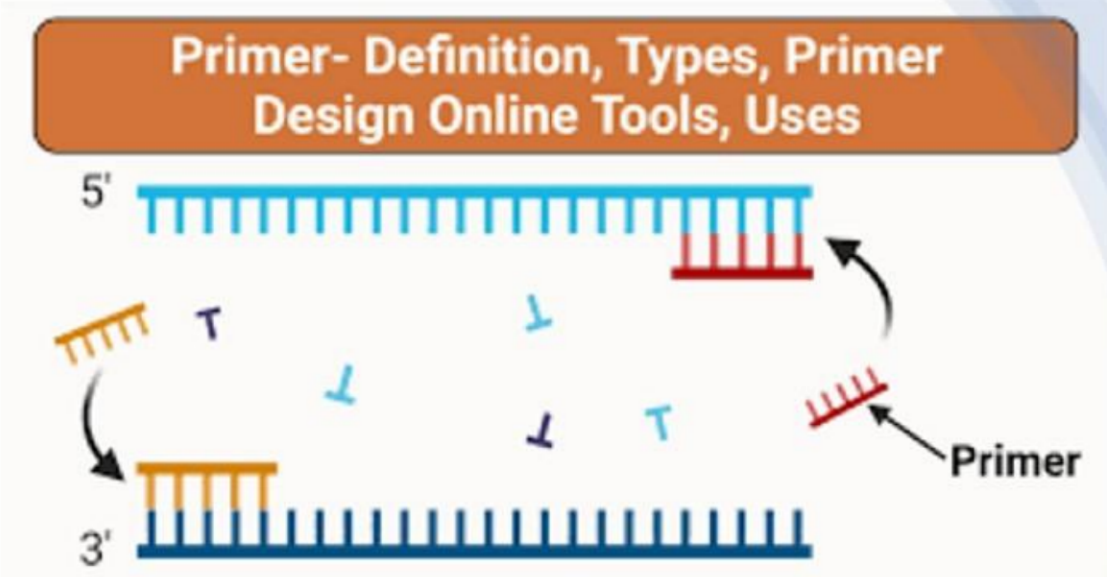


PCR Primer Design

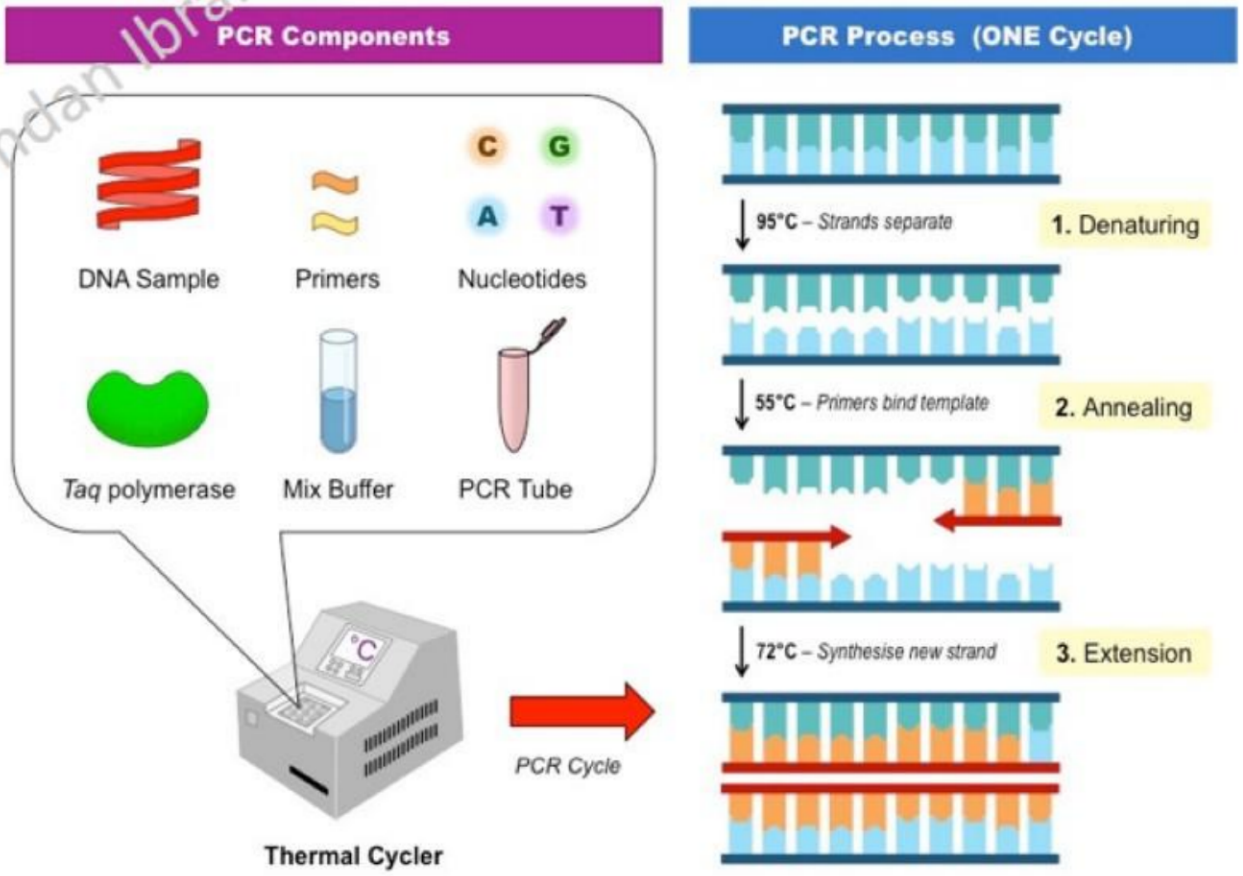
Bioinformatics / Lec 6

By

Dr Delveen R. Ibrahim

What is PCR?

Polymerase chain reaction (PCR) is a method used to selectively amplify a DNA sequence and has uses throughout biotechnology.



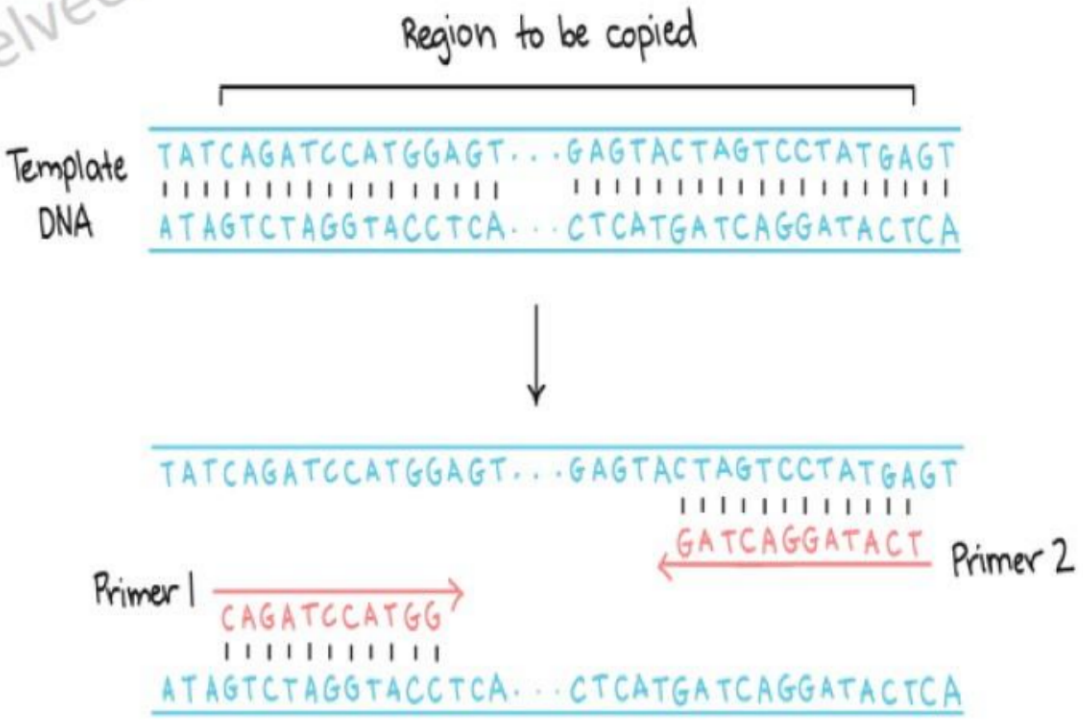
So why do we use PCR to amplify a DNA fragment?

- To clone a gene into a vector – Molecular biotechnology
- For diagnostic purposes – Medicine
- To find out how the gene is expressed through quantitative PCR – Genetics
- To discover novel DNA sequences – Genetics/genomics

PCR Primers

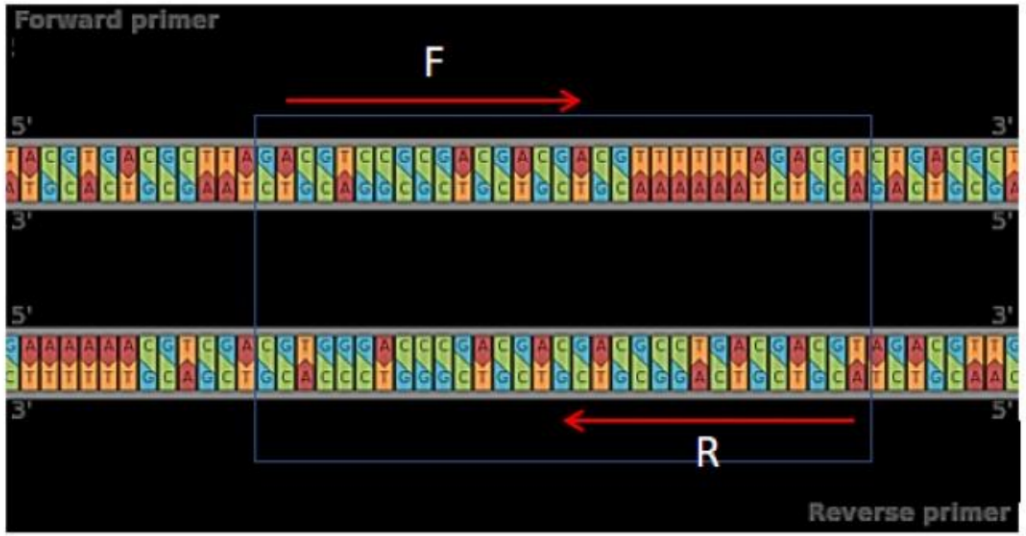
- PCR primers are short, single-stranded segments of DNA that are designed to be complementary to the target sequence that will be amplified.

- In a PCR, it is the primers that command exactly what sequence of DNA gets copied.



- In a **PCR** experiment, there are two types of **primers** use in PCR, **forward** and **reverse** primers. designed to match to the segment of DNA you want to copy.

- One **primer** attaches to the top strand at one end of your segment of interest, and the other **primer** attaches to the bottom strand at the other end.



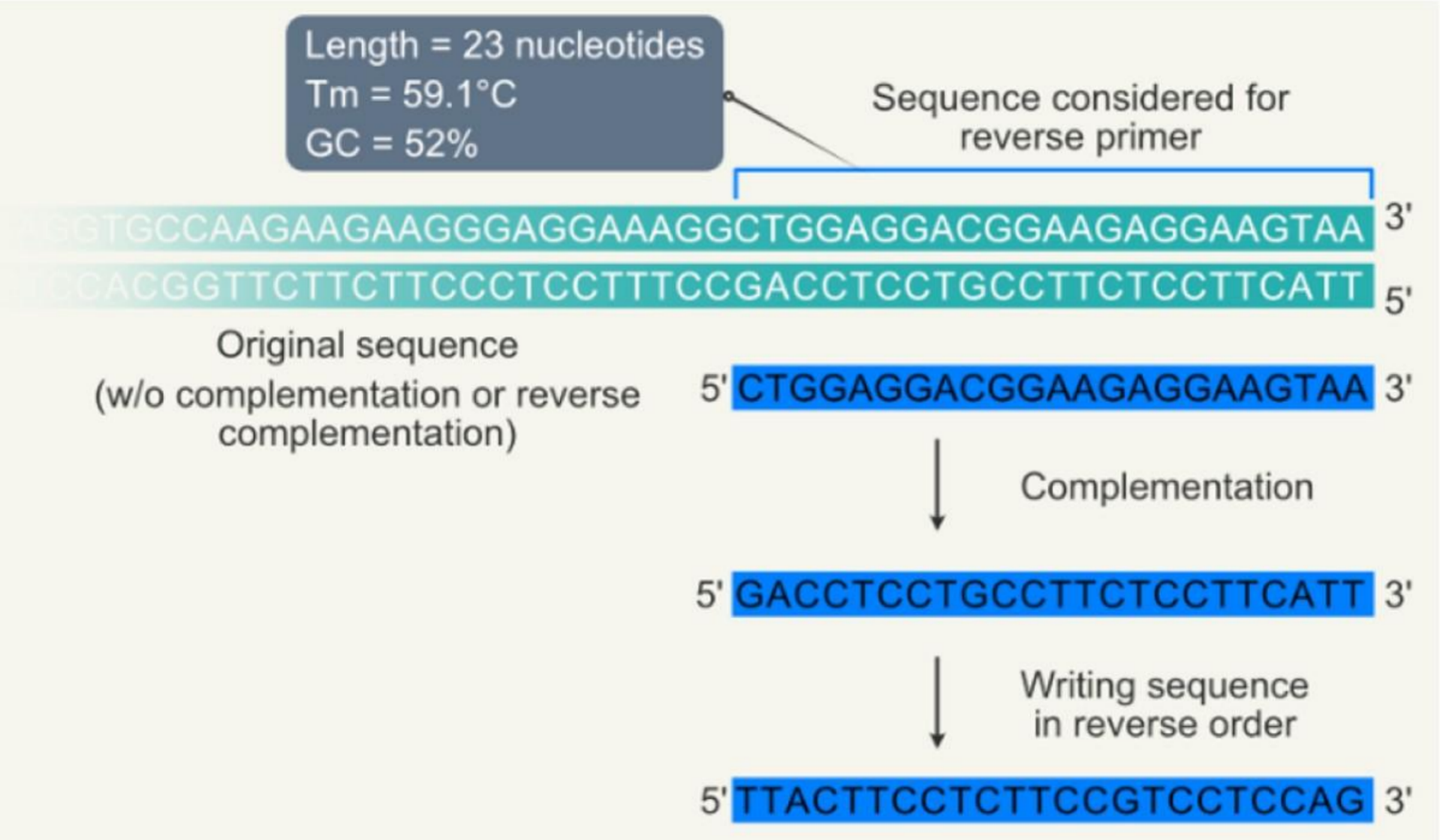
Remember the second primer (reverse) is the reverse complement of your stand

- Ex:

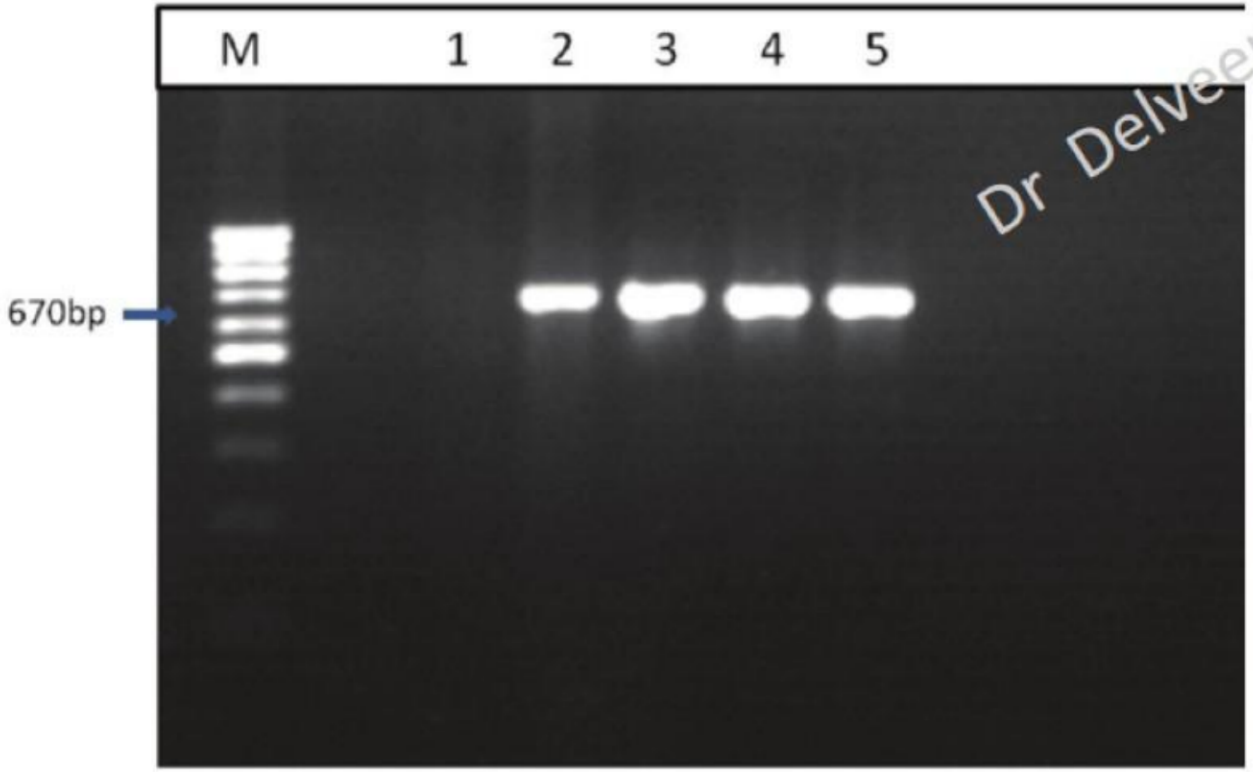
5'CTCTTCTGGTCCC**CACAGACTCAGAGAGAACC**CACCATGGTGCTGTCTCCTGCCGACAAGACCAACGTCAAGGCCGCCTGAACGCCGTGGCGCGT
GGACGACATGCCCAACGCGCTGTCCGCCCTGAGCGACCTGCACGCGCACAAGCTTCGGGTGGACCCGGTCAACTTCAAGCTCCTAAGCCACTGCCTG
CTGGTGACCCTGGCCGCCCACCTCCCCGCCGAGTTCACCCCTGCGGTGCACGCCTCCCTGGACAAGTTCCTGGCTTC**TGTGAGCACCGTGCTGAC**CTC
CAAATACCGTTAAGCTGGAGCCTCGGTGGCCATGCTTCTTGCCCCTTGGGCCTCCCCCCAGCCCCTCCTCCCCTTCCTGCACCCGT 3'

- Forward primer: **CACAGACTCAGAGAGAACC**
- Reverse primer: **GTCAGCACGGTGCTCACA**
- **So how you get the reverse primer??**
- **Take the complement for the selected second primer, then reverse it:**
- The selected sequence for the second primer is: TGTGAGCACCGTGCTGAC
- The complement is : ACACTCGTGGCACGACTG
- Reverse complement is : **GTCAGCACGGTGCTCACA**

Steps for the reverse primer design:



Designing primers for PCR

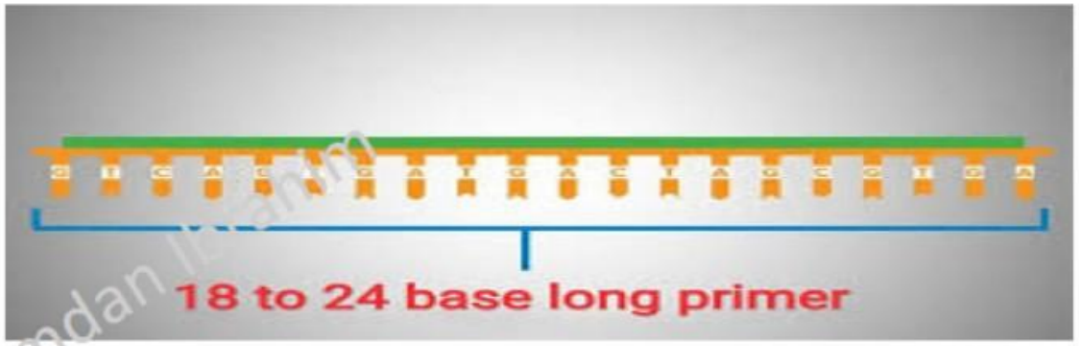


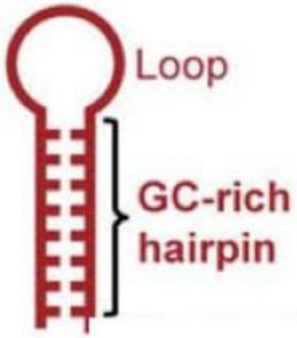
- Successful amplification of the desired target DNA with PCR relies on well-designed oligonucleotide primers. The effectiveness of PCR primers can be improved by adhering to a few primer-design guidelines:

Basics or parameters of PCR Primer Design

1. Primer Length:

- The optimal length of PCR primers is 18-24 bases.
- Shorter primers may lead to non-specific PCR amplification
- Longer primers are more specific however they contain secondary structure such as hairpin loops.





2. Primer Melting Temperature:

- The temperature at which one half of the DNA duplex will dissociate and become single stranded DNA.
- The optimal melting temperatures of primers range from 52-60°C.
- Primers with melting temperatures above 65°C have a tendency for nonspecific amplification (secondary structure).
- The guanine-cytosine (GC) content of the sequence gives a fair indication of the primer Tm.

Methods to calculate the melting temperature for a specific primer:

There are many formula can be used to calculate the melting temperature for a primer , here are two examples:

1. **(Wallace Formula)**

$$Tm = [4(G + C) + 2(A + T)]\ °C$$

e.g., primer composition :GACTGCGTTAGGATTGGC; count the number of G+C and A+T and enter into the formula.

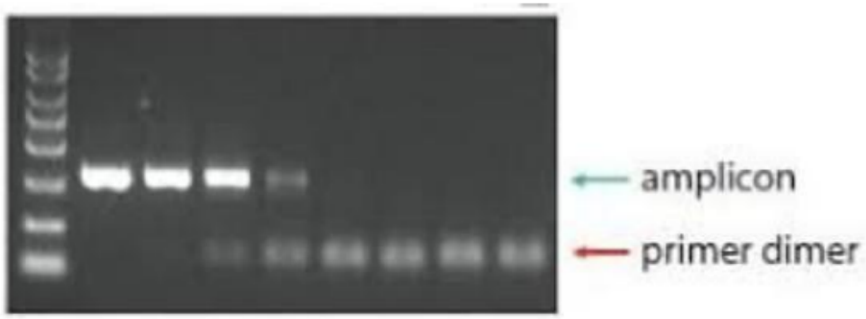
Hence, Tm = [4 (10)+ 2 (8)] °C = (40+16) °C = 56°C

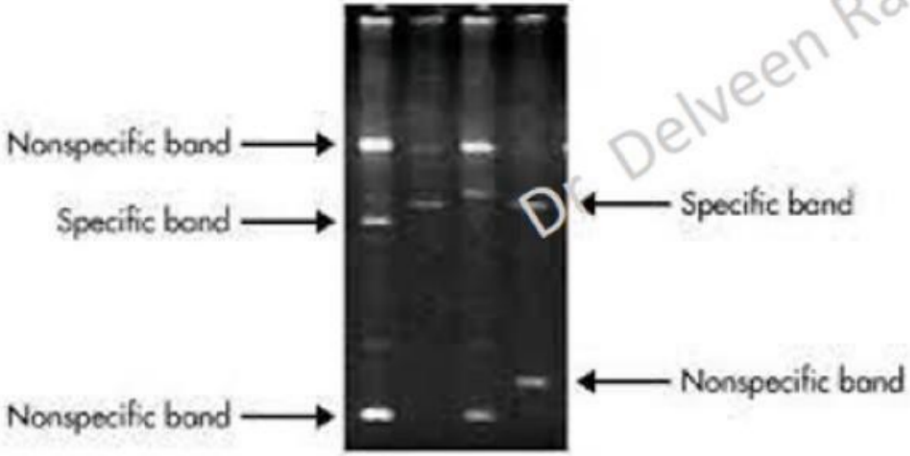
2. Nearest-Neighbor Model:

$$Tm = \frac{\Delta H}{\Delta S + R \ln C} - 273.15$$

- In this modle, Each base pair (e.g., A-T or G-C) contributes to the stability of the DNA duplex.
- The contribution is not just from the base pair itself but also from the identity of the adjacent base pair—hence the term "nearest-neighbor
- Since the calculations are complex, tools like **OligoAnalyzer (IDT) and** , **Primer3** use the nearest-neighbor model automatically.

Feature	Wallace Rule	Nearest-Neighbor Model
Accuracy	Low (good for short primers)	High (good for all primers)
Considers GC content?	Yes	Yes
Considers base stacking effects?	No	Yes
Considers salt concentration?	No	Yes
Considers mismatches?	No	Yes
Best for primer length	< 20 bases	Any length





Nonspecific bands

3. Optimum Annealing temperature (T_a Opt):

- The temperature at which the maximum amount of primer is bound to its target.
- The annealing temperature should be 5°C lower than primer melting temperature.
- Low annealing temperature leads to unspecific product.

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%.

$$\%GC = \left(\frac{G+C}{A+T+G+C}\right) \times 100$$

5. GC Clamp:

In primer design, avoiding repetitive C and G at the 3' end is generally recommended, as the presence of C and G can increase the likelihood of forming secondary structures such as hairpins. However, in some cases, a few G or C bases at the 3' end can be beneficial for primer stability, especially when you want a strong binding for a specific target.

6. Avoid Secondary structure:

- Secondary structures are formed if the primer has regions of self-homology or inter-primer homology.
- Secondary structures such as hairpin structure or primer dimer
- The stability of these template secondary structures depends largely on their free energy (ΔG)and melting temperatures(T_m).
- It is better to avoid secondary structures as the primers are unable to bind to the template and the yield of PCR product is significantly affected.

Hairpin

```
GTGT
A
TGCAGCAT
```

Cross Dimer

```
5' TGTGATGCAGCATCACGCACAC 3'
3' CTACGTCGACTCTGATAGCTACG 5'
```

7. Avoid Repeats and runs:

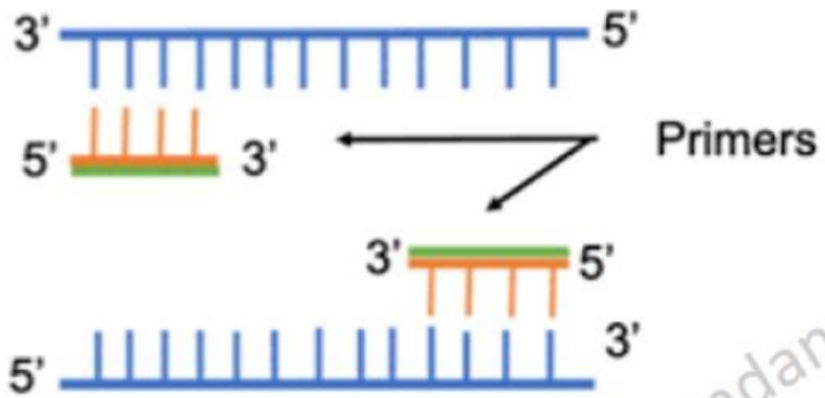
A primers with long run of a single base or repeated sequences of four or more nucleotide should be avoided because they can misprime.

For example: AATAATAATAAT nucleotide repeats

For example, AGC**GGGGG**ATGGGG has runs of base 'G' of value 5 and 4. A maximum number of runs accepted is 4 bp.

no runs of at least 5 for same base: 4 **points**

5'-CAACAT~~AAAAA~~CGACAACACTAGA-3'



Practical Part/ Lec 7

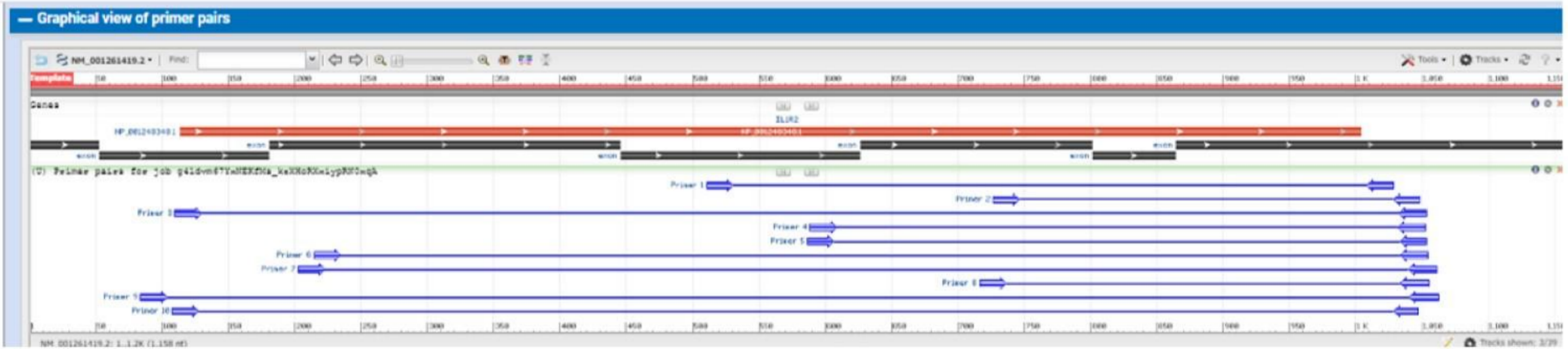
Websites and tools for primer design:

There are many online tools and software's for designing primers and for checking the quality of the designed primers:

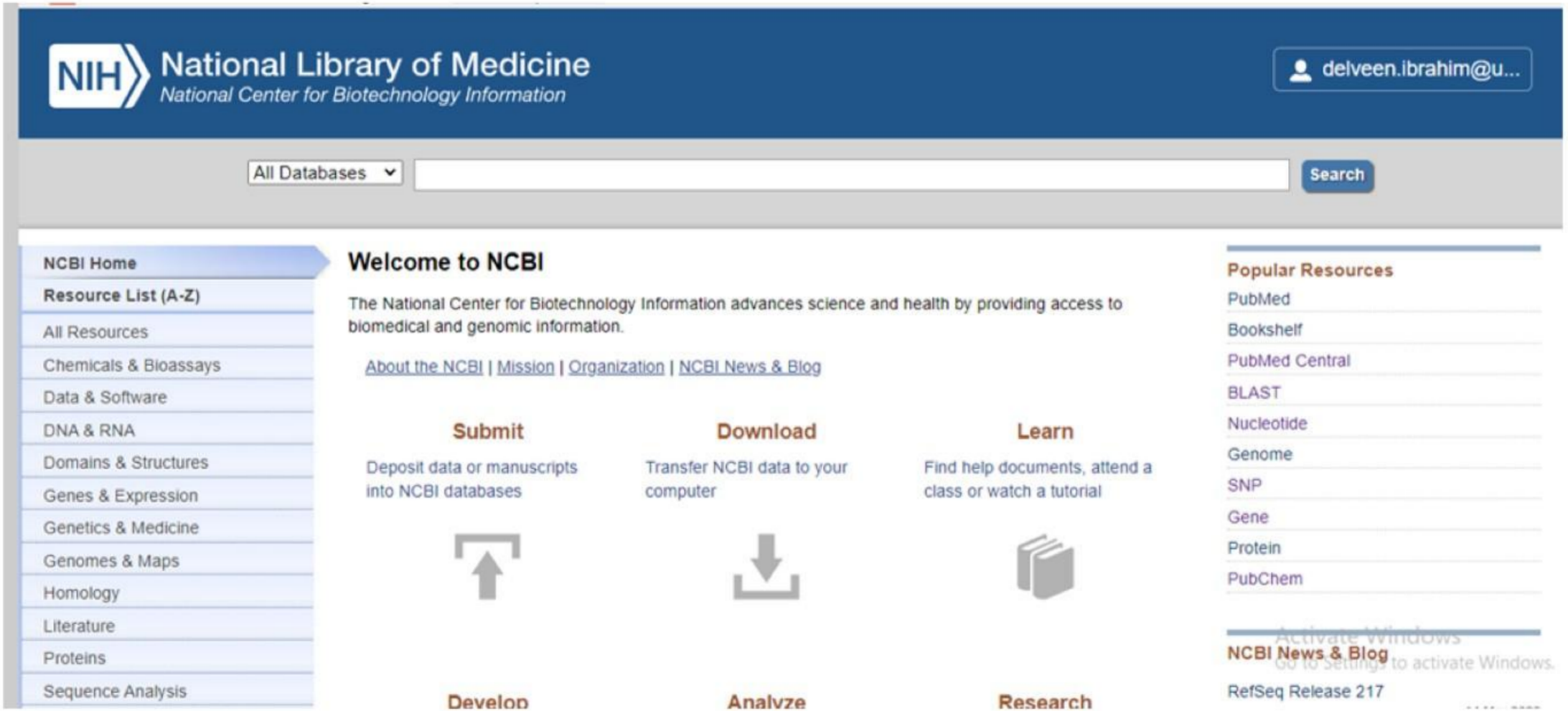
- **Primer-BLAST (Online tool)**
- **Primer3 (software and web : primer3web & primer3plus)**
- **GenScript Pcr Primer Design (Real-time PCR Primer Design online tool)**
- **Eurofins Genomics' Primer Design Tools (Online tool)**

Primer-BLAST

- Primer-BLAST was developed by NCBI and utilizes the Primer3 platform in combination with the BLAST algorithm.
- It gives you an informative visual output for where the primers will bind on the target sequence.



Type NCBI in your browser



National Library of Medicine
National Center for Biotechnology Information

Log in

Search NCBI

NM-001261419 Search

Results found in 9 databases (1 error)

Accession no. : NM_001261419

NUCLEOTIDE SEQUENCE

Homo sapiens interleukin 1 receptor type 2 (IL1R2), transcript variant 3, mRNA

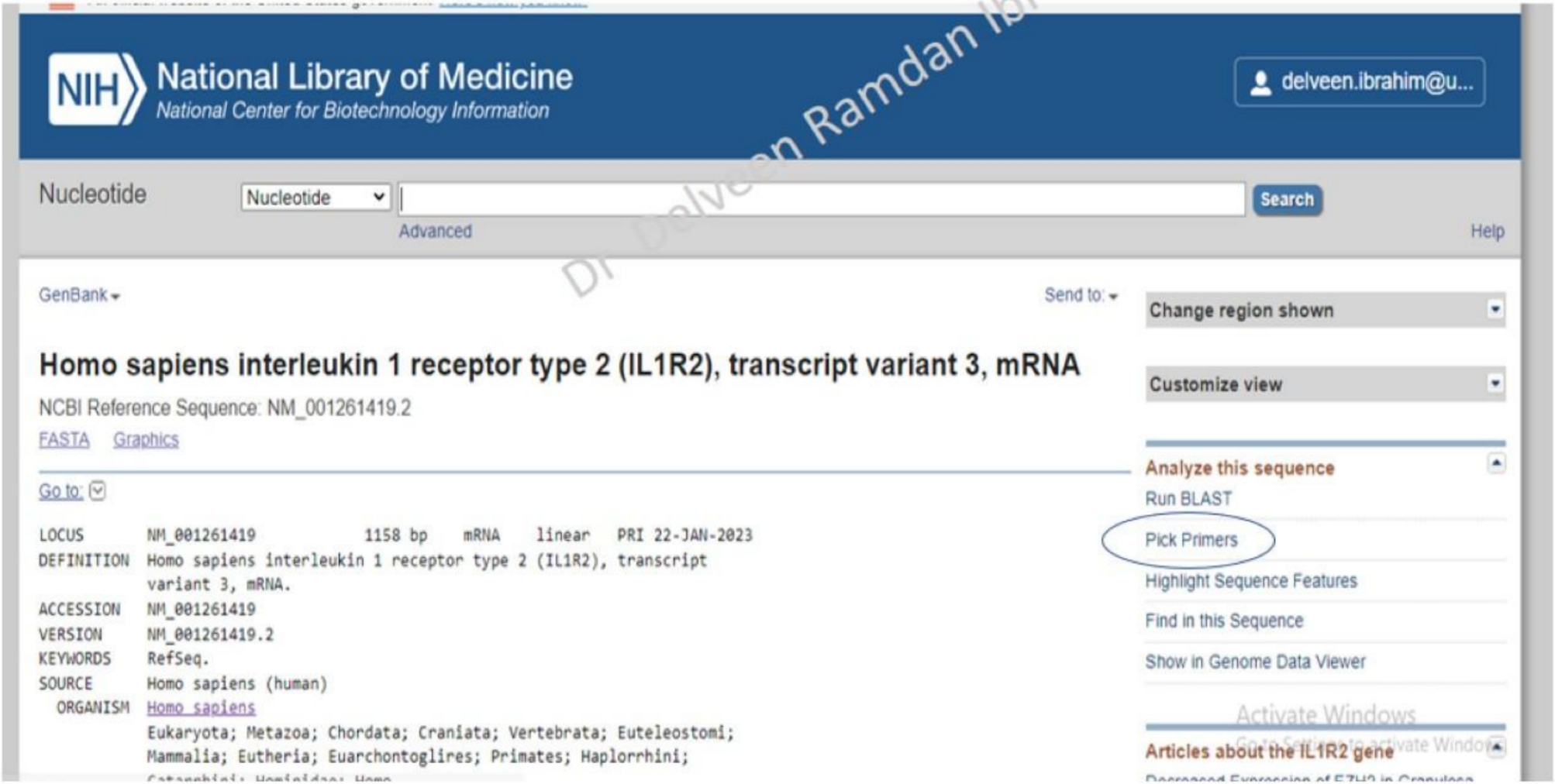
Homo sapiens
1,158 bp mRNA sequence
NM_001261419.2

FASTA Gene

BLAST Download

Literature		Genes		Proteins	
Bookshelf	0	Gene	1	Conserved Domains	0
MeSH	0	GEO DataSets	0	Identical Protein Groups	1

Chose Pick Primers, which represents Primer-BLAST



Primer-BLAST

A tool for finding specific primers

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

Primers for target on one template | Primers common for a group of sequences

Retrieve recent results | Publication | Tips for finding specific primers

Save search parameters | Reset page

PCR Template

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

NM_001261419.2

Or, upload FASTA file: Choose File No file chosen

Range Clear

	From	To
Forward primer		
Reverse primer		

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: 10

	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

Min 5' match	Min 3' match	Max 3' match
7	4	8

Minimal and maximal 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	Max
1000	10000

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 Min 5' match: 7 | Min 3' match: 4 | Max 3' match: 8

Minimal and maximal 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: 10000

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 Automatic

Database Refseq mRNA

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

Organism 9606 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.

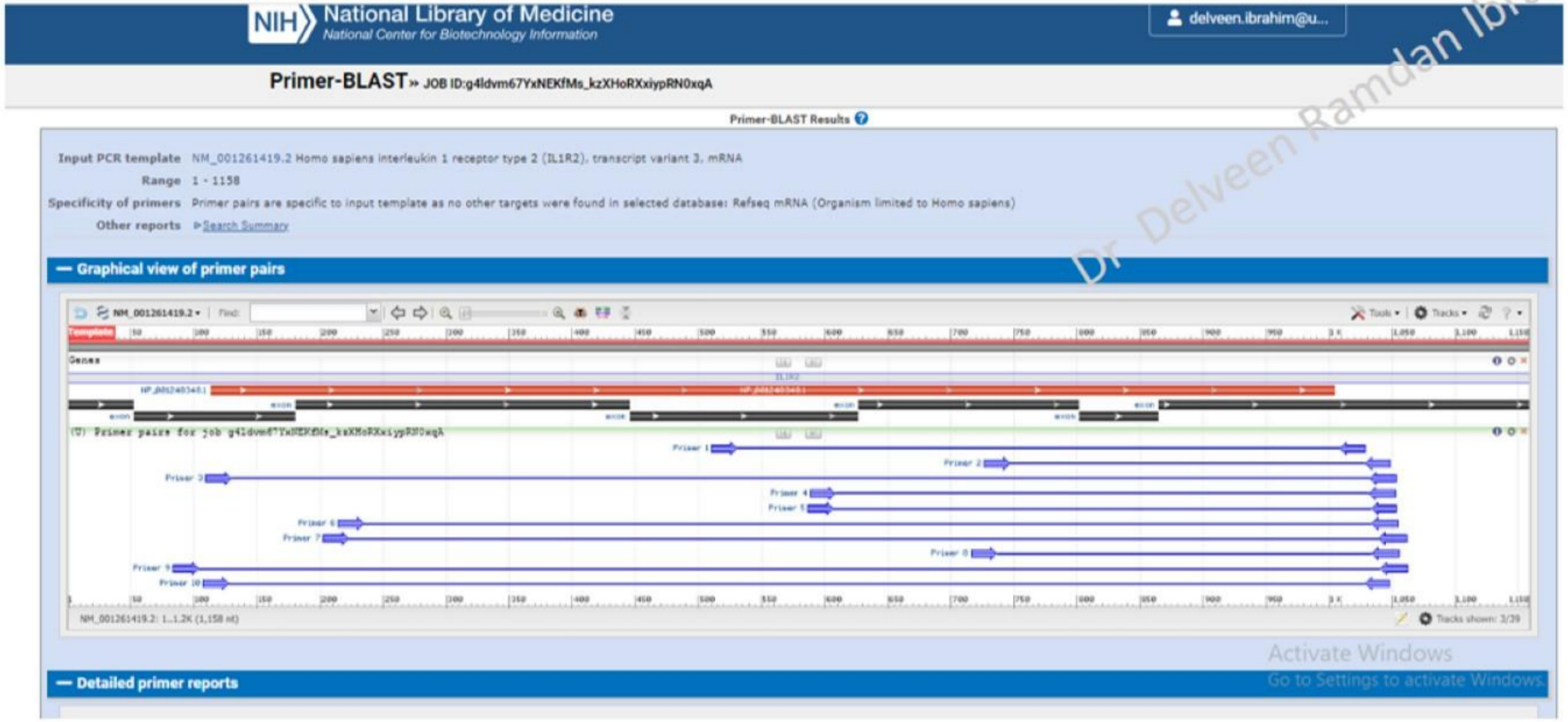
Ignore targets that have 6 or more mismatches to the primer.

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

Graphic view of the primers sets



10 Sets of Primers will show off , usually the first set is the best one, but we must check them using specific software's

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGCCGTTCATCTCATACCCG	Plus	20	511	530	59.90	55.00	2.00	2.00
Reverse primer	TCTCAACAGAAGACCCTGGC	Minus	20	1028	1009	59.31	55.00	3.00	2.00
Product length	518								
Total intron size	8243 (between pos. 102016051 and 102024533 on NC_000002.12)								

Products on intended targets

>NM_001261419.2 Homo sapiens interleukin 1 receptor type 2 (IL1R2), transcript variant 3, mRNA

```
product length = 518
Forward primer  1    TGCCGTTCATCTCATACCCG  20
Template        511  ....................  530

Reverse primer  1     TCTCAACAGAAGACCCTGGC  20
Template        1028  ....................  1009
```

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ACCGCTGTGTCCTGACATTT	Plus	20	727	746	59.89	50.00	6.00	2.00
Reverse primer	AAGAGCGAAACCCACAGAGT	Minus	20	1048	1029	59.24	50.00	2.00	1.00
Product length	322								
Total intron size	4657 (between pos. 102019812 and 102024533 on NC_000002.12)								

Products on intended targets

>NM_001261419.2 Homo sapiens interleukin 1 receptor type 2 (IL1R2), transcript variant 3, mRNA

```
product length = 322
Forward primer  1    ACCGCTGTGTCCTGACATTT  20
Template        727  ....................  746

Reverse primer  1     AAGAGCGAAACCCACAGAGT  20
Template        1048  ....................  1029
```

Primer pair 3

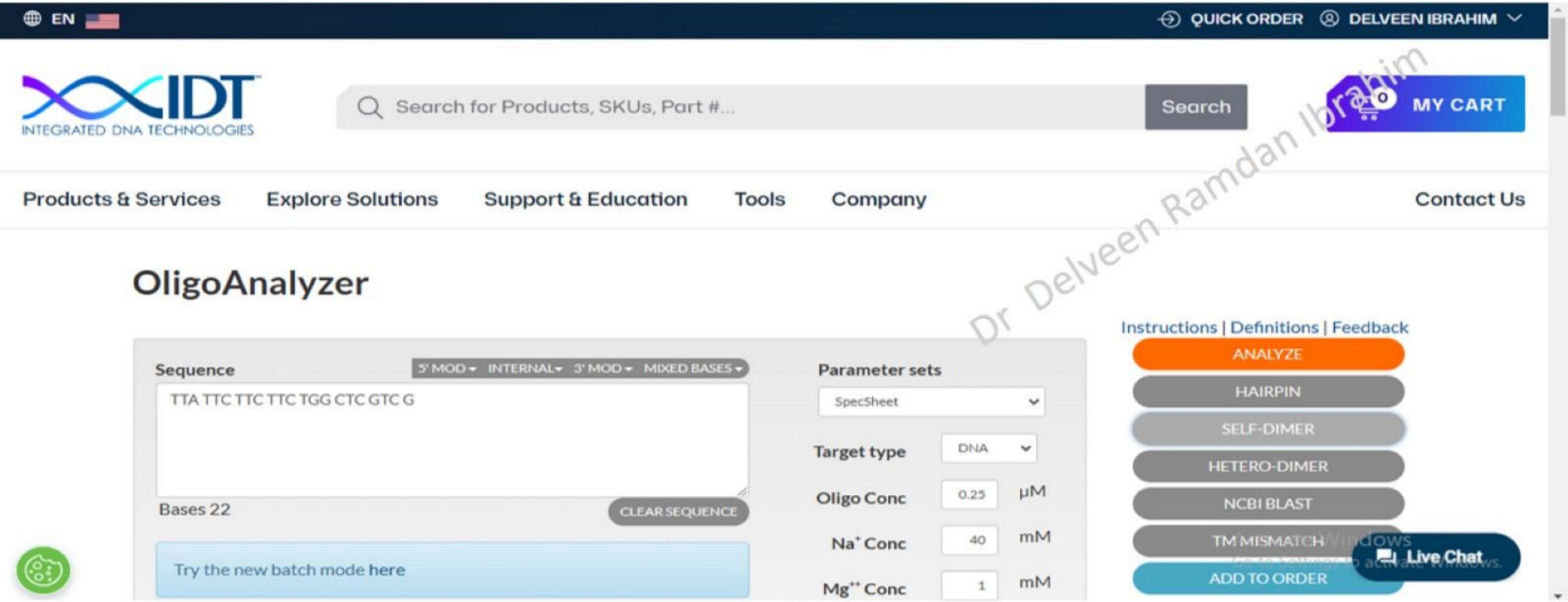
	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGCAATGTTGCGCTTGTACG	Plus	20	110	129	60.11	50.00	6.00	2.00
Reverse primer	AAAGGAAGAGCGAAACCCACA	Minus	21	1053	1033	60.13	47.62	2.00	0.00
Product length	944								
Total intron size	15206 (between pos. 102008642 and 102024533 on NC_000002.12)								

Products on intended targets

>NM_001261419.2 Homo sapiens interleukin 1 receptor type 2 (IL1R2), transcript variant 3, mRNA

```
product length = 944
Forward primer  1    AGCAATGTTGCGCTTGTACG  20
```

- **PCR primer analysis (quality):** There are many software to analysis the primers quality for your PCR reaction
  you can use OligoAnalyzer tool (IDT) for more detailed analysis



Task 1:

Use the following accession no. NM_000700.3 for designing a primer :

1: To which gene does this accession no. belongs.

2: Provide 10 sets of primers that shows good parameters

3. Select the best set based on the parameters that you learned from the lecture.

See the video for revision https://youtu.be/1rBWi5yKG88