# Sea Cucumber RAD Library Prep Protocol, Part 3Macintosh HD:Users:natalielowell:Desktop:Screen Shot 2016-11-29 at 4.05.22 PM.png

## Step 7: Blunt-end Repair

WARNING: From here, you must continue through until completion of Step 12.

1. Create the Blunt End Repair Master Mix using the following table:

|  |  |  |  |
| --- | --- | --- | --- |
| **STEP 7 - BLUNT END REPAIR** |  |  |  |
|  |  |  |  |
| **Reagent** | **Stock Concentration** | **uL per rxn** | **uL for Master Mix** |
| Blunting Buffer | 10X | 2.5 | 30.25 |
| dNTP mix | 1mM | 2.5 | 30.25 |
| Blunting Enzyme mix | NA | 1 | 12.1 |
|  |  |  |  |
| Total |  | 6 | 72.6 |

1. Add 6 uL to each sublibrary, for a total volume of 26 uL
2. Incubate at room temperature for 1 hour, in the coldest room

## Step 8: Bead Clean-up, .9X

1. Ensure beads are at room temperature & homogenized before use and prepare fresh 80% ethanol
2. Add the volume of beads from the table below to each library, and mix by pipetting carefully 10 times. If you find liquid on the wall of the tube, then spin down for only a second[[1]](#footnote-0)

|  |  |
| --- | --- |
| **STEP 8 - BEAD CLEAN-UP** | |
|  |  |
| **Library Volume** | 26 |
| **.9X Bead Volume** | 23.4 |

1. Incubate at room temperature for 5 minutes
2. Place tubes on magnetic plate and incubate for 2 minutes or until the supernatant clears
3. Aspirate and discard supernatant
4. Wash beads with 200 uL 80% ethanol, and rinse up and down by pipetting
5. Incubate for 30 seconds
6. Aspirate ethanol and discard
7. Repeat steps 6,7, & 8 for a second wash
8. Use a P10 to remove any remaining drops of ethanol
9. Air dry for ~5 minutes, until all ethanol has disappeared
10. Add 43 uL elution buffer and mix by inversion until beads resuspended
11. Incubate for 2 minutes in a tube rack
12. Place back on magnetic rack for 2 minutes or until supernatant clears
13. Transfer 41 uL supernatant to clean labeled tubes

## Step 9: Add A Overhang

1. Prepare A Overhang Master Mix with the following table

|  |  |  |  |
| --- | --- | --- | --- |
| **STEP 9 - ADD A OVERHANG** |  |  |  |
|  |  |  |  |
| **Reagent** | **Stock Concentration** | **uL per rxn** | **uL for Master Mix** |
| **NEBuffer 2** | 10X | 5 | 60.5 |
| **dATP** | 10mM | 1 | 12.1 |
| **Klenow fragment** | 5000 U/mL | 3 | 36.3 |
|  |  |  |  |
| **Total** |  | 9 | 108.9 |

1. Add 9uL master mix to each sample, for a total volume of 50 uL
2. Incubate at 37C for at least 1 hour in the shaker incubator

## Step 10: Bead Clean-up, .9X

1. Ensure beads are at room temperature & homogenized before use and prepare fresh 80% ethanol
2. Add the volume of beads from the table below to each library, and mix by pipetting carefully 10 times. If you find liquid on the wall of the tube, then spin down for only a second[[2]](#footnote-1)

|  |  |
| --- | --- |
| **STEP 10 - BEAD CLEAN-UP** | |
|  |  |
| **Library Volume** | 50 |
| **.9X Bead Volume** | 45 |

1. Incubate at room temperature for 5 minutes
2. Place tubes on magnetic plate and incubate for 2 minutes or until the supernatant clears
3. Aspirate and discard supernatant
4. Wash beads with 200 uL 80% ethanol, and rinse up and down by pipetting
5. Incubate for 30 seconds
6. Aspirate ethanol and discard
7. Repeat steps 6,7, & 8 for a second wash
8. Using a P10 to remove any remaining drops of ethanol
9. Air dry for ~5 minutes, until all ethanol has disappeared
10. Add 45 uL elution buffer and mix by inversion until beads resuspended
11. Incubate for 2 minutes in a tube rack
12. Place back on magnetic rack for 2 minutes or until supernatant clears
13. Transfer 43 uL supernatant to clean labeled tubes

## Step 11: Ligate P2 Adapters

1. Create the P2 Ligation Master Mix with the following table

|  |  |  |  |
| --- | --- | --- | --- |
| **STEP 11 - LIGATE P2 ADAPTERS** |  |  |  |
|  |  |  |  |
| **Reagent** | **Stock Concentration** | **uL per rxn** | **uL for Master Mix** |
| **NEBuffer 2** | 10X | 5 | 60.5 |
| **P2 Adapter** | 10uM | 1 | 12.1 |
| **rATP** | 100uM | 0.5 | 6.05 |
| **T4 DNA Ligase** | 2000000 U/mL | 0.5 | 6.05 |
|  |  |  |  |
| **Total** |  | 7 | 84.7 |

1. Add 7uL to each sublibrary, for a total volume of 50 uL
2. Incubate sublibraries at room temperature, in the coldest room, for 1 hour and 20 minutes

## Step 12: Double Bead Clean-up, .7 or .8X[[3]](#footnote-2)

1. Ensure beads are at room temperature & homogenized before use and prepare fresh 80% ethanol
2. Add the volume of beads from the table below to each library, and mix by pipetting carefully 10 times. If you find liquid on the wall of the tube, then spin down for only a second[[4]](#footnote-3)

|  |  |
| --- | --- |
| **STEP 12 - DOUBLE BEAD CLEAN-UP** | |
|  |  |
| **Library Volume** | 50 |
| **.7X Bead Volume** | 35 |
| **.8X Bead Volume** | 40 |

1. Incubate at room temperature for 5 minutes
2. Place tubes on magnetic plate and incubate for 2 minutes or until the supernatant clears
3. Aspirate and discard supernatant
4. Wash beads with 200 uL 80% ethanol, and rinse up and down by pipetting
5. Incubate for 30 seconds
6. Aspirate ethanol and discard
7. Repeat steps 6,7, & 8 for a second wash
8. Using a P10 to remove any remaining drops of ethanol
9. Air dry for ~5 minutes, until all ethanol has disappeared
10. Add 52 uL elution buffer and mix by inversion until beads resuspended
11. Incubate for 2 minutes in a tube rack
12. Place back on magnetic rack for 2 minutes or until supernatant clears
13. Transfer 50 uL supernatant to clean labeled tubes, and repeat 1-12 for a second clean
14. Repeat steps 1-15, except add 54 uL elution buffer and transfer 52 to clean tubes; this is so you can use 2 uL for PCR and gel verification





1. Do not centrifuge samples with magnetic beads unless there is obvious sample on the sides of the tube. Only spin for a second. [↑](#footnote-ref-0)
2. Do not centrifuge samples with magnetic beads unless there is obvious sample on the sides of the tube. Only spin for a second. [↑](#footnote-ref-1)
3. Use .7x if you are trying to get ride of more small fragments, ~200 bp, and use .8x if you want to keep things 200bp or and up [↑](#footnote-ref-2)
4. Do not centrifuge samples with magnetic beads unless there is obvious sample on the sides of the tube. Only spin for a second. [↑](#footnote-ref-3)