DNA Sequencing
- a practical guide.

Functional Genomics and Proteomics Facility

Requirements

DNA – **Always** elute your DNA in molecular biology grade water. Commercial elution buffers contain salts that inhibit the PCR reaction.

Primers – Avoid repeated freeze/thawing of your primers by making aliquots of your concentrated stock and storing at -20°C. Make a fresh working stock from the concentrate each time.

Water – Use good quality Molecular grade water. **Impurity=Inhibition**

10µl sample volume – **More important than you might think!** We add 10µl BigDye Reaction Mixture (BDRM) to your sample which includes the buffer at 2X. If the ratio of sample to BDRM is not 1:1 then the buffer is > or < than 1X, and the PCR reaction will be inhibited or inefficient.

Sample Preparation.

Template Quantity.

Template	Recommended Quantity
PCR Product:	
100-200bp	1-3ng
200-500bp	3-10ng
500-1000bp	5-10ng
1000-2000bp	10-40ng
>2000bp	40-100ng
Single–Stranded DNA	50-100ng*
Double-Stranded DNA	200-500ng*
Bacterial genomic DNA	2-3ug

*Because the DNA Analyser is a highly sensitive instrument, you may not need this much template.

Reactions.

Reagent	Quantity
Template	See Table Above
Primer	3.2 pmol
Molecular grade water	q.s
Total Volume 10ul	

Genomics Facility will add Terminator Reaction Mix to your total volume of 10ul to give a final volume of 20ul.

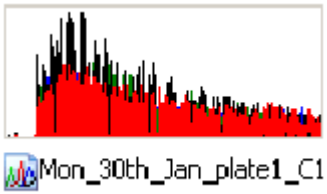
PCR Primers.

The standard protocol used requires the use of primers with annealing temperatures of between 50°C and 65°C.

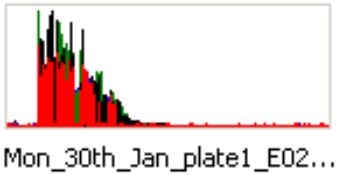
Troubleshooting results in Sequence Scanner v.1.0

(Sequence Scanner free to download from Applied Biosystems website)

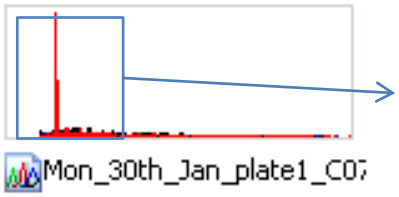
Look at the thumbnail of your trace.

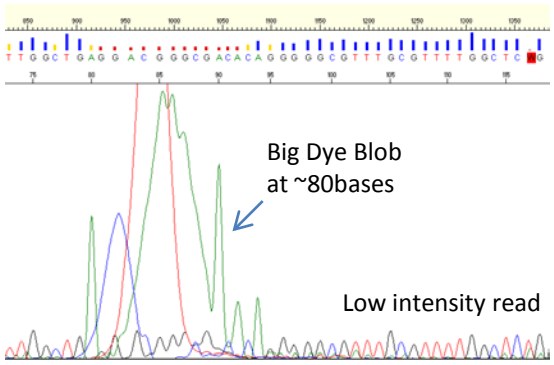


Example 1. Good reaction and read. Trace always tails off a little.

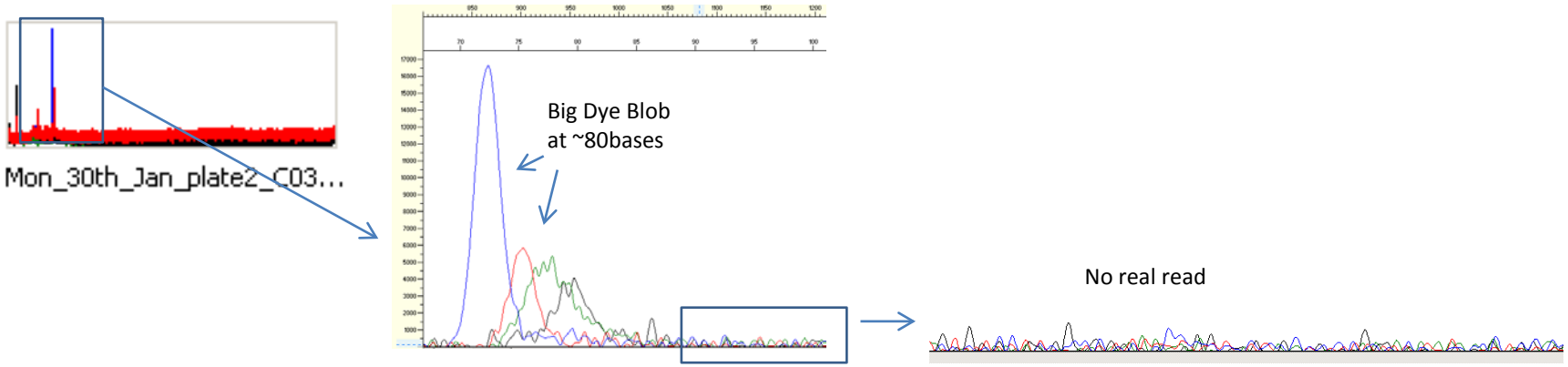


Example 2. Too much template. No big dye blob – which indicates all the nucleotides have been used up. But the steepness in which the trace tails off indicates that with each cycle the nucleotides are being used up to start the extension process but are running out before the full sequence (900-1000 bases) can be fulfilled. **Action** – reduce template.





Example 3. Too little template. BigDye blob indicates left over nucleotides, not used up in the PCR reaction. But there is a read, just at a low intensity. But you get more mixed reads as noise can confuse the base caller. **Action** – increase template.

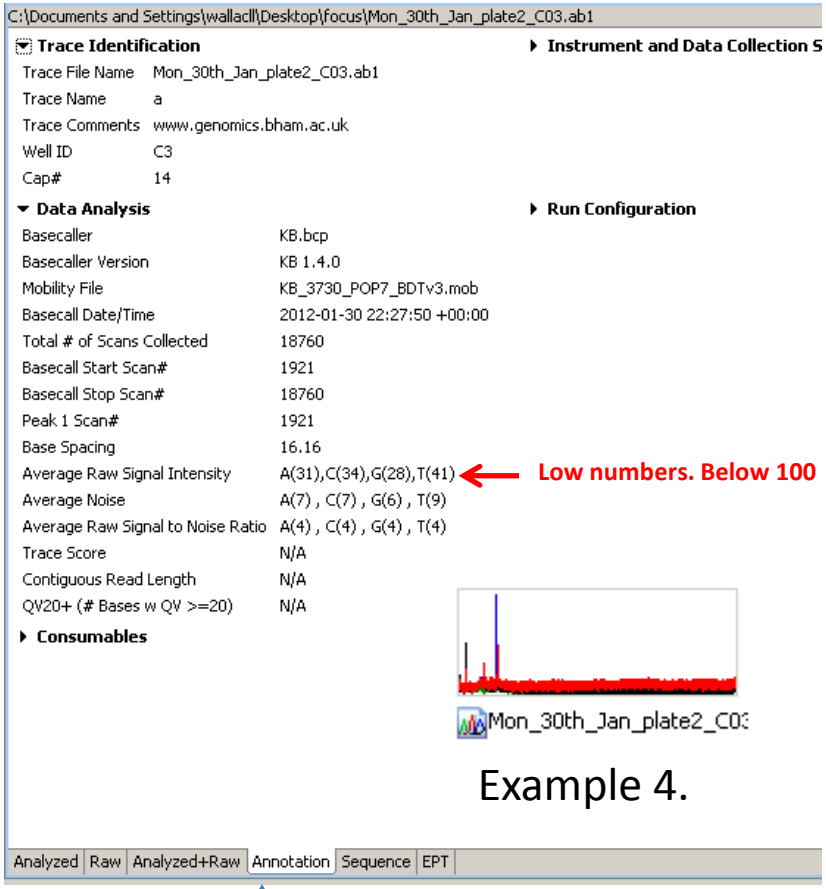


Example 4. No read at all. Big Dye blob indicates the nucleotides have not been used. No evidence of a clear read, just noise. Software will try to find a signal and bring up the background.

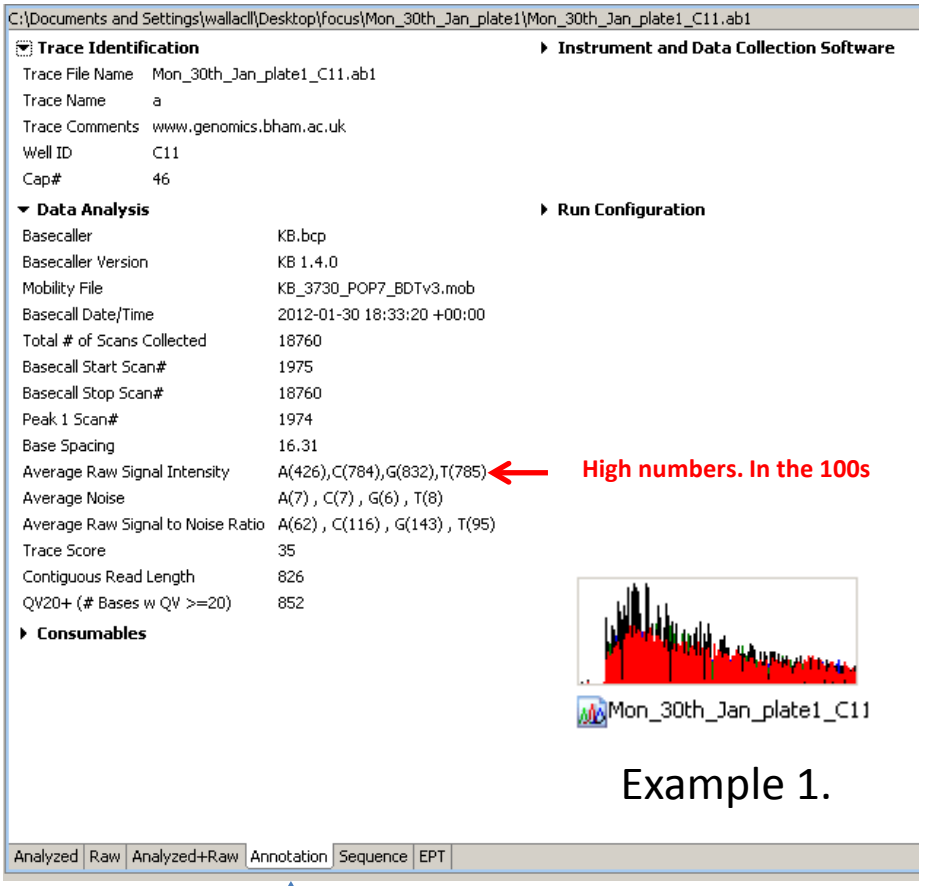
Causes – Not enough template
Primers not annealing
Primers not at right concentration or degraded
Inhibition by impurities
GC-Rich sample

Action – Increase template (check concentration on gel or nandrop)
Check annealing temperature and cross reference with our PCR protocol
Make fresh primer
Clean up DNA
Elute DNA in molecular grade water
Longer denaturing step

Additional Useful Information



Example 4.

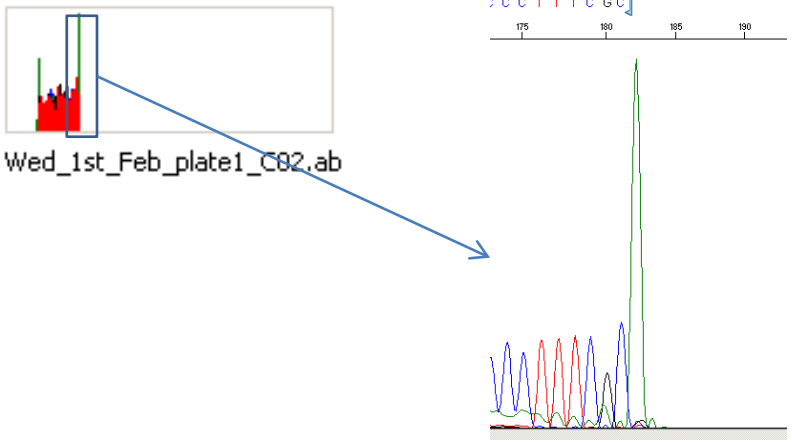


Example 1.

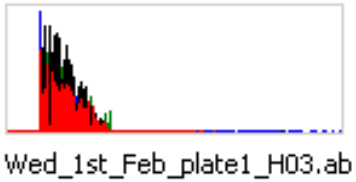
In support of the visual read there is useful information in the Annotation tab of your file.

Low Average Raw Signal intensity is a good indicator of reaction success, figures should be in their 100's for a confident read.

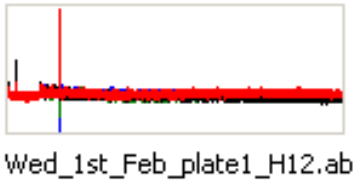
Examples of sequence characteristics.



Example 5. A natural stop or short fragment of a sequence. No trailing off of read, just a "dead stop".



Or



Example 6. GC-rich sequences.
If your sequence is rich in G and C nucleotides then the corresponding labelled nucleotides in the BDRM get used up too quickly and lead to a steep tail off of the read.
G and C bonds are stronger and if in high abundance sometimes requires longer denaturing times, or the addition of 5% formamide to reduce the tertiary DNA structure and allow primers to anneal and extension to occur.
Action: See Genomics Staff