MICROMON SANGER SEQUENCING

COMMON REASONS FOR SEQUENCING FAILURE

The causes of Sanger DNA sequencing failure are often complex and sometimes very difficult to pinpoint. Failure or poor quality data may be caused by one factor alone or by a combination of several contributing variables.

Inadequate DNA template concentration

The use of spectrophotometry - rarely does this give a true determination of the template DNA concentration due to the presence of contaminating RNA, chromosomal DNA, proteins, etc. The optimal methodology is to use visualisation of the stained DNA on an agarose gel against a standard of similar size and known concentration. If your standard is linear then you must also linearise your plasmid for this determination. Once experienced, reasonable estimates can be made without the use of a standard.

Inadequately purified DNA Template

- **Plasmid**

Aside from contaminating RNA, genomic DNA, proteins, DNA minipreps will also contain carbohydrates, lipids and salts, all of which can inhibit the activity of the Taq DNA Polymerase during the cycle sequencing reaction. This often shows up as a sharply fading read ('ski slope effect'). Purification columns remove most of these unwanted contaminants but if overloaded, the purification devices will not be effective. Not all columns give the same performance results and some trial and error is needed to find the products that are most suited to your needs. Often, a simple Ethanol precipitation step following the column purification elution can clean up the DNA prep significantly.

- **PCR Product**

If the PCR product is not purified, the PCR reaction components will be transferred to the cycle sequencing reaction and will lead to a less than optimal result. Carry over dNTPs further dilute the vital fluorescent dideoxy terminators. The non-purified PCR reaction may contain multiple products which can bind the sequencing primer leading to multiple reads.

Failure to streak clones to single-colony
Results in a template that does not contain the expected primer binding site.

Failure to fully characterise a new plasmid construct
Results in a template that does not contain the expected primer binding site or contains an altered binding site. This can also be the case when designing cross species primer pairs where the sequence of the new species is not accurately known.

Calculation error in primer concentration
Too little primer will result in low amounts of extension products being generated, showing up as a low signal, and a low resolution read. Too much primer is generally not a problem but should be avoided.

Primer melting temperature [Tm] too low
Will result in a less stringent primer during the annealing step and a higher likelihood that the primer will bind to other sites on the template.

Secondary structure in the DNA template
Mostly as hairpin loops but includes any secondary base pairing along the DNA molecule. This structure can restrict or even prevent the Taq DNA Polymerase from moving along the DNA template at the point of the structure. The use of DNA relaxing agents like Dimethylsulphoxide (DMSO) and Betaine in the cycle sequencing reaction can often open up the secondary structure.

Multiple priming sites present
Primer binds to more than one site and the resultant reads are overlayed.

Sequencing incorrect PCR products
PCR product extracted from the agarose gel is not the target product.

Primer-Dimer formation
Primer sequence is such that it can anneal to itself resulting in minimal binding to the DNA template.

Sample handling error
Wrong template/primer combination used in the cycle sequencing reaction.