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PathoSeq Bio Hybrid Capture with ONT Minion Sequencing R9 Chemistry

 [Pathogens](#)

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Manuscript citation:

Coming Soon!

We understand ONT is phasing out R9 chemistry in favor of more advanced R10 chemistry. This protocol is being validated and updated to be compatible with R10 flow cells and will be uploaded ASAP.

Please stay tuned for release of a **custom bioinformatic analysis pipeline** that accompanies this sequencing protocol.

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Protocol status: Working

We and others have used this protocol and it is working.

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Disclaimer

This protocol is provided as-is without any guarantees or warranties. Use of this protocol is at the user's own risk, and the author assumes no liability for its application or any associated outcomes.

MinKNOW software undergoes routine updates and changes. Setting up a MinKNOW sequencing run may look a little different than the steps outlined in this protocol. Future versions of this protocol will be published with the "current at that time" MinKNOW set up instructions.

Abstract

Procedure to prepare and enrich libraries for next generation sequencing on Oxford Nanopore platform. The method uses Illumina's DNA prep with enrichment using cDNA followed by barcoding and MinION loading and sequencing.

Attachments



Workflow_with_times....

1.3MB



Workflow_with_times...

1.9MB

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Attached workflow images were created using BioRender (<https://BioRender.com>)

Guidelines

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Please stay tuned for release of a custom bioinformatic analysis pipeline that accompanies this sequencing protocol.

Materials

Reagents, Consumables, and Equipment

	Reagent	Description	Storage Temp
		NEBNext First Strand Synthesis Reaction Buffer	-20°C
		Random Primers	-20°C
		NEBNext First Strand Enzyme Mix	-20°C
		NEBNext Second Strand Reaction Buffer	-20°C
		NEBNext Second Strand Synthesis Enzyme Mix	-20°C
		KAPA Magnetic Beads	+4°C
		Twist Biotinylated probes	-20°C
		Molecular Grade H2O	Room temperature
		Nextera_ONT- PCR primers	-20°C
		2X Reaction Buffer	-20°C
		dNTPs	-20°C
		10X Basic E-Mix	-20°C
		20X Core Reaction Mix	-20°C
		280mM MgOAc	-20°C
	TWB	Tagmentation Wash Buffer	Room temperature
	TB1	Tagmentation Buffer 1	-20°C
	ST2	Stop Tagmentation Buffer 2	+4°C
	SMB	Streptavidin magnetic beads	+4°C
	RSB	Resuspension Buffer	-20°C
	NHB2	New hybridization buffer 2	-20°C
	HP3-NaOH	Sodium hydroxide	-20°C



	Reagent	Description	Storage Temp
	EtOH	Fresh 80% Ethanol	Room temperature
	ET2	Elute Target buffer	+4°C
	EPM	Enhanced PCR Mix	-20°C
	EHB2	Enrich Hyb Buffer 2	+4°C
	EEW	Enhanced Enrichment Wash	-20°C
	EE1	Enrichment Elution buffer 1	-20°C
	eBLTs	Enrichment Bead linked Transposomes	+4°C
	BP#	BP Barcode	-20°C

	A	B	C
	Consumables		
	ITEM	VENDOR	CAT#
	NEBNext® Ultra™ II RNA First Strand Synthesis Module	NEB	E7771L
	NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module	NEB	E611L
	Illumina® DNA Prep with Enrichment, (S) Tagmentation (16 Samples)	Illumina	20025523
	Illumina® DNA Prep, (S) Tagmentation (96 Samples)	Illumina	20025520
	TwistAmp® Basic	TwistDx	TABAS03 KIT
	Twist biotinylated probes, such as but not limited to the Comprehensive Viral Research Panel or a custom made probe panel	Twist Bioscience	Varied 12 rxns of comprehensive panel = 103547
	Nextera-MinION-PCR-FWD TTTCTGTTGGTGCT	IDT	standard primers



A	B	C
GATATTGCGTCTCGTGGGC TCGG		
Nextera-MinION- PCR_REV ACTTGCCTGTC GCTCTATCTTCTCGTCGGC AGCGTC	IDT	standard primers
PCR-cDNA Barcoding Kit	ONT	SQK- PCB111.24
SPRI beads (Agencourt AMPure XP purification beads, Kapa beads or similar)	multiple	
1.5 ml or 2 ml microcentrifuge tubes	General lab supplier	
10 µl pipette tips	General lab supplier	
10 µl multichannel pipettes	General lab supplier	
10 µl single channel pipettes	General lab supplier	
20 µl pipette tips	General lab supplier	
20 µl multichannel pipettes	General lab supplier	
20 µl single channel pipettes	General lab supplier	
200 µl pipette tips	General lab supplier	
200 µl single channel pipettes	General lab supplier	
200 µl multichannel pipettes	General lab supplier	
1000 µl pipette tips	General lab	



A	B	C
	supplier	
1000 µl single channel pipettes	General lab supplier	
Hard-Shell 96-well PCR plates	Bio-Rad	HSP-9601
Microseal 'B' adhesive seals	Bio-Rad	MSB-1001
RNase/DNase-free 8-tube strips and caps	General lab supplier	
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR	89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	General lab supplier	
Nuclease-free water	General lab supplier	
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific	Q32850
Qubit Assay Tubes	Thermo Fisher Scientific	Q32856
Flow cells R9		
Equipment		
ITEM	VENDOR	CAT#
Thermocycler capable of holding Fully skirted 96 well plates		
Magnetic Stand-96 for skirted plates	Thermo Fisher Scientific	AM10027
Microcentrifuge General lab supplier	General lab supplier	



	A	B	C
	Microplate centrifuge General lab supplier	General lab supplier	
	Qubit Fluorometer 4.0/Qubit Flex	Thermo Fisher Scientific	Q33216
	Vortexer	General lab supplier	
	MinION sequencer	ONT	
	Computer capable of basecalling and performing bioinformatics analysis		

Before start

If you use this protocol, your average final library size is approximately 300 nt which can be used to calculate the fmol at the end for flow cell loading.

If you are unsure about the computing requirements required for ONT MinION sequencing, please review here: <https://nanoporetech.com/document/requirements/minion-it-reqs> or reach out to us for a recommendation.

A. cDNA Synthesis

1 Denature RNA

A	B	C	D	E	F
Item		Storage	Instructions		
NEBNext First Strand Synthesis Reaction Buffer		-20°C	Thaw on ice. Vortex to mix and spin down.		
Random Primers		-20°C	Thaw on ice. Vortex to mix and spin down.		

- 1.1 Thaw NEBNext First Strand Synthesis Reaction Buffer and Random Primers on ice, vortex to mix, and centrifuge briefly.
- 1.2 Combine the following volumes to prepare the Denaturation master mix (**NOTE:** the following volumes **do not** account for pipette error. Consider making an additional reaction. MM=mastermix):

A	B	C	D	E	F	G	H
Reagent		Volume for 1x (Does not consider pipette error)		To adjust for pipetting error make MM with an addition rxn, (i.e., for 8 rxns make 9rxns)		16rxns	24 rxns
NEBNext First Strand Synthesis Reaction Buffer		4 µl		36 µl		68 µl	100 µl
Random Primers		1 µl		9 µl		17 µl	25 µl

- 1.3 Store the Denaturation master mix on ice until use.
- 1.4 On ice, combine 5 µl of Denaturation mastermix with 13 µl of RNA, if you don't have 13 µl of RNA, make up the difference with nuclease free H₂O.
- 1.5 Seal the tubes/plate and transfer onto a thermocycler.



1.6 Run the following thermocycler protocol: **DENATURE**

A	B	C
Step	Temp	Time
1	65°C	5 minutes
2	4°C	Hold
Preheat Lid: 100°C		
Reaction Volume: 18 µl		

1.7 Once the run is complete, **immediately** place the tubes/plate on ice.



2 **First Strand Synthesis**

A	B	C	D	E
Item		Storage	Instruction	
NEBNext First Strand Synthesis Enzyme Mix		-20°C	Keep on ice. Invert to mix and spin down.	

2.1 Place the NEBNext First Strand Synthesis Enzyme Mix on ice, invert to mix and spin down.

2.2 On ice, add 2 µl of NEBNext First Strand Synthesis Enzyme Mix to the 18 µl of primed/denatured RNA.

2.3 Mix samples by pipetting and spin down

2.4 Run the following thermocycler protocol: **FIRST STRAND**



A	B	C
Step	Temp	Time
1	25°C	10 minutes
2	42°C	50 minutes
3	70°C	15 minutes
4	4°C	Hold
Preheat Lid >= 80°C		
Reaction Volume: 20 µl		

2.5 While the FIRST STRAND Protocol is running, prepare the Second Strand Mastermix listed in **Step 3**.

2.6 Once the run is complete, ***immediately*** place the tubes/plate on ice.



3 Second Strand Synthesis

A	B	B	C	E
Item		Storage	Instruction	
NEBNext Second Strand Synthesis Reaction Buffer		-20°C	Thaw on ice. Vortex to mix and spin down	
NEBNext Second Strand Synthesis Enzyme Mix		-20°C	Keep on ice. Invert to mix and spin down.	

3.1 Thaw NEBNext Second Strand Synthesis Reaction Buffer on ice, vortex to mix and spin down. Take out the NEBNext Second Strand Synthesis Enzyme Mix when buffer is thawed and place on ice, invert to mix and spin down.

3.2 Combine the following volumes to create the Second Strand master mix (**NOTE:** the following volumes ***do not*** account for pipette error. Consider making an additional reaction):



	A	B	C	D	E
	Reagent			Volume for 1x (Does not consider pipette error)	
	Nuclease-free Water			48 µl	
	NEBNext Second Strand Synthesis Reaction Buffer			8 µl	
	NEBNext Second Strand Synthesis Enzyme Mix			4 µl	
	Total			60 µl	

3.3 Store the Second Strand master mix on ice until use

3.4 On ice, add 60 µl of Second Strand master mix each 20 µl First Strand Synthesis reaction.

3.5 Pipette slowly to mix and spin down

3.6 Run the following thermocycler protocol: **SECOND STRAND**

	A	B	C
	Step	Temp	Time
	1	16°C	60 minutes
	2	4°C	Hold
	Preheat Lid: Off or < 40°C		
	Reaction Volume: 80 µl		

3.7 While the run is in-progress, be sure to prep KAPA Beads, RSB, and 80% Ethanol required for **Step 4**. As well, you can prep the master mixes for **Section B Tagmentation** during this incubation.

3.8 Once the run is complete, proceed **immediately** into cleanup.



4 Clean up cDNA

	A	B	B	D	E
	Item	Storage	Instruction		
	KAPA Beads	4°C	Let stand at room temperature for 30 minutes. Vortex vigorously.		
	RSB	4°C	Thaw at room temperature. Vortex to mix.		

- 4.1 Vortex beads to resuspend.
- 4.2 Add 96 µl (1.2X) of resuspended KAPA magnetic beads to the second strand synthesis reaction.
- 4.3 Mix by shaking at **1800 rpm** for 1 minute or by slowly pipetting up and down at least 10 times.
- 4.4 Incubate for 5 minutes at room temperature.
- 4.5 Briefly centrifuge the plate/tubes to collect all the sample at the bottom.
- 4.6 Place the plate/tubes on a magnetic stand to separate beads from the supernatant (about 5 minutes).
NOTE: The volume of sample with KAPA beads added is quite large, and depending on the magnet you are using, it may be difficult for the magnet to collect the KAPA beads. Consider using a skirted 96-well plate to pull beads down or transferring to 1.5 mL tubes (if you are working with a small number of samples).
- 4.7 After the solution is completely clear, carefully remove and discard the supernatant without disturbing the beads.
- 4.8 Wash **two times** as follows:
 - A. With the plate on the magnetic stand, add 200µl fresh 80% ethanol. Do not mix.
 - B. Incubate for 30 seconds, remove and discard supernatant.
 - C. Using a 20 µl multichannel pipette, remove all remaining ethanol from samples

- 4.9 Air-dry ethanol on a magnetic stand for up to 2 minutes (**Ensure that beads do not dry out**).
- 4.10 Remove plate/tubes from the magnetic stand and add 19.5 µl RSB to the beads.
- 4.11 Mix by pipetting 10 times and/or shake at 1800 rpm for 2 minutes
- 4.12 Incubate at room temperature for 2 minutes.
- 4.13 Place plate/tubes on magnet for 2 minutes or until solution is clear.
- 4.14 Transfer 17.5 µl supernatant into a new plate/set of tubes.
- 4.15 The remaining 2 µl of cDNA can be quantified with Qubit HS 1x dsDNA kit but it is possible there is not enough DNA to quantify at this step.
- 4.16 **SAFE STOPPING POINT:** Store cDNA in a sealed plate or tubes at either at 4°C for next day use or at -20°C for long term storage.



B. Library Preparation using eBLTs

5 Prepare Tagmentation and PCR master mixes

	A	B	B	B	E	F
	Item		Storage	Instruction		
	eBLT		4°C	Bring to room temperature. Vortex to mix.		
	TB1		-20°C	Bring to room temperature. Vortex to mix and spin down.		
	EPM		-20°C	Thaw on ice, invert to mix, then spin down.		



	A	B	B	B	E	F
	cDNA NF-ONT primer mix (10µM)		-20°C	Thaw at room temperature. Vortex to mix.		

5.1 Bring eBLT and TB1 to room temperature and vortex vigorously to resuspend.

5.2 Combine the following volumes to prepare the Tagmentation master mix:

	A	B	B	D
	Reagent		Volume for 1x (this volume **already** accounts for pipetting error)	
	eBLT		11.5 µl	
	TB1		11.5 µl	
	Nuclease-free water		14.5 µl	

6 Tagmentation

6.1 Vortex Tagmentation master mix thoroughly to resuspend.

6.2 Add 32.5µl of Tagmentation master to 17.5 µl of cDNA and mix by pipetting.

6.3 Seal the plate/tubes and place them on the thermocycler.

6.4 Run the following thermocycler protocol: **TAGMENTATION** (or **TAG**)

	A	B	C
	Step	Temp	Time
	1	55°C	5 minutes

A	B	C
2	10°C	Hold
Preheat Lid: 100°C		
Reaction Volume: 50 µl		

- 6.5 While Tagmentation is occurring combine the following volumes to prepare the PCR master mix:

A	B	B	D
Reagent		Volume for 1x (this volume **already** accounts for pipetting error)	
Nuclease-free water		27.5 µl	
EPM		22 µl	
cDNA NF-ONT primer mix (10µM)		5.5 µl	

- 6.6 Keep PCR master mix on **ice** until **ONT Adapter Amplification on Tagmented DNA** (Step 8).



7 Post Tagmentation Cleanup

A	B	B	D	E
Item	Storage	Instruction		
ST2	15°C or RT	If precipitates are observed, heat at 37°C for 10 minutes, bring to room temperature and vortex		
TWB	15°C or RT	Bring to room temperature.		

- 7.1 Remove plate from thermocycler and incubate at room temperature for 2 minutes.

- 7.2 Shake at **2200 rpm** for 1 minute or slowly pipette each sample 10 times.

- 7.3 Incubate at room temperature for 5 minutes.
- 7.4 Briefly centrifuge.
- 7.5 Place plate/tubes onto a magnetic stand for 2 minutes or until clear.
- 7.6 Remove and discard the supernatant while leaving the beads on the wall of the tube/well.
(**NOTE:** The **tagmented DNA is attached to the beads**).
- 7.7 Remove the plate/tubes from the magnetic stand.
- 7.8 Wash **two times** as follows:
A. Add 100 µl of TWB directly onto the beads
B. Slowly pipette until well mixed or shake briefly at 1800 rpm
C. Place tubes/plate on a magnetic stand and allow to sit for 2 minutes or until clear.
D. Remove and discard 100 µl of supernatant
- 7.9 Remove the plate/tubes from the magnetic stand and add 100 µl of TWB directly on to the beads
- 7.10 Slowly pipette until well mixed or shake briefly at 1800 rpm.
- 7.11 Place plate/tubes on a magnetic stand for 2 minutes, or until clear.
- 7.12 **NOTE:** Keep the beads in the wash solution and on the magnetic stand until you are ready to proceed with adding PCR master mix. This will prevent overdrying of the beads



8 **ONT Adapter amplification on tagmented DNA**

- 8.1 Mix PCR master mix by inverting/flicking and briefly centrifuge.

- 8.2 With the plate/tubes on the magnetic stand, remove 100µl of wash supernatant from each well and discard.
- 8.3 Remove plate/tubes from the magnetic stand and add 50 µl of PCR master mix directly onto the beads.
- 8.4 Seal plate and shake at **1600 rpm** for 2 minutes or slowly pipette 10 times and briefly centrifuge.
- 8.5 Run the following thermocycler protocol: TAG PCR

	A	B	C	D	E	F
	Step	Cycles	Note		Temperature	Time
	1	1	Gap Fill		72°C	3 minutes
	2	1	Initial Denaturation		98°C	3 minutes
	3	17	Denaturation		98°C	20 seconds
			Annealing		60°C	30 seconds
			Extension		72°C	1 minute
	4	1	Final Extension		72°C	3 minutes
	5	1	Temperature Hold		10°C	Hold
	Preheat Lid: 100°C					
	Reaction Volume: 50 µl					

- 8.6 **SAFE STOPPING POINT:** Keep thermocycler at 4°C overnight, or store libraries at -20°C for long term use.



9 Clean up Libraries

A	B	B	D	E
Item	Storage	Instruction		
KAPA Beads	4°C	Let stand at room temperature for 30 minutes. Vortex vigorously.		



	A	B	B	D	E
	RSB	4°C	Place at room temperature. Vortex to mix.		

- 9.1 Centrifuge the plate/tubes to collect all contents at the bottom.
- 9.2 Place the plate/tubes on a magnetic stand for 2 minutes or until clear.
- 9.3 Transfer 45 µl supernatant from each well into a new plate/tubes.
- 9.4 Add 81 µl (1.8X) KAPA beads to each sample.
- 9.5 Shake at 1800 rpm for 2 minutes or slowly pipette 10 times.
- 9.6 Incubate at room temperature for 5 minutes. Prepare fresh 80% ethanol solution.
- 9.7 Place plate/tubes on a magnetic stand for 2 minutes or until clear.
- 9.8 Without disturbing the beads, remove and discard supernatant.
- 9.9 Wash **two times** as follows:
A. With the plate/tubes on a magnetic stand, add 175µl fresh 80% ethanol (Do not mix)
B. Incubate for 30 seconds, remove and discard supernatant.
- 9.10 Using a 20 µl multichannel pipette, remove all remaining ethanol from samples.
- 9.11 Air-dry ethanol on the magnetic stand for 2 minutes **(Ensure beads do not dry out)**.
- 9.12 Remove plate/tubes from the magnetic stand and add 17 µl RSB directly onto the beads.



- 9.13 Mix by pipetting 5 times or shake at 1800 rpm for 2 minutes
- 9.14 Incubate at room temperature for 2 minutes
- 9.15 Place plate/tubes on a magnetic stand for 2 minutes or until clear.
- 9.16 Transfer 15 µl supernatant into a new plate, if going to processed directly to hybridization you can pipet the 9 µl directly from tubes with the beads to the tubes containing the NHB2, probes, and EHB2.
- 9.17 **SAFE STOPPING POINT:** Store at -20°C in sealed plate/tubes, or immediately continue to library enrichment.



10 **Validate libraries**

- 10.1 Validate libraries using a Qubit HS 1x dsDNA assay to determine whether you have amplified any libraries.
- 10.2 Additional QC at this step could include determining the average length of your libraries using an Agilent Tapestation, Bioanalyzer, or running a gel to ensure correct size of the libraries.



C. Library Enrichment

11 **Hybridization of Probes**

	A	B	B	B	E	F
	Item		Storage	Instruction		
	EHB2		4°C	Bring to room temperature. Vortex and spin down.		
	Enrichment Probe		-20°C	Thaw on ice. Vortex and spin down.		
	NHB2		-20°C	Thaw at room temperature. Once at RT, preheat at 50°C for 5 minutes. Vortex and centrifuge. Check for precipitates, vortex again if needed.		

11.1 Thaw the EHB2 and NHB2 at room temperature and vortex to mix. For NHB2, be sure to heat at 50°C for 5 minutes to dissolve any precipitates. Thaw the enrichment probes on ice and vortex to mix.

11.2 In a new set of tubes/plate, combine the reagents in the following order:

A	B	B
Reagent		Volume
Cleaned up Library from previous step 9		9 µl
NHB2		12.5 µl
Probes		1 µl
EHB2		2.5 µl

11.3 Mix by pipetting or shake at 1600 rpm for 1 minute.

11.4 Centrifuge briefly and then place tubes on a thermocycler

11.5 Run the following thermocycler protocol: **HYBRIDIZATION** (or **HYB**):

	A	B	C	D	E	E
	Step	Cycles	Note	Temperature		Time
	1	1	Denaturatio n	95°C		5 minutes
	2	18	Ramp Down**	94°C **(Decrease by 2°C per cycle)**		1 minutes
	3	1	Hybridizati on	58°C		90 minutes
	4	1	Temperatur e Hold	58°C		Hold
	Preheat Lid: 100°C					
	Reaction Volume: 25 µl					

- 11.6 **SAFE STOPPING POINT:** The **minimum** hybridization time at 58 °C is **90** minutes. But, the protocol can be held overnight to provide increased sensitivity and a convenient safe stopping point during the protocol. **NOTE:** Hybridization has only been tested up to **16** hours.



12 Hybridization Capture

	A	B	B	D	E	F
	Item	Storage	Instruction			
	ET2	4°C	Bring at room temperature. Vortex to mix and centrifuge.			
	SMB3	4°C	Let stand for 30 minutes at room temperature. Vortex to mix.			
	EE1	-20°C	Thaw at room temperature. Vortex to mix and centrifuge.			
	HP3	-20°C	Thaw at room temperature. Vortex to mix and centrifuge.			
	EEW	-20°C	Thaw at room temperature. Vortex 3 times for 30 seconds.			

- 12.1 ****IT IS IMPORTANT TO KEEP SAMPLES AT 58°C DURING THE HYBRID CAPTURE STEPS. ALLOWING SAMPLE TO GO LOWER THEN 58°C CAN LEAD TO NON-SPECIFIC BINDING AND LOWER SENSITIVITY****



- 12.2 Ensure that SMB3 has been equilibrated at room temperature for at least 30 minutes before ending the hybridization.

- 12.3 Aliquot and pre-heat EEW at 58°C

A. The wash steps will require a total of 200 µl of EEW per sample.

B. The EEW can be heated on either a heating block or a thermocycler held at 58°C.

- 12.4 Once the SMB3 and EEW have been prepared, remove the plate/tubes containing the library from the thermocycler and centrifuge.

- 12.5 Add 62.5 µl of SMB3 to each sample.

- 12.6 Seal the plate/tubes and shake at **1200 rpm** at 58°C for 4 minutes. **NOTE** If using a **Bioshake IQ**, leave the Bioshake at 58°C indefinitely and increase the shaking during



**sharing steps and use a timer to time the incubation.**

12.7 Incubate the samples on a thermocycler or heating block at 58°C for 15 minutes.

12.8 During the incubation, prepare the Elution master mix and store on ice:

	A	B
	Reagent	Volume for 1x
	EE1	7.12 µl
	HP3	0.37 µl

13 Wash and Transfer

13.1 Once the incubation is complete, remove the plate/tubes from the heating system and centrifuge.

13.2 Place plate/tubes on a magnetic stand for 2 minutes or until clear

13.3 Remove and discard the supernatant from each well.

13.4 First Wash

1. Remove plate/tubes from the magnetic stand.
2. Add **50 µl** of preheated EEW to each sample and return the EEW to the heating system.
3. Seal the plate/tubes and shake at **1600 rpm at 58°C for 4 minutes**.
4. Incubate the libraries on the heating system held at **58°C for 5 minutes**.
5. Briefly centrifuge and place tubes/plate on a magnetic stand for 2 minutes or until clear.
6. **Carefully** remove and discard the **50 µl** of supernatant

13.5 Second and Third Washes

1. Remove the plate/tubes from the magnetic stand.
2. Add **50 µl** of preheated EEW to each sample and return the EEW to the heating system.
3. Seal the plate/tubes and shake at **2000 rpm at 58°C for 1 minute**.



4. Incubate the libraries on the heating system held at **58°C for 5 minutes**.
5. Briefly centrifuge and place tubes/plate on a magnetic stand for 2 minutes or until clear.
6. **Carefully** remove and discard the 50 µl of supernatant.
7. Repeat these steps for the **third** wash.

13.6 Transfer Wash

1. Remove the plate/tubes from the magnetic stand.
2. Add **50 µl** of preheated EEW to each sample and return the EEW to the heating system.
3. Seal the plate/tubes and shake at **2000 rpm at 58°C for 1 minute**.
4. Once the shaking is complete, transfer the entire volume (including the beads) to a new plate/tubes. *This will minimize carry over from previous washes.*
5. Incubate the libraries on the heating system held at **58°C for 5 minutes**.
6. **NOTE:** If proceeding directly to **Isothermal ONT Adapter Amplification**, begin thawing reagents for that step (**step 14**) and the subsequent cleanup step (**step 15**).
7. Briefly centrifuge and place tubes/plate on a magnetic stand for 2 minutes or until clear.
8. **Carefully** remove and discard the 50 µl of supernatant.
9. Use a 20 µl pipette to remove any residual liquid from the beads.
10. Proceed to Elution **immediately** to avoid overdrying the beads.

14 Elution

- 14.1 Remove the plate/tubes from the magnetic stand.
- 14.2 Add **6 µl** of Elution master mix (prepared during **step 12**)
- 14.3 Shake at **2000 rpm** for 1 minute.
- 14.4 Incubate at room temperature for 2 minutes.
- 14.5 Briefly centrifuge and place plate/tubes on magnetic stand for 2 minutes or until clear
- 14.6 Transfer **5.5 µl** of supernatant to a new plate/set of tubes.



14.7 Add **1 µl** of ET2 to each sample and mix by pipetting.

14.8 Briefly centrifuge.

14.9 **SAFE STOPPING POINT:** Store sealed plate/tubes overnight at 4°C or at -20°C for long term storage



15 Isothermal ONT Adapter Amplification of Enriched Libraries

	A	B	B	D	E	F
	Item		Storage	Instructions		
	KAPA beads		4°C	Take out and leave at room temperature for use after isothermal reaction		
	RSB		4°C	Thaw at room temperature. Vortex to mix and spin down.		
	Primer Free Rehydration buffer		-20°C	Thaw on ice, invert to mix, tap bottle on desk to get liquid to bottle, ensure it is completely thawed		
	BP barcodes		-20°C	Thaw on ice, vortex to mix and spin down.		
	TwistAmp Basic reaction		-20°C	NOTE: Reagent is lyophilized		
	280mM MgOAc		-20°C	Thaw on ice, invert to mix, and spin down.		

15.1 Mix the following volumes to create the Isothermal Amplification master mix:

	A	B	C	B
	Reagent			Volume for 1x
	Primer Free Rehydration buffer			29.5 µl
	Nuclease Free H2O			10.5 µl

15.2 Combine 40 µl of Isothermal Amplification master mix with 6.5 µl of sample.

- 15.3 Add 1 μ l of a unique BP Barcode to each sample.
- 15.4 Add the sample (now 47.5 μ l with master mix, sample, and barcode combined) to a TwistAmp Basic Reaction Tube.
- 15.5 Add 2.5 μ l of 280 mM MgOAc to each reaction tube. Mix by inverting and flicking.
- 15.6 Briefly centrifuge.
- 15.7 Incubate the reaction tube at 40°C for 10 minutes
1. It is important to continue to clean up **immediately** after the 10 minute incubation as the enzyme will continue amplifying until removed via cleanup.
 2. Ensure that KAPA Beads and RSB have equilibrated to room temperature to proceed immediately.



16 Clean up Libraries

A	B	B	D	E	F
Item	Storage	Instructions			
KAPA Beads	4°C	Let stand at room temperature for 30 minutes. Vortex vigorously.			
RSB	4°C	Thaw at room temperature. Vortex to mix.			

- 16.1 Add **50 μ l** KAPA beads to each well containing supernatant.
- 16.2 Shake at **1800 rpm** for 2 minutes or slowly pipette 10 times.
- 16.3 Incubate at room temperature for **5 minutes**. Prepare fresh 80% Ethanol.
- 16.4 Place plate/tubes on a magnetic stand for **3-5** minutes or until clear.

- 16.5 Without disturbing the beads, remove and discard supernatant.
- 16.6 Wash two times as follows:
1. With the plate/tubes on the magnetic stand, add 175 μ l fresh 80% ethanol. Do not mix.
 2. Incubate for 30 seconds, remove and discard.
- 16.7 Using a 20 μ l multichannel pipette, remove all remaining ethanol from samples.
- 16.8 Air-dry on the magnetic stand for **2 minutes** (**Ensure that beads do not dry out**). 
- 16.9 Remove plate/tubes from the magnetic stand and add **20 μ l RSB** to the beads.
- 16.10 Mix by pipetting **10** times and shake at **1800 rpm** for **2** minutes.
- 16.11 Incubate at room temperature for **2** minutes.
- 16.12 Place plate on magnet for **2** minutes or until solution is clear.
- 16.13 Transfer **17.5 μ l** supernatant into a new plate.
- 16.14 **SAFE STOPPING POINT:** Store sealed plate/tubes *overnight* at **4°C** or at **-20°C** for *long term*  

17 Quantify Libraries

- 17.1 At this point, libraries will need to be quantified prior to loading. Libraries should be quantified using a Qubit HS 1xdsDNA kit as well as a Tapestation or BioAnalyzer if the size of your library is unknown. These QC steps will provide us with a mass and length value, which we can use to calculate the number of DNA molecules present in the sample. For Oxford Nanopore Sequencing, the number of DNA molecules is important, as the molecules of DNA loaded are individually sequenced (unlike other "sequencing by synthesis" approaches). 

17.2 To calculate the number of mols in each sample, use the following formula:

$$(Concentration(ng/\mu L))/(660 \times averagelibrarysize(bp)) \times 10^6 = Concentration(nM)$$

17.3 The nM concentration is equivalent to the number of fmols per μ l of sample:

$$nM = nmol/L \approx fmol/\mu L$$

17.4 For this assay ONT recommends loading 50 fmols, we have seen loading this amount you **tend to underload**. So we start by **loading 100 fmols**, which is **equivalent to 20 ng** of a pooled library to start. Adjust the ng of pooled library to maximize flow cell output by observing things like pore occupancy overtime.

17.5 Example Calculations:

A	B	C	D
Mass		Fmol if length of library was equal to 300 nt	
1 ng of a pooled library		5.1 fmol	
5 ng of a pooled library		25.3 fmol	
10 ng of a pooled library		50.5 fmol	
15 ng of a pooled library		75.8 fmol	
20 ng of a pooled library		101 fmol	

17.6 Example of library pooling:

A	B	C	D	E	F	G
Library name	Concentration after enrichment PCR (ng/μl)	Lib added to pool		EB added to pool		Total ng
Lib 1	5	4 μ l		6 μ l		20
Lib 2	4	5 μ l		5 μ l		20

	A	B	C	D	E	F	G
	Lib 3	2	10 µl		0 µl		20
		Sum	19 µl		11 µl		60
	Total volume				30 µl		
	Concentration				2 ng/µl		

For example above you would add 10 µl of the pooled library and 1 µl of additional EB for a total of 11 µl to be loaded on the flow cell.

D. Loading the Flow Cell

18 Loading the MinION Flow cell

	A	B	B	D	E	F	G
	Item		Storage	Instructions			
	RAP T (Rapid Adapter T)		-20°C	Invert to mix, spin down and place on ice.			
	RDB (RAP Dilution Buffer)		-20°C	Thaw at room temperature. Pipette to mix and spin down.			
	FB (Flush Buffer)		-20°C	Thaw at room temperature. Vortex to mix and spin down.			
	FLT (Flush Tether)		-20°C	Thaw at room temperature. Vortex to mix and spin down.			
	SBII (Sequencing Buffer II)		-20°C	Thaw at room temperature. Vortex to mix and spin down.			
	LBII (Loading Beads II)		-20°C	Thaw at room temperature. Vortex to mix and spin down.			
	EB (Elution Buffer)		-20°C	Thaw at room temperature. Vortex to mix and spin down.			

18.1 Make sure MinION Flow cell has been equilibrated for at least 30 minutes at Room Temperature.





- 18.2 Prior to loading, be sure to run Hardware and Flow cell checks on the MinkNOW software.
- 18.3 Combine 30 μ l FLT into an entire tube of FB (or 1.17 ml of FB) to create the priming solution and marked tube that FLT was added.
- 18.4 Label four new 1.5 ml Eppendorf DNA LoBind tube, Diluted RAP-T, Library pool, Pool plus RAP-T, and Load
- 18.5 In a the Diluted RAP-T tube, dilute RAP T by preparing the following mix:

	A	B
	Reagent	Volume for 1x
	RDB	5.7 μ l
	RAP T	0.9 μ l

- 18.6 Mix by pipetting and spin down and place back on ice. (this can be stored for xx[WM1])
- 18.7 In the Library pool tube combine barcoded libraries in equal representations determined above.
1. A. Example if you added 3 ul of Library pool, add 20 of ONT EB to make the final volume 23 ul
- 18.8 Add 1 μ l of the Diluted RAP-T to the Pool plus RAP-T tube.
- 18.9 Mix well by pipetting and spin down.
- 18.10 Incubate for 5 min at RT.
- 18.11 Remove any air from the Flow cell by opening the priming port and removing approximately 1-2 μ l of buffer by following the procedure below:
1. On the Flow Cell, slide the priming port open.
 2. Remove any air from the Flow cell by setting a P1000 to 500 μ l and inserting a new p1000 tip into the priming port and dialing the pipette up until a small amount of liquid



enters the tip (do not go past 530 μ l when dialing the pipette).

18.12 Next, load 800 μ l of priming solution into the priming port by picking up 900 μ l of priming solution and dialing down the pipette until you reach 100 μ l. Put the remaining priming solution back into the Priming solution tube.

18.13 Close the priming port and incubate for 5 minutes at room temperature.

18.14 During the incubation, combine the following volumes in a 1.5 ml tube to create the Loading mix:

	A	B
	Reagent	Volume for 1x
	Pool plus RAP-T	14.5 μ l
	SBII	35 μ l
	LBII	25.5 μ l

18.15 After the 5 minutes incubation, open the priming port.

18.16 Remove any air from the Flow cell by setting a P1000 to 500 μ l and inserting a new p1000 tip into the priming port and dialing the pipette up until a small amount of liquid enters the tip (do not go past 530 μ l when dialing the pipette).

18.17 Next, open the SpotOn port.

18.18 With both the SpotON and Priming ports open, pipette 300 μ l of priming solution and slowly add 200 μ l of the priming solution into the priming port by dialing the pipette down until 100 μ l.

18.19 Mix the Loading mix by pipetting prior to loading (as the loading beads settle very quickly).

18.20 Load all 75 μ l of loading mix in a dropwise manner on the SpotOn.

18.21 Close the SpotOn and Priming ports.



E. Setting up the Sequencing Run in MinKNOW

- 19 Open the MinKNOW Software
- 19.1 On the left-hand menu, select "Start", and then select "Start Sequencing".
- 19.2 Be sure to select the correct device (if multiple devices are plugged in at one time).
A. This can be confirmed by comparing the MinION serial number (found on the inside of the device lid) to the number shown on the screen and/or by comparing the Flow Cell serial number shown on the screen to the number on the Flow Cell.
- 19.3 Choose a name for the sequencing run. We recommend beginning the name with the date of the sequencing run, and then providing a brief description of the run in the title (Example: 20230101-PathoSeq_Bio-Project_Name-Run1)
- 19.4 Once completed, proceed to "Kit Selection".
- 19.5 On the kit selection screen, choose the "PCR-cDNA Barcoding Kit" or "SQK-PCB111.24".
A. This selection tells Guppy/Dorado (the demultiplexing software) to look for the correct barcode sequences.
- 19.6 Once completed, proceed to "Run Options".
- 19.7 On the Run Options screen, set the minimum read length to **20bp**.
- 19.8 Once completed, proceed to "Basecalling".
- 19.9 On the Basecalling screen, there will be 3 separate portions (Basecalling, Barcoding, and Alignment). Modify the following options:
A. Under Basecalling, choose "Edit options" and select "High-accuracy basecalling". This will increase basecalling accuracy and make use of the GPU-basecalling functionality.
B. Under Barcoding, choose "Edit options" and turn on "Trim Barcodes".
C. Proceed **without** Alignment enabled
- 19.10 Once complete, proceed to "Output"



- 19.11 On this screen, leave the output folder default (as MinKNOW has been found to have issues writing to other places on the computer).
- 19.12 Additionally, in the circular box containing “Quality Score”, select “Options”, and drop the **Quality Score to 8**.
- 19.13 Once completed, continue to “Final Review”.
- 19.14 Check over the parameters you set to ensure they match this guide.
- 19.15 Once you have validated this, start the run.
Note: while it is generally safe to navigate the on-screen graphics during the run, best practice is to never stay on the interactive graphs indefinitely during the sequencing run as this requires substantial memory and can cause MinKNOW to become unresponsive or crash. After monitoring the graphs, back out to the default MinKNOW run screen menu
- 19.16 After your desired run time, stop the sequencer and proceed to data analysis.



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