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Automated Amplicon Library Preparation Using the Echo 525 and KingFisher Apex with a 384-well Destination Plate V.1

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Protocol status: Working
We use this protocol and it's working

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Abstract

We developed a miniaturized, automated amplicon library preparation protocol for 96 samples. This protocol uses the Echo 525 Acoustic Liquid Handler for liquid transfers and the KingFisher Apex for automated magnetic bead cleanup. This protocol is designed to use a 384-well destination plate with the Echo. After library preparation, these samples will be ready for amplicon sequencing.

Attachments

DOCX

Automated Amplicon L...

592KB

Troubleshooting

Amplicon PCR

1 NOTES:
Total volume = 5 µL for each sample/well
Source plate = Echo 384-well polypropylene (384PP) Source plate
Destination plate = 384 well qPCR plate

2 Dilute the DNA samples to 5 to 10 ng/µL into 25 µL total volume in the top left corner of the Source plate (A1-H12) according to the Source plate figure in step 4.

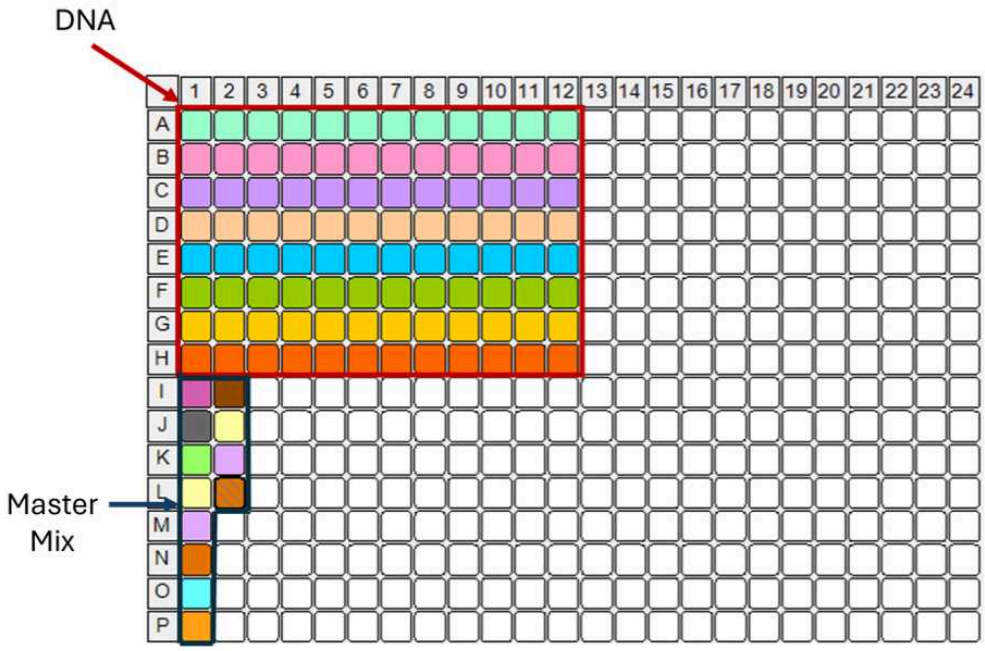
3 Prepare a master mix including the following reagents, if PNAs are not added substitute that volume with water:

	A	B	C
	Reagent	1 Reaction (µL)	150 Reactions (µL)
	Amplicon PCR Forward Primer (2 µM)	0.5	75
	Amplicon PCR Reverse Primer (2 µM)	0.5	75
	2X KAPA HiFi HotStart ReadyMix	2.5	375
	mPNA 50 µM (if necessary)	0.2	30
	pPNA 50 µM (if necessary)	0.2	30
	Water	0.6	90
	Total	4.5	675

Reagents and volumes used per reaction and calculated for 150 reactions for Amplicon PCR.

- The Echo 525 Acoustic Liquid Handler will add 4.5 µL of master mix and 0.5 µL of sample DNA to each well of the Destination plate.

4 Pipet 55 µL of the master mix into the wells I1-P1 and I2-L2.



Source plate layout. Sample DNA is in wells A1-H12. Master mix is in wells I1-P1 and I2-L2.

5 Once the Source plate is prepared, carefully tap it a couple of times to get rid of bubbles. Examine the plate and ensure the liquid has been distributed evenly in all wells and that there are no bubbles.

6 Make a protocol in Echo Plate Reformat to transfer 4.5 µL of master mix and 0.5 µL of sample DNA to each corresponding well of the Destination plate.

- If already made, follow the protocol. Click on the protocol, then run. It will ask to place a Source plate and Destination plate inside the robotic arms. Provide both and proceed to run the protocol.
- The Destination plate was designed in the Echo software to skip a well between columns, so a multichannel pipet can be used to transfer between 384-well plates and 96-well plates in further steps.

Destination plate layout after running the Echo protocol.

7 Once the protocol is over, remove both the Source and the Destination plate and check that the Destination plate has equal amounts of liquid in each well.

8 The software will ask you to see an exception report, open it, and examine if all wells have the appropriate volume of 5 µL transferred. If something didn't transfer, the software will generate an exceptions report. Here, the Transfer Volume = the volume it should have transferred, and Actual Volume is what was actually transferred. Use this to manually subtract the Actual Volume from the Transfer Volume.

8.1 Save this exceptions report in a folder on the desktop. Open Echo Cherry Pick and select New.

8.2 Change the Source plate to 384PP and the Destination plate to the plate type used.

8.3 In the Pick List tab, import the updated exceptions report. Unselect Sample ID and Name, then click Import.

8.4 If the issue was not enough volume in the master mix wells, add more master mix to the Source plate and then run the Cherry Pick protocol. Save the run in the same folder.

If the issue was not enough volume in the sample DNA wells, manually add 0.5 µL of sample DNA to the corresponding wells. The minimum volume required for the Echo to

transfer is 10 µL, so it may not transfer if there isn't enough volume or bubbles are present.

Other issues may arise during transfer. Check the exceptions report for issues and either add more master mix to the wells or manually transfer master mix and sample DNA to the corresponding wells in the Destination plate.

9 Before running the Amplicon PCR1, cover the plate with a plastic PCR seal and centrifuge the plate at 1,000 xg for 1 minute. Amplicon PCR reaction set up:

- The PCR reaction will proceed in the Applied Biosystems QuantStudio 6 Flex Real-Time PCR machine
- Seal the 384-well plate and perform Amplicon PCR in the qPCR machine using the following program:

1. 95°C for 3 minutes
2. 25-35 cycles of:
   i. 95°C for 30 seconds
   ii. 55°C for 30 seconds
   iii. 72°C for 30 seconds
3. 72°C for 5 minutes
4. Hold at 4°C

10 After Amplicon PCR is complete, run 2-4 samples on the TapeStation BioAnalyzer using the High Sensitivity D1000 DNA ScreenTapes.

- Transfer 0.5 µL of Ladder and samples into 2 µL of water and 2 µL of TapeStation Buffer.
- Follow the remaining TapeStation protocol as usual.

11 Optional: Store at -20°C if not performing Amplicon PCR bead cleanup right away.

Amplicon PCR Bead Cleanup in the KingFisher Apex

12 Before starting, run UV sterilization on the King Fisher for at least 5 minutes.

13 Before you begin using the King Fisher make sure that:

- The 96-deep well magnetic head (the smallest magnetic head) on the KingFisher Apex instrument is installed (there are 3 magnetic heads in total).

14 Prepare the processing plates: bead slurry, wash 1 and wash 2 plates, and elution plate using the table below. All 96-well plates used are the Thermo Armadillo High Performance 96-well PCR Plates.

	A	B	C
	Plate Name	Reagent	Volume per well (µL)
	Sample	Magnetic Beads	10
	Wash Plate 1	70% ethanol	30
	Wash Plate 2	70% ethanol	30
	Elution Plate	Molecular-Grade Water	15
	Tip Comb	Place a tip comb in a plate	NA

Processing plates to complete bead cleanup.

15 Make sure to take the magnetic beads out of the fridge and get to room temperature. This will take at least 30 minutes. Magnetic beads used are the Omega Biotek Mag-Bind TotalPure NGS.

16 Preparing fresh 70% ethanol before starting the cleanup protocol is crucial for optimal results. Make 10 mL with 7 mL of 200 proof EtOH and 3 mL of molecular-grade water. Add 30 µL of 70% to each well of two 96-well plates using a trough and multichannel pipet. These correspond to Wash Plate 1 and Wash Plate 2.

17 Pipet 100 µL of magnetic beads into 8 PCR strip tubes. Then, in a new 96-well plate (Sample Plate), pipet 10 µL of beads into each well using a multichannel pipet.

18 Pipet 50 µL of water into 8 PCR strip tubes. Then use a multichannel pipet to transfer 5 µL of water into each well of the 96-well Sample Plate.

19 Using a multichannel pipet, transfer 5 (all) µL of sample DNA to the corresponding wells of the 96-well Sample Plate containing beads and water. Don't mix the samples after adding the sample DNA.

20 Prepare the Elution Plate. Pipet 250 µL of water into 8 PCR strip tubes. Use a multichannel pipet to transfer 15 µL into each well of a new 96-well plate (Elution Plate).

21 Prepare the Tip Comb and plate. In a new 96-well plate, place a new Tip Comb.

22 Then run the PCR cleanup protocol on the instrument.

Count denotes the number of times the magnetic head moves in the wells to collect the beads.

Collect time sets the time that the magnetic head stays at the bottom of the well to collect beads.

	A	B	C	D
	Step	Mixing	Count	Time
	Pick up 96-well PCR tip comb	NA	NA	NA
	Bind to magnetic beads in each sample	00:06:00	10	00:00:20
	Wash 1 with 70% EtOH	00:00:15	NA	NA
	Wash 2 with 70% EtOH	00:00:15	NA	NA
	Collect beads	NA	10	00:00:30
	Dry beads	NA	NA	00:01:00
	Elute	00:04:00	NA	NA
	Bead collect	00:02:00	10	00:00:30
	Bead collect	NA	10	00:00:30
	Leave 96-well PCR tip comb	NA	NA	NA

Bead cleanup steps and time configured on the KingFisher Apex.

23 Start the run, then load the prepared plates in the appropriate positions when prompted by the instrument.

Note: The instrument will scan each plate, starting from the last (Elution Plate) to the first needed (Tip Comb). It will prompt the loading of each plate. Make sure to place the plates where A1 is in the top left. Make sure the Tip Comb barcode is facing toward the center of the machine.

24 Once the last plate is loaded, close the door on the instrument.

Note: The run proceeds for approximately 42 minutes.

25 At the end of the run, the instrument will prompt the user to remove the plates one by one. Check if any beads remain in the Elution Plate and Sample Plate.

- If there are a lot of beads remaining in the Sample Plate, re-run the entire bead cleanup protocol. If only a few beads are remaining in the Sample Plate, proceed normally.

- If beads are remaining in the Elution Plate, collect the beads again until there are no visible beads in the wells.

26 Remove the Elution plate. Discard the other plates.

27 After bead cleanup is complete, run the same 2-4 samples as Amplicon PCR on the TapeStation BioAnalyzer using the High Sensitivity D1000 DNA ScreenTapes.
- Transfer 0.5 µL of Ladder and samples into 2 µL of water and 2 µL of TapeStation Buffer.
- Follow the remaining TapeStation protocol as usual.

28 Seal the plate for storage. Samples can be stored at -20°C until further steps.

Index PCR

29 NOTES:
Total volume = 10 µL for each sample/well
Source plate = Same Echo 384 well source plate from Amplicon PCR
Destination plate = Same 384 well qPCR plate from Amplicon PCR, but will use the second half of the plate.

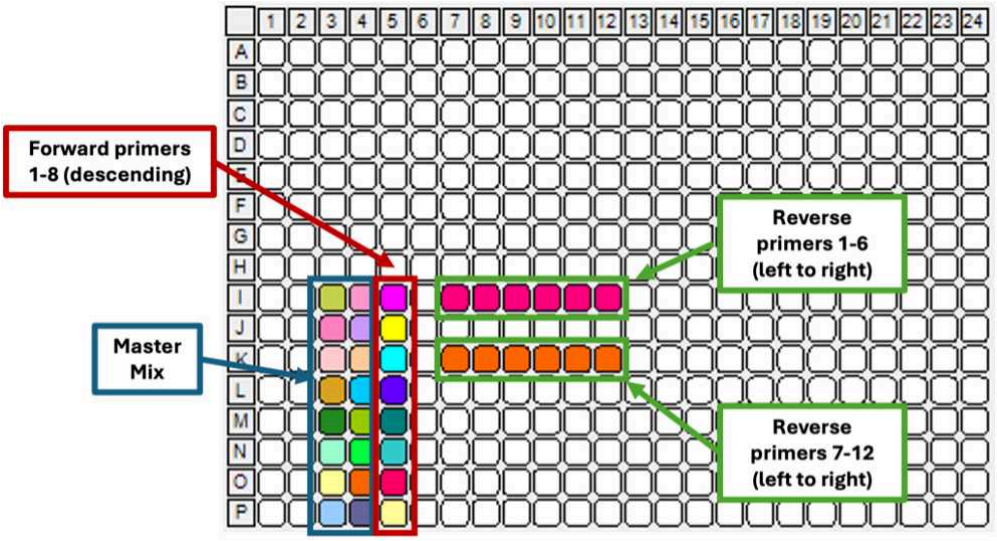
30 Prepare a master mix including the following reagents:

	A	B	C
	Reagent	1 Reaction (µL)	150 Reactions (µL)
	2X KAPA HiFi HotStart ReadyMix	5.0	750
	Water	1.0	150
	Total	6.0	900

Reagents and volumes used per reaction and calculated for 150 reactions for Index PCR.

- The Echo will add 1 µL of forward and reverse Nextera index primers and 6 µL of master mix. The user will pipet 2 µL of clean Amplicon PCR product in step 28 by hand to complete the total 10 µL volume necessary for Index PCR.

31 Pipet 55 µL of the master mix into the wells I3-P3 and I4-P4.



Source plate layout. Master mix is in wells I3-P3 and I4-P4, forward primers are in wells I5-P5, and reverse primers are in wells I7-I12 and K7-K12.

32 Pipet 35 µL of each of the 8 Forward Nextera index primers of the corresponding index plate in wells I5-P5.

33 Pipet 35 µL of each of the 12 Reverse Nextera index primers of the corresponding index plate on wells I7-I12 and K7-K12.

34 Once the source plate is prepared, carefully tap it a couple of times to get rid of bubbles. Examine the plate and ensure the liquid has been distributed evenly in all wells and that there are no bubbles.

35 Make a protocol in Echo Plate Reformat to transfer 1 µL of forward and reverse Nextera index primers and 6 µL of master mix to each corresponding well of the Destination plate.

- If already made, follow the protocol. Click on the protocol, then select run. Start and add the source and destination plate when prompted. Proceed to run the protocol.
- The destination plate containing the Index PCR reaction is the same one used for Amplicon PCR and was also designed in the Echo software to skip a well between columns, so a multichannel pipet can be used to move between 384-well plates and 96-well plates in further steps.

Destination plate layout after running the Echo protocol.

36 Once the protocol is over, remove both, the Source and the Destination plate and check that the Destination plate has equal amounts of liquid on each well.

37 Same as with Amplicon PCR, the software will automatically ask you to see an exception report, open it, and examine if all wells have the appropriate volume 10 µL. If something doesn't transfer, it will give an exception report. Here, the Transfer Volume = the volume it should have transferred, and the Actual Volume is what was actually transferred. Use this to manually subtract the Actual Volume from the Transfer Volume.

37.1 Save this exceptions report in a folder on the desktop. Open Echo Cherry Pick and select New.

37.2 Change the Source plate 384PP and Destination plate to the plate type used.

37.3 In the Pick List tab, import the updated exceptions report. Unselect Sample ID and Name, then click Import.

37.4 If the issue was not enough volume in the master mix wells, add more master mix to the Source plate and then run the Cherry Pick protocol. Save the run in the same folder.

Other issues may arise during transfer. Check the exceptions report for issues and either add more master mix to the wells or manually transfer master mix and sample DNA to the corresponding wells in the Destination plate.

38 Spin down the 96-well plate containing the clean Amplicon PCR product. Gently pipet up and down 10 times to mix, then with a multichannel pipet, add 2 µL of the clean Amplicon PCR product into each corresponding well of the destination plate. Cover the plate with a seal and centrifuge the plate at 1,000 xg for 1 minute.

39 Perform Index PCR using the following program:

- The PCR reaction will proceed in the Applied Biosystems QuantStudio 6 Flex Real-Time PCR machine
- Seal the 384-well plate and perform Index PCR in the qPCR machine using the following program:

1. 95°C for 3 minutes
2. 8-11 cycles of:
   i. 95°C for 30 seconds
   ii. 55°C for 30 seconds
   iii. 72°C for 30 seconds
3. 72°C for 5 minutes
4. Hold at 4°C

40 After Index PCR is complete, run the same 2-4 samples as Amplicon PCR on the TapeStation BioAnalyzer using the High Sensitivity D1000 DNA ScreenTapes.

- Transfer 0.5 µL of Ladder and samples into 2 µL of water and 2 µL of TapeStation Buffer.
- Follow the remaining TapeStation protocol as usual.

41 Optional: Store at -20°C if not performing Index PCR bead cleanup right away.

Index PCR Bead Cleanup in the KingFisher Apex

42 Before starting, run UV sterilization on the King Fisher for at least 5 minutes.

43 Before you begin using the King Fisher make sure that:

- The 96-deep well magnetic head (the smallest magnetic head) on the KingFisher Apex instrument is installed (there are 3 magnetic heads in total).

44 Prepare the processing plates: bead slurry, wash 1 and wash 2 plates, and elution plate using the table below. All 96-well plates are the Thermo Armadillo High Performance 96-well PCR Plates.

	A	B	C
	Plate Name	Reagent	Volume per well (µL)

	A	B	C
	Sample	Magnetic Beads	10
	Wash Plate 1	70% ethanol	30
	Wash Plate 2	70% ethanol	30
	Elution Plate	Molecular-Grade Water	25
	Tip Comb	Place a tip comb in a plate	NA

Processing plates to complete bead cleanup.

45 Make sure to take the magnetic beads out of the fridge and get to room temperature. This will take at least 30 minutes. Magnetic beads used are the Omega Biotek Mag-Bind TotalPure NGS.

46 Preparing fresh 70% ethanol before starting the cleanup protocol is crucial for optimal results. Make 10 mL with 7 mL of 200 proof EtOH and 3 mL of molecular-grade water. Add 30 µL of 70% to each well of two 96-well plates using a trough and multichannel pipet. These correspond to Wash Plate 1 and Wash Plate 2.

47 Pipet 130 µL of beads into 8 PCR strip tubes. Then, in a new 96-well plate (Sample Plate), pipet 10 µL of beads into each well using a multichannel pipet.

48 Using a multichannel pipet, transfer 10 (all) µL of sample DNA to the corresponding wells of the 96-well Sample Plate containing beads. Don't mix the samples after adding the sample DNA.

49 Prepare the Elution Plate. Pipet 300 µL of water into 8 PCR strip tubes. Use a multichannel pipet to transfer 25 µL into each well of a new 96-well plate (Elution Plate).

50 Prepare the Tip Comb and plate. In a new 96-well plate, place a new Tip Comb.

51 Then run the same PCR cleanup protocol as used in Amplicon PCR bead cleanup on the KingFisher Apex.

52 Start the run, then load the prepared plates in the appropriate positions when prompted by the instrument.

Note: The instrument will scan each plate, starting from the last (Elution Plate) to the first needed (Tip Comb). It will prompt the loading of each plate. Make sure to place the

plates where A1 is in the top left. Make sure the Tip Comb barcode is facing toward the center of the machine.

53 Once the last plate is loaded, close the door on the instrument.

Note: The run proceeds for approximately 42 minutes.

54 At the end of the run, the instrument will prompt the user to remove the plates one by one. Check if any beads remain in the Elution Plate and Sample Plate.
- If there are a lot of beads remaining in the Sample Plate, re-run the entire bead cleanup protocol. If only a few beads are remaining in the Sample Plate, proceed normally.
- If beads are remaining in the Elution Plate, collect the beads again until there are no visible beads in the wells.

55 Remove the Elution plate. Discard the other plates.

56 After bead cleanup is complete, run the same 2-4 samples as Amplicon PCR on the TapeStation BioAnalyzer using the High Sensitivity D1000 DNA ScreenTapes.
- Transfer 0.5 µL of Ladder and samples into 2 µL of water and 2 µL of TapeStation Buffer.
- Follow the remaining TapeStation protocol as usual.

57 Seal the plate for storage. Samples can be stored at -20°C until further steps.

Quantification with Qubit and Diluting to 4 nM

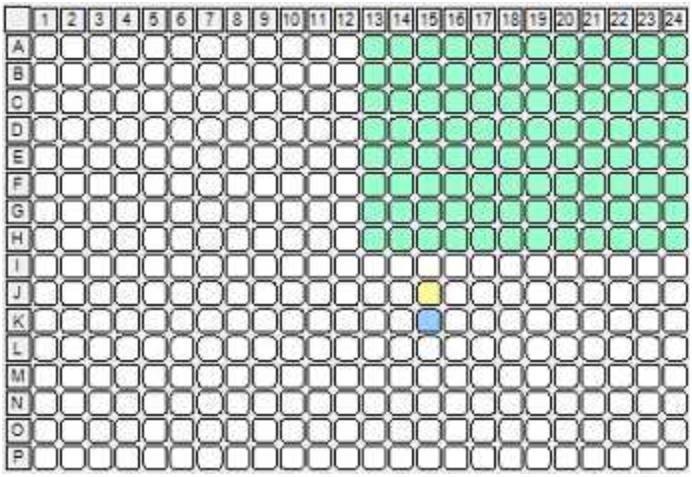
58 Make sure to take the Qubit 1X dsDNA HS Working Solution Buffer and Standards 1 and 2 out of the fridge and allow them to get to room temperature before starting.

59 Transfer 25 (all) µL of samples from the elution plate from step 57 to corresponding Source Plate wells A13-H24.

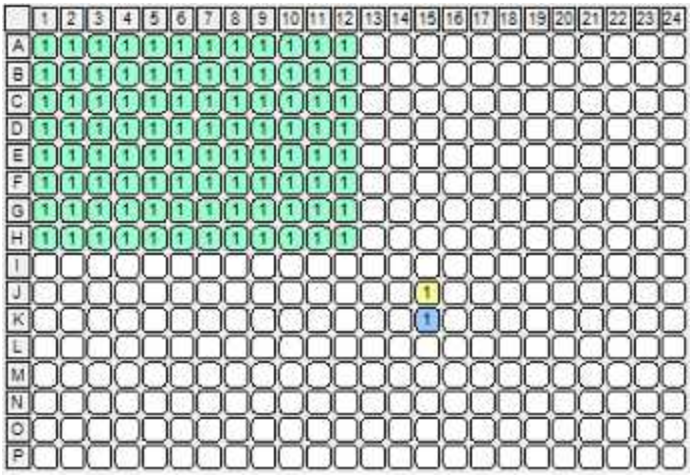
60 Depending on which side of the black-edged 384-well plate you will be using, perform the following steps:
- If using wells A1-H12 of the black-edged 384-well plate, add 20 µL of Standard 1 to well J15 and 20 µL of Standard 2 to well K15 of the Source Plate.
- If using wells A13-H24 of the black-edged 384-well plate, add 20 µL of Standard 1 to well J13 and 20 µL of Standard 2 to well K13 of the Source Plate.

61 Make a protocol in Echo Plate Reformat to transfer 0.6 µL of sample DNA to each well of the black-edged 384-well Destination plate.

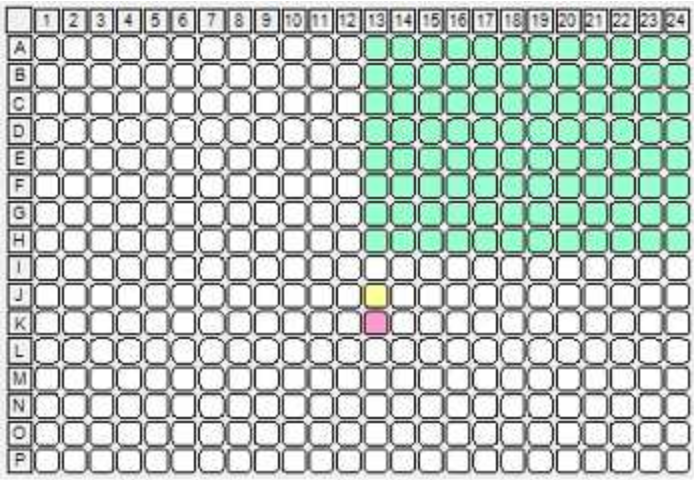
- If already made, follow the correct protocol depending on which side of the Destination plate to be used. Click on the protocol, then select run. Start and add the Source and Destination plate when prompted. Proceed to run the protocol.



Source plate layout if using wells A1-H12 of the black-edge 384-well plate.



Destination plate layout if using wells A1-H12 of the black-edged 384-well plate.



Source and Destination plate layout if using wells A13-H24 of the black-edged 384-well plate.

62 After the Echo protocol is finished transferring, move the black-edged 384-well Destination plate back into the hood.

63 Transfer 6.5 mL of the Qubit 1X dsDNA HS Working Solution Buffer to a sterile trough.

64 With a multichannel pipet, manually add 61.4 µL of the Qubit 1X dsDNA HS Working Solution Buffer to wells A1-H12 or A13-H24 of the black-edged plate, depending on which side is used.

- Use four tips at a time to pipet to every other well. Pipet up and down 10 times to mix.
- Transfer 61.4 µL to wells J13 and K13 or J15 and K15 for the standards.

65 Cover the plate from light and let it sit for 2 minutes after adding the Qubit buffer to each well.

66 Make a protocol to read the Qubit measurements in the Cytation5. In Read Method, select "Fluorescence Intensity" in Detection Method, "Endpoint/Kinetic" in Read Type, and "Monochromators" in Optics Type. In Read Step, Select 1 Wavelength and set the Excitation to 500 and Emission to 530. Select the "BD 384 well Black/Clear Flat 120 µL" in Plate Type. Update the plate layout to denote where samples and standards will be according to the plate layout used.

- If already made, follow the correct protocol depending on which side is used in the black-edged plate. Run the protocol and read the plate in the Cytation5. Keep the lid on and make sure "Use Lid" is selected.

67 When the Cytation5 is done reading, export the results as an excel file.

- This will contain the Qubit reads as well as the quantification in ng/µL. Use this to calculate the concentration in nM, and then to calculate dilutions to get samples to 4 nM.

Optional: Store at -20°C if not diluting right away.

68 Calculate the volume needed to dilute each sample to 4 nM with a total of 25 µL using the concentrations in nM.
- Equation:
(4nM*25µL)/sample concentration in nM

69 Dilute samples to 4 nM with molecular-grade water according to calculations into a new 96-well plate.
- If any samples are below 4 nM, transfer all 25 µL to the new 96-well plate.

70 After diluting, pool 5 µL from each sample.
- Use a multichannel pipet to pool 5 µL of samples into 8 PCR-strip tubes. Then use a single-channel pipet to transfer entire volumes into one 1.7 mL Eppendorf tube.

71 After pooling, measure the concentration of pooled samples with the 1X dsDNA HS Qubit Kit.
- Transfer 190 µL of Qubit 1X dsDNA HS buffer into two tubes for Standards 1 and 2, and
198 µL of buffer into two tubes for pooled samples (use 2 replicates).
- Transfer 10 µL of each standard and 2 µL of pooled samples into their corresponding tubes.
- Wait for 2 minutes while continuously flicking the tubes to mix, then measure on Qubit.
- Calculate their concentrations in nM.

72 Store pooled libraries at -20C until sequencing.