Outline of Steps (per 96-well Sample Plate)

1. Sample Receipt & Aliquot (~20 minutes)
2. RNA Extraction (~2 hours and 15 minutes)
    a. User setup (45 minutes)
    b. Method (1 hour 30 minutes)
3. RT and cDNA generation (~1 hour and 20 minutes)
    a. User setup (35 minutes)
    b. Method (25 minutes)
    c. Thermocycler Run Time (20 minutes)
4. Target Amplicon generation (~5 hour and 10 minutes)
    a. User setup (20 minutes)
    b. Method (50 minutes)
    c. Thermocycler Run Time (4 hours)
5. QC Checkpoint: Gel Electrophoresis (~2 hours and 20 minutes)
    a. User Setup (40 minutes)
    b. Electrophoresis Run Time (1 hours and 40 minutes)
6. Ampure XP Bead Clean-up (~2 hours)
    a. User setup (45 minutes)
    b. Method (1 hr 15 minutes)
7. Sample Dilution (~25 minutes)
    a. User setup (5 minutes)
    b. Method (20 minutes)
8. Library Preparation (~4 hours and 15 minutes)
    a. User setup (45 min)
    b. Method (3 hours and 30 minutes)
9. Quantification (~1 hour and 10 minutes)
    a. User Setup (30 minutes)
    b. Method (30 minutes)
    c. Plate Reader Run Time (10 minutes)
10. Sample Dilution (~25 minutes)
    a. User setup (5 minutes)
    b. Method (20 minutes)
11. Pooling (~30 minutes)
    a. User Setup (5 minutes)
    b. Method (30 minutes)

Sample Receipt & Aliquot

Hands on time: 20 min.

Materials Needed

- ☐ Barcoded Sterile 96-DeepWell plate

- [ ] Bucket of ice

User Preparation

1. Thaw inactivated sample plate if needed on ice, and transfer 200uL from each well into a barcoded sterile 96-DeepWell Plate. This is your **RNA Sample Plate (96w Deep Well Plate)**
2. Keep on ice until ready to process through the RNA Extraction method, or store sealed at -80C.

RNA Extraction

Total time: 2 hrs 15 min.
Hands on time: 45 min.

Materials Needed

- [ ] **RNA Sample Plate (96w Deep Well Plate)**
- [ ] MagMAX™-96 Viral RNA Isolation Kit
  - [ ] MagMAX™ Viral/Pathogen Proteinase K
  - [ ] MagMAX™ Viral/Pathogen Binding Solution
  - [ ] MagMAX™ Viral/Pathogen Wash Solution
  - [ ] MagMAX™ Viral/Pathogen Elution Solution
  - [ ] MagMAX™ Viral/Pathogen
- [ ] Corning® 50 mL PP Centrifuge Tubes (2)
- [ ] 300mL ThermoFisher Scientific Robotic Reservoirs (6)
- [ ] 60mL Hamilton Reagent Reservoir
- [ ] CO-RE 1000uL filtered Hamilton tip sets (6)
- [ ] CO-RE 300uL filtered Hamilton tip sets (4)
- [ ] Barcoded Sterile 96-DW plate
- [ ] Applied Biosystems 96-well Microtiter Plate (1)
- [ ] Molecular Biology Grade 200 proof Ethanol
- [ ] DNase/RNase Free Water (not DEPC-Treated)
- [ ] Alpaqua Magnum FLX Magnet Plate

User Preparation (~45 minutes)

1. Clean benchtop and Hamilton robot with DNase Away, RNase Away, and 70% ethanol.
2. Add 5uL of proteinase K to each sample.
3. Prepare reagent troughs and reservoirs for each of the following reagents:
   Reference: Automated_RNA_Extraction_(MagMax_Kit)_Reagent_Preparation.pdf

a. **Binding Bead Mix**
   i. Vortex or invert MagMAX™ Viral/Pathogen Binding Beads to ensure that the bead mixture is homogeneous.
   ii. Prepare the Binding Bead Mix according to the table and instructions below. Mix well by inversion, and then store at room temperature until needed.

1. You will need a total of **2** Binding Bead Mix Conical Tubes for 1 96-well plate of samples.
2. Prepare the Binding Bead Mix in a 50 mL Centrifuge Tube as follows, and then dump both tubes into the labeled 300mL Robotic Reservoir to achieve 66mL in total:

Component	**Total Volume in Each 50mL Conical**
MagMAX™ Viral/Pathogen Binding Solution	31.8 mL
MagMAX™ Viral/Pathogen Binding Beads	1.2 mL
Total Volume per 50mL Conical	33 mL
Total Volume added to Robotic Reservoir	66mL

b. Wash Buffer

i. Measure out 68mL of the MagMAX™ Viral/Pathogen Wash Solution into a labeled 300mL Robotic Reservoir.

c. Elution Buffer

i. Measure out 8.3mL of the MagMAX™ Viral/Pathogen Elution buffer into a labeled 60mL Reagent Reservoir.

d. Nuclease Free Water

i. Measure out 87.2mL of DNase/RNase Free Water into a labeled 300mL Robotic Reservoir.

e. 80% Ethanol

i. Prepare a total of three 80% ethanol troughs required using Ethanol, Absolute, Molecular BIology Grade, and Nuclease-free water troughs using three 300mL Robotic Reservoirs
   1. First Wash - **Reservoir 1**: 68 ml
   2. Second Wash - **Reservoir 2**: 68 ml
   3. Third Wash - **Reservoir 3**: 88 mL

Automated Method (~1.5 hrs)

4. Load ***COVID-19 Extraction v1.2.0_DAMP*** method, and follow user prompts to set up the Hamilton deck as follows:
   a. Ensure liquid and sharp carboys are less than half full.
   b. Input user initials
   c. Input '**1**' into value box for 'Number of plates'
   d. Select '**Autoload Scan**' or '**Manual Scan**' depending on if the deck is equipped with an autoload or not. If 'Manual Scan' is selected, you will have to individually

scan each barcode for every set of tips and every plate into the designated text box. If 'Autoload Scan' is selected, keep carriers on the autoload deck and the autoload will pull in and scan the carriers.

e. Check only the box next to HHS 1.
f. In Site 2 of Track -4-1, load one sterile 96-well DeepWell plate and then carefully place a rack of CO-RE 1000uL filtered Hamilton tips inside so each tip is inside one well of the 96-well plate.
g. Use the dialog box to drag and highlight the number of CO-RE 1000uL filtered Hamilton tips available in Site 1 of Track 33-38. Scroll to zoom if needed. If first time running the method or there are no tips already present in Site 1, load a fresh rack of CO-RE 1000uL filtered Hamilton tips and highlight all tips.
h. Load 4 sets of CO-RE 1000uL filtered Hamilton tips in Sites 2-5 of Track 33-38.
i. In Sites 2-5 of Track 39-44, load four sets of CO-RE 300uL filtered Hamilton tips.
j. In Site 2 of Track 2-7, ensure there is an Alpaqua Magnum FLX Magnet Plate
k. In Site 2 of Track 27-32, load one empty sterile 96-well Microtiter plate. A1 should be in the back left corner. This will be your final **96-well RNA Elution Plate.**
l. Enter lot numbers of all kit reagents.
m. In Track 21-26, load the troughs in the Sites 1-5 respectively:
    i. **Binding Bead Mix**
    ii. **Wash Buffer**
    iii. **80% Ethanol Reservoir 1**
    iv. **80% Ethanol Reservoir 2**
    v. **80% Ethanol Reservoir 3**
n. In Site 5 of Track 45, load the **Elution Buffer** reservoir. Ensure to uncap the reservoir.
o. In Site 1 of Track 15-20, load the **Nuclease Free Water** trough
p. In Site 2 of Track 15-20, load your **RNA Sample Plate (96w Deep Well Plate).** A1 should be in the back left corner.

5. Method will now begin, est. runtime 1 hour, 30 minutes.
6. Once finished, a textbox will popup stating the method's completion.
7. Clean-up deck & immediately place the final **96-well RNA Elution Plate** on an isofreeze or on ice.

cDNA Generation

Total time: 1 hr.
Hands on time: 35 min.
Equipment run time: 20 min.

Materials Needed

- ☐ **96-well RNA Elution Plate** (from Step 7)
- ☐ LunaScript RT Super Mix
- ☐ DNase/RNase Free Water
- ☐ Applied Biosystems 96-well Microtiter Plates (2)
- ☐ Fisherbrand 2mL Microcentrifuge Tubes

- [ ] 96-well Thermal Cycler
- [ ] ThermoFisher Adhesive PCR Plate Seals
- [ ] Bucket of ice
- [ ] CO-RE 50uL filtered Hamilton tips (2)

User Preparation (~30 min)

1. Spin down sealed purified RNA elution plate (from Step 6). Keep on ice. This is your **Sample Plate**.
2. Make the master mix using the 20% overage column in the following table in a 2mL tube. Keep on ice.
    a. RT / cDNA Mastermix Calc

	Per reaction	96 samples	Hamilton 20% overage
DNase/RNase Free Water	11 uL	1056 uL	1267.2 uL
LunaScript RT Super Mix	4 uL	384 uL	460.8 uL
Total	15 uL	1440 uL	1728 uL

3. In a sterile 96-well PCR plate, aliquot 216uL of the master mix into the first column's eight wells (A1-H1). Seal, and spin down. Keep on ice. This is the **Reagent Plate**.

Automated Method (~20 min)

1. Load ***cDNA_Generation_V1.0.0*** and follow user prompts to set up the Hamilton deck.
2. In the first user prompt, enter the following:
    a. 'How many samples are you processing?' : '**96**'
    b. 'What is the total reaction volume' : '**20**'
    c. What is the volume of RNA/Sample being added to each well : '**5**'
    d. 'What is the volume of Mastermix being added to each well?' : '**15**'
3. Load two racks of CO-RE 50uL filtered Hamilton tips onto the deck as prompted.
4. Load the **Reagent Plate**, **Sample Plate**, and an empty sterile 96-well PCR plate onto the deck as prompted. The empty sterile 96-well PCR plate will be your **cDNA Plate**, which will have the sample and mastermix combined.
5. Run method.
6. Once method is complete, load the **cDNA Plate** onto a 96-well thermal cycler using the following conditions:

Step	Temperature (˚C)	Time (minutes)
1. Primer annealing	25	2
2. cDNA synthesis	55	10
3. Heat inactivation	95	1
4. Hold	4	Hold

7. Clean up the deck, and store the **Sample Plate** in -80C.

🛑 SAFE STOPPING POINT: can safely store **cDNA Plate** plates in -20C 🛑

Amplicon Generation

Total time: 5 hours 10 min.
Hands on time: 20 min.

Materials Needed

- ☐ **cDNA Plate**
- ☐ NEB Q5® Hot Start High-Fidelity 2X Master Mix
- ☐ ARTIC SARS-CoV-2 Amplicon Panel
- ☐ DNase/RNase Free Water
- ☐ Eppendorf Tubes 5.0mL, screw cap (2)
- ☐ Applied Biosystems 96-well Microtiter Plates (2)
- ☐ 96-well Thermal Cyclers (2)
- ☐ ThermoFisher Adhesive PCR Plate Seals
- ☐ Bucket of ice
- ☐ CO-RE 50uL filtered Hamilton tips (2)

User Preparation (~20 min)

1. Label two sterile 5mL Eppendorf tubes “Pool 1” and “Pool 2,” and place on ice.
2. Make the Pool 1 and Pool 2 master mixes, following the Hamilton 20% overage column for each Pool as follows:

	Per reaction (uL)	96 samples (uL)	Hamilton 20% overage (uL)
NEB Q5® Hot Start High-Fidelity 2X Master Mix	12.5 uL	1200 uL	1440 uL
ARTIC SARS-CoV-2 Amplicon Panel **Pool 1** or **Pool 2**	2.5 uL	480 uL	576 uL
Nuclease-free water	5 uL	240 uL	288
Total (uL)	20 uL	1920 uL	2304

3. In a sterile 96-well Microtiter plate, aliquot 288uL of the Pool 1 master mix into the first column’s eight wells (A1-H1). In the same 96-well Microtiter plate, aliquot 288uL of the Pool 2 master mix into the last columns eight wells (A12-H12). Seal, and spin down. Keep on ice. This is your **Reagent Plate.**

Automated Method (~50 min)

8. Load ***Amplicon_Generation_Connor_V1.0.0*** and follow user prompts to set up the Hamilton deck.
9. In the first user prompt, enter the following:
    a. 'How many samples are you processing?' : '**96**'
    b. 'What is the total reaction volume' : '**25**'
    c. What is the volume of RNA/Sample being added to each well : '**5**'
    d. 'What is the volume of Mastermix being added to each well?' : '**20**'
10. Load two racks of CO-RE 50uL filtered Hamilton tips onto the deck as prompted.
11. Load the **Reagent Plate**, **cDNA Plate** ('Sample Plate'), and an empty sterile 96-well PCR plate onto the deck as prompted. The empty sterile 96-well PCR plate will be your **Pool 1 Amplicon Plate**, which will have the sample and mastermix combined. Orient all plates so that A1 is in the top left corner.
12. Run method, est. run time ~25 minutes.
13. Once method is complete, load the **Pool 1 Amplicon Plate** onto a 96-well thermal cycler using the following conditions:

Step	Temperature (˚C)	Time
1. Hot-start	98	30 sec
2. Denaturation	98	15 sec
3. Anneal and extend	65	5 min
4. Cycle	Repeat steps 2-3 for a total of 40 cycles	
5. Hold	4	Hold

14. Exit method, but do not take off the **cDNA Plate** or the **Reagent Plate**. Reload Amplicon_generation_Connor_V1.0.0. Follow user prompts again to set up the Hamilton deck.
    a. 'How many samples are you processing?' : '**96**'
    b. 'What is the total reaction volume' : '**25**'
    c. What is the volume of RNA/Sample being added to each well : '**5**'
    d. 'What is the volume of Mastermix being added to each well?' : '**20**'
15. Load two racks of CO-RE 50uL filtered Hamilton tips onto the deck as prompted.
16. Load an empty sterile 96-well PCR plate onto the deck as prompted. The empty sterile 96-well PCR plate will be your **Pool 2 Amplicon Plate**.
17. Re-orient only the **Reagent Plate** so H12 is in the top left, to use the Pool 2 Master Mix.
18. Run method, est. run time ~25 minutes.
19. Once the method is complete, load the **Pool 2 Amplicon Plate** onto another thermal cycler using the same conditions as Step 13.
20. Clean the deck, and store **cDNA Plate** in -20C for long term storage.

🛑 SAFE STOPPING POINT: can safely store **Pool 1 Amplicon Plate** & **Pool 2 Amplicon Plate** at 4C overnight 🛑

QC Gel Electrophoresis (Optional)

Total time: 2 hr 20 min.

Materials Needed

- ☐ **Pool 1 Amplicon Plate** & **Pool 2 Amplicon Plate**
- ☐ Agarose powder

- [ ] 50x TAE Buffer
- [ ] SYBR Safe DNA Gel Stain
- [ ] 1kb Plus DNA Ladder
- [ ] 6X Loading Dye
- [ ] DI water
- [ ] Applied Biosystems 96-well Microtiter Plate (1)
- [ ] 25-well 1.5mm gel combs (8)
- [ ] Large gel cassettes (2)
- [ ] Casting tray (2)
- [ ] Gel electrophoresis chamber (2)
- [ ] Gel electrophoresis power box (1)
- [ ] Sterile 500mL flask (1)
- [ ] Sterile 1L screw-cap bottle (1)

User Preparation (40 min hands-on)

1. Create 1x TAE Buffer by combining 1960mL of DI water with 40mL 50x TAE Buffer in a sterile 1L bottle.
2. In a 500mL flask, measure out 200mL of 1x TAE Buffer. Set aside.
3. Using a weighboat and scale, first tare out the boat and then measure out 2.5g of agarose powder. Pour into the 500mL flask with the 1x TAE Buffer. Swirl to mix.
4. Microwave using 30 second intervals until agarose is completely dissolved.
5. Add 20uL of SYBR Safe DNA Gel Stain into the flask (1:10000 dilution). Swirl to mix until light pink in color.
6. Set the two large gel cassettes into the casting tray side by side, keeping the rubber ends flush with the top and bottom sides of the tray. The first slot for the comb should be at the top.
7. Pour and split the 200mL from the flask evenly between the two cassettes. Pop any bubbles.
8. Place four 25-well 1.5mm gel combs into the designated slots of the two cassettes, casting 100 wells in each gel.
9. Let set on the benchtop for 1 hour.
10. While setting, load the two gel chambers with 1X TAE Buffer until the designated fill line.
11. In a sterile 96-well Microtiter plate, aliquot 0.5uL of 6X loading dye and 2.5uL of sample from the Pool 1 Amplicon plate into each well. Mix well, seal, spin down, & set aside.
12. Repeat Step 11 in a separate 96-well PCR plate, using the Pool 2 Amplicon plate for sample input.
13. Once the gels are finished setting, place into chambers and carefully pull out the combs.
14. In the first well of each 25-well row, load 5uL of the 1kb Plus DNA Ladder. In total, there will be four ladders per gel.
15. In one gel, load 3uL of the Pool 1 Amplicons mixed with the 6X loading dye in each well, going down the columns sequentially.
16. Repeat for the Pool 2 Amplicons mixture in the other gel.

17. Store the **Pool 1 Amplicon Plate** & **Pool 2 Amplicon Plate** in the 4C until downstream steps, or in the -20C for longer term storage.
18. Secure the electrode covers for both gels and attach to the power box, with the negative electrode at the top of the gel.
19. Run at 130V for 40 minutes.
20. When finished, image both gels on a gel imager. Ensure bands are around the 300-400bp mark.

Amplicon Cleanup

Total time: 1 hour 15 min.
Hands on time: 30 min.

Materials Needed

- ☐ **Pool 1 Amplicon Plate** & **Pool 2 Amplicon Plate**
- ☐ Ampure XP Beads
- ☐ Molecular Biology Grade 200 proof Ethanol
- ☐ DNase/RNase Free Water
- ☐ Magnetic Stand (24-well) with 3D printed adaptor (File: "**Magnet_Riser v2.step**")
- ☐ Sterile screw-cap 100mL media bottle
- ☐ 2mL screw-cap microtubes (2)
- ☐ 60mL Hamilton Reagent Reservoir
- ☐ 12-Channel Reservoirs for Automation
- ☐ Abgene™ 96 Well 0.8mL Polypropylene DeepWell™
- ☐ Barcoded Sterile 96-DW plate
- ☐ Applied Biosystems 96-well Microtiter Plate (2)
- ☐ CO-RE 50uL filtered Hamilton tips (6)
- ☐ CO-RE 300uL filtered Hamilton tips (4)
- ☐ CO-RE 1000uL filtered Hamilton tips (1)

User Preparation (~30 min)

1. Allow the Ampure XP Beads to thaw at room temperature for 30 minutes prior to use.
2. Make a 60mL working stock of 80% ethanol by combining 40mL of Molecular Biology Grade 200 proof Ethanol with 20mL of DNase/RNase Free Water in a sterile screw-cap 100mL media bottle. Mix by inversion.
3. In a 60mL Hamilton Reagent Reservoir, transfer 52.2mL of 80% ethanol from the working stock.
4. Once thawed, thoroughly vortex the beads and aliquot 1750uL into two 2mL screw-cap microtubes.
5. In Column 3 of a 12-channel robotic reservoir, transfer 9,480uL of DNase/RNase Free Water.
6. Spin down **Pool 1 Amplicon Plate** & **Pool 2 Amplicon Plate**

Automated Method (~45 minutes)

1. Load ***Amplicon_cleanup_v1.1.1*** method, and follow user prompts to set up the Hamilton deck as follows:

a. Set the Number of Columns to '**12**', Pool 1 Volume (uL) to '**22**', Pool 2 Volume (uL) to '**22**', and Bead Volume (uL) to '**35**'
b. Load 5 sets of CO-RE 50uL filtered Hamilton tips in Sites 1-5 of Track 6-11
c. Load 4 sets of CO-RE 300uL filtered Hamilton tips in Sites 1 and Sites 3-5 of Track 12-17
d. Load 1 set of CO-RE 50uL filtered Hamilton tips in Site 2 of Track 12-17
e. Load 1 set of CO-RE 1000uL filtered Hamilton tips in Site 1 of Track 12-17
f. In Site 3 of Track 18-23, load the **Pool 1 Amplicon Plate**
g. In Site 4 of Track 18-23, load the **Pool 2 Amplicon Plate**
h. Load an empty Applied Biosystems 96-well Microtiter plate in Site 5 of Track 18-23
i. Load a Barcoded Sterile 96-DW ('DW Plate') in Site 1 of Track 24-29
j. Load the 12-Channel Reservoir for Automation ('12-well DW Plate') with DNase/RNase Free Water in Site 2 of Track 24-29
k. Load an Abgene™ 96 Well 0.8mL Polypropylene DeepWell™ ('MIDI Plate') in Site 3 of Track 24-29
l. In Track 31-37, load an empty Applied Biosystems 96-well Microtiter plate in Site 4 ('Empty PCR') on top of a Hamilton CPAC. This will be your **Cleaned Amplicons Plate**.
m. In Track 31-37, place the 24-well magnetic stand equipped with the 3D printed adaptor in Site 5.
n. Use the dialog box to drag and highlight the number of CO-RE 1000uL filtered Hamilton tips available in Site 1 of Track 12-17. Scroll to zoom if needed. If first time running the method or there are no tips already present in Site 1, load a fresh set and highlight all tips.
o. Use the dialog box to drag and highlight the number of CO-RE 300 uL filtered Hamilton tips available in Site 1 of Track 12-17. Scroll to zoom if needed. If first time running the method or there are no tips already present in Site 1, load a fresh set and highlight all tips.
p. Use the dialog box to drag and highlight the number of CO-RE 50 uL filtered Hamilton tips available in Site 2 of Track 12-17. Scroll to zoom if needed. If first time running the method or there are no tips already present in Site 2, load a fresh set and highlight all tips.
q. Load the 60 mL Hamilton Reagent Reservoir with 80% Ethanol in Site 1 of Track 30.
r. Load the two 2 mL screw-cap microtubes with beads in Site 5 of Track 38-44 in the top left slot and the left second row slot as depicted. Ensure the tubes are pressed all the way into the slot.

2. Run method, est.run time ~45 minutes.
3. Once the method is complete, clean up the deck and either proceed to Sample Dilution or store **Cleaned Amplicons Plate** in the -20C.

🛑 SAFE STOPPING POINT: can safely store **Cleaned Amplicons Plate** in -20C 🛑

Sample Dilution

Total time: 20 min.
Hands on time: 5 min.

Materials Needed

- ☐ **Cleaned Amplicons Plate**
- ☐ IDTE pH 8.0 (1X TE Solution)
- ☐ 60mL Hamilton Reagent Reservoir
- ☐ 96-well BioRAD skirted PCR plate (HSP Plate)
- ☐ CO-RE 50uL filtered Hamilton tips (1)
- ☐ CO-RE 300uL filtered Hamilton tips (1)

User Preparation (5 min)

1. Spin down **Cleaned Amplicons Plate**. If needed, transfer the contents of the **Cleaned Amplicons Plate** into a 96-well BioRAD skirted PCR plate (HSP) plate.
2. Transfer Hamilton prompt provided volume of 1X TE Solution into a 60mL Hamilton Reagent Reservoir.

Automated Method (15 min)

4. Load ***Dilution Method V1.0*** method, and follow user prompts to set up the Hamilton deck.
    a. Enter '**12**' for 'How many columns are you running?'
    b. Enter '**10**' for 'What dilution ratio would you like?'
    c. Enter '**30**' for 'What final sample volume would you like?'
    d. In Sites 4 and 5 of Track 12-17, load one set of CO-RE 50uL filtered Hamilton tips and one set of CO-RE 300uL filtered Hamilton tips, respectively.
    e. In Site 4 of Track 18-23, load an empty 96-well BioRAD skirted PCR plate (HSP) plate. This will be your **1:10 Diluted Amplicon Plate.**
    f. In Site 5 of Track 18-23, load the **Cleaned Amplicons Plate.**
    g. In Site 5 of Track 30, load the 60mL Hamilton Reagent Reservoir with 1X TE Solution.
5. Run method, est.run time ~30 minutes.
6. Once the method is complete, clean up the deck, store the **Cleaned Amplicons Plate**
7. in the -20C for long term storage, and proceed to Library Prep with the **1:10 Diluted Amplicon Plate.**

Library Preparation

Total time: 4 hours 15 min.
Hands on time: 45 min.

Materials Needed

- ☐ **1:10 Diluted Amplicon Plate**
- ☐ Ampure XP Beads
- ☐ Molecular Biology Grade 200 proof Ethanol

- [ ] NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (Any Set 1 - 5)

- [ ] NEBNext UltraExpress® FS DNA Library Prep Kit
- [ ] DNase/RNase Free Water
- [ ] IDTE pH 8.0 (1X TE Solution)
- [ ] Corning® 50 mL PP Centrifuge Tubes (1)
- [ ] Alpaqua Magnum FLX® Magnetic 96-Well Stand
- [ ] 96-well BioRAD skirted PCR plate (HSP Plate) (7)
- [ ] Axygen® 96-well Clear V-Bottom 500 µL Polypropylene Deep Well Plate (4)
- [ ] Abgene™ 96 Well 0.8mL Polypropylene DeepWell (1)
- [ ] Barcoded Sterile 96-DW plate (2)
- [ ] 60mL Hamilton Reagent Reservoir (3)
- [ ] 2mL screw-cap microtubes (16)
- [ ] CO-RE 1000uL filtered Hamilton tips (1)
- [ ] CO-RE 300uL filtered Hamilton tips (4)
- [ ] CO-RE 50uL filtered Hamilton tips (5)

User Preparation (45 min.)

1. Allow the Ampure XP Beads to thaw at room temperature for 30 minutes prior to use.
2. Make a 30mL working stock of 0.1X TE Buffer by combining 3mL of **1x TE Buffer** with 27mL DNase/RNase Free Water in a Corning 50mL PP Centrifuge tube. Transfer 22,248uL into a 60mL Hamilton Reagent Reservoir.
3. Make a 60mL working stock of **80% Ethanol** by combining 40mL of Molecular Biology Grade 200 proof Ethanol with 20mL of DNase/RNase Free Water. Mix by inversion and transfer 52,200uL into a 60mL Hamilton Reagent Reservoir.
4. Transfer 60mL of **DNase/RNase Free Water** into a 60mL Hamilton Reagent Reservoir.
5. Place all NEBNext UltraExpress FS DNA Library Prep Kit reagents to thaw on ice, including the Adaptor and USER enzyme that come with the NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) plate.
    a. Once thawed, perform the following:
        i. **End Prep Mix**: combine 416uL of the FS Reaction Buffer and 104uL of the FS enzyme mix into one 2mL screw-cap tube. Vortex well.
        ii. **Adaptor**: aliquot 232uL into one 2mL screw-cap tube
        iii. **Ligation Master Mix**: aliquot 2mL into one 2mL screw-cap tube
        iv. **USER enzyme**: aliquot 328uL into one 2mL screw cap tube
        v. **MSTC High Yield Master Mix** (PCR MM): aliquot 1150uL into four 2mL screw-cap tubes.
        vi. **NEB Bead Reconstitution Buffer**: aliquot 2.04mL into four 2mL screw-cap tubes.
        vii. **Ampure XP Beads** (not supplied with kit): Thoroughly vortex and aliquot 1750uL into four 2mL screw-cap microtubes
6. Ensure the **NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) plate** is thawed and spun down. Carefully remove the foil using a razor blade so all column wells needed are exposed.

Automated Method (3.5 hrs)

7. Load ***NEB_Next_UltraExpress_v1.1.2*** method.

8. Select '**UltraExpress FS**' radio button, and input '**12**' into the Number of Columns box.
9. Input '**1**' for the first column of the primer plate to use, and '**12'** for the last column of the primer plate to use.
    a. Make note of which primer plate columns are combined with which sample plate columns to inform the i7/i5 index sheet for sequencing.
10. Follow user prompts to set up the Hamilton deck:
    a. Select the radio button for 'UltraExpress FS'
    b. Input '**12**' for Number of Columns
    c. Input '**1**' for 'What is the first column of the primer plate to use?'
    d. Input '**12**' for 'What is the last column of the primer plate to use?'
    e. In Sites 1-3 respectively of Track 6-11, load one full or partial set of CO-RE 1000uL filtered Hamilton tips, CO-RE 300uL filtered Hamilton tips, and CO-RE 50uL filtered Hamilton tips. Confirm the tip positions of these racks in the next dialog box by dragging to select present tips.
        i. Ensure the CO-RE 50uL filtered Hamilton tip set is **at least half full.**
    f. In Sites 1-5 respectively of Track 7-17, load three full sets of CO-RE 300uL filtered Hamilton tips and two full sets of CO-RE 50uL filtered Hamilton tips.
    g. In Sites 1 of Track 18-23, load one full set of CO-RE 50uL filtered Hamilton tips.
    h. In Site 3 of Track 18-23, load the **NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) plate**. Ensure the foil is removed from all columns and exposes all wells needed.
    i. Ensure to spin down the **1:10 Diluted Amplicon Plate** before removing its seal and loading it onto the Hamilton deck in the 'Load Position' in Site 5 of Track 18-23.
    j. In Sites 1 and 2 of Track 24-29, load two empty Barcoded Sterile 96-DW plates ('DW Plate Waste') ('DW Plate Rinse')
    k. In Site 3 of Track 24-29, load an empty Abgene™ 96 Well 0.8mL Polypropylene DeepWell ('MIDI Plate').
    l. Ensure there is an Alpaqua Magnum FLX® Magnetic 96-Well Stand placed on Site 5 of Track 32-37.
    m. In Site 3 of Track 47-53, load four stacked Axygen® 96-well Clear V-Bottom 500 µL Polypropylene Deep Well Plates ('Axygen Plate')
    n. In Site 2 of Track 47-53, load one 96-well BioRAD skirted PCR plate ('HSP Plate')
    o. In Site 1 of Track 47-53, load six 96-well BioRAD skirted PCR plates ('HSP Plates')
    p. Load the following into the designated tube slots in Slot 5 of Track 38-44, ensure the screw-cap is off and they are pushed all the way into the slot:
        i. Column 1, Slot 1: **End Prep Mix**
        ii. Column 1, Slot 2: **Adaptor**
        iii. Column 1, Slot 3: **Ligation Master Mix**
        iv. Column 1, Slot 4: **USER enzyme**
        v. Column 2, Slots 1-4: **MSTC High Yield Master Mix**
        vi. Column 5, Slots 1-4: **Ampure XP Beads**

vii. Column 6, Slots 1-4: **NEB Bead Reconstitution Buffer**

11. Run method, est. runtime ~4.5 hours
    a. Several dialog boxes will popup during the method as thermocycling occurs off deck while parallel processes occur on the Hamilton.
        i. When prompted, move the **1:10 Diluted Amplicon Plate** off the deck from the load/offload position and into -20C storage.
        ii. When prompted, remove the plate from the load/offload position of the deck, and load onto a thermal cycler with the **heated lid set to 75C or greater.** Once on the thermal cycler, press 'OK' to continue with parallel processing on the Hamilton.

Step	Temperature (˚C)	Time (minutes)
1	37	20
2	65	15
3	4	Hold

        iii. When prompted, load the sample plate back in the load/offload position of the deck from the thermal cycler if complete. Click 'OK' to continue.
        iv. When prompted, click 'OK' to begin the ligation reaction timer and continue with parallel processing.
        v. When prompted, click 'OK' to resume once the ligation reaction is complete.
        vi. When prompted, click 'OK' to begin the USER incubation timer and continue with parallel processing.
        vii. When prompted, click 'OK ' to resume once the USER incubation is complete.
        viii. When prompted, remove the sample plate from the load/offload position and load onto a thermal cycler with the **heated lid set to 105C or greater.** Once on the thermal cycler, press 'OK' to continue with parallel processing on the Hamilton.

Step	Temperature (˚C)	Time
1. Initial Denaturation	98	30 sec
2. Denaturation	98	10 sec
3. Anneal and extend	65	75 sec
4. Cycle	Repeat steps 2-3 for a total of 6 cycles	
5. Final Extension	65	5 min
5. Hold	4	Hold

        ix. When prompted, reload the sample plate from the thermal cycler if complete back onto the deck in the load/offload position. Press 'OK' to continue to bead cleanup.

8. Run method, est. runtime ~3.5 hours.
    8. Once the method is complete, clean up the deck, proceed to the downstream steps or store the **Final Libraries Plate** in the -20C, which will be in the load/offload position at the end of the method.

Quantification

*method only accommodates up to 88 samples or 11 columns, to allow for standard curve

Total time: 1 hr.
Hands on time: 30 min.
Equipment run time: 10 min.

Materials Needed

- ☐ **Final Libraries Plate**
- ☐ DNase/RNase Free Water
- ☐ Invitrogen Qubit dS HS Assay Kit
  - ☐ Qubit™ dsDNA HS Buffer
  - ☐ Qubit™ dsDNA HS Reagent (200X)
- ☐ 60mL Hamilton Reagent Reservoir
- ☐ Amber 50mL conical tube
- ☐ Black 96-well flat-bottomed microplate (2)
- ☐ CO-RE 50uL filtered Hamilton tips (2)
- ☐ Applied Biosystems 96-well Microtiter Plate (1)
- ☐ Plate Reader

User Preparation (20 min)

1. Since the quantification method can only accommodate 11 columns, transfer column 12 of the Final Libraries plate to the first column of a sterile 96-well PCR plate.
2. Aliquot standards into empty column 12 (A-H) using Nuclease-free water and 10ng/ul standard provided by kit in **two** sterile black-walled 96-well plates:
   a. A12: 10uL 10ng/uL standard
   b. B12: 5uL 10ng/uL standard + 5uL DNase/RNase Free Water
   c. C12: 2.5uL 10ng/uL standard + 7.5uL DNase/RNase Free Water
   d. D12: 1.25uL 10ng/uL standard + 8.75uL DNase/RNase Free Water
   e. E12: 0.626uL 10ng/uL standard + 9.37uL DNase/RNase Free Water
   f. F12: 0.312uL 10ng/uL standard + 9.68uL DNase/RNase Free Water
   g. G12: 10uL DNase/RNase Free Water
   h. H12: 10uL DNase/RNase Free Water
3. Create Working Solution by combining 31,063.9uL of Qubit™ dsDNA HS Buffer with 156.1uL of Qubit™ dsDNA HS Reagent (200X) into an Amber 50mL conical tube. Mix well by vortexing.
4. Pour contents of Amber 50mL conical tube into a 60mL Hamilton Reagent Reservoir.
5. Spin down **Final Libraries Plate**.

Automated Method (30 min.)

6. Load ***Quantification Prep_V3.0*** method.
7. Input '**11**' columns.
8. Follow user prompts to set up the Hamilton deck.
9. Run method, est. runtime ~30 minutes.
10. Once the method is complete, immediately load the black-walled plate onto a plate reader using the following settings:
    a. Fluorescence Endpoint
    b. Excitation: 485

c. Emission: 535
d. Optics: Top
e. Gain: AutoScale
f. Light Source: Xenon Flash
g. Lamp Energy: High
h. Standard Dynamic Range
i. Read Speed: Normal
j. Delay: 100 msec
k. Measurements/Data Point: 10
l. Read Height: 7mm

11. Exit and reload method, repeat above steps with second black-walled 96-well plate loaded but change Input to '1' column.
12. Once the method is complete, load the second black-walled plate onto the plate reader using the above settings, clean the deck and store the Final Library plate in the -20C.

DNA Concentration Calculations

1. Copy and paste raw plate reader data (RFU) into the following spreadsheet, updating the slope and y value boxes. The values located in Row 45-52 are your sample ng/uL concentrations. The values located in Row 70-77 are the uL amounts of each sample you will add to the pool, these are the values you will copy and paste into Column C of the following csv template, corresponding to each sample's well. Export and save as a csv file to be used downstream for sample pooling.

2. Option to proceed without spreadsheet as detailed below:
    a. Obtain average of the blanks from G12 and H12, subtract from all raw data cells.
    b. Plot blanked standards and ng/uL concentration as a linear regression to obtain y = mx + b equation
        i. A12 = 10ng/uL
        ii. B12 = 5ng/uL
        iii. C12 = 2.5ng/uL
        iv. D12 = 1.25ng/uL
        v. E12 = 0.625ng/uL
        vi. F12 = 0.312ng/uL
    c. Multiply the blanked RFU samples by 5 in order to compare against the standard curve (which is at 5x concentration relative to samples).
    d. Plug in new RFU values to the equation, substituting the x to obtain our y value.
    e. This y value is now your sample's ng/uL.
    f. Determine the desired weight (in ng) of DNA to add into the library pool from each sample. The default is 25 ng.
    g. Divide the desired weight by the sample's ng/uL concentration. This is the uL amount of that sample that will be added into the pool.
    h. Cap each sample's max volume added to 18uL (as to preserve some library).
    i. Copy and paste these values into Column C of the following csv template, corresponding to each sample's well. Export and save as a csv file to be used downstream for sample pooling.

3. Please read the important disclaimer below in bold before proceeding.

IMPORTANT: **The Pooling automated method can only accurately accommodate samples with concentrations <1.7ng/uL when setting the default DNA ng in the pool to 25; this is to keep aspirated/dispensed volumes of the liquid handler to >1uL. Proceed to the optional Sample Dilution section if your concentrations are larger than 1.7ng/uL or the uL amounts of samples added are <1uL. Optional also to up the ng of DNA in the pool greater than 25 so sample volumes are >1uL.

a. By default, the Sample Dilution method below outlines a 1:5 dilution. Ensure this dilution works for the majority of your samples using the following spreadsheet to ensure equimolarity of samples in the pool.

i. Input the sample ID and well ID into Columns A & B.

ii. Input 25 into Column H, or change this number to the DNA ng you desire of each sample in your pool.

iii. Change E2 to the dilution factor you want (5 equates to a 1:5 dilution which is the default)

iv. Input associated sample concentrations into Column C.

v. In Column D, the original uL amount of each sample you would have added to the pool will populate.

vi. In Column F, update the final volume to 50uL (or your preferred volume)

vii. Column E displays the uL amount of sample you will be needing for the dilution (recommended amount is 10uL as there is only 30uL of sample in total).

viii. Column J displays the total ng of sample that will be going into the pool based on the dilution factor. Ideally, this number matches or is close to the number in Column H.

ix. The highlighted column I gives you the new uL amount of each sample you will add to the pool once diluted. **IMPORTANT: Change any value higher than 50 uL to 50**. **This number cannot go above 50 as the Hamilton tips in the Pooling method cannot aspirate over 50 uL.**

x. Column I is what you will then copy and paste into Column C of the following csv template instead to use for the Pooling automated method.

b. Proceed to the Sample Dilution step below as outlined if continuing with a 1:5 dilution, or adjust based on the dilution factor you chose.

Sample Dilution (Optional)

Total time: 20 min.
Hands on time: 5 min.

Materials Needed

- ☐ **Final Libraries Plate**
- ☐ IDTE pH 8.0 (1X TE Solution)
- ☐ 60mL Hamilton Reagent Reservoir
- ☐ 96-well BioRAD skirted PCR plate (HSP Plate)
- ☐ CO-RE 50uL filtered Hamilton tips (1)
- ☐ CO-RE 300uL filtered Hamilton tips (1)

User Preparation (5 min)

3. Spin down **Final Libraries Plate**.
4. Transfer 4,224uL of 1X TE Solution into a 60mL Hamilton Reagent Reservoir.

Automated Method (15 min)

9. Load ***Dilution Method V1.0*** method, and follow user prompts to set up the Hamilton deck.
    a. Enter '**12**' for 'How many columns are you running?'
    b. Enter '**5**' for 'What dilution ratio would you like?'
    c. Enter '**50**' for 'What final sample volume would you like?'
    d. In Sites 4 and 5 of Track 12-17, load one set of CO-RE 50uL filtered Hamilton tips and one set of CO-RE 300uL filtered Hamilton tips, respectively.
    e. In Site 4 of Track 18-23, load an empty 96-well BioRAD skirted PCR plate (HSP) plate. This will be your **1:5 Diluted Final Libraries Plate.**
    f. In Site 5 of Track 18-23, load the **Final Libraries Plate.**
    g. In Site 5 of Track 30, load the 60mL Hamilton Reagent Reservoir with 1X TE Solution.
10. Run method, est.run time ~30 minutes.
11. Once the method is complete, clean up the deck, store the **Final Libraries Plate**
12. in the -20C for long term storage, and proceed to Library Prep with the **1:5 Diluted Final Libraries Plate.**

Pooling

Total time: 30 min.
Hands on time: 5 min.

Materials Needed

- ☐ **Final Libraries Plate** or **1:5 Diluted Final Libraries Plate**
- ☐ 1.5mL Snap Cap Low Retention Microcentrifuge Tube
- ☐ CO-RE 50uL filtered Hamilton tips
- ☐ Magnetic Stand (24-well) with 3D printed adaptor (File: "**Magnet_Riser v2.step**")

User Preparation (5 min)

1. Spin down **Final Libraries Plate**.
2. On the desktop associated with the Hamilton to be used, open file: '**Hamilton_Pooling input - export_csv.csv**'
3. Copy and paste the data from the csv file saved above (in the Quantification section) to replace the data in the template csv file.
4. Save and exit.

Automated Method (25 min)

1. Load ***Normalization & Pooling_V1.0*** method.
2. Select 'Magnet' radio button.
3. Set up the Hamilton deck as per the follow:
    a. In Site 1 of Track 38-43, load one set of CO-RE 50uL filtered Hamilton tips.
    b. In Site 2 of Track 26-31, load an empty 1.5mL Eppendorf tube into the bottom left slot. Ensure the tube is pushed all the way into the slot.
    c. In Site 5 of Track 2-7, place the Final Libraries Plate onto the 24-well magnet equipped with the 3D printed adaptor. This will be your **Final Pool Tube**.

4. Run method, est. runtime ~25 minutes.
5. Once complete, store the **Final Libraries Plate** in the -20C as well as the **Final Pool Tube** until ready to send to sequencing.
6. Optional to fragment analyze the final library at a 1:20 dilution using a high sensitivity assay to confirm peak sizes ~320 bp.