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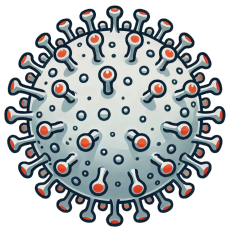
Twist-ONT: Twist Comprehensive Viral Research Panel for Nanopore Sequencing



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Abstract

The Twist comprehensive viral research panel is a probe based amplification kit allowing for whole genome sequencing of all known pathogenic viruses. This Twist-ONT protocol provides step by step instructions for the use of this kit together with Oxford Nanopore Technologies based sequencing on GridION, MinION or PromethION instruments.

Image Attribution

Jonathan Haars using DALL-E through ChatGPT.

Safety warnings

NUCLISENS easyMAG Lysis Buffer contains 50 % guanidine thiocyanate, <2 % Triton X-100, <1 % EDTA. This buffer should never come in contact with bleach based cleaning products as it creates toxic gas.

Ethics statement

The study during which this protocol was developed was approved by the Swedish Ethical Review Authority under the case number 2024-05546-01.

DNA and RNA extraction using EMAG

1h 11m

1 **Materials:**
- Sample

Consumables:
- NUCLISENS easyMAG Extraction Buffer 1
- NUCLISENS easyMAG Extraction Buffer 2
- NUCLISENS easyMAG Extraction Buffer 3
- NUCLISENS easyMAG Magnetic Silica
- NUCLISENS easyMAG Lysis Buffer
- NUCLISENS easyMAG Disposable
- EMAG TIPS 1000µL

Equipment:
- EMAG Nucleic Acid Extraction System
- Vortex mixer
- P1000 pipette with tips
- P200 pipette with tips
- P10 pipette with tips

2 Note: The DNA and RNA extraction method used here can be replaced by other extraction methods compatible with Oxford Nanopore Technologies (ONT) sequencing.

3 Warning: NUCLISENS easyMAG Lysis Buffer contains 50% guanidine thiocyanate, <2% Triton X-100, <1% EDTA. This buffer should never come in contact with bleach based cleaning products as it creates toxic gas.

4 Add 200 µL of the liquid sample to a tube with 2 mL of NUCLISENS easyMAG Lysis Buffer.

1m

5 Vortex the tube.

6 Repeat for all samples you are extracting.

7 Incubate all samples for at least 10 minutes at room temperature.

10m

8 Perform EMAG extraction according to the manual from the manufacturer.

1h

9 Use elution volume 60 µL.

cDNA synthesis

1h 39m

10 **Materials:**

- Extracted RNA or DNA/RNA mixture

Consumables:

- LunaScript RT SuperMix
- 5X Sequenase reaction buffer
- Sequenase Version 2.0 DNA polymerase
- Sequenase dilution buffer
- AMPure XP beads
- 70% ethanol (mixed from absolute ethanol)
- Nuclease-free water

Equipment:

- Benchtop centrifuge
- Rotator mixer (such as HulaMixer)
- Vortex mixer
- P1000 pipette with tips
- P200 pipette with tips
- P10 pipette with tips

Note: Whenever possible, keep the samples on ice.

11 Note: Prepare tubes with LunaScript and sequenase-mixes in a pre-PCR room. Sample material should be added in a different room, this is to reduce the risk of contamination.

12 In a 0,2 mL strip tube, add 4 µL LunaScript RT SuperMix to a well for each sample you have. 5m

13 Prepare a Sequenase-mix-1 mastermix in a 1,5 mL LoBind tube by mixing the following: 5m

	A	B
	Reagent	Volume/sample
	5X Sequenase reaction buffer	2 µL
	Nuclease-free water	7.7 µL

	A	B
	Sequenase Version 2.0 DNA Polymerase	0.3 µL
	Total	10 µL

14 Mix by pipetting then spin down.

15 Prepare Sequenase-mix-2 mastermix in a 1,5 mL LoBind tube by mixing the following: 5m

	A	B
	Reagent	Volume/sample
	Sequenase Dilution Buffer	0.9 µL
	Sequenase Version 2.0 DNA Polymerase	0.3 µL
	Total	1.2 µL

16 Mix by tapping the tube then spin down.

17 Add 16 µL sample to each well of the strip tube with LunaScript for a final volume of 20 µL. 5m

18 Mix by tapping the tube then spin down.

19 Incubate the samples in a Thermal cycler using the following program: 15m

	A	B	C	D
	Step	Temperature	Time	Cycles
	Primer annealing	25°C	2 minutes	1
	cDNA synthesis	55°C	10 minutes	1
	Heat inactivation	95°C	1 minute	1

	A	B	C	D
	Hold	4°C	∞	-

20 Add 10 µL Sequenase-mix-1 to each incubated sample and transfer to a new 1,5 mL LoBind tubes. This can be done in PCR strip tubes if you want to perform the incubation steps below in a PCR instrument. 3m

21 Mix by tapping the tube then spin down. 1m

22 Incubate the samples at 37°C for 8 minutes. 8m

23 Add 1,2 µL of Sequenase-mix-2 to each incubated sample. 3m

24 Mix by tapping the tube then spin down.

25 Incubate the samples at 37°C for 8 minutes. 8m

26 Vortex AMPure XP beads.

27 Add 45 µL AMPure XP beads to new 1.5 mL LoBind tubes. 3m

28 Transfer the incubated samples to the tubes with AMPure XP beads.

29 Incubate the sample at room temperature with mixing for 5 minutes using a rotator mixer. 5m

30 During the incubation time, prepare 500 µL 70% ethanol per sample by mixing 350 µL absolute ethanol with 150 µL nuclease free water. For multiple samples you can prepare a larger batch of 70% ethanol.

31 Spin down samples and place in a magnetic rack. If you have many samples, you may need to perform this step and the following washes in batches. 5m

32 When the supernatant is clear (No beads in the supernatant), keep the tube in the magnetic rack and discard the supernatant.

33 With the tube still in the magnetic rack, add 200 µL 70% ethanol on the opposite side of the magnetic beads, then pipette off and discard the ethanol. 5m

34 Repeat the previous step.

35 Spin down the sample and place in a magnetic rack.

36 Pipette off all the liquid still in the sample tube with a P10 pipette and discard the liquid.

37 Allow the magnetic beads to air dry for ~30 seconds, the pellet should not dry to the point of cracking. The timing of this step is dependent on the humidity of the working environment.

38 Remove the tube from the magnetic rack and add 60 µL nuclease free water, resuspend the magnetic beads with a pipette.

39 Incubate for at least 2 minutes at room temperature. If doing multiple samples, leave them at this stage until you have finished all your samples. 3m

40 Place the sample in the magnetic rack until the liquid is clear and colorless. 5m

41 Transfer 50 µL eluate to a new 1,5 mL LoBind tube. Discard the pellet of magnetic beads. 5m

42 Quantify 1 µL of each sample with the Qubit dsDNA HS Assay Kit. 10m

43 Freeze samples unless you are continuing directly to the next step.

Twist comprehensive viral research panel part 1. Perform DNA fragmentation

1h 30m

44 In this section you will perform enzymatic fragmentation of input DNA and subsequent end repair and dA-tailing to generate dA-tailed DNA fragments. Note that Twist Bioscience offers a kit for mechanical fragmentation as well, if mechanical fragmentation is preferred check their original protocol to find possible modifications needed.

45 **Materials:**

- 40 µL of DNA sample.

Consumables:

- Nuclease-free water

Equipment:

- Benchtop centrifuge
- Vortex mixer
- Thermal cycler with heated lid
- P200 pipette and tips
- P10 pipette and tips

46 Ensure that you have a thermal cycler ready with all necessary PCR programs.

47 Prepare ice in an ice box.

48 Defrost your samples on ice, then move them to 0.2 mL PCR tubes. Mark sample tubes and keep on ice.

49 In a pre-PCR room, defrost Frag/AT Buffer and Frag/AT Enzymes.

50 While waiting for reagents to defrost, start the enzymatic fragmentation PCR-cycler program (see settings below) to pre chill the PCR instrument.

35m

Step	Temperature	Time
1	4°C	HOLD
2	30°C	5 minutes
3	65°C	30 minutes
4	4°C	HOLD

Enzymatic fragmentation PCR-cycler program.

The heated lid should be set to 105°C.

51 In a pre-PCR room, vortex the Frag/AT Buffer for 5 seconds. Spin down.

52 Invert Frag/AT Enzymes a minimum of 10 times to homogenize or briefly vortex to ensure complete mixing. Spin down.

53 Prepare an enzymatic fragmentation mix in a 1.5 mL microfuge tube on ice. Use the volumes listed below. Homogenize the master-mix with moderate vortexing for 5 seconds, flicking, or pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles). Prepare a master-mix if you have multiple samples. 5m

Reagent	Volume Per Reaction	Volume for 8 Samples
Frag/AT Buffer	4 µL	32 µL
Frag/AT Enzymes	6 µL	48 µL
Total	10 µL	80 µL

54 Outside the pre-PCR room. Add 10 µL enzymatic fragmentation mix to each 40 µL sample tube. Homogenize with moderate vortexing for 5 seconds or by pipetting a minimum of half the total volume up and down 10 times (avoid formation of bubbles). Cap the tube(s) and keep the reaction on ice. 5m
Note: Complete mixing is critical to achieve consistent fragment lengths.

55 Spin down the samples and transfer immediately to the pre-chilled thermal cycler running the enzymatic fragmentation PCR-cycler program then end the hold at 4°C by going to the next step of the PCR program. The program takes 35-40 minutes. 45m

While the program is running, you can prepare the reagents for the next section of this protocol.

56 When the thermal cycler program is complete and the sample block has returned to 4°C, remove the samples from the block and place them on ice.

57 Proceed immediately to the next section of the protocol.

Twist comprehensive viral research panel part 2. Ligate universal adapters and purify

2h 55m

58 **Materials:**

- Samples from the previous step (dA-tailed DNA fragments)

Consumables:

- Ethanol
- Nuclease-free water
- 10 mM Tris HCl pH 8 or Buffer EB (optional, for elution)

From the Twist Library Preparation EF Kit 1, 2.0:

- Ligation Master Mix

From the Twist (HT) Universal Adapter System:

- Twist Universal Adapters
- From the Twist Library Preparation Kit 2:
- DNA Purification Beads

Equipment:

- Benchtop centrifuge
- Vortex mixer
- Thermal cycler with heated lid
- P1000 pipette with tips
- P200 pipette with tips
- P10 pipette with tips

59 Defrost and store on ice:

- Twist Universal Adapters (tube; utilized for all samples)
- Ligation Master Mix

60 Add 5 µL Twist Universal Adapters into each sample well or tube containing the dA-tailed DNA fragments from the previous section of the protocol. This is a tube stored in the box together with the barcoding plates. Keep on ice. 5m

61 Add 20 µL ligation master mix to the sample from the previous step and mix well by gentle pipetting. 5m

62 Seal or cap the tubes and pulse-spin to ensure all solution is at the bottom of the tube.

63 Incubate the ligation reaction at 20°C for 1 hour in a thermal cycler, then move the samples to the bench top. 1h
Note: the heated lid should be turned off or set to the minimum temperature for this step.

64 Equilibrate DNA Purification Beads to room temperature for at least 30 minutes (For use in both this section and the next section of the protocol).

65 Prepare 2 mL 80% ethanol for each sample.

66 Vortex the pre-equilibrated DNA purification beads until well mixed.

67 Add 80 µL of homogenized DNA purification beads to each ligation sample. This can be done in 0.2 mL PCR tubes or new 1.5 mL LoBind tubes. 5m

Note: Choose the tube type that you are most comfortable with using.

68 Incubate the samples for 5 minutes at room temperature. 5m

69 Place the samples on a magnetic plate/rack for 5-10 minutes or until the supernatant is clear. 10m

70 The DNA purification beads form a pellet, leaving a clear supernatant. Without removing the tubes from the magnetic plate/rack, remove and discard the supernatant.

71 Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol. 5m

72 Repeat the wash once, for a total of two washes, while keeping the samples on the magnetic plate/rack. 5m

73 Carefully remove the remaining ethanol with a 10 µL pipette, making sure not to disturb the bead pellet.

Note: If needed, the bead pellet may be briefly spun down to collect the ethanol at the bottom of the tube, then place the tube back on the magnetic plate/rack and remove the remaining ethanol.

74 Air-dry the bead pellet on the magnetic plate/rack for 5 minutes or until the bead pellet is dry. 5m

Note: Do not overdry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

75 Remove the tubes from the magnetic plate/rack and add 37 µL 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized. 5m

76 Incubate at room temperature for 2 minutes. 2m

77 Place the tubes on a magnetic plate/rack and let stand for 3 minutes or until the beads form a pellet. 3m

78 Transfer 35 µL of the clear supernatant containing the ligated and indexed libraries to a clean 0.2 mL PCR tube, making sure not to disturb the bead pellet. 5m

Note: If you are not comfortable with doing magnetic bead washes in 0.2 mL tubes you can transfer to a 1.5 mL LoBind tube instead.

79 **Size Selection**

80 Prepare diluted DNA purification beads (35% w/v) as indicated in the table below. 5m

	A	B	C
	Reagent	8 reactions	Larger mix
	DNA purification beads	453.25 µL	3.5 mL
	10 mM Tris-HCl pH 8	841.75 µL	6.5 mL
	Total	1295 µL	10 mL

DNA purification beads (35% w/v)

81 Add 129.5 µL of diluted DNA purification beads (3.7x, 35% w/v) to each ligation sample. Mix well by gently flicking the tube. Spin down. 5m

82 Incubate the samples for 5 minutes at room temperature. 5m

83 Place the samples on a magnetic plate for 5-10 minutes or until the supernatant is clear. 10m

84 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the tubes from the magnetic plate, remove and discard the supernatant.

85 Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol. 5m

86 Repeat the wash once, for a total of two washes, while keeping the sample on the magnetic plate. 5m

87 Carefully remove all the remaining ethanol with a 10-µL pipette, making sure not to disturb the bead pellet.

Note: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the plate or tube and returned to the magnetic plate.

88 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the bead pellet is dry. Do not overdry the pellet. 5m

89 Remove the tubes from the magnetic plate and add 37 µL 10 mM Tris-HCl pH 8 to each sample. Mix by pipetting until homogenized. 5m

90 Incubate at room temperature for 2 minutes. 2m

91 Place the tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet. 3m

92 Transfer 36 µL of the clear supernatant containing the ligated and indexed libraries to a clean 0.2 mL PCR tube, making sure not to disturb the bead pellet. 5m

Twist comprehensive viral research panel part 3. Pre-capture PCR Amplify, Purify and Perform QC 2h 35m

93 **Materials:**

- Ligated libraries from the previous step

Consumables:

- 80% Ethanol (from A.2)
- Equilibrated DNA Purification Beads (from A.2)
- Nuclease-free water
- KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs

- Twist UDI Primers

Equipment:

- Benchtop centrifuge
- Vortex mixer
- Thermal cycler with heated lid
- P200 pipette and tips
- P10 pipette and tips

94 Thaw Twist UDI Primers (96-well plate with single use primers) on ice.

95 Thaw KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer and dNTPs in the pre-PCR room.

96 In the pre-PCR room, prepare a PCR mix in a 1.5 mL LoBind tube according to the table below, Mix well by gentle pipetting. 10m

	A	B	C
	Reagent	Volume (1 sample)	Volume (8 samples+5%)
	Water (chilled)	10 µL	84 µL
	2X XtremeBuffer	100 µL	840 µL
	dNTPs (2mM each)	40 µL	336 µL
	KOD Xtreme Hot Start DNA Polymerase	4 µL	33.6 µL
	Total	154 µL	1293.6 µL

97 Add 10 µL Twist UDI Primers from the provided 96-well plate to each of the ligated libraries from the previous section and mix well by gentle pipetting. Add 154 µL PCR master mix to each sample. Pulse-spin the tubes. 5m

98 Transfer 100 µL of the 200 µL reaction to another tube for each sample and then immediately transfer it to the thermal cycler and start the pre-capture PCR program. 1h 40m

Note: The separate tubes for each sample are referred to as the 1st and 2nd PCR reactions.

	A	B	C	D
	Step	Temperature	Time	# Cycles
	1 Initialization	94°C	2 minutes	1
	2 Denaturation	98°C	10 seconds	8
		58.8°C	30 seconds	
		68°C	10 minutes	
	3 Final Extension	68°C	10 minutes	1
	4 Final Hold	4°C	HOLD	-

Pre-capture PCR program

Heated lid should be set to 105°C.

99 Remove the samples from the thermal cycler when the program is finished.

100 Vortex the pre-equilibrated DNA Purification Beads until mixed.

101 Add 50 µL (0.5X) homogenized DNA Purification Beads to the 1st and 2nd PCR reaction. Mix well by flicking the tube. Spin down. 5m

102 Incubate the samples for 5 minutes at room temperature. 5m

103 Place the samples on a magnetic plate for 1 minute. 1m

104 The DNA Purification Beads form a pellet, leaving a clear supernatant. Without removing the tubes from the magnetic plate, remove and discard the supernatant. 2m

105 Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet), incubate for 1 minute at room temperature, then remove and discard the ethanol. 2m

106 Repeat this wash once, for a total of two washes, while keeping the samples on the magnetic plate. 5m

107 Carefully remove all remaining ethanol with a 10 µL pipette, making sure not to disturb the bead pellet.

Note: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the tube and returned to the magnetic plate.

108 Air-dry the bead pellet on the magnetic plate for 5 minutes or until the pellet is dry. Do not overdry the bead pellet. 5m

109 Remove the plate or tubes from the magnetic plate and add 22 µL water to the 1st PCR reaction. Mix by pipetting until homogenized.

Note: The 2nd PCR reaction will be eluted using the elution from the 1st PCR reaction.

110 Incubate at room temperature for 2 minutes. 2m

111 Place the tubes on a magnetic plate and let stand for 3 minutes or until the beads form a pellet. 3m

112 Transfer 22 µL of the supernatant to the 2nd PCR reaction and repeat the two previous steps for the 2nd PCR reaction.

113 Transfer 21 µL of the clear supernatant containing the amplified, indexed libraries from both PCR reactions to a clean 0.2 mL PCR tube, making sure not to disturb the bead pellet.

114 Quantify the concentration of each sample by using 1 µL sample with the Qubit dsDNA High Sensitivity Quantification Assay. 10m

115 Optional: It is possible to perform additional quality control with the Agilent Femto Pulse gDNA 165 kb Analysis Kit. *

116 If not proceeding immediately, the amplified indexed libraries can be stored at -20°C. This is typically a good stopping point. ⏸

Twist comprehensive viral research panel part 4. Prepare Libraries for Hybridization 1h 15m

117 The Twist protocol supports single or multiplex (up to 8-plex) hybridization capture. We will typically multiplex 8 samples.

Materials:
- Amplified indexed libraries

Consumables:
- 80% Ethanol
- Nuclease-free water
- DNA Purification Beads
- From Twist Universal Blockers
- Universal Blockers
- Blocker Solution

Equipment:
- Rotator mixer (such as HulaMixer)
- Vortex mixer
- Benchtop centrifuge
- P1000 pipette with tips
- P200 pipette with tips
- P10 pipette with tips

118 Equilibrate DNA Purification Beads for at least 30 minutes. Vortex until well mixed before use. 30m

119 Prepare 500 µL fresh 80% ethanol. 5m

120 Use the concentration of each amplified, indexed library to calculate the volume (in µL) of each library needed for hybridization:
- Determine the amount of each indexed library per pool from the table below.
- Divide the amount of each indexed library per pool by the concentrations measured in ng/µL from the library preparation Qubit measurements.

For example: If multiplexing eight libraries per hybridization reaction, the amount of each library will be 500 ng and the total mass of the pool will be 4,000 ng.

	A	B	C
	Number of indexed samples per pool	Amount of each indexed library per pool	Total mass per pool
	1	1,500 ng	1,500 ng
	2	1,500 ng	3,000 ng
	3	1,000 ng	3,000 ng

	A	B	C
	4	1,000 ng	4,000 ng
	8	500 ng	4,000 ng

Note: If the amount of library you have is insufficient, you can use a smaller amount. More than 4 µg total DNA may lead to reduced performance of the enrichment.

121 Transfer the calculated volumes from each amplified indexed library to a 1.5 mL LoBind tube (indexed library pool tube). 5m

122 Pulse-spin the indexed library pool tube to minimize the amount of bubbles present.

123 **DNA concentration**
DNA is concentrated here using magnetic beads, however, a vacuum concentrator can be used instead using low or no heat.

124 Thaw Universal Blockers and Blocker Solution on ice, then pulse-vortex for 2 seconds to mix and then spin down.

125 Add 1.8x homogenized DNA Purification Beads to the tube containing the DNA libraries. 5m

Note: If multiplexing 8 samples and using the full volume (20 µL) for each library then 288 µL DNA Purification Beads should be added.

Note: If the indexed library pool has a volume of less than 10 µL, bring the volume up to 10 µL with nuclease-free water.

126 Incubate for 5 minutes at room temperature on a rotator mixer. 5m

127 Spin down to ensure that all solution is at the bottom of the tube then place the tube in a magnetic rack for 3 minutes or until the solution is clear. 3m

128 Without removing the tube from the magnetic rack, remove and discard the clear supernatant. 2m

129 Wash the bead pellet by gently adding 200 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol. 5m

130 Repeat the previous step, for a total of two washes, while keeping the tube on the magnetic rack. 5m

131 Carefully remove all remaining ethanol using a 10 µL pipette, making sure not to disturb the bead pellet.

Note: If necessary, pulse spin to ensure complete removal of ethanol.

132 Air-dry the bead pellet on a magnetic rack for 1-5 minutes or until the bead pellet is dry. Do not overdry the bead pellet. 5m

133 Remove the tube from the magnetic rack and add 7 µL Universal Blockers and 5 µL Blocker Solution. Mix by pipetting until homogenized. 5m

Note: There should be magnetic beads left in the tube.

Note: Blocker solution is species specific. The standard one only works for human samples.

Twist comprehensive viral research panel part 5. Hybridize Capture Probes with Pools 16h 30m

134 **Materials:**

- Indexed library pool mixed with Universal Blockers and Blocker Solution from the previous step

Consumables:

- Twist Comprehensive Viral Research Panel
- From Twist Hybridization Reagents:
- Hybridization Mix
- Hybridization Enhancer

Equipment:

- Thermal cycler with heated lid
- Heating block
- Vortex mixer
- Benchtop centrifuge
- P200 pipette with tips
- P10 pipette with tips

135 Thaw the Twist Comprehensive Viral Research Panel, Hybridization Mix and Hybridization Enhancer on ice, then pulse-vortex for 2 seconds to mix and then spin down.

136 Preheat a thermal cycler at 95°C (lid 105°C) and preheat a heating block at 95°C.

137 Prepare a probe solution in a 0.2 mL PCR tube following the table below. Mix by flicking the tube. 10m

Note: Hybridization Mix is very viscous. Pipette slowly to ensure accurate pipetting. Small white particles may be present in the Twist panel tube, this will not affect the final capture product.

	A	B
	Reagent	Volume (1 hybridization reaction, up to 8 multiplexed samples)
	Hybridization Mix	20 µL
	Twist Comprehensive Viral Research Panel	4 µL
	Nuclease-free water	4 µL
	Total	28 µL

138 Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes. 7m

Note: Do not turn the thermal cycler program off. Switch to a 70°C program with 85°C lid.

139 While the probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a heating block, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes. 10m

140 Add the indexed library to a new 0.2 mL tube.

141 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool.

142 Mix well by vortexing.

143 Pulse-spin the tube to ensure all solution is at the bottom of the tube.

144 Add 30 µL Hybridization Enhancer to the top of the entire capture reaction. 3m

145 Pulse-spin the tube to ensure there are no bubbles present. Ensure that the tube is well sealed tightly to prevent excess evaporation over the 16-hour incubation.

146 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C. 16h

Note: Halting hybridization between 15-17 hours will not affect downstream capture quality.

Twist comprehensive viral research panel part 6. Bind Hybridized Targets to Streptavidin Beads 2h 10m

147 **Materials:**

- Hybridization reaction from the previous section of the protocol

Consumables:

- From the Twist Wash Buffers:
- Binding Buffer
- Standard Wash Buffer 1
- Wash Buffer 2
- From the Twist Library Preparation Kit 2:
- DNA Purification Beads
- Invitrogen Dynabeads M-270 Streptadivin Beads
- 2 N NaOH (Same as 2 M NaOH)
- 200 mM Tris-HCl pH 8

Equipment:

- Thermal cycler with heated lid
- 2 heating blocks
- Magnetic rack
- Vortex mixer
- Benchtop centrifuge
- P200 pipette with tips

- P10 pipette with tips

148 Start one heat block at 48°C and one heat block at 68°C.

149 Preheat the following tubes at 48°C until any precipitate is dissolved:
- Binding Buffer
- Standard Wash Buffer 1
- Wash Buffer 2

150 Equilibrate Dynabeads M-270 Streptadivin Beads and DNA Purification Beads to room temperature for at least 30 minutes. 30m

151 Make fresh 0.2 N NaOH using 20 µL of 2 N NaOH into 180 µL nuclease-free water.

152 Equilibrate Binding Buffer to room temperature.
Equilibrate Std Wash Buffer 1 to 68°C.
Leave Wash Buffer 2 at 48°C.

153 **Prepare the Beads**
Vortex the pre-equilibrated Streptadivin Binding Beads until mixed.

154 Add 100 µL Streptavidin binding beads to a 1.5 mL LoBind tube.

155 Add 200 µL Binding Buffer to the tube and mix by pipetting. 2m

156 Place the tube on a magnetic rack for 1 minute, then remove and discard the clear supernatant. Make sure not to disturb the bead pellet. Remove the tube from the magnetic stand. 3m

157 Repeat the wash (the previous two steps) two more times for a total of three washes. 10m

158 After removing the clear supernatant from the third wash, add a final 200 µL Binding Buffer and resuspend the beads by vortexing until homogenized.

159 Heat the resuspended beads at 68°C on a heating block for at least 10 minutes before continuing to the next step. 10m

160 **Bind the Target** 5m

After the 16 hour hybridization step is complete, open the thermal cycler lid and directly transfer the volume of the hybridization reaction into the tube with preheated Streptadivin Binding Beads. Mix by pipetting and flicking.

Critical step: Rapid transfer directly from the thermal cycler at 70°C is a critical step for minimizing off-target binding. Do not remove the tube of hybridization reaction from the thermal cycler or otherwise allow it to cool to less than 70°C before transferring the solution to the washed Streptadivin Binding Beads. Allowing the tube to cool to room temperature for less than 5 minutes will result in as much as 10-20% increase in off-target binding.

161 Incubate the tube of the hybridization reaction with the Streptadivin Binding Beads for 5 minutes in a heat block at 68°C, agitation is not required. 5m

Note: **Do not vortex**. Aggressive mixing is not required.

162 Remove the tube containing the hybridization reaction with Streptadivin Binding Beads from the heat block and pulse-spin to ensure all solution is at the bottom of the tube. 2m

163 Place the tube on a magnetic rack for 1 minute. 1m

164 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet. 2m

Note: Some Hybridization Enhancer may be visible after supernatant removal and throughout each wash step. It will not affect the final capture product.

165 Remove the tube from the magnetic rack and add 200 µL 68°C pre-heated Standard Wash Buffer 1. Mix by pipetting. 3m

Note: Ensure that beads are well mixed by pipetting or flicking.

166 Incubate the tube for 5 minutes at 68°C. 5m

167 Pulse-spin to ensure all solution is at the bottom of the tube. 1m

168 Transfer the entire volume from the previous step into a new 1.5 mL LoBind tube. 2m

Note: This step reduces background from non-specific binding to the surface of the tube.

169 Place the tube on a magnetic rack for 1 minute. 1m

170 Remove and discard the clear supernatant. Make sure not to disturb the bead pellet. 1m

171 Remove the tube from the magnetic rack and add 200 µL of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube. Place Wash Buffer 2 back on the 48°C heat block. 2m

172 Incubate the tube for 5 minutes at 48°C. 5m

173 Place the tube in a magnetic rack for 1 minute. 1m

174 Remove and discard the clear supernatant. Make sure not to disturb the bead pellet. 1m

175 Repeat the wash (the previous four steps) two more times, for a total of three washes. 18m

176 After the final wash, use a 10 µL pipette to remove all traces of supernatant. Proceed immediately to the next step. Do not allow the beads to dry. 1m

Note: Before removing supernatant, you may pulse-spin to collect supernatant at the bottom of the tube then return it to the magnetic rack.

177 Remove the tube from the magnetic rack and add 12 µL nuclease-free water. Mix by pipetting until homogenized, then place this solution, hereafter referred to as the Streptadivin Binding Beads slurry on ice. 5m

178 Add 12 µL 0.2 N NaOH to the Streptavidin Binding Bead slurry. Mix well by flicking the tube then spin down. 5m

179 Incubate the mixture from the previous step at room temperature for 5 minutes. 5m

180 Add 24 µL 200 mM Tris-HCl pH 8 to the mixture from the previous step. Mix well by flicking the tube then spin down. 2m

181 Place the mixture from the previous step in a magnetic rack and transfer 50 µL of the supernatant into a new 0.2 mL PCR tube, store on ice. Discard the beads. 2m

Note: You may need to pipette the supernatant in two steps, first 48 µL and then the final 2 µL with a 10 µL pipette to avoid disturbing the beads.

Twist comprehensive viral research panel part 7. Post-capture PCR Amplify 4h 45m

182 **Materials:**
- Supernatant from the end of the previous section.

Consumables:
- Ethanol
- Nuclease-free water
- DNA purification beads
- KOD Xtreme Hot Start DNA Polymerase, 2X XtremeBuffer, dNTPs
- Twist Amplification Primers
- Qubit dsDNA High Sensitivity Quantification Assay

Equipment:
- Thermal cycler with heated lid
- Qubit fluorometer
- Vortex mixer
- Benchtop centrifuge
- P200 pipette and tips
- P10 pipette and tips

183 Make sure you have a PCR cycler ready with the post-capture PCR program

184 If the supernatant from the previous section of the protocol is not already in a 0.2 mL PCR tube then move it to one. Keep on ice until ready to use in the next step. 3m

Note: If you recover <50 µL, add nuclease free water to bring the total volume to 50 µL.

185 In a pre-PCR room, prepare the post capture PCR mixture by mixing the reagents in the table below in a 1.5 mL LoBind tube. 8m

	A	B
	Reagent	Volume Per Reaction

	A	B
	Twist Amplification primers	6 µL
	2X XtremeBuffer	100 µL
	dNTPs (2mM each)	40 µL
	KOD Xtreme Hot Start DNA Polymerase	4 µL
	Total	150 µL

186 Add the post-capture PCR mixture to the sample tube. Mix by pipetting. 2m

187 Split the 200 µL reaction into two 100 µL tubes. 2m

188 Pulse-spin the tubes, transfer to the thermal cycler and start the post-capture PCR program below. 4h 30m

	A	B	C	D
	Step	Temperature	Time	# of cycles
	1. Initialization	94°C	2 minutes	1
	2. Denaturation	98°C	10 seconds	20
	Annealing	58.8°C	30 seconds	
	Extension	68°C	10 minutes	
	3. Final Extension	68°C	10 minutes	1
	4. Final Hold	4°C	HOLD	-

Post-capture PCR program

Note: The number of cycles for step 2 of the PCR program can be adjusted depending on concentrations and Twist panel size. 20 cycles works for this Nanopore sequencing protocol. Should be reduced if possible to reduce PCR bias and to save time if you see

an over abundance of PCR product. If low yield is observed, the number of cycles can be increased (3-5 additional cycles).

Twist comprehensive viral research panel part 8. Purify PCR-product and measure concentration

45m

189 The steps of this section should be performed in a post-PCR room.

190 Prepare tubes and reagents for measurements with the Qubit HS Assay kit. 10m

191 Prepare a new tube of 2 mL 80% ethanol by mixing 1600 µL absolute ethanol with 400 µL nuclease free water. This will be used for both this section as well as the next section of this protocol. 5m

192 When the thermal cycler program is complete, immediately start with the purification.

193 **Purify**
Vortex the pre-equilibrated DNA purification beads until well mixed. 1m

194 Combine the two separated 100 µL reactions into one 1.5 mL LoBind tube. 1m

195 Add 100 µL (0.5X) DNA purification beads. Mix well by flicking the tube. Spin down. 1m

196 Incubate for 5 minutes at room temperature. 5m

197 Place the tube on a magnetic rack for 1 minute or until the supernatant is clear. 1m

198 The DNA purification beads form a pellet, leaving a clear supernatant. Without removing the tube from the magnetic plate, remove and discard the clear supernatant.

199 Wash the bead pellet by gently adding 500 µL freshly prepared 80% ethanol (do not disturb the pellet). Incubate for 1 minute at room temperature, then remove and discard the ethanol. 2m

200 Repeat this wash once, for a total of two washes, while keeping the tube in the magnetic rack. 2m

201 Carefully remove all remaining ethanol using a 10 µL pipette, making sure to not disturb the bead pellet. 2m

Note: Before pipetting, the bead pellet may be briefly spun to collect ethanol at the bottom of the tube and returned to the magnetic rack.

202 Air-dry the bead pellet in the magnetic rack for 5 minutes or until the bead pellet is dry. Do not overdry the bead pellet. 5m

203 Remove the tube from the magnetic rack and add 42 µL nuclease-free water. Mix by pipetting until homogenized. 2m

Note: 10 mM Tris-HCl pH8 or EB buffer can be used instead of nuclease-free water.

204 Incubate at room temperature for 2 minutes. 2m

205 Place the tube in the magnetic rack and let stand for 3 minutes or until the beads fully pellet. 3m

206 Transfer 40 µL of the clear supernatant containing the enriched library to a clean 0.2 mL PCR tube, making sure not to disturb the bead pellet. 3m

207 (Optional) Validate and quantify the library with the Femto Pulse gDNA 165 kb Analysis Kit. *

Sequencing library preparation

208 **Materials:**

- Library from the previous section

Consumables:

- NEBNext Ultra II End Repair / dA-tailing Module
- DNA purification beads
- 80 % Ethanol
- Nuclease-free water
- 0.2 mL PCR tubes
- 1.5 mL LoBind tubes

Equipment:

- Thermal cycler
- Benchtop centrifuge
- Magnetic rack
- Rotator mixer (such as HulaMixer)
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P10 pipette and tips
- Qubit fluorometer

209 Take a flow cell out of the fridge.

210 Insert the flow cell into the GridION and perform a flow cell check.

211 Defrost all reagents. If ice is available, store the reagents on ice.

212 Flick and/or invert the reagents to ensure they are well mixed.

Note: Do not vortex the Ultra II End Prep Enzyme Mix.

213 Always spin down tubes before opening for the first time each day.

214 The Ultra II End Prep Buffer may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for 30 seconds to solubilise any precipitate.

Note: It is important that the NEBNext Ultra II End Prep Reaction Buffer is mixed well by vortexing.

215 Keep up to 800 ng of DNA in the 0.2 mL tube. Store any left over library in a new 1.5 mL LoBind tube.

216 Adjust the volume to 50 µL with nuclease-free water. Mix thoroughly by pipetting up and down or by flicking the tube. Spin down.

217 In the 0.2 mL PCR tube, mix the following:
Between each addition, pipette the mix 10-20 times.

	A	B
	Reagent	Volume
	DNA + Water	50 µL
	Ultra II End-prep Reaction Buffer	7 µL
	Ultra II End-prep Enzyme mix	3 µL
	Total	60 µL

218 Thoroughly mix the reaction by gently pipetting and spinning down.

219 Using a thermal cycler, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

220 Resuspend the AMPure XP beads (AXP) by vortexing.

221 Transfer the DNA sample to a clean 1.5 mL LoBind tube.

222 Add 60 µL of the resuspended AMPure XP beads (AXP) to the end-prep reaction and mix by flicking the tube.

223 Incubate on a rotator mixer for 5 minutes at room temperature. If a rotator mixer is not available, invert the tube manually several times during the incubation period.

224 Spin down the sample and pellet on a magnetic stand until the supernatant is clear and colorless. Keep the tube in the magnetic rack and pipette off the supernatant.

225 Keep the tube in the magnetic rack and wash the beads with 200 µL 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

226 Repeat the previous step.

227 Spin down and the tube back in the magnetic rack. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

228 Remove the tube from the magnetic rack and resuspend the pellet in 61 µL nuclease-free water.

229 Incubate for 2 minutes at room temperature.

230 Pellet the beads in a magnetic rack until the eluate is clear and colorless, for at least 1 minute.

231 Remove and retain 61 µL of eluate into a clean 1.5 mL LoBind tube.

232 (Optional) If you want to quality check the end-prep and cleanup you can measure 1 µL of eluate with the Qubit dsDNA High Sensitivity Quantification Assay.

233 Either continue directly with adapter ligation or store the sample at 4°C. Oxford Nanopore technologies states that this can be stored overnight. Storing the sample for longer may affect the final result negatively.

234 **Nanopore Ligation Kit Adapter Ligation and Clean-up**

Materials:

- Ligation Adapter (LA) from the Ligation Sequencing Kit
- Ligation Buffer (LNB) from the Ligation Sequencing Kit
- Short Fragment Buffer (SFB)
- AMPure XP Beads (AXP)
- Elution Buffer (EB) from the Oxford Nanopore sequencing kit

Consumables:

- Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
- Qubit Assay Tubes
- NEBNext Quick T4 DNA Ligase (Sold as part of the NEBNext Quick Ligation Module, NEB E6056)
- 1.5 mL Eppendorf DNA LoBind tubes

Equipment:

- Qubit fluorometer
- Benchtop centrifuge
- Magnetic rack
- Vortex mixer
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips

- P10 pipette and tips

The Ligation Adapter included in the Ligation Sequencing Kit is not interchangeable with other sequencing adapters according to the original protocol by Oxford Nanopore Technologies.

Oxford Nanopore Technologies recommends in their original protocol that their Ligation Buffer (LNB) is used instead of the buffer supplied with NEBNext Quick T4 DNA Ligase in the NEBNext Quick Ligation Module.

235 Spin down Ligation Adapter (LA) and Quick T4 Ligase, place on ice (if available).

236 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice (if available) immediately after thawing and mixing.

237 Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Spin down and place on ice (if available).

238 Thaw Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Spin down and place on ice (if available).

239 In a 1.5 mL LoBind tube, mix in the following order:
Between each addition, pipette mix 10-20 times.

	A	B
	Reagent	Volume
	DNA sample from the previous step	60 µL
	Ligation Buffer (LNB)	25 µL
	NEBNext Quick T4 DNA Ligase	10 µL
	Ligation Adapter (LA)	5 µL
	Total	100 µL

240 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

241 Incubate the reaction for 10 minutes at room temperature.

242 Resuspend the AMPure XP Beads (AXP) by vortexing.

243 Add 40 µL of resuspended AMPure XB Beads (AXP) to the reaction and mix by flicking the tube.

244 Incubate on a rotator mixer for 5 minutes at room temperature. If a rotator mixer is not available, invert the tube manually several times during the incubation period.

245 Spin down the sample and pellet in a magnetic rack. Keep the tube in the magnetic rack, and pipette off the supernatant when clear and colorless.

246 Wash the beads by adding 250 µL Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

247 Repeat the previous step.

248 Spin down and place the tube back in the magnetic rack. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

249 Remove the tube from the magnetic rack and resuspend the pellet in 15 µL Elution Buffer (EB). Spin down and incubate for 10 minutes at room temperature.

250 Pellet the beads on the magnetic rack until the eluate is clear and colorless, for at least 1 minute.

251 Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 mL LoBind tube. Dispose of the pelleted beads.

252 Quantify 1 µL of eluated sample using a Qubit fluorometer.

253 Calculate how much library needs to be added for a final weight of 200 ng DNA. If the calculated volume is less than 12 µL, move the calculated volume to a new LoBind tube

and top it up with Elution Buffer (EB) to reach 12 µL. Store any leftover library in the -20°C freezer, this can be used to rerun the library if needed.

Calculation:
200 ng / Concentration (ng/µL) = Volume of library to use (µL)
Example: 200 ng / 19.7 ng/µL = 10.2 µL

Note about storage:
For short term storage, 4°C in LoBind tubes is recommended.
For long term storage of more than 3 months, -80°C in LoBind tubes is recommended.

254 Store the library at 4°C until ready to load.

255 Store the library at 4°C until ready to load.

256 **Priming and Loading the Flow Cell**

Materials:
- Flow Cell Flush (FCF)
- Flow Cell Tether (FCT)
- Library Solution (LIS)
- Library Beads (LIB)
- Sequencing Buffer (SB)

Consumables:
- MinION/GridION Flow Cell
- 1.5 ml Eppendorf DNA LoBind tubes

Equipment:
- MinION or GridION device
- MinION Flow Cell Light Shield
- Vortex mixer
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

257 Place the flow cell into the MinION or GridION instrument.

258 Start a flow cell check in the MinKNOW software.

259 Defrost sequencing buffer (SB), library beads (LIB), flow cell tether (FCT) and flow cell flush (FCF) at room temperature.

260 Mix SB, LIB, FCT and FCF with a vortex then spin down.

261 Prepare the “flow cell priming mix” by mixing Flow Cell Flush (FCF) and Flow Cell Teher (FCT) according to the table below. FCF comes in a large bottle and can be portioned out in 1.5 mL LoBind tubes and refrozen. Write +FCT on this tube after adding FCT to the tube of FCF. Mix with a vortex and keep the tube at room temperature.

	A	B
	Reagents	Volume per flow cell
	Flow Cell Flush (FCF)	1,170 µL
	Flow Cell Tether (FCT)	30 µL
	Final total volume in tube	1,205 µL

262 Open the priming port on the flow cell.

263 Remove air from the flow cell using a 1000 µL pipette set to 200 µL that is twisted to around 220-230 µL while being held against the priming port. A small amount of liquid should be seen entering the pipette tip. Check that the sensor array area of the flow cell is covered with buffer.

264 Load 800 µL priming mix via the priming port, use reverse pipetting. Avoid the introduction of any air bubbles as they damage the flow cell.

265 Wait 5 minutes before adding anything more to the flow cell.

266 Vortex Loading Beads (LIB) well. Then mix the following in a 1.5 mL LoBind tube:

	A	B
	Reagent	Volume
	Sequencing Buffer (SB)	34 µL
	Loading Beads (LIB), mixed immediately before use	25.5 µL
	Nuclease-free water	4.5 µL
	DNA library	11 µL
	Total	75 µL

267 Lift the SpotOn port on the flow cell.

268 Add 200 µL priming mix via the priming port, use reverse pipetting. Avoid the introduction of any air bubbles as they damage the flow cell.

269 Carefully mix the prepared DNA library with a pipette then add it drop by drop to the flow cell via the SpotOn port. Each drop should flow into the flow cell before you add the next one.

270 Close the SpotOn port and the priming port. Place the lightshield on the flow cell and close the cover of the GridION/MinION.

Sequencing

3d

271 On the GridION instrument or the computer controlling a MinION instrument, start MinKNOW.

272 Name the run.

273 Choose the Nanopore ligation sequencing kit V14 (SQK-LSK114) kit that was used for library prep.

274 Choose the run time 72 hours.

Note: The majority of the data will be generated in the first 48 hours. If you are looking to reduce the time the protocol takes, reducing the run time is a good starting point.

275 Choose high accuracy or super accurate basecalling.

276 Set Min Q score to 9.

Note: This value can be reduced if lower amounts of higher quality data is preferred.

277 Set minimum read length to 0.2 kb.

278 Save output as POD5 and FASTQ (compression can be used).

279 Start the run. 3d

Protocol references

This protocol was adapted from the following protocols provided by Twist Bioscience: Long Read Library Preparation and Standard Hyb v2 Enrichment, DOC-001320 REV 2.0 and Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter System, DOC-001239 REV 5.0

Nanopore library preparation was based on the following protocol from Oxford Nanopore Technologies: Ligation sequencing V14 - low input by PCR (SQK-LSK114 with EXP-PCA001) (Document version: LWP_9183_v114_revL_12Dec2024)

cDNA synthesis was based on Alcolea-Medina et al. (2024): https://doi.org/10.1038/s43856-024-00554-3

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