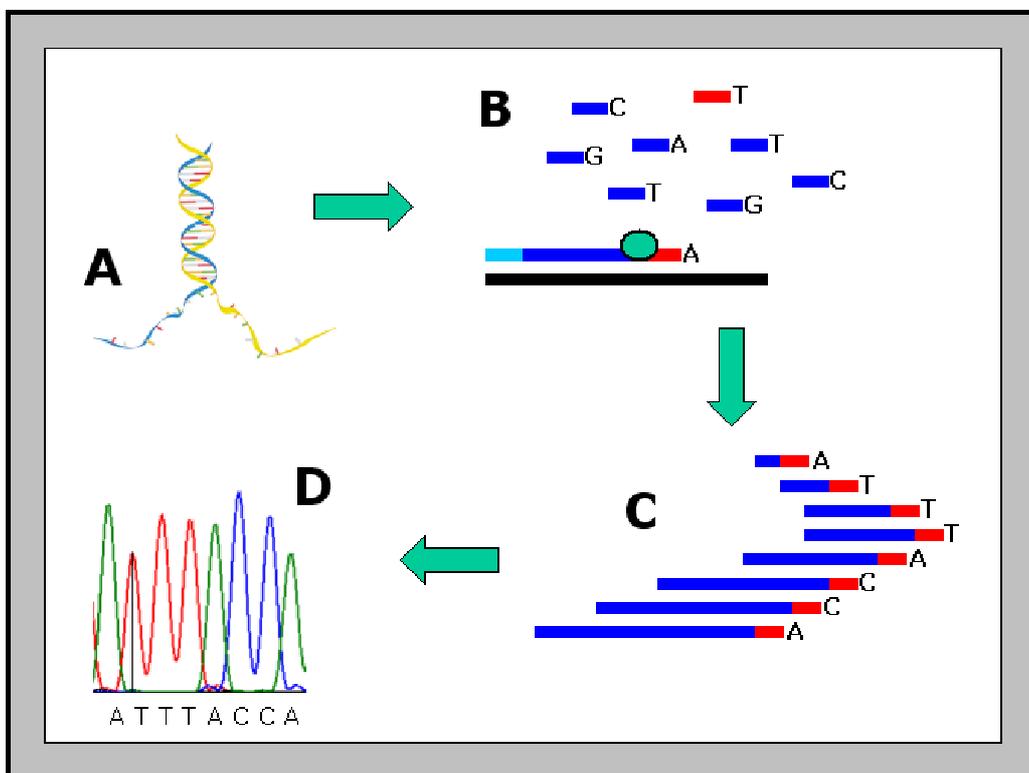




## Sequencing at Micropathology Ltd

### Sequencing technology

We use an ABI 3130xl genetic analyser in combination with ABI BigDye 3.1 technology to perform our in-house sequencing. This technology, referred to as Sanger sequencing, is a variant of the chemistry first described by Fred Sanger in 1977 and is based on the protocol described below:



- (A) A sequencing reaction mix containing amplified template DNA, oligonucleotide primers and a mixture of 'normal' dNTPs and fluorescently tagged terminator dNTPs is heated to 95°C to denature the double stranded DNA template.
- (B) The temperature is reduced and short, complementary primers bind to the now single-stranded template. A subsequent extension step at an enzyme-specific temperature allows DNA polymerase to replicate the template. The inclusion of

chain-terminating fluorescently tagged nucleotide analogues leads to the random termination of growing DNA molecules.

- (C) Appropriately concentrated reaction mixtures will produce a pool of molecules containing representatives that have been terminated at every position of the original template amplicon.
- (D) This pool of DNA strands of varying lengths is then separated chromatographically in hair-thin capillaries and the sequence read by analysing the emerging fluorescent tags as they pass the capillary terminus.

We routinely employ sequencing technology to aid the identification, genotyping and drug resistance testing of some pathogens, as described below:

### ***Identification of unknown pathogens***

There are certain circumstances under which diagnostic tests for specific infecting organisms cannot be applied. This occurs most frequently where infections are culture negative or caused by organisms which grow slowly, are fastidious in their growth requirements or are identified only rarely in human infection.

We perform both **16S rRNA** analysis, targeting a genetic locus common to all bacterial species, and a similar test targeting the **18S rRNA** locus found in fungi. Although these regions are universal to a wide range of organisms, there is sufficient nucleotide sequence variation to enable identification at the genus and sometimes species level. This is accomplished by searching against the NIH annotated genetic sequence database Genbank, the international repository for all publicly available sequence data.

We also perform genus-wide assays for Mycobacteria and Mycoplasma. These tests target regions specific to the genera in question and sequence analysis of the resulting product is usually sufficient to permit species identification.

### ***Viral genotyping***

Many viral species have been classified into genotypes on the basis of clustering by nucleotide sequence identity. This information is frequently of clinical relevance as treatment response or severity of infection in an individual may differ with infecting genotype. We routinely perform **hepatitis B and C genotyping** by searching specific, curated databases for these organisms with nucleotide sequence data obtained from genotyping assays. These assays are designed to target regions which exhibit some level of conservation sufficient to allow successful amplification across the range of observed genotypes but are diverse enough to allow their distinction by sequence analysis. **Genotyping of influenza A, human papilloma virus and adenovirus** is performed by querying Genbank.

### ***Drug resistance testing***

Where therapeutic options for the treatment of chronic bacterial and viral infections exist, the development of drug resistance is frequently inevitable. This process is dependent upon a number of factors including patient adherence to their treatment regimen and the genetic barrier to the development of a drug resistant phenotype.

Phenotypic drug resistance is initiated by genetic mutations that lead to changes in the structure, binding capability or function of pathogen proteins targeted by the drug in question. Where sufficient data is available, particular mutations can be assigned predictive values representing their likely effect on drug susceptibility phenotype. Genotyping assays are frequently quicker and easier to perform than phenotyping but novel mutations or resistant phenotypes caused by complex genotypic combinations may not be identified.

We routinely test for **resistance to multiple drugs commonly used to treat HBV and HIV infections, for ganciclovir resistance in CMV and rifampicin resistance in TB.** Once an appropriately predictive amplicon has been sequenced, analysis can be performed using one of two pipelines. A curated database can be queried if it exists for the organism in question (e.g. HIV) or alternatively the consensus sequence can be compared to a reference list of mutations known to be associated with drug resistance.

### ***Next generation sequencing***

During the last decade, sequencing technologies using methods that diverge significantly from traditional Sanger sequencing have proliferated. These 'next-generation' methods can vastly increase the number of sequence reads obtained in a single run and are thus commonly described as being massively parallel.

We have invested in a GS Junior system from Roche diagnostics, which utilises 454 sequencing technology. Individual DNA molecules within an amplicon or genomic library preparation are bound to beads suspended within emulsion droplets and amplified clonally. The beads are then distributed across hundreds of thousands of wells within a picotitre plate and reagents are flowed across the plate, initiating an enzyme cascade when nucleotides are incorporated into the extending strand of DNA. This reaction is luciferase-coupled and a CCD camera records the resulting light flashes which form the basis of the sequence read.

This technology will be used for research projects describing bacterial population biology, genetic variation within the virulence factors of a single species, diagnostic analysis of challenging mixed infections and the detection of very low level drug-resistance associated mutations.