MASARYKOVA UNIVERZITA

Design sekvence PCR primerů

Hana Konečná

CEITEC – MU Centrální laboratoř - Proteomika

Přírodovědecká fakulta NCBR

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY OP Vzdělávání pro konkurenceschopnost

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

SYNTETICKÉ OLIGONUKLEOTIDY

MASARYKOVA UNIVERZITA

OLIGONUKLEOTIDY

PCR primery

- definice
- aplikace
- modifikace
- syntéza

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

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

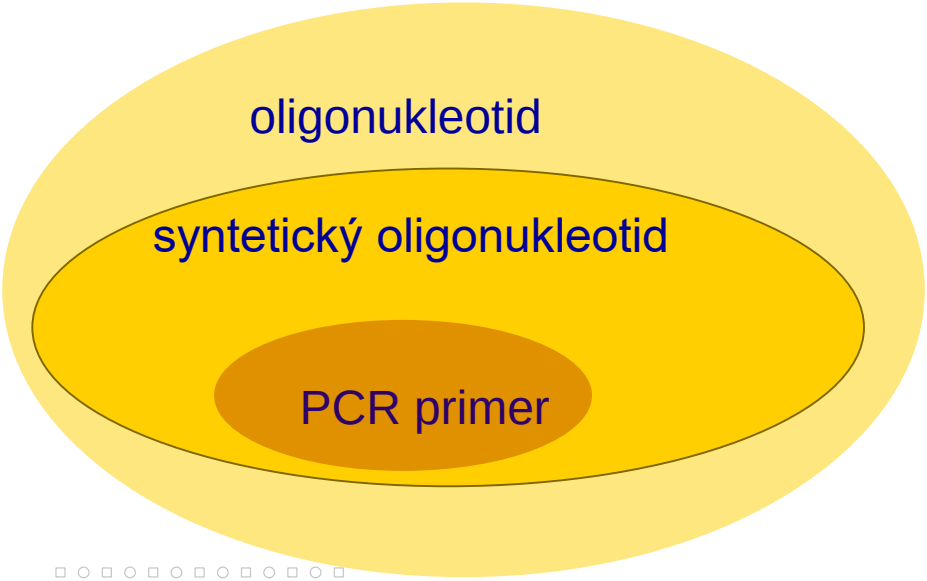
OP Vzdělávání pro konkurenceschopnost

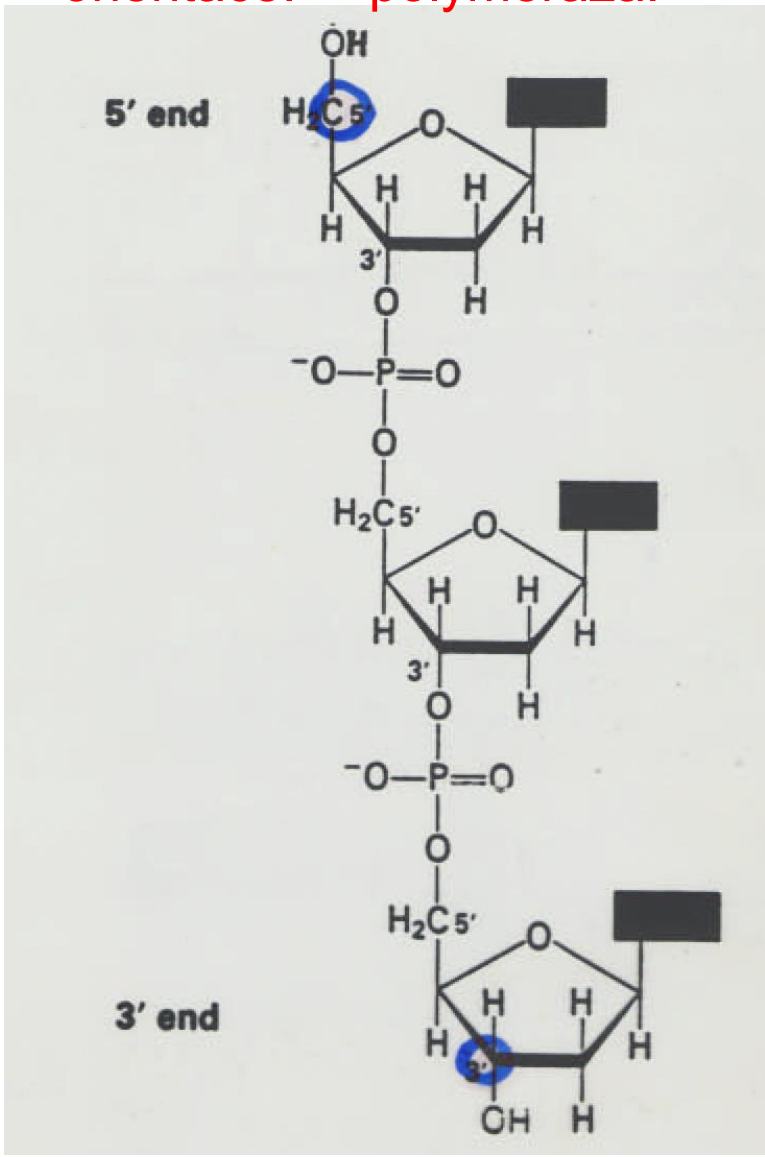
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

orientace! polymeráza!

oligonukleotid

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



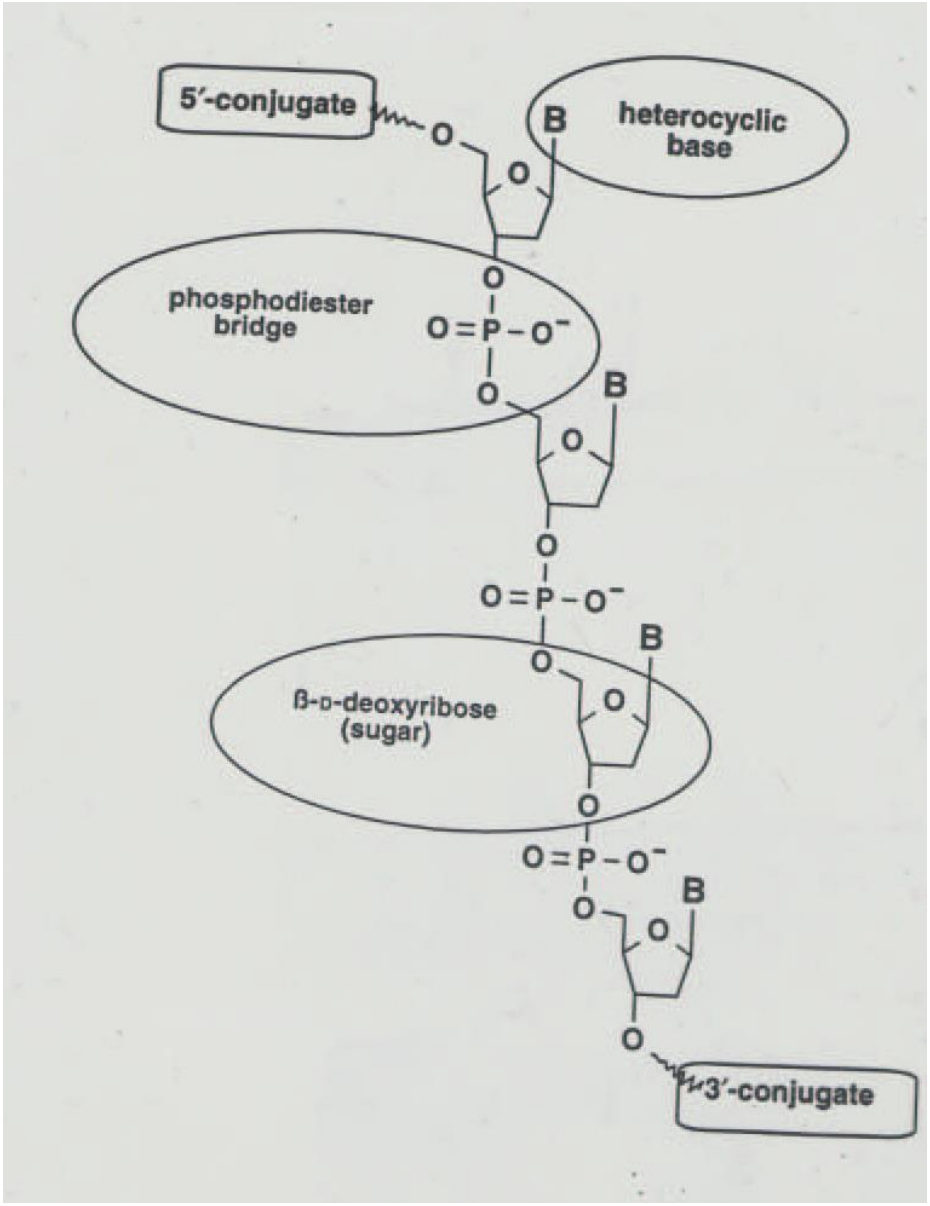


Aplikace syntetických oligonukleotidů

- primery pro syntézu komplementární DNA
  PCR, Real-Time PCR
- syntéza genů a rekombinantní proteiny
- hybridizační sondy pro klonování
- místně cílená mutageneza
- sekvenování a genetické profilování
- diagnostika – testy a biosensory
- gene arrays
- blokace genové exprese *antisense oligo*
- potenciální léčiva a DNA vakcíny
- NMR studia interakcí DNA-protein
- strukturální rentgenová analýza NA

Modifikace

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



Modifikace na 5´- konci

postsyntetické modifikace →

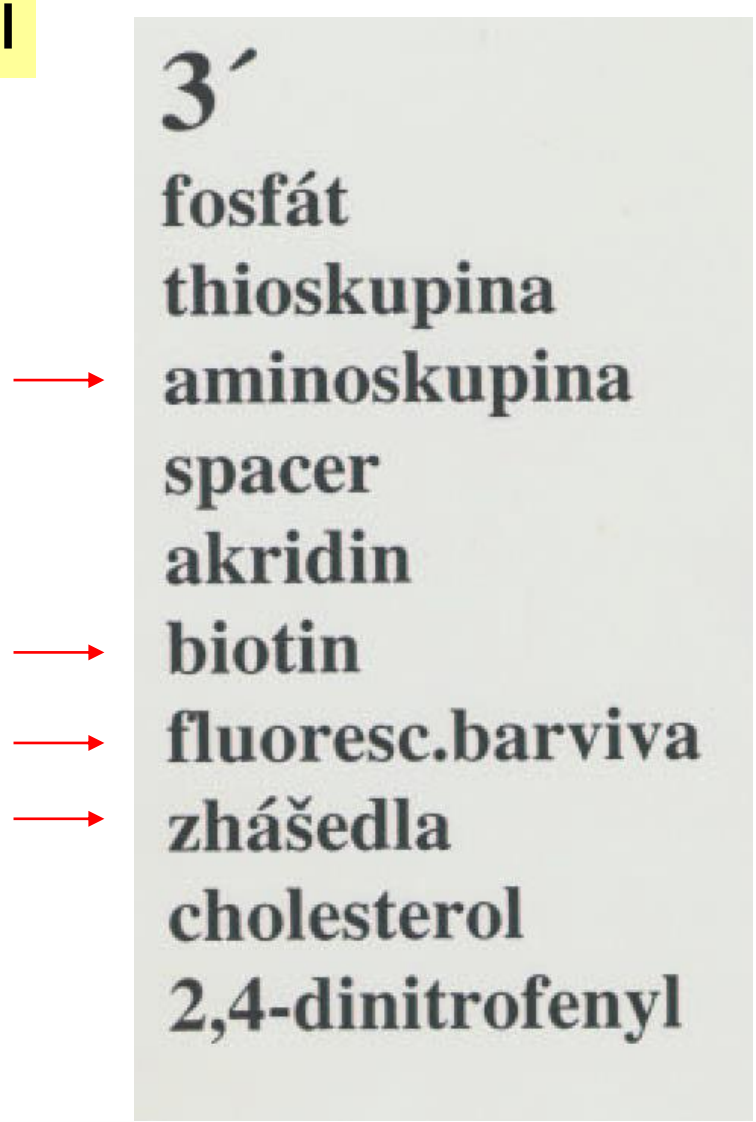
sekvenování →
fragmentační analýza
gene arrays
Real-Time PCR

5´

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

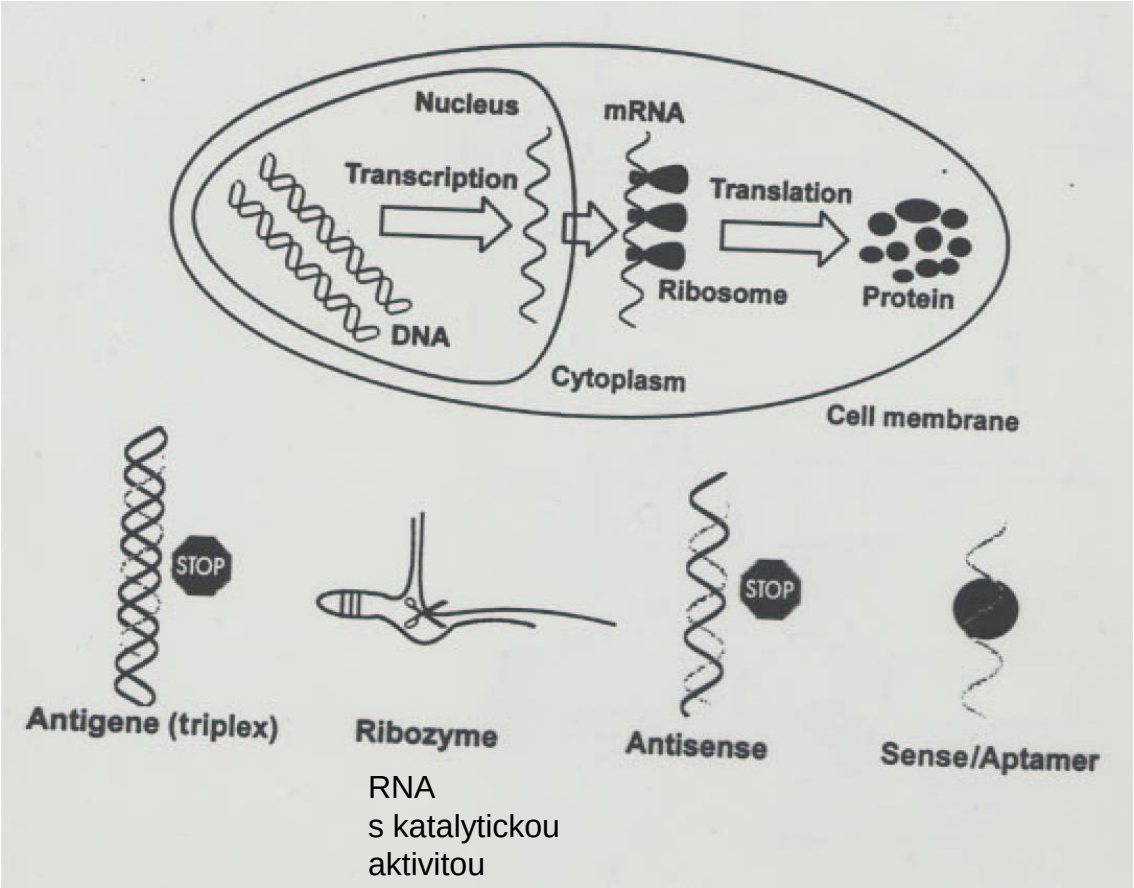
Modifikace na 3´- konci

derivatizovaná matrice



ANTISENSE oligonukleotid

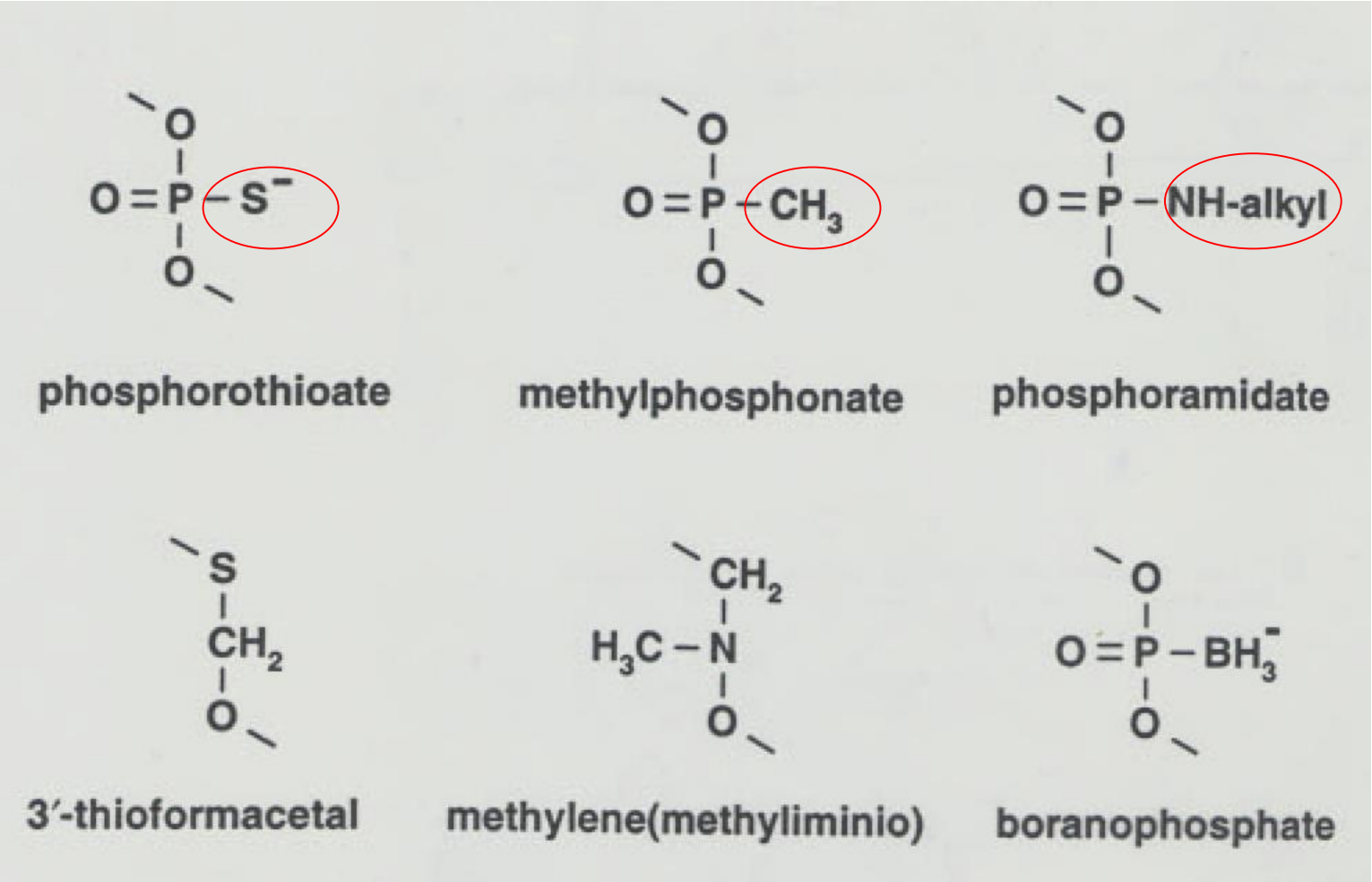
- oligonukleotid nebo analog
- komplementární k segmentu RNA nebo DNA
- vazbou inhibuje jejich normální funkci



RNA
s katalytickou
aktivitou

Terapeutika

→ nedegradována nukleázami!
modifikace fosfodiesterové vazby



TIDES: Oligonucleotide and Peptide Therapeutics

2018

- Messenger RNA Therapeutics
- CRISPR-Cas9 and Applications of Genome Editing
- Risk-based Approaches to CMC Development of Therapeutics Oligonucleotides
- Opportunities for Extending to Larger Disease Populations
- Stereochemical Control of Antisense Oligonucleotides Enhances Target Efficacy
- Mechanisms of Action and Quantification of Biodistribution of Oligonucleotides
- Alternative Manufacturing Technologies to Reduce Cost of Goods in Oligonucleotide Manufacturing: Application of Enzymatic Manufacturing
- Late Stage Drug Product Development and Drug Product Manufacturing
- Nonclinical Case Studies in Oligonucleotide Development
- Regulatory Authority Expectations

Degenerované oligonukleotidy

symbol AA nukleotidy

amino acid	amino acid symbol	nucleotide sequence (with degeneracy)	complement (for designing reverse primers)
methionine	M	ATG	TAC
tryptophan	W	TGG	ACC
cysteine	C	TGY	ACR
aspartic acid	D	GAY	CTR
glutamic acid	E	GAR	CTY
phenylalanine	F	TTY	AAR
histidine	H	CAY	GTR
lysine	K	AAR	TTY
asparagine	N	AAY	TTR
glutamine	Q	CAR	GTY
tyrosine	Y	TAY	ATR
isoleucine	I	ATH	TAD
alanine	A	GCN	CGN
glycine	G	GGN	CCN
proline	P	CCN	GGN
threonine	T	ACN	TGN
valine	V	GTN	CAN
leucine	L	YTN	RAN
arginine	R	MGN	KCN
serine	S	WSN	WSN

kód báze	zahrnuje
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

Degenerované oligonukleotidy

CODEHOP
Consensus Degenerate Hybrid Oligonucleotide Primers

HYDEN
HighlY DEgeNerate primers

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

2-deoxyinosin

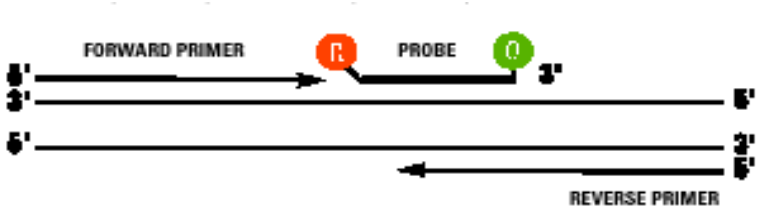
Degenerované oligonukleotidy

Příklady:

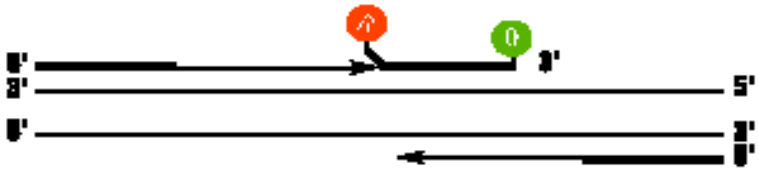
ACG TAC GTA CGT ACG TAC	nedegenerovaný
ACG TA**M** GTA CGT ACG TAC	M = A/C
ACG TAC GTA C**D**T ACG TAC	D = A/G/T
ACG TAC GTA CGT ACG **N**AC	N = A/C/G/T

Real-Time PCR

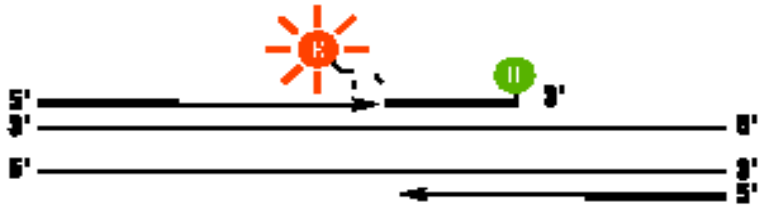
- 2x značená sonda
- REPORTER
- QUENCHER



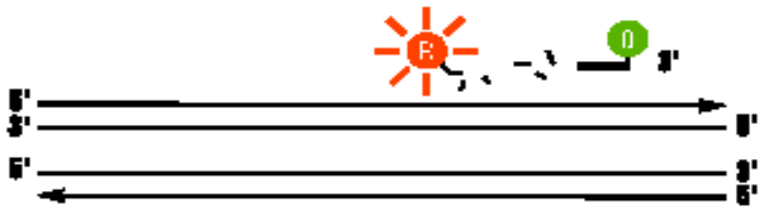
2. **Strand displacement:** When the probe is intact, the reporter dye emission is quenched.



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



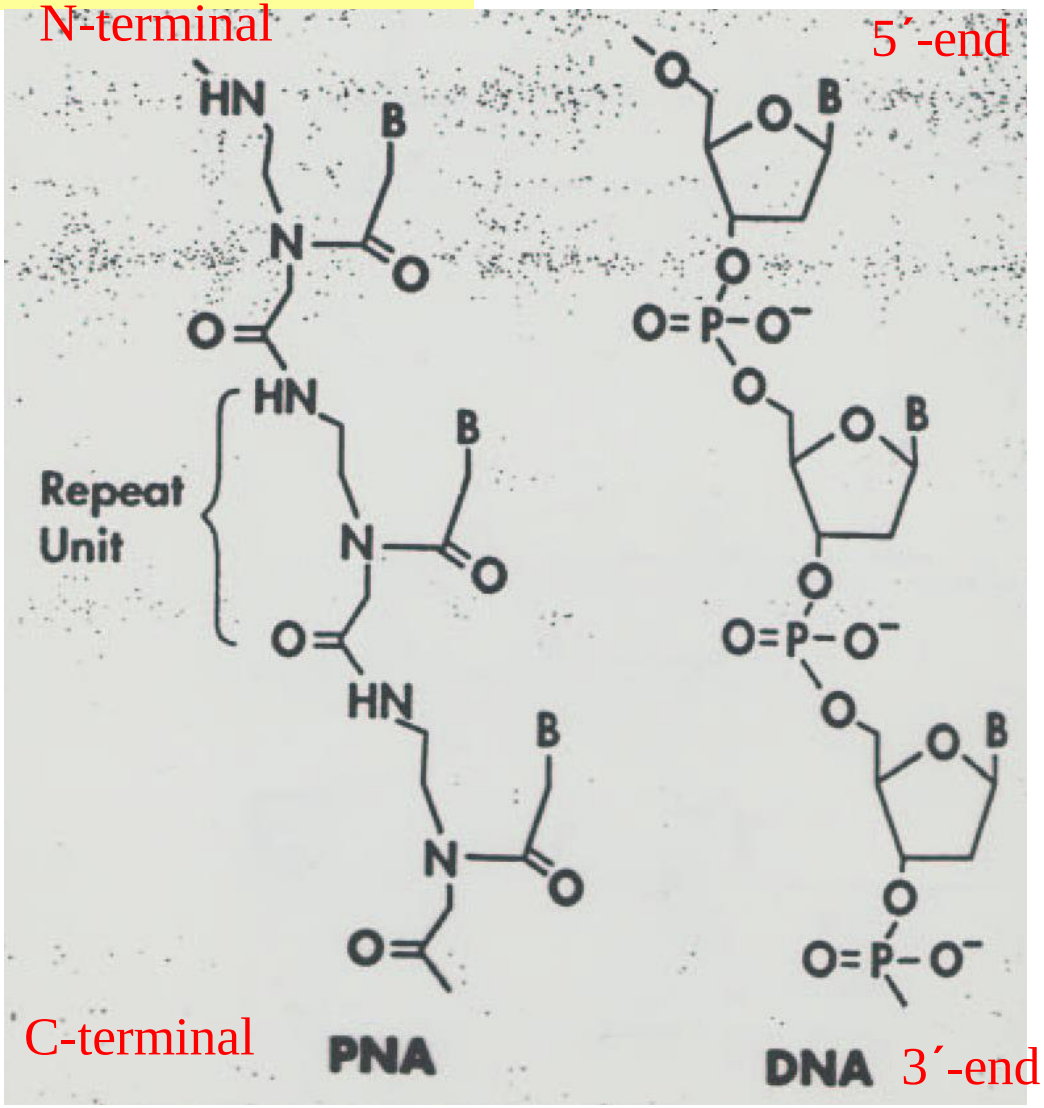
4. **Polymerization completed:** Once separated from the quencher, the reporter dye emits its characteristic fluorescence.



Peptidonukleová kyselina PNA

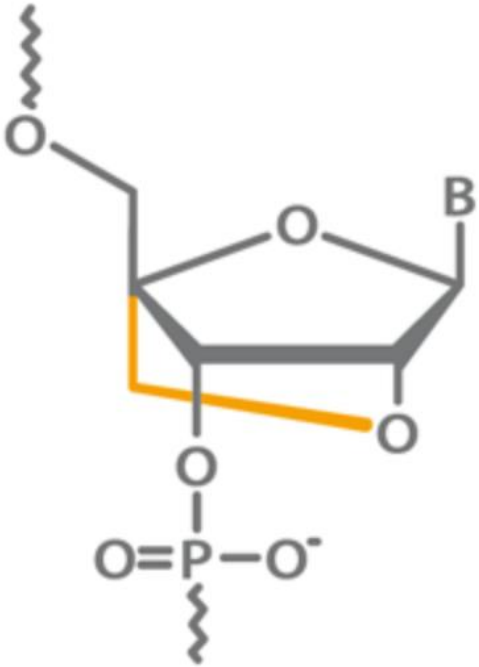
- nenabitá molekula
- vazba k DNA/RNA

N-(2-aminoethyl)-glycin →



DNA

LNA



Locked **N**ucleic **A**cid

2'-O, 4'-C methylenový můstek
potlačená flexibilita ribofuranózového kruhu
struktura je zamčena do rigidní C3-endo konformace
zlepšená hybridizace
výjímečná biostabilita

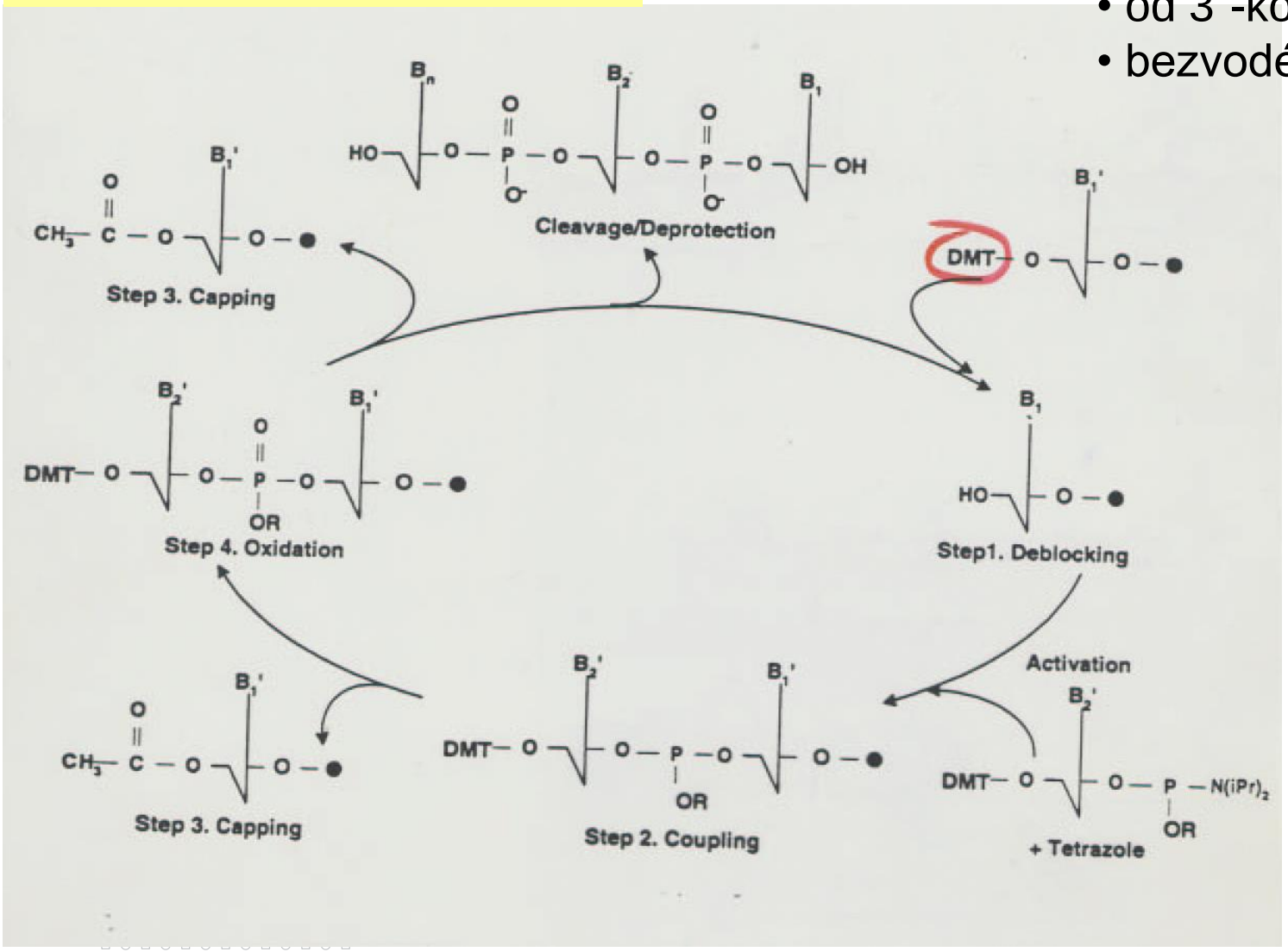
OLIGONUKLEOTIDY

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

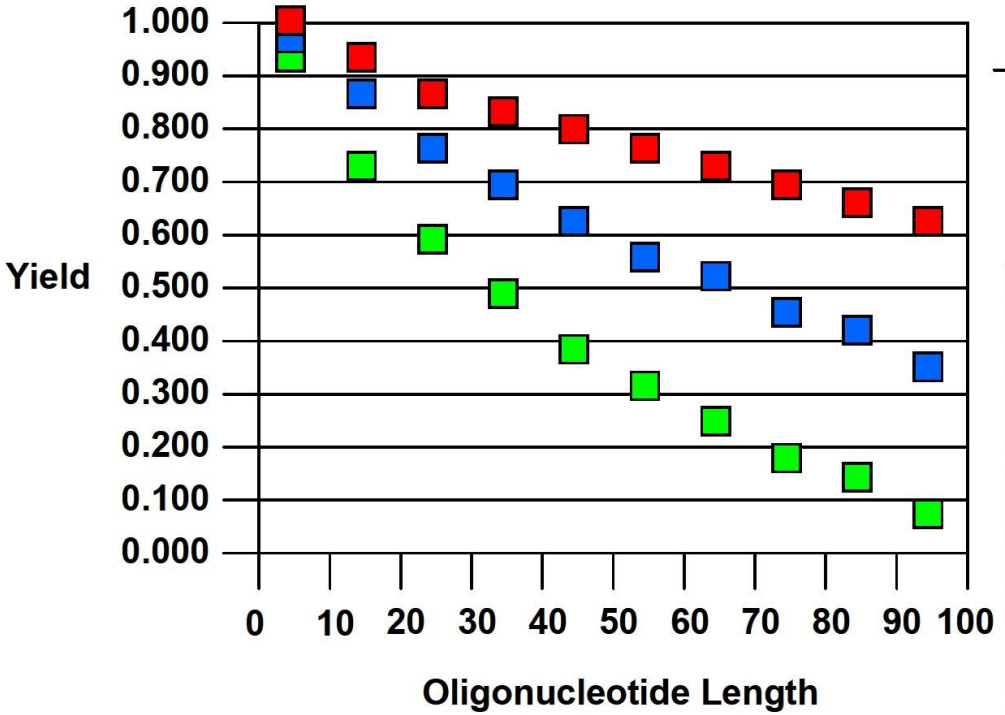
EXPEDITE 8909

Syntéza oligonukleotidu

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



VÝTĚŽEK



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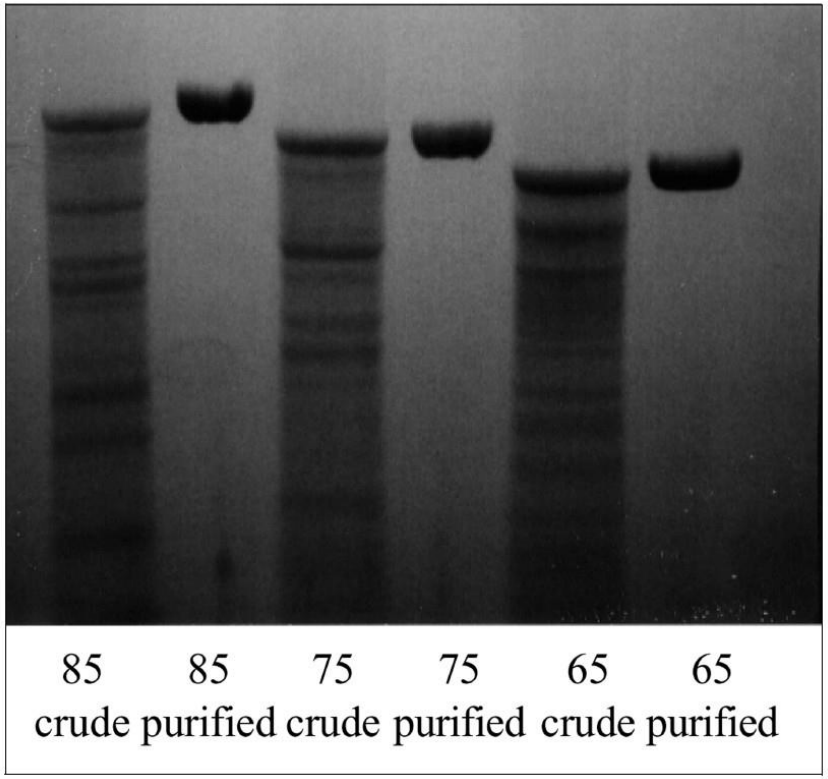
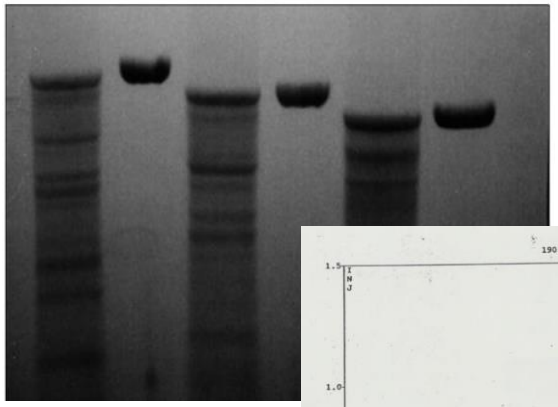
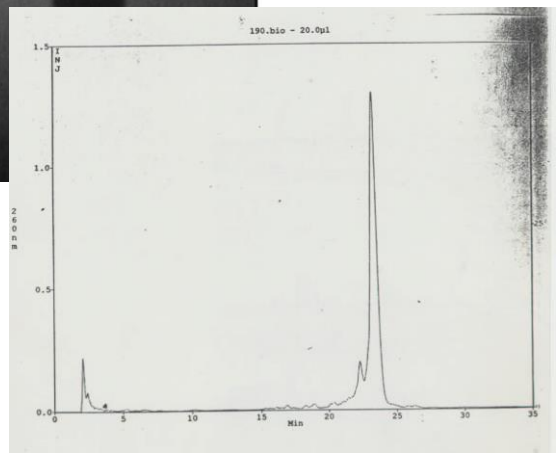


PURIFIKACE a QC

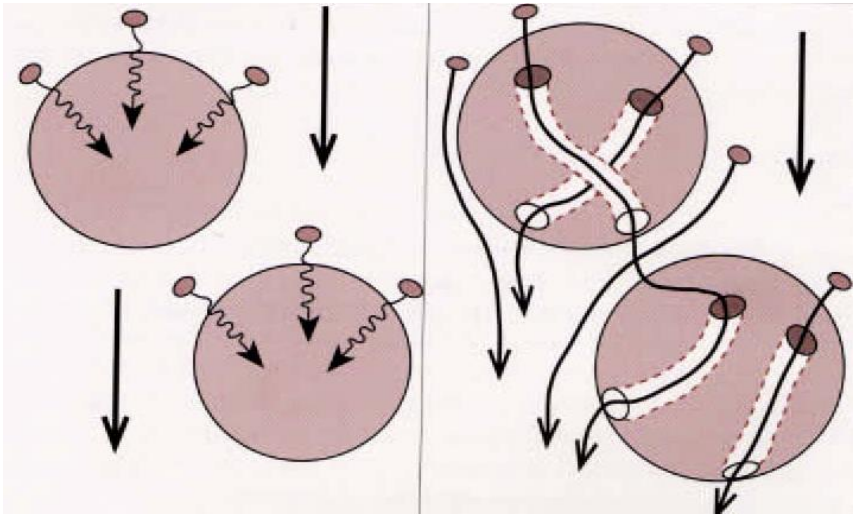
- Sephadex minispin kolonka
- RP cartridge
- HPLC
- Gelová elektroforéza

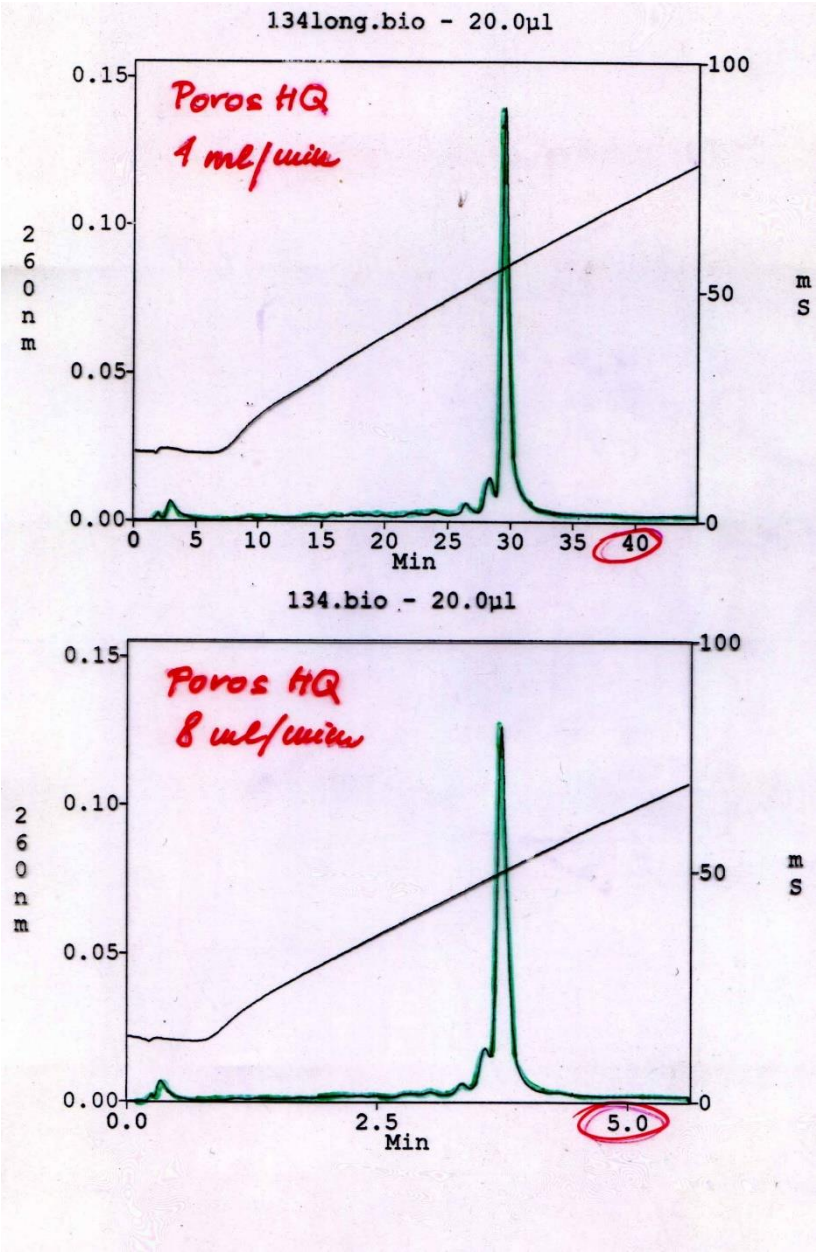




Perfúzní chromatografie

klasický HPLC sorbent POROS





Jaký typ purifikace vybrat?

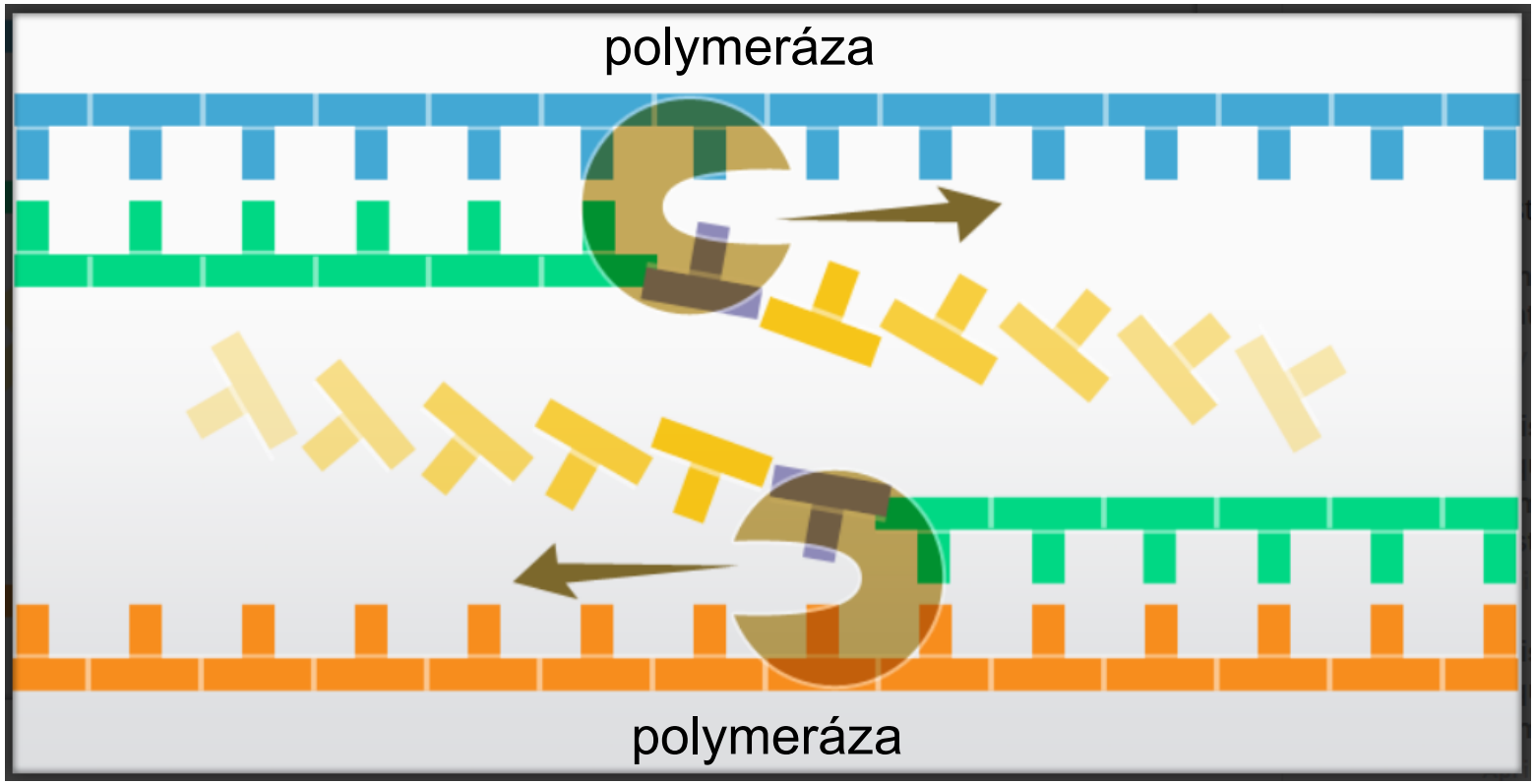
- aplikace oligo
- délka oligo
- modifikace oligo
- výtěžek oligo

PCR nebo sekvenování	standardní odsolení
Klonování . Mutageneza . Gel shift *	HPLC . PAGE
Modifikované oligonukleotidy	HPLC . ~~PAGE~~

* affinity electrophoresis for protein-DNA or protein-RNA interactions

PCR primery

Methods in Enzymology 529 (2013)
Laboratory Methods in Enzymology:
DNA Explanatory Chapter: PCR Primer Design



http://www.genomecompiler.com/tips-for-efficient-primer-design/

DESIGN OLIGONUKLEOTIDU

- manuální
- počítačový

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

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1526718/pdf/1746-4811-2-4.pdf

https://academicjournals.org/journal/AJB/article-full-text-pdf/D84CFC59144

Hlavní kritéria pro sekvenci PCR primeru

- vysoce specifické – zejména 3´konec
- netvoří dimery a vlásenky
- stabilní duplexy s aktivní sekvencí
- nepříliš stabilní 3´-konec
- obvykle 18 – 25 nt
- 40% až 60% GC

www.genomecompiler.com/tips-for-efficient-primer-design/

OLIGO 6

- PCR primery,
- hybridizační sondy
- sekvenační primery

OLIGO 7 (od roku 2008)

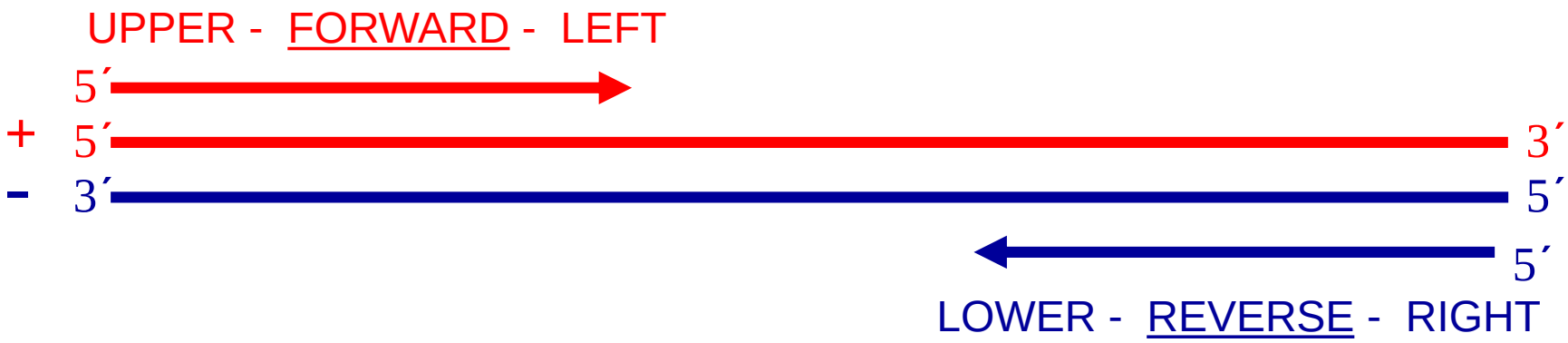
- TaqMan sondy
- primery pro *nested PCR*
- *molecular beacons*
- siRNA

Oligo 7 Power Point Presentation http://www.oligo.net/tutorials.html

Terminologie PCR primerů

forward primer… část sekvence + vlákna

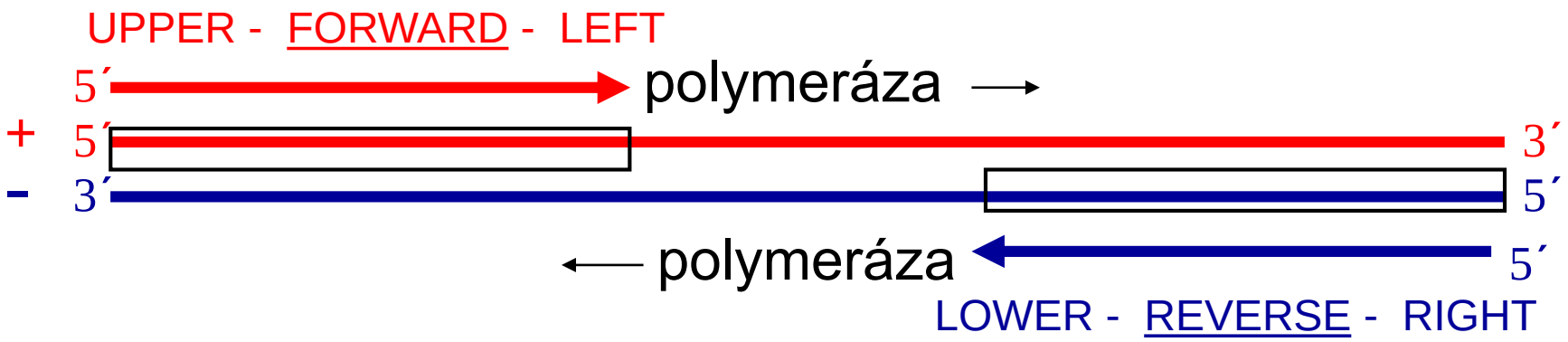
reverse primer… část sekvence - vlákna



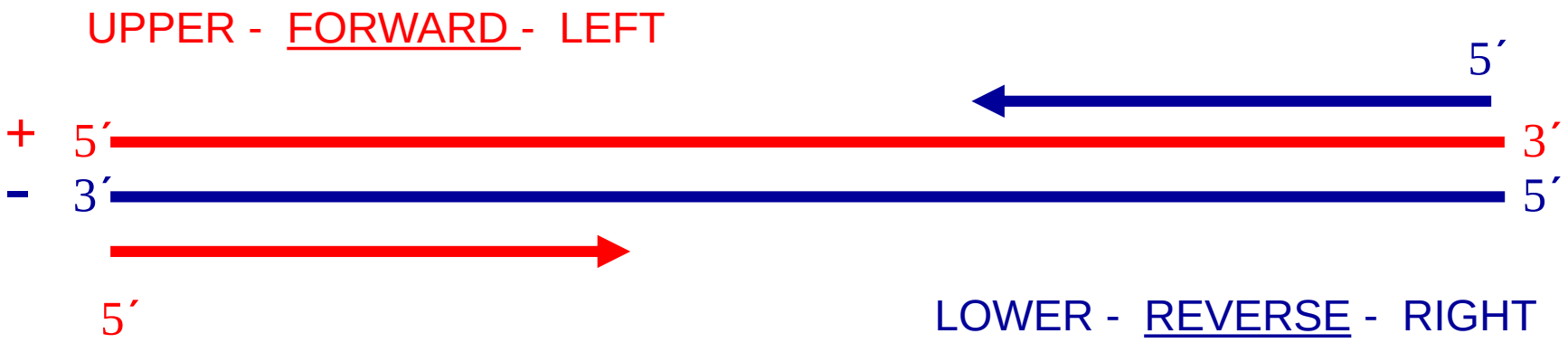
Terminologie

forward primer… část sekvence + vlákna

reverse primer… část sekvence - vlákna



Nasedání PCR primerů



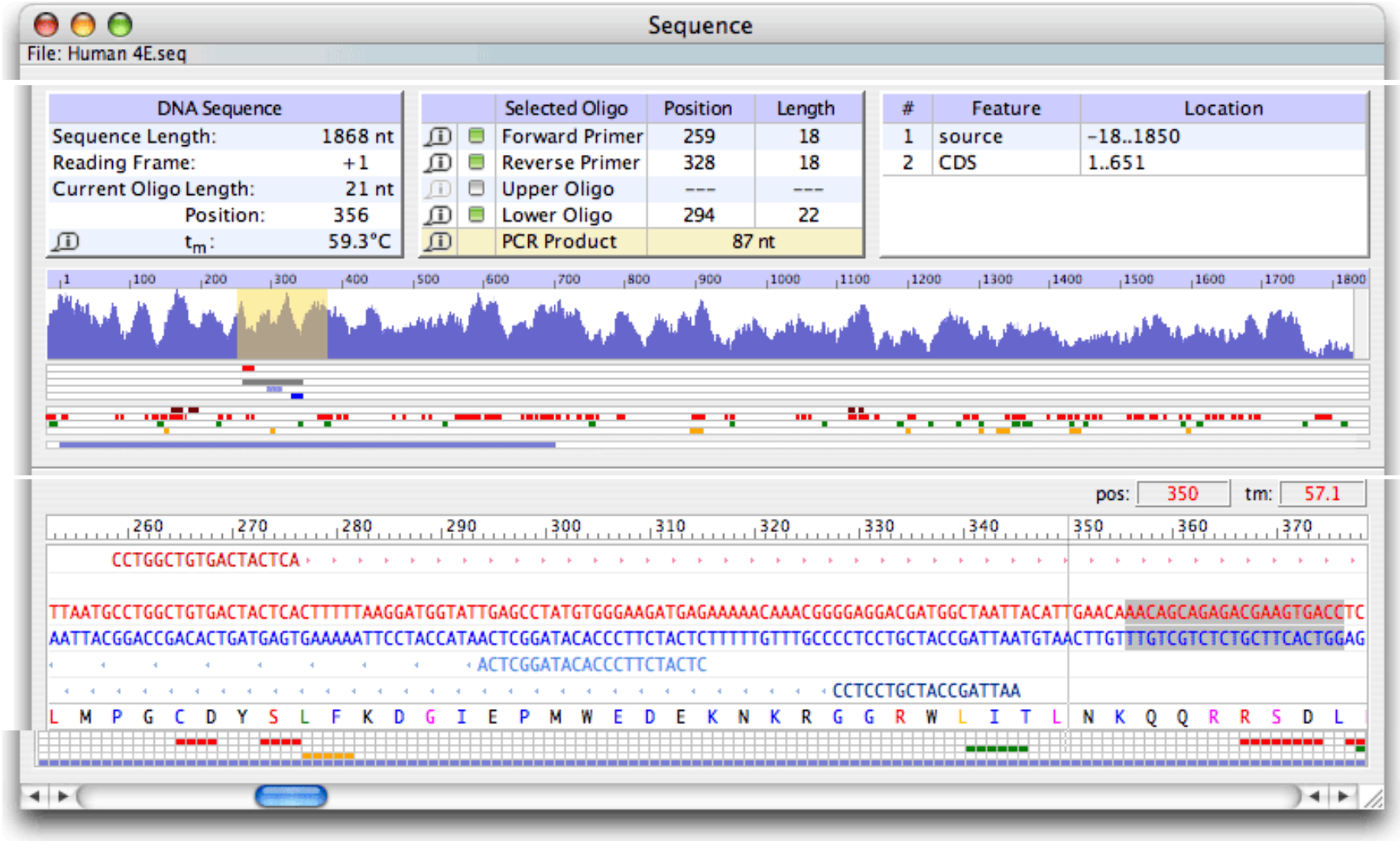
5´ CTT CTG CTC AAT CTT TCT AC 3´ **FORWARD**

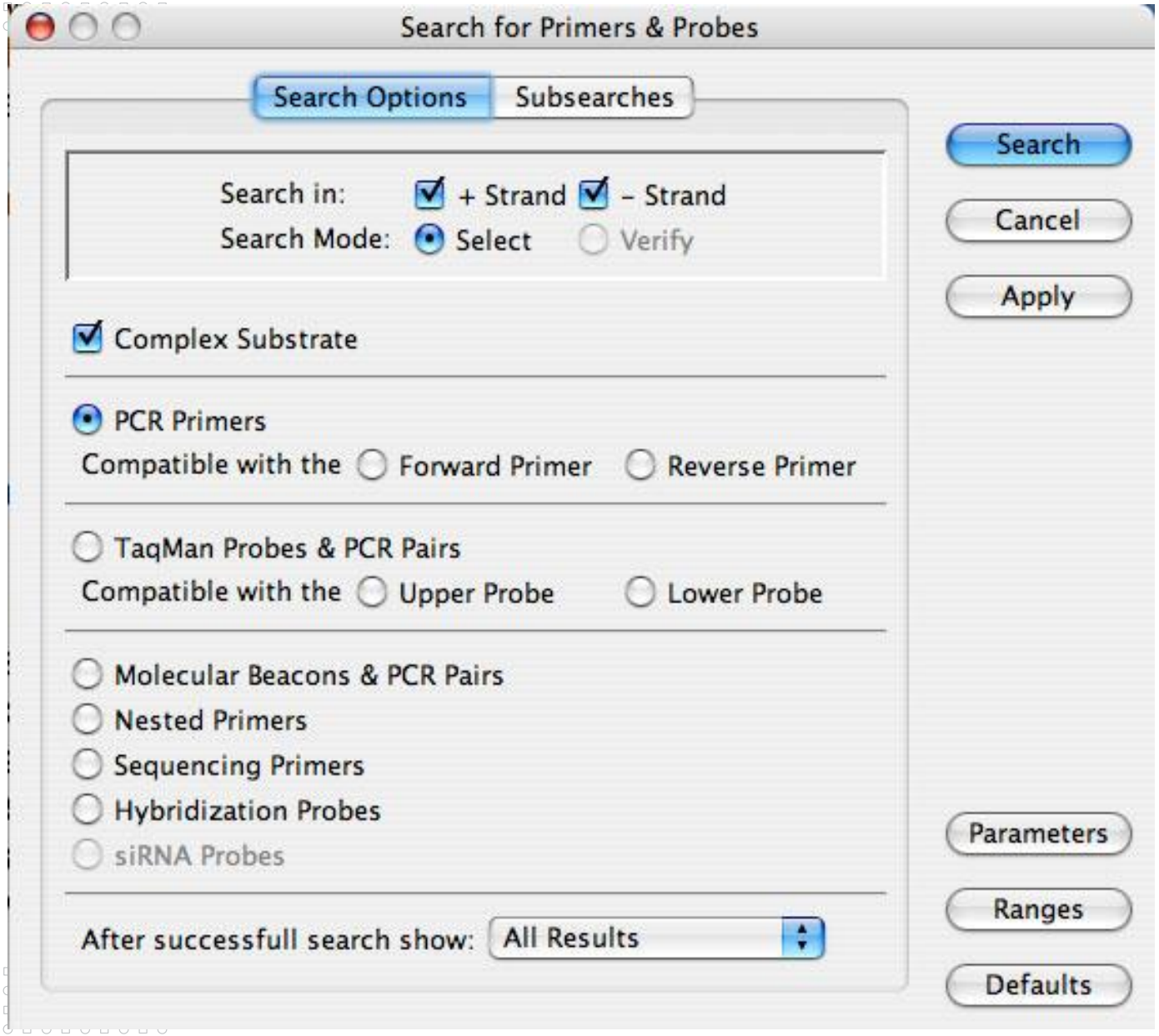
\+ 5´

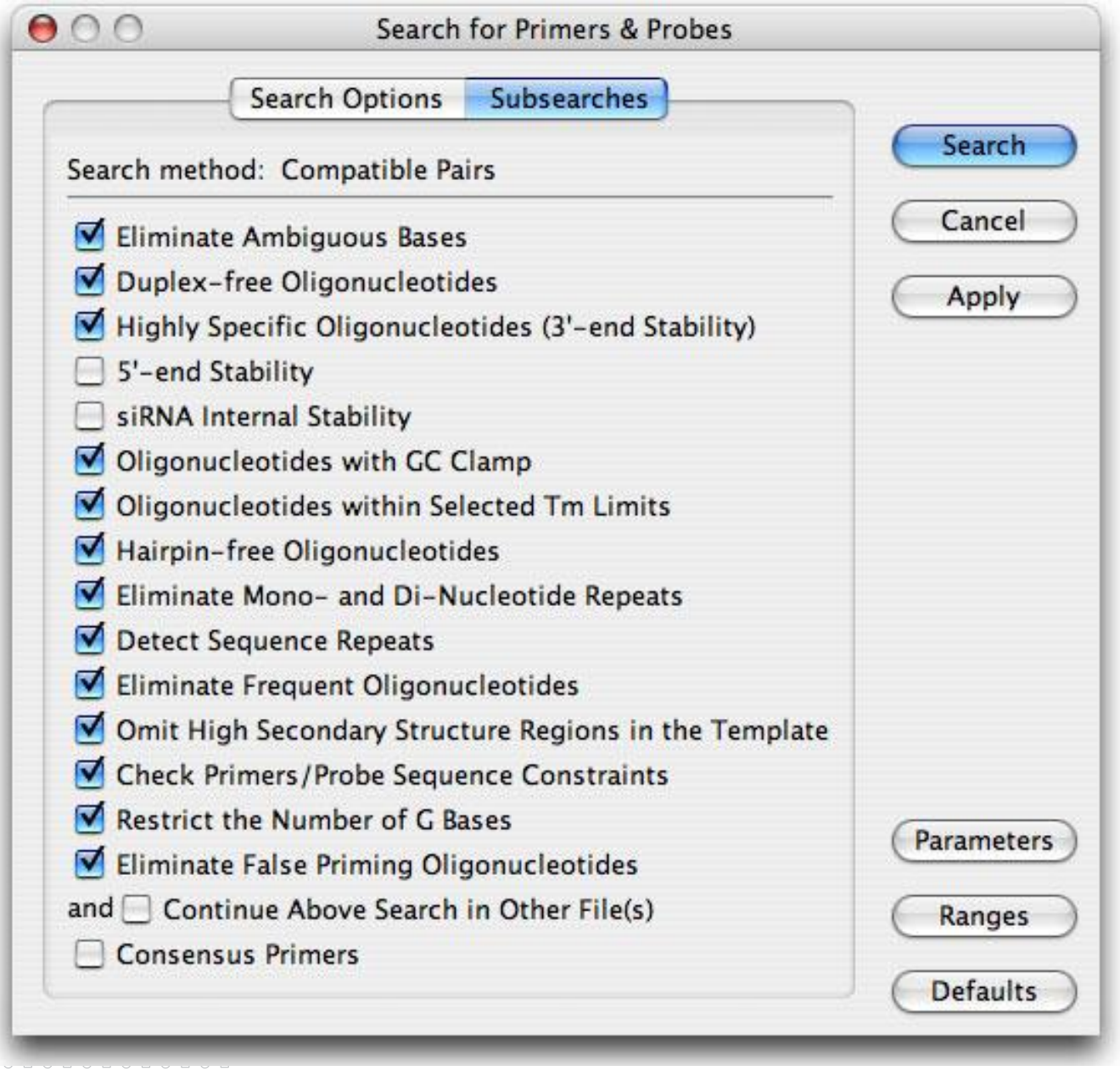
```
   1 ATGGCTTCTG CTCAATCTTT CTACAACCAA AGCTCTGTCT TGAAAATCAA
  51 TGTCATGGTT GTGGACGATG ATCATGTTTT CCTTGATATC ATGTCACGCA
 101 TGCTTCAACA CTCCAAATAC AGAGGTAATT AAATATTATT ATCATATTAT
 151 ATATAATATG TTATTGATTT TTTGTTTGTG ATTTCATTTA GATTTTTATT
 201 TCTATGATTT CTTAGCATGA AATACAATTT TTGGAGAAAC AACTAGCAGT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

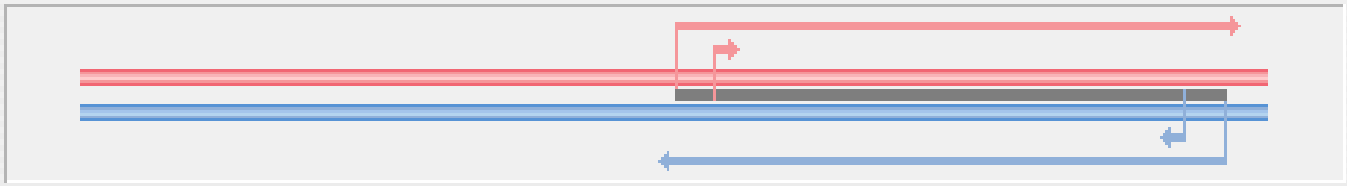

Sequence
File: Human 4E.seq
DNA Sequence
Sequence Length: 1868 nt
Reading Frame: +1
Current Oligo Length: 21 nt
Position: 356
tm: 59.3°C
Selected Oligo | Position | Length
Forward Primer | 259 | 18
Reverse Primer | 328 | 18
Upper Oligo | --- | ---
Lower Oligo | 294 | 22
PCR Product | 87 nt
| Feature | Location
1 | source | -18..1850
2 | CDS | 1..651
pos: 350
tm: 57.1
CCTGGCTGTGACTACTCA
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

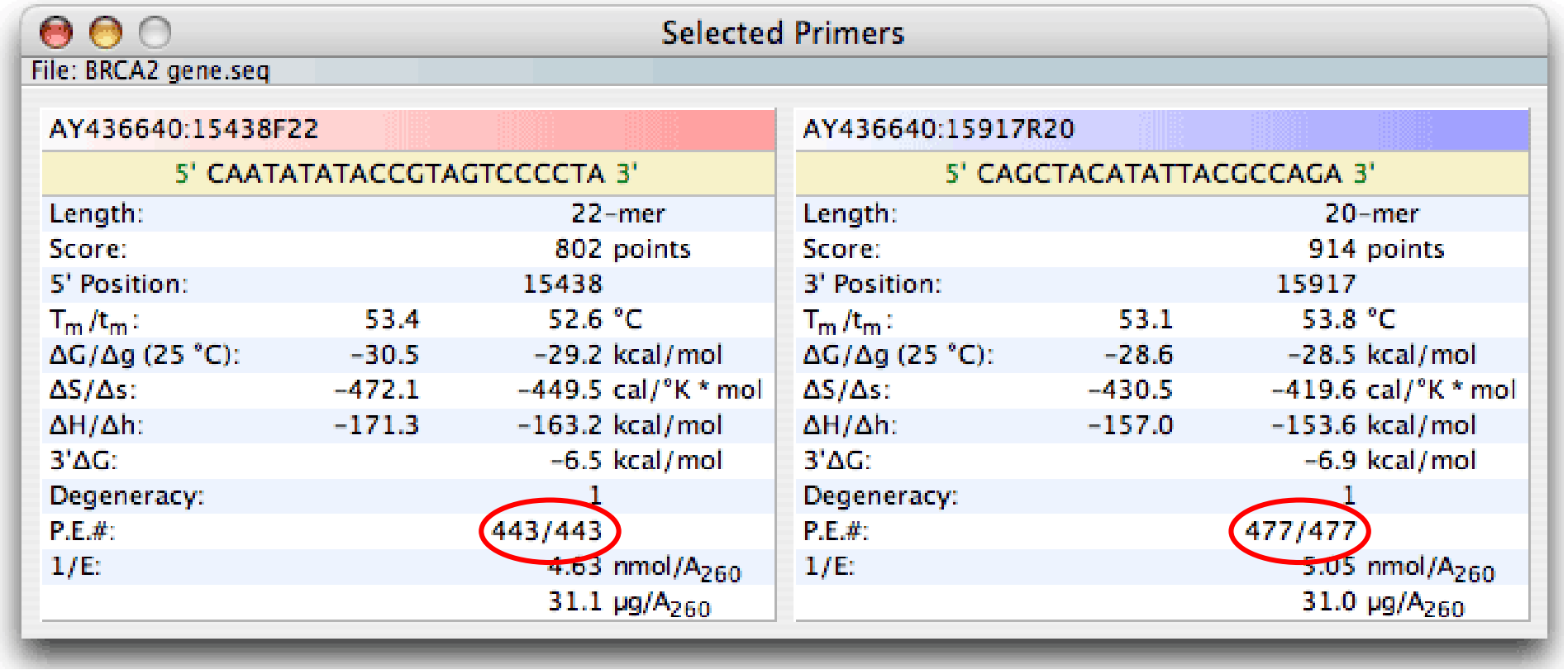
Product T_m – Reverse Primer T_m: 23.6 °C

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

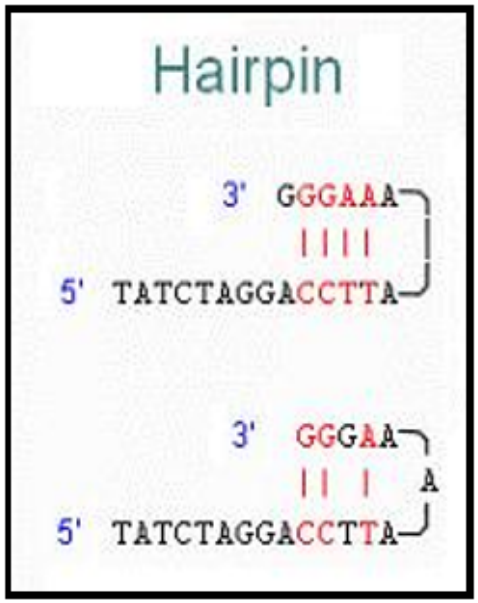
Total Na[+] Equivalent: 155.8 mM



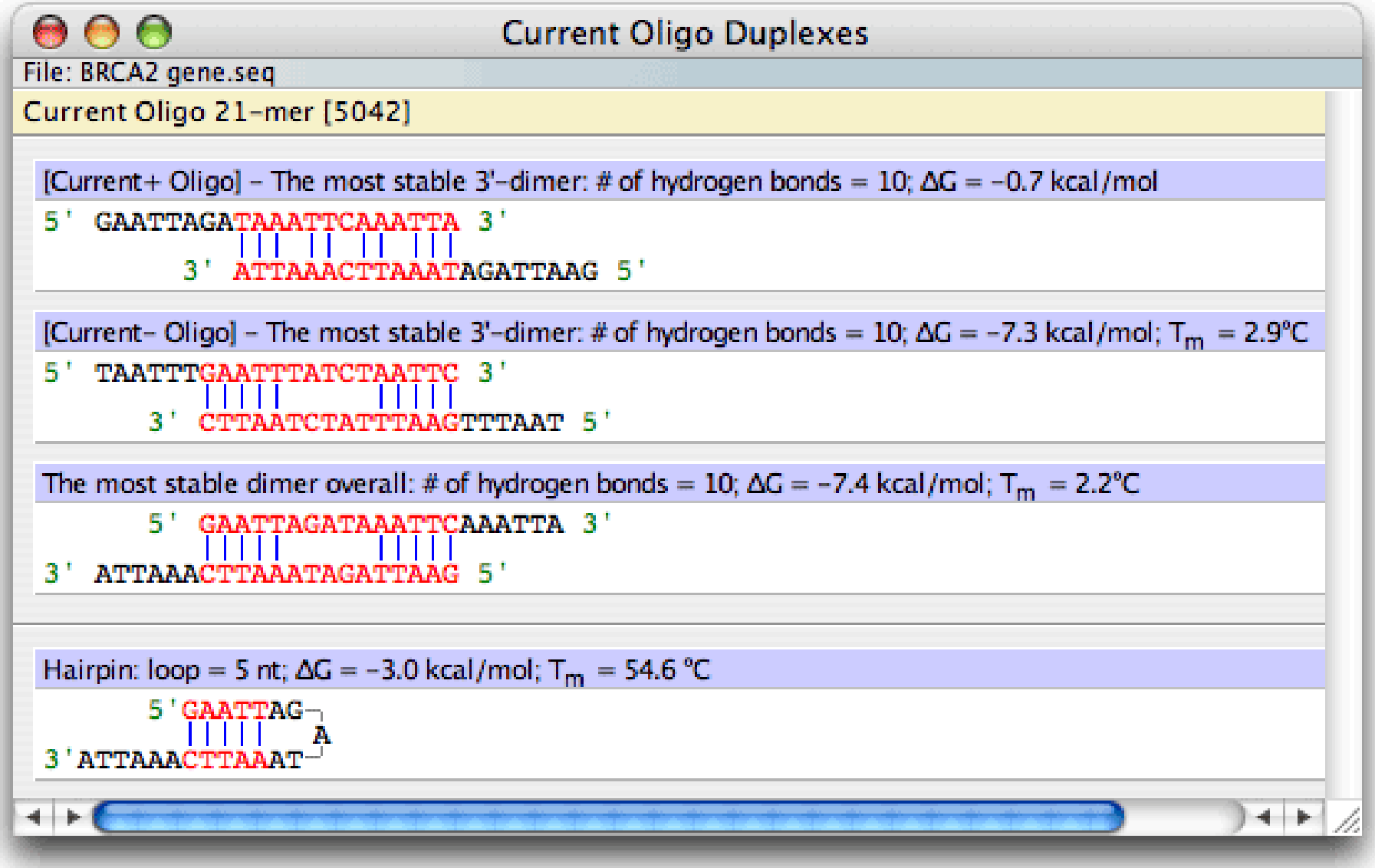
Priming Efficiency PE Score

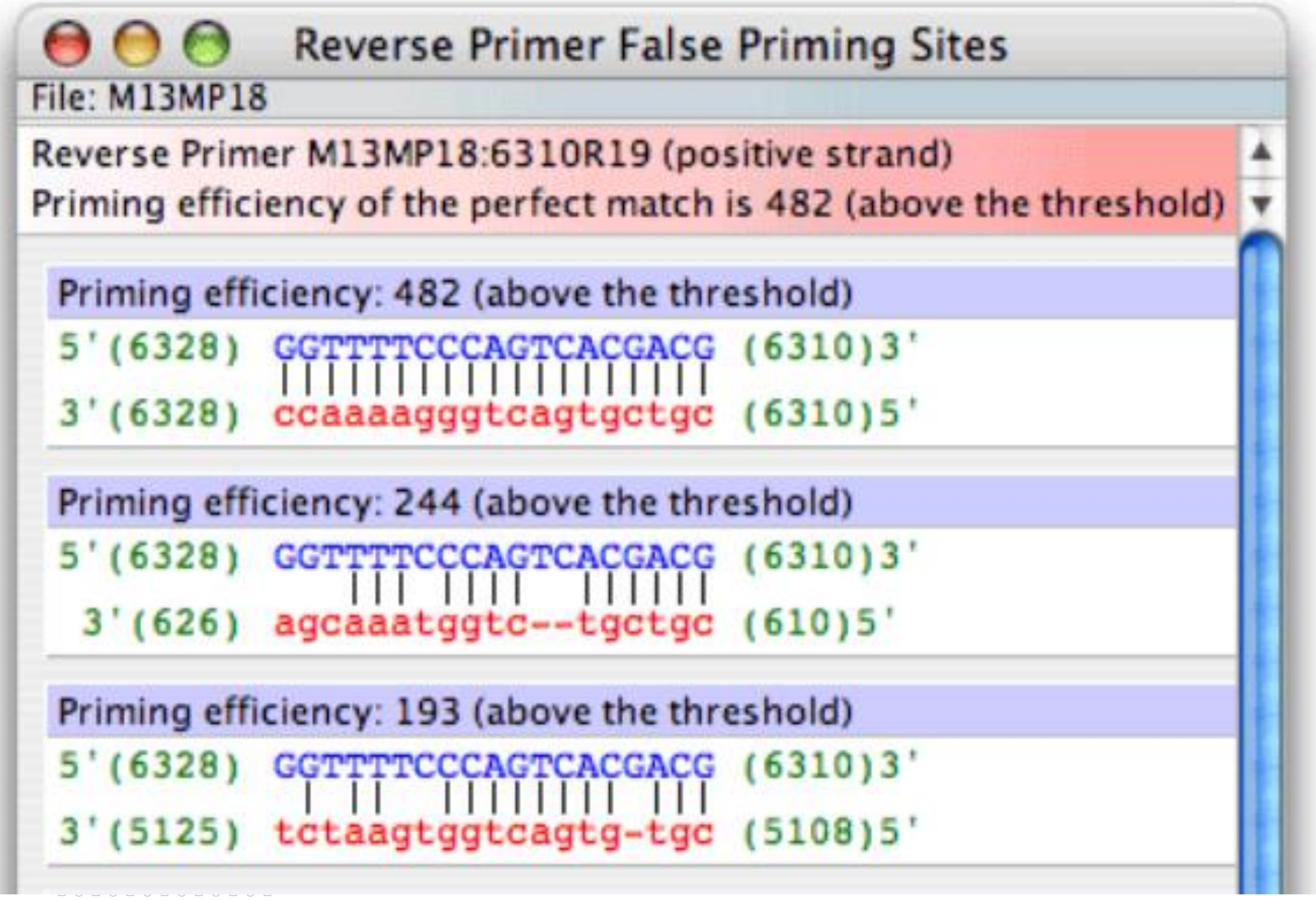
Sekundární struktury

- **HAIRPIN intramolekulární**
- **DIMER intermolekulární**



- **HAIRPIN intramolekulární**
- **DIMER intermolekulární**




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'

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

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

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AHP2ex_up

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AHP2ex_low

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

LITERATURA

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Visual OMP™. http://dnasoftware.com/tabid/108/Default.aspx

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

Marcel Proust

Tato prezentace vznikla s podporou projektu **OP VK** „*Rozvoj týmu pro výuku, výzkum a aplikace v oblasti funkční genomiky a proteomiky*“(CZ.1.07/2.3.00/09.0132)

Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf

MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY

OP Vzdělávání pro konkurenceschopnost 2007-13

UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ