**Sequence Capture using AFLP-generated Baits**

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1. **AFLP Bait Preparation**

**1.1 Restriction digestion**

1. Start with 1 μg high molecular weight DNA and digest it using the FastDigest MluI (Thermo Scientific) and FastDigest SbfI (Thermo Scientific). To do so, prepare a reaction on ice as the follow.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **Final concentration**  **in 20-μL reaction** |
| FastDigest Buffer (10×) | 2 | 1× |
| FastDigest MluI | 1 |  |
| FastDigest SbfI | 1 |  |
| DNA + ddH2O | 16 |  |
| Total | 20 |  |

2) Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3) Incubate at 37°C in a water bath for 20 minutes.

**1.2 Cleanup Using AMPure XP® Beads** **(Beckman Coulter, Inc.)**

1) Vortex AMPure XP Beads to resuspend.

2) Add 36 μL (1.8X) of resuspended AMPure XP Beads to the reaction. Mix

thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

3) Incubate for 5 minutes at room temperature.

4) Quickly spin the tube and place the tube on an appropriate magnetic stand to

separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

5) Add 200μL of 80% freshly prepared ethanol to the tube while in the magnetic

stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6) Repeat Step 5) once

7) Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.**! CAUTION Do not overdry the beads. This may result in lower recovery of DNA target**

8) Remove the tube from the magnet. Elute the DNA target from the beads into50 μL of ddH2O. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.

9) Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 50 μL of the supernatant to a fresh, sterile PCR tube.

**1.3 Y adapter Ligation**

1. Mix the following components in a sterile tube.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **Final concentration**  **in 60-μL reaction** |
| T4 DNA ligase buffer (10x) | 6 | 1× |
| ATP (100 mM) | 0.6 | 1 mM |
| MluI adapter (10 μM) | 1 | 0.167 μM |
| SbfI adapter (10 μM ) | 1 | 0.167 μM |
| DNA fragment + ddH2O | 50.4 |  |
| Total | 59 |  |

2) Spin down the liquid by brief centrifugation, then incubate for 5 min at 45 °C in a thermal cycler.

*\*It is necessary to improve the efficiency of cohesive end ligation by making two adapters separate in 45℃.*

3) Quickly transfer the reactions to ice bath, add 1μL of **T4 DNA ligase (5U/μL)** to the reaction and mix well, take the reaction back to the thermocycler, with the heated lid on, and run the following program 30 minutes @ 25℃.

**1.4** **Size selection Adaptor Ligated DNA Using AMPure XP Beads**

*The following size selection protocol is for resulting fragments with a target ranged between 500bp and 2000 bp. The size selection protocol is based on a starting volume of 60 μL.*

1. Vortex AMPure XP Beads to resuspend.

2) Add **15 μL** of resuspended AMPure XP Beads to the 60 μL ligation reaction.

Mix thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

3) Incubate for 5 minutes at room temperature.

4) Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube. Discard the beads that contain the un-wanted large fragments.**! CAUTION Do not discard the supernatant**

5) Add **20μL** resuspended AMPure XP Beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

6) Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.**! CAUTION Do not discard the beads**

7) Add 200μL of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

8) Repeat Step 7) once.

9) Air dry the beads for 5 minutes while the tube is on the magnetic stand with the lid open.

**! CAUTION Do not overdry the beads. This may result in lower recovery of DNA target**

10) Remove the tube from the magnet. Elute the DNA target from the beads into50 μL of 1x TE. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.

11) Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 50μL of the supernatant to a fresh, sterile PCR tube for amplification. Measure the concentration using Nanodrop 2000. The final concentration of the product should be 1ng/μL.

**1.5 Preamplification**

1) Mix the following components in a sterile tube.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **Final concentration**  **in 25-μL reaction** |
| TIANGEN HiFi buffer (10×) | 2.5 | 1× |
| dNTPs (10 mM each) | 0.5 | 200 μM |
| TIANGEN HiFi Taq (5U/μL) | 0.25 | 0.05 U/μL |
| MluI-F (10 μM ) | 0.5 | 0.2 μM |
| SbfI-R (10 μM ) | 0.5 | 0.2 μM |
| DNA (1ng/μL ) | 5 |  |
| ddH2O | 15.75 |  |
| Total | 25 |  |

2) Using the following PCR program:

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycle number** | **Denature** | **Anneal** | **Extent** |
| 1-25 | 94°C, 30s | 56°C, 1 min | 72 °C, 1 min |

3) Dilute the pre-ampliﬁcation reaction product obtained in step 2) 10-fold with 1x TE buffer. These diluted reaction products serve as templates for the final selective amplification reactions using primers with one selective bases in both primers.

**1.6** **Selective Amplification**

1) Selective amplification can be accomplished separately using selective primer combinations. Mix the following components in a sterile tube.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **× *n*** | **Final concentration**  **in 25-μL reaction** |
| TIANGEN HiFi buffer (10×) | 2.5 |  | 1× |
| dNTPs (10 mM each) | 0.5 |  | 200 μM |
| TIANGEN HiFi Taq (5U/μL) | 0.25 |  | 0.05 U/μL |
| Forward selective primer*\** (10 μM ) | 0.5 |  | 0.2 μM |
| Reverse selective primer*\** (10 μM ) | 0.5 |  | 0.2 μM |
| DNA (1ng/μL ) | 5 |  |  |
| ddH2O | 15.75 |  |  |
| Total | 25 |  |  |

*\** *Four* *selective primer combinations are shown in the following list:*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Combination** | **1** | **2** | **3** | **4** |
| Forward selective primer | MluI-F-SA | MluI-F-SA | MluI-F-ST | MluI-F-ST |
| Reverse selective primer | SbfI-R-SC | SbfI-R-SG | SbfI-R-SC | SbfI-R-SG |

2) Using the following PCR program:

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycle number** | **Denature** | **Anneal** | **Extent** |
| 1-13 | 94°C,30s | 65°C,1 min  (reduced each cycle by 0.7 °C) | 72 °C,1 min |
| 14–36 | 94°C,30s | 56°C,1 min | 72 °C,1 min |

**1.7 AMPure XP Beads Purification and Agarose Gel Electrophoresis**

1) Vortex AMPure XP Beads to resuspend.

2) Add 45 μL (1.8X) of resuspended AMPure XP Beads to the reaction. Mix

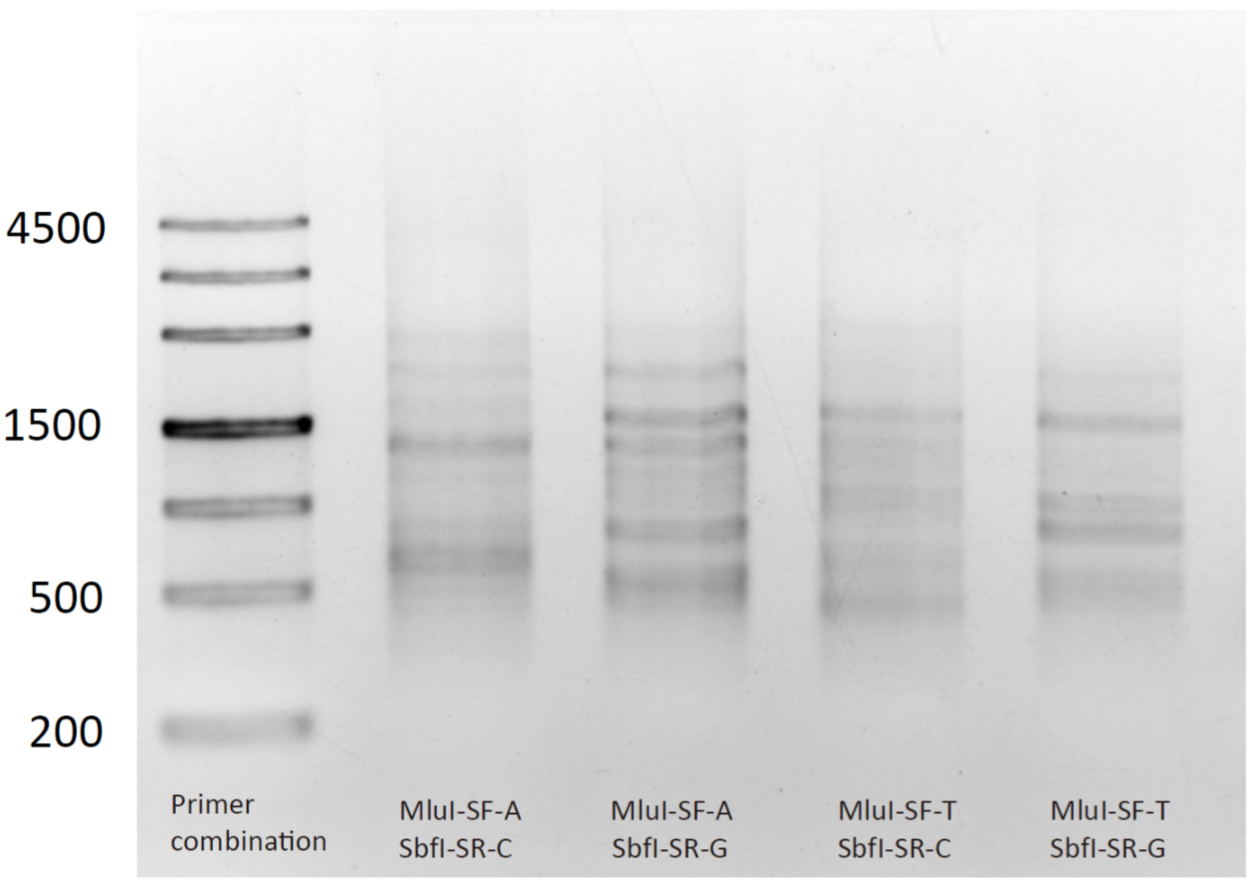
thoroughly on a vortex mixer or by pipetting up and down at least 10 times.

Repeat 1.2 3) -9)

10) Measure the concentration using Nanodrop 2000. The final concentration of the product should be 50 ng/μL.

11) Run 1μL to check the selective amplification product (1.2% agarose, 1 x TAE gel).

*\*The result of agarose gel electrophoresis, as shown below.*



**1.8 Bait immobilization**

*Four different selective amplification purification products were pooled 2* *μg for 10 capture reactions*

1) Combine 2 μg (40μl) of the double-strand biotinylated AFLP bait with 40 μL **2X BWT Buffer** in a PCR tube.

2) Heat the mixture for 5 min at 95ºC, put on ice immediately and keep cold until further use.

3) Add 800μL of **1X BWT Buffer** (at RT) to 50 μL **Dynabeads Myone streptavidin beads** (Life Technologies) to wash. Vortex tube for 5–10 s, place on magnetic particle stand for 2 min to pellet the beads and remove and discard supernatant.

*\*Discard any loose beads that are not tightly attracted to the wall of the tube.*

4) Add 800μL **TET buffer** (at RT) to wash beads. Vortex tube for 5–10 s, place on magnetic particle stand for 2 min to pellet the beads and remove and discard supernatant.

*\*Discard any loose beads that are not tightly attracted to the wall of the tube.*

5) Add the bait mixture that is kept on ice to the beads and resuspend the beads.

6) Rotate the tube for 20 minutes at room temperature (25ºC) to allow the biotinylated AFLP baits to bind to the beads.

7) Collect the beads with a magnet on the tube wall and remove the supernatant, which now contains the baits that did not bind to the beads.

8) Wash the beads **four** times with 800μL of the **PWB buffer** heated to **65ºC** to remove non-biotinylated strands.

9) Resuspend the beads in 50 μL of **TET buffer**, transfer the suspension to a 1.5 ml tube and store it at 4ºC until the hybridization mixture is ready.

1. **Illumina Library Preparation**

**2.1 DNA Fragmentation**

1) Start with 100 ng genomic DNA and fragment it to ***200 - 400 bp***range using the

NEB dsDNA Fragmentase. To do so, prepare a reaction ***on ice*** as the follow.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Volume (****μL)**  **per sample** | **× *n*** | **Final concentration**  **in 20-****μL reaction** |
| dsDNA Fragmentase Buffer (10×) | 2 |  | 1× |
| NEB dsDNA Fragmentase | 1 |  |  |
| DNA + ddH2O | 17 |  |  |

2) Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3) Place in a thermocycler, with the heated lid on, and run the following program:

10-25 minutes\* @ 37°C.

*\*Always do a pre-experiment to determine the optimized time.*

4) Quickly transfer the reactions to ice bath, add 2 μL of 0.5 M EDTA to the reaction and mix well.

5) Run 2 μL to check the fragmented DNA size.

**2.2** **Cleanup Using AMPure XP Beads**

Repeat 1.2 1) - 7)

8) Remove the tube from the magnet. Elute the DNA target from the beads into30

μL of 1x TE. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.

9) Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 30 μL of the supernatant to a fresh, sterile PCR tube.

**2.3** **Illumina Library preparation using** **NEBNext Ultra DNA Library Prep Kit.**

*Libraries can be prepared in advance and store in -20°C.*

**3. Capture Hybridization on Beads**

**3.1** **Prepare Library Mix & Hybridization Buffer**

1) The final reaction system in a PCR tube:

|  |  |
| --- | --- |
| Reagent | Volume (μL) per sample |
| **Library Mix** | 26.5 |
| 500ng of pooled indexed libraries |  |
| Human Cot1 and Blocking oligos |  |
| **200 ng Bait-immobilized beads** | 5 |
| **HYB Buffer (5x SSPE)** | 18.5 |
| Total | 50 |

2) Prepare **Library Mix** as follow for the number of samples needed:

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **× *n*** | **Final concentration**  **in 50-μL reaction** |
| Human Cot1 (1 µg/µl) | 2.5 |  | 0.05µM |
| BO1.P5.F (100 µM ) | 0.5 |  | 1 µM |
| BO3.P7.part1.F (100 µM ) | 0.5 |  | 1 µM |
| BO5.P7.part2.F (100 µM ) | 0.5 |  | 1 µM |
| 500ng of pooled indexed libraries | 22.5 |  |  |
| Total | 26.5 |  |  |

3) Mix the sample by pipetting gently. Collect the liquid at bottom of the tube by briefly centrifuging. Set aside in a refrigerator until step 3.2 2).

4) Prepare **HYB Buffer** for the number of samples needed as follow:

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Volume (μL) per sample** | **× *n*** | **Final concentration**  **in 50-μL reaction** |
| 20X SSPE | 12.5 |  | 5x |
| 0.5 M EDTA | 0.5 |  | 0.005 M |
| 50× Denhardt’s | 5 |  | 5x |
| 10% SDS | 0.5 |  | 0.1% |
| Total | 18.5 |  |  |

5) Mix the regents by pipetting gently and collect the liquid at the bottom of the tube by briefly centrifuging. Transfer 18.5μL of **HYB Buffer** to an empty PCR tube. Set aside in a refrigerator until step 3.2 3).

**3.2** **Incubation**

1) For regular samples, set the following program on a thermal cycler: 95 °C for 5 min, 65 °C for 5 min, 65 °C for 6 hours, 62 °C for 6 hours, 59 °C for 6 hours, 56 °C for 6 hours, 53 °C for 6 hours, 50 °C for 6 hours, and 50 °C forever. It is ok to keep the samples on the machine at 50 °C for a few hours after the program ends.

2) Place the tube containing the **Library Mix** to the thermo cycler and start the program set in step 1. This will denature the DNA for 5 minutes at 95 °C.

3) Once the thermo cycler program reaches step 2 (temperature = 65 °C), place the tube containing the **HYB Buffer** and **Bait-coated beads** to the thermo cycler. Still leave the **Library Mix** in the thermo cycler. This will pre-warm the **HYB Buffer** and **Bait-immobilized beads** for 5 minutes at 65°C.

4) While keeping tubes at 65 °C, transfer all of the **Library Mix** tube (26.5μL) into the **HYB Buffer** and **Bait-immobilized beads** tube, and mix via pipetting up and down.

5) Seal the tube lid with parafilm. Keep the hybridize solution on the thermal cycler until the program end.

**! CAUTION It is important that the tube used is tightly sealed.**

*\*It is better to check if there is leakage on lids on hour after the hybridization start and vortex it two or more times per 6-h period.*

**3.3 Beads Washing and Elution**

1) Place the **hybridization solution** tube on magnetic stand to separate the beads

from the supernatant and remove supernatant completely.

2) Add 200μL **Wash Buffer 1** (1× SSC, 0.1% SDS) to the beads and pipette up and down to resuspend. Incubate for 10 min at room temperature. Collect the liquid at the bottom of the tube by brief centrifugation. Pellet beads with magnetic particle stand for two min and remove supernatant. Repeat this step one more time for a total of **two** low stringency washes. In the meantime, preheat Wash Buffer 2 (0.1× SSC, 0.1% SDS) to 50°C.

3) Add 200μL 55°C **Wash Buffer 2** (0.1× SSC, 0.1% SDS) to the beads and pipette up and down to mix the sample. Incubate for 10 min at 50°C. Pellet beads with at the magnetic plate for two min and remove supernatant.

4) Repeat step 3) 2 times for a total of **three** higher stringency washes at 50°C.

After the last wash, make sure all additional buffer is removed.

5) Add 30μL 1x TE to the beads and store in -20°C.

**3.4 Pre-hybridization PCR (off-beads amplification)**

*This avoids the need to denature and elute the captured target from the baits using sodium hydroxide. The procedure is less problematic and results in more captured products.*

1) Mix the following components in a sterile tube.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (μL)**  **per sample** | **Final concentration**  **in 25-μL reaction** |
| TIANGEN HiFi buffer (10×) | 2.5 | 1× |
| dNTPs (10 mM each) | 0.5 | 200 μM |
| TIANGEN HiFi Taq (5U/μL) | 0.25 | 0.05 U/μL |
| Universal PCR Primer (10 μM ) | 0.5 | 0.2 μM |
| Index Primer (10 μM ) | 0.5 | 0.2 μM |
| DNA | 5 |  |
| ddH2O | 15.75 |  |

2) PCR condition

|  |  |  |  |
| --- | --- | --- | --- |
| **Cycle number** | **Denature** | **Anneal** | **Extent** |
| 1-14 | 98°C, 30s | 65°C, 30 s | 72 °C, 45 s |

3) Run 1 μL to check the size of the captured library.

**3.5 Cleanup Using AMPure XP Beads**

Repeat 1.2 1) - 7)

8) Remove the tube from the magnet. Elute the DNA target from the beads into30

μL of ddH2O. Mix well on a vortex mixer or by pipetting up and down. Incubate for 2 minutes at room temperature.

9) Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 30 μL of the supernatant to a fresh, sterile PCR tube.

**4.** **Pooling multiple samples for sequencing**

1) Pooling multiple samples with same concentration. For Illumina HiSeq X-ten sequencing, recover DNA fragments of 300-500 bp range by using Gel-cutter method. The final concentration of the pooled library should be around~10ng/μL.

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**APPENDIX A**

**Oligo hybridization buffer (10X)**

500mM NaCl

10 mM Tris-Cl, pH 8.0

1 mM EDTA, pH 8.0

**MluI adapter (10μM)**

|  |  |  |
| --- | --- | --- |
| Reagent | Volume (μL) | Final concentration in 100 μL reaction |
| MluI adapter F (100 μM) | 10 | 10 μM |
| MluI adapter R (100 μM) | 10 | 10 μM |
| Oligo hybridization buffer (10×) | 10 | 1× |
| ddH2O | 70 |  |

Mix and incubate the reactions in a thermal cycler for 3min at 95 °C, followed by a ramp from 95 °C to 12 °C at a rate of 0.1 °C/sec.

**SbfI adapter (10μM)**

|  |  |  |
| --- | --- | --- |
| Reagent | Volume (μL) | Final concentration in 100 μL reaction |
| SbfI adapter F (100 μM) | 10 | 10 μM |
| SbfI adapter R (100 μM) | 10 | 10 μM |
| Oligo hybridization buffer (10×) | 10 | 1× |
| ddH2O | 70 |  |

Mix and incubate the reactions in a thermal cycler for 3min at 95 °C, followed by a ramp from 95 °C to 12 °C at a rate of 0.1 °C/sec.

**BWT buffer (2X)**

2 M NaCl

10 mM Tris-Cl, pH 8.0

1 mM EDTA, pH 8.0

0.1% Tween-20

**TET Buffer**

10mM Tris-Cl, pH 8.0

1 mM EDTA, pH 8.0

0.05% Tween 20

**PWB buffer**

0.1 M NaCl

5 mM Tris-Cl, pH 8.0

0.5 mM EDTA, pH 8.0

0.05% Tween-20

**Wash Buffer 1**

1X SSC

0.1% SDS

**Wash Buffer 2**

0.1X SSC

0.1% SDS

**APPENDIX B**

**MluI Adapter**

**MluI adapter-F (5’-3’)**

AGTCGTCTGACAGTTACCCGATACACCA\*A

**MluI adapter-R (5’-3’)**

Phosphate-CGCGTTGGTGTATCGATCTGGACTAGCCTCTAC

**SbfI Adapter**

**SbfI adapter-F (5’-3’)**

GGGTGTGACAGAAGCGAAGCCGAAGAGTCCTGC\*A

**SbfI adapter-R (5’-3’)**

Phosphate-GGACTCTTCGGCCATCTTAGGTGTGGTTTG

**Preamplification Primer:**

**MluI-F (5’-3’)**

AGTCGTCTGACAGTTACC

**SbfI-R (5’-3’)**

CAAACCACACCTAAGATG

**Selective Amplification Primer:**

**MluI-F-SA (5’-3’)**

GTTACCCGATACACCAACGCGT**A**

**MluI-F-ST (5’-3’)**

GTTACCCGATACACCAACGCGT**T**

**SbfI-R-SC (5’-3’)**

Biotin-GATGGCCGAAGAGTCCTGCAGG**C**

**SbfI-R-SG (5’-3’)**

Biotin-GATGGCCGAAGAGTCCTGCAGG**G**

**Blockers Sequence:**

**BO1.P5.F (5’-3’)**

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-phosphate

**BO3.P7.part1.F (5’-3’)**

AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-phosphate

**BO5.P7.part2.F (5’-3’)**

ATCTCGTATGCCGTCTTCTGCTTG-phosphate