No recognisable sequence

![Graph showing nucleotide sequence with peaks and valleys indicating signal strength.

No analysed data is present because the signal strength is below the threshold for analysis page 7.10

Possible Causes

**Template**
- Insufficient template
- Inhibitory contaminant in the template

**Primer**
- Insufficient primer
- Primer has no annealing site
- Mutation present in the primer binding site
- Poor primer design or primer

**Sequencing reaction**
- Missing reagent
- Old or mishandles reagents
- Thermal cycler power failure or incorrect cycling conditions

**Sequencing cleanup**
- Extension products lost during reaction cleanup

Recommended Actions

- Quantitate DNA template page 3.17
- Increase amount of DNA in the sequencing reactions
- Clean up the template page 3.16
- Quantitate the primer 3.19
- Increase amount of primer in the sequencing reactions
- Use a primer that is complementary to the template 7.19
- Use different primer or sequence complementary strand
- Redesign primer page 3.18
- Carefully repeat reaction page 3.21
- Use fresh reagents page 3.20
- Repeat the reactions
- Calibrate thermal cycler regularly
- Use correct tubes for thermal cycler page 3.20
- Set rate to 1°C/sec
- Check centrifugation speed and times page 3.33
- Check ethanol concentrations
Noisy data throughout sequence with low signal strength

Noisy data is characterised by a high background and peaks under peaks causing ambiguities in basecalling page 7.10

**Possible Causes**

**Template**
- Insufficient template
- Degraded template

**Primer**
- Poor primer binding due to low melting temperature of primer
- Primer anneals poorly due to secondary structure

**Sequencing reaction**
- Old or mishandles reagents
- Thermal cycler power failure or incorrect cycling conditions

**Recommended Actions**

- Quantitate DNA template page 3.17
- Increase amount of DNA in the sequencing reactions
- Prepare fresh template
- Redesign primer page 3.18
- Use fresh reagents page 3.20
- Repeat the reactions
- Calibrate thermal cycler regularly
- Use correct tubes for thermal cycler page 3.20
- Set rate to 1°C/sec
Noisy data throughout sequence with good signal strength

Noisy data is characterised by a high background and peaks under peaks causing ambiguities in basecalling page 7.11

Possible Causes

**Template**
- Multiple templates in reaction

**Inhibitory contaminant in template**

**Primer**
- Multiple priming sites
- Multiple primers in reaction
- Primer with N-1 contamination

Recommended Actions

- Check PCR template on gel for single band page 3.16
- Re-isolate the DNA from a pure colony and re-sequence page 3.16
- Prepare fresh template page 3.16
- Make sure primer only has one priming site page 3.18
- Ensure only one primer has been used
- Purify PCR template to remove excess primers page 3.12
- Use HPLC purified primers
Noise up to or after a specific point in the sequence

In this example noise starts after the multiple cloning region of the vector because more than one colony was purified. Bacteria with no insert or a different insert were included page 7.12

Possible Causes

**Template**
- Mixed plasmid preparation
- Multiple PCR products
- Frame shift mutation

**Primer**
- Primer-dimer contamination in PCR sequencing

Recommended Actions

- Make sure you have only one template page 3.6
- Make sure you have only one PCR product by agarose gel electrophoresis page 3.12
- Use a different primer after mutation or sequence complementary strand
- Optimise PCR amplification page 3.10
- Make sure there is no sequence complementarity between the two PCR primers
- Make sure your sequencing primer does not overlap the sequences of the PCR primers
- Use a Hot Start technique
- Try alternative sequencing chemistry
- Use an anchored primer page 7.36
# Early signal loss

Early signal loss can be gradual or abrupt page 7.14

## Possible Causes

<table>
<thead>
<tr>
<th>Template</th>
<th>Recommended Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradual signal loss due to nucleotide repeat sequence</td>
<td>♦ Sequence complementary strand</td>
</tr>
<tr>
<td>Gradual or abrupt signal loss due to excess of template in reaction causing top heavy data</td>
<td>♦ Quantitate template page 3.17</td>
</tr>
<tr>
<td>Abrupt signal loss due to secondary structure in the template</td>
<td>♦ Sequence complementary strand</td>
</tr>
<tr>
<td></td>
<td>♦ Use a primer that anneals at a different position</td>
</tr>
<tr>
<td></td>
<td>♦ Incubate the reaction at 96 degrees C for 10 minutes before cycling</td>
</tr>
<tr>
<td></td>
<td>♦ Increase the extension temperature by 2 to 3 degrees C</td>
</tr>
<tr>
<td></td>
<td>♦ Increase denaturation temperature to 98 degrees C</td>
</tr>
<tr>
<td></td>
<td>♦ Add DMSO to a final concentration of 5%</td>
</tr>
<tr>
<td></td>
<td>♦ Double all reaction components and incubate at 98 degrees for 10 minutes before cycling</td>
</tr>
<tr>
<td></td>
<td>♦ Linearise the DNA with a restriction enzyme</td>
</tr>
<tr>
<td></td>
<td>♦ Shear the insert into smaller fragments (&lt;200bp) and subclone</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer</th>
<th>Recommended Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gradual or abrupt signal loss due to excess of primer in reaction causing top heavy data</td>
<td>♦ Quantitate primer page 3.19</td>
</tr>
</tbody>
</table>
Excess dye peaks at beginning of sequence

Incomplete removal of excess dyes during the post cycle sequencing cleanup can obscure data at the beginning of the sequence page 7.27

Possible Causes

Sequencing cleanup
Poor removal of unincorporated dye terminators

Recommended Actions

- Use ethanol/EDTA precipitation protocol to remove unincorporated dye terminators page 3.33
- With microfuge tubes, aspirate the supernatant rather than decanting
- Use correct concentration of ethanol
- Do not use denatured alcohol
- Use ethanol at room temperature not 4ºC
- Do not leave reactions precipitating overnight
Sequence composition

High G or C template or templates containing repetitive sequences can be difficult to sequence.

Possible Causes

Template
Gradual signal loss due to nucleotide repeat sequence
Abrupt signal loss due to secondary structure in the template

Recommended Actions

♦ Sequence complementary strand
♦ Use a primer that anneals at a different position
♦ Incubate the reaction at 96 degrees C for 10 minutes before cycling
♦ Increase the extension temperature by 2 to 3 degrees C
♦ Increase denaturation temperature to 98 degrees C
♦ Add DMSO to a final concentration of 5%
♦ Double all reaction components and incubate at 98 degrees for 10 minutes before cycling
♦ Linearise the DNA with a restriction enzyme
♦ Shear the insert into smaller fragments (<200bp) and subclone
Multiple sequences

The presence of more than one template in a reaction will result in multiple, overlapping sequences in the data page 7.21

### Possible Causes

**Template**
- Mixed plasmid preparation
- Multiple PCR products

**Primer**
- Multiple priming sites
- Multiple primers in reaction
- Primer with N-1 contamination (primer that is one base shorter than the desired primer)

### Recommended Actions

- Make sure you have only one template page 3.6
- Make sure you have only one PCR product by agarose gel electrophoresis page 3.12
- Use gel purification techniques
- Make sure primer only has one priming site page 7.21
- Ensure only one primer has been used
- Purify PCR template to remove excess primers page 3.11
- Use HPLC purified primers
Homopolymer regions

Long homopolymer T regions (or A regions) can cause Problems due to enzyme “slippage” page 7.35

Possible Causes

**Template**
Long homopolymer T (or A) regions causing slippage of the enzyme during sequencing

Recommended Actions

♦ Use an anchored primer (i.e., a sequencing primer that is polyT containing a A, C, or G base at the 3’ end of a poly A region). The 3’ base will anchor the primer into place at the end of the homopolymer region