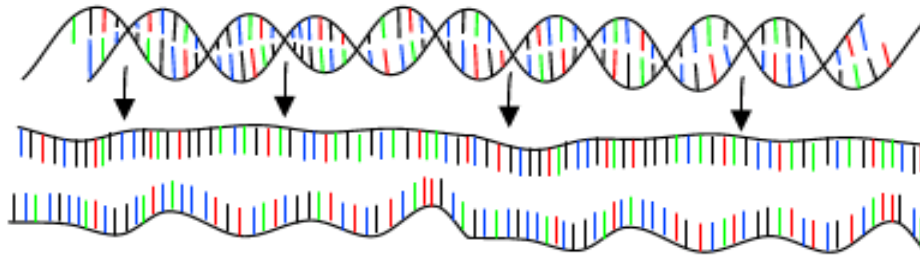


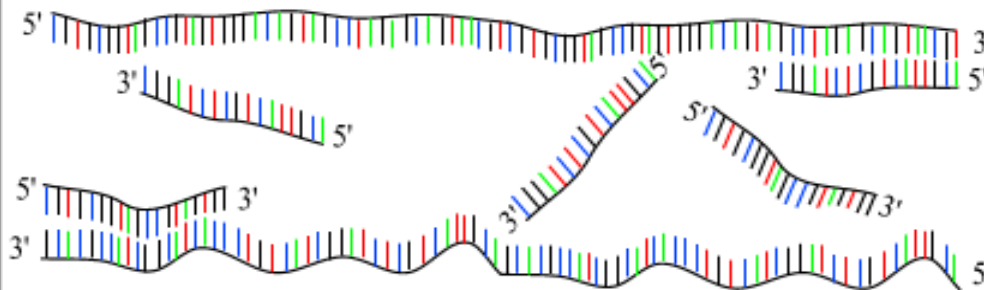
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

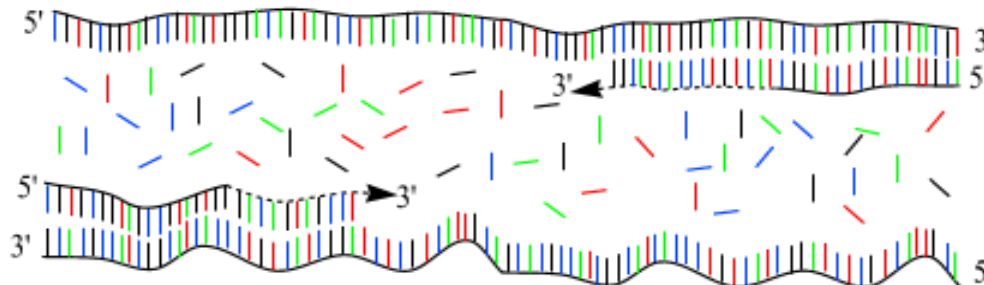
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!

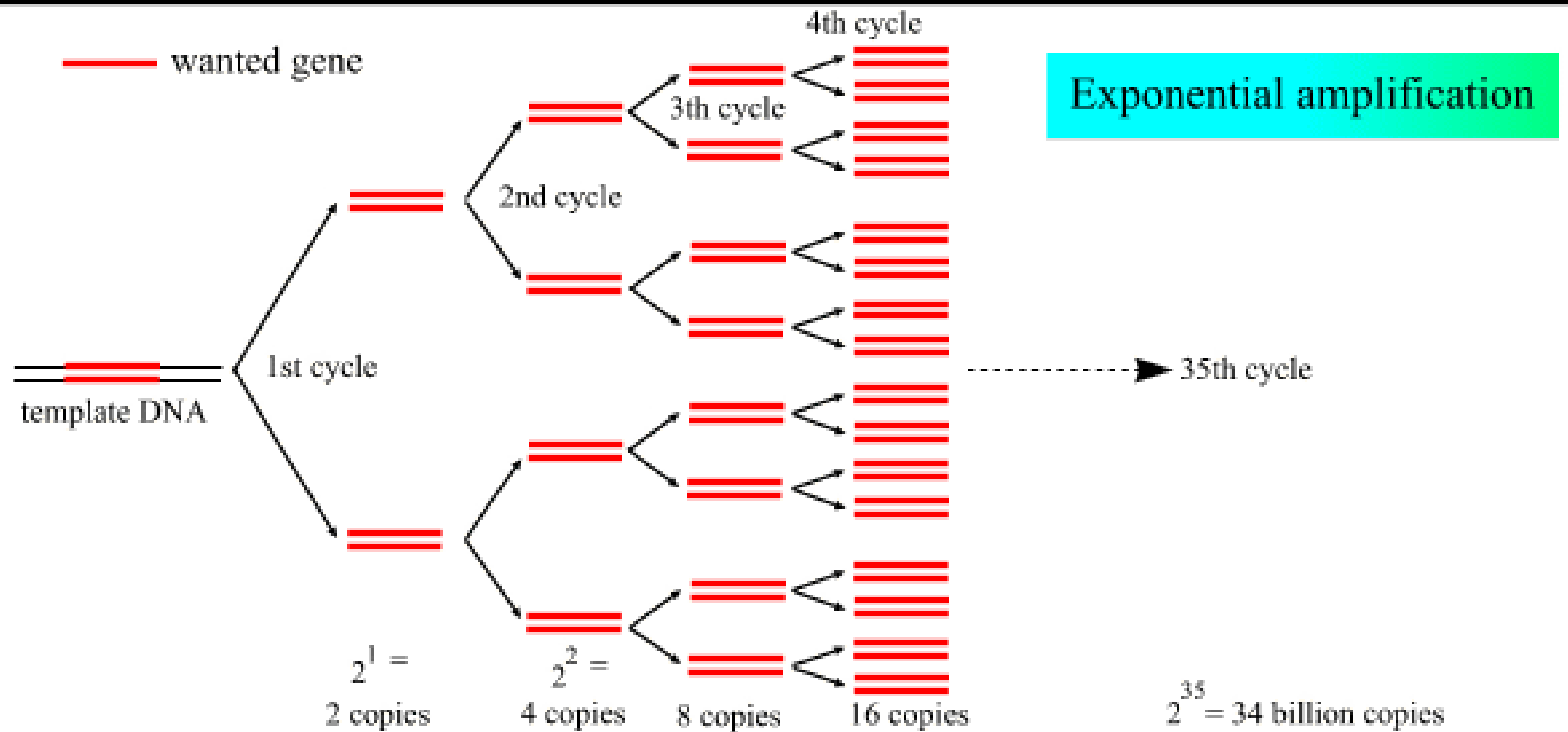


Step 3 : extension

2 minutes 72 °C

only dNTP's

PCR

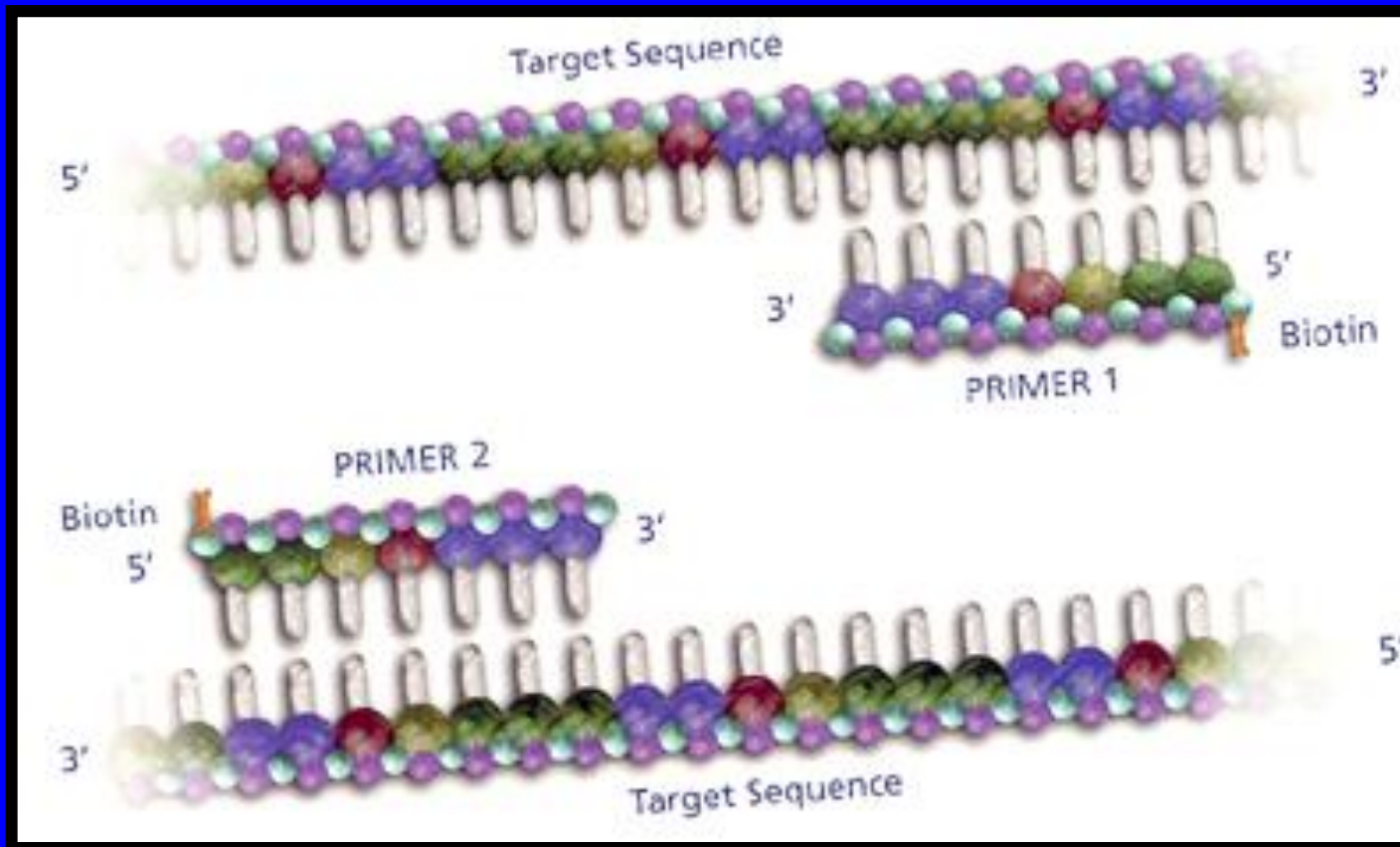


(Andy Vierstraete 2001)

Primer (molecular biology)

- A primer is a strand of short nucleic acid sequences (generally about 10 base pairs) that serves as a starting point for DNA synthesis.
- The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand.
- PCR primer design is the creation of short nucleotide sequences for use in amplifying a specific region of DNA.

PCR Primer Design



<http://www.modares.ac.ir/elearning/mnaderi/Genetic%20Engineering%20course%20II/images/2a.jpg>

Summary ~ Primer Design Criteria

1. Primer Length:

It is generally accepted that the optimal length of PCR primers is 18-22 bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature.

The Base composition: average (G+C)

- **Optimal G/C content: 45-55%**
- **Common G/C content range: 40-60% ,**
- **and avoid long (A+T) and (G+C) rich region if possible;**

Summary ~ Primer Design Criteria

2. Primer Melting Temperature:

the one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. Primers with melting temperatures in the range of 52-58 °C generally produce the best results. The GC content of the sequence gives a fair indication of the primer T_m .

- The melting temperature (T_m) of the primers used are not more than 5°C different from each other.
- Calculate T_m with this formula:

$$T_m = 4(G + C) + 2(A + T)^{\circ}\text{C}$$

Summary ~ Primer Design Criteria

3. Primer Annealing Temperature:

The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a may possibly lead to non-specific products caused by a high number of base pair mismatches

$$T_a = 0.3 \times T_m(\text{primer}) + 0.7 T_m(\text{product}) - 14.9$$

Summary ~ Primer Design Criteria

4. GC Content: The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.

5. GC Clamp: The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

Summary ~ Primer Design Criteria

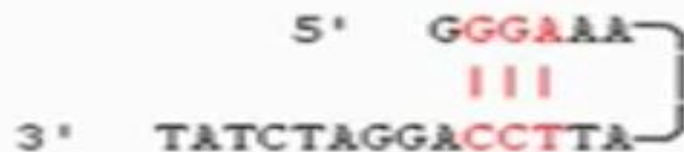
6. Primer Secondary Structures: Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification. They greatly reduce the availability of primers to the reaction.

6. Primer Secondary Structures

A.) Hairpins: It is formed by intramolecular interaction within the primer and should be avoided.

Hairpin

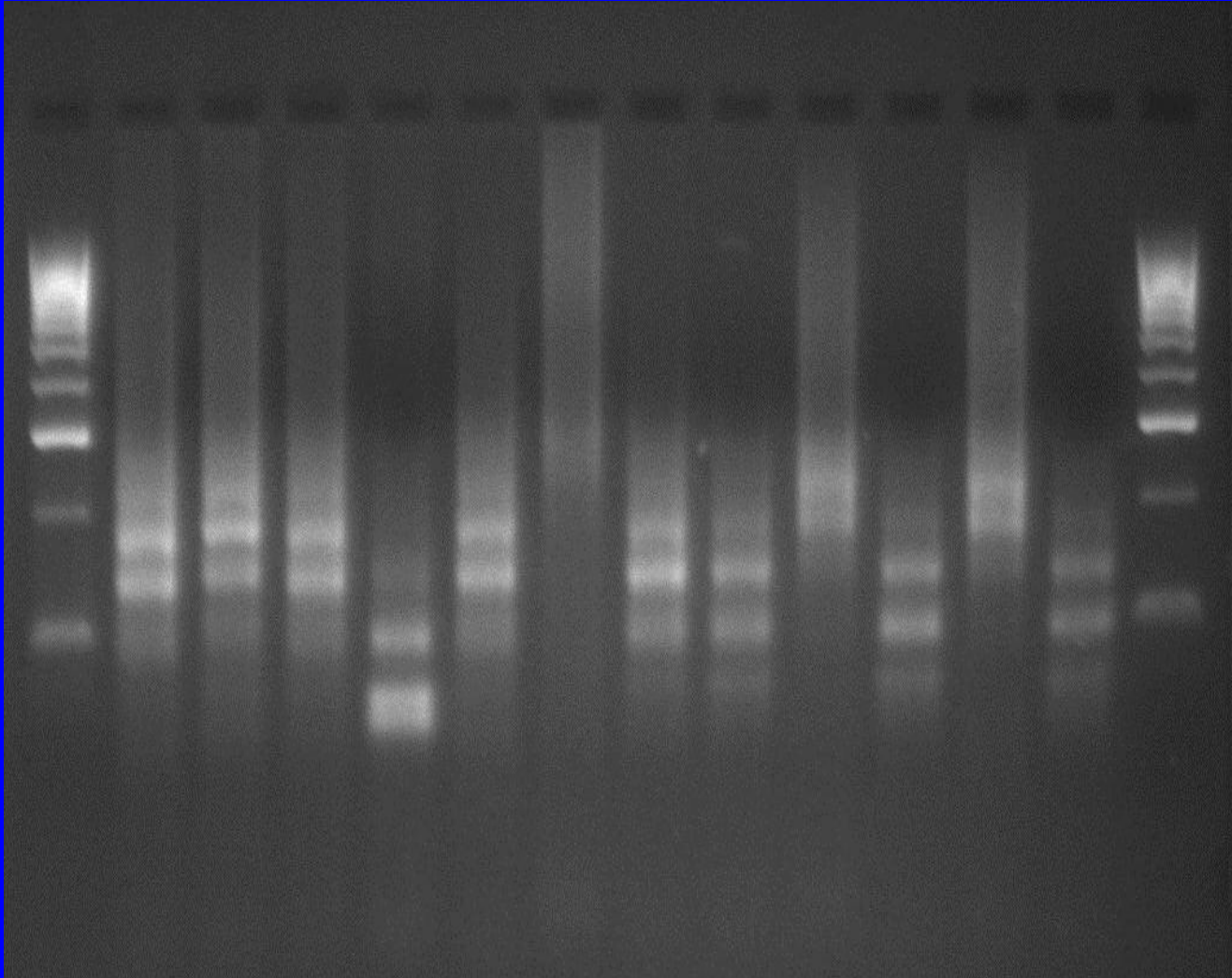
Oligo, 3 bp (Loop=4), $\Delta G = -0.1 \text{ kcal/mol}$



Oligo, 2 bp (Loop=3), $\Delta G = 2.1 \text{ kcal/mol}$



A- Hairpins



6. Primer Secondary Structures

B) Self Dimer: A primer self-dimer is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Generally a large amount of primers are used in PCR compared to the amount of target gene. When primers form intermolecular dimers much more readily than hybridizing to target DNA, they reduce the product yield.

B- Self-dimer

Self-Dimer

4 bp, delta G = -6.6 kc/m (bad!) (worst= -36.6)

5' GGGAAAATTCCAGGATCTAT 3'

|||| |

3' TATCTAGGACCTTAAAAGG 5'

4 bp, delta G = -5.4 kc/m (bad!) (worst= -36.6)

5' GGGAAAATTCCAGGATCTAT 3'

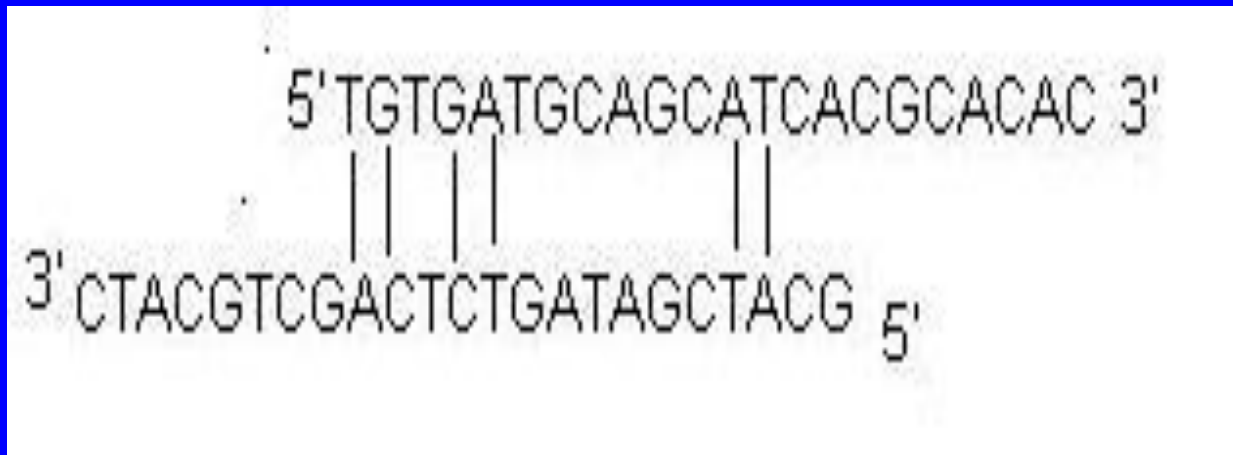
||||

3' TATCTAGGACCTTAAAAGG 5'

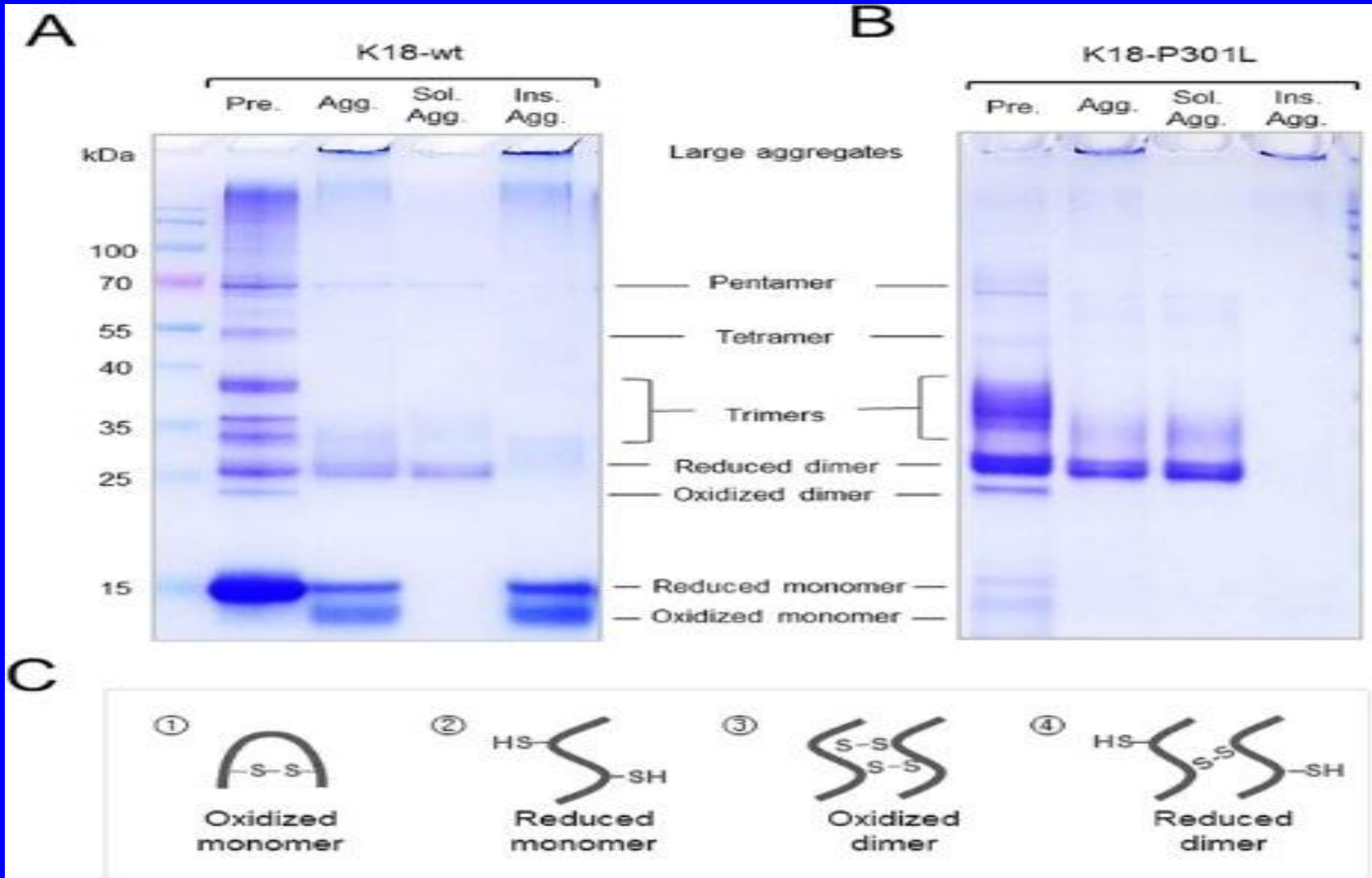


6. Primer Secondary Structures

C) Cross Dimer: Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous.



C- Cross Dimer



Computer-Aided Primer Design

Some primer design programs are :-

❖ **Primer3**

❖ **Primer3Plus**

❖ **PrimerZ**

❖ **PerlPrimer**

❖ **Vector NTI Advantage 10**

Steps of primer design

<http://www.ncbi.nlm.nih.gov/>

<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>

<http://www.mutationdiscovery.com/md/MD.com/screens/optimase/OptimaseInput.html?action=none>

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Search results

Items: 1 to 20 of 5969

See also 4684 discontinued or replaced items.

Name/Gene ID	Description	Location	Aliases
<input type="checkbox"/> ECU11_1520 ID: 860112	similarity to HYPOTHETICAL METHYLTRANSFERASE-LIKE PROTEIN YGGH_ECOLI [<i>Encephalitozoon cuniculi</i> GB-M1]	Chromosome XI, NC_003237.1 (194605..195336)	ECU11_1520
<input type="checkbox"/> ECU07_1170 ID: 859476	similarity to HYPOTHETICAL PROTEINS YJBN_ECOLI and Y926_YEAST [<i>Encephalitozoon cuniculi</i> GB-M1]	Chromosome VII, NC_003234.1 (140583..141656)	ECU07_1170
<input type="checkbox"/> ECU07_0510 ID: 859408	similarity to HYPOTHETICAL PROTEIN YBET_ECOLI [<i>Encephalitozoon cuniculi</i> GB-M1]	Chromosome VII, NC_003234.1 (67846..69222, complement)	ECU07_0510
<input type="checkbox"/> piIV ID: 24955882	similar to SHU1_ECOLI,sp:P09745 [<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i>]	NC_023290.1 (97119..98555, complement)	p9134dT_0460
<input type="checkbox"/> traG ID: 24955878	similar to TraG of plasmid R64, 41 pct identical(1 gap) to 174 residues of 200 aa protein sp:AIS_ECOLI [<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i>]	NC_023290.1 (91425..92009, complement)	p9134dT_0430
<input type="checkbox"/> resA ID: 24955835	similar to REDM_ECOLI,sp:P18021 [<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i>]	NC_023290.1 (32553..33332)	p9134dT_0145

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Find items

Search details
ecoli[All Fields] AND alive[prop]
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ECU11_1520 similarity to HYPOTHETICAL METHYLTRANSFERASE-LIKE PROTEIN YGGH_ECOLI [*Encephalitozoon cuniculi* GB-M1]

Gene ID: 860112, updated on 26-Jun-2015

Summary

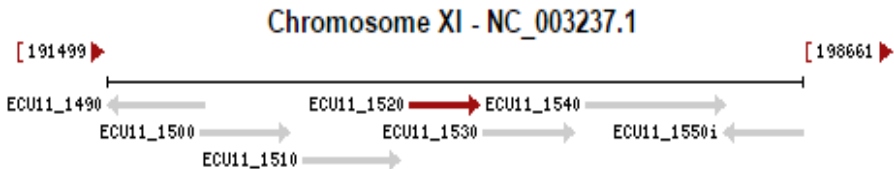
Gene symbol	ECU11_1520
Gene description	similarity to HYPOTHETICAL METHYLTRANSFERASE-LIKE PROTEIN YGGH_ECOLI
Locus tag	ECU11_1520
Gene type	protein coding
RefSeq status	PROVISIONAL
Organism	Encephalitozoon cuniculi GB-M1 (strain: GB-M1)
Lineage	Eukaryota; Fungi; Microsporidia; Unikaryonidae; Encephalitozoon

Genomic context

Location: chromosome: XI

Exon count: 1

Sequence: Chromosome: XI; NC_003237.1 (194605..195336)



Genomic regions, transcripts, and products

Genomic Sequence: NC_003237.1



Go to [reference sequence details](#)

Go to nucleotide: [Graphics](#) [FASTA](#) [GenBank](#)

FASTA ▾

Send: ▾

Encephalitozoon cuniculi GB-M1 chromosome XI, complete sequence

NCBI Reference Sequence: NC_003237.1

[GenBank](#) [Graphics](#)

>gi|17158053:194605-195336 Encephalitozoon cuniculi GB-M1 chromosome XI, complete sequence

```
ATGCAGATGGGAAATGGGATCAAGAAAAGCCTTTACAGACAAAGAGCCCACGCCAATCCATTCAAGGACA
GCAACATCACCGTTCTCTCCGAGCCCTCAGCTTATAGACTGGTCTTCTTACTTCAGAATGAATAGAAGGCC
GGATTTTCGTTGATATCGGGTGTGGATACGGAAAGTTCCTTATGAAGGTTGCCGAGAAGAATCCAGAGCAC
AGCATACTGGGCCTCGAGATCAGAGACAAAGTGTGTGAGTATGTTAAGGCAAGGATAGAAGCCGCCGGGA
TTCCAAATGCCGGGGTCATGAGAACAAATGCATTGATTTTCTCCCGAACATATTCAGTAGAGGCCAGCT
GACCAAGATCTTTATTTTATTCCCCGACCCTCACTTCAAAAAGAGGAAGCAAAAGGGGCGCATTGTGTGC
CGGCAGATGATGGAGATGTACGAGTACCTGCTAGCGGACGGGGGAAAGTTGTATATATCTACCGACGTGA
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AGTGGATGGAGATGAGTTGTTCCATATGATTTCTAGGGACACGGACGAGGCGCTGAGGGCCGGAGCCAAG
GCAGGAAAGGTGTTTTTGAAGGTTTTTGAATAAAGAAAGATTGGCACTACTCCTTTCATCTTCGACTCC
AGAGGAGTATTGGGCAAAGCAGCTCCTTTTGA
```

copy to
Primer3Plus



<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>

Primer3Plus

pick primers from a DNA sequence

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Load server settings:

Default

Activate Settings

Task: generic

Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.

Pick Primers

Reset Form

Main

General Settings

Advanced Settings

Internal Oligo

Penalty Weights

Advanced Sequence

Sequence Id:

Paste template sequence below

Or upload sequence file:

Choose File

No file chosen

Upload File

sequence

```
ATGCAGATGGGAAATGGGATCAAGAAAAGCCTTTACAGACAAAGAGCCCACGCCAATCCATTCAAGGACA
GCAACATCACCGTTCCTCCGAGCCCTCAGCTTATAGACTGGTCTTCTTACTTCAGAATGAATAGAAGGCC
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TTCCAAATGCCGGGGTCATGAGAACAAATGCATTGATTTTCTCCCGAACATATTCAGTAGAGGCCAGCT
GACCAAGATCTTTATTTTATTTCCCGACCCTCACTTCAAAAAGAGGAAGCAAAAGGGGCGCATTGTGTGC
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AGTGGATGGAGATGAGTTGTTCCATATGATTTCTAGGGACACGGACGAGGCGCTGAGGGCCGGAGCCAAG
GCAGGAAAGGTGTTTTGAAAGGTTTTGAAATAAAGAAAGATTGGCACTACTCCTTTCATCTTCGACTCC
AGAGGAGTATTGGGCAAAGCAGCTCCTTTTGA
```

Mark selected region:

< >

[]

{ }

Clear

Save Sequence

Excluded Regions:

<

>

Targets:

[

]

Included Region:

{

}

Primer overlap positions:

-

click

< Back

☒ Pair 1: Primer

Left Primer 1: GCATACTGGGCCTCGAGATC

Start: 220 Length: 20 bp Tm: 60.0 C GC: 60.0 % Any: 5.5 End: 0.0 TB: 6.0 HP: 0.0 3' Stab: 2.8 Penalty: 0.038

Right Primer 1: CAAAAGGAGCTGCTTTGCCC

Start: 739 Length: 20 bp Tm: 60.3 C GC: 55.0 % Any: 21.3 End: 0.0 TB: 7.0 HP: 0.0 3' Stab: 5.4 Penalty: 0.320

Pair: Product Size: 520 bp Any: 0.0 End: 0.0 TB: 13.0 Penalty: 0.358

Send to Primer3Manager

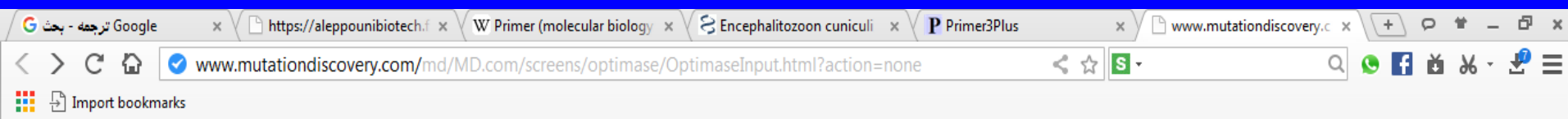
Reset Form

1	Sequence	ATGCAGATGGGA	AATGGGATCA	AGAAAAGCCT	TTACAGACAA
51		AGAGCCCACG	CCAATCCATT	CAAGGACAGC	AACATCACCG
101		CCCTCAGCTT	ATAGACTGGT	CTTCTTACTT	CAGAATGAAT
151		ATTTCTGTTGA	TATCGGGTGT	GGATACGGAA	AGTTCCTTAT
201		GAGAAGAATC	CAGAGCACAG	CATACTGGGC	CTCGAGATCA
251		GTGTGAGTAT	GTTAAGGCAA	GGATAGAAGC	CGCCGGGATT
301		GGGTCATGAG	AACAAATGCA	TTGATTTTCC	TCCCGAACAT
351		GGCCAGCTGA	CCAAGATCTT	TATTTTATTC	CCCGACCCTC
401		GAGGAAGCAA	AAGGGGCGCA	TTGTGTGCCG	GCAGATGATG
451		AGTACCTGCT	AGCGGACGGG	GGAAAGTTGT	ATATATCTAC
501		GAGTTGTTTA	ACTGCATGAT	GGAGGCCGTT	CTGGGACATG
551		GGCATTGTCT	GAGAACGAAG	TGGATGGAGA	TGAGTTGTTC
601		CTAGGGACAC	GGACGAGGCG	CTGAGGGCCG	GAGCCAAGGC
651		TTTTCGAAGG	TTTTTGAAAT	AAAGAAAGAT	TGGCACTACT
701		TCGACTCCAG	AGGAGTATTG	GGCAAAGCAG	CTCCTTTTGA

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ProtocolWriter™

☐ Select all Primers

<http://www.mutationdiscovery.com/md/MD.com/screens/optimase/OptimaseInput.html?action=none>



Optimase ProtocolWriter™

This tool will generate PCR protocols for use with Optimase, the high-fidelity polymerase product from Transgenomic. To generate a PCR protocol, enter your primers and anticipated PCR product length, and select a PCR protocol type. When you click on "Develop PCR protocol", this software will generate the appropriate PCR protocol.

Forward primer sequence:

Reverse primer sequence:

PCR product length: bp

Protocol type: Simple 3-step PCR ▾

Develop PCR Protocol

Note: In the sequence fields above, include only the template-specific portion of the primer--do not include any GC clamps or tails. For DHPLC applications, we recommend that the product length be between 150 and 700 bp in length.

Optimase and ProtocolWriter are trademarks of Transgenomic, Inc.



Optimase ProtocolWriter™

This tool will generate PCR protocols for use with Optimase, the high-fidelity polymerase product from Transgenomic. To generate a PCR protocol, enter your primers and anticipated PCR product length, and select a PCR protocol type. When you click on "Develop PCR protocol", this software will generate the appropriate PCR protocol.

Forward primer sequence: GCATACTGGGCGCTCGAGATC Tm = 58.3°C

1

Reverse primer sequence: CAAAAGGAGCTGCTTTGCCC Tm = 56.3°C

2

PCR product length: 520 bp

3

Protocol type: Simple 3-step PCR ▾

Develop PCR Protocol

Note: In the sequence fields above, include only the template-specific portion of the primer--do not include any GC clamps or tails. For DHPLC applications, we recommend that the product length be between 150 and 700 bp in length.

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click

PCR protocol

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www.mutationdiscovery.com/md/MD.com/screens/optimize/OptimaseProtocol.jsp?action=create

Import bookmarks

Optimase ProtocolWriter™

The following PCR protocol is designed for use with Optimase, the high-fidelity polymerase product from Transgenomic.

Forward primer sequence: GCCGAGAAGAATCCAGAGCA (Tm = 56.3°C)
Reverse primer sequence: AGGAGCTGCTTTGCCCAATA (Tm = 54.2°C)
PCR product length: 538 bp
Protocol type: Simple 3-step PCR protocol

Step 1: 95°C, 2 min.
Step 2: 95°C, 30 sec.
Step 3: 58.3°C, 30 sec.
Step 4: 72°C, 60.0 sec.
Step 5: Repeat steps 2-4 29 more times
Step 6: 72°C, 5 min.
Step 7: 4°C, forever

[Purchase Optimase Polymerase kit](#)

Purchase of Optimase is accompanied by a limited license to use it in the PCR process for research and development purposes, in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by the up-front license fee, either by payment to Applied Biosystems, or as purchased in an authorized thermocycler.

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EN | 11:23 م | ٢٠١٥/١٢/٢٦