LoopCap™ Multiplex Viral Target Capture (ONT)

User Guide

ML5120 (96 reactions)
ML5220 (384 reactions)

Molecular Loop Proprietary

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Protocol Description

LoopCap™ Viral Target Capture Kits are used to prepare next-generation sequencing libraries for whole genome or targeted sequencing of DNA and RNA viruses. This protocol contains instructions for library preparation for sequencing on Oxford Nanopore Technologies (ONT) platforms, with ON probe designs. Refer to product labeling for the probe design code, which contains ON in the descriptor, as well as the probe fill size and the design version number.

Highly multiplexed designs, such as those used for pan-viral applications, can result in high levels of background noise which limit yield of the target product. This protocol describes the recommended workflow for use with pan-viral designs but may also be useful in single-virus designs with very low input copy numbers.

Required Kits

The table below lists the kits required for preparation of **96 reactions**. Index primers are supplied as ready-to-use premixes in 96-well plates. Refer to the ML2100-ON Index Primer Kit Product Data Sheet for plate layout and primer sequences.

Part Number	Reagent	Quantity	Storage
ML5120	Viral Target Capture Kit (96 reactions)	1	-15 to -25 °C
ML2100-ON	Index Primer Kit, ONT (1 x 96-reaction plate)	2	-15 to -25 °C
ML0004	PCR Mix Kit (96 reactions)	1	-15 to -25 °C

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Reagents, Consumables, and Equipment

The items listed below are recommended when this protocol is performed manually in 96-well plates. Reactions may also be performed in single PCR tubes or strip tubes. Contact support@molecularloop.com for assistance when using liquid handling automation.

User-supplied reagents

Item	Vendor	Catalog #
KAPA Pure Beads	Roche	KK8002
80% Ethanol (for bead purification)	Various	N/A
Bioanalyzer DNA 7500 Assay	Agilent	5067-1506
1X TE Buffer, pH 7.0 - 7.5	Various	N/A
1X TE Buffer, pH 8.0	Various	N/A

User-supplied consumables

Item	Vendor	Catalog #
96-well PCR plates, white/clear	Bio-Rad	HSP9601
Microseal B film	Bio-Rad	MSB1001
1.5 ml low-bind microcentrifuge tubes	Eppendorf	22431021
Pipette tips, filtered (various sizes)	Various	Various

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Equipment

Item	Vendor	Catalog #
Centrifuge (5810 or similar)	Eppendorf	5811000017
Centrifuge (5418 or similar)	Eppendorf	5418000017
Mastercycler X50s	Eppendorf	6311000010
Microfuge	VWR	C1413V-115V
Pipettes (P10, P20, P200, P1000)	Eppendorf / Gilson	Various
Film sealing roller for PCR plates	Bio-Rad	MSR0001
PCR workstation, AC600 series	Airclean	AC624LFUV
DynaMag-2 tube magnet	Thermo Fisher	12321D
Bioanalyzer 2100 (or similar)	Agilent	G2939A

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Input Recommendations

This protocol has been optimized for multiplex viral applications. Depending on the application, variable amounts of input nucleic acid may be appropriate. Best results will be achieved if reactions contain at least 1,000 copies of input nucleic acid. To reduce inter-sample performance variability, all samples in a batch should be quantified using the same method and normalized to the same concentration.

NOTE: *The relationship between input copies and qPCR Ct value may vary and must be empirically determined by the user. Higher amounts of input nucleic acid (lower Ct values) will generally produce superior results.*

Purified nucleic acid should be resuspended in nuclease-free water or 1X TE Buffer (10 mM Tris-HCl, 1 mM EDTA), pH 7.0 - 7.5. Contaminants including ethanol, sodium azide, sodium acetate, and guanidine salts may affect performance. The presence of small amounts of host nucleic acid should not affect performance.

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Thermal Cyclers and Programs

This protocol is sensitive to evaporation, particularly during the hybridization step. It is therefore important to use thermal cyclers with proper lid engagement that form a tight seal against the reaction plate. In addition, the cycler lid should be heated to 105 °C for all steps except 4 °C holds, where it may be omitted. Note that some thermal cyclers may not allow the use of a heated lid for lower block temperatures, such as the 25 °C step during Reverse Transcription. Should this be the case, use a heated lid for all steps where it is permissible by the instrument to do so.

Program and Steps	Temperature and Time (hh:mm:ss)
RTHybFill Reverse Transcription Denature Hybridize and Fill Hold	**Heated lid 105 °C** 25 °C 00:10:00, 50 °C 00:50:00 95 °C 00:01:00 55 °C 24:00:00 55 °C ∞
CleanupPCR1 Cleanup Denature PCR Hold	**Heated lid 105 °C** 45 °C 00:30:00 95 °C 00:03:00 **8** x [98 °C 00:00:15, 55 °C 00:00:15, 72 °C 00:01:30[1]] 4 °C ∞
PCR2 Denature PCR Hold	**Heated lid 105 °C** 95 °C 00:03:00 **N**[2] x [98 °C 00:00:15, 55 °C 00:00:15, 72 °C 00:01:30[1]] 4 °C ∞

[1] An extension time of 1 min 30 sec is recommended for 675 bp fill designs; for larger designs (e.g. 1125 bp fill), increase the extension time to 2 min 30 sec.

[2] The number of PCR cycles to run in PCR 2 (N) should be adjusted based on the amount of input RNA, the number of probes, the hybridization timespan, and the sensitivity of the quantification method. On-target yield (an amplicon of ≥700 bp) will change approximately linearly with respect to both input RNA and number of probes. A nonspecific amplicon of ~100-150 bp may also be observed, especially in reactions with lower RNA input. Recommended cycle numbers are provided on the next page.

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PCR Cycle Number Recommendations

The recommended cycle numbers in the table below are based on a 16 hour hybridization with quantification using an Agilent Bioanalyzer 2100 DNA 7500 assay. More sensitive quantification methods may allow for fewer PCR cycles.

The relationship between number of input copies and qPCR Ct value may vary and must be empirically determined by the user. If samples are unnormalized with highly variable Cts or unknown copy numbers, assume a copy number of at least 10,000 and employ a high sensitivity quantitation method (with proportionally fewer PCR cycles) to limit overamplification of higher input samples. In general, minimizing PCR cycles will produce more on-target and uniform coverage, so run as few cycles as necessary to achieve precise and reproducible quantification.

The probe count included in product labeling is the **total number** of probes in the assay. In multiplexed designs, only a subset of those probes will be active when analyzing samples with a single infection. Refer to the information provided with multiplexed assays for average target probe counts and use those to determine the appropriate cycle numbers to use for the assay.

PCR	# Probes	1,000 copies	10,000 copies	100,000 copies
1	**All**	8	8	8
2	**128**	23	20	17
	256	22	19	16
	512	21	18	15
	1,024	20	17	14
	2,048	19	16	13
	4,096	18	15	12
	8,192	17	14	11
	16,384	16	13	10
	32,768	15	12	9
	65,536	14	11	8

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Best Practices

Although not always stated at all steps in this protocol, these best practices should be followed throughout to maximize performance.

Samples

- Nucleic acid samples should be stored at -80 °C (RNA) or -20 °C (DNA) and thawed on ice before use.
- All work surfaces and gloves should be sanitized with RNaseZap (or equivalent) prior to reaction setup.
- For most consistent performance, all samples in a batch, including control samples, should be from the same sample type and extracted with the same RNA extraction procedure.
- A no-RNA control is recommended.
- Upon thawing frozen samples, briefly vortex and spin down prior to use.

Master Mixes

- Prepare master mixes in a PCR workstation.
- The PCR workstation should be UV-irradiated after each setup. If unsure, UV-irradiate the workstation before setting up a master mix. *Do not turn on the UV light when reagents are in the workstation.*
- Master mixes are prepared in 1.5 ml or 2 ml microfuge tubes. Briefly vortex to mix and spin down.
- All master mixes for the batch should be prepared in the same manner unless specified otherwise in this protocol.
- Master mixes can be warmed to room temperature immediately before and during use but should be stored at -20 °C when not actively in use.

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Reaction Setup

- **Set up RT-hybridization reactions on ice or a 96-well cold block.** Do not allow the RNA samples to warm to room temperature prior to beginning the incubation in the thermal cycler.
- **Set up subsequent reactions (fill-in, cleanup/PCR1, and PCR2) at room temperature.** After beginning the RT-hybridization reaction, do not cool the reaction plate below room temperature at any time until a safe stopping point has been reached (i.e. after the Cleanup, PCR, or combined Cleanup & PCR steps). This includes centrifugation steps - refer to the note in the Reaction Plates section below.

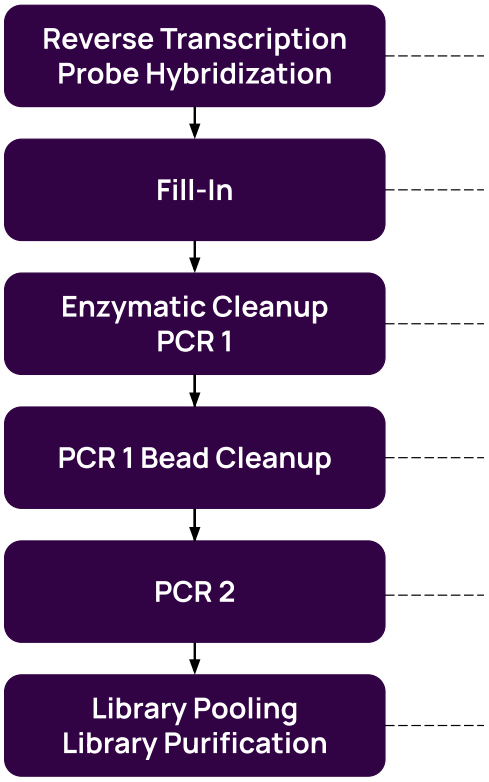
Reaction Plates

- Always seal plates with microseal B film (clear adhesive) seals. Foil seals are not recommended for any step in this protocol. However, they can be used for plates that will be placed in the freezer for storage.
- Using a roller for microseal B film, apply firm pressure and seal over the tops of all wells. Visually inspect all wells, particularly the edges and corners of the plate, to confirm that the seal is in contact with the plate. If not, apply firm pressure and roll until the film is in contact with the plate.
- When removing plate seals, a heated plate sealer can be used if desired to briefly warm the seal and loosen the adhesive.
- **Pulse centrifuge** in an Eppendorf 5810 fitted with a swinging bucket plate rotor up to maximum rpm for a few seconds. Balance plates with a blank if spinning an odd number of plates.

  NOTE: *Many centrifuges have a cooling setting. Since reactions should not be cooled below room temperature at any point after beginning the RT and Probe Hybridization step, turn the cooling off and centrifuge at room temperature.*
- After centrifugation, inspect the bottom of the plate to ensure the expected volume is present in every well.
- It is particularly important to spin down the Index Primer Plate(s) to ensure that the required volume can be easily pipetted from the bottom of the plate, and limit potential cross-contamination between adjacent wells. Wells may appear empty prior to centrifugation.

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Workflow



	16 hr Hybridization	
	Hands-on Time	Turnaround Time
Reverse Transcription Probe Hybridization	15 min	17 hr 15 min
Fill-In	15 min	1 hr 15 min
Enzymatic Cleanup PCR 1	15 min	1 hr 15 min
PCR 1 Bead Cleanup	45 min	45 min
PCR 2	15 min	45 min
Library Pooling Library Purification	30 min	30 min
	2 hr 15 min	**21 hr 45 min**

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Procedure

1. Reverse Transcription and Probe Hybridization

1.1. Set up the RTHybFill program on a thermal cycler:

Program and Steps	Temperature and Time (hh:mm:ss)
RTHybFill Reverse Transcription Denature Hybridize and Fill Hold	**Heated lid 105 °C** 25 °C 00:10:00, 50 °C 00:50:00 95 °C 00:01:00 55 °C 24:00:00 55 °C ∞

1.2. Prepare labware and reagents

- Label one 96-well PCR plate to be used for the capture reactions and place it on ice or on a cold block.
- Retrieve nucleic acid samples from frozen storage.
- Retrieve RT Mix and Probe Mix from frozen storage.

1.3. Add sample RNA to reaction plate

- Per reaction, pipette **6 µl** of nucleic acid into a well of the reaction plate. Adhere to guidelines described under Input Recommendations.

1.4. Prepare RT-Hybridization master mix

- Once thawed, vortex and spin down the RT Mix and Probe Mix.
- Prepare the RT-Hybridization master mix as follows:

Component	Per reaction	96 reactions
Probe Mix	1.6 µl	176 µl
RT Mix	0.4 µl	44 µl
TOTAL	**2.0 µl**	**220 µl**

NOTE: *The 96 reaction volumes already include the required overage. The RT Mix is viscous; exercise care when pipetting.*

- Vortex to mix, and spin down the tube.

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1.5. Add RT-Hybridization master mix to reactions

- Pipette **2 µl** of RT-Hybridization master mix into each sample well. The master mix is viscous; exercise care when pipetting.
- **Mix the contents of each well** by pipetting up and down 3 times, then seal the plate as described in Best Practices. Alternatively, seal and then pulse vortex the plate. Pulse centrifuge the plate briefly.
- The reactions should now be a homogenous **pale blue** color.

NOTE: *The plate will be incubated at a high temperature for an extended period of time. Improper sealing may result in significant volume loss due to evaporation.*

1.6. Perform Reverse Transcription and Probe Hybridization

- Place the reaction plate in the thermal cycler and run the RTHybFill program.
- Make a note of the thermal cycler start time.
- Allow the program to run for the desired hybridization time. An overnight hybridization (12 - 20 hours) is recommended.

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2. Fill-In

Start this procedure roughly 20 minutes prior to the end of the hybridization to ensure that the Fill-in Mix is fully thawed and at room temperature when hybridization is completed.

NOTE: *Do not remove the reaction plate from the thermal cycler until the Fill-in Mix is fully thawed. Correct timing is important to maximize result quality.*

2.1. Retrieve Fill-in Mix from frozen storage. Allow to thaw and equilibrate to room temperature, then vortex and spin down.

2.2. Add Fill-in Mix to reactions

- Remove the reaction plate from the thermal cycler, but leave the RTHybFill program running. Close the lid of the thermal cycler so the temperature of the block does not fluctuate.

NOTE: *Do not end the thermal cycler program. The thermal cycler should remain at 55 °C for the Fill-in reaction.*

- Pulse centrifuge the plate briefly and carefully remove and discard the seal.
- Add **2 µl** of Fill-in Mix to each reaction well in the reaction plate.
- **Mix the contents of each well** by pipetting up and down 3 times, then seal the plate as described in Best Practices. Alternatively, seal and then pulse vortex the plate. Pulse centrifuge the plate briefly.
- The reactions should now be a homogenous **pale green** color.

2.3. Place the reaction plate back in the thermal cycler and allow the RTHybFill program to continue for 60 minutes.

NOTE: *Record the time the reaction plate was returned to the thermal cycler; correct timing at this step is important to maximize result quality.*

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3. Cleanup and PCR 1

Start this procedure roughly 20 minutes prior to the end of the fill-in reaction.

NOTE: *Correct timing is critical to maximize result quality. Ensure that master mixes and index primers are fully thawed before use.*

3.1. Set up the CleanupPCR1 program on a thermal cycler:

Program and Steps	Temperature and Time (hh:mm:ss)
CleanupPCR	**Heated lid 105 °C**
Cleanup	45 °C 00:30:00
Denature	95 °C 00:03:00
PCR	8 x [98 °C 00:00:15, 55 °C 00:00:15, 72 °C 00:01:30*]
Hold	4 °C ∞

* An extension time of 1 min 30 sec is recommended for 675 bp fill designs; for larger designs (e.g. 1125 bp fill), increase the extension time to 2 min 30 sec.

3.2. Retrieve the following reagents from frozen storage:

- Cleanup Mix
- PCR Mix
- ML2100-ON Index Primer Plate

3.3. Allow reagents to thaw and equilibrate to room temperature, then vortex and spin down.

NOTE: *It is particularly important to spin down the Index Primer Plate(s) to ensure that the required volume can be easily pipetted from the bottom of the plate, and limit potential cross-contamination between adjacent wells. Wells may appear empty prior to centrifugation.*

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3.4. Prepare the **Cleanup and PCR master mix** in a microcentrifuge tube by combining kit components as follows:

Component	Per reaction	96 reactions
Cleanup Mix	2 µl	204 µl
PCR Mix	12 µl	1224 µl
TOTAL	**14 µl**	**1428 µl**

NOTE: *The 96 reaction volumes already include the required overage.*

- Vortex to mix, and spin down the tube.

3.5. End the RTHybFill program and remove the reaction plate from the thermal cycler.

3.6. Pulse centrifuge the plate briefly and carefully remove and discard the seal. The volume of the capture reaction product at this step, net of evaporation, should be **~7.6 µl**.

3.7. Set up Cleanup and PCR reactions:

- Add **14 µl** of **Cleanup and PCR master mix** to each reaction
- Add **2.4 µl** of the appropriate **index primer** to each reaction.

NOTE: *The total reaction volume in each well should be approximately 24 µl.*

- **Mix the contents of each well** by pipetting up and down 3 times, then seal the plate as described in Best Practices. Alternatively, seal and then pulse vortex the plate. Pulse centrifuge the plate briefly.
- The reactions should now be a homogenous **magenta color**.

3.8. Perform Cleanup and PCR by placing the plate in the thermal cycler, and run program CleanupPCR1.

NOTE: *This program may be run overnight, but the reactions should remain at 4 °C until no later than the following day.*

3.9. After the CleanupPCR1 program completes, store the reaction plate at -20 °C or proceed directly to Step 4, PCR 1 Bead Cleanup.

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4. PCR 1 Bead Cleanup

4.1. Remove the reaction plate from the thermal cycler (or -20 °C storage).

4.2. Perform **two consecutive 0.67X bead cleanups** on each reaction using KAPA Pure Beads or an equivalent product:

- Add 16 µl of beads to each PCR 1 product.
- Mix thoroughly by pipetting up and down. Incubate at room temperature for 5 minutes.
- Place the reaction plate on a 0.2 ml magnetic plate rack for 2 - 3 minutes to pellet the beads. Carefully remove and discard the supernatant.
- With the plate still on the magnet, perform the first bead wash by adding 200 µl of freshly prepared 80% ethanol to each well. Incubate for 30 seconds at room temperature, then remove and discard the supernatant.
- Repeat the step above for the second bead wash.
- Allow the beads to air-dry for 3 - 5 minutes at room temperature or until all the residual ethanol has evaporated. Be sure not to let the bead pellet over-dry. Remove the reaction plate from the magnet.
- Add 30 µl of 1X TE Buffer or PCR-grade water to each reaction. Resuspend the beads by pipetting up and down, ensuring that the beads are fully resuspended. Incubate for 2 minutes at room temperature.
- Place the reaction plate back on the magnet and allow the beads to pellet for 2 - 3 minutes.
- Transfer 24 µl of the supernatant to a fresh reaction plate.
- Repeat the process above for the second bead cleanup; perform the final resuspension of the washed, air-dried beads in 15 µl of 1X TE Buffer, pH 8.0 or PCR-grade water, and transfer 9.6 µl to a fresh reaction plate.

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5. PCR 2

5.1. Retrieve reagents from frozen storage:

- PCR Mix Kit
- ML2100-ON Index Primer Plate

5.2. Allow reagents to thaw and equilibrate to room temperature, then vortex and spin down.

NOTE: *It is particularly important to spin down the Index Primer Plate(s) to ensure that the required volume can be easily pipetted from the bottom of the plate, and limit potential cross-contamination between adjacent wells. Wells may appear empty prior to centrifugation.*

5.3. Set up the PCR2 program on a thermal cycler:

Program and Steps	Temperature and Time (hh:mm:ss)
PCR2 Denature PCR Hold	**Heated lid 105 °C** 95 °C 00:03:00 N[1] x [98 °C 00:00:15, 55 °C 00:00:15, 72 °C 00:01:30[2]] 4 °C ∞

[1] Refer to page 9 for PCR cycle recommendations.

[2] An extension time of 1 min 30 sec is recommended for 675 bp fill designs; for larger designs (e.g. 1125 bp fill), increase the extension time to 2 min 30 sec.

5.4. Set up PCR 2 reactions by adding the following to each 9.6 µl purified PCR 1 product:

- Add **12 µl** of **PCR Mix** to each reaction
- Add **2.4 µl** of the **same index primer that was used for PCR1** to each reaction.
- **Mix the contents of each well** by pipetting up and down 3 times, then seal the plate as described in Best Practices. Alternatively, seal and then pulse vortex the plate. Pulse centrifuge the plate briefly.

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5.5. Perform PCR 2 by placing the plate in the thermal cycler and run program PCR2.

NOTE: *This program may be run overnight, but the reactions should remain at 4 °C until no later than the following day.*

5.6. After the PCR2 program completes, store the reaction plate at -20 °C or proceed directly to Step 6, Library Pooling.

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6. Library Pooling

6.1. Remove the reaction plate from the thermal cycler (or -20 °C storage). If frozen, allow the plate to thaw and equilibrate to room temperature.

6.2. Spin down the reaction plate and carefully remove and discard the seal.

CAUTION: *This plate contains highly concentrated DNA that could contaminate subsequent experiments. Be very careful when handling this plate to avoid spilling or splashing.*

6.3. Prepare the library pool by transferring an equal volume of each reaction into a single microcentrifuge tube. A minimum of 10 µl per reaction is recommended, and the total pool volume should be at least 120 µl (e.g. transfer ≥15 µl per reaction for 8 reactions) to allow for a 10-fold concentration during the subsequent bead cleanup that follows (Step 7, Library Purification).

NOTE: *If input quantity and/or quality differs significantly between samples, bin samples with similar input into pools, or vary the volume of each library in a pool to be representative of the expected library yield. Failure to do so may result in significant over- or under-representation of libraries in the final sequencing results.*

6.4. Proceed directly to Step 7, Library Purification, or store the tube at -20 °C.

NOTE: *If library QC will include individual reactions and be performed immediately after purification, the reaction plate may be stored at room temperature temporarily. If not, seal the plate and store at -20 °C.*

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7. Library Purification

7.1. Perform library purification using KAPA Pure Beads (or an equivalent product) on the pooled library, with a 0.67X bead ratio (i.e. 67 µl of beads per 100 µl of pooled library).

NOTE: *The bead ratio may be adjusted depending on the amplicon size and the desired purification stringency and yield. A higher ratio may increase overall yield but slightly reduce the fraction of on-target amplicons. A lower ratio may produce a higher fraction of on-target amplicons but reduce overall yield and sensitivity to deletion-containing amplicons that are smaller than expected.*

7.2. Elute the pooled, purified library DNA in 1X TE Buffer (10 mm Tris-HCl, 1 mM EDTA), pH 8.0, using an elution volume 10-fold smaller than the original pooled library volume (i.e. for each 100 µl of pooled library, elute in 10 µl of 1X TE Buffer).

7.3. The purified library is ready for quantification, dilution, and sequencing preparation according to the sequencer manufacturer's instructions, but should be stored at -20 °C until use.

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8. Library Quantification/QC

Quantification can be performed using electrophoresis- or fluorescence-based methods. An example of a purified library pool and no-template control analyzed using the Agilent 2100 Bioanalyzer DNA 7500 Assay is shown in Figure 1 below.

NOTE: *When loading the chip, only go to the first stop of the pipette to prevent air bubbles from being introduced into the DNA 7500 chip. This achieves better accuracy. Add the DNA/ladder directly after adding the matrix.*

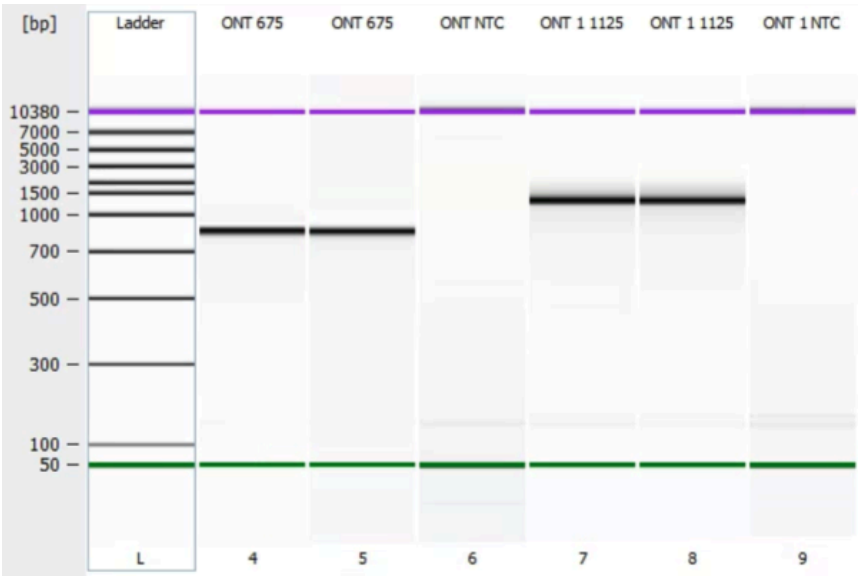


Figure 1. Agilent Bioanalyzer 2100 DNA 7500 Assay results for two ONT assays with fill sizes of 675 bp (lanes 4 - 6) and 1125 bp (lanes 7 - 9). The first two lanes for each assay are duplicate measurements of libraries prepared using 10,000 input copies, while the third lanes are no-template controls (NTCs). The strong peaks at ~800 and ~1300 bp represents the desired product for 675 and 1125 bp fill designs, respectively. One or more small peaks of ~100-150 bp may or may not be present, especially in no-template controls; these likely represent self-ligated, unfilled circles.

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Data Analysis

Targets are captured by probes containing synthetic sequences at their 5’ and 3’ ends. These synthetic sequences will appear at the 5’ (and sometimes also the 3’) end of each read generated by the NGS system. These synthetic sequences should be trimmed from the reads prior to alignment or genotype calling.

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