MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

MASSEY GENOME SERVICE

Sanger Sequencing and Genotyping using Applied Biosystems™ 3500xL Genetic Analyzer

SEQUENCING TECHNICAL BULLETIN
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BULLETIN INCLUDES

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Sanger Sequencing using Applied Biosystems™ 3730 and 3500xL Genetic Analyzer

TECHNICAL BULLETIN

Quality Values and Mixed Bases

Quality Values

The Sequencing Analysis Software used by the Massey Genome Service to analyse customers sequencing data uses an algorithm that calculates quality values (QVs) for pure and mixed bases. The QV is a per-base estimate of the basecaller accuracy. The per-base QVs are calibrated on a scale corresponding to:

QV=-10log10(Pe), where Pe is the probability of error.

The range of a QV is 1-50, with 1 being low confidence and 50 being high confidence.

Quality Value (QV)	Probability the Basecall is incorrect
10	10%
20	1%
30	0.1%
40	0.01%
50	0.001%

QVs are displayed as bars above each base in the sample electropherogram. You will see QVs on your electropherogram printouts only. EditView, Chromas and Sequencher will not show QVs. DNA Sequencing Analysis Software® version 5.0, Sequence Scanner® v1.0 and SeqScape® Software will show QVs.

The colour of a QV bar indicates its value.

QV Bar	Default Colour and Range		The range identifies data that is:
Low	Red	0 to 14	Not acceptable
Medium	Yellow	15 to 19	Needs manual review
High	Blue	20 to 50	Acceptable

Mixed Bases

The Sequencing Analysis Software uses an algorithm called “KB basecaller” for calling bases. Mixed bases are one-base positions that contain two bases. The KB basecaller assigns A, C, G, or T to pure bases and an IUB code to mixed bases. The IUB codes are in the table below.

IUB codes – International Universal Base coding

Code	Translation
A	Adenosine
C	Cytidine
G	Guanine
T	Thymidine
B	C, G, or T
D	A, G, or T
H	A, C, or T
R	A or G (puRine)
Y	C or T (pYrimidine)
K	G or T (Keto)
M	A or C (amino)
S	G or C (Strong -3H bonds)
W	A or T (Weak -2H bonds)
N	ANy base
V	A, C, or G

Information included in the Analysis Report

You will be sent an analysis report of the sequencing data, if you tick this on your online submission. The report will show the success and/or failure of your sequencing data. The report displays a summary of QVs and length of reads (LORs), as well as individual sample information and errors. The report is used to help troubleshoot and provide easy assessment of data quality.

The report contains four tables of information:

- **Summary:**
  - Displays the number of samples in the report
  - Displays the number of samples that contain QVs (sequences anlysed with the KB basecaller.
  - Displays the definition of the QV ranges.
- **Length of Read (LOR):**
  - Displays the definition of the LOR ranges and colours, and the number of samples in each range.
- **Sample Details:**
  - This part of the report contains a list of each sample and its associated analysis information. The following information is given:
    - Well - The well position in the plate that the sample was in.
    - Cap# - The capillary number that the sample was run on.
    - Peak1- The scan number that represents the first data point in the file that is from the sample, not including primer peaks.
    - Base Spacing -The values represent the calculated base spacing for the sample. Base spacing is defined by the number of scan points from the crest of one peak to the crest of the next peak.
    - Bases with Low, Medium, or High QVs - The number of bases where the QVs are in the low, medium, or high range.
    - Sample Score - The average quality value of the bases in the clear range sequence for that sample.
    - LOR - The usable range of high-quality or high-accuracy bases, as determined by quality values.
    - A, G, C, or T S/N - The value represents the average signal/average noise of a bases in a sample.
    - Avg S/N -The value represents the average signal/noise value of all the bases in a sample.
- **Errors Table:**
  - This part of the report displays the errors that occurred during analysis and post processing.
  - This part of the report gives the name of the sample that the error occurred in, where in Basecalling or post processing the error occurred, and a description of the error.

Primer Design

The following recommendations are provided to help optimize primer selection:

- Primers should be at least 18 bases long to ensure good hybridization.
- Avoid runs of an identical nucleotide, especially runs of four or more Gs.
- Keep the G-C content in the range 30-80%, preferably 50-55%.
- For cycle sequencing, primers with T_M>45°C produce better results than primers with lower T_M using Applied Biosystems Inc. thermal cycling parameters.
- For primers with G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_M>45°C.
- Use of primers longer than 18 bases also minimizes the chances of having a secondary hybridization site on the target DNA.
- Avoid primers that hybridize to form dimers.
- Avoid palindromes because they can form secondary structures.
- The primer should be as pure as possible, preferably purified by HPLC.

Sequencing Reaction Recommendations

The following is useful information if you are using the **Capillary Separation Service, Capillary Separation Service with reaction clean-up, Plate Service,** or **Plate service with reaction clean-up**, and are doing your own sequencing reactions using BigDye™ Terminator chemistries.

The Massey Genome Service recommends doing a 20µl total volume sequencing reaction.

If you are diluting the sequencing reaction mix and are using the 5x sequencing reaction buffer supplied with the BigDye™ Terminator chemistries, please follow the dilution guidelines below for doing the dilutions:

Reaction	1X	½ X	¼ X	1/8 X
BDT	8	4	2	1
5X Seq. Buffer	0	2	3	3.5
Primer	3.2 pmol	3.2 pmol	3.2 pmol	3.2 pmol
Template	-	-	-	-
Water	To 20µl	To 20µl	To 20µl	To 20µl

For cleanup of your florescent labelled sequencing products, and remove the unincorporated fluorescent ddNTPs, the Massey Genome Service recommends using the following commercial kits:

- X-Terminator™: Supplied by Applied Biosystems Inc.
- SupreDye™ XT Purification Kit
- Agencourt Cleanseq™: Supplied by Beckman Coulter

With capillary sequencing where the ions in the sample are electrokenetically injected into the capillaries, it is critical to remove all unincorporated labelled ddNTPs when purifying your extension products, because they can be preferentially injected.

You may also choose to use ethanol precipitation for cleaning up your sequencing reactions. This method is much less costly than using a commercial kit but is less effective in the removal of the unincorporated fluorescent ddNTPs from sequencing reactions.

Ethanol/EDTA/Sodium Acetate Precipitation method

NOTE: This method is for 20µl total volume sequencing reactions.

- If you did the sequencing reaction in 0.2ml strip tubes or 0.2ml individual PCR tubes, pipette the full contents of the sequencing reaction into a 0.5ml or 1.5ml tube. If you did your sequencing reactions in a 96 well plate, carry out the ethanol precipitation in this plate.
- Make up a 1:1 premix solution of 3M sodium acetate: 125mM EDTA.
- Add 4µl of the premix solution to each of the sequencing reactions.
- Add 50µl of 100% ethanol to each sequencing reaction. Vortex and spin down briefly so that the contents is at the bottom of the tube.
- Incubate at room temperature for 15 minutes.
- Spin at 13000rpm for 15 minutes at room temperature.
- Aspirate off the supernatant using a pipette. Make sure that when aspiring that all of the liquid is removed, before proceeding to the next step. If there is any supernatant left after aspiration, then give the tube a quick spin and pipette the rest of the supernatant off.
- Add 70µl of 70% ethanol to each tube. Vortex and spin at 13000rpm for 10 minutes. Aspirate off the supernatant using a pipette. Make sure that when aspiring that all of the liquid is removed, before proceeding to the next step.
- Perform a second wash with 70µl of 70% ethanol and spin for another 10 minutes at 13000rpm.
- Aspirate off the supernatant using a pipette. Make sure that when aspiring that all of the liquid is removed, before proceeding to the next step.
- Make sure the tubes are completely dry before submitting to the Massey Genome Service for capillary separation.

NOTE: When using ethanol precipitation make sure that all liquid is removed after each spin, before proceeding to the next step. If precipitating in tubes, aspirate off the supernatant with a pipette. If there is any supernatant left after aspiration, then give the tube a quick spin and pipette the rest of the supernatant off. If precipitating in a plate, tip the plate upside down and remove the supernatant onto a lint-free tissue. Then spin the plate upside down at 185 X g for 1 minute, to remove the rest of the supernatant.

Make sure you make up regular stocks of 3M sodium acetate pH5.2, 125mM EDTA, and 70% ethanol, for the ethanol/EDTA precipitation and the ethanol/EDTA/sodium acetate precipitation methods.

To send samples in PCR tubes or plates

The Massey Genome Service requires customers who are using the Full Sequencing Service to send the template and primers premixed in 0.2ml individual PCR tubes or 0.2ml strip tubes.

For the Sequencing Capillary Separation Service and Sequencing Capillary Separation Service with reaction cleanup please send the sequencing reactions in 0.2ml individual PCR tubes or 0.2ml strip tubes and follow the guideline in section "**Sequencing Reaction Recommendations**" for setting up your sequencing reactions.

For the Sequencing Plate Service and Sequencing Plate Service with reaction cleanup, please send the sequencing reactions in a 96 well plate, sealed with strip tubes, plastic seal or foil seal. The strip tubes provide the best result.

Template and primer concentration requirements

Template type	Template total quantity (in final volume)	Primer total quantity* (in final volume)	Final volume required #
PCR product: 100-200bp 200-500bp 500-1000bp 1000-2000bp >2000bp Rule: For PCR products use **4ng** of template for every 100bp.	Quantity: **4-8ng** **8-20ng** **20-40ng** **40-80ng** **80-200ng**	4pmol	20ul if using your own primer, 19ul if using MGS primer
Single-stranded plasmid	**100-200ng**	4pmol	20ul if using your own primer, 19ul if using MGS primer
Double-stranded plasmid	**400-1000ng**	4pmol	20ul if using your own primer, 19ul if using MGS primer
Cosmid, BAC DNA, Lambda DNA	**1-2µg**	6.4pmol	20ul if using your own primer, 18ul if using MGS primer
Bacterial genomic DNA	**4-6µg**	6.4pmol	20ul if using your own primer, 18ul if using MGS primer

* Make sure only one primer is added to the template/primer premix

Make the template/primer premix up to the final volume with filtered molecular grade water.

Example of requirements

You have a 500bp PCR product at a concentration of 7ng/µL and your primer is at a concentration of 2pmol/µL.

For PCR products use **4ng** of template for every 100bp. So, for a 500bp product you will need to add 20ng to the template/primer premix. At a concentration of 7ng/µL you will need to add 3µL template to the premix to get 21ng for your PCR template.

For the primer you need 4pmol total amount in the template/primer premix. So, at a concentration of 2 pmol/µL you will need to add 2 µL primer to the premix. You then need to make the premix up to a final volume of 20 µL by adding 15 µL of filtered molecular grade water to the premix to get the final volume of 20 µL.

Using spectrophotometer or fluorometer for quantification

Relying solely on spectrophotometer readings (e.g. NanoDrop) for quantification can be misleading if contaminants are present. Either too little or too much DNA template can cause sequencing failure. I recommend quantifying the sample using a Qubit fluorometer to get more accurate concentration readings if you have access to one. Also check the OD 260/280 and 260/230 ratios on the NanoDrop. For high-quality DNA, a Nanodrop 260/280 ratio should be between 1.8 and 2.0 to indicate low protein

contamination, while the 260/230 ratio should be in the 1.8–2.2 range to show low contamination from other organic compounds like phenol. A 260/280 ratio lower than 1.8 suggests protein or phenol contamination, and a 260/230 ratio below 1.8 indicates the presence of other contaminants that could interfere with downstream applications.

MGS recommend using a fluorometer (e.g. Qubit) if you have access to one. Qubit will generate a more accurate concentration reading.