DNA Footprinting with an automated DNA Analyzer: does the shoe fit?

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DNA Footprinting with an Applied Biosystems 3730 DNA Analyzer: does the shoe fit ...........yes!
Development of DNA Footprint Analysis Techniques and Goals of This Study

- First performed in 1977, and utilized radioactively labeled DNA, slab gels, and autoradiography.
- In 1994, dyes and fluorescent gel imager were used instead of radioisotopes and autoradiography but the slab gel remained.
- In 2000, the slab gel and imager were replaced by an automated capillary electrophoresis instrument: 310 DNA Analyzer.
- In 2004, ..........
  - Demonstrate the feasibility by reproducing a protection assay previously done with autoradiography and a slab gel.
  - Demonstrate that each peak could be accurately identified.
  - Apply this method to a previously uncharacterized protein.
DNA Footprint analysis with protein: Autoradiography vs Electropherogram
DNA Footprint analysis without protein: Electropherogram vs Autoradiography
Fluorescent Primer Design

- For use in: (1) the DNA sequencing reactions which act as a standard/ladder and (2) the PCR reaction to make the DNA probe
- Used routine DNA sequencing guidelines:
  - 18 -25mer.
  - Tm at 55 – 60C.
  - about 50% GC.
- Primer purified by HPLC
- 5’ label with FAM or VIC which worked well; HEX is okay
DNA sequencing reaction and Analysis

- DNA probe made by PCR performed with labeled primers followed by a cleaned up protocol, e.g. Qiagen PCR column

- Used ThermoSequenase Dye Primer DNA Sequencing Kit (USB Corp.) with the manufacturers protocol but at maximal values and amounts.

- For each base/tube of a sequencing reaction used 0.2 to 2ul in 10ul HiDi and 0.1ul LIZ 500 standards (Applied Biosystems)

- Analyze with 3730 DNA Analyzer (Applied Biosystems) using default GeneMapper50-POP7-1 Run module, but in addition increased injection voltage to 3kV and 30sec from 1.5kV and 15sec respectively to increase signals

- All electropherograms were aligned with GeneMapper v3.5 or 3.7
DNA sequencing Reactions
Confirmation of Sequence by BigDye sequencing
Detailed DNA footprint protocol for CbbR

- **CbbR**
  - LysR-type activator with a Helix-turn-helix motif that binds to the *cbbI* operon promoter of the photosynthetic bacterium *Rhodobacter sphaeroides*
  - Necessary for regulating carbon fixation genes in regards to redox state and carbon dioxide concentration

- **DNA fragment for digestion**
  - One primer labeled with 6-FAM and other HEX; 285bp long
  - PCR protocol: 25x of 30sec/95C – 30sec/50C – 60sec/72C
  - PCR products were purified by Qiagen spin column and quantified by UV spectrophotometry or by measuring FAM dye in spectrofluorometer.

- **DNA footprint protocol**
  - Recombinant CbbR was purified by several chromatography columns, and refolding
  - 100 to 500ng of labeled probe was incubated with various amounts of CbbR protein (30mM potassium glutamate; 10mM Tris pH 8.5; 1mM DTT; 30% glycerol; 5mM MgAcetate; 2mM CaCl2; BSA 0.125mg/ml);
    - Not a pure protein preparation so amount is unknown.
  - Nuclease digestion was performed with various Kunitz units of DNase I (Worthington Biochemicals) per 500µl reaction for 5 – 30 min.
  - The reaction was stopped with heat (5min at 95C)
  - DNA fragments were purified in a Qiagen spin column.
  - Footprint required constant optimization of the amount of CbbR, DNasel concentration, and digestion time due to protein instability
  - Dialysis performed to reduce salt inhibition during electrokinetic injection:
    - Millipore membrane (P/N VSWP02500) protocol
  - For each digestion 1 – 5µl (from 50µl elutant) in 10µl HiDi with 0.1µl LIZ-500 standards
DNA Footprint of CbbR and cbbI promoter operon: sense strand

Red – without cbbR; black – with cbbR
FAM labeled
DNA Footprint of CbbR and cbbI promoter operon: antisense strand

Red – without cbbR; black – with cbbR
Labeled with HEX
Detailed DNA footprint protocol for HrpY

**HrpY**
- FixJ-type activator that has a Helix-turn-helix motif that binds to the *hrpS* promoter of the phytopathogen *Pantoea stewartii*.
- Part of a two component system that is necessary for sensing a host, maize, and activating the expression of the *hrp* proteins which are necessary for infection.

**DNA fragment for digestion**
- One primer labeled with 6-FAM; 386bp long
- PCR protocol: 25x of 30sec/95C – 30sec/50C – 60sec/72C
- PCR products were purified from gel and quantified by UV spectrophotometry.

**DNA footprint protocol**
- Recombinant HrpY purified with a His tag column
- 45 ng of labeled probe were incubated with amounts of HrpY protein ranging from 0 to 140 uM in binding buffer (150 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 8% glycerol in 10 mM Tris-HCl pH 8.0).
- Nuclease digestion was performed with 0.0025 Kunitz units of DNase I (Worthington Biochemicals) per 20µl reaction for 5 min.
- The reaction was stopped with 0.25 M EDTA and extracted with phenol-chloroform-isoamyl alcohol (25:24:1).
- DNA fragments were purified in a Qiagen spin column.
- For each digestion 1 – 5µl (from 50µl elutant) in 10µl HiDi with 0.1µl LIZ-500 standards.
Optimization of DNase I digestion conditions for \textit{hrpS} promoter

K units of DNase I

- 0.05 (over digested)
- 0.005 (over digested)
- 0.0005 (good)
- 0.00005 (under digested)
HrpY DNA footprint analysis with varying concentrations of transcription activator HrpY
DNA footprint Analysis: alignment with DNA sequence

- Green box – protected regions
- Red box – hypersensitive regions
- I and II are distinct regions that fit binding motif:
  - 1) T- N<sub>11</sub>-A
  - 2) centered about -70 from transcription start site
Region I overlayed with GeneMapper 3.7.

Upper panel: red – without HrpY; blue - with HrpY
Lower panel: red – t; green –a; blue – c; black - g
Region II overlayed with GeneMapper 3.7.

Upper panel: red – without HrpY; blue - with HrpY
Lower panel: red – t; green –a; blue – c; black - g
DNA primer extension analysis to determine the transcription start site for hrpS gene of *Pantoea stewartii*

Two transcription start sites were identified (purple peaks; capitalized text). Red – t; green – a; black – g; blue – c.
1) RNA prepared from Ethanol/phenol stopped cultures using Promega Total RNA kit
2) Add 30-50 ug Total bacterial RNA per reaction
3) Mix 50 ug RNA with 100 pmol 6FAM primer in 30 ul total
4) Heat at 90C for 3 min, then slow cool to 30C in PCR machine
5) Synthesize cDNA as follows:
   a) mix 6 ul 0.1M DTT, 12 ul 1st strand buffer (GIBCO) 1.5 uL 25 mM dNTP, 4 uL Superscript II, 2 ul RNasin, 0.5 ul water
   b) incubate at 42C for 1 h
   c) add 2 ul superscript II
   d) incubate at 42C for 1 h
6) Degrade RNA adding 10 ul 1 M NaOH and by heating at 70C x10min
7) Neutralize with 10 ul 1 M HCl
8) Purify with Qiagen PCR prep kit (Minelute version) in 15 ul water.
9) Combine 1 to 5ul with 10ul HiDi/0.1ul LIZ 500 standards
10) Analyze with 3730 DNA Analyzer
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