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AAU-nCoV-2019_Tailed_Long_Amplicon_Sequncing V.2

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

This protocol describes the preparation of long read amplicon libraries of nCoV-2019 for sequencing with the Oxford Nanopore MinION platform. The protocol is based on samples of rRNA extracts of the nCoV-2019 virus.

Sequences for primers can be found under guidelines, currently using V3.1 from CJ020.

The protocol is based on the ARTIC Networks nCoV-2019 sequencing protocol and modified to our lab at Aalborg Univeristy. https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w

Guidelines

Primers - Spreadsheet.xlsx

artic_primer_pools_long-tailed_CJ0...

Section 1: Preparations

1 **Create journal**

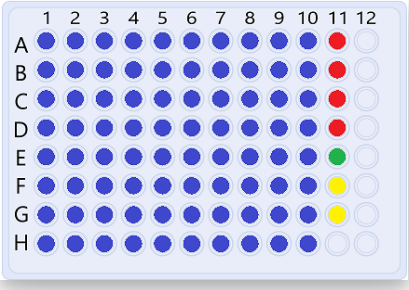
Create a journal with associated unique journal number (CJXXX) inculding metadata for the samples to be processed.
Metadata must include reference to a sample registration journal and a sample overview of the samples to be processed. For reference see SOP for setting up journals.

Sample preparations

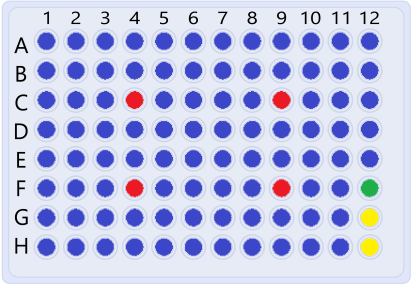
The protocl describes the workflow as pr 96-well plate basis. A full plate must include 4 negative controls (NFW), a positive control and two empty well for Qubit DNA concentration measurement. Depending on the sample organiziation of the received samples these control samples may be included in varying empty well IDs to minimize any risk of potential mix-ups. (e.g. for a plate with 80 samples, keep the original organiziation and include controls in empty wells).

For full sample plates, 7 samples should be skipped to be processed later, preferably in positions C4, F4, C9, F9, F12, G12 and H12 to include the control samples and space for Qubit standards.

- C4, F4, C9, F9: Negative controls of sterile nuclease free water
- F12: Positve control RNA sample
- G12 and H12: Empty for Qubit measurement



Example of plate with empty wells
Red: Negative
Green: Positive
Blue: Sample
Yellow: Empty for Qubit measurement



Example of full plate
Red: Negative
Green: Positive
Blue: Sample
Yellow: Empty for Qubit measurement

Section 2: cDNA preparation

2 **Cleaning pre-PCR laminar flow cabinet**

Should always be cleaned before and after ues as follows:

- Wipe down all items in the cabinet; pipettes, tip boxes, trash bin rack, etc. with 70 % ethanol and allow to dry.
- Wipe down all items in the cabinet; pipettes, tip boxes, trash bin rack, etc. with RNAse away

Consumables for hexamer annealing and cDNA synthesis

Place the following items and reagents in the LAF-bench for UV-treatment (pr plate basis)

- 1 pcs. of skirted PCR-plate - labeled CJXXX_cDNA_pX (journal number and plate number)
- 12 pcs. cap-strips (wipe down plastic bag/container)
- 1 pcs. yellow ice-block (for reagents)
- 1 pcs 96-plate ice-block
- 1 pcs. 8-strip PCR-tubes (for cDNA mastermix aliquots)
- 1 pcs. PCR-strip rack
- 1 pcs of 1.5 mL Eppendorf tube (for cDNA mastermix mixing)
- 1 pcs. reservoir (for mixing of hexamer mastermix)
- Sufficient amount of each reagent depending on the amount of samples to be processed in the plate (should be calculated in template excel sheets and added to the journal)
- Ensure sufficient pipette tips are present (wipe new boxes down)

Expose the hood to UV light for 15 minutes.

UV sensitive tubes should be wiped down when entering and leaving the cabinet. Gloves should be changed each time entering and leaving the laminar flow cabinet. Production reagents must NEVER be opened outside the laminar flow cabinet.

Sheet for calculating **Annealing Mastermix**

Annealing Mastermix.xlsx

Sheet for calculating **cDNA synthesis Mastermix**

cDNA Synthesis Mastermix.xlsx

3 **Mastermix for annealing of random hexamers to RNA template**

Mastermix should be made up in the pre-PCR laminar flow cabinet in the resevoir and aliquoted into the skirted 96 well plate.

Mix the following components in a reservoir and alliqout to the 0.2 mL 96 well plate stored in the 96-well ice-block:

	Component	Volume pr sample	96-sample mastermix
	Nuclease-free water	6 µL	619.2 µL
	50 µM random hexamers	1 µL	103.2 µL
	10 mM dNTPs mix (10 mM)	1 µL	103.2 µL
	Total	8 µL	825.6 µL

For mastermix of multiple reations an overhead of ~7.5 % is added to ensure sufficient material for all samples.

Seal the plates with strip-caps and note the specific batch number of applied reagents in your journal.

4 **Template addition and controls**

Clean the pre-PCR area and transfer your mastermix plates and RNA samples plates to this area.
The pre-PCR area should be cleaned before and after use as follows:

- Wipe down bench, pipettes, racks, vortex, tubes, etc. in 70 % ethanol and allow to dry
- Wipe down bench, pipettes, racks, vortex, tubes, etc. with RNAse away

Spin down all template samples to avoid splashing of areosls when handling. Keep the RNA sample plate and cDNA synthesis mastermix plate on ice.

Transfer **5 µL template RNA** from the sample plate to the **mastermix plate containing the annealing mastermix** for a total volume of 13 µL in each well. Gently mix by pipetting, seal the plate and spin down to collect liquid at the bottom of the wells. For the negative controls transfer 5 µL of nuclease free water and for the positive control transfer 5 µL of EXT-CJ001-4A.

5 **Annealing of hexamers**

Incubate the plate in PCR-machine with the following program (/protocols/COVID-19/Corona - Random hexamers annealing.js):

	Temperature	Time
	65 °C	5 min

Should be move to ice as the incubation finishes. A 4 °C hold has been added to the PCR program to ensure cooling as security.

After incubation move samples to ice and leave for at least 1 minute.
Random hexamers have now annealed to the template RNA

6 **Master mix for cDNA synthesis**

In the pre-PCR/master mix cabinet prepare mastermix for cDNA synthesis in a Eppendorf tube as follows and alliqout to an 8-PCR-tube strip 0.2 mL:

	Component	Volume pr sample	96-samples
	SSIV Buffer	4 µL	422.4 µl
	100 mM DTT	1 µL	105.6 µL
	RNaseOUT RNase Inhibitor	1 µL	105.6 µL
	SSIV Reverse Transcriptase	1 µL	105.6 µL
	Total	7 µL	739.2 µL

For mastermix of multiple reations an overhead of ~10 % is added to ensure sufficient material for all samples.

Seal the tubes, place on ice and note the specific batch number of applied reagents in your journal

7 Transfer the RNA samples and cDNA synthesis mastermix to the pre-PCR area.
For each sample add 7 µL cDNA synthesis mastermix to the plate containing template RNA with hexamers for a total volume of 20 µL in each well.

Gently mix by pipetting, seal the plate and spin down to collect liquid at the bottom of the wells.

8 **cDNA synthesis**
Transfer the plate to the PCR-machine and Incubate the reaction as follows (/protocols/COVID-19/Corona - cDNA synthesis.js):

	Temperature	Time
	42 °C	50 min
	70 °C	10 min
	5 °C	-

9 **cDNA storage**
If proceeding with the protocol the same day, temporay store the cDNA plate on ice otherwise transfer to storage at -18 °C. The plate must be labeled cDNA_CJXXX_pX if not done already.

Section 3: Primer pool preparation

10 **Primer scheme**
To effectivly amplify the entire virus genome two different primer pools with overlapping ends are needed. The primer pools used in this protocol is based on the primer sequences of the artic network version 3 (https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V3) with a custom scheme generating ~1200 bp amplicons with tails for easy barcoding.

The primer scheme and sequences can be found under guidelines or in journals for preparing primer stocks. Pay attention to which version of the primer pool is used. For production use the newest validated version, currently V3.1.

11 **Primer pool 1 and 2 (if out of stock solution)**

All work with stock primers must be done in the laminar flow cabinet.
Primer stocks of lyophilised primers are resuspended at a concentration of 100 µM each.

Primer pool stocks are generated by adding 10 µL of each primer to a 1.5 mL Epppendorf tube labelled either:
- COVID-19 long-tailed primer pool1 V3.X, 100 µM 2020-XX-XX CJXXX
- COVID-19 long-tailed primer pool2 V3.X, 100 µM 2020-XX-XX CJXXX

See guidelines/journals for preparing primer pool stocks for which primers should be included in the different pools/versions. It is recommend that multiple aliquots of each primer pool are made in case of degradation or contamination. OBS all new primer pools must be undergo QC before being applied in production.

12 **Working primer solution (if out of working solution)**
Stock primer pools needs to be diluted 1:10 before use. This can be done just before preparing the PCR mastermix (step 14) if no more working solution is available. Dilution should be 1:10 in sterile UV treated TE buffer or NFW, to generate 10 µM primer pool working solutions.

Section 4: PCR1 amplification

13 **PCR1 preparations**
Clean pre-PCR/mastermix cabinet as in step 2

Consumables for PCR1
Place the following items and reagents in the LAF-bench for UV-treatment (pr plate basis)
- 2 pcs. of tear-away PCR-plates - labeled CJXXX_pX_poolX (journal, plate and pool number)
- 24 pcs. cap-strips (wipe down plastic bag/container)
- 1 pcs. yellow ice-block (for reagents)
- 2 pcs 96-plate ice-block
- 2 pcs of 1.5 mL Eppendorf tube (for primer-pool dilution)
- 2 pcs. reservoir (for mixing of the two mastermix, one for each primer pool)
- Sufficient amount of each reagent depending on the amount of samples to be processed in the plate (should be calculated in template excel sheets and added to the journal)
- Ensure sufficient pipette tips are present (wipe new boxes down)

Expose the hood to UV light for 15 minutes.

UV sensitive tubes should be wiped down when entering and leaving the cabinet. Gloves should be changed each time entering and leaving the laminar flow cabinet. Production

reagents must NEVER be opened outside the laminar flow cabinet.

Sheet for calculating **Mastermix - PCR1 amplification**

Mastermix - PCR1 amplification.xlsx

14 **Mastermix for targeting PCR**

In the pre-PCR/master mix cabinet prepare **2 mastermix** for targeting PCR as follows and alliqout to an 0.2 mL 96 well plate (tear-away) (one mastermix for each primer pool):

	Component	Pr sample	96 samples
	Nuclease free water	13.15 µL	1357.08 µL
	5X Super Fi™ Buffer	5 µL	516 µL
	10 mM dNTP mix	0.5 µL	51.6 µL
	Long-tailed primer pool (1 or 2) 10 µM	3.6 µL	371.52 µL
	Platinum™ Super Fi™ DNA Polymerase (2 U/µL)	0.25 µL	25.8 µL
	Total	**22.5 µL**	**2322 µL**

For mastermix of multiple reations an overhead of ~7.5 % is added to ensure sufficient material for all samples.

Seal the plates with strip-caps and note the specific batch number of applied reagents in your journal

Spin down to collect liquid at the bottom of the wells

15 **Template addition**

Clean cDNA area as follows:

- Wipe down bench, pipettes, racks, vortex, tubes, etc. in 70 % ethanol and allow to dry
- Wipe down bench, pipettes, racks, vortex, tubes, etc. with RNAse away

Transfer the two 96 well plates containing the mastermix for the PCR1 reaction to the cDNA work area.
Spin down the plate with cDNA samples to avoid splashing of areosls when handling.

For each sample, transfer 2.5 µL cDNA sample to each well in the mastermix plate, for a final volume of 25 µL. Do so in a one column fashing to minimze risk of cross contamination:

- Un-seal one column of the mastermix plate and sample plate
- Transfer the 2.5 µL sample using a multi-pipette
- Re-seal the column of the mastermix and sample plate

Each sample should be present in both pools with matching well IDs.
Re-seal the cDNA plate and move to storage at -18 °C.

Briefly vortex the PCR sample plates and spin down to collect liquid at the bottom of the wells.

16 **PCR1 amplification conditions**

Set-up the following program on two PCR machines (one for each pool), for PCR1 amplification (/protocols/COVID-19/Corona - PCR1 amplficiation.js):

	Step	Temperature	Time	Cycles
	Heat Activation	98 °C	30 sec	single step
	Denaturation	98 °C	15 sec	5
	Annealing/ Extension	65 °C	5 min	5
	Hold	4 °C	indefinite	single step

Sufficient tailed target amplicons are now generated for later amplification and barcoding PCR

Section 5: PCR1 clean-up

17 **Combine pool 1 and 2**

Clean the barcoding area as follows:

- Wipe down bench, pipettes, racks, vortex, tubes, etc. in 70 % ethanol and allow to dry
- Wipe down bench, pipettes, racks, vortex, tubes, etc. with RNAse away

At the barcoding area combine the contents of the PCR1 products from Pool 1 and Pool 2 for each biological sample in one of the two 96 well plates.

18 **Clean-up**

Clean up amplicons using cleanNGS SPRI beads based on the following protocol:

Step by step:

- Homogenize SRPI bead solution by vortexing (CleanNGS, Netherlands)
- Un-seal one column of samples
- Add 50 µL bead SPRI solution to each sample for a ratio of 1:1
- Mix by carefully pipetting up and down
- Repeat the three previous steps untill beads have been added to all samples
- Seal the plate with foil and briefly spin down to collect liqued at the bottom of the tube (avoid pelleting of beads)
- Incubate at room temperature for 00:03:00
- Place tube on a magnetic rack and wait until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)
- Unseal and discard foil
- Carefully discard the supernatant (keep tube on the magnetic rack)
- Wash beads by adding 190 µL fresh Ethanol (80 % v/v) along the opposite side of the beads
- Wait 00:00:30 and discard the ethanol
- Repeat the two previous washing steps
- Seal the plate by applying foil
- Spin down plate, place it back on the magnetic rack, unseal and to remove residual ethanol
- Let beads air dry for no more than 00:00:30 and remove tube from the magnetic rack
- Resuspend the purified DNA by adding 18 µL of nuclease free water.
- Re-seal plate with new sterile strip caps
- Vortex the plate for mixing and lighly spin down the plate (aviod pelleting of beads)
- Incubate at room temperature for 00:02:00

- Move plate to the magnetic rack until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)

Section 6: PCR2 Barcoding and amplification

19 **PCR2 preparations**

Clean pre-PCR/mastermix cabinet as in step 2

Consumables for PCR2

Place the following items and reagents in the LAF-bench for UV-treatment (pr plate basis)

- 1 pcs. of tear-away PCR-plates - labeled CJXXX_pX_poolX (journal, plate and pool number)
- 12 pcs. cap-strips (wipe down plastic bag/container)
- 1 pcs. yellow ice-block (for reagents)
- 1 pcs. 96-plate ice-block
- 1 set of IDT/ILM DUAL-INDEX plate (primer with unique barcode)
- 1 pcs. reservoir (for mixing of the of the mastermix)
- Sufficient amount of each reagent depending on the amount of samples to be processed in the plate (should be calculated in template excel sheets and added to the journal)
- Ensure sufficient pipette tips are present (wipe new boxes down)

Expose the hood to UV light for 15 minutes.

UV sensitive tubes should be wiped down when entering and leaving the cabinet. Gloves should be changed each time entering and leaving the laminar flow cabinet. Production reagents must NEVER be opened outside the laminar flow cabinet.

Sheet for calculating **Mastermix - PCR2 Barcoding and amplification**

Mastermix - PCR2 barcoding and a...

20 **Mastermix for multiplex PCR2**

In the pre-PCR/master mix cabinet prepare mastermix for barcoding PCR as follows and alliqout 8 µL to each 0.2 mL well in the 96 well plate (tear-away):

	Component	Pr sample	96 samples
	Nuclease	2.25 µL	232.2 µL

	free water		
	5X Super Fi™ Buffer	5 µL	516 µL
	10 mM dNTP mix	0.5 µL	51.6 µL
	ILM/IDT primer mix (10 µM)	2	-
	Platinum™ Super Fi™ DNA Polymerase (2 U/µL)	0.25 µL	25.8 µL
	Total	**10 µL**	**825.6 µL**

For mastermix of multiple reations an overhead of ~7.5 % is added to ensure sufficient material for all samples.

From the barcode set (plate containing the ILM/IDT primer mix) transfer 2 µL of each seperate barcode primer to each seperate position in the mastermix plate, for a total volume of 10 µL.

Seal the plates with strip-caps and note the specific batch number of applied reagents in your journal.
Spin down to collect liquid at the bottom of the wells.
Note the specific barcodes for each position of the mastermix template together with the associated sample ID in the journal.

21 Transfer the 96 well plate containing the mastermix for the barcoding PCR2 to the barcoding work area containing
the magnetic rack with the cleaned product of PCR1.

For each sample transfer 15 µL without disturbing the beads to the barcode mastermix plate for a total volume of 25 µL. Do so in a one column fashion to minimze risk of cross

contamination:
- Un-seal one column of the mastermix plate and sample plate
- Transfer the 15 µL sample
- Re-seal the column of the mastermix and sample plate

Vortex the sealed plate to mix and spin down the plate to collect liquid at the bottom of the wells.

22 **PCR2 conditions**
Set-up the following program on the PCR machine, for barcoding PCR (/protocols/COVID-19/Corona - PCR2 Barcoding Long-fragment):

	Step	Temperature	Time	Cycles
	Heat Activation	95 °C	30 sec	single step
	Denaturation	95 °C	15 sec	25
	Anneaing	65 °C	15 sec	25
	Extension	72 °C	1 min	25
	Final extension	72 °C	1 min	single step
	Hold	4 °C	indefinite	single step

The samples are now amplified and barcoded.

Section 7: PCR2 clean-up

23 **Preparations for clean-up**
Clean the barcoding area as follows:
- Wipe down bench, pipettes, racks, vortex, tubes, etc. in 70 % ethanol and allow to dry
- Wipe down bench, pipettes, racks, vortex, tubes, etc. with RNAse away

At the barcoding area clean up the barcoded amplicons using cleanNGS SPRI beads based on the following protocol:

Step by step:
- Homogenize SRPI bead solution by vortexing (CleanNGS, Netherlands)

- Un-seal one column of samples
- Add 17.5 µL bead SPRI solution to each sample for a ratio of 0.7:1
- Mix by carefully pipetting up and down
- Repeat the three previous steps untill beads have been added to all samples
- Seal the plate with foil and briefly spin down to collect liqued at the bottom of the tube (avoid pelleting of beads)
- Incubate at room temperature for 00:03:00
- Place tube on a magnetic rack and wait until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)
- Unseal and discard foil
- Carefully discard the supernatant (keep tube on the magnetic rack)
- Wash beads by adding 190 µL fresh Ethanol (80 % v/v) along the opposite side of the beads
- Wait 00:00:30 and discard the ethanol
- Repeat the two previous washing steps
- Seal the plate by applying foil
- Spin down plate, place it back on the magnetic rack, unseal and to remove residual ethanol
- Let beads air dry for approximately 00:00:30 and remove tube from the magnetic rack
- Resuspend the purified DNA by adding 20 µL of nuclease free water.
- Re-seal plate with new sterile strip caps
- Vortex the plate for mixing and lighly spin down the plate
- Incubate at room temperature for 00:02:00
- Move plate to the magnetic rack until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)

Section 8: Quantification, integrity and normalization

24 **DNA quantification**
Measure the DNA sample concentration using the TECAN instrument with the Qubit 1X dsDNA HS assay kit (Thermo Fisher Scientific) using 1 µL sample.

25 In a 96 well microtiter plate for the TECAN instrument prepare standard 1 and standard 2 by mixing:

Standard 1, in well G12 (for full plates) add the following:

190 µL	Qubit™ 1X dsDNA HS Working Solution (Component A)
10 µL	Qubit™ 1X dsDNA HS Standard #1 (Component B)

Standard 2, in well H12 (for full plates) add the following:

190 µL	Qubit™ 1X dsDNA HS Working Solution (Component A)
10 µL	Qubit™ 1X dsDNA HS Standard #2 (Component C)

26 For each sample mix the following in each of the remaining wells:

Sample:

199 µL	Qubit™ 1X dsDNA HS Working Solution (Component A)
1 µL	Sample

27 **Measuring**

Seal the plate with foil and mix by vigorously vortexing for 3-5 seconds followed by a quick spin down to collect the liquid at the bottom of the wells. Allow to incubate at room temperature for 00:02:00.

Carefully remove foil from the plate avoiding splashing.
On the TECAN instrument press the display button to open the instrument and add the plate.
On the computer open the controller software select the DNA concentration measurement script.
Mark which wells to measure and assign standards (G12 and H12 for full plates).
Press start.

To calculate the concentrations from the measured RFU values make a standard curve from the two standards.
Save the excel file in the format CJXXX_YYYY-MM-DD_PCR2 in the destination /Desktop/EB/Covid19

Add concentrations to the matching sample and barcode IDs in the journal metadata sheet

28 **DNA integrity**

Measure the DNA integrity by gel electrophoresis on a Agilent 2200 Tapestation using D5000 screentapes (Agilent Technologies).
The tape should always include the positive control and two negative control samples as awell as an ladder.
Select up to 10 samples with DNA concentrations above 1.0 ng/µL for measurement. For low concentration samples the D5000 HS assay can be used to evaluate integrity.

29 Prepare the ladder in the first position of the tube-strip by mixing:

10 µL	D5000 Sample Buffer
1 µL	D5000 Ladder

For each sample mix:

10 µL	D5000 Sample Buffer
1 µL	Sample

30 Vortex at 2000 rpm for 00:01:00 and spin down the tubes to collect the centents at the bottom of the tube.

31 **Measuring**
Insert the sample into the 2200 TapeStation Instrument.
Ensure the tip-array is full with none in the trash.
Select the required sample postions on the 2200 Tapestation Controller Software.
Click **Start** and save in the format CJXXX_YYYY-MM-DD_PCR2

Section 10: Normalization

32 **Normalization**
0.7 ng/µL is substracted from each sample concentration before normalization to account for dimer product.

Every sample's DNA concentration is normalized to 1 ng/uL in a dilution plate by transfering 5 µL sample to an adjustable amount of nuclease free water. Diltution plates should be made with using nuclease free water with an volume calculated based on the concentratons from the metadata sheet. Skip dilution for samples with lower concentration than 1 ng/µL.

33 **Pooling for sequncing**
For each normalized sample transfer and pool 3 µL (3 ng of DNA). For the negative controls and possibly other sample with lower concentrations transfer and pool up to 10 µL for a maximum of 3 ng of DNA.

Transfer the remaining undiluted DNA sample volumes to a skirted storage plate. Label this plate CJXXX_PCR, seal with foil and transfer to storage at -18.

Concentrate the pool with a 0.7:1 SRPI bead clean-up and elute in 50 µL:

Step by step:
- Homogenize SRPI bead solution by vortexing (CleanNGS, Netherlands)
- Add X µL bead SPRI solution to the pooled sample for a ratio of 0.7:1
- Mix by flicking the tube
- Briefly spin down to collect liqued at the bottom of the tube (avoid pelleting of beads)
- Incubate at room temperature for 00:03:00
- Place tube on a magnetic rack and wait until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)
- Carefully discard the supernatant (keep tube on the magnetic rack)
- Wash beads by adding 400 µL fresh Ethanol (80 % v/v) along the opposite side of the beads

- Wait 00:00:30 and discard the ethanol
- Repeat the two previous washing steps
- Spin down tube, place it back on the magnetic rack, and remove residual ethanol
- Let beads air dry for approximately 00:00:30 and remove tube from the magnetic rack
- Resuspend the purified DNA by adding 50 µL of nuclease free water
- Vortex the tube for mixing and lighly spin down the tube
- Incubate at room temperature for 00:02:00
- Move tube to the magnetic rack until beads have settled on the side of tube and the supernatant is completely clear (~00:00:20)

34 **Library preparation**

The pooled samples are prepared for MinION sequencing following the LSK-SQK 109 amplicon by ligation protocol from Nanopore using 48 µL pooled library skipping CS DNA. Keep 1 µL for integrity QC of the sample prefore and after library preparation.

amplicon-sqk-lsk109-ACDE_9064_...