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Automated QIAseq FX DNA Library Preparation on the Opentrons FLEX

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Opentrons. QIAseq FX 48× FLEX protocol for Opentrons (no date). Opentrons Library. Retrieved, from <https://library.opentrons.com/p/QIAseq-FX-48x>

QIAGEN. *QIAseq FX DNA Library Kit Handbook*. Version 12/2022, QIAGEN, 2022.
<https://www.qiagen.com/us/resources/resourcedetail?id=3c85f7e7-7a6b-4d55-b7b2-8e6b94dc94e2&lang=en>.

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We use this protocol and it's working

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Abstract

This workflow automates DNA-library construction for next generation Illumina short read sequencing by integrating the QIAseq FX DNA Library Kit with the Opentrons FLEX liquid-handling platform. A single FLEX script performs fragmentation, end-repair/A-tailing, adapter ligation, and bead clean-ups, eliminating manual pipetting and standardizing large-scale library preparation in 96-well plate format. The protocol yields consistent, high-quality libraries ready for short read sequencing while requiring very little hands-on time.

Guidelines

Always use sterile technique when interacting with the Opentrons.

Always keep plates wrapped or lidded when transferring plates on and off the Opentrons deck.

Keep hands out of the Opentrons when the robot is running.

Always keep enzymes cold and minimize freeze thaw cycles



Materials

☒ Ethanol, Absolute (200 Proof), Molecular Biology Grade **Fisher Scientific Catalog #BP28184**

☒ Nuclease-free water **Invitrogen Catalog #10977**

☒ NEST 96 Well Plate 100 μ L PCR Full Skirt **Opentrons Catalog #402501**

☒ NEST 2 mL 96-Well Deep Well Plate, V Bottom **NEST Biotechnology Catalog #503501**

☒ Opentrons Flex Filter Tips, 50 μ L **Opentrons Catalog #991-00104**

☒ Opentrons Flex Filter Tips, 200 μ L **Opentrons Catalog #991-00105**

Protocol materials

☒ QIAseq FX DNA Library Kit **Qiagen Catalog #180475**

☒ QIAseq Beads **Qiagen Catalog #333923**

☒ Buffer EB **Qiagen Catalog #19086**

☒ Ethanol, Absolute (200 Proof), Molecular Biology Grade **Fisher Scientific Catalog #BP28184**

☒ Nuclease-free water **Invitrogen Catalog #10977**

☒ NEST 96 Well Plate 100 μ L PCR Full Skirt **Opentrons Catalog #402501**

☒ NEST 2 mL 96-Well Deep Well Plate, V Bottom **NEST Biotechnology Catalog #503501**

☒ Opentrons Flex Filter Tips, 50 μ L **Opentrons Catalog #991-00104**

☒ Opentrons Flex Filter Tips, 200 μ L **Opentrons Catalog #991-00105**

Safety warnings

- ⚠ Although the robot will automatically pause when the door is open, never put hands inside the robot when it is moving.
Do not place anything outside of the specified deck locations (A1 - D4).
Do not place uncalibrated/unfamiliar labware into the Opentrons until they are properly calibrated and tested.

Before start

Resuspend and allow the SPRI beads to reach room temperature 20-60 mins before starting the plate prep. Keep cold blocks on hand to use during the protocol.

Instrumentation, Reagents, and Consumables

1 Equipment

Equipment

Opentrons Flex NGS Workstation	NAME
Automated liquid-handling workstation (liquid handler)	TYPE
Opentrons	BRAND
991-00354	SKU
https://opentrons.com/products/opentrons-flex-ngs-workstation	LINK

Equipment

Thermocycler Module GEN2	NAME
On-deck thermocycler (PCR thermal cycler)	TYPE
Opentrons	BRAND
991-00114	SKU
https://opentrons.com/products/thermocycler-module-1	LINK

Equipment

Heater-Shaker Module GEN1 NAME

Microplate heater-shaker TYPE

Opentrons BRAND

991-00115-FL-UN SKU

<https://opentrons.com/products/heater-shaker-module> LINK

Equipment

Temperature Module GEN2 NAME

Temperature-control plate (thermoelectric cooler/heater) TYPE

Opentrons BRAND

991-00350-0 SKU

<https://opentrons.com/products/temperature-module-gen2> LINK

Equipment	
Magnetic Block GEN1	NAME
Magnetic separation block (96-well)	TYPE
Opentrons	BRAND
999-00204	SKU
https://opentrons.com/products/opentrons-flex-magnetic-block-gen1 ^{LINK}	

Equipment	
Deck Expansion Kit	NAME
Deck-expansion accessory	TYPE
Opentrons	BRAND
999-00203	SKU
https://opentrons.com/products/opentrons-flex-deck-expansion-set-4-count ^{LINK}	

Equipment

Aluminum Block – 96-well PCR Plate

NAME

Plate adapter

TYPE

Opentrons

BRAND

991-00208

SKU

<https://opentrons.com/products/aluminum-block-96-well-pcr-plate>^{LINK}

Equipment

Opentrons Flex 8-Channel Pipette (50 uL)

NAME

Automated Liquid Handling

TYPE

Opentrons

BRAND

999-00188

SKU

<https://opentrons.com/products/opentrons-flex-8-channel-pipette?sku=999-00188>^{LINK}

Equipment

Opentrons Flex 8-Channel Pipette (1000 uL)	NAME
Automated Liquid Handling	TYPE
Opentrons	BRAND
999-00189	SKU
https://opentrons.com/products/opentrons-flex-8-channel-pipette?sku=999-00189	LINK

Equipment

Opentrons Flex Waste Chute	NAME
Automated Liquid Handling	TYPE
Opentrons	BRAND
999-00227	SKU
https://opentrons.com/products/opentrons-flex-waste-chute-gen1	LINK

2 Reagents

The major reagents for this protocol are listed here. A full list of all reagents and supplies needed is provided in the Materials section and in the following Consumables section.

 QIAseq FX DNA Library Kit **Qiagen Catalog #180475**

 QIAseq Beads **Qiagen Catalog #333923**

 Buffer EB **Qiagen Catalog #19086**

3 Consumables

- NEST 96 Deep Well Plate (2 mL)
- Opentrons Flex 96 Tip Rack (50 µL)
- Opentrons Flex 96 Tip Rack (200 µL)
- Opentrons Tough 96 Well Plate 200 µL PCR Full Skirt

Note

Alternatives for these consumables are available but equipment calibration may be required prior to use.

Preparation, system set-up, and system parameters

35m

4 Pre-protocol preparation

10m

- Prepare fresh 80% ethanol.
- Prepare 60% bleach, if needed for deep sterilization.
- Pre-chill the consumable 96-well plates and Opentrons adapters at -20 °C.
- Refer to original QIAseq FX DNA Library Kit Handbook for details on all reagents.
- Handle reagents in a sterile environment (e.g. biosafety hood) with pipettes cleaned with DNase Away or equivalent.
- Thaw reagents slowly on ice and keep cold throughout use.
- Determine the fragmentation time for the input DNA. Table 1 relates total ng of input DNA and the desired fragment peak size (bp) to fragmentation time in minutes.

DNA Input	250 bp	350 bp	450 bp	550 bp
50 pg - 1 ng	14	4	1	-
10 ng	24	16	14	10
100 ng	16	10	8	6
1000 ng	14	8	6	4

Table 1. Fragmentation time as a function of input DNA and desired fragment size.

- Determine the number of PCR cycles dependent on the size of the input DNA. Table 2 relates the amount of input DNA to the number of PCR cycles.

Input DNA (ng)	Number of PCR cycles
100	6
10	10
1	12
0.1	14
0.02	16

Table 2. Input DNA in ng and the recommended number of PCR cycles

5 Opentrons Flex deck: equipment set-up

5m

- Set up the deck of the NGS Flex according to the the diagram below.



Figure 1. Opentrons Flex Deck Configuration.

6 Sterilization

5m

- Wipe down all surfaces with 70% ethanol. Do not spray directly on the deck, instead you a ethanol soaked paper towel to wipe down the surfaces.



- Using a fresh ethanol soaked paper towel wipe down the rubber seal on the thermocycler.
- Turn on HEPA air filter and UV sterilization (see Figure 3 in step 7). Sterilization will automatically turn off after 15 minutes.

Note

For deep sterilization, first wipe the the deck with 60% bleach solution before moving on to the procedure listed above.

7 Powering on the Opentrons Flex

5m

- Power on the Opentrons Flex with the switch on the back of the machine, as seen in Figure 2.



Figure 2. Opentrons Flex power panel with the power switch outlined in red.

- Power on the HEPA/UV filter with the switch on the back of the machine, as seen in Figure 3.



Figure 3. HEPA/UV module power panel with the power switch outlined in red.

- The thermocycler module has a separate power-block outside the Opentrons Flex. Power on the thermocycler with the power switch as seen in Figure 4



Figure 4. External thermocycler power block with the power switch outlined in red.

- Using the switch on the deck inside the Opentrons Flex, power on the temperature block and heater-shaker modules with the power switches as seen in Figure 5.

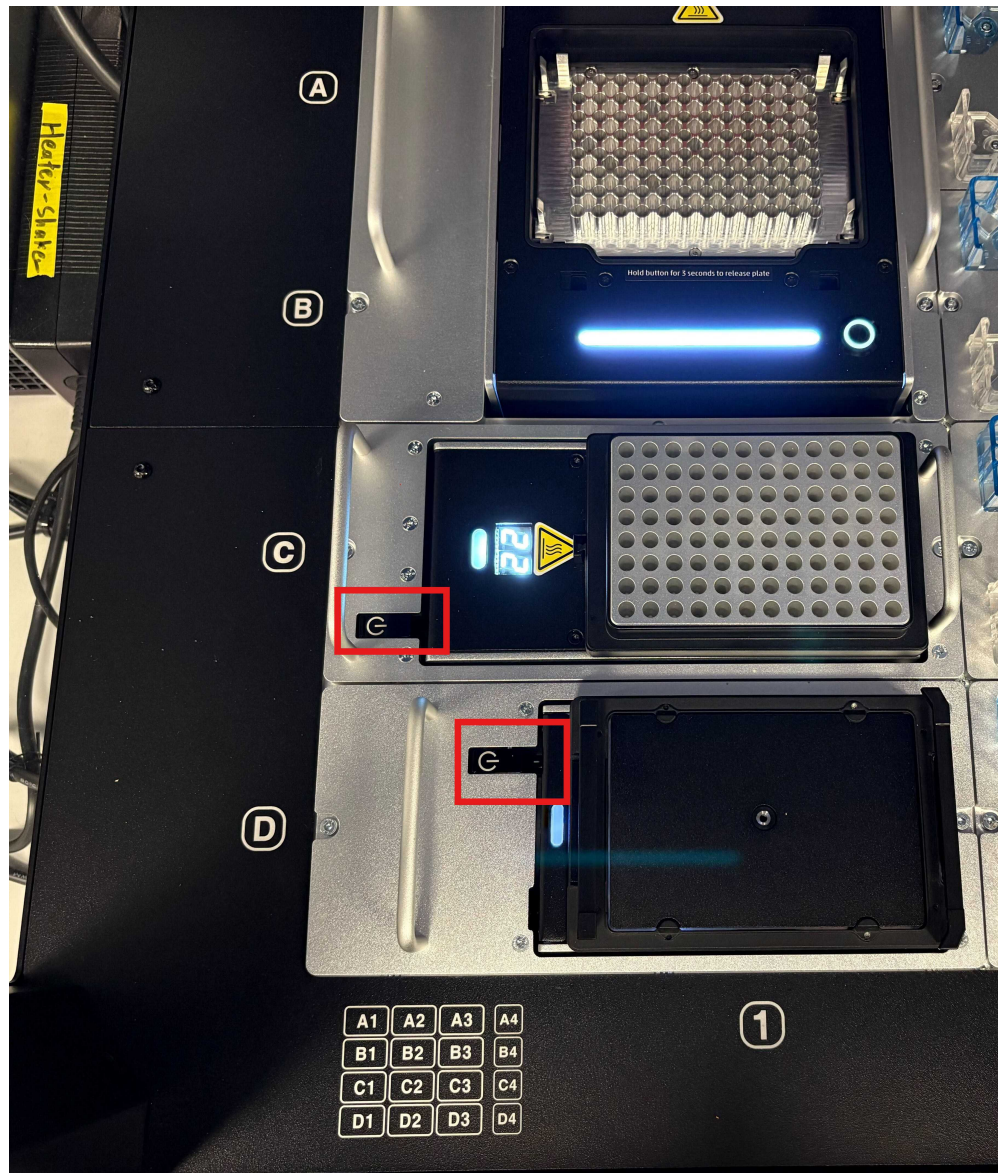


Figure 5. Opentrons Flex modules: temperature block module in deck slot C1 and heater-shaker in deck slot D1 with power buttons outlined in red.

8 Python script uploading

10m

- Install the Opentrons app onto a suitable computer: <https://opentrons.com/ot-app>.
- Download the python script associated with this protocol from this GitHub repository: https://github.com/exfab/Opentrons_Flex_ExFAB
- In the Opentrons app, under the "Protocols" tab, click import and upload the python script, as seen in Figure 6.
- Connect the Opentrons Flex to a computer running the app through either a wired USB connection or a WIFI connection, which requires setup not described in this

protocol. Once connected the "Devices" tab will display the connected Opentrons Flex as shown in Figure 7.

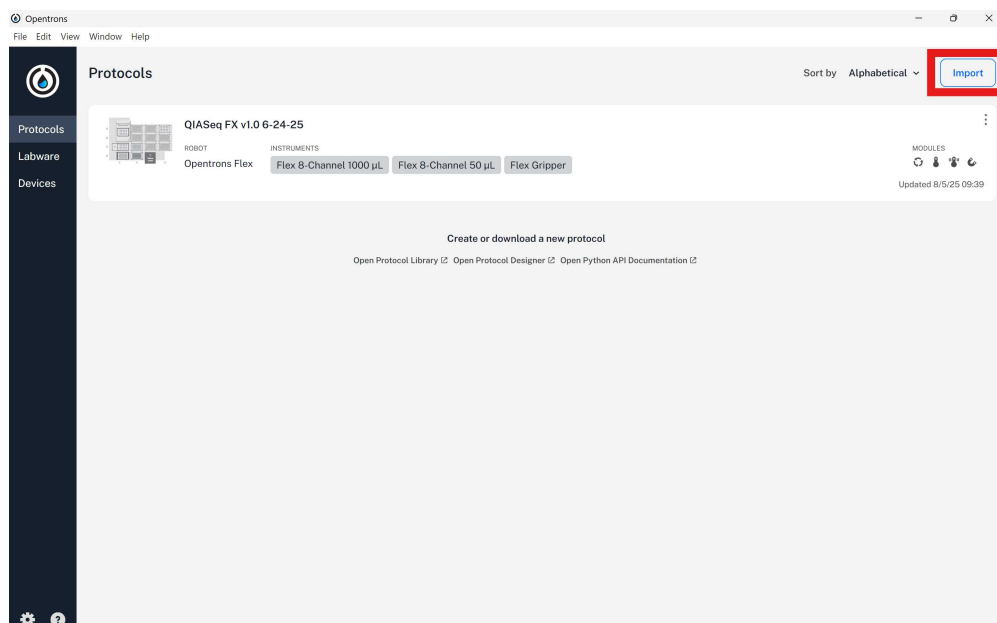


Figure 6. Opentrons app "Protocols" tab screen with the import button outlined in red.

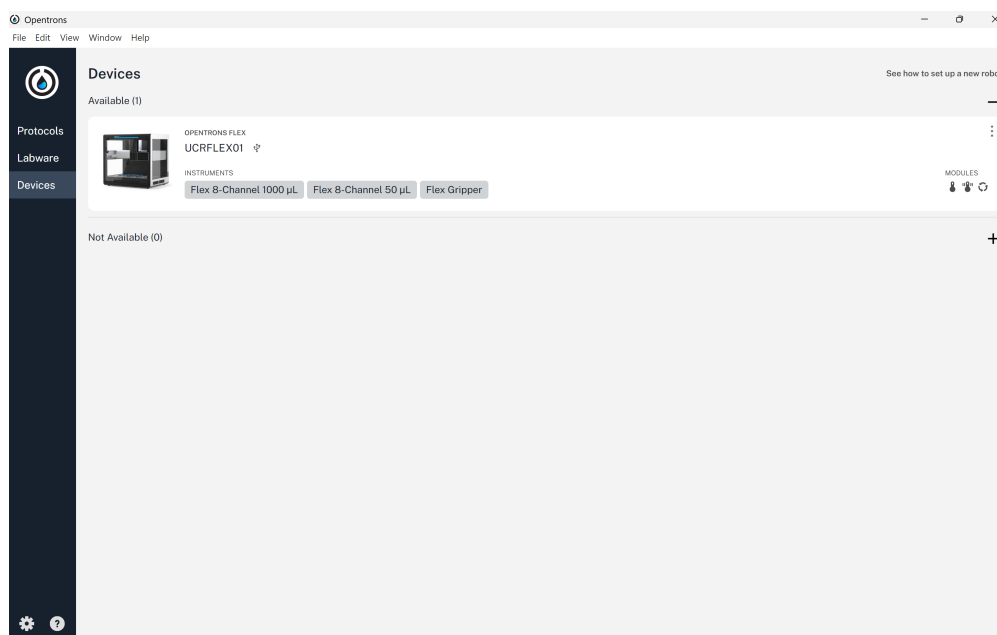


Figure 7. Opentrons app "Device" tab screen with a connected Opentrons Flex

9 Thermocycler and temperature block set-up

Note

Thermocycler parameters are set in the protocol script. These settings are further described in the QIAseq FX DNA Library Kit Handbook. No action is needed here; parameter values are provided for completeness.

- Set the thermocycler lid to 70°C.
- Set the temperature block module to 4°C.

	Step	Temperature (°C)	Time
	Pre-cooling	4	3 min
	DNA fragmentation	32	Determined by the fragment peak size in step 4.
	Enzyme inactivation	65	30 min
	Hold	4	∞

Table 3. Thermocycler parameter settings for fragmentation.

	Time per step	Temperature (°C)	Time
	2 min	98	1
	20 s	98	
	30 s	60	See Table 2.
	30 s	72	
	1 min	72	1
	∞	4	Hold

Table 4. Thermocycler parameter settings for PCR amplification.

Sample, reagent, and reservoir plate set-up

40m

10 Sample and sample plate preparation

10m

**Note**

- To keep the sample plate free of any DNases or contaminating DNA, all work must be performed in a biosafety cabinet or PCR hood.
- This protocol is designed for 48 samples, modify the layout as needed to accommodate other sample counts.

- For each genomic DNA sample, prepare 100 ng of DNA in 35 μ L of QIAGEN's Buffer EB (0.1x TE, DNase free water and 10 mM Tris are also acceptable).
- Using the DNA prepared above, aliquot 35 μ L of each sample into a single well of the pre-chilled 96-well PCR plate.
- The DNA samples should be aliquoted as shown in Figure 8 below. Samples to be processed should be placed in the first 6 columns leaving the second 6 columns empty and available for subsequent steps.

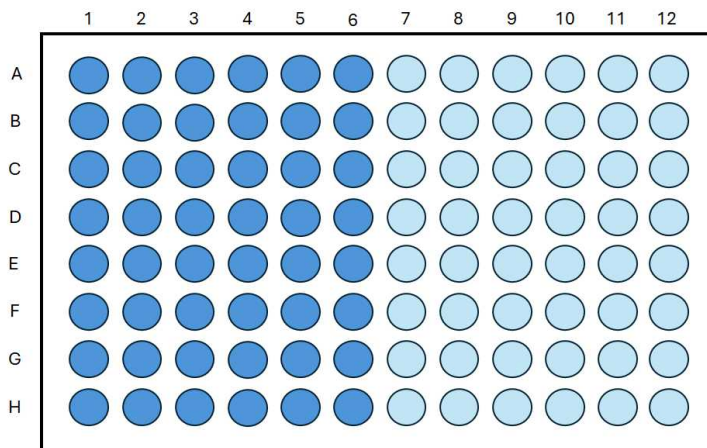


Figure 8. Initial plate configuration. Dark blue = DNA samples; Light blue = empty

11 Reagent and reagent plate preparation

15m

**Note**

- Ensure all enzymes and buffers are stored between -30°C and -15°C.
- All master mix solutions should be made in a sterile, DNase- and RNase-free microcentrifuge tubes.
- Aliquot Qiagen kit reagents to minimize freeze-thaw cycles.
- Vortex thawed reagents prior to use, with the exception of the ligation buffer mix.
- Tips must be changed after each aliquot.
- 10% extra reagent is included in the protocol below to accommodate for any potential errors in pipetting.
- Column 6 of the reagent plate will not be used

- Create reagent solutions and aliquot reagents into a standard, low-format 96-well plate as described in the steps below.
- Refer to Table 5 below for a summary of the compositions and volume of each reagent used to create the various solutions.
- Refer to Figure 9 below for a summary of the locations of various reagent solutions.

Column 1: Fragmentation master mix

- Reagent solution: Add 264 μL of FX buffer and 528 μL of FX Enzyme in a 1.5 mL microcentrifuge tube.
- Aliquot 99 μL of fragmentation mix to all wells in column 1.

Column 2-3: Ligation master mix

- Reagent solutions: Make two ligation mix reagent solutions, each one in a 2 mL microcentrifuge tube by adding 528 μL of ligation buffer, 264 μL of DNA ligase, and 396 μL of nuclease free water into each tube.
- Aliquot 148 μL of the ligation mix into each well in columns 2 and 3.

Column 4: Primer mix

- Reagent solution: Add 79.2 μL of primer mix and 184.8 μL of nuclease free water into a 1.5 mL microcentrifuge tube.
- Aliquot 33 μL of primer mix into each well of column 4.

Column 5: PCR master mix

- Use the PCR master mix tube provided in the Qiagen kit.
- Aliquot 165 μL of the PCR mix into each well of column 5.

Column 7-12: Barcodes and adapters

- Barcodes are provided in a foil-sealed 96-well PCR plate in the Qiagen kit.
- Break the foil seal of each well that will be used and aliquot 5.5 μL into columns 7 through 12.
- Note the barcode used in each well.

Reagent	Single Reaction (μL)	48 Reactions (μL)
Column 1: Fragmentation Mix		
FX Buffer	5.5	264
FX Enzyme	11	528
Total	16.5	792
Aliquot/Well		99
Column 2: Ligation Mix		
Ligation Buffer	22	1056
DNA Ligase	11	528
H2O	16.5	792
Total	49.5	2376
Aliquot/Well		297
Column 4: Primer Mix		
Primer	1.65	79.2
H2O	3.85	184.8
Total	5.5	264
Aliquot/Well		33
Column 5: PCR Mix		
PCR	27.5	1320
Aliquot/Well		165
Column(s) 7-12: Barcoded Adapters		
Barcode Column(s)	5.5	Columns: 7-12
Aliquot/Well		5.5

Table 5. Reagent composition. This table is color-coded to match Figure 9.

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

Figure 9. Reagent plate setup. Colors in this figure correspond to colors on the volume table, Table 3.

12 Reservoir plate preparation

15m

Note

- Use fresh 80% ethanol solution for each run.
- SPRI beads used in this section must be equilibrated to room temperature for at least 20 minutes at before use.
- Use care when re-suspending the beads; tilt and gently swirl to re-suspend in solution.
- Columns 3, 7, 8, and 9 of the reservoir plate are not used.

- The reservoir plate prepared in this step will be used in the magnetic bead extraction steps described below.
- Refer to Table 6 below for a summary of the compositions and volume of each reagent used to create the various solutions.
- Refer to Figure 10 below for a summary of the locations of various reagent solutions.

Column 1: SPRI Beads

- Use the SPRI beads bottle provided by Qiagen.
- In a sterile 96 deep well plate aliquot 1188 μ L of SPRI beads into all wells of column 1.



Column 2: Resuspension Buffere (RSB)

- Use Buffer EB provided by Qiagen.
- Aliquot 646.8 μL of RSB into all wells of column 2.

Column 4-6 : 80% ethanol

- Use freshly prepared 80% molecular biology grade ethanol.
- Aliquot 1980 μL into all wells of columns 4, 5 and 6.

Reagent	Single Reaction (μL)	48 Reactions (μL)
Column 1: Cleanup Beads		
AMPure	82.5	3960
Aliquot/Well		495
Column 2: RSB		
RSB	38.5	1848
Aliquot/Well		231
Columns 4-6: 80% EtOH		
80% EtOH	330	15840
Aliquot/Well	Columns 4-6	660
Columns 10-12: Trash Wells		

Table 6. Reservoir reagent composition. This table is color coded to match Figure 10.

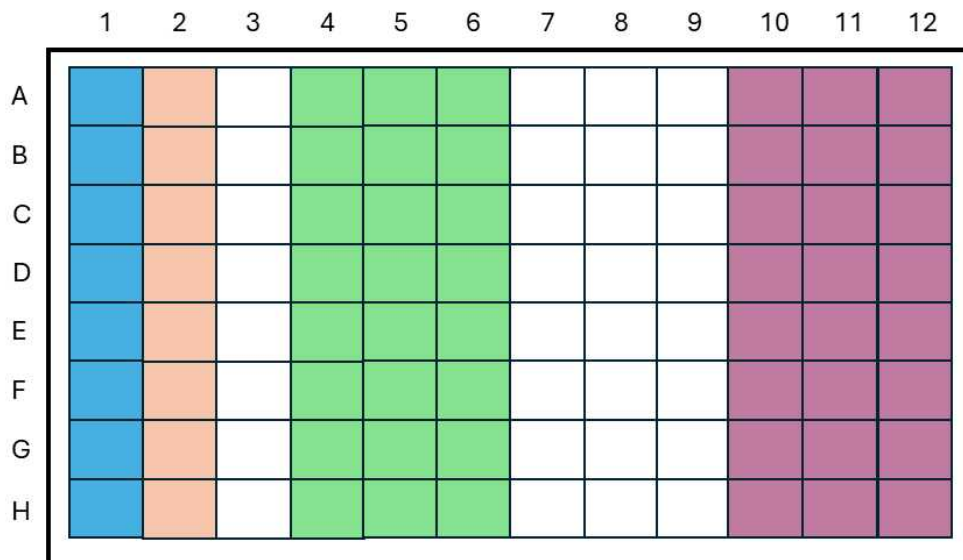


Figure 10. Reservoir plate setup. Colors in this figure correspond to the colors on Table 6.

Protocol set-up and operation

4h 55m

13 Executing the NGS library preparation protocol

10m

- Click on the previously uploaded protocol and select the "Start setup" button, as seen in Figure 11.

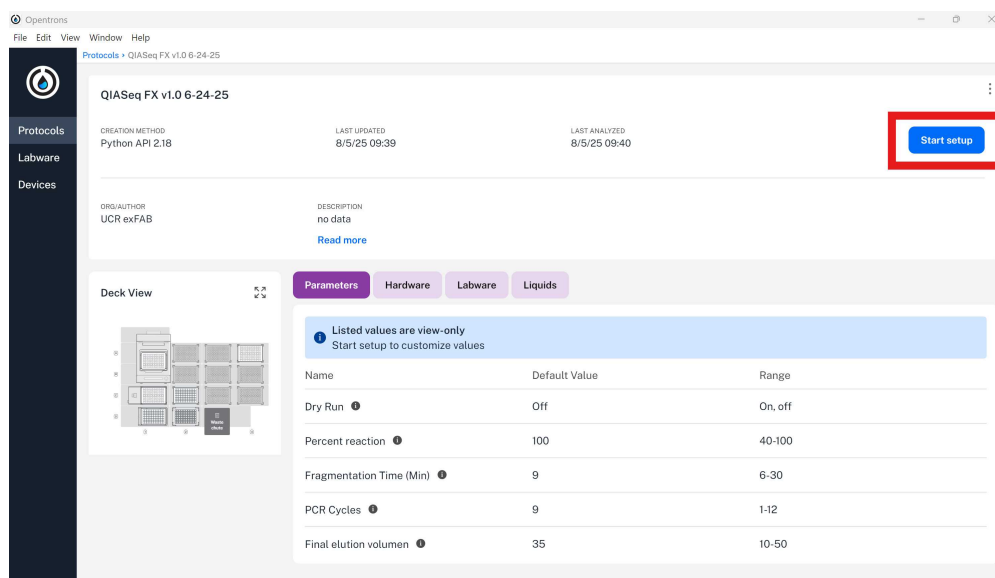


Figure 11. Opentrons control software: Start setup button outlined in red.

- A user prompt will appear in which the user can select whether the protocol provided will be a dry (test) or regular run. Other input parameters include, percent reagent to be used, fragmentation time in minutes, number of PCR cycles, and final elution volumes. These parameters are shown in Figure 12.

Dry run - If switched on, the protocol will skip all thermocycler preheating, cycles, and sample shaking. Switching this on and running this protocol with water is recommended for preliminary testing.

Percent reagent - This value will automatically calculate how much of each reagent to add to reactions. This protocol is written for 100% reagent use however the protocol can reliably run at 40% reagent use with high quality DNA.

Fragmentation time - This will determine the time the thermocycler will run the fragmentation reaction after adding fragmentation reaction mix. See Table 1 for reference.

PCR cycles - This sets the number of PCR cycles the thermocycler will run after adding the PCR primers and master mix. See Table 2 for reference.

Final elution volumes - This will set the final elution buffer volume added to the magnetic bead product. This value will affect the concentration of resulting DNA, a smaller elution volume will result in a lower volume but higher DNA concentration.

- Once parameters are input, select "Confirm values."

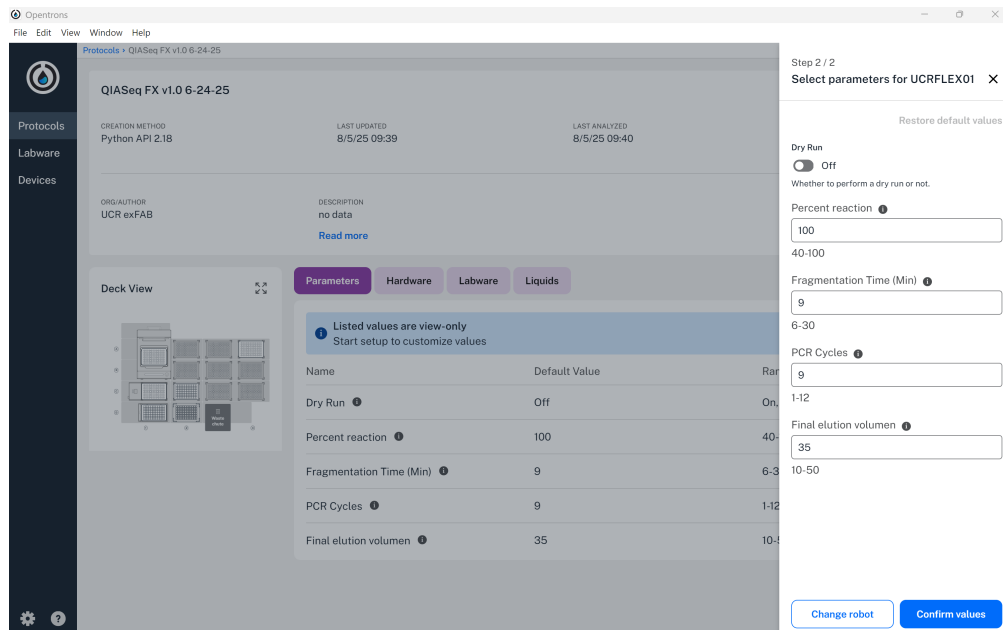


Figure 12. User parameter selection; Dry run switch, percent reaction, fragmentation time, PCR cycles, final elution volume.

- The protocol will load onto the Flex workstation and the same options will appear on the Opentrons app and the on-deck touchscreen display, as seen in Figure 13.



Figure 13. On-deck touchscreen display.

- Resolve any location conflicts and enter labware offsets according to the on-screen tutorials.

14 Reagent set-up and protocol start

15m

- Place reagent, sample, and reservoir plates on the deck as shown in Figure 14.

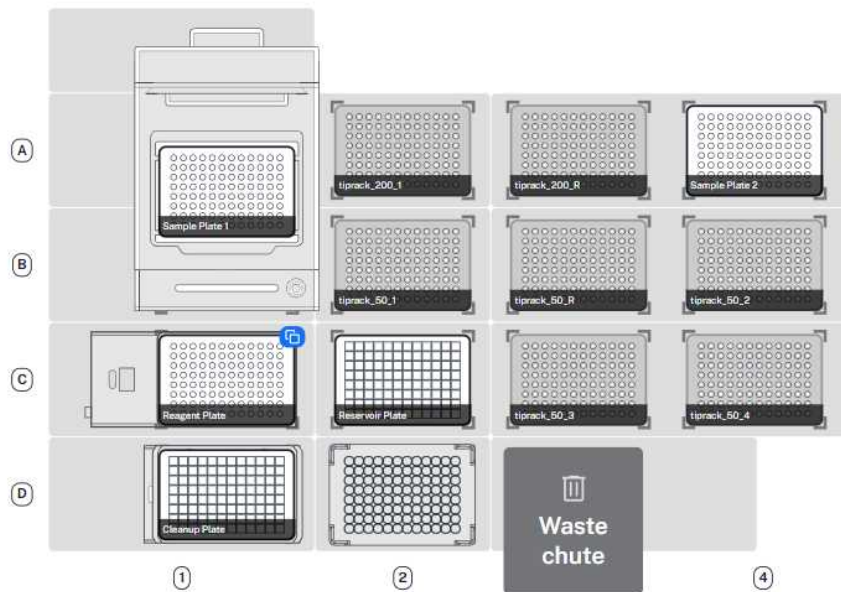


Figure 14. Opentrons Flex deck set-up prior to executing the protocol.

- Once the deck is loaded and the protocol parameters are set, the blue start button can be selected and the protocol started.

Note

- When the Opentrons Flex finishes the protocol, the resulting libraries will be located in "Sample Plate 2", depicted in deck location A4 on Figure 14. Cover the plate with a plastic or foil seal and store at -20°C.

15 NGS library preparation protocol

4h 30m

Note

- This section summarizes the actions undertaken after initiating the protocol in step 14. The details here are provided for completeness.



- After clicking the blue start button, the Opentrons Flex system will conduct the following procedures.

Fragmentation

- Fragmentation master mix is dispensed into designated wells in the sample plate loaded on to the thermocycler, the thermocycler lid is closed, and the temperature program is executed.

Ligation

- Barcodes, adapters, and ligation master mix are added to the working columns of the sample plate. The samples are incubated at 20 °C for 15 min to complete ligation.

Bead Cleanup

- The Flex transfers the samples to a plate on the Heater-Shaker Module to mix with SPRI beads, then moves the plate to the Magnetic Block. After binding, the supernatant is discarded, two ethanol washes are performed, and the DNA is re-suspended. The sample is transferred to a separate portion of the plate and the beads are left behind.

Library Amplification

- Primers and HiFi PCR master mix are introduced to the sample wells and the sample plate is moved to the thermocycler. The lid is closed and the temperature program executed.

Second Bead Cleanup

- The Flex transfers the samples to a plate on the Heater-Shaker Module to mix with SPRI beads, then moves the plate to the Magnetic Block. After binding, the supernatant is discarded, two ethanol washes are performed, and the DNA is re-suspended.

Clean up and shut down procedure

15m

- | | | |
|----|---|----|
| 16 | The final plate will be moved to the heater-shaker, seal the plate and store it in a -20 °C refrigerator, this will stay stable for 3 months in the fridge, or alternatively move on to quantification and quality control. | 5m |
| 17 | Dispose of any plates and tips by double bagging into biohazard bags to be autoclaved and disposed of. | 5m |
| 18 | Repeat the sterilization method in step 6 and turn off all modules and robots as described in step 7 | 5m |



Library quantification and quality control

- 19 Quantification is recommended to be done as soon as library preparation is completed to avoid freeze thaw cycles.

Protocol references

Opentrons. QIAseq FX 48× FLEX protocol for Opentrons (no date). Opentrons Library. Retrieved, from <https://library.opentrons.com/p/QIAseq-FX-48x>

QIAGEN. *QIAseq FX DNA Library Kit Handbook*. Version 12/2022, QIAGEN, 2022.

<https://www.qiagen.com/us/resources/resourcedetail?id=3c85f7e7-7a6b-4d55-b7b2-8e6b94dc94e2&lang=en>.

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