**PrimerID NGS Library Prep Protocol v1.2.1 – 04052023**

Adapted from “Zhou, S. 2021. Lab Protocol v1.2.”

**Purpose:** Prepare a library of HIV-1 infected plasma for downstream PrimerID-NGS (Illumina MiSeq.)

**Primer List**

|  |  |  |  |
| --- | --- | --- | --- |
| **Regions** | **cDNA PID primer** | **PCR1 Forward** | **Stock Primer Concentration (µM)** |
| PR | R2614\_PID | F2163AD | 100  |
| RT | R3284\_PID11 | F2620\_AD | 100 |
| INT | R4752\_PID11 | F4383\_AD | 100 |
| V3 | V3R\_Buni\_11 | V1F\_AD | 100 |

Making 10uM Multiplexed Primer Mixes: cDNA and PCR1 Forward (from 100uM Stock)

1. Mix 10 µL of each cDNA or PCR1-F primer + 60 µL sterile water in a sterile, RNase-free 1.7 mL microcentrifuge tube. Label accordingly and store at -20C when not in use.

Making 10uM Adapter Mixes: PCR1 and PCR2 Adapters (from 100uM Stock)

1. Adapter 2A / P1:
	1. Mix 10 µL of either adapter (ADPT\_2A or P1, 100 µM stock) with 90 µL sterile water in a sterile, RNase-free 1.7 mL microcentrifuge tube. Label accordingly and store at -20C when not in use.
2. Indexed Adapters:
	1. Mix 10 µL of indexed adapter (100 µM stock concentration) with 90 µL sterile water in a sterile, RNase-free 1.7 mL microcentrifuge tube. Label accordingly and store at -20C when not in use.

Making Carrier RNA Aliquots

1. Add 560 µL of DEPC-treated water to the tube of desiccated carrier RNA (Qiagen Viral RNA Mini Kit) and pipet mix.
2. Use a repeater pipet to aliquot 10 µL of rehydrated carrier RNA to RNase-free 1.7 mL tubes. Store at -20C until ready for use.

**RNA extraction: PCR Clean Room**

Ultracentrifuge Plasma Specimens

1. Clean a biosafety cabinet (BSC) with 10% bleach then 70% ethanol. Place a double-bagged autoclave biohazard waste bin inside with paper towels lining the bottom.
2. Start the ultracentrifuge with the rotor inside at **300 rpm / 4˚C** and allow to cool to **4˚C**.
3. While the ultracentrifuge cools down:
	1. Fully thaw plasma specimens and quality control (QC2).
	2. Label a set of RNase-free 1.7 mL tubes.
4. Transfer **500 µL** **of plasma specimen (up to 1.5mL)** to an RNase-free 1.7 mL tube. Include a negative control (500 µL DEPC-treated water).
5. Remove the rotor from the ultracentrifuge and place inside the BSC.
6. Transfer the 500 µL of plasma, QC2 and negative control to the rotor.
7. Screw the lid onto the rotor **INSIDE** **the BSC** then place in the ultracentrifuge.
8. Run the ultracentrifuge at **22,000 rpm / 4˚C for 2 hours**.
9. Return remaining plasma to -80C storage.

Prepare for Viral RNA Extraction and Remove Plasma Supernatants

1. While the ultracentrifuge pellets the plasma, prepare the necessary tubes for the extraction/storage:
	1. Two sets of RNase-free 1.7 mL tubes, one for plasma supernatant storage (“SUP”) and one for purified viral RNA (“vRNA”).
	2. One set of spin columns (Qiagen Viral RNA Mini Kit)
	3. Four sets of collection tubes (Qiagen Viral RNA Mini Kit).
	4. One set of RNase-free 0.6 mL PCR tubes for the cDNA synthesis step.
2. Clean in the Qiagen Viral RNA Mini-Kit reagents:
	1. Buffer AVL (Viral lysis buffer)
	2. 100% Ethanol
	3. Buffer AW1 (Wash 1)
	4. Buffer AW2 (Wash 2)
	5. Buffer AVE (Elution buffer)
	6. Carrier RNA aliquots
3. Once the ultracentrifuge completes its spin cycle, carefully remove the rotor.
	1. It is very important you do not disturb the pelleted plasma. Do NOT shake or invert ultracentrifuge rotor.
4. **Remove the lid of the rotor** **inside the BSC** and transfer plasma specimens to a rack. Remove the rotor and disinfect thoroughly with 70% ethanol.
5. Carefully remove **360 µL** **of plasma supernatants** by slowly aspirating from the top of the liquid, leaving behind a 140 µL pellet. If volume of the plasma is greater than 500 µL, calculate the volume to aspirate to ensure 140 µL of plasma left in the tube with the pellet
6. Dispense the supernatants into the designated 1.7 mL “SUP” tubes and transfer to -80C storage.

**RNA extraction: PCR Clean Room**

Purify viral RNA using the Qiagen Viral RNA Mini Kit

1. Mix **560 µL** of **Buffer AVL** with a **carrier RNA** **aliquot**. Transfer the AVL + carrier RNA to the 140 µL plasma pellet, then pulse-vortex for 15 seconds to mix.
2. Use a mini centrifuge inside the BSC to remove any liquid from inside the tube lid.
3. Incubate at room temperature for 10 minutes.
4. Add **560 µL** of **100% ethanol** to each sample. Pulse-vortex for 15 seconds to mix.
5. Use a mini centrifuge inside the BSC to remove any liquid from inside the tube lid.
6. Transfer **630 µL** of the sample to a spin column without wetting the rim. Close the spin column.
7. Transfer samples to a benchtop centrifuge rotor inside the BSC. Tighten the lid, remove from the BSC, wipe with 70% ethanol and place back onto the benchtop centrifuge.
8. **Centrifuge at 6000 g for 1 minute.**
9. Place the rotor back in the BSC and remove the specimens. Transfer the spin columns to clean collection tubes, discarding the filtrate.

\*\*Repeat steps **6-9**.

1. Add **500 µL** of **Buffer AW1** to the spin column.
	1. Transfer samples to the rotor inside the BSC and tighten the lid. Return the rotor to the centrifuge.
	2. **Centrifuge at 6000 g for 1 minute.**
	3. Place the rotor back in BSC and remove the specimens. Transfer the spin columns to clean collection tubes, discarding the filtrate.
2. Add **500 µL** of **Buffer AW2** to the spin column.
	1. Transfer samples to the rotor inside the BSC and tighten the lid. Return the rotor to the centrifuge.
	2. **Centrifuge at 20,000 g for 3 minutes.**
	3. Place the rotor back in the BSC and remove the specimens. Transfer the spin columns to clean collection tubes, discarding the filtrate.
3. Re-load the empty spin columns in the rotor and transfer back to the centrifuge.
	1. **Centrifuge at 20,000 g for 1 minute.**
4. Transfer the spin columns to the designated 1.7 mL “vRNA” tubes.
5. Add **50 µL** of **Buffer AVE** to each spin column. Incubate at room temperature for 2 minutes.
	1. Load the vRNA tubes with the spin columns into the rotor and return to the centrifuge.
	2. **Centrifuge at 10,000 g for 1 minute.**
6. Return the rotor to the BSC. Discard the spin columns, leaving behind 50 µL of purified viral RNA. Proceed with the cDNA synthesis. Store any remaining RNA at -80C immediately after use.

**cDNA Synthesis: PCR Clean Room**

Generate First Strand, PrimerID-Tagged cDNA Products from Purified Viral RNA

1. Label a set of 0.6 mL RNase-free PCR tubes for cDNA reactions.
2. Use two RNase-free 1.7 mL tubes to prepare **cDNA1** and **cDNA2 master mixes**.
3. Remove the following reagents from -20C storage and thaw completely.

|  |  |  |  |
| --- | --- | --- | --- |
| **Manufacturer** | **Reagent**  | **Heat block, 32C** | **Room temperature** |
| KAPA | dNTP Mix | ✓ |   |
|  | cDNA Primer Mix |  | ✓  |
| Invitrogen | 5x First-Strand Buffer | ✓ |  |
| Invitrogen | DTT |  ✓ |  |

Reagents may be thawed at room temperature or using a low-temperature heat block, but it is recommended to keep them cold/on ice while handling.

1. Pulse-vortex the **dNTPs, 5x First-Strand Buffer** and **DTT** 2 times to homogenize. Briefly spin down the dNTP mix, 5x First-Strand Buffer and DTT via. mini centrifuge before use.
2. Prepare the **cDNA1 master mix** by adding the following reagents in the order listed. The volume of each reagent per sample and total master mix volume are listed below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **cDNA1: REAGENTS** | **Stock Conc.** | **Final Conc.** | **Master Mix Volume (µL)\*** | Volume per sample (µL) |
| DEPC-Treated Water |  |  | 78 | 3.0 |
| dNTP Mix | 10 mM each | 0.5 mM | 52 | 2.0 |
| PrimerID cDNA Primer Mix  | 10 µM | 0.25 µM | 26  | 1.0 |

\* Master mix volume calculation is sufficient for 25 reactions with 1% overage for pipet error.

1. Pipet-mix the cDNA 1 master mix 15 times, then aliquot **6 µL** to each 0.6 mL PCR tube.
2. Add **23 µL** of purified RNA to each 0.6 mL PCR tube and pipet-mix 15 times.
3. **Place cDNA1 reactions on a thermal cycler with the following conditions:**
	1. **65°C for 5 minutes**
	2. **2˚C for 2 minutes**
4. Prepare the **cDNA2 master mix** by adding the following reagents to in the order listed. The volume of each reagent per sample and total master mix volume are listed below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **cDNA2: REAGENTS** | **Stock Conc.**  | **Final Conc.**  | **Master Mix Volume (µL)\*** | Volume per sample (µL) |
| 5x First-Strand Buffer  | 5x | 1x | 208 | 8.0 |
| DTT  | 100 mM | 5 mM | 52 | 2.0 |
| RNase OUT  | 40 U/µL | 0.5 U/µL | 13 | 0.5 |
| Superscript III RT | 200 U/µL | 2.5 U/µL | 13 | 0.5 |

\* Master mix volume calculation is sufficient for 25 reactions with 1% overage for pipet error. Return all reagents and enzyme to -20C storage immediately after use.

1. Remove the cDNA reactions from the thermal cycler then add **11 µL** of the cDNA2 master mix. Pipet-mix 15 times.
2. **Place cDNA2 reactions on a thermal cycler under the following conditions:**
	1. **50°C for 60 minutes 🡪 Increase to 55°C for 60 minutes.**
	2. **Increase to 70°C for 15 minutes** to inactivate the Superscript III RT.

**RNase H Digestion: PCR Clean Room**

Perform RNase H Digestion on cDNA Reactions using NEB RNase H

1. Remove the **RNase H Reaction Buffer** from -20C storage.
2. Thaw completely at room temperature or at 32C using a heat block. Pulse-vortex 2 times to mix.
	1. Leave the RNase H enzyme at -20C until ready to use.
3. Use a 1.7 mL tube to prepare the **RNase H master mix** by adding the following reagents in the order listed. The volume of each reagent per sample and total master mix volume are below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **RNase H: REAGENTS** | **Stock Conc.** | **Final Conc.** | **Master Mix Volume (µL)\*** | Volume per sample (µL) |
| Water, sterile/DEPC-treated  |  |  | 117 | 4.5 |
| RNase H Reaction Buffer  | 10x | 1x | 130  | 5.0 |
| RNase H Enzyme | 5 U/µL | 0.05 U/µL | 13 | 0.5 |

\* Master mix volume calculation is sufficient for 25 reactions with 1% overage for pipet error. Return all reagents and enzyme to -20C storage immediately after use.

1. Add **10 µL** of the RNase H master mix to each cDNA reaction and pipet-mix15 times.
2. **Place RNase H reactions on a thermal cycler for the following conditions:**
	1. **37°C for 20 minutes**
3. Proceed with the cDNA purification once the thermal cycler has been started. If samples must be stored before proceeding, store at 4C for up to 24 hours, then transfer to -20C for longer-term storage.

**cDNA Purification: PCR Clean Room**

Prepare Aliquots of Agincourt RNAClean XP Magnetic Beads

1. Take out 40 mL stock bottle of RNAClean XP magnetic beads.
2. Resuspend beads into the solution by shaking. Ensure that no clumps of beads are left in the bottle and the solution appears homogenous.
3. Using a repeater pipette, aliquot **1.0 mL** of the magnetic bead solution into sterile 1.7 mL tubes for later use.
	1. Store bead aliquots at 4C until ready for use. Once all beads have been aliquoted, start cDNA purification from **step 4**.

cDNA Double-Purification using Agincourt RNAClean XP Bead Aliquots

1. Remove **two** of the **1 mL** aliquots of RNAClean XP magnetic beads. Allow to sit at room temperature for **30 minutes** before use.
2. Create a fresh **50 mL** aliquot of **70% Ethanol** in a sterile conical tube.
3. Label two sets of sterile 1.7 mL tubes and one new set of 0.6 mL PCR tubes.
4. After 30 minutes at room temperature, resuspend the 1 mL bead aliquots by shaking or vortexing. The solution should appear homogenous with no clumps of beads. Centrifuge briefly to remove any solution from the tube cap.

**cDNA Purification Part 2: PCR Clean Room**

cDNA Double-Purification using Agincourt RNAClean XP Bead Aliquots

1. Add magnetic beads to both sets of 1.7 mL tubes: **35 µL** **of bead solution per tube**.
2. Transfer the total **50 µL** of each cDNA reaction to the first set of beads. Pipet mix 15 times.
	1. Do **NOT** vortex the beads and sample to mix.
	2. Set aside the second set of magnetic beads for now. They will be used for the second cDNA purification.
3. Incubate samples at room temperature for **20 minutes**, then place on the magnetic rack for **5 minutes** to allow all the beads to collect in a pellet on the side of the tube.
	1. Ensure each sample tube is pressed firmly against the magnetic rack.

\*\*Seps 4-7 should be performed **while the tubes are on the magnetic rack**.

1. Slowly aspirate the cleared solution from the tube and discard. **This should be done while the tube is still in the magnetic rack.**
2. Dispense **400 µL** of 70% ethanol into each sample tube. Incubate at room temperature for 30 seconds, then remove ethanol and discard.
	1. Repeat step **5**.
3. Use a pipet to remove any trace amounts of ethanol left in the bottom of the sample tubes.
4. Allow the beads to dry with the tube cap left open until the last visible traces of ethanol has disappeared. **This should take** **up to 10 minutes maximum.**
	1. Over-drying the beads can result in lower recovery when performing the elution step.

1. Remove the sample tube from the rack and re-suspend the magnetic bead pellet in **50 µL sterile/DNase-free water** by pipet-mixing ~15 times. The solution should be homogenous with no bead clumps.
	1. Over-dried beads will not always fully re-suspend, and there will be small clumps of beads left in solution.
2. Once re-suspended, close the tube cap and place back onto the magnetic rack for 3 minutes to allow the beads to separate from the solution.
3. Slowly aspirate the 50 µL elution and **transfer to the second set of 1.7 mL tubes with magnetic beads.** Pipet-mix 15 times.

\*\*Proceed with the double purification by repeating steps **3-7**, then move to step **11.**

1. Remove the sample tube from the rack and re-suspend the magnetic bead pellet in **24 µL sterile/DNase-free water** by pipet-mixing 15 times. Close the tube cap and place back onto the magnetic rack for **3 minutes** to allow the beads to separate from the solution.
2. Transfer the **23.5 µL purified cDNA** elution to the 0.6 mL PCR tubes and proceed with the PCR1 reaction.

**PCR 1: PCR clean room and main lab room**

Prepare First-Round PCR Reactions using the KAPA 2G Robust Hotstart Kit

1. Remove the following reagents from -20C storage and thaw completely.
2. Leave the KAPA 2G Robust polymerase at -20C until ready to use.

|  |  |  |  |
| --- | --- | --- | --- |
| **KAPA Kit** | **Reagent**  | **Heat block, 32C** | **Room temperature** |
| KAPA 2G Robust | 5x Buffer A, KAPA 2G Robust | ✓ |   |
| KAPA 2G Robust | dNTP Mix | ✓ |   |
| KAPA 2G Robust | Enhancer |  | ✓ |
|  | Universal Adapter (ADPT\_2A) |   | ✓ |
|  | PCR1-Forward Primer mix |  | ✓ |

Reagents may be thawed at room temperature or using a low-temperature heat block, but it is recommended to keep them cold/on ice while handling.

1. Pulse-vortex the **5x Buffer A** and **dNTPs** 2 times to homogenize. Briefly spin down the 5x buffer, Enhancer, dNTP mix and 2A Adapter via. mini centrifuge before use.
2. Use a new 1.7 mL tube to prepare the **PCR1 master mix**.
3. Add the following reagents to the master mix tube in the order listed. The volume of each reagent needed per sample and total master mix volume calculations are included below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **REAGENT** | **Stock Conc.**  | **Final Conc.** | **Master Mix Volume (µL)\*** | Volume per sample (µL) |
| 5x Buffer A  | 5x | 1x | 260 | 10.0 |
| Enhancer  | 5x | 1x | 260 | 10.0 |
| dNTP mix | 10 mM | 0.2 mM | 26 | 1.0 |
| PCR1-Forward Primer mix | 10 µM | 0.5 µM | 65 | 2.5 |
| 2A Adapter (ADPT\_2A) | 10 µM | 0.5 µM | 65 | 2.5 |
| KAPA 2G Robust polymerase  | 5 U/µL | 2.5 U | 13 | 0.5 |

\* Master mix volume calculation is sufficient for 25 reactions with 1% overage for pipet error. Return all reagents and enzyme to -20C storage immediately after use.

1. Mix the PCR1 master mix by pulse-vortexing 2 times. Spin down using mini centrifuge.
2. Aliquot **26.5 µL** of **PCR1 master mix** to each purified cDNA reaction and pipet mix 15 times.
3. Transfer PCR1 reactions to a tube rack located outside the PCR clean room. Return PCR1 reagents to -20C storage, clean the hood and take PCR1 reactions to the **main lab room.**
4. **Place PCR1 reactions on a thermal cycler under the following conditions:**

|  |  |
| --- | --- |
| 95˚C | 1 min |
|  |  |
| 95˚C | 15 s |
| 58˚C | 1 min |
| 72˚C | 30 s |
| 25 cycles |
|  |
| 72˚C | 3 min |
| 4˚C | On hold |

**PCR 1 Purification: Main lab room**

Create 1 mL Aliquots of Ampure XP Magnetic Beads

1. Take out 40 mL bottle of Ampure XP Magnetic Beads. Vortex thoroughly to resuspend beads into the solution. Ensure the solution is homogenous and there are no clumps of beads left in the bottle.
2. Using a repeater pipette, aliquot **1.0 mL** the magnetic bead solution into sterile 1.7 mL tubes for later use. Store beads at 4C until ready for use.

PCR1 Purification using Ampure XP Bead Aliquots

1. Remove a **1 mL** aliquot of Ampure XP magnetic beads from 4C storage. Allow to sit at room temperature for **30 minutes** before use.
2. While the beads are thawing, label a set of sterile 1.7 mL tubes.
3. Create a fresh **50 mL** aliquot of **70% Ethanol** in a sterile conical tube.
4. After 30 minutes at room temperature, resuspend the beads by **vortexing**. Ensure that there are no clumps of beads left in the tube before using.
5. Transfer **40 µL** of magnetic bead solution to each 1.7 mL tube. Transfer all 50 µL of each PCR1 reaction to the 40 µL magnetic bead aliquots and mix by pulse-vortexing 2 times.
6. Incubate the samples at room temperature for **5 minutes**, then place on the magnetic rack for **5 minutes**.
	1. Ensure each sample tube is pressed firmly against the magnetic rack.
	2. Check to make sure all beads have been collected against the side of the sample tube in a pellet before proceeding.

\*\*Seps 7-10 should be performed **while the tubes are on the magnetic rack**.

1. Open each sample tube by holding the tube against the rack and gently lifting the lid. Avoid disturbing the magnetic beads.
2. Slowly aspirate the cleared solution from the tube and discard. **This should be done while the tube is still in the magnetic rack.**
3. Dispense **400 µL** of 70% ethanol into each sample tube. Incubate at room temperature for 30 seconds, then remove ethanol and discard.
	1. Repeat step **5**.
4. Use a pipet to remove any trace amounts of ethanol left in the bottom of the sample tubes.
5. Allow the beads to dry with the tube cap left open until the last visible traces of ethanol has disappeared. **This should take** **up to 10 minutes maximum.**
	1. Over-drying the beads can result in lower recovery when performing the elution step.

1. Remove the sample tube from the rack and re-suspend the magnetic bead pellet in **50 µL sterile/DNase-free water** by pipet-mixing ~15 times. The solution should be homogenous with no bead clumps.
	1. Over-dried beads will not always fully re-suspend, and there will be small clumps of beads left in solution.
2. Once re-suspended, close the tube cap and place back onto the magnetic rack for 3 minutes to allow the beads to separate from the solution.
3. Slowly aspirate the **45 µL** elution and **transfer to a clean set of 1.7 mL tubes**.
4. Proceed to PCR2, or store PCR1 products at -4C for up to 24 hours, then transfer to -20C for long-term storage.

**PCR 2: PCR clean room and main lab room**

Prepare Second-Round PCR Reactions Using the KAPA HiFi Hotstart Kit

Return to the **PCR clean room** to create the PCR2 master mix:

1. Remove the following reagents from -20C storage and thaw.
2. Leave the KAPA HiFi Hotstart polymerase at -20C until ready to use.

|  |  |  |  |
| --- | --- | --- | --- |
| **KAPA Kit** | **Reagent**  | **Heat block, 32C** | **Room temperature** |
| KAPA HiFi | 5x Buffer, KAPA HiFi | ✓ |   |
| KAPA HiFi | dNTP Mix | ✓ |   |
|  | Universal Adapter aliquots (ADPT\_P1) |   | ✓ |
|  | Indexed Adapter aliquots (1-24) |  | ✓ |

Reagents may be thawed at room temperature or using a low-temperature heat block, but it is recommended to keep them cold/on ice while handling.

1. Pulse-vortex the **5x Buffer** and **dNTPs** 2 times to homogenize. Briefly spin down the 5x buffer, dNTP mix and P1 adapter via. mini centrifuge before use.
2. Label a set of 0.6 mL PCR tubes, including the indexed adapter number for each sample tube.
3. Use a new 1.7 mL tube to prepare the **PCR2 master mix.**
4. Add the following reagents in the order listed. The volume of each reagent needed per sample and total master mix volume calculations are included below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **REAGENT** | **Stock Conc.** | **Final Conc.** | **Master Mix Volume (µL)\*** | Volume per sample (µL) |
| Water, sterile |  |  | 377 | 14.5 |
| 5x HiFi Buffer  | 5x | 1x | 130 | 5.0 |
| dNTP mix | 10 mM | 0.4 mM | 26 | 1.0 |
| P1 Adapter | 10 µM | 0.4 µM | 26 | 1.0 |
| KAPA HiFi Polymerase | 1 U/µL | 0.5 U | 13 | 0.5 |

\*Master mix volume calculation is sufficient for 25 reactions with 1% overage for pipet error. Return all reagents and enzyme to -20C storage immediately after use.

Mix the PCR2 master mix by pulse-vortexing 2 times. Spin down using mini centrifuge.

1. Aliquot **22 µL** of the master mix to each 0.6 mL PCR tube using a repeater pipette.
2. Add **1 µL** of indexed adapter (1 µM) to each 0.6 mL PCR tube containing the master mix.
3. Place PCR2 master mix aliquots in a clean rack and move them to the **main lab room**.
4. Add **2 µL** of PCR1 product to each PCR2 master mix aliquot. Thoroughly mix the PCR2 reactions by increasing pipet volume to **20 – 22 µL** and pipet mixing **15 times**.
5. **Place PCR2 reactions on a thermal cycler, set to the following conditions:**

|  |  |
| --- | --- |
| 95˚C | 2 min |
|  |  |
| 98˚C | 20 s |
| 63˚C | 15 s |
| 72˚C | 30 s |
| 25 - 35 cycles |
|  |
| 72˚C | 3 min |
| 4˚C | On hold |

**PCR 2 Test Gel Imaging: Main lab room**

Record PCR2 Product Amplification via. Carestream Gel Imaging Software

1. Prepare a 1% agarose gel using 1X TAE with 0.001% Ethidium Bromide and 16-well combs.
2. Transfer gel to a clean electrophoresis box and fill with 1X TAE just until the agarose gel has been covered.
3. In a 96-well assay plate, mix 2 µL of 5X loading dye and 2 µL of PCR2 products.
4. Add a 1kb ladder to the first and/or last well(s) of each row on the gel. Transfer 2 µL of each reaction to separate wells in the test gel.
5. Run the 1% agarose gel for **30 minutes at E = 4 V/cm**.
6. Transfer the test gel to a gel imager.
7. Validate that the positive and negative controls are correct:
	1. Negative control should have absolutely **NO** amplification besides the bottom primer dimer band, well below 500/250 kb.
	2. Positive control/QC should have the target sequence band(s) appear in the 500-600 bp range, validated by comparison via. the 1kb ladder.
8. Save a digital copy of the test gel and print a physical copy to attach to lab notebook.

**Gel Extraction Part 1: Main lab room**

Extract PCR2 Products from Agarose Gel

1. Prepare a 1.2% agarose extraction gel using 1X TAE with 0.001% Ethidium Bromide and 12-wide well combs.
2. Transfer gel to a clean electrophoresis box and fill with 1X TAE just until the agarose gel has been covered.
3. Add **4 µL** of loading dye to each PCR2 reaction tube and pipet mix.
4. Add a 1kb ladder to the first and/or last well(s) of each row on the gel. Transfer all remaining volume of each reaction to separate wells in the extraction gel.
5. Run the extraction gel for **30 minutes at E = 4 V/cm.**
6. Remove the gel and transfer to a blue-light transilluminator.
7. Using a clean scalpel blade, physically remove the target sequence band(s) for each sample from the gel and place into separate, labeled sterile 1.7 mL tubes.
	1. The target band(s) should be in the 500-600 bp range when compared to a 1 kb ladder and will appear as a bright single or double band.
	2. Some samples will not amplify as well as the positive control. Even if no clear bands are seen, excise the gel fragment where the target **should** appear. Use the positive control and ladder as guides.
	3. The negative control, once validated as having no contamination, does not need to be excised and can be removed from the run from this point onward.
8. Proceed with the MinElute Gel Extraction or store excised gel fragments at 4C for up to 48 hours.

**Gel Extraction Part 2: Main lab room**

QIAGEN MinElute Gel Extraction

1. Prepare and label the following before continuing:
	1. One set of MinElute spin columns
	2. One set of 1.7 mL tubes
	3. Three sets of Qiagen 2 mL collection tubes
2. Weigh the gel fragments. Add a **3X volume** (microliters**) Buffer QG** to **mass** (grams) of **gel fragment**. An example conversion is found below:

**0.6 mL** (Buffer QG): **0.20 g** (gel fragment)

1. Incubate at 50 ˚C for 10 minutes to completing dissolve. Vortex every 2-3 minutes to help dissolve.
	1. Check the color of gel solution (should be yellow, otherwise add 10 µl 3M sodium acetate).
2. Add total volume of dissolved gel fragment to the MinElute spin column.
	1. Centrifuge for 1 minute.
	2. Transfer the MinElute column to a new collection tube, discarding the filtrate.
3. Add **500 µl buffer QG** to the MinElute spin column.
	1. Centrifuge for 1 min.
	2. Transfer the MinElute column to a new collection tube, discarding the filtrate.
4. Add **700 µL** of **buffer PE** to the spin column and incubate for 5 minutes at room temperature.
	1. Centrifuge for 1 minute.
	2. Transfer the MinElute column to a new collection tube, discarding the filtrate.
5. Centrifuge the spin column **without any buffer** for additional 3 minutes.
6. Place the MinElute spin columns into the labeled 1.7 ml tubes.
7. Add **10 µl buffer EB** to the spin column. Incubate at room temperature for 4 minutes.
	1. Centrifuge for 2 minutes.
	2. Discard the spin column and close the 1.7 mL tube, now containing concentrated, purified DNA.
8. Proceed with the QuBit concentration assay. If DNA needs to be stored, keep at 4C for up to 24 hours, then transfer to -20C for extended storage.

**Quantification and Pooling: Main lab room**

Quantify Library Concentrations using QuBit DS-BR Assay Kit

1. Full methods for using the QuBit DS-BR Assay Kit can be found at the following link:

<https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_BR_Assay_UG.pdf>

1. Remove QuBit BR standards 1 and 2 from 4C storage and allow to thaw to room temperature for 30 minutes.
2. While standards are thawing, label a set of 0.6 mL PCR tubes: One for each sample, and one for each QuBit BR standard.
	1. Ensure the PCR tubes are clear, thin-walled, and unfrosted (no tube label on the side).
3. Make a 1:200 master mix of BR assay reagent to BR assay buffer for each sample. Calculations for 25 sample tubes + ~.9% overage is below:

22 samples + QC + 2 standards = 25 tubes 🡪 28 µL BR Reagent: 5574 µL BR Buffer

1. Pulse-vortex the master mix 3 times.
2. Aliquot 190 µL of master mix to the 0.6 mL tubes labeled for standard 1 and standard 2.
3. Aliquot 199 µL of master mix to each sample tube.
4. Add 10 µL of standard 1 and 2 to their respective tubes, then vortex to mix.
5. Add 1 µL of purified DNA libraries to each sample tube. Mix all tubes by pulse-vortexing 2 times.
6. Read the concentrations of standard 1 and 2 first. Ensure standard range is correct:
	1. Standard 1: 250 – 350
	2. Standard 2: 25000 – 35000
7. Read and record the stock concentrations of each sample (ng/µL) and record in **Appendix IV: Library pooling and submission sheet**.
	1. Libraries with concentrations below 5 ng/µL should be marked as failed and excluded from the library pool.

Pool Purified Libraries in Equal Concentrations

1. Appendix IV contains an excel spreadsheet which will automatically calculate the necessary volumes of each sample to pool together based on the concentrations inputted into the spreadsheet.
	1. Record the concentration of each purified library under the “Concentration” column.
	2. Once the spreadsheet is filled, the “µL to Use” column will show the correct volume needed to pool all samples in equal concentration.
2. Label a new, sterile, low-binding 1.7 mL tube with the name of the library pool.
3. Carefully add the calculated volume for each purified library to the new 1.7 mL tube.
4. Proceed to the final pooled library purification step or store the pooled library at 4C for up to 24 hours, then freeze at -20C for long-term storage.

**Final Purification: Main lab room**

Purify the Pooled Library Using Ampure XP Magnetic Beads

1. Perform a magnetic bead purification using AMPure XP beads as detailed under “PCR1 Purification using Ampure XP Bead Aliquots”.**\***

**\*DNA to beads ratio: 1 to 0.6** (microliters). Elute in **20** to **30** µL of **elution buffer** (Qiagen MinElute Gel Extraction Kit).

**Final Quantification: Main lab room**

Calculate Average Fragment Size using the Agilent Tapestation/D5000 Reagent Kit

1. Remove **D5000 screentape**, **D5000 reagent** and **ladder** from 4C storage and allow to adjust to room temperature for **30 minutes**.
2. Using an 8-well optical tube strip, add **10 µL** of D5000 reagent to the first and second wells.
	1. The top well will be used for the ladder, and second well is for the pooled library.
3. Add **1 µL** of ladder to the first well, and **1 µL** of purified library to the second well.
4. Place cap on the tube strip and label the ladder and purified library wells.
5. Vortex at 2000 rpm for 1 minute, then centrifuge briefly.
6. Turn on the Agilent Tapestation and load Tapestation controller software.
7. Load the Tapestation:
	1. Open the Tapestation lid and **add the** **tube strip to row A**, located next to the tip cartridge.
	2. Remove the tube strip cap.
	3. Add the D5000 screentape to the Tapestation, behind the tip cartridge and tube strip. The **QR code located on the screentape should be oriented to the** **bottom right**, **facing away from you**. A sensor in the Tapestation will scan the QR code.
	4. Check the tip cartridge and ensure it is fully stocked. If tips are missing, replace them.
	5. Close the lid.
8. In the Tapestation controller software:
	1. Use the cursor to **select row A** **and B of column 1** by left-clicking the icons.
	2. Verify at the bottom of the screen that the D5000 screentape has been scanned and has not expired (green checkmark).
	3. Select the “Start” button at the bottom right of the screen to begin the run.
9. Once the run is complete, load the Agilent Tapestation analysis software.
	1. Select **“Region”** in the **Home tab**, located in the top left under “View”.
	2. Select the column with the pooled library. Ensure there are no primer dimer peaks, usually located at the lower standard around 150-250 bp.
	3. Right-click on the graph and select **“Add Region”.** Use the mouse to scroll the upper and lower bounds to trim the region and include only the peak from the pooled library.
	4. If you have two peaks, select the smaller fragment size (the left peak).
	5. Record the average fragment size, located underneath the region graph.
10. Remove the optical tube strip and screentape. Replace any tips used and discard waste.