IMPORTANT PRIMER INFO:

cDNA primers must be ordered from IDT using handmixing for the random bases. Otherwise they are not as random as then need to be.

18 samples - starting concentrations

ASSIGN AN ILLUMINA INDEX NUMBER TO EACH SAMPLE - THE SAME INDEXED PRIMER CAN BE USED FOR BOTH THE RANDOM REVERSE PRIMER ASSAY AND THE COMMON K-MER PRIMER ASSAY. AFTER SEQUENCING, READS FOR EACH ASSAY CAN BE DISTINGUISHED BY THEIR PRIMERS.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample name | Illumina # | [] in ng/µl | µl for 2000ng | dH2O to 22.0 µl |
| Ctrl\_24 - 1 | 1 | 486 | 4.12 | 17.88 |
| Ctrl\_24 - 2 | 2 | 537.4 | 3.72 | 18.28 |
| Ctrl\_24 - 3 | 3 | 475.8 | 4.20 | 17.80 |
| A2B1\_24 - 1 | 4 | 390.1 | 5.13 | 16.87 |
| A2B1\_24 - 2 | 5 | 376.2 | 5.32 | 16.68 |
| A2B1\_24 - 3 | 6 | 375.8 | 5.32 | 16.68 |
| A3\_24 - 1 | 7 | 54.2 | 22.00\* | 0.00 |
| A3\_24 - 2 | 8 | 185.9 | 10.76 | 11.24 |
| A3\_24 - 3 | 9 | 225.5 | 8.87 | 13.13 |
| Ctrl\_48 - 1 | 10 | 269.4 | 7.42 | 14.58 |
| Ctrl\_48 - 2 | 11 | 491 | 4.07 | 17.93 |
| Ctrl\_48 - 3 | 12 | 568 | 3.52 | 18.48 |
| A2B1\_48 - 1 | 13 | 309 | 6.47 | 15.53 |
| A2B1\_48 - 2 | 14 | 286.5 | 6.98 | 15.02 |
| A2B1\_48 - 3 | 15 | 400 | 5.00 | 17.00 |
| A3\_48 - 1 | 16 | 144 | 13.89 | 8.11 |
| A3\_48 - 2 | 17 | 151.7 | 13.18 | 8.82 |
| A3\_48 - 3 | 18 | 206.7 | 9.68 | 12.32 |

**\*this concentration can't allow for 2000ng, but this is the max amount that can be used for each RR and Common K-mer, if we do both assays.**

**cDNA for nested Random Reverse Assay - SEE NEXT PAGE IF SAMPLES ARE CONCENTRATED ENOUGH TO USE SMALLER VOLUMES**

**Master Mix – Random Reverse Short Primer**

|  |  |  |
| --- | --- | --- |
|  | 1x | 27x MM |
| dNTP Mix | 2 ul | 54 ul |
| uni\_rp\_sh\_**40nM** | 2 ul | 54 ul |

**NOTICE THE 4X CONCENTRATION OF UNI\_RP\_SH! THIS PRIMER WILL BIND ALL THE RNA IN THE SAMPLE AS IT IS NON-SPECIFIC, THEREFORE MORE IS NEEDED TO AVOID PRIMER INSUFFICIENCY**

Pipette the following components into a 0.5 ml RNase-free tubes

Total volume = 26 ul. RNA + dH2O = 22ul + 4 ul MM

Place tube in 65°C thermocycler for 5 minutes,reduce to cDNA temp.

Use Veriti #9: AEcDNA-long\_65\_to\_cDNA

Centrifuge briefly and add the following components to each tube (add as 14 uL of mastermix):

|  |  |  |
| --- | --- | --- |
|  | 1x | 21xMM |
| 5x buffer | 8 ul | 168 |
| DTT .1M | 2 ul | 42 |
| RNaseOUT | 2 ul | 42 |
| SSIII RT | 2 ul | 42 |

1. Mix by pipetting gently and incubate at 55°C for 1 hr- then add 2ul SSIII and incubate one more hour.
2. Inactivate SSIII RT by heating at 70°C for 15’.

To each tube, add 2 μl RNase H, incubate at 37°C for 20 minutes.

**cDNA for nested Random Reverse Assay - USE THIS PAGE IF RNA CONCENTRATIONS ARE SUFFICIENT**

**Master Mix – Random Reverse Short Primer**

|  |  |  |
| --- | --- | --- |
|  | 1x | 27x MM |
| dNTP Mix | 1 ul | 27ul |
| uni\_rp\_sh\_**40nM** | 1 ul | 27ul |

Pipette the following components into a 0.5 ml RNase-free tubes

Total volume = 13 ul. RNA + dH2O = 11ul + 2 ul MM

Place tube in 65°C thermocycler for 5 minutes,reduce to cDNA temp.

Use Veriti #9: AEcDNA-long\_65\_to\_cDNA

Centrifuge briefly and add the following components to each tube (add as 7 uL of mastermix):

|  |  |  |
| --- | --- | --- |
|  | 1x | 21xMM |
| 5x buffer | 4 ul | 84 |
| DTT .1M | 1 ul | 21 |
| RNaseOUT | 1 ul | 21 |
| SSIII RT | 1 ul | 21 |

1. Mix by pipetting gently and incubate at 55°C for 1 hr- then add 1ul SSIII and incubate one more hour.
2. Inactivate SSIII RT by heating at 70°C for 15’.

To each tube, add 1 μl RNase H, incubate at 37°C for 20 minutes.

**RANDOM REVERSE PRIMER**

Purify cDNA using Agencourt RNAClean X – in 4O fridge in Rm. 142

1. Resuspend the beads. You need 1x ul of beads for each tube + some extra. Keep in room temperature for at least **30 minutes** before use.
2. Resuspend the beads. Add **40 µl** Agencourt RNAClean XP beads to each cDNA reaction and mix by vortexing
3. Let the tubes incubate at room temperature for **20 minutes** before proceeding to the next step.
4. Transfer the reactions to a bead clean up plate.
5. Place the plate onto the magnetic plate rack for 5 minutes to separate the beads from solution
6. Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the plate is situated on the rack. Do not disturb the magnetic beads.
7. Dispense 300 μL of 70% ethanol into each well and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of **Four washes.** It is important to perform these steps with the plate situated on the rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
8. Let the reaction tube air-dry ~ 10 minutes on the rack with the lid off.
9. Elute the purified product from the beads with DNase-free water **22.6 ul** for random reverse assay. Add water and resuspend the beads by sliding the plate back and forth on the benchtop.
10. Put the plate back on the magnetic rack and give the beads time to stick to the side.
11. Pipette the eluant from the plate while it is situated on the magnetic tube rack.  **21.1 to PCR1 input**

**cDNA for nested Common K-mer Assay - SEE NEXT PAGE IF RNA SAMPLES ARE CONCENTRATED ENOUGH TO USE SMALLER VOLUMES**

**Master Mix – Random Reverse Short Primer**

|  |  |  |
| --- | --- | --- |
|  | 1x | 27x MM |
| dNTP Mix | 2 ul | 54 ul |
| ComK\_deG | 2 ul | 54 ul |

Pipette the following components into a 0.5 ml RNase-free tubes

Total volume = 26 ul. RNA + dH2O = 22ul + 4 ul MM

Place tube in 65°C thermocycler for 5 minutes,reduce to cDNA temp. shs

Use Veriti #9: AEcDNA-long\_65\_to\_cDNA

Centrifuge briefly and add the following components to each tube (add as 14 uL of mastermix):

|  |  |  |
| --- | --- | --- |
|  | 1x | 21xMM |
| 5x buffer | 8 ul | 168 |
| DTT .1M | 2 ul | 42 |
| RNaseOUT | 2 ul | 42 |
| SSIII RT | 2 ul | 42 |

1. Mix by pipetting gently and incubate at 55°C for 1 hr- then add 2ul SSIII and incubate one more hour.
2. Inactivate SSIII RT by heating at 70°C for 15’.

To each tube, add 2 μl RNase H, incubate at 37°C for 20 minutes.

**cDNA for nested Common K-mer Assay -USE THESE AMOUNTS IF RNA CONCENTRATIONS ARE SUFFICIENT**

**Master Mix – Random Reverse Short Primer**

|  |  |  |
| --- | --- | --- |
|  | 1x | 27x MM |
| dNTP Mix | 1 ul | 27ul |
| ComK\_deG | 1 ul | 27ul |

Pipette the following components into a 0.5 ml RNase-free tubes

Total volume = 13 ul. RNA + dH2O = 11ul + 2 ul MM

Place tube in 65°C thermocycler for 5 minutes,reduce to cDNA temp. shs

Use Veriti #9: AEcDNA-long\_65\_to\_cDNA

Centrifuge briefly and add the following components to each tube (add as 7 uL of mastermix):

|  |  |  |
| --- | --- | --- |
|  | 1x | 21x MM |
| 5x buffer | 4 ul | 84 |
| DTT .1M | 1 ul | 21 |
| RNaseOUT | 1 ul | 21 |
| SSIII RT | 1 ul | 21 |

1. Mix by pipetting gently and incubate at 55°C for 1 hr- then add 1ul SSIII and incubate one more hour.
2. Inactivate SSIII RT by heating at 70°C for 15’.

To each tube, add 1 μl RNase H, incubate at 37°C for 20 minutes.

**COMMON K-MER PRIMER**

Purify cDNA using Agencourt RNAClean X – in 4O fridge in Rm. 142

1. Resuspend the beads. You need 1x = 40 ul of beads for each tube + some extra. Keep in room temperature for at least **30 minutes** before use.
2. Resuspend the beads. Add **40 µl** Agencourt RNAClean XP beads to each cDNA reaction and mix by vortexing
3. Let the tubes incubate at room temperature for **20 minutes** before proceeding to the next step.
4. Transfer the reactions to a bead clean up plate.
5. Place the plate onto the magnetic plate rack for 5 minutes to separate the beads from solution
6. Slowly aspirate the cleared solution from the tube and discard. This step should be performed while the plate is situated on the rack. Do not disturb the magnetic beads.
7. Dispense 300 μL of 70% ethanol into each well and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of **Four washes.** It is important to perform these steps with the plate situated on the rack. Do not disturb the separated magnetic beads. Be sure to remove all of the ethanol from the bottom of the well as it may contain residual contaminants.
8. Let the reaction tube air-dry ~ 10 minutes on the rack with the lid off.
9. Elute the purified product from the beads with DNase-free water **22.6 ul** for Common K-mer assay. Add water and resuspend the beads by sliding the plate back and forth on the benchtop.
10. Put the plate back on the magnetic rack and give the beads time to stick to the side.
11. Pipette **21.1 µl**  from the plate to new tubes while it is situated on the magnetic tube rack - this is the templat cDNA and these tubes are used directly in PCR I

FIRST ROUND PCR: NESTED RR ASSAY AND NESTED COMMOM K-MER ASSAY - 36 SAMPLES TOTAL

Forward Primer: idx\_fp\_nest

Reverse Primer: RR\_adapter

All primers are at 10 uM

|  |  |  |
| --- | --- | --- |
|  | 1x | 38 x Master Mix |
| A buffer | 10 ul | 380ul |
| dNTPs | 1ul | 38ul |
| idx\_fp\_nest | 2.5ul | 95ul |
| RR\_adapter | 2.5ul | 95ul |
| KAPA robust HS Pol | .4ul | 15.2ul |
| 5% DMSO | 2.5ul | 95ul |
| Enhancer 1 | 10 ul | 380ul |
| cDNAs | <21.1 ul> |  |
| Total volume | 28.9 | OK |

cDNA = 21.1µl

Add 28.9 ul MM to each sample cDNA tube

PCR Cycle: Touchdown PCR\_RR - **30 cycles total**

95 5:00

3x

95 :30

72 15

72 2:00

Then 3x each with annealing temp at 70, 68, 66, 64, 62,

**12x 60**

72 10:00

4 hold

Purify PCR products using AmpureXP PCR cleanup kits. Use 80 ul beads for each 50 ul PCR tube. 4 washes in 70% EtOH. Elute in 30ul.

SECOND ROUND PCR FOR NESTED RANDOM REVERSE ASSAY AND NESTED COMMON K-MER

Forward Primer: idx\_fp

Reverse Primer: RR\_adapter

All primers are at 10 uM

Reverse Primer: RR\_adapter

All primers are at 10 uM

|  |  |  |
| --- | --- | --- |
|  | 1x | 38x Master Mix |
| A buffer | 10 ul | 380ul |
| dNTPs | 1ul | 38ul |
| idx\_fp | 2.5ul | 95ul |
| RR\_adapter | 2.5ul | 95ul |
| KAPA robust HS Pol | .4ul | 15.2ul |
| 5% DMSO | 2.5ul | 95ul |
| Enhancer 1 | 10 ul | 380ul |
| dH2O | 16.1 | 611.8ul |
| 5 µl PCR I template DNA | <5 ul> |  |
| Total volume | 45.0 | OK |

Add 45 ul MM to each sample cDNA tube

PCR Cycle: Touchdown PCR\_RR

95 5:00

3x

95 :30

72 15

72 2:00

Then 3x each with annealing temp at 70, 68, 66, 64, 62,

the **12x at 60**

72 10:00

4 hold

Purify PCR products using AmpureXP PCR cleanup kits. Use 80 ul beads for each 50 ul PCR tube. 4 washes in 70% EtOH. Elute in 30ul.

THIRD ROUND PCR FOR NESTED RANDOM REVERSE ASSAY AND COMMON K-MER ASSAY

Primers are:

Forward Primer: ADPT\_P1 (Uni Adapter)

Reverse Primers: Illumina Indexed Adapters

|  |  |  |
| --- | --- | --- |
|  | 1x | 38x Master Mix |
| A buffer | 10 ul | 380ul |
| dNTPs | 1ul | 38ul |
| ADPT\_P1 | 2.5ul | 95ul |
| KAPA robust HS Pol | .4ul | 15.2ul |
| 5% DMSO | 2.5ul | 95ul |
| Enhancer 1 | 10 ul | 380ul |
| dH2O | 16.1 | 611.8 |
| Total volume | 42.5 | OK |

|  |  |
| --- | --- |
|  | per tube |
| MasterMix | 42.5 |
| Indexed Adaptor | 2.5 |
| Template DNA: 2nd round  PCR Products\* | 5 |
| Total volume | 50ul |

PCR Cycle: Touchdown PCR\_RR - **35 cycles total**

95 5:00

3x

95 :30

72 15

72 2:00

Then 3x each with annealing temp at 70, 68, 66, 64, 62,

the **17x** at 60

72 10:00

4 hold

Check on 2% agarose gel

**WHAT YOU'RE LOOKING FOR HERE IS A SMEAR OF VARIOUS SIZED TRANSCRIPTS THAT ARE NOT PRIMER DIMERS (>200 )**

**THE POINT OF THE NEXT SECTION IS TO TITRATE THE PCR REACTIONS PRIOR TO SIZE SELECTION WITH THE BLUE PIPPIN. THE GOAL IS TO END UP WITH APPROXIAMATELY EQUAL AMOUNTS OF ALL SAMPLES. TRY TO GET THE SAME AMOUNTS FOR EACH SAMPLE, BUT DON'T INCLUDE PRIMER DIMERS IN YOUR ESTIMATE, AS THEY WILL (HOPEFULLY) BE FILTERED OUT.**

**THIS IS A SAMPLE GEL, FOLLOWED BY THE TITRATION ESTIMATES.**

A close up of a paper

Description automatically generated

**RANDOM REVERSE: Pre-Pippin titration**

**SHOWN ARE THE AMOUNTS USED FROM THE PREVIOUS GEL**

**In this unusual case, they all amplified the same amount so I used 30 ul of each**

|  |  |  |
| --- | --- | --- |
| Sample name | Illumina # | µl to use in pre-Pippin titration mix |
| Ctrl\_24 - 1 | 1 | ALL 30 ul |
| Ctrl\_24 - 2 | 2 |  |
| Ctrl\_24 - 3 | 3 |  |
| A2B1\_24 - 1 | 4 |  |
| A2B1\_24 - 2 | 5 |  |
| A2B1\_24 - 3 | 6 |  |
| A3\_24 - 1 | 7 |  |
| A3\_24 - 2 | 8 |  |
| A3\_24 - 3 | 9 |  |
| Ctrl\_48 - 1 | 10 |  |
| Ctrl\_48 - 2 | 11 |  |
| Ctrl\_48 - 3 | 12 |  |
| A2B1\_48 - 1 | 13 |  |
| A2B1\_48 - 2 | 14 |  |
| A2B1\_48 - 3 | 15 |  |
| A3\_48 - 1 | 16 |  |
| A3\_48 - 2 | 17 |  |
| A3\_48 - 3 | 18 |  |

**RANDOM REVERSE**

**COMBINE ALL THE SAMPLES AND THEN DO A BEAD CLEAN UP PRIOR TO RUNNING THE BLUE PIPPIN**

FOR RANDOM REVERSE

Bead clean of of mixed samples:

Volume = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl

1.8x beads = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl beads

Elute in 155 µl

Qubit = \_\_\_\_\_\_\_\_\_\_\_\_\_\_ng/µl

Blue Pippin –1.5% gel with internal standard, i.e., 5 lanes with 2ug each = 10 ug

10 µg = \_\_\_\_\_\_\_\_\_\_\_\_\_µl

+ \_\_\_\_\_\_\_\_\_\_\_\_\_\_µl dH2O to 150 µl total

Follow Pippin protocol

Collect in range 380 to 1325

DO NOT use high pass

Bead concentrate:

Total volume = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl

X1.8x beads = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl beads

elute in 50 µl or more if needed.

Leave tube with eluant in bottom drawer overnight to condense - SERIOUSLY - JUST LEAVE IT IN A SAFE PLACE OVERNIGHT WITH THE LID OPEN.

Qubit [] = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

ng of each sample = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**COMMON K-MER: Pre-Pippin Titration**

**SHOWN ARE THE AMOUNTS USED FROM THE PREVIOUS GEL**

|  |  |  |
| --- | --- | --- |
| Sample name | Illumina # | µl to use in pre-Pippin titration mix |
| Ctrl\_24 - 1 | 1 | 30 |
| Ctrl\_24 - 2 | 2 | 30 |
| Ctrl\_24 - 3 | 3 | 40 |
| A2B1\_24 - 1 | 4 | 20 |
| A2B1\_24 - 2 | 5 | 20 |
| A2B1\_24 - 3 | 6 | 20 |
| A3\_24 - 1 | 7 | 25 |
| A3\_24 - 2 | 8 | 25 |
| A3\_24 - 3 | 9 | 25 |
| Ctrl\_48 - 1 | 10 | 30 |
| Ctrl\_48 - 2 | 11 | 30 |
| Ctrl\_48 - 3 | 12 | 30 |
| A2B1\_48 - 1 | 13 | 30 |
| A2B1\_48 - 2 | 14 | 30 |
| A2B1\_48 - 3 | 15 | 30 |
| A3\_48 - 1 | 16 | 30 |
| A3\_48 - 2 | 17 | 40 |
| A3\_48 - 3 | 18 | 40 |

**COMMON K-MER**

**FOR COMMON K-MER ASSAY**

Bead clean of of mixed samples:

Volume = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl

1.8x beads = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl beads

Elute in 155 µl

Qubit = \_\_\_\_\_\_\_\_\_\_\_\_\_\_ng/µl

Blue Pippin –1.5% gel with internal standard, i.e., 5 lanes with 2ug each = 10 ug

10 µg = \_\_\_\_\_\_\_\_\_\_\_\_\_µl

+ \_\_\_\_\_\_\_\_\_\_\_\_\_\_µl dH2O to 150 µl total

Follow Pippin protocol

Collect in range 400 to 1350

DO NOT use high pass

Bead concentrate:

Total volume = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl

X1.8x beads = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_µl beads

elute in 50 µl or more if needed.

Leave tube with eluant in bottom drawer overnight to condense

Qubit [] = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

ng of each sample = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

combine all samples

qubit final [] = \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

PROCESSING THE SEQUENCING DATA

files are most easily processed on the computer cluster, but can be done on a laptop if you have enough memory.

navigate to the folder with the sequencing files.

unzip the sequencing files - these programs all run on unzipped files

This can be done with this command:

gunzip \*

For each sample, separate the random reverse primer reads from the common k-mer primer reads using this code, replacing the R1 and R2 files with the names of your sample:

ruby SeparateRandomReversePlusCommonKmerDeG\_NL4\_3.rb R1.fastq R2.fastq

This will create two sets of files, one with the random reverse reads and one with the common k-mer reads.

process the random reverse files using this command:

ruby 2024\_01\_CatagorySortRR\_NL4-3\_Cryptics.rb RR\_R1.fastq RR\_R2.fastq

For each sample there are three text file outputs. You probably only want the one with the splicing info. The others contain primer binding data and PID (primer ID) data.

process the common K-mer files using this command:

ruby 2023\_06\_RNAseq\_sort\_NL4-3\_CommonKmerNested.rb CommK\_R1.fastq CommK\_R2.fastq

If your computer cluster uses a Slurm scheduler, then the slurm scripts file has code to produce the Slurm required input for all samples.