Sequencing Service Sample Submission Guidelines

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Online Sample Submission Portal

Please use our Sample Submission Portal (MSSS) available at https://gmg-submit.gimr.garvan.org.au/#/login to submit samples. Find instructions on how to use the sample submission portal in the document linked on our portal called "GMG Sample Submission Instructions". There is also a short "How to submit samples" video.

Further information on our services can be found at our website https://www.garvan.org.au/research/capabilities/molecular-genetics/sequencing-and-fragment-analysis. New clients need to create an account (please follow instructions after clicking on the 'Create Account' button on the landing page), existing clients log into their account by entering their login details and selecting the button 'Login for non clinical samples', see Figure 1

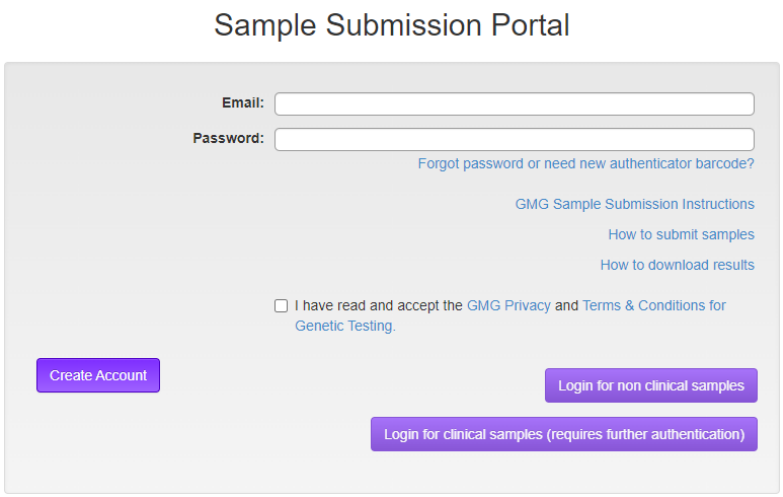

Figure 1: SEQ service login screen

After logging in the next window will show your account where you can submit new samples and view previous submissions, To submit a new manifest select 'New Mouse Genotyping Manifest' from the options available, see Figure 2

Figure 2: Manifest choice

In the popup window choose the type of service (there are three options, please see below for more information) and select the container type and how many samples will be submitted.

Premix Sequencing and Fragment Analysis Services please select:

a) Premix service module & samples in 8-strip tubes

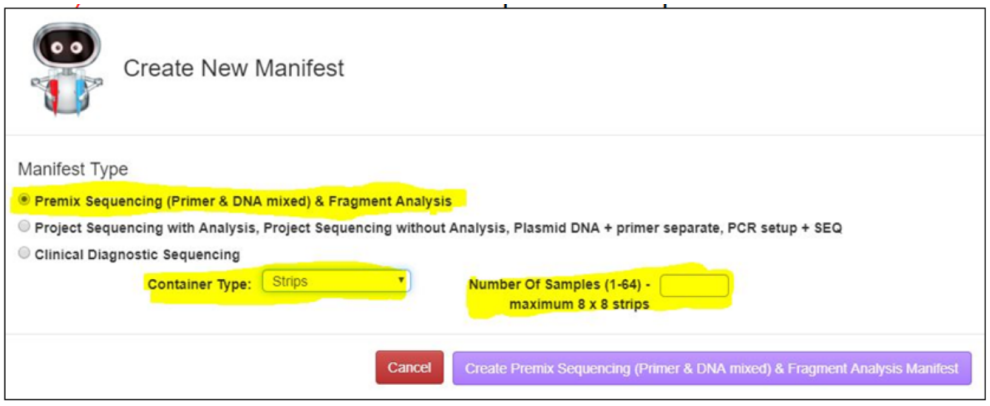

Figure 3: Premix service module & samples in 8-strip tubes

b) Premix service module & samples in a 96 well plate

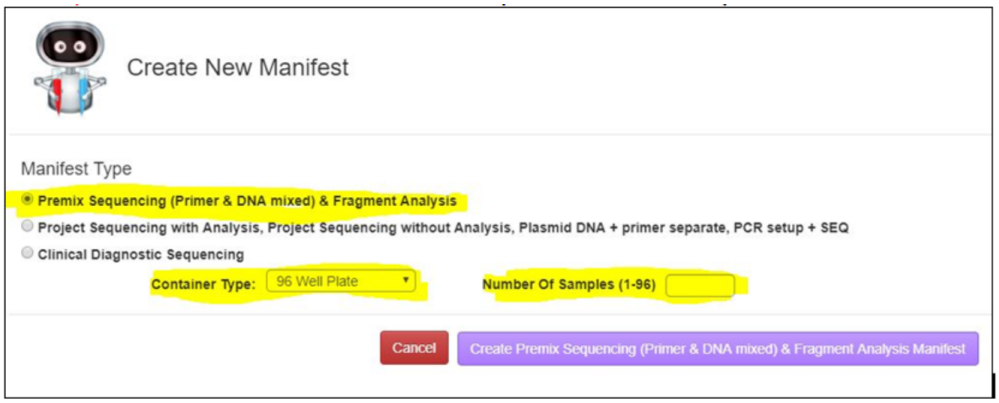

Figure 4: Premix service module & samples in a 96 well plate

After clicking on the “Create Premix Sequencing & Fragment Analysis Manifest” button the new Manifest will open, see Figure 5

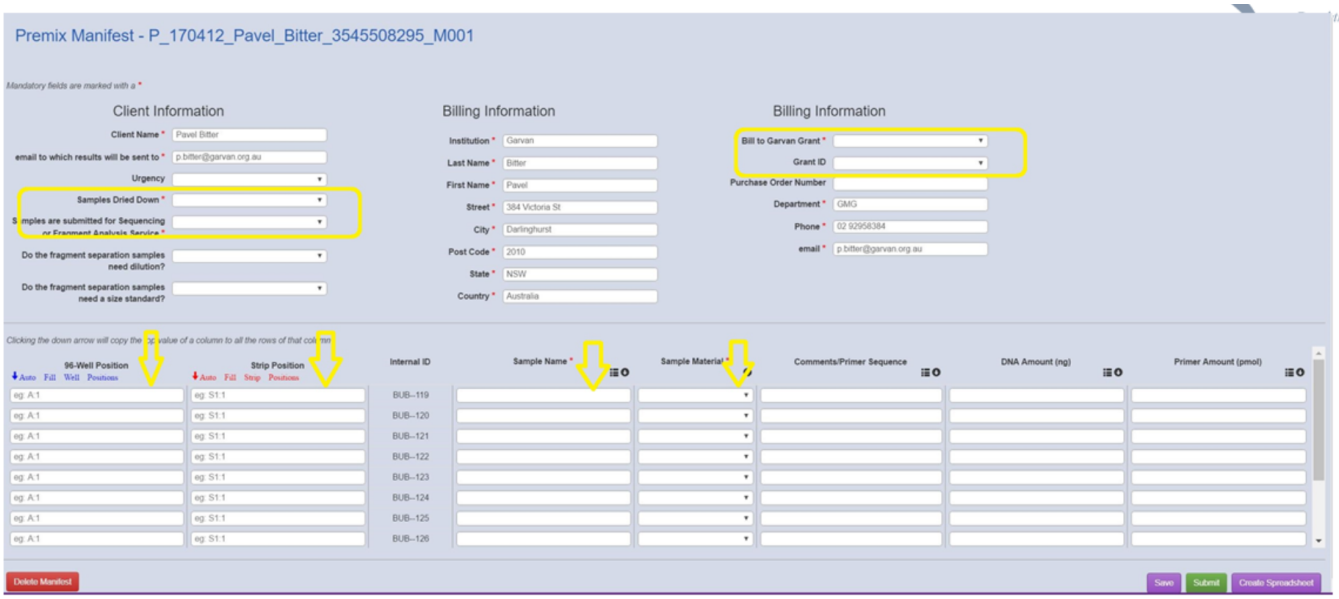

Figure 5: Create Premix Sequencing & Fragment Analysis Manifest Page

All yellow highlighted fields need to be filled in before submission. You can automatically fill the sample locations by clicking on the **blue arrow down button** (for a 96 well plate) or on the **red arrow down button** for strip tubes. Alternatively you can give other locations for your samples manually in the format A:1 etc, see Figure 6

Figure 6: Fields need to be filled in before submission

Premix PCR Product (or Plasmid) + Primer & SEQ / Fragment Analysis

Type of Service

Samples can be submitted for the **Sequencing Service** or the **Fragment Analysis Service**. If the "Fragment Analysis" is selected you can choose whether we add a size standard to each of your samples or not, please choose from the dropdown menu in the manifest accordingly. There is another dropdown menu to select which size standard you wish that we add (GS 500 or GS 600) and there is another drop down menu to select whether samples need to be diluted or have been diluted and are ready to be processed (options: need 1/10 dilution; need 1/20 dilution; ready for loading).

Sample Submission in Tubes or Plates

You can submit your samples in 0.2mL 8x strip tubes (recommended: *Interpath Services Pty. Ltd. Cat# 3131-00; 0.2mL 8-Strip PCR Tubes with Caps pkt/ 1000*) or you can use 96-well semi skirted PCR plates covered with a seal (recommended: *Bio-Rad Cat # HSS-9901*). Single 0.2mL tubes easily brake and are hard to label legibly, please use a strip even if you submit only one sample.

Drying Down Samples

We recommend drying down the liquid of your samples for sending samples without cooling. Please spin the DNA + primer mix to the bottom of the well and place it in a heatblock or Thermocycler for 10min at 80°C with open lids until no volume is left in the tubes/plates. Then close the tubes with the caps or cover the 96-well plate with a seal. Please indicate in the Sample Submission Manifest in the dropdown menu what status your samples are when submitted.

Sample Names

Please do not use any special characters in the sample names like hyphen (-), space ( ) or dots (.) or any unusual characters (%&$@#). The submission software does not accept sample names with special characters other than underscore (_), letters and numbers. If you enter a name with special characters MSSS will not let you submit your manifest and a warning will appear saying: “Invalid Sample Name”. Sample names must contain more than one number or letter that means the minimum sample name must contain two characters (you could use your initials and a number for example). You can copy and paste sample names from Excel by using the table icon (popup window will open) or you can fill down a column by using the arrow down icon.

Sample Material

Please select from the dropdown menu what material you are sending us, the options are “Plasmid”, “PCR product” or “Other”. You can fill down the first selection of the column by using the arrow down icon.

Comments

In the field “Comments” additional information can be given supplementary to sample names, vector name, primer used etc. Please do not use special characters as they can cause problems with our instruments.

Clean DNA Samples for Premix Sequencing

Please clean your DNA (PCR products via post-PCR clean up kits or Exonuclease I / Shrimp Alkaline Phosphatase (Exo/SAP) digestion. Plasmids via mini spin column kits) and quantify your DNA via Nanodrop, gel or fluorescent intercalation. The quality of your input DNA has a significant effect on the quality of your results, see Appendix 1.

Sample Preparation for Sequencing Samples

Please dilute your stock primer (see Appendix 2) on the day of sample preparation to 3.2pmol/µL (freeze thawing the primer at this concentration will unavoidably lead to poor sequencing data). Add 1µL of this primer dilution to your DNA (please refer to the table in Figure 7 for DNA amount). Submit samples either dried down or in a volume <20µL. When submitting samples in 96-well plates please fill the plate from column to column (A1 to H1, then A2 to H2 etc).

Sequencing Reaction of:	DNA amount		Primer amount
Plasmid DNA	20-80ng	+	3.2pmol
PCR Product	10-30ng		

Figure 7: Sample Preparation for Sequencing Samples

Labelling of tubes or 96-well plates

Samples need to be positioned and labelled according to the cells they are named in the manifest. Please label the 8x strip tubes with a permanent marker on the side of the tube itself and on the lids as shown in the pictures. Please label each 8x strip tube with your initials!!! Please make sure that the position of the samples in the tubes corresponds with the position of that sample in the Manifest: write number “1” for your first sample, number “2” for the second one, etc., see Figure 8. Do not write your sample names, just consecutive numbers. Plates can be labelled with the name of the submitting person and the date of submission.

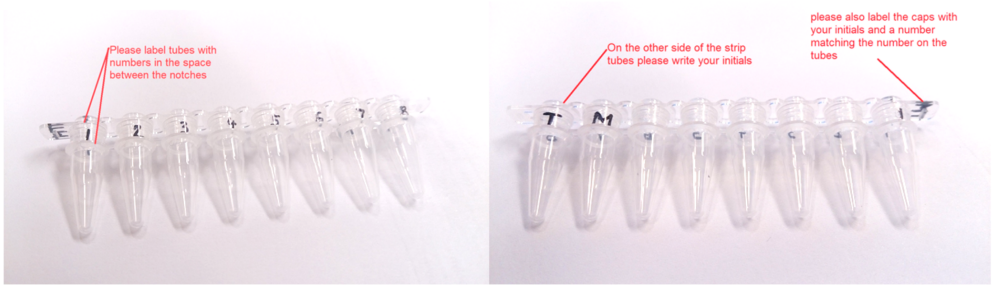

Figure 8: Labeling of tubes

Project Sequencing Service

For Project Sequencing Services please select, see Figure 9

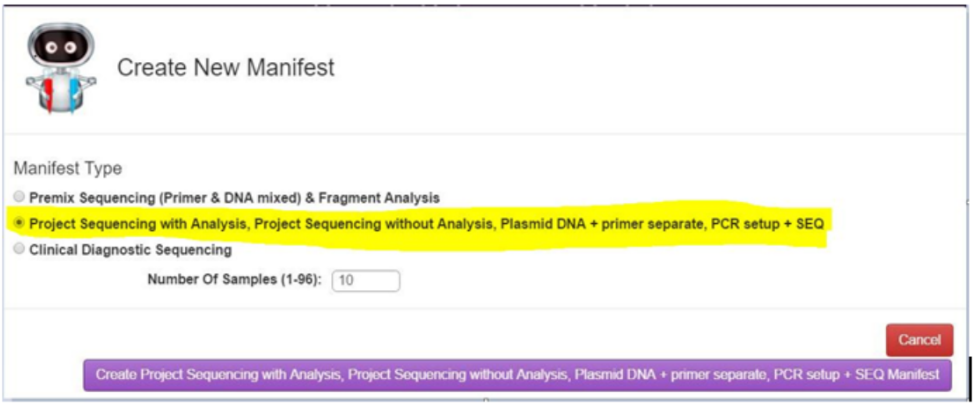

Figure 9: Project Sequencing Service Manifest

Then in the manifest choose one of these options:

Plasmid Mix & SEQ

For this service you can submit your plasmid DNA and nominate a primer from the dropdown options in the field called “Primer to be used”. If your primer is unique and cannot be found in the dropdown options you can enter the sequence of the primer in the “Comments” field and choose the option “to be ordered by GMG according to sequence given in Comments field” from the dropdown menu. We will then order this primer for you and use it for sequencing your plasmid sample. Or you can send us your primer tube(s) with your samples and select the option “Submitted with samples” in the dropdown menu. Please make sure you submit at least 100ng of plasmid DNA for each sample and sequencing direction (either dried down or in a volume <20µL). The quality of your submitted DNA is crucial for the quality of your results.

PCR Mix & SEQ

For this service you can submit your PCR product. In the dropdown options in the field called “Primer to be used” choose either “to be ordered by GMG according to sequence given in Comments field” and enter the sequence of the primer in the “Comments” field or choose the option called “to be designed by GMG from your submitted SEQ file” and send us a sequence of your PCR product via email and we will design and order a primer for you. Or you can send us your primer tube(s) with your samples and select the option “Submitted with samples” in the dropdown menu. Please make sure you submit at least 100ng of PCR product for each sample and sequencing direction (either dried down or in a volume <20µL). The quality of your submitted DNA is crucial for the quality of your results.

Project Sequencing with Analysis or without Analysis

Please contact us via email or phone to discuss the turnaround time, costs and regions of interest for your PCR Setup project. We will then use your sequence file information to design primers and amplify your genomic DNA or cDNA via PCR and sequence the PCR product. Please make sure you submit at least 100ng of genomic DNA for each sample (either dried down or in a volume <20µL). The quality of your submitted DNA is crucial for the quality of your results. We will align and analyse the results for you and identify mutations and create a report. If you choose the option “without Analysis” we will send you the raw data .abi files and you can analyse the data yourself.

Research Diagnostic Technical Sanger Sequencing Report (Confirmatory, level 1, ISO 17025)

Our sequencing facility is NATA ISO 17025 accredited to perform Sanger Sequencing for confirmation of defined mutations or polymorphisms or detecting heterozygous loci. We can only perform confirmatory sequencing which means the results must have been generated by whole genome or exome sequencing or microarray analysis before the samples are submitted to our service. We will then extract DNA from patient blood, design PCR primers, amplify the DNA, set up Bigdye reaction, perform capillary separation and analyse the results. We will issue a technical report which can be used for research purposes only.

This service module does not require a patient consent form and you need to make sure you do NOT send us any patient information that is confidential under our privacy policy!

Sending or Delivering Samples

Samples need to be accompanied by a printed version of your Sample Submission Manifest. There is an option to print your manifest when clicking on the button called “Create Spreadsheet” in the Manifest view of the Online Sample Submission Portal. Please place this printout into a patted envelope with the samples. A padded envelope is highly recommended as we have seen damaged tubes due to rough transport. Please send DNA that is not dried-down cooled at 4°C to us, dried down DNA can be sent without cooling. Blood, cells or tissue samples also need to be sent cooled at 4°C.

Shipping Address:

Please address your samples to:

Garvan Institute / GMG

Loading Dock West Street (off Burton Street)

Darlinghurst, NSW, 2010

or drop your samples off:

Fridge in the Garvan Molecular Genetics facility

in front of room 8.05 (behind the lifts)

Sequencing Service reception tray

Dock Times: 8am to 4pm

Phone: 02 9295 8640

Pricing

Pricing information is available on our website at GMG Prices - Molecular Genetics Shop.

Results

Our online sample submission portal will send you an email with a link to download your results within an approximate turnaround time of 48h for the Premix service (unless you have specified a different turnaround time). Non Premix Service samples will take between 10 days to 3 weeks depending on sample numbers and complexity of the project. For more information please see our webpage (https://www.garvan.org.au/research/capabilities/molecular-genetics/cell-line-identification)

For analysis of and troubleshooting your results see Appendix 3 and 4.

Contact Staff

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Garvan Molecular Genetics

Phone: 02 92958384

NATA accreditation according to standard ISO17025 (biological) and ISO15189 (clinical)

Accreditation No: 18464 (category S)

Figure 10: NATA Accredited for technical competence

Appendix

1) PCR Clean-up protocols

Residual primers and dNTPs must be removed from PCR reactions prior to sequencing. There are various commercial spin column kits (Qiagen, Invitrogen etc) or magnetic bead systems (Dynabeads, Agencourt) available. Alternatively an Exonuclease I / Shrimp Alkaline Phosphatase (Exo/SAP) digestion can be used.

MinElute PCR Purification Kit (50)	Qiagen	28004	~$ 200
PureLink™ PCR Purification Kit	Invitrogen	K3100-01	~$ 150
Agencourt AMPure 5 mL Kit	Beckman	A50850	~$ 150

Exo/SAP Method

1. Transfer 4µl of a 20ul PCR reaction to a 0.5ml tube.
2. In a total volume of 8ul,
3. Add 2 units each of Exonuclease I and Shrimp Alkaline Phosphatase.
4. Incubate at 37°C for 1 hour.
5. Heat denature at 75°C for 15min.
6. Use 4 µl in BigDye Terminator v3.1 cycle sequencing reaction.

Exonuclease I USB Cat# E70073Z 2500 units ($151.00)

Shrimp Alkaline Phosphatase USB Cat# E70092Y 500units ($202.00)

Poor template quality is **the most common** cause of sequencing problems. Excess PCR primers, dNTPs, enzyme, and buffer components (from PCR) are the most likely contaminations for PCR products in sequence setup. The following are characteristics of poor quality templates:

- Noisy data or peaks under peaks
- No usable sequence data
- Weak signal

Plasmid Prep Clean-up

The quality of your plasmid kit will determine sequence results. So, any salt in the plasmid prep will compete with the plasmid DNA when injecting into the capillary. Any mini-prep carryover reagent will harm the efficiency of the Sequenase enzyme. Commercially available mini prep kits will give good sequencing results.

QIAprep Spin Miniprep Kit (50)	Qiagen	27104	~ $200

2) Adding Primer for Premix Sequencing

Whilst you adjust the DNA amount, it is recommended to keep the primer concentration constant. Use this seemingly odd amount: **3.2pmol of primer**. This amount of primer can be easily added to the sequence reaction if you have it in a concentration of 3.2pmol/µL and add 1µL of your primer. For this you can dilute a primer stock of usually 100pmol/µL (=100uM) in the following way:

> Add **1µL of your stock** (100uM) dilution **into 31µL water**.
>
> Add **1µL of this to your DNA** then dry down the mix.

Keep in mind, that a dilution of this primer at 3.2pmol/µL is **not very stable** if repeatedly frozen and thawed, so throw out this dilution and make a **fresh one each time you set up sequences.**

3) Analysing your Results

We will send you two files for each sample. One is a file with the extension **.abi** and the other is a file with the extension **.seq**. Please do not simply use the file with the extension **.seq**. You will have to check your sequence and look at the chromatogram in the **abi** file to verify what part of the data you can trust. If you encounter problems please check the troubleshooting section below or call us.

> To open the **abi** file you need either the ABI software installed on your computer or a similar program. There are many options which can be found with this link:
>
> http://www.dnaseq.co.uk/chromatogram_viewers.html
>
> An excellent program, which we recommend to use is called **FinchTV.** It can be downloaded for either Macintosh or PC users with the link:
>
> http://www.geospiza.com/finchtv/

4) Troubleshooting

You should be aware that we always run a control with our sequence reactions. Therefore we can exclude that the sequence setup, clean-up or capillary separation was insufficient or sub-standard. When you receive "bad" sequences that means that our instrument and whatever we have done to the sample has performed well for our internal control, a pGEM plasmid sequencing. On request we can send the control data file to you. Have a look at the below electropherograms which one resembles yours and read up what might be the cause, please ask us for help too…

No sequence

Many clients are not aware that there are different ways to look at the result data file. The electropherogram is only one way to look at the data, this view is the result of many mathematical algorithms that convert the raw data into readable sequence information. For the purpose of trouble shooting you should also look at your raw data information which you can also see in FinchTV under "View">raw data.

If your electropherogram looks similar to Figure 11 the raw data might tell you what happened.

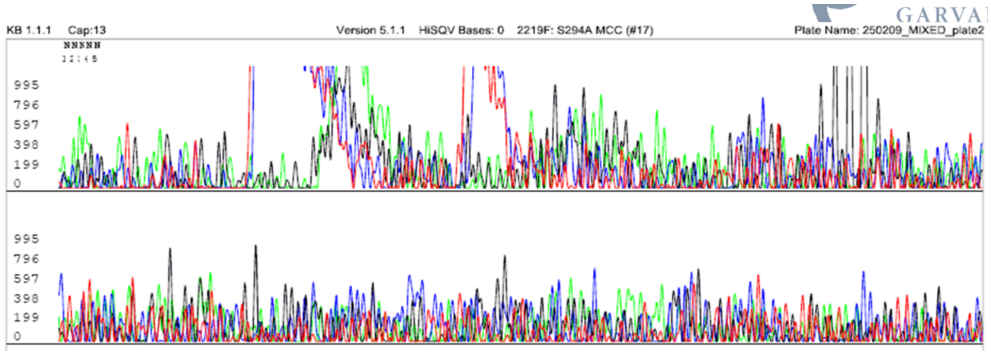

Figure 11: No sequence

If your raw data file looks like Figure 12. That means there was no sequence reaction injected. Most likely there was no template DNA, or the DNA was lost when cleaning up the cycle sequencing reaction, the algorithms of the software will amplify the background noise into a seeming sequence result that you then see in the electropherogram. You can also see that the intensity (y-axis) of the electropherogram is not the true intensity of the signal, which you can only see in the raw data. Always check the raw data view!

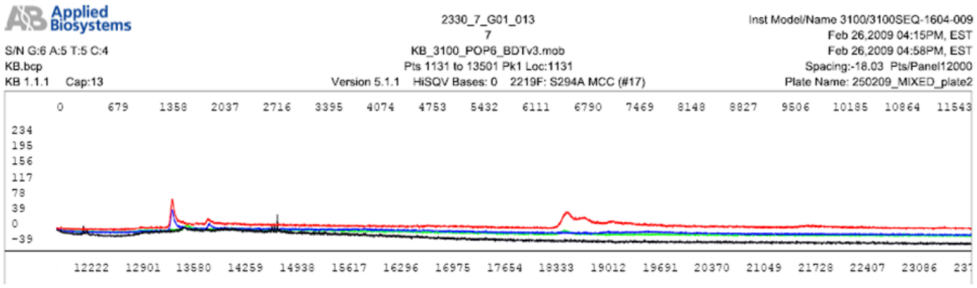

Figure 12: Raw data

Homopolymer Regions

Long homopolymer T regions (or A regions) can cause problems in DNA sequencing reactions due to “slippage” in the region of the homopolymer. Although the sequence data can be clean through the homopolymer region, the data after this region is noisy due to the presence of multiple sequences, see Figure 13. The exact mechanism of slippage is not known. Presumably, the two strands do not stay paired correctly during polymerization through the homopolymer region. This generates fragments with homopolymer regions of different length that have the same sequence after that region.

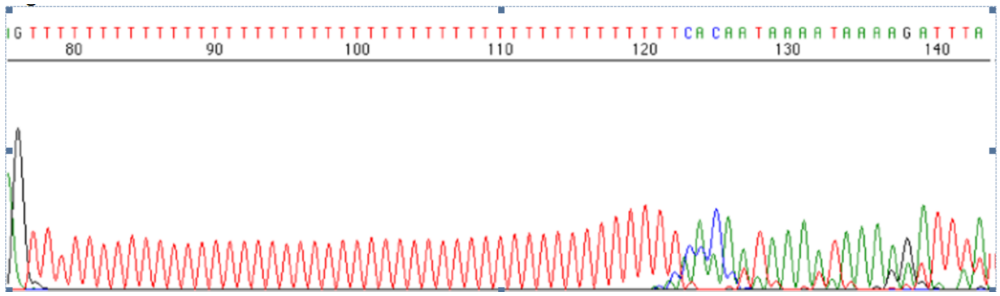

Figure 13: Homopolymer

The occurrence of slippage is length dependent and short homopolymer regions are rarely problematic in sequencing reactions. Slippage is more of a problem with T regions (A in the template strand) in BigDye terminator reactions due to the use of dUTP in the deoxynucleotide mixture.

'Good sequence data immediately past a polyA region can be obtained by using an anchored primer, a sequencing primer that is polyT containing an A, C, or G base at its 3´ end (Figure 7-47). The 3´ base anchors the primer into place at the end of the homopolymer region' (*Khan et al., 1991; Thweatt et al., 1990; Thomas et al., 1993*).

With PCR Templates

Slippage also occurs in PCR amplification and is a common problem when sequencing PCR products. Slippage can occur at regions that normally are not problematic to sequence with cloned DNA. Sequence data is shown in Figure 14 from a plasmid clone. After the homopolymer G region, the sequence data is unusable for the amplified template.

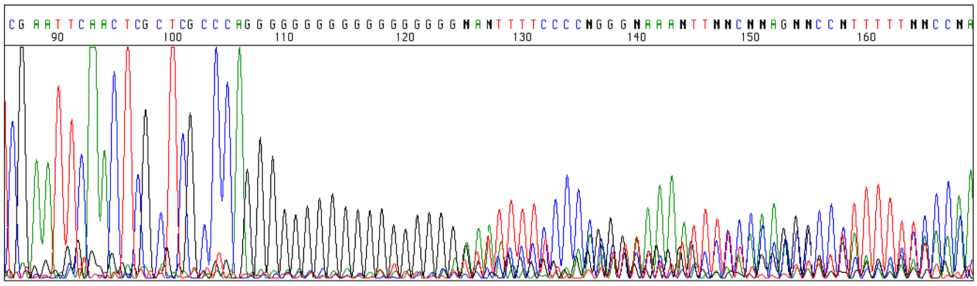

Figure 14: Homopolymer 2

At the present time there is no easy solution for the problem of slippage in PCR amplifications. There are three approaches that can be used to obtain the sequence data after such a region in PCR-generated templates:

- Anchored primers can sometimes be used to obtain sequence data after homopolymer T regions
- Sequence the complementary strand. This will give good sequence for the ambiguous region up to the homopolymer region, but the same problem will occur afterwards. It can be difficult to determine the exact number of bases present in the homopolymer region.
- Clone the PCR product. In many cases the cloned product will not show any evidence of slippage when sequenced. Multiple clones need to be sequenced to be certain that the correct species has been identified. The main disadvantage of this is the work required to isolate and sequence a reasonable number of clones.

Imbalance of primer and DNA ratio

This is the most common cause for a good start and a sudden drop of readable sequence. It is due to the primer binding to excessive template in the first few rounds of cycle sequencing when primarily short fragments are produced and later in cycle sequencing all primer is used up and no longer fragments are formed. The ratio of the DNA to primer is incorrect due to improper quantification of the template DNA. The raw data looks like Figure 15

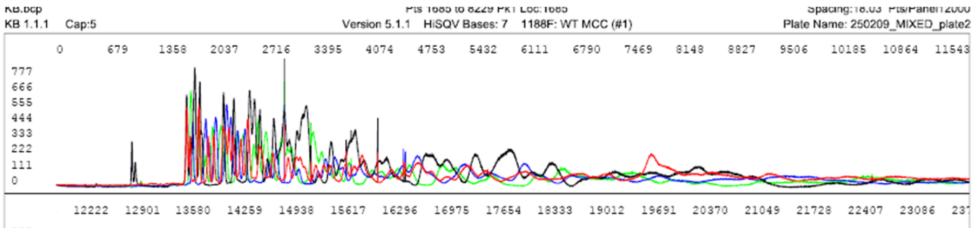

Figure 15: Primer imbalance

And the electropherogram has this typical signature, see Figure 16

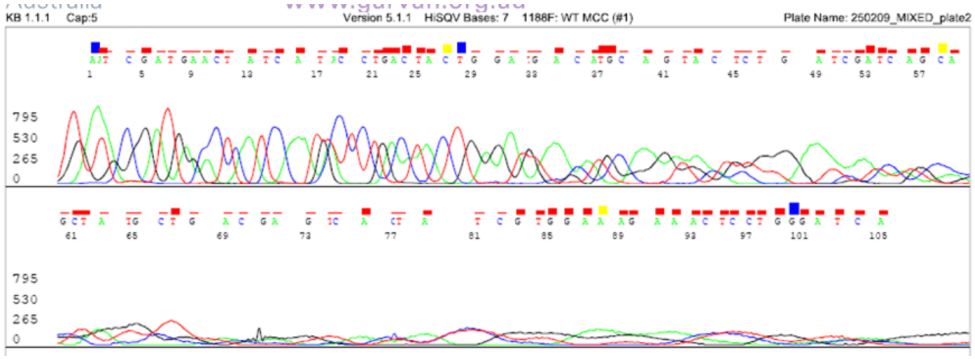

Figure 16: Primer imbalance electropherogram

Noisy sequence

Some background noise is always present in sequencing data. Noisy data is characterized by a **high background** and **peaks under peaks**. Noise can be grouped into several categories, including the following:

- Noise throughout the sequence
- Noise up to or after a specific point in the sequence

Noisy data looks like Figure 17

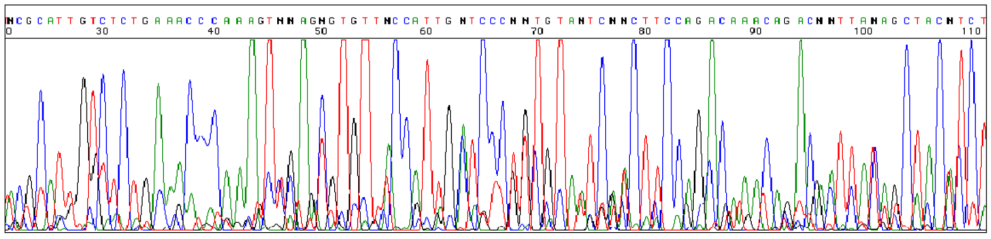

Figure 17: Noisy sequence

Noisy sequence results can be caused by the following:

Not enough template DNA present

Wrong quantification of the template DNA because only spectrophotometer, not gel is used to quantify or, contaminants present, or DNA is degraded or lost.

Nonspecific PCR Products

Nonspecific PCR products include primer-dimer artefacts and secondary PCR products. The presence of any significant quantity of either in a PCR product can result in poor quality sequencing data. Nonspecific PCR products behave as templates in the sequencing reaction and produce extension products, which results in noisy data. These products often can be visualized on an agarose gel before sequencing. If they are present, the PCR amplification should be optimized and repeated before sequencing. Use of a nested or semi-nested sequencing primer can also allow good sequence data to be obtained. Alternatively, the PCR product of interest can be purified by agarose gel electrophoresis

Other reasons for Noisy data

Not all noisy data is caused by low signal. In other cases, the signal strength and the raw data can appear normal, so other possibilities should be considered. Potential causes for noise throughout the sequence include the following:

- Low signal strength, as in the example above
- High signal strength, saturating the detector (leading to high background noise, check the intensity of the raw data if it exceeds 6000 you should request a dilution of the template and rerun)
- Contaminated template (as discussed above: other PCR products, other plasmids because you picked two colonies, or genomic DNA contamination in the prep etc)
- Multiple priming sites (your primer binds at 50C which we use for cycle sequencing to more than one specific site, the generated multiple sequence products overlay creating noise in “each other”.
- Multiple primers (carryover from PCR reaction, not cleaned up perfectly after PCR amplification)

Abrupt ending of sequence then noise

The next electropherogram Figure 18 shows data from a plasmid clone sequenced with the BigDye terminators. Because the noise starts after the multiple cloning region of the vector (base 62, see arrow below), the probable cause was picking a colony that was not well isolated and also had bacteria with no insert or a different insert in the plasmid. The raw data (not shown) appears normal. This kind of noise can have the following causes:

- Mixed plasmid or PCR preparation, as in the example above
- Frame shift mutation
- Primer-dimer contamination in PCR sequencing
- Slippage after homopolymer region in template

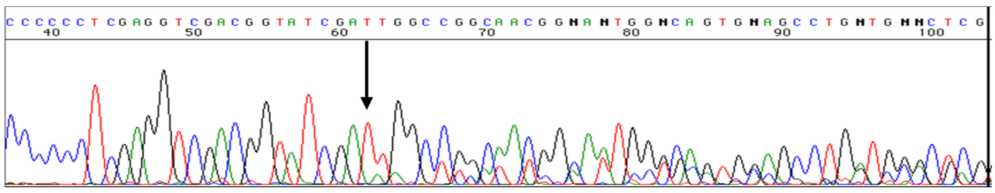

Figure 18: Slippage

Sudden drop of sequence

The next electropherogram Figure 19 shows a sudden drop of the sequence reaction read

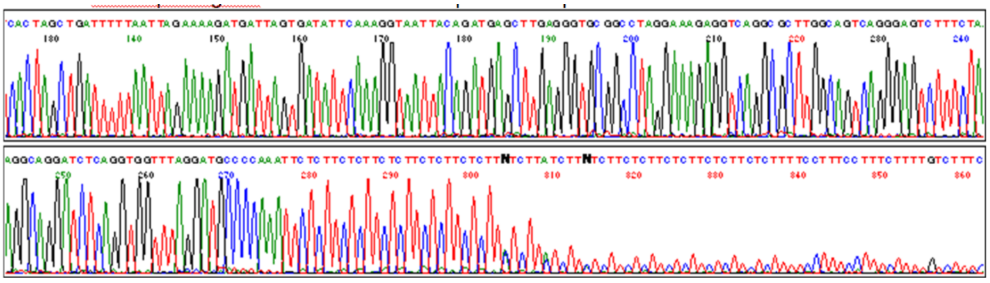

Figure 19: Sudden drop

The raw data view will show something like Figure 20

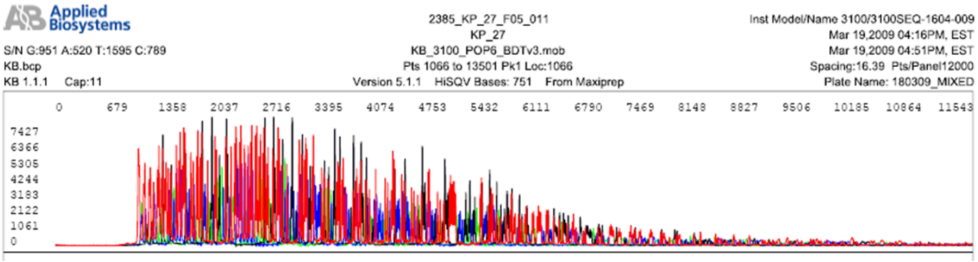

Figure 20: Raw data sudden drop

Gradual and abrupt signal losses have many causes, including the following:

- **MOST LIKELY: Poor quantitation of primer and/or template,** leading to top heavy data. An imbalance of the delicate interplay of 3.2pmol of primer with the target DNA will lead to the amplification of only short fragments. You need to reduce the DNA amount and sequence again, best is to prepare a series dilution.
- The sequenase enzyme hits a region of repeats that cause the enzyme to fall off, the only way around that is to design the reverse primer for this region.
- Region of secondary structure in the template, only solution is choosing a sequence primer closer to the region or try the reverse strand

Degraded template DNA

If the electropherogram looks like Figure 21

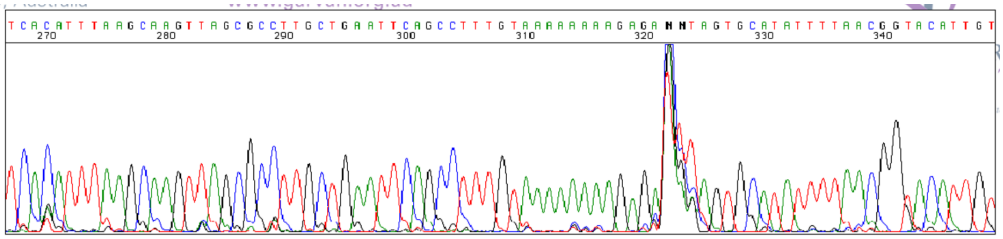

Figure 21: Degraded

Nuclease contamination in a template preparation and repeat freeze-thaw cycles can result in degradation of DNA over time. The electropherogram above shows sequence data from a BigDye primer reaction done with an old template preparation. There is a large stop peak present in the sequence data after base 320.

Primer-Related Problems

There are several primer-related problems that can affect the data obtained in sequencing reactions. These can be divided into three categories based on the type of problem seen in the data:

- No recognizable sequence
- Very weak signal

- Two or more sequences present in the electropherogram

No Recognizable Sequence

If there is no priming site for the primer in the template, no sequence data will be obtained. The raw data will show only a flat line except for the primer peak in primer reactions (see above). This would also happen if the wrong primer is used for a particular vector, or if a mutation is present in the primer binding site in the vector that results in the primer not working effectively.

Very Weak Signal

The signal in sequencing reactions can be very weak if the primer anneals poorly because of a low melting temperature (Tm). Generally primers should have a Tm above 45°C. In some cases, however, lowering the annealing temperature in the reaction can help in obtaining good signal. The primer used to generate the next electropherogram sequence data Figure 22 was 15 bases long and had a Tm of 41°C.

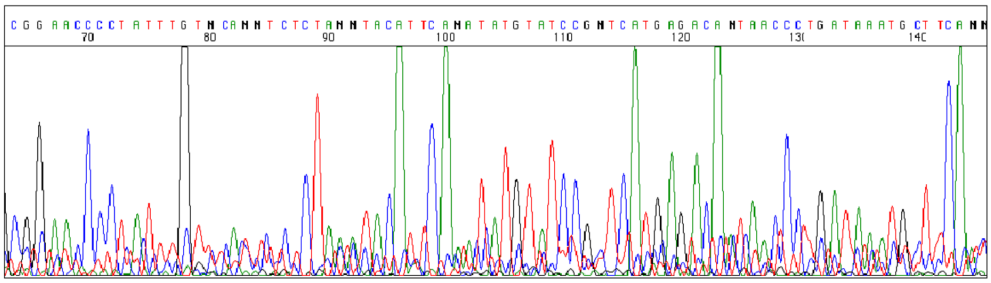

Figure 22: Very weak

The raw data for this sequence reaction looked like Figure 23

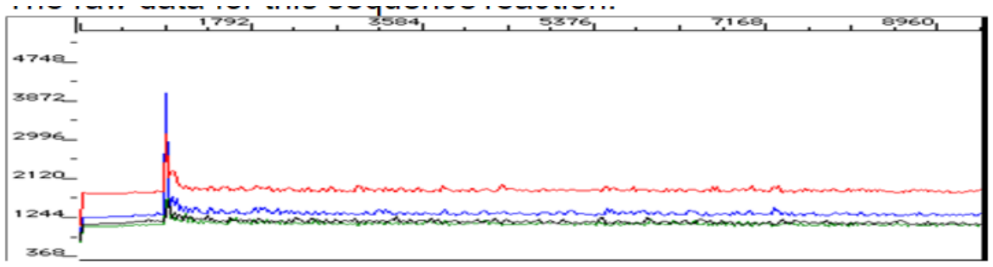

Figure 23: Very weak raw data

The same template was re-sequenced with a 30-mer primer that had a melting temperature of 58°C, see Figure 24

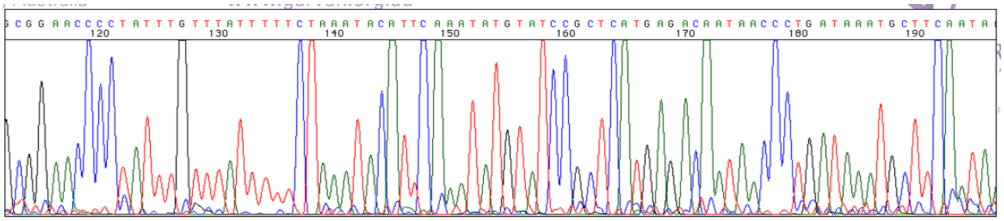

Figure 24: Higher annealing temp

And the raw data for this new file looked like Figure 25

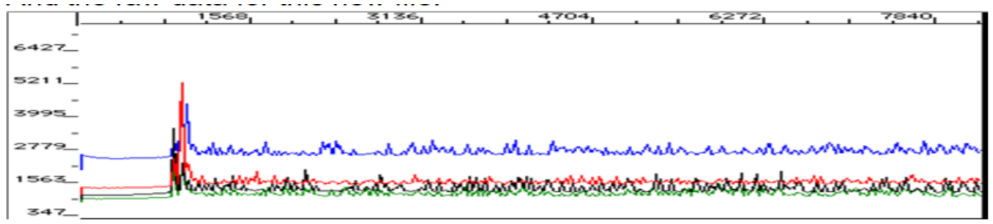

Figure 25: Higher temp raw data

Weak signal can also result if the primer anneals poorly due to secondary structure, particularly at the 3´ end. Whether or not a primer is likely to have significant secondary structure can be determined by analysing its sequence with one of several primer design programs that are available, such as Primer Express™ software.

The presence of more than one primer in a sequencing reaction can be a problem when sequencing PCR products. Since two primers are present in the PCR reaction, failure to completely remove the unincorporated primers from the PCR product will result in the carryover of some of these primers into the sequencing reaction. When using dye primer chemistries, fragments that extend from these residual PCR primers will be unlabelled. If the concentration of these fragments is not too high, there should not be a significant impact on the reaction. With dye terminator chemistries, however, the extension products from the residual primers will also be labelled and will result in a second sequence being present in the data, see Figure 26

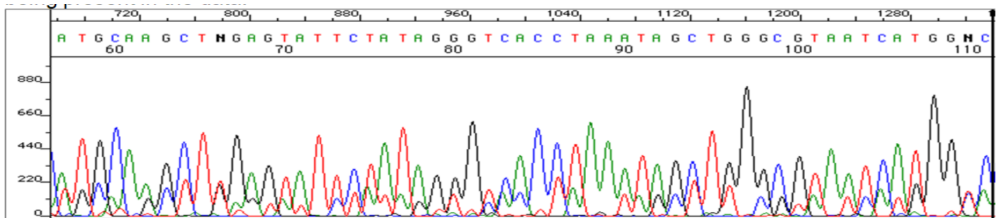

Figure 26: Higher temp raw data

Complete removal of the unincorporated primers from the PCR amplification before sequencing will prevent this. If a secondary hybridization site for the primer is present in the template, two sequences will be detected, resulting in noisy data.

False Stops in Dye Primer Chemistry

One of the advantages of cycle sequencing is that the high extension temperature discourages the formation of template secondary structures. Certain templates, particularly GC-rich sequences, can still form intra-strand complexes through which AmpliTaq® DNA Polymerase, FS has difficulty extending. In dye terminator sequencing, extension products are labelled only if a dye-labelled dideoxynucleotide is incorporated. If the enzyme falls off the template at a region of secondary structure and no dye-labelled dideoxynucleotide is incorporated, the fragment is not detected, see Figure 27

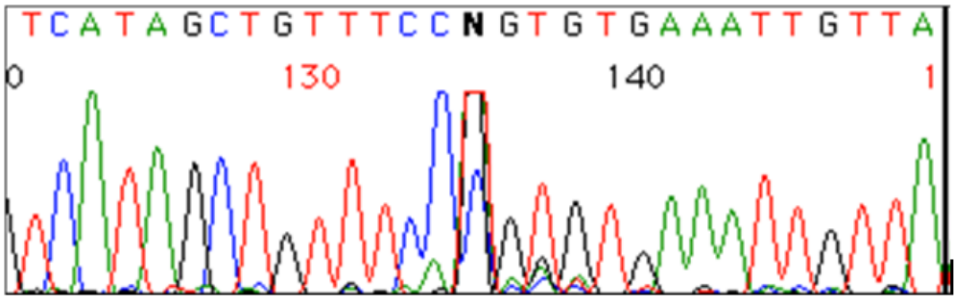

Figure 27: False stop

Compressions

Band compressions in DNA sequencing result from the formation of secondary structures in the DNA fragments that are not eliminated by the denaturing conditions in cycle sequencing, see Figure 28

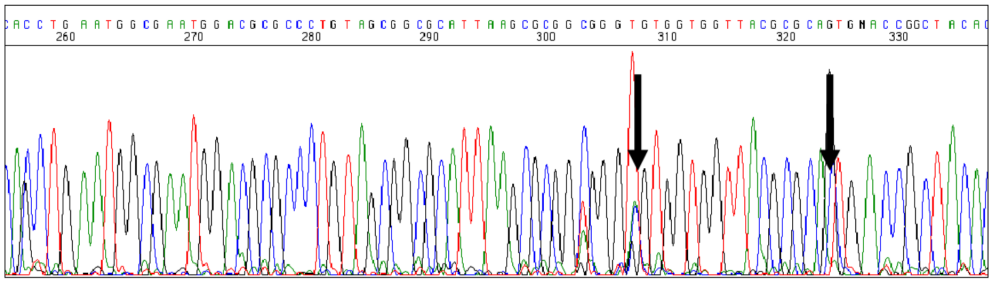

Figure 28: Compression

When problems are encountered with compressions in dye primer data, there is only way to resolve them: Sequence the complementary strand, if possible. Compressions rarely occur at the same position in both strands of a template.

GC-Rich Templates

Templates with a GC content greater than 70% can be difficult to sequence when using the standard reaction conditions. This is probably related to the higher melting temperature of the DNA caused by the higher proportion of GC base pairs. Even a template that has a fairly average base composition overall can have a very GC-rich region that affects its ability to be sequenced. The most common problem seen with GC-rich templates is weak signal which in the following electropherogram of a GC-rich sequence you can notice because of the increase in background signal.

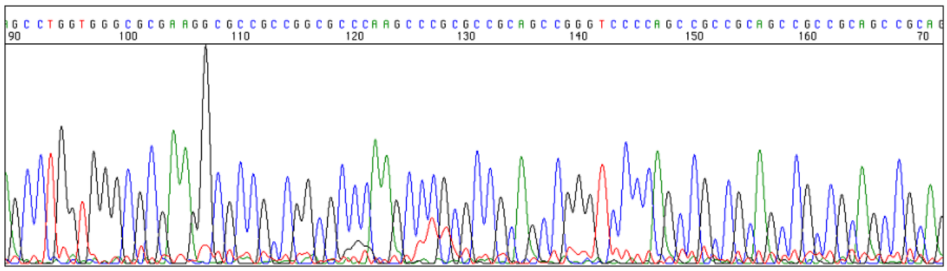

Figure 29: GC rich

Suggested Approaches for GC-Rich Templates

- Add DMSO to a final concentration (v/v) of 5% (*Burgett et al., 1994; Landre et al.,1995*)
- Addition of a mixture of 5% DMSO and 5% glycerol
- Add betaine to a final concentration of 1M (*Henke et al., 1997; Baskaran et al.,1996*)
- Double all reaction components and incubate at 98 °C for 10 minutes before cycling
- Add 5–10% formamide or 5–10% glycerol to the reactions
- Linearize plasmids with a restriction enzyme
- Amplify the DNA with substitution of 7-deaza-dGTP for 75% of the dGTP in the PCR