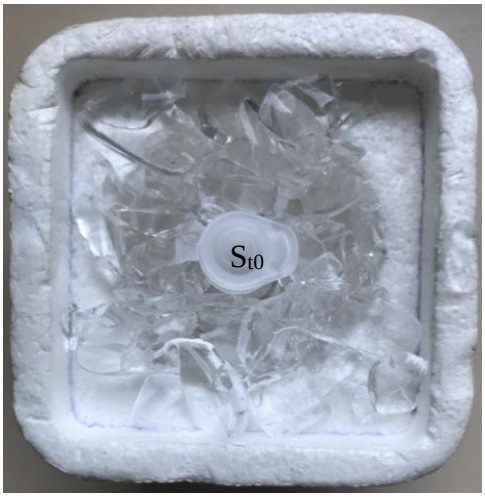
Protocol CRISPR + Nanopore Sequencing

1. gRNA-Synthesis

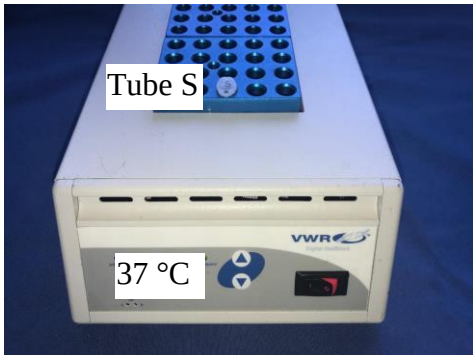
1.1 Pipet following reagents your empty tube S for gRNA-synthesis.

Tube	Inhalt	Vol.
H_2O	Nuclease free H_2O	16 µL
NTP	NTP buffer mix	10 µL
D	DNA Duplex	2 µL
T7	T7 RNA Polymerase	2 µL
Total Volume in tube S		***30 µL***

1.2 As the blanc control for gRNA-synthesis pipet 5 µL out of your tube S in a new tube marked with S_{t0} and your group number and put it on ice.

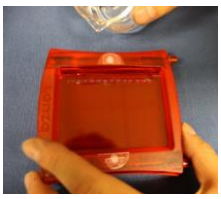


1.3 Incubate your tube S at 37 °C for 1 hour.



2. Check of produced gRNA by Gel-Electrophoresis

2.1 Open gel cassette and moisten the wells with H_2O_{dest}.



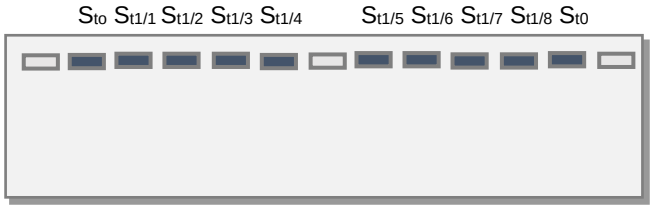
2.2 Aspirate excess water by using a kitchen roll.

2.3 Insert the flash gel cassette in the flash gel dock.

2.4 Pipet 5 µL out of your gRNA-synthesis tube (S) in a new tube marked with S_{t1} and your group number.

2.5 Add into both tubes S_{t0} and S_{t1} 1 µL of loading buffer (LB).

2.6 Pipet according to the following scheme 6 µL of gRNA-synthesis tubes $S_{t1/1}$ – $S_{t1/8}$ and 2 groups S_{to} in the wells of the gel:

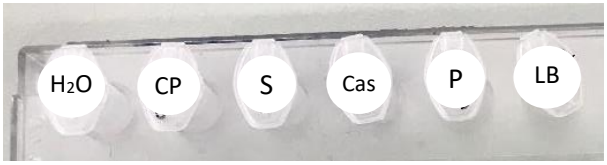


2.7 Connect flash gel dock to power supply and start electrophoresis at 180 V.

2.8 Watch progress of electrophoresis by switched on UV lamp of the dock.

Task: Evaluate your gel regarding gRNA-synthesis.

3. Cleavage of Plasmid pBR322 by Nuclease Cas9



3.1 Pipet samples 1 – 3 according to following table:

Sample	**1**	**2**	**3**
	pBR322 supercoiled	pBR322+ supercoiled Cas9 (- gRNA)	pBR322 supercoiled + Cas9 + gRNA
H_2O (Nuclease free)	25 µL	24 µL	23 µL
CP (Buffer)	3 µL	3 µL	3 µL
S (synthesized gRNA)	0 µL	0 µL	1 µL
Cas9 Nuclease	0 µL	1 µL	1 µL
Mix by tapping the tubes and incubate for 10 minutes at room temperature			
P (pBR322 supercoiled 0,5 µg/µL)	2 µL	2 µL	2 µL
Mix by tapping the tubes and incubate for 15 minutes at 37 °C			
Total Sample Vol.	**30 µL**	**30 µL**	**30 µL**

4. Proof of Cas9 Cleavage in Plasmid ($pBR322_{lin}$) by Gel Electrophoresis

4.1 Pipet 8.5 µL out of your samples 1 – 3 in 3 new tubes and mark the tubes again with 1, 2 and 3.

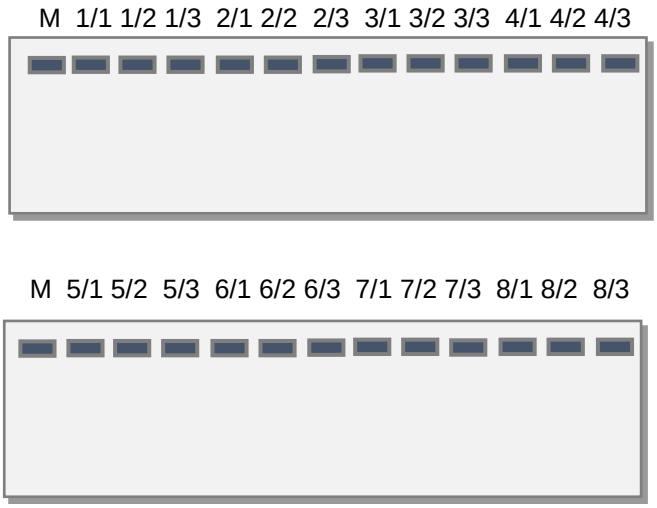
4.2 Add to each of the 3 new tubes 1.5 µL of loading buffer (LB) and mix by snipping.

4.3 Open your gel cassette and moisten the wells with H_2O_{dist}.

4.4 Aspirate excess water by using a kitchen roll.

4.5 Insert the flash gel cassette in the flash gel dock.

4.6 Pipet according to the following pipetting scheme in each well of your gel cassette
- 4 µL marker (M),
- 10 µL of your samples 1 - 3



4.7 Connect flash gel dock to the power supply and start gel electrophoresis at 180 V.

4.8 Watch progress of electrophoresis by switched on UV lamp of the dock.

Task: Evaluate your gel regarding nuclease Cas9 cleavage.

5. Preparation of DNA-Library

Reagents in the Rapid Barcoding Kit RBK004:

Name	Shortcut	Cap colour	Tubes	Vol. (µL)
Fragmentation Mix	RB01-RB12	colourless	12	20
Rapid Adapter	RAP	green	1	10
Loading Beads	LB	pink	1	360
Sequencing Buffer	SQB	red	1	300

5.1 Pipet **7,5 µL of CRISPR/Cas9 cutted plasmid-DNA from test approach 3** in a 1,5 mL tube marked with B (B stands for barcoding).

5.2 Add in tube B **2,5 µL out of one of 12 fragmentation mixes** (RB01-12) and mix by pipetting up and down.

5.3 For barcoding incubate your tube B for 1 minute at 30 °C which is the optimal temperature of the enzyme transposase.

5.4 For denaturation of the transposase incubate tube B at 80 °C for 1 minute.

5.5 Cool down tube B for 3 minutes on ice.

5.6 **Pool all 10 µL volumes of all work groups in a 1,5 mL tube marked with P** (P stands for Pool)**.**

Only 1 group is going on working:

5.7 Pipet **10 µL out of tube (P) in a new 1,5 mL tube L** (L stands for Library)**.**

5.8 Add **1 µL rapid adapter (RAP) to 10 µL of barcodet DNA in tube L** and mix it by pipetting up and down (Vol. = 11 µL).

5.9 Incubate tube L for 5 minutes at 20 °C and measure DNA-concentration by fluorometer.

6. Concentration Measurement of DNA-Library (L)

6.1 In a 0,5 mL tube pipet **200 µL Quanti-Fluor®ONE dsDNA Dye and 1 µL of DNA-Library (L)**.

6.2 After an incubation of 5 minutes in darkness DNA concentration in 0,5 mL tube will be measured in the fluorometer.

Select fluorometer set-up:
- Sample Vol. 200 µL
- Units ng/µL

Measured DNA-concentration should be between 10 – 400 ng/µL.

7. Finalizing of DNA-Library (L) for Loading in the Flongle Flow Cell

7.1 Homogenize loading beads (LB) in Rapid Barcoding Kit RBK004 by vortexing.

7.2 Pipet following reagents in a 1,5 mL tube, again marked with L (for DNA-Library) and mix it by pipetting up and down:

Reagents	**Tube cap**	**Volume**
Sequencing buffer	red	15 µL
Loading Beats	pink	10 µL
DNA-Library	L	5 µL
Total Vol.	**L**	**30 µL**

MinKNOW should already run, Flongle Flow Cell check and priming should be done.

7.3 Move off the sticker of the Flongle Flow Cell and **pipet to avoid air bubbles inside the primed array only 25 µL out of 30 µL library L** in the loading port of the Flongle Flow Cell.

7.4 Enter sequencing set-up in MinKNOW and start sequencing.

Preparation of Flongle Flow Cell and Flow Cell Check

1. **Insert the Flongle Adapter in the MinION (Wear gloves to avoid contaminations!)**
   Open MinION and take out the configuration test cell (CTC).

		Take CTC protec-tion out of the Flongle adapter. Push the Flongle adapter under the stainless steel clip till it snaps in place.

Click on the picture to see a movie about the procedure. →

Alternative: Open following link in your browser
https://www.youtube.com/embed/Wnx59DhrUe8?feature=oembed

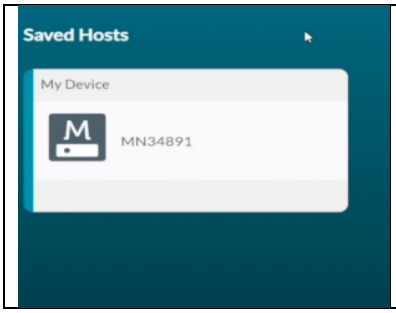
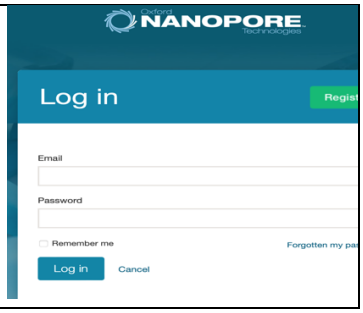
2. **Connect MinION with the PC**

	Connect PC with MinION by USB cable. → MinION gets access to electricity and LED is shining red.

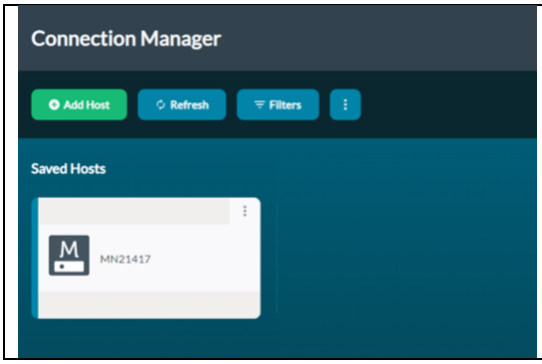
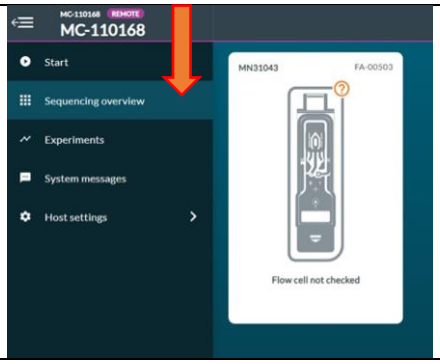
3. **Insert Flongle Flow Cell in the Flow Cell Adapter**

		Push the Flongle Flow Cell in arrow direction under the stainless steel clip of the MinION and press it down on the other side. → with a click Flongle Flow Cell snapps in.

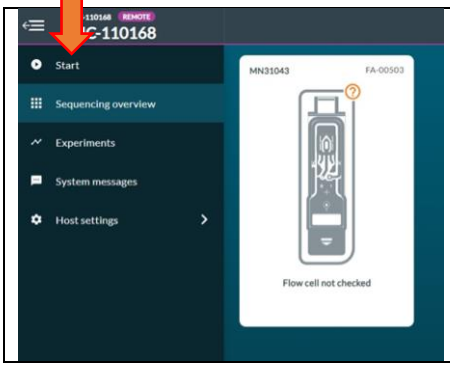
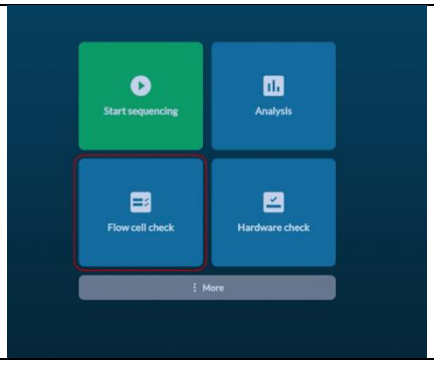
4. **By a double click MinKNOW program is started.**
   Log in with your Nanopore account (Email and password)**.**

	Click the following link to log in Nanopore homepage: https://community.nanoporetech.com/support Log in with your Email and password.	

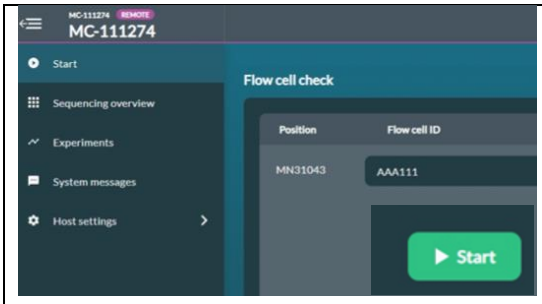
5. **Select in the Connection Manager the sequencing device which is connected to your PC.**

		In the sequencing overview Flongle Flow Cell is shown.

6. **Navigate to Start and select „Flow Cell Check“**

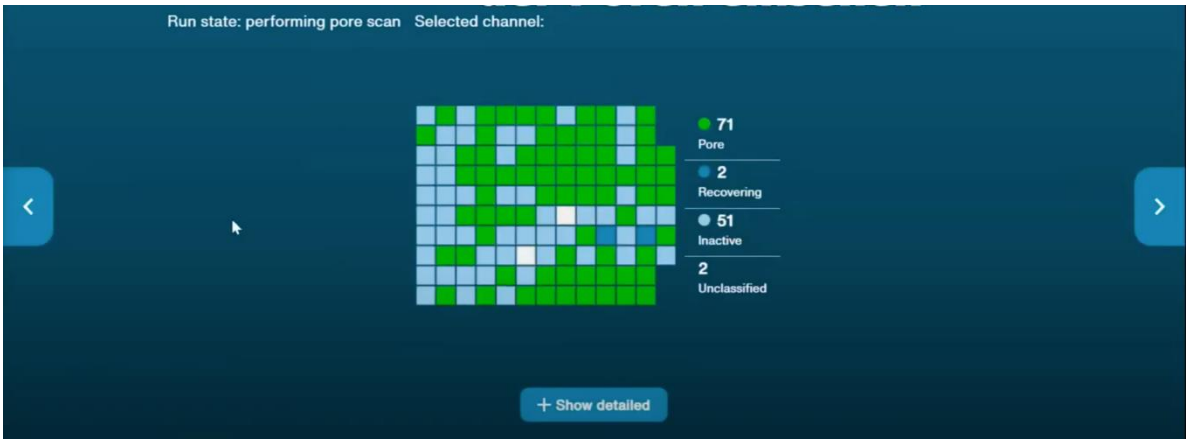
		Click button Flow Cell Check.

7. **Add Flongle Flow Cell ID and select Flow Cell Type FLO-FLG001.**

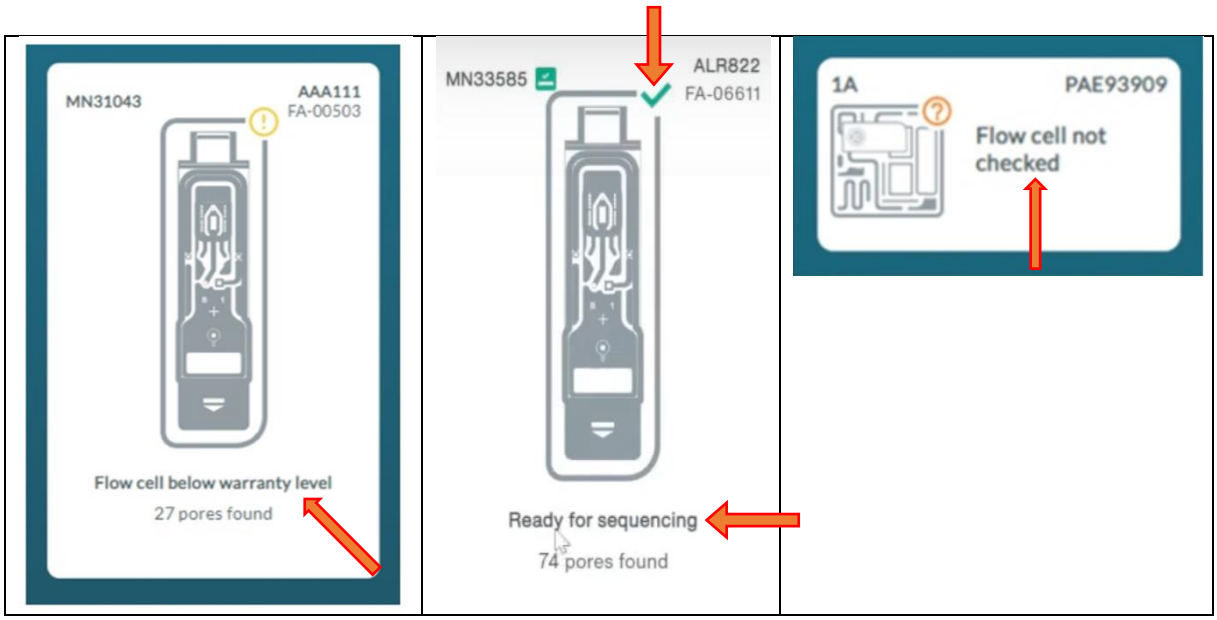
		Add Flongle Flow Cell ID (3 letters and 3 numbers without a space). Select type **FLO-FLG001**. Click on **start** Flow Cell Check.

Flow Cell Check takes some minutes.

Click on **Experiments** and the **current Flow Cell Check** to see parameters during the Check:



At the end of the Flow Cell Check one of the 3 following results will be shown:



The green arrow shows „Ready for sequencing"
A result of the Flongle Flow Cell check with more than 50 pores found is fine.

Flongle Flow Cell Priming

After the Flongle Flow Cell check the storage buffer inside the Flongle must be substituted by the sequencing buffer. This should be done directly before start of sequencing.

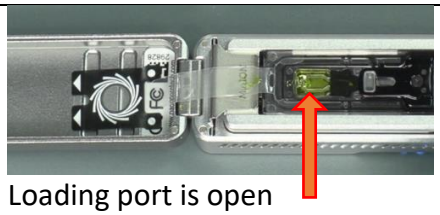
1. Preparation of Flongle Flow Cell Priming Buffer (FLB)

Pipet from Flow Cell Priming Kit (EXT-FLP002) in a 1,5 mL tube:
117 µl Flush Buffer (FB) + 3 µl Flush Tether (FLT) = 120 µl FLB

Video about Flongle Flow Cell Priming Click on the picture → For alternative click the following link: https://www.youtube.com/watch?v=ExTMvDuOGK4	

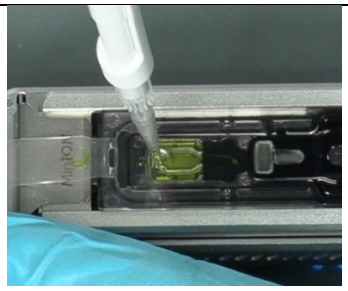
2. Priming of Flongle Flow Cell

2.1 Pull off the sticker of the Flongle Flow Cell

 Pull off the sticker	 Loading port is open	Pull off the sticker of the Flongle Flow Cell in the direction marked with arrows and fix the sticker inside the lid of the MinION.

2.2 Substitution of storage buffer in the Flongle Flow Cell by Priming Buffer (FLB)

Take up 120 µl priming buffer (FLB) air bubbles free in your pipet.
Place the pipet tip in the loading port of the Flongle Flow Cell. Check that the pipet tip fits inside the loading port.
Pipet air bubbles free in the Flongle Flow Cell array 110 µl FLB out of 120 µl.

 Pipet tip filled with 120 µl FLB air bubbles free	 Loading 110 µl FLB in loading port of a Flongle	 Rest of FLB liquid (may be 5 – 10 µl)	**Attention:** - Only 110 µl of priming-buffer (FLB) is necessary to fill in the array of the Flongle Flow Cell! - To avoid air bubbles in the array of the Flongle Flow Cell a rest of liquid stays in the pipet tip!

Flongle Flow Cell is ready to use now for loading a DNA-library (see page 3; 7.3) and start sequencing.

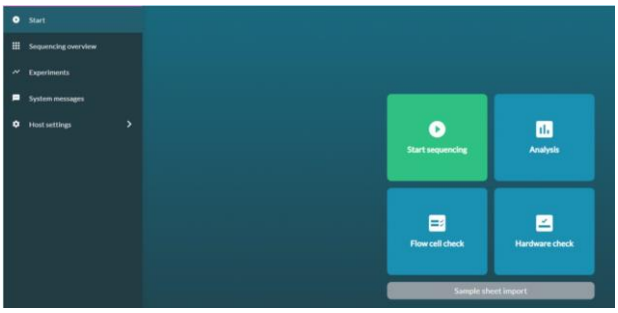
Start Sequencing

Program MinKNOW is still running and Flow Cell Priming was successful

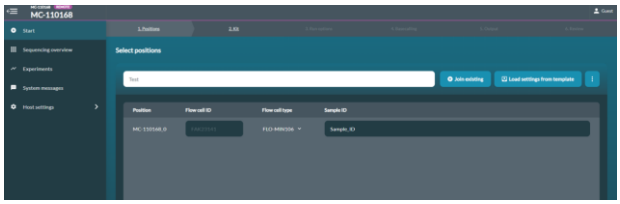
Program MinKNOW controls.the MinION Flow Cell, the raw data record, the basecalling and the barcode demultiplexing.

Select run options

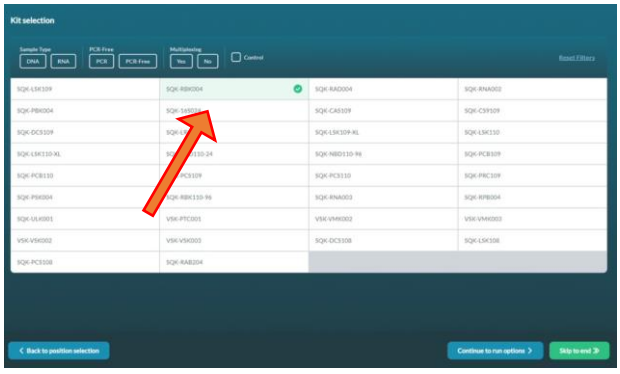
1. Select the Flongle Flow cell connected with the PC and click the start button to enter the sequencing set-up.



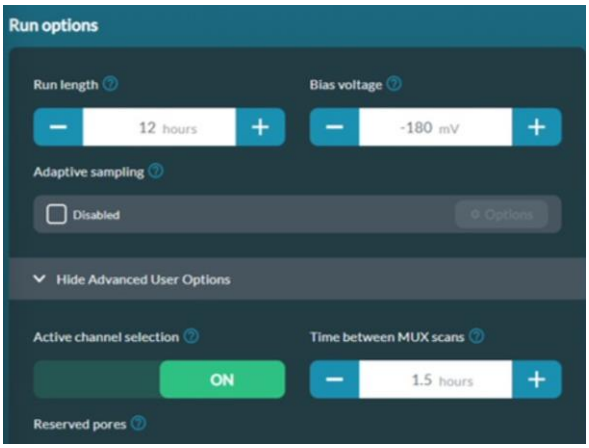
2. Select positions
   Select „1. Positions“.
   Name your experiment and select an approach number.



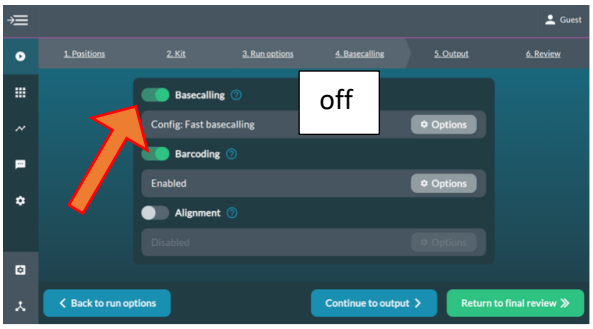
3. Click button „Continue to kit selection“
   In the Kit selection table click on **SQK-RBK004.**



4. Click button „Continue to run options“
   Run options are fixed → don`t change!
   - run length: 24 h
   - active channel selection: on

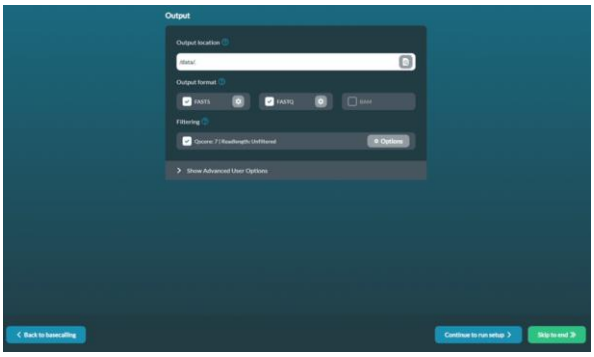


5. Click button “Continue to basecalling



Under „Continue to Basecalling“ basecalling must be active switched off (must be gray).
If not recorded data volume during basecalling is to high for the PC.

6. Click button „Continue to output“
   Select on PC where the data packages should be stored (e.g.: C/data/ + name)



7. Review your sequencing set-up according to the shown table.
   → Click button: Start
   After 15 min. a first raw data package with 1000 reads will be stored in c/data/.. as a Fast5 file.