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# BABS3121

## Basic Techniques 1

**DNA structure and properties**

- A polymer with 2 strands forming a helical structure.
- 1 turn on the helix (3.4nm) spans 10.5 bps.
- Narrow angle (120 degrees) between the sugars on one edge of the base pairs generates a minor groove. The large angle (240 degrees) on the other edge generates the major groove.
    - Major groove, main interaction site of DNA and protein (TFs).
        - Because more chemical properties of DNA are exposed.
- Two strands interact non-covalently through hydrogen bonds.
- Two strands are anti-parallel.
- **DNA forms:** can exist in 3 forms.
    - A DNA: occurs during DNA-protein interactions; similar to dsRNA. Helix has 11bp per turn. The major groove is narrower and deeper than in B form. Right handed helix.
    - B DNA: Common in cells. Formed in the nucleus. Has 10.5bp per turn. Right handed helix.
    - Z DNA: left handed helix transiently formed during transcription.
- Rosalind Franklin's X-ray diffraction image of DNA crystals, revealing Maltese cross confirming the helical structure. The white diamond areas also indicate the phosphate backbone.
- **Summary:**
    - DNA is not a perfectly regular double helix. Is non-uniform.
    - Variations from the ideal occur, but average structure is close to ideal.
    - Variations in DNA structure are dependent on DNA sequence.
    - Random movement also occurs: NMR studies.
- **Measuring DNA**
    - DNA and RNA absorbs UV light. Max at 260nm.
        - An $OD_{260}$ of 1 = 50ug/ml of dsDNA = 40 ug/ml of ssDNA and RNA = 33ug/ml of dNTPs.
        - ssDNA absorbs more UV. As in dsDNA, the bases are hidden due to stacking. (Hyperchromic effect)
    - Proteins (esp. aromatic AAs) absorb UV at max 280 nm.
    - Organics (phenolate ions, thiocyanates) absorb UV at max = 230 nm.
    - For pure RNA, the $A_{230:260:280}$ should be 1:2:1
    - For pure DNA, the $A_{230:260:280}$ should be 1:1.8:1.
- **Feature of DNA**
    - Separate complementary strands at 100 degrees C, or under high pH, denaturation.
        - A reversible process, as complementary bases will reanneal at low temp.
    - Melting temperature (Tm): the temperature at which the DNA strands are half denatured.
    - Tm increases with GC content, and ionic strength.
    - Tm decreases with other agents: formamide and DMSO (binds to GC rich areas and facilities denaturation and primer annealing)
- **PCR**

- The size of PCR product is the distance between the 5’ end of primers.
- Note: primers are included in the PCR product.

**PCR Primer Design**

- **Guidelines**
  - Usually 18-22bp long.
    - Long enough for adequate specificity.
    - Short enough for primers to bind easily to template.
  - Primer melting temp should be set at 52-58 degrees depending of GC content.
  - Primer annealing temp should be no more than 5 degree below the Tm of your primers.
  - Maintain GC content at 40-60%. Should not contain regions of 4 or more consecutive G residues.
  - Avoid sequences with propensity to form secondary structures.
  - Prevent primer dimer formation.
    - When the 3 ends of the primers complement each other.
  - 3’ end of primer most important for specificity.
  - Middle section of primer can have base mismatch (for site-directed mutagenesis)
  - 5’ end of primer less important (can engineer restriction enzyme sites at 5’ end)
- Reverse transcription PCR (RT-PCR)
  - Amplify RNA. Can’t use Taq polymerase, as it’s a DNA polymerase.
  - Use reverse transcriptase. A DNA polymerase that is RNA dependent.
  - It will require a primer. Use the poly A tail to attach primer.
  - Use DNA polymerase on single stranded cDNA to form 2$^{nd}$ strand.
  - When designing primers, remember there are no introns in mRNA.

**DNA Cloning**

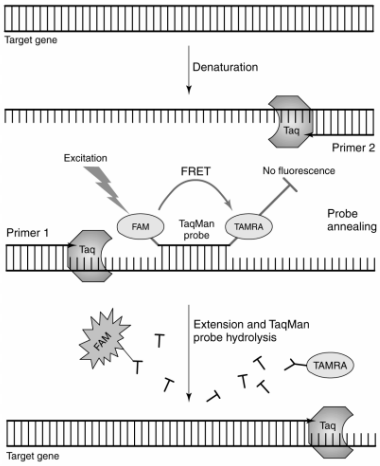
- Restriction enzymes leave phosphate groups at 5’ ends.
- Selectable markers: provide features of selective growth for cells that contain the vector. Use antibiotic, or ability to synthesize an essential component as selection process.
  - Prokaryote: Ampicillin, Tetracycline, Kanamycin, Streptomycin, Zeocin
  - Eukaryotic: Hygromycin, Histidinol, Neomyin (G418), Uracil.
- pGLO expression plasmid core elements
  - Origin of replication
  - Selectable markers
  - Promoter controlled by activator/repressor system. (Arabinose)
  - Multiple cloning site
- Problem when cloning PCR products as Taq polymerase adds a non-templated A to the 3’ end. Removing it with nuclease proved unreliable.
  - Solutions:
    - Vector with 3’ thymine overhangs: used pGEM-T vector based cloning.
    - Engineer restriction enzyme sites at ends of PCR products.

## Basic techniques 2

**Reverse Transcription quantitative PCR (qPCR)**

- Mostly used to quantify RNA levels.
- Composed of two techniques:
    - Reverse transcription: convert rna to cDNA.
        - If RNA does not have polyA tail (i.e. not from eukaryote) use hexamer oligonucleotide method.
    - And PCR
- Normal PCR has 3 stages:
    - Exponential: Exact doubling of product is accumulated at every cycle. Reaction is very specific and precise.
    - Linear: reaction components are being consumed and the reaction is slowing. Products are starting to degrade.
    - Plateau: End point, the reaction has stopped, no more products being made.
- qPCR measures amount of product at the exponential phase.
- **Threshold Cycle**
    - Central concept to calculate your qPCR product.
    - $C_T$ : The amount of PCR cycles required before products fluoresces at the threshold level.
    - The higher the $C_T$ number, the smaller the initial PCR product.
    - From the $C_T$ number, you can calculate the initial copy number, with standard curve.
- During qPCR, the machine can measure increasing fluorescence as the products duplicate.
- Called the fluorescent reporter, either in the form of
    - fluorescent DNA-Binding dye
    - Fluorescent oligonucleotide primers.
- Only fluoresce when associated with the amplicon (PCR product), the increase in fluorescence is directly proportional to the amount of amplified product.
- SYBR Green I: will only interact with ds-DNA.
    - Advantages:
        - not sequence specific, so it can be used for any gene.
        - A cheap alternative to fluorescent probes.
        - Temperature stable and doesn't interfere with DNA polymerase.
    - Disadvantages:
        - Will also bind to primer dimers.
        - Long amplicons can generate very strong signal, thus causing saturation of camera. Thus 200-300bp long amplicons are recommended.
- Fluorescent probes: sequence-specific.
    - DNA sequence-specific oligonucleotide probes. (approx. 20bp)
    - Contain fluorogenic dye and a quencher dye and are designed to hybridise to the target gene.
    - When fluorescent dye is in close proximity to a quencher dye the fluorescent signal generated by the fluorescent dye is absorbed by the quencher dye.
    - This is known as fluorescence resonance energy transfer (FRET).
    - When PCR amplification occurs, the fluorescent dye and the quenching dye become spatially separated, and thus no FRET, and fluorescence occurs.

- TaqMan probes:
  - A gene specific dye that contains fluorescent dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end.
  - During annealing stage, both primer and probes anneal to target gene. But no fluorescence generated as quencher dye is near the fluorescent dye.
  - During extension, the Taq polymerase displaces the probe, and results in the loss of dye contact, thus fluorescent signal produced.
  - Advantages:
    - Sequence specific hence once designed, little optimisation is needed.
  - Disadvantages:
    - Probe synthesis expensive
    - To perform qPCR, requires 3 oligonucleotides.
    - Probe can produce high background fluorescence.




- Molecular beacons:
  - Adopts a hairpin structure with each dye at each end. (No fluorescence)
  - At annealing stage, the structure dissolves and it anneals to the amplicon resulting in loss of FRET.
  - Advantages:
    - Lower background fluorescence, and greater specificity than taqMan probes.
  - Disadvantages:
    - Difficult to design and optimise, due to hairpin structure.
- Scorpion probe:
  - Attaches to the 5' end of target gene specific primer.
  - During annealing and extension, it will anneal with DNA, and primer is extended.
  - A blocker on the prober prevents the Taq from reading it.
  - On the next denaturation step, the probe will dissolve and curl back to hybridise with the target sequence and loss FRET.
  - Advantages:
    - The signal is stronger and background lower comparing to TaqMan probes and molecular beacons.
  - Disadvantages:
    - Difficult design and expensive synthesis.

- **Absolute quantification** using standard curve.
  - Compare unknown to a standard curve created with known samples.
  - There is a variety of different standards that can be used, including in vitro reverse transcribed mRNA, in vitro synthesized single-stranded DNA, or purified plasmid DNA.
  - Only 1 sample needed.
  - Extra work needed to create a standard curve.
- **Relative quantification** using comparative threshold (CT) method
  - Compare 2 samples. A control and experimental.

- Compares Ct values of unknown to a control (calibrator) such as RNA from untreated sample.
- Ratio or fold, change of expression can be established.
- E.g. If Ct value of control is 17 and 22 for experimental.

$$\text{Ratio Experiment : Control} = 2^{\Delta Ct} = 2^{(22-17)} = 2^5 = 32$$

(32-fold decrease in amount or expression)

- **Warning (bias):**
  - There can be a different initial concentration in template. A higher concentration will result in more.
  - Reverse transcription is an imperfect process, not very robust and will convert less cDNA than it should.
- Normalisation using double delta CT
  - The most acceptable way of correcting for differences in input RNA and reverse transcription efficiencies is based on normalization of the target gene to a housekeeping gene.
  - Housekeeping genes are ubiquitously expressed in all cells and tissues and constitute a basal transcriptome for the maintenance of fundamental cellular functions. E.g.
    - beta-actin (ACTB)
    - glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
    - 16S ribosomal RNA.
  - Co-amplify with the housekeeping gene.
  - $$2^{-[(Ct_{Exp}\text{-}Ct_{Cont})\text{-}(Ct_{Exp}\text{-}Ct_{Cont})]}$$ (Gene of Interest; Reference)
  - $$\text{Corrected Ratio} = 2^{\Delta\Delta Ct} = 2^{-(22-17)-(16-15)} = 2^{-(5-1)} = 2^{-4} = 1/16$$
  - (16-fold decrease in amount or expression)