

Design of PCR Primers

```
GGCCTTCTGCTCAATCTTTCTACAACCAAAGCTCTGTCTTGAA  
GTCATGGTTGTGGACGATGATCATGTTTTCTTGATATCATGT  
GCTTCAACACTCCAATACAGAGGTAATTAAATATTATTATCA  
ATATAATATGTTATTGATTTTTGTTTGTGATTTCAATTA  
GATTTCTATGATTTCTTAGCATGAATAACAATTTTGGAGAAAC  
AACTTTAAAAACA  
AACTTGAATTTTGAGAAAT  
TCAAAGATGTATA  
GTCAAAATT  
TAACAATTATCTTCTAAATCATCCGGATTCCGT  
ATACACATCTACAATTTTCAATTGAGGTATCTTGTTTTG  
ATGCACGAATAGTTGATTGATAAAAAAATTC  
TAACCAATATGATAATTTATTTT  
GTCAAACCACTACTTTATACTATGTA  
ACTTTAGATTATTG  
AAAAATAGTTTATTTATAAAA  
TAGTAACCTA  
TTGTTAAAA  
AAAAA  
AATTTGTAAATCGTGTT  
TGCAAACGACATGTCTTAGTTT  
AAACTAGCTGATTTCTCA  
AATCGACTGTCTTATCAACCA  
TTAGCATGAA  
TCAAAATAAAA  
TTGTAAACAC  
TTCAATGGTGATTT  
TAAAGAATATGTTTTACTTA  
TGTTATGAAC  
TATCTGTGAAATA  
TTTCATAACTAATGTGGAAA  
ACTATATAAC  
CCCTAAACGTAAG  
TAAAATTTATGAAATCCTAT  
CATTTTTTAAA  
GGTTATCAAAAAGT  
AATAATTCTTGGTACTTGCA  
ATATTTTTTGT  
CATTAGTTTTATTA  
ATTTTTATTTGATTAAATGG  
TTTTAGATCC  
ATCAGAGATCGCAG  
TTATAGCTGTAGACGATCCG  
AAGAAAGCAT  
TATAAAAATTCAA  
CGAGACAATA  
TAGATCTCAT  
AATCACAGAT  
TATCTGGTATGAA  
CGGTTTACAA  
CTCAAAAAAC  
AAATCACTCA  
GGAATTTACCGG  
TCTTAGGTAA  
CATTTTTTGT  
TCTTTACAAC  
TTAA
```

Hana Konečná

CEITEC Central European Institute of Technology

NCBR National Centre for Biomolecular Research



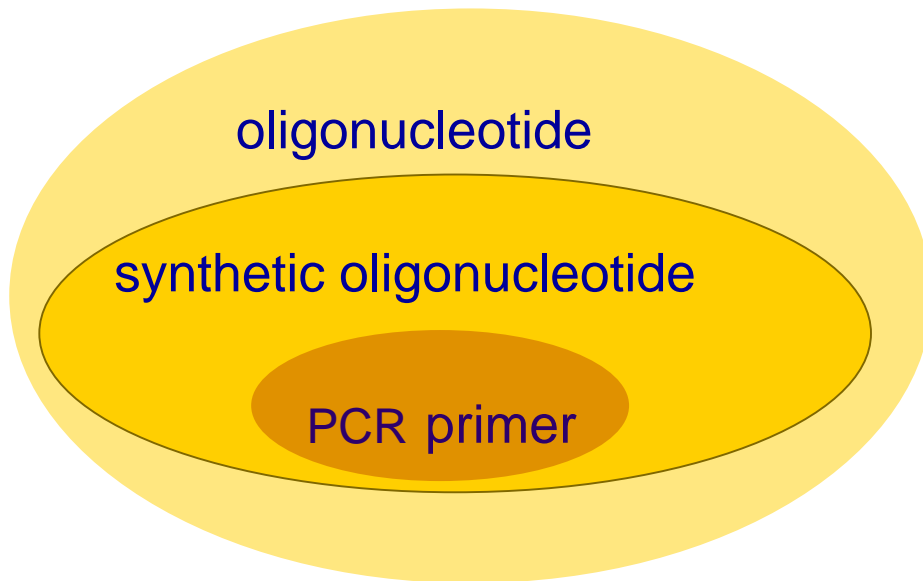
OLIGONUCLEOTIDES

- definition
- applications
- modifications
- synthesis
- purification
- quality control

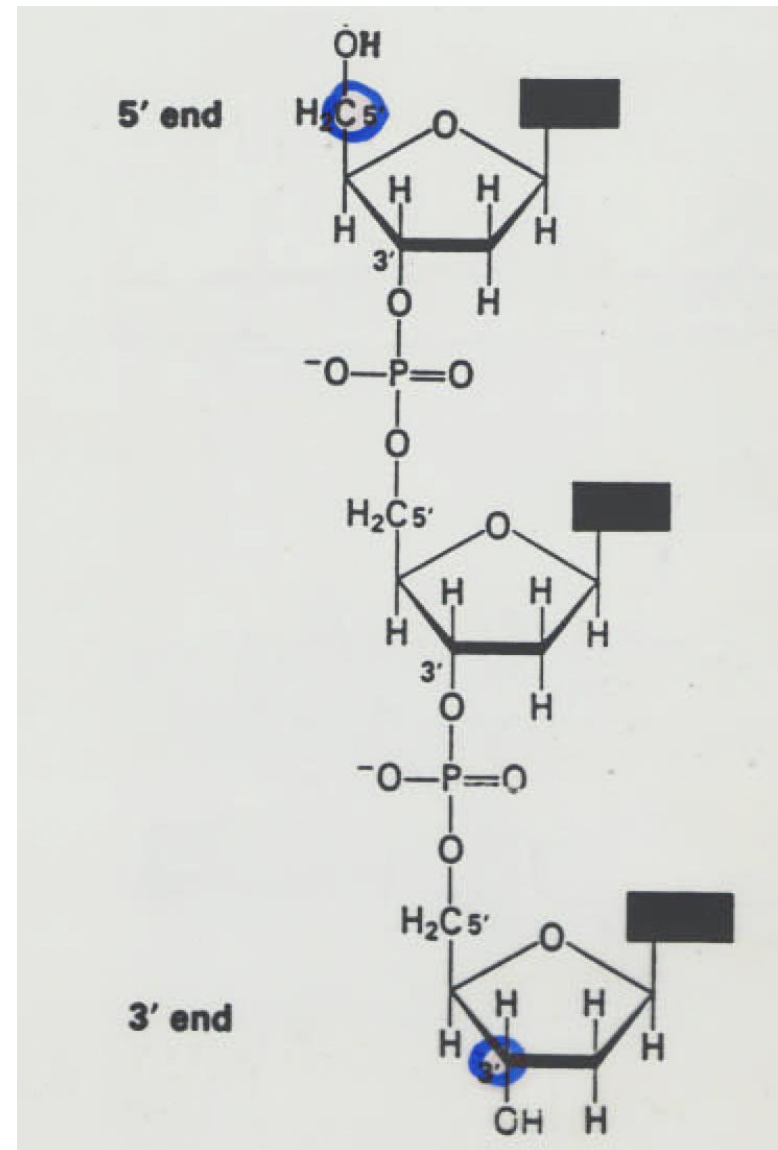
- design of sequence
- rules
- software OLIGO 7
- example

oligonucleotide

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



orientation! polymerase!

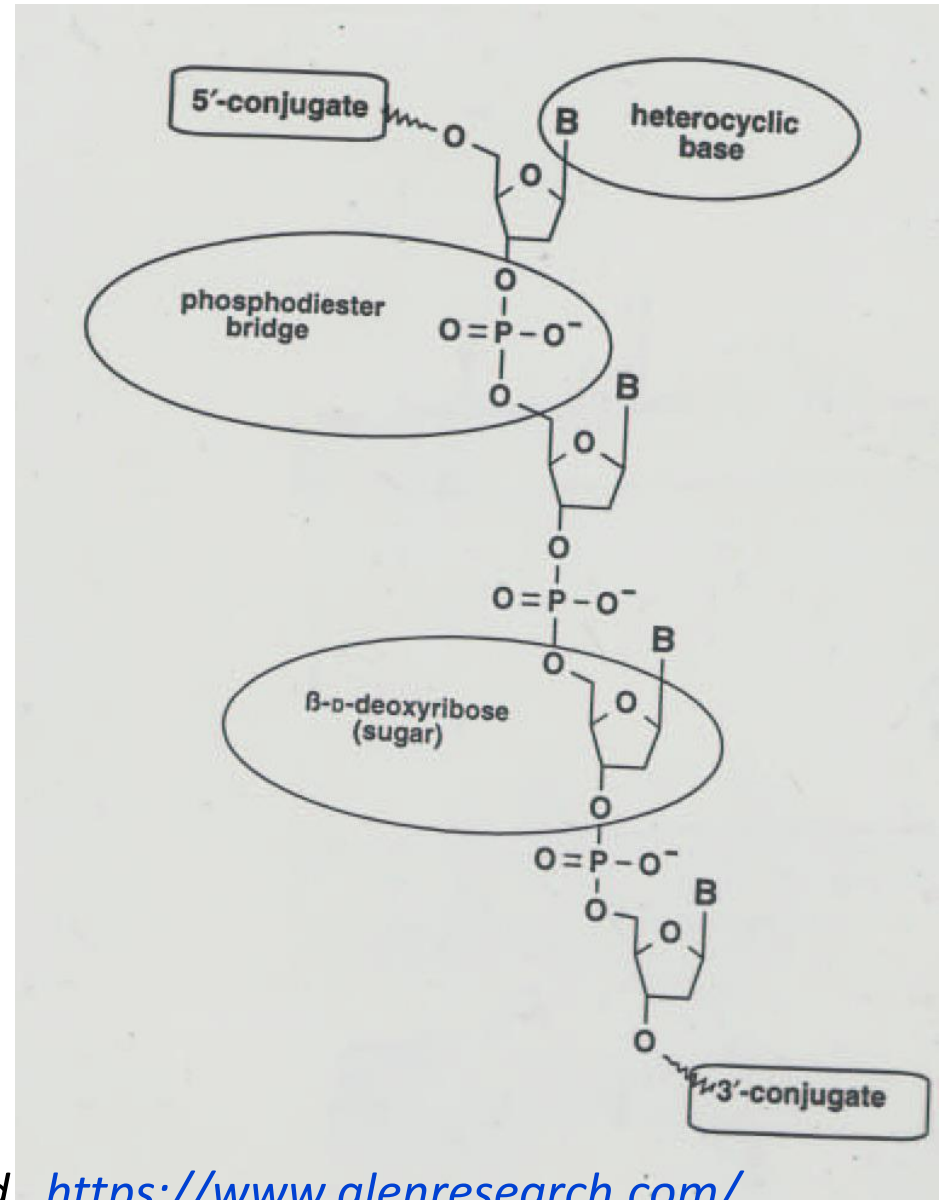


Applications of synthetic oligonucleotides

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

Modifications

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



Degenerated oligonucleotides

ACG TAC GTA CGT ACG TAC

non-degenerated

ACG TAM **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



Phosphorylation

Amino group

Thio group

Digoxigenin

Biotin

Enzymes

Psoralen

Acridine

Cholesterol

Fluorescent dyes

Quenchers

2,4- dinitrophenyl

Spacer

Branching

Block

sequencing

fragmentation analysis

gene arrays

Real-Time PCR



Modifications on 3'-end

derivatized matrix



Phosphate
Thio group
Amino group
Spacer



Biotin



Fluorescent dyes



Quenchers

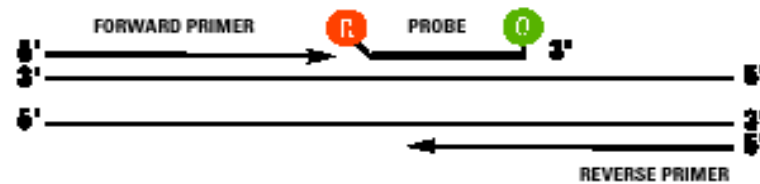
Cholesterol

2,4 dinitrophenyl

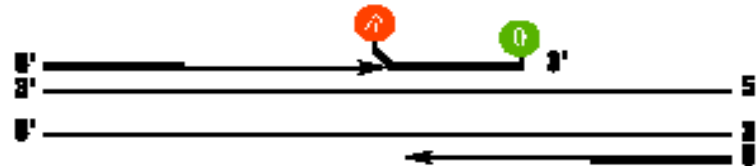
* Additional resources for interested <https://www.glenresearch.com/>

Real-Time PCR

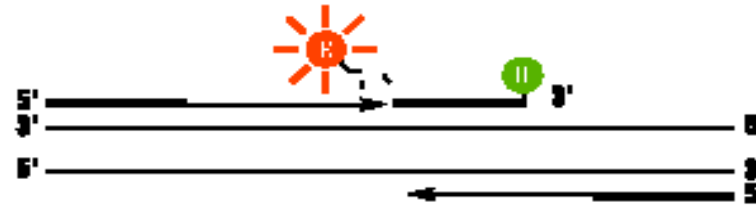
- 2x labeled probe
- REPORTER
- QUENCHER



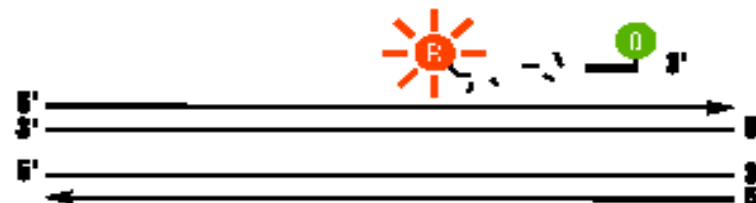
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



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.



* Additional resources for interested <https://www.glenresearch.com/>

Other modifications

Phosphorothioates
Phosphorodithioates
H-phosphonates
Methylphosphonates

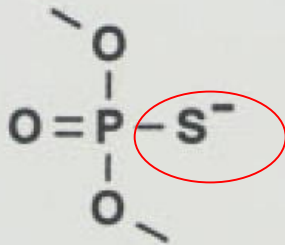
← backbone

carbohydrate →

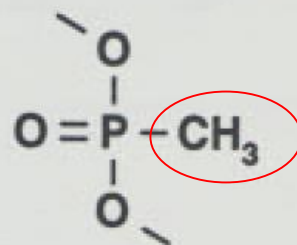
Modifications in 2' - position
Ribose modification

Therapeutics

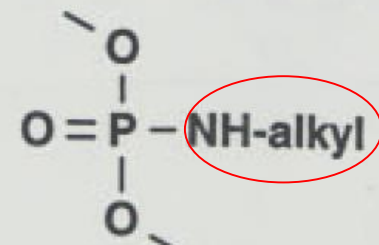
→ **Nondegradable by nucleases!**
Modification of phosphodiester



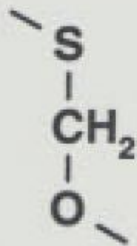
phosphorothioate



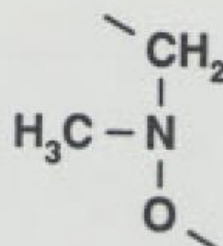
methylphosphonate



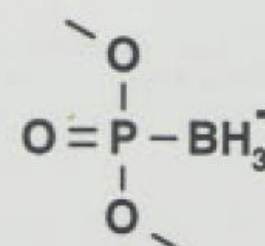
phosphoramidate



3'-thioformacetal



methylene(methyliminio)

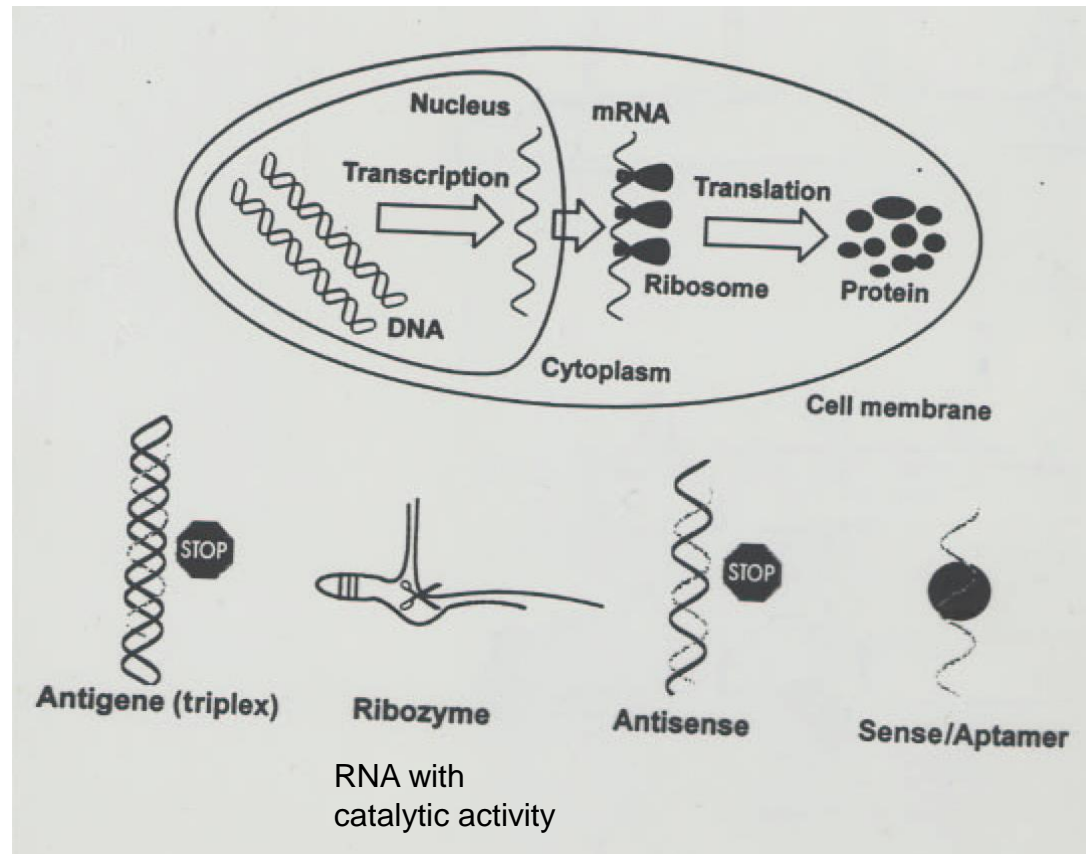


boranophosphate

* Additional resources for interested <https://www.glenresearch.com/>

ANTISENSE oligonucleotide

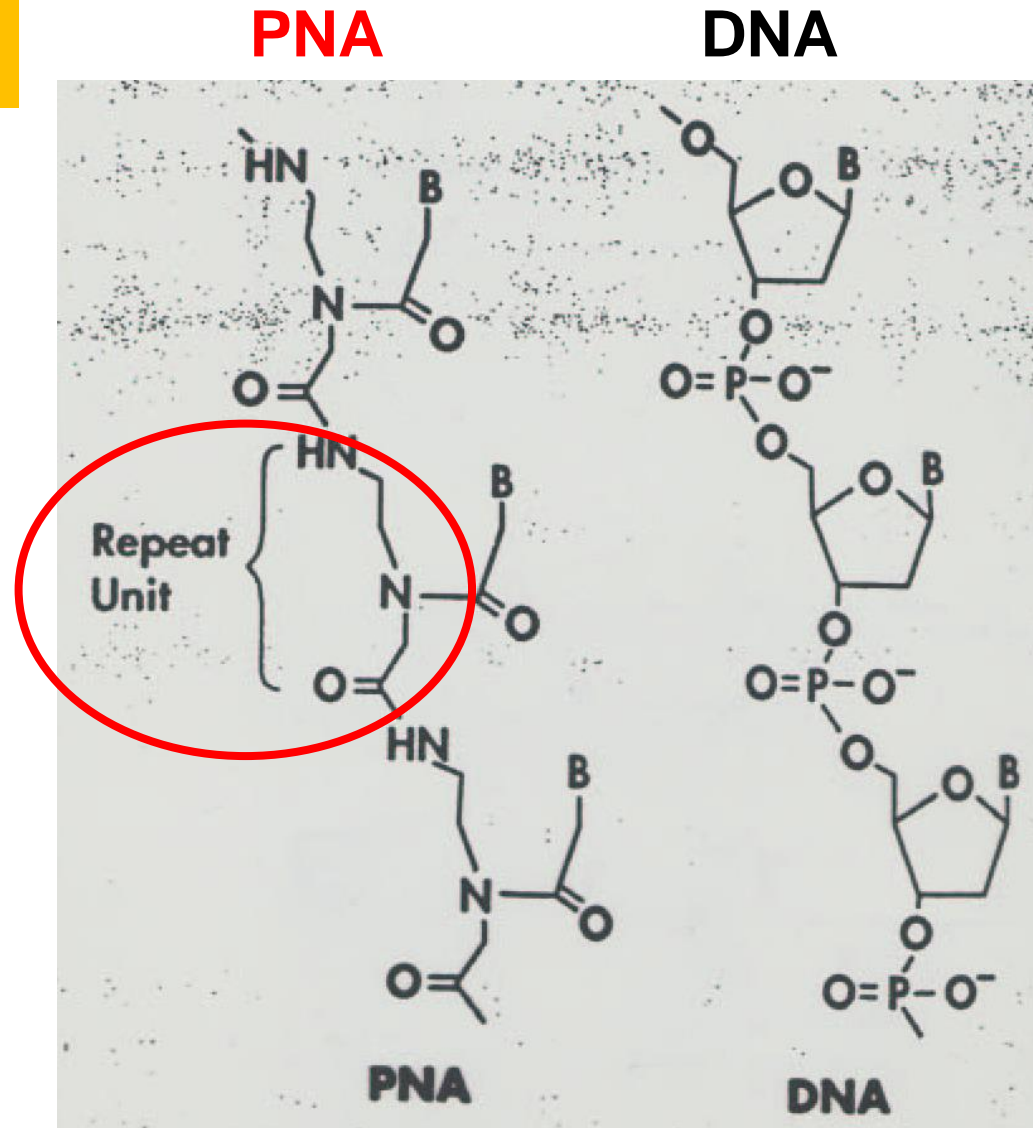
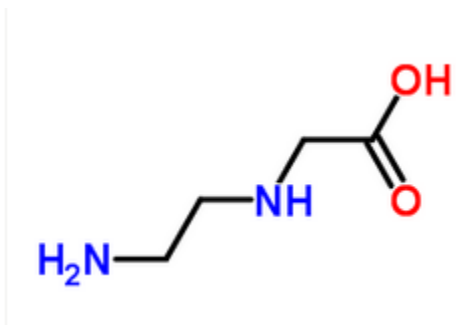
- oligonucleotide or analogue
- complementary to segment of RNA or DNA
- inhibition of normal function due to coupling



Peptidonucleic acid

- noncharged molecule
- binding with DNA/RNA

N-(2-aminoethyl)-glycine \rightarrow



Why PNA

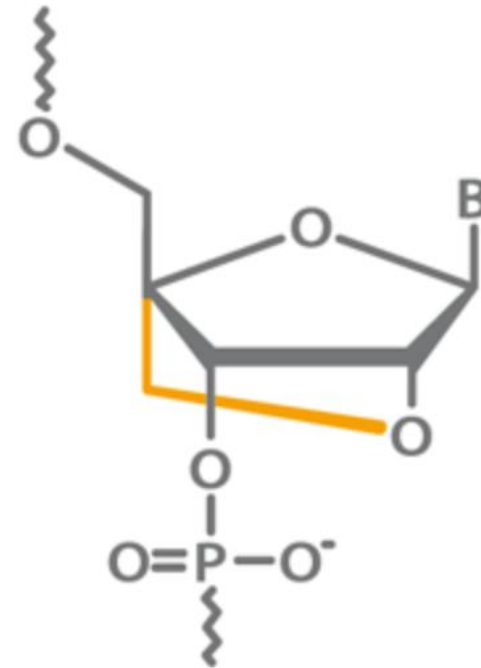
- thermostable
- T_m not depending on salts
- high specificity
- high affinity
- resistant towards enzymes

* *Additional resources for interested* [Gambari R. *Expert Opin Ther Pat.* 2014, 24\(3\):267-94.](#)
Peptide nucleic acids: a review on recent patents and technology transfer.

LNA

Locked Nucleic Acid

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



* Additional resources for interested <https://www.glenresearch.com/>
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3412486/>

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

OLIGONUCLEOTIDES

design

synthesis

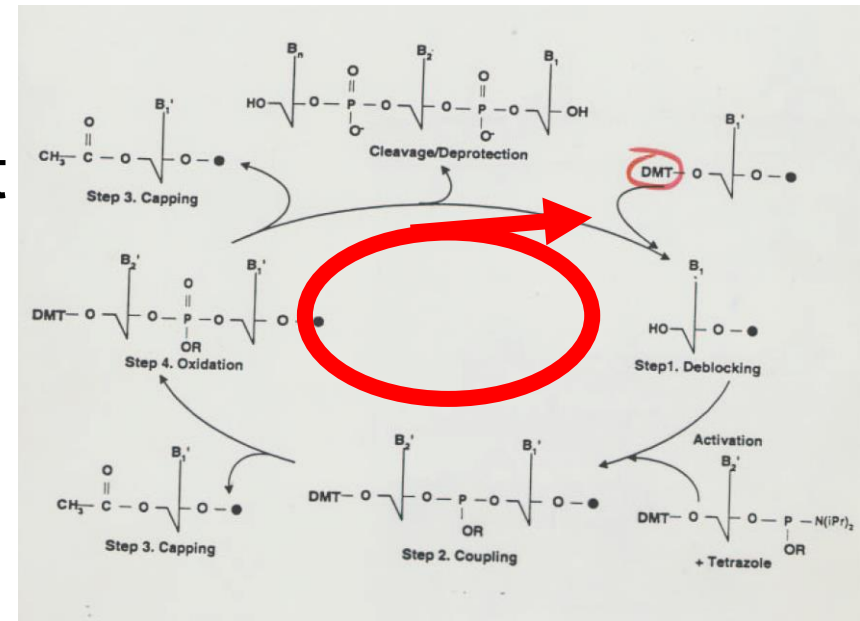
purification



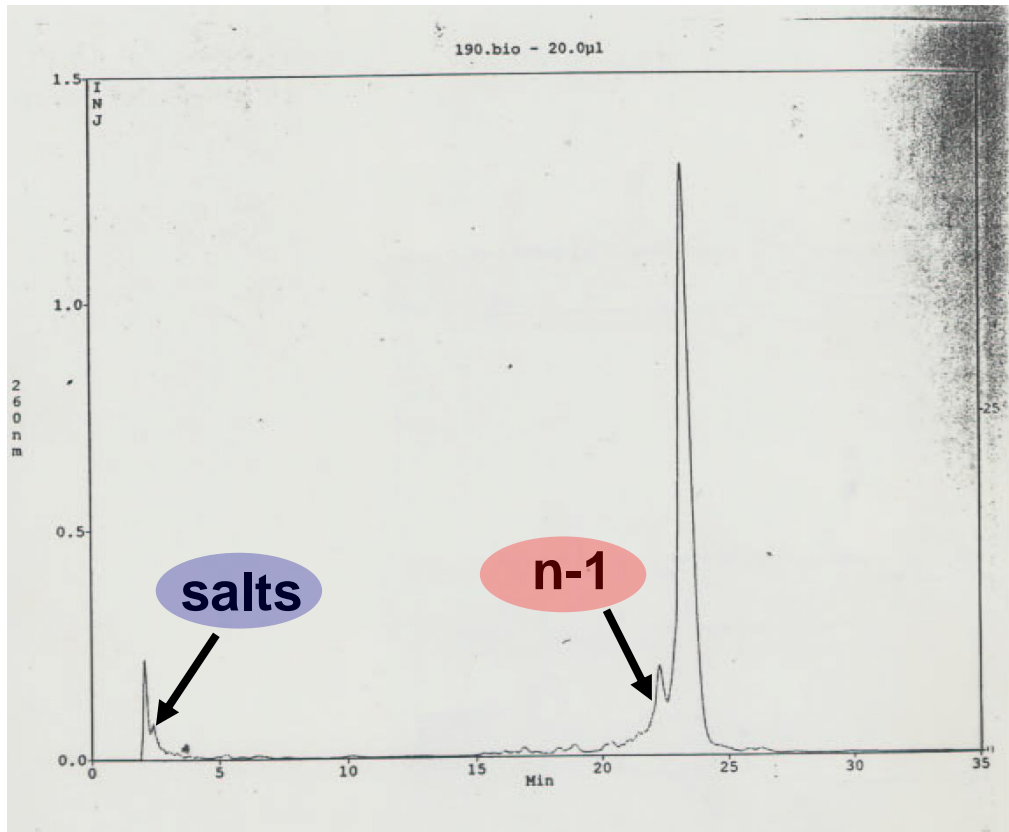
EXPEDITE 8909

Oligonucleotide Synthesis

- no enzyme
- organic synthesis on solid matrix
- adds new base in **cycles**
- from 3'-end to 5'-end
- in anhydrous environment



Quality Control

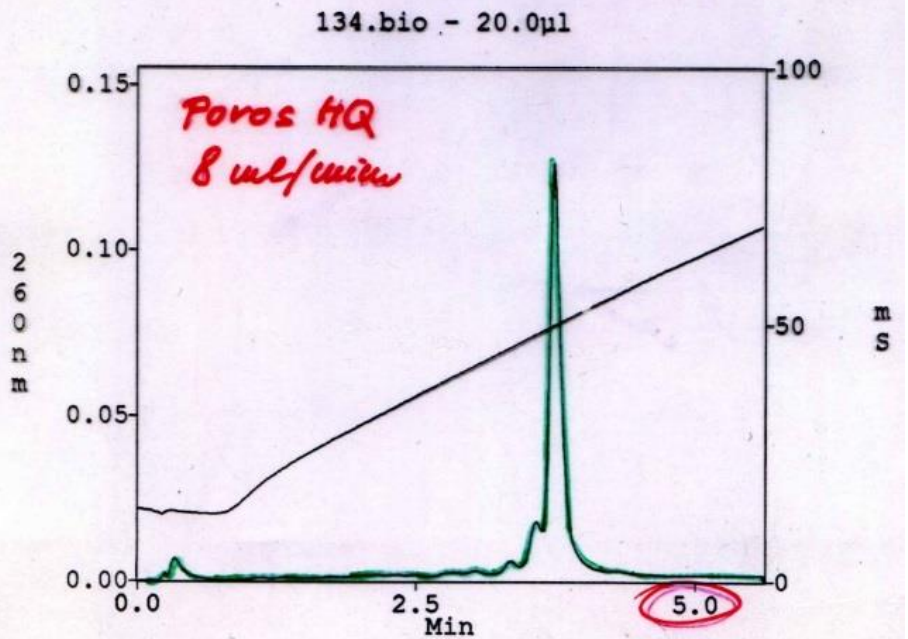
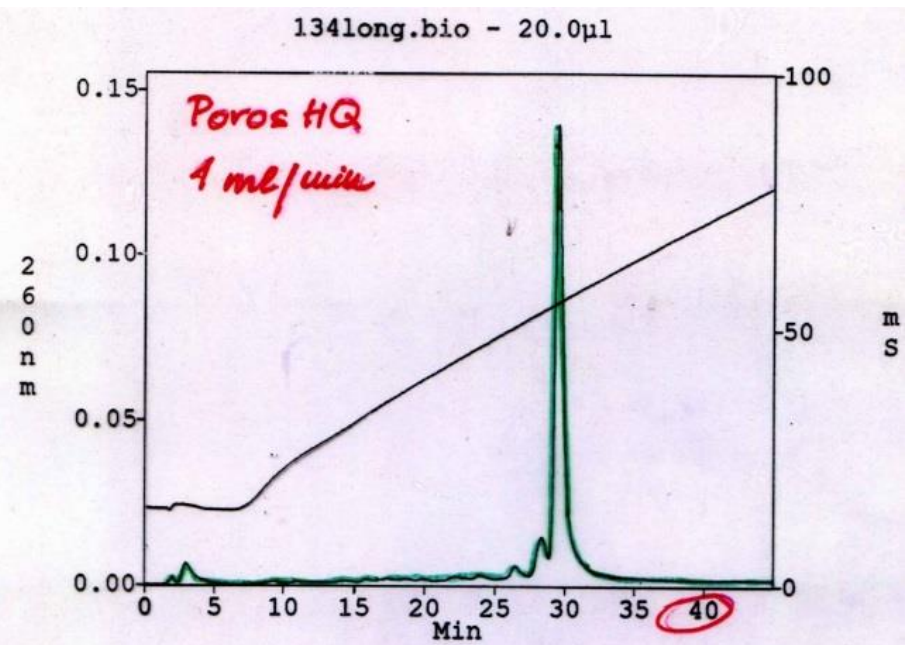
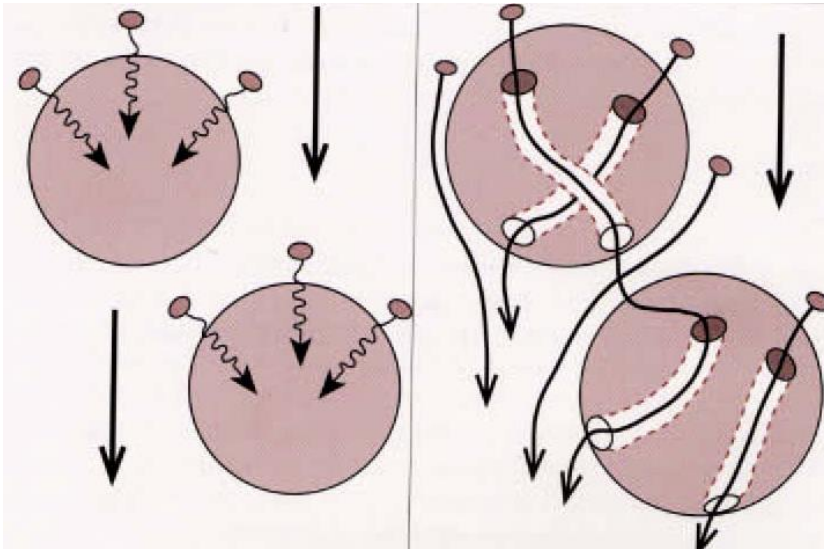


Liquid Chromatography

LC option: Perfusion Chromatography

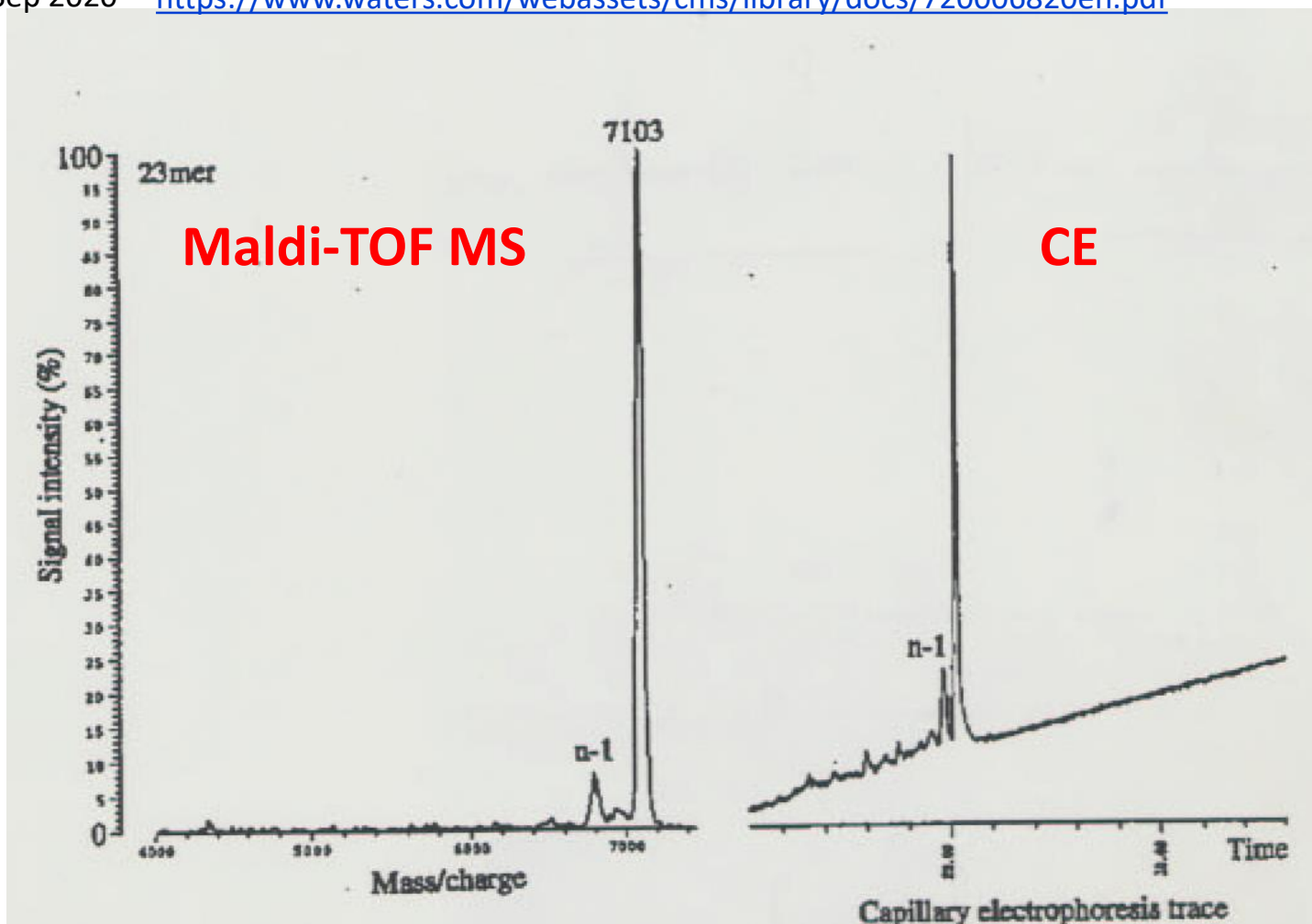
classical sorbent

POROS

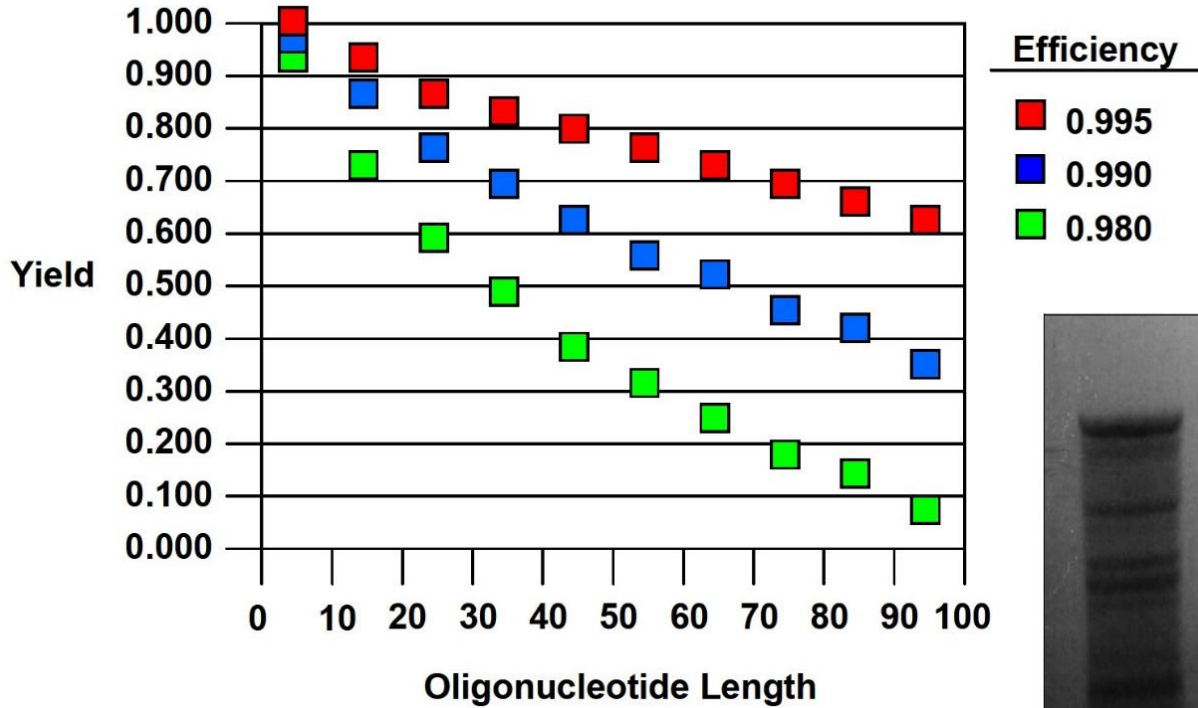


LC-MS **additional literature for interested*

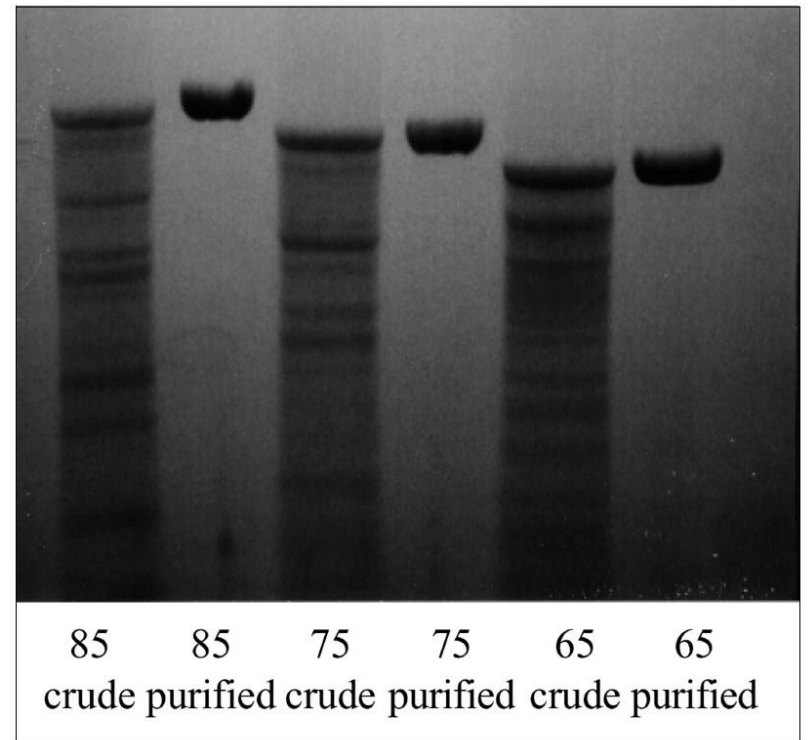
An automated compliance-ready LC-MS workflow for mass confirmation of both modified and unmodified oligonucleotides, Sep 2020 <https://www.waters.com/webassets/cms/library/docs/720006820en.pdf>

PAGE

YIELD

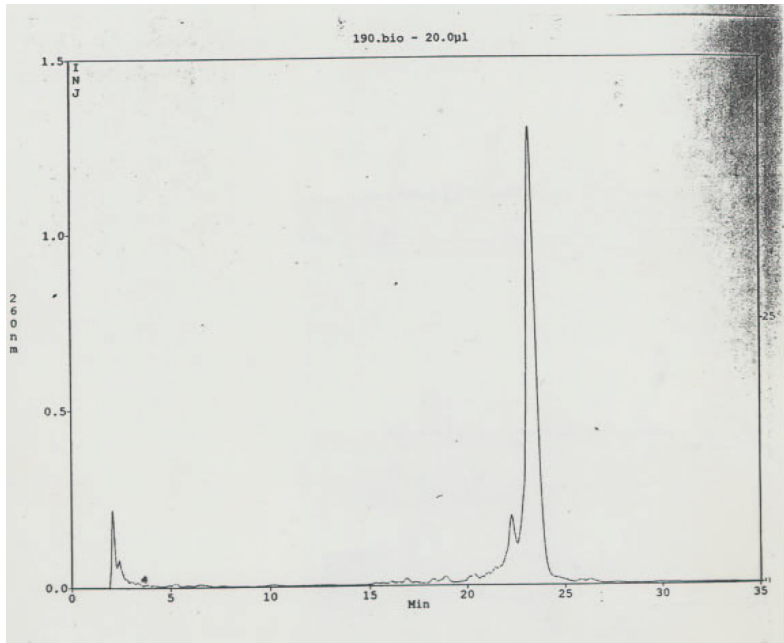


PAGE of long-mers



PURIFICATION

- Sephadex
- RP cartridge
- HPLC



OLIGONUCLEOTIDE DESIGN

- manual
- computer assisted

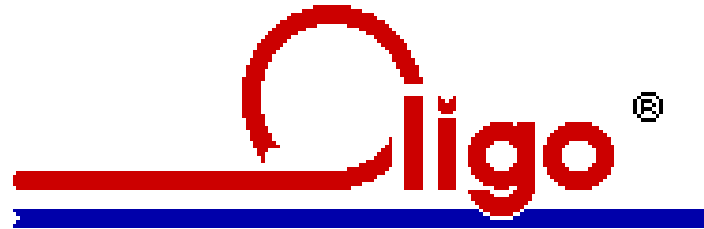
**Additional literature for interested*

<https://www.sciencedirect.com/science/article/pii/S2001037019300844>

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)

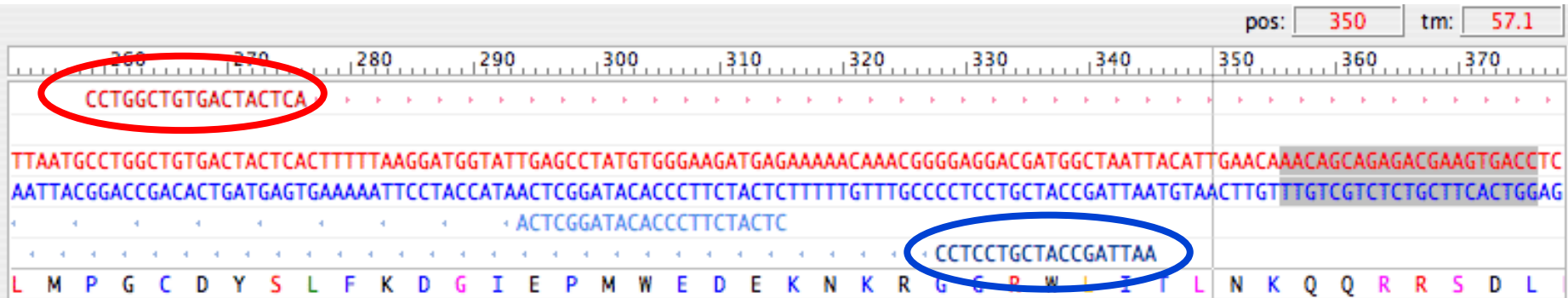
- TaqMan probes
- primers for *nested PCR*
- *molecular beacons*
- siRNA

* *Additional resources for interested* <http://oligo.net/tutorials.html>

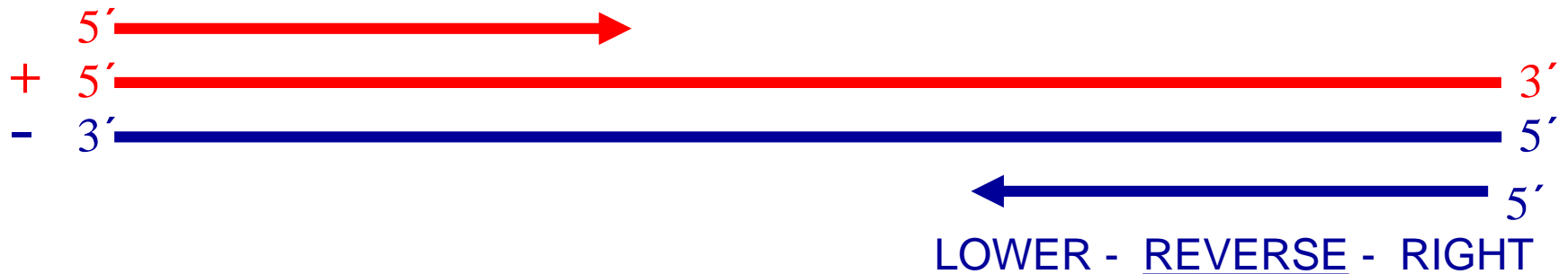
Terminology of PCR primers

forward primer... part of the + string

reverse primer... part of the - string



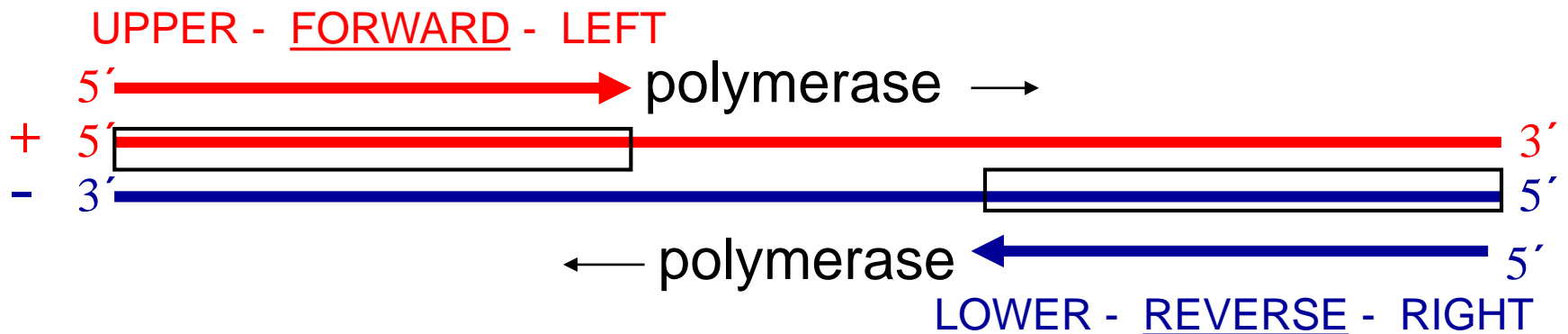
UPPER - FORWARD - LEFT



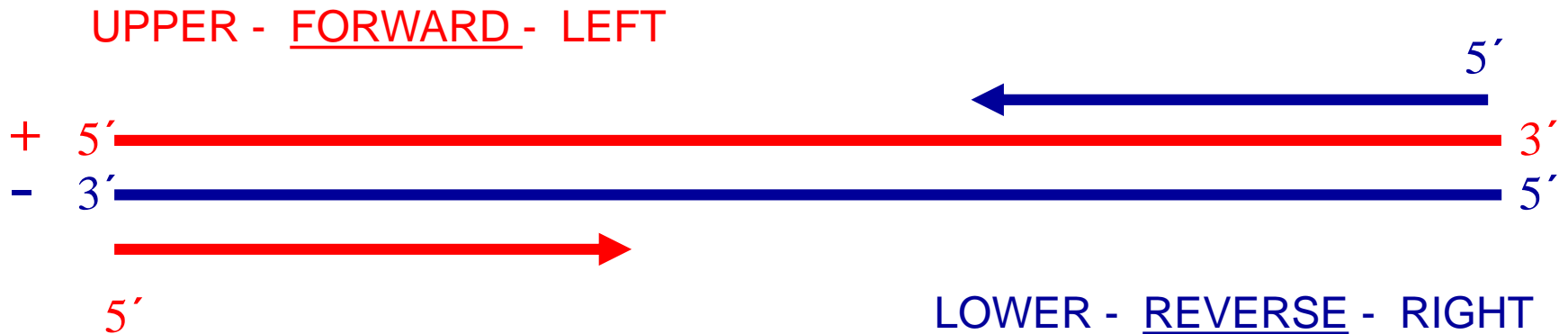
Terminology

forward primer... part of the + string

reverse primer... part of the - string



Annealing of PCR primers



MUNI

5' CTT CTG CTC AAT CTT TCT AC 3' **FORWARD**
read from left to right

+ 5' 1 ATGG **CTTCTG CTCAATCTTT CTAC** AACCAA AGCTCTGTCT TGAAAATCAA
51 TGTCATGGTT GTGGACGATG ATCATGTTTT CCTTGATATC ATGTCACGCA
101 TGCTTCAACA CTCCAAATAC AGAGGTAATT AAATATTATT ATCATATTAT
151 ATATAATATG TTATTGATTT TTTGTTTGTG ATTTCAATTA GATTTTTATT
201 TCTATGATTT CTTAGCATGA AATACAATTT TTGGAGAAAC AACTAGCAGT
251 TTTAAAAACA AAAC TTGAAT TTTGAGAAAT TCAAAGATGT TATATATATA
301 TGTCAAAATT TAACAATTAT TCTTCTAAAT CATCCGGATT CCGTTTACAT
351 GTACACATCT ACAATTTTCA ATTGAGGTAT TCTTGTTTTG ATGCCTTTGA
401 GACGAATAGT TTGATTGATA AAAAAAATTC TAACCAATAT GATATATAAA
451 GTTTTTTTTT TTTTTGTCAA ACCATACTTT ATACTATGTA ACTTTTTTTAA
501 GAGATTATTG AAAATAGTTT ATTTATAAAA TAGTAACCTA TTGTTGAATT
551 AAAAAAAAAA AAAAAATTGT AAATCGTGTG TGCAAACGAC ATGTGATTTA
601 TCTTAGTTTA **AACTAGCTG 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 CATTTTTTAAA GGTAAACCA
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 TTA AATTTAAA

3' **REVERSE**
read from right to left
complementary

Sequence

File: Human 4E.seq

DNA Sequence		Selected Oligo		Position	Length	#	Feature	Location
Sequence Length:	1868 nt	<input checked="" type="checkbox"/>	Forward Primer	259	18	1	source	-18..1850
Reading Frame:	+1	<input checked="" type="checkbox"/>	Reverse Primer	328	18	2	CDS	1..651
Current Oligo Length:	21 nt	<input type="checkbox"/>	Upper Oligo	---	---			
Position:	356	<input type="checkbox"/>	Lower Oligo	294	22			
t _m :	59.3°C	<input checked="" type="checkbox"/>	PCR Product	87 nt				

pos: 350 tm: 57.1

CCTGGCTGTGACTACTCA >

TTAATGCCTGGCTGTGACTACTCACTTTTAAAGGATGGTATTGAGCCTATGTGGGAAGATGAGAAAAACAACGGGGAGGACGATGGCTTTTACATTGAACAAACAGCAGAGACGAAGTGACCTC
 AATTACGGACCGACTGATGAGTGAAAAATTCCTACCATAACTCGGATACACCCCTCTACTCTTTTGTGGCCCTCTGCTACGATTAATGTAACCTGTTGTCGCTCTGCTTCACTGGAG
 > ACTCGGATACACCCCTCTACTC > 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

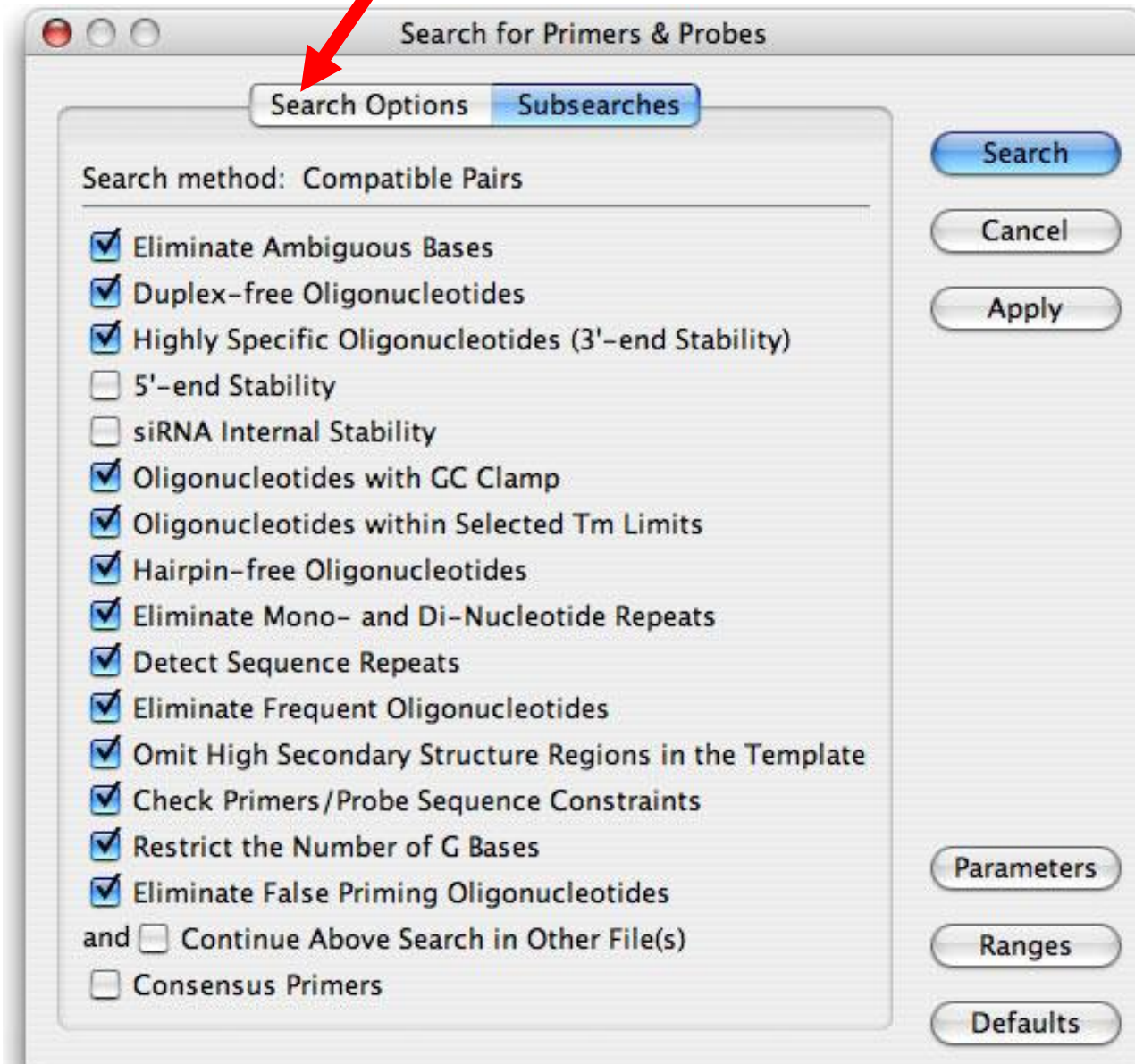
TaqMan Probes & PCR Pairs
Compatible with the Upper Probe Lower Probe

Molecular Beacons & PCR Pairs
 Nested Primers
 Sequencing Primers
 Hybridization Probes
 siRNA Probes

After successfull search show: All Results

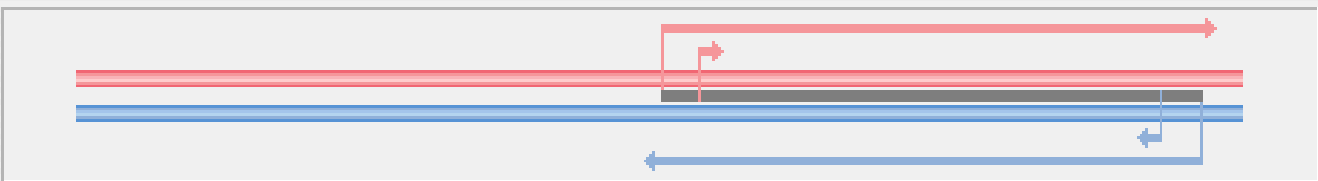
Search
Cancel
Apply

Parameters
Ranges
Defaults



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	852		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


Selected Primers

File: BRCA2 gene.seq

AY436640:15438F22		AY436640:15917R20	
5' CAATATATACCGTAGTCCCCTA 3'		5' CAGCTACATATTACGCCAGA 3'	
Length:	22-mer	Length:	20-mer
Score:	802 points	Score:	914 points
5' Position:	15438	3' Position:	15917
T_m/t_m :	53.4 52.6 °C	T_m/t_m :	53.1 53.8 °C
$\Delta G/\Delta g$ (25 °C):	-30.5 -29.2 kcal/mol	$\Delta G/\Delta g$ (25 °C):	-28.6 -28.5 kcal/mol
$\Delta S/\Delta s$:	-472.1 -449.5 cal/°K * mol	$\Delta S/\Delta s$:	-430.5 -419.6 cal/°K * mol
$\Delta H/\Delta h$:	-171.3 -163.2 kcal/mol	$\Delta H/\Delta h$:	-157.0 -153.6 kcal/mol
3' ΔG :	-6.5 kcal/mol	3' ΔG :	-6.9 kcal/mol
Degeneracy:	1	Degeneracy:	1
P.E.#:	443/443	P.E.#:	477/477
1/E:	4.63 nmol/A ₂₆₀ 31.1 µg/A ₂₆₀	1/E:	5.05 nmol/A ₂₆₀ 31.0 µg/A ₂₆₀

Priming Efficiency PE
Score

Secondary structures


Current Oligo Duplexes

File: BRCA2 gene.seq

Current Oligo 21-mer [5042]

[Current+ Oligo] - The most stable 3'-dimer: # of hydrogen bonds = 10; $\Delta G = -0.7$ kcal/mol

```

5' GAATTAGATAAAATTCAAATTA 3'
      |||||
3' ATTAAACTTAAATAGATTAAG 5'
      
```

DIMER intermolecular

[Current- Oligo] - The most stable 3'-dimer: # of hydrogen bonds = 10; $\Delta G = -7.3$ kcal/mol; $T_m = 2.9^\circ\text{C}$

```

5' TAATTTGAATTTATCTAATTC 3'
      |||||
3' CTTAATCTATTTAAGTTTAAT 5'
      
```

The most stable dimer overall: # of hydrogen bonds = 10; $\Delta G = -7.4$ kcal/mol; $T_m = 2.2^\circ\text{C}$

```


5' GAATTAGATAAAATTCAAATTA 3'
      |||||
3' ATTAAACTTAAATAGATTAAG 5'
      
```

Hairpin: loop = 5 nt; $\Delta G = -3.0$ kcal/mol; $T_m = 54.6^\circ\text{C}$

```

5' GAATTAG
      |||||
3' ATTAAACTTAAAT
      
```

HAIRPIN intramolecular



Current Oligo Hairpin Stems

File: BRCA2 gene.seq

Current Oligo 21-mer [5042]

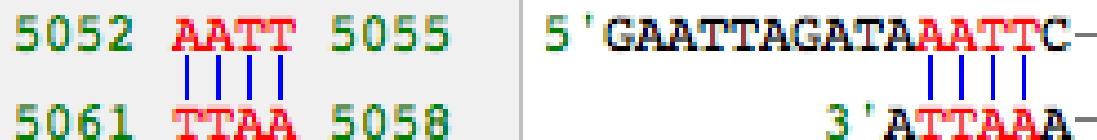
1. # of paired bases = 5; loop = 5 nt; $\Delta G = -3.0$ kcal/mol; $T_m = 54.6$ °C



2. # of paired bases = 6; loop = 5 nt; $\Delta G = 0.2$ kcal/mol; $T_m = 21.7$ °C



3. # of paired bases = 4; loop = 2 nt; $\Delta G = 0.9$ kcal/mol; $T_m = 8.7$ °C



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) GGT TTT CCC AGT CAC GAC G (6310) 3'
          ||| ||| ||| ||| ||| ||| |||
3' (6328) ccaaaagggtcagtgctgc (6310) 5'
    
```

Priming efficiency: 244 (above the threshold)

```

5' (6328) GGT TTT CCC AGT CAC GAC G (6310) 3'
          ||| ||| ||| ||| ||| |||
3' (626)  agcaa atggtc--tgctgc (610) 5'
    
```

Priming efficiency: 193 (above the threshold)

```

5' (6328) GGT TTT CCC AGT CAC GAC G (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]
$\mu\text{g}/\text{OD}$	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%]

Oligonucleotide Database

File: NewDatabase.odb



of Records: 29

#	Date	ID Number	Sequence	3'-Dim. ΔG		P.E. / p.e.		Tm / t _m	
<input type="checkbox"/> 21	12/02/06	AY436640:5916R19	AATGCCTGCCTTTAGTCTG	-	SC	430	430	54.1	54.5
<input type="checkbox"/> 22	12/02/06	AY436640:5916R20	CAATGCCTGCCTCTAGTCTG	0.3	SC	366	450	50.9	57.2
<input type="checkbox"/> 23	12/02/06	AY436640:5937R21	TCAATTTCTTTAGCTTGGCAT	0.3	SC	449	449	54.7	53.1
<input checked="" type="checkbox"/> 24	12/02/06	AY436640:5937R22	TTCAATTTCTTTAGCTTGGCAT	0.3	SC	458	458	55.9	53.8
<input type="checkbox"/> 25	12/02/06	AY436640:4695U22	TGCCTTAACAAAAGTAATCCAT	0.3	SC	432	432	54.5	53.0
<input type="checkbox"/> 26	12/02/06	AY436640:5325U22	AATTACGTCTTTCTTATGCCAA	0.3	SC	453	453	53.3	53.0
<input type="checkbox"/> 27	12/02/06	AY436640:5786L23	CTCTGCCTAGAACATTATCACTC	-0.3	SC	451	451	54.8	55.0
<input type="checkbox"/> 28	12/02/06	AY436640:5860L19	AACAACCAAAGCCAACCTG	-0.9	SC	444	444	55.3	55.9

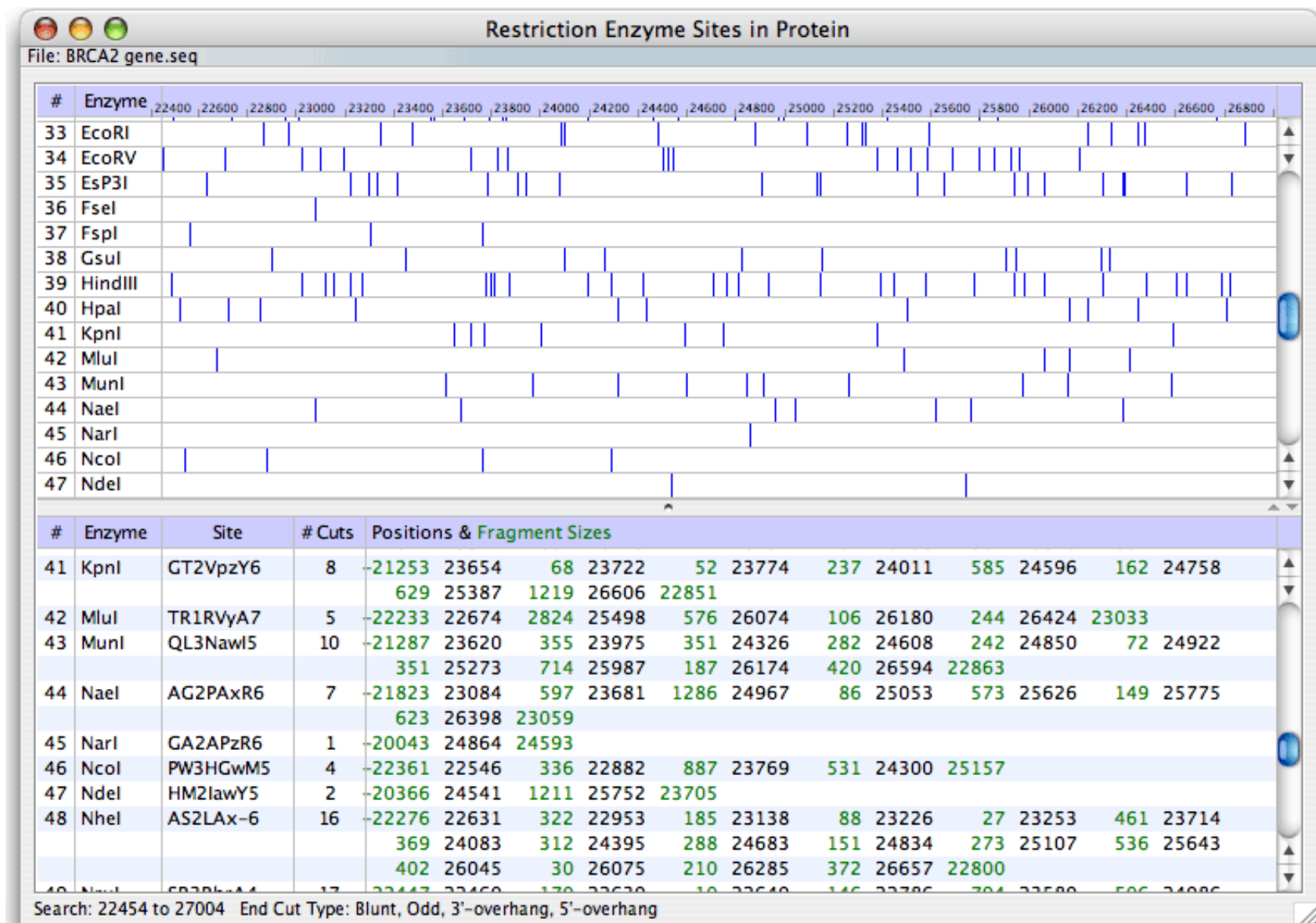
Selected oligo

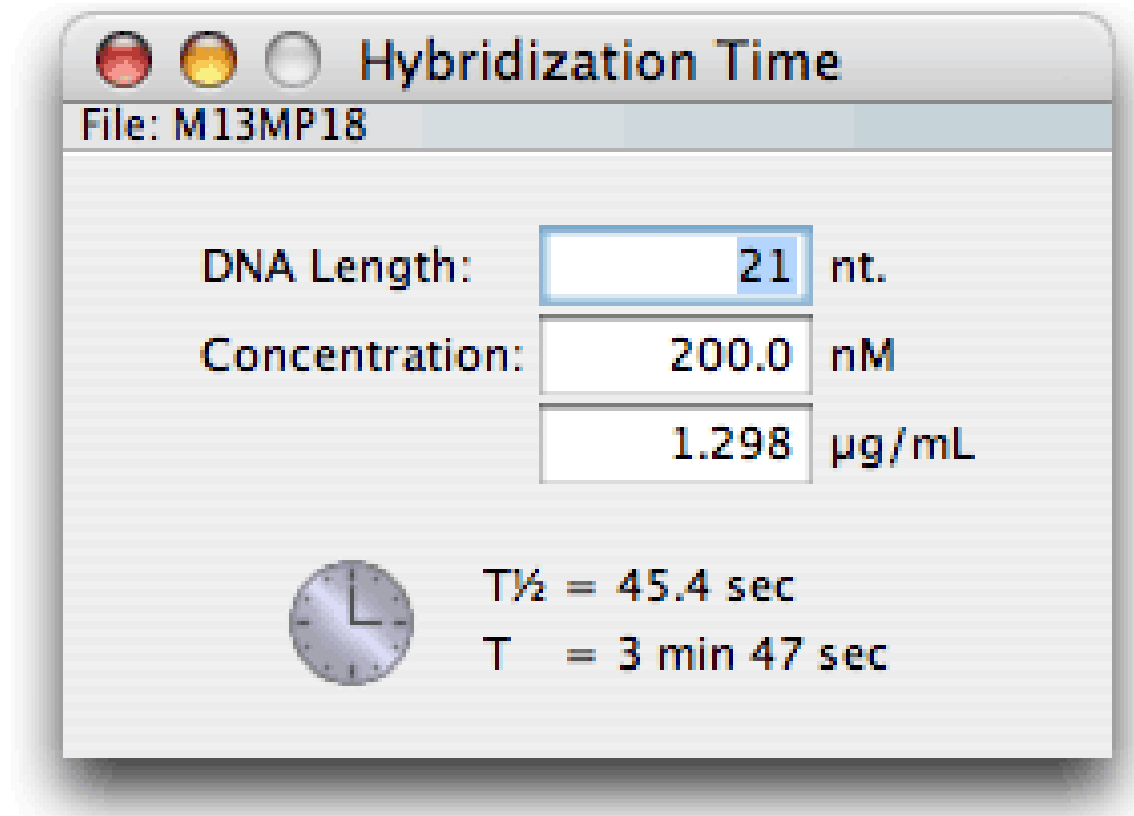
Oligonucleotide Sets (64)

#	Forward Primer	Reverse Primer	Upper Oligo	Lower Oligo
	1	2	3	4
<input type="checkbox"/> 36	8	23	25	28
<input type="checkbox"/> 42	8	24	25	28
<input checked="" type="checkbox"/> 47	9	14	25	27
<input type="checkbox"/> 39	9	15	25	27
<input type="checkbox"/> 33	9	16	25	27
<input type="checkbox"/> 61	9	17	25	27
<input type="checkbox"/> 48	9	18	25	27

Checked Set of nested primers

This database is linked to BRCA2 gene.seq





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

µg
 or OD(260)
 or nmol
 in µL
 yields 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 TCTCATGGAC GCTCTCATTG CTCAGCTTCA
151 GAGACAATTT CGTGATTACA CCATTTCTCT CTACCAACAG GGGTTTTTTGG
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
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
```

MUNI

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 TCAAACTTT GTGTGTTAGC TTCAAGGAAT GTTGTGAAGC
451 TAAGA ACTAC GAAGGGTGTG TGAGATGTTT GCAGCAAGTG GATATTGAGT
501 ACAAGGCGTT AAAGACAAAG CTTCAAGATA TGTTC AATCT 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'

MUNI

101 CCCAATCTCC GCTTCACTCT TCTC**ATGGAC GCTCTCATTG CTCAG**CTTCA
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 AGTT**CTCAA GTGGATATTA ACTAA**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'

- **Computational and Structural Biotechnology Journal 17, 2019, 1056-1065. In-silico Design of DNA Oligonucleotides: Challenges and Approaches.** *includes a list of oligo design tools*
<https://www.sciencedirect.com/science/article/pii/S2001037019300844>
- [www.protocol-online.org/prot/Research Tools/Online Tools/Oligo Design/index.html](http://www.protocol-online.org/prot/Research%20Tools/Online%20Tools/Oligo%20Design/index.html)
- **OLIGO 7 Power Point Presentation** <http://oligo.net/tutorials.html>
- **OLIGO Primer analysis software, Version 7** <http://oligo.net/tutorials.html>
- <https://langdalelab.files.wordpress.com/2015/07/degenerate-primer-design.pdf>
- <http://www.genomecompiler.com/tips-for-efficient-primer-design/> (2015)
- Nature Methods 2014, 11 (5): 499. Large-scale de novo DNA synthesis: technologies and applications
- *Expert Opin Ther Pat.* 2014, 24(7):801-19. Oligonucleotide delivery: a patent review (2010 - 2013).
- Laboratory Methods in Enzymology 529: DNA Explanatory Chapter: PCR Primer Design (2013)
- PCR Primer Design; IMBB Workshop, N. Ndegwa (2013)
- PCR Primer Design; A. Yuryev (2010)
- *AAPS Journal* 2009, 11(1): 195 - 203. Targeted Delivery Systems for Oligonucleotide Therapeutics
- **iCODEHOP** (COnsensus-DEgenerate Hybrid Oligonucleotide Primer)PCR primer design (2009)
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2703993/>
- Bioinformatic tools and guideline for PCR primer design. Kamel A. Abd-Elsalam (2003)
- Artificial DNA: Methods and Applications; Khudyakov, Y.E., Fields, W.A., Ed. (2003)
- PCR Primer: A Laboratory Manual (2003)

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

Marcel Proust