Sanger Sequencing: Sample Preparation Guide

Use this as a guide to prepare your samples for Sanger sequencing at AGRF

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1 Overview

The Australian Genome Research Facility (AGRF) is accredited to ISO/IEC 17025:2005 in the field of Biological Testing by the National Association of Testing Authorities (NATA). (Accreditation Number: 14332).

AGRF offers high throughput Sanger sequencing using Applied Biosystems 3730 and 3730*xl* capillary sequencers. These automated platforms use Big Dye Terminator (BDT) chemistry version 3.1 (Applied Biosystems) under standardised cycling PCR conditions. Sequence data is provided as:

- ***.ab1:** The raw chromatogram trace file
- ***.seq:** A text file of the sequence, as generated by the sequencing instruments
- ***.fa:** A quality trimmed FASTA formatted text file
- ***.bn:** A BLAST file (GenBank) of the quality trimmed FASTA file

Additionally, one extra file per batch is generated. This is your batch summary report, which outlines the quality scores and signal intensities for each sample submitted (further information in Section 9).

The flowchart on the following page outlines the process and data outputs (for more information on these file types, see section 9 of this documents).

Four sequencing services are routinely offered:

1.1 Capillary Separation (CS) or electrophoretic separation:

- Client performs the BDT sequencing reaction and removes unlabelled dyes through a reaction clean-up, and the purified labelled DNA is submitted as a dried down pellet for resuspending and loading directly onto the AB 3730xl instrument.
- Turnaround time is 1-2 working days after receipt of samples at AGRF (for less than 200 samples).

1.2 Unpurified BDT reaction (CS+):

- Client performs the BDT sequencing reaction and the unpurified labelled reaction is submitted as a 20µl solution, for clean-up and sequencing.
- Turnaround time is 1-2 working days after receipt of samples at AGRF (for less than 200 samples).

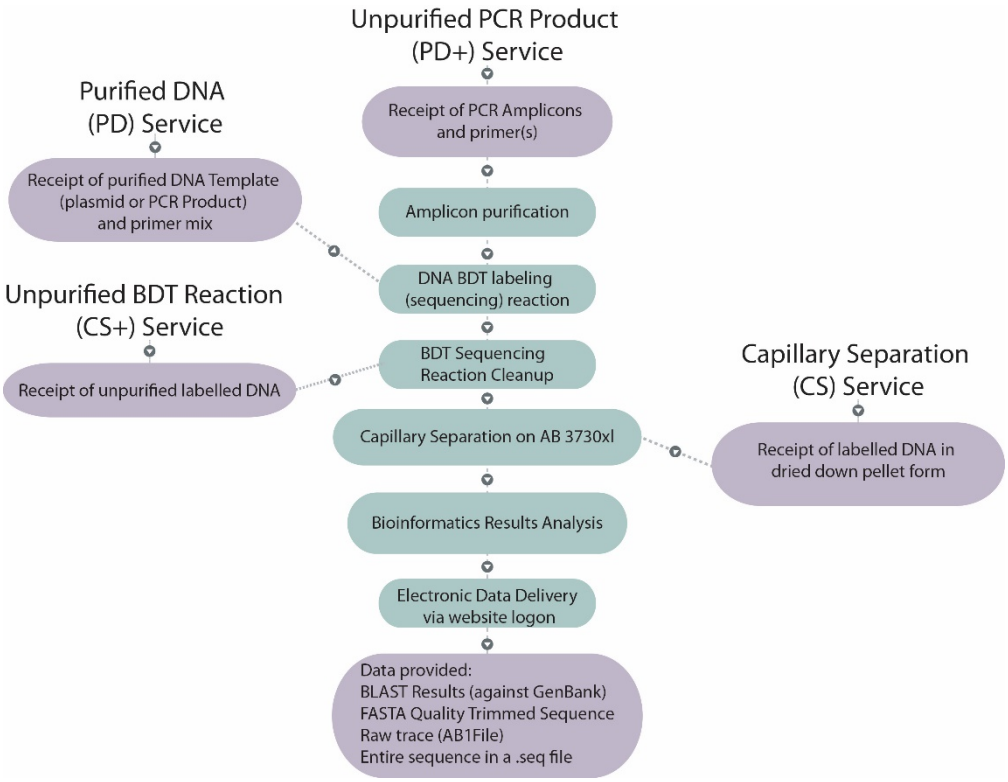
1.3 Purified DNA (PD):

- Purified DNA template (plasmid or PCR product) is pre-mixed with the appropriate primer, and submitted for BDT labelling, purification and sequencing.
- The turnaround time is between 2 – 3 working days after receipt of samples at AGRF (for less than 200 samples).

1.4 Unpurified PCR product (PD+):

- This service is only suitable for established PCR reactions that consistently give strong, clear PCR amplicons of equal consistency between samples. **Please be aware that no sample normalisation is provided as part of this service.**
- The PD+ service is designed for users requiring high-volume purification and sequencing of PCR amplicons
- PCR Amplicon is submitted for purification and sequencing by AGRF
- The turnaround time is between 4 and 7 working days after receipt of samples at AGRF (for less than 400 samples).
- Please submit in plate format where possible (for submissions of >10 samples)

2 Sanger Routine Sequencing Service Process:



3 Three Simple Steps to using AGRF Sanger Sequencing Service

1. Complete your details via the client login on the AGRF website (**www.agrf.org.au**). Your user name and password will be automatically generated and emailed to you
2. Log in to submit your sample information online
3. Print off your submission recept and send it along with your samples to AGRF

You will receive an email when your samples have been received at AGRF, and again when your sequencing data is available for download from the secure FTP site.

4 References

- AGRF: www.agrf.org.au
- AGRF BDT Order form: http://www.agrf.org.au/docs/bdtv3.1-order-form-oct2014.pdf
- Thermo Fisher: www.thermofisher.com
- AB Sequencing Chemistry Guide: http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041258.pdf
- AB Basecaller Software Frequently Asked Questions document: http://docs.appliedbiosystems.com/pebiodocs/04362968.pdf
- NATA: www.nata.asn.au

5 DNA Sample Preparation

The two most important factors in Sanger sequencing are the quality and quantity of both the DNA template and primer.

5.1 Template Quality:

Automated sequencing on the AB3730 platforms can be affected by the quality of DNA in the reaction. The AB3730 is much more sensitive to many contaminants compared to gel based systems (AB377) and older capillary systems (AB3700). Contaminants may include:

- RNA
- Proteins, Carbohydrates and Lipids
- Ethanol
- Buffer salts
- Elution Buffers (e.g. Qiagen EB buffer)
- Purification column resins
- ExoSAP residual chemistry

Templates should only be resuspended and submitted in water. To ensure good DNA quality, templates should be analysed by both:

a. **Agarose gel electrophoresis** using a known mass standard where a visible band should be present on the gel at the expected quantitated level.

b. **Spectrophotometer** to ensure OD_{260}/OD_{280} range is between 1.8 and 2.0
$OD_{260}/OD_{280} < 1.8$ may indicate protein contamination
$OD_{260}/OD_{280} > 2.0$ may indicate RNA contamination

5.2 Template Quantity:

It is very important to know how much DNA template is being used in order to ensure reliable, reproducible results. The quantity of DNA template required is dependent on its size. Table 1 outlines the quantities required by AGRF for samples and primers that are being sent for the PD and CS services.

AGRF prefers template DNA to be quantified by gel electrophoresis, as spectrophotometry tends to overestimate the concentration of the template DNA.

5.3 Primer Quality and Quantity:

Sequencing primers should be non-degenerate, homologous to the target region, and have a T_m between 55°C and 60°C. Primers that have been defrosted/frozen many times will degrade, resulting in poorer sequencing performance

Table 1: Recommended amounts of template and primer for sequencing reactions

Template	**Recommended Quantity for PD Samples (in 12µL)**	**Recommended Quantity for CS Samples (BDT set up in your own lab)**
PCR Product 100 – 200 bp	3 - 8 ng	1 - 3 ng
PCR Product 200 – 400 bp	6 - 12 ng	2 - 4 ng
PCR Product 400 – 600 bp	12 - 18 ng	4 - 6 ng
PCR Product 600 – 800 bp	18 - 30 ng	6 - 10 ng
PCR Product >800 bp	30 - 75 ng	10 - 25 ng
Plasmid, Single-stranded	150 - 300 ng	50 - 100 ng
Plasmid, Double-stranded	600 - 1500 ng	200 - 500 ng
Primer Quantity (one primer per reaction)	10pmol* (0.8 pmol/µl)	3.3 pmol

**this equates to 1µL of a 10µM stock, or 2uL of a 5µM stock*

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

5.4 DNA labelling preparation for clients using the CS/CS+ service:

Reactions should be prepared according to Table 2. BDT must be completely thawed before use. It is also recommended that BDT be aliquoted into appropriate working volumes and stored frozen until needed to avoid more than 3 freeze / thaw cycles.

Table 2: Recommended amounts of template for sequencing reaction

Components	Half Volume Reactions (0.125x)	Half Volume Reactions (0.25x) *	Full Volume Reactions (0.5x)	Full Volume Reactions (1x)
Template (see Table 1) Primer (0.8 pmol/µl) MilliQ Water (if necessary)	7.75µL	7.5µL	14µL	12µL
BDT v3.1	0.5µL	1µL	4µL	8µL
5X BDT dilution Buffer	1.75µL	1.5µL	2µL	-
TOTAL	**^10µL**	**^10µL**	**20µL**	**20µL**

* Preferred reaction set up for AGRF CS sequencing service

^ For CS+, reaction should be diluted to 20uL prior to submission

Samples should be free of oil as this may affect the quality of the sequencing data. Large DNA templates and bacterial genomic DNA can be submitted (please refer to AB Sequencing Chemistry Guide for protocols regarding these large templates).

AGRF accepts samples that have been precipitated in either tubes or plates. These dry sequencing products are stable for 48 hours at room temperature or ten days at 4ºC.

Cycle Sequencing: The cycling conditions are the same as those outlined in Table 3.

Table 3: AGRF Cycling Conditions for PD sequencing service

Temperature and Time	Number of Cycles
96°C for 2 min	1
96°C for 10 secs 50°C for 5 secs 60°C for 4 mins	30
4°C	Hold

Post Reaction Clean-up Considerations: It is critical to remove all unincorporated dye terminators from the cycle sequencing reaction prior to analysis by capillary electrophoresis to prevent basecalling errors.

AGRF currently uses a magnetic bead based method for all internal BDT cleanups. Suitable commercial clean up protocols include CentriSep (ABI), DyeEX (Qiagen) and MicroSpin G-50 (Amersham/Pharmacia). Non-commercial clean-up methods may also be used and protocols for these methods are listed in the Appendix.

5.5 DNA sample preparation for clients using the PD service:

The template and sequencing primer (one primer per submission) should be pre-mixed in a total volume of 12µL, according to Table 1.

As AGRF operates a high throughput facility, all PD samples are run using the same cycling conditions (Table 3). Therefore melting temperatures for primers should match these cycling conditions. If primers are not able to match these conditions, sequencing data quality may be compromised.

5.6 PCR Sample preparation for clients using the PD+ service:

Each sample submitted for PD+ must be of a consistent size and concentration. AGRF recommends that a portion of the PCR reaction is visualised using **agarose gel electrophoresis** against a known mass standard, where clear single bands of a consistent intensity should be visualised on the gel at the expected size range.

Table 4: Recommended amounts of template required for PD+ sequencing

Template	Minimum quantity of PCR amplicon required for PD+ (20µL)
PCR Product 100 – 200 bp	1.5 ng/uL
PCR Product 200 – 400 bp	3 ng/uL
PCR Product 400 – 600 bp	4.5 ng/uL
PCR Product 600 – 800 bp	6 ng/uL
PCR Product 800 bp – 1200bp	7.5 ng/uL
PCR Products > 1200bp	Not recommended

Please note that these template amounts are guides only and optimisation may be required. AGRF will not process plates that show significant variations in amplicon size and/or intensity following GelQC.

PD+ turn-around times
The turn-around time for this service is between 4-7 working days, for submissions of four or less 96-well plates. Larger submission may require longer to process, and estimates of turnaround times can be provided on request. Anything that may cause a delay beyond the expected turnaround time will be communicated to the sample submitter as soon as possible.

6 Submission formats

6.1 Submitting Plates:

AGRF places a positive control in every plate tested. For most nodes, the positive control is in well H1, and so the maximum number of samples that may be submitted per plate is 95. Samples should be inputted across the plate i.e. A1 - A12, then B1 - B12 etc. (no gaps please). **AGRF processes each plate as a batch, so please ensure that the minimum sample number requirements for the service are met for each plate.**

6.2 Preferred Plate Type:

- ABI Prism 96-well Optical Reaction Plate with Barcode (P/N 4306737)
- ABI Prism 384-well Clear Optical Reaction Plate with Barcode (P/N 4309849)
- Axygen PCR-96 M2-HS-C
  Note: if plates will be shipped by air, a thermal seal should be used to prevent sample leakage. Adhesive seals are insufficient.

6.3 Preferred Tube Type:

- 1.5ml flip-cap tube (snap lock and boil proof are preferred)

6.4 Sample Labelling:

- **Tubes**: Please keep sample names simple with a maximum of 8 characters long. Clearly label 1.5 mL tubes both on the lid and on the side. If there is room you may wish to number your samples in the order that you submitted them (this helps clearly identify your sample order during reception and QC).
- **Plates:** A batch ID will be generated for each set of samples you submit through the AGRF sample submission form. Label the plate with this batch ID, as well as print off the sample submission form to accompany the plate during transit.

6.5 Sample Presentation:

- **CS Service:** CS samples should be sent to AGRF in a dried down pellet format. These samples must be precipitated (cleaned) post-cycling so they are dried in the process (e.g. through ethanol washing). Where a column-based clean-up method has been used, the eluted sample must be dried before submission (e.g. speedy-vac or ethanol precipitation). Samples can be precipitated in either tubes or plates. These dry sequencing products are stable for 48 hours at room temperature or ten days at 4°C.
- **CS+ Service:** CS + samples should be submitted as a 20µl solution following the BDT sequencing reaction that is performed prior to sending to AGRF.
- **PD Service:** The template and primer should be pre-mixed in a total volume of 12µL.
- **PD+ Service:** Samples should be submitted in tubes or plates in a total volume of 20µL.

6.5 Primer submission for PD+ Service:

- **For single-direction sequencing:** For each sample, submit 2 µL of a 3.2uM primer solution, in a 1.5mL tube. For full plates, submit 200 µL.
- **For dual-direction sequencing:** For each sample, submit 2µL of your forward primer (3.2µM), and 2µL of your reverse primer (3.2µM), in separate 1.5mL tubes. Label these primer tubes with "–F" and "–R" suffixes. For full plates, submit 200 µL.
- **For mixed-primer (complex) plates:** If you require different primers to be used for different samples across a plate, you must submit a primer plate, with each well position of the primer matching the well position of the correct sample. 5 µL of primer (3.2uM) is required in each well position (this is to allow for transit loss and pipette limits). Label the primer plate with the corresponding batch ID, followed by "-PRIMER", and a "-F" or "-R" suffix. E.g. sample plate "**AGRF23**" would be run using the primers from "**AGRF23-PRIMER-F**".

7 Appendix:

Protocols for the clean-up of sequencing reactions for submitting samples to AGRF for the CS Service

7.1 Protocol 1: Magnesium sulphate clean-up protocol

a. Allow your plates or tubes to equilibrate at room temperature following thermal cycling
b. Add 75µL of 0.2mM $MgSO_4$ Ethanol solution (see below) to each well/tube. This volume applies to sequencing reactions between 10 – 20µL in total volume
c. Mix thoroughly by vortexing and allow to sit at room temperature for a minimum of 15 minutes to allow precipitation of your labelled products
d. Spin at room temperature for a minimum of 15 minutes (longer incubation and spinning times will increase the precipitation of labelled products but may also precipitate un-incorporated dyes)
    I. plates at >2000 x *g*
    II. tubes at max speed in a bench top centrifuge
e. Remove samples from the centrifuge and gently invert plates/tubes over paper towels for 1-2 minutes.
f. Add 100µL of 70% EtOH to each tube to wash the pellet (do not vortex), and re-spin for 15 minutes using the same conditions as the first spin.
g. Remove samples from the centrifuge and gently invert plates/tubes over paper towels for 1-2 minutes. If using plates, place paper towels in centrifuge plate container (to prevent spatter), place plates upside down on the paper towels and spin for 1 minute at 1000 rpm
h. If using tubes, allow them to air dry or place in a 37^{0}C oven, but do not allow your samples to over-dry, as they will be difficult to resuspend if this occurs. Do NOT aspirate remaining solution as the pellet is easily dislodged
i. When samples are dry submit them to the AGRF for re-suspension and analysis

0.2mM $MgSO_4$ ethanol solution:

- 30 mL of MilliQ water
- 70 mL of absolute ethanol
- 20 µL 1M $MgSO_4$

Stock solutions should be prepared fresh on a fortnightly basis. Store and use at room temperature (do not refrigerate or freeze).

7.2 Protocol 2: Ethanol/EDTA Precipitation Clean-up Protocol as per the AB Sequencing Guide

(http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041258.pdf)

a. Remove the 96-well reaction plate from the thermal cycler
b. Remove the cover from the reaction plate
c. Prepare the ethanol / EDTA solution:
   i) **For half-volume reactions**, add the following to each 10µL reaction in the following order:
      1. 2.5µL of 125 mM EDTA
      2. 30µL of 100% ethanol or 35µL of non-denatured 95% ethanol

   ii) **For full-volume reactions**, add the following to each 20µL reaction in the following order:
      1. 5µL of 125 mM EDTA
      2. 60µL of 100% ethanol or 70µL of non-denatured 95% ethanol

d. Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminium foil tape. Press the foil onto the wells to prevent any leakage

   IMPORTANT! If you are using heat-seal film (PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (PN 4311971). The residual glue will interfere with the heat-sealing process.

e. Invert the reaction plate four times or vortex for 15 sec to mix
f. Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products
g. Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*:
   i) 1400 to 2000 × *g*: 45 min
   ii) 2000 to 3000 × *g*: 30 min
   Note: The reaction plate can withstand 3000 × *g* for 30 min.

   IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

h. Discard the supernatant as follows:
   i) Without disturbing the precipitates, remove the adhesive tape
   ii) Invert the reaction plate onto a paper towel folded to the size of the plate
   iii) Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g*. Then remove the plate from the centrifuge

IMPORTANT! The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators remain in the samples. Unincorporated dye terminators will significantly affect the quality of the sequencing read for approximately the first 100 bases.

i. Perform a 70% wash
   i) For half-volume reactions (10 µL), add 30 µL of 70% ethanol to each pellet
   ii) For full-volume reactions (20 µL), add 60 µL of 70% ethanol to each pellet

j. Seal the wells as in step d, then invert the reaction plate a few times or vortex for 15 sec to mix

k. Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × *g*

IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the reaction plate for 2 min immediately before performing the next step.

l. Repeat step h. except in step iii, place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × *g* for 1 min. Then remove from the centrifuge

Note: Start timing when the rotor begins to move

m. Remove the reaction plate from the centrifuge and discard the paper towel

IMPORTANT! Make sure the wells are dry. Use a Speed-Vac for 15 min to dry the plate.

IMPORTANT! Make sure the samples are protected from light while they are drying.

n. Seal the wells as in step d for storage and keep in the dark at −15°C to −25°C until ready to send to AGRF for re-suspension and analysis

7.3 Protocol 3: Ethanol/EDTA/Sodium Acetate Precipitation Clean-up Protocol as per the AB Sequencing Guide

(http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041258.pdf)

a. Remove the 96-well reaction plate from the thermal cycler
b. Remove the cover from the reaction plate
c. Prepare the ethanol/EDTA/sodium acetate solution:
   i) **For half-volume reactions**, add the following to each 10µL reaction in the following order:
      1. 1µL of 125 mM EDTA
      2. 1µL of 3 M sodium acetate, pH 4.6
      3. 25µL of 100% ethanol or 29µL of non-denatured 95% ethanol

   ii) **For full-volume reactions**, add the following to each 20µL reaction in the following order:
      1. 2µL of 125 mM EDTA
      2. 2µL of 3 M sodium acetate, pH 4.6
      3. 50µL of 100% ethanol or 58µL of non-denatured 95% ethanol

d. Seal the wells with a piece of 3M Scotch Tape 431 or 439 adhesive backed aluminum foil tape. Press the foil onto the wells to prevent any leakage

   IMPORTANT! If you are using heat-seal film (Applied Biosystems PN 4337570) to cover samples prior to electrokinetic injection, do not use the Optical Adhesive Covers (Applied Biosystems PN 4311971). The residual glue will interfere with the heat sealing process.

e. Invert the reaction plate four times or vortex for 15 sec to mix
f. Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products
g. Place the reaction plate in a centrifuge with a plate adaptor and spin at the maximum speed, which must be ≥1400 × g but <3000 × g:
   i) 1400 to 2000 × g: 45 min
   ii) 2000 to 3000 × g: 30 min

   Note: The reaction plate can withstand 3000 × g for 30 min

   IMPORTANT! Proceed to the next step immediately. If this is not possible, then spin the reaction plate for an additional 2 min immediately before performing the next step.

h. Discard the supernatant as follows:
   i) Without disturbing the precipitates, remove the adhesive tape
   ii) Invert the reaction plate onto a paper towel folded to the size of the plate
   iii) Place the inverted reaction plate and paper towel into the centrifuge and spin up to 185 × g. Then remove from the centrifuge

   IMPORTANT! The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the wells, the more unincorporated dye terminators will remain in the samples. Unincorporated dye terminators will significantly affect the quality of the sequencing read for approximately the first 100 bases.

i. Perform a 70% wash
   i) For half-volume reactions (10µL), add 35µL of 70% ethanol to each pellet
   ii) For full-volume reactions (20µL), add 70µL of 70% ethanol to each pellet
j. Seal the wells as in step d, then invert the reaction plate a few times or vortex for 15 sec to mix
k. Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × *g*
l. Repeat step h.
m. Remove the reaction plate from the centrifuge and discard the paper towel

   Note: Pellets may or may not be visible. Vacuum drying of the samples is not necessary

n. Seal the wells as in step 4 for storage and keep in the dark at − 15°C to −25°C until ready to send to AGRF for re-suspension and analysis