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Automated Amplicon Library Preparation Using the Echo 525 and KingFisher Apex with a 96-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 96-well destination plate with the Echo. After library preparation, these samples will be ready for amplicon sequencing.

Attachments



[Automated Amplicon L...](#)

509KB

Amplicon PCR

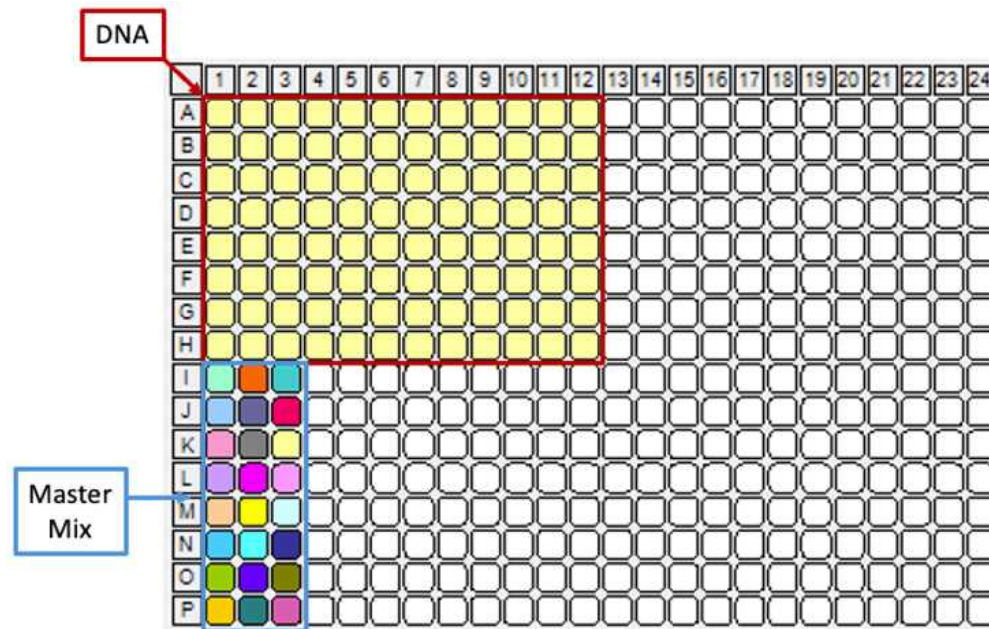
- 1 **NOTES:**
 Total volume = 10 μ L for each sample/well
 Source plate = Echo 384-well polypropylene (384PP) Source plate
 Destination plate = Thermo Armadillo High Performance 96-well PCR Plate
- 2 Dilute the DNA samples to 5 to 10 ng/ μ L into 25 μ L total volume in the top left corner of the Source 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 (μ L)	150 Reactions (μ L)
	Amplicon PCR Forward Primer (2 μ M)	1.0	150
	Amplicon PCR Reverse Primer (2 μ M)	1.0	150
	2X KAPA HiFi HotStart ReadyMix	5.0	750
	mPNA 50 μ M (if necessary)	0.4	60
	pPNA 50 μ M (if necessary)	0.4	60
	Water	1.2	180
	Total	9.0	1350

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

- The Echo 525 Acoustic Liquid Handler will add 9 μ L of master mix and 1 μ L of sample DNA to each well of the Destination plate.

- 4 Pipet 55 μ L of the master mix into the wells I1-P1, I2-P2, and I3-P3.



Source plate layout. Sample DNA is in wells A1-H12. Master mix is in wells I1-P1, I2-P2 and I3-P3.

- 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 9 μ L of master mix and 1 μ L 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 a Destination plate inside the robotic arms. Provide both and proceed to run the protocol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	1	1	1	1	1	1	1	1	1
D	1	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	1	1	1	1	1	1	1	1
F	1	1	1						1	1	1	1
G	1	1	1						1	1	1	1
H	1	1	1	1	1	1	1	1	1	1	1	1

E3
Source[1] N1-N1 9000 nL
Source[1] E3 1000 nL

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 10 μ L 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 96_Twin.Tec_Skirted_PCR.
 - 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 1.0 μ L of sample DNA to the corresponding wells. The minimum volume required for the Echo to transfer is 10 μ L, 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 PCR, 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 Mastercycler epGradient Eppendorf thermocycler. Double-check the parameters are correct, then proceed.
 - Seal the 96-well plate and perform Amplicon PCR in the thermocycler 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	18
	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 beads into 8 PCR strip tubes. Then pipet 10 μL of beads into each well of the Sample plate from Amplicon PCR step 11 using a multichannel pipet. Don't mix the samples after adding the magnetic beads.
- 18 Pipet 70 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. Don't mix the samples after adding the water
- 19 Prepare the Elution Plate. Pipet 250 μL of water into 8 PCR strip tubes. Use a multichannel pipet to transfer 18 μL into each well of a new 96-well plate (Elution Plate).
- 20 Prepare the Tip Comb and plate. In a new 96-well plate, place a new Tip Comb.
- 21 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.

- 22 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.

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

Note: The run proceeds for approximately 42 minutes.

- 24 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.

- 25 Remove the Elution plate. Discard the other plates.
- 26 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.
- 27 Seal the plate for storage. Samples can be stored at -20°C until further steps.

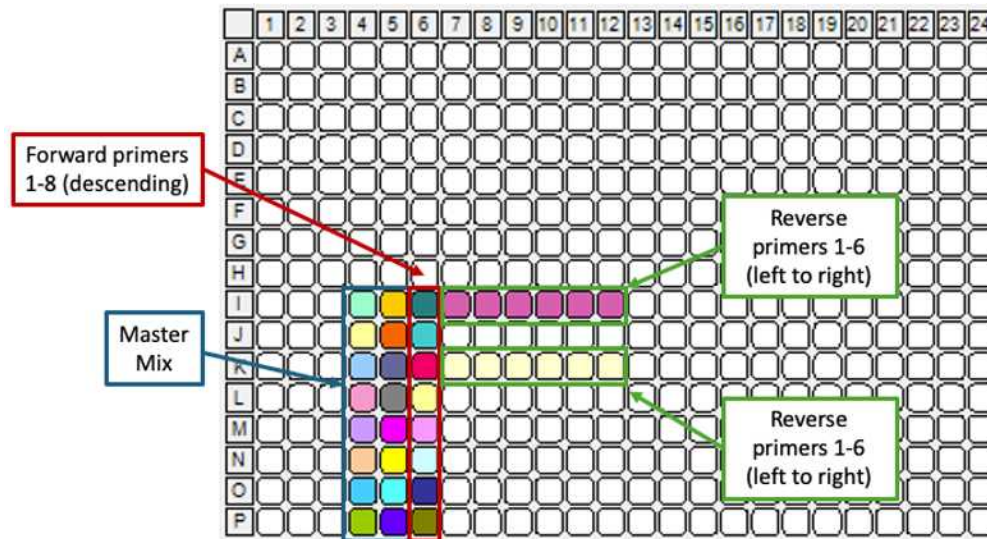
Index PCR

- 28 NOTES:
 - Total volume = 10 μL for each sample/well
 - Source plate = Same Echo 384 well source plate from Amplicon PCR
 - Destination plate = Thermo Armadillo High Performance 96-well PCR Plate
- 29 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 26 by hand to complete the total 10 μL volume necessary for Index PCR.
- 30 Pipet 55 μL of the master mix into the wells I4-P4 and I5-P5.



Source plate layout. Master mix is in wells I4-P4 and I5-P5, forward primers are in wells I6-P6, and reverse primers are in wells I7-I12 and K7-K12.

- 31 Pipet 35 μ L of each of the 8 Forward Nextera primers of the corresponding index plate on wells I6-P6.
- 32 Pipet 35 μ L of each of the 12 Reverse Nextera primers of the corresponding index plate on wells I7-I12 and K7-K12.
- 33 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.
- 34 Make a protocol in Echo Plate Reformat to transfer 1 μ L of forward and reverse Nextera index primers and 6 μ L 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 a Thermo Armadillo High Performance 96-well PCR plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	1	1	1	1	1	1	1	1	1	1
B	1	1	1	1	1	1	1	1	1	1	1	1
C	1	1	1	1	1	1	1	1	1	1	1	1
D	1	1	1	1	1	1	1	1	1	1	1	1
E	1	1	1	1	1	1	1	1	1	1	1	1
F	1	1	1	1	1	1	1	1	1	1	1	1
G	1	1	1	1	1	1	1	1	1	1	1	1
H	1	1	1	1	1	1	1	1	1	1	1	1

Destination plate layout after running the Echo protocol.

- 35 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.
- 36 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 μ L. 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.
 - 36.1 Save this exceptions report in a folder on the desktop. Open Echo Cherry Pick and select New.
 - 36.2 Change the Source plate 384PP and Destination plate to 96_Twin.Tec_Skirted_PCR.
 - 36.3 In the Pick List tab, import the updated exceptions report. Unselect Sample ID and Name, then click Import.
 - 36.4 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.

- 37 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 μ L 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.
- 38 Perform Index PCR using the following program:
 - The PCR reaction will proceed in the Mastercycler epGradient Eppendorf thermocycler. Double-check the parameters are correct, then proceed.
 - Seal the 96-well plate and perform Amplicon PCR in the thermocycler 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
- 39 After Index PCR is complete, run 2-4 samples on the TapeStation BioAnalyzer using the High Sensitivity D1000 DNA ScreenTapes.
 - Transfer 0.5 μ L of Ladder and samples into 2 μ L of water and 2 μ L of TapeStation Buffer.
 - Follow the remaining TapeStation protocol as usual.
- 40 Optional: Store at -20°C if not performing Index PCR bead cleanup right away.

Index PCR Bead Cleanup in the KingFisher Apex

- 41 Before starting, run UV sterilization on the King Fisher for at least 5 minutes.
- 42 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).
- 43 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 (μ L)



	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 the plate	NA

Processing plates to complete bead cleanup.

- 44 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.
- 45 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 μ L 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.
- 46 Pipet 130 μ L of beads into 8 PCR strip tubes. Then pipet 10 μ L of beads into each well of the Sample plate from Index PCR step 40 using a multichannel pipet. Don't mix the samples after adding the magnetic beads.
- 47 Prepare the Elution Plate. Pipet 300 μ L of water into 8 PCR strip tubes. Use a multichannel pipet to transfer 25 μ L into each well of a new 96-well plate (Elution Plate).
- 48 Prepare the Tip Comb and plate. In a new 96-well plate, place a new Tip Comb.
- 49 Then run the same PCR cleanup protocol as used in Amplicon PCR bead cleanup on the KingFisher Apex.
- 50 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.

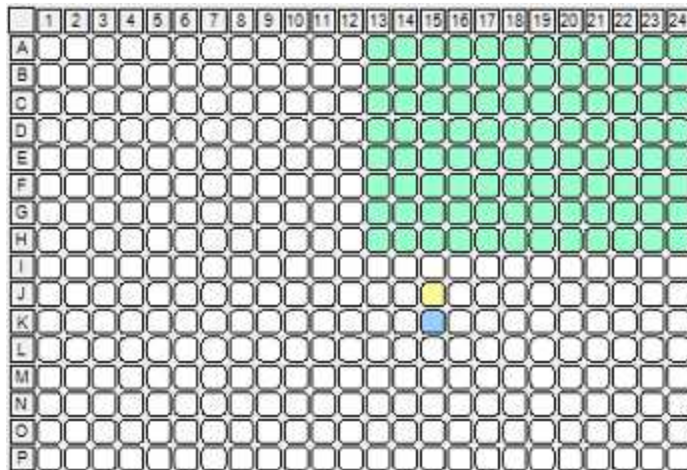
- 51 Once the last plate is loaded, close the door on the instrument.

Note: The run proceeds for approximately 42 minutes.

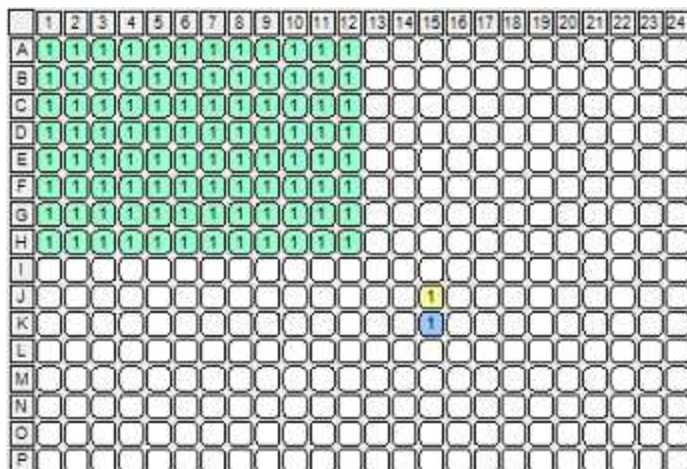
- 52 At the end of the run, the instrument will prompt the user to remove the plates one by one. Check if any beads remain in the Elution Plate and Sample Plate.
- If there are a lot of beads remaining in the Sample Plate, re-run the entire bead cleanup protocol. If only a few beads are remaining in 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.
- 53 Remove the Elution plate. Discard the other plates.
- 54 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 μ L of Ladder and samples into 2 μ L of water and 2 μ L of TapeStation Buffer.
 - Follow the remaining TapeStation protocol as usual.
- 55 Seal the plate for storage. Samples can be stored at -20°C until further steps.

Quantification with Qubit and Diluting to 4 nM

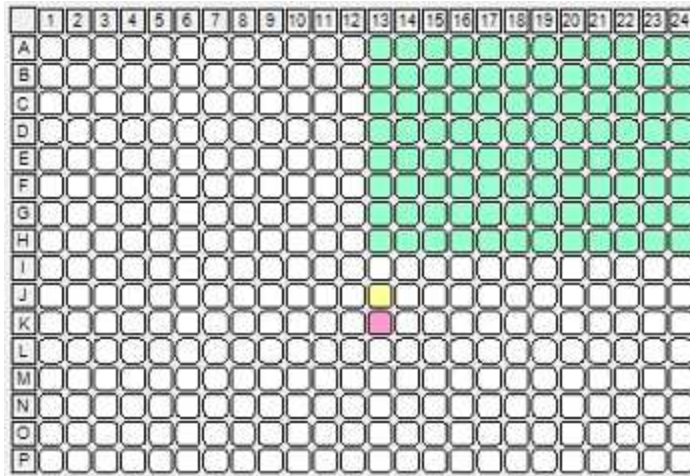
- 56 Make sure to take the Qubit 1X dsDNA HS Working Solution Buffer and Standards 1 and 2 out of the fridge and allow them to get to room temperature before starting.
- 57 Transfer 25 (all) μ L of samples from the elution plate from step 57 to corresponding Source Plate wells A13-H24.
- 58 Depending 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 μ L of Standard 1 to well J15 and 20 μ L of Standard 2 to well K15 of the Source Plate.
 - If using wells A13-H24 of the black-edged 384-well plate, add 20 μ L of Standard 1 to well J13 and 20 μ L of Standard 2 to well K13 of the Source Plate.
- 59 Make a protocol in Echo Plate Reformat to transfer 0.6 μ L 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.

- 60 After the Echo protocol is finished transferring, move the black-edged 384-well Destination plate back into the hood.
- 61 Transfer 6.5 mL of the Qubit 1X dsDNA HS Working Solution Buffer to a sterile trough.
- 62 With a multichannel pipet, manually add 61.4 μ L 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 μ L to wells J13 and K13 or J15 and K15 for the standards.
- 63 Cover the plate from light and let it sit for 2 minutes after adding the Qubit buffer to each well.
- 64 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 μ L" 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.
- 65 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.

- 66 Calculate the volume needed to dilute each sample to 4 nM with a total of 5 μL using the concentrations in nM.

- Equation:
$$(4\text{nM} \times 5\mu\text{L}) / \text{sample concentration in nM}$$

NOTE: If a sample requires more than 5 μL, only add 5 μL of sample and no water. Do not add more PCR product of that sample.

- 67 Copy calculated dilution values into a list form going from A1-A12, B1-B12, etc.

- 68 Set up an excel file for use in Echo Cherry Pick on the Echo Liquid Handler. Make sure to include the following columns:

Source Plate Name	Source Plate Barcode	Source Well	Destination Plate Name	Destination Plate Barcode	Destination Well	Transfer Volume
Source[1]	384PP_AQ_BP	A13	Destination[1]	96Well_V-Bottom - Costar	A1	1107
Source[1]	384PP_AQ_BP	A14	Destination[1]	96Well_V-Bottom - Costar	A1	1454
Source[1]	384PP_AQ_BP	A15	Destination[1]	96Well_V-Bottom - Costar	A1	1446
Source[1]	384PP_AQ_BP	A16	Destination[1]	96Well_V-Bottom - Costar	A1	988
Source[1]	384PP_AQ_BP	A17	Destination[1]	96Well_V-Bottom - Costar	A1	3146

Layout of excel file to be used for diluting and pooling using Echo Cherry Pick.

- Source Plate Name, Source Plate Barcode, Destination Plate Name, and Destination Plate Barcode will not be changed.
- When updating this file, ensure that the Source Well matches the correct sample for Transfer Volume concentrations.
- Destination plate is a 96-well plate where everything will get pooled into one column. For example, everything in column A on the Source plate will be pooled into A on the Destination plate, and so on.
- Transfer Volume is the amount the Echo will transfer from the Source plate to the Destination plate. Copy the list of calculated dilution values in the Transfer Volume column and multiply every calculation by 1000 to get to nL instead of μL.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

Source Plate layout for pooling and diluting to 4 nM.

- 69 After transferring with the Echo, all of the library will be in 8 wells on a 96-well plate. Manually pool these together into one 1.7 mL Eppendorf tube.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Destination Plate layout for pooling and diluting to 4 nM.

- 70 Calculate how much water to add to the pooled libraries to dilute to 4 nM.
- First, add the calculated dilution values of all samples together in μL .
 - Subtract this by 480 μL ($5 \mu\text{L} * 96$ samples) to determine the amount of water to add to the pooled tube.
- 71 Add the calculated amount of water to the pooled library tube to dilute to 4 nM.



- 72 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.
- 73 Store pooled libraries at -20°C until sequencing.