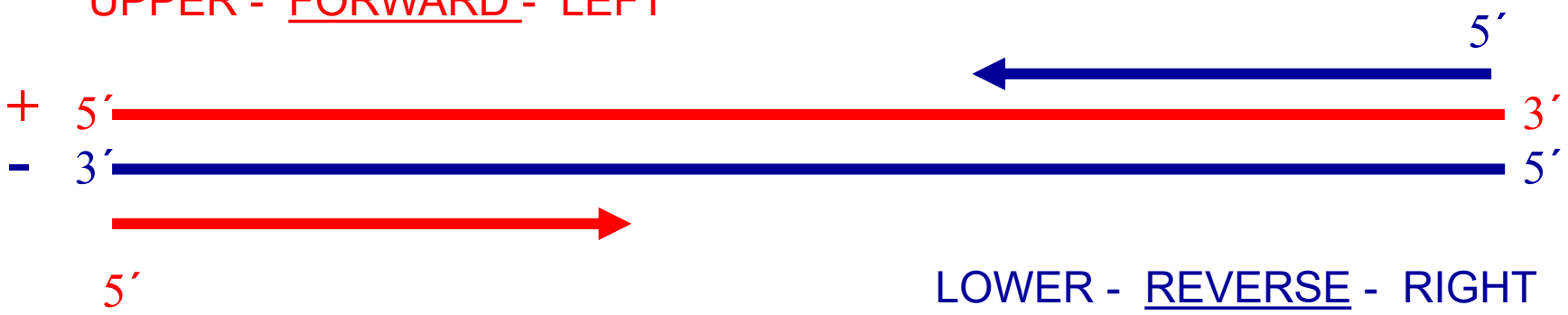


Annealing of PCR primers

UPPER - FORWARD - LEFT



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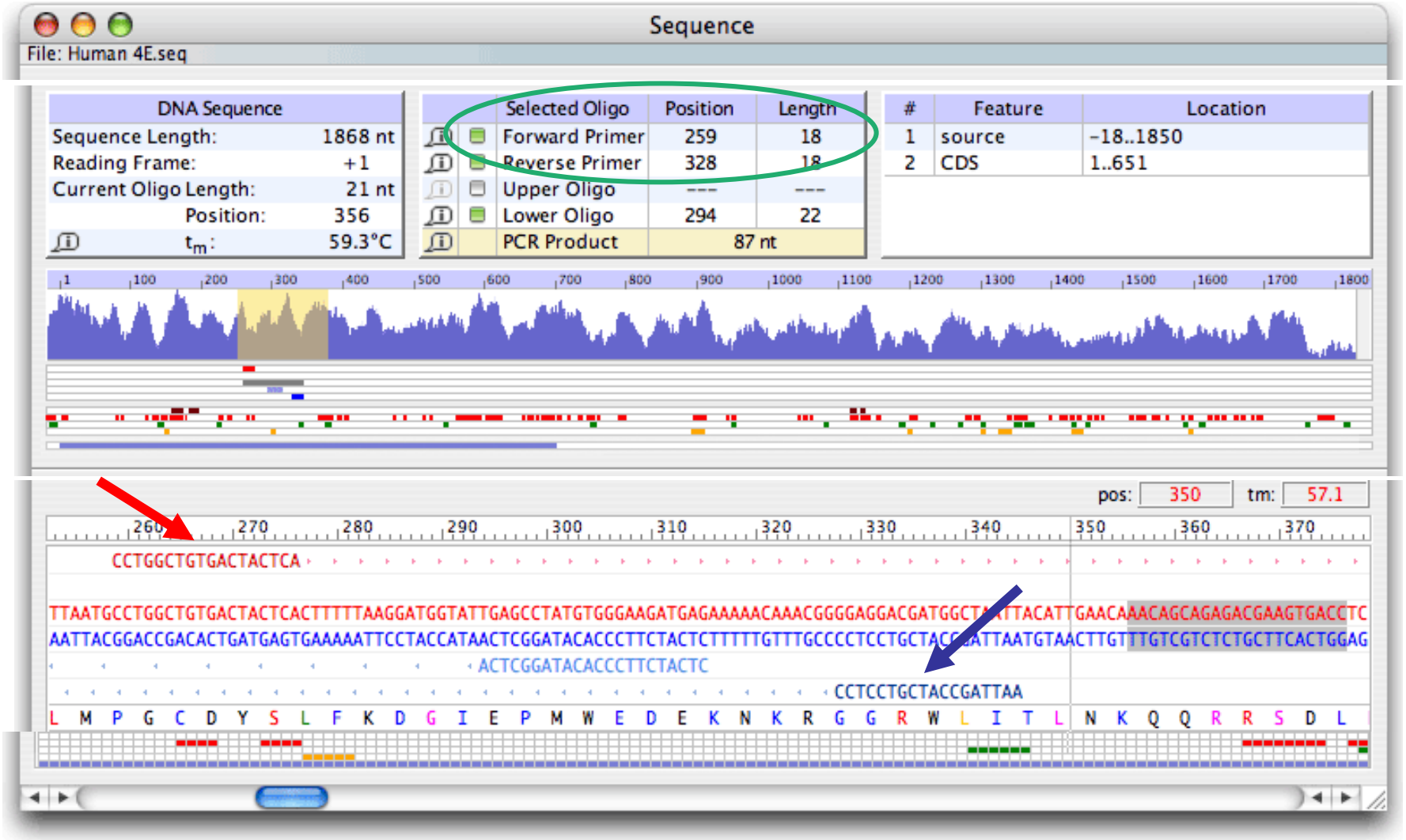
5´ CTT CTG CTC AAT CTT TCT AC 3´ **FORWARD**

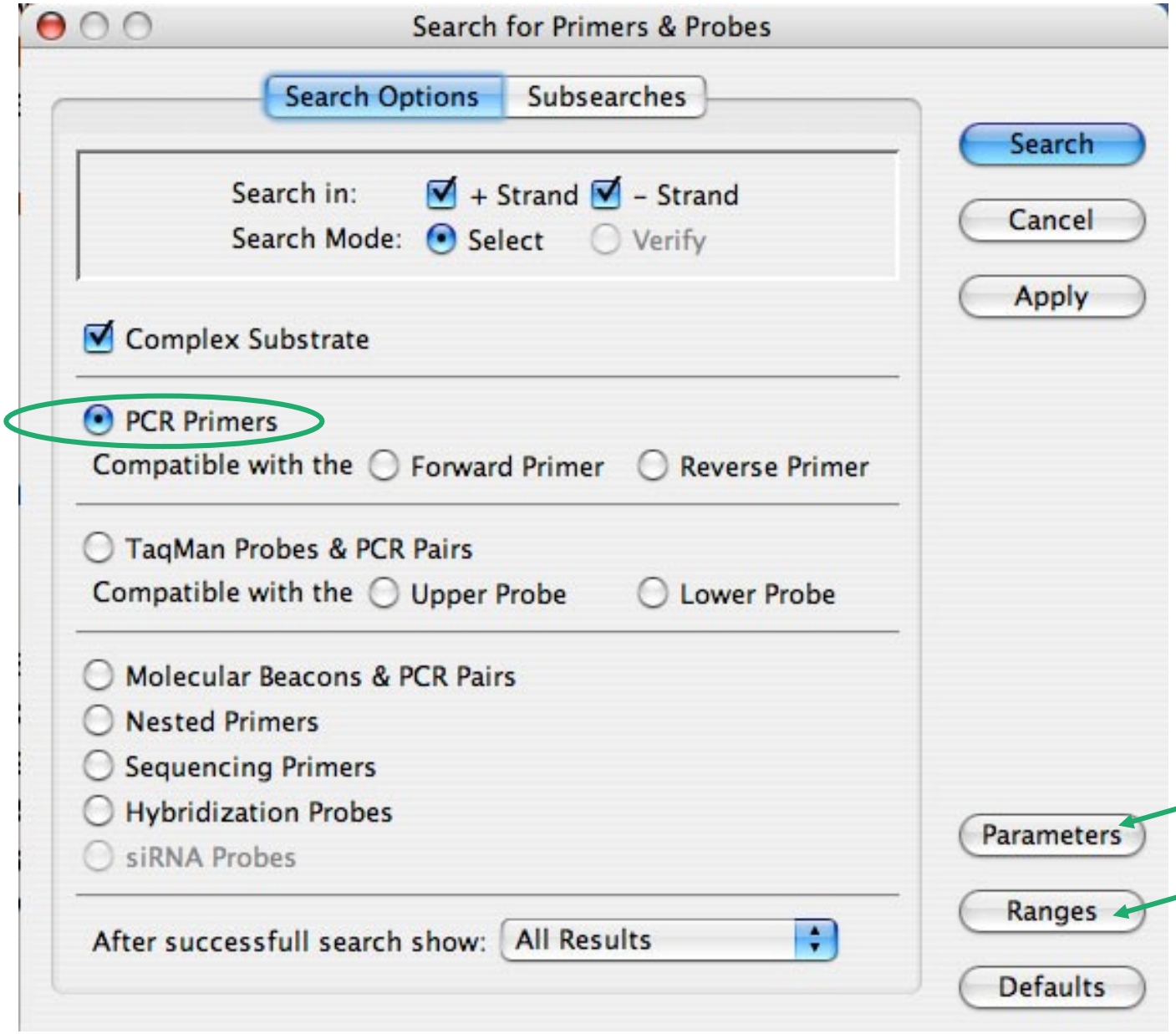
+ 5´

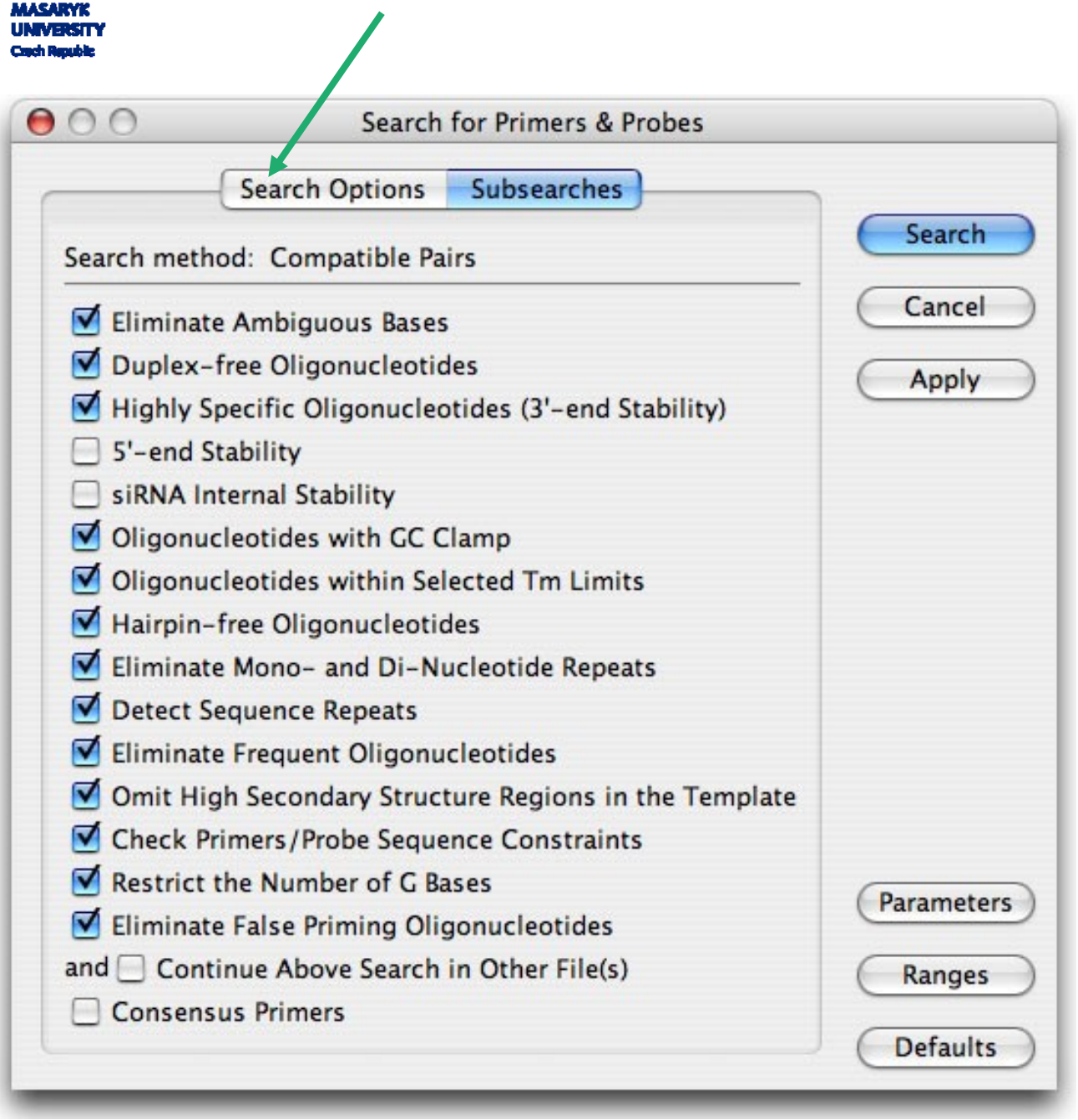
```
   1 ATGGCTTCTG CTCAATCTTT CTACAACCAA AGCTCTGTCT TGAAAATCAA
  51 TGTCATGGTT GTGGACGATG ATCATGTTTT CCTTGATATC ATGTCACGCA
 101 TGCTTCAACA CTCCAAATAC AGAGGTAATT AAATATTATT ATCATATTAT
 151 ATATAATATG TTATTGATTT TTTGTTTGTG ATTTCATTTA GATTTTTATT
 201 TCTATGATTT CTTAGCATGA AATACAATTT TTGGAGAAAC AACTAGCAGT
 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
tm: 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
ACTCGGATACACCCTTCTACTC
CCTCCTGCTACCGATTAA


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

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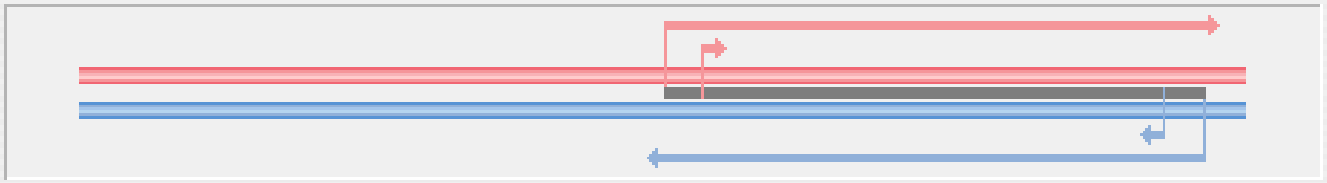
PCR

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

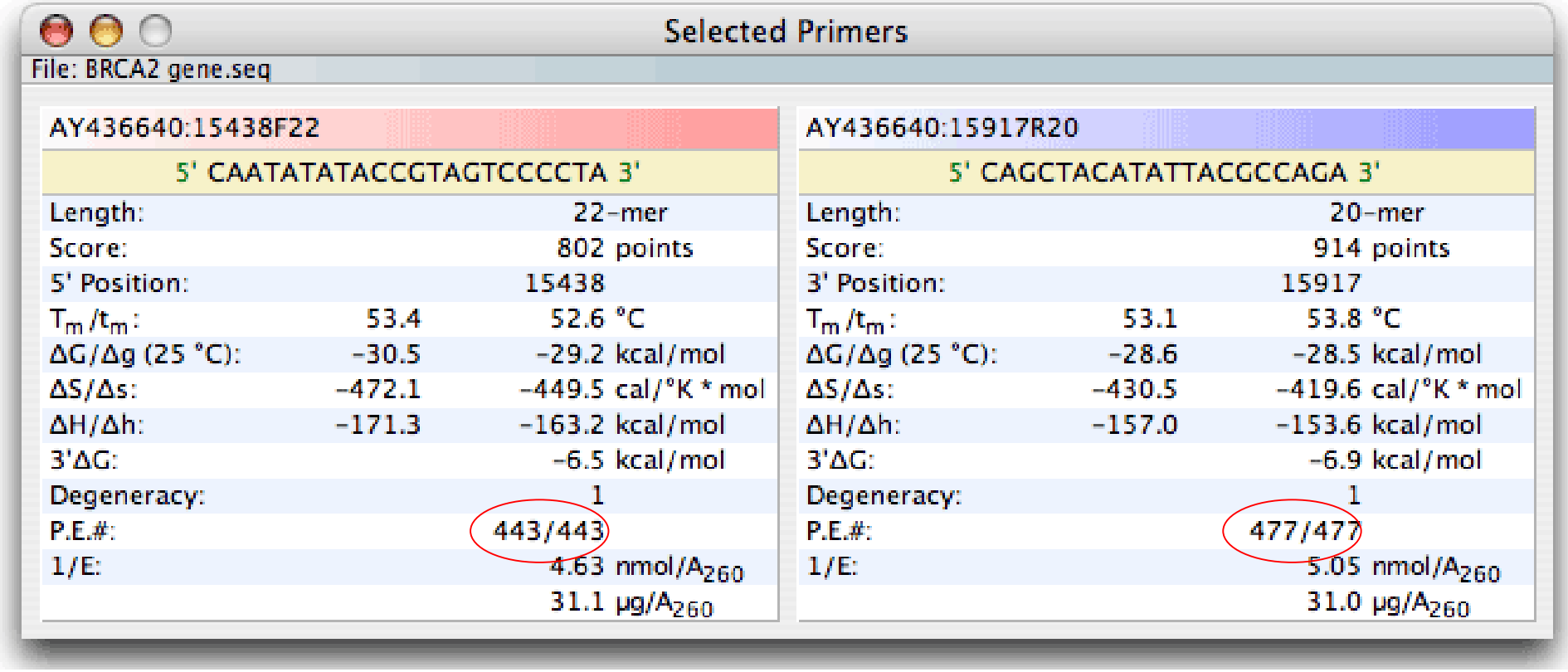
Product T_m – Reverse Primer T_m: 23.6 °C

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

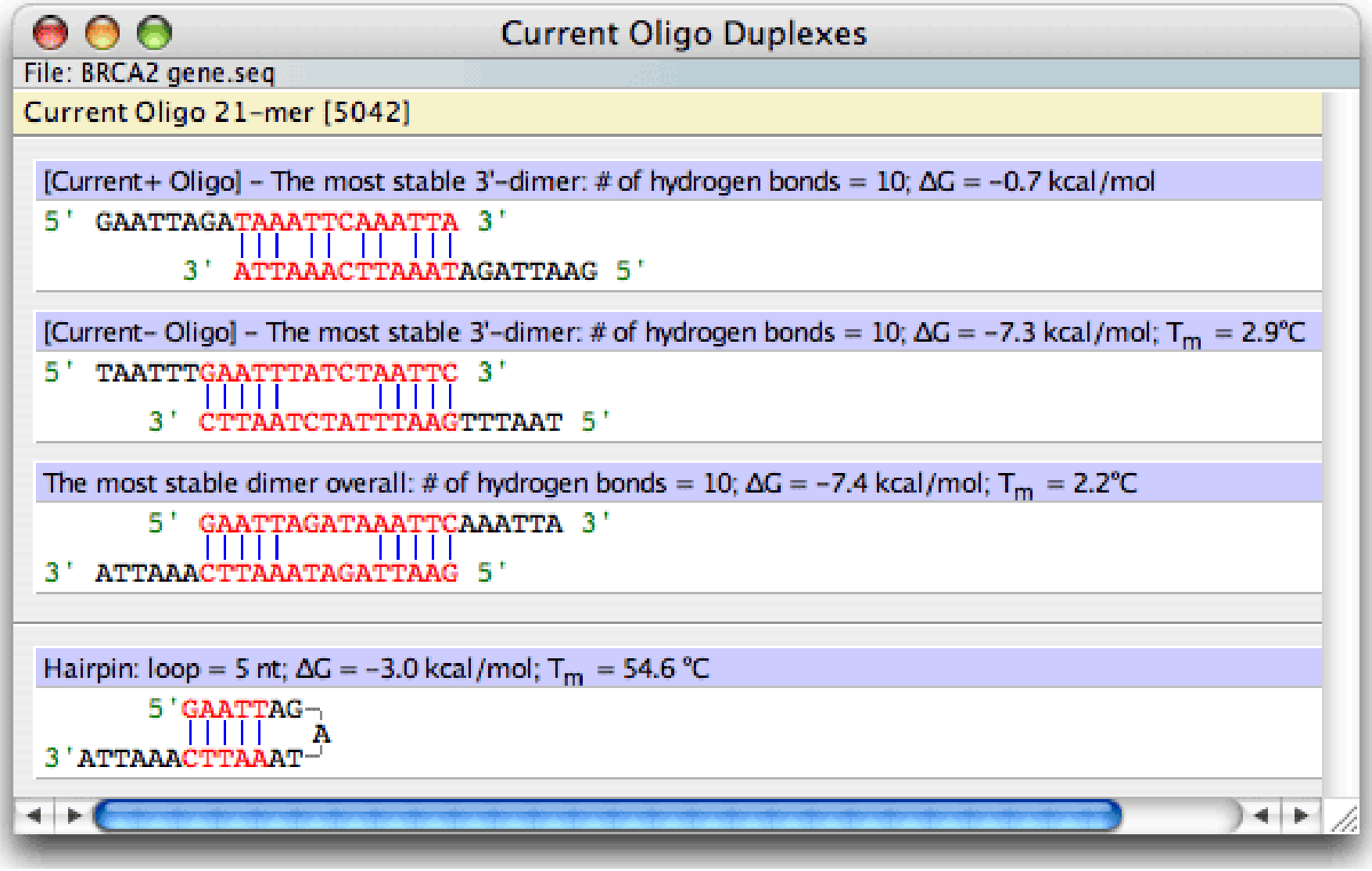
Total Na[+] Equivalent: 155.8 mM

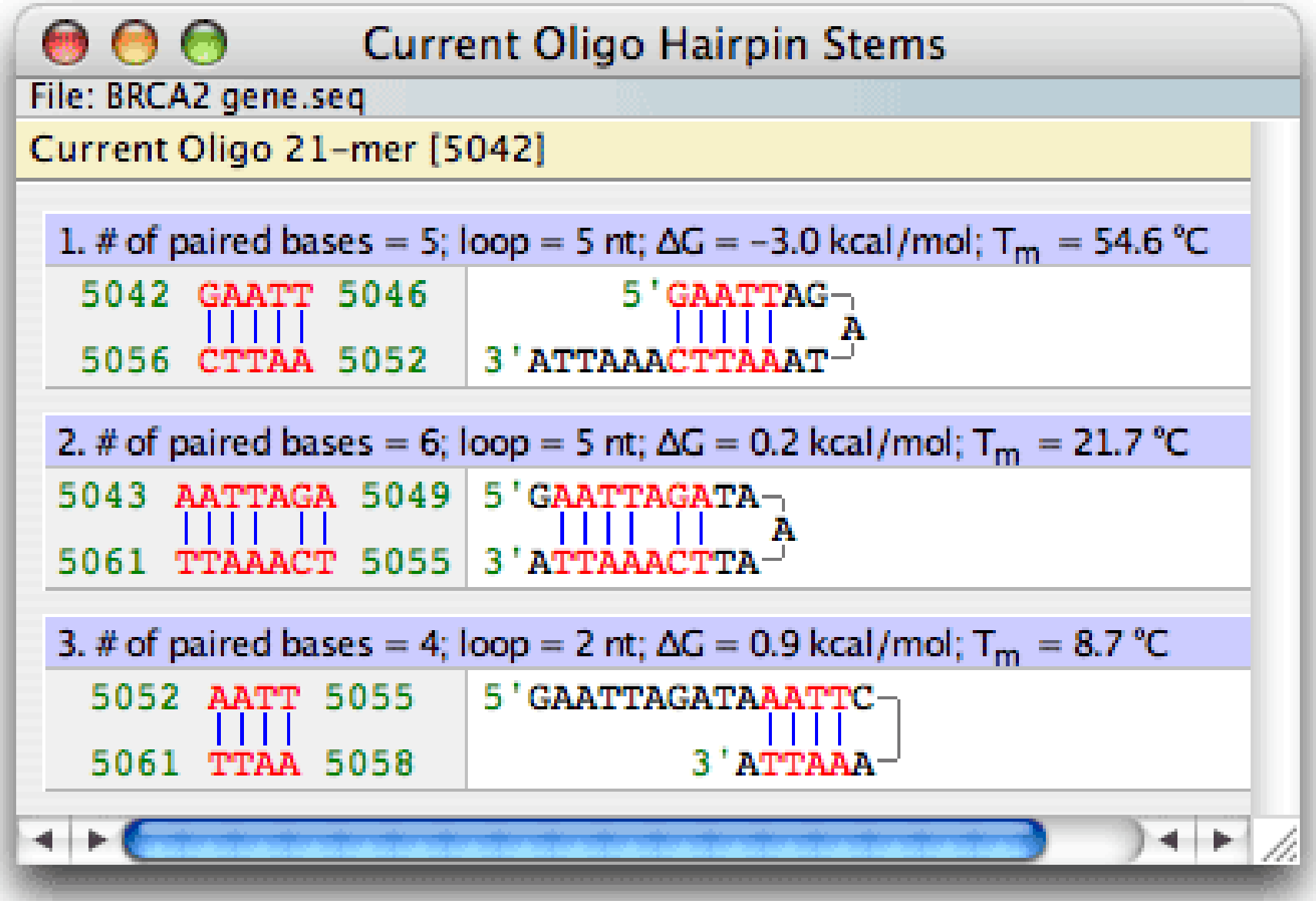


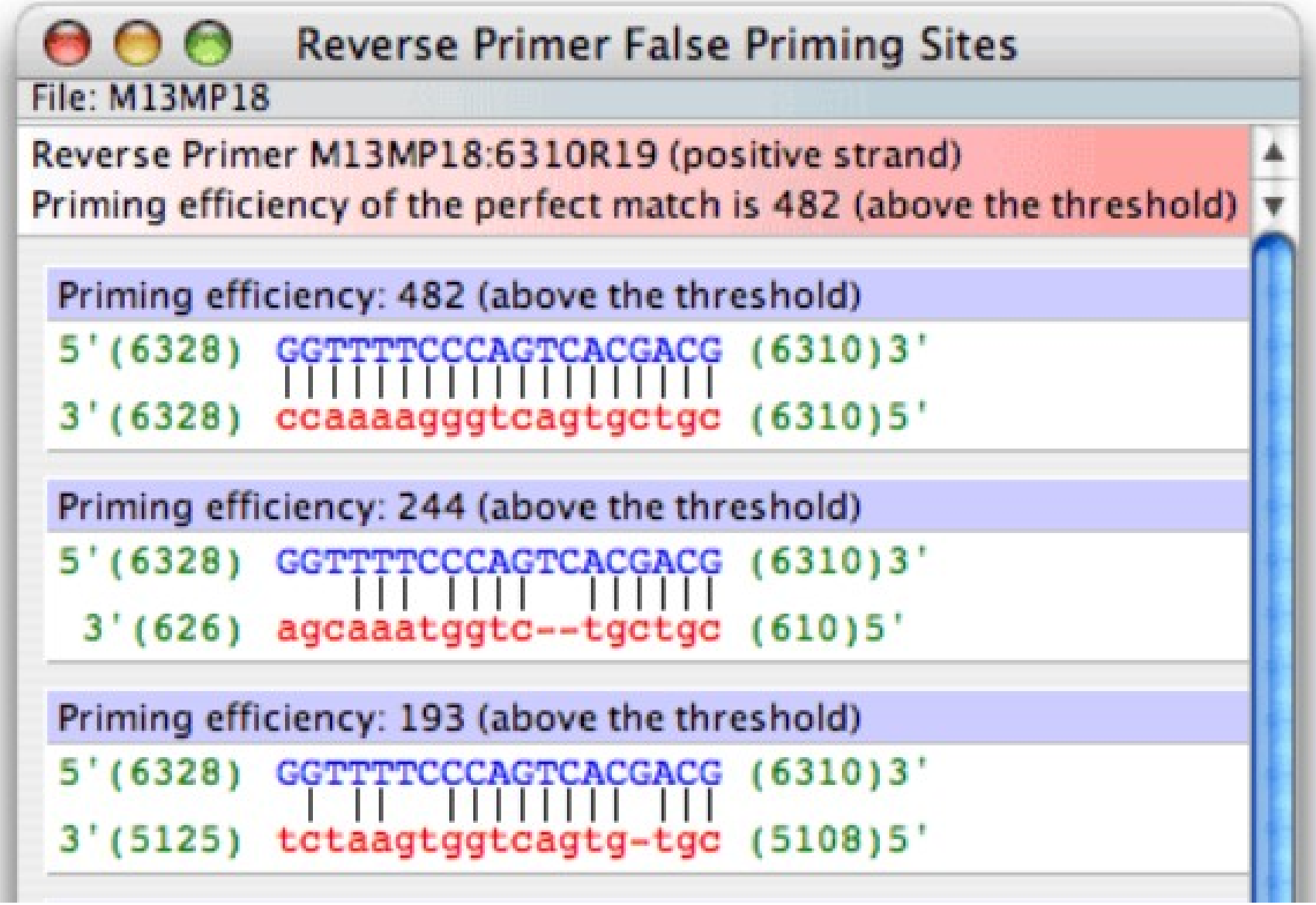
Priming Efficiency PE Score

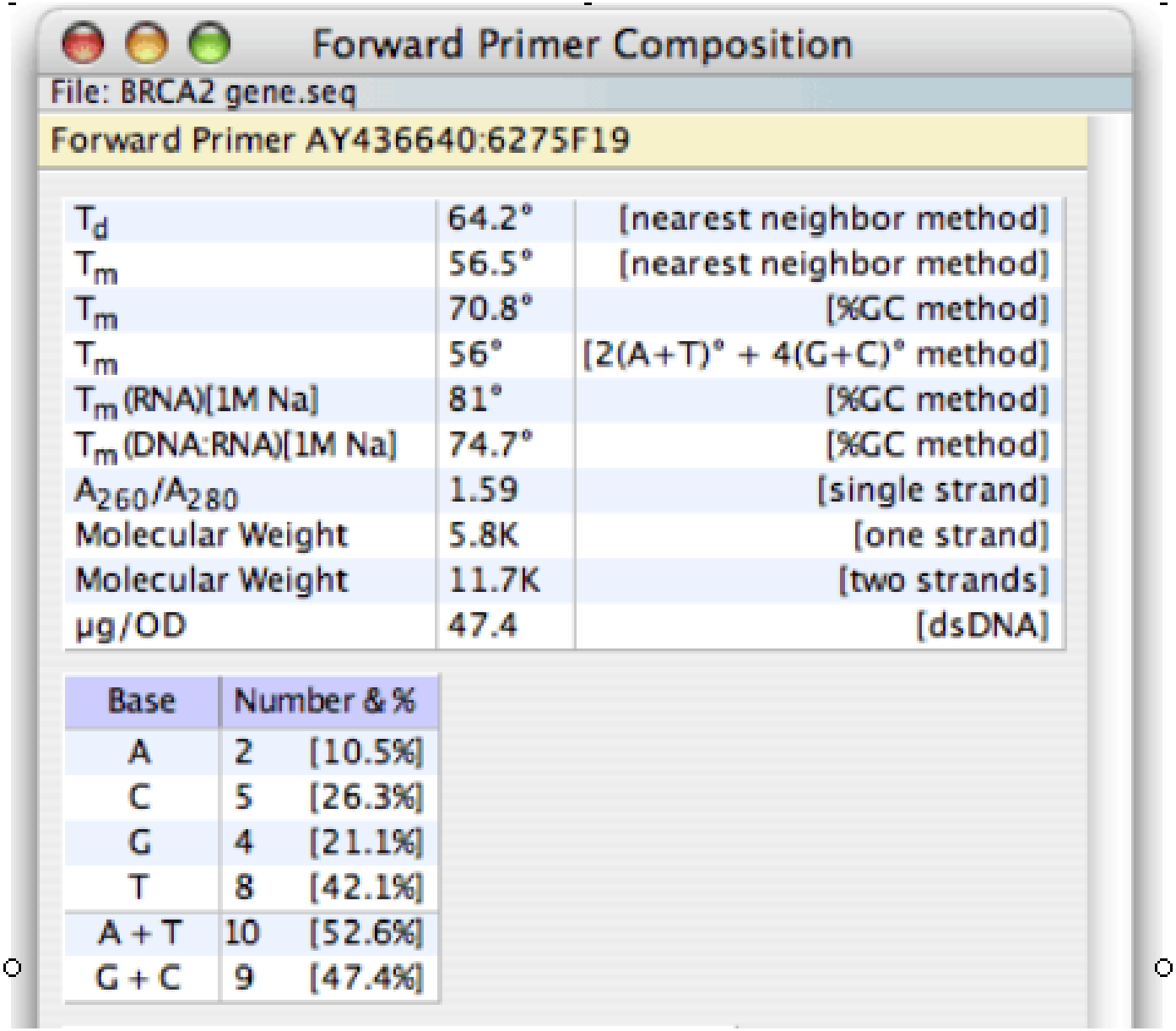
Secondary structures

- HAIRPIN intramolecular
- DIMER intermolecular




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'



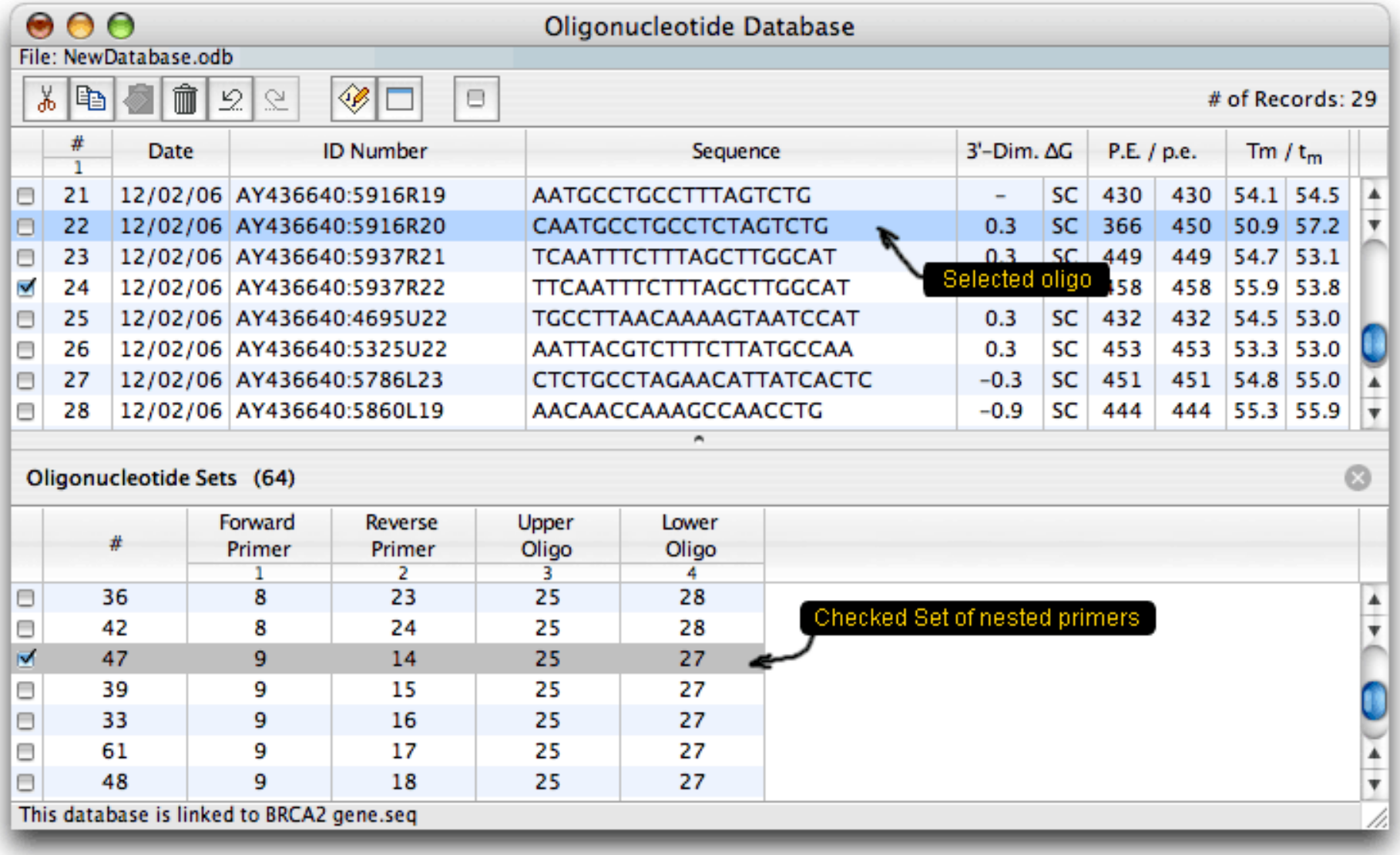
Forward Primer Composition

File: BRCA2 gene.seq

Forward Primer AY436640:6275F19

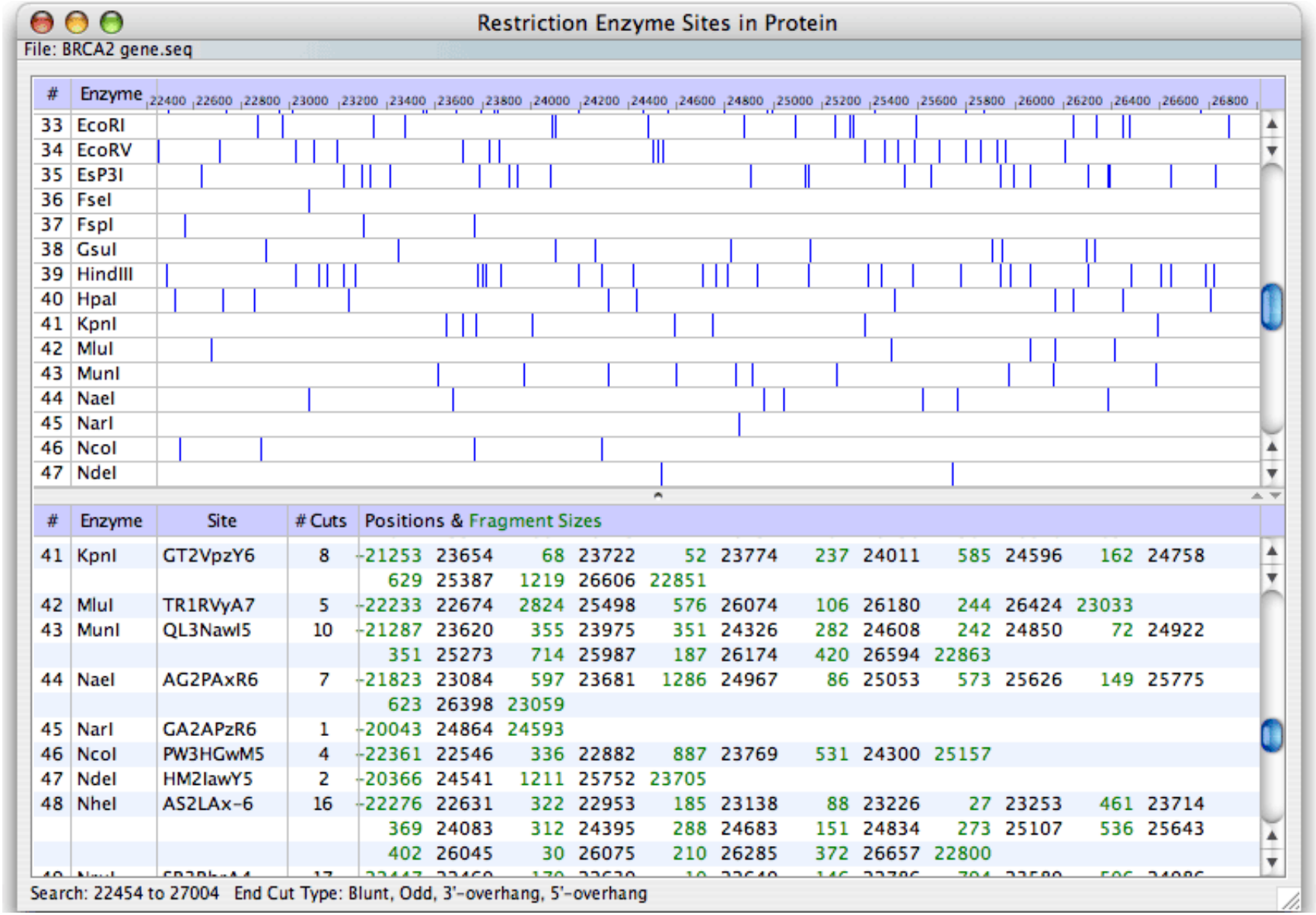
T_d	64.2°	[nearest neighbor method]
T_m	56.5°	[nearest neighbor method]
T_m	70.8°	[%GC method]
T_m	56°	[2(A+T)° + 4(G+C)° method]
T_m (RNA)[1M Na]	81°	[%GC method]
T_m (DNA:RNA)[1M Na]	74.7°	[%GC method]
A_{260}/A_{280}	1.59	[single strand]
Molecular Weight	5.8K	[one strand]
Molecular Weight	11.7K	[two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

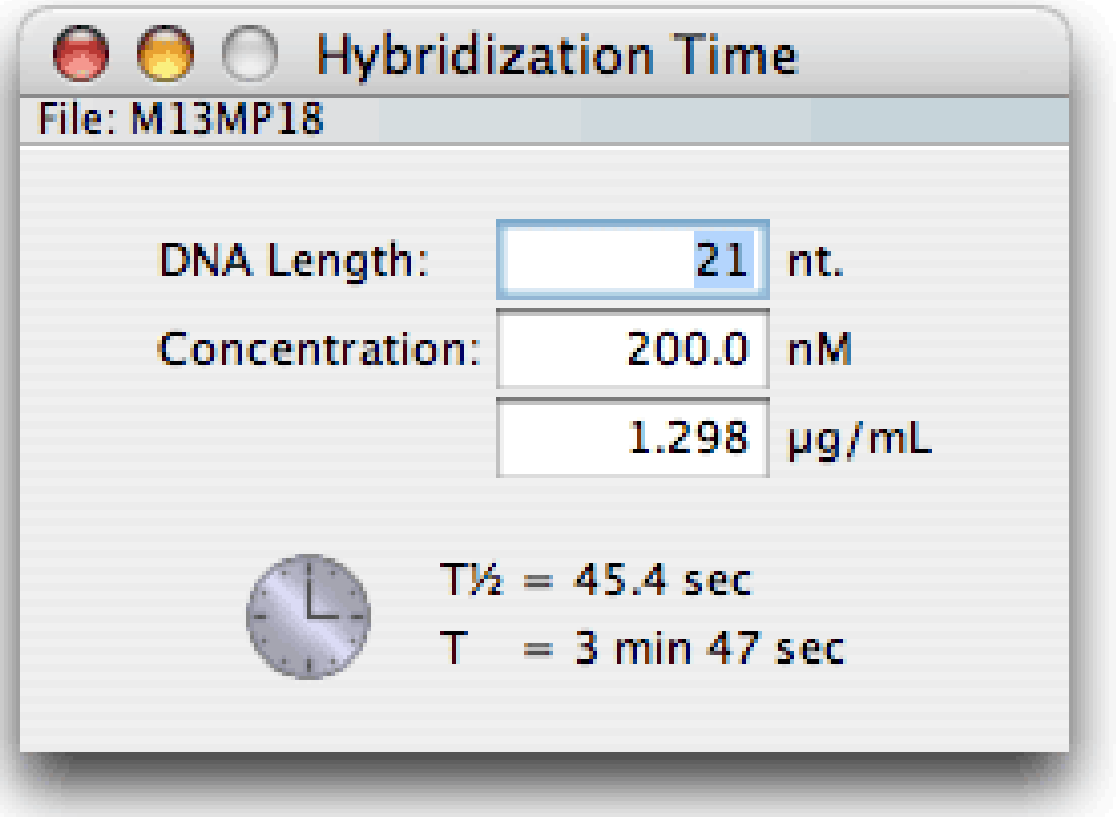
Base	Number & %	
A	2	[10.5%]
C	5	[26.3%]
G	4	[21.1%]
T	8	[42.1%]
A + T	10	[52.6%]
G + C	9	[47.4%]

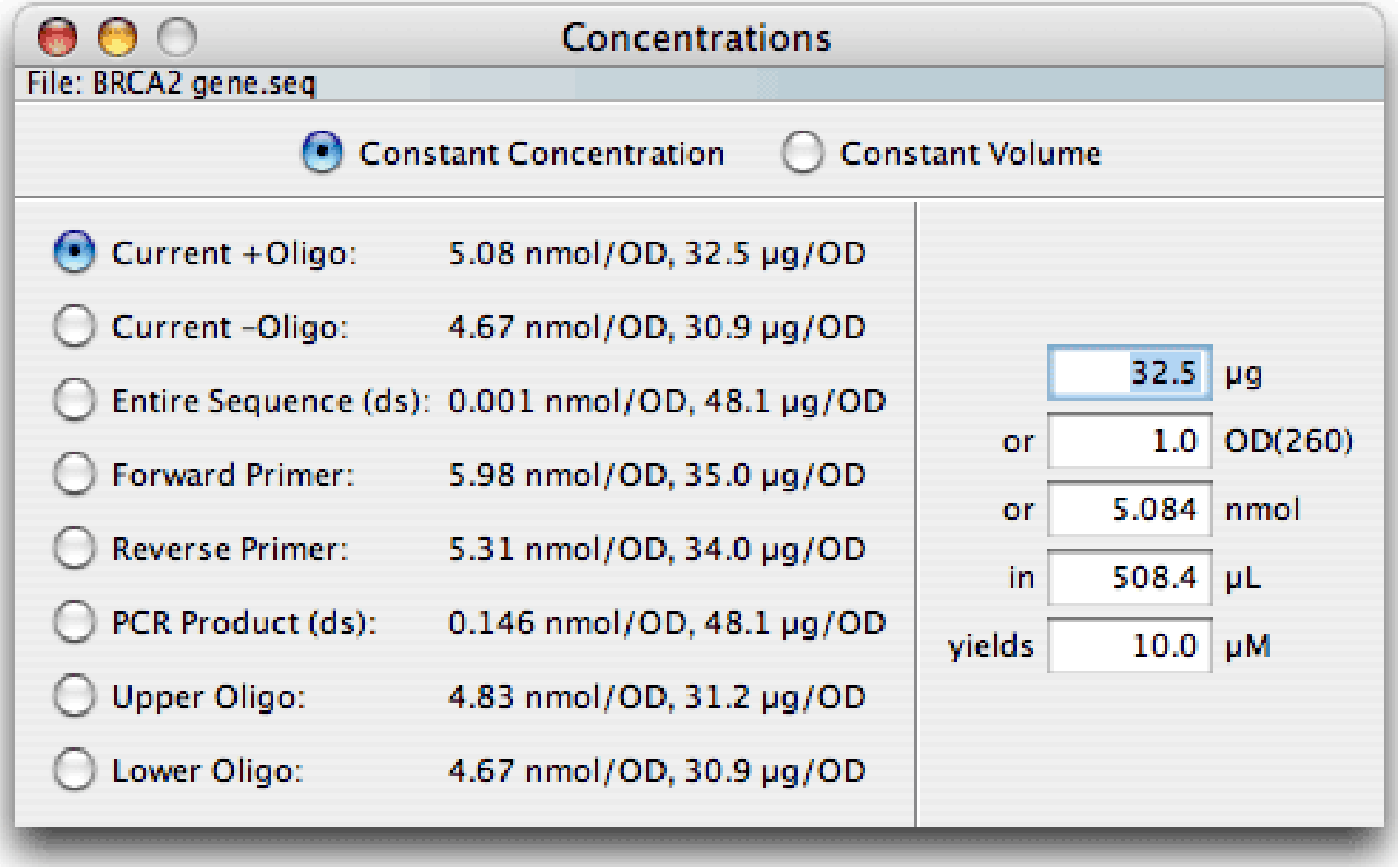


#	Date	ID Number	Sequence	3'-Dim. ΔG		P.E. / p.e.		Tm / t_m	
1									
21	12/02/06	AY436640:5916R19	AATGCCTGCCTTTAGTCTG	-	SC	430	430	54.1	54.5
22	12/02/06	AY436640:5916R20	CAATGCCTGCCTCTAGTCTG	0.3	SC	366	450	50.9	57.2
23	12/02/06	AY436640:5937R21	TCAATTTCTTTAGCTTGGCAT	0.3	SC	449	449	54.7	53.1
24	12/02/06	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

#	Forward Primer	Reverse Primer	Upper Oligo	Lower Oligo
	1	2	3	4
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


Restriction Enzyme Sites in Protein
File: BRCA2 gene.seq
22400 22600 22800 23000 23200 23400 23600 23800 24000 24200 24400 24600 24800 25000 25200 25400 25600 25800 26000 26200 26400 26600 26800
33 EcoRI
34 EcoRV
35 EsP3I
36 FseI
37 FspI
38 GsuI
39 HindIII
40 HpaI
41 KpnI
42 MluI
43 MunI
44 NaeI
45 NarI
46 NcoI
47 NdeI
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

AHP2 cDNA (TAIR database)

Sequence: AT3G29350.1 Date last modified 2007-04-17 Name AT3G29350.1 Tair Accession Sequence:4010737427 Sequence Length (bp) 827

1 ACAATTCGCG AGAAAGACAA AACACAAGTT TCTTCTTCTT GGGATTGGCT
51 ATTTCCAGAA ATCCAAGTCA ATAATCAAAG TCCAAACAAA AAAATCCTCT
101 CCCAATCTCC GCTTCACTCT TCTC**ATG**GAC GCTCTCATTG CTCAGCTTCA
151 GAGACAATTT CGTGATTACA CCATTTCTCT CTACCAACAG GGGTTTTTGG
201 ATGATCAATT TACTGAGTTG AAAAAGCTAC AAGATGATGG AAGTCCTGAT
251 TTTGTGTCTG AAGTGCTTTC ACTTTTCTTT GAAGATTGTG TGAAGCTTAT
301 CAGTAACATG GCTAGAGCTT TGGACACGAC AGGAACTGTA GATTTTAGTC
351 AGGTAGGTGC TAGTGTGCAT CAATTGAAGG GTAGTAGCTC AAGTGTTGGT
401 GCCAAGAGGG TCAAAACTTT GTGTGTTAGC TTCAAGGAAT GTTGTGAAGC
451 TAAGAACTAC GAAGGGTGTG TGAGATGTTT GCAGCAAGTG GATATTGAGT
501 ACAAGGCGTT AAAGACAAAG CTTCAAGATA TGTTCAATCT TGAGAAACAG
551 ATCATTCAAG CTGGTGGTAT AGTTCCTCAA GTGGATATTA AC**TAA**AGAGA
601 CTAGTCCATA AGAAGAAAAA AGATGATGAC TTTCTTTCTT TAGTTTCTCT
651 TCTAAATTAT TTTGGATTTG GTGTTTGCTC AAAAACTCAA TAAAATATGT
701 GCAAAAAGAA ACAAAAACAA GTGATGGTTG TTTATAAATC AGTAGTATGT
751 ATTGTTTGAT CTCATCCGAG AAAATTGAAA CCATTGGACT AATGAATGTG
801 ATGATAATAT ATATTGGTTT GCTTCTG

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101 CCCAATCTCC GCTTCACTCT TCTC**ATG**GAC GCTCTCATTG CTCAGCTTCA
151 GAGACAATTT CGTGATTACA CCATTTCTCT CTACCAACAG GGGTTTTTGG
201 ATGATCAATT TACTGAGTTG AAAAAGCTAC AAGATGATGG AAGTCCTGAT
251 TTTGTGTCTG AAGTGCTTTC ACTTTTCTTT GAAGATTGTG TGAAGCTTAT
301 CAGTAACATG GCTAGAGCTT TGGACACGAC AGGAACTGTA GATTTTAGTC
351 AGGTAGGTGC TAGTGTGCAT CAATTGAAGG GTAGTAGCTC AAGTGTTGGT
401 GCCAAGAGGG TCAAAACTTT GTGTGTTAGC TTCAAGGAAT GTTGTGAAGC
451 TAAGAACTAC GAAGGGTGTG TGAGATGTTT GCAGCAAGTG GATATTGAGT
501 ACAAGGCGTT AAAGACAAAG CTTCAAGATA TGTTCAATCT TGAGAAACAG
551 ATCATTCAAG CTGGTGGTAT AGTTCCTCAA GTGGATATTA AC**TAA**AGAGA

EcoRI restriction site

5′……G| AATTC……3′
3¨……CTTAA| G……5′

Design of primers

AHP2ex_up

5’- CCG GAA TTC ATG GAC GCT CTC ATT GCT CAG – 3’

AHP2ex_low

5’- CCG GAA TTC TTA GTT AAT ATC CAC TTG AGG – 3’

101 CCCAATCTCC GCTTCACTCT TCTCATGGAC GCTCTCATTG CTCAGCTTCA
151 GAGACAATTT CGTGATTACA CCATTTCTCT CTACCAACAG GGGTTTTTGG
201 ATGATCAATT TACTGAGTTG AAAAAGCTAC AAGATGATGG AAGTCCTGAT
251 TTTGTGTCTG AAGTGCTTTC ACTTTTCTTT GAAGATTGTG TGAAGCTTAT
301 CAGTAACATG GCTAGAGCTT TGGACACGAC AGGAACTGTA GATTTTAGTC
351 AGGTAGGTGC TAGTGTGCAT CAATTGAAGG GTAGTAGCTC AAGTGTTGGT
401 GCCAAGAGGG TCAAAACTTT GTGTGTTAGC TTCAAGGAAT GTTGTGAAGC
451 TAAGAACTAC GAAGGGTGTG TGAGATGTTT GCAGCAAGTG GATATTGAGT
501 ACAAGGCGTT AAAGACAAAG CTTCAAGATA TGTTCAATCT TGAGAAACAG
551 ATCATTCAAG CTGGTGGTAT AGTTCCTCAA GTGGATATTA ACTAAAGAGA

EcoRI restriction site

5′……G| AATTC……3′
3¨……CTTAA| G……5′

Design of primers

AHP2ex_up

5’- CCG GAA TTC ATG GAC GCT CTC ATT GCT CAG – 3’

AHP2ex_low

5’- CCG GAA TTC TTA GTT AAT ATC CAC TTG AGG – 3’

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LITERATURE

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- PCR Primer: A Laboratory Manual (2003)
- OLIGO Primer analysis software, Version 7
- *Expert Opin Ther Pat.* 2014, 24(7):801-19.
  Oligonucleotide delivery: a patent review (2010 - 2013).
- *AAPS Journal* 2009, 11(1): 195 - 203.
  Targeted Delivery Systems for Oligonucleotide Therapeutics
- Large-scale de novo DNA synthesis: technologies and applications
  Nature Methods 2014, 11 (5): 499

Discovery is not in seeking new landscapes,
but in having new eyes…

Marcel Proust