SERVICE GUIDE

Sanger Sequencing Service

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Service Guide
Sanger Sequencing

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1.0 Overview and Turnaround times

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

Five Sanger Sequencing services are routinely offered:

1.1 Capillary Separation Purified or electrophoretic separation:

You perform the BDT sequencing reaction and remove unlabeled dyes through a reaction clean-up, then 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 Capillary Separation Unpurified:

You perform the BDT sequencing reaction and the unpurified labelled reaction is submitted as a 20 µL 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 Template and primer:

You pre-mix your purified DNA template (plasmid or PCR product) with the appropriate primer, and submit for BDT labelling, purification and sequencing. The turnaround time is 2-3 working days after receipt of samples at AGRF (for less than 200 samples).

1.4 Unpurified PCR products:

This service is only suitable for established PCR reactions that consistently give strong, clear PCR amplicons of equal consistency between samples. This service is ideal for users requiring high-volume purification and sequencing of PCR amplicons but can be used for small batches of samples. Your PCR amplicon is submitted for purification and sequencing by AGRF. The turnaround time is between 3-5 working days after receipt of samples at AGRF (for less than 400 samples). Please submit in plate format >47 samples).

1.5 PCR Re-Sequencing (Full-Service Sanger):

Send us your gDNA (or samples for DNA extraction) and we will PCR amplify, purify and sequence the PCR products. You can provide us with a primer design, or our bioinformatics team can design the primers for you. If you are planning a large PCR-based sequencing project, this option lets us take care of the whole process.

This service is commonly used for the identification of somatic mutations and genetic variability, such as SNPs. If you add our extraction services and custom bioinformatics analysis options, we can take you from raw tissue to analysed data.*

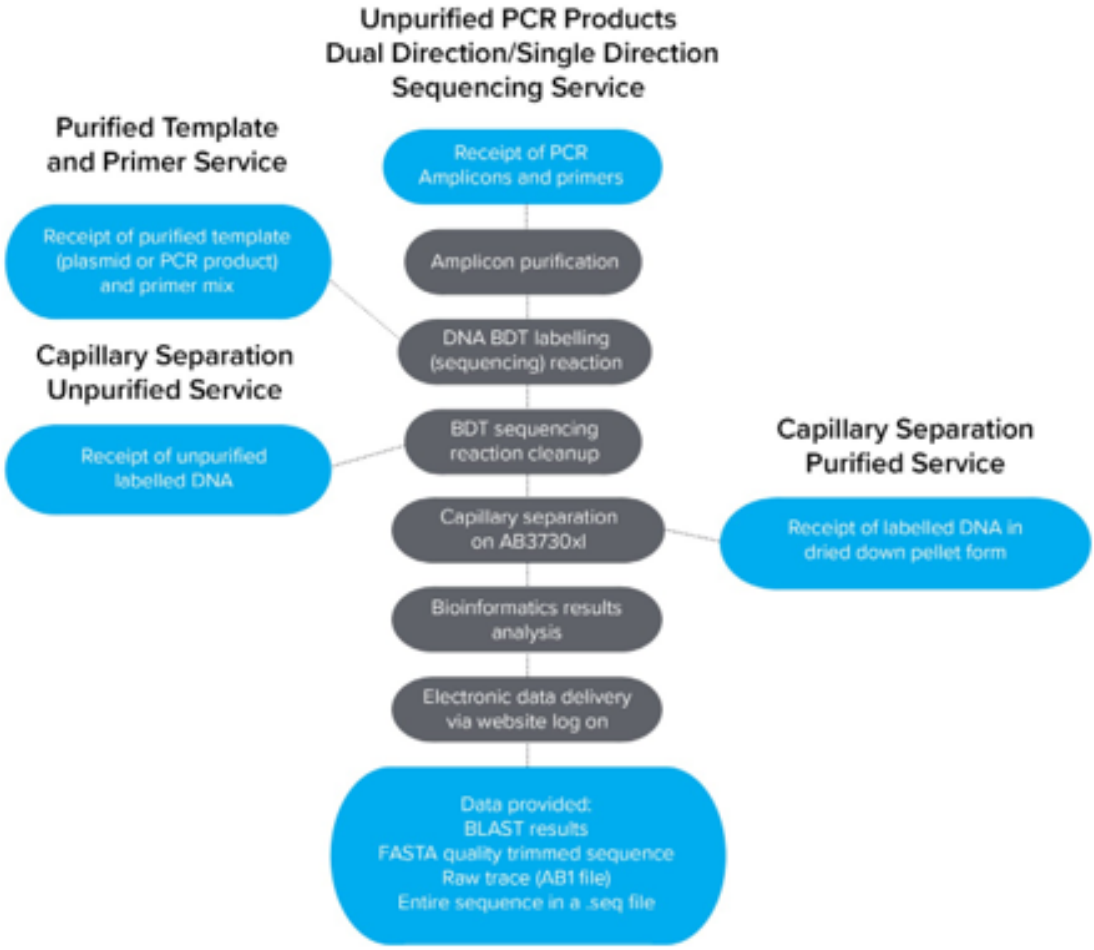
*Note: minimum project sizes apply.

PCR Resequencing Conditions of Service

- AGRF will assess the amplicon success on gel visualization. If less than 85% of all targeted amplimers fail to produce clear, single amplicons (when visualized using gel electrophoresis), a second attempt at PCR amplification will be undertaken. If this also fails to generate more than 85% amplicon success, then the client will be notified, and the project will be closed. The client will only be charged the cost of the primers. If more than 85% of amplimers are successful, the project will proceed to sequencing of all amplicons, and the full project price will be invoiced upon completion.

- Sequencing may fail due to sequence specific anomalies such as but not limited to: G/C, A/T rich sequence bias, repeat sequences, homopolymer tracts, oligo design etc. Such sequence anomalies, which affect the quality of individual reads are considered inherent to the nature of the DNA and are not the responsibility of the AGRF. gDNA and primers submitted or purchased for the project will be stored by AGRF for 3 months after project completion, and then discarded unless the client requests AGRF to return the original materials. Returns will require a quote and should be discussed at the commencement of a project.

Figure 1. AGRF Sanger Routine Sequencing Service Process



2.0 Online Sample Submission

- On the AGRF website, click Login at the top of the page, then choose the Submit Your Samples button.
- Log in using your AGRF account details.
- Choose Submit Samples tab.
- Select your routine agreement from the Agreement ID dropdown menu, (if your account has more than one agreement).
- Select “Sanger Sequencing” as the Service Type.
- Select your chosen Service from the dropdown menu (refer to section 1 for the available services).
- Complete submission format, product type and base call method options.
- If you require AGRF to add a primer to your sample, tick the Primers box and choose a primer from the dropdown menu (see table 2).
- If requesting a rerun, tick the Rerun box and select the original batch name. Enter the specific samples for rerunning by entering their original samples name in the grid below.
- Use the Notes, section to add any additional information, such as outlining primer addition for the Unpurified PCR Products service or any other requirements, for example - addition of DMSO or Betaine for the GC rich samples.

- Enter samples names either by filling in the grid, or completing the template from the Download Template link, saving it to your computer, then uploading it using the Browse button.
  - Sample names can be up to 40 characters in length.
  - Where possible, keep sample name simple. Using your initials and a sequential number is preferred.
  - You can use numbers, letters, underscores and hyphens.
  - The first character must be a letter or number.
  - For example: Sample-1_b
- Complete Safety and Regulatory information and Delivery and Packaging information.

Submit and print a paper copy of your sample submission receipt that will be generated as a PDF file.

This receipt must be included with your sample package.

3.0 Outputs

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.

4.0 DNA sample Preparation

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

4.1 Template Quality

Sanger sequencing can be significantly affected by the quality of DNA in the reaction. Contaminants that can decrease the quality of your result may include:

- RNA
- Proteins, carbohydrates and lipids
- Ethanol
- Buffer salts
- Elution buffers (e.g. Qiagen EB buffer)
- Purification column resins
- ExoSAP residual chemistry

If you are preparing or purifying DNA for Sanger sequencing, they should only be resuspended or eluted and submitted in water.

To ensure good DNA quality, templates should be analysed by both:

- Agarose gel electrophoresis, using a known mass standard where a visible band should be present on the gel at the expected size and intensity.
- Spectrophotometer to ensure OD260/OD280 range is between 1.8 and 2.0. OD260/OD280 < 1.8 may indicate protein contamination.
- OD260/OD280 > 2.0 may indicate RNA contamination.

4.2 Template Quantity

It is important to know how much DNA template is being used to ensure reliable and reproducible results. The quantity of DNA template required is dependent on its size, as outlined in Sections 6.1 and 6.3. AGRF requests template DNA to be quantified by gel electrophoresis, as spectrophotometry tends to overestimate the concentration of the template DNA. Insufficient template is the most common fault encountered in Sanger sequencing. Accurate measurement of sample volumes is important, and where possible volumes of 2 µL or greater should be used when hand pipetting.

4.3 Primer Quality and Quantity

Sequencing primers should be non-degenerate, homologous to the target region, and have a temperature between 55°C and 60°C. Primers that have been through multiple freeze/thaw cycles will degrade, resulting in poorer sequencing performance.

5.0 Submission Formats

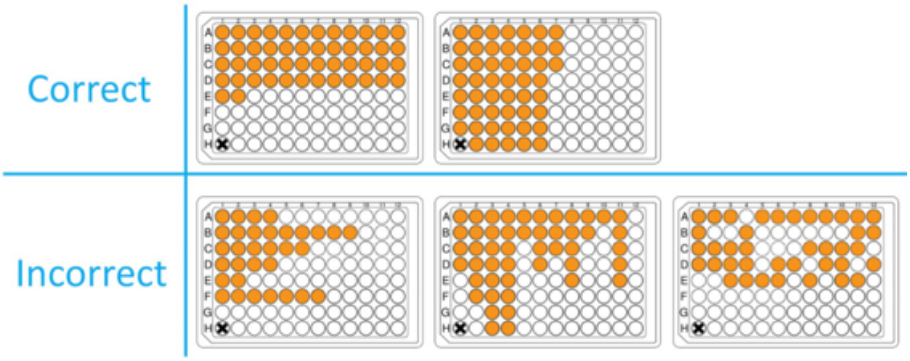
5.1 Submitting Tubes

Please use 1.5 mL flip-cap tubes, NOT PCR tubes. Snap-lock and boil-proof tubes are preferred, and use clean DNase-free tubes that have NOT been autoclaved - autoclaving weakens the tube seal. Do not use Parafilm or similar seals.

5.2 Submitting Samples in 96-Well PCR Plates

AGRF places a positive control in every plate tested. The positive control is in well H1, and so the maximum number of samples that may be submitted per plate is 95. Samples may be pipetted across or down the plate, such as A1-A12 or A1-G1 (H1 left empty). Please do not skip wells (besides H1) or leave gaps on the plate, as demonstrated in Figure 2.

Figure 2: Preferred plate sample layout



The following plate types are preferred.

- ABI Prism 96-wellOptical Reaction Plate with Barcode (P/N 4306737)
- Axygen PCR-96 M2-HS-C

Adhesive seals for transporting plates must be strong adhesive seals. If plates will be shipped by air, a thermal seal should be used to prevent sample leakage. Many standard PCR seals are insufficient.

5.3 Labelling Sample Tubes

Keep sample names simple. We advise that you limit labels to your initials and a number, and clearly label your 1.5 mL tubes on the lid and side of the tube.

e.g. RD001, RD002,RD003.

If submitting primers for the Unpurified PCR service, label the primer tubes with “–F” and “–R” suffixes as appropriate.

5.4 Labelling Plates

A batch ID (e.g. IanMalcolm271) will be generated for each set of samples you submit to AGRF. Label the plate with this batch ID. If you have submitted more than one plate, the plates will be given a sequential alphabetical assignment (A, B, C etc). Include this letter on the plates to distinguish them.

e.g. IanMalcolm271A and IanMalcolm271B.

Additionally, when submitting samples and primer plates for the Unpurified PCR service, label the appropriate plate with the word “samples”, “F primer” or “R primer”.

6.0 Sample Preparation

6.1 Purified Template and Primer

To submit for the Purified Template and Primer service, samples should be submitted as pre-mixed template and primer in a total volume of 12 µL. Each sample should contain only one primer. If you require sequence from both forward and reverse primers, submit separately as two samples. Table 1 outlines the recommended template amount based on product size and the necessary primer quantity. The plasmid quantities are for a range of plasmid size (4-10kb), use an amount in the range roughly appropriate for your plasmid size (a rough rule is divide your plasmid size by 20 then times by 3). If your plasmid is >10kb in size, please contact the team to discuss submission options.

Table 1: Recommended amounts of template and primer for sequencing reactions. Please note that these template amounts are guides only and optimisation may be needed.

Template	Recommended Quantity for Purified Template & Primer Samples
PCR Product 100 – 200 bp	3 - 8 ng
PCR Product 200 – 400 bp	6 - 12 ng
PCR Product 400 – 600 bp	12 - 18 ng
PCR Product 600 – 800 bp	18 - 30 ng
PCR Product >800 bp	30 - 75 ng
Plasmid, Single-stranded	150 - 300 ng
Plasmid, Double-stranded	600 - 1500 ng
Primer Quantity (one primer per reaction)	10pmol* (0.8 pmol/µl)

**this equates to 1µL of a 10µM stock, or 3µL of a 3.3µM stock*

As AGRF operates a high throughput facility, all Purified Template and Primer samples are run using the same cycling conditions (Table 6). 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.
If necessary, AGRF can add one of our in-house primers to your samples prior to processing. The available primers are listed below in Table 2. When requesting a primer to be added to your samples, please submit your template in a total volume of 9 µL, following the template quantity recommendations in Table 1.

Table 2: List of AGRF in-house primers that can be added to your samples prior to processing.

Oligo	Sequence	Site/Vector
M13 (-21)_F	TGTAAAACGACGGCCAGT	Common vector primer
M13_R	CAGGAAACAGCTATGACC	Common vector primer
SP6	ATTTAGGTGACACTATAG	SP6 promoter
T3	GCAATTAACCCTCACTAAAGG	T3 promoter
T7prom_F	TAATACGACTCACTATAGGG	T7 promoter
T7term_R	GCTAGTTATTGCTCAGCGG	T7 promoter
pGEX_F	CCAGCAAGTATATAGCATGGCC	pGEX vector
pGEX_R	CTCCGGGAGCTGCATGTG	pGEX vector
eGFP-C_F	CATGGTCCTGCTGGAGTTCGTG	3′ end of EGFP
eGFP-N_R	CGTCGCCGTCCAGCTCGACCAG	5′ end of EGFP
dsRed-C_F	TGGACATCACCTCCCACAACGAGG	3′ end of DsRed1, also suitable for mCHERRY
dsRed-N_R	GATGTCCCAGGCGAAGGG	5′ end of DsRed1, also suitable for mCHERRY
CMV_F	CGCAAATGGGCGGTAGGCGTG	Human CMV promoter
bGH_R	TAGAAGGCACAGTCGAGG	Mammalian expression vector
LucN_R	CCTTATGCAGTTGCTCTCC	5′ end of luciferase

6.2 Unpurified PCR Product

Each sample submitted for the Unpurified PCR product service must contain only a clean single amplicon with a minimum concentration determined by size. See Table 3 below for minimum amplicon concentrations. Please note that these template amounts are guides only and optimisation may be required.

Table 3: Recommended amounts of template required for Unpurified PCR Products sequencing

Template	Minimum quantity of PCR amplicon required for PD+ (20 µL)
PCR Product 100 – 200 bp	1.5 ng/µL
PCR Product 200 – 400 bp	3 ng/µL
PCR Product 400 – 600 bp	4.5 ng/µL
PCR Product 600 – 800 bp	6 ng/µL
PCR Product 800 bp – 1200 bp	7.5 ng/µL
PCR Products > 1200 bp	Contact AGRF team

AGRF recommends that a portion of the PCR reaction is visualised using agarose gel electrophoresis against a known mass standard, verifying the expected size range.

AGRF will not process plates that show multiple bands and/or very low intensity following clean-up and GelQC.

If you are having issues generating a strong PCR band or are not sure your PCR is of high enough concentration, please contact your local AGRF laboratory for advice.

Samples should be submitted in tubes or plates in a total volume of 20 µL along with primers in separate tubes. Labelling is described in section 5.3.

- For single-direction sequencing: For each sample, submit 3 µL of a 3.2 µM primer solution (minimum primer volume 10 µL) in a 1.5mL tube. For full plates, submit 200 µL.
- For dual-direction sequencing: For each sample, submit 3 µL of your forward primer (3.2 µM), and 3 µL of your reverse primer (3.2 µM), in separate 1.5 mL tubes (minimum volume 10 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 (3.2 µM) is required in each well position (this is to allow for transit loss and pipette limits).

6.3 Capillary Separation Unpurified

Capillary Separation Unpurified samples should be submitted as a 20 µL solution of the completed BDT sequencing reaction that is performed prior to sending to AGRF. Table 4 outlines the recommended amount of template used for your BDT reactions.

Table 4: Recommended amounts of template and primer for sequencing reactions. Please note that these template amounts are guides only and optimisation may be needed.

Template	Recommended quantity for BDT reactions
PCR Product 100 – 200 bp	1 - 3 ng
PCR Product 200 – 400 bp	2 - 4 ng
PCR Product 400 – 600 bp	4 - 6 ng
PCR Product 600 – 800 bp	6 - 10 ng
PCR Product >800bp	10 - 25 ng
Plasmid, Single-stranded	50 - 100 ng
Plasmid, Double-stranded	200 - 500 ng
Primer Quantity (one primer per reaction)	3.3 pmol

Reactions should be prepared according to Table 5. 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. Cycling conditions are provided in Table 6.

Table 5: Recommended primer quantity and quality

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) and MilliQ Water (if necessary)	7.75 µL	7.5 µL	14 µL	12 µL
BDT v3.1	0.5 µL	1 µL	4 µL	8 µL
5X BDT dilution Buffer	1.75 µL	1.5 µL	2 µL	-
TOTAL	**^10 µL**	**^10 µL**	**20 µL**	**20 µL**

*Preferred reaction set up for AGRF Capillary Separation sequencing service
^For Capillary Separation Unpurified, reaction should be diluted to 20 µL prior to submission

Table 6: AGRF Cycling Conditions for BDT reactions

Temperature and Time	Number of Cycles
96°C for 2 minutes	1
96°C for 10 seconds	30
50°C for 5 seconds	
60°C for 4 minutes	
4°C	Hold

Samples need to 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 the Thermo Scientific Sequencing Chemistry Guide for protocols regarding these large templates).

6.4 Capillary Separation Purified

Following the BDT reactions outlined in Section 6.3, Capillary Separation Purified 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). It is critical to remove all unincorporated dye terminators from the cycle sequencing reaction prior to analysis by capillary electrophoresis to prevent base calling errors.

AGRF uses a magnetic bead-based method for all internal BDT cleanups. Suitable commercial clean up protocols include:

- CentriSep (ABI)
- DyeEX (Qiagen)
- MicroSpin G-50 (Amersham/Pharmacia)

Non-commercial clean-up methods may also be used. Protocols for these methods are listed in the Appendix.

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.

7.0 References

- AGRF: www.agrf.org.au
- Thermo Fisher: www.thermofisher.com
- AB Sequencing Chemistry Guide: http://tools.thermofisher.com/content/sfs/manuals/cms_041003.pdf
- AB Basecaller Software Frequently Asked Questions document: http://tools.thermofisher.com/content/sfs/manuals/cms_079032.pdf

8.0 Appendix-Cleanup protocols

8.1 Protocol 1: Ethanol/EDTA Precipitation Clean-up Protocol as per the AB Sequencing Guide

This protocol provides cleaner signal but loses some smaller fragments. For small amplicons or if sequence required closer to the primer use Protocol 2 : Ethanol/EDTA/Sodium Acetate.

- 1. Remove the 96-wellreaction plate from the thermal cycler.
- 2. Remove the cover from the reaction plate.
- 3. Prepare the ethanol / EDTA solution:

◊For half-volume reactions, add the following to each 10 µL reaction in the following order:

  - i. 2.5 µL of 125 mM EDTA
  - ii. 30 µL of 100% ethanol or 35µL of non-denatured 95% ethanol

◊For full-volume reactions, add the following to each 20 µL reaction in the following order:

  - i. 5 µL of 125 mM EDTA
  - ii. 60µL of 100% ethanol or 70 µL of non-denatured 95% ethanol

- 4. 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 (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.

- 5. Invert the reaction plate four times or vortex for 15 sec to mix.
- 6. Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
- 7. 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 minutes immediately before performing the next step.

- 8 .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.
  - Remove the plate from the centrifuge.

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, which will affect the quality of the sequencing reads for the first 100 bases.

- 9. 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.
- 10. Seal the wells as in step 4, then invert the reaction platea few times or vortex for 15 sec to mix.
- 11. Place the reaction plate in the centrifuge and spin for 15 min. at 1650 × g.

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

- 12. Repeat step 8, 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.

- 13. Remove the reaction plate from the centrifuge and discard the paper towel.

Make sure the wells are dry. Use a Speed-Vac for 15 min to dry the plate. Ensure the samples are protected from light while they are drying.

14. 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.

8.0 Protocol 2: Ethanol/EDTA/Sodium Acetate Precipitation Cleanup Protocol as per AB Sequencing Guide.

Use this protocol for smaller fragments or if targeting sequence close to the primer, may give increased amount of dye blob, (left over unincorporated dye labelled nucleotides).

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

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

- 4. 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.

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.

- 5. Invert the reaction plate four times or vortex for 15 sec to mix.
- 6. Leave the reaction plate at room temperature for at least 15 min to precipitate the extension products.
- 7. 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.

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

- 8. 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.

Remove from the centrifuge.

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, which will affect the quality of the sequencing reads for the first 100 bases.

- 9 . Perform a 70% wash.
  - i.For half-volume reactions (10µL), add 35 µL of 70% ethanol to each pellet
  - ii. For full-volume reactions (20µL), add 70 µL of 70% ethanol to each pellet
- 10. Seal the wells as in step 4, then invert the reaction plate a few times or vortex for 15 sec to mix.
- 11. Place the reaction plate in the centrifuge and spin for 15 min at 1650 × g.
- 12. Repeat step 8.
- 13. 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.

- 14. 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.

9.0 Quality Statement

Non-Clinical Work

All non-clinical activities at AGRF Ltd adhere to the stringent requirements outlined in ISO17025: 2005. AGRF Ltd holds accreditation in the field of Biological Testing, specifically DNA Analysis, in accordance with the ISO17025: 2005 standard by the National Association of Testing Authorities (NATA).
Our dedicated staff and analytical processes strictly follow Standard Operating Procedures (SOPs), which clearly outline responsibilities and quality checks to consistently achieve the reported standards. Compliance is actively monitored through regular reviews and internal audits. To ensure the highest standards, all work is conducted under the supervision of qualified personnel, with ongoing checks at various stages, both in progress and upon completion, to confirm adherence to the necessary ISO17025: 2005 standards.