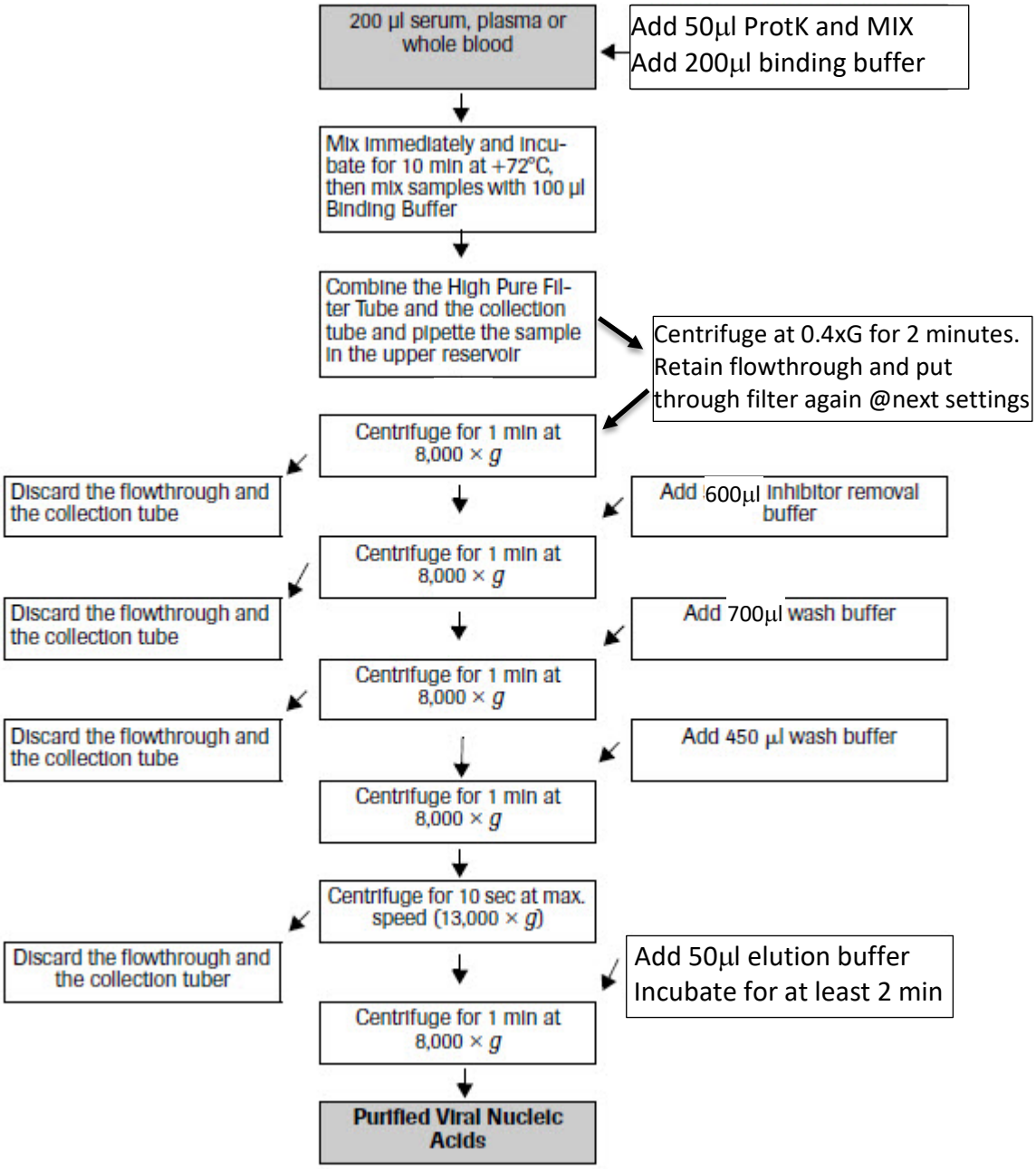
MPXV: DNA isolation and amplicon sequencing

1. High pure viral nucleic acid kit

Optional step: Centrifuge sample at 5000xg for 3 min and transfer 200 µl of supernatant



2. Long MPXV amplicon sequencing (2.5 kbp amplicons – preferred option for Nanopore sequencing)

Two Primer pools (1 and 2) per sample:

Reagents	Pool 1 (odd)	Pool 2 (even)
Water (nuclease free)	15.2	15.2
$MgCl_2$ (25nM)	0.75	0.75
Primer pool 1 (odd) (100µM)	1.06 (**1**)	-
Primer pool 2 (even) (100µM)	-	1.06 (**2**)
Q5 reaction buffer (5x)	5	5
Polymerase (Q5)	0.33	0.33
dNTPs (10nM)	0.8	0.8
DNA	3	3

PCR cycling (volume 25µl, lid at 105°C) :

98°C 45 sec
98°C 15 sec
65°C 5 min } **35x cycles**
65°C 2 min
Hold: Overnight @ ∞10°C; Same day @ ∞16°C

For each sample, combine amplified Pool 1 and Pool 2 reactions (2x25 µl) together.

SPRI clean-up

Make sure the Ampure XP beads are on room temperature and mixed thoroughly.

1. Add 22.5 µl SPRI beads (mixed well and on RT) to the 50 µl of sample (0.45:1 ratio) and mix
2. Let samples mixed with AMPure XP beads stand for 5 minutes at room temperature.
3. Place the Plate onto a magnet for 2 minutes to separate the beads from solution. Do not disturb the bead pellet. For odd numbered columns, the magnet will pull the bead pellet to the right. Therefore, sharply angle the pipette tip to the left when aspirating.
4. Leaving the purification plate on the magnet, dispense 150µL of freshly made 80% ethanol to each well, and incubate for 30 seconds.
5. Remove and discard all ethanol.
6. Leaving purification plate on magnet, dispense 150µL of freshly made 80% ethanol to each well, and incubate for 30 seconds.
7. Remove and discard all ethanol. Step 9. – 12 should be performed fast to avoid over drying of beads
8. Take a 20 µl pipette and try to remove all traces of ethanol
9. Allow the plate to air-dry on the magnet for 30 seconds. Do not allow the bead rings to dry for longer than 3 minutes.
10. Remove plate from the magnet
11. Add 35 µl of nuclease free water to each well. Resuspend with pipette or place strip caps over active wells, and vortex the plate for 5 seconds. (Ensure that each cap snaps into place.).

12. Incubate at room temperature on the counter for at least 2 minutes Then place back on the magnet for at least 2 minutes or until the solution is clear.
13. Transfer 34 µl into a new container

Quantification with Qubit (ds DNA)

Prepare the working solution per sample: 199 µl buffer and 1 µl dye (photo sensitive)

- Standards: Add 10 µl Standard 1 to 190 µl of working solution; Add 10 µl Standard 1 to 190 µl of working solution.
- Sample: Add 1 µl of sample to 199 µl working solution

Mix by vortexing and incubate for 2 min in a drawer
Measure on Qubit (It should be 3 ng/ul or higher, negative control should be below 1 ng/ul)

3. Short MPXV amplicon assay (~350 bp amplicon size) [back-up]

Two Primer pools (1 and 2) per sample:

	1x	1x
LongAmp® Hot Start Taq 2x Master Mix	13	13
Primer pool 1	5	-
Primer pool 2	-	5
H_2O	3	3
$MgCl_2$ (25nM)	1	1
DNA	3	3

PCR cycling (volume 25µl, lid at 105°C) :

95°C 2 min
94°C 30 sec } **36x cycles**
60°C 4 min }
Hold: Overnight @ ∞10°C; Same day @ ∞16°C

For each sample, combine amplified Pool 1 and Pool 2 reactions (2x25 µl) together.

SPRI clean-up

1. Add 40 µl SPRI beads (mixed well and on RT) to the 50 µl of sample **(0.8:1 ratio)** and mix
2. Let samples mixed with AMPure XP beads stand for 5 minutes at room temperature.
3. Place the Plate onto a magnet for 2 minutes to separate the beads from solution. Do not disturb the bead pellet. For odd numbered columns, the magnet will pull the bead pellet to the right. Therefore, sharply angle the pipette tip to the left when aspirating.
4. Leaving the purification plate on the magnet, dispense 150µL of freshly made 80% ethanol to each well, and incubate for 30 seconds.

5. Remove and discard all ethanol.
6. Leaving purification plate on magnet, dispense 150µL of freshly made 80% ethanol to each well, and incubate for 30 seconds.
7. Remove and discard all ethanol. Step 9. – 12 should be performed fast to avoid over drying of beads
8. Take a 20 µl pipette and try to remove all traces of ethanol
9. Allow the plate to air-dry on the magnet for 30 seconds. Do not allow the bead rings to dry for longer than 3 minutes.
10. Remove plate from the magnet
11. Add 35 µl of nuclease free water to each well. Resuspend with pipette or place strip caps over active wells, and vortex the plate for 5 seconds. (Ensure that each cap snaps into place.).
12. Incubate at room temperature on the counter for at least 2 minutes Then place back on the magnet for at least 2 minutes or until the solution is clear.
13. Transfer 34 µl into a new container

Quantification with Qubit (ds DNA)

Working solution per sample: 199 µl and 1 µl dye (photo sensitive)

- Standard: Add 10 µl Standard ½ and 190 µl of working solution
- Sample: Add 1 µl of sample to 199 µl working solution

Mix and incubate for 2 min

Measure on Qubit

4. Perform library preparation

Ligation Sequencing Kit will provide the best output.

Apply new v14 chemistry on the R.10 flowcells starting the new ONT protocols: Ligation sequencing amplicons - Native Barcoding Kit 96 V14 (SQK-NBD114.96).

For questions:

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Old library prep protocol (SQL-LSK109/110 end of life) for 2.5 kbp amplicon size

Based on old v10 chemistry/ R.9 flow cells. Use SQL-LSK 114 and R10 for upcoming runs with standard protocol for amplicons from Nanopore page!

DNA repair and end-prep

For the amplicon size (2.5 kbp) results in less ends → reagents can be halved (initially)
Start library prep with 250 ng of amplicon product. For small amplicon start with 175 ng.

Reagent	Volume
DNA	25.5 µl
Ultra II End-prep reaction buffer	3 µl
Ultra II End-prep enzyme mix	1.5 µl
Total	**30 µl**

PCR machine: 7.5 minutes 20°C, 7.5 minutes 65°C

SPRI bead clean-up

Add 30 µl beads to the 30 µl of sample (1:1 ratio) and mix well

5 min incubation

Follow steps 3 – 13 from SPRI cleanup on page 3 but elute in 12 µl water

Native barcode ligation

Reagent	Volume
End-prepped DNA	11 µl
Native Barcode	1.75 µl
Blunt/TA Ligase Master Mix	12.5 µl
Total	**25 µl**

Mix by pipetting and incubate 15 minutes RT. Slow pipetting of Master Mix - highly viscous

SPRI bead clean-up

Add 20 µl beads and mix (0.8:1 ratio)
Follow steps 3 – 13 from SPRI cleanup on page 3 but elute in 12 µl water

Qubit

Measure and calculate for a 250 ng pool (e.g. 12,5ng per sample for 20 samples).

Adapter ligation and clean-up

Thaw one tube of Short Fragment Buffer (SFB) and elution buffer (EB)

Reagent	Volume
250 ng pooled barcoded sample DNA	48.5 µl
NEBNext Quick Ligation Reaction Buffer (5x)	15 µl
Adapter Mix II (AMII)	4 µl
Quick T4 DNA Ligase	7.5 µl
Total	**75 µl**

Mix by flicking the tube and incubate 15min @ RT.

Bead clean-up (<u>NO ETHANOL</u>)

Add 60 µl beads to the 75 µl of sample (0.8:1 ratio)

5 min incubation

Resuspend beads in **100 µl** Long fragment buffer (**LFB**)

Put back on magnet (2 min) + Remove supernatant

Resuspend with 100 µl short fragment buffer (SFB)

Put back on magnet (2 min) + Remove supernatant

Remove rest liquid (Beads shouldn't dry out)

Elute in 14 µl **ELUTION BUFFER** with 10 minutes incubation time

Load library (roughly 125 ng @ 100 fmol for 2.5 kbp amplicons)

5. MinION preparation

Remove a flowcell from the fridge
Store the reagents on ice.

*<u>Flow cell priming mix was prepared (take to the 5th floor):</u>

	One reaction (µl)
FLT	30
1 tube FB	
Total	

Note: Please keep remnants of FLT+FLB mix and store in at 4`C (reuse of old flowcells)

* <u>The library was prepared for loading **[NEW flowcell]:**</u>

	One reaction (µl) (>26h runtime)
SQB	37.5
LB (mix before adding)	25.5
DNA library (~75 ng)	12
Total	**75**

	A	B
1	**Component**	**Volume**
2	SQB	37.5 µL
3	LB	25.5 µL
4	Library	12 µL
5	**Total**	**75 µL**

*<u>The library was loaded on to the flow cell:</u>

- Place the flow cell on the grid ion
- Flip back the MinIon lid and slide the priming port cover clockwise so that the priming port is visible
- Draw back a small volume to remove any bubbles
  1) Set a P1000 pipette to 200 µl
  2) Insert the tip into the priming port
  3) Turn the wheel until the dial shows 220-230 µl (to get some volume out of flow cell)
- Load 800 µl of the **priming mix** into the flow cell, avoid air bubbles
- Wait 5 min.

- Gently lift the SpotOn sample port cover to make the SpotOn sample port accessible
- Load 200 µl of the priming mix into the flow cell via the **priming port**
- Mix the prepared library gently by pipetting up and down.
- Add 59.4 µl of sample to the flow cell via the **SpotOn port (drop wise).**
- Close the ports
- Start the run:
    - name run: Viro_Run_nr (check database for nr)
    - Select 22h run time (run options)
    - Select the kit used (LSK109/LSK110)
    - Run length: 22h
    - Minimal reads length: 200bp
    - **Barcode on both sides**
    - Basecalling: on (high accuracy)
    - Alignment: off
    - FAST5: off **[Or on and move on a hard drive? → Nanopolish]**
    - FASTQ: om (GZip, 32.000 reads per file)

For OLD/Washed flow cells (Low cost)

Add 600 µl LB and 15 µl FLT or use leftover prepared LB mix

	One reaction (µl) **OLD FLOWCELL**
SQB	22.75
LB (mix before adding)	12.75
DNA library (~75 ng)	4
LB + FLT mix	10.5
Total	**50**

Open port
Remove air
Add 350ul LB mix
Open Spot on and add 50ul LB mix
Close both ports and remove storage buffer wasteport 1
Incubate 5 min
Open ports
Add 200 LB
Close and remove storage buffer
Open ports and add library