

Procedures for DNA Sequencing at the **Plant-Microbe Genomics Facility** **Ohio State University**

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Hours: 9:00 am to 5:00 pm Monday through Friday except for University holidays

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Introduction

For DNA sequencing, PMGF uses an Applied Biosystems 3730 DNA Analyzer and BigDye™ cycle sequencing terminator chemistry. The sequencing reaction utilizes dye labeled-dideoxynucleotides, A heat-stable DNA polymerase and a thermal cycler to generate the extension products that are separated by capillary electrophoresis on the Analyzer. The extension products are detected by exciting the unique dyes attached to each dideoxynucleotide with a laser, followed by a measurement of the fluorescent emission with a CCD (charge-coupled device) camera. Subsequently, the signal is interpreted by the Applied Biosystems Sequencing Analysis program in order to determine the sequence of the nucleotides in the DNA template.

Preparation of Template DNA

The quality of the sequencing results is directly proportional to the quality of the template, *i. e.* clean DNA is absolutely critical. The 3730 DNA Analyzer offers an extremely rapid and efficient means to automate DNA sequencing, however DNA of the highest quality is required because of the potential problems associated with capillary electrophoresis (which is used to separate extension products by the 3730 DNA Analyzer). The capillary tubes are easily fouled or even blocked by contaminating organic solvents, proteins, carbohydrates, and detergents. The samples are injected by electrokinesis so any residual contaminating ions such as RNA, SDS, or salt will decrease the amount of extension products entering the capillary and therefore decrease the signal. The template concentration is also critical so the concentration should be measured by a quantitative method such as (1) absorbance at 260 nm, (2) fluorescence in a fluorometer, or (3) fluorescence in an agarose gel with standards.

Double-stranded DNA

For plasmids, cosmids, and BACs the strain of *E. coli* used as the host can have a significant impact on the quality of the template DNA. Strains HB101 and DH5alpha are the best, while XL-1 and MV1190 are okay. The JM101 series of strains are inconsistent at best, and strains optimized for protein expression should be avoided entirely. Template DNA should be prepared using a solid phase, DNA binding extraction kit from a manufacturer such as Qiagen, Promega, or Applied Biosystems. Traditional chemical methods can work, but are usually very inconsistent.

The template should be provided in water or 10mM Tris buffer, **but not TE since EDTA will inhibit the sequencing reaction.** Please send the following amounts at the appropriate concentration for each sequencing reaction. These amounts are sufficient to repeat the reaction if there is a problem with the initial reaction.

<u>Template</u>	<u>Quantity (ng)</u>	<u>Concentration (ng/μl)</u>
Plasmid (<25 kb)	600	50-200
Phage, Cosmid, BAC	2000	100-500
Bacterial genomic	3000	200-1000

Symmetric PCR Product

PCR products must be purified to remove residual primers, nucleotides and salts. The following products are examples of what can be used for PCR purification: (1) Centricon-100 columns (P/N N930-2119), or (2) QIAquick PCR Purification Kit (P/N 28104). Please send the following amounts at the appropriate concentration for each sequencing reaction. These amounts are sufficient to repeat the reaction if there is a problem with the initial reaction.

<u>PCR Product(bp)</u>	<u>Quantity(ng)</u>	<u>Concentration(ng/μl)</u>
100-200	2-4	1-4
200-500	3-10	1-10
500-1000	5-20	2-20
1000-2000	10-40	2-40
>2000	40-100	5-100

Primers

Standard Primers

The Facility will provide the primers listed below at no additional cost. The use of standard primers is recommended, when possible, since custom primers can introduce variability due to (1) the nature of the priming site, and (2) the quality of the primer. When requesting a PMGF primer you *must* use the designated abbreviation found in the drop down menu of the DNA Sequencing Request Form page.

<u>Primer</u>	<u>Abbreviation</u>	<u>Sequence (5' to 3')</u>
M13/pUC forward	F	CGCCAGGGTTTTCCAGTCACGAC
M13/pUC reverse	R	AGCGGATAACAATTCACACAGG
T7 promoter	T7P	TAATACGACTCACTATAGGG
T3 promoter	T3	ATTAACCCTCACTAAAGGGA
SP6 promoter	SP	TATTTAGGTGACACTATAG
T7 terminator	T7T	TGCTAGTTATTGCTCAGCGGTG
polyT mixture	polyTmix	TTTTTTTTTTTTTTTTTTTV*

*V = equal mixture of primers with A, C and G; to be used for samples that have the problem of homoT slippage.

Custom Primers

The recommendations below are provided to help design DNA sequencing primers:

- 1) The primer should be at least 20 bases from the area of interest.
- 2) The primer should be at least 18 bases long and preferably 20 to 25.
- 3) Avoid runs of identical nucleotides and especially 4 or more guanines.
- 4) Keep the G/C content in the range of 30 to 80% and preferably about 50%.
- 5) The T_m should be above 45 C.
- 6) For primers with a G/C content lower than 50%, it may be necessary to extend the primer to keep the T_m greater than 45 C.

- 7) The use of primers longer than 18 bases minimizes the chance of secondary hybridization to the template DNA.
- 8) The terminal base at the three prime end should be a G or C.
- 9) Several computer programs for primer selection are available free on the internet and can be useful in identifying potential secondary structure problems, possible primer dimer formation, and if a secondary hybridization site exists on the target DNA.

Primer Concentration and Volume

Custom primers must be provided by the customer. **Ten pmoles of primer in water or 10mM Tris buffer pH 8,0 are required for each reaction, and it must be at a concentration of 2pmol/μl (or 2μM).** If the template is a BAC or genomic DNA, then provide 50 pmoles at a concentration of 10pmol/μl. For 48 and 96-well plates please provide 10μl of primer at 100μM, if the Facility is to add the primer to the reactions. Correct primer concentration is important to get optimal read lengths.

Preparing and Submitting an Order

The template DNA and primers can be sent to the above address by mail or by special courier, *i. e.* yourself, and placed in the refrigerator marked for DNA Sequencing Samples in room 420 of the Biological Sciences Building from 9:00 am to 5:00 pm Monday through Friday. For remote drop off locations on the OSU main campus see the following webpage: http://www.biosci.ohio-state.edu/~pmgf/services_dna_sequencing_remote-location.html. For instructions on how to send samples from off campus locations please see the following website: http://www.biosci.ohio-state.edu/~pmgf/documents/Shipping_Samples_Recommendation_9-08.pdf.

Individual Reactions

A sequencing reaction is defined as one template and one primer. An online DNA sequencing request must be completed for each order in order to properly process the request and receive the results. Instructions and means to submit a DNA sequencing request can be found at the following internet address: <http://pmgf.biosci.ohio-state.edu/>. **The template and primer names on the form must be identical to the names on the tubes.** We will not assume which template and primer are to be used together. The samples must be submitted in a 1.5ml or 0.6ml microcentrifuge tube, *i.e.* do not use 0.2ml tubes. The size of the template refers to the total size of the molecule and not the insert size, because it is important to know the approximate molar concentration of the priming site in order to do the sequencing reaction properly.

There are two options for special requests: dGTP_kit and Templphi. The dGTP kit utilizes an alternative sequencing chemistry to help get through difficult regions, *i.e.* secondary structure or high G/C content. This option should be selected when you know it will help or a staff member recommends its use. The Templphi option utilizes a kit that will amplify the template prior to sequencing with alternative nucleotides to aid extremely low concentration or extremely difficult regions that do not work with dGTP. This option requires an additional day and additional costs.

96-well and 48-well Format

An on line DNA sequencing request must be completed for each order in order to properly process the request and receive the results. Instructions and means to submit a DNA sequencing request can be found at the following internet address: <http://pmgf.biosci.ohio-state.edu/>. For this service please place the order through the "Upload and Import File" link on the dnaLIMS User Tools page. For each

plate be sure the **Customer order number** and **customer name** are written on the side of each plate. The Facility will provide our preferred plates and films at no additional cost.

96-well and 48-well Format Sequencing

For high through put needs, samples may be submitted in a 96-well V-bottom plate that is covered with an Aluminum PCR foil seal and labeled. We encourage you to obtain the plates and seals from the Facility at no additional cost to you. For 96-wells, the plate positions must be filled by columns, *e.g.* fill column one positions A through H, then column 2 positions A through H, etc. Position H12 must be empty to accommodate the Facility's positive control reaction; therefore 1 to 95 reactions may be done per plate. For 48-wells, the odd columns should have sample, *i.e.* 1, 3, 5, 7 etc. Leave well H11 empty for the positive control reaction. **Each well should have a total volume of 10 μ l.**

Template

Each well should have 200 - 300ng plasmid or the appropriate amount of PCR product that is listed above.

Primer

Each well should have 5pmoles of primer. If there is more than 1 primer for the plate, then the customer must add the primer(s) to each well. If only one custom primer is to be used for all of the reactions in a plate, then the primer can be added by the Facility. In order for the Facility to do this provide 10 μ l of primer at 100 μ M. If the primer is to be added at PMGF, then clearly request this on the order form. The total volume should still be 10 μ l per well.

384-well Format Sequencing

Please inquire for details.

Receiving Results

You will be notified by email when your sequences are finished. The files can be copied from the dnaLIMS server (<http://pmgf.biosci.ohio-state.edu>) by following the instructions on the web page. For single reactions a color print out of the electropherogram can be sent to you by mail when requested on the order form. You should receive the results in 2 to 3 days unless there are unforeseen delays. In that event, you will be notified as soon as possible after the samples arrive at the facility.

If the above instructions are not followed there will be delays in receiving your results. The most common problems that cause delays include the tubes (1) having an insufficient amount or concentration of template and/or primer, (2) being improperly labeled, or (3) being the incorrect tube size. Also, a lack of a printout of the online order form and/or inaccurate billing information on the order form is common causes of delays.

Sequencing Data

For a DNA sequencing reaction of good quality the Analyzer will read 800 to 1000 nucleotides accurately with the first 30 bases having very low quality. Individual sequencing reaction files will be named with the following format: name of template, underscore, name of primer, for example plasmid1_T7P.ab1. For 48-well and 96-well plates the file names will have the following format: well position, hyphen, template name, underscore, primer name for example E6-plasmid1_T7P.ab1.

The results for each sequence will be provided in two different files: (1) a generic text file (.seq) with the base sequence, and (2) an ABI Sample file (.ab1) that contains the nucleotide sequence, electropherogram, as well as other information about the run conditions. The generic text file can be opened by any word processing programs whereas the ABI sample files require specific software to view, print and edit the sequence. Please see the following website for the latest list of free programs for .ab1 file: http://www.biosci.ohio-state.edu/~pmgf/services_dna_sequencing.html.

Problems with Results

If you have any questions or concerns, then please do not hesitate to contact the staff at the Facility. In addition there is a Guide to Interpreting the Electropherogram at the following website: http://www.biosci.ohio-state.edu/~pmgf/guide_DNA_electropherogram.htm. The Facility will repeat a reaction **once (with the following exception)** at no additional cost after consulting with the Facility staff in order to determine the cause or nature of the problem and the best scenario to solve the problem. If the Q20 values of the results exceed 800 bases or go to the end of the PCR product, then the criteria for a good result has been met. Therefore it is at the Facility's discretion as to whether a free repeat reaction will be done. To facilitate doing repeat reactions all remaining sample is stored at -20C for 4 to 8 weeks. To help with the occasional but inevitable problem of lost or destroyed data the Facility keeps all results on a removable storage media.