

Guide to Sanger Sequencing at RAMAC



**Ramaciotti Centre
for Genomics**

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1 INTRODUCTION

Welcome to the Ramaciotti Centre for Genomics Sanger Sequencing Guide. We are committed to providing you with a world-class sequencing service and assisting you in achieving your research goal. This guide will provide an overview of the Sanger sequencing process and our services and walk you through each of the steps involved in preparing your samples for sequencing.

This guide is not intended to be comprehensive and is provided as a starting point. If you need in depth information, please refer to:

- i. [DNA Sequencing by Capillary Electrophoresis \(3rd edition\)](#) - Applied Biosystems/ThermoFisher Scientific
- ii. Molecular cloning: a laboratory manual - Joseph Sambrook, David W. Russell

If you are an experienced user of this technology, please go straight to [Appendix II](#) for details on how to prepare your samples for submission to our sequencing service.

2 OPTIONS FOR YOUR SEQUENCING NEEDS

We can provide an end to end service or we are ready to step in when you are ready to go hands-off with your samples. The following overview shows the different submission points. A detail submission guideline for our standard services (Core Prep, Core Prep Plus and User Prep) is in [Appendix II](#). For all other submission, please [contact us](#) for further information or to make an appointment.

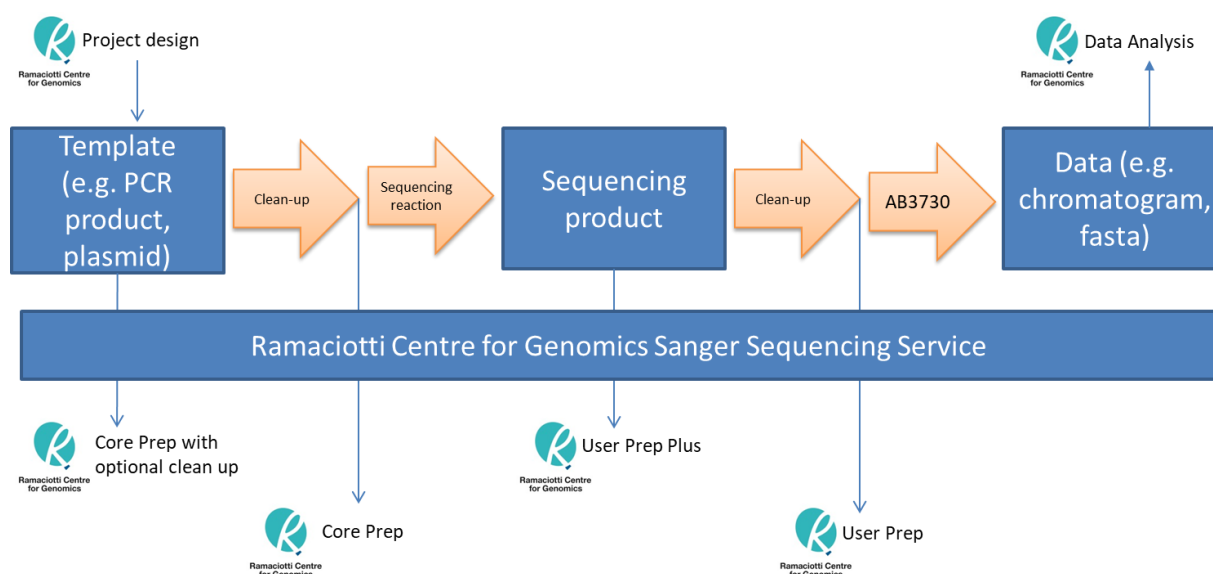


Figure 1 - Overview of Sanger sequencing service at Ramaciotti Centre



3 WHAT IS SANGER SEQUENCING?

Sanger sequencing by capillary electrophoresis is the gold-standard DNA sequencing technique that is used in many life sciences laboratories. Sanger sequencing is a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. By including primers that flank the region of interest, PCR can generate thousands of copies of the specific target.

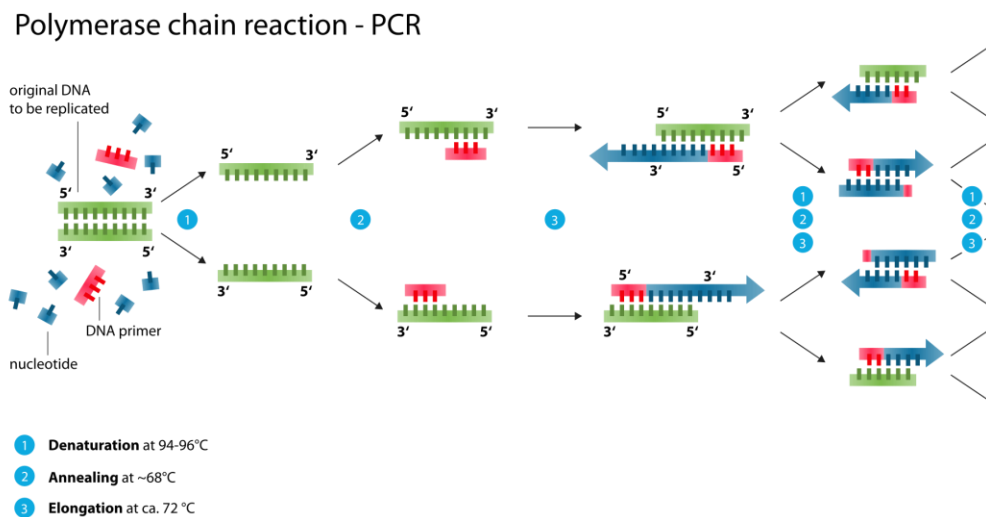


Figure 2 - Overview of PCR (en.wikipedia.org)

A sequencing reaction is essentially a PCR except only a single primer is used and fluoresce-labelled chain-terminating dideoxynucleosides are used in the reaction. This will generate thousands of multi-length fragments which match the target of interest.

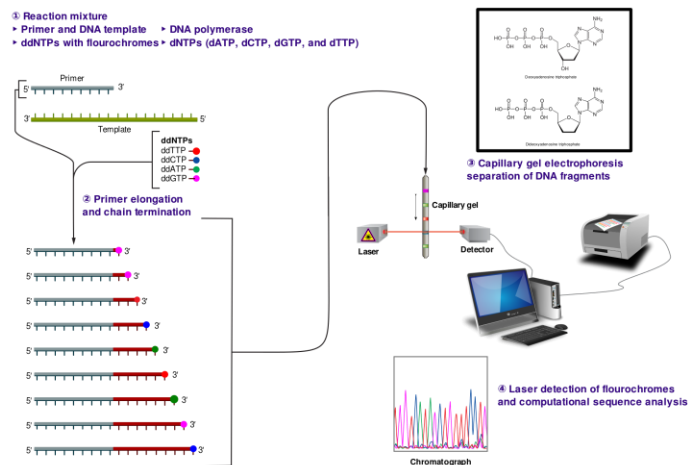


Figure 3 - Overview of Sanger Sequencing (en.wikipedia.org)

4 TEMPLATE PREPARATION

One of the most important factors that contribute to successful sequencing is template quality. In this section we discuss how to prepare templates for sequencing and assess their quality. In this guide we focus on Sanger sequencing from plasmid and PCR templates. More information on other template types can be found in the [DNA Sequencing by Capillary Electrophoresis \(3rd edition\)](#). Please note that direct sequencing on genomic DNA (gDNA) is not recommended (except bacterial gDNA) due to the low ratio of the target to background DNA in gDNA.

4.1 PLASMID TEMPLATE

When extracting plasmid DNA you can use commercial kits (*Table 2-1*) or use general methods such as alkaline lysis (refer to resources such as *Molecular cloning: a laboratory manual* by Joseph Sambrook, David W. Russell). The host strain used in the cloning step can affect template quality; it is therefore advisable that you consult the manufacturer's user guide to confirm compatibility before proceeding to plasmid extraction.

In addition to using the appropriate extraction method to obtain high quality plasmid DNA, it is essential that a single colony is used for extraction. A mixed population of plasmids (mixed template) will sequence well but produce unusable data that is a combination of sequence from the two populations.

After extracting plasmid DNA you must ensure that it is intact and free of contaminants before proceeding to sequencing. Please consult our [Nucleic Acid Quality Control Document](#) for further information on the standard quality control steps that should be performed after DNA extraction.

Table 4-1 Examples of commercial products for preparing vector-based DNA (from DNA Sequencing by Capillary Electrophoresis)

Product	Source	Purpose
CosMCPrep® Kit	Agencourt Bioscience Corporation	To purify high-copy and low-copy plasmids, BACs, cosmids, and fosmids
NucleoBond Plasmid Purification Kits	Clontech Laboratories	To purify high-copy and low-copy plasmids, cosmids, BACs, PACs, and YACs
Aurum™ Plasmid Purification Kits and Quantum Prep Plasmid Purification Kit	Bio-Rad Laboratories	To purify plasmid DNA
ProPrep™ BAC 96	Biotech Support Group	To miniprep BAC DNA in 96-well format
SeqPrep™ Plasmid Prep Kit	Edge Biosystems	To purify plasmid DNA in 96-well or 384-well formats
illustra TempliPhiDNA Sequencing Template Preparation Kit	GE Healthcare	To amplify single- or double-stranded circular DNA templates from bacterial cells containing a plasmid, purified plasmids, or intact M13 phage
ChargeSwitch®-Pro Plasmid Miniprep Kit	Invitrogen	To purify small-scale plasmid DNA
Montage Plasmid Miniprep _{HTS} 96 Kit	Millipore	To purify plasmid or BAC DNA in 96-well format
PSI [®] Clone BAC DNA Kits	Princeton Separations	To purify BAC DNA
Wizard® Plus Minipreps DNA Purification Systems	Promega	To purify plasmid DNA
Wizard SV 96 and SV 9600 Plasmid DNA Purification Systems	Promega	To purify plasmid DNA
QIAprep® Spin Miniprep Kits	QIAGEN	To purify plasmid DNA in 8-well or 96-well formats
GenElute™ Plasmid Miniprep Kit	Sigma-Aldrich	To purify small-scale plasmid DNA

4.2 PCR TEMPLATE

PCR is one of the most common methods for obtaining targeted template for Sanger sequencing. By designing target-specific primers you can selectively amplify the target region to obtain sufficient template for sequencing. Successful PCR requires good quality input DNA and good primer design. In most situations PCR will require optimisation to produce a strong single band product for sequencing (i.e. high efficiency and high specificity). Generally, the higher the quality of your input DNA and the better optimized the PCR protocol the more time you will save in the long run. This document does not provide a guide for PCR optimization, please refer to the PCR manufacturer's instructions or a source such as the [Life Technologies website](#).

i. DNA extraction

It is critical to use good quality genomic DNA that is free of contaminants in your PCR. There are several different extraction methods and kits tailored to different sample types (*Table 2-2*). It is important to select the right method/kit for your sample type. Use the recommended amount of input material specified in the protocol to get best result.



Following DNA extraction, you must ensure that it is intact and free of contaminants before proceeding to PCR. Please consult our [Nucleic Acid Quality Control Document](#) for further information on the standard quality control steps that should be performed after extraction.

Table 4-2 Examples of commercial kits for DNA extraction (from DNA Sequencing by Capillary Electrophoresis)

Product	Source	Purpose
MELT™ Total Nucleic Acid Isolation System	Ambion (PN AM1983)	To purify genomic DNA from tissue samples
RecoverALL™ Total Nucleic Acid Isolation for FFPE Tissues	Ambion (PN AM1975)	To purify genomic DNA from FFPE tissue
TRI Reagent®	Ambion (PN AM9738)	To isolate total RNA or rRNA, DNA, and protein from a variety of fresh or frozen biological tissue or cells
NucPrep® DNA Isolation Chemistry Starter Kit	Applied Biosystems (PN 4340274)	To purify genomic DNA from cultured and fixed cells, gram-negative bacteria, plant and animal tissue, and whole blood and bone marrow cells
BloodPrep® DNA Starter Kit	Applied Biosystems (PN 4346860)	To purify genomic DNA directly from whole fresh and frozen blood or isolated blood cells of many animal species, and from tissue-cultured cells or buccal swab samples
Bacterial Genomic DNA Purification Kit	Edge Biosystems	To purify genomic DNA from bacterial cultures
PUREGENE® DNA Purification Kits	QIAGEN	To purify high molecular weight genomic DNA
QIAamp® DNA Blood Mini/Midi/Maxi Kits	QIAGEN	To purify genomic DNA from blood and blood-derived samples

ii. Primer design

A successful PCR for Sanger sequencing is one that produces a strong single product of the correct size. To obtain this primers must be designed to ensure that they have excellent specificity. There are online tools such as [Primer3](#) to assist with design and provide you with more information on design. Regardless of the utility of online tools you must optimize the PCR in the lab, as no predictive tools can guarantee the success of the primer design for PCR and sequencing. Depending on the circumstances you may need to use other approach such as nested/semi-nested PCR. Please note that if you want to use the same set of primers for sequencing the annealing temperature should be comparable with the cycle conditions of the sequencing enzyme (50°C).

Note: We offer a PCR design and optimization service, please [contact us](#) for a project-based pricing.

iii. PCR cleanup

It is essential that reagents used in the PCR are removed prior to sequencing. There are a number of different ways to clean PCR products prior to sequencing, see below. Please select the method most appropriate to your situation.

a) Gel purification – Can be used to isolate and purify the target PCR band in a multi band PCR. Commercial kits are available (e.g. Qiagen).



b) Enzymatic purification –Removes excess PCR primers and unincorporated dNTPs. It is simple and cost effective. Commercial kits are available (e.g. Exosap-IT).

c) Bead clean up –Removes excess PCR primers and unincorporated dNTPs. It is simple to use, however it requires a magnetic stand. Commercial kits are available (e.g. PCR clean up beads from Beckman).

d) Ethanol purification – The most cost-effective way to clean up your samples, may be used for the removal of salts and phenol. However, inexperienced users are at a higher risk of losing template. A protocol for this method can be found in Appendix I.

e) Column purification - Removes excess PCR reagents, unused primers and unincorporated dNTPs. May be used for salt removal. Commercial kits are available (e.g. Qiagen).

Note: We offer Exosap-IT clean up as a part of our standard service and a bead-based cleanup is available for project-based submission. Please contact us for details.

iii. Quality checking your PCR

Before submitting your PCR product for sequencing there are two quality checks that should be performed to ensure a successful outcome:

- Checking that the PCR product is a single band
- Testing that the product is free of contamination

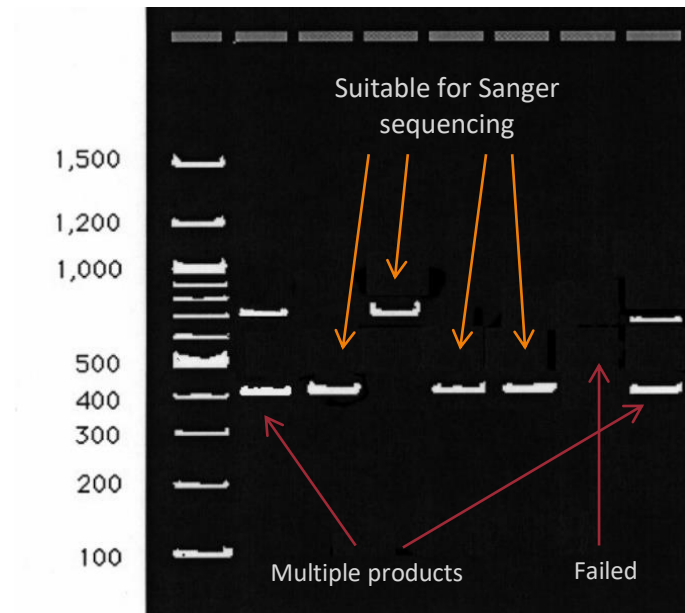
Gel electrophoresis is simple way to visualize your PCR prior to sequencing. Gel electrophoresis reveals the specificity and quantity of your PCR product. A good PCR produces a single product at a detectable quantity (*Figure 2-1*). It does not however reveal information about other contaminants such as proteins. It is recommended that you also read your sample on a spectrophotometer to detect contaminants such as proteins and other organic chemicals. It is important to note that neither of these methods will reveal contamination by salts, which can cause noisy data.

Contaminants that can interfere with sequencing:

- Protein
- RNA
- Chromosomal DNA
- Excess PCR primers, dNTPs, enzyme and buffer
- Residual salts
- Residual organic chemicals
- Residual detergents
- Agarose (from gel purification)



Figure 4-1 Example of PCR gel electrophoresis



5 SEQUENCING REACTION (WITH BDTV3.1)

There are a number of different sequencing kits available. We routinely use [BigDye Terminator \(BDT\) version 3.1](#) from Life Technologies. In our experience, BDTV3.1 can sequence a wide range of templates of varying quality. However, the rule still stands, the better template quality the better data! As the sequencing reaction is finely balanced adding more template to the reaction does not mean a better outcome. The recommended input for sequencing reactions using BDT is outlined in Table 3-2. Template input may require optimization.

The recommended thermal cycling protocol for sequencing reactions with BDT is outlined in Table 3-3. Further details on the sequencing reaction can be found in Chapter 4 of the [DNA sequencing guide from Life Technologies](#). The standard protocol should work for most samples as the BDT chemistry is very well developed. However, the quality of the sample and the primer design will determine the outcome: the better the input, the better the output. Note that the standard annealing temperature for this kit is 50°C. Sequencing primers that require a significantly different annealing temperature may yield poor results. It is possible to customize the cycling conditions of the sequencing reaction; you can do it via our User Prep Service or request a project-based quote.

Table 5-2 Recommended input for sequencing reaction with BDT

Template	BigDye Terminator v1.1 and v3.1
PCR product:	
100 to 200 bp	1 to 3 ng
200 to 500 bp	3 to 10 ng
500 to 1000 bp	5 to 20 ng
1000 to 2000 bp	10 to 40 ng
>2000 bp	40 to 100 ng
Bisulfite converted genomic DNA-PCR product	3 to 10 ng
Single-stranded DNA	50 to 100 ng
Double-stranded DNA	200 to 500 ng
Cosmid, BAC	0.5 to 1.0 µg
Bacterial genomic DNA	2 to 3 µg



Table 5-3 Recommended protocol for sequencing reaction with BDT

Mode	Thermal Cycling Conditions			
9600 emulation mode 9700 standard mode 9800 standard mode - Veriti® 96-Well Thermal Cycler	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	4 min
	3	Hold	4	Indefinite hold
9800 Fast mode - Veriti® FAST 96-Well Thermal Cycler	Stage	Description	Temp. (°C)	Time
	1	Denaturation	96	1 min
	2	Amplification: 25 cycles	96	10 sec
			50	5 sec
			60	75 sec
	3	Hold	4	Indefinite hold

6 SEQUENCING REACTION CLEANUP

After the sequencing reaction, the final product must be purified before being run on the sequencer (e.g. AB3730XL). This purification step is essential to remove both unlabeled and dye-labeled components that can interfere with the electro-kinetic injection, electrophoresis and data analysis. The purification can be done using various methods. The Ramaciotti Centre for Genomics offers the bead-cleanup method as part of our User Prep Plus Service.

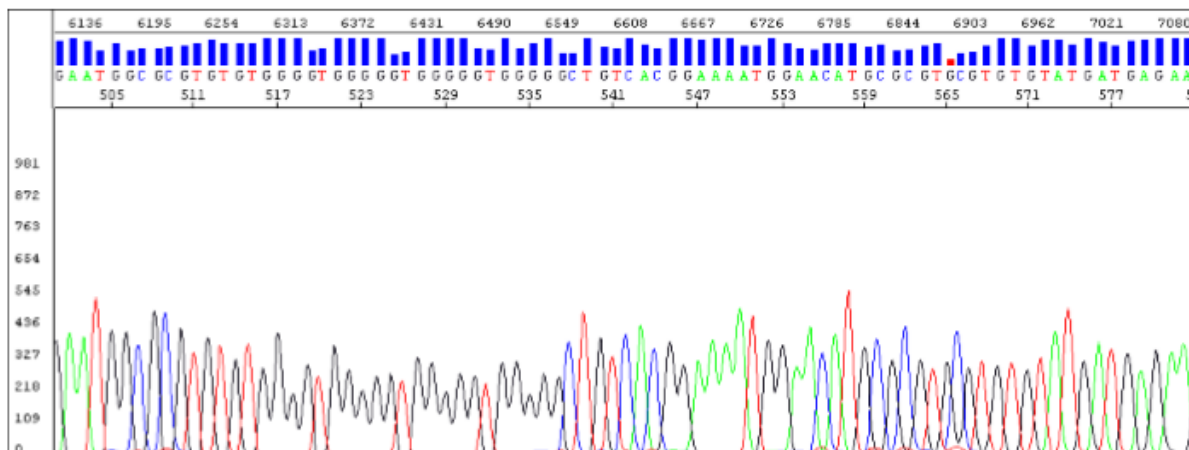


Figure 6-1 Example of the chromatogram of a clean sequence

Examples of alternative methods are BigDye Xterminator Purification (Life Technologies), spin-column purification (e.g. Qiagen) and ethanol precipitation. These methods had different advantages and disadvantages, please refer to the manufacturer's protocol. Ethanol precipitation is a very cost-effective, however it can be challenging for new users with the final product being lost in the cleanup process. Please refer to Appendix I for ethanol precipitation protocols that we have successfully used.

7 POST CAPILLARY ELECTROPHORESIS/DATA ANALYSIS

Once the data is ready, you will receive an email from our server to inform you the data is ready for download via your login. On the download page, you will have the choice of downloading the data in various formats (e.g. chromatogram, text, fasta).

The data collection software does a very good job of translating the raw fluorescence signals into base calls nonetheless it is best if you download the chromatogram and manually check your data before performing any downstream analysis. By checking the chromatogram manually, you will be able to ensure correct base calling and get information on the success of the sequencing reaction. Please refer to our [DNA sequencing software page](#) for further information on software that can be used to view chromatograms.



8 TROUBLESHOOTING

Troubleshooting for Sanger sequencing can be split into two main areas; the template and the sequencing reaction. It is of utmost importance that the input template is of good quality and at the right quantity; refer to the previous sections on these topics. When you are confident that the input to the reaction is good and not causing any issue, you can refer to the very informative sequencing troubleshooting guide can be found in Chapter 8 of the [DNA Sequencing by Capillary Electrophoresis 3rd edition](#).

The Centre runs a sequencing clinic whereby users of our service can book one on one time with a Sanger sequencing expert. Please [contact us](#) for further information or to make an appointment.

8.1 COLLECTION OF COMMON PATTERNS IN CHROMATOGRAMS

(Extracted from Chapter 8 of the [DNA Sequencing by Capillary Electrophoresis 3rd edition](#))

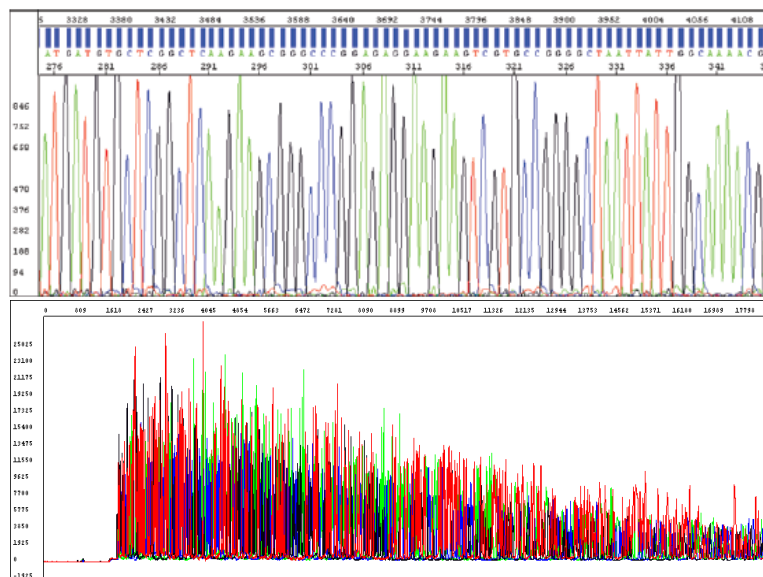
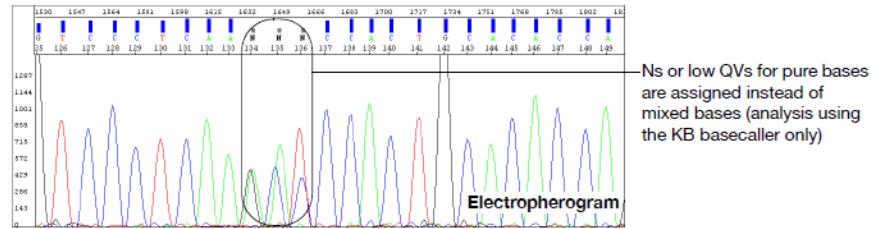
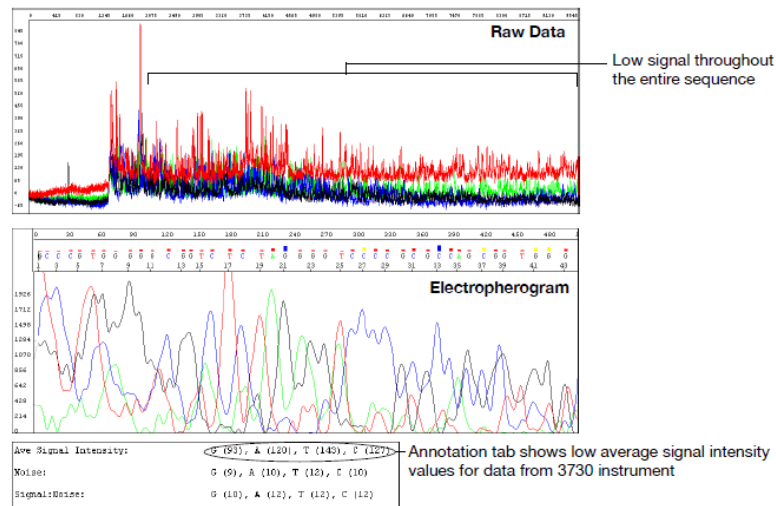


Figure 2 – High quality sequence (TOP: Chromatogram; BOTTOM: Raw trace)

Mixed base not called correctly



Low signal throughout



No signal

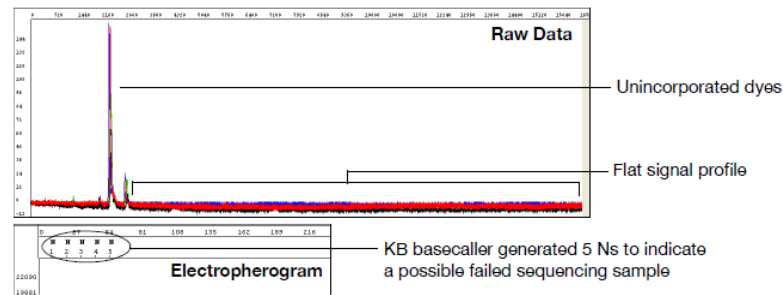


Figure 7 - Examples of common problematic chromatograms and traces

9 APPENDIX I – SUGGESTED STARTING PROTOCOLS

i) Sequencing Protocol for ABI 3730 Capillary Sequencer:

100ng -500ng template (plasmid)

or

20-50ng PCR product

1ul Big DyeTerminator V3.1

3.2pmol primer

3.5ul 5x buffer

dH2O to 20uL

Note: ABI supply enough buffer for 1.5ul of buffer per reaction. Additional buffer can be purchased separately.

Cycling conditions:

- Repeat the following for 25 cycles, on maximum ramp speed:
 - 96°C for 10 secs
 - 50°C for 5 secs
 - 60°C for 4mins
- Hold at 4°C and hold until ready to purify.

ii) Purification Method - Ethanol/EDTA Precipitation (Plates):

This method is good for removal of excess dye terminators (unincorporated dye labeled terminators).

While this method produces the cleanest signal, it may cause loss of small molecular weight fragments.

To precipitate 20ul sequencing reactions in a 96 well reaction plate:

- Remove the 96 well reaction plate from the thermal cycler and spin briefly
- Add the following to each of the wells:
 - 5ul of 125mM EDTA
 - 60ul of 100% ETOH
- Make sure EDTA reaches the bottom of the wells.
- Seal the plate with aluminum tape and mix by inverting 4 times.
- Incubate at room temperature for 15 mins to precipitate the extension products.

Note: Precipitation times shorter than 15 mins will result in loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

- Use a plate adapter and spin plate at the maximum speed as follows:
- 1,400-2,000 x g for 45 min or 2,000-3,000 x g for 30 min



Note: Proceed to the next step immediately (if not possible, spin for an extra 2 mins before performing next step).

- Invert the plate to decant supernatant. Keep the plate inverted and spin up to 185 x g (line the plate with papertowel), then remove from the centrifuge. The timing starts when the rotor starts moving.

Note: Supernatant must be removed completely or unincorporated dye terminators will remain in the samples and interfere with sequencing.

- Add 60ul of freshly made 70% ethanol to each well.
- With the centrifuge set at 4°C, spin at 1,650 x g for 15 min.
- Invert the plate to decant supernatant. Keep the plate inverted and spin up to 185 x g (line the plate with paper towel), then remove from the centrifuge.
- Cover with aluminum foil, and store at 4°C until submission.

Note: Make sure wells are dry. You may use a speed-vac for 15 mins to dry. Make sure samples are protected from light while drying.

iii) Purification Method - Ethanol/EDTA Precipitation (1.5ml tubes):

This method is good for removal of excess dye terminators (unincorporated dye labeled terminators). While this method produces the cleanest signal, it may cause loss of small molecular weight fragments. To precipitate 20ul sequencing reactions in 1.5ml microfuge tubes:

- Pipette entire contents (20µl) of the extension reaction into a 1.5ml microfuge tube
- Add the following to each well:
5ul of 125mM EDTA, making sure the EDTA reaches the bottom of the wells
60ul of 100% ETOH to each
- Close the tubes and vortex briefly.
- Leave the tubes at room temperature for 15 mins to precipitate the extension products.

Note: Precipitation times shorter than 15 mins will result in loss of very short extension products. Precipitation times longer than 24 hours will increase the precipitation of unincorporated dye terminators.

- Place tubes in micro-centrifuge and spin for 20 mins at $\geq 14,000 \times g$.

Note: Proceed to the next step immediately (if not possible, spin for an extra 2 mins before performing next step).

- Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible.



Note: The supernatants must be removed completely or else unincorporated dye terminators will remain in the samples.

- Add 250ul of the freshly made 70% ethanol to the tubes and vortex briefly.
- Place the tubes in the microcentrifuge in the same orientation as in the previous spin step and spin for 10 mins at $\geq 14,000 \times g$ (set the centrifuge temp at 4°C for this step).
- Aspirate the supernatants carefully as previously.
- Dry the samples in a vacuum centrifuge for 10 -15 mins or to dryness (alternatively, place the tubes with the lids open in a heat block or thermal cycler at 90°C for 1 minute.
- Close tubes and store at 4°C until submission.



10 APPENDIX II – SUBMITTING SAMPLES FOR OUR SERVICE

Once you have set up your dnaLIMS account, please refer to the submission procedure base on the batch size and required service type for your order. For all orders under 40 samples, please follow the small batch size submission procedures (see 10.1). For above 40 samples, please follow the large batch size submission procedures (see 10.2).

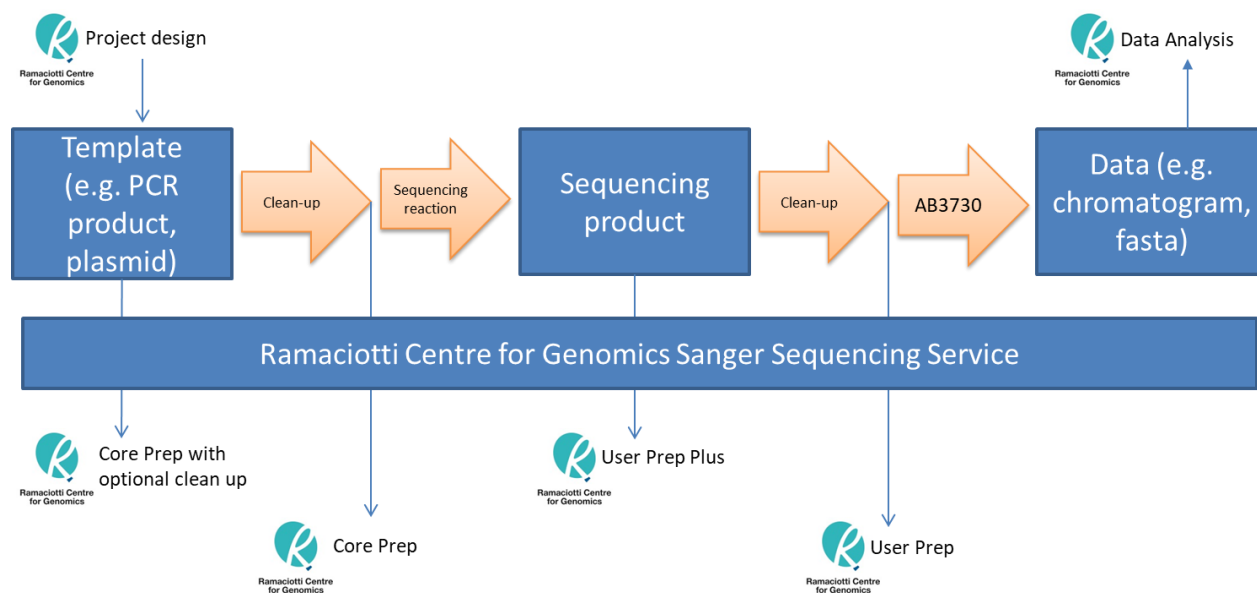


Figure 4 - Overview of Sanger sequencing service at Ramaciotti Centre

Summary of available services (selectable on dnaLIMS):

Small batch size: Individual tube samples submission (<40 samples):

User_Prep – 10.1.1

Core_Prep – 10.1.2

Large batch size: Plate submissions (>40 samples):

Plate-User_Prep – 10.2.1

Plate-User_PrepPlus – 10.2.2

Plate-Core_Prep – 10.2.3 (with/without ExosapIT cleanup)

Plate-Core_PrepBead40 – 10.2.4 (with bead cleanup)

Plate-Core_PrepBead96 – 10.2.4 (for full plate with bead cleanup)

Submission for arranged project work only:

Project_PCR2DIR – do not use unless arranged prior (contact us).



10.1 SMALL BATCH SIZE SUBMISSION: ANY ORDERS UNDER 40 SAMPLES

10.1.1 Individual User Prep – Use 1.5mL microfuge tubes

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Enter Individual DNA Sequencing Requests” or “Upload and Import DNA Tube File”.
 - Select “User Prep” for Service Request.
 - Fill in all information.
- Perform sequencing reaction and clean up using protocols suitable for AB3730 (see Appendix I for our suggested protocols).
- Label the sample tubes with the order number (generated at submission on dnaLIMS) and consecutive sample number on the lid. For example, for order number 1234 with 3 samples, label tubes as “1234-1”, “1234-2” and “1234-3”.



- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.

10.1.2 Individual Core Prep or Core Prep with ExosapIT – Use 1.5mL microfuge tubes

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Enter Individual DNA Sequencing Requests” or “Upload and Import DNA Tube File”.
 - Select “Core Prep” for Service Request.
 - Fill in all information.
- For **Core Prep**: Select No for EXOSAPIT cleanup.

Purified template and primer for each sample should be combined in one tube, with minimum final volume of 10ul in 1.5ml microfuge tubes. Spin down tubes.
- For **Core Prep with ExosapIT cleanup**: Select Yes for EXOSAPIT cleanup.



PCR products template and primers should be sent separately in 1.5mL microfuge tubes.

Refer to the table below as a starting guide for template and primer concentration. The final ratio of template:primer for submission may require optimisation.

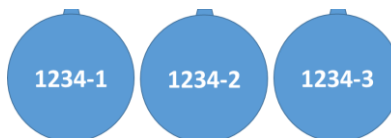
	VOLUME PER SAMPLE	PCR PRODUCT <1kB	Final Concentration (in 10ul)
Template	8 µL	20-50 ng/µL	16-40ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.

	VOLUME PER SAMPLE	PCR PRODUCT >2KB AND DS PLASMID	Final Concentration (in 10ul)
Template	8 µL	100-200 ng/µL	80-160 ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.

- Label tubes with the order number (generated at order lodgment processing in dnaLIMS) and consecutive sample number on the lid. For example, for order number 1234 with 3 samples, label tubes as “1234-1”, “1234-2” and “1234-3”.



- A tube of primer should be sent per sample, labelled by the order number and the primer name.
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm. Please use “Core_Prep **no** Exosap” box for orders with no cleanup or “Core_Prep Exosap” box if your order require Exosap-IT cleanup.



- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.

10.2 LARGE BATCH SIZE SUBMISSION: ANY ORDERS OVER 40 SAMPLES

10.2.1 Plate User Prep – Use MicroAmp™ Optical 96-Well Reaction Plate

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Upload and Import DNA Plate File”.
 - Select “Plate-User_Prep” for Service Request.
 - Fill in all information.
- Perform sequencing reaction and clean up using protocols suitable for AB3730 (see Appendix I for our suggested protocols). Place samples in consecutive **columns**.
- Send dried samples using MicroAmp™ Optical 96-Well Reaction Plate (Life Technologies) with appropriate labelling.
- Label plates with the order number or plate number (generated at order lodgment processing in dnaLIMS).
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.

10.2.2 Plate User Prep Plus – Use any 96-Well V-bottom PCR Plate

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Upload and Import DNA Plate File”.
 - Select “Plate-User_PrepPlus” for Service Request.
 - Fill in all information.
- Perform sequencing reaction using sequencing reaction protocol suitable for AB3730 (see Appendix I for our suggested protocol). Place samples in consecutive **columns**.



- Send 12ul of the sequencing reaction product per sample using 96-Well V-bottom PCR plates with appropriate labelling. **Seal well.**
- Label plates with the order number or plate number (generated at order lodgment processing in dnaLIMS).
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.

10.2.3 Plate Core Prep or Core Prep with ExosapIT cleanup – Use any 96-Well V-bottom PCR Plate

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Upload and Import DNA Plate File”.
 - Select “Plate-Core_Prep” for Service Request.
 - Fill in all information.
- For **Plate-Core Prep**: Select No for both EXOSAPIT cleanup and Bead cleanup.
Purified template and primer for each sample should be combined in each well, with minimum final volume of 10ul. Use 96-Well V-bottom PCR plates with appropriate labelling. Spin down plate. **Seal well.**

Place samples in consecutive **columns**.

- For **Plate-Core Prep with ExosapIT**: Select Yes for EXOSAPIT cleanup and No for Bead cleanup.

The standard recommendation for PCR product is ExosapIT cleanup.

For ExosapIT cleanup, the minimum sample volume is 10ul/sample. Send templates using 96-Well V-bottom PCR plate with appropriate labelling. Spin down plate. **Seal well.**

Place samples in consecutive **columns**.

Send primer separately in 1.5mL microfuge tube.



Plate submission is default to single primer per plate. If multiple primers are used in a single plate submission, please group your samples by each primer on the plate and put the primers layout in the comment box. Send primers separately in 1.5mL microfuge tubes.

Refer to the table below as a starting guide for template and primer concentration. The final concentrations for each project may require optimisation.

	VOLUME PER SAMPLE	PCR PRODUCT <1kB	Final Concentration (in 10ul)
Template	8 µL	20-50 ng/µL	16-40ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.

	VOLUME PER SAMPLE	PCR PRODUCT >2KB AND DS PLASMID	Final Concentration (in 10ul)
Template	8 µL	100-200 ng/µL	80-160 ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.

- Label plates with the order number or plate number (generated at order lodgment processing in dnaLIMS).
- Primer should be sent using 1.5mL microfuge tube, labelled by the order number and the primer name.
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.



10.2.4 Plate Core Prep with Bead cleanup – Use any 96-Well V-bottom PCR Plate

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Upload and Import DNA Plate File”.
 - Select “Plate-Core_PrepBead40” for Service Request. Or
 - Select “Plate-Core_PrepBead96” for Service Request for full plate submission.
 - Fill in all information.
- For **Plate-Core Prep with Bead**: Select No for EXOSAPIT cleanup and No for Bead cleanup.
The bead cleanup addition is pre-set in these service request options.

For Bead cleanup, the minimum sample volume is 25ul/sample.
Send templates using 96-Well V-bottom PCR plate with appropriate labelling. Spin down plate. **Seal well.**

Place samples in consecutive **columns**.

Send primer separately in 1.5mL microfuge tube.

Plate submission is default to single primer per plate. If multiple primers are used in a single plate submission, please group your samples by each primer on the plate and put the primers layout in the comment box. Send primers separately in 1.5mL microfuge tubes.

Refer to the table below as a starting guide for template and primer concentration. The final concentrations for each project may require optimisation.

	VOLUME PER SAMPLE	PCR PRODUCT <1kB	Final Concentration (in 10ul)
Template	8 µL	20-50 ng/µL	16-40ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.



	VOLUME PER SAMPLE	PCR PRODUCT >2KB AND DS PLASMID	Final Concentration (in 10ul)
Template	8 µL	100-200 ng/µL	80-160 ng/ul
Primer	2 µL	10uM	2pmol/ul

Please note that these template amounts are guides only and optimisation may be needed.

- Label plates with the order number or plate number (generated at order lodgment processing in dnaLIMS).
- Primer should be sent using 1.5mL microfuge tube, labelled by the order number and the primer name.
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.



10.3 FRAGMENT ANALYSIS (PLATE SUBMISSION ONLY, MINIMUM 40 SAMPLES)

Fragment analysis measures the size of DNA fragments by comparing the unknown fragment to DNA fragments with known lengths (the size standard). The size standard is combined with the sample of interest and co-injected into the capillary electrophoresis system. Common applications include: DNA fingerprinting, parentage analysis, microsatellites analysis, AFLP and ARISA.

To ensure that you obtain high quality data, it is essential that you have a robust PCR reaction that requires considerable dilution before running your samples on the AB3730 machine (e.g. 1 in 100). This will dilute out most of the excess PCR reagents, salt and potential contaminants in your samples. Excess reagents and salt can interfere with the electrokinetic injection of the machine and lower the quality of the data. Other contaminants, such as residual protein or detergents carried over from DNA extraction can adhere to the capillaries of the array thereby adversely reducing the quality and life span of the array. If considerable dilution of the sample is not possible we require our customers to perform a cleanup step (e.g. ethanol precipitation) prior to submission.

Very strong signals are difficult to analyse and can cause similar negative impacts on the array as other contaminants, so it is suggested that a 1:20 to 1:150 dilution series is completed on a small number of samples to optimise prior to submitting large numbers of samples. If size ranges do not overlap within a dye colour PCR products may be pooled or multiplex PCR used.

10.3.1 Options for Fragment Analysis:

Size standards:

LIZ500
LIZ600
LIZ1200

Supported fluorescence labels:

6-FAM (blue)
VIC (green)
NED (yellow)
PET (red)

Note: Customizable primers with these labels are available from Life Technology.

10.3.2 Plate Fragment Analysis – Use MicroAmp™ Optical 96-Well Reaction Plate

- Submit your order request online using the dnaLIMS system (see LIMS user guide).
 - Use “Upload and Import Fragment Analysis File”.
 - Select “Plate-FA” for Service Request.
 - Select appropriate size standard.
 - Fill in all information.



- Prepare a 1-3ul aliquot of each sample in MicroAmp™ Optical 96-Well Reaction Plate (Life Technologies). Spin down plate. **Seal well.**

Place samples in consecutive **columns**.

Note: All samples are required to be diluted 10-fold or more, otherwise a cleanup step is required to remove excess dye and reagents.

- Label plates with the order number or plate number (generated at order lodgment processing in dnaLIMS).
- Please send samples and a copy of your submission confirmation. Send order by either courier or express post (Australia Post) to ensure prompt delivery.

Alternatively, you can drop off your samples at our sample reception area weekdays between 8am and 6pm.

- You will receive an email when your results are available. You can access your results via dnaLIMS as per the LIMS user guide.

