DNA detection techniques:

- Quantitative polymerase chain reaction (PCR) + Electrophoresis:
  - Reagents required:
    - DNA
    - 2* Primers (antiparallel structure)
    - Taq Polymerase
    - Polynerase buffer
    - dNTP
    - Water
  - PCR cycle:
    - DNA double strand separation (95ºC) 1min
    - Primer annealing (~ 65ºC) 30sec
    - DNA elongation (~ 75ºC) 30sec + 5min
- Electrophoresis separate DNA fragments based on relative molecular mass.
  - Electric current applied to agarose gel
  - Fragments placed in wells at negative end, attracted by positive side.
  - Speed of movement inversely correlated to fragment size.
  - Visualised using intercalating agent stains.

- Sanger sequencing - Low throughput sequencing
  - Components:
    - Template ssDNA
    - Primers
    - DNA polymerase I
    - 4 dNTPs
    - 4 ddNTP (small amounts labelled in different colours)
    - ddNTP terminates sequence generation results in different length fragment
  - Radioactive labelling (all dNTP and ddNTP are radioactive to improve intensity on polyacrylamide gel)
    - Different termination length separated by electrophoresis. Deduce sequence from last nucleotide
  - Fluorescence labelling (only ddNTP are fluoresent tagged)
    - Colour of fragment shows last nucleotide, capillary gel electrophoresis allow drawing of electropherogram.

Next Generation Sequencing (NGS)

Characteristics: Lower costs, faster, higher accuracy, more sensitive.

- Illumina sequencing (sequencing by synthesis):
  - Library preparation: Fragmentation of DNA, adaptors are ligated onto the ends, complementary to immobilised adaptors on solid surface
  - Cluster generation: bridge amplification of DNA, create clusters of identical immobilised DNA segments
  - Sequencing by synthesis:
    - A, T, C, G dNTP are added with an attached fluorescent terminator, DNA segments elongate by one nucleotide.
    - Imaging: laser illumination causes each ATCG dNTP to emit different colour fluorescence, image recorded.
    - Reset: fluorescent terminator sequence cleaved off, cycle continues.
  - Analysis: Fluorescent images of each cluster sequenced, sequences aligned and assembled into longer sequence

Genetic engineering

Restriction endonuclease:

- Three types of endonuclease (I II III), type I and III have different recognition site and cleavage site, type II have same.
    - Recognise short palindromic sequence (~6bp)
    - Catalyse hydrolysis of phosphodiester bonds (Type II RE does not require ATP for hydrolysis)
    - Can create staggered cuts, blunt cuts

Cloning

- Cleavage of plasmid
- Segment of desirable gene cleaved ou tusing RE
- Incorporation of segment into the plasmid, sealed by ligase
- Transformation of recombinant vector into the host cell, allow replication
- Selection of cells that successfully uptaken the vector. Allow multiply

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

- CRISPR locus contains:
    - Transactivating CRISPR RNA (TracrRNA)
    - Cas gene
    - AT-rich leader sequence (promotor)
    - Short semi-palindromic sequences (~20bp), with spacers in between, spacers are specific foreign DNA sequences acquired by the bacteria, incorporated into the array using cas9.
- Upon viral invasion:
    - RNA polymerase bind to leader sequence, transcription of CRISPR RNA (crRNA) precursors
    - Transcription and translation of cas genes into cas proteins.
    - Trans-activating crRNA (tracrRNA) transcribed, bind to crRNA precursor, cleaved by RNase III, form gRNA
    - gRNA incorporate with cas nuclease protein - form CRISPR complex
    - Spacer RNA in crRNA recognise foreign sequence, cas induce double stranded breaks
    - Before cutting, cas9 check for protospacer adjacent motif (PAM), a sequence downstream of complementary part (XGG for cas9).
- Applications: can be used to create sticky ends, allow deletion, insertion of segments

- Prime editing
    - Cas9 nuclease replaced by cas9 nickase, which only induce single stranded breaks, bound to reverse transcriptase
    - gRNA replaced by prime editing gRNA(pegRNA), also contains a primer binding sequence and a edited desired RNA template
    - crRNA recognise sequence, nickase induce ssb, nicked ssDNA bind with primer binding sequence, RT transcribe desired DNA.
    - Desired DNA sequence incorporated back into gap created by nickase, unedited strand fixed with cellular mechanism

Means of delivery

Ex-vivo delivery: Cell extracted from the host, edited in culture solution then replaced

In-vivo delivery: Editing complex introduced into host via viral factor; nanoparticle; microinjection; lipofection etc

6 PCR components

3 PCR cycle

Electrophoresis

Sanger sequencing (2 types)

4 NGS characteristic

4 stages Illumina sequencing

Type Restriction endonuclease, actions

Cloning

4 components of the CRISPR locus

Viral infection

Prime editing

Delivery