



DNA SEQUENCING SANGER: TECHNICALS SOLUTIONS GUIDE

We recommend for the sequence visualization the use of software that allows the examination of raw data in order to determine quantitatively how good has been the sequencing reaction.

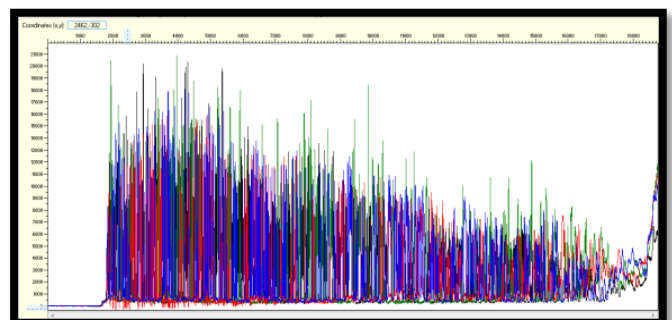
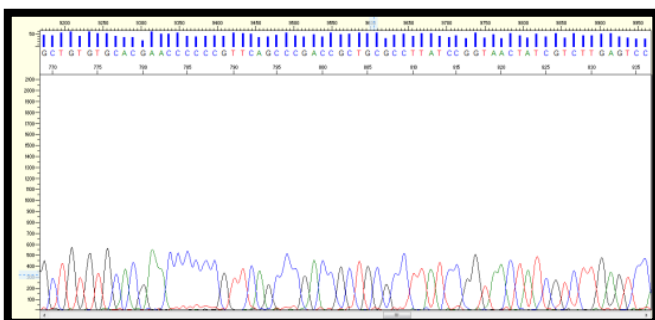
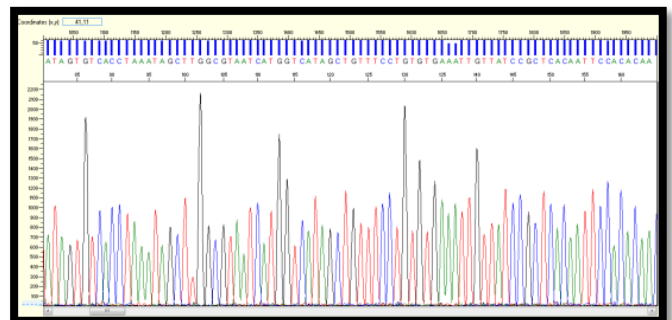
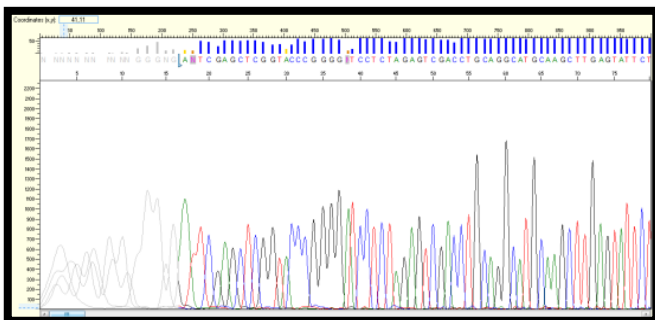
We recommend the use of Sequence Scanner Software from Applied Biosystems.

SUCCESSFUL DNA SEQUENCING READ

Electrophoregram shows correctly formed and separated peaks with good quality scores. Usually there is a small area at the beginning of the run where the peaks are not well defined. Raw data has intensities over 1000 units of fluorescence.

Example.

PLASMID



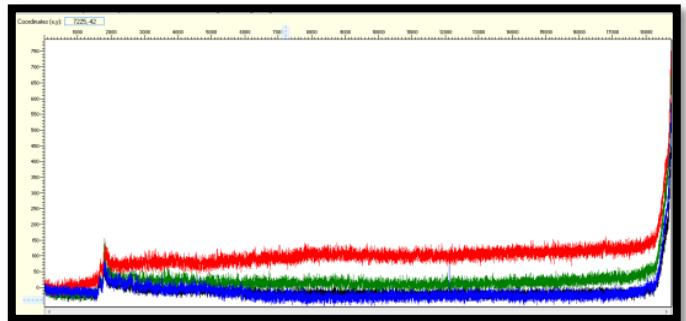
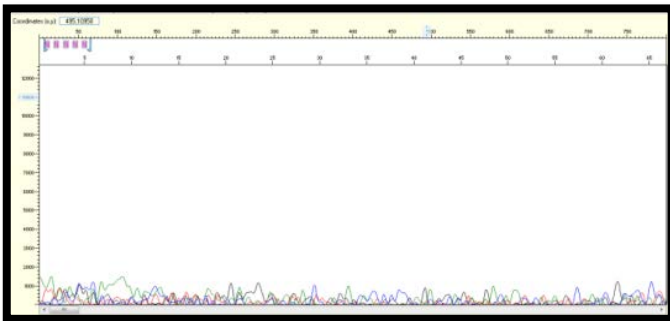
FAILED DNA SEQUENCING REACTION OR “DIRTY” SEQUENCE

Sequence Appearance.

- Electrophoregram has mixed peaks or is mostly blank.
- Many ‘N’s in the sequence, if bases are called at all.
- The read sequence file yields a no recognizable sequence.
- Raw data has signal intensity in the low hundreds.

Example.

NO REACTION



Possible causes.

The causes for these kind of results can be several, the most common are the following ones:

- DNA Template concentration is too low.
- Note: measuring DNA concentration by UV absorption is often inaccurate and concentration is frequently overestimated. Agarose gels or espectrofluotometry are a much better method to estimate quality and quantity of DNA samples. In this case the Raw Data signal is low, around 100, due to poor DNA sequencing reaction.
- No correct primer was added to the reaction, so binding between the DNA and the primer cannot occur. No DNA sequencing reaction, and hence Raw Data signal usually below 100 units.
- Template purification is not working properly. Sometimes the sample preparation is contaminated with ethanol or other molecules that inhibit the DNA Polymerase, yielding no sequencing reaction.

Treatment.

- Check the concentration of the template by agarose gel or espectrofluorometry to ensure that it is in the ranges described in the Sample Submission Guidelines.
- Check the primer sequence against the template sequence to ensure that there is a proper binding site.
- Improve the quality of the template preparation, mainly for the primers making fresh dilution each time in case problems were observed.

DROP OFF

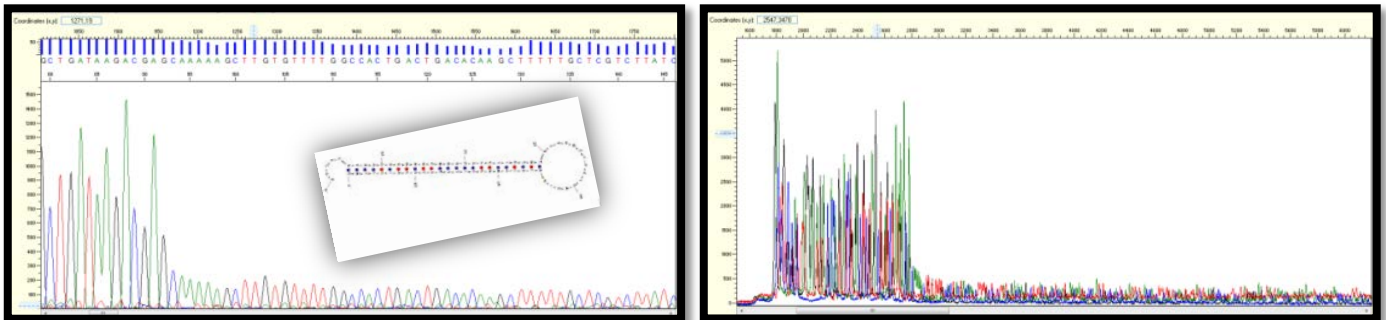
Sequence Appearance.

- The DNA sequence suddenly stops or peak intensity drops off substantially. This is due to a strong secondary structure or repeats.

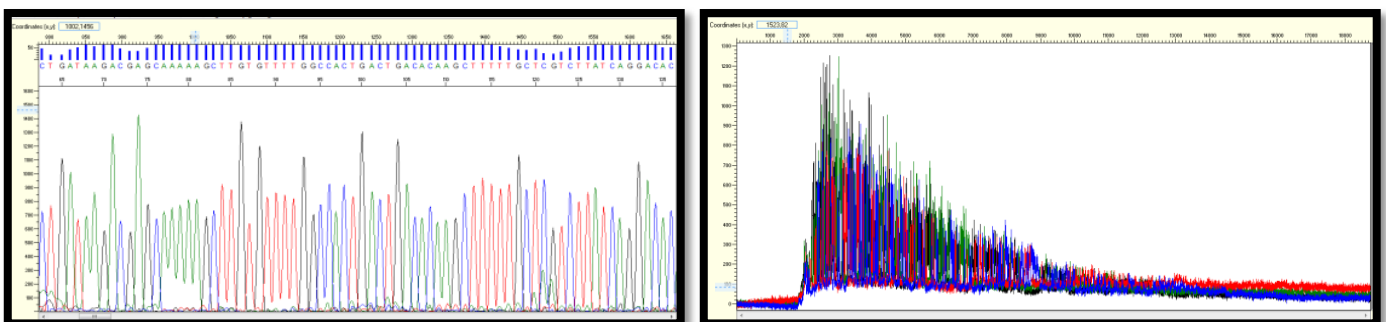
Note: Some cloning vectors have palindromes flanking their linker and may show this drop-off effect when sequenced.

Example.

SECONDARY STRUCTURE IN PLASMID SEQUENCED UNDER STANDARD CONDITIONS



SECONDARY STRUCTURE IN PLASMID SEQUENCED UNDER SPECIAL CONDITIONS





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Possible causes.

- Strong secondary structures in the DNA template. Some cloning vectors have palindromes flanking their linker and may show this drop-off effect when sequenced due to a hairpin formation.
- Region rich in GC or GT repeats.
- Sample contains an siRNA construct.

Treatment.

- Apply special protocols for secondary structures or repeats rich regions.

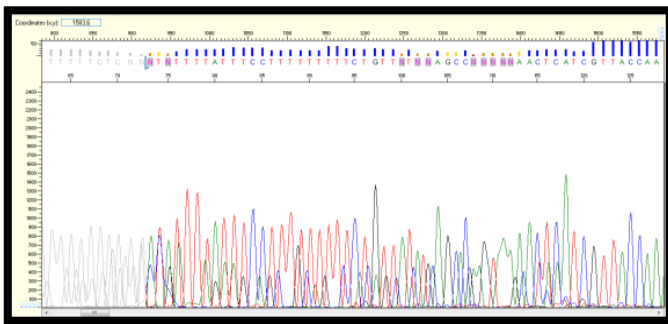
DOUBLE SEQUENCE

Sequence Appearance.

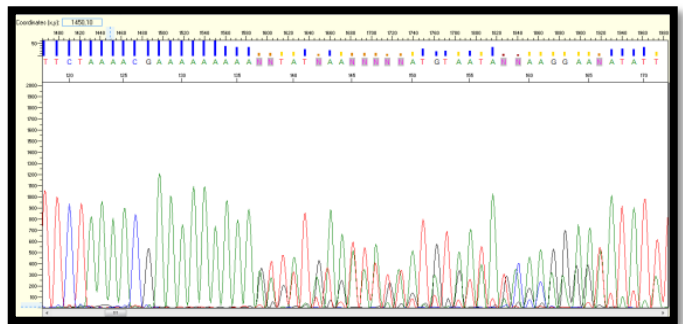
- Double peaks are observed. Multiple peaks with the same or different height overlapping one another.
- Raw data has adequate signal intensity, indicating that the DNA sequencing reaction yielded product, so the double peaks are not due to weak signal and/or background noise.

Example.

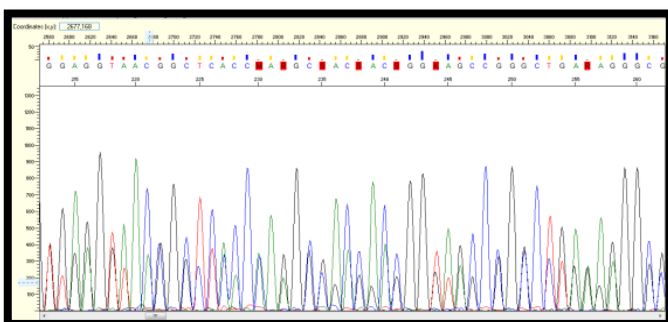
PRIMER DIMER



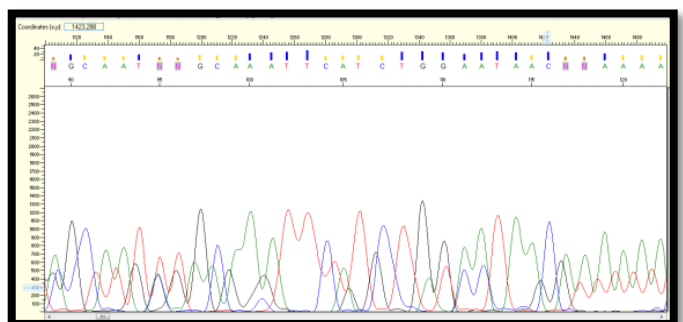
INDEL



CLONE CONTAMINATION



TWO PRIMERS



Possible causes.

- PCR products were not purified (or the purification was not performed properly). In this case, residual PCR primers or primer dimers may participate in the sequencing reaction.
- PCR template may be heterozygous due to indels present in a diploid (or polyploid) organism.
- Clone contamination. In this case, the beginning of the sequence is often clean and becomes double as the sequence progresses into the two inserts.
- Two primers may have been mistakenly added to the sequencing reaction.
- The PCR reaction yields more than a product, and both are sequenced.
- There may be another region in the molecule with enough homology to the primer.

Treatment.

- If clone contamination is expected, please plate again the bacteria onto selection media, and select clones again.
- Where PCR template heterozygosity is suspected, examination of the 'dirty' sequence may yield some indication of the area of heterozygosity. Newly designed PCR primers that yield shorter products may allow you to discern where the troublesome area is. Alternatively, you may decide to redesign the sequencing primer close to the area where the problem first arises hoping to find a primer that will sequence one of the product species. If the PCR product is large, subcloning the template into shorter pieces may also provide a strategy for discerning the true sequence of the area of interest.
- Run the PCR reaction in an agarose gel in order to check if more than one amplicon is present.
- If you suspect that two different primers have been added to the reaction, repeat the reaction. If the working solution for the sequencing primer may be contaminated then going back to your original uncontaminated stock solution to prepare a new working solution or resynthesize the primer.
- Check your PCR purification protocol and the solutions used in the clean-up reaction.
- Check the primer sequence against the template sequence to ensure that there is a single binding site. Where sequence is unknown, you may need to switch to a different sequencing primer to eliminate the problem.

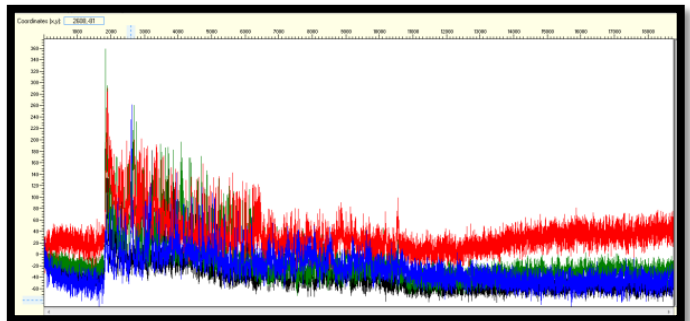
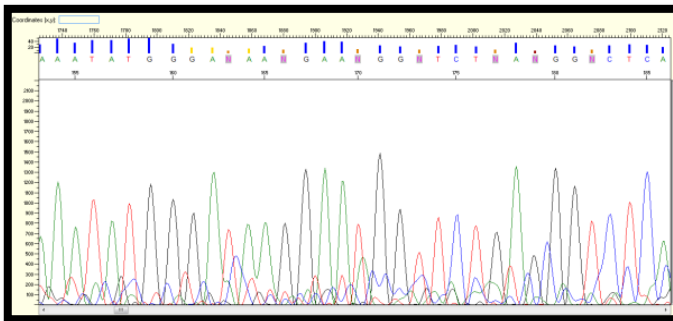
NOISY BACKGROUND

Sequence Appearance.

- Background noise and odd peaks are present underneath the main sequence peaks, along with “N” dispersed in the sequence. Low raw signal.

Example.

BACKGROUND



Possible causes.

- The most common cause for this is that the low signal above background is due to an insufficient template or primer input or an inefficient sequencing reaction for enzyme inhibition. A typical example is ethanol contamination in the template preparation.

Treatment.

- Quantify properly template and primer; be sure you don't have ethanol carry over from the purification columns.

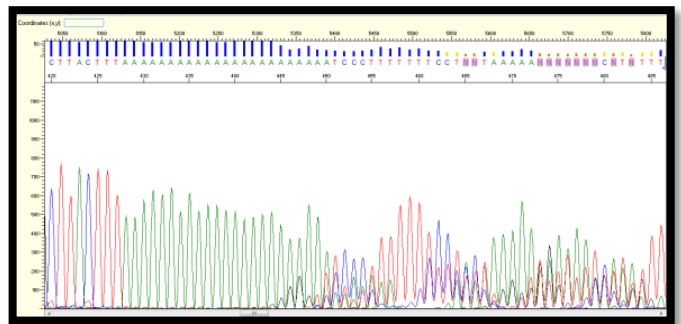
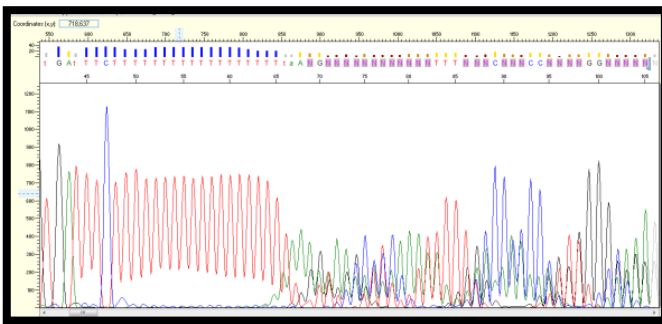
STUTTERING AFTER MONONUCLEOTIDE STRETCHES

Sequence Appearance.

- Sequencing data quality is poor after stretches of 10 or more nucleotides of the same base due to mixed bases.

Example.

“STUTTERING”



Possible causes.

- Polymerase slippage during DNA synthesis. This is a recognized limitation of the Sanger method.

Treatment.

- Sequence from the reverse direction.
- Use a poly-mononucleotide primer with a degenerate base (wobble) at the 3' end.



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GRADUAL DIMINUTION OF SIGNAL

Sequence Appearance.

- Sequencing signal gradually drops off.

Possible causes.

- Excess DNA template or primer, disbalanced reaction.
- GC or GT rich template (from bisulfide treated DNA, for example)

Treatment.

- Carefully quantify your DNA template and primer prior to sequencing.
- Sequence from the reverse direction.



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DNA SPIKES

Sequence Appearance.

- Sharp, high-intensity, multicolored peaks that randomly appear in the sequence.

Possible causes.

- The cause of spikes is not completely understood. One possible explanation is that an impurity or micro bubble passes through the camera view of the sequencing instrument, scattering light.

Treatment.

- The surrounding sequence should still be correct and accurate if of good quality, please request your sample to be rerun if necessary.