PCR Primers Design

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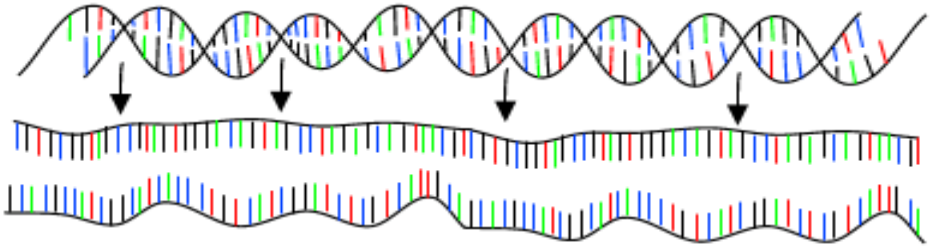
PC

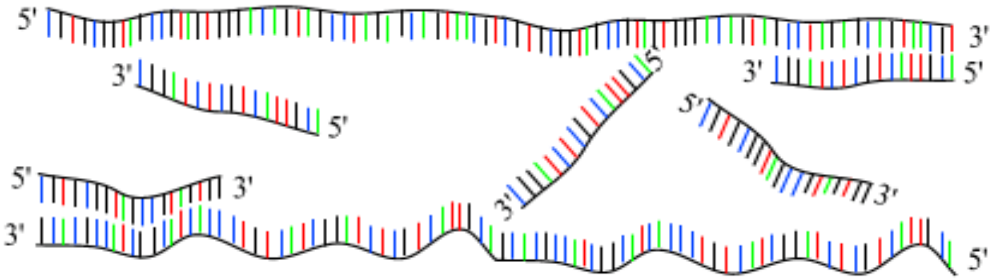
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

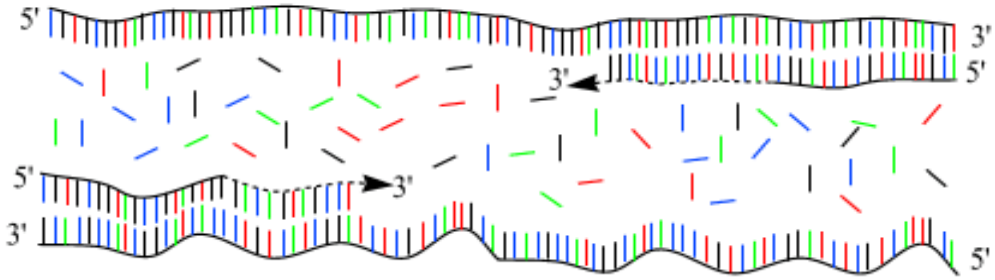
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



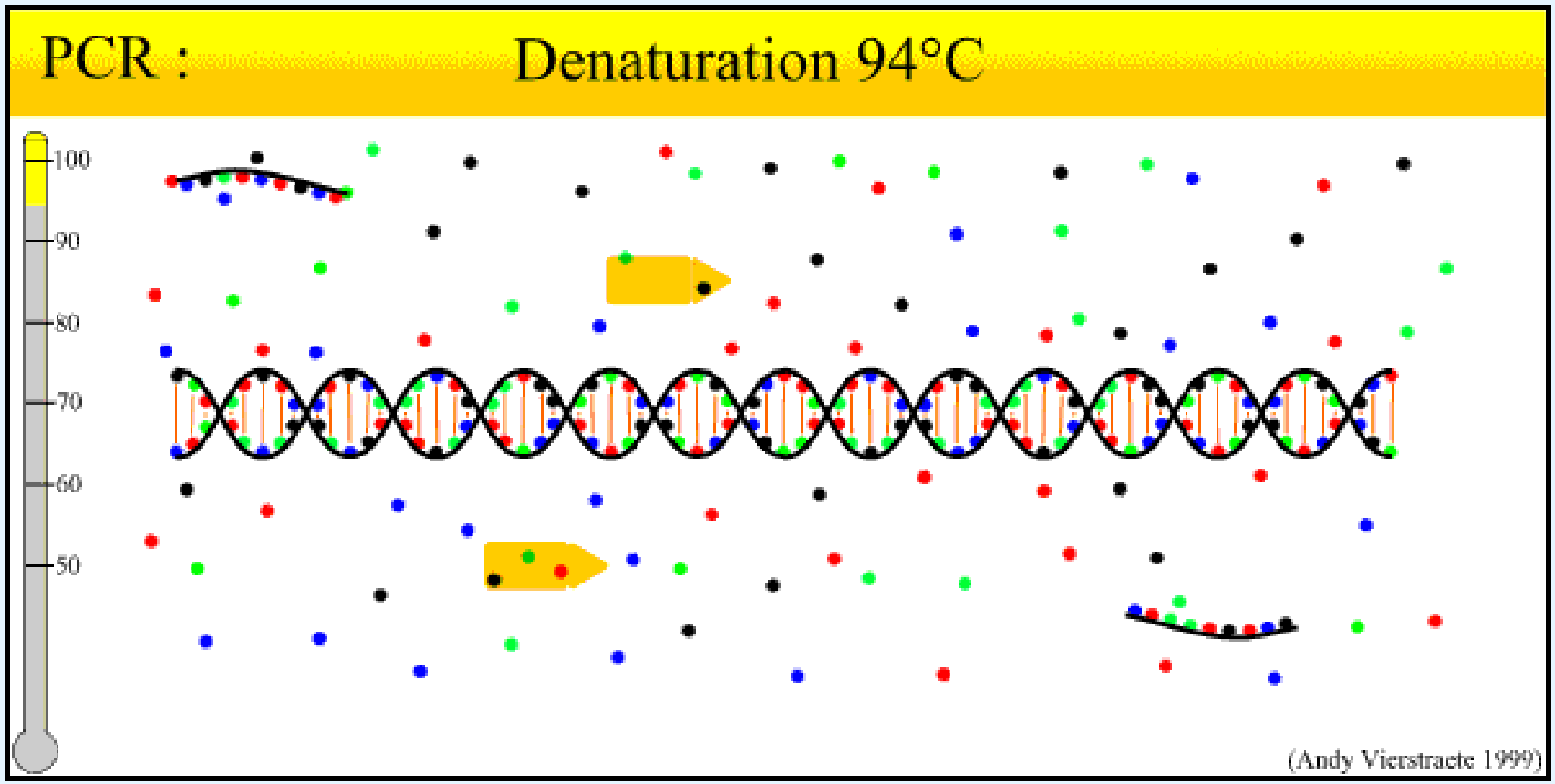
Step 3 : extension

2 minutes 72 °C

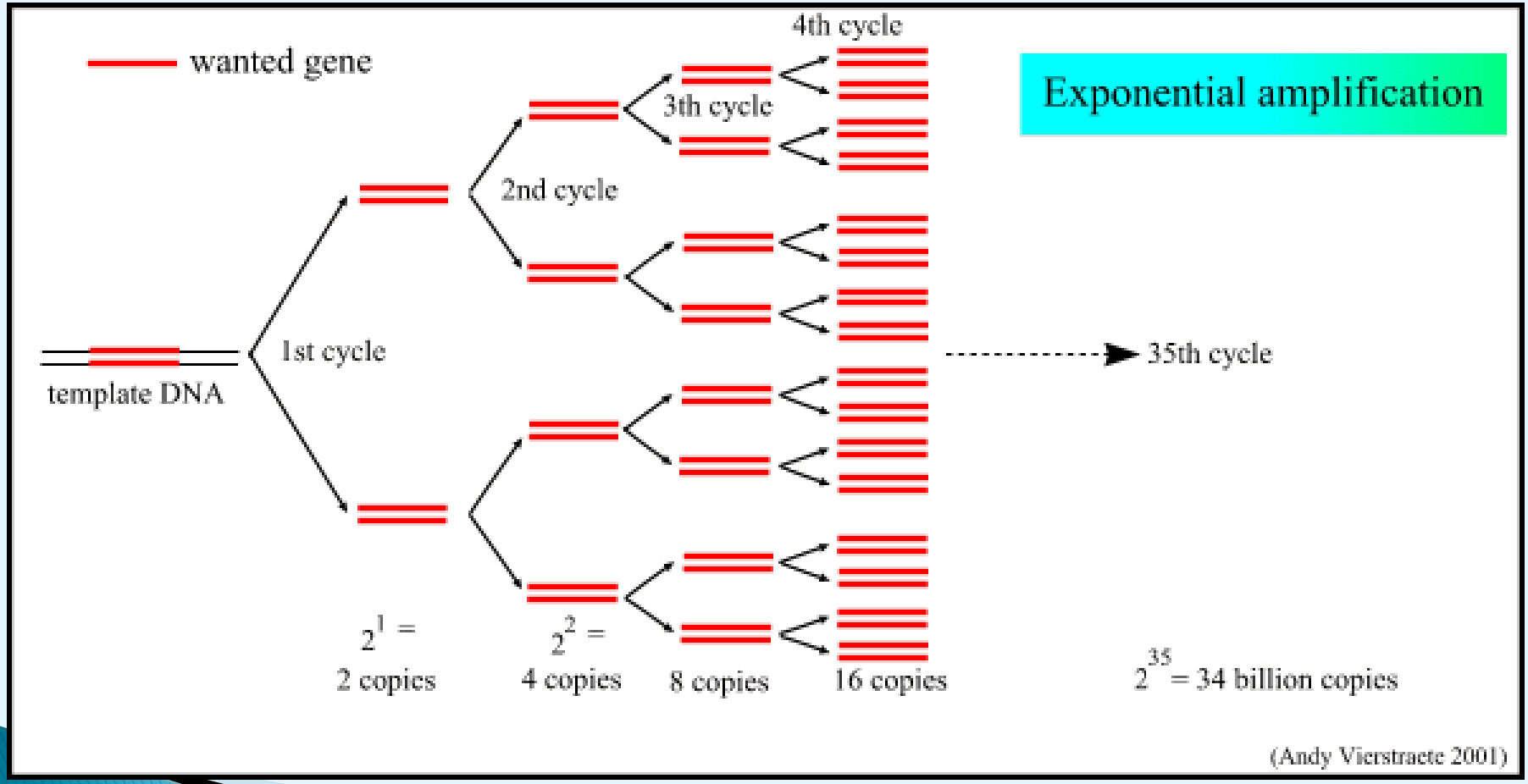
only dNTP's

(Andy Vierstraete 1999)

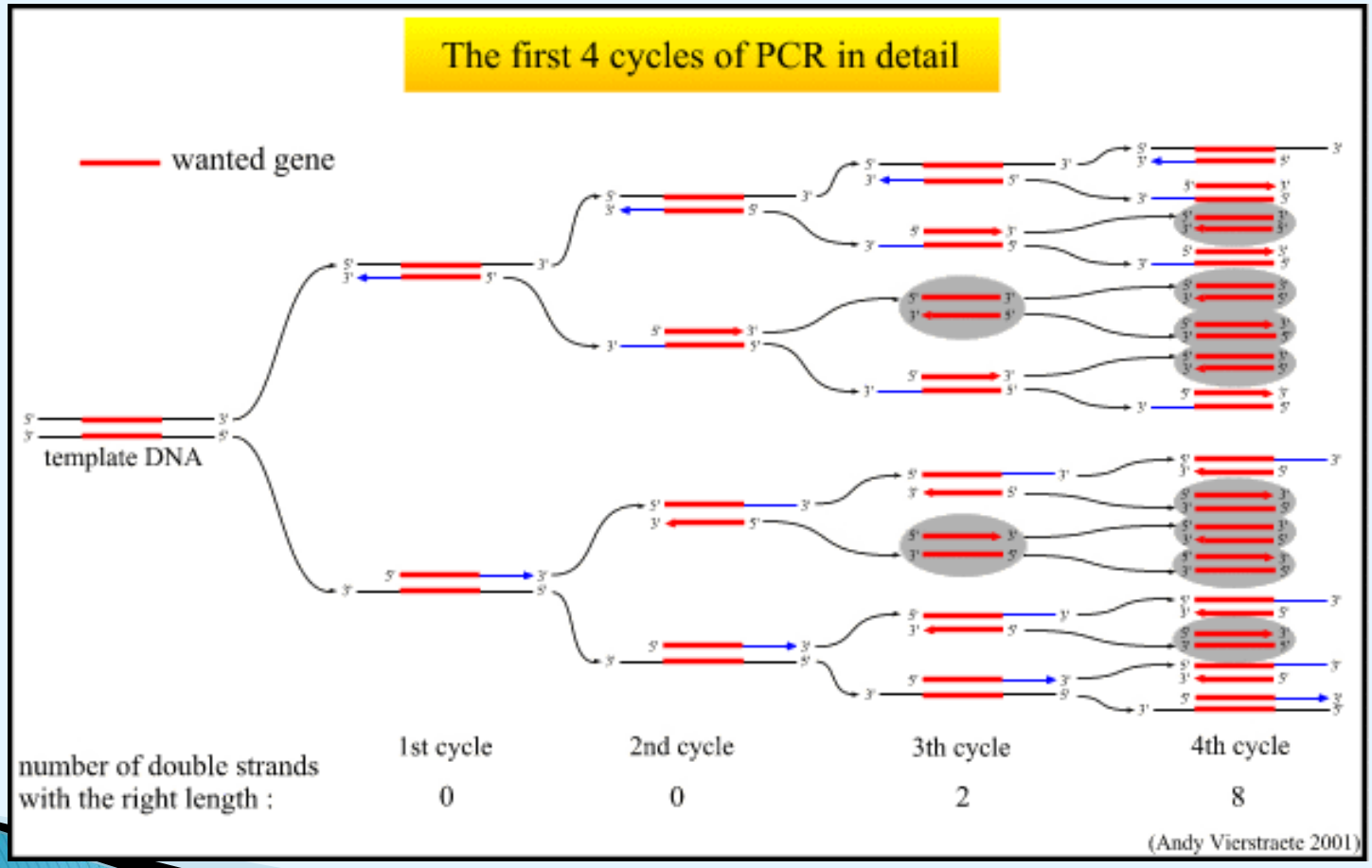
PCR


PCR :
Denaturation 94°C
100
90
80
70
60
50
(Andy Vierstraete 1999)

PCR


wanted gene
4th cycle
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
Exponential amplification
2^1 =
2 copies
2^2 =
4 copies
8 copies
16 copies
2^{35} = 34 billion copies
(Andy Vierstraete 2001)

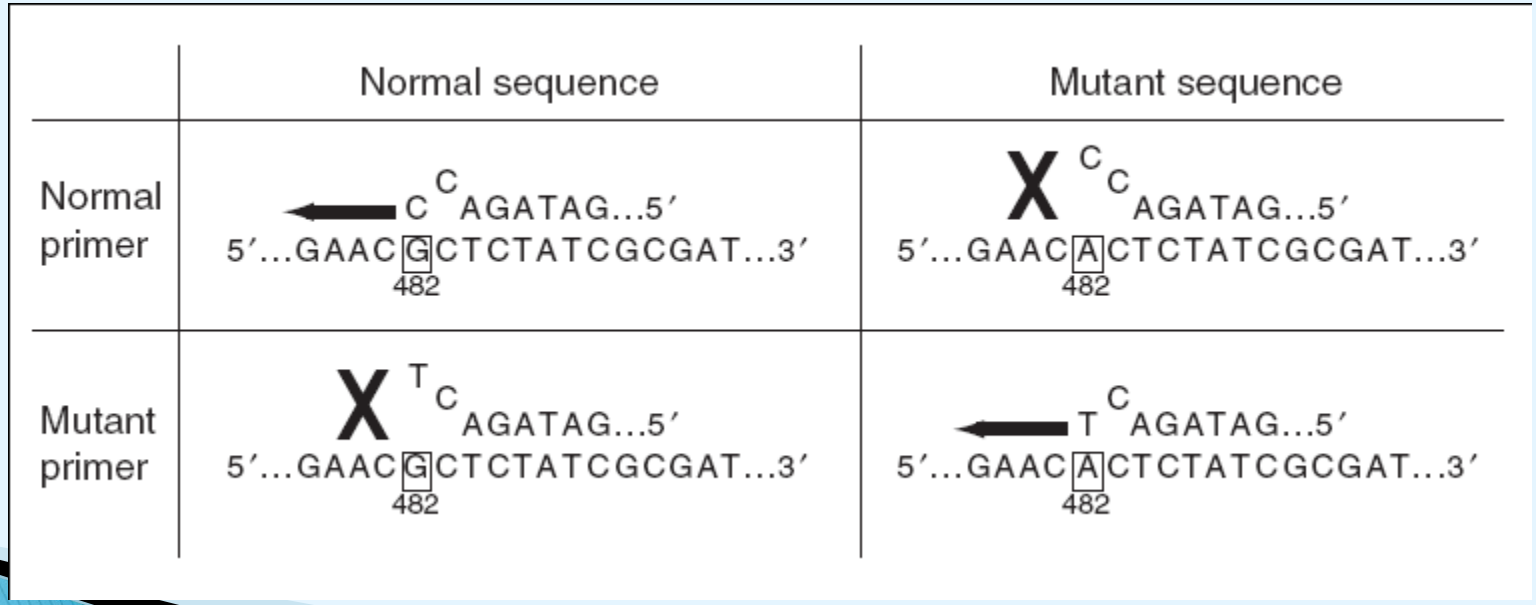
PCR


The first 4 cycles of PCR in detail
wanted gene
template DNA
1st cycle
2nd cycle
3th cycle
4th cycle
number of double strands with the right length :
0
0
2
8
(Andy Vierstraete 2001)

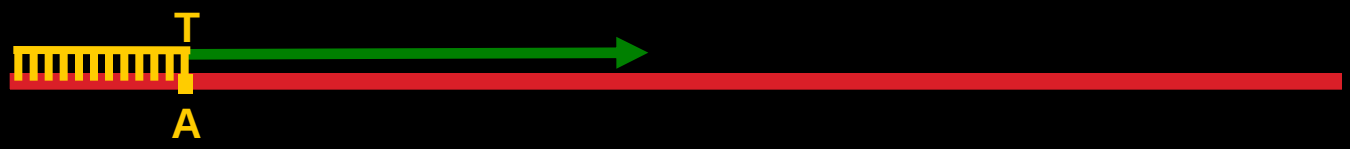
ARMS-PCR

Points in primer design

- ARMS primer sequences for a single nucleotide
- normal and mutant DNA sequences
- also known as allele-specific PCR (ASPCR)
- presence or absence of a PCR product is diagnostic for the presence or absence of the target allele

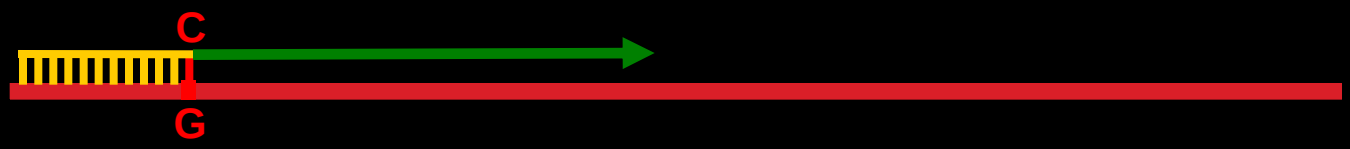


Wt primer + Wt Sample DNA: PCR product



Wt primer + Mutant Sample DNA: No Production

Mutant primer + Mutant Sample DNA: PCR product

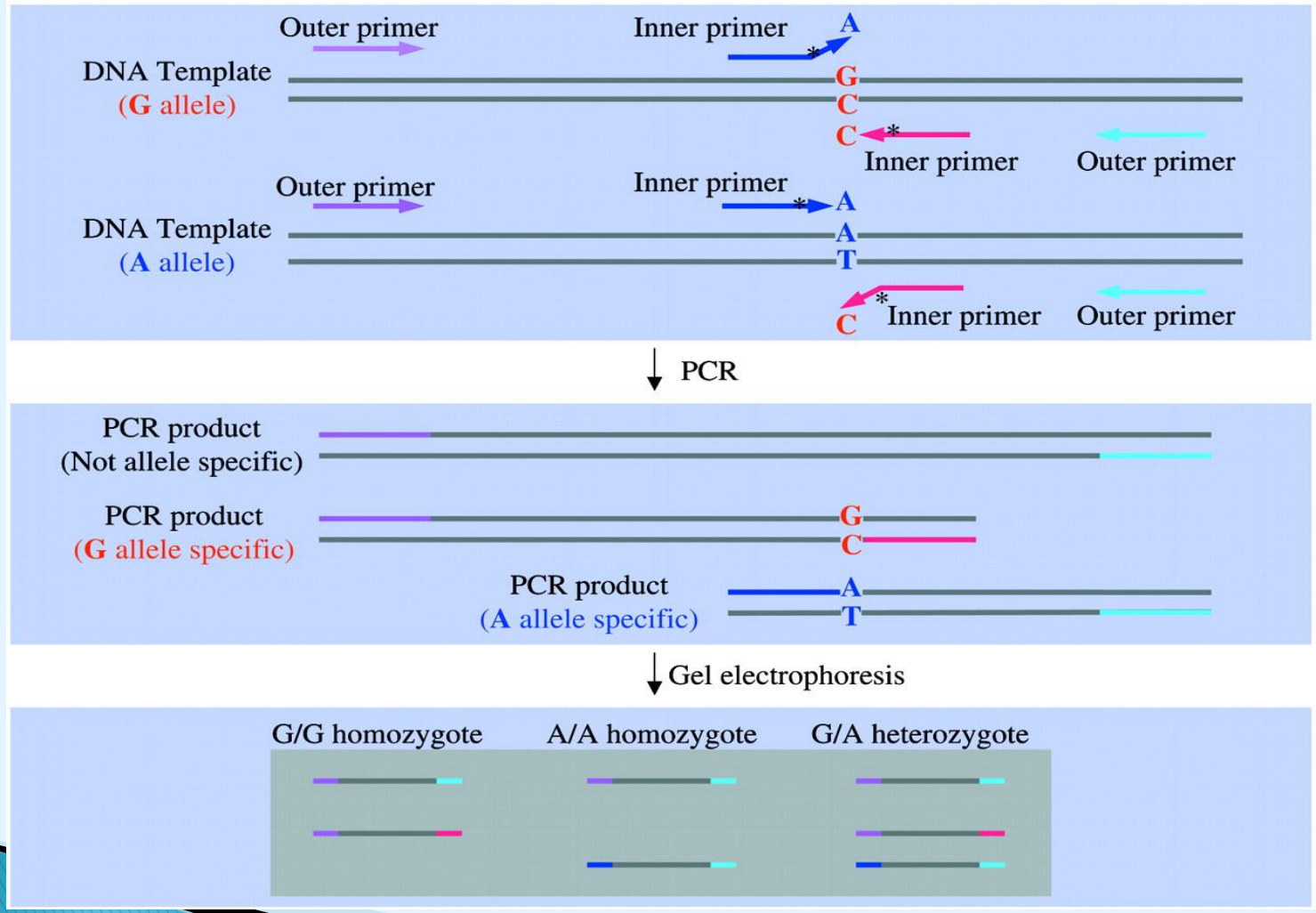


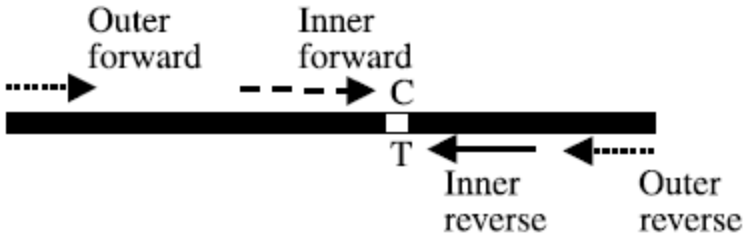
Mutant primer + Wt Sample DNA: No Production

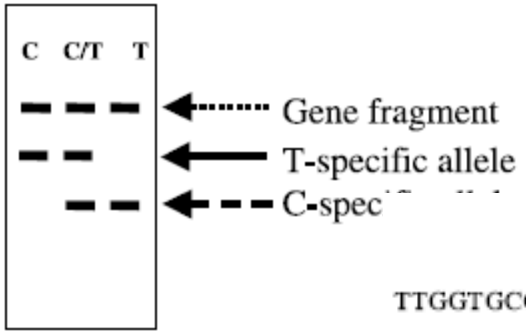
- oligonucleotides of around 30 or more bases
- Primers less than 28 bases long should be avoided
- Longer primers (up to 60-mers) may be used.
- For the mutant-specific primer (M), the 3 ' terminal base of the ARMS primer should be complementary to the mutation
- for the normal-specific primer (N), the 3 ' terminal base should be complementary to the corresponding normal sequence

- To enhance the specificity: a destabilizing mismatch incorporated at the 3rd nucleotide from the 3'terminus in each primer
- to increase the specificity of the ARMS reaction
- A single mismatch at the penultimate base is not sufficient to prevent extension
- A primer with two mismatches: is not extended

Tetra-primer ARMS- PCR







……..TTGTG TCAAGCATAT CGGTTGCTCT TGCTATCGTT

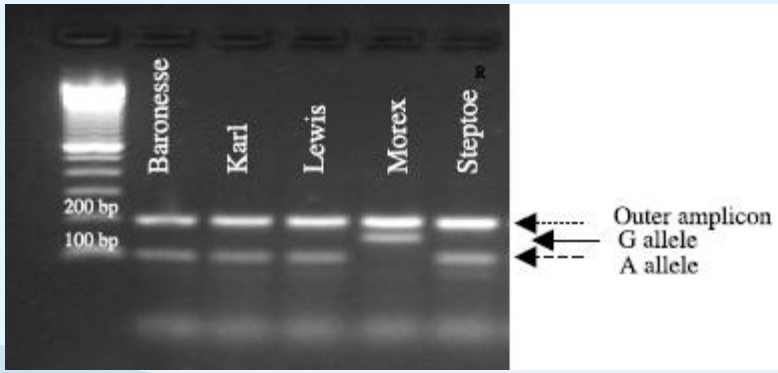
TTGGTGCGCA A TCTATGGGG G TAT GCATAC AGCAA CGACA AGGAAGT GGT GGAGT ACATT

TCA AGAATTA TGCCAATTA T TGGCGTGGC **R** TTCTTGTTTG ATGACATGCA GTGTGTTCTT

TCA GGTATTA TA AGGAGGAT CCTA TTGTTT TCGAACGTGC TGGAAATA TG CGACTTTA TT

AATA AGCATA TTTTTGCAGG TA TTGTTA GG GGCTGCGGCT TTCAAAAGAT TGGCTCCTA T

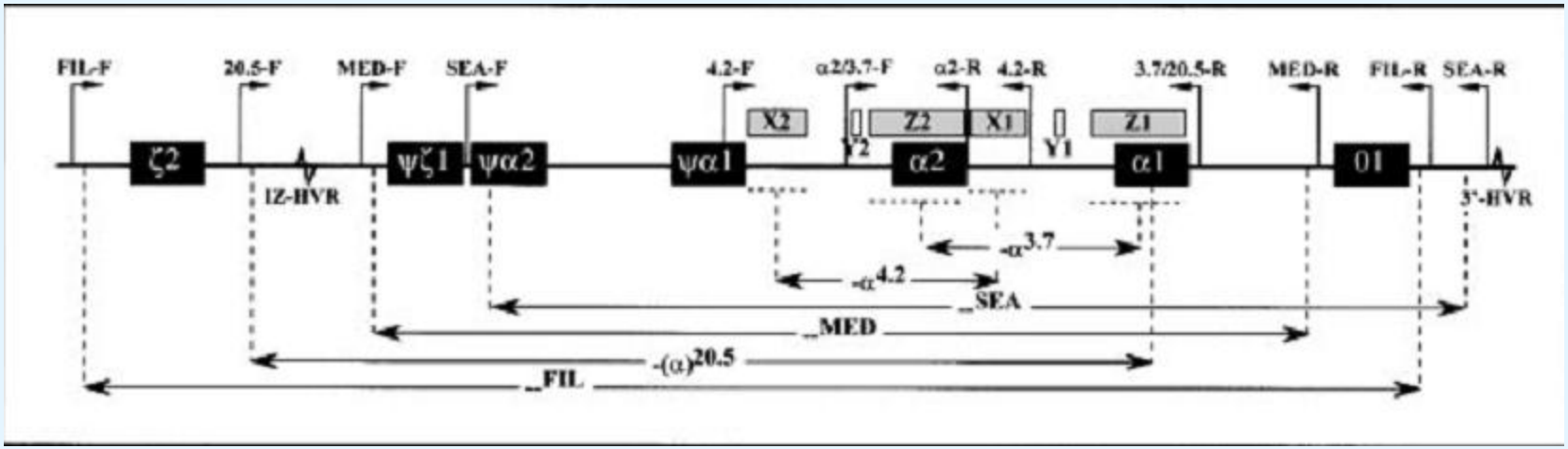
GTCAAT CTTA GTGCGTACTA CCT.....

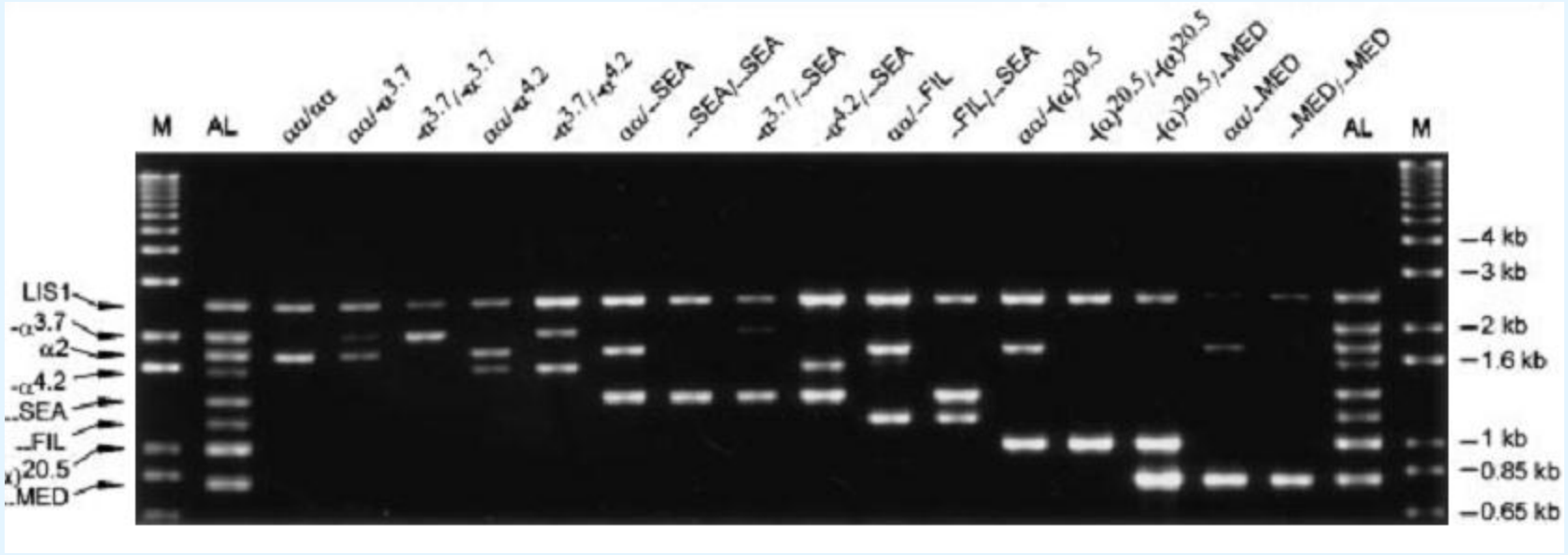


Gap PCR

Points in primer design

- using oligo-primers flanking deletion breakpoints
- to detect known deletion mutations
- based upon the inability of PCR primers that are far apart to direct amplification
  - unless a deletion brings them closer together
- primer pairs are designed to flank a known deletion
- primer in border of deletion
  - mostly in normal area and 2 to 3 nucleotide in deletion area
- generating a unique amplicon that will be smaller in the mutant sequence compared with the wild type
- presence or absence of PCR product is detected by electrophoresis


FIL-F
20.5-F
MED-F
SEA-F
4.2-F
α2/3.7-F
α2-R
4.2-R
3.7/20.5-R
MED-R
FIL-R
SEA-R
ζ2
ψζ1
ψα2
ψα1
X2
Z2
X1
Z1
Y2
Y1
α2
α1
01
IZ-HVR
3'-HVR
-α3.7
-α4.2
--SEA
--MED
-(α)20.5
--FIL


M
AL
αα/αα
αα/-α3.7
-α3.7/-α3.7
αα/-α4.2
-α3.7/-α4.2
αα/--SEA
--SEA/--SEA
-α3.7/--SEA
-α4.2/--SEA
αα/--FIL
--FIL/--SEA
αα/-(α)20.5
-(α)20.5/-(α)20.5
-(α)20.5/--MED
αα/--MED
--MED/--MED
AL
M
LIS1
-α3.7
α2
-α4.2
--SEA
--FIL
-(α)20.5
--MED
4 kb
3 kb
2 kb
1.6 kb
1 kb
0.85 kb
0.65 kb

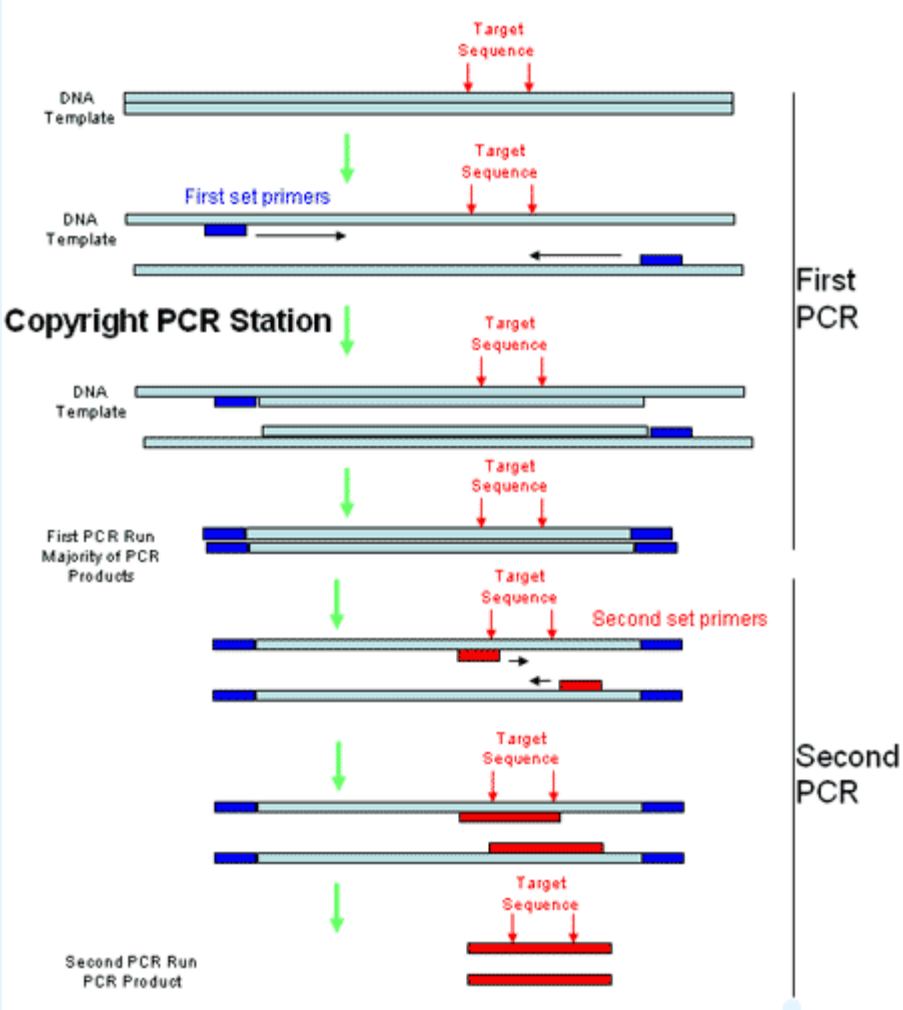
Nested PCR

Points in primer design

Nested PCR

- second round of amplification on a fragment which is already the result of a PCR reaction
- In the "2nd" round reaction, the set of primers will be internal to those of the first round, thus amplifying a slightly shorter fragment

Nested polymerase chain reaction



Nested polymerase chain reaction

- intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites
- Nested PCR:
  - two sets of primers
  - used in two successive runs of PCR
  - the second set intended to amplify a secondary target within the first run product

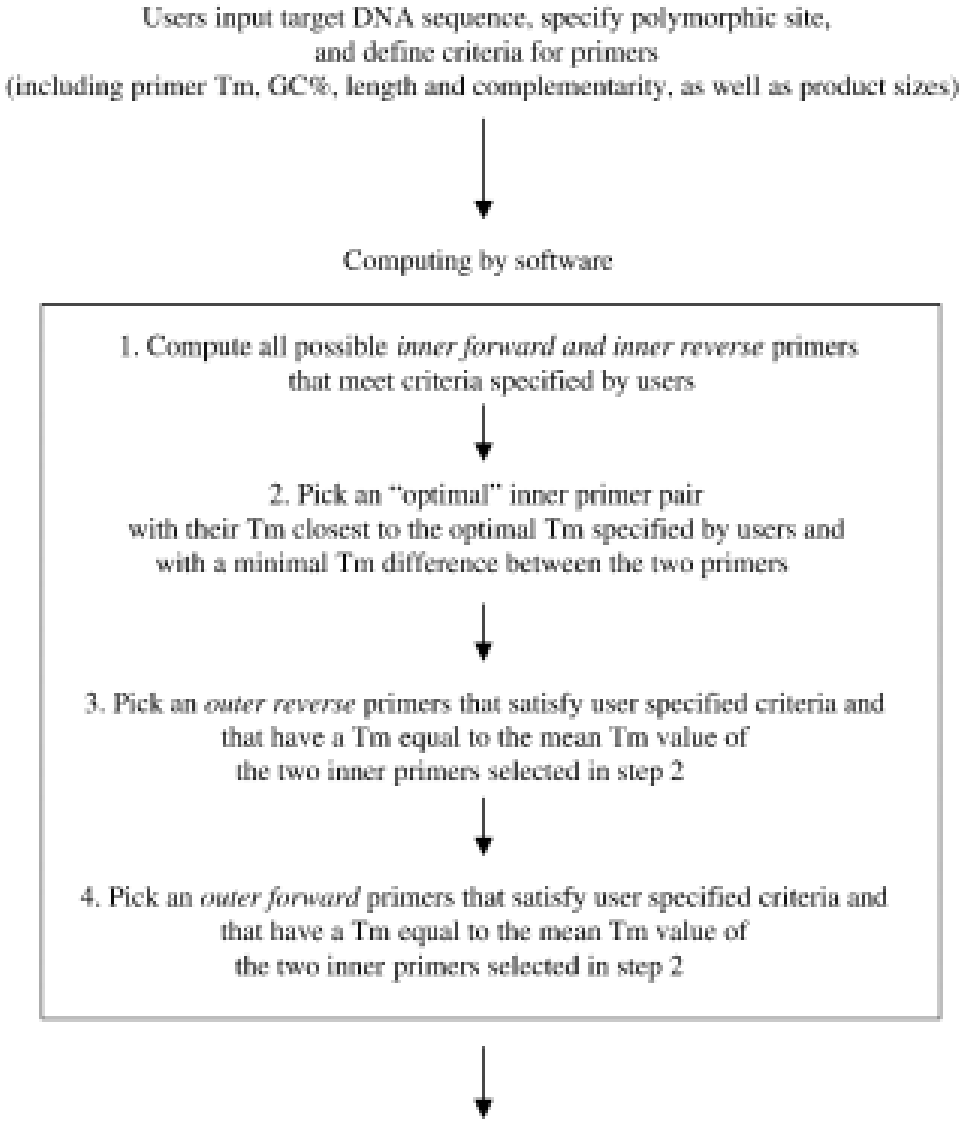
- target DNA undergoes the first run of PCR with the first set of primers
- ensuring the product from the second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences

Multiplex PCR

- Multiple primer pairs can be added in the same tube to do the PCR
- Good for amplifying multiple sites
- Design difficulty
  - Melting temperatures should be similar
  - No primer dimer
  - Consider primer interaction

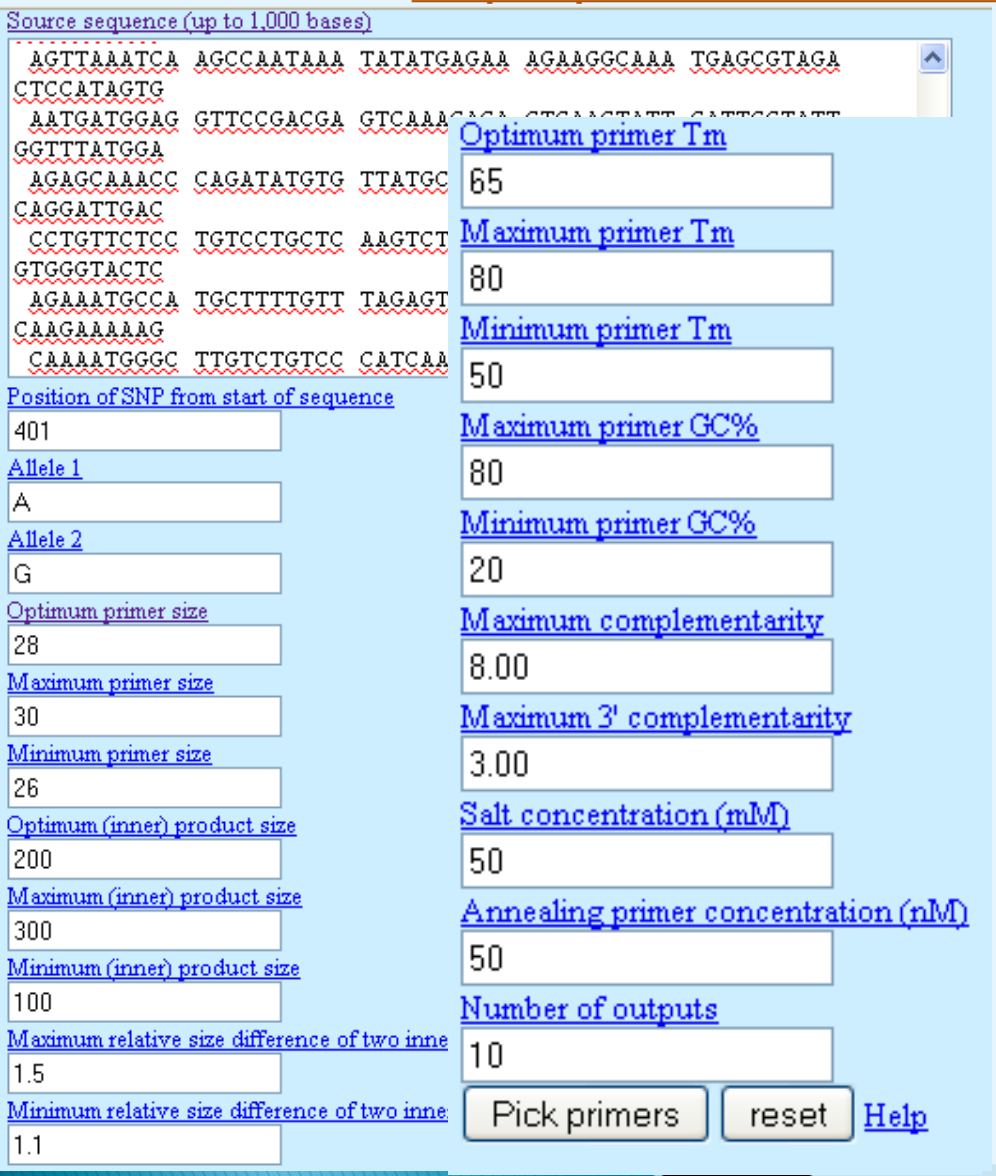
Primer 1

Designing primer for ARMS-PCR


Users input target DNA sequence, specify polymorphic site,
and define criteria for primers
(including primer Tm, GC%, length and complementarity, as well as product sizes)
Computing by software
1. Compute all possible inner forward and inner reverse primers
that meet criteria specified by users
2. Pick an "optimal" inner primer pair
with their Tm closest to the optimal Tm specified by users and
with a minimal Tm difference between the two primers
3. Pick an outer reverse primers that satisfy user specified criteria and
that have a Tm equal to the mean Tm value of
the two inner primers selected in step 2
4. Pick an outer forward primers that satisfy user specified criteria and
that have a Tm equal to the mean Tm value of
the two inner primers selected in step 2
Output:
Either primer details or error messages

PRIMER1: primer design for tetra-primer ARMS-PCR

http://primer1.soton.ac.uk/primer1.html



- Input target DNA sequence (FASTA)
- specify the polymorphic site
- define criteria for the primers (T_m, %GC, length and complementarity) and product sizes

```
**********OUTPUT 1**********
Forward inner primer (G allele):                    Melting temperature
372 GTCTGGTATATCTAACTAATCATATAACCG 401                       57

Reverse inner primer (A allele):
427 CATGCTACACAGTCTAAGATGAAATAT 401                          57

Forward outer primer (5' - 3'):
162 CTGATTTTAAGCATTAAGTATGGATATC 189                         57

Reverse outer primer (5' - 3'):
565 AATGAATAGTTCAGTGTCTTTGACTC 540                           57

Product size for G allele: 195
Product size for A allele: 266
Product size of two outer primers: 404
```

Primer 3

Designing primer for ARMS-PCR

BatchPrimer 3

- http://probes.pw.usda.gov/batchprimer3/

- Choose primer type
- Tetra Primers ARMS-PCR primers
- Input Sequences: Fasta/upload/browse/ Upload sequence file in FASTA format
- IUPAC code
- Pick Primers

BatchPrimer3

a high-throughput web tool for picking PCR and sequencing primers

Choose primer type: Tetra-primer ARMS-PCR primers

Design tetra primer ARMS PCR primers.

Pick Primers

Reset the entire form

Input Sequences: (the maximum of 500 sequences at a time will be processed)

Upload sequence file in FASTA format: :\Documents and Settings\Guity\Desktop\rs7202116.txt Browse...

OR copy/paste source sequences in FASTA format. Example sequences | Pre-analysis of input sequences | Clear sequence

```
>gnl|dbSNP|rs7202116|allele
Pos=401|totalLen=801|taxid=9606|snpclass=1|alleles='A/G'|mol=Genomic|build=135

 GAAGTGGGAA AGCAGGTTAA TAGGTTCTTC TTAATGGAAA ATGCAGCCAA TATTGGCCAA
 CTTACTTTGA TTTCGGTAGT CATAACACCA CCCTGGAAGG CACCCTAGAT AGAGGTCACT
 TGCTACCACT CATTTTACAG ATCAGGATAC TAAGGATTTT CCTGATTTTA AGCATTAAGT
 ATGGATATCC CTGTTGGTTG AAGTTAAATT GGTCAACTAG AATTTAAAAG CAAAAATTAA
 AAAAAAATTA TTTTGTATTA GGTTTCAAAG GAATTGTTGT CAGTAGGAGA AGCCTGATTG
 TTCCTTTTAC GCTGACTCAT ACAGTTTCAG CAGATTACAT TTGAGGCCTA ATGTTGAAAT
 CTCATCTGTA AGTCTGGTAT ATCTAACTAA TCATATAAAC
 R
```

Tetra Primer ARMS PCR Primer Settings

Primer Size	Min: 20	Opt: 25	Max: 30		
Primer Tm	Min: 60	Opt: 68	Max: 80		
Max Tm Difference:	5				
Primer GC%	Min: 20	Max: 80			
Inner product size	Min: 100	Opt: 200	Max: 400		
Relative size difference between inner product sizes	Min: 1.0	Max: 1.6			
Max #N's:	0				
Salt Concentration:	50	DNA Concentration:	50		
Max Self Complementarity:	8	Max 3' Self Complementarity:	3		

Report of BatchPrimer3 Primer Design

Help | View primers in HTML table format | View primers in tab-delimited table format
Save as a tab-delimited text file | Save as an Excel file | Primer Report Statistics | Download entire results (a zip file)

Primer type: Tetra-primer ARMS PCR primers

Sequence Index: 1
Sequence ID: gnl|dbSNP|rs7202116|allelePos=401|totalLen=801|taxid=9606|snpclass=1|alleles='A/G'|mol=Genomic|build=135

Outer primers for tetra-primer ARMS PCR:

	Orientation	Start	Len	Tm	GC%	Any compl	3' compl	Primer Seq	Product Size	Seq Size	Included Size	Pair_any	Pair_3'
1	FORWARD	369	24	46.71	29.17	6.00	0.00	TAAGTCTGGTATATCTAACTAATC	71	801	801	4.00	1.00
	REVERSE	439	19	47.35	47.37	5.00	2.00	GTCTACTAGGGACATGCTA					

Inner primers for tetra-primer ARMS PCR:

	Orientation	Start	Len	Tm	GC%	Any compl	3' compl	Score	SNP	Pos	Primer Seq	Product Size	Seq Size	Included Size	Pair_any	Pair 3'
1	FORWARD	377	25	46.93	24	6.00	2.00	76.18	A	401	GTATATCTAACTAATCATATAACCA	63	801	801	3.00	1.00
2	REVERSE	425	23	47.97	34.78	5.00	1.00	71	G	401	CTACACAGTCTAAGATGAAATAC	55	801	801	5.00	3.00

Primer Report Statistics

Total sequences input: **1**

Number of sequences with sucessful primer sets: **2**

Number of sequences without primer picked: **0**

Total primer sets picked: **1**

Used time: **1** seconds.

Primer BLAST

Designing primer for PCR

Design PCR primers and check them for specificity

- **A TARGET TEMPLATE SEQUENCE OR ACCESSION NUMBER**
- Go to the Primer BLAST submission form.
- Enter the target sequence in FASTA format or an accession number of an NCBI nucleotide sequence in the PCR Template section of the form
- Click the "Get Primers" button to submit the search and retrieve specific primer pairs.