Design Primer & Probe

Course: MICROBIAL GENOME & MICROBIOME ANALYSIS - 2024
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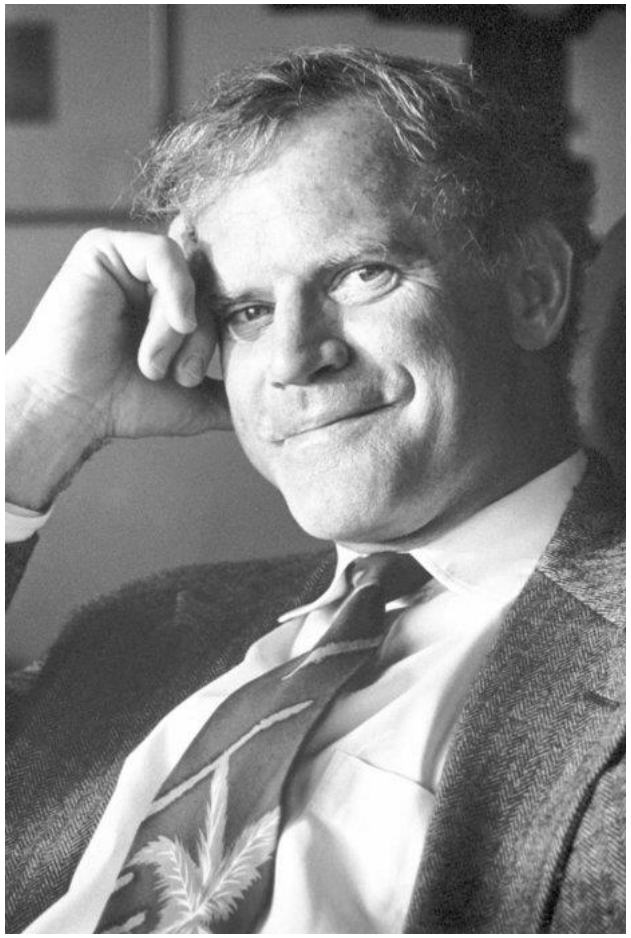
The Nobel Prize in Chemistry 1993



Kary B. Mullis

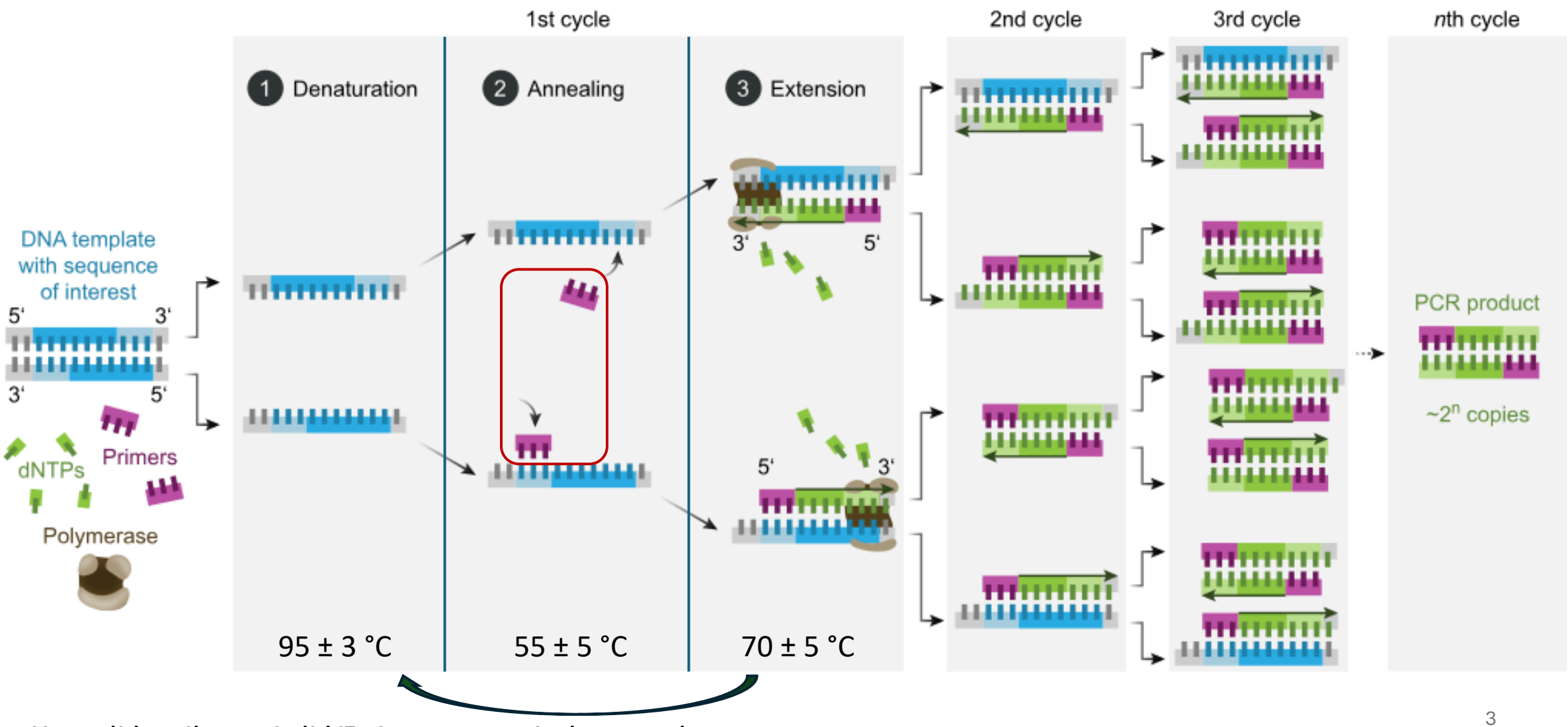
The Nobel Prize in Chemistry 1993
Born: 28 December 1944, Lenoir, NC, USA
Died: 7 August 2019, Newport Beach, CA, USA
Prize motivation: “for his invention of the polymerase chain reaction (PCR) method”
Prize share: ½

Work

An organism's genome is stored inside DNA molecules, but analyzing this genetic information requires quite a large amount of DNA. In 1985, Kary Mullis invented the process known as polymerase chain reaction (PCR), in which a small amount of DNA can be copied in large quantities over a short period of time. By applying heat, the DNA molecule's two strands are separated and the DNA building blocks that have been added are bonded to each strand. With the help of the enzyme DNA polymerase, new DNA chains are formed and the process can then be repeated. PCR has been of major importance in both medical research and forensic science.

https://www.nobelprize.org/

What are primer and probe? Why do we need to design for our own?



https://en.wikipedia.org/wiki/Polymerase_chain_reaction

General Rules for Design of Oligonucleotides

With four possibilities (A, C, G, T) for selection of nucleotides at each position of the sequence, 4^18 (~10^11) possibilities exist for the design of an 18 nucleotide oligonucleotide.

Lengths of PCR Primers and Products

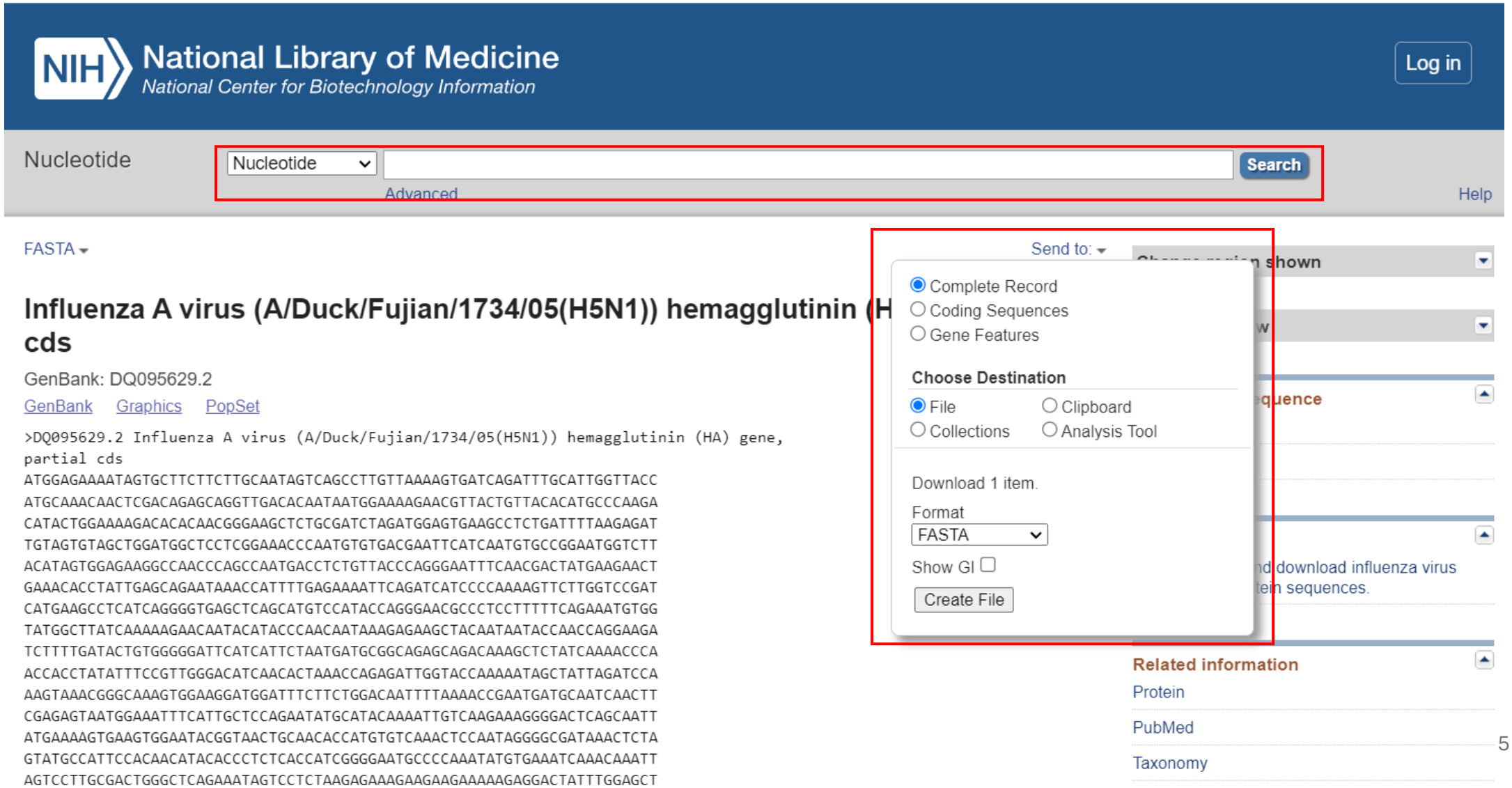
- PCR primers in length: 18 and 24 nt
  - PCR primers of 15 nucleotides or shorter are only used for arbitrary or random short priming in the mapping of simple genomes.
- Melting temperature (Tm): approximately 60 °C(59±2 °C) for qPCR but may vary (55±5 °C) for conventional PCR
- Tm difference between primers: ≤ 5 °C
  - To match the Tm of both forward and reverse primers, the length of a primer with high GC content needs to be shorter compared to one with less GCs.
- Primer pairs' GC content: 40–60% GC
- Product in length: approximately 200 to 1500 bp for conventional PCR and < 200 bp for qPCR
- Primer dimers (hairbin, seft-dimer, hetero-dimer): ΔG ≥ –2.0 kcal
  - Any primer with both a terminal ΔG < –2.0 kcal and an extendable 3'-end (5'-overlap) should be avoided.
  - Avoid 3' clamping (examine the 5 bases of the 3' and accept 3 of these as A or T and 2 as G or C)
  - The strongest overall dimer should be unstable (ΔG ≥ –6.0 kcal)

Lengths of Oligonucleotide Hybridization Probes

- Hybridization probes have been applied with lengths from around 20 nt up to several hundreds of nt.
  - At least one or two nucleotide differences are needed for separation of target from nontarget sequences.
  - Mismatches near the end of probes are less destabilizing than internal mismatches
- For microarray experiments, the usual size is 25 nt, but probes of 50–70 nt are also used on certain applications

Example 1: Design primer for Virus H5N1

- DNA sequence of the hemagglutinin (HA) gene of strain 1734 with serotype H5N1 isolated from duck in Fujian in 2005 with GenBank/EBI/DDBJ acc. no. DQ095629
- https://www.ncbi.nlm.nih.gov/nuccore/



Example 1: Design primer for Virus H5N1

- https://bioinfo.ut.ee/primer3-0.4.0/

Primer3 (v. 0.4.0) Pick primers from a DNA sequence.	Checks for mispriming in template.	disclaimer	Primer3 Home
	Primer3plus interface	cautions	FAQ/WIKI

There is a newer version of Primer3 available at http://primer3.ut.ee

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library): NONE

```
ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGCCTTGTTAAAAGTGATCAGATTTGCATTGGTTACCATGCAAACAACTCGACAGAGCAGGT
TGACACAATAATGGAAAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAGATGGAGTGA
AGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATA
GTGGAGAAGGCCAACCCAGCCAATGACCTCTGTTACCCAGGGAATTTCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTT
TGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTCATCAGGGGTGAGCTCAGCATGTCCATACCAGGGAACGCCCTCCT
TTTTCAGAAATGTGGTATGGCTTATCAAAAAGAACAATACATACCCAACAATAAAGAGAAGCTACAATAATACCAACCAGGAAGATCTTTTGATA
```

Pick left primer, or use left primer below:	Pick hybridization probe (internal oligo), or use oligo below:	Pick right primer, or use right primer below (5' to 3' on opposite strand):

Pick Primers | Reset Form

Sequence Id:		A string to identify your output.	
Targets:		E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.	
Excluded Regions:		E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the source sequence with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.	
Product Size Ranges	100-300		
Number To Return	5	Max 3' Stability	9.0
Max Repeat Mispriming	12.00	Pair Max Repeat Mispriming	24.00
Max Template Mispriming	12.00	Pair Max Template Mispriming	24.00

Pick Primers | Reset Form

General Primer Picking Conditions

Example 1: Design primer for Virus H5N1

Primer3 Output

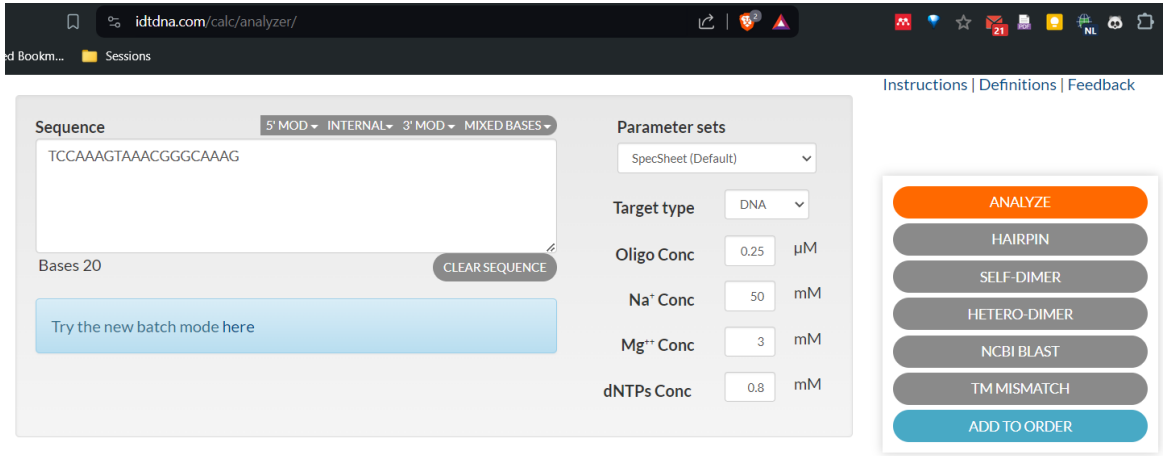
```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%    any    3'  seq
LEFT PRIMER        697   20   60.10    45.00   2.00  0.00 TCCAAAGTAAACGGGCAAAG
RIGHT PRIMER       899   20   59.93    50.00   4.00  3.00 GCCCCTATTGGAGTTTGACA
SEQUENCE SIZE: 1698
INCLUDED REGION SIZE: 1698

PRODUCT SIZE: 203, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

    1 ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGCCTTGTTAAAAGTGATCAGATTTGC

   61 ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGAAAAGAACGTT

  121 ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA

  181 GATGGAGTGAAGCCTCTGATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGAAAC

  241 CCAATGTGTGACGAATTCATCAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAAC

  301 CCAGCCAATGACCTCTGTTACCCAGGGAATTTCAACGACTATGAAGAACTGAAACACCTA

  361 TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTCTTGGTCCGAT

  421 CATGAAGCCTCATCAGGGGTGAGCTCAGCATGTCCATACCAGGGAACGCCCTCCTTTTTC

  481 AGAAATGTGGTATGGCTTATCAAAAAGAACAATACATACCCAACAATAAAGAGAAGCTAC

  541 AATAATACCAACCAGGAAGATCTTTTGATACTGTGGGGGATTCATCATTCTAATGATGCG

 1501 ACGTATGACTACCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGTGGA

 1561 GTAAAATTGGAATCAATAGGAACTTACCAAATACTGTCAATTTATTCAACAGTGGCGAGT

 1621 TCTCTAGCACTGGCAATCATGGTGGCTGGTCTATCTTTATGGATGTGCTCCAATGGGTCG

 1681 TTACAATGCAGAATTGCA

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                    start  len      tm      gc%    any    3'  seq

 1 LEFT PRIMER        82   20   60.03    55.00   4.00  1.00 TCGACAGAGCAGGTTGACAC
   RIGHT PRIMER      234   20   59.83    60.00   4.00  0.00 GAGGAGCCATCCAGCTACAC
   PRODUCT SIZE: 153, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

 2 LEFT PRIMER        82   20   60.03    55.00   4.00  1.00 TCGACAGAGCAGGTTGACAC
   RIGHT PRIMER      282   20   59.82    50.00   4.00  2.00 GTAAGACCATTCCGGCACAT
   PRODUCT SIZE: 201, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

 3 LEFT PRIMER        572   20   60.13    45.00   4.00  2.00 TGTGGGGGATTCATCATTCT
   RIGHT PRIMER      716   20   60.10    45.00   2.00  0.00 CTTTGCCCGTTTACTTTGGA
   PRODUCT SIZE: 145, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

 4 LEFT PRIMER        150   20   60.16    50.00   2.00  2.00 AAAGACACACAACGGGAAGC
   RIGHT PRIMER      414   20   59.90    45.00   7.00  1.00 CCAAGAACTTTTGGGGATGA
   PRODUCT SIZE: 265, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

Statistics
        con   too    in    in          no    tm    tm  high  high        high
        sid  many   tar  excl   bad    GC   too   too   any    3'  poly   end
       ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab     ok
Left  14722     0     0     0    44     0  6567  3702     1    10     0   186   4212
Right 14582     0     0     0    44     0  6424  3736     0     4     0   202   4172
Pair Stats:
considered 790, unacceptable product size 772, ok 18
primer3 release 1.1.4
```

Example 1: Design primer for Virus H5N1

- https://www.idtdna.com/calc/analyzer/

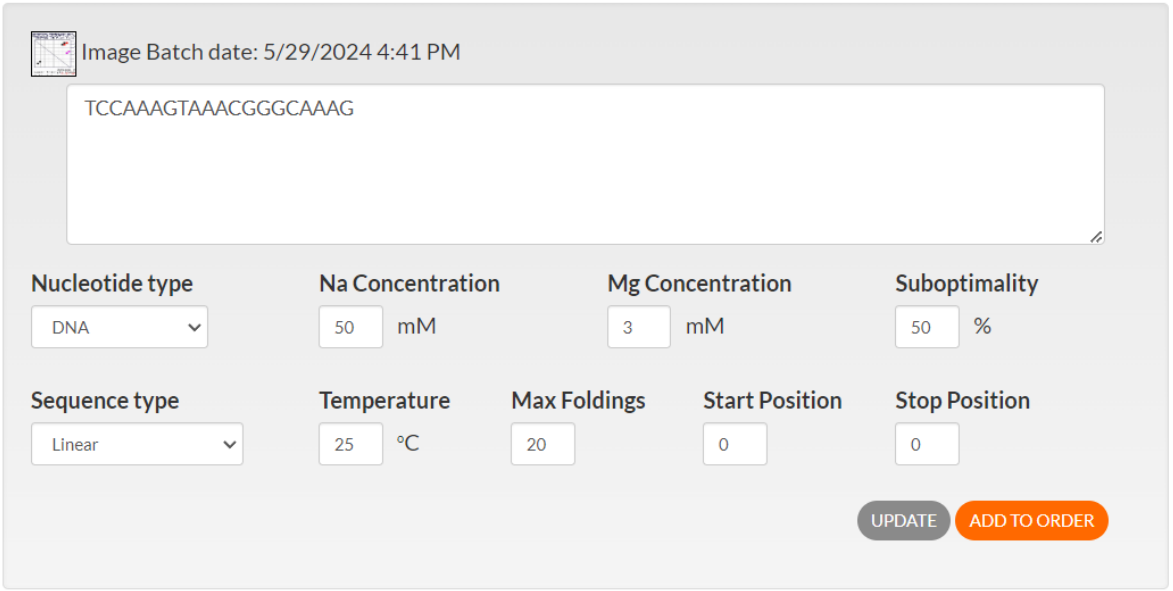
Left Primer – TCCAAAGTAAACGGGCAAAG

Hairpin



Homo-Dimer Analysis

The delta G is calculated by taking into account the longest stretch of complementary bases. These pairs of complementary bases are represented by a solid line. Dotted lines represent additional complementary bases for that dimer structure, but their presence does not impact calculated delta G values. Actual delta G values may vary based on presence of additional complementary bases. The Maximum Delta G value refers to the free energy of the oligo sequence binding to its perfect complement.



Structures

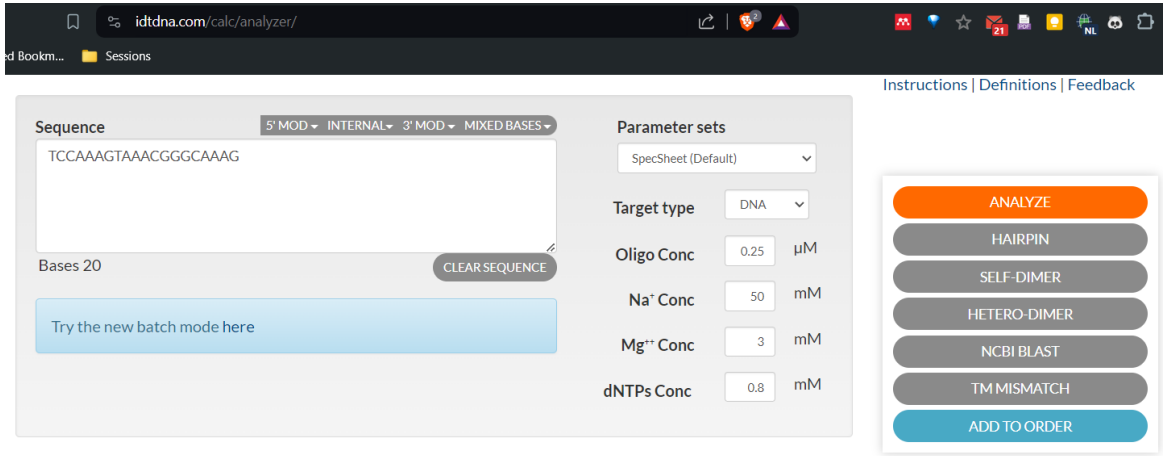
structure	Image	ΔG (kcal.mole^{-1})	T_m (°C)	ΔH (kcal.mole^{-1})	ΔS (cal.K^{-1}mole^{-1})	Output
1		0.24	21	-17.9	-60.86	Ct Det
2		0.45	16.3	-15.2	-52.5	Ct Det
3		1.23	0.9	-14	-51.09	Ct Det

Example 1: Design primer for Virus H5N1

- https://www.idtdna.com/calc/analyzer/

Left Primer – TCCAAAGTAAACGGGCAAAG

Seft-Dimer



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Delta G: -3.61 kcal/mol **Base Pairs:** 2

```
5' TCCAAAGTAAACGGGCAAAG
          :    ||    :
3'      GAAACGGGCAAATGAAACCT
```

Delta G: -3.14 kcal/mol **Base Pairs:** 2

```
5' TCCAAAGTAAACGGGCAAAG
                ||
3'           GAAACGGGCAAATGAAACCT
```

Delta G: -3.07 kcal/mol **Base Pairs:** 2

```
5'       TCCAAAGTAAACGGGCAAAG
          ||          ::
3' GAAACGGGCAAATGAAACCT
```

Delta G: -3.07 kcal/mol **Base Pairs:** 2

```
5'      TCCAAAGTAAACGGGCAAAG
         ||     ::     ::
3' GAAACGGGCAAATGAAACCT
```

Delta G: -1.34 kcal/mol **Base Pairs:** 2

```
5'   TCCAAAGTAAACGGGCAAAG
      :      ||  ::      :
3' GAAACGGGCAAATGAAACCT
```

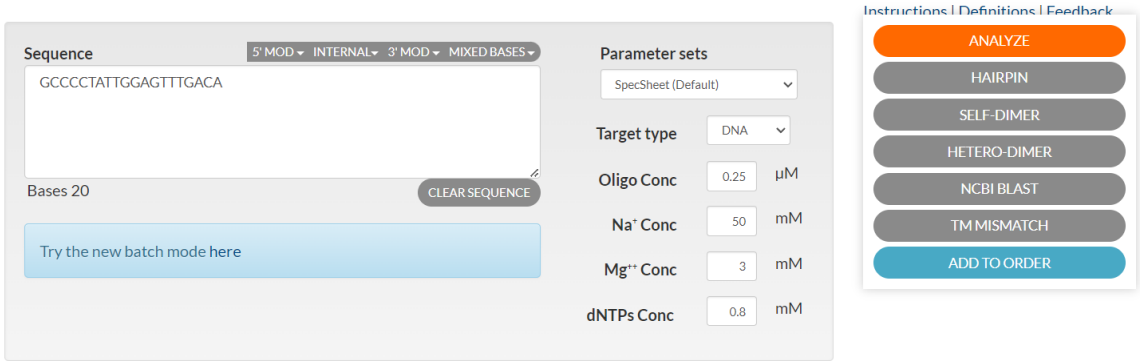
Example 1: Design primer for Virus H5N1

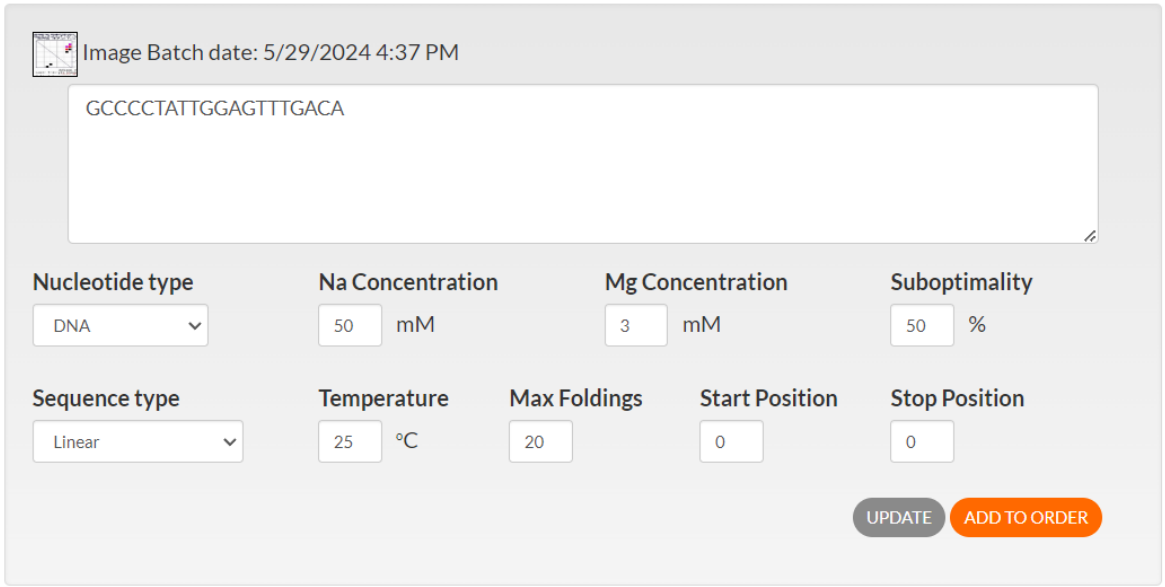
- https://www.idtdna.com/calc/analyzer/

Right Primer – GCCCCTATTGGAGTTTGACA

Hairpin

OligoAnalyzer





Hetero-Dimer Analysis

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Structures

structure	Image	ΔG (kcal.mole^{-1})	T_m (°C)	ΔH (kcal.mole^{-1})	ΔS (cal.K^{-1}mole^{-1})	Output
1		-1.1	42.5	-19.8	-62.73	Ct Det
2		-0.99	43.5	-16.9	-53.38	Ct Det
3		-0.53	34.3	-17.6	-57.24	Ct Det

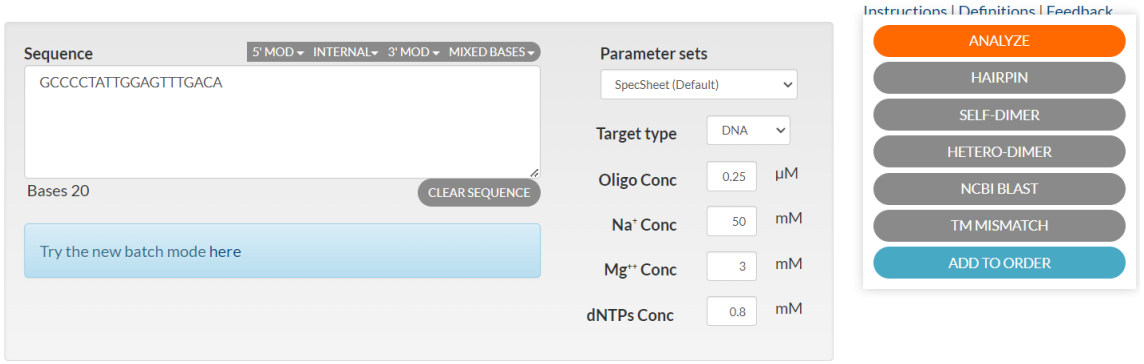
Example 1: Design primer for Virus H5N1

- https://www.idtdna.com/calc/analyzer/

Right Primer – GCCCCTATTGGAGTTTGACA

Seft-Dimer

OligoAnalyzer



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Delta G: -3.14 kcal/mol **Base Pairs:** 2

```
5'                  GCCCCTATTGGAGTTTGACA
                    ||
3' ACAGTTTGAGGTTATCCCCG
```

Delta G: -1.95 kcal/mol **Base Pairs:** 2

```
5' GCCCCTATTGGAGTTTGACA
          ||        ::
3'        ACAGTTTGAGGTTATCCCCG
```

Delta G: -3.07 kcal/mol **Base Pairs:** 2

```
5'         GCCCCTATTGGAGTTTGACA
           ||  ::  ::
3' ACAGTTTGAGGTTATCCCCG
```

Delta G: -1.95 kcal/mol **Base Pairs:** 2

```
5' GCCCCTATTGGAGTTTGACA
                ||  ::
3'              ACAGTTTGAGGTTATCCCCG
```

Delta G: -3.07 kcal/mol **Base Pairs:** 2

```
5'        GCCCCTATTGGAGTTTGACA
          ||     ::
3' ACAGTTTGAGGTTATCCCCG
```

Delta G: -1.6 kcal/mol **Base Pairs:** 2

```
5'    GCCCCTATTGGAGTTTGACA
        ||     ::
3' ACAGTTTGAGGTTATCCCCG
```

Delta G: -3.07 kcal/mol **Base Pairs:** 2

```
5'       GCCCCTATTGGAGTTTGACA
         : || :: :: :
3' ACAGTTTGAGGTTATCCCCG
```

Delta G: -1.34 kcal/mol **Base Pairs:** 2

```
5' GCCCCTATTGGAGTTTGACA
             ||   ::
3'           ACAGTTTGAGGTTATCCCCG
```

Example 1: Design primer for Virus H5N1

- https://www.idtdna.com/calc/analyzer/

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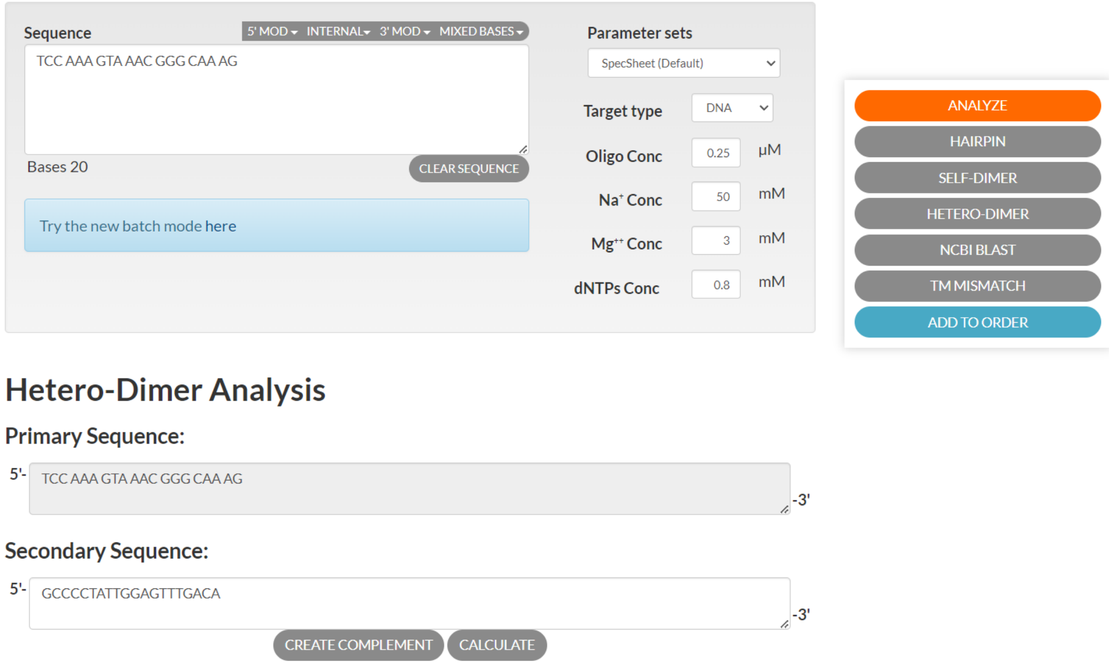
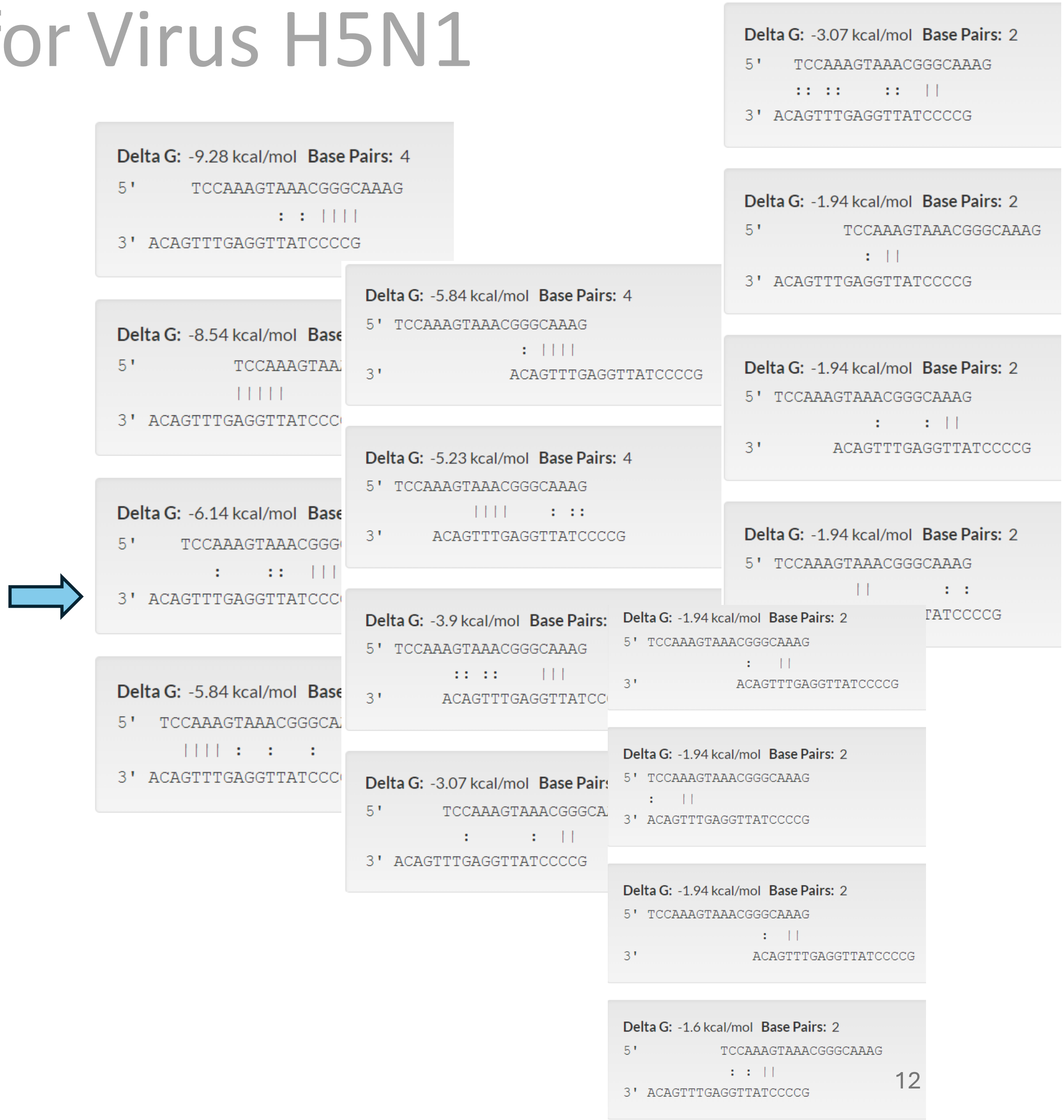
Right Primer – GCCCCTATTGGAGTTTGACA
Hetero-Dimer





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  - Avoid 3' clamping (examine the 5 bases of the 3' and accept 3 of these as A or T and 2 as G or C)
  - The strongest overall dimer should be unstable (ΔG ≥ –6.0 kcal)

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- Hybridization probes have been applied with lengths from around 20 nt up to several hundreds of nt.
  - At least one or two nucleotide differences are needed for separation of target from nontarget sequences.
  - Mismatches near the end of probes are less destabilizing than internal mismatches
- For microarray experiments, the usual size is 25 nt, but probes of 50–70 nt are also used on certain applications

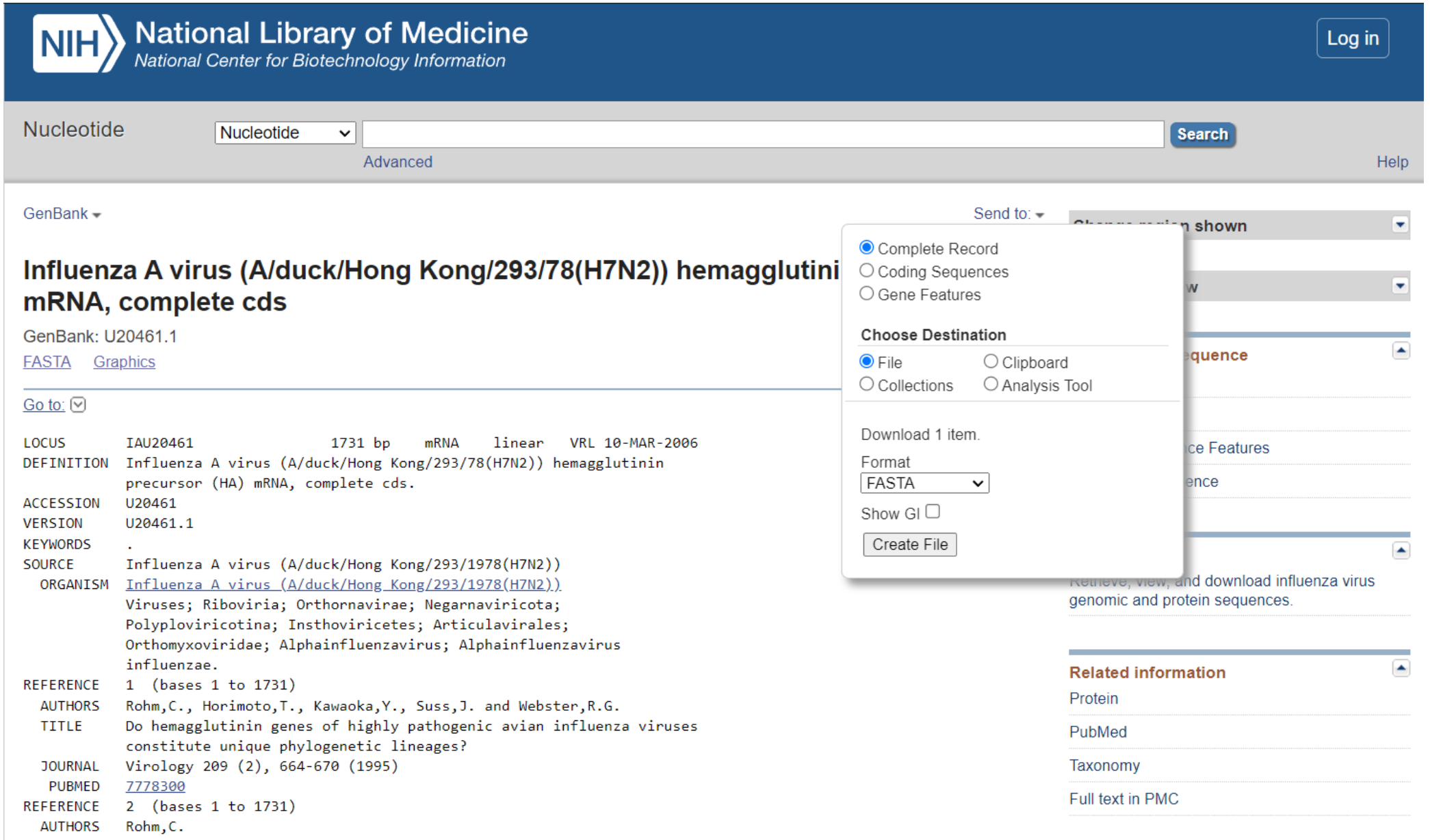
Homework 1

1. Between global and local alignments, which algorithm do we use for designing primer/probe?
2. Is the primer pair appropriate for the hemagglutinin (HA) gene of strain 1734 with serotype H5N1 and Why?

Based on '**General Rules for Design of Oligonucleotides**' to answer the question 2.

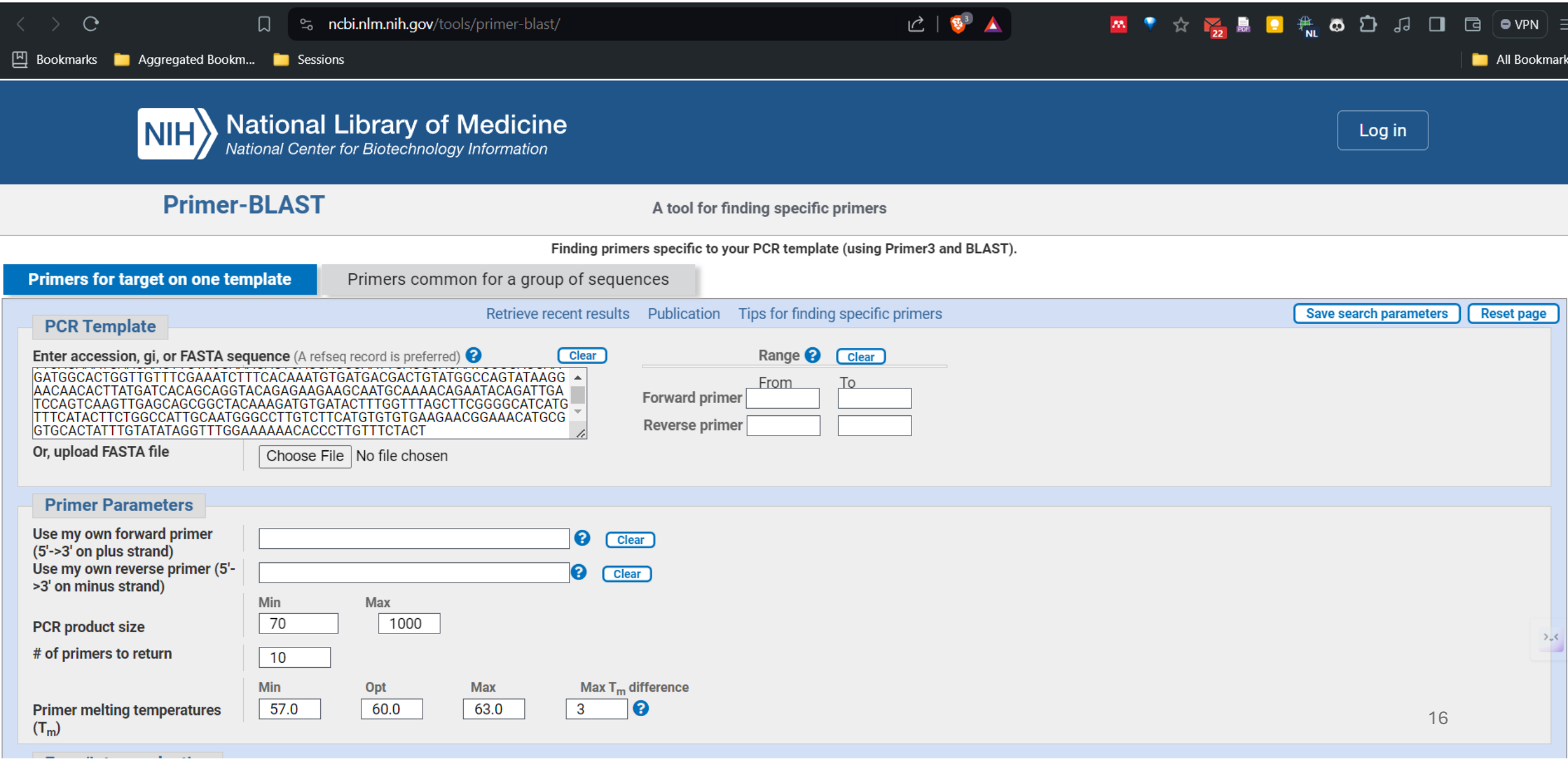
Example 2: Design primer for Virus H7N2

The serotype H7N2 of influenza virus: acc. no. U20461



Example 2: Design primer for Virus H7N2

https://www.ncbi.nlm.nih.gov/tools/primer-blast/



Example 2: Design primer for Virus H7N2

Note: Parameter values that differ from the default are highlighted in yellow

Primer Pair Specificity Checking Parameters

Specificity check — Enable search for primer pairs specific to the intended PCR template

Search mode — User guided

Database — nr

Exclusion — Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences

Organism — Influenzavirus A [Add organism]

Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type.

Entrez query (optional)

Primer specificity stringency

Primer must have at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end.

Ignore targets that have 6 or more mismatches to the primer.

Max target amplicon size — 4000

Allow splice variants — Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)

Get Primers

Show results in a new window Use new graphic view

Example 2: Design primer for Virus H7N2

Primer-BLAST — A tool for finding specific primers

Finding primers specific to your PCR template (using Primer3 and BLAST)

Input PCR template lcl|Query_1

Range 1 - 1731

Your PCR template is highly similar to the following sequence(s) from the search database. To increase the chance of finding specific primers, please review the list below and select all sequences (within the given sequence ranges) that are intended or allowed targets.

Select: All None Selected:9

Accession	Title	Identity	Alignment length	Seq. start	Seq. stop
☐ U20461.1	Influenza A virus (A/duck/Hong Kong/293/78(H7N2)) hemagglutinin precursor (HA) mRNA, complete cds	100%	1731	1	1731
☑ CY006029.1	Influenza A virus (A/dk/Hong Kong/293/1978(H7N2)) segment 4, complete sequence	99.94%	1731	2	1732
☑ CY067686.1	Influenza A virus (A/swine/KU/16/2001(H7N2)) segment 4 sequence	99.36%	1731	2	1732
☑ AB302789.1	Influenza A virus (A/duck/Hong Kong/301/1978(H7N2)) HA gene for haemagglutinin, complete cds	99.82%	1698	1	1698
☑ KX130802.1	Influenza A virus (A/twite/Gangcha/02/2006(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.76%	1683	1	1683
☑ KX130803.1	Influenza A virus (A/tree sparrow/Gangcha/02/2006(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.7%	1683	1	1683
☑ KX130801.1	Influenza A virus (A/twite/Gangcha/01/2006(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.7%	1683	1	1683
☑ KX130820.1	Influenza A virus (A/pika/Maduo/01/2009(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.64%	1683	1	1683
☑ KX130811.1	Influenza A virus (A/pika/QH-Maduo/02/2006(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.64%	1683	1	1683
☑ KX130809.1	Influenza A virus (A/ground jay/Maduo/01/2006(H7N2)) segment 4 hemagglutinin (HA) gene, complete cds	99.64%	1683	1	1683
☐ DQ003216.1	Influenza A virus (A/duck/Hongkong/301/72(H7N1)) hemagglutinin (HA) gene, complete cds	99.58%	1682	1	1682

Submit ☐ Show results in a new window

Example 2: Design primer for Virus H7N2

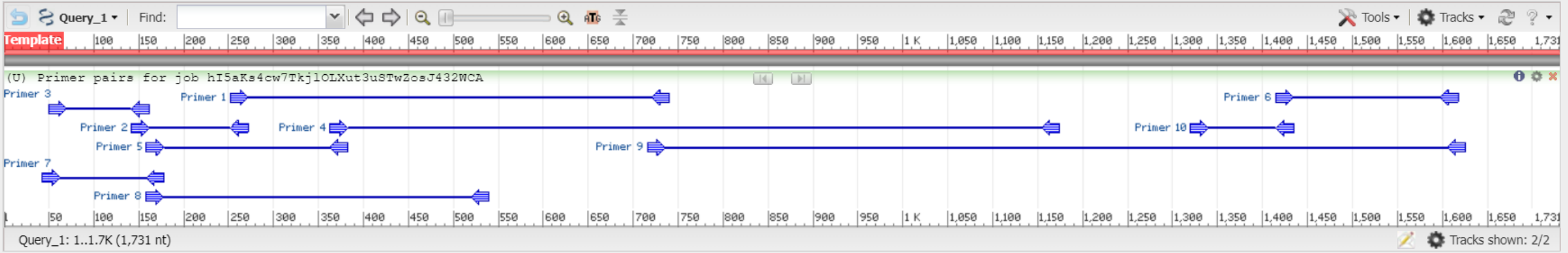
Input PCR template lcl|Query_1

Range 1 - 1731

Specificity of primers Primers may **not** be specific to the input PCR template as targets were found in selected database:Nucleotide collection (nt) (Organism limited to Alphainfluenzavirus)...help on specific primers

Other reports ▷Search Summary

— Graphical view of primer pairs



— Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers Submit

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTCACTGGACCACCTCAGTG	Plus	20	252	271	59.97	60.00	5.00	3.00
Reverse primer	ATCGATCCGTCCAGATTGGC	Minus	20	740	721	59.97	55.00	6.00	2.00
Product length	489								

Homework 2

1. Are all primers of ‘Products of intended targets’ for Primer pair 1 matching the type H7N2?
2. Are all ‘Products of unintended targets’ forming mismatches for Primer pair 1 that are expected not to result in generation of product?

Based on ‘**General Rules for Design of Oligonucleotides’** to answer this question.

Reference

- *https://www.sigmaaldrich.com/NL/en/technical-documents/technical-article/genomics/pcr/assay-optimization-and-validation*
- *https://www.sigmaaldrich.com/NL/en/technical-documents/protocol/genomics/pcr/pcr-qpcr-dpcr-assay-design*
- https://www.idtdna.com/pages/education/decoded/article/designing-pcr-primers-and-probes
- https://www.idtdna.com/pages/education/decoded/article/design-efficient-pcr-and-qpcr-primers-and-probes-using-online-tools
- Christensen, Henrik, and John Elmerdahl Olsen. "Primer Design: Design of Oligonucleotide PCR Primers and Hybridization Probes." *Introduction to Bioinformatics in Microbiology* (2018): 81-102.
- Nolan, Tania, Jim Huggett, and Elena Sanchez. "Good practice guide for the application of quantitative PCR (qPCR)." Teddington: LGC (2013).

Tm Calculations

- Estimation of Tm by Formula
  - For oligonucleotides of ≤ 20 nt: Tm = 4 (G + C + 2(A–T)
  - For oligonucleotides of 10–50 nt: Tm = 81.5 + 16.6logM + 0.41(G + C%) – (820/(length of probe)
  - For oligonucleotides of ≥ 50 nt: Tm = 81.5°C + 16.6logM + 0.41(G + C%) – (500/length of probe)

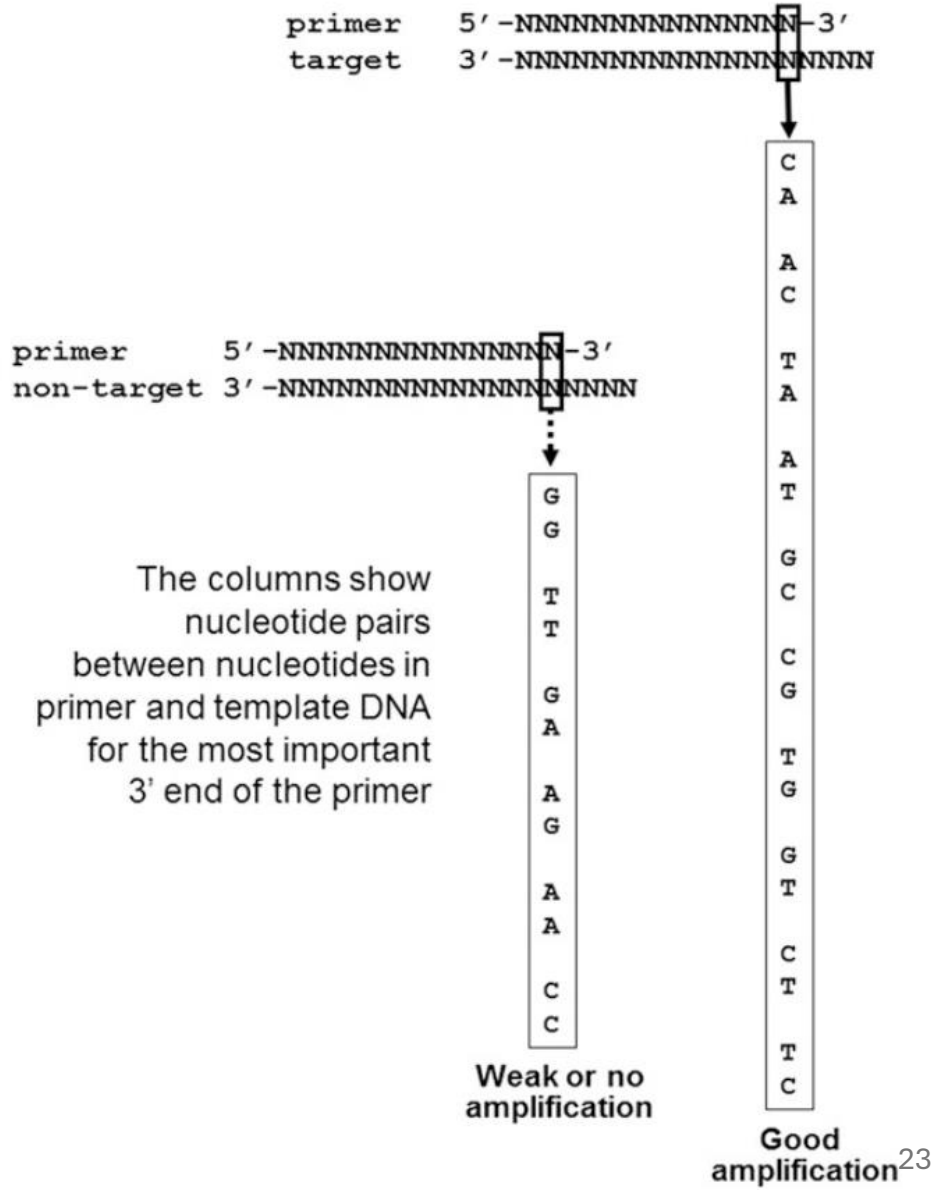
- Formamide Considerations
  - 1% formamide reduces Tm by 0.6°C

- Estimation of Tm by Nearest Neighbor Prediction
  - Td = ΔH/(ΔS + RlnC) + 16.6log10 [M]

- Combination of Formula and nearest neighbor calculations
  - Ta = 0.3 Tm primer +0.7 Tm product – 14.9

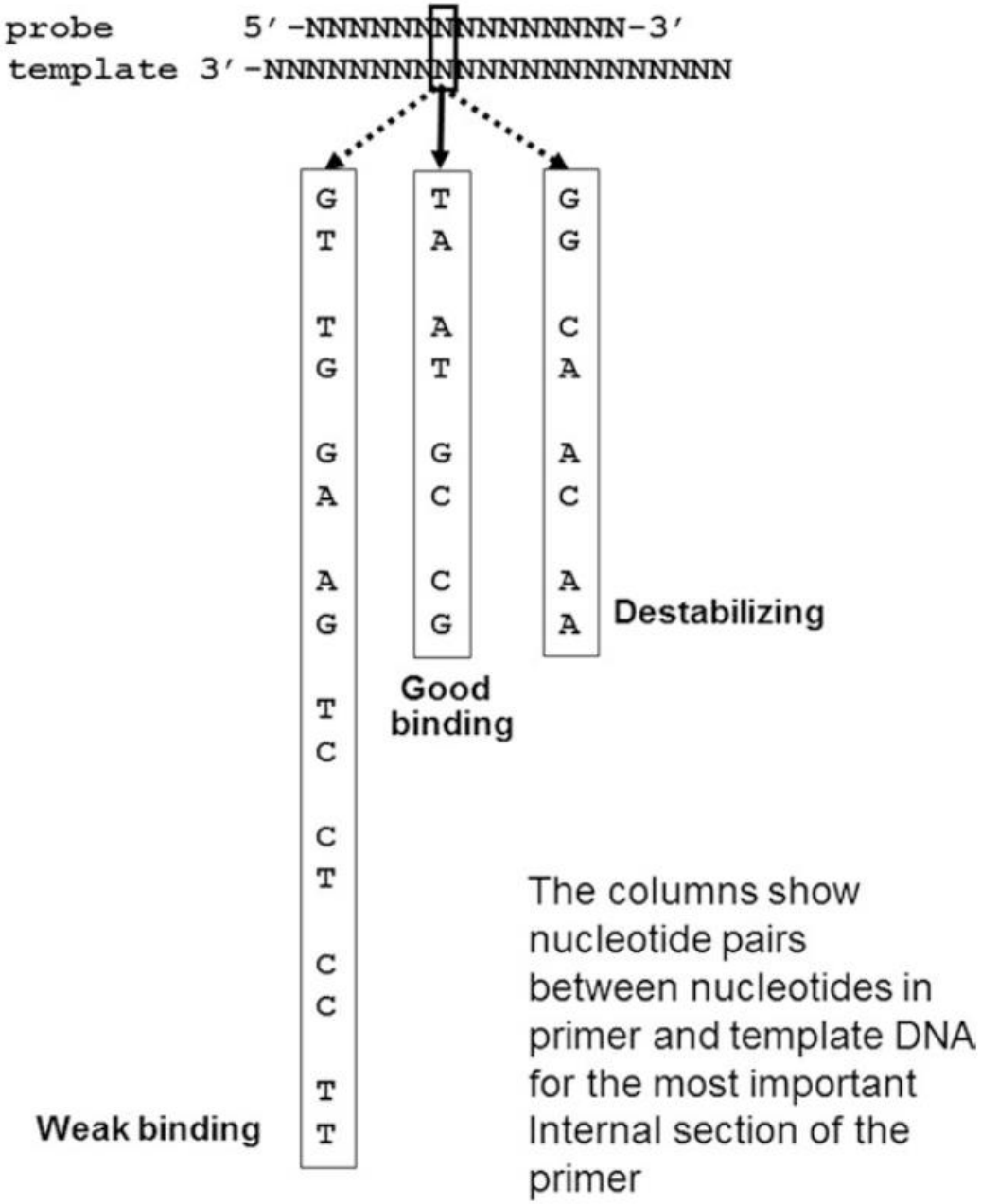
Design of Primers for PCR and “Kwok’s Rules”

Prediction of amplication from interactions between PCR oligonucleotide primer at the 3 end and template (based on Kwok et al. 1990). Low degree of mismatch is assumed between oligonucleotide and template for the remaining part of sequence



Design of Probes for Hybridization

Prediction of binding in hybridization between probe and template. A low degree of mismatch formation is assumed between oligonucleotide and template for the remaining part of the sequence



Program	Description	URL and reference
Amplifx2.1	PCR design program for MacOS X	https://macdownload.informer.com/amplifx/
FastPCR	PCR primer design including multiplex PCR	http://primerdigital.com/fastpcr.html
Netprimer	Numerous parameters of single PCR primers	http://www.premierbiosoft.com/netprimer/index.html
Oligo	Nearest neighbor calculations of secondary structures and Tm	https://www.oligo.net/ Rychlik and Rhoads (1989)
OligoAnalyzer	Nearest neighbor parameters. Hair-pin and primer-dimer analysis	https://eu.idtdna.com/pages/tools Owczarzy et al. (2008)
OligoCalc	Physical properties of oligonucleotides, self complementarity and hairpin loop formation	http://biotools.nubic.northwestern.edu/OligoCalc.html Kibbe (2007)
OligoFaktory	DNA microarrays, primers for PCR, siRNAs	http://www.bioinformatics.org/oligofaktory/
Pride and Genome pride	PCR and microarray	http://pride.molgen.mpg.de/ (Staden package)
Pria	Search for primers in multiple alignments	https://services.birc.au.dk/pria/ Fredslund et al. (2005)
Primegens	Primer design	http://primegens.org/ Xu et al. (2002)
Primer3	The most frequently used program see also Activity 5.1	http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm Rozen and Skaletsky (2000)
Primer3Plus	Simpliaed version of Primer3	http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/
Primer Design Assistant (PDA)	Primer design with all parameters	http://dbb.nhri.org.tw/primer/ Chen et al. (2003)
PrimerPremier	PCR primer design, multiplex, degeneracies and nested PCRs	http://www.premierbiosoft.com/primerdesign/index.html
PrimerQuest	PCR and hybridization probes. Based on Primer3	https://eu.idtdna.com/PrimerQuest/Home/Index
Primer Search (EMBOSS)	Matches between primers-pairs and DNA template by string comparisons	http://emboss.sourceforge.net/ Rice et al. (2000)
Primo Oligo	Calculation of Tm	http://www.changbioscience.com/primo/oligo.html
Web Primer	PCR primer design	https://www.yeastgenome.org/cgi-bin/web-primer
SciTools	A series of tools	http://www.idtdna.com/SciTools/Scitools.aspx Owczarzy et al. (2008)

Program	Description	URL and reference
ARB	Design of rRNA targeted probes	http://www.arb-home.de Ludwig et al. (2004).
ArrayDesigner	Oligo-arrays, cDNA arrays and SNP arrays	http://www.premierbiosoft.com/dnamicroarray/index.html
Assembly PCR oligo maker	PCR-based construction of long DNA molecules for RNA molecules by T7 RNA polymerase	http://www.yorku.ca/pjohnson/AssemblyPCRoligomaker.html
Beacon designer™	Real-time PCR	http://www.premierbiosoft.com/molecular_beacons/index.html
BLOCKMAKER and CODEHOP	Degenerate primers to genes of proteins	https://4virology.net/virology-ca-tools/j-codehop/ Rose et al. (2003)
Expeditor	QTL design	https://www.animalgenome.org/cgi-bin/expeditor/expeditor2
Geneasher2	Degenerate PCR primers based on multiple aligned sequences	https://bibiserv.cebitec.uni-bielefeld.de/geneasher2/
MFOLD	Evaluates probes	http://www.unafold.org/mfold/applications/rna-folding-form-v2.php
OligoPicker	microarray design of oligonucleotides	https://pga.mgh.harvard.edu/oligopicker/index.html
Pira-PCR	SNP	http://primer1.soton.ac.uk/primer2.html Ke et al. (2001)
PrimerD	Degenerate primer pairs	http://mblab.wustl.edu/software.html#primerdLink
Primer exmplore	LAMP primers	http://primerexplorer.jp/e/
PrimerX	Site-directed mutagenesis	http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi
ProDesign	Oligonucleotide design for microarray	http://wwwlabs.uhnresearch.ca/tillier/ProDesign/ProDesign.html Feng and Tillier (2007)