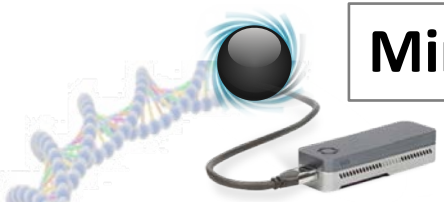
EvoPAD Summer School „Bioinformatics“

MinION Genome Sequencing

Jürgen Schmitz
Martin Kiefmann

10-13. September 2019 K6 Seminarhotel, Kirschallee 6, 38820 Halberstadt

MinION – Nanopore Genome Sequencing



The Targets

Sequencing Techniques

Nanopores

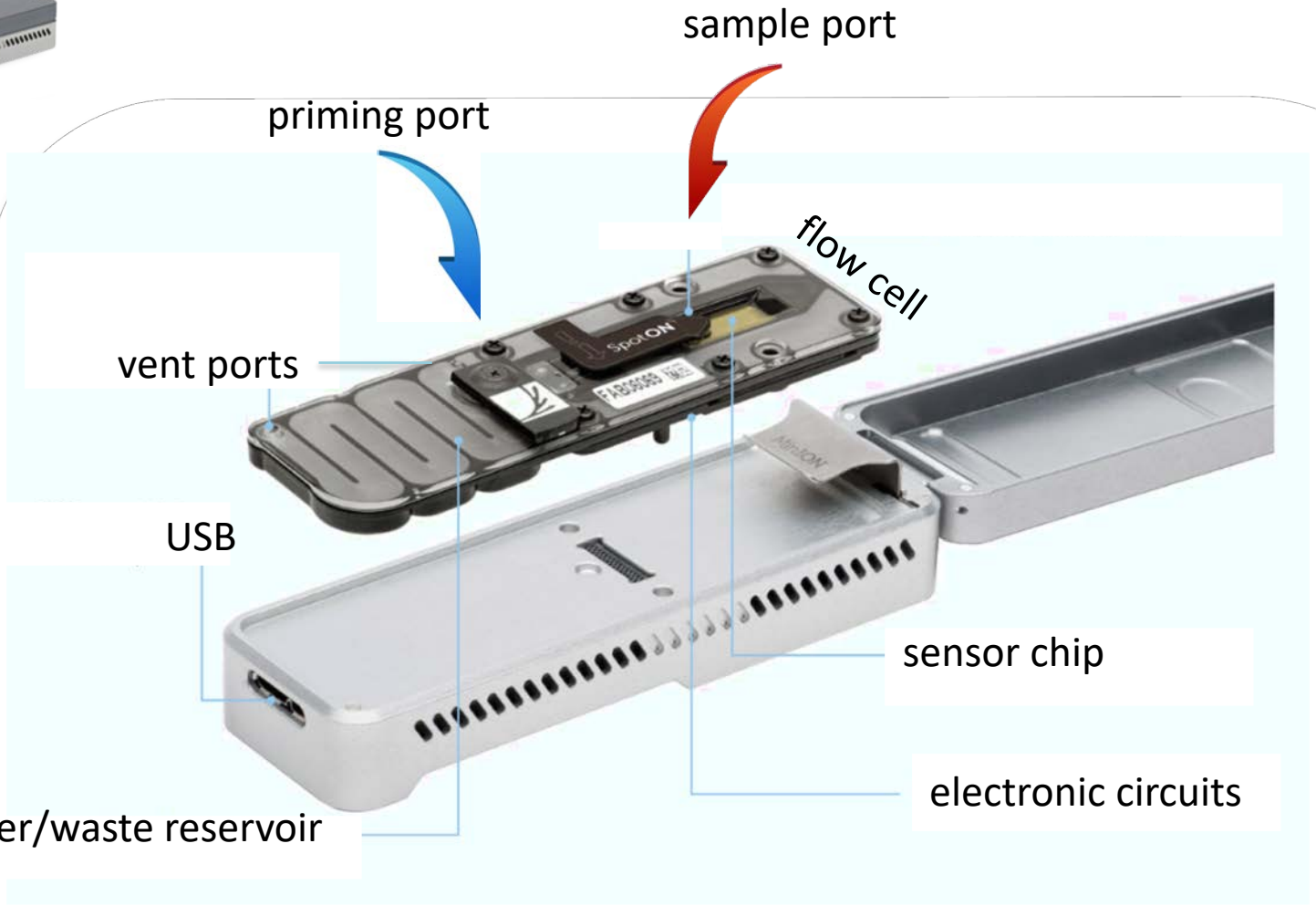
Library Construction

Loading Samples

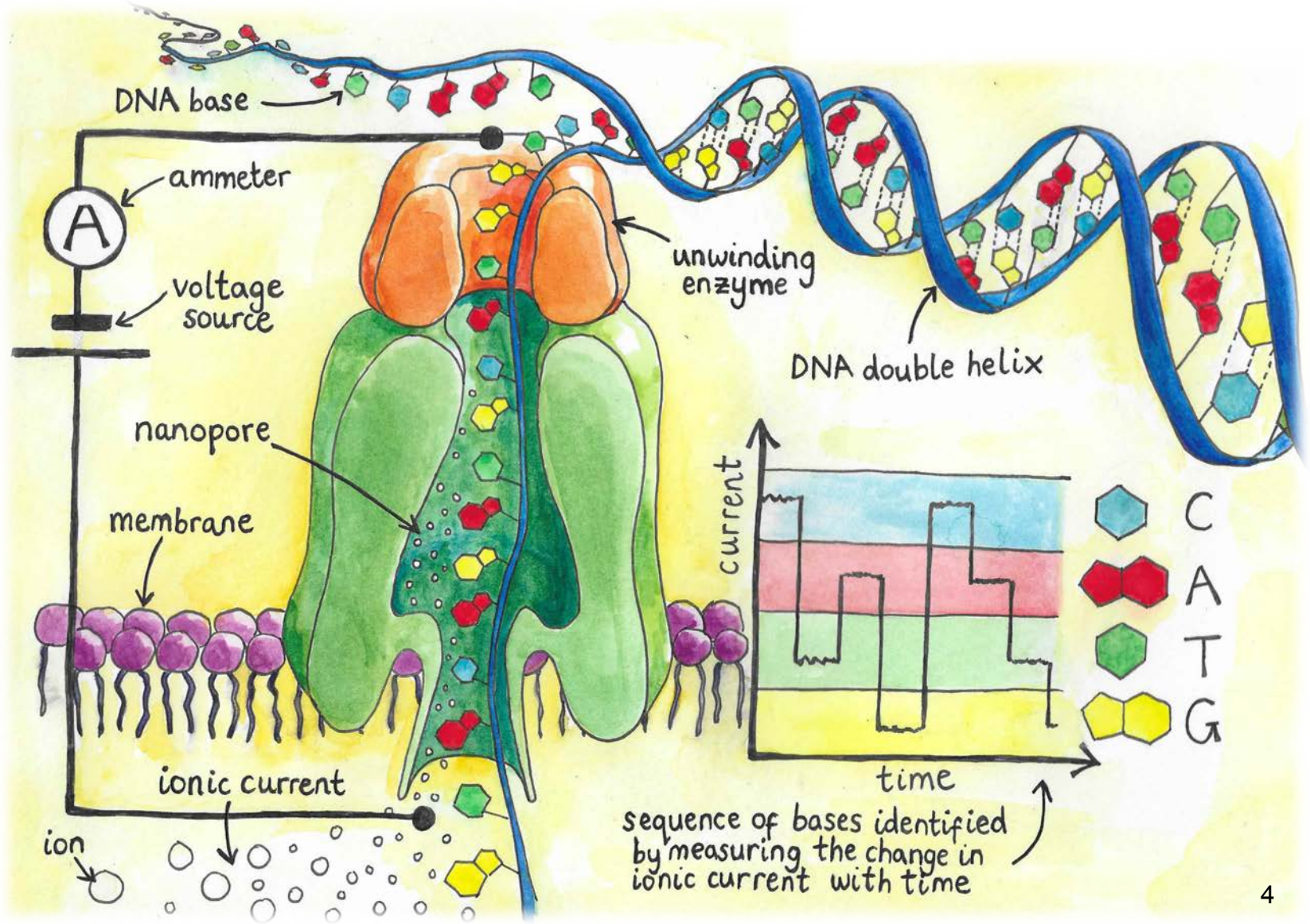
MinION Run

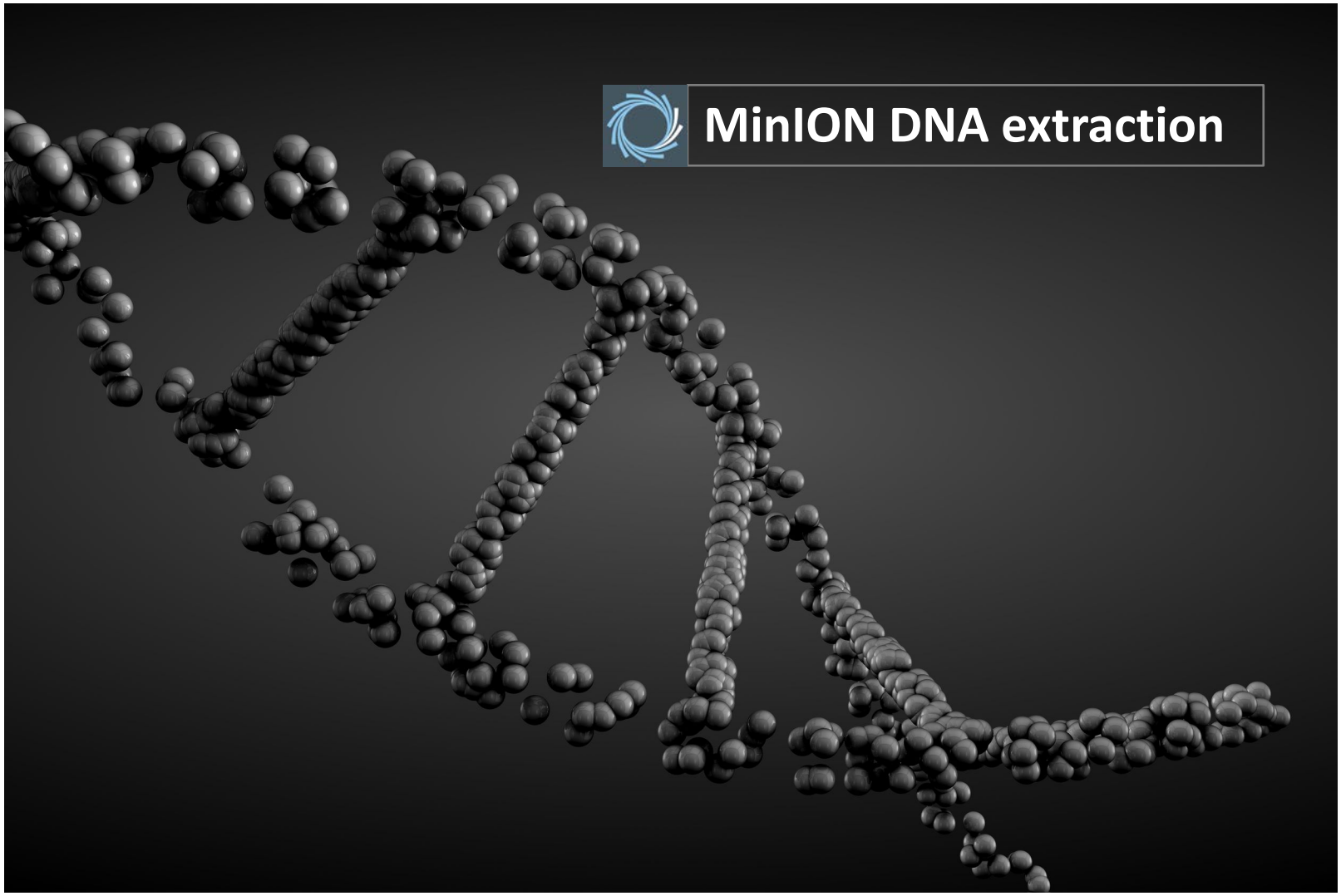
Data Analysis

PLUGANDPLAY



Principles of Nanopore Sequencing

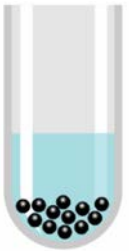



MinION DNA extraction

- Magnetic beads for efficient DNA binding
- Minimal sharing
- High range, high quality DNA for Nanopore (200-500kb)

Smart DNA prep (m) for MinION

Proteolytic Lysis of Tissue (1-100 mg)



1. Cut the tissue into small pieces and place in a 1.5 ml tube
2. Add 400 µl Lysis Solution CBV and 40 µl Proteinase K
3. Vortex shortly and incubate at 55°C for 1-3 hs in thermal shaker (1,200 rpm)
4. After lysis centrifuge 5 min (max speed), transfer supernatant to a new tube
5. Add 1 µl RNase A (10 mg/ml), digestion 10 min at RT, transfer to **SE tube**

Binding DNA to SE Smart Extraction Macro Beads

6. Add 40 µl Binding Optimizer and 350 µl 2-Propanol in the **SE tube**
7. Place SE tube into a thermal shaker (3 min; 1,400 rpm)
8. Place SE tube into magnetic rack for separating the SE Macro Beads
9. Discard supernatant

Washing and removing of alcohol

10. Add 800 µl Wasching Solution LS (invert rack 5X)
11. Discard supernatant – leaving SE tubes in magnetic rack

2X

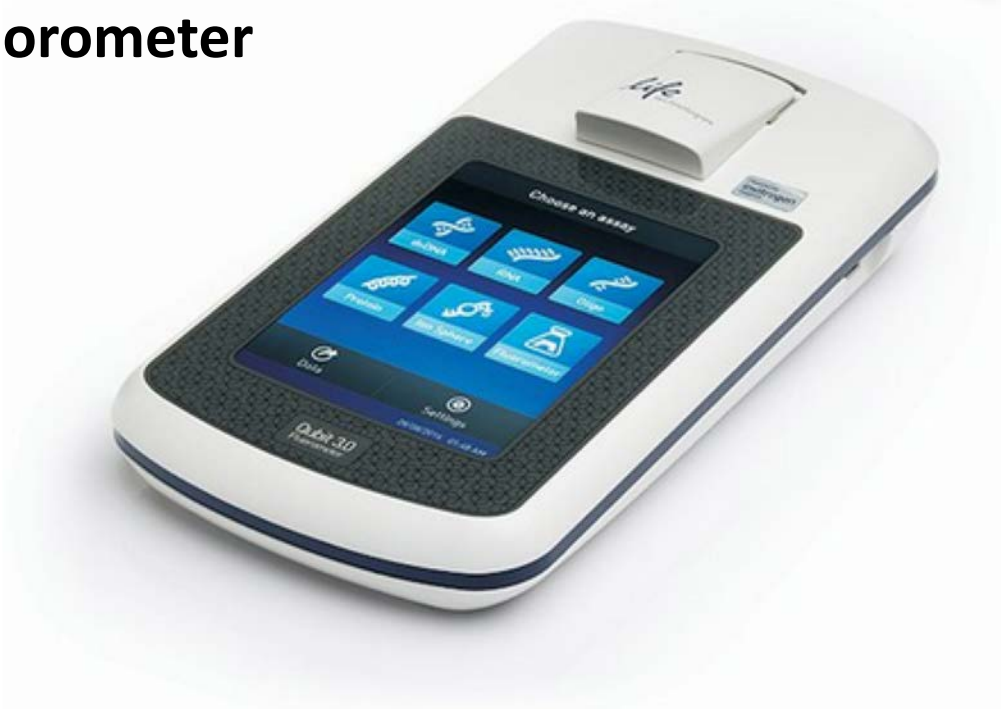
12. Add 800 µl of 80% ethanol and wash beads (invert rack 5X)
13. Discard supernatant – leaving SE tubes in magnetic rack

Remove the ethanol 15 min 65°C in thermal cycler, lid open 400 rpm

Elution of DNA

14. Add 200 µl – 1,000 µl Elution Buffer 15 min at 65°C, thermal cycler 1,000 rpm
15. Place SE tube into the magnetic rack and transfer DNA supernatant into a new tube

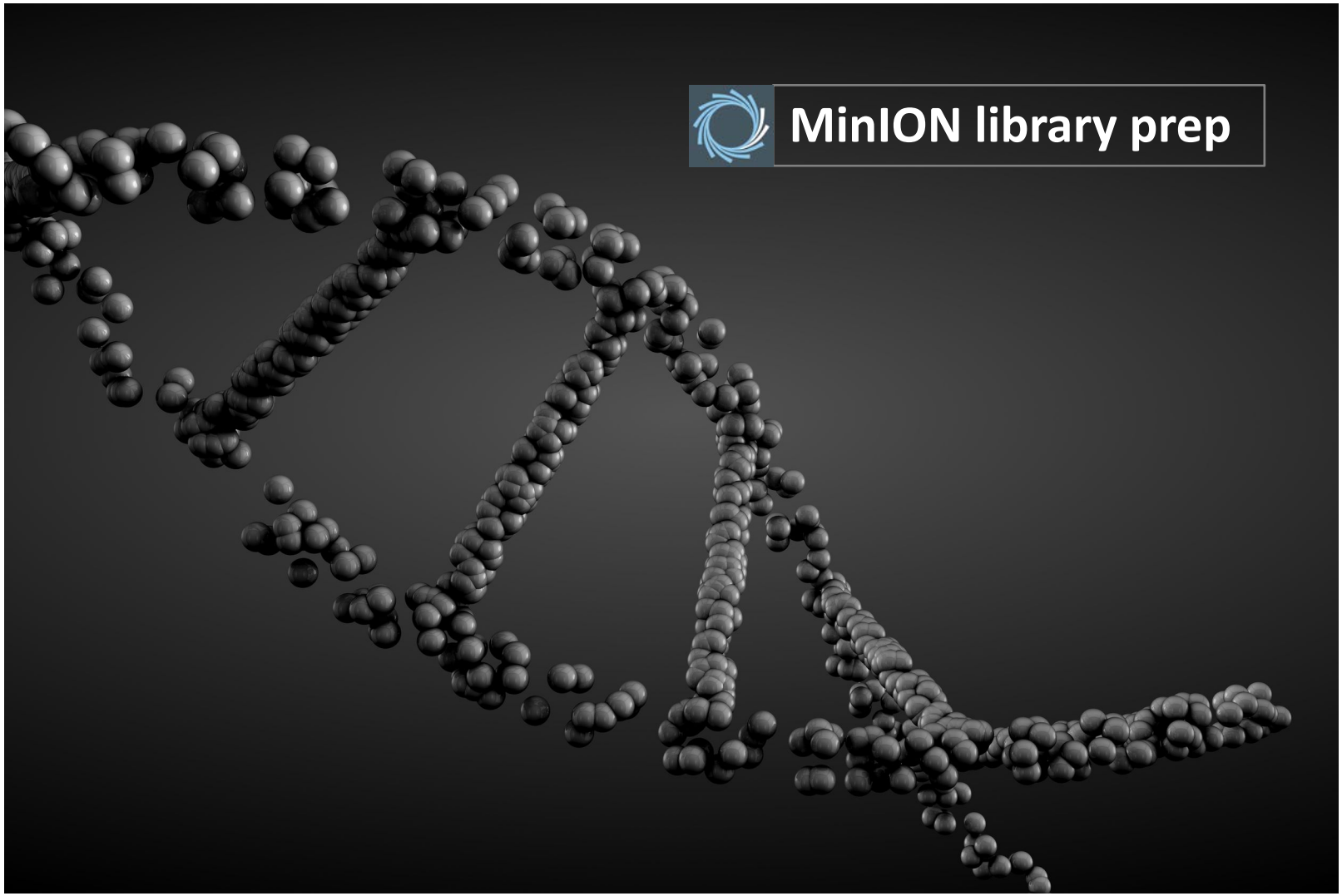
- **Qubit™ 4 Fluorometer**



Quantify 1 µl eluted sample

(15.) Average fragment size > 30 kb (electrophoresis)

16. Input mass (Qubit 1 µg)


MinION library prep

MinION library prep

SQK-LSK109

Ligation Sequencing Kit

High molecular weight gDNA

Optional fragmentation
(for <100 ng input)

repair
and end-prep

Ligation of sequencing
adapters

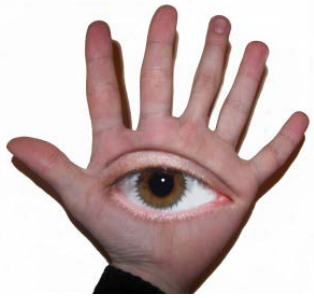
Loading

60 min

1D (directional) PCR-free gDNA

MinION library prep

Prepare the DNA



1. 1 µg genomic DNA in a DNA LoBind tube
2. Adjust volume to 49 µl with Nuclease-free water
3. Mixing by inversion (avoid sharing)
4. Spin down briefly in a microfuge

End-prep and nick repair

5. Thaw DNA CS (DCS) at RT, spin down, mix by pipetting, place on ice
6. Prepare the NEBNext FFPE DNA Repair Mix and NEB Next End repair/dA-tailing Module reagents and place on ice (see 5.)

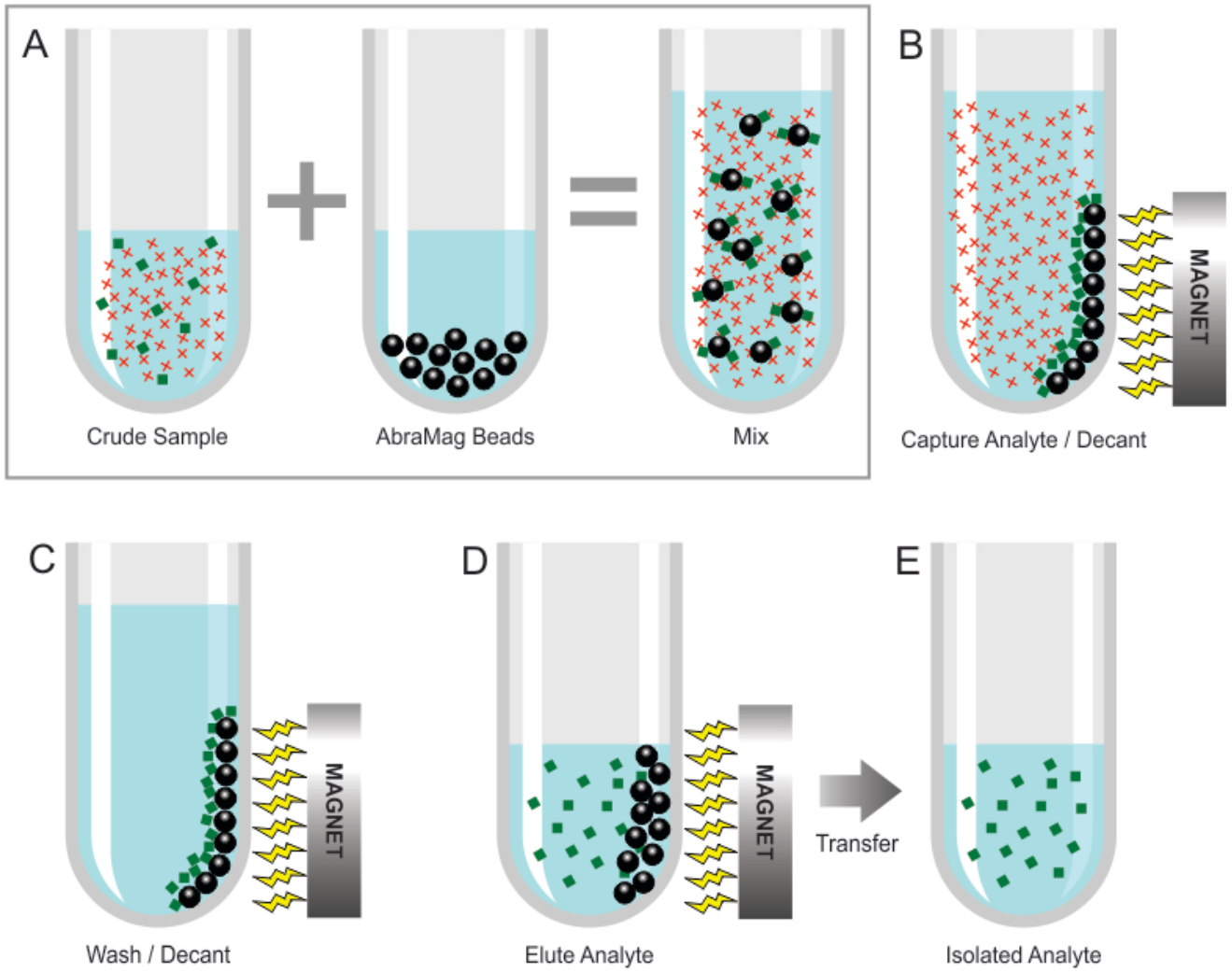
Mix in a 0.2 ml thin-walled PCR tube:

1 µl	DNA CS
47 µl	DNA
3.5 µl	NEBNext FFPE DNA Repair Buffer
2 µl	NEBNext FFPE DNA Repair Mix
3.5 µl	Ultra II End-prep reaction buffer
3 µl	Ultra II End-prep enzyme mix

Mix gently by flicking the tube, and spin down
Using a thermal cycler, icubate at 20°C for 5 Min and 65°C 5 min

MinION library prep

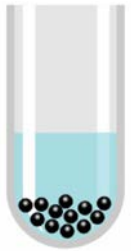
- Magnetic beads and magnetic rack



MinION library prep

3

Clean and extract the repaired DNA



1. Resuspend the AMPure XP beads by vortexing
2. Transfer the DNA sample to a clean 1.5 ml DNA LoBind tube
3. Add 60 µl of resuspended AMPure XP beads to the end-prep reaction
4. Mix by flicking the tube
5. Incubat on a Hula mixer (rotating) for 5-15 min at RT
6. Prepare 500 µl of fresh 70% ethanol in Nuclease-free water
7. Spin down the sample and pellet on a magnet
8. Keep the tube on the magnet and pipette off the supernatant

2X

9. Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet
10. Remove the 70% ethanol using a pipette and discard

11. Spin down and place the tube back on the magnet
12. Pipette off any residual ethanol. Allow to dry for ca. 30 seconds
13. Remove tube from magnetic rack and resuspend pellet in 61 µl water
14. Incubate for 5 min at RT
15. Pellet the beads on a magnet until the eluate is clear and colourless
16. Remove and retain 61 µl of eluate into a clean 1.5 ml LoBind tube

- Safe stop point 4°C -

- **Quantification**

Quantify 1 µl eluted sample using a Quibit fluorometer

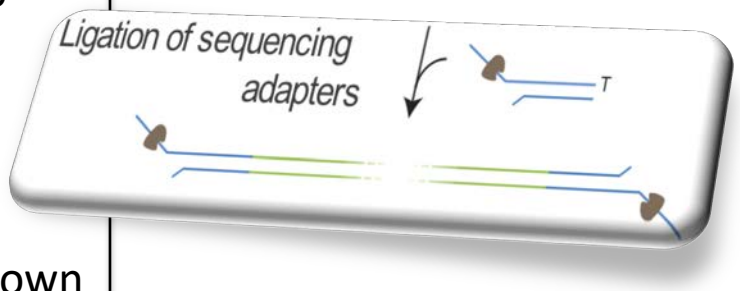
Ligation of sequencing adapters

1. Spin down Adapter Mix (AMX) and T4 Ligase (E6056). Place on ice
2. Thaw Ligation Buffer (LNB) at RT, spin down and mix by pitpetting. Place on ice
3. Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down. Place on ice

4. To enrich DNA fragements of 3 kb or longer, thaw one tube of L Buffer (LFB)
   To retain DNA fragemts shorter than 3 kb, thaw one tube of S Buffer (SFB)
5. Mix by vortexing, spin down and place on ice

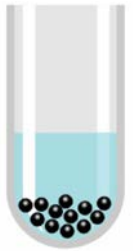
6. In a 1.5 LoBind tube mix:
   - 60 µl DNA sample from previous step
   - 25 µl Ligase Buffer (LNB)
   - 10 µl NEBNext Quick T4 DNA Ligase
   - 5 µl Adapter Mix (AMX)

   Mix gently by flicking the tube, and spin down
   Incubate 10 min at RT



MinION library prep

Clean and extract the adapter-ligated DNA



7. Prepare the AMPure XP beads for use. Resuspend by vortexing
8. Add 40 µl of resuspended AMPure XP beads to the reaction and flick tube
9. Incubate on Hula mixer for 5-45 min at RT
10. Spin down the sample and pellet on a magnet. Keep the tube on the magnet
11. Remove the supernatant using a pipett and discard

2X

12. Wash the beads by adding either
    250 µl Long Fragment Buffer (LFB)
    or 250 µl Short Fragment Buffer (SFB)
    - Flick the beads to resuspend, then return the tube to the magnetic rack
    - allow the beads to pellet
    - Remove the supernatant using a pipett and discard

13. Spin down and place the tube back on the magnet
14. Pipette off any residual supernatant
15. Allow to try for ca. 30 seconds
16. Remove the tube from the magnetic rack and resuspend pellet in 15 µl EB buffer
17. Incubate for 10-30 min at 37°C
18. Pellet the beads on a magnet until the eluate is clear and colourless

MinION library prep

Quantify

19. Remove and retain 15 µl of eluate (Lib) into a clean 1.5 µl LoBind tube
20. Dispose of the pelleted beads
21. Quantify 1 µl eluted sample using a Quibit fluoromter

22. The prepared library is used for loading into the flow cell
23. Store the library on ice until ready to load

MinION library prep

SQK-LSK109

Ligation Sequencing Kit

High molecular weight gDNA

Optional fragmentation
(for <100 ng input)

repair
and end-prep

Ligation of sequencing
adapters

Loading

60 min


MinION loading

MinION loading

Loading strategy

first 6 h next 6 h

Flow cell 1

first 6 h next 6 h

Flow cell 2

MinION – Set up the MinION flow cell and host computer

1. Open the MinKNOW GUI from the desktop icon
2. Establish a local internet connection (firmware confirmation)
3. Turn off sleep mode of computer
4. Choose the flow cell type from the selector box (FLO-MIN106)
5. Click Start test
6. Check the number of active pores available in System History panel
   - should be >800 from 4 X 512

Priming and loading the SpotON flow cell

1. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and Flush Buffer (FLB) at RT, place on ice
2. Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing
3. Spin down the Flush Tether (FLT) tube, mix by pipetting, return to ice

4. Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible

priming port

5. Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all time. Removing more than 20-30 µl damaging the pores in the array
6. After opening the priming port, check for small bubble under the cover Draw back a small volume to remove any bubble (a few µls)
7. Set a P1000 pipette to 200 µl
8. Insert the tip into the priming port
9. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip

MinION loading

10. Prepare the flow cell priming mix: add
    30 µl of thawed and mixed flush Tether (FLT) directly to the tube
    of thawed and mixed Flush Buffer (FLB), and mix by pipetting

11. Load 800 µl of the priming mix into the flow cell via the priming port

12. Awoid air bubbles, wait for 5 min

13. Throughly mix the content of the Loading Beads (LB) by pipetting
    The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed!!! immediately before use

14. In a new tube, prepare the library for loading as follows:

37.5 µl	Sequencing Buffer (SQB)
25.5 µl	Loading Beads (LB), mixed immediately before use
12 µl	DNA library
75 µl	

MinION loading

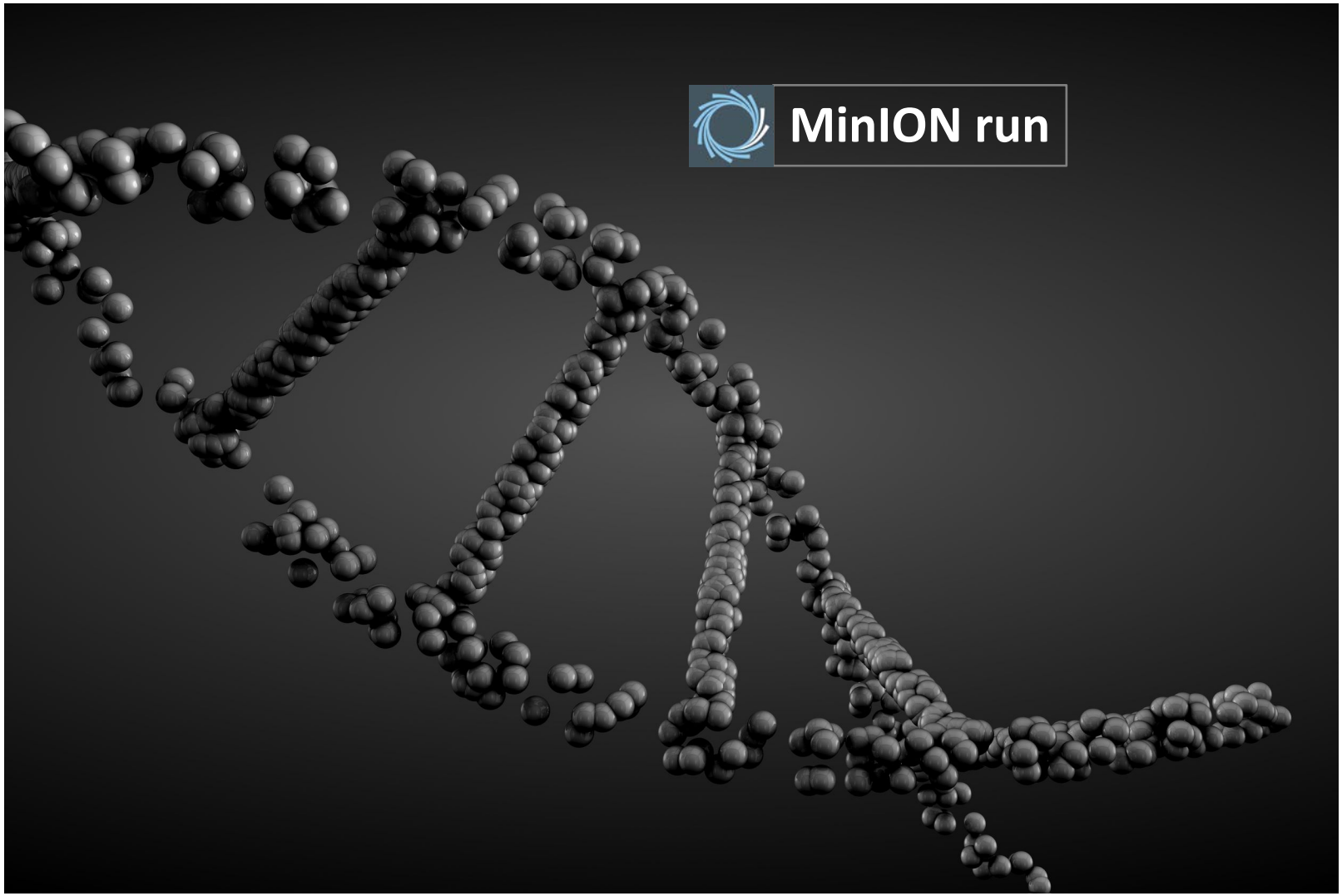
15. Gently lift the SpotON sample port cover to make the SpotON sample port accessible

16. Load slowly!!! 200 µl of the priming mix into the flow cell via the priming port!!!!! (NOT the SpotON sample port). Avoiding airbubles

17. Mix the prepared library gently by pipetting up and down just prior loading

18. Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next

19. Gently close the spotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.


MinION run

MinION run

- ***Data strategy***

Names:

Data deposited: /Library/MinKNOW/data

MinION run

1. Double-click the MinKNOW icon at the desktop to open the MinKNOW GUI
2. Choose the flow cell type from the selector box. Then check „Available“ box

   FLO-MIN106
3. Click the „New Experiment“ button at the bottom left of the GUI

On the new experiment popup screen, select the running parameters for your Experiment from the individual tabs

Output settings – FASTQ: The number of basecalls that MinKNOW will write in a single file. By default this is set to 4000

Output settings – FAST5: The number of files that MinKNOW will write to a single folder. Default 4000

Basecalling tab to ON – if using basecalling via MinKNOW (abalone)

Basecalling tab to Off – if using basecalling via guppy later

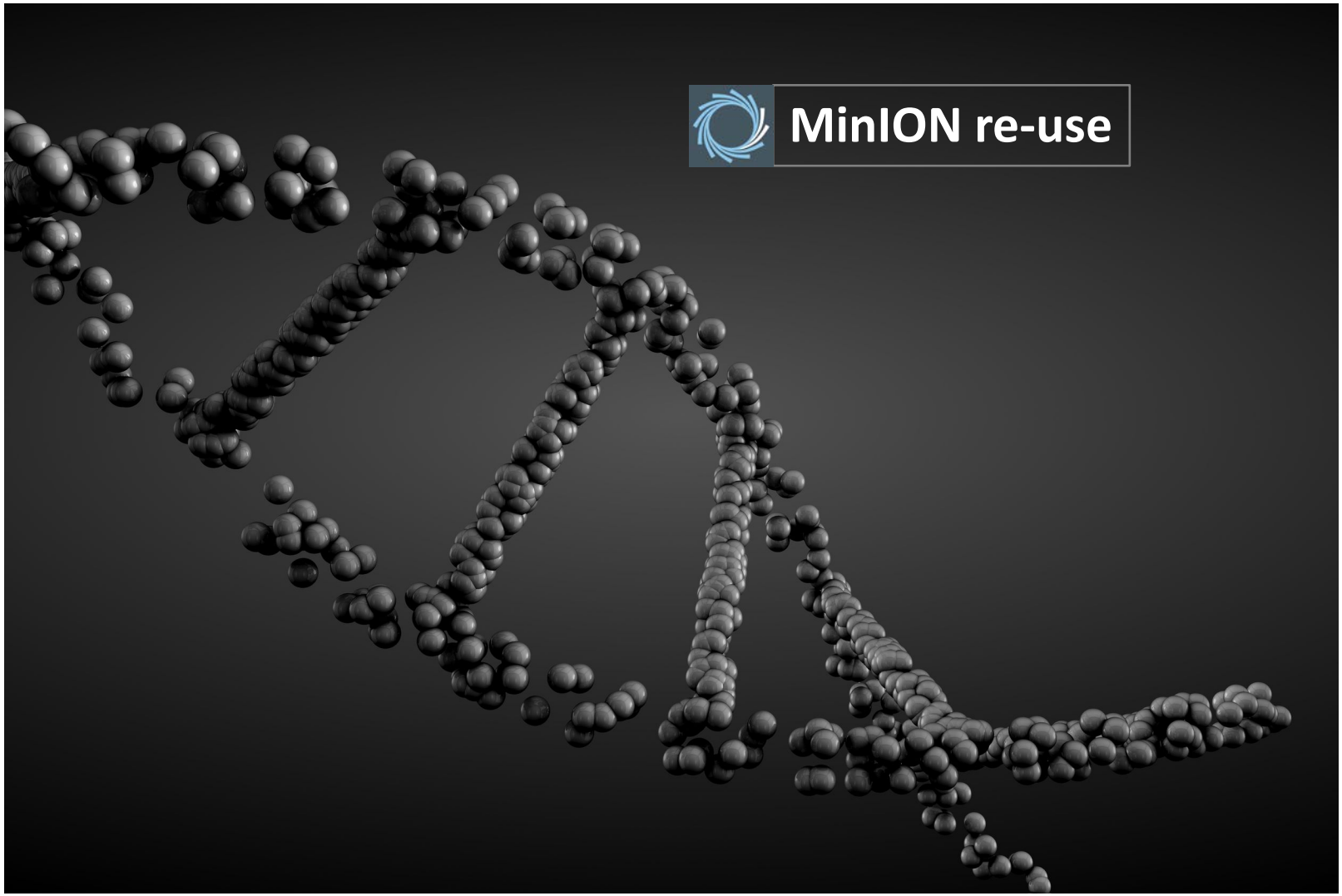
Click start run

Allow the script to run to completion

The MinKNOW Experiment page will indicate the progress of the script

This can be accessed through the „Experiment“ tab (top right of the screen)

Monitor messages in the message panel in the MinKNOW GUI


MinION re-use

MinION re-use flow cell

Wash Kit (EXP-WSH002) – to re-using flow cells

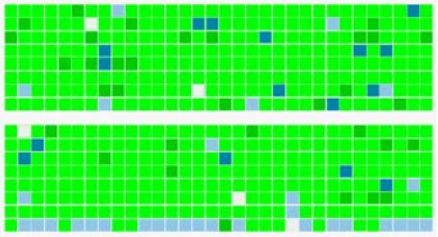
Solution A	1000 µl	2 tubes	12X	sequesters the library strands from membrane
Solution B	500 µl	4		removes the sequestered library refreches FC
Storage Buffer	1600 µl	4		

1. Open the priming port cover to check that buffer is continuous
2. Ensure that the SpotON sample loading port is closed
3. Gilson P1000 adding 150 µl Solution A through priming port
6. Wait 10 min
7. Add 150 µl Solution B
8. Start a new MinKNOW experiment

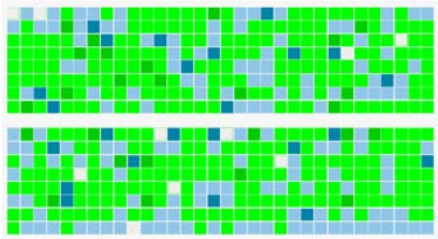
Later use

7. Slowly add 500 µl of Storage Buffer through priming port
8. Close priming port. Remove buffer from the waste section of the flow cell through either of waste ports
9. Store flow cell at 4-8°C

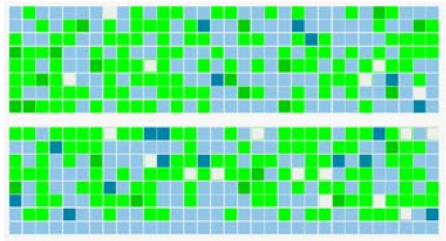
pore active/512



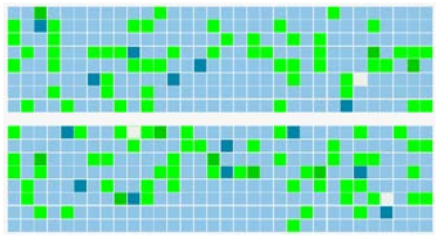
5 h



18h

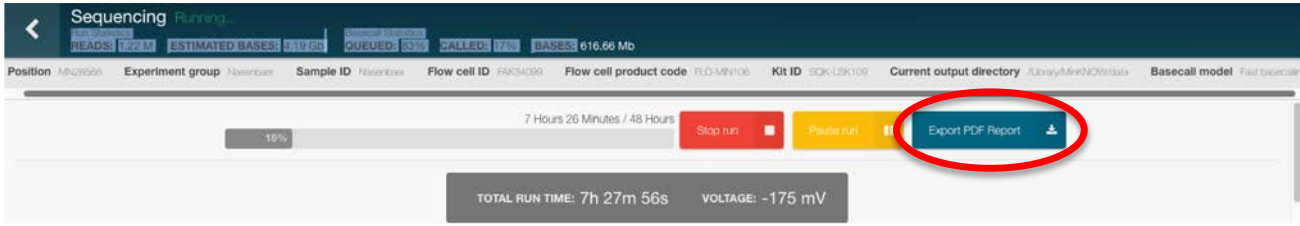


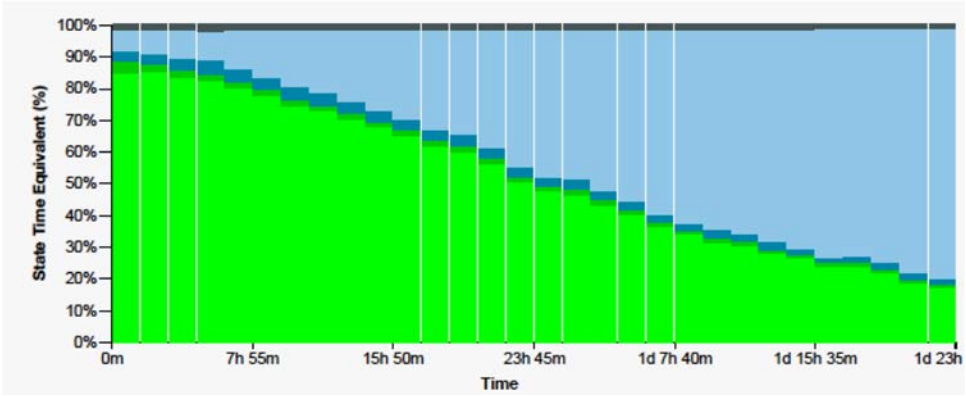
28h

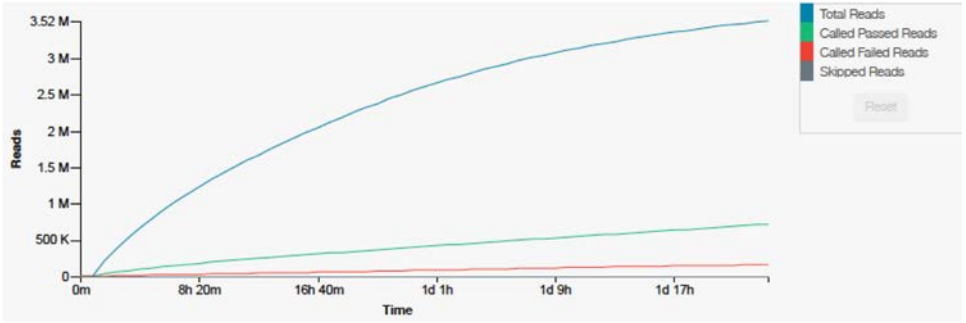


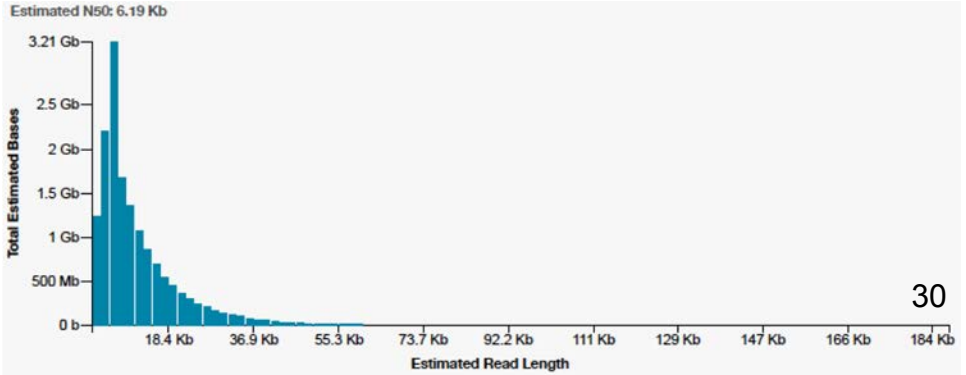
45h

pore not longer available/512

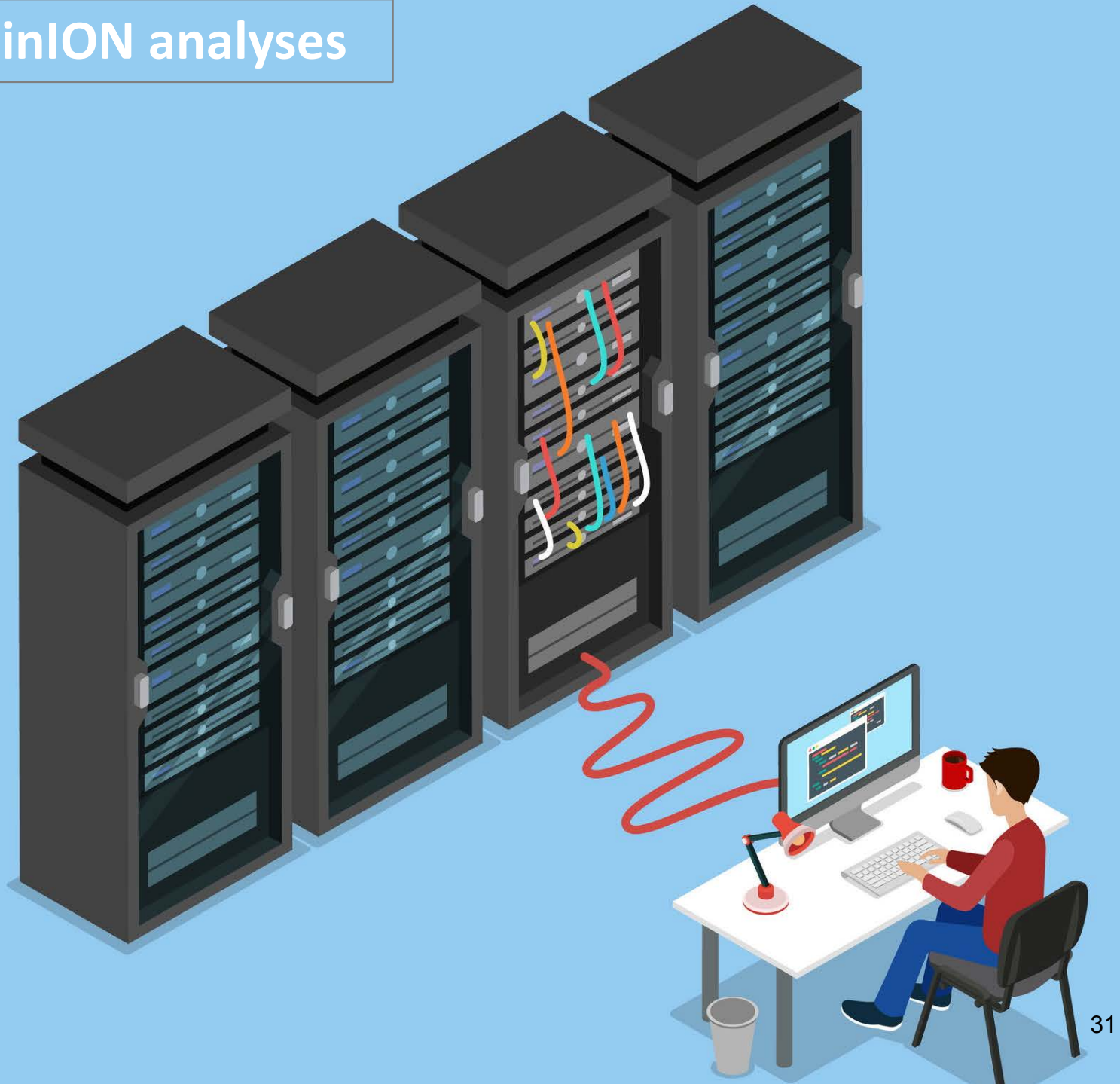




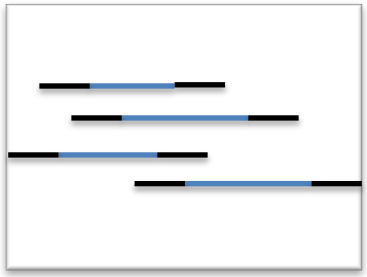




MinION analyses



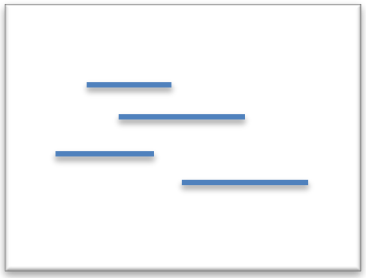
MinION analyses



MinION
Sequencing

reads
fast5

48 h

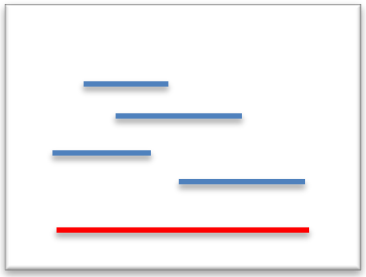


MinKNOW/Guppy
Quality control

reads
fastq

7 d

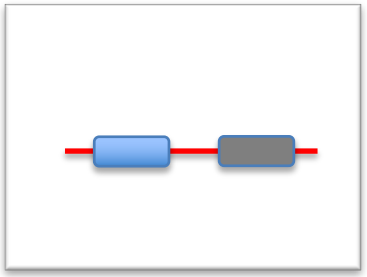
GPUs 



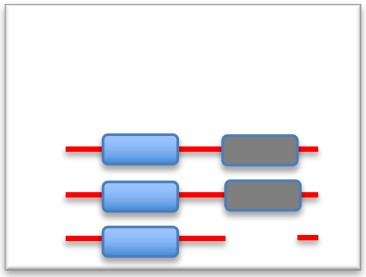
Canu
Assembly

contigs
fasta

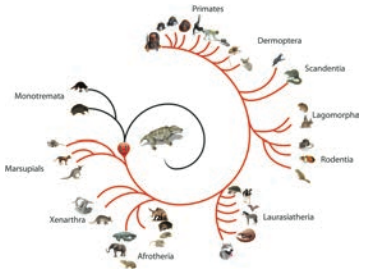
10 d



BLAST-RM
Annotation



UCSC
Comparison



MinION analyses

Server access

ssh -p 40044 extern@retrogenomics2.uni-muenster.de
password: eXtern01
location: /home/extern

terminal

Transfer > server (terminal client)

scp -o "Port 40044" -r /Users/juergenschmitz/Desktop/Transfer/*.* extern@retrogenomics2.uni-muenster.de:/home/extern/data/coati/fast5

source client — target server

Transfer < server (terminal client)

scp -o "Port 40044" extern@retrogenomics2.uni-muenster.de:/home/extern/data/coati/fast5/*.* /Users/juergenschmitz/Desktop/Transfer

source server — target client

ssh = secure shell network protocol
scp = secure copy protocol

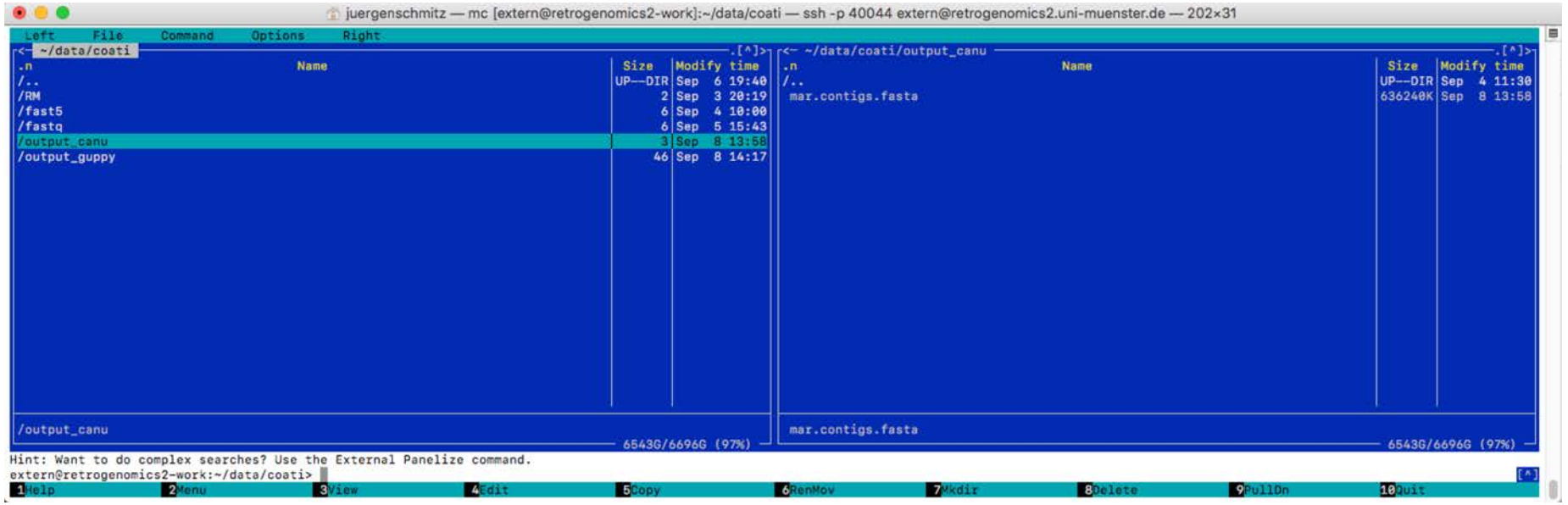
-o = option
-r = copy all files in a folder recursively

VPN:
zmbeprak
ZMBE.2019n

MinION analyses

- ***UNIX commands:*** mc visual shell

terminal

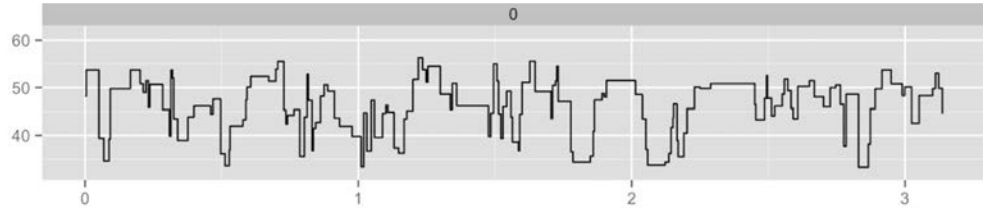


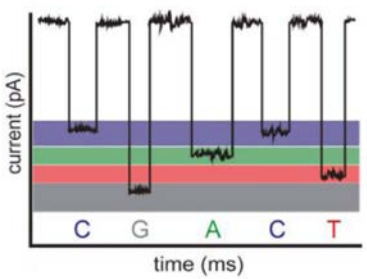
MinION analyses

FAST5 files

Data out of Oxford Nanopore Technology (ONT)

Raw electronical signal in HDF5 (Hierachical Data Format)
Main data: „squiggles“ = pico-amp measurements at micropores





MinION analyses

FASTQ files

@0243e9e9-5442-4df3-aa14-fdfd6d2ba214 flow_cell_id=FAK33926 protocol_group_id=Maria1 sample_id=maria1
ATCAGTATTGCTTCGTTCGGTTACGTATTGCTGGCAGCAGGTGCGGGCGTACTTCAGCCTGGCGTTCTGTGACTTCTGCCTTAAGTTT
+
'-8#.,(+%%+1631:66#0(:,54<96<564593-14)+&()&*&%$)*+-7>50%3++02-23>999:9788>9<:;05/,&++<<89<6-.*'(2,+3;1

@ … = sequence identifier and description

ACTG … = sequence
\+ = separator

!) 3 =… = quality value

ASCII	p
!	1
)	0.1
3	0.01
=	0.001
H	0.0001

Probability of incorrect base

MinION analyses

- ***FASTA files***

```
>coati_FAK34099_9e4d2b4f8a8663b7a95159919ce03578e435d1d4_0
ATCAGTATTGCTTCGTTCGGTTACGTATTGCTGGCAGCAGGTGCGGGCGTACTTCAGCCTGGCGTTCTGTGACTTCTGCCTTAAGTTT
```

> … = header

ACTG … = sequence

- **Transfer FASTQ to FASTA:** sed -n '1~4s/^@/>/p;2~4p' INFILE.fastq > OUTFILE.fasta

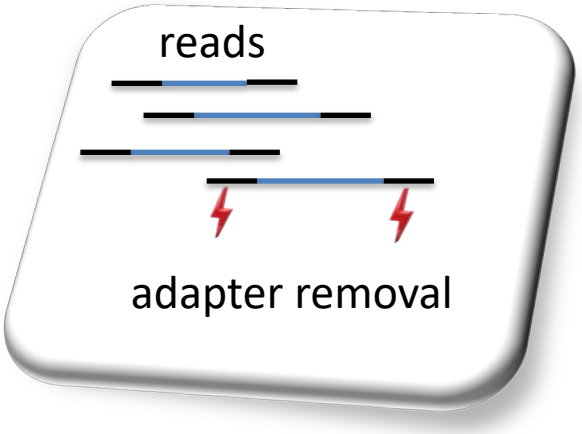
MinION analyses

Guppy basecalling

Adapter Strand Trimming: Removal of sequencing adapter

Input: .fast5 files containing raw data

Output: .fast5 and FASTQ files basecalled



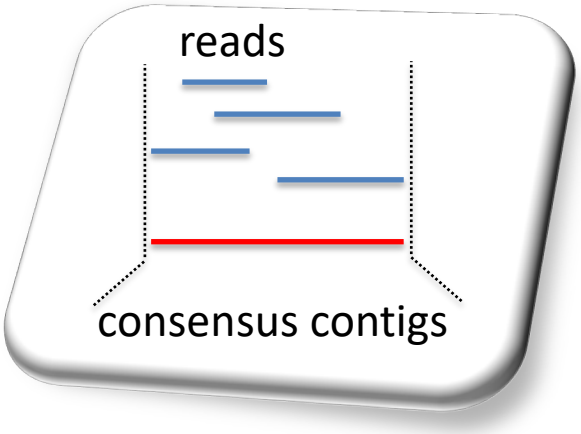
Command line (server):

source fast5 output

guppy_basecaller --input_path /home/extern/data/coati/fast5/ --save_path /home/extern/data/coati/output_guppy --config/appl/src/guppy/ont-guppy-cpu/data --flowcell FLO-MIN106 --kit SQK-LSK109 --num_callers 10 --recursive

guppy config file | used flowcell | used kit | # threads | including subfolders

Canu assembly



Reads-to-contigs (derive consensus sequences)
Input: .fastq or fasta
Output: .fasta

Command line:

> 6X coverage	1 node	genome size	memory/threads restrictions	MinION settings	save folder

canu stopOnLowCoverage=6 useGrid=false genomeSize=2.4g maxMemory 256 maxThreads 24 correctedErrorRate=0.16 -p coatiCANU -d /home/extern/data/coati/output_canu -nanopore-raw /home/extern/data/coati/fastq/for_coati/ *.fastq

save folder	data folder

10 days - 56 threads - 521 Gb

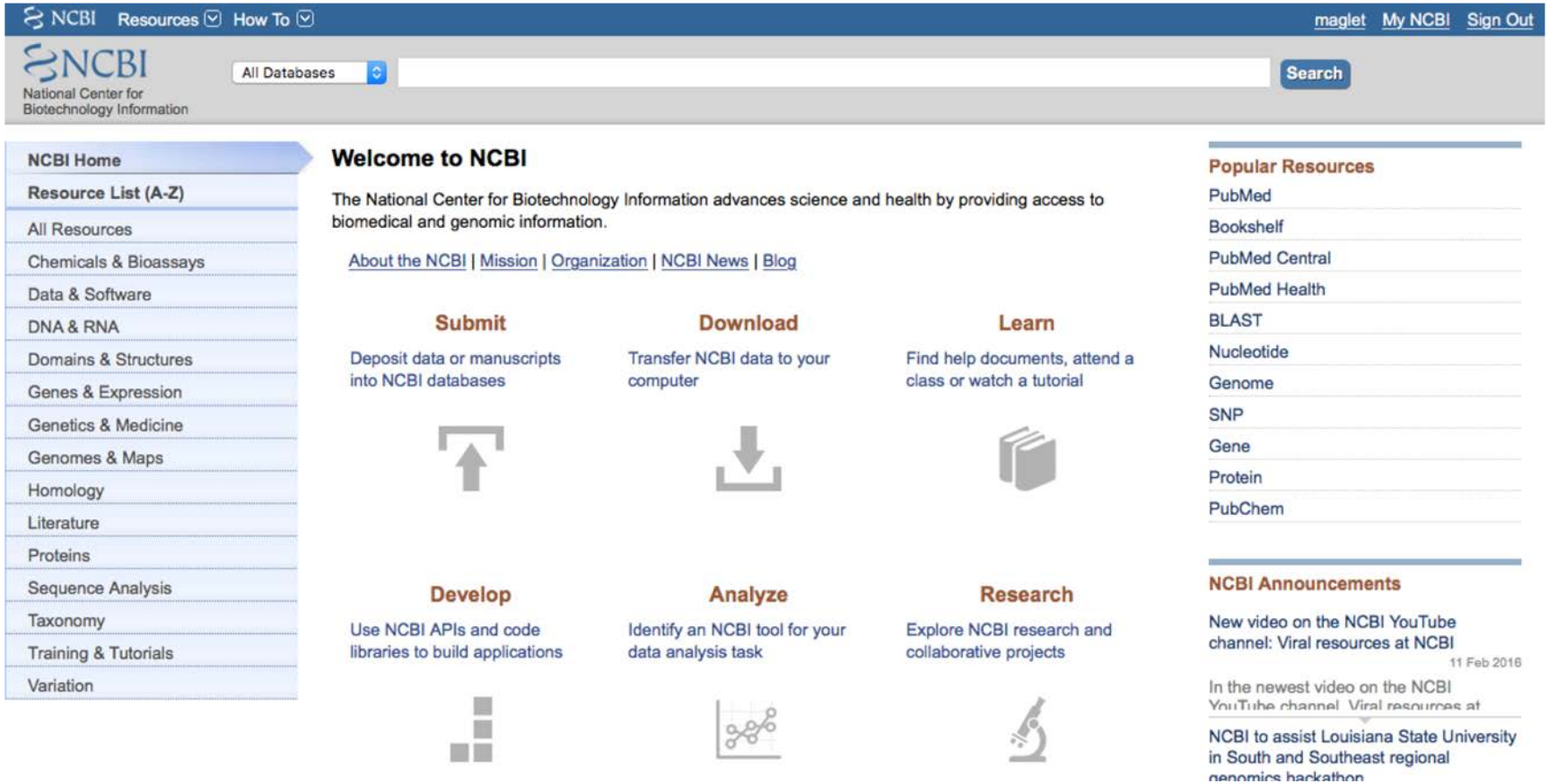
```
-- Finished on Sat Sep  7 19:43:48 2019 (10 seconds) with 6100.424 GB
----------------------------------------
-- Finished stage 'generateOutputs', reset canuIteration.
--
-- Assembly 'mar' finished in '/data/projects/ASSEMBLY/nasenbaer'.
--
-- Summary saved in 'mar.report'.
--
-- Sequences saved:
--   Contigs         -> 'mar.contigs.fasta'
--   Unassembled     -> 'mar.unassembled.fasta'
--   Unitigs         -> 'mar.unitigs.fasta'
--
-- Read layouts saved:
--   Contigs         -> 'mar.contigs.layout'.
--   Unitigs         -> 'mar.unitigs.layout'.
--
-- Graphs saved:
--   Contigs         -> 'mar.contigs.gfa'.
--   Unitigs         -> 'mar.unitigs.gfa'.
--
-- Bye.
```

0.6 Gb contigs – 0.6 Gb unique reads

```
drwxrwxr-x   2 juergen  user         688 Sep  7 19:43 canu-logs
drwxrwxr-x   2 juergen  user           2 Aug 30 13:35 canu-scripts
drwxrwxr-x   6 juergen  user          10 Sep  6 19:02 correction
drwxrwxr-x   2 juergen  user           2 Aug 30 13:35 haplotype
-rw-rw-r--   1 juergen  user   651509421 Sep  7 19:43 mar.contigs.fasta
-rw-rw-r--   1 juergen  user     8498310 Sep  7 19:43 mar.contigs.gfa
-rw-rw-r--   1 juergen  user  9524804661 Sep  7 19:41 mar.contigs.layout
-rw-rw-r--   1 juergen  user    59123961 Sep  7 19:41 mar.contigs.layout.readToTig
-rw-rw-r--   1 juergen  user    51970305 Sep  7 19:41 mar.contigs.layout.tigInfo
-rw-rw-r--   1 juergen  user  3435934573 Sep  6 19:05 mar.correctedReads.fasta.gz
-rw-rw-r--   1 juergen  user       27307 Sep  7 19:43 mar.report
drwxrwxr-x   3 juergen  user          29 Sep  7 06:11 mar.seqStore
-rw-rw-r--   1 juergen  user      226520 Aug 30 13:39 mar.seqStore.err
-rw-rw-r--   1 juergen  user      224202 Aug 30 13:35 mar.seqStore.ssi
-rw-rw-r--   1 juergen  user  2536456933 Sep  7 06:14 mar.trimmedReads.fasta.gz
-rw-rw-r--   1 juergen  user  4090474584 Sep  7 19:43 mar.unassembled.fasta
-rw-rw-r--   1 juergen  user     2230197 Sep  7 19:43 mar.unitigs.bed
-rw-rw-r--   1 juergen  user   663418894 Sep  7 19:43 mar.unitigs.fasta
-rw-rw-r--   1 juergen  user    16870520 Sep  7 19:43 mar.unitigs.gfa
-rw-rw-r--   1 juergen  user  1365885494 Sep  7 19:42 mar.unitigs.layout
-rw-rw-r--   1 juergen  user    20378745 Sep  7 19:42 mar.unitigs.layout.readToTig
-rw-rw-r--   1 juergen  user     2865999 Sep  7 19:42 mar.unitigs.layout.tigInfo
drwxrwxr-x   5 juergen  user           7 Sep  7 06:12 trimming
drwxrwxr-x  10 juergen  user          16 Sep  7 19:38 unitigging
```

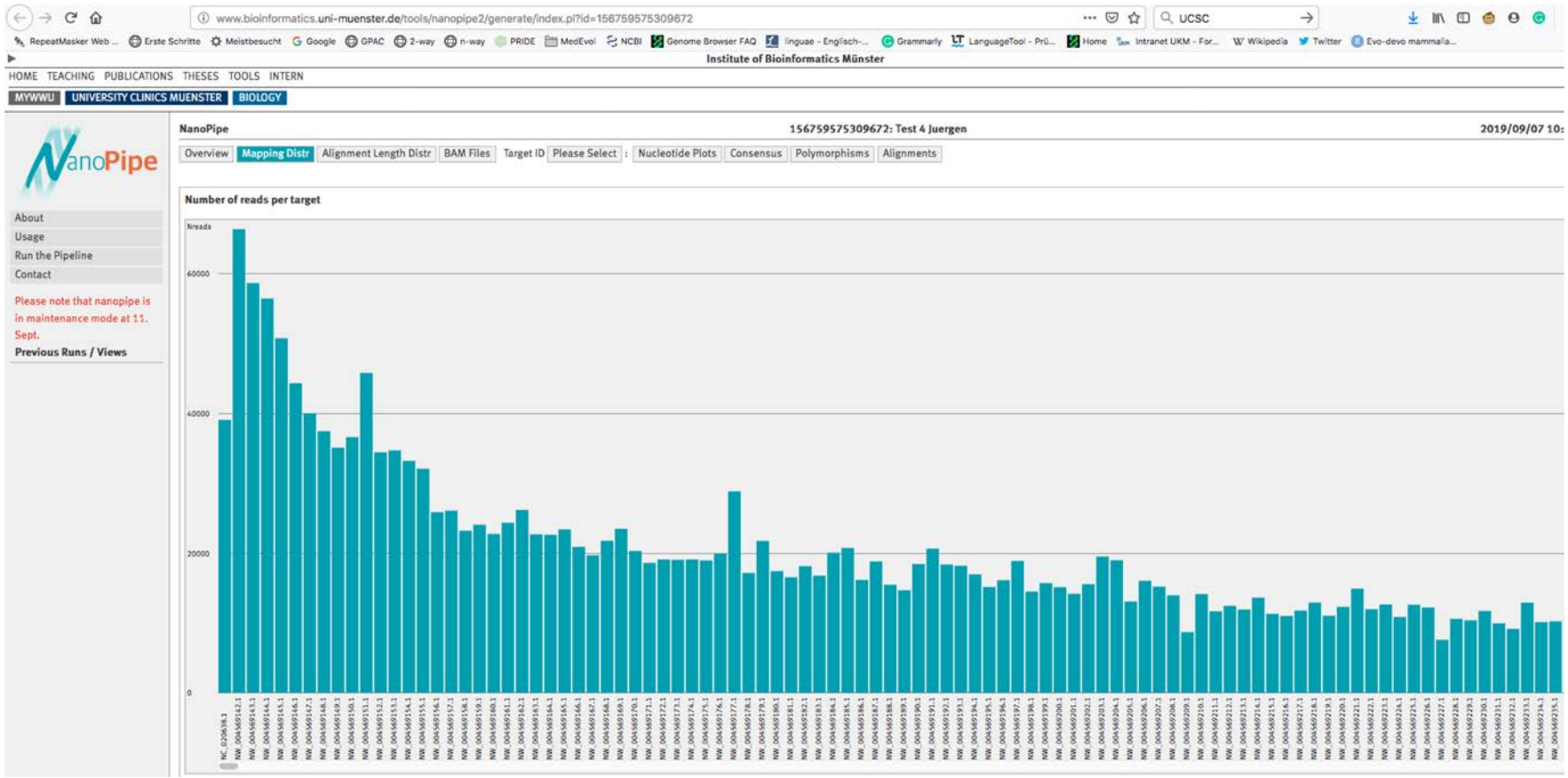
MinION analyses

NCBI



MinION analyses

NanoPipe

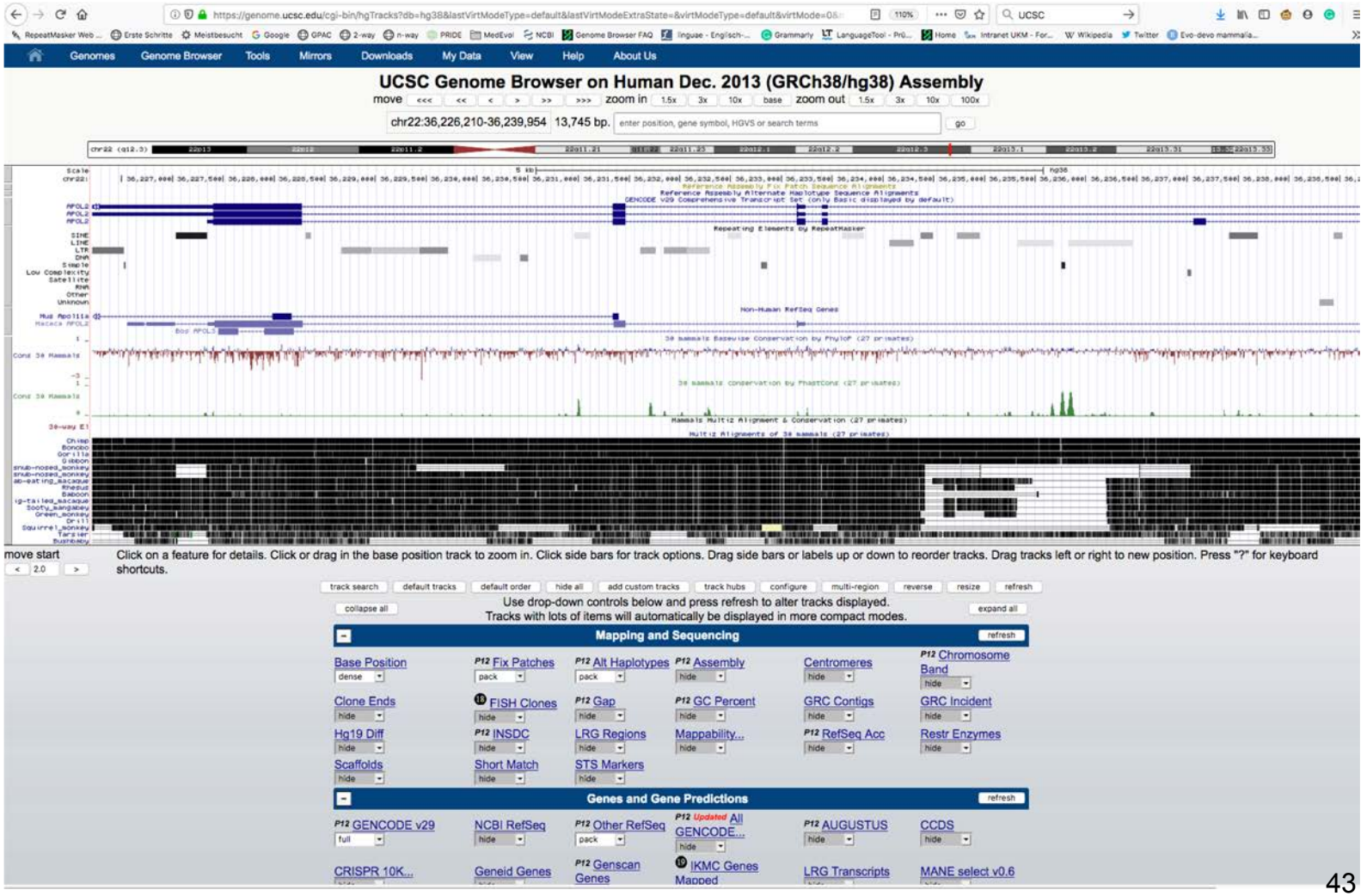


MinION analyses

PhyDE

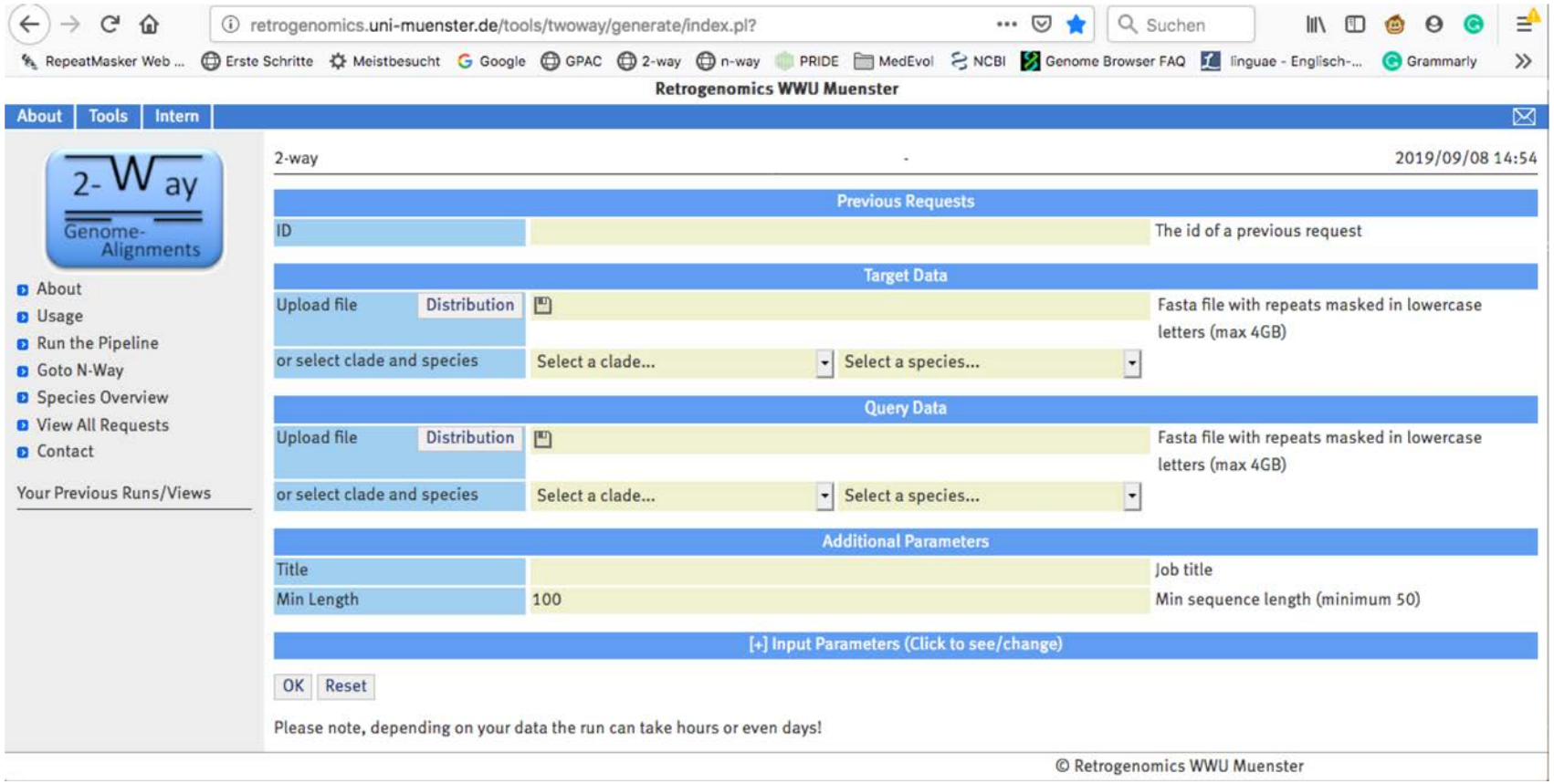
MinION analyses

UCSC comparative genomics



MinION analyses

Generate 2-way genome alignments



MinION analyses

RepeatMasking

./repeatmasker -nolow -xsmall -pa 10 -species mammalia /home/extern/coati/RM

./repeatmasker -nolow -xsmall -pa 10 -species rodentia /home/extern/guinea_pig/RM

./repeatmasker -nolow -xsmall -pa 10 -species human /home/extern/human/RM

./RepeatFinder.pl -f <input file in multiple fasta format> -i <identification name>


FUN
AHEAD
GGTTGTTTCTGTTGGTGCTGATATTGCTTAAGAAGCCAAGGC