Oligomers design (primers and probes)

Bioinformatics teachings

http://bioinfomed.fr - Olivier Croce

Summary

* Use of PCR - Concept of primers and/or probes

* Sensibility, specificity and other thermodynamic constraints

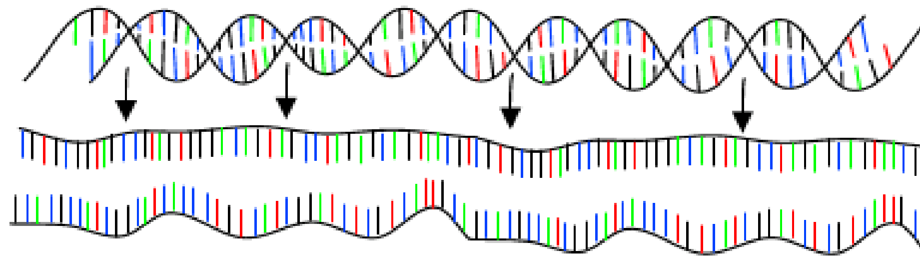
* Examples of softwares used for oligomers design.

* Exercise : Searching of primers/probes for *Bacillus anthracis*, cya

Use of PCR - Concept of primers and/or probes

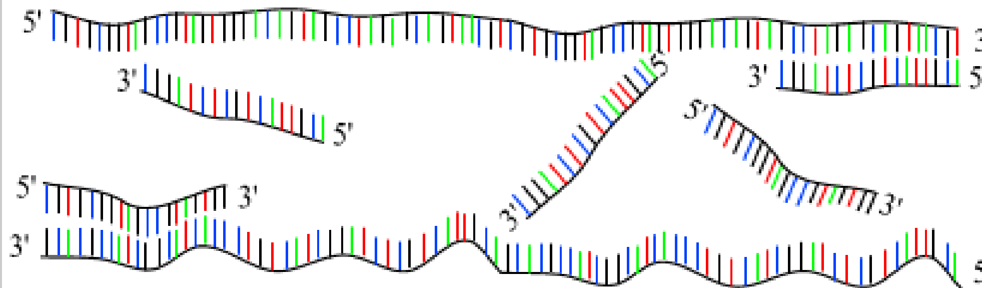
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

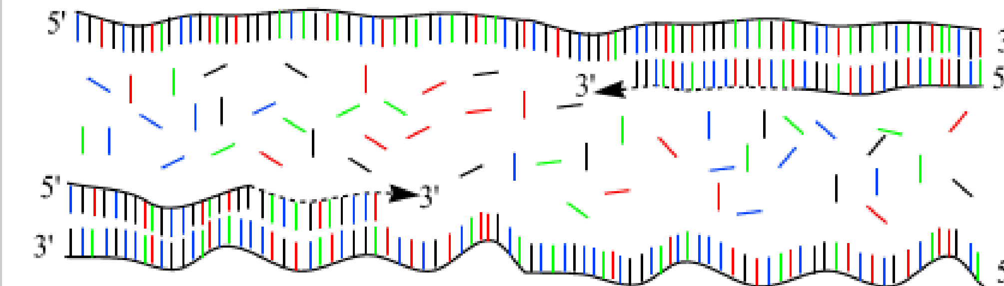
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



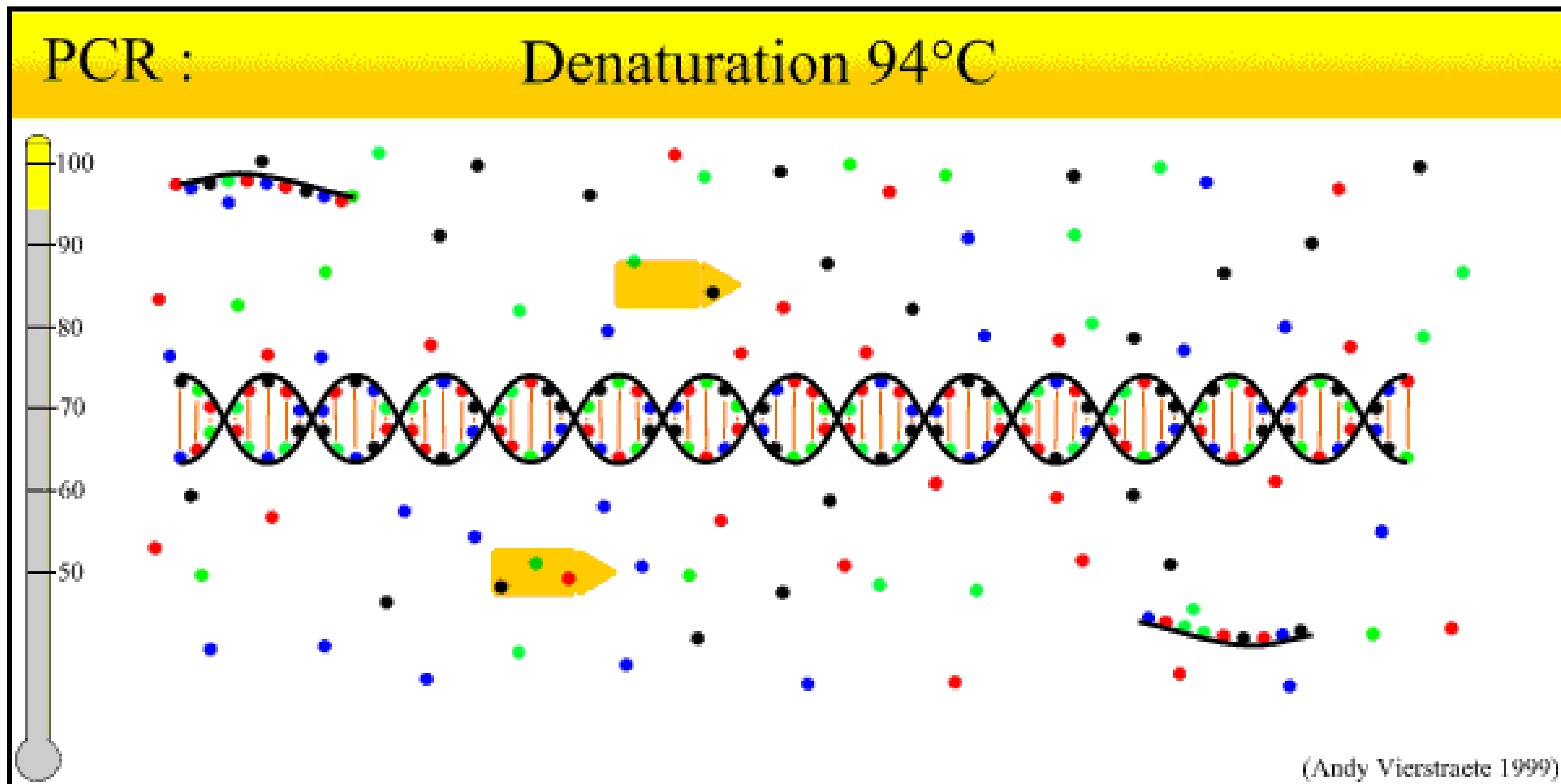
Step 3 : extension

2 minutes 72 °C

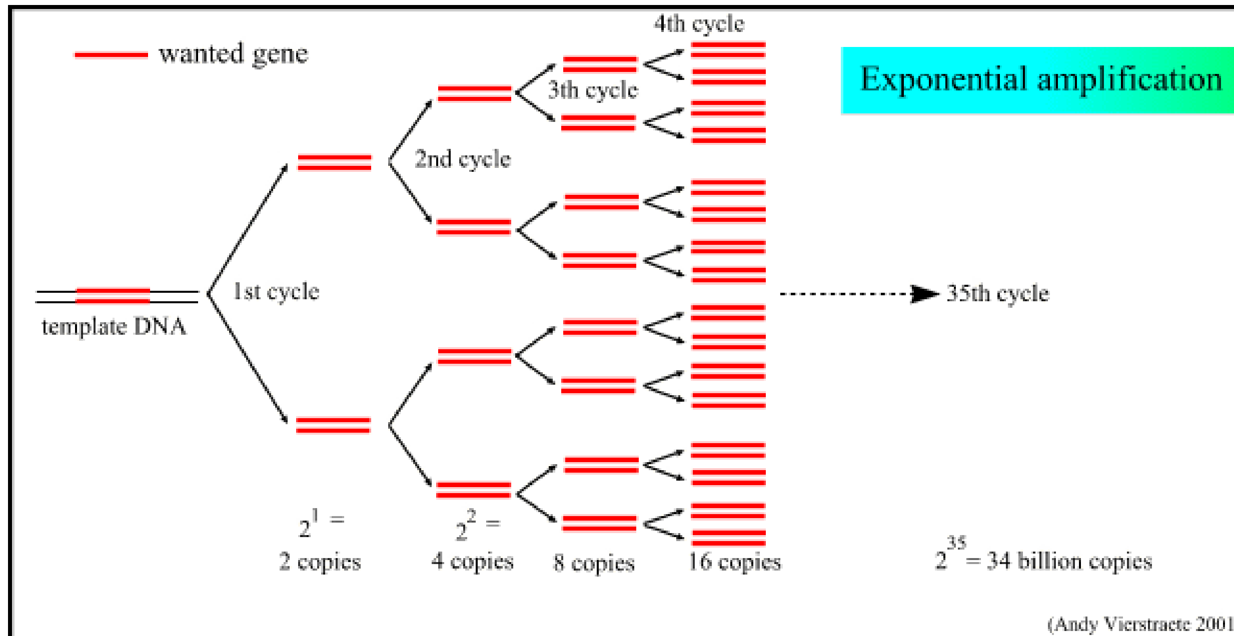
only dNTP's

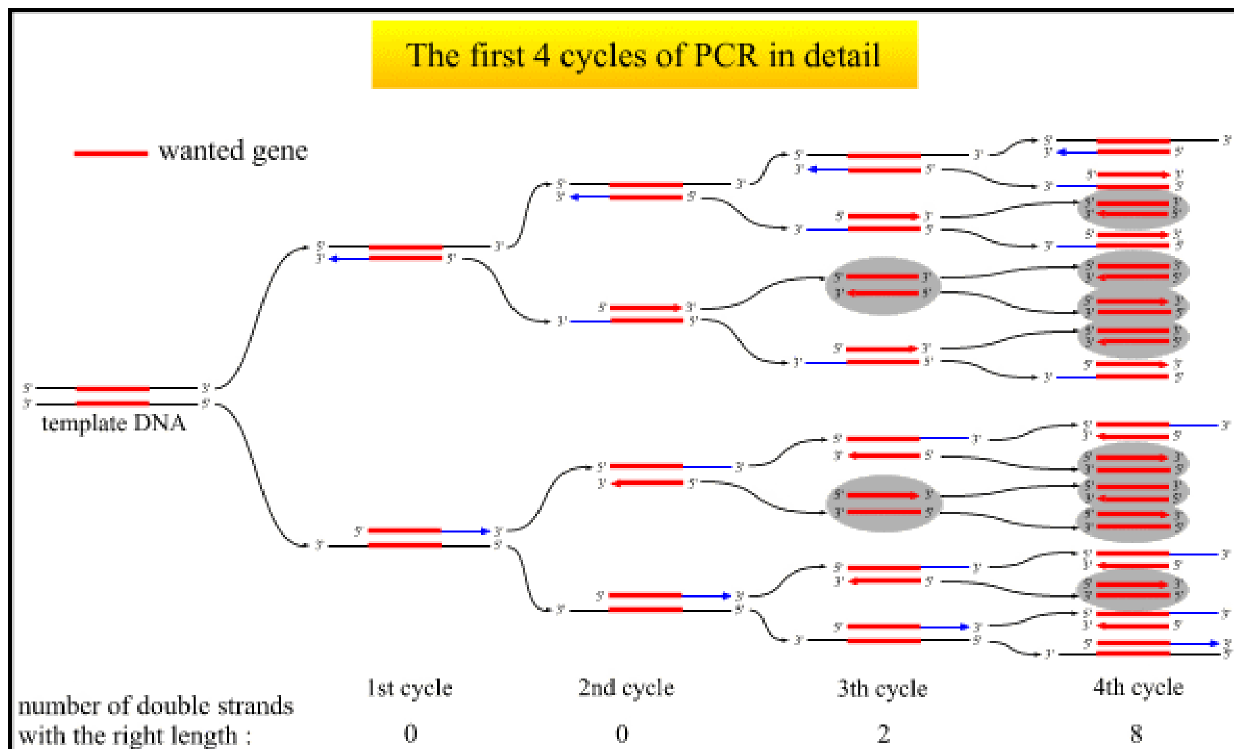
(Andy Vierstraete 1999)

Use of PCR - Concept of primers and/or probes



Use of PCR - Concept of primers and/or probes





Sensibility, specificity and other thermodynamic constraints

Sensibility

- A melting temperature (Tm) in the range of 52 C to 65 C
- Absence of dimerization capability
- Absence of significant hairpin formation (>3 bp)
- Lack of secondary priming sites
- Low specific binding at the 3' end (ie. lower GC content to avoid mispriming)

Specificity

- Must be **only one** target site in the template DNA where the primer binds

  => the primer sequence shall be unique in the template DNA.

- No annealing site in possible contaminant sources, such as human, rat, mouse, unwanted bacteria, etc.

Sensibility, specificity and other thermodynamic constraints

Length

- Primer length has effects on uniqueness and melting/annealing temperature.

    => the longer the primer, the more chance that it's unique

    => the longer the primer, the higher melting/annealing temperature.

- Length of primer has to be at least 15 bases to ensure uniqueness.

- Usually, primers of 17-28 bases long.

    => range varies based on if you can find unique primers with appropriate annealing temperature within this range

Sensibility, specificity and other thermodynamic constraints

Base composition

- affects hybridization specificity and melting/annealing temperature.
- Random base composition is preferred.
- Avoid long (A+T) and (G+C) rich region if possible.
- Usually, average (G+C) content around 50-60% will give the right melting/annealing temperature for ordinary PCR reactions, and will give appropriate hybridization stability.

  => However, melting/annealing temperature and hybridization stability are affected by other factors (see later). Therefore, (G+C) content is allowed to change

Annealing Temperature (T_{anneal})

=> the temperature at which primers anneal to the template DNA. It can be calculated from T_m .

$$T_{anneal} = T_{m_primer} - 4^{\circ}C$$

Sensibility, specificity and other thermodynamic constraints

Melting Temperature (Tm)

=> the temperature at which half the DNA strands are single stranded and half are double-stranded. Tm is characteristics of the DNA composition (ie. Higher G+C content DNA has a higher Tm due to more H bonds).

Basic : Marmur and Doty, 1962

$$Tm = 64.9 + 41.0 \times \left(\frac{yG + zC - 16.4}{wA + xT + yG + zC} \right)$$

Salt Adjusted : Howley et al., 1979

$$Tm = 100.5 + 41.0 \times \left(\frac{yG + zC - 16.4}{wA + xT + yG + zC} \right) - \left(\frac{820.0}{wA + xT + yG + zC} \right) + 16.6 \log([Na^+])$$

Nearest-neighbor thermodynamic : SantaLucia, 1998

$$Tm = \frac{\sum(\Delta H_d) + \Delta H_i}{\sum(\Delta S_d) + \Delta S_i + \Delta S_{self} + R \times \ln \frac{C_T}{b}} + C_{Na^+}$$

- Tm formula : http://www.basic.northwestern.edu/biotools/oligocalc.html

- or see "DNAmate" (many servers available, => google)

Sensibility, specificity and other thermodynamic constraints

Primer Pair Matching

• Primers work in pairs – forward primer and reverse primer. Since they are used in the same PCR reaction, it shall be ensured that the PCR condition is suitable for both of them.

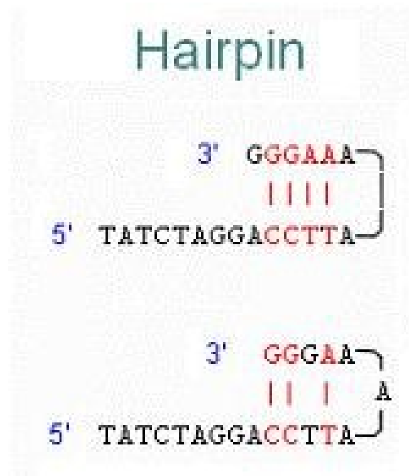
• One critical feature is their annealing temperatures, which shall be compatible with each other.

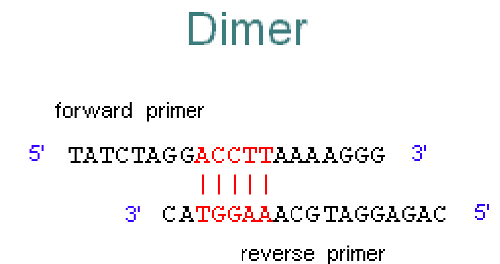
=> The maximum difference allowed is 3 °C. The closer their T_{anneal} are, the better.

Sensibility, specificity and other thermodynamic constraints

Internal Structure (= secondary structure)

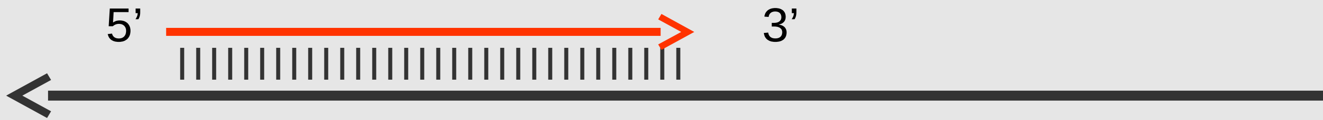
=> If primers can anneal to themselves, or anneal to each other rather than anneal to the template, the PCR efficiency will be decreased dramatically. They shall be avoided.

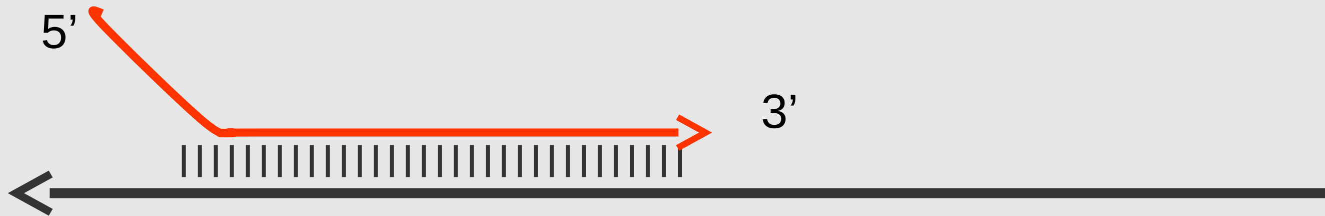


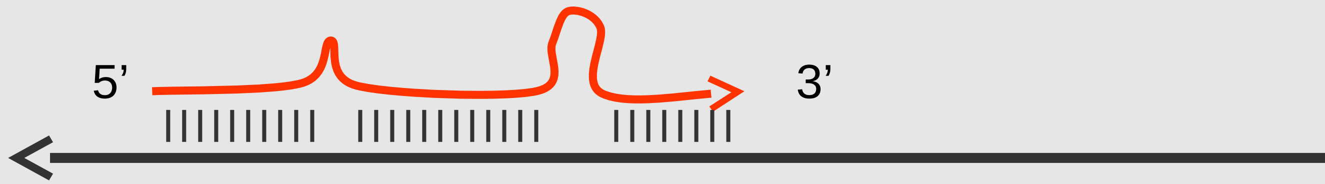


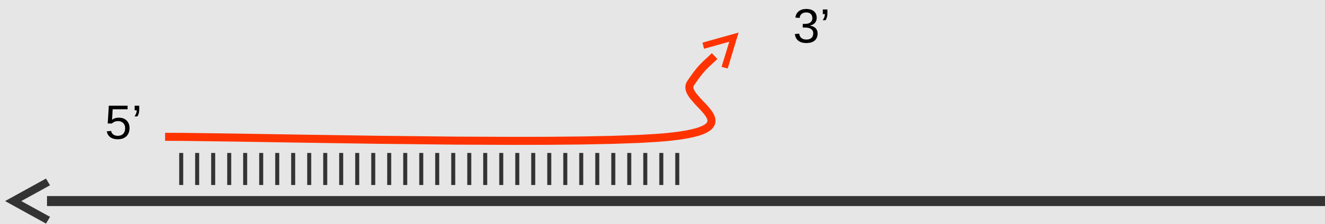
However, sometimes these 2° structures are harmless when the annealing temperature does not allow them to take form. For instance, some dimers or hairpins form at 30 °C while during PCR cycle, the lowest temperature only drops to 60 °C.

Sensibility, specificity and other thermodynamic constraints









Sensibility, specificity and other thermodynamic constraints

Summary of required criteria

- Uniqueness: ensure correct priming site;
- Length: 17-28 bases. This range varies;
- Base composition: average (G+C) content around 50-60%; avoid long (A+T) and (G+C) rich region if possible;
- Optimize base pairing, in particular 3' extremity should have high stability
- Melting Tm between 55-80 °C are preferred;
- Assure that primers at a set have annealing Tm within 2 – 3 °C of each other.
- Minimize internal secondary structure: hairpins and dimmers shall be avoided.

Sensibility, specificity and other thermodynamic constraints

Multiplex PCR

- Multiple primer pairs can be added in the same tube to do the PCR
- Good for amplifying multiple sites
- Application example: genome identification/ finishing
- Design difficulty

Difficulties:

=> Melting temperatures should be similar

=> No dimer formulation

Sensibility, specificity and other thermodynamic constraints

Universal primers

Primers can be designed to amplify only one specific product.

Or, primers can also be designed to amplify multiple products.

=> “universal primers”. For example, design primers to amplify all 16S genes of a given clade (genera, families, etc.).

Semi-universal primers

- Primers can be designed to amplify only a subset of template sequences from a large group of similar sequences.

For example, design primer to amplify HPV type 1 and type 6 gene, but not other types.

- Or, if a couple can not amplify all your set

=> you may design > 2 couples to fulfill the set

Sensibility, specificity and other thermodynamic constraints

Strategy for a small set of sequences:

- Groups sequences into a set of "wanted sequences", and a set of "unwanted sequences"

- Align groups of sequences.

- Find the most conservative primers for ”wanted sequences” and divergent for “unwanted sequences”

- Test forward and reverse primers to find the best pair in terms of thermodynamical constraints.

- Avoid hybridizations with unwanted sequences
    - compared alignment with your unwanted sequences set
    - or blast primers on a a larger set of unwanted sequences that you may construct
    - or Blast on nt-like database, and check for unwanted (not always very efficient...)

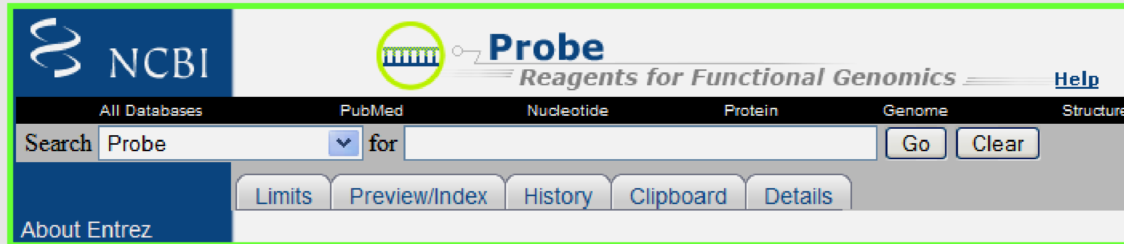
Strategy for a big set of sequences (classes or phyla) :

- Could be very problematic....

Examples of softwares used for oligomers design

Before using software : do not reinvent the wheels !

- Search oligomers in publications
- Databases of primers



- NCBI Probe Database
- RTPrimerDB
- Primer Bank
- qPrimerDepot
- PCR-QPPD
- PerlPrimer

qPrimerDepot ---- A quantitative real time PCR primer database

Quantitative PCR Primer Database
QPPD

RTPrimerDB
Real Time PCR Primer and Probe Database

> DNA Methylation analysis PCR Primer Database
Quick Search / Advanced Search
Gene | Substring | Exact phrase | Organism | All | Search | methPrimerDB ID

MASSACHUSETTS GENERAL HOSPITAL
CCIB The Center for Computational and Integrative Biology
HARVARD MEDICAL SCHOOL
Primer Bank
PCR Primers for Gene Expression Detection and Quantification

Examples of softwares used for oligomers design

- Primer/probes design by a skill human beings is far better done by automatics softwares !

Some primer design softwares:

- Primer3 (http://frodo.wi.mit.edu/) - many servers existing through the web => google
- Primer3Plus (similar as Primer3)
- PrimerZ
- PerlPrimer
- Vector NTI Advantage 10
- PrimerX
- Oligo (Life Science Software)
- GCG:(Accelrys)
- Others: GeneFisher, Primer!, PrimaClase, Codhop, Web Primer, etc.

Some primer checker softwares and Tm calculation softwares:

- PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)
- Oligoanalyzer (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/)
- OHM (OligoHeatMap) (http://bioinfo.unice.fr)
- DNAMate (http://melolab.org/dnaMATE/tm-pred.html)
- BioMath (http://www.promega.com/techserv/tools/biomath/calc11.htm)

Examples of softwares used for oligomers design

Primer-Blast

Primer-BLAST — A tool for finding specific primers

NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST). More... Tips for finding specific primers

Reset page · Save search parameters · Retrieve recent results

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear

Range: From / To — Forward primer; Reverse primer — Clear

Or, upload FASTA file [Choisir...]

Primer Parameters

Use my own forward primer (5'->3' on plus strand)				Clear
Use my own reverse primer (5'->3' on minus strand)				Clear
	Min	Max		
PCR product size	70	1000		
# of primers to return	5			
	Min	Opt	Max	Max T_m difference
Primer melting temperatures (T_m)	57.0	60.0	63.0	3

Exon/intron selection

A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span: No preference

Exon junction match: Exon at 5' side: 7; Exon at 3' side: 4

Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

Intron inclusion: ☐ Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range: Min 1000; Max 1000000

Primer Pair Specificity Checking Parameters

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

Database: Refseq mRNA

Organism: Homo sapiens

Enter an organism name, taxonomy id or select from the suggestion list as you type.

Add more organisms

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

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.

Misprimed product size deviation: 4000

Splice variant handling: ☐ 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

Examples of softwares used for oligomers design

Primer-Blast

▼ Advanced parameters

Primer Pair Specificity Checking Parameters

Parameter	Value
Max number of Blast target sequences	50000
Blast expect (E) value	30000
Blast word size	7
Max primer pairs to screen	1000

Primer Parameters

Parameter	Min	Opt	Max
PCR Product Tm			
Primer Size	15	20	25

Parameter	Min	Max
Primer GC content (%)	20.0	80.0

Parameter	Value
GC clamp	0
Max Poly-X	5
Max 3' Stability	9
Max GC in primer 3' end	5
Secondary Structure Alignments	○ Use Thermodynamic Secondary Structure Alignments ⦿ Use Old Secondary Structure Alignments

Warning: Thermodynamic Secondary Structure Alignments can be very slow.

Parameter	Primer	Pair
Max Template Mispriming	12.00	24.00

Parameter	Any	3'
Max Self Complementarity	8.00	3.00
Max Pair Complementarity	8.00	3.00

Parameter	Value
Excluded regions	
Concentration of monovalent cations	50.0
Concentration of divalent cations	1.5
Concentration of dNTPs	0.6
Salt correction formula	SantaLucia 1998
Table of thermodynamic parameters	SantaLucia 1998
Annealing Oligo Concentration	50.0
SNP handling	☐ Primer binding site may not contain known SNP
Repeat filter	Automatic — Avoid repeat region for primer selection by filtering with repeat database
Low complexity filter	☑ Avoid low complexity region for primer selection

Internal hybridization oligo parameters

Parameter	Value
Hybridization oligo	☐ Pick internal hybridization oligo

Parameter	Min	Opt	Max
Hyb Oligo Size	18	20	27
Hyb Oligo tm	57.0	60.0	63.0
Hyb Oligo GC%	20.0	50	80.0

Exercise :

- **Goal : detection of a given organism using a specific PCR applification :**

Searching specific primers/probes for « *Bacillus anthracis* », *gene* « cya »

- **Step 1: build your sets of sequences** : NCBI Entrez, Blast, SRS, Acnuc, or in publications (pubmed) !

  - targets : homologues sequences of *Bacillus anthracis* cya

  - non-targets : closed sequences of *Bacillus anthracis* cya (Blastn ...)

- **Step 2: find the potential primers and probes**:

  1) find existing primers in publications (using http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) or dedicaced website/databases

  2) design new couples of primers and/or probes (ex. PrimerBlast or other, using the "targets" set of sequences)

Exercise :

3) Check « by eyes » the primers against the aligned sequencing of targets **and** also against the « non targets » sequences (so, primers which are aligned with non targets should be remove).

Example of aligners of alignment viewer tools : Clustalw, Clustal Omega, Jalview, Seaview, Mega, etc..

4) Check again your (set of) primers using “Blast” or “Oligo Heat Map” (they should match the target seq and not the non-targets). Remove suspicion oligos

5) Check potential self hybridization or cross hybridization (ex. OligoAnalyzer, OligoCalc, etc..)