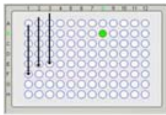
Sequencing on the ABI 3100xl

Instructions for sample submission

The ABI Prism 3100xl genetic analyzer is a 16-capillary instrument using fluorescence based electrophoresis technology for DNA sequencing and fragment analysis. For sequencing we routinely use the 3'-BigDye dideoxynucleotide triphosphates labeling chemistry.

- Download the "***Sequencing Submission Form***" (Excel format), fill it in completely, and e-mail it as an attachment to: mcic_seq@osu.edu.
- Limit sample name to 5 characters and do not use spaces, comas, period or other punctuation in the sample name, or do not start the name with the letter "M", because the sequencing software does not accept them.
- If you have 8 or more reactions, submit samples in microtiter plates or tube strips.
- Bring your samples to the facility, Selby Hall room #009 and place the samples in the refrigerator with the sign 'Sequencing samples'.



For samples submitted in a microtiter plate list samples following the columns in the submission form

Bring the following quantities:

1. ***Submission of templates to be sequenced with custom primers***:
   6ul of DNA (see concentrations in the table below)
   3ul custom primer (2 pmoles/ul, which is app. 5ng/ul)
2. ***Submission of templates to be sequenced with primers available at the center***:
   6 ul of template DNA in H2O or 10mM TrisCl pH8.0 buffer (no EDTA!) at the concentrations indicated in the table below.
3.

Concentrations/amounts of DNA needed

Type of template	Concentration of 6 ul	Amount in 6 ul
PCR products	app. 0.6 ng/ul per 100 bp*	3.6 ng per 100 bp
Plasmid	30 ng/ul **	180 ng
Phage, cosmid, BAC DNA	200 ng/ul	1.2 ug
Bacterial genomics DNA	400 ng/ul	2.4 ug

*the amount of DNA submitted needs to be proportional to the PCR fragment size. For example for a 300 bp fragment submit 1.8 ng/ul, and for a 700 bp fragment submit 4.2 ng/ul.
** this amount is for an approximately 6 Kb dsDNA plasmid. For larger fragments/plasmids you need to provide proportionally higher concentrations.

Primers available at the center:

M13F	5'-CGC-CAG-GGT-TTT-CCC-AGT-CAC-GAC-3'
M13R	5'-AGC-GGA-TAA-CAA-TTT-CAC-ACA-GGA-3'
T3	5'-ATT-AAC-CCT-CAC-TAA-AGG-G-3'
T7	5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-3'
SP6	5'-ATT-TAG-GTG-ACA-CTA-TAG-3'

Tips for sequencing sample preparation

General recommendation for primer design

Your decisions concerning primer sequence, method of primer synthesis, and approach to primer purification can have a significant effect on the quality of the sequencing data. The purpose here is to provide you with some guidelines to follow in making those decisions.

- Primers should be at least 18-20 nucleotides in length to minimize secondary hybridizations.
- Primers with long runs of a single base should be generally avoided; it is especially important to avoid 3 or more G or C runs.
- Primers with melting temperature above 50 degree C generally produce better results than primers with lower melting temperatures.
- Primers should have G/C content between 50 and 60%.
- For primers with a G/C content of less than 50%, it may be necessary to extend the length beyond 18 bases.
- Primers should be “stickier” on their 5’ end than on their 3’ end. A “sticky” 3’ end, as indicated by a high G/C content, could potentially anneal and prime the polymerization reaction at multiple sites.
- A G or a C is desirable at the 3’ end of the primer.
- Primers should not contain complementary sequences (palindromes).
- Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or to other primer molecules.
- Run a computer search against the vector and insert DNA sequences to verify that the primer and especially the 8-10 bases of its 3’ end are unique.
- Do not design degenerate primers, and do not request inosine for sequencing primers.

Template preparation

The quality of template DNA has a major impact on the quality of the sequence data. Inadequate template preparation is the most common cause of sequencing problems.

Methods for plasmid template purification:

QIAGEN DNA isolation (QIAGEN Inc., Chatsworth, CA; http://www1.qiagen.com/Products/DnaCleanup/PcrCleanup.aspx) and alkaline lysis with PEG precipitation (Sambrook, Fritsch and Maniatis, Molecular Cloning, A Laboratory Manual, p. 1.38 - 1.41 and p. 4.29 - 4.32) have proven to be the most reliable DNA preparation techniques for sequencing. These two methods generally provide good yields and clean DNA. Alkaline lysis followed by PEG precipitation is also inexpensive, the ingredients are readily available and the yields are generally higher that using other methods. Qiagen also offers single reaction kits or higher throughtput/96 well formats. There are many other methods and kits available on the market (5’ 3’ Perfect Prep; Promega Wizard Prep; Qiagen QiaWell and Tips; Nucleobond AX; .....). With these methods better results can be obtained by re-precipitating the final solution, rinsing the DNA pellet with 70% EtOH and resuspending the DNA in deionized water. The final DNA concentration and purity has to be accurately measured.

Host Strain variability in template preparation:

The host strain used for plasmid preparation also impacts template quality. The following information may help you in choosing a host strain: HB101 and DH5a host strains consistently produce good results; XL Blue, MV1190 and JM109 host strains show some variability in result quality; JM101 generally does not work well.

Cleaning a failed template preparation: It is sometimes possible to clean up a template which failed to give good sequencing data. Here are three commonly used methods:

- Extract DNA 2x with 1 volume of chloroform:isoamyl alcohol (24:1, vol:vol ratio). Precipitate the aqueous layer with EtOH and wash the DNA pellet with 70% EtOH.
- Precipitate with PEG. Add 0.16 volumes of 5M NaCl and 1 volume of 13% PEG 8000. Incubate 20 minutes on ice, centrifuge at 4oC for 20 minutes. Wash with 70% EtOH.

- Purify the DNA by ultrafiltration (for example, with Centricon-100, Micro-Concentrator columns, Perkin-Elmer Biosystems, Foster City, CA).

Sequencing PCR products

PCR products can be sequenced without cloning. After PCR amplification, the resulting PCR product exists in the presence of PCR primers, dNTPs, enzyme and buffer components. Dye terminator cycle sequencing requires rigorous PCR product purification, because the presence of residual primer or RNA will result in high background due to unspecific elongation products. Contamination with enzymes (nucleases, polymerases), salts, nucleotides, and ethanol decreases read length and signal intensity. PCR products can be purified directly from the PCR reaction using one of the following techniques, as long as only one product is present:

- Column purification, using commercially available kits, such as the QIAquick PCR purification columns, available for single reaction or 96-well format (QIAGEN Inc., Chatsworth, CA; http://www1.qiagen.com/Products/DnaCleanup/PcrCleanup.aspx), and Centricon-100 Micro-Concentrator columns (P/N N930-2119, Perkin-Elmer Biosystems, Foster City, CA).
- PCR products can be also purified by eluting them from the gel using electroelution, glass resin or other standard methods (QIAGEN or Promega kits are available).
- PEG precipitation is a very cost effective way for PCR fragment purification ("Fractionation of DNA Fragmebnts by Polyethylene Glycol Induced Precipitation" by John T. Lis, Methods in Ezymology, Vol.65, 1980. pp. 347 - 353). *Get protocol.*
- ExoSap (USB, cat.# 78200; http://www.usbweb.com/category.asp?cat=144&id=78200) is a mixture of Exonuclease I and Shrimp Alkaline Phosphatase, which respectively degrades the primers and hydrolysis the nucleotides used for the PCR reaction, and is added directly to the PCR reaction. After the digestion and inactivation of the enzymes, the product is ready to sequence. This method is slightly more costly, but it requires very little manipulation and it produces excellent results.

If more than one PCR product is present, the desired product needs to be eluted from the gel, or the PCR reaction needs to be optimized to obtain a single product. PEG precipitation might work, as it is selectively precipitates DNA fragment of a certain sizes.

Chromatogram viewing software

The chromatograms from the ABI 3100xl are saved as .ab1 or .scr files, and can be viewed with one of following software (click on the name to download the software):

- TraceViewer for Windows or Mac
- Xplorer for Windows or Mac
- Finch for Windows or Mac
- Chromas145 for Window/Window NT
- 4Peaks for Macintosh OsX and higher
- Sequence Scanner

How to retrieve your sequence data

Once the sequencing run is completed you will be alerted via e-mail and receive instructions of how to access your data.