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PathoSense

2025

CONFIDENTIAL

Wet lab flow diagnostics PathoSense

WET LAB I - Enrichment

Viral enrichment via Benzonase-Nuclease

Timing ~ 45 min

PathoSense highly recommends to perform the enrichment step **daily**, so that samples are stabilised as soon as possible after arriving in the lab.

Host ≠ Cat/Dog

Reagents

- IC + HMB, provided by PathoSense, aliquoted by Partner Lab
- 10 nM EDTA, provided by PathoSense
- Benzonaze® Nuclease, Purity > 90%, 70746 Millipore = enzyme, so keep on -20°C!

REAGENT	QUANTITY per sample	HANDLING CONDITIONS
IC+HMB	1 aliquot	on ice
Benzonase nuclease	3.5 µL	-20 °C (on cool rack)
10 nM EDTA	12.5 µL	on ice

Materials

- 1,5 mL Eppendorf tube
- 1,2 µm filter
- PathoSense Sample

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated sample preparation area.

Preparations

- Pre-heat the **heat block at 37°C**
- Thaw a 1.5 mL Eppendorf tube with **an aliquot FeCVII internal control (IC) + 20x Homemade Buffer (HMB)** on ice **just before adding the sample** (because the IC contains a virus)

Protocol

1. **Label** a **1.5 mL Eppendorf tube** for each sample
2. Connect a **filter to the syringe** containing the sample
3. **Flush the sample through the filter** in the corresponding labeled Eppendorf tube
4. Take a **1.5 mL Eppendorf tube with an aliquot FeCVII internal control (IC) + 20x Homemade Buffer (HMB)** for each sample

5. Transfer **200 µL of the sample** to the Eppendorf with the aliquot FeCVII internal control (IC) and 20x homemade buffer
6. Add **3.5 µL Benzonase -Nuclease** to each tube
7. Gently **mix by inverting tube 3 times** (DO NOT VORTEX) and quick spin to collect all droplets
8. Incubate the tubes for **30 minutes** at **37°C**
9. Add **12.5 µL 10nM EDTA** to each tube to stop enzymatic reaction
10. Gently **mix by inverting tube 3 times** (DO NOT VORTEX) and quick spin to collect all droplets

Safe stopping point

If the samples cannot be processed immediately, Benzonase-treated samples can be stored at -70°C for 1 month

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Keep Benzonase-treated samples on ice and proceed with

WET LAB II EXTRACTION

Host = Cat/Dog

Reagents

- HMB, provided by PathoSense, aliquoted by Partner Lab
- 10 nM EDTA, provided by PathoSense
- Benzonaze® Nuclease, Purity > 90%, 70746 Millipore = enzyme, so keep on -20°C!

REAGENT	QUANTITY per sample	HANDLING CONDITIONS
HMB	12.5 µL	on ice
Benzonase nuclease	3.5 µL	-20 °C (on cool rack)
10 nM EDTA	12.5 µL	on ice

Materials

- 1,5 mL Eppendorf tube (2)
- 1,2 µm filter
- PathoSense Sample

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated sample preparation area.

Preparations

- Pre-heat the **heat block at 37°C**

Protocol

1. **Label** a **1.5 mL Eppendorf tube** for each sample
2. Connect a **filter to the syringe** containing the sample
3. **Flush the sample through the filter** in the corresponding labeled Eppendorf tube
4. Take a **1.5 mL Eppendorf tube** and transfer **234 µL of the sample**
5. Add **12.5µL 20x Homemade Buffer** (HMB) for each sample to the Eppendorf tube
6. Add **3.5 µL Benzonase -Nuclease** to each tube
7. Gently **mix by inverting tube 3 times** (DO NOT VORTEX) and quick spin to collect all droplets
8. Incubate the tubes for **30 minutes** at **37°C**
9. Add **12.5 µL 10nM EDTA** to each tube to stop enzymatic reaction
10. Gently **mix by inverting tube 3 times** (DO NOT VORTEX) and quick spin to collect all droplets

Safe stopping point

If the samples cannot be processed immediately, Benzonase-treated samples can be stored at -70°C for 1 month

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Keep Benzonase-treated samples on ice and proceed with

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WET LAB II - Extraction

Viral and bacterial lysis and isolation of viral and bacterial DNA/RNA

Timing ~ 45 min / 6 samples

Reagents

- 2x DNA/RNA Shield, Zymo Research (200 µL)
- Viral DNA/RNA Buffer, Zymo Research* (800 µL)
- Viral RNA Wash Buffer, Zymo Research** (1000 µL)
- 95 - 100 % Ethanol (500 µL)
- DNase/RNase free water (35 µL)

*Before first use of a new bottle, add 0.5% (v/v) Beta-mercaptoethanol to the viral DNA/RNA buffer (125 µL/25mL)

**Before first use of a new bottle, add 96 mL 100% ethanol to Viral RNA Wash Buffer

Materials

- Benzonase-treated sample
- Zymo-Spin IIC-XL Column
- Collection tubes (6)
- DNA-Lobind 1.5 mL Eppendorf tube

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated sample preparation area.

Protocol

1. Add **200 µL DNA/RNA Shield** to each sample and mix well by pipetting
2. Add **800 µL Viral DNA/RNA Buffer** to each sample and mix well by pipetting

3. Transfer 600 µL into a **Zymo-Spin IIC-XL Column** in a collection tube and centrifuge for 2 minutes at 16,000 xg

4. Transfer the column into a **new collection tube** and **reload with the leftover 600 µL** and centrifuge for 2 minutes at 16,000 xg

5. Transfer the column into a new collection tube and add 500 µL **Viral RNA Wash Buffer** to the column and centrifuge for 30 sec at 16,000 xg

6. Transfer the column into a new collection tube and add 500 µL **Viral RNA Wash Buffer** to the column and centrifuge for 30 sec at 16,000 xg

7. Transfer the column into a new collection tube and add 500 µL **95-100% ethanol** to the column and centrifuge for 1 minute at 16,000 xg

8. Transfer the column into a new collection tube and centrifuge for 1 minute at 20,000 xg (= **dry spin**)

9. Transfer the column into a **newly labeled 1.5 mL DNA Lobind tube** and add **35 µL DNase/RNase water** directly onto the centre of the column matrix, without touching the column with the tip and **incubate for 1 minute** at room temperature

10. Centrifuge* closed column in DNA-Lobind tube for **30 sec** at **16,000 xg**

*Place the cap of the tube in the opposite direction as the arrow, along with the movement of the centrifuge. This to avoid breaking off the cap of the tube. If this does happen, transfer the eluate to a new tube.

Keep eluted viral nucleic acids (RNA and DNA) on ice and proceed (preferentially) immediately with

WET LAB III - cDNA generation and PCR amplification

Safe stopping point

If the samples cannot be processed immediately, viral nucleic acids can be stored at -70°C for 1 month

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WET LAB III - PCR assay

cDNA generation and PCR amplification

Timing for 12 samples:

- **30 min manual work**
- **1h25 waiting time**
- **10 min manual work**
- **1h45 waiting time**

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated area:
  - **For preparation of Master Mix > MM area***
  - **For working with samples > DNA area**

*Change gloves before working in MM area to prevent contaminating primers and enzymes

Overview

Denaturation

Preparation of the primer/dNTP Master Mix

Primer binding

Preparation of the SuperScript IV Master Mix

Reverse Transcription and Template Switching Reaction

Preparation of the KAPA Hifi Hotstart Master Mix

KAPA Hifi Hotstart Reaction

Denaturation ~ DNA area

Reagents and Materials

- DNA/RNA extract
- 0.2 mL PCR tube (1 per sample + 3)
- deoxynucleotide (dNTP) Solution Mix, N0447L NEB
- SuperScript IV, 18090200 Invitrogen
- KAPA Hifi Hotstart ReadyMix, KK2602 Roche
- Primers, provided by PathoSense
  - nonamer_TSO primer
  - TSO_SS primer

- TSO_PCR primer
- random hexamer

Protocol

1. **Label** a new 0.2 mL PCR tube (strip) for each sample and add **5 µL of the DNA/RNA extracts**
2. **Quick spin** to collect all droplets
3. Incubate at **95°C for 2 minutes** in a PCR thermocycler

Immediately transfer to ice
DO NOT LET COOL TO 4°C IN THE THERMOCYCLER!

Preparation of the primer/dNTP Master Mix ~ MM area

Change gloves before preparation of the Master Mix

Reagents

REAGENT	1 RXN	13 RXN*
DNase/RNase free water	4 µL	52 µL
Nonamer_TSO primer	1 µL	13 µL
dNTP	1 µL	13 µL
Total	6 µL	78 µL

*For 12 samples prepare master mix for 13 RXN, to take account of loss

Protocol

4. Take a PCR tube for the mix and add **4 µL/sample DNase/RNase free water**
5. Vortex and quick spin the **dNTPs** and the **Nonamer TSO** primer

6. Add **1 µL/sample dNTPs** and **1 µL/sample Nonamer_TSO primer** to the mix
7. Vortex and quick spin mix

Primer binding ~ DNA area

8. Add **6 µL of the primer/dNTP mix** to each sample
9. Gently mix the suspension by tapping the tube with your fingers and quick spin
10. Incubate at **65°C for 5 minutes** in a PCR thermocycler to allow proper primer binding

Immediately transfer to ice
and keep for at least 1 minute

Preparation of the SuperScript IV Master Mix ~ MM area

Change gloves before preparation of the Master Mix

Reagents

REAGENT	1 RXN	13 RXN*
DNase/RNase free water	2 µL	26 µL
SuperScript IV buffer 5x	4 µL	52 µL
DTT (100 mM)	1 µL	13 µL
TSO_SS primer (10 µM working stock)	1 µL	13 µL
SuperScript IV enzyme KEEP ON COOL RACK!	1 µL	13 µL
Total	9 µL	117 µL

*For 12 samples prepare master mix for 13 RXN, to take account of loss

Protocol

11. Take a PCR tube for the mix and add **2 µL/sample DNase/RNase free water**
12. Vortex and quick spin the SuperScript Buffer, DTT and TSO_SS primer
13. Add **4 µL/sample SuperScript Buffer, 1 µL/sample DTT** and **1 µL/sample TSO_SS primer** to the mix
14. Quick spin Superscript IV enzyme (DO NOT VORTEX!)
15. Add **1 µL/sample SuperScript IV Enzyme** to the mix
16. Gently mix the suspension by tapping the tube with your fingers and quick spin

Reverse Transcription and Template Switching Reaction ~ DNA area

17. **Quick spin** to collect all droplets
18. Add **9 µL of the SuperScript IV Master Mix** to each sample

19. Gently mix the suspension by tapping the tube with your fingers and quick spin

20. Incubate in a PCR thermocycler according to the program below:

50°C	**42°C**	**80°C**	**4°C**
60 min.	10 min.	10 min.	hold

Preparation of the KAPA Hifi Hotstart Master Mix ~ MM area

Change gloves before preparation of the Master Mix

REAGENT	**1 RXN**	**13 RXN***
DNase/RNase free water	3.5 µL	45.5 µL
KAPA Hifi Hotstart	25 µL	325 µL
TSO_PCR primer (10 µM working stock)	0.5 µL	6.5 µL
random hexamer (10 µM working stock)	1 µL	13 µL
Total	30 µL	390 µL

*For 12 samples prepare master mix for 13 RXN, to take account of loss

Protocol

21. Take a PCR tube for the mix and add **3.5 µL/sample DNase/RNase free water**

22. Add **0.5 µL/sample TSO_PCR primer** and **1 µL/sample Random Hexamer primer** to the mix

23. Quick spin the KAPA Hifi HotStart ReadyMix

24. Add **25 µL/sample KAPA Hifi Hotstart ReadyMix** to the mix and pipet up and down to mix the Master Mix

25. Quick spin the KAPA Hifi HotStart Master Mix

KAPA Hifi Hotstart Reaction ~ DNA area

26. **Quick spin** to collect all droplets

27. Add **30 µL of the KAPA Hifi Hotstart Master Mix** to each sample

28. Gently mix the suspension by tapping the tube with your fingers and quick spin

29. Incubate in a PCR thermocycler according to the program below:

Denaturation	Denaturation	Annealing	Elongation	Final elongation
98°C	98°C	67°C	72°C	72°C
3 min.	20 sec.	15 sec.	4 min.	5 min.
	18 cycles	18 cycles	18 cycles	

Take the samples from the PCR thermocycler, quick spin to collect all droplets, and keep on ice.

Proceed immediately with

WET LAB IV - DNA clean-up

Critical step

Do **not freeze** the DNA as this results in additional fragmentation. If the samples cannot be processed immediately, the DNA can be kept overnight at 4°C.

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WET LAB IV - DNA Clean Up

Viral and bacterial DNA clean up using CleanNA magnetics beads

Timing ~ 60 min / 12 samples

Reagents

- 70% Ethanol* (400 µL / sample)
- CleanNA magnetic beads (50 µL / sample, 1:1 ratio)
- DNase/RNase free water

*It is important to prepare fresh 70% ethanol each time, since ethanol attracts water when kept too long

Materials

- DNA-Lobind 1.5 mL Eppendorf tubes (3 / sample)
- Magnetic rack
- Wide-bore tips

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated sample preparation area.

Protocol

1. **Label** three **1.5 mL Eppendorf tubes** for each sample
2. **Vortex** the CleanNA magnetic beads until all beads are suspended
3. Use **wide-bore tips** to add **50 µL CleanNA magnetic beads** to the first labeled eppendorf (1:1 ratio)
4. Add **50 µL sample** to each corresponding labeled eppendorf containing the magnetic beads
5. **Gently mix** the suspension by tapping the tube with your fingers and **quick spin**
6. **Incubate** the suspension for **5 minutes** at **room temperature**

In the meanwhile prepare the fresh 70% ethanol:

- It is important to prepare **fresh** 70% ethanol each time, since ethanol attracts water when kept too long
- Add **3.64 ml 100% ethanol** (Molecular grade) and **1.56 ml DNase/RNase- free water** in a 15 mL Falcon tube.

7. Place the sample centered into the **magnet holder**, and incubate for **2 minutes**
8. **Remove the supernatant** using a P200 pipette tip, without disturbing the beads
9. Gently **wash the beads** using **200 µL fresh 70% ethanol**, without disturbing them
10. **Remove the ethanol**, and **repeat** the wash step (9)
11. **Remove the ethanol**, and spin the sample down
12. Place sample in **magnet holder** and **remove the residual of the ethanol** with P20 pipette tip
13. **Incubate** the tubes with open lids for **MAX 5 minutes at 50°C** to evaporate all the ethanol and to dry the beads

    Incubate until a '**dry**' pellet is observed and no more ethanol is visible
14. When a 'dry' pellet is observed **add 25 µL DNase/RNase-free** water straight on the pellet (DO NOT PIPET)
15. **Tap the tube** to dissolve the magnetic beads, quick spin
16. Incubate at **room temperature for 2 minutes** to elute the DNA
17. Incubate for **2 more minutes** on the **magnetic rack**
18. **Transfer the supernatant (25 µL)** to a new labeled eppendorf tube
19. Place the tube once more on **the magnetic rack for 2 minutes**
20. Again **transfer the supernatant (20 µL)** to the 3rd labeled eppendorf tube
21. The **concentration and quality of the DNA** in the sample can be checked with an appropriate assay such as Nanodrop or Quantifluor using 2 µL of the sample

Ideally, samples are processed immediately for library preparation. They can be stored at 4°C for up to 5 days.

Keep samples on ice and proceed with

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WET LAB V - ONT

Preparation of the library and loading of the flow cell

Timing ~ 45 min

Overview

DNA fragmentation and barcoding

DNA clean-up using AMPure XP beads

Adapter Ligation and loading

DNA fragmentation and barcoding

Reagents

- SQK-RBK114.24 ONT
- Ethanol
- Bovine Serum Albumine (no recombinant!)

Materials

- DNA-Lobind 1.5 mL Eppendorf tubes (3)
- 0.2 mL thin-walled PCR tubes (per sample +1)
- Magnetic rack
- Wide-bore tips

- All surfaces should be cleaned with Hibiscrub, 70% Ethanol, and 10% Bleach in this order of cleaning. Prevent contamination.
- Aerosol-barrier tips should be used throughout the entire procedure.
- Perform all steps in the dedicated sample preparation area.

Preparations

- Pre-heat the **heat block to 50°C**

Protocol

1. Transfer **15 µl sample** in a 0.2 ml thin-walled PCR tube
2. Quick spin the **Fragmentation Mix RB01-24,** one barcode for each sample
3. Add **2.5 µl Fragmentation Mix RB01-24** to each sample (add a different barcode to each sample)
4. **Mix gently** by tapping the tube with your fingers, and spin down
5. Incubate the tube at **30°C for 2 minutes** and then at **80°C for 2 minutes** in a thermocycler

Immediately transfer to ice

DNA clean-up using AMPure XP beads

6. Quickly spin the samples
7. Pool the samples together in a 1.5 mL Eppendorf LoBind tube
8. Resuspend the **AMPure XP beads** by vortexing and quick spin
9. Use wide-bore tips to add an equal volume of resuspended **AMPure XP beads** to the pooled sample (1:1 ratio, 210 µL for 12 samples)
10. **Gently mix** the suspension by tapping the tube with your fingers and **quick spin**
11. **Incubate** the suspension for **5 minutes** at **room temperature**

    In the meanwhile prepare the fresh 80% ethanol:

    - It is important to prepare **fresh** 80% ethanol each time, since ethanol attracts water when kept too long
    - Add **800 µL 100% ethanol** (Molecular grade) and **200 µL DNase/RNase- free water** in a 1.5 mL Eppendorf tube.

12. Place the sample centered into the **magnet holder**, and incubate for **2 minutes**
13. **Remove the supernatant** using a P200 pipette tip
14. Gently **wash the beads** using **500 µL fresh 80% ethanol**, without disturbing them
15. **Remove the ethanol**, and **repeat** the wash step (14)
16. **Remove the ethanol**, and spin the sample down

17. Place the sample in **magnet holder** and **remove the residual of the ethanol** with a P20 pipette tip

18. **Incubate** the tubes with open lids for **MAX 5 minutes at 50°C** to evaporate all the ethanol and to dry the beads

    Incubate until a '**dry**' pellet is observed and no more ethanol is visible

19. When a 'dry' pellet is observed **add 13 µL Elution buffer** straight on the pellet (DO NOT PIPET)

20. **Tap the tube** to dissolve the magnetic beads, quick spin

21. Incubate at **room temperature for 2 minutes** to elute the DNA

22. Incubate for **2 more minutes** on the **magnetic rack**

23. Transfer the **13 µL of eluate** to a newly labeled Eppendorf Lobind tube

24. Place the tube once more on **the magnetic rack for 2 minutes**

25. **Transfer 11 µL of the supernatant** to a 0.2 mL thin-walled PCR tube

Library preparation - Adapter Ligation and loading

26. Quick spin the **Rapid Adapter (RA)** and the **Adapter Buffer (ADB)**

27. In a new 1.5 mL Eppendorf DNA LoBind tube, add **1.5 µL Rapid Adapter (RA)** and **3.5 µL Adapter Buffer (ADB)**, mix by tapping with fingers and quick spin

28. Add **1 µL of the RA/ADB mix** to 11 µL of barcoded DNA, mix by tapping with fingers and quick spin

29. Incubate the tube for **5 minutes at 21°C** in the thermocycler

30. Vortex and quick spin **Flow Cell Flush (FCF)** and **Flow Cell Tether (FCT)**

31. Add **30 µL Flow Cell Tether (FCT)** to the tube with **1.17 mL Flow Cell Flush (FCF)**

32. Add **5 µL Bovine Serum Albumine (BSA)** to the tube and put the tube on ice

33. Vortex **Library Beads (LIB)** and **Sequencing Buffer (SB).** Quick spin the sequencing buffer, do not spin the library beads.

34. Add **37.5 µL Sequencing Buffer (SB)** to a new tube

35. Add **25.5 µL Library Beads (LIB)** to the tube using wide bore tips

36. Add **12 µL DNA Library** to the tube

37. Open the **MinION** device lid and slide the flowcell under the clip

38. Perform a **flow cell check**.
    a. Go to the 'start' tab in the MinKNOW software
    b. Click on 'Flow cell check'
    c. Select the correct flow cell position
    d. Click on 'Start'

39. Open the device lid and **open the priming port** by sliding the priming port cover clockwise

40. Set a **P1000 pipette to 800 µl** and insert the tip into the **priming port**

41. Turn the wheel until the dial shows 820-830 µl, to **draw back 20-30 µl**, or until you can see a small volume of buffer entering the pipette tip

42. Load **800 µl of the priming mix** into the flow cell via the priming port, avoiding the introduction of air bubbles

43. Close the priming port and **wait for 5 minutes**

44. **Open the priming port** and gently **lifting the SpotON sample port** cover to make the SpotON sample port accessible

45. Load **200 µl of the priming mix** into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles

46. **Mix the library** gently by shaking it or pipetting it up and down before loading

47. Add **75 µl of sample** to the flow cell via the SpotON sample port in a dropwise fashion

48. Gently **replace the SpotON sample port** cover, making sure the bung enters the SpotON port, **close the priming port** and **close the MinION device lid**

Set up a sequence run on MinKNOW, proceed with

WET LAB VI - RUN SET-UP

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WET LAB VI - RUN SET-UP

Set-up of a sequencing run on the MinKNOW software and generation of a sequencing run file

Timing ~ 5 min

1. Open the MinKNOW software and click on '**Start sequencing**'
2. Fill in the **experiment name**
3. Click on the position of the correct flow cell
4. Copy the **experiment name** and paste it in the Sample ID
5. Click on '**Continue to kit selection**'
6. Select the used kit (Rapid Barcoding Kit 24, **SQK-RBK114.24**)
7. Click on '**Continue to run options**'
8. Click on '**Options**', change '**Run time**' to **'9' Hrs** and click on '**Apply rules**'
9. Click open '**Advanced options**', turn **off** '**Reserve pores**' and change '**Time between pore scans**' to **'1' Hours**
10. Click on '**Continue to analysis**'
11. In the section 'Basecalling', click on '**Edit options**', change '**Basecalling model**' to '**Super-accurate basecalling**' and click on '**Apply**'
12. In the section 'Barcoding', click on '**Edit options**', turn on '**Trim barcodes**' and click on '**Apply**'
13. Click on '**Continue to output**'
14. Select the correct output location (**PathoSense_Diagnostics**)
15. Click on '**Options**', change '**Qscore**' to '**1**' and click on '**Apply**'
16. Click on '**Continue to final review**'
17. Click on '**Start**'
18. While the flow cell is getting to temperature, go to cloud.pathosense.com and log in with your credentials.

19. On the Runs page, make a new run by setting the "**Size of run**" to the correct size and pressing "**+ New run**"

20. Select the run by clicking anywhere on the run and add the samples by clicking them
    Note: *only arrived samples can be added, if the samples are in "En route", set the analysis to "Arrived" on the analysis page*

21. Click the green **save icon** and select the used ONT barcode for each of the samples

22. Press "**Download**" to download the run file

23. Press the blue "**Start run**" button to move the run to "**Running**"

24. When the flowcell has reached it's temperature, the sequencing output folder should appear in the "**PathoSense_Diagnostics**" folder

25. Copy the downloaded run file and paste it in the sequencing folder, to the same directory that contains the "**fastq_pass**" and "**pod5_pass**" folders