

Procedures and Recommendations for MSACE: mRNA Start Site Analysis by Automated Capillary Electrophoresis

Plant-Microbe Genomics Facility Ohio State University

420 Biological Sciences Building,
484 W. 12th Ave., Columbus OH 43210
Telephone: 614/247-6204
FAX: 614/247-8696
E-mail: pmgf@osu.edu
Internet: www.pmgf.osu.edu

Version 2.1

July, 2009

1. Introduction

1.1. *Overview:* The first base of a mRNA molecule, *i.e.* transcription start site, is commonly determined by a primer extension assay which employs an oligonucleotide (complementary to the coding region) which is extended to the end of the mRNA molecule by Reverse Transcriptase. Traditionally, the extension product or cDNA is labeled with a radioactive isotope, and the size is determined by gel electrophoresis and autoradiography. In addition a DNA sequencing reaction is performed and analyzed in the same gel to determine the terminal 5' base of the mRNA. The Facility can do the same experiment by replacing the radioactive isotope and gel electrophoresis/autoradiography with a fluorescent dye and automated capillary electrophoresis respectively. In summary the client provides the dye-labeled cDNA product along with template and primer for the DNA sequencing reaction, and the Facility will then analyze the extension products on a 3730 DNA Analyzer. Following is the reference for an example of this type of analysis performed at the Facility: Merighi, et al (2006) Journal of Bacteriology. 188(14): 5089-5100.

1.2. *Pre-experimental Considerations:* Prior to the Facility starting any work, we request two things: (1) a free face-to-face consultation or, at the minimum, a direct phone consultation and (2) a completed Promoter Characterization order form) for each request of services. This initial communication is necessary to clarify the customer's goals. In order for the Facility to best serve the customer, it is essential that the customer have a clear understanding of the service's capabilities and the input required. A critical point that must be made clear is that **optimization is almost inevitable** due to the specific characteristics of diverse samples and the requirements of individual customers. There are, however, steps that can be taken during sample preparation to minimize the need for lengthy optimization. The following are guidelines and recommendations, as well as specific information, provided to maximize the value of the Facility's services. The guidelines have been provided for the benefit of the customer; they are, of course, not strict rules.

2. Reverse Transcription and DNA Sequencing

- 2.1. *Primer Design:* A dye labeled primer is required for the reverse transcription reaction and the DNA sequencing reaction. Design the primer such that the 3' terminal base is 50 to 80 bases 3' of the translation start site, *i.e.* complementary to the coding sequence. Design the primer to have: (1) 20 – 27 bases for robust annealing and (2) minimal self-hybridization. The dye should be labeled with either FAM or VIC on the 5' end of the oligonucleotide. A high quality oligonucleotide is important so we recommend ordering the primer from Applied Biosystems Inc.
- 2.2. *Template Design:* A template is necessary for the DNA sequencing reaction and includes the reverse transcribed sequence from above as well as the entire promoter region, *i.e.* potential transcription start site(s). The template can either be cloned DNA in a plasmid or a purified PCR product, and should include 400 to 500 bases upstream (5') of the translation start codon. At best this type of DNA sequencing reaction can read about 500 bases, so this size maximizes the analysis capabilities of the kits and instruments. If you decide to use a PCR product as the template, then the same primer from above can be used to generate the PCR product although the primers should not be labeled with a dye.
- 2.3. *DNA Sequencing Reaction:* The DNA sequencing reaction will be performed by the Facility with the Thermo Sequenase Dye Prime Manual Cycle Sequencing Kit (USB Corporation; P/N 79620) according to the manufacturer's protocol.
- 2.4. *Reverse Transcription Reaction:* Below is a protocol that has been used successfully by other previous clients (M. Merighi and A. Septer). Other protocols may work equally as well and in those cases follow the manufacturer's protocol with the exception of adding the maximal or even more RNA than recommended since signal intensity can be an issue.

Primer extension:

- 2.4.1. RNA prepared from Ethanol/phenol stopped cultures using Promega Total RNA kit
- 2.4.2. use 5-50 ug Total bacterial RNA per reaction
- 2.4.3. Mix 50 (or 5) ug RNA with 100 pmol 6FAM primer in 30ul total
- 2.4.4. heat at 90C for 3 min, then slow cool to 30C in PCR machine
- 2.4.5. Synthesize cDNA as follows:
- 2.4.5.1. Mix 6ul 0.1M DTT, 12ul 1st Strand buffer, 1.5ul 25mM dNTP, 4ul Superscript II, 2ul RNasin, 0.5ul water
- 2.4.6. Incubate 42C for 2 hours
- 2.4.7. Degrade RNA adding 10ul 1M NaOH and by heating 70C for ten min.
- 2.4.8. Neutralize with 10ul 1M HCl

2.4.9. Purify with Qiagen PCR prep kit (Minelute version) in 15ul water.

2.4.9.1. Note: Make certain the cDNA product is of appropriate size to be properly bound and eluted from the solid phase matrix.

Because many mRNA molecules have strong secondary structures a high temperature reverse transcriptase may be useful, e.g. Thermoscript (Invitrogen, Inc.), which can transcribe at 50C to 65C. Purification of the final cDNA product is critical as the salts from the primer extension reaction will interfere with electrophoresis. Most solid phase PCR purification kits will work, but the Qiagen products are known to be effective. The PCR product should be eluted in **water** in a minimal volume in order to maximize cDNA concentration. Water is important since any buffer or salt can interfere with electrophoresis.

3. Submission of Samples

3.1. *Samples*: Send the dye-labeled primer(s) at a concentration of 2 μ M (2pmoles/ul) in a total volume of 10 μ l. If providing a plasmid for sequencing, then send 1 μ g at a concentration of 50 to 200ng/ μ l. If providing a PCR product for sequencing, then send 20ng at a concentration of 2 to 5ng/ μ l. The primer, plasmid and PCR product can be shipped in water or 10mM Tris, pH 8.0. Please provide 10 to 20ul of each cDNA sample in water. Send all samples in 0.6 or 1.5ml microcentrifuge tubes that are clearly labeled with the name, concentration, date and your initials. In addition we require the information described below in order to properly analyze the samples. Please send printed versions along with the samples and electronic versions to the following email address: pmgf@osu.edu.

3.1.1. Primer: sequence, T_m, dye, concentration, volume

3.1.2. Template: plasmid or PCR product, concentration, volume, size (bp), sequence with map (features such as promoter region, translation start codon, etc.)

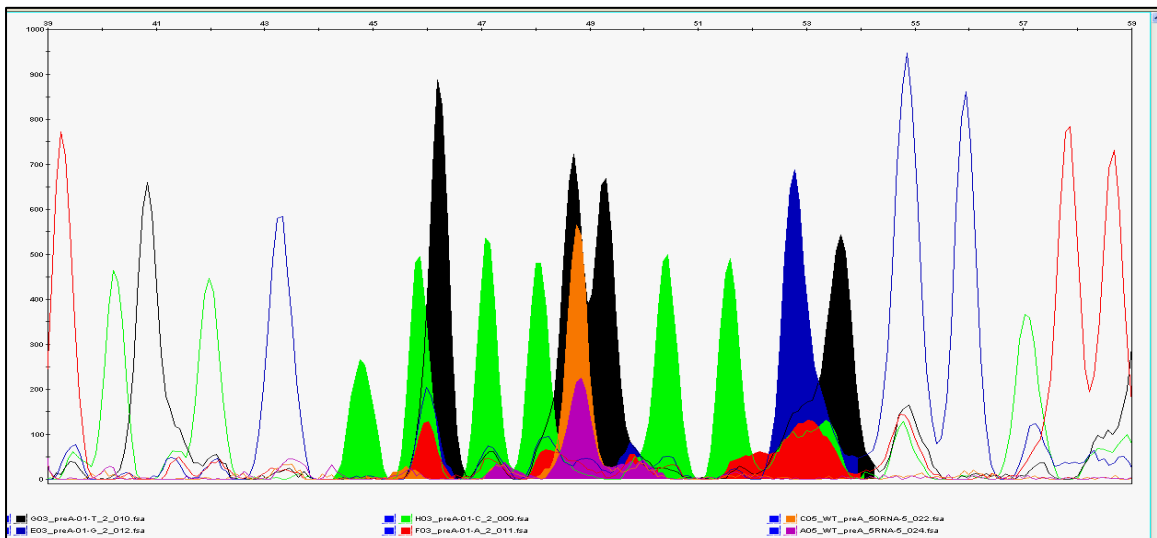
3.2. *Shipment*: Tubes should be wrapped in parafilm to prevent the caps from opening during transit. Samples have been sent successfully by overnight courier at ambient temperature, but we recommend shipping samples on wet or dry ice. For all shipment methods the tubes should be in a protective layer, e.g. bubble wrap, to prevent physical damage to the tubes from handlers, ice blocks, etc. A completed order form (http://www.biosci.ohio-state.edu/~pmgf/documents/orderform_PromoterCharacterization.pdf) must be included with each order. All tubes and their samples names should be listed on the form. Also include which primer is to be used with which template for the sequencing reaction and which template will be used for analysis of each cDNA sample. Ship the samples to the address above.

3.3. *Client Account*: For first time clients please create an account in dnaLIMS (<http://pmgf.biosci.ohio-state.edu>). This is required for you to receive the results, and must be done prior to processing the samples at the Facility. Please do not

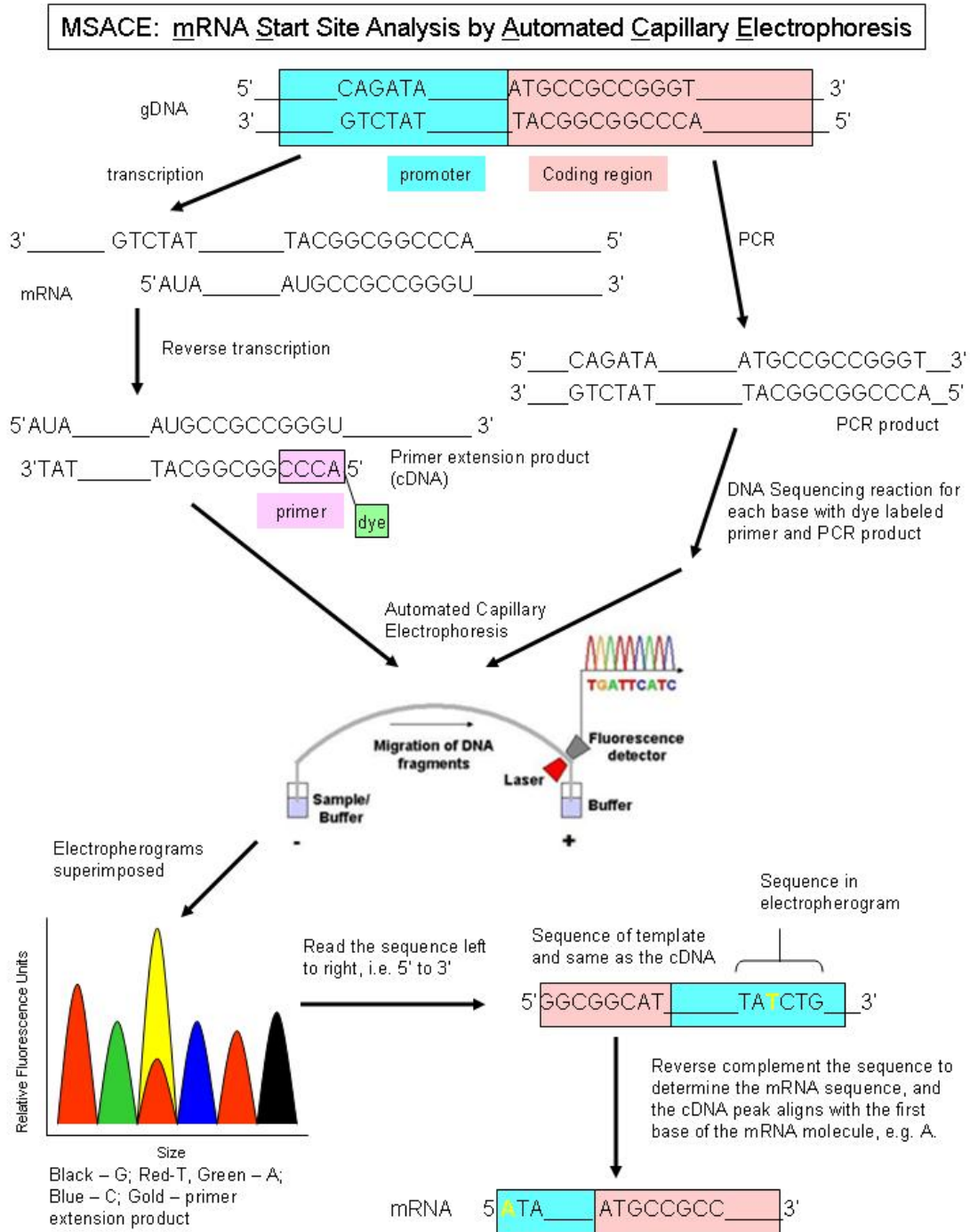
place orders through the online software as the Facility staff does this for proper processing of MSACE samples.

4. Data and Analysis

- 4.1. *Software:* The extension products from the DNA sequencing reaction and Reverse Transcription reaction will be analyzed with the 3730 DNA Analyzer (Applied Biosystems) and the software GeneMapper. By overlaying the appropriate electropherograms with GeneMapper the peaks patterns can be read manually to determine the DNA sequence and the 5' terminal base of the mRNA molecule. Typically the analysis is done with the client present in order to facilitate data interpretation, but this is not necessary.
- 4.2. *Data Format:* The files produced by the 3730 DNA Analyzer (*.fsa) will be available on line through dnaLIMS and have a proprietary format, therefore GeneMapper, PeakScanner or the online dnaLIMS FSA viewer is required to view the files. Unfortunately the latter two programs do not allow electropherograms to be superimposed to aid analysis. The Facility staff will superimpose and analyze the electropherograms in consultation with the client, and the resulting data will be sent to the client as pdf documents by email. In addition, the Facility can generate images of the electropherograms with a JPEG format to aid handling of the data by the client for papers, presentations, etc.
- 4.3. *Example of Results:* Sequence of the mRNA is: 5'CTTCTTGTCTA... as derived from the reverse complement of the sequence below starting with the black/gold/pink peak. G = Black; C = Blue; A = Green; T = Red; Primer extension product: 50ug RNA = Gold, and 5ug RNA = Pink.



4.4. Diagram of MSACE:



Using the electropherogram data (an example is above) determine the sequence of the mRNA molecule by starting at the primer extension peak and then read the sequence from right to left simultaneously complementing each base.