Title: Sanger Sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit			
SOP #: 8.1.1.1.20	Version: 01	Revision Date: 2020-12-25	Effective Date: 2020-12-25
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FILE NAME: 8.1.1.1.20-Sanger Sequencing using BigDye.docx

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of the SOP is to provide a standard laboratory procedure for performing in-lab dye-terminator sequencing. Normally, for small- to medium-sized sequencing orders, we pay the Cornell Biotechnology Resource Center (BRC) to perform these reactions. For large quantities of sequencing, however, it makes economic sense to perform this reaction ourselves.

1.2 Scope

This SOP applies to the Food Safety Laboratory and the Milk Quality Improvement Program Laboratory.

1.3 Definitions

Sanger or Dye Terminator Sequencing: a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years. It was first commercialized by Applied Biosystems in 1986. More recently, higher volume Sanger sequencing has been replaced by "Next-Gen" sequencing methods, especially for large-scale, automated genome analyses. However, the Sanger method remains in wide use, for smaller-scale projects, and for validation of Next-Gen results. It still has the advantage over short-read sequencing technologies (like Illumina) in that it can produce DNA sequence reads of > 500 nucleotides. For a visual demonstration, please watch this video: https://www.youtube.com/watch?v=e2G5zx-OJIw.

dideoxy-NTPs (ddNTPs): chain-elongating inhibitors of DNA polymerase, used in the Sanger method for DNA sequencing. They are also known as 2',3' because both the 2' and 3' positions on the ribose lack hydroxyl groups, and are abbreviated as ddNTPs (ddGTP, ddATP, ddTTP and ddCTP). Dideoxynucleotides are useful in the sequencing of DNA in combination with electrophoresis. A DNA sample that undergoes PCR (polymerase chain reaction) in a mixture containing all four deoxynucleotides and one dideoxynucleotide will produce strands of length equal to the position of each base of the type that complements the type having a dideoxynucleotide present. Therefore, if the sample then undergoes electrophoresis, there will be a band present for each length at which the complement of the dideoxynucleotide is present.

1.4 Safety

No specific safety concerns. Observe all normal laboratory precautions.

SECTION 2 MATERIALS

- 96-well semi-skirted PCR plates (Room 358B)
- Sterile reagent reservoirs (Room 358B)
- Multichannel pipette (Room 358B)
- Adhesive PCR Plate Foils (Room 358B; ThermoFisher Scientific item AB0626)
- Adhesive PCR Plate Seals (plastic; Room 358B; ThermoFisher Scientific item AB0558)
- Eppendorf 5810R centrifuge
- ABI 2720 Thermal Cycler

- Sterile dH_2O (Room 358B and Room 350A)
- Forward and reverse PCR primers for amplified gene, 10 µM concentration
- BigDye™ Terminator v3.1 Cycle Sequencing Kit (Chest freezer in Room 352C; ThermoFisher Scientific item 4337455)
- BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer (Chest freezer in Room 352C; ThermoFisher Scientific item 4336697)
- DMSO (Room 352, “Dumb” freezer)
- Crushed ice

SECTION 3 PROCEDURES

1. Decide whether or not performing your own dye terminator sequencing reactions makes economic sense using the tables and decision tree below:

Sequencing Method Cost Comparison, 2020-12-25

Prices current as of 2020-12-25 from Cornell eShop and Biotechnology Resource Center Price List – See Wiki for downloadable version of spreadsheet if prices need to be updated.

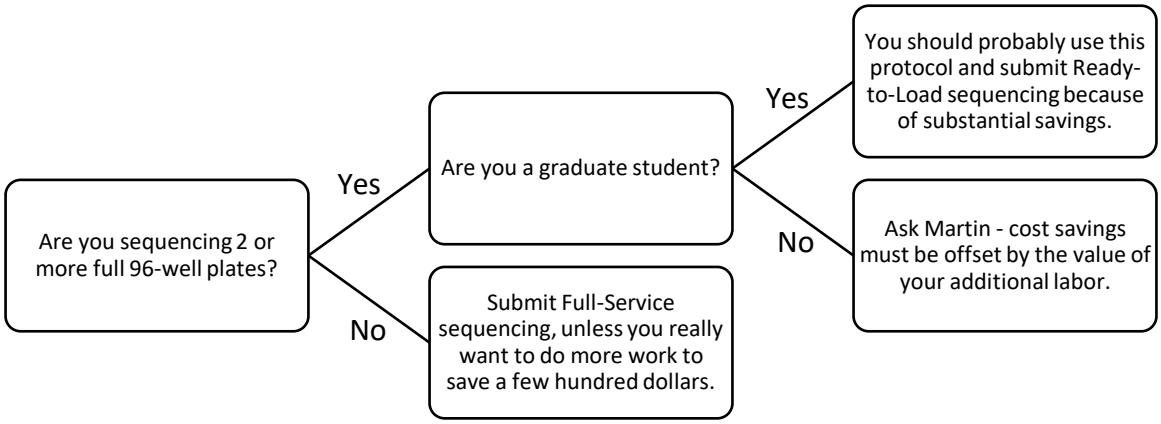
Item	Cost	
Full-Service Sequencing (72-94 Samples; per plate)	$	315.00
Full-Service Sequencing (< 72 samples; per reaction)	$	4.30
Ready-to-Load Sequencing at BRC (per 96-well plate)	$	102.50
Per-plate Charge	$	42.50
Edge Biosystems 96-well dye terminator removal	$	60.00
Ready-to-Load Sequencing Reagents (per 1,600 reactions)	$	1,391.79
BigDye Terminator v3.1 Cycle Sequencing Kit	$	1,245.27
BigDye Terminator v1.1 and v3.1 5X Sequencing Buffer, 2 mL	$	146.52

Number of Reactions	Full-Service Cost	Ready-to-Load Cost
2	$ 8.60	$ 104.24
4	$ 17.20	$ 105.98
6	$ 25.80	$ 107.72
8	$ 34.40	$ 109.46
10	$ 43.00	$ 111.20
12	$ 51.60	$ 112.94
14	$ 60.20	$ 114.68
16	$ 68.80	$ 116.42
18	$ 77.40	$ 118.16
20	$ 86.00	$ 119.90
22	$ 94.60	$ 121.64
24	$ 103.20	$ 123.38
26	$ 111.80	$ 125.12
28	$ 120.40	$ 126.86
30	$ 129.00	$ 128.60
32	$ 137.60	$ 130.34
34	$ 146.20	$ 132.08
36	$ 154.80	$ 133.82
38	$ 163.40	$ 135.56
40	$ 172.00	$ 137.29
42	$ 180.60	$ 139.03
44	$ 189.20	$ 140.77
46	$ 197.80	$ 142.51
48	$ 206.40	$ 144.25
50	$ 215.00	$ 145.99
52	$ 223.60	$ 147.73
54	$ 232.20	$ 149.47
56	$ 240.80	$ 151.21
58	$ 249.40	$ 152.95
60	$ 258.00	$ 154.69
62	$ 266.60	$ 156.43
64	$ 275.20	$ 158.17
66	$ 283.80	$ 159.91
68	$ 292.40	$ 161.65
70	$ 301.00	$ 163.39
72	$ 309.60	$ 165.13
74	$ 315.00	$ 166.87

76	$	315.00	$	168.61
78	$	315.00	$	170.35
80	$	315.00	$	172.09
82	$	315.00	$	173.83
84	$	315.00	$	175.57
86	$	315.00	$	177.31
88	$	315.00	$	179.05
90	$	315.00	$	180.79
92	$	315.00	$	182.53
94	$	315.00	$	184.27
96	$	315.00	$	186.01

Though Ready-to-Load sequencing is technically less expensive for as few as ~30 or more reactions, we do not recommend submitting less than 1 full 96-well plate for Ready-to-Load sequencing.

Sequencing Method Decision Tree:



2. Prepare 2 Master Mixes for the sequencing reactions in appropriately sized centrifuge tubes, using the reagents listed in the table below. Invert contents to mix and pipette entire volume of each into separate sterile reagent reservoirs.
    a. *Note: Only 1 primer is added to each Master Mix, not 2. You will need to make 2 separate Master Mixes (1 using the forward primer, and 1 using the reverse primer) to sequence each set of PCR products.*

Reagent	Volume needed for 1 reaction (µL)	Volume needed for 1 96-well plate (100 reactions; µL)
Water	4.75	475
5X Buffer	2.5	250
DMSO	0.5	50
Primer, forward or reverse (10 µM)	0.25	25
Big Dye v3.1 Master Mix	0.5	50
PCR Product	3.5	-
Total Volume	**12.0**	**850**

3. Using a multichannel pipette, aliquot 8.5 µL of the forward Master Mix into the desired number of wells on 1 or more sterile 96-well PCR plates and do the same for the reverse Master Mix on 1 or more additional PCR plates.

4. Loosely seal the plates containing the Master Mixes with foil covers. Place the plates on crushed ice. Protect them from bright light and direct sunlight.

5. Outside of the PCR prep room, add 3.5 µL of PCR product to each well in the forward and reverse plates containing the Master Mix using a multichannel pipette.

6. Pipette 12 µL of ddH_2O into any unused wells in the plates. This step is necessary for the plate processing done after submission by the BRC.

7. Seal the plates securely with plastic covers. Always keep the plates on ice prior to thermocycling.
8. Centrifuge the PCR plates in the Eppendorf 5810R bucket centrifuge at 4°C and 4,000 rpm for 1 minute to collect all liquid at the bottom of the plate and eliminate any air bubbles.
9. Perform the sequencing reaction for each plate in the thermocycler using the following reaction conditions:

Temperature (°C)	Time (mm:ss)	Cycle Description
96	04:00	Denaturation
96 50 60	00:10 00:05 3:00	25 cycles (denaturation, annealing, extension)
4	∞	Hold

10. Store the plates at either 4°C or at -20°C until ready to submit.
11. Order and submit plates to the Cornell BRC as "Ready-to-Load." For more information on submitting Ready-to-Load plates, see https://www.biotech.cornell.edu/sites/default/files/2020-07/Ready-to-Load_Sanger_Handbook.pdf.

12. MAKE SURE THAT THE ORDER INCLUDES THE FOLLOWING NOTES:

   a. **Please perform post-sequencing cleanup on Edge Biosystems columns.**
   b. **Please use KB Basecaller software for trace processing, with mixed base identification set at a 70% detection level.**

13. The above step is very important! Your sequencing reaction will fail if the clean-up reaction post-sequencing is not performed. The BRC will perform the reaction, but only if you tell them to in the "notes" section when submitting the plates.

SECTION 4 TROUBLESHOOTING

- If the sequencing reactions fail (short sequence reads or noisy reads), try reducing the amount of PCR product added to each sequencing reaction. If the concentration of PCR product is too high, the chain-terminating nucleotides will be exhausted before full-length sequence reads can be produced.
- To verify that there is not a problem with the BigDye™ Terminator v3.1 Cycle Sequencing Kit or our thermocyclers, try submitting the PCR product from a failed sequencing reaction for Full-Service sequencing. If it is successful, this could indicate:
    - A problem with template concentration – see first troubleshooting note.
    - A problem with our thermocyclers – see SOP 5.4.2-Thermal Cycler Calibration Verification and Temperature Non-Uniformity Testing on the Food Safety Wiki for instructions on how to troubleshoot the thermocyclers
    - A problem with the BigDye™ Terminator v3.1 Cycle Sequencing Kit, such as too many freeze-thaw cycles or product expiration. Replace the kit and retry.

SECTION 5 REFERENCES

- https://www.biotech.cornell.edu/sites/default/files/2020-07/Ready-to-Load_Sanger_Handbook.pdf
- https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2F4337035_BDTv31CycSqKt_RUO_UG.pdf&title=VXNlciBHdWlkZTogQmlnRHllIFRlcm1pbmF0b3IgdjMuMSBDeWNsZSBTZXF1ZW5jaW5nIEtpdA==

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	2020-12-25	sjr267	Adapted, expanded, and updated from old SOP titled "8.1.1.1.13-PCR and Sanger Sequencing Reactions in 96-Well Plates."
Version 2			
Version 3			