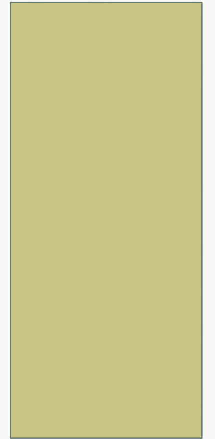
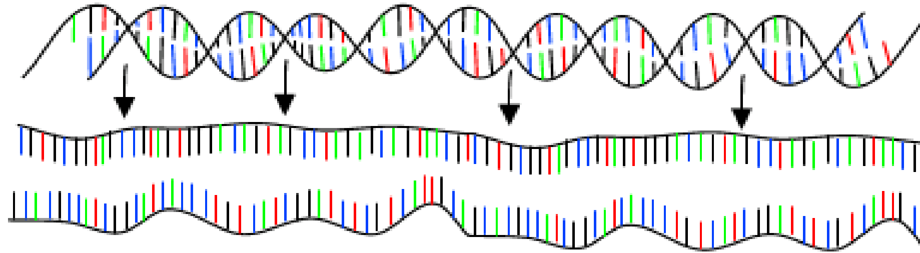
PCR, PRIMER DESIGN AND DNA SEQUENCING



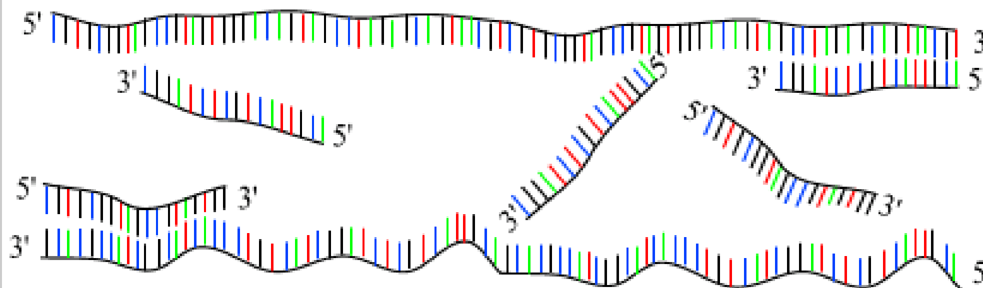
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

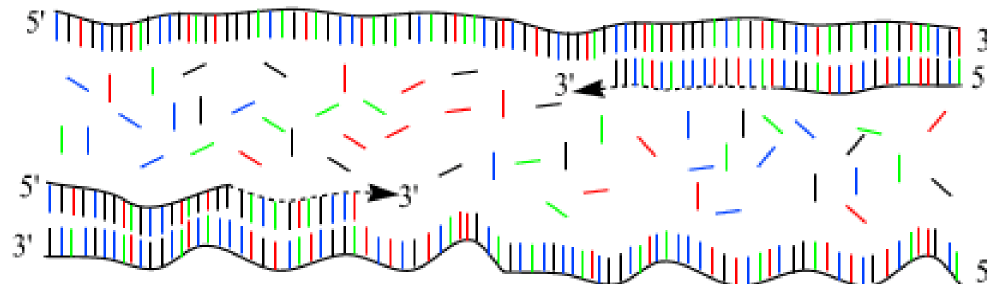
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

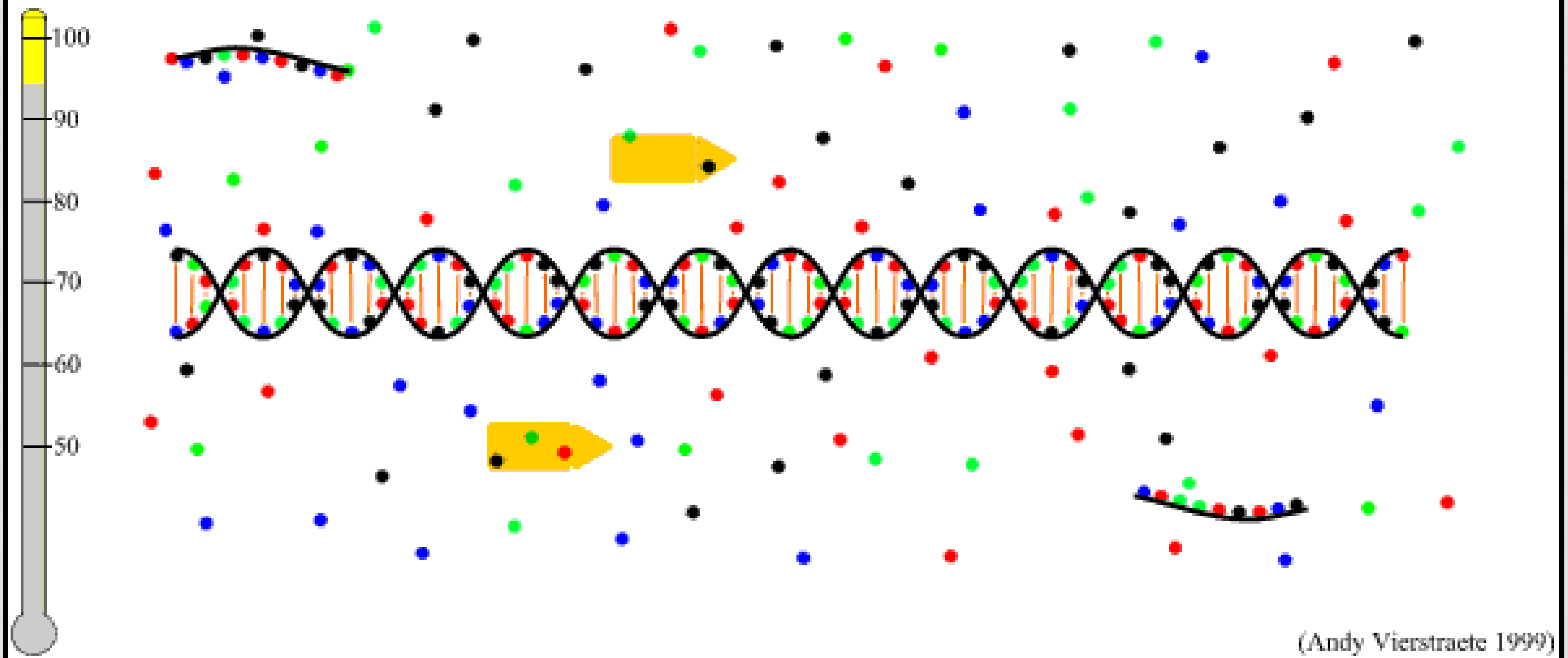


Step 3 : extension

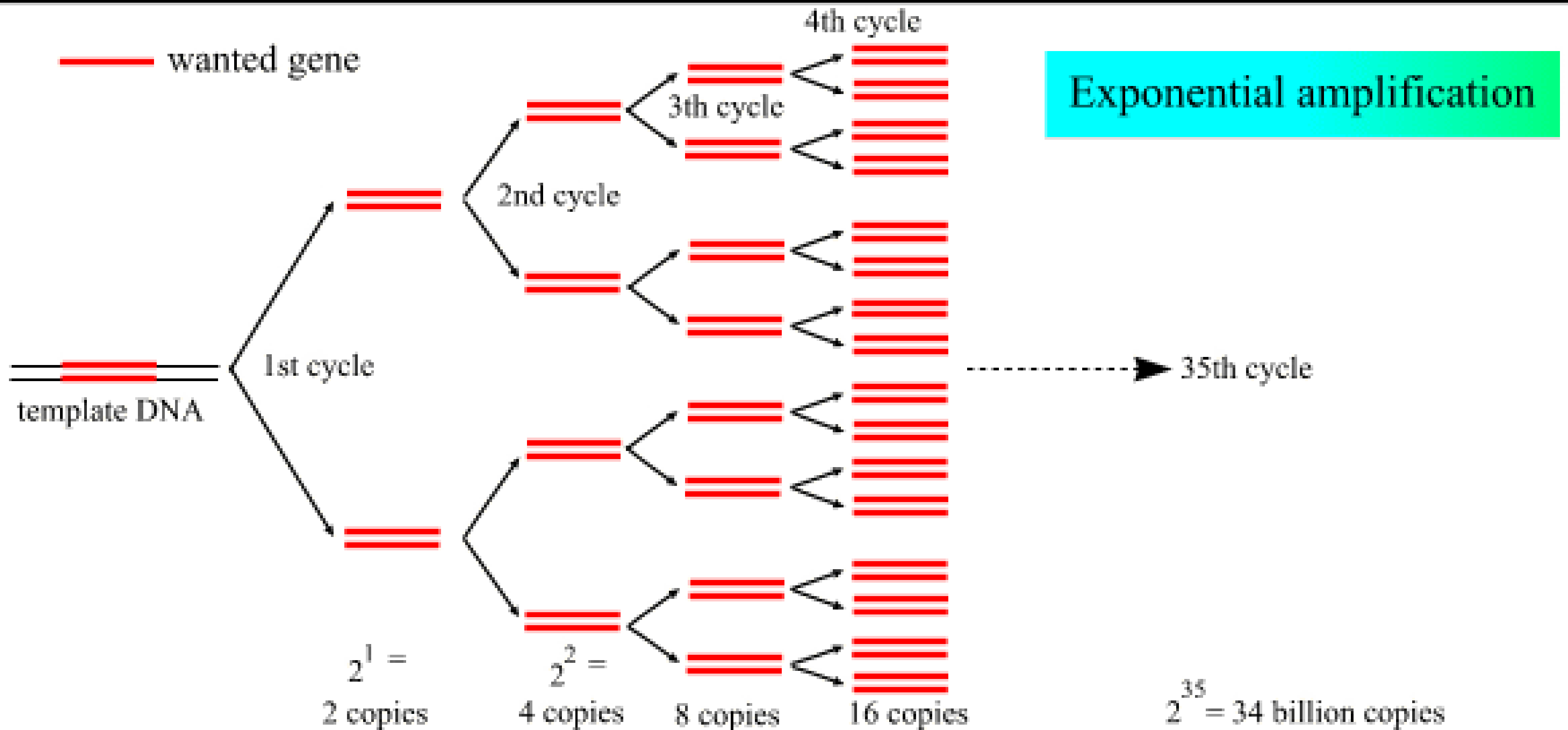
2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)

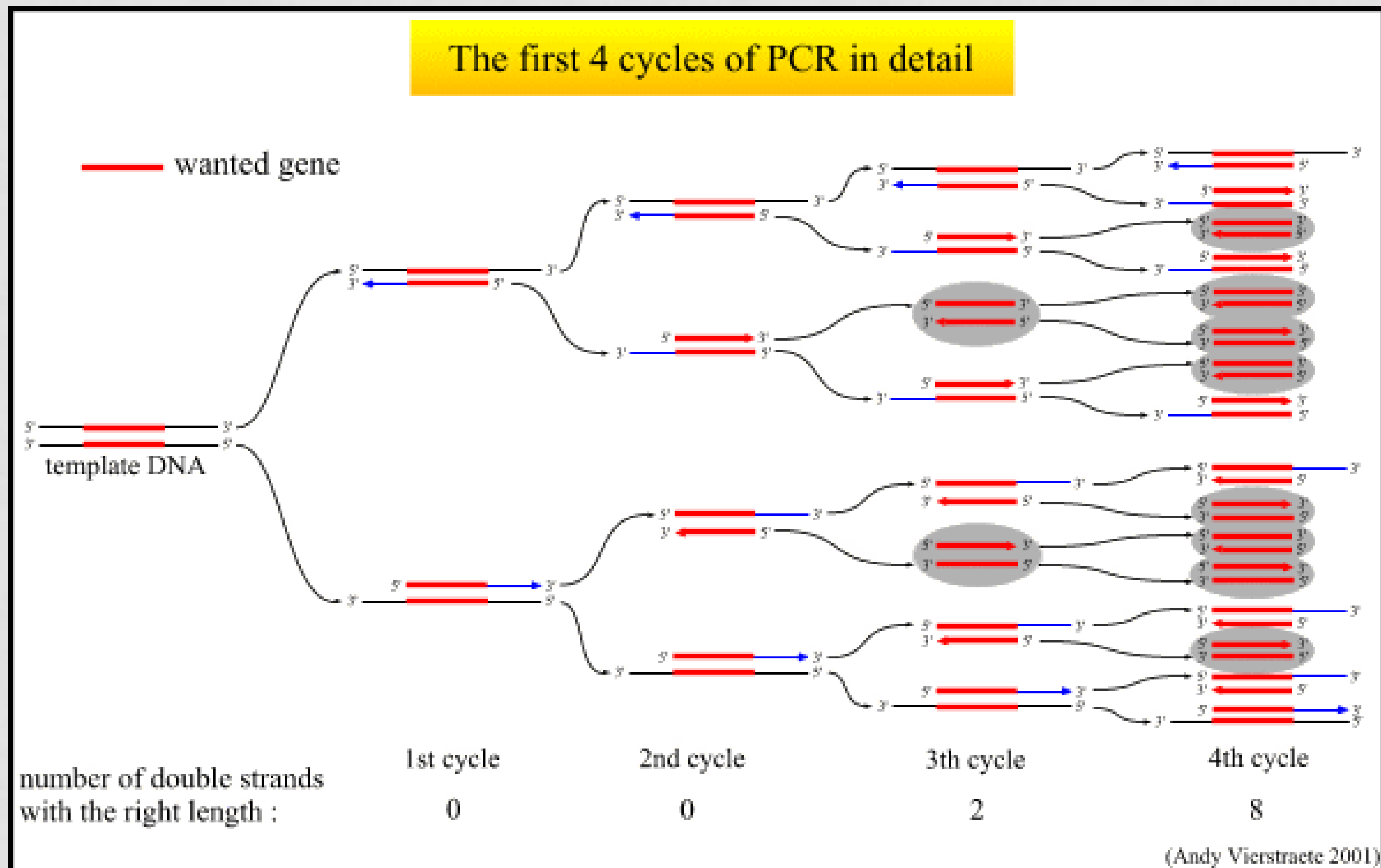
PCR



PCR



PCR



DEFINITION

- PCR primer design is the creation of short nucleotide sequences for use in amplifying a specific region of DNA.

EXAMPLES

- PCR primers are designed to:
  - Highly conserved DNA regions
  - Protein-coding regions with low degeneracy
  - More conserved regions that flank variable regions

NON-EXAMPLES

- PCR primers are not designed to:
  - Repeat regions
  - Regions with secondary structure
  - Regions that can form primer-dimers
  - Regions with low G/C content

APPLICATIONS

- Primer design is used for:
  - Finding new genes
  - Developing new identification tools
  - Optimizing PCRs

PRIMER DESIGN CRITERIA

- Target: Conserved nucleotide or protein regions
- Length: Usually 18 - 24 bases
- Purine:pyrimidine content of around 1:1
- End with 1-2 GC pairs, if possible
- No inter- or intra-primer interactions
- Check with databases for specificity
- Cycling conditions and buffer concentrations should be adjusted for each primer pair (see PCR troubleshooting)

GOOD PRIMER'S CHARACTERISTIC

- 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)

UNIQUENESS

There shall be one and only one target site in the template DNA where the primer binds, which means the primer sequence shall be unique in the template DNA.

There shall be no annealing site in possible contaminant sources, such as human, rat, mouse, etc. (BLAST search against corresponding genome)

Template DNA

5'...TCAACTTAGCATGATCGGGTA...GTAGCAGTTGACTGTACAACTCAGCAA...3'

TGCTAAGTTG CAGTCAACTGCTAC TGCT A AGTTG

Primer candidate 1 5'-TGCTAAGTTG-3' NOT UNIQUE!

Primer candidate 2 5'-CAGTCAACTGCTAC-3' UNIQUE!

LENGTH

Primer length has effects on uniqueness and melting/annealing temperature. Roughly speaking, the longer the primer, the more chance that it's unique; the longer the primer, the higher melting/annealing temperature.

Generally speaking, the length of primer has to be at least 15 bases to ensure uniqueness. Usually, we pick primers of 17-28 bases long. This range varies based on if you can find unique primers with appropriate annealing temperature within this range.

BASE COMPOSITION

Base composition affects hybridization specificity and melting/annealing temperature.

• Random base composition is preferred. We shall avoid long (A+T) and (G+C) rich region if possible.

Template DNA

5′...TCAACTTAGCATGATCGGGCA...AAGATGCACGGGCCTGTACACAA...3′

TGCCCGATCATGCT TGCCCGATCATGCT

• Usually, average (G+C) content around 50-60% will give us 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, which we'll discuss later. Therefore, (G+C) content is allowed to change.

MELTING TEMPERATURE

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; Higher G+C content DNA has a higher Tm due to more H bonds.

Calculation

Shorter than 13: Tm= (wA+xT) * 2 + (yG+zC) * 4

Longer than 13: Tm= 64.9 +41*(yG+zC-16.4)/(wA+xT+yG+zC)

(Formulae are from http://www.basic.northwestern.edu/biotools/oligocalc.html)

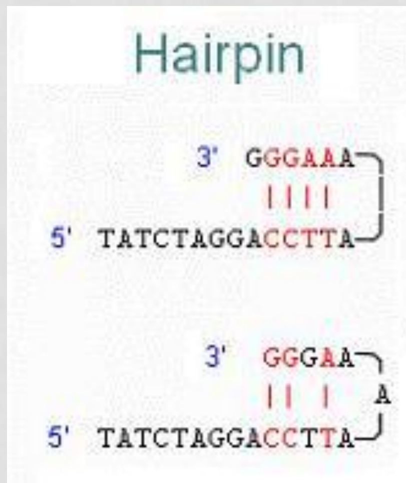
ANNEALING TEMPERATURE

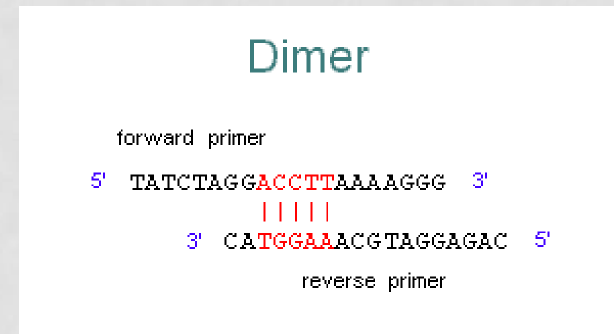
Annealing Temperature, T_{anneal} – the temperature at which primers anneal to the template DNA. It can be calculated from T_m .

$$T_{anneal} = T_{m_primer} - 4°C$$

INTERNAL 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 example, some dimers or hairpins form at 30 °C while during PCR cycle, the lowest temperature only drops to 60 °C.

General rules for primer design
-- Primer secondary structures

Hairpins

- **Formed via intra-molecular interactions**
- **Negatively affect primer-template binding, leading to poor or no amplification**
- **Acceptable ΔG (free energy required to break the structure): >-2 kcal/mol for 3'end hairpin; >-3 kcal/mol for internal hairpin;**

Self-Dimer (homodimer)

- **Formed by inter-molecular interactions between the two same primers**
- **Acceptable ΔG: >-5 kcal/mol for 3'end self-dimer; >-6 kcal/mol for internal self-dimer;**

Cross-Dimer (heterodimer)

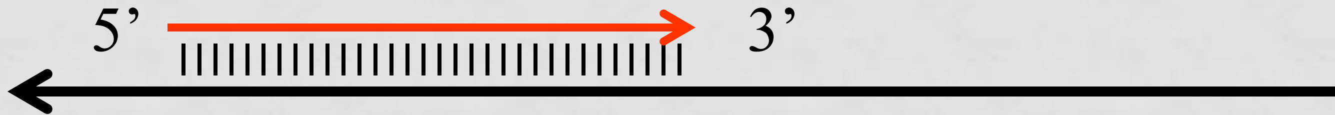
- **Formed by inter-molecular interactions between the sense and antisense primers**
- **Acceptable ΔG: >-5 kcal/mol for 3'end cross-dimer; >-6 kcal/mol for internal cross-dimer;**

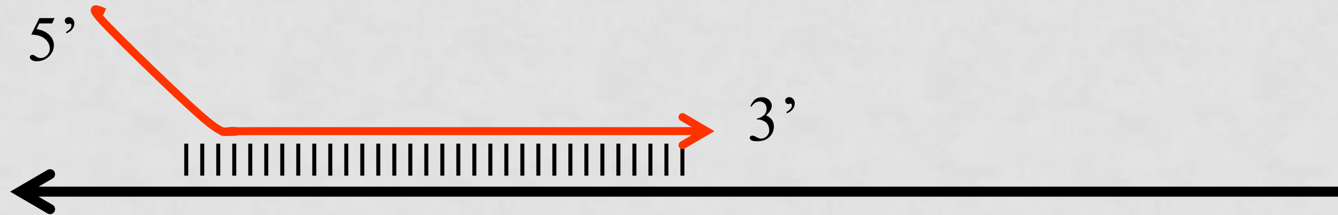
PRIMER PAIR MATCHING

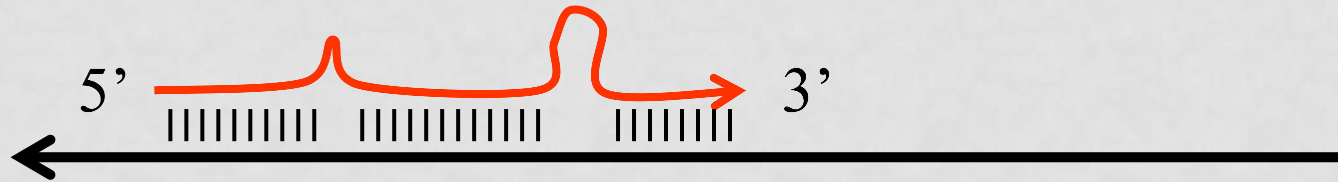
Primers work in pairs – forward primer and reverse primer. Since they are used in the same PCR reaction, it shall be ensured that the PCR condition is suitable for both of them.

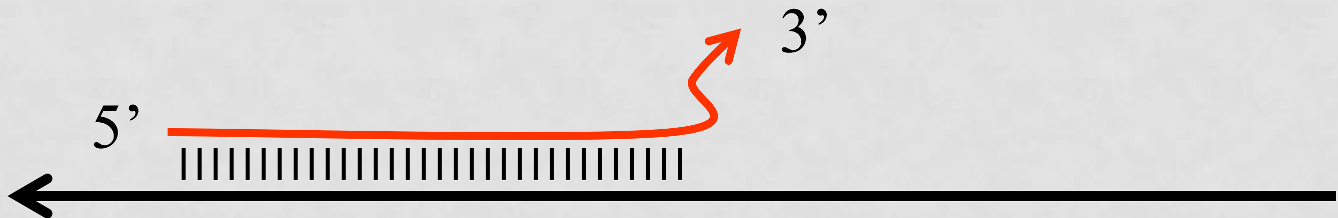
One critical feature is their annealing temperatures, which shall be compatible with each other. The maximum difference allowed is 3 °C. The closer their T_{anneal} are, the better.

SUMMARY ~ WHEN IS A "PRIMER" A PRIMER?









SUMMARY ~ PRIMER DESIGN CRITERIA

1. Uniqueness: ensure correct priming site;
2. Length: 17-28 bases.This range varies;
3. Base composition: average (G+C) content around 50-60%; avoid long (A+T) and (G+C) rich region if possible;
4. Optimize base pairing: it's critical that the stability at 5' end be high and the stability at 3' end be relatively low to minimize false priming.
5. Melting Tm between 55-80 °C are preferred;
6. Assure that primers at a set have annealing Tm within 2 – 3 °C of each other.
7. Minimize internal secondary structure: hairpins and dimmers shall be avoided.

COMPUTER-AIDED PRIMER DESIGN

Primer design is an ***art*** when done by human beings, and a ***far better done by machines***.

Some primer design programs we use:

- **Oligo:** Life Science Software, standalone application
- **GCG**: Accelrys, ICBR maintains the server.
- **Primer3:** MIT, standalone / web application
  http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
- **BioTools:** BioTools, Inc. ICBR distributes the license.
- **Others:** GeneFisher, Primer!, Web Primer, NBI oligo program, etc.

Melting temperature calculation software:

- **BioMath**: http://www.promega.com/biomath/calc11.htm

UNIVERSAL PRIMERS

Primers can be designed to amplify only one product.

Primers can also be designed to amplify multiple products. We call such primers "universal primers". For example, design primers to amplify all HPV genes.

Strategy:

1. Align groups of sequences you want to amplify.
2. Find the most conservative regions at 5' end and at 3' end.
3. Design forward primer at the 5' conservative region.
4. Design reverse primer at the 3' conservative regions.
5. Matching forward and reverse primers to find the best pair.
6. Ensure uniqueness in all template sequences.
7. Ensure uniqueness in possible contaminant sources.

SUMMARY ~ ADVANCED PRIMER DESIGN

Primers can be designed to serve various purposes. Universal primer, semi-universal primer, guessmers are some of them. There are many more fields where primer design skills are required, such as real-time PCR, population polymorphism study (microsatellite, AFLP, SNP …), internal probe design, and so on.

However, the basic rules always apply –
achieve the appropriate hybridization specificity and stability.

General rules for primer design
-- Annealing temperatures and other considerations

❖ T_a (Annealing temperature) vs. T_m

- **T_a is determined by the T_m of both primers and amplicons: optimal T_a=0.3 x T_m(primer)+0.7 x T_m(product)-25**
- **General rule: T_a is 5°C lower than T_m**
- **Higher T_a enhances specific amplification but may lower yields**
- **Crucial in detecting polymorphisms**

❖ Primer location on template

- **Dictated by the purpose of the experiment**
- **For detection purpose, section towards 3' end may be preferred.**

❖ When using composite primers

- **Initial calculations and considerations should emphasize on the template-specific part of the primers**
- **Consider nested PCR**

http://www.hsls.pitt.edu/guides/genetics/obrc

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OBRC: Online Bioinformatics Resources Collection

OBRC

- Email Suggestions
- Recommend a New Resource

The Online Bioinformatics Resources Collection (OBRC) contains annotations and links for 1768 bioinformatics databases and software tools.

primer* [Search]

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Search examples: transcription factors, promoters, RNAi

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Search the Online Bioinformatics Resources Collection (OBRC) at the Health Sciences Library System of University of Pittsburgh

Find open-access databases and software in this collection of over 1700 bioinformatics resources.

[Search OBRC] (e.g. **PCR primer**, **transcription factor**)

Search tips and more information on OBRC

http://www.usc.edu/hsc/nml/lib-services/bioinformatics/index.html

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Bioinformatics Tools

86 results retrieved for the query **"primer*"**

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   Use this suite of programs to design different types of PCR **primers**.
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   Search for pre-designed and tested PCR **primers** for amplification of exons, genes and SNPs of almost all sequenced genomes.
   ...more info
3. Primaclade -- a flexible tool to find conserved PCR **primers** across multiple species [clusters]
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4. PrimerX -- Automated design of mutagenic **primers** for site-directed mutagenesis [clusters]
   Design PCR **primers** for site-directed mutagenesis using DNA or protein sequences.
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5. PerlPrimer -- cross-platform, graphical **primer** design for standard, bisulphite and real-time PCR [clusters]
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6. RTPrimerDB -- The Real-Time PCR **primer** and probe database [clusters]
   Search for validated real time PCR **primer** and probe sequences.
   ...more info
7. PrimerStation -- a highly specific multiplex genomic PCR **primer** design server for the human genome [clusters]

http://search.hsls.pitt.edu/vivisimo/cgi-bin/query-meta?input-form=molbio-simple&query=pcr+primer&v%3Asources=OBRC&v%3Aproject=molbio

Resources for General Purpose PCR Primer Design

- Primer3
- Primer3Plus
- PrimerZ
- PerlPrimer
- Vector NTI Advantage 10

General Purpose PCR Primer Design Tool– Primer3

Name	**Primer3 -- an online tool for PCR primer design**
Type	Web-based software
Key Functions	Design PCR primers and hybridization probes.
Publication Info	Methods Mol Biol 2000
Times Cited	823
Pros	The original and most widely used PCR primer design program; uses sequence as input; a huge number of options for customizing primer design;
Cons	busy interface;
Note	In OBRC; the program has been widely adopted by many primer design software.
YiBu's Rating	**4 out of 5**

Web Site:

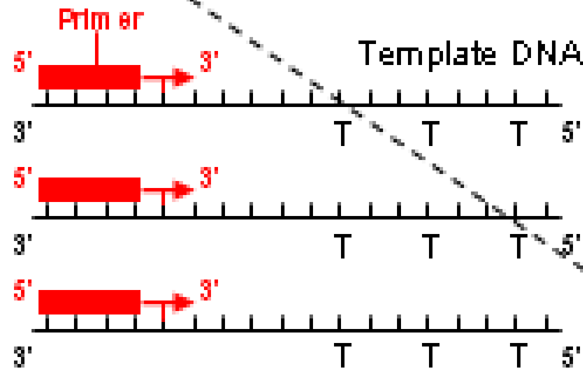
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

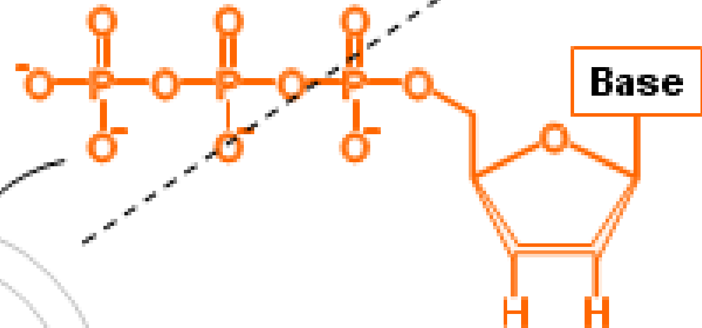
More Info:

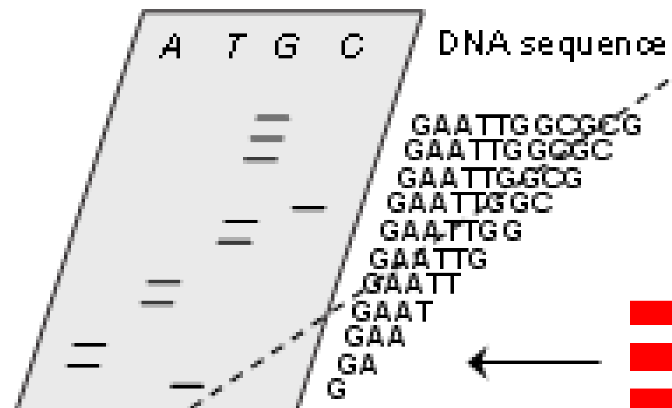
http://www.hsls.pitt.edu/guides/genetics/obrc/dna/pcr_oligos/URL1043858198/info

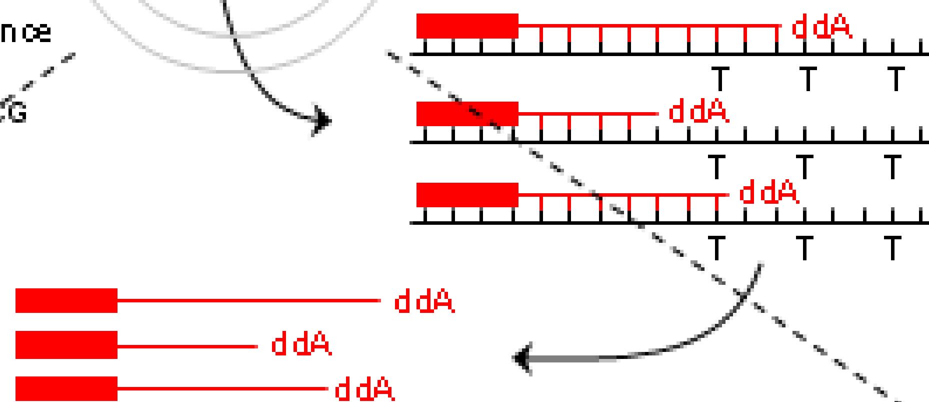
DNA Sequencing

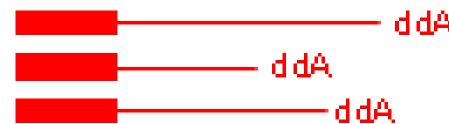
Chain termination DNA sequencing











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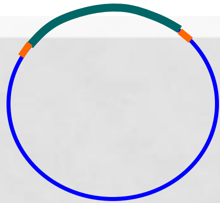
SANGER METHOD: GENERATING READ



1. Start at primer (restriction site)
2. Grow DNA chain
3. Include ddNTPs
4. Stops reaction at all possible points
5. Separate products by length, using gel electrophoresis

A C G T

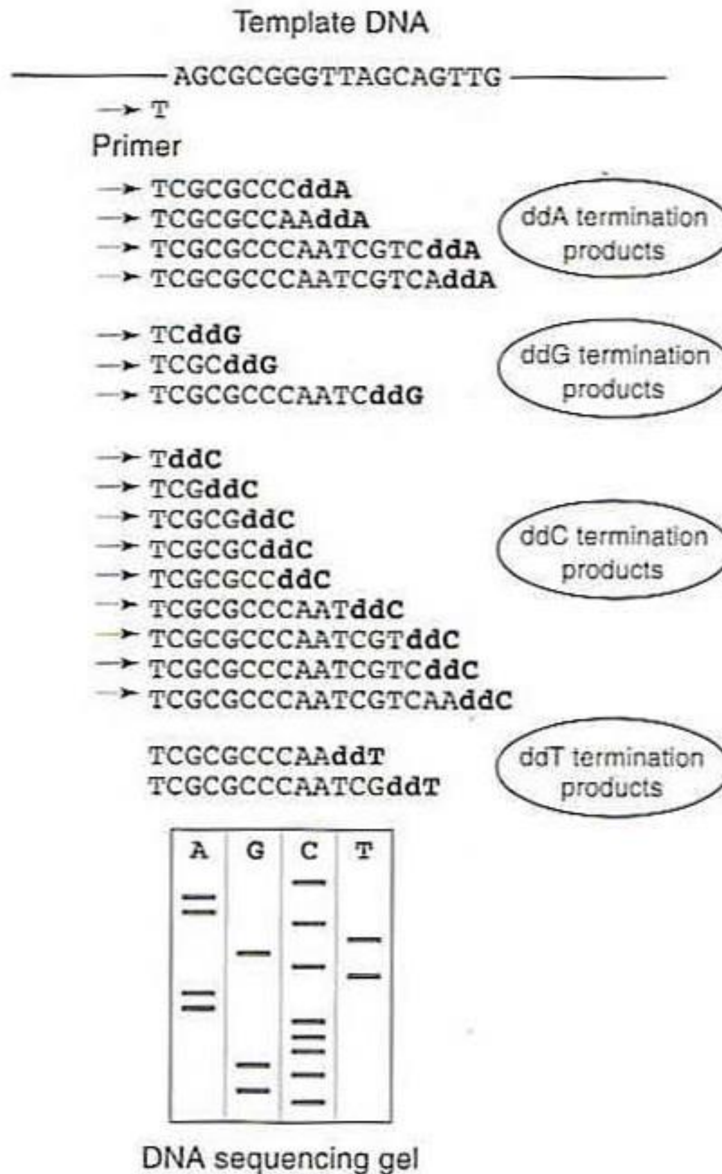
DNA Length

G G G T C A T T T G T A C T A G T G G A G G A A T

template strand

primer

Dideoxynucleotide chain termination method of DNA sequencing



- Originally four separate sets of DNA, primer and a single different DD nucleotide were produced and run on a gel.

- Modern technology allows all the DNA, primers, etc to be mixed and the fluorescent labeled DDnucleotide ‘ends’ of different lengths can be ‘read’ by a laser.

- In addition, can sequence directly from PCR products

- Additionally, the gel slab has been replaced by polymer filled capillary tubes in modern equipment

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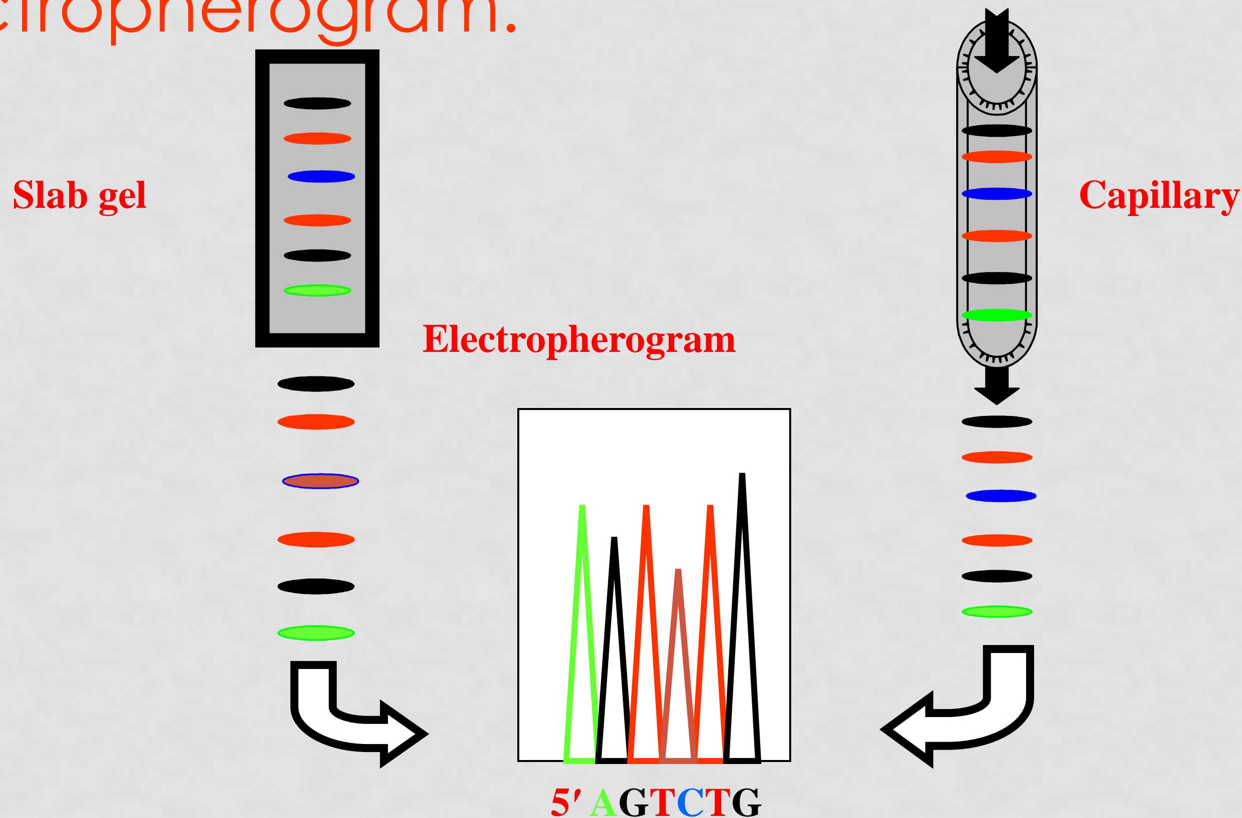
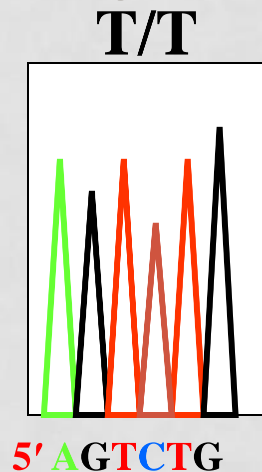
DYE TERMINATOR SEQUENCING

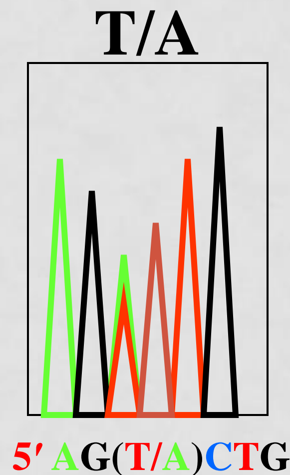
- The DNA ladder is read on an electropherogram.

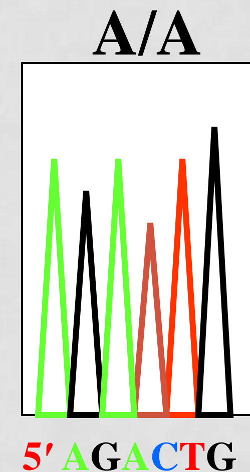


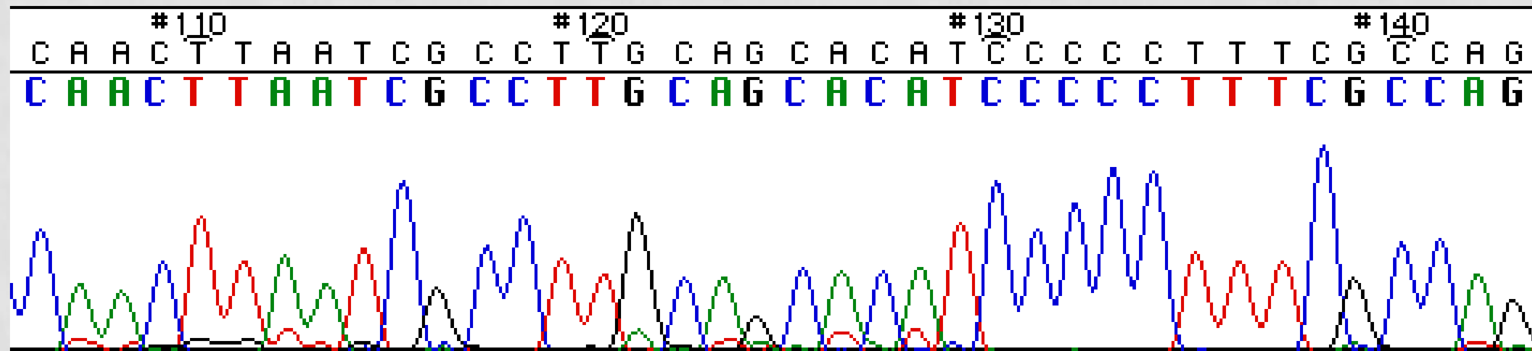
AUTOMATED SEQUENCING

- Dye primer or dye terminator sequencing on capillary instruments.
- Sequence analysis software provides analyzed sequence in text and electropherogram form.
- Peak patterns reflect mutations or sequence changes.

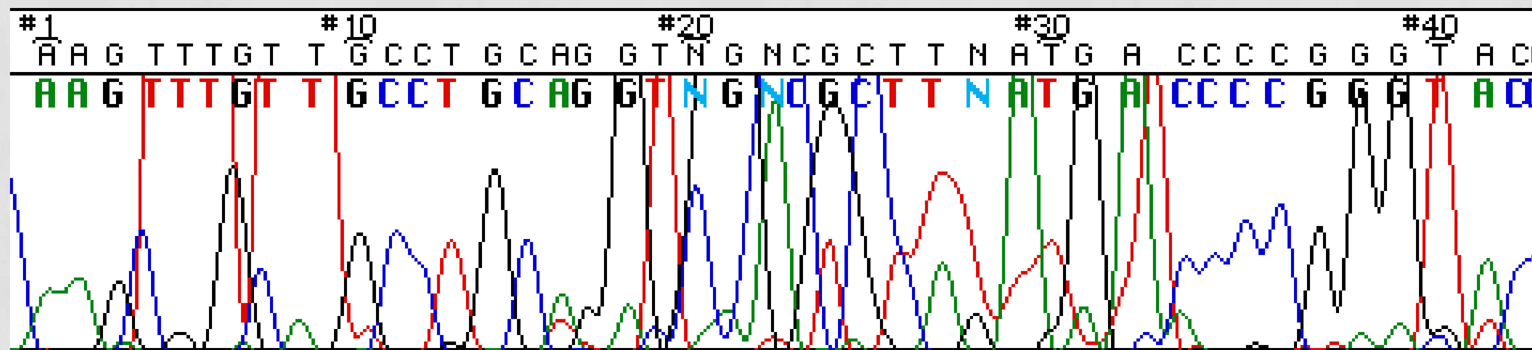




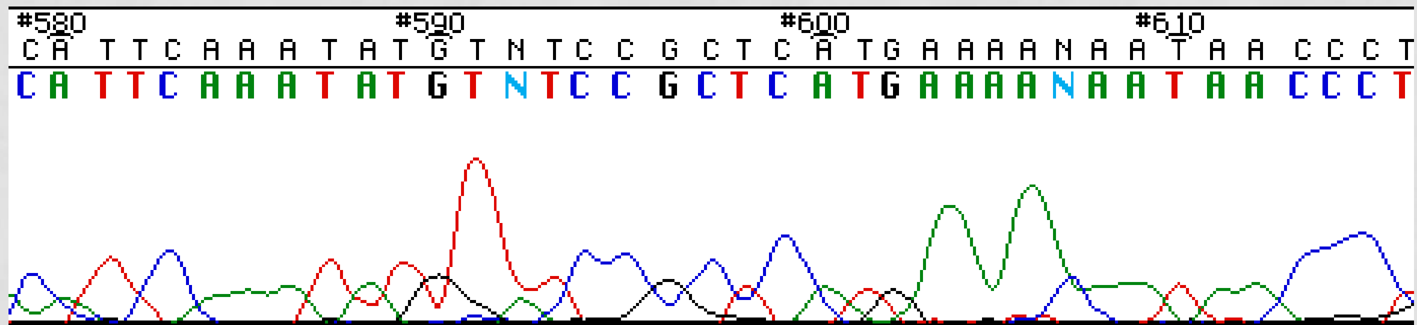




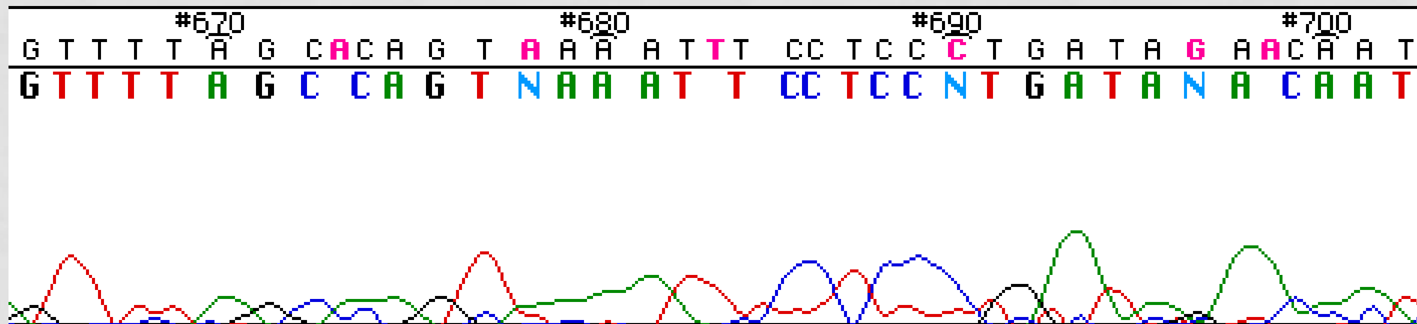
This is an example of a good chromatogram showing well-resolved peaks and no ambiguities. Generally the first several hundred bases of a chromatogram will look like this.



Start of a chromatogram showing peaks corresponding to unincorporated dye-terminators (dye-blobs) superimposed over and partially obscuring the real peaks.



Region of a chromatogram fairly far along the sequence where some bases in runs of 2 or more are no longer visible as single peaks



This is a region of a chromatogram where the traces have become too ambiguous for accurate base calling. While some parts of this region of the chromatogram can be useful for linking to existing sequences following manual editing, it should not be considered accurate.