

## BAC-end Sequencing Protocol

1. Add the following components after the BAC DNA is isolated using Qiagen Kit and then quantified and then quality analyzed by running the BAC DNA on 0.8% agarose gel.

|                                |                     |
|--------------------------------|---------------------|
| <b>1. BAC DNA</b>              | <b>-1ul (300ng)</b> |
| <b>2. Primer (3.2 pmol)</b>    | <b>-1ul</b>         |
| <b>3. DMSO (50%)</b>           | <b>-1ul</b>         |
| <b>4. ABI Big dye v 3.1</b>    | <b>-1ul</b>         |
| <b>5. 5X sequencing buffer</b> | <b>-1ul</b>         |
| <b>6. ddH<sub>2</sub>O</b>     | <b>-x ul</b>        |
| <b>TOTAL</b>                   | <b>-10ul</b>        |

Note: Do everything on ICE

2. Run the sequencing reaction on PCR

**Step1: 98<sup>0</sup>C for 5 mins**  
**Step2 96<sup>0</sup>C for 10 seconds**  
**Step3 50<sup>0</sup>C for 5 seconds**  
**Step4 60<sup>0</sup>C for 4 mins**  
**Step5 GO to step 2 for 40 cycles**  
**Step6 4<sup>0</sup>C for ∞**

Note: Never freeze the product keep at 4<sup>0</sup>C in dark in using later.

3. Precipitate the products by adding 5ul of 25mM EDTA to the reaction mix. Make sure that the EDTA reaches the bottom of the tube.
4. Add 36 ul of **1X ethanol/ sodium aceate mix**. Seal the plate and mix by inverting 4-5 times. Incubate at room temperature for 15 min.
5. Spin the plate at 2000-3000 xg for 30 min. Proceed to the next step immediately. If this is not possible, then spin the plate for 2 min immediately before performing the next step.
6. Invert the plate on a paper towel and spin at 185 xg for 5 min.
7. Add 60 ul of 70% ethanol to each well. Seal the plate and mix by inverting 4-5 times.
8. Spin the plate at 1650 xg for 15 min.
9. Invert the plate on a paper towel and spin at 185 xg for 1 min, and then remove from the centrifuge (Note: start timing when rotor starts moving).
10. Air dry samples for 40 min with minimum exposition to light under a laminar flow.
11. Resuspend the samples in 12ul of deionized formamide HI-Di Formamide.

**Reagents:**

1. ABI big sequence dye v 3.1 : **ABI cat# 4337455** (for 100 reactions)
2. 5X Sequencing buffer 28 ml: **ABI cat# 4336699**
3. 96 well Sequencing PCR plates: ISC bioexpress cat# **T-3085-1** semi skirted 25/pack
4. Hi-Di formamide: **ABI cat# 4311320**

**ABI : 1-800327-3002****1X ethanol/ sodium aceate mix:**

1.4ul      3M sodium aceate pH 5.2 exactly  
34.5ml     100% ethanol

**100X ethanol/ sodium aceate mix:**

140ul      3M sodium aceate pH 5.2 exactly  
3450ml     100% ethanol

**DMSO (50%):**

5ml DMSO  
5ml of ddH<sub>2</sub>O autoclaved water  
Note: store in dark bottle

**Most commonly used primers:**

M13 forward (-20) 5'-GTAAAACGACGGCCAGT-3' (on the T7 side) and  
M13 reverse 5'-AGCGGATAACAATTTTCACACAGG-3' (on the Sp6 side)  
give consistently good results.

pIB FP = pIndigoBAC-5 Forward Sequencing Primer: 5'  
GGATGTGCTGCAAGGCGATTAAGTTGG 3'

pIB RP = pIndigoBAC-5 Reverse Sequencing Primer: 5'  
CTCGTATGTTGTGTGGAATTGTGAGC 3'