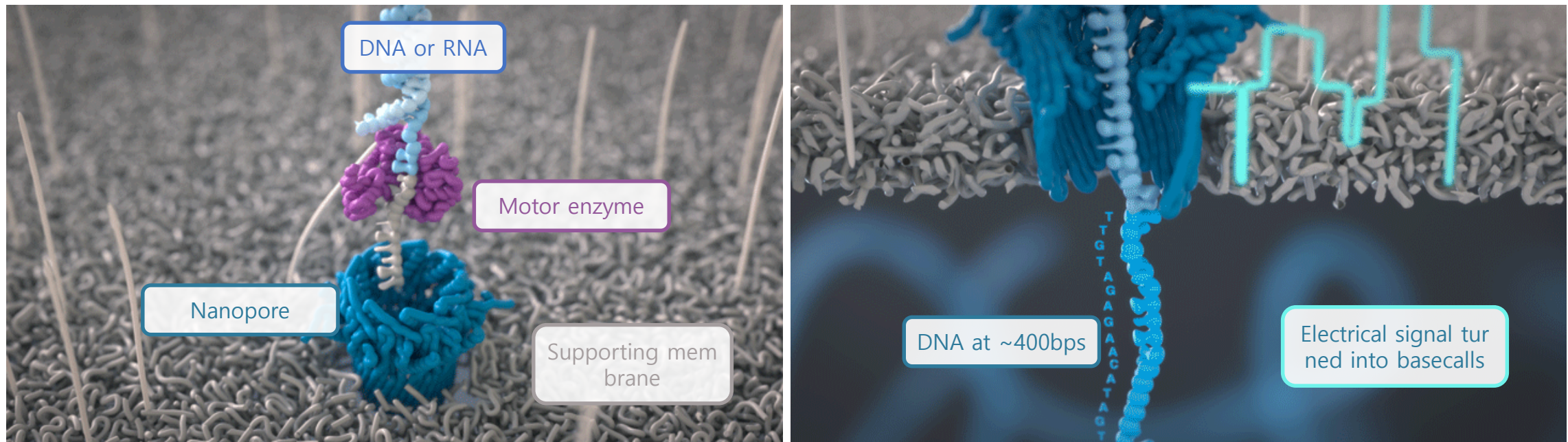


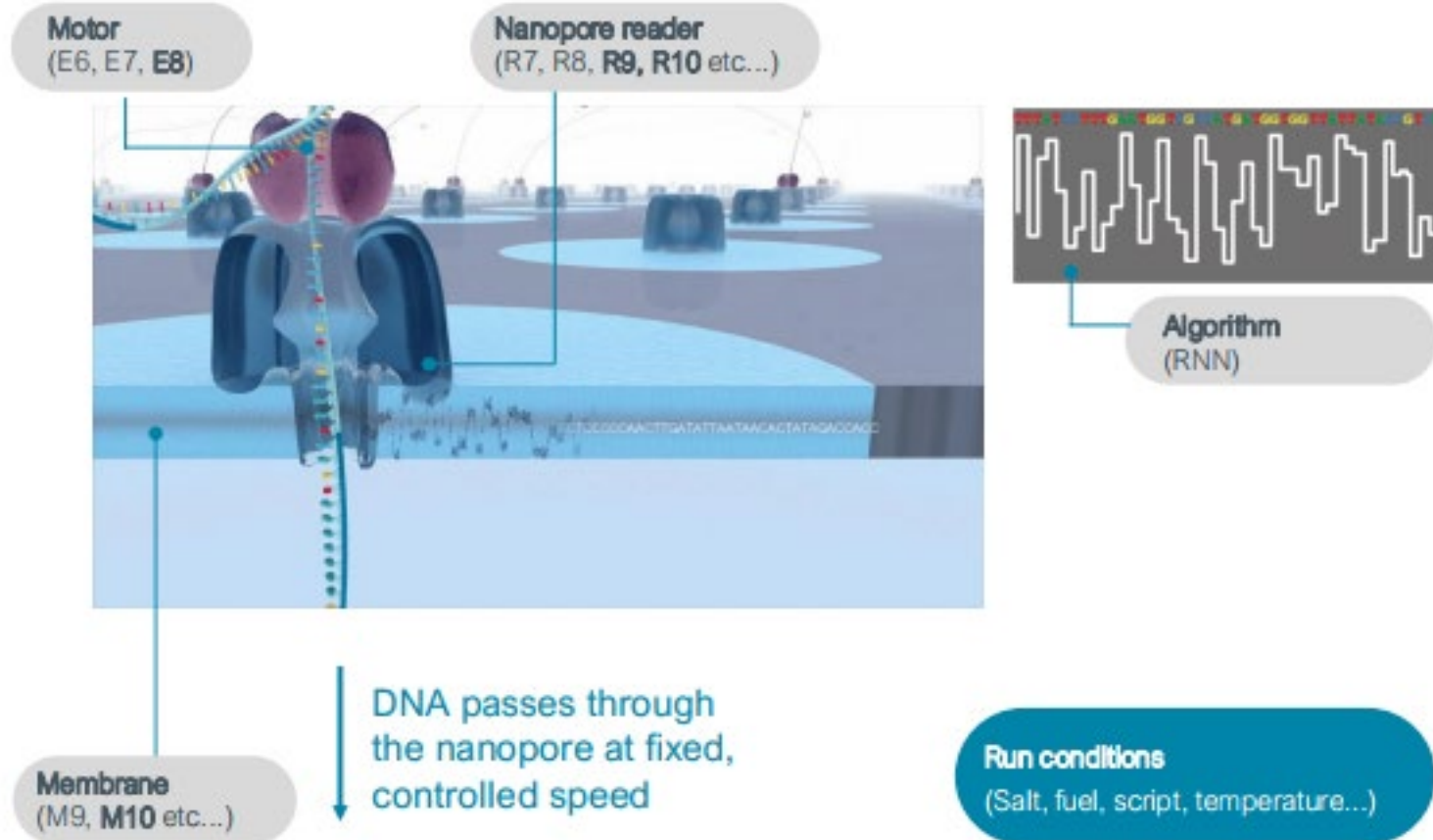
Oxford Nanopore Sequencing

Nanopore sequencing의 원리

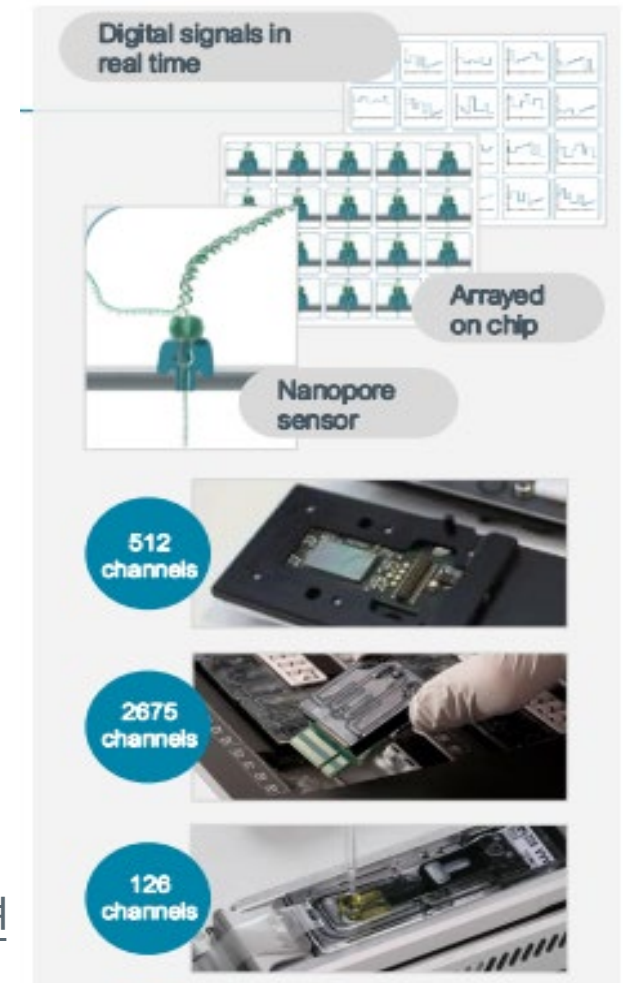


- Adapter가 결합된 DNA/RNA strand가 nanopore를 통과할 때, 각각의 nucleotide에 의해 서로 다르게 변화되는 전기적 신호를 인식하여 염기서열을 sequencing 하는 원리
- DNA의 경우 400~450 bps의 속도로, RNA의 경우 70~130 bps의 속도로 sequencing 가능

Nanopore sequencing chemistry



- Chemistry version에 따라 nanopore의 protein 구조 변화 및 화학조성의 변화로 sequencing에 대한 효율성이 개선 및 향상됨.
- 따라서 library preparation kit 및 flow cell의 호환성에 맞게 사용하여야 함



A close-up, low-angle shot of a microscope's objective lenses, which are dark and metallic. The lenses are slightly out of focus, with some text like 'HCX' and '63x' visible on them. The background is a deep blue gradient.

Plant

Genome assembly

SV and transgene resolution

Genomic methylation

Challenging to sequence using traditional technology

Because plant genomes are...

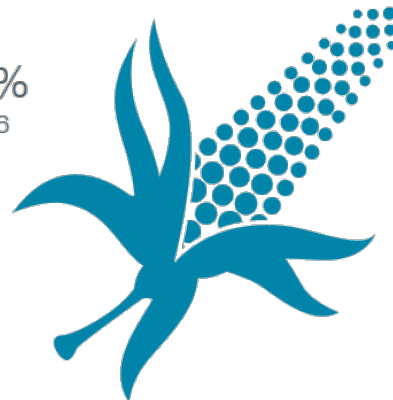
Human		3 Gb
Wheat		15 Gb
Tulip		34 Gb
Paris japonica		152 Gb

...large

...highly repetitive

Example:

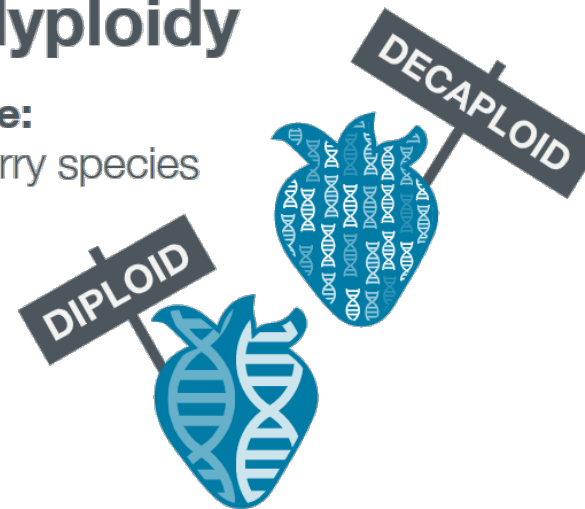
Maize over 80% repetitive DNA⁶



...polyploidy

Example:

Strawberry species



The challenges of plant sequencing

Plants are difficult to sequence and assemble using traditional technology

Very large size of genome

- 신뢰성 있는 충분한 coverage를 얻기 위해서, 식물의 경우 더 많은 양의 sequencing data가 필요함
- 기존의 Short read 기반의 sequencing으로는 genome assembly에 대한 한계가 존재함

Highly repetitive sequence

- Short read는 large repetitive regions를 cover 할 수 없으며, 따라서 잘못된 genome assembly를 진행할 수 있음
- Repetitive sequence 구간은 PCR이 잘 되지 않아, 해당 서열의 sequencing 분석에 제한이 있을 수 있음

Highly polyploid

- 식물의 경우 다배체인 경우가 대부분이며, 서로 매우 유사한 haplotype을 가지고 있기 때문에 short read로는 haplotype을 구분하는 것이 어려움.

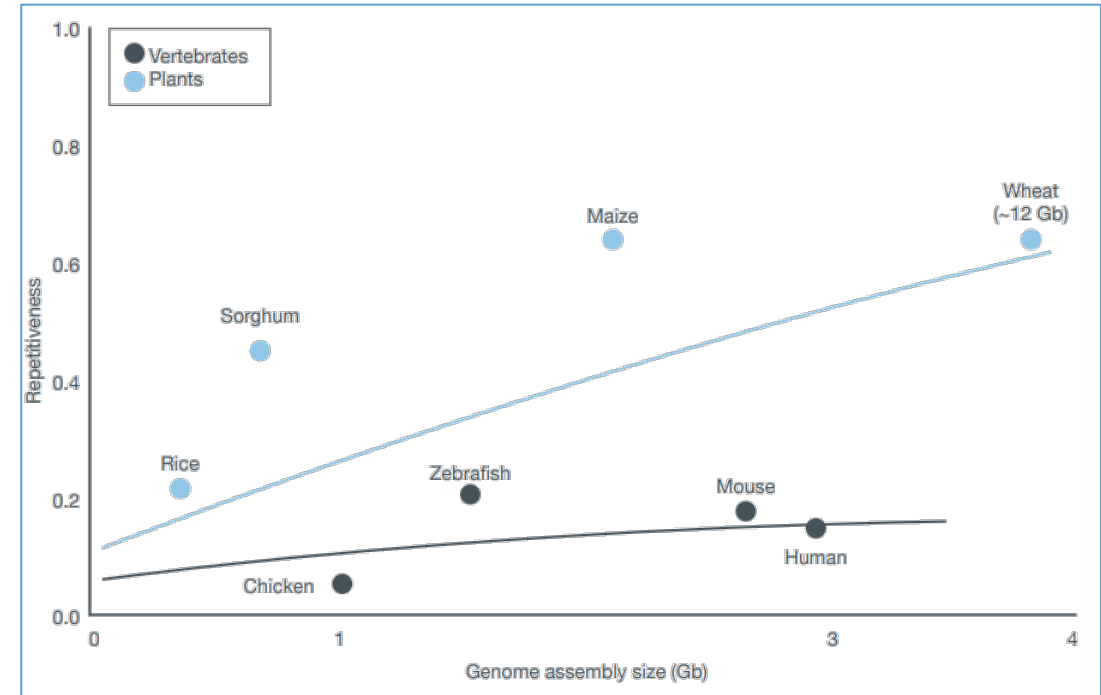


Figure: plant genomes can be large and highly repetitive, making sequencing with traditional methods challenging. Repetitive content is seen to increase with genome size.

Why use nanopore sequencing technology in plant genomics research?

Assemble highly contiguous genomes with unrestricted read length

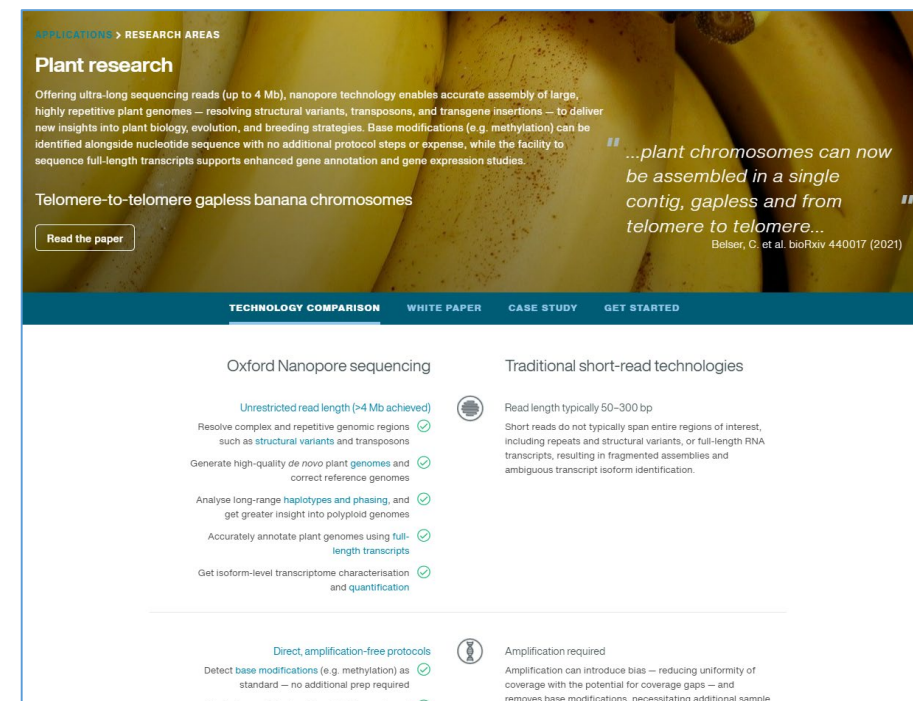
- 식물 genome에서 나타나는 large repetitive region을 완전히 cover하여 분석할 수 있음
- Chromatin conformation capture (Pore-C) 기술을 접목하여 genome assembly의 정확성과 완성도를 높일 수 있음

Comprehensively resolve genetic variation

- SV, transposon, 그리고 transgene insertion 등을 명확하게 분석이 가능
- 4 Mb 이상의 ultra long read도 sequencing이 가능하여, variant region 전체를 mapping할 수 있으며, SV, SNV를 whole genome 수준에서 식별하고, phasin까지 가능함

Simultaneously assess methylation

- 별도의 PCR 과정 없이 native DNA/RNA sequencing이 가능하며, 별도의 샘플 전처리 과정 없이 methylation 분석이 가능



Plant research

Offering ultra-long sequencing reads (up to 4 Mb), nanopore technology enables accurate assembly of large, highly repetitive plant genomes — resolving structural variants, transposons, and transgene insertions — to deliver new insights into plant biology, evolution, and breeding strategies. Base modifications (e.g. methylation) can be identified alongside nucleotide sequence with no additional protocol steps or expense, while the facility to sequence full-length transcripts supports enhanced gene annotation and gene expression studies.

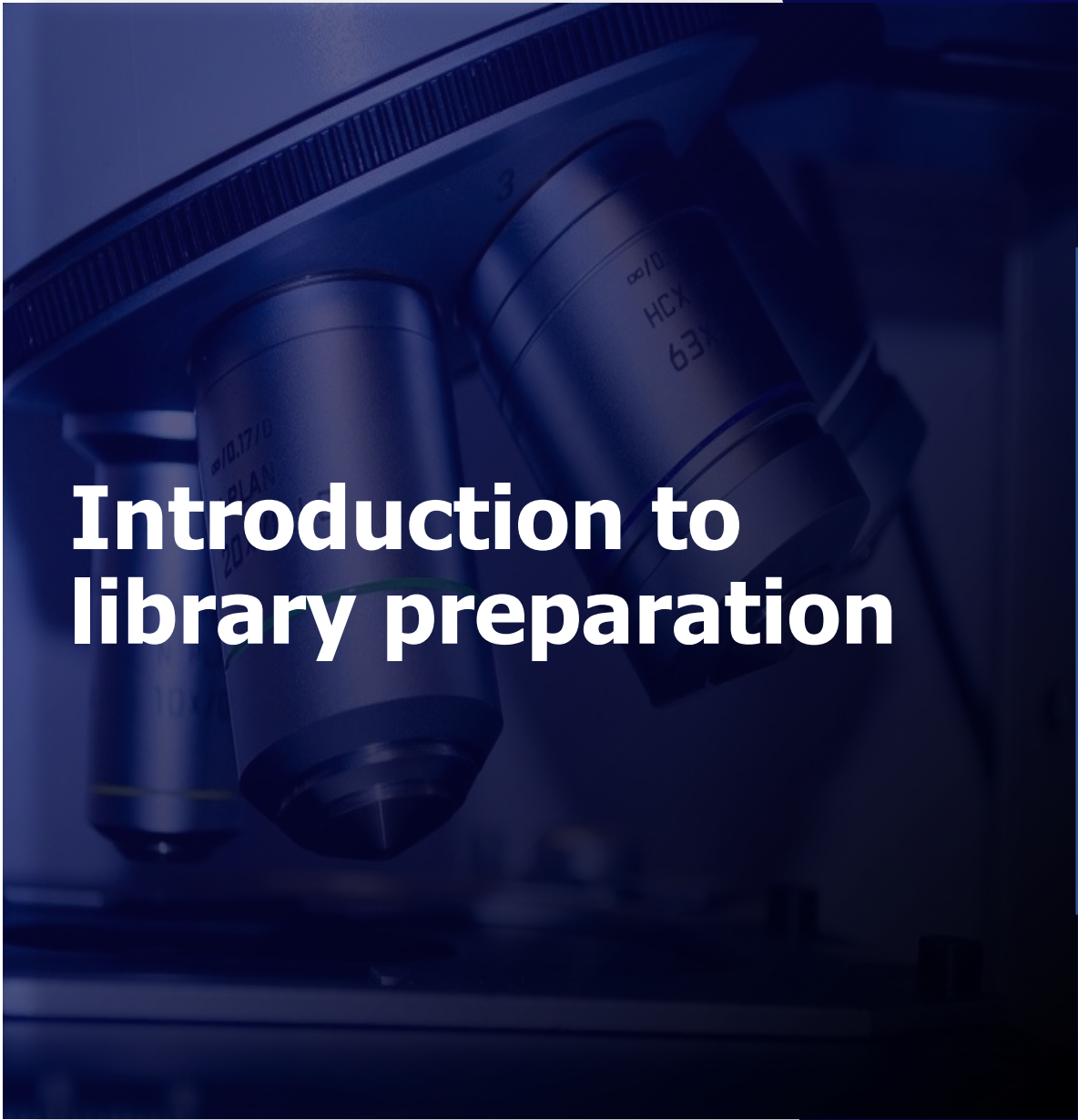
Telomere-to-telomere gapless banana chromosomes

[Read the paper](#)

“...plant chromosomes can now be assembled in a single contig, gapless and from telomere to telomere...”
Belser, C. et al. bioRxiv 440017 (2021)

TECHNOLOGY COMPARISON	WHITE PAPER	CASE STUDY	GET STARTED
Oxford Nanopore sequencing			
Unrestricted read length (>4 Mb achieved)			
Resolve complex and repetitive genomic regions such as structural variants and transposons			
Generate high-quality de novo plant genomes and correct reference genomes			
Analyse long-range haplotypes and phasing and get greater insight into polyploid genomes			
Accurately annotate plant genomes using full-length transcripts			
Get isoform-level transcriptome characterisation and quantification			
Direct, amplification-free protocols			
Detect base modifications (e.g. methylation) as standard — no additional prep required			
Traditional short-read technologies			
Read length typically 50–300 bp			
Short reads do not typically span entire regions of interest, including repeats and structural variants, or full-length RNA transcripts, resulting in fragmented assemblies and ambiguous transcript isoform identification.			
Amplification required			
Amplification can introduce bias — reducing uniformity of coverage with the potential for coverage gaps — and removes base modifications, necessitating additional sample			

Find out more at nanoporetech.com/applications/plant-research

A close-up, low-angle shot of microscope objective lenses, with the text 'Introduction to library preparation' overlaid in white. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible on them. The background is a dark blue gradient.

Introduction to library preparation



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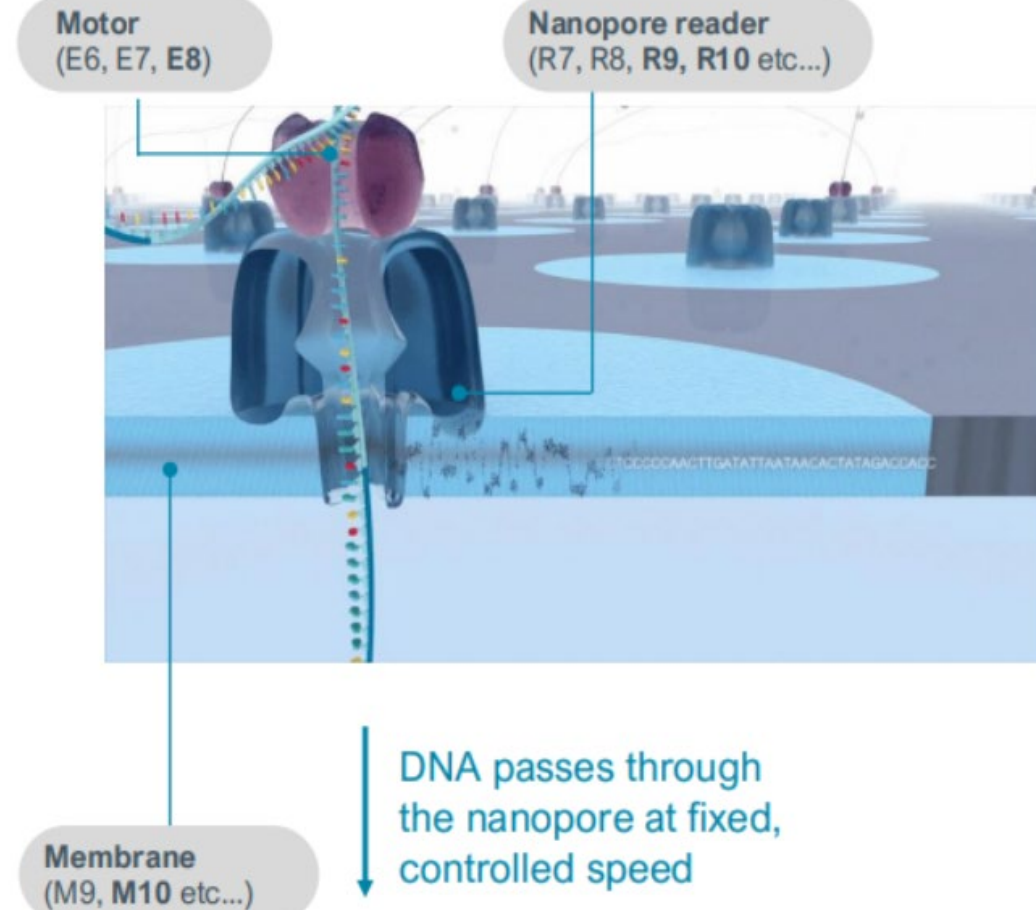
- DNA 또는 RNA 샘플을 nanopore sequencing과 호환이 가능하게 변환하는 과정

Library prep for nanopore sequencing

- Sequencing adapter를 부착하는 과정

Adapter

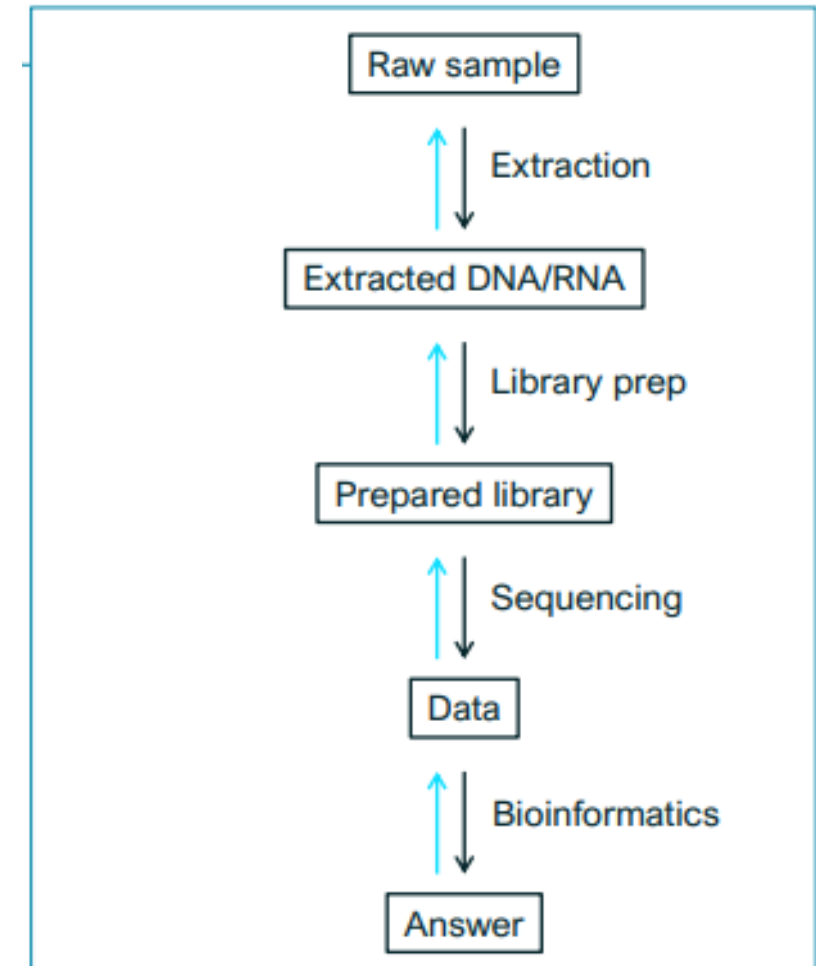
- Motor protein이 부착되어 있으며, 이 motor protein이 nanopore와 binding하여, DNA/RNA strand가 nanopore 안으로 들어가게 해줌. (Tethering)
- 5' end of DNA에 binding



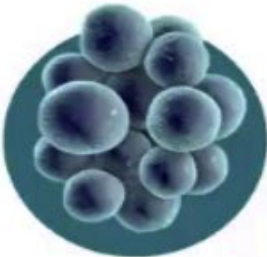
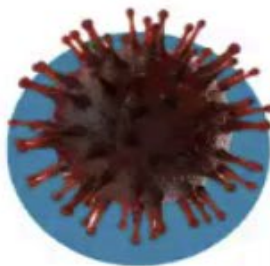
Planning a nanopore sequencing experiment

Sequencing 시에 고려해야 하는 사항

- Required sequencing depth, coverage
 - 일반적으로 신뢰성 있는 sequencing data를 얻기 위해서는 20x~50x depth, 30x~60x coverage가 필요
- Read length distribution
- Choice of library preparation protocol
 - Library preparation kit에 필요한 input sample 양
 - Sequencing을 통해 어떠한 분석을 진행할 것인지 (methylation, SNVs, SNP, InDel...)
- Sample에 따라 권장되는 nucleic acid extraction protocol



Planning a nanopore sequencing experiment

What is it? What is in it?	Is it changing? Responding?	Is it healthy or diseased?	What is the disease?	Is it pathogenic?	How does it vary from others?
 Metagenome Mixed species	 Virus Eg Influenza A 8 segments of RNA ~13.5 Kb (13,500 base pairs)	 Bacteria Eg E.coli 4.6Mb (4.6 million base pairs)	 Mammal Eg cow 3Gb x 2 (3 billion base pairs x 2)	 Human 3Gb x 2	 Crop Eg wheat 17Gb x 6

1. DNA/RNA total length: Human whole genome: ~ 3.1 Gb

2. Coverage: 신뢰성 있는 data를 얻기 위해서 ONT에서 권장하는 coverage는 30x~60x

- Human whole genome의 경우, $3.1 \text{ Gb} \times 30 = \sim 90 \text{ Gb}$
따라서, 90 Gb 이상의 data를 얻어야 신뢰성 있는 data 생성이 가능

3. Sequencing device and flow cell 선택

- Human whole genome 분석을 위해서는 90 Gb 이상의 data 생성이 가능하여야 하므로, promethION을 추천

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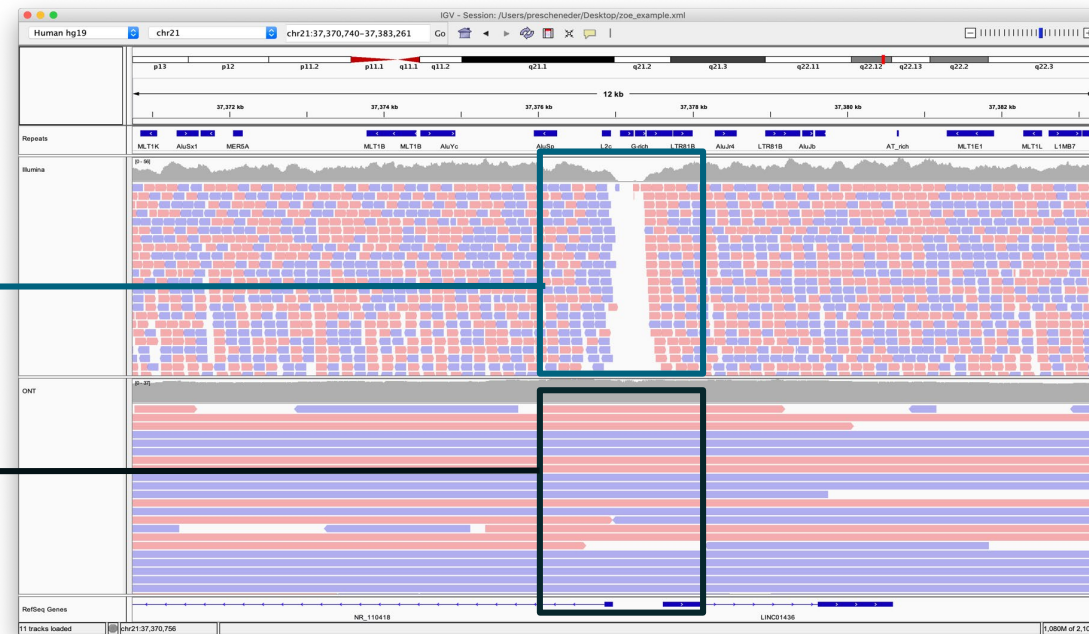
Accuracy는 genome의 coverage 측면에서 고려되어야 함 Coverage

- 특정 유전체 영역이 얼마나 많이 sequencing 되었는지를 나타내는 지표
- 일반적으로 "depth of coverage" 또는 "Sequencing depth"로 표현됨
- 특정 유전자나 유전체 영역이 sequencing 된 read의 평균 수
- Coverage가 높을수록 sequencing data의 신뢰도가 높아지며, variants 검출이 가능

Human genome의 약 8% 정도는 short read로는 sequencing이 불가능 한 것으로 추정. 하지만 이 영역에는 생물학적으로 중요한 variant가 포함되어 있음

Nanopore는 Long read sequencing을 통한 완전한 genome assembly가 가능하며, 생물학적으로 중요한 variant까지 분석이 가능함

Internal data set and ILMN data mapped to Chr.21/HG 38



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible. The background is a deep blue gradient.

Sample QC

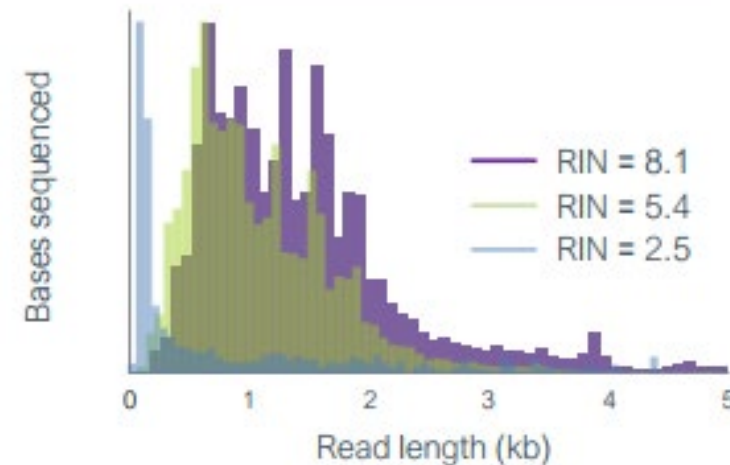
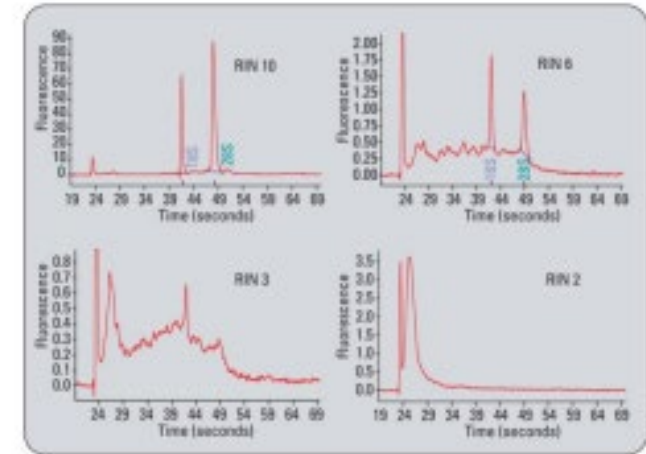
Quantification: Qubit fluorimeter

Purity: Nanodrop

- Pure RNA의 OD 측정값:
- $A260/A280 = \sim 2.0$
- $A260/A230 = \sim 2.0-2.2$

Quality: Agilent Bioanalyzer

- RNA Integrity Number (RIN) 값을 측정
- RIN 값이 높을수록 RNA quality가 좋으며, 낮은 RIN 값은 data output을 감소시킴
- $1 \leq RIN \leq 10$
- Recommendation: $7 \leq RIN$



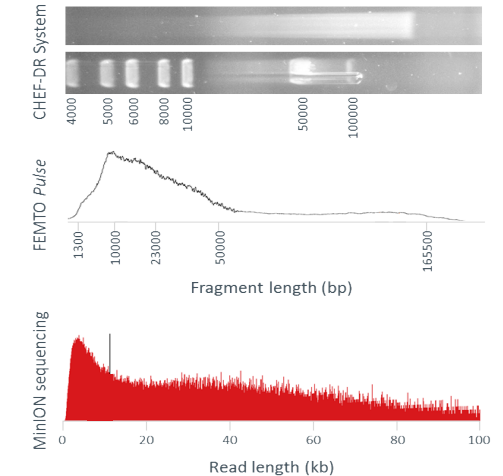
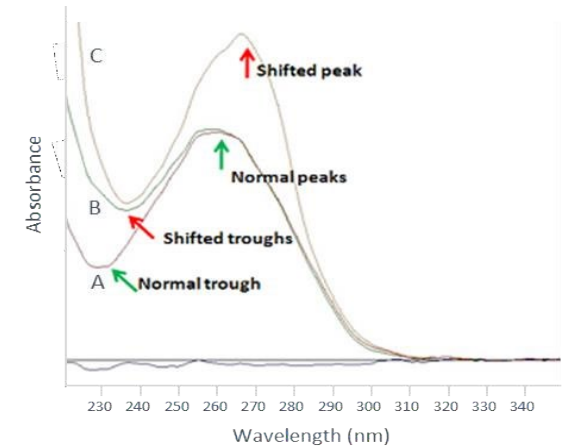
Quantification: Qubit fluorimeter

Purity: Nanodrop

- Pure DNA의 OD 측정값:
- $A_{260}/A_{280} = \sim 1.8$
- $A_{260}/A_{230} = \sim 2.0-2.2$

Quality: Standard gel-based analysis, Agilent Bioanalyzer/Tapestation

- Fragment length size 측정은 정확한 DNA 정량을 위해 필수적인 요소

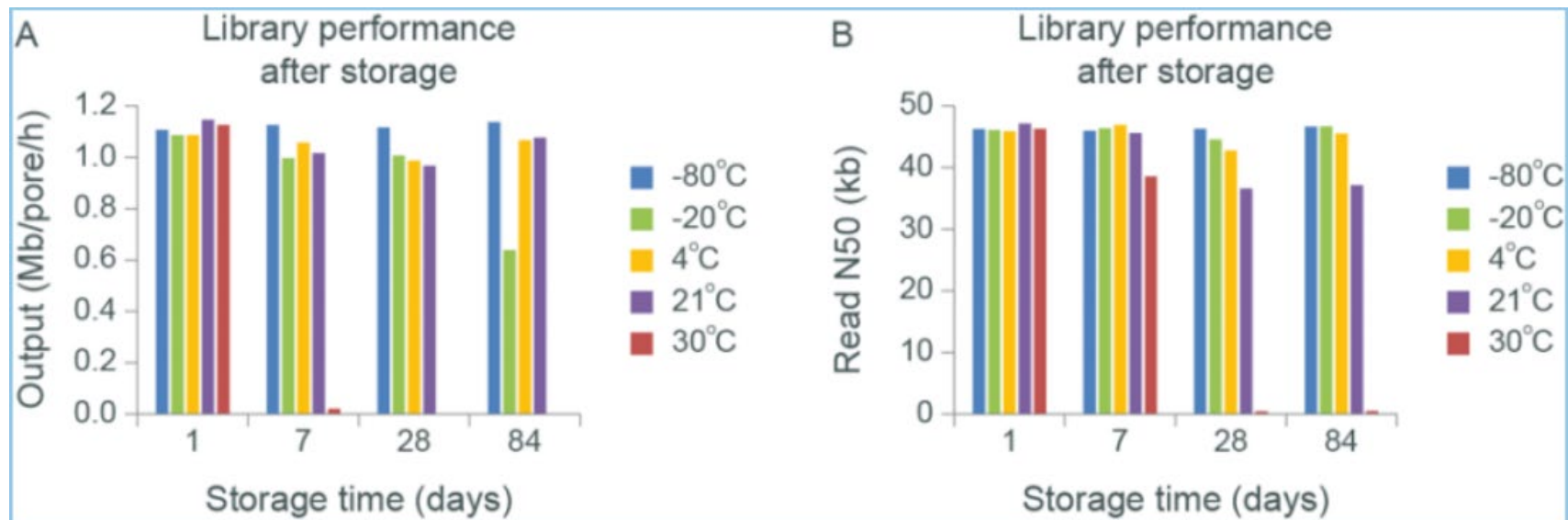


A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible on them. The background is dark and out of focus.

Library storage

Optimizing library stability during stored

- DNA library의 마지막 단계는 prepared library를 Elution buffer(EB)에서 Elution하는 단계이며, 이 단계에서 library 보관이 가능함.
- **Recommendation**
 - 단기간 보관 또는 library 재사용 시: Eppendorf DNA LoBind tube에서 4°C 보관
 - 한 번 사용하거나 또는 3 개월 이상 장기간 보관 시: -80°C 보관



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A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text visible on them, including 'HCX' and '63'. The background is dark and out of focus.

Flow cell warranty



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Nanopore sequencing flow cells



MinION / GridION Flow cell

- MinION과 GridION device와 호환 가능
- Reusable with sensor array (512 channels)
- Library loading 양: 75 ul
- Pore: R10.4/RNA
- Output: 20 Gb +



PromethION Flow cell

- PromethION 장비 (P2 solo, P2I, P24 & P48) 호환 가능
- Reusable with sensor array (2675 channels)
- Library loading 양: 200 ul
- Pore: R10.4/RNA
- Output: 100 Gb +

Warranty 기간

- MinION/GridION flow cell (FLO-MIN114): 납품 후 12주 이내, flow cell QC check 진행
- PromethION flow cell (FLO-PRO114M): 납품 후 12주 (3개월) 이내, flow cell QC check 진행

** RNA version일 경우[MinION/GridION flow cell (FLO-MIN004RA), PromethION flow cell (FLO-PRO004RA)]:
납품 후 4주 (1개월) 이내, flow cell QC check 진행

Flow cell	Minimum number of active pores covered by warranty
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible on them. The background is dark and out of focus.

Flow cell priming and handling

Flow cell light shield

Maximizing the output

- The light shield는 flow cell에 포함이 되어 있으며, light shield를 부착하면 sequencing의 output이 3배 정도 증가 됨.
- 최대의 Data output을 얻기 위해서는 Sequencing을 시작하기 전에 반드시 flow cell에 light shield를 부착하는 것이 필수적임

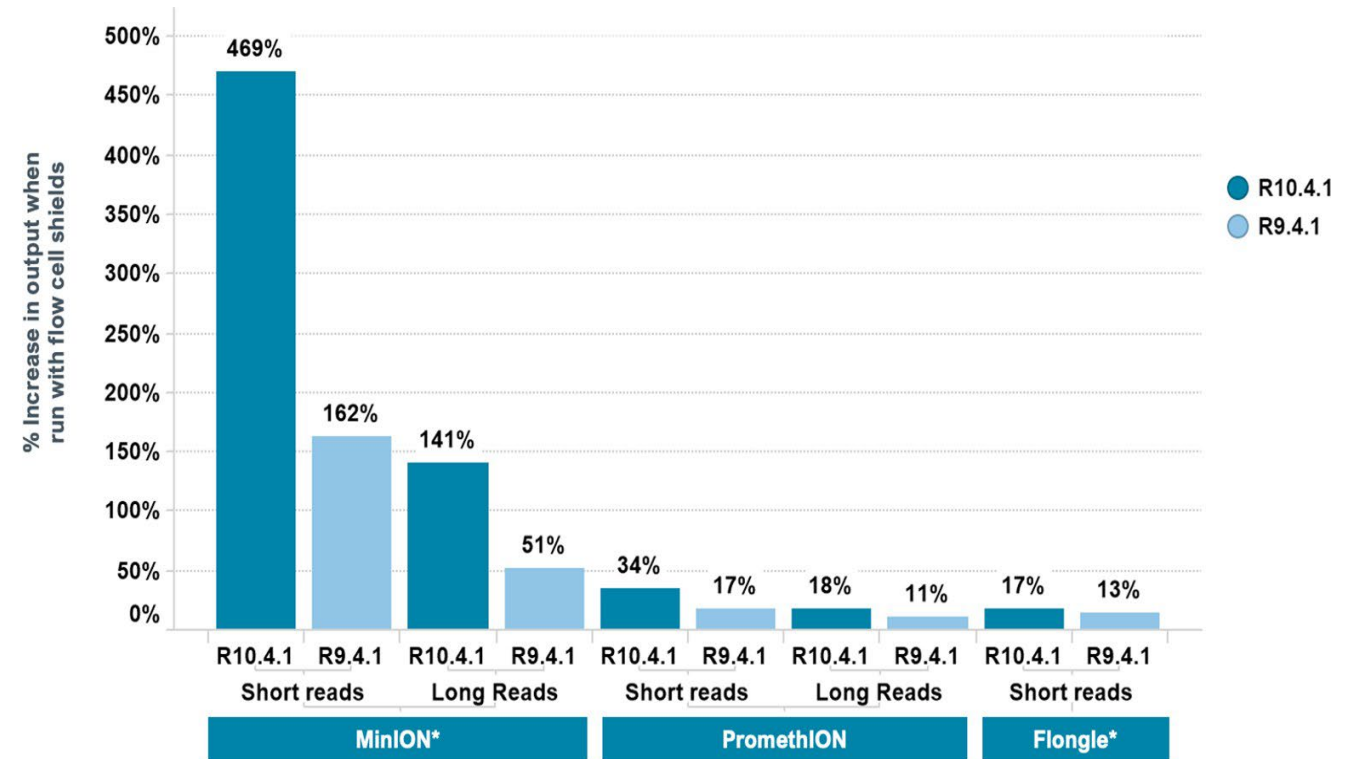


Figure: Example % Increase in output from flow cells where the array is shielded from light during sequencing in internal experiments. R10.4.1 and flow cells with short fragment libraries observe the most benefit to running in the dark. Short reads = 200bp amplicon. Long reads = 30Kb N50 human native DNA. Samples prepped with SQK-LSK114 (R10.4.1) or SQK-LSK110 (R9.4.1) reagents. Shielding of light with flow cell light shields. *MinION and Flongle Flow Cells were run on a GridION device.

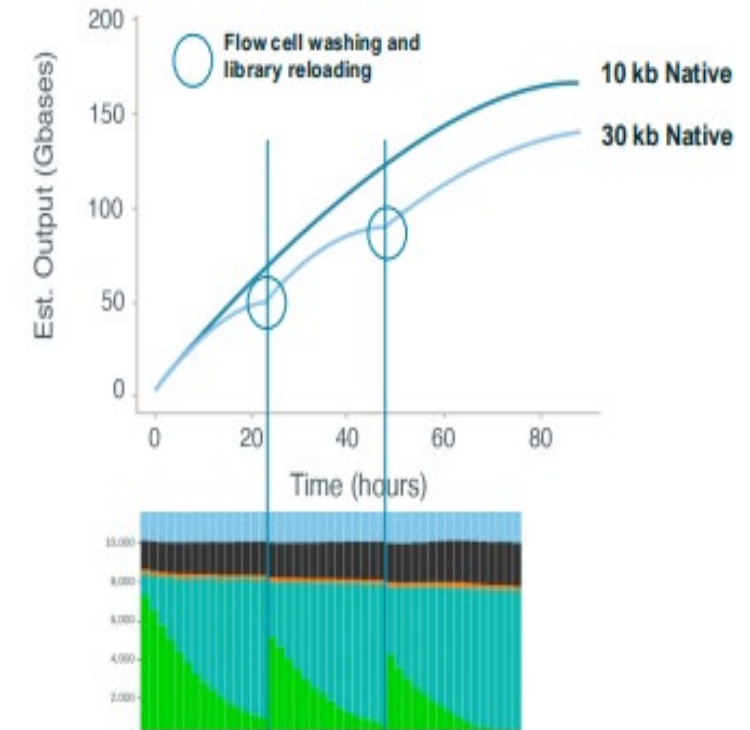
Flow cell washing

- Flow cell을 최대로 사용하여, 최대의 data output을 얻기 위해 진행함.

Flow cell washing kit

- Library strand를 분해하는 nuclease로 flow cell washing을 수행하게 되면 막힌 pore에 존재하는 최대 99.9%의 DNA template가 제거되며, 따라서 동일한 flow cell에 새로운 library를 loading하고 다시 sequencing을 수행할 수 있게 됨.
- DNA에만 적용이 가능하며, RNA의 경우 washing kit은 별도로 존재하지 않음. 따라서 RNA의 경우, flow cell에 한번 library를 loading하면 재사용이 불가능 하기 때문에, 72시간까지 sequencing을 running하여 최대의 data output을 내야함

Washing the FC increases the output of longer reads libraries

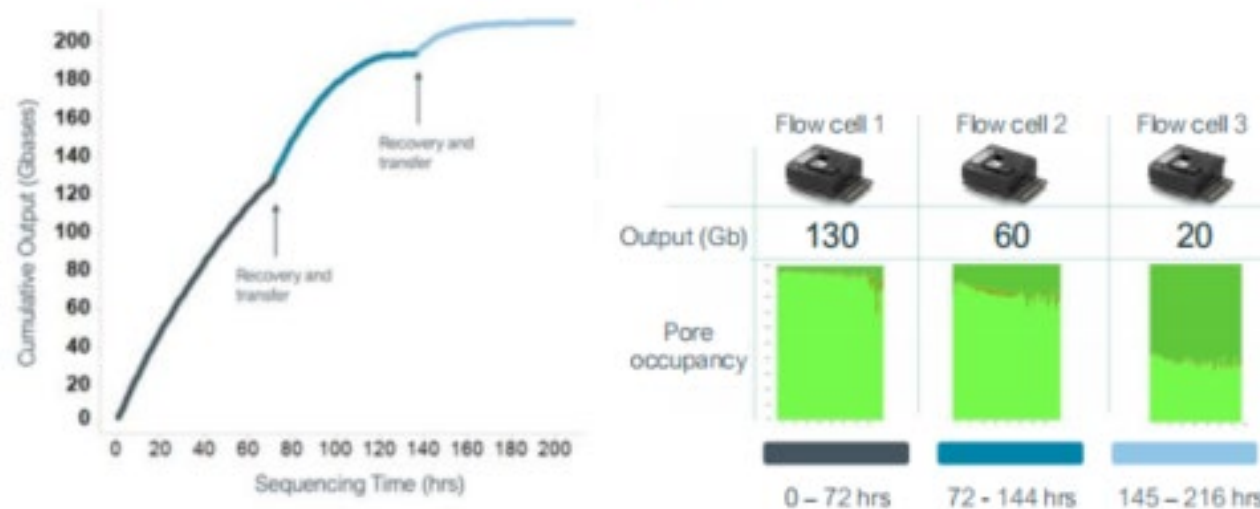


Recover DNA library and reuse it

하나의 Library를 이용하여 sequencing output을 극대화

- Run – Recover library – 새로운 flow cell에 run
- Sequencing을 진행할 수 있는 sample이 한정적일 때 사용
- Flow cell이 sequencing 도중 문제가 발생하거나, flow cell loading 중에 문제가 발생한 경우
- MinION 및 PromethION flow cell에서 적용 가능
- Oxford nanopore 사의 DNA library preparation kit으로 생성된 모든 library에 적용이 가능

Output of a single DNA library across 3 PromethION Flow Cells



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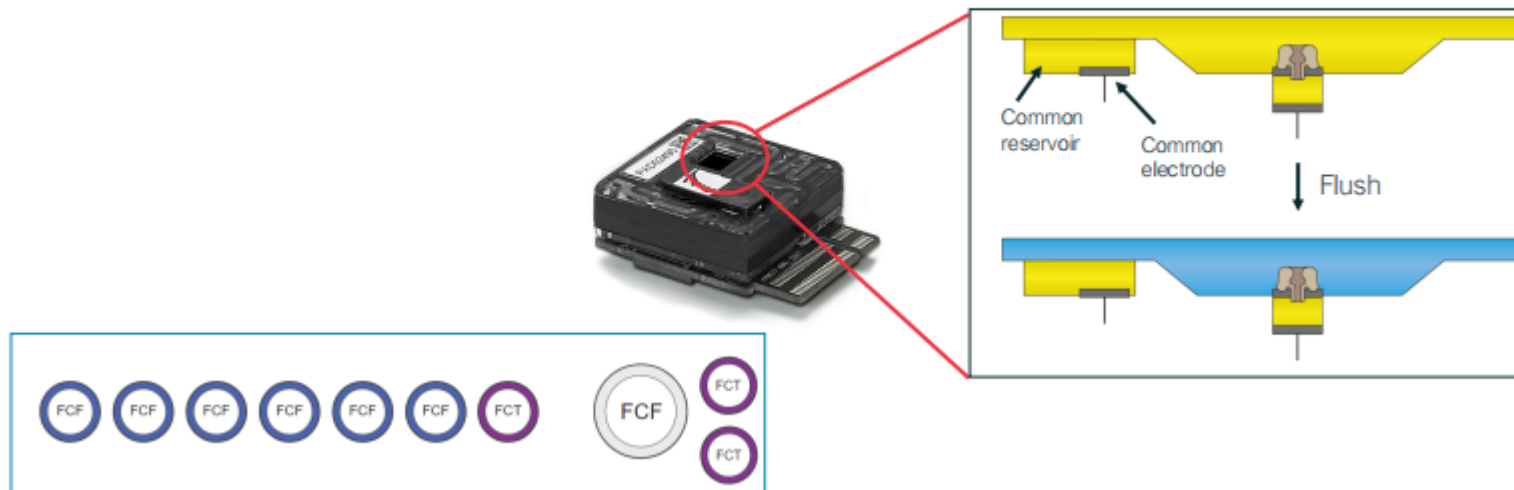
Priming a flow cell

Priming

- 새로운 flow cell에는 storage buffer가 포함되어 있음. 따라서 library를 loading 하기 전에, sequencing에 필요한 효소 및 기타 화학 물질을 활성화시켜 sequencing에 필요한 환경을 조성하기 위해 수행

Priming mixture

- Flush buffer와 tether를 혼합하여 조성됨



Priming and loading the MinION and GridION Flow cell

1. Ligation sequencing kit reagents

Label	Full name
LIB	Library Bead
LIS	Library Solution
FCF	Flow Cell Flush
FCT	Flow Cell Tether

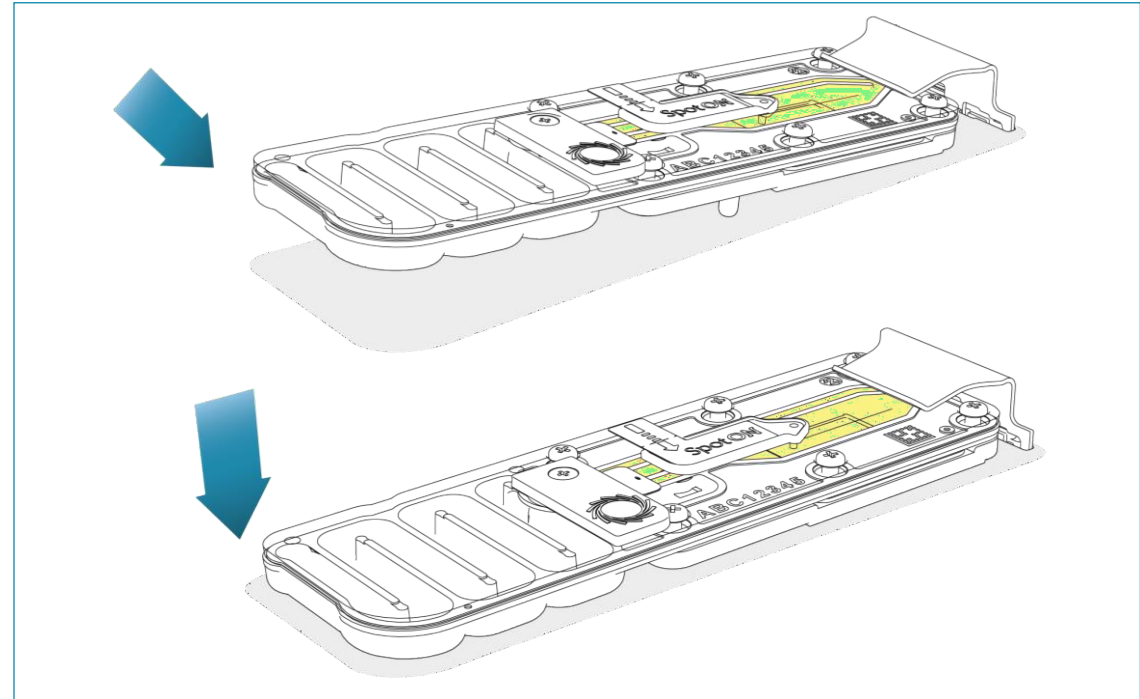
Recommend using Library beads (LIB) for most sequencing
However, the Library solution (LIS) is available for more
viscous libraries

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 μ l
Bovine Serum Albumin (BSA) at 50 mg/ml	5 μ l
Flow Cell Tether (FCT)	30 μ l
Total volume	1,205 μl

2. MinION and GridION Flow cell

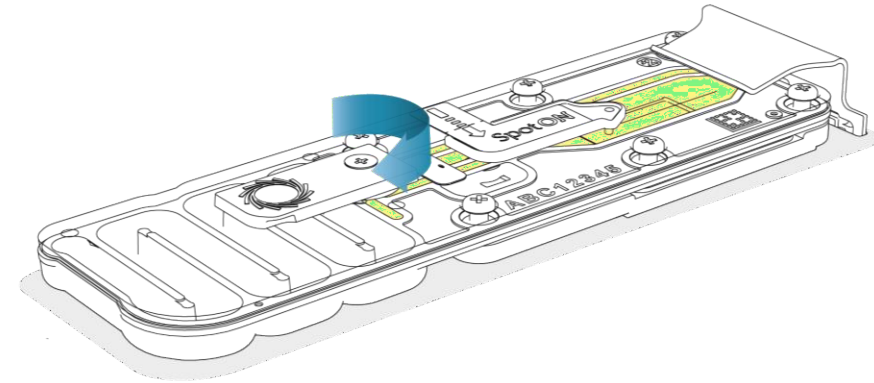
2. How to priming and loading flow cells

1. Insert the flow cell into the device



2. How to priming and loading flow cells

2. Slide open the priming port 90°



2. How to priming and loading flow cells

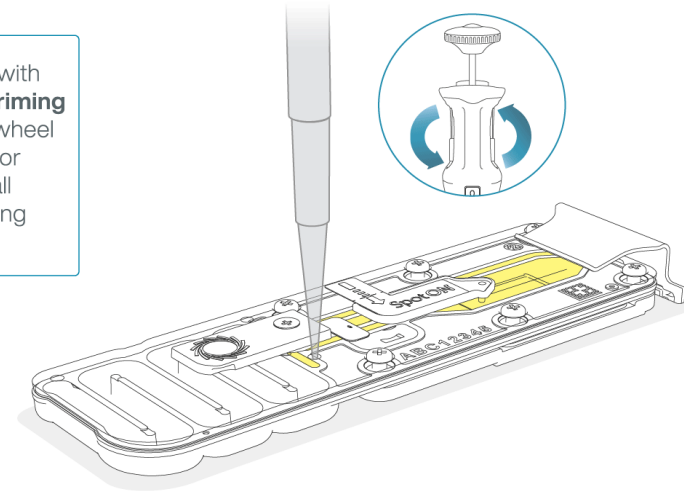
3. Remove the air bubble

1. Set a P1000 pipette to 200 μ l
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 μ l, to draw back 20-30 μ l, or until you can see a small volume of buffer entering the pipette tip

Note: Visually check that there is continuous buffer from the priming port across the sensor array.

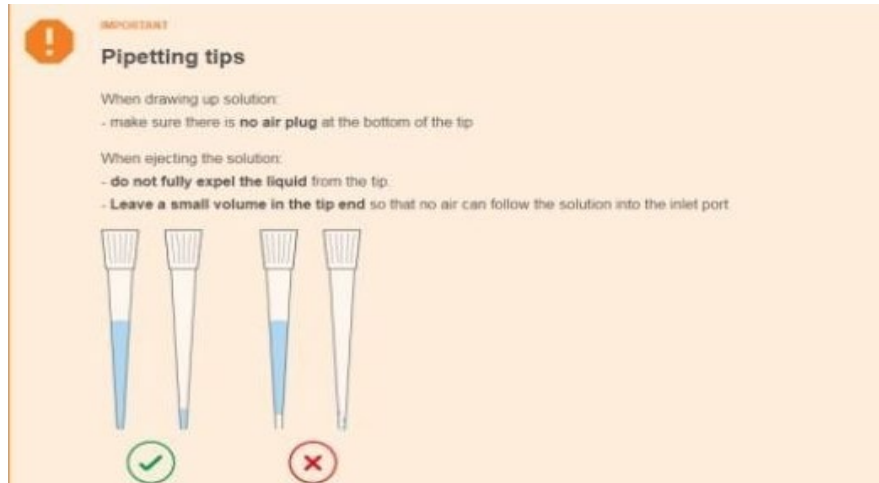
3

Insert a P1000 pipette with an empty tip into the **Priming port**. Turn the pipette wheel to draw back 20-30 μ l or until you can see a small volume of buffer entering the pipette tip.



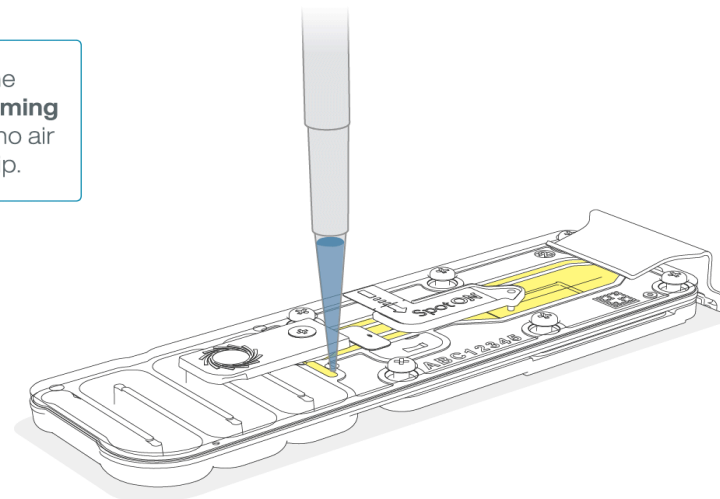
How to priming and loading flow cells

4. Load 800 μ l of priming buffer through the priming port. Wait 5 min before proceeding



4

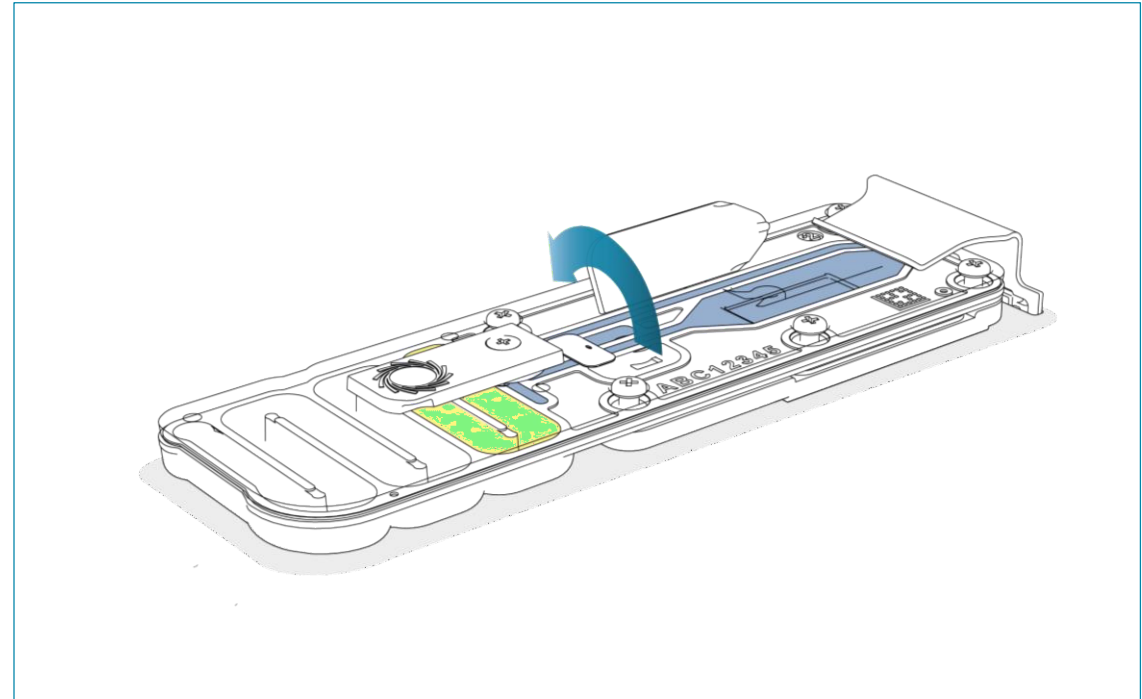
Slowly load 800 μ l of the priming mix into the **Priming port**. Ensure there are no air bubbles in the pipette tip.



Wait 5 minutes before proceeding to the next step.

2. How to priming and loading flow cells

5. Open the SpotOn port

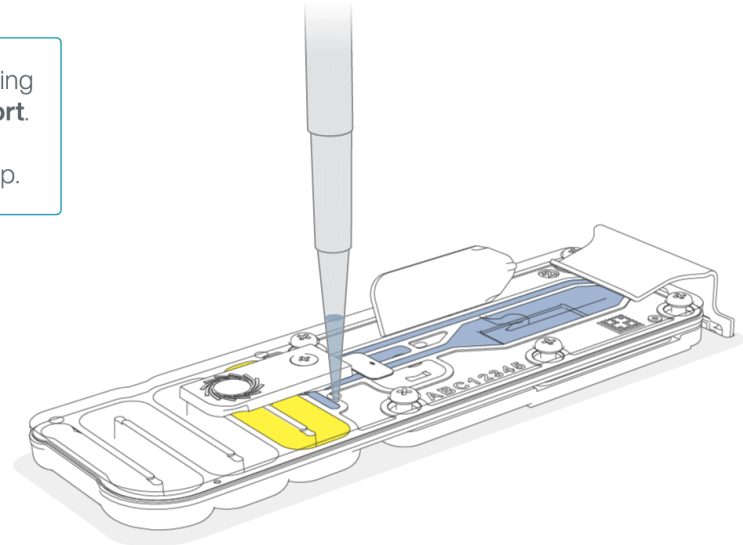


2. How to priming and loading flow cells

6. Load 200 μ l of priming buffer through the priming port

6

Load 200 μ l of the priming mix into the **Priming Port**. Ensure there are no air bubbles in the pipette tip.



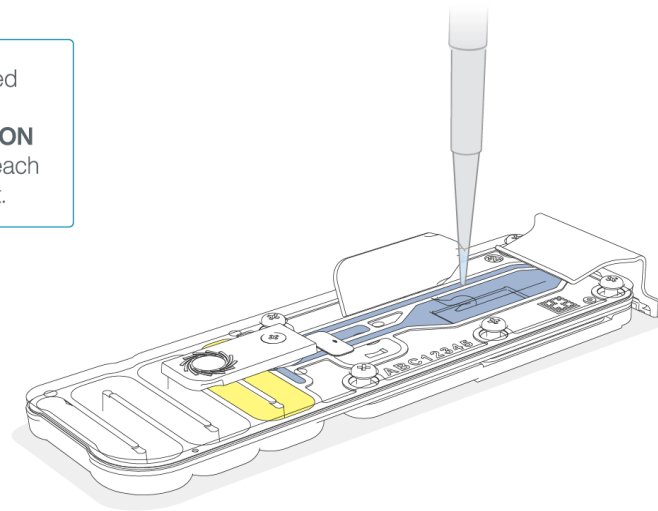
2. How to priming and loading flow cells

Reagent	Volume per flow cell
Sequencing Buffer (SB)	37.5 μ l
Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 μ l
DNA library	12 μ l
Total	75 μl

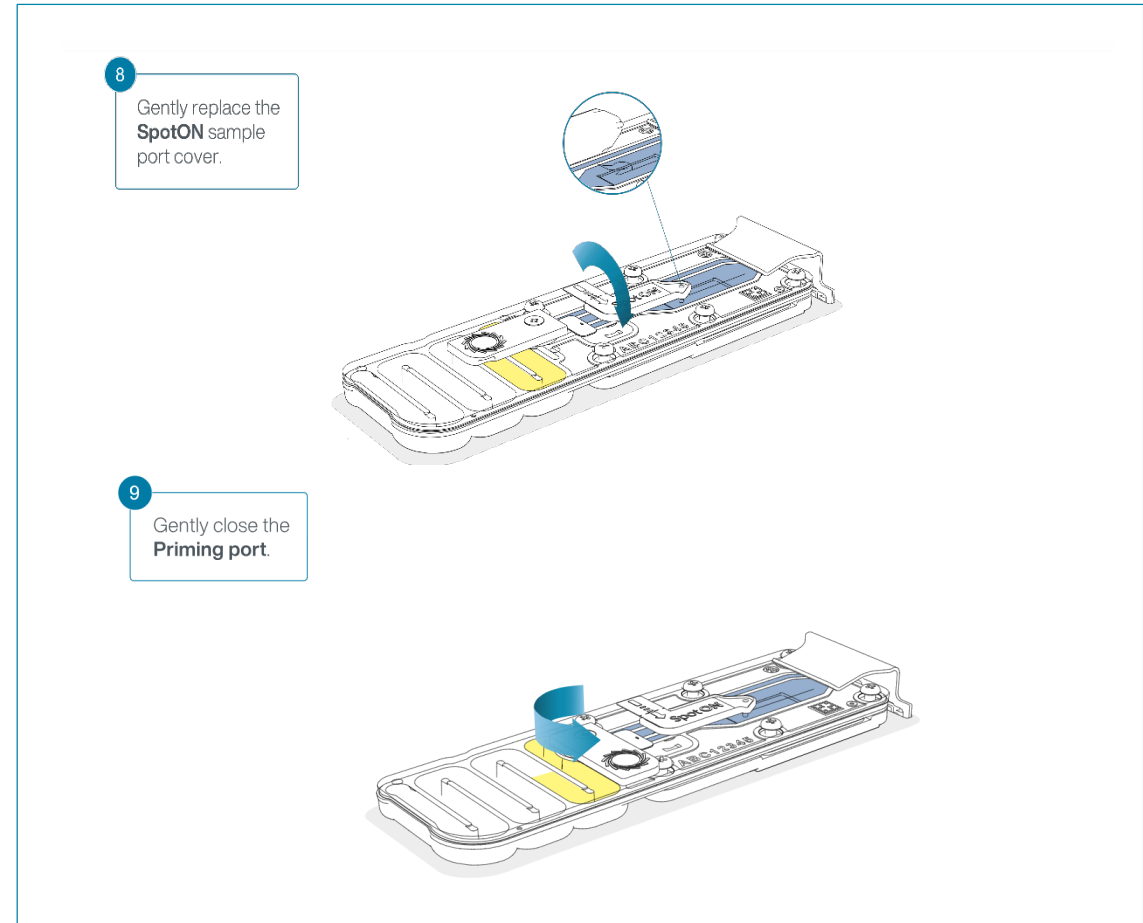
7. Load 75 μ l of sequencing mix through the SpotON port **dropwise**

7

Pipette mix the prepared library and load 75 μ l dropwise into the **SpotON** sample port, ensuring each drop flows into the port.



2. How to priming and loading flow cells



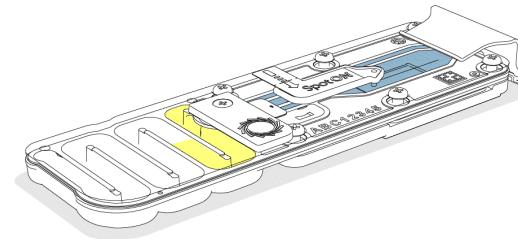
8. Gently replace the SpotON sample port cover and priming port

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2. How to priming and loading flow cells

10

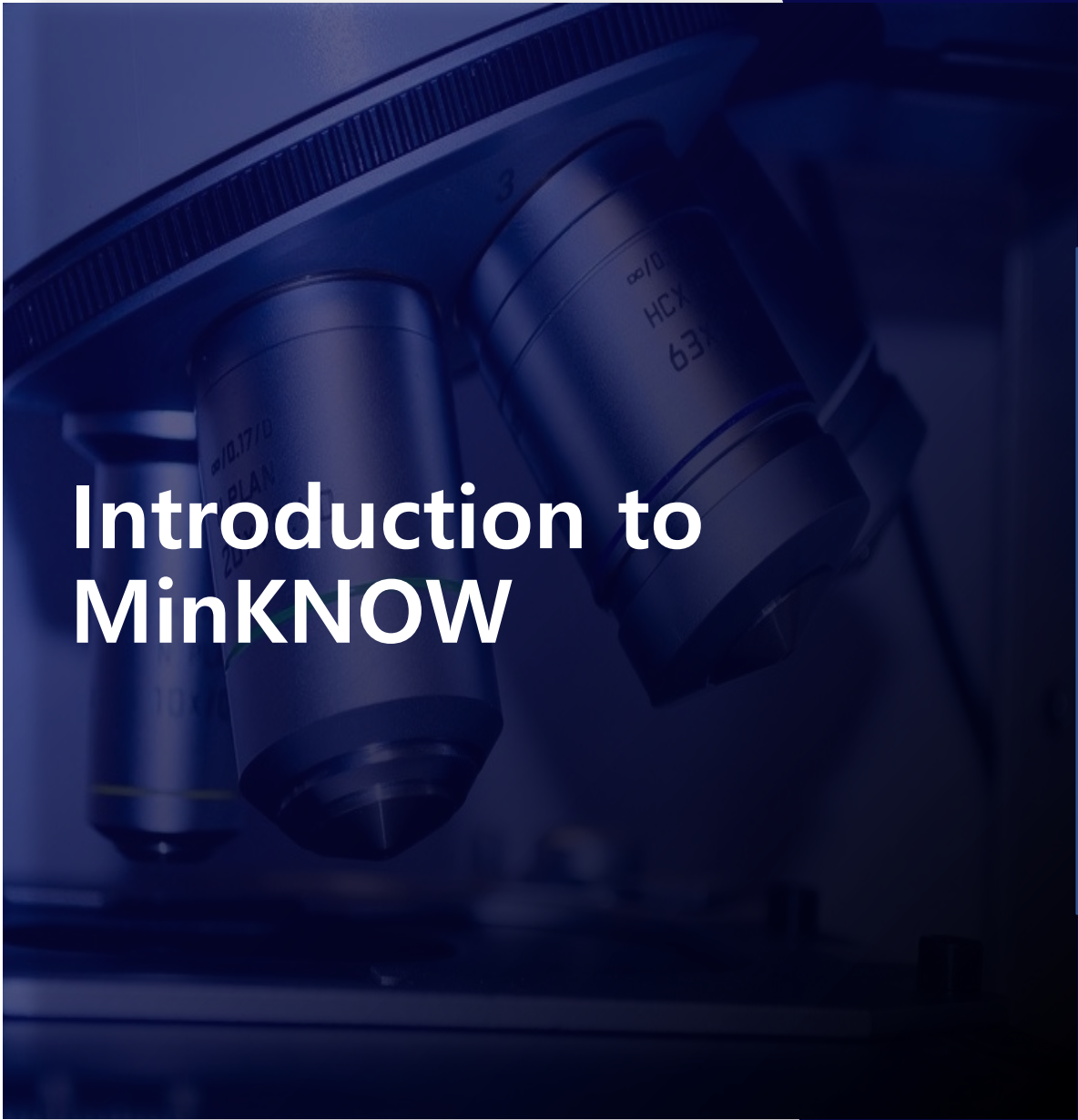
Carefully align the **light shield** against the clip and lower onto the flow cell.



The MinION Flow cell Light Shield is not secured to the flow cell and careful handling is required after installation!!

9. Install the light shield on flow cell

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A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible on them. The background is a deep blue gradient.

Introduction to MinKNOW



www.philekorea.kr

Device control

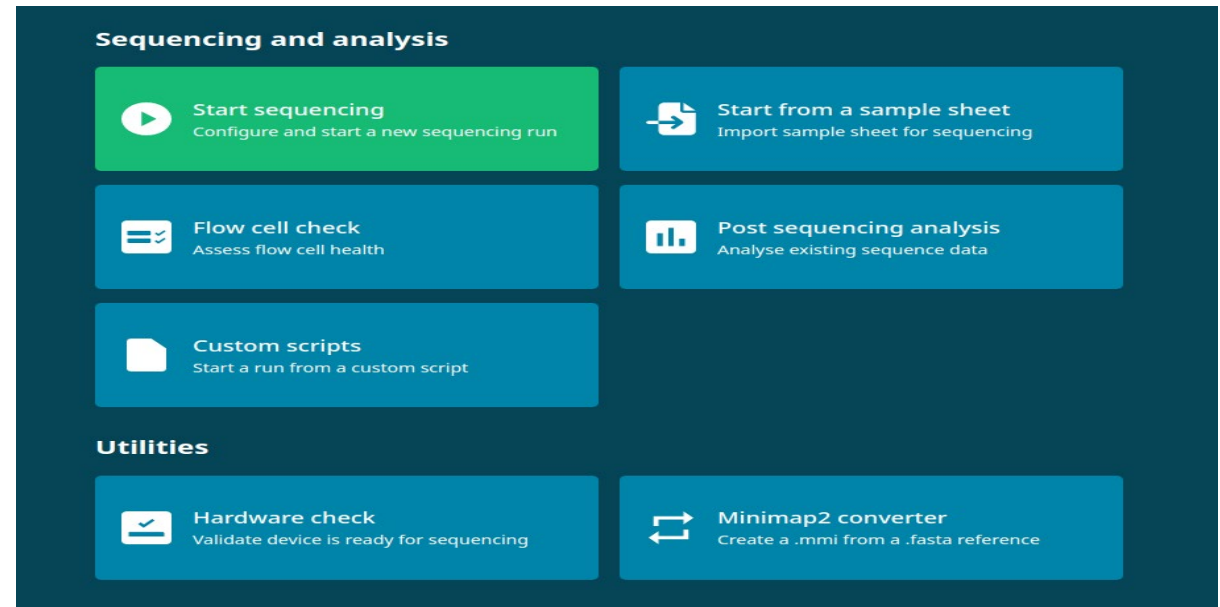
Data collection

Live basecalling

Analysis

호환성

- MinION, GridION 또는 PromethION device를 구동할 수 있는 software
- 운영 체제: Windows/MacOS/Linux



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Device control

Data collection

Live basecalling

Analysis

4가지 주요 기능

1. Hardware check

- Software와 device의 작동에 이상유무를 확인

2. Flow cell check

- Flow cell의 membrane integrity와 pore수를 확인

3. Start sequencing

- Sequencing run의 step 및 start 진행

4. Analysis

- Post-run base-calling, de-multiplexing, alignment를 진행

Sequencing and analysis



Start sequencing

Configure and start a new sequencing run



Start from a sample sheet

Import sample sheet for sequencing



Flow cell check

Assess flow cell health



Post sequencing analysis

Analyse existing sequence data



Custom scripts

Start a run from a custom script

Utilities



Hardware check

Validate device is ready for sequencing



Minimap2 converter

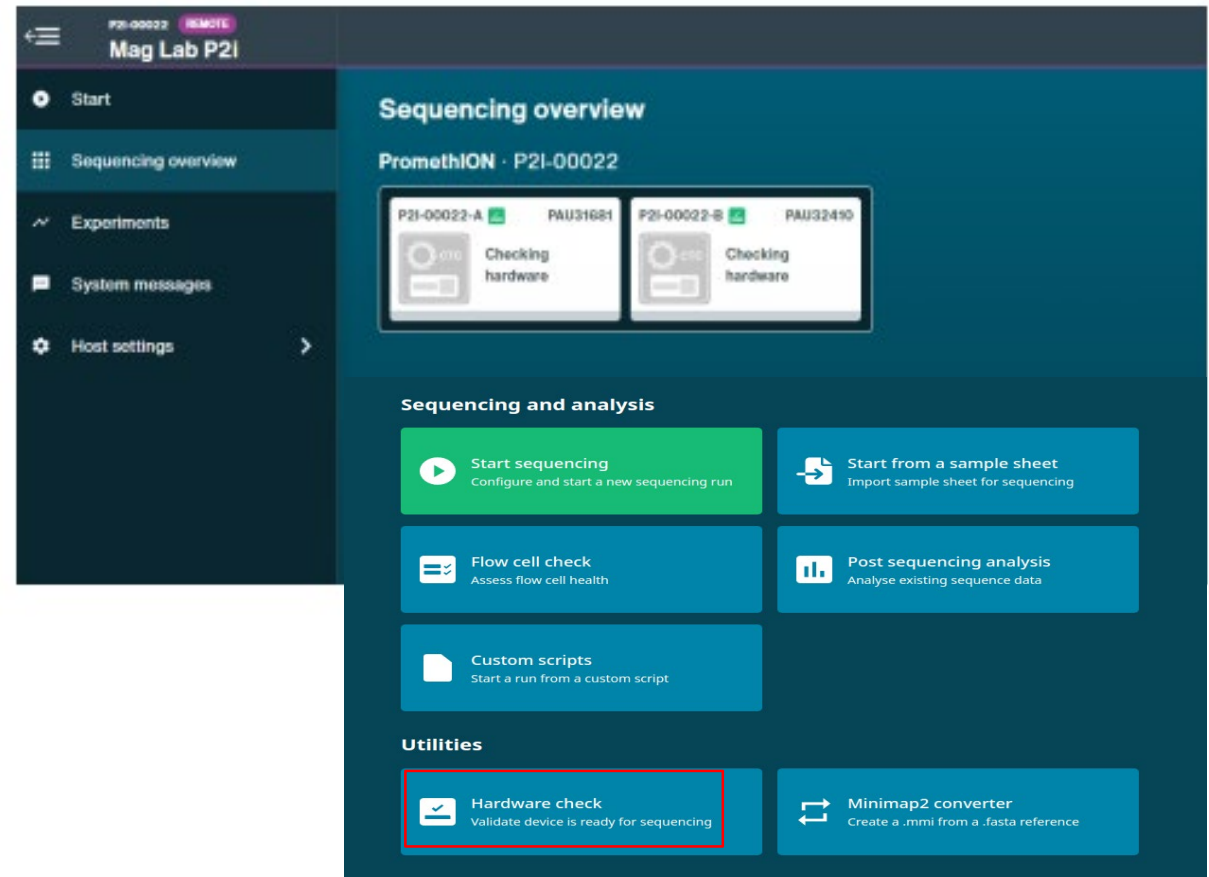
Create a .mmi from a .fasta reference

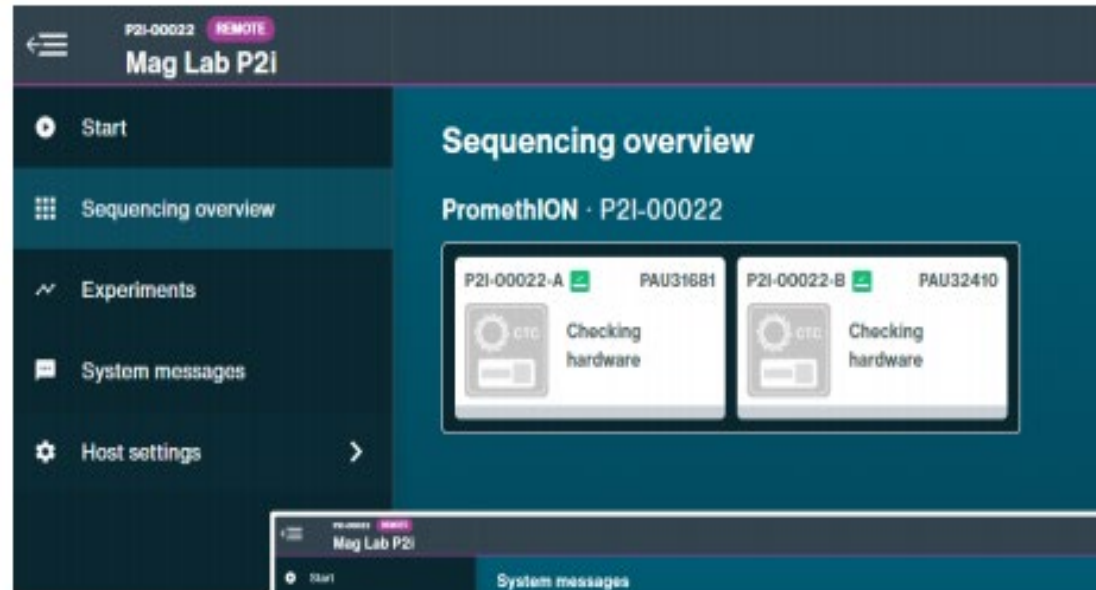
A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible. The background is a deep blue gradient.

Hardware check

Hardware check 과정

1. Hardware check 아이콘 클릭
2. CTC를 device에 삽입 (하얀색)
3. MinKNOW software에서 device 각각의 position에 CTC를 인식
4. "Start" button 클릭





Hardware check 과정

1. Hardware check 시 1분 정도 시간이 소요됨
2. 이상이 없다면 position에 초록색 check 표시가 나타남 (Hardware check passed)



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text visible on them, including 'HCX' and '63'. The background is dark and out of focus.

Flow cell check

Flow cell check

Sequencing and analysis

-  **Start sequencing**
Configure and start a new sequencing run
-  **Start from a sample sheet**
Import sample sheet for sequencing
-  **Flow cell check**
Assess flow cell health
-  **Post sequencing analysis**
Analyse existing sequence data
-  **Custom scripts**
Start a run from a custom script

Utilities

-  **Hardware check**
Validate device is ready for sequencing
-  **Minimap2 converter**
Create a .mmi from a .fasta reference



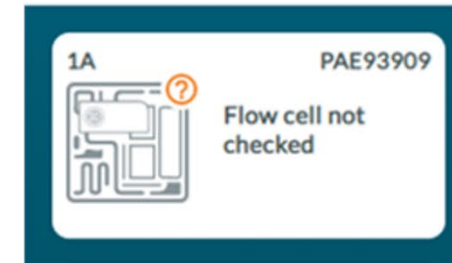
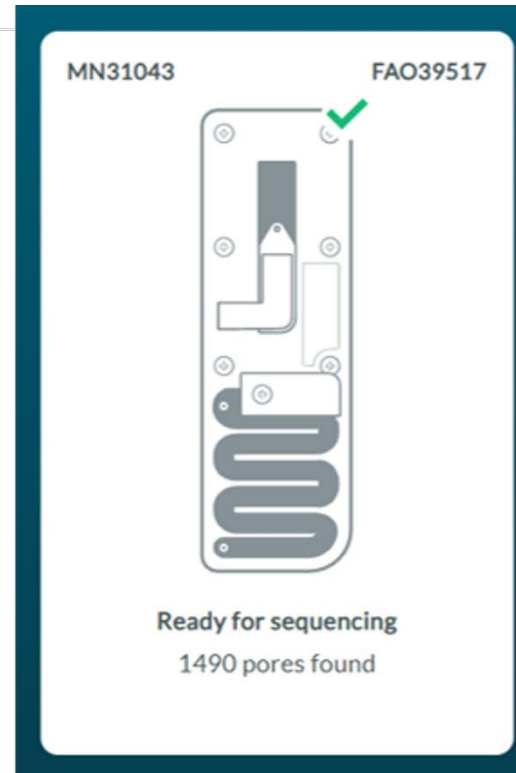
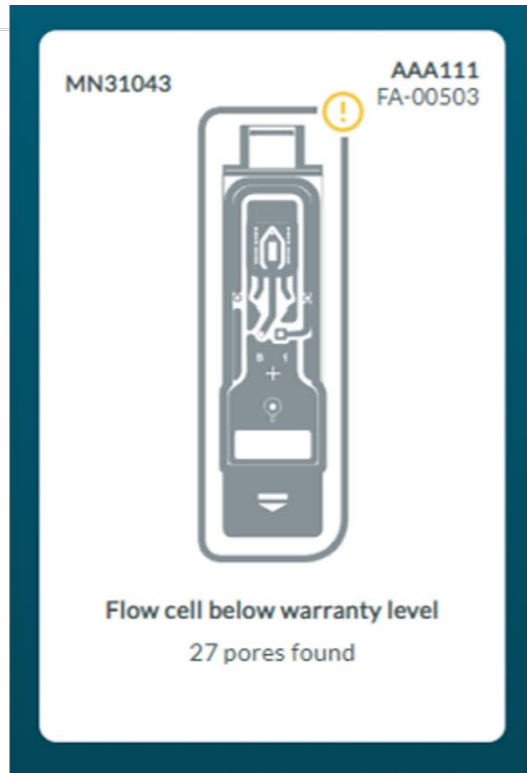
Flow cell check

PromethION position: PromethION - P21-00522

PromethION - P21-00522	Position	Flow cell ID	Flow cell type
Select all available positions	P21-00522-A	P21-00522-A	P21-PRO468 -
	P21-00522-B	P21-00522-B	P21-PRO468 -

[Back to start](#) [Start](#)

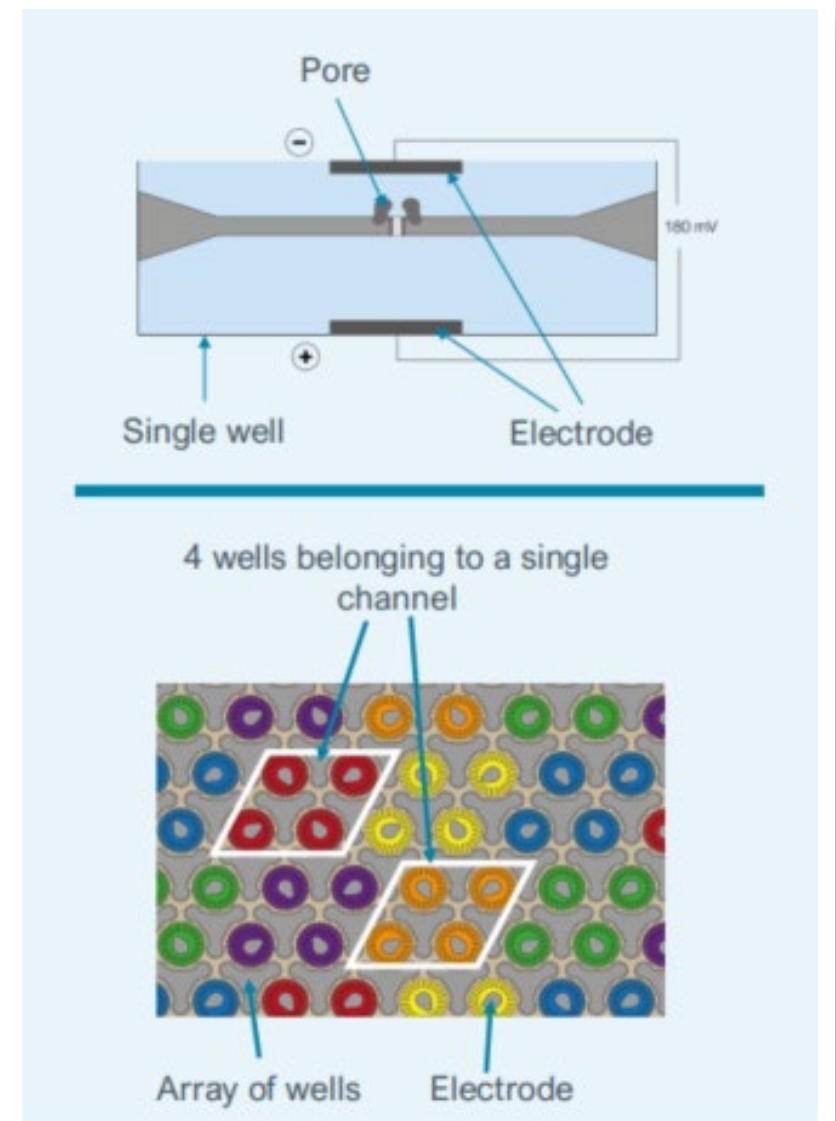
Flow cell check



Mark	Status of sequencing pores
Yellow exclamation (!)	The number of sequencing pores is below warranty (Fail)
Green tick (v)	The number of sequencing pores is above warranty and ready to sequencing (Pass)
Question (?)	A flow cell check has not been run on the flow cell during this MinKNOW session (restart)

Mux scan and flow cell check의 의미

- Flow cell은 wells와 electrodes로 구성
- 각각의 well은 각각의 electrode를 가지고 있으며, 하나의 개별적인 nanopore를 포함함
- MinION flow cell의 경우, 512개의 nanopore sensor (channel)로 구성
- Mux scan - Sequencing 진행 시에 각 channel에서 발생하는 current를 개별적으로 recording 후, 각각의 well을 testing하여 먼저 sequencing을 수행할 1개의 well을 선택하는 과정
- 모든 sequencing script는 Mux scan으로 시작



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text visible on them, including 'HCX' and '63'. The background is dark and out of focus.

Starting a sequencing run

Starting a sequencing run

Sequencing and analysis



Start sequencing

Configure and start a new sequencing run



Start from a sample sheet

Import sample sheet for sequencing



Flow cell check

Assess flow cell health



Post sequencing analysis

Analyse existing sequence data



Custom scripts

Start a run from a custom script

Utilities



Hardware check

Validate device is ready for sequencing



Minimap2 converter

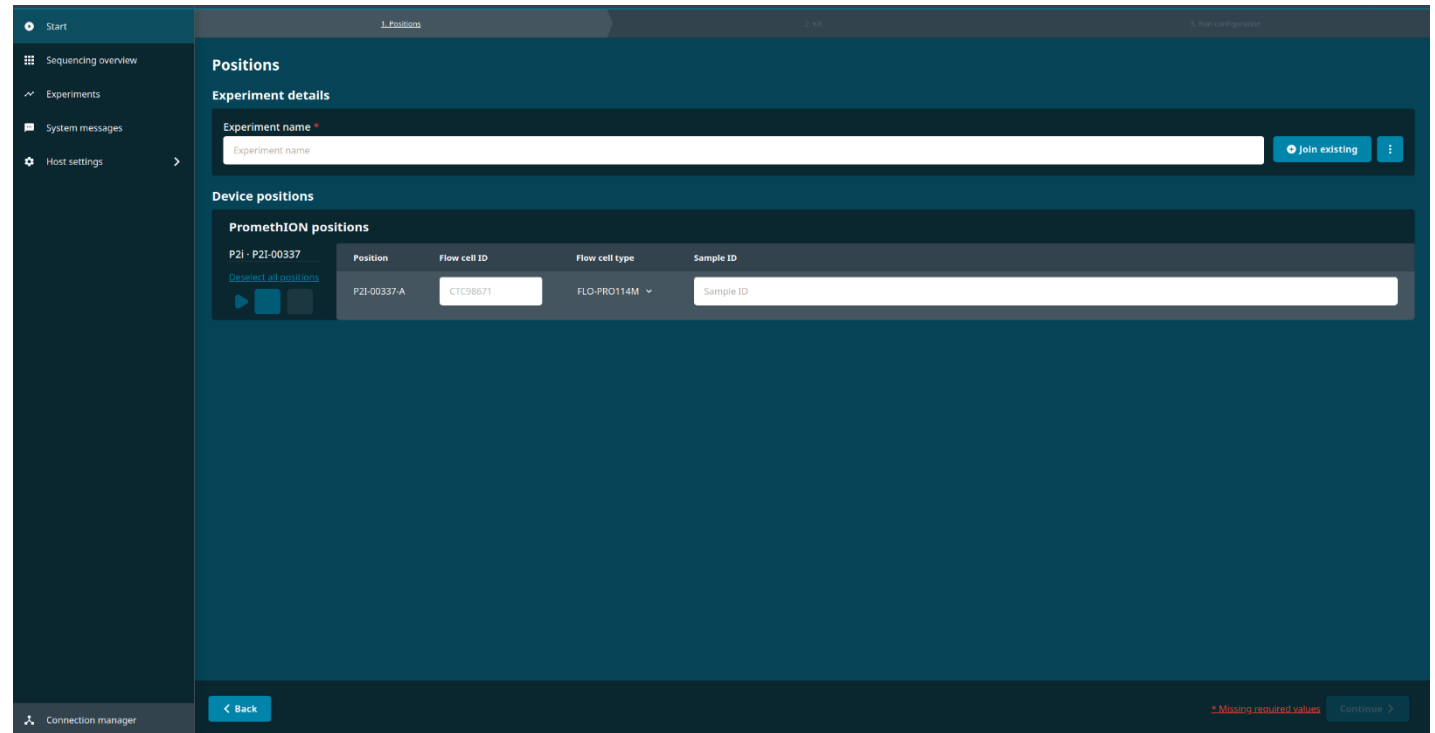
Create a .mmi from a .fasta reference

Start sequencing run

Positions/Experiment name/Sample name

1. Positions

- Experiment name 입력
- Sample ID 입력
- 저장된 설정 불러오기
- Flow cell type 확인
 - 자동으로 감지됨
- Flow cell ID 확인
 - MinION/GridION flow cell & PromethION flow cell의 경우에는 자동으로 Flow cell ID가 입력이 됨



Start

1. Positions

2. Run

3. Run configuration

Sequencing overview

Experiments

System messages

Host settings

Positions

Experiment details

Experiment name *

Experiment name

Join existing

Device positions

PromethION positions

P21 - P21-00337

Position

Flow cell ID

Flow cell type

Sample ID

Detected all positions

P21-00337-A

CTC98671

FLO-PRO114M

Sample ID

Connection manager

Back

*Missing required values

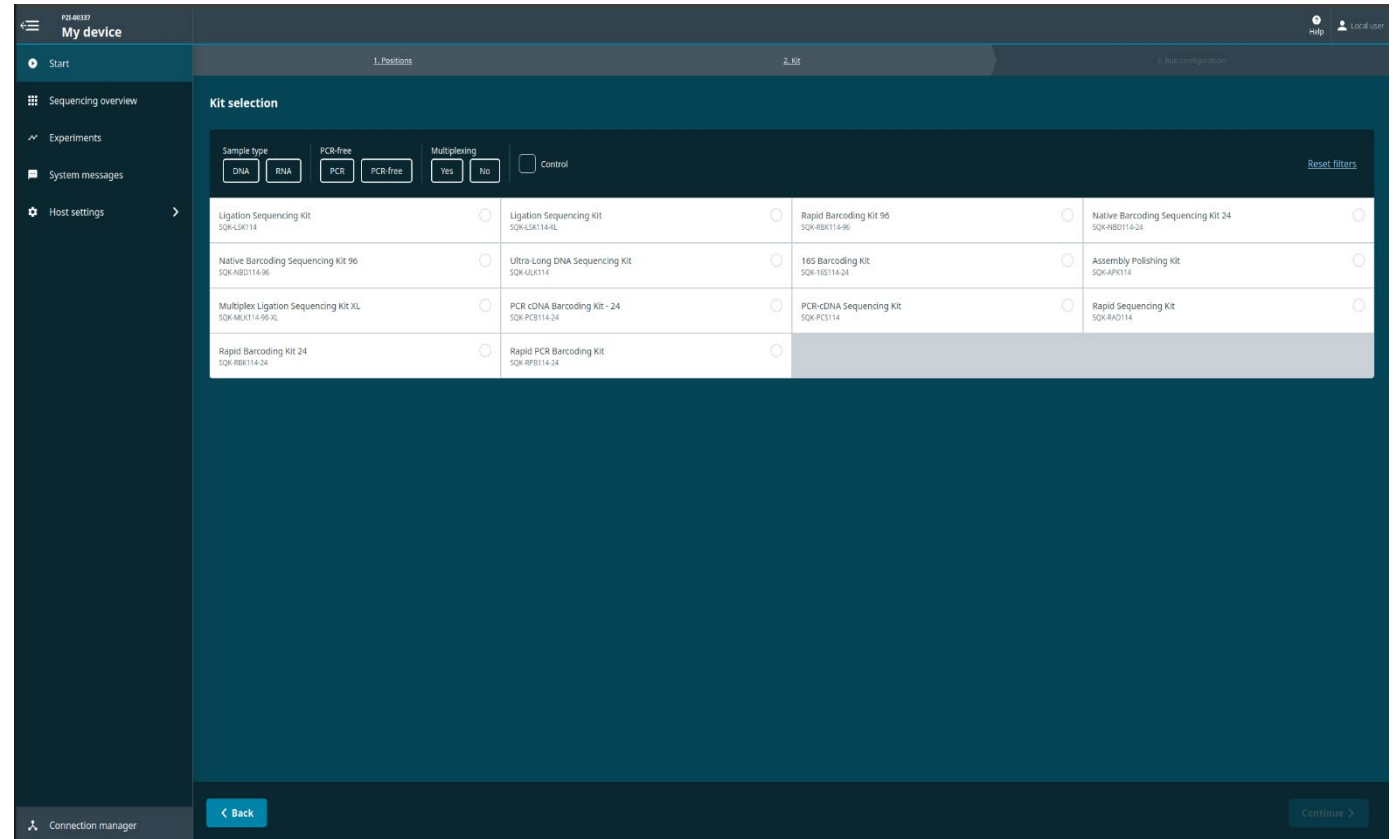
Continue

Start sequencing run

Library preparation kit

2. Kits

- Library preparation에 사용한 kit 선택
- Barcode expansion pack 사용 시, 사용한 barcode expansion pack 선택
- Filtering 기능
 - Sample type: DNA or RNA
 - PCR free or PCR
 - Multiplexing
 - Control experiment



The screenshot displays the 'Kit selection' interface within the PhileKorea sequencing run software. The interface is divided into three main sections: '1. Positions', '2. Kit', and '3. Run configuration'. The '2. Kit' section is currently active, showing a 'Kit selection' screen. On the left, there is a sidebar with navigation options: 'Start', 'Sequencing overview', 'Experiments', 'System messages', and 'Host settings'. The main area contains a 'Sample type' filter with buttons for 'DNA' and 'RNA'. Below this, there are checkboxes for 'PCR-free', 'PCR', 'Multiplexing' (Yes/No), and 'Control'. A table lists various sequencing kits with their respective IDs and selection radio buttons. The kits are arranged in a grid. At the bottom, there are 'Back' and 'Continue' buttons.

Kit Name	Kit ID	Selection
Ligation Sequencing Kit	SQK-LSK114	☐
Native Barcoding Sequencing Kit 96	SQK-NBD114-96	☐
Multiplex Ligation Sequencing Kit XL	SQK-MLX114-96-XL	☐
Rapid Barcoding Kit 24	SQK-RBK114-24	☐
Ligation Sequencing Kit	SQK-LSK114-96	☐
Ultra-Long DNA Sequencing Kit	SQK-GLX114	☐
PCR cDNA Barcoding Kit - 24	SQK-PCB114-24	☐
Rapid PCR Barcoding Kit	SQK-RPB114-24	☐
Rapid Barcoding Kit 96	SQK-RBK114-96	☐
16S Barcoding Kit	SQK-16S114-24	☐
PCR cDNA Sequencing Kit	SQK-PCB114	☐
Native Barcoding Sequencing Kit 24	SQK-NBD114-24	☐
Assembly Polishing Kit	SQK-APB114	☐
Rapid Sequencing Kit	SQK-RAD114	☐

Start sequencing run

Sequencing and analysis

3. Sequencing options

- Basecalling model 선택
- Alignment option 설정
- Barcoding option 설정
- Adaptive sampling 설정
- Advanced sequencing option 설정

Basecalling

Basecalling model
High-accuracy basecalling

Modified bases [?](#)
☐ ShmC and 5mC in CG contexts

Cancel Save

Basecalling

Basecalling model
High-accuracy basecalling

☐ Fast basecalling
☒ High-accuracy basecalling
☐ Super-accurate basecalling

Cancel Save

Barcoding

☐ Trim barcodes [?](#)
☐ Barcode both ends [?](#)
☐ Custom barcodes for demultiplexing [?](#)

Cancel Save

Alignment

Reference for alignment *

Optional file enumerating regions

Cancel Save

Advanced sequencing options

Time between pore scans
- 1.5 hours +

☒ Reserve pores [?](#)

Simulated playback

Cancel Save

Run configuration Load configuration

Experiment name test
Sample ID -
Flow cell type FLO-PRO114M
Sequencing kit Native Barcoding Sequencing Kit 24 (SQK-NBD114-24)

Sequencing and analysis

Basecalling ☒ [?](#)
Model High-accuracy - v4.3.0 - 400bps
Modified bases Off

Barcoding ☒ [?](#)
Trim Off
Barcode both ends Off
Selected barcodes All

Alignment ☐ [?](#)

Adaptive sampling ☐ [?](#)

Advanced sequencing options [?](#)

Data targets [?](#)

Conditions
Run limit Stop run when sequencing reaches 72 hours

Output [?](#)

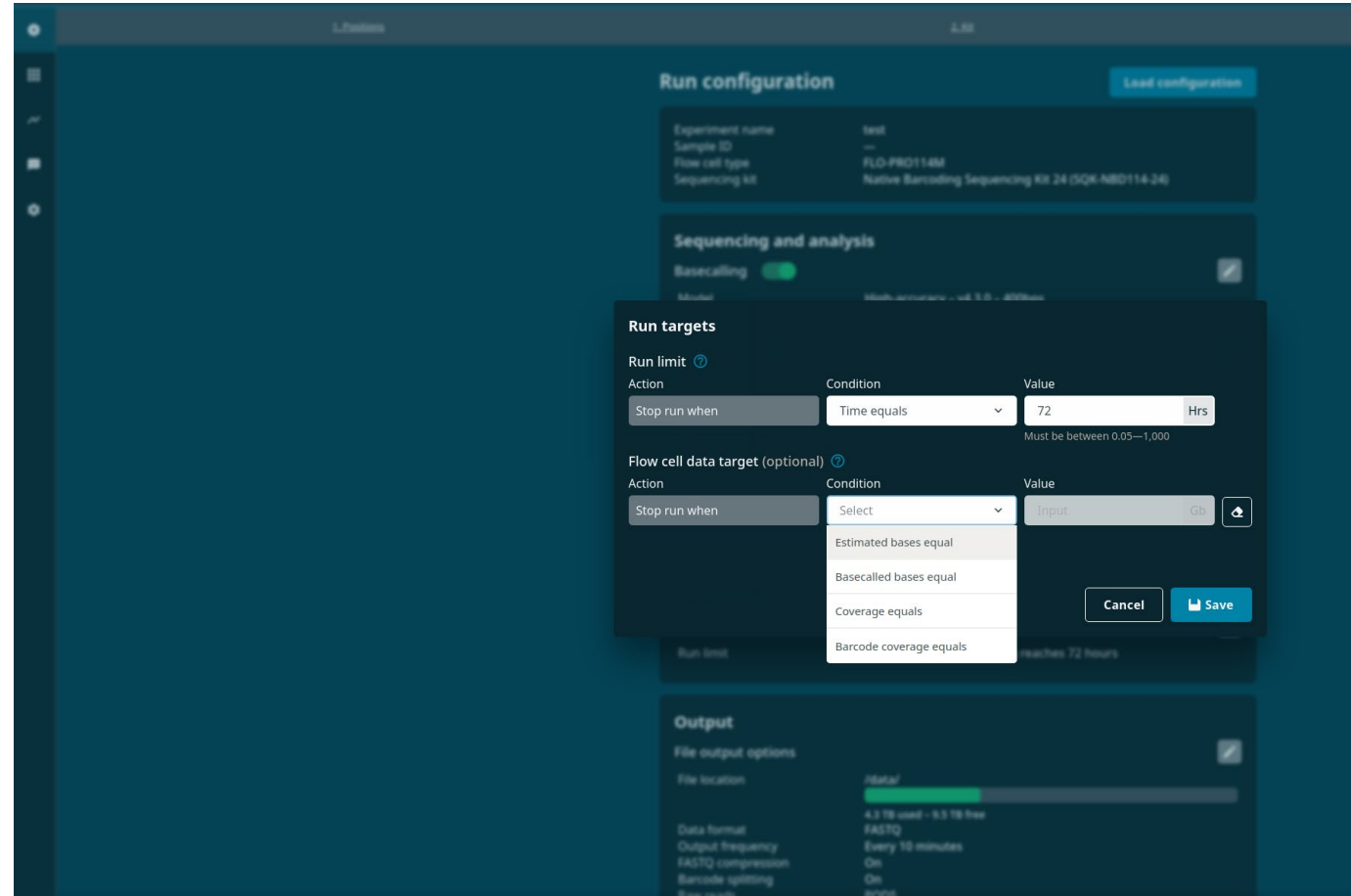
File output options
File location /data/
4.3 TB used - 9.5 TB free

Data format FASTQ
Output frequency Every 10 minutes
FASTQ compression On
Barcode splitting On
Raw reads PDS

Data targets

4. conditions

- Sequencing running time 설정
 - Default 값: 72 hr
- Data output에 따라 sequencing stop 설정
 - Estimated bases equal
 - Basecalled bases equal
 - Coverage equals
 - Barcode coverage equals



Start sequencing run

Output

5. Output

- Output 저장 위치 지정
- Output file 양식 설정
- 추가 옵션 설정

Output files

- POD5/FAST5 files (with basecall information in them)
- FASTQ files
- BAM files (aligned or unaligned)

Additional options include:

- Number of reads per file
- Frequency save FASTQ file

Based on

Time elapsed ▼

☒ Time elapsed

☐ Number of reads

Frequency ⓘ

Every 10 minutes ▼

☐ Every 1 minute

☒ Every 10 minutes

☐ Hourly

☐ End of run

Output settings

Data saved as

test/

Saved output file location

/data/ 🔍

4.3 TB used – 9.5 TB free

Basecalled output type ⓘ

☐ .BAM

☒ .FASTQ

Based on

Time elapsed ▼

Frequency ⓘ

Every 10 minutes ▼

Barcoding

☒ Split files by barcode ⓘ

FASTQ options

☒ Compression ⓘ

Downstream informatics applications may not work with FASTQ Gzip compression

Raw reads output ⓘ

☒ Raw reads

☐ .POD5

☐ .FAST5

Raw reads options

☐ Split .POD5 files by barcode

> Show advanced options

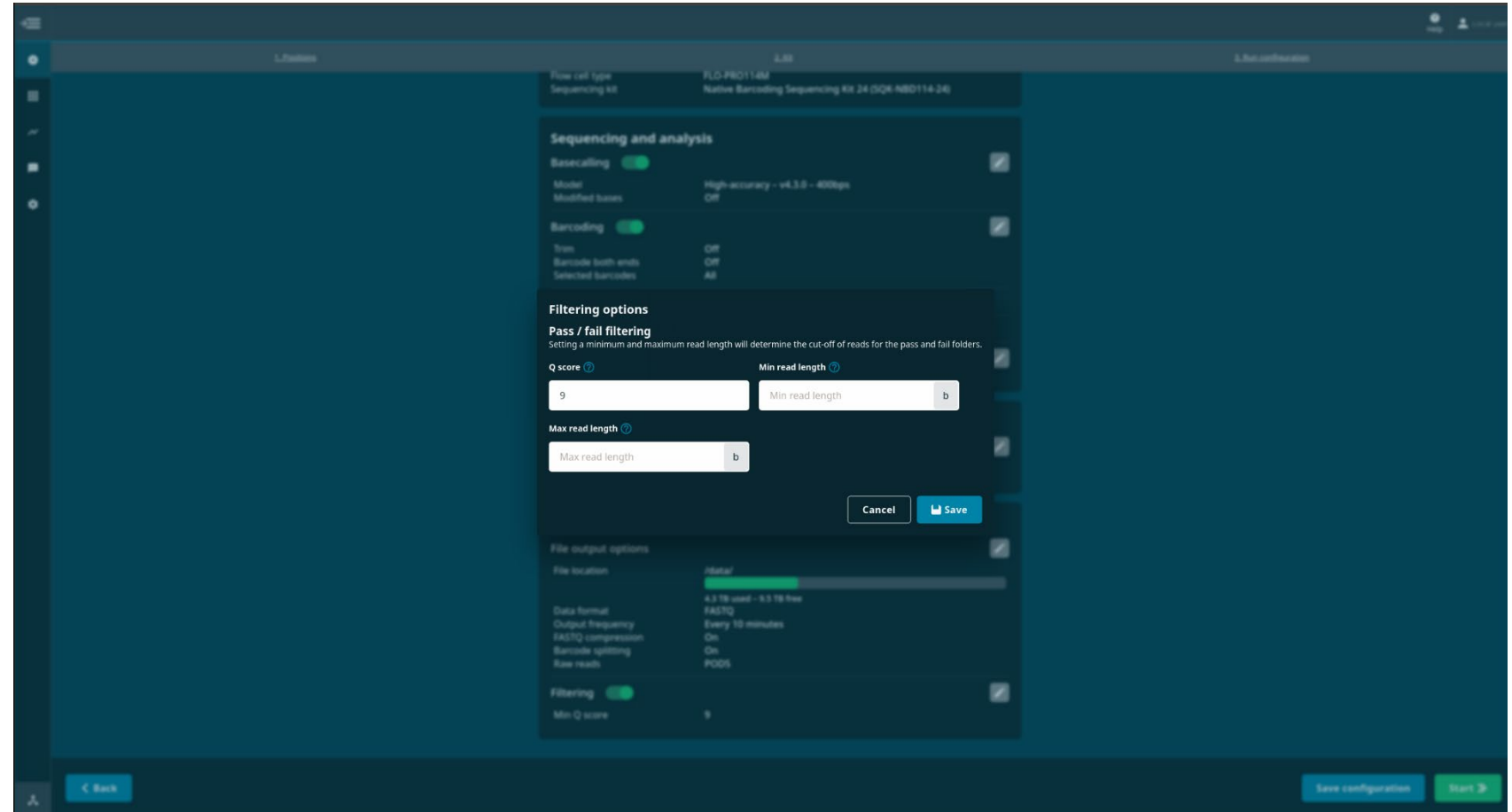
Cancel Save

Start sequencing run

Filtering options

6. Filtering options

- Q score filtering
- Min/Max read length

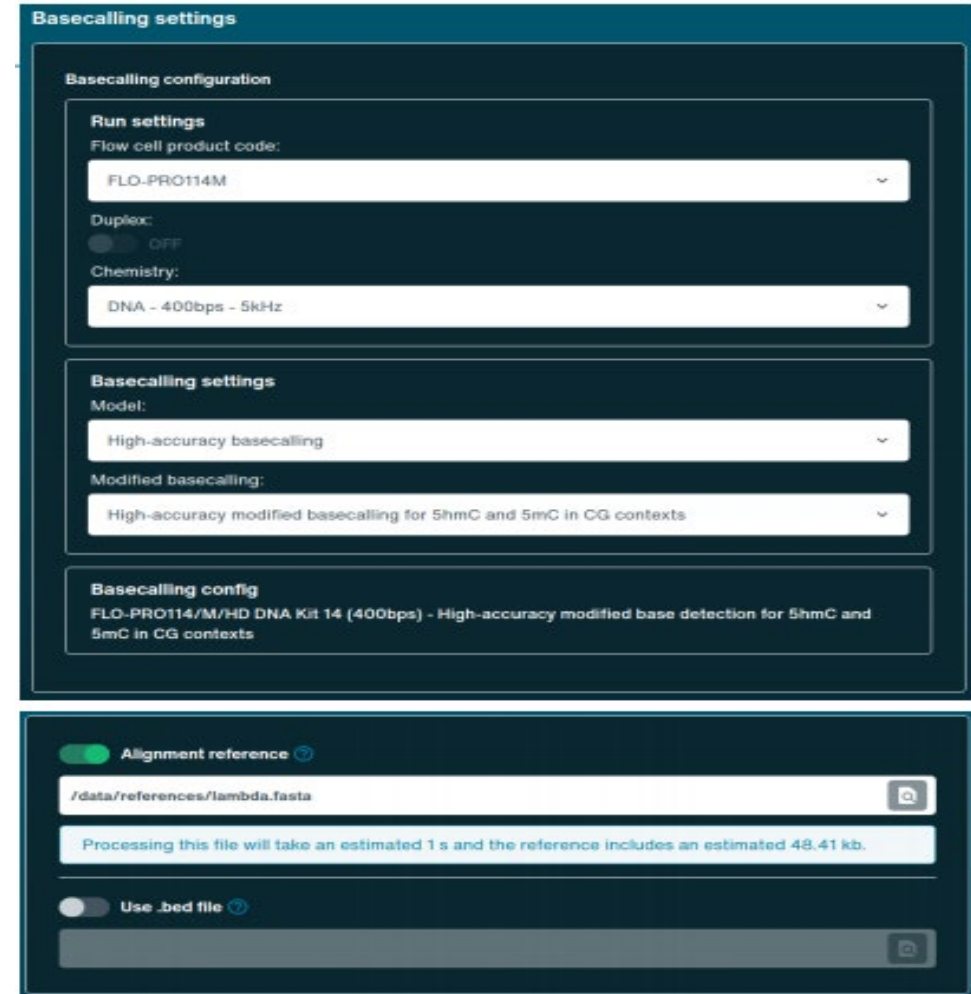
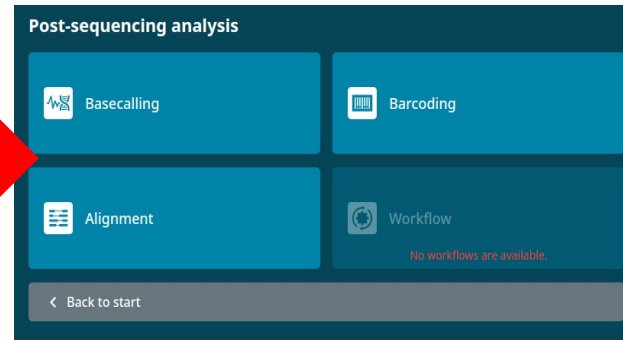
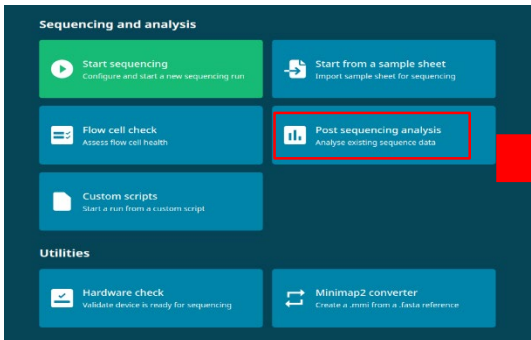


The screenshot displays the 'Filtering options' dialog box within the Oxford Nanopore sequencing software. The dialog is titled 'Filtering options' and includes a sub-section 'Pass / fail filtering' with the instruction: 'Setting a minimum and maximum read length will determine the cut-off of reads for the pass and fail folders.' The 'Q score' is set to 9, and the 'Min read length' is set to 'b'. The 'Max read length' is also set to 'b'. Below these settings, there are 'Cancel' and 'Save' buttons. The background shows the 'Sequencing and analysis' section with various options like 'Basecalling', 'Model', 'Modified bases', 'Barcoding', 'Trim', 'Barcode both ends', and 'Selected barcodes'. At the bottom of the screen, there are 'Back', 'Save configuration', and 'Start' buttons.

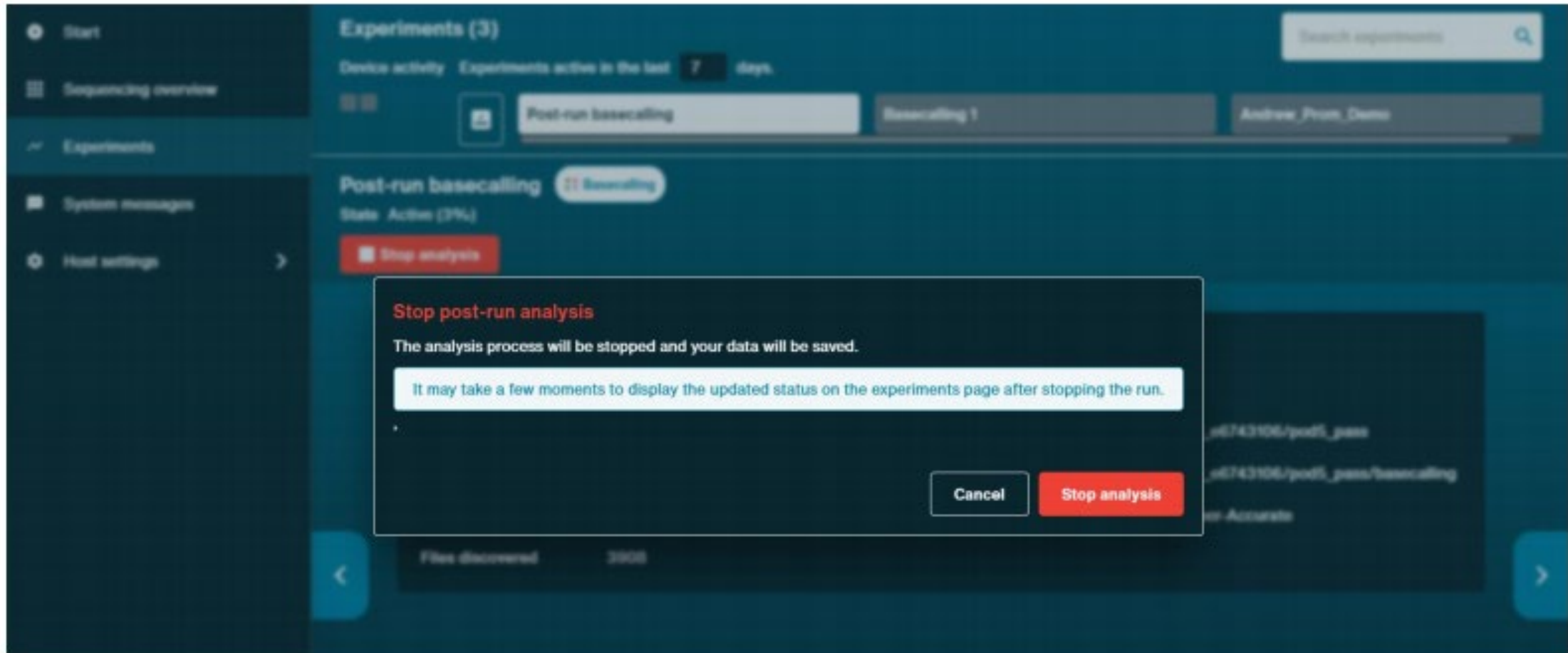
Post-run basecalling options

Analysis

- Input folder 및 output folder 선택
- 선택 사항:
- Basecalling models
- Barcoding kits for demultiplexing
- Alignment



Post-run basecalling options



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63' visible on them. The background is dark and out of focus.

Adaptive Sampling

1. Enrichment takes place during sequencing

- 별도의 sample 전처리 과정이 필요하지 않음.
- Target gene의 수에 대한 제한이 없음

2. PCR free

- Read length에 제한이 없이 sequencing이 가능
- Base modification regio도 분석이 가능
- 특정 Repetitive sequence 또는 GC rich region의 분석이 가능

Adaptive sampling

Enrich/deplete regions of interest during sequencing

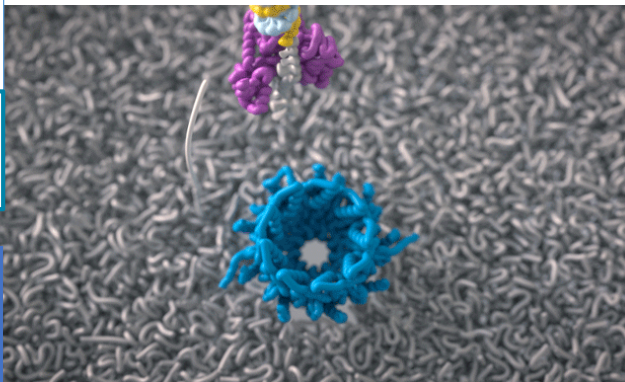
Adaptive sampling의 장점

- Read length 또는 target number에 대한 제한이 없이 적용이 가능

[nanopore_adaptive_seq_comic.bed]									
1	chr1	2226695	2310119	SKI	28.67	61424			
2	chr1	2556366	2563829	THFRSF14	11.35	7463			
3	chr1	3049211	3438421	PRDM14	30.67	369410			
4	chr1	6185020	6189612	SPZ22	26.96	14592			
5	chr1	6785324	7769706	CANTAL	33.13	984382			
6	chr1	11106535	11262507	MTOR	31.63	155972			
7	chr1	13704855	13825079	PRDM2	36.52	120224			
8	chr1	15492270	15524295	CASF9	30.93	32025			
9	chr1	15847864	15940460	SPEN	32.57	92596			
10	chr1	17018722	17084170	SOXB	30.83	35448			
11	chr1	17539035	17697849	ARHGEF10L	32.24	158034			
12	chr1	18631006	18736138	PAX7	31.2	105132			
13	chr1	23557918	23559794	ID3	22.95	1876			
14	chr1	23581495	23640568	ND3	33.43	59073			
15	chr1	26696033	26782110	ARID1A	34.14	86077			
16	chr1	32251239	32286165	LCK	36.92	34926			
17	chr1	35182552	35193148	SFEQ	34.06	10216			
18	chr1	36224416	36305357	THRAP3	37.17	80941			
19	chr1	36466043	36483278	CSF3R	26.94	17235			
20	chr1	38595426	38902013	MYCL	26.67	6587			
21	chr1	43337849	43352772	NPL	34.28	14923			
22	chr1	45329242	45340358	MUTYH	27.67	11146			

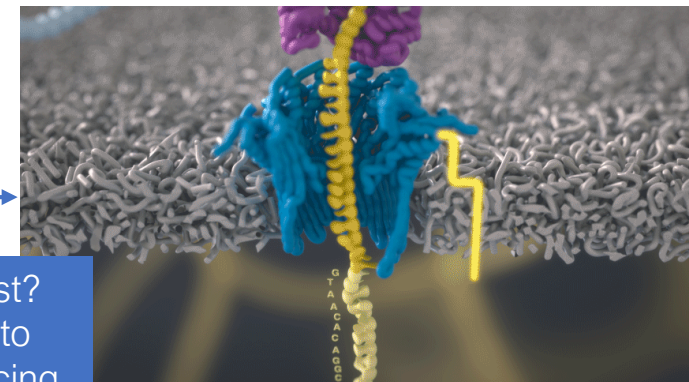
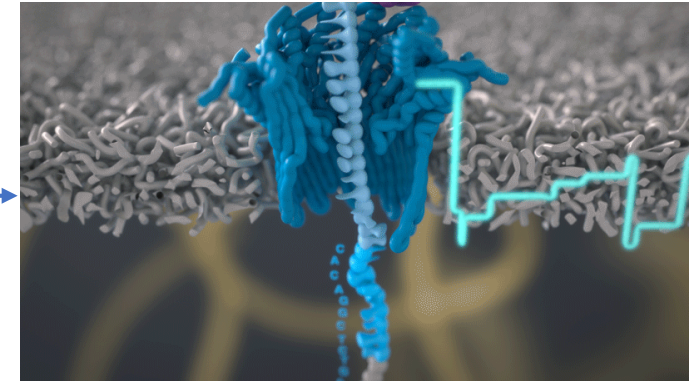
Upload reference file to MinKNOW™

Strand approaches nanopore and sequencing starts



Real-time basecalling and alignment

Region of interest?
Strand allowed to continue sequencing



Adaptive sampling in MinKNOW

Straightforward file-based setup

Sequencing and analysis

Basecalling ☒

Modified bases Off

Duplex Off

Model Fast basecalling

Barcoding ☐

Alignment ☐

Adaptive sampling ☐

Advanced sequencing options

Adaptive sampling

☒ Enrich

☐ Deplete

☐ Barcode balancing Beta

Reference for alignment *

Optional file enumerating regions

Accepted file format: .bed

[Show advanced options](#)

Cancel

Save

Two modes of adaptive sampling

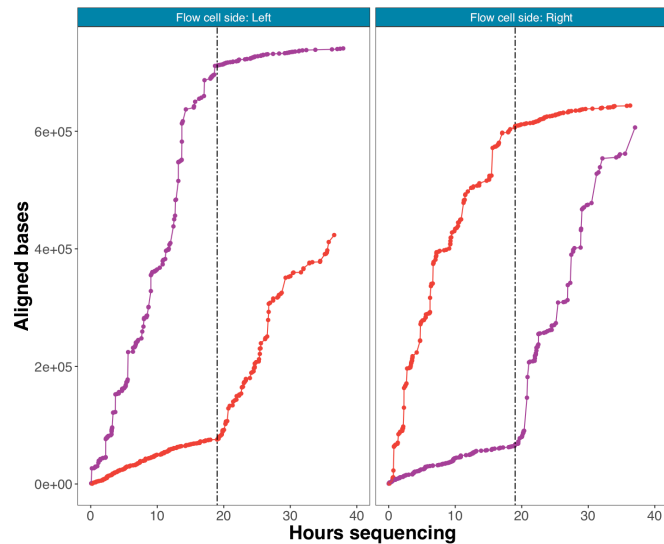
- **Enrichment**
 - Target file에 있는 sequence만 포함하여 sequencing
- **Depletion**
 - Target file에 있는 sequence만 제외하고 sequencing

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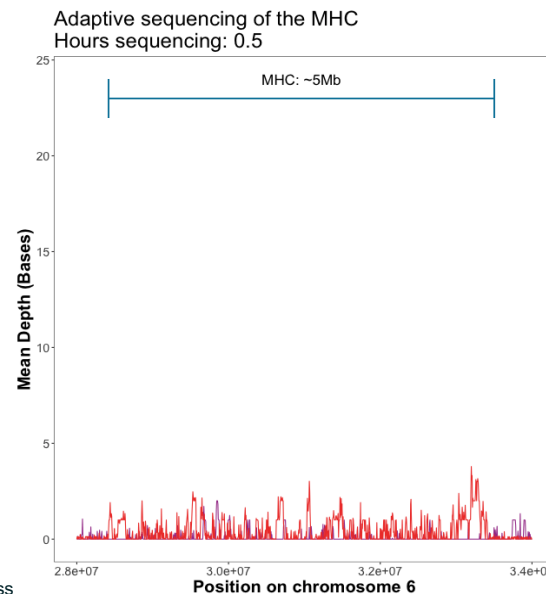


Adaptive sampling applications

- 148 gene cancer panel to 30 X depth on a MinION (Kovaka *et al.*)
- 170 gene exome panel to 32 X depth on MinION (Payne *et al.*)
- 253 host response targets associated with COVID-19 (ONT)
- Enrich for BRCA targets (ONT)
- Focus coverage on MHC (ONT)



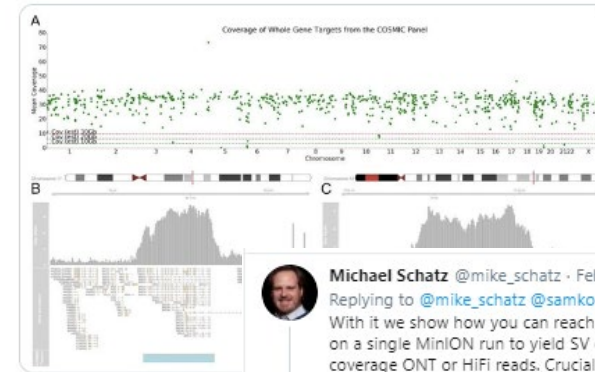
Oxford Nanopore Technologies products are not intended for use for health assess



Sequencing type



Read Until to enrich >700 genes to mean 30x coverage on @nanopore single flowcell. Custom panels (up to 25,600 targets so far) as easy as providing a coordinate file. Read more at biorxiv.org/content/10.1101/111111 - thanks to @alexomics, @Rorymatics and @DeepSeqNotts team.



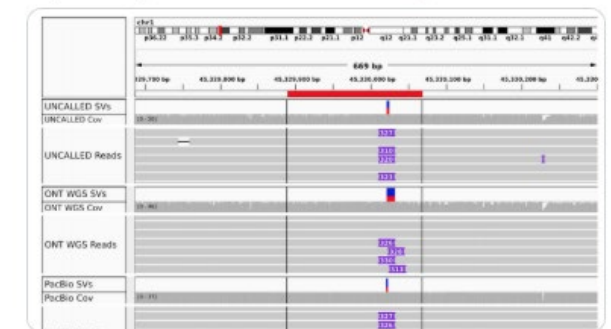
3:13 PM · Feb 3, 2020 · [Twitter Web](#)



Michael Schatz @mike_schatz · Feb 3

Replying to @mike_schatz @samkovaka and 2 others

With it we show how you can reach 30x coverage of a 148 gene cancer panel on a single MinION run to yield SV calling 100% concordant to deep coverage ONT or HiFi reads. Crucially, we find twice the number of SVs using long reads compared to short reads in these cancer genes.



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text like 'HCX' and '63x' visible on them. The background is a deep blue gradient.

Basecalling

Electrical signal to base pair

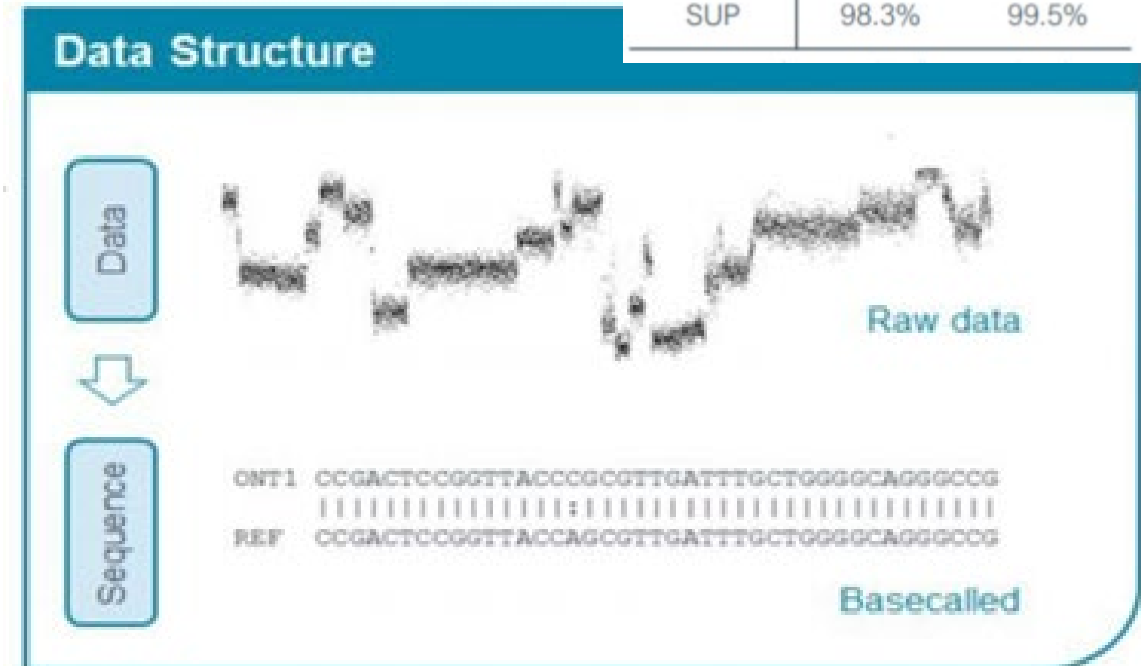
Base calling의 정의

- 전기적인 신호를 nucleotide sequence로 변환하는 과정
- Input: POD5 or FAST5
- Output: FASTQ and/or BAM

Base calling DNA and RNA

- **Fast model**
 - Good accuracy, keeps up with data generation
- **High accuracy (HAC) model**
 - 8x slower than Fast model
- **Super-accuracy (SUP) model**
 - 3x slower than HAC model

Model	Raw read Accuracy	
	R9.4.1 Kit 10	R10.4.1 Kit 14
Fast	95.8%	96.8%
HAC	97.8%	99.1%
SUP	98.3%	99.5%



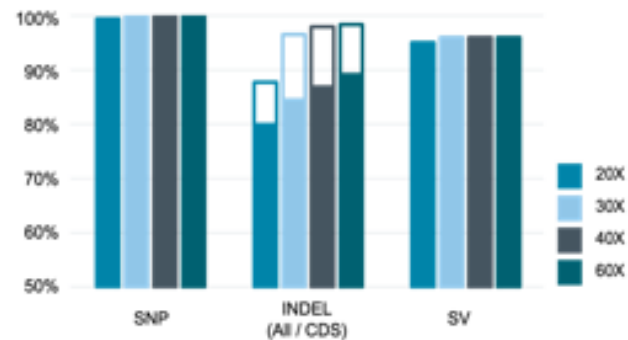
HAC (High Accuracy basecalling)

'Fast' base calling keeps up on all Oxford Nanopore devices
'High accuracy' (HAC) balances performance and accuracy
'Super accuracy' (SUP) on R10.4.1 achieves up to **99.5%**

HAC model is recommended as an efficient model for methylation and variant calling.

SUP basecalling is expected to require a further 4-5X more compute.

High accuracy basecalling model (HAC)



Accurate data at 30x coverage with Q20+ real-time basecalling:

Raw read accuracy



Simplex



Methylation
(5mC)

Variants accuracy at 30x



SNPs



INDELs
(CDS)



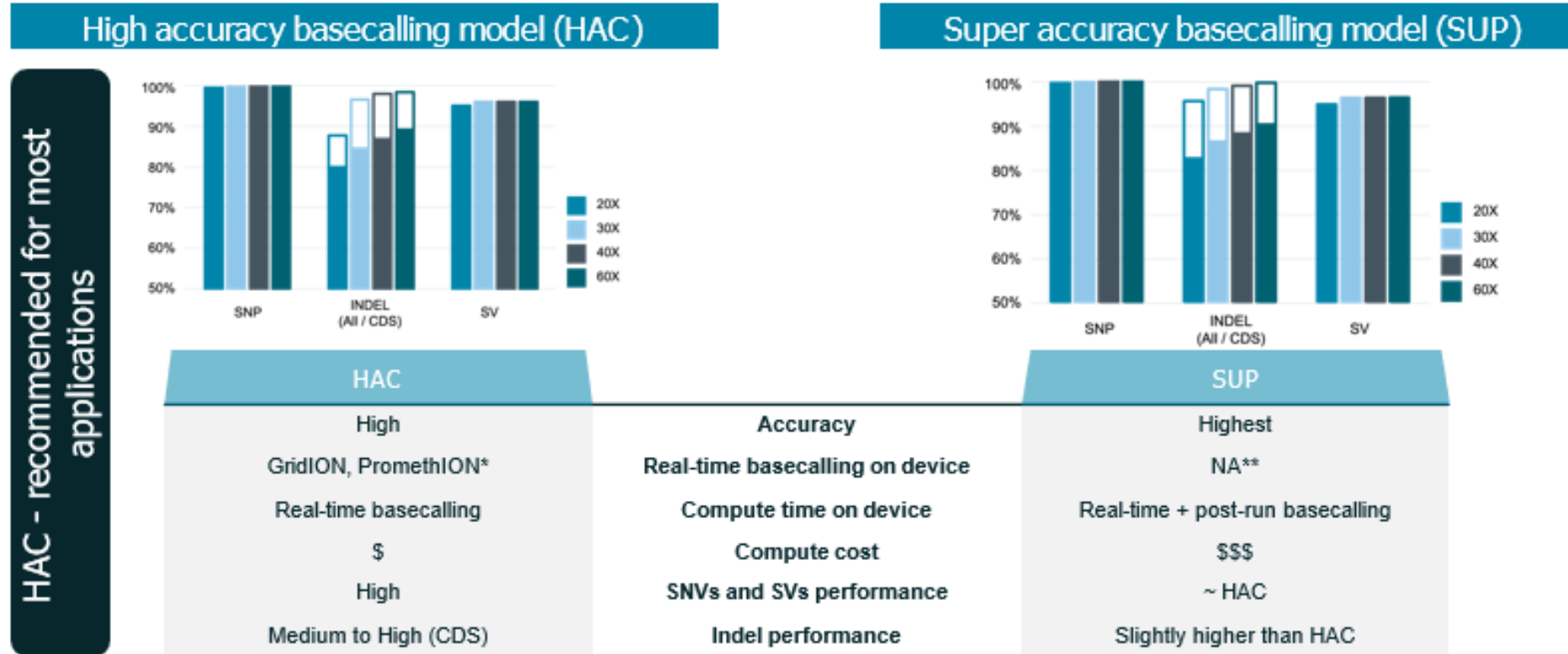
SVs

		Simplex	Methylation (5mC)	SNPs	INDELs (CDS)	SVs
	HAC	99.0%	99.7%	99.6%	95.5%	96%
	SUP	99.5%	99.7%	99.6%	98.5%	96%



For further information about specific tools and methods used to generate these results please visit nanoporetech.com/accuracy

Comparison of HAC and SUP



Shaded bars in the graphs refer to CDS regions

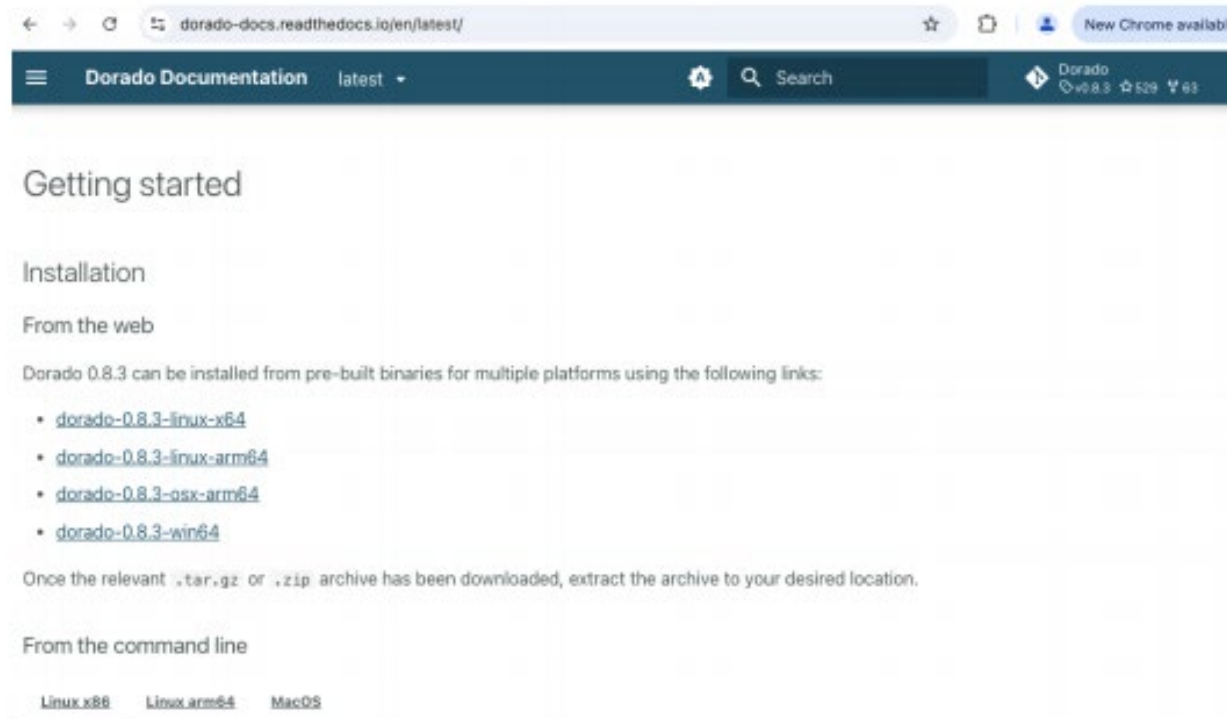
* Compatible with real-time basecalling on GridION™ and soon on PromethION™ 24, with Dorado basecaller on a A-series PromethION tower. Expected integration Q3 2023.

** Can start real-time but 3x HAC runtime for basecalling

Find out more: Dataset release (EPI2ME Labs): <https://labs.epi2me.io/njab-2023.05/>

Dorado

- Oxford nanopore read에 대한 사용하기 쉽고, 고성능의 open source base caller
- Command line 기반의 applicatio이며, MinKNOW software (v5.7~)에도 통합이 되어 있음
- 대부분의 사용자는 MinKNOW software를 통해 Dorado를 사용하며, 일부 BI 전문가들은 compute cluster 등에서 독립형 Dorado base caller 사용이 가능함



A close-up, low-angle shot of microscope objective lenses, with a dark blue overlay. The lenses are metallic and have technical markings like 'HCX' and '63x'.

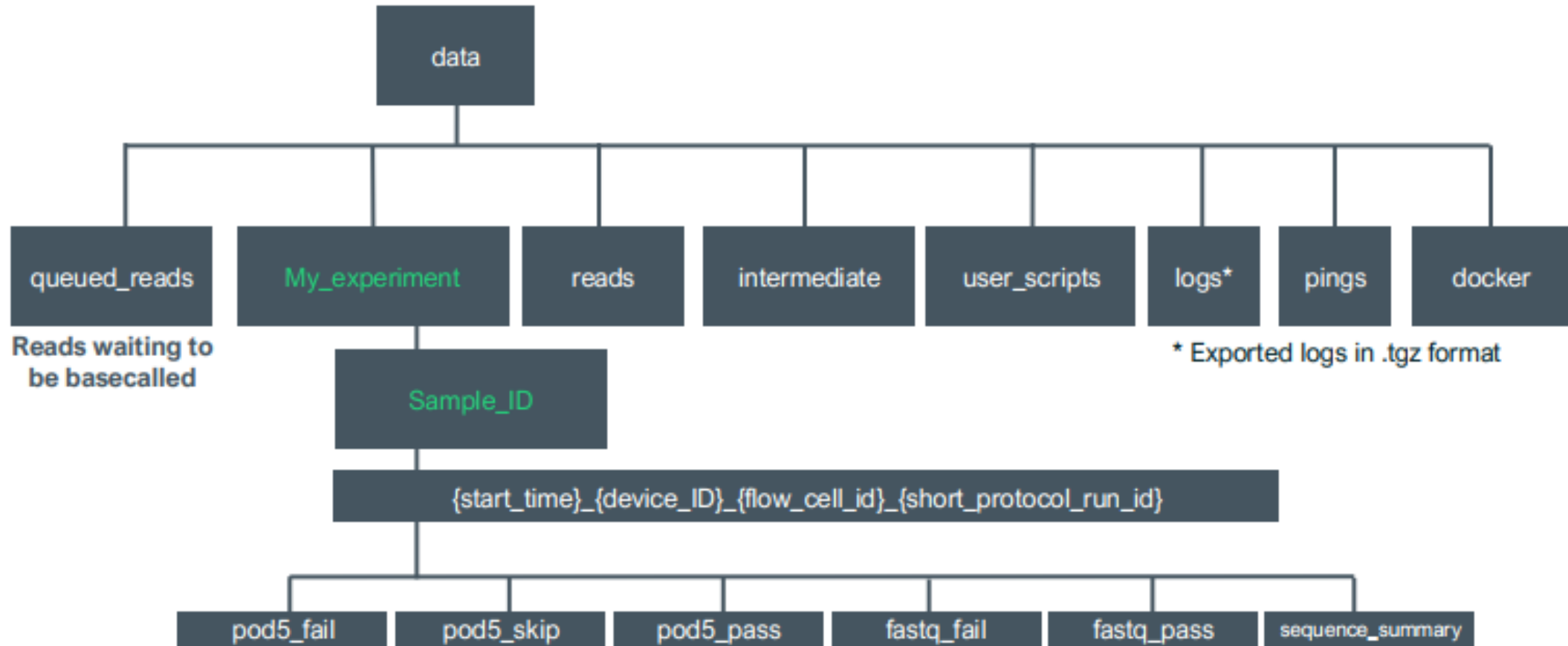
MinKNOW folder structure

Files are output using the following folder structure:

- {output_dir}/{experiment_id}/{sample_id}/{start_time}_{device_ID}_{flow_cell_id}_{short_protocol_run id}/

- **output_dir** configured output directory
- **experiment_id** user-entered identifier for a group of runs
- **sample_id** user-entered value for a specific sample or run. It is intended that multiple sample_ids may exist for an individual experiment_id
- **start_time** time at which the protocol started in YYYYMMDD_HHMM format
- **device_id** serial ID of the MinION or device position for GridION/PromethION
- **flow_cell_id** flow cell id (eg: FAW12345), either programmed on the ASIC or entered by the user
- **short_protocol_run_id** unique identifier of 7 characters from the protocol ID

Where is my data?



A close-up, low-angle shot of a microscope's objective lenses. The lenses are dark and metallic, with some text visible on them, including 'HCX' and '63'. The background is a deep blue gradient.

Monitoring a sequencing run

Monitoring a run

Experiment page

- Sequencing 및 장비 check에 관한 정보
- Experiment graph 확인



Blue panel

- All experiment 및 post run analysis 개요
- Reads 수, Base 수 및 active run 확인
- 정해진 기간 동안의 experiment 표시
- "Check box" 아래에서 run 상태 확인



White panel

- Experiment의 모든 sequencing run 상태와 sequencing run 정보를 제공
- Sequencing run 상태, pore 상태 및 read 수를 포함한 모든 요약 정보를 확인할 수 있음
- Run control에서 수동으로 pore scan 및 sequencing run을 멈추거나 일시정지 시킬 수 있으며, run report 추출이 가능함.

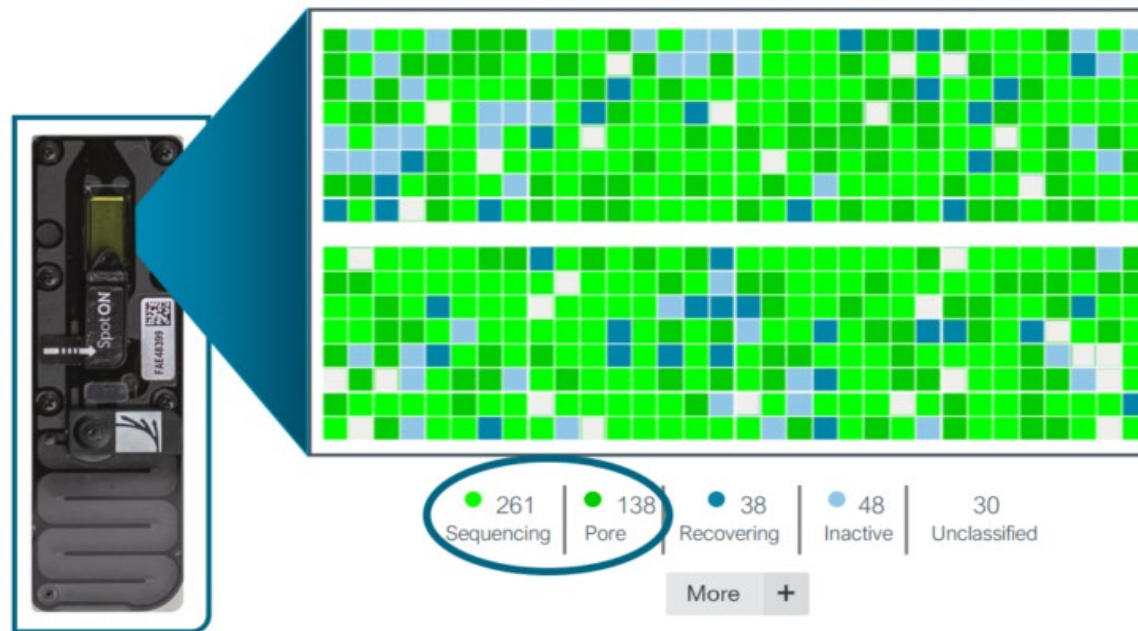
Run controls									
   Start pore scan Export run report 									
Position	Flow cell ID	Sample ID	Health	Available pores	Run time	Run state	Reads	Estimated bases	Basecalled
X3	FAW97004	RRMS_mouse_v55_rep_12kb		 770 / 2048	 1d 1h / 3d 0h	Active	3.24 M	7.16 Gb	7.12 Gb
X2	FAW97779	RRMS_mouse_v55_rep_10kb		 1122 / 2048	 1d 1h / 3d 0h	Active	4.24 M	8.81 Gb	8.74 Gb
X1	FAW97206	RRMS_mouse_v55_rep_5kb		 742 / 2048	 1d 1h / 3d 0h	Active	7.24 M	9.22 Gb	9.33 Gb

Monitoring a run



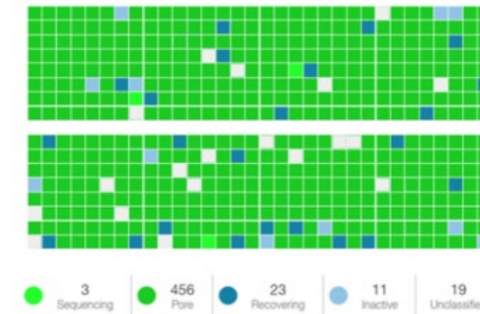
Assessing the performance of run

Some examples



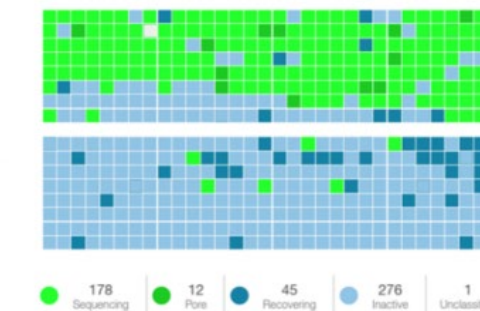
Success full library preparation

- Sequencing이 진행되고 있는 active pore의 비율이 높음
- Recovery pore와 inactive pore가 적음



Low pore accuracy

- Sequencing이 진행되고 있는 active pore의 비율이 낮음
- Recovery pore와 inactive pore의 비율이 낮음



Introducing air bubbles on sensor array

- Inactive channel이 많음
- 공간적으로 Inactive channel의 명확한 pattern이 나타남

High pore occupancy maximizes output

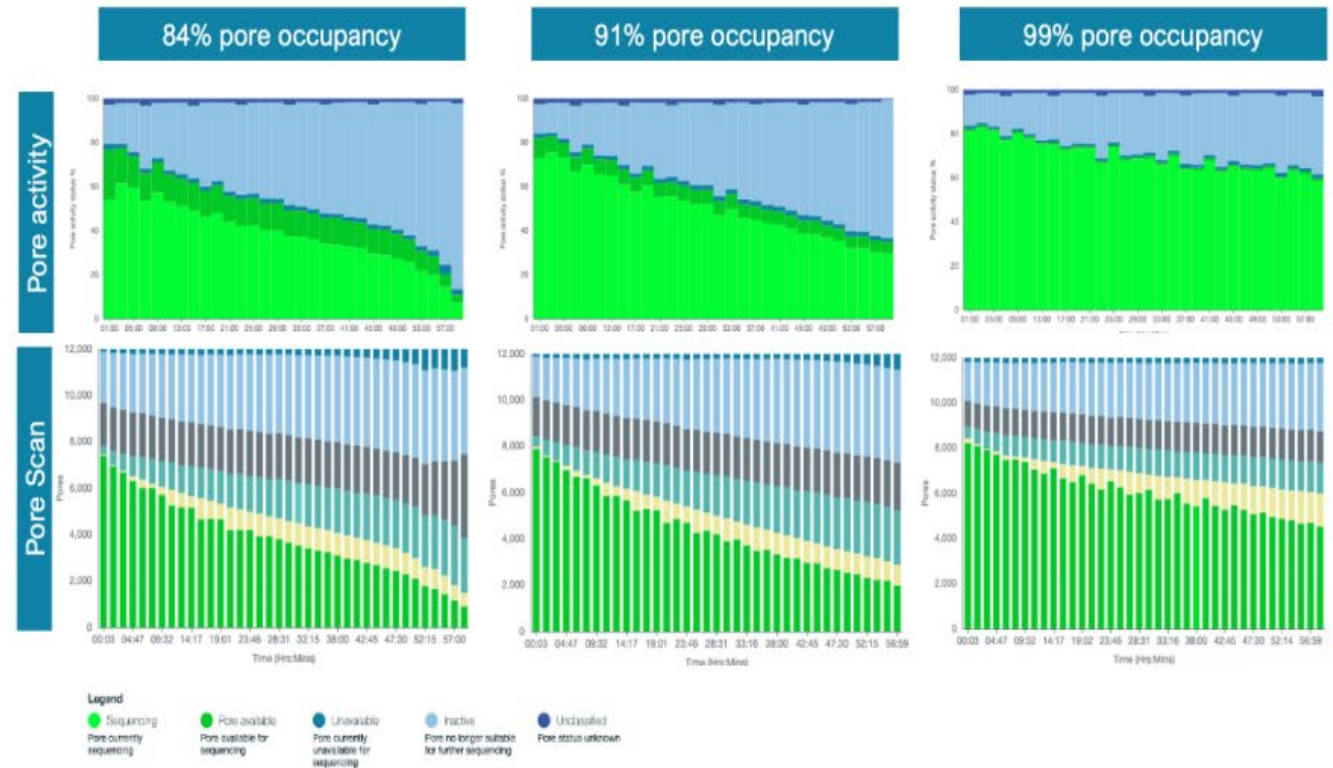
High pore occupancy :

- 시간 당 더 많은 양의 **sequencing data** 생성
- 더 많은 **active pore** 유지



A good occupancy is obtained with a good libraries:

- 충분한 양의 **DNA library loading**
- 효율적인 **sequencing Adapter attachment**



Monitoring a sequencing run

The five main channel states

- Sequencing, Pore, Recovering, Inactive, and Unclassified
- DNA/RNA library의 quality가 좋은지, 그리고 Flow cell이 잘 작동하는지에 대한 지표

Recommendation

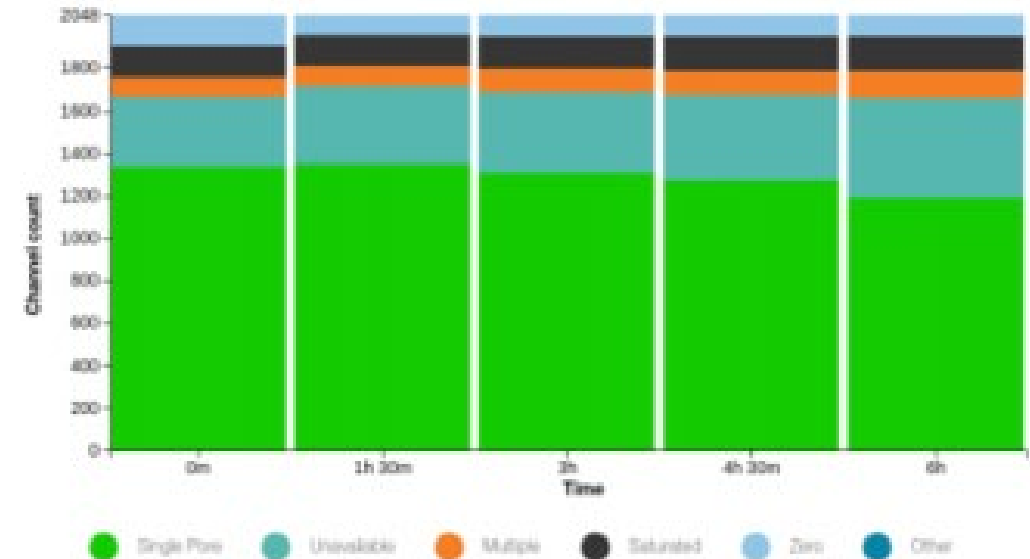
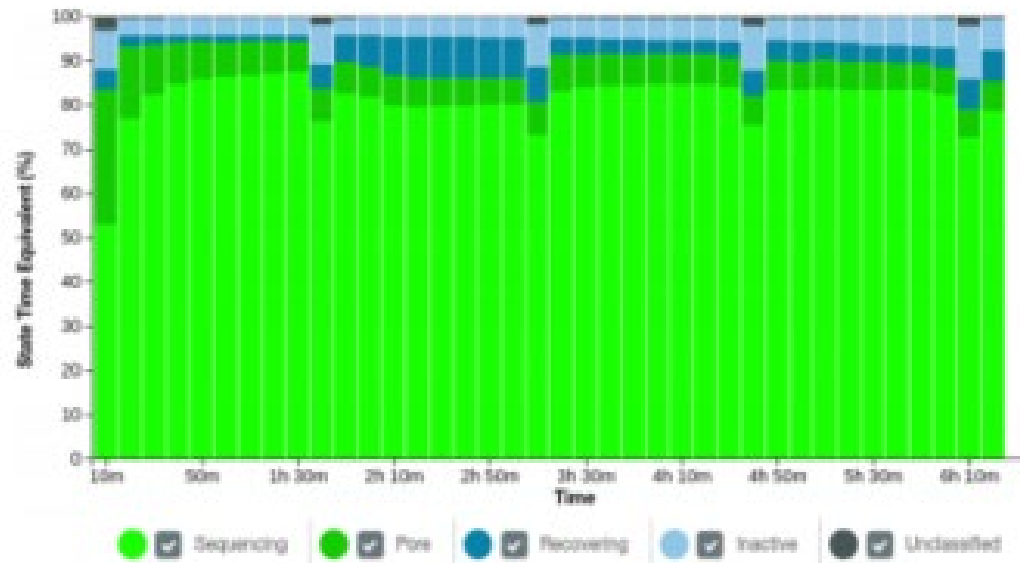
- Sequencing 진행 후, 초기 30분에서 1시간 사이에 pore activity plot을 확인

Monitoring a sequencing run: Good library

Result in most of the pores being in the “Sequencing” state

The five main channel states

- Very few in “Pore”, “Recovering” or “Inactive”
- A library is likely to give a good sequencing output.



Monitoring a sequencing run: channel blocking

Result in channels may be become blocked and therefore unable to sequence.

The five main channel states

- 라이브러리에 오염물이 존재하는 등 sequencing 시간이 증가할 수록 “**Unavailable**” channel 수가 증가

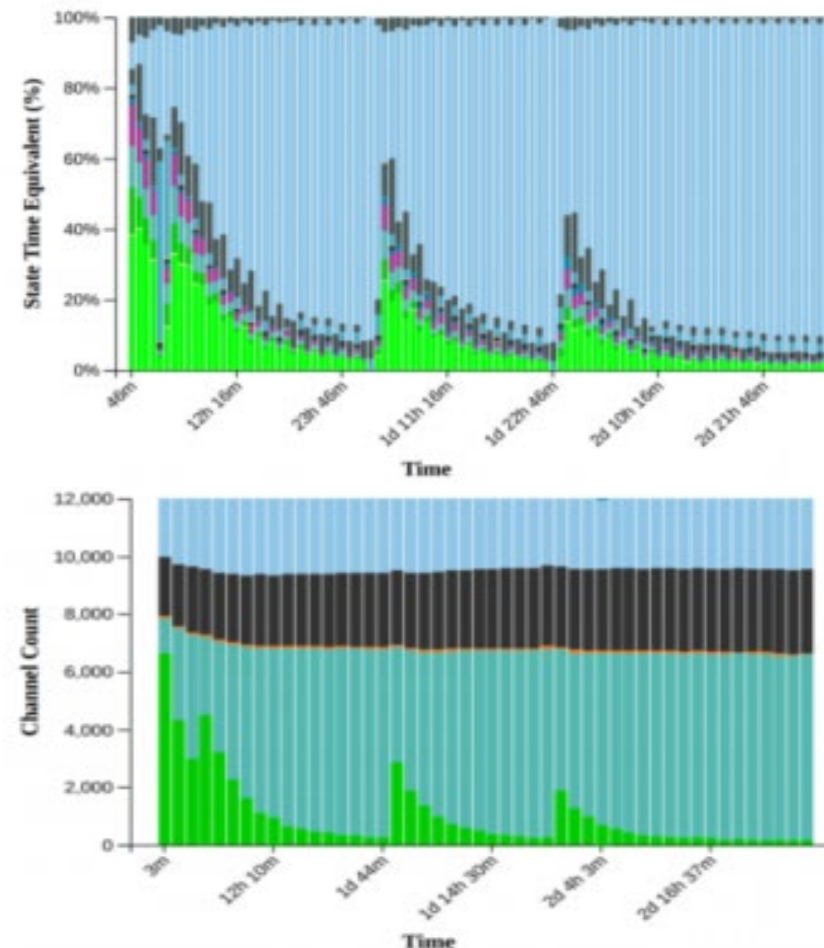
Recommendation

1. Library가 충분한 수의 read를 생성하는 경우

- 시퀀싱 실험을 계속 진행

2. Library가 부족한 수의 read를 생성하는 경우

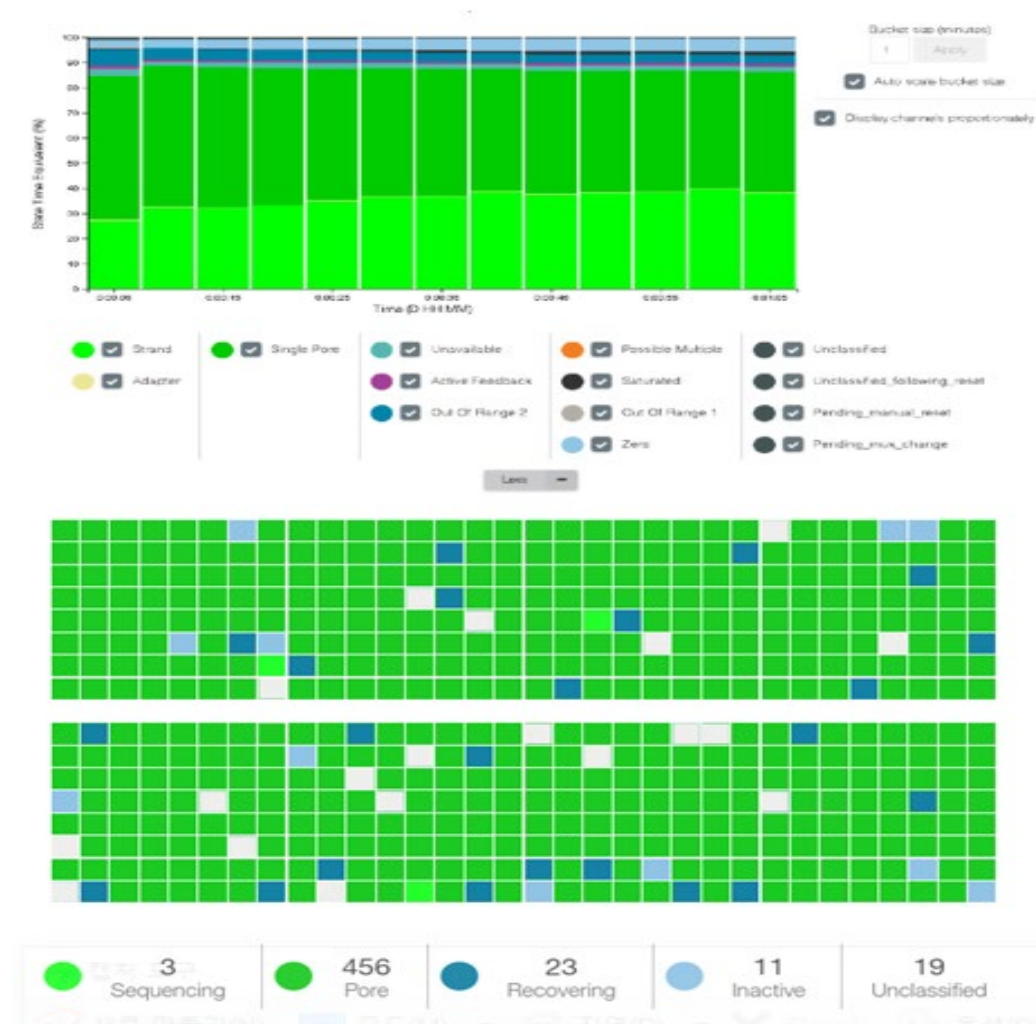
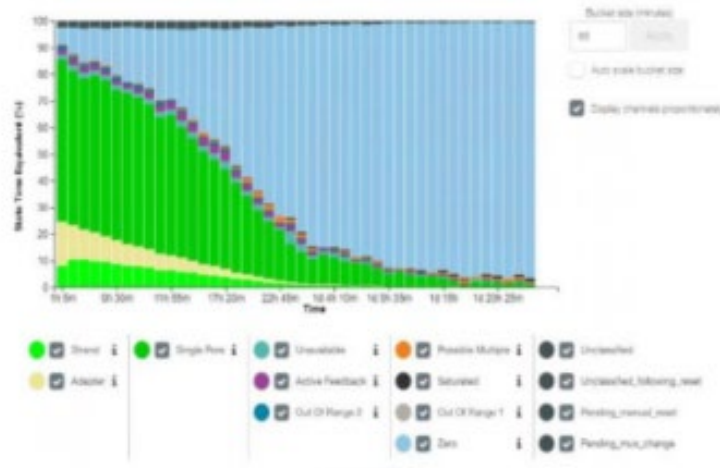
- Sequencing을 중지하고, Flow Cell Wash Kit으로 Flow cell을 washing 한 후 새로운 library를 준비하여 sequencing을 재 진행
- 일반적으로 active pore 개수가 ~20% 미만일 때 flow cell washing을 진행



Monitoring a sequencing run: Low pore occupancy

The five main channel states

- Active pore보다 available pore 상태가 더 많이 관찰됨.
이는 sequencing이 가능한 전체 pore 중, 일부 pore만 DNA/RNA strand가 통과하여 sequencing이 진행되고 있다는 의미
- DNA/RNA input이 충분하지 않거나, library preparation 과정 중 일부 sample이 loss되었거나, sequencing adapter가 strand 끝에 제대로 binding 되지 않았을 경우



Monitoring a sequencing run: Low pore occupancy

Recommendation

- **DNA/RNA로 생성한 library의 yield 확인**
 - 새로운 library를 준비하고, protocol에서 권장하는 loading input양을 기준으로 flow cell에 다시 loading
 - Library 농도가 낮다면 library kit에 권장된 input DNA/RNA 양을 더 증가시켜 library를 다시 준비한 후 loading.
 - Library preparation 과정 중, Flow cell priming mix에 flush tether를 반드시 첨가
- Ligation 기반의 library preparation kit의 경우, sequencing adapte를 정확하게 ligation 후, short or long fragment buffer를 사용하여 clean-up을 수행하는 등 protocol을 정확하게 따라야 함
- Sequencing run이 진행 중이라면, 일시정지 후 준비된 library에 추가적으로 DNA/RNA 200 ~ 300 ng 정도의 library를 flow cell에 추가 loading 후 sequencing을 진행
 1. 추가적인 DNA/RNA library에 sequencing buffer 및 loading bead를 mixing
 2. Sequencing running을 pause 후, spotON 또는 inlet port를 열어 library를 추가적으로 넣음
 3. SpotON 또는 inlet port를 닫고, sequencing running을 재진행

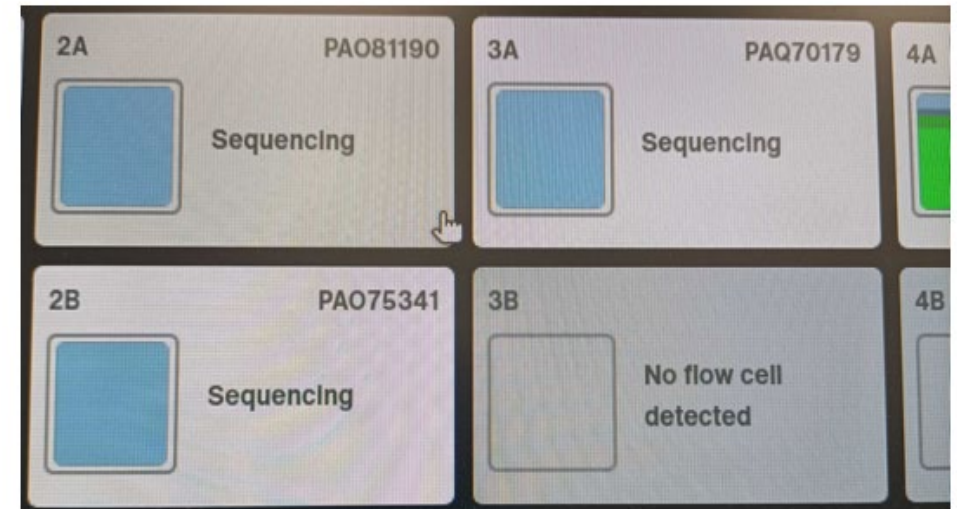
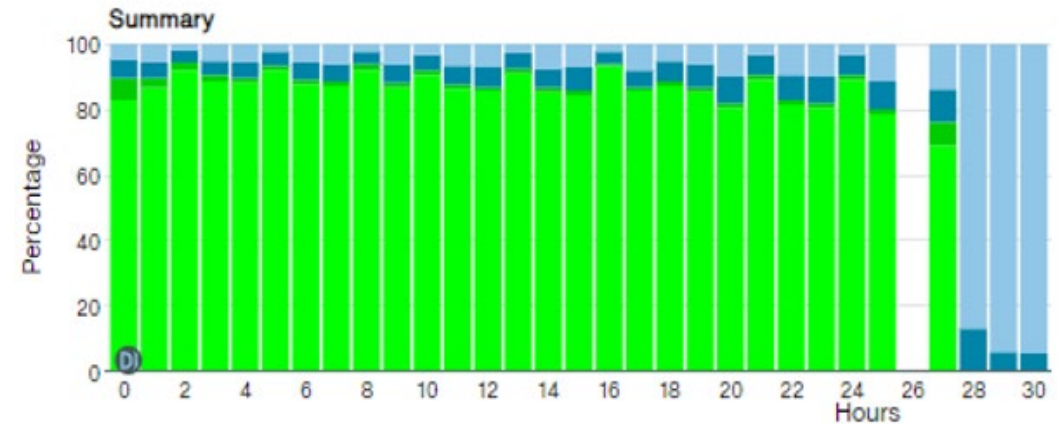
Monitoring a sequencing run: Pore lost during sequencing

The five main channel states

- Pore scan 후에, Available pore가 갑자기 급격하게 감소함

Recommendation

- **MinKNOW software 재부팅**
 - Ctrl + R
 - MinKNOW 창을 close 후, 다시 open
- **Experiments tab에서 Pore scan을 수동으로 다시 실행**
- 위의 2가지 방법으로 해결되지 않을 때, **sequencing run을 중지하고 flow cell을 device의 다른 position에 위치시킨 후 sequencing을 진행**
 - 더 많은 pore가 감지되고 flow cell에서 sequencing이 되면, device에서 log file을 추출하여 원인 파악을 위해 log file을 ONT 본사에 전달. ONT 본사에서 원인을 식별하는 동안 이 position은 사용을 금하여야 함.
 - 다른 position에서도 같은 문제가 발생할 경우, electrode와 array 사이에 bubble이 있을 수 있으므로, bubble 제거 후 sequencing 재 실행



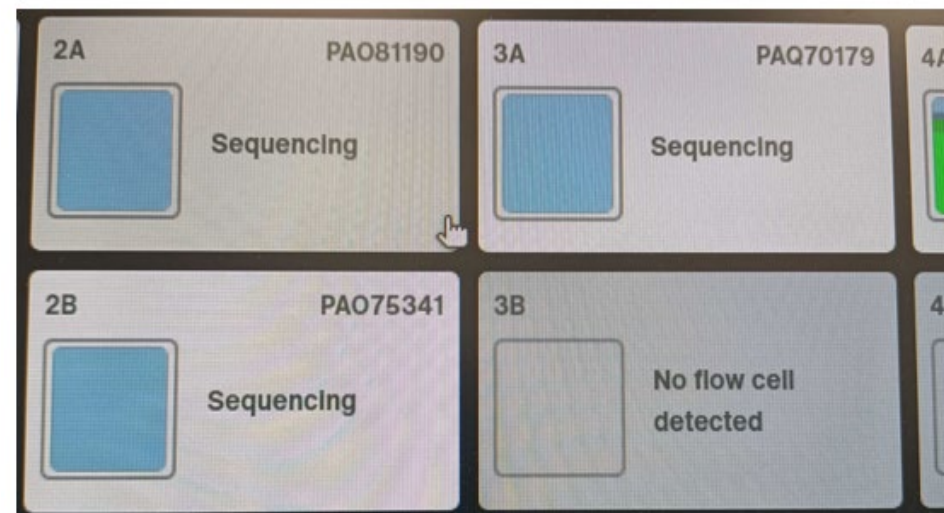
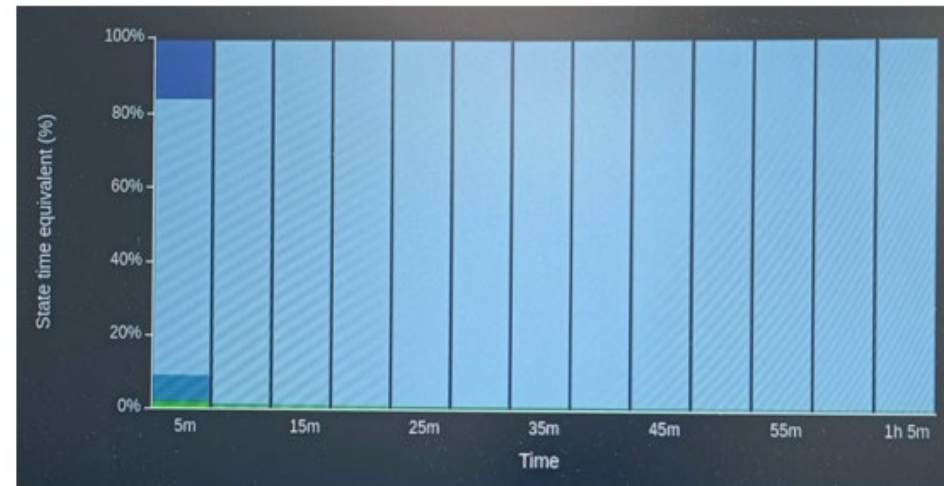
Monitoring a sequencing run: Low pore availability or low pore occupancy

The five main channel states

- 새 Flow cell로 sequencing running을 시작하자마자, available pore가 거의 소멸되었을 경우

Recommendation

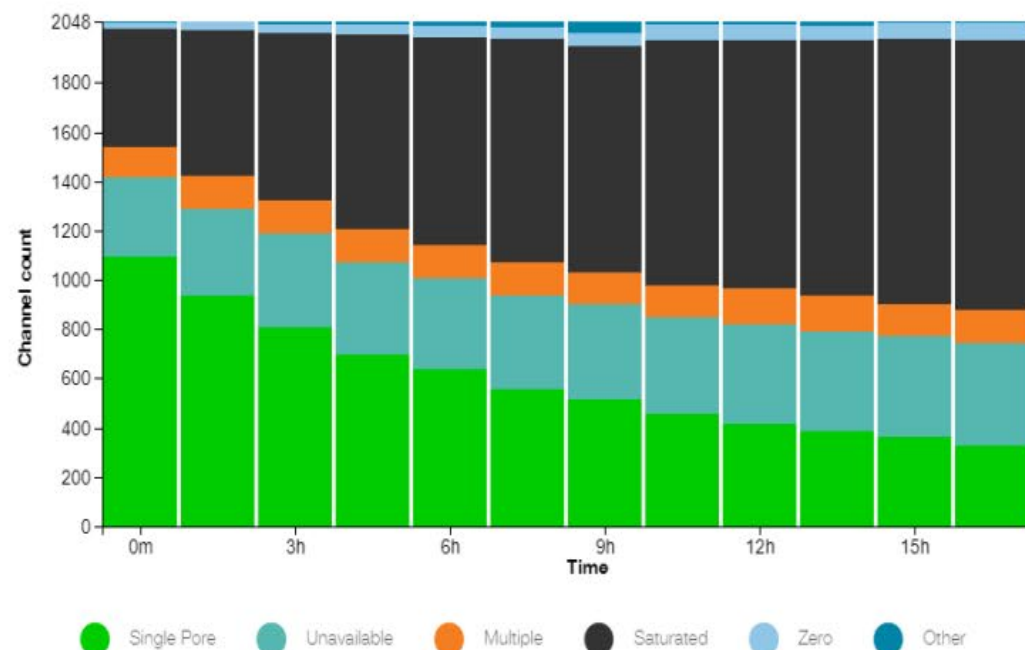
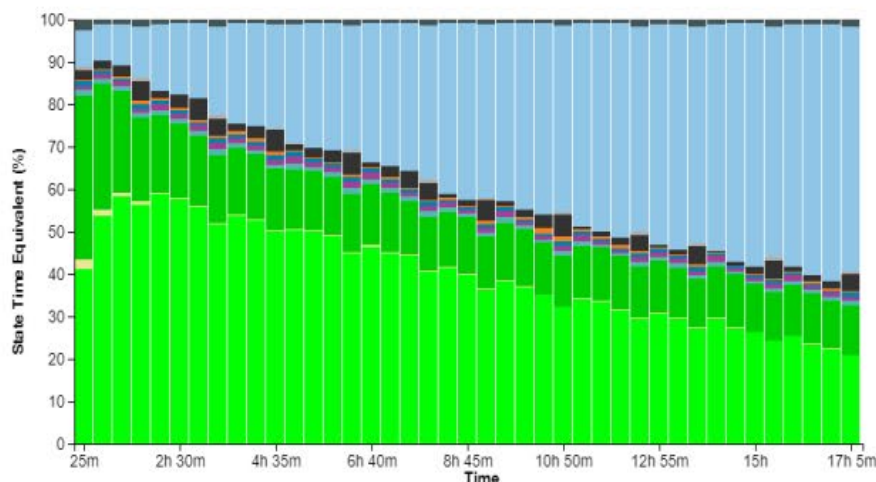
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 - 더 많은 pore가 감지되고 flow cell에서 sequencing이 되면, device에서 log file을 추출하여 원인 파악을 위해 log file을 ONT 본사에 전달. ONT 본사에서 원인을 식별하는 동안 이 position은 사용을 금하여야 함.
 - 다른 position에서도 같은 문제가 발생할 경우, electrode와 array 사이에 bubble이 있을 수 있으므로, bubble 제거 후 sequencing 재 실행



Monitoring a sequencing run: Osmotic imbalance

The five main channel states

- Sequencing running이 진행될수록, Inactive channel이 증가함
- Mux scan 결과, saturating well이 증가하는 양상을 나타냄
- Salt concentration, Surfactant, pH 등 추가적인 membrane stress가 존재한다는 의미



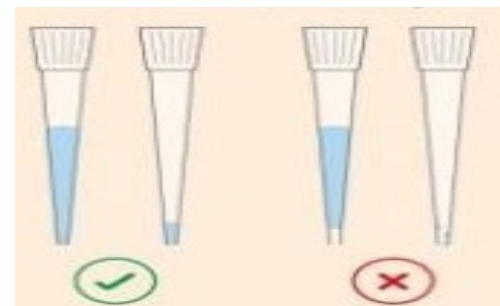
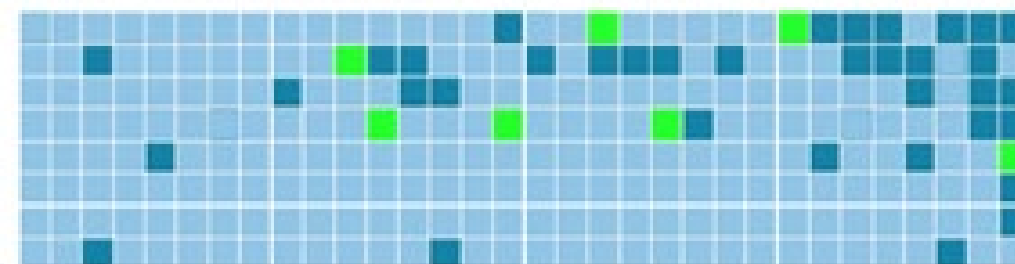
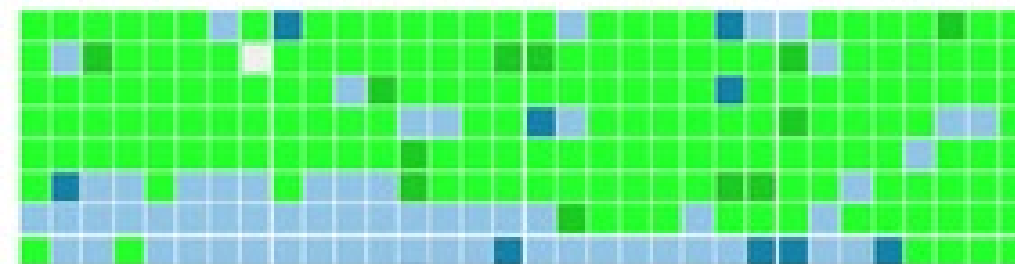
Monitoring a sequencing run: Introducing air bubbles on sensor array

The five main channel states

- Mux scan 직 후, 거의 대부분 Inactive channel로 관찰됨
- 공간적으로 Inactive channel의 명확한 pattern이 나타남

Recommendation

- Flow cell에 priming mix 또는 library loading 시에 air bubble이 유입되지 않게 protocol에 따라 loading 진행
 - Priming mix를 loading하기 전, P1000 파이펫을 200 ul로 설정 후, Priming port (MinION flow cell) or Inlet port (PromethION flow cell)에서 storage buffer를 20~30 ul를 twist 방법으로 제거 및 loading
- Priming mix 또는 library volume을 취할 때, tip안에 bubble이 생성되어 있지 않은지 반드시 확인 후, loading 진행
- Priming mix 또는 library를 loading 할 때, 전체 volume을 무리하게 loading하지 않고, 일부 volume은 남겨두고 loading을 진행



Stop

- Sequencing run을 stop하고 싶을 때, “Stop”을 선택 후 stop할 active run을 선택
- “Stop sequencing and basecalling”을 선택하면 sequencing이 바로 stop되고 일부 POD5 file은 basecalling 되지 않은 상태로 저장됨 (Skipped POD5)
- “Stop sequencing”을 선택하면 sequencing은 즉시 stop되지만, basecalling은 계속 진행이 됨. 즉, sequencing을 진행한 모든 read에 대해 basecalling을 완료하여 FASTQ file로 저장한 후 완전히 stop
- Run report는 언제든지 export가 가능

