# Library Amplification and Size Selection for Sequencing

## Materials

Phusion Plus Polymerase
Forward PCR primer
Barcoded reverse PCR primer
dNTPs
Ultra Low Range DNA Ladder
6% PAGE-TBE gel
SYBR Gold
Gel breaker tubes
Centrifugal filter tubes
10 mM Tris-HCl (pH 7.0)

## Procedure

### PCR amplification of the library

### PCR Amplification

1. Prepare the following 20 µL PCR reaction mixture.

| **Reagent** | **Volume** |
|---|---|
| HF Phusion Plus Buffer | 4 µL |
| cDNA template | 3 µL |
| Forward primer (20 µM) | 0.4 µL |
| Barcoded reverse primer (20 µM) | 0.4 µL |
| dNTPs (10 mM) | 0.4 µL |
| dd$H_2O$ | 11.6 µL |
| Phusion Plus Polymerase | 0.2 µL |
| **Total** | **20 µL** |

2. Use 9, 11, 13 PCR cycles in the current protocol.

### PCR Cycling Conditions

| **Step** | **Temperature** | **Time** | **Cycles** |
|---|---|---|---|
| Initial denaturation | 98 °C | 30s | 1 |
| Denaturation | 98 °C | 10s | 9 or 11 or 13 or 15 |
| Annealing | 60 °C | 10s | 9 or 11 or 13 or 15 |
| Extension | 72 °C | 5s | 9 or 11 or 13 or 15 |
| Final extension | 72 °C | 5min | 1 |

**Final PAGE Purification**

1. Load the PCR products together with an Ultra Low Range DNA Ladder onto a 6% non-denaturing PAGE-TBE gel.
2. Run the gel at the condition:

| Parameter | Condition |
|---|---|
| Running buffer | 1x TBE |
| Voltage | 100 V |
| Running time | About 1 h |

3. Visualize the DNA bands by SYBR Gold
4. Choose the proper band from the 3 cycles. Excise the target library band at 172–192 bp.
5. Recover the DNA using the crush-and-soak method described above.

**RPF Gel Excision**

1. Stain the gel using SYBR Gold.
2. Visualize the RNA bands using a fluorescence transilluminator.
3. Excise fragments between 20 and 40 nt.

**RPF Gel Extraction**

1. Transfer the excised gel pieces into a gel breaker tube.
2. Centrifuge for 2 min at 17000 × g.
3. Add 0.5 mL 10 mM Tris-HCl, pH 7.0 to the fragmented gel.
4. Incubate at 75 °C for 15 min with agitation.
5. Transfer the sample into a centrifugal filter tube.
6. Centrifuge at 15,000 × g for 2 min at room temperature.
7. Collect the eluate.

**RPF Precipitation**

1. Add the following reagents to the eluate.

| Reagent | Amount |
|---|---|
| Isopropanol | 1× eluate volume |
| 3 M sodium acetate, pH 5.5 | 0.1× eluate volume |
| GlycoBlue | 2 µL |

2. Mix thoroughly.
3. Incubate at −80 °C overnight.

**RPF Recovery**

1. Centrifuge at 17,000 × g, 4 °C, for 60 min.
2. Remove the supernatant.
3. Wash the pellet with 80% ethanol.
4. Centrifuge again using 17,000 × g, 4 °C, for 10 min.
5. Remove residual ethanol completely and repeat washing and 10 min-centifuge

6. Briefly air-dry the pellet.

**Final Library Delivery**
Send the final library to GENEWIZ for QC and sequencing using Illumina Nova PE150.

**Oligonucleotides**
The PCR primers were synthesized by GenScript.

| **Oligonucleotide** | **Sequence** |
|---|---|
| Forward PCR primer | 5'-AATGATACGGCGACCACCGAGATCTACACN NNNNNNNACACTCTTTCCCTACACGACGCT-3' |
| Barcoded reverse primer | 5'-CAAGCAGAAGACGGCATACGAGATNNNNNN NNGTGACTGGAGTTCAGACGTG-3' |

N in the primer sequences represent the index sequence.