**Lecture 17: DNA Stability, PCR - Polymerase Chain Reaction**

**DNA Stability:** DNA can be reversibly denatured - converting the double stranded DNA duplex to single stranded DNA. The temperature at which the DNA is ½ denatured (50:50 double and single stranded) is call the melting temperature, or TM.

* If DNA is heated above the melting temperature it will be completely converted to single stranded DNA (ssDNA).
* If this solution is cooled below the melting temperature the DNA will anneal, forming double stranded DNA again, with Watson-Crick hydrogen bonds- in the above example the shorter fragment anneals at the indicated position due to WC H-bonding.

**Review of DNA Polymerases:**

* Bind to primer-template.
* Add dNTPs to 3’OH of primer.
* Extend to the end of template.

**PCR (Polymerase Chain Reaction):** A way to amplify a specific region of a DNA template.

We will mix the following:

i. Double stranded DNA template

ii. Excess “right” and “left” primers

iii. dNTPs

iv. *Thermostable* DNA polymerase (so it doesn’t denature)

and then do many (20) cycles of the following:

**D = denaturation** – all double stranded DNA becomes single stranded

**A = annealing**, the temperature is low enough that primers will anneal to the template.

**P = polymerization** – the DNA polymerase will add dNTPs to the primer, until the end of the template.

**Example:**

Template GGCGTAACGATATGCGGGATTAA

CCGCATTGCTATACGCCCTAATT

Primers, 5’AAC, 5’TCC

PCR Product: AACGATATGCGGGA

 TTGCTATACGCCCT

*What is the relationship between the template, primers, and the PCR product?*

* The left (upstream) primer is the sequence of the top strand at the left boundary.
* The right (downstream) primer is the sequence of the bottom strand at the right boundary.
* The final product contains the DNA between the two primers **and** the primers themselves.

**Example**: Develop PCR primers that are 8 bases long that will amplify the following gene during PCR, from the start codon to the stop. The amplified product should not have any additional bases on the end.

5’-GCG**ATGGTGGCGCAATCGCTA--TATGCTGAAACGCTGTCAGAT--ATATGCAGCTCGCTACACTAA**CGCG-3’

3’-CGCTACCACCGCGTTAGCGAT--ATACGACTTTGCGACAGTCTA--TATACGTCGAGCGATGTGATTGCGC-5’

 **Met**ValAlaGlnSerLeu--TyrAlaGluThrLeuSerAsp IleCysSerSerLeuHisStp

Left Primer: Right Primer:

These will give the following PCR product:

 **ATG**GTGGCGCAATCGCTA--TATGCTGAAACGCTGTCAGAT--ATATGCAGCTCGCTACACTAA

 TACCACCGCGTTAGCGAT--ATACGACTTTGCGACAGTCTA--TATACGTCGAGCGATGTGATT

**How PCR works – Following PCR Cycles.**

5’--------------------AAC---------------------------GGA-----------------------3’(+)

3’--------------------TTG---------------------------CCT-----------------------5’(-)

Left (upstream) primer: AAC Right (downstream) primer: TCC

Cycle 1:

5’--------------------**AAC---------------------------GGA**-----------------------3’ (+)

3’--------------------**TTG---------------------------CCT**-----------------------5’ (-)

 Denaturation

5’--------------------**AAC---------------------------GGA**-----------------------3’(+)

3’--------------------**TTG---------------------------CCT**-----------------------5’(-)

 Primer Annealing

5’--------------------**AAC---------------------------GGA**-----------------------3’(+)

 CCT5’

 5’AAC

3’--------------------**TTG---------------------------CCT**-----------------------5’(-)

 Polymerization

5’--------------------**AAC---------------------------GGA**-----------------------3’(+)

3’--------------------**TTG---------------------------CCT**5’

 **5’AAC---------------------------GGA**-----------------------3’

3’--------------------**TTG---------------------------CCT**-----------------------5’(-)

Cycle 2 (after denaturation step) (Focusing on the new DNA strands that were made in cycle 1).

5’--------------------**AAC---------------------------GGA**-----------------------3’

3’--------------------**TTG---------------------------CCT5’(-)**

 5’AAC---------------------------GGA-----------------------3’(+)

3’--------------------TTG---------------------------CCT-----------------------5’

 Primer Annealing

 5’AAC

3’--------------------TTG---------------------------CCT5’(-)

 5’AAC---------------------------GGA-----------------------3’(+)

 CCT5’

 Polymerization

 **5’AAC---------------------------GGA3’**

3’--------------------TTG---------------------------CCT5’(-)

  **5’AAC---------------------------GGA**-----------------------3’(+)

 **3’TTG---------------------------CCT5’**

**Cycle 3: (After denaturation step) (Focusing on the new DNA strands that were made in cycle 1)**

 **5’AAC---------------------------GGA3’(+)**

3’--------------------TTG---------------------------CCT5’

  **5’AAC---------------------------GGA**-----------------------3’

 **3’TTG---------------------------CCT5’(-)**

 Primer Annealing

 **5’AAC---------------------------GGA3’(+)**

 CCT5’

 5’AAC

 **3’TTG---------------------------CCT5’(-)**

 Polymerization

 **5’AAC---------------------------GGA3’**

 3’TTG---------------------------CCT5’

 5’AAC---------------------------GGA3’

 **3’TTG---------------------------CCT5’**

**Note: Typo on pg 1 – corrected sequences are:**

Template GGCGTAACGATATGCGGGATTAA

CCGCATTGCTATACGCCCTAATT

Primers, 5’AAC, 5’TCC [No change]

PCR Product: AACGATATGCGGGA

 TTGCTATACGCCCT

**Applications of PCR**

a) DNA Repeat Disease - Huntington’s Disease – neurological disease

* CAG is repeated multiple times within the huntington protein, coding for glutamine.
* There are 26 or less repeats in normal people
* There are greater than 40 repeats in affected individuals
* The DNA sequence outside of the CAG repeat is not repetitive.

MetTyrThrAlaIleAspGlnGln..(Gln)N..GlnGlnLeuTyrLeuGluSerMet…

ATGTATACGGCGATCGAT**CAGCAG**..(CAG)N..**CAGCAG**TTATACCTAGAGTCAATGC…

TACATATGCCGCTAGCTAGTCGTC.........GTCGTGAATATGGATCTCAGTTACG…

How can we use PCR and electrophoresis to determine whether someone has Huntington’s disease?

b) Amplification of Genetic Material for Sequencing

* HIV virus is present in relatively low levels
* Difficult to get sufficient nucleic acid (viral RNA) for sequencing
* PCR can be used to amplify the region of the vRNA that encodes HIV protease.

**Modification of DNA sequences by PCR:**

**A.** Addition of bases to the ends of the PCR product.

* Any non-complementary DNA sequences that are on the 5’ end of the primers will be added to the PCR product because the entire primer is incorporated into the PCR product.

**Example:**

Template GGCGTAACGATATGCGGGATTAA (+)

CCGCATTGCTATACGCCCTAATT (-)

Primers, 5’CCAAC, 5’ GCTCC

PCR Product: CCAACGATATGCGGGAGC

 GGTTGCTATACGCCCTCG

*What is the relationship between the template, primers, and the PCR product?*

Cycle 1 (prime (–) strand with left primer):

 CC CC

 AAC AACGATATGCGGGATTAA (+)

CCGCATTGCTATACGCCCTAATT(-) CCGCATTGCTATACGCCCTAATT (-)

Cycle 2 (prime (+) strand from last cycle with right primer):

CCAACGATATGCGGGATTAA (+) CCAACGATATGCGGGATTAA (+)

 CCT GGTTGCTATACGCCCT (-)

 CG5’ CG5

Cycle 3 (prime (-) strand from last cycle with left primer):

 CCAAC 5’CCAACGATATGCGGGAGC

 GGTTGCTATACGCCCTCG5’(-) GGTTGCTATACGCCCTCG5

**B.** Site directed mutagenesis – changing any base (and therefore any amino acid).

Four primers are required:

* Two to define the boundaries
* Two to generate the mutation

1. First make two PCR products, each one made with one boundary primer and one mutagenic primer.

2. These products are then combined and they act as primers for each other to generate the complete product.

Example change:

GGCGTAACGATATGCGGGATTAA(+)

CCGCATTGCTATACGCCCTAATT(-)

 to

 AACGAT**C**TGCGGGA

 TTGCTA**G**ACGCCCT

Left primer: 5’AAC, this will be paired with AGA, the bottom mutagenic primer.

Right primer: 5’TCC, this will be paired with TCT, the top mutagenic primer.

Mutagenic primers: 5’TCT & 5’AGA – these bind to the template with a mismatch. The mutation will be incorporated into the PCR product, e.g: TCT

 CCGCATTGCTATACGCCCTAATT (-)

1. Two separate PCR reactions.

GGCGTAACGATATGCGGGATTAA

CCGCATTGCTATACGCCCTAATT

 ACC + AGA TCT + TCC

AACGATCT TCTGCGGGA

TTGCTAGA AGACGCCCT

2. Combine products to “self-prime” and then form dsDNA due to polymerase activity.

5’AACGATCT3’ 5’TCTGCGGGA3’

 3’AGACGCCCT5’ 3’TTGCTAGA5’

5’AACGATCTGCGGGA3’

3’TTGCTAGACGCCCT5’