

# Procedures and Recommendations for Transcription Start Site Analysis

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Version 1.3

February, 2008

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## 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 a reference and 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 ([http://www.biosci.ohio-state.edu/~pmgf/documents/orderform\\_PromoterCharacterization.pdf](http://www.biosci.ohio-state.edu/~pmgf/documents/orderform_PromoterCharacterization.pdf)) for each new ordering of services. This initial communication is necessary to clarify the customer's goals. The order can be found on the Facility's website. 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.

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## 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 60 to 100 bases 3' of the translation start site, *i.e.* complementary to the coding sequence. Design the primer to have: (1) 22 – 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. **The dye primer must be provided to the Facility at a concentration of 2 $\mu$ M in a total volume of 20 $\mu$ l water or 10mM Tris, pH 8. Please label the tube(s) with the name and concentration.**

**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. **If providing a plasmid for sequencing, then send 1 $\mu$ g at a concentration of 100 to 200ng/ $\mu$ l. If providing a PCR product for sequencing, then send 20ng at a concentration of 2 to 5ng/ $\mu$ l. Please label the tube(s) with the name and concentration.**

**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 1<sup>st</sup> 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 optimize cDNA concentration, and please provide 10 to 20ul to the Facility for analysis.

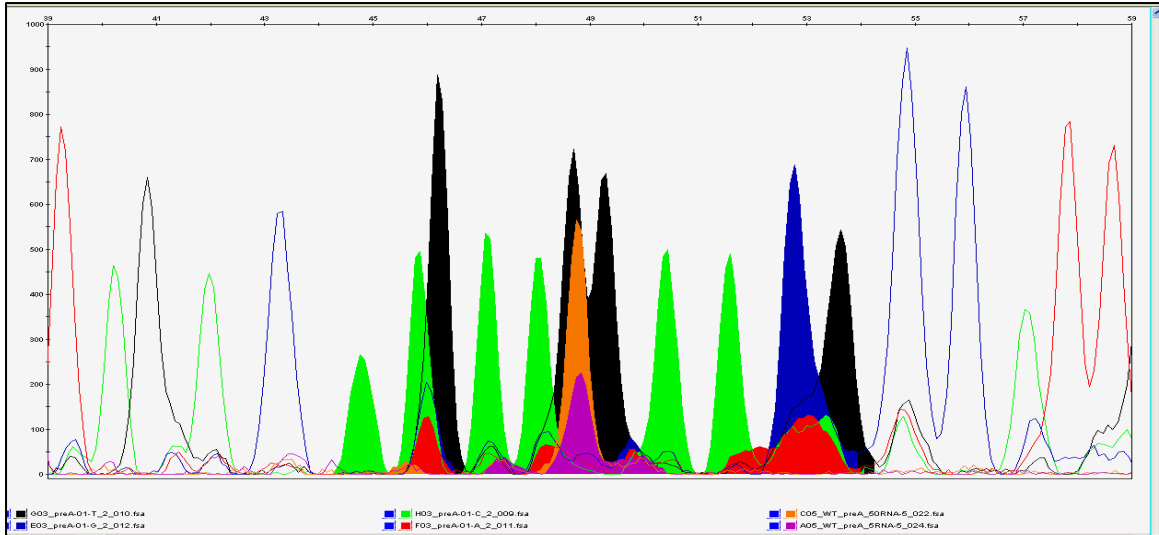
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### 3. Analysis of Data

- 3.1. *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.
- 3.2. *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. In addition, the Facility can generate images of the electropherograms with a JPEG format to aid handling of the data by the Client. Below is an example of data from a jpg file.

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.



(mrz; 11-07)