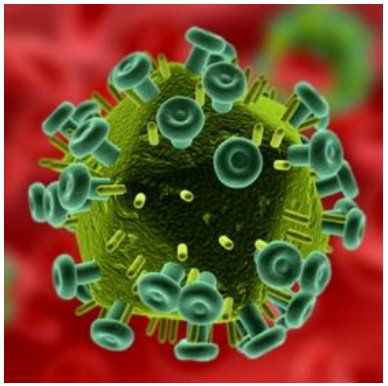


1. Introduction

Cyprinid herpesvirus 3 (CyHV-3)

The cyprinid herpesvirus 3 (CyHV-3, or KHV)

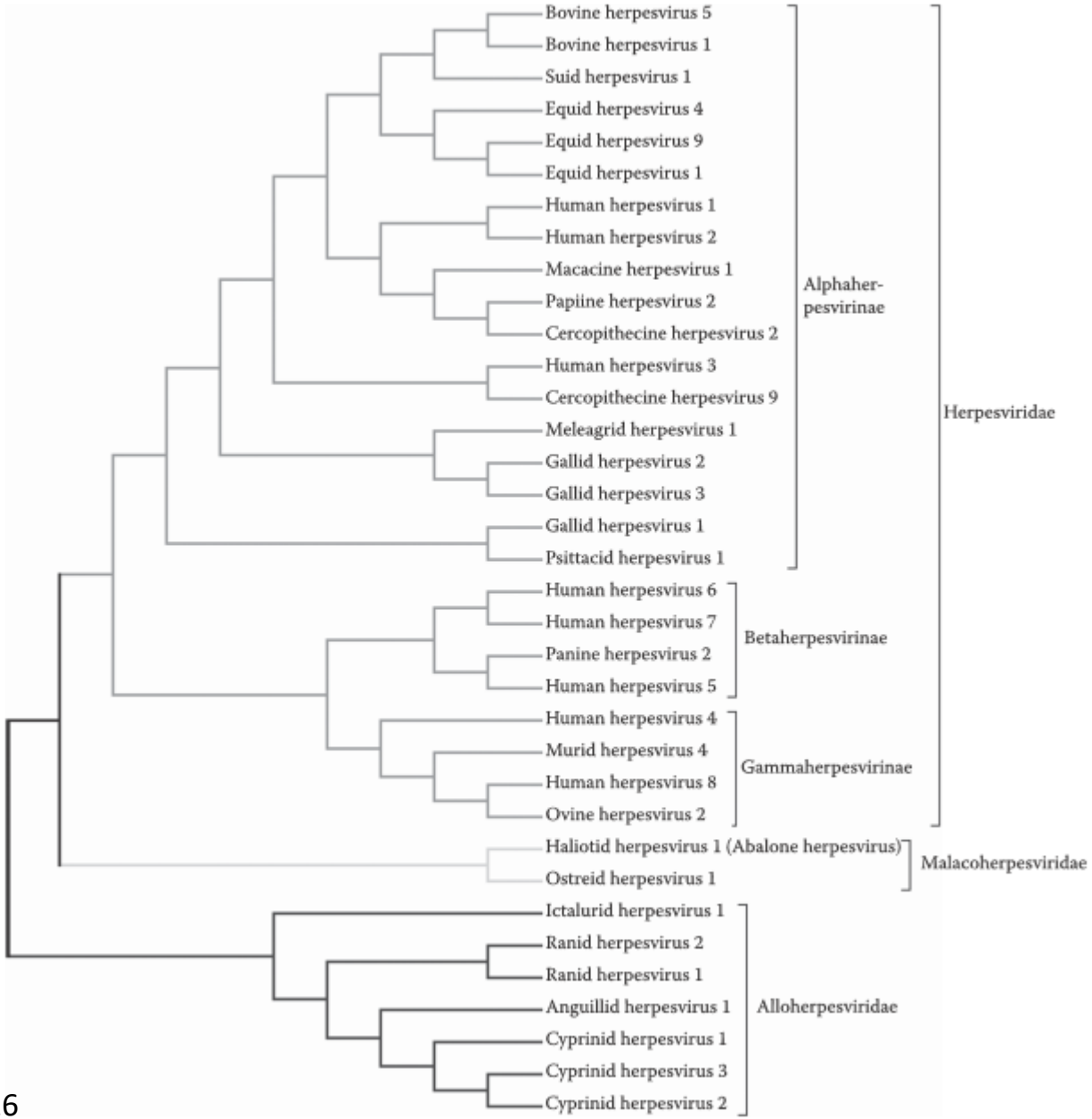
- Described for the first time in 1998
- Has spread over > 30 countries worldwide
- Etiological agent of the koi herpesvirus disease (KHVD)
- Highly contagious virus that causes significant morbidity and mortality

Cyprinus carpio koi

Cyprinus carpio carpio

- Has been acknowledged as an economically and sociologically important pathogen
- KHVD has been placed on the list of serious diseases notifiable to the World Organisation for Animal Health (OIE) and the European Union (EU)

Classification of CyHV-3 within the order Herpesvirales



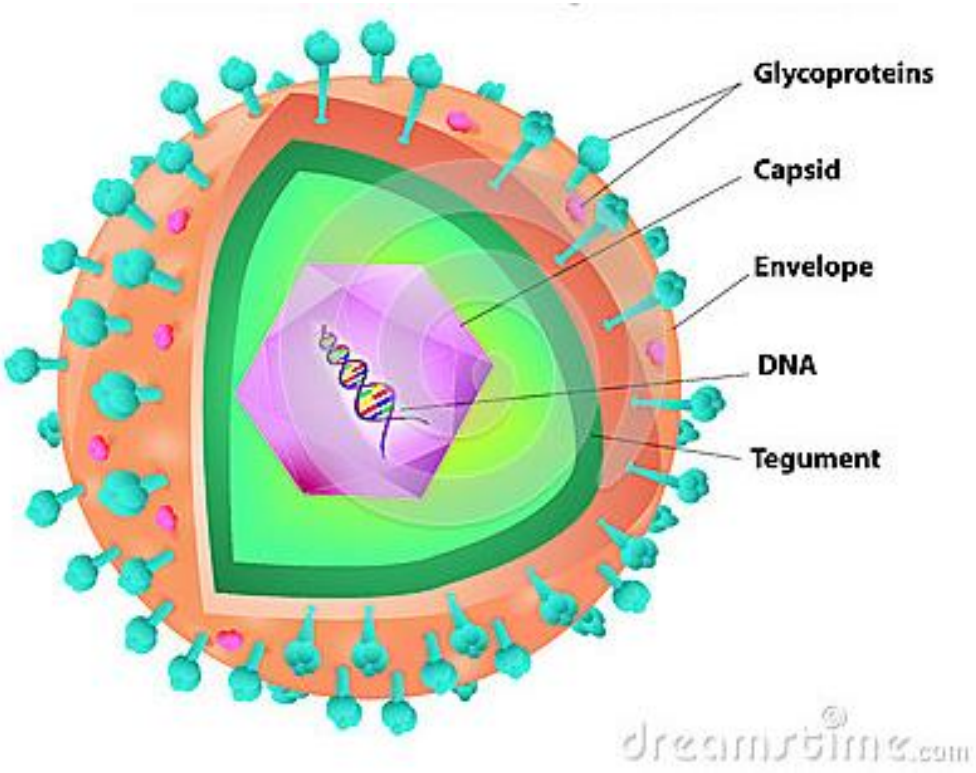
Source: McColl et al, 2016

Members of the *Alloherpesviridae* family

	Family: *Alloherpesviridae*
	Genus: *Batrachovirus*
★	**Species:** *Ranid herpesvirus 1*
	Species: *Ranid herpesvirus 2*
	Species: *Ranid herpesvirus 3*
	Genus: *Cyprinivirus*
	Species: *Anguillid herpesvirus 1*
	Species: *Cyprinid herpesvirus 1*
	Species: *Cyprinid herpesvirus 2*
★	**Species:** *Cyprinid herpesvirus 3*
	Genus: *Ictalurivirus*
	Species: *Acipenserid herpesvirus 2*
★	**Species:** *Ictalurid herpesvirus 1*
	Species: *Ictalurid herpesvirus 2*
	Genus: *Salmonivirus*
★	**Species:** *Salmonid herpesvirus 1*
	Species: *Salmonid herpesvirus 2*
	Species: *Salmonid herpesvirus 3*

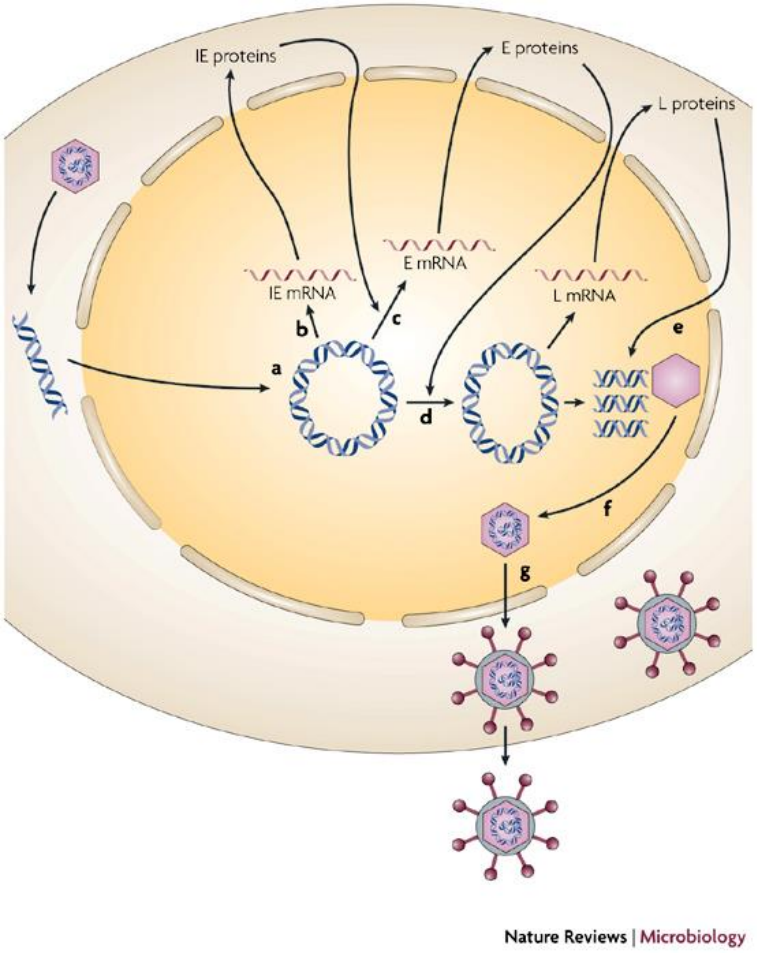
International Committee on Taxonomy of Viruses (2018)

Structure of a herpesvirus



http://www.dentalnotebook.com/human-herpesvirus-1/

Overview of a herpesvirus lytic infection cycle



a | Parental viral DNA enters the host cell nucleus and rapidly circularizes

b | The first genes to be expressed are the immediate–early (IE) genes

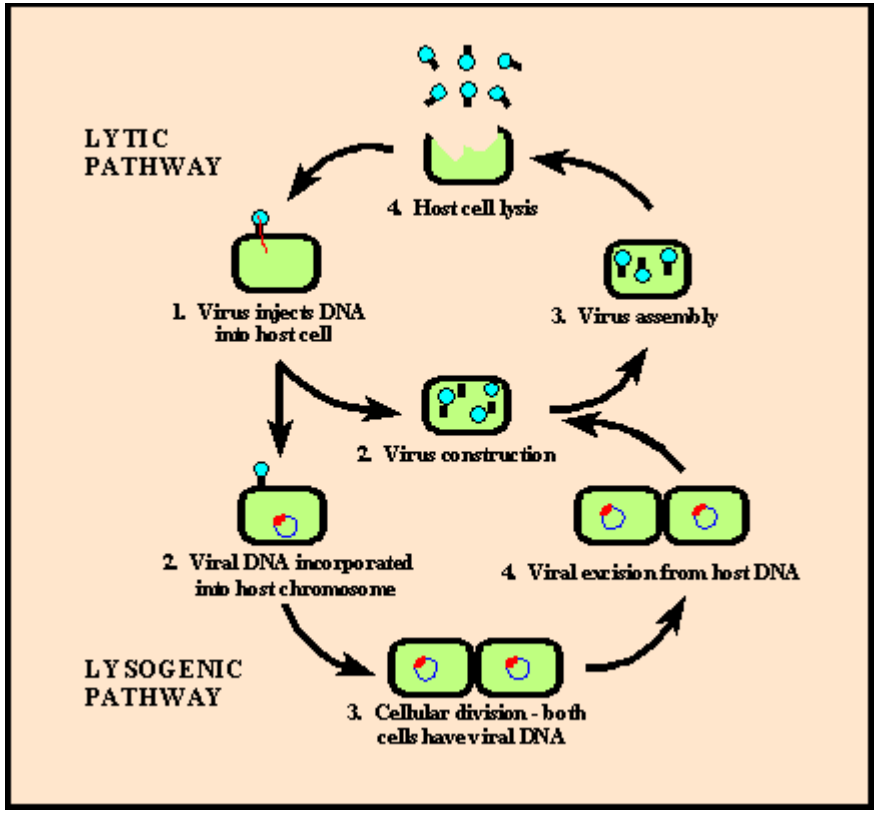
c | IE proteins are transported into the nucleus and transactivate early (E) gene expression

d | DNA replication stimulates the expression of the late (L) genes

e,f | Viral capsid assembly and progeny DNA encapsidation take place in the nucleus

g | Virions egress from the nucleus and the cell

Lytic *versus* latent cycles



http://www.uic.edu/classes/bios/bios104/mike/bacteria01.htm

CyHV-3 has a latent cycle: it is present in the host, but its replication and protein expression is extremely reduced

First full sequences of CyHV-3 : 2007

JOURNAL OF VIROLOGY, May 2007, p. 5058–5065 Vol. 81, No. 10
0022-538X/07/$08.00+0 doi:10.1128/JVI.00146-07

Genome Sequences of Three Koi Herpesvirus Isolates Representing the Expanding Distribution of an Emerging Disease Threatening Koi and Common Carp Worldwide[▽]

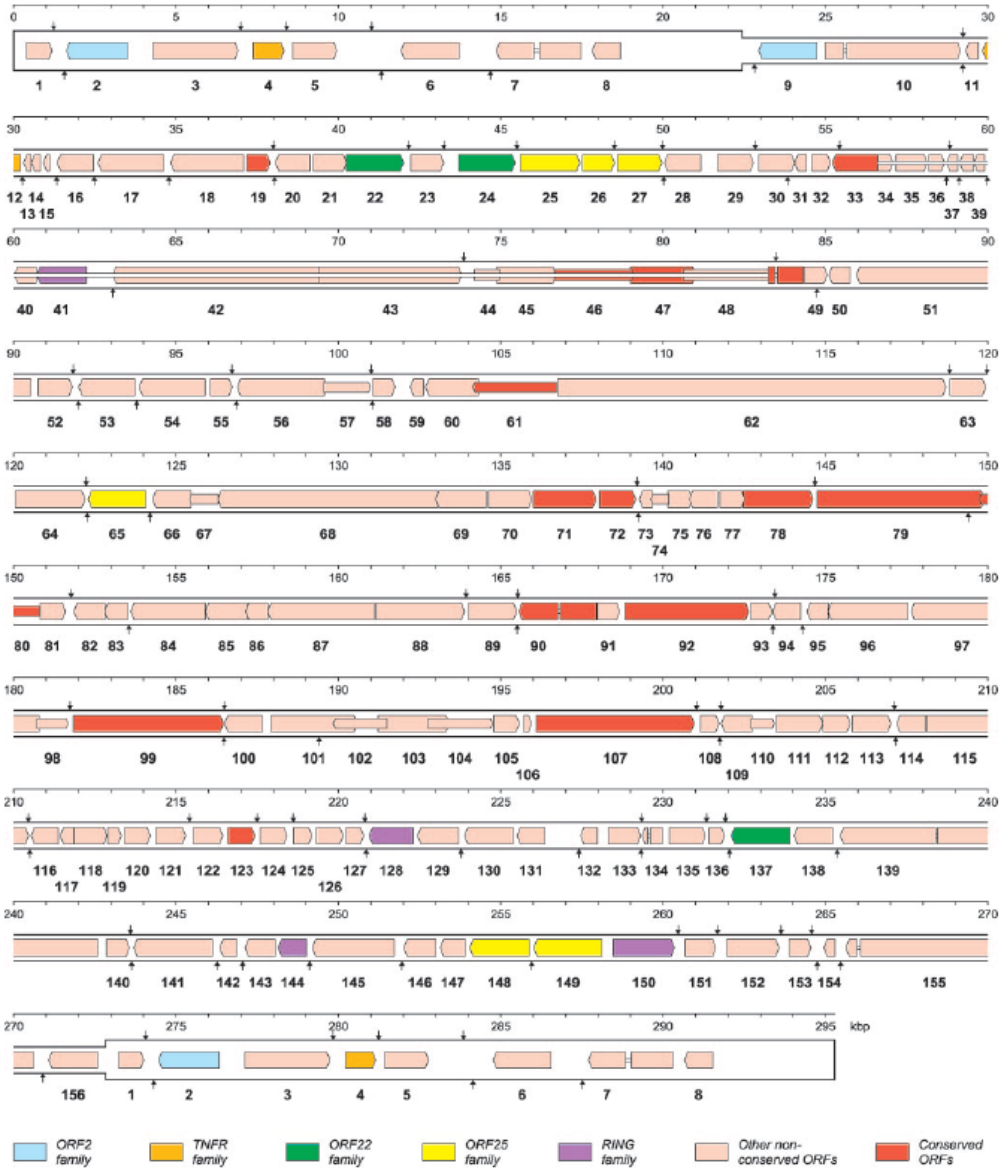
Takashi Aoki,[1] Ikuo Hirono,[1] Ken Kurokawa,[2] Hideo Fukuda,[1] Ronen Nahary,[3] Avi Eldar,[4] Andrew J. Davison,[5]* Thomas B. Waltzek,[6] Herve Bercovier,[3] and Ronald P. Hedrick[6]

Genome annotation

-156 predicted open reading frames (ORF)

-Only 13 with homologs within the family *Halloherpesviridae*

-The majority of them with unknown function



Source: Aoki *et al*, 2007

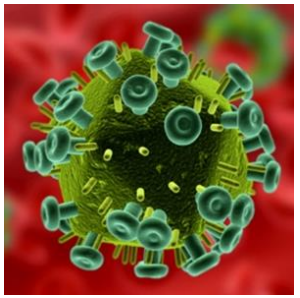
Genome comparison of the 3 sequenced strains

- Nine ORFs harbor mutations associated with loss of function
- Six of these genes encode membrane glycoproteins

Hypothesis:

Emergence of virulent strains of CyHV-3 would be associated with gene fragmentation (loss of function)

Objectifs pédagogiques du TP



Objectif global:

Comprendre et maîtriser les principales étapes du séquençage, de l'échantillon à l'assemblage du génome

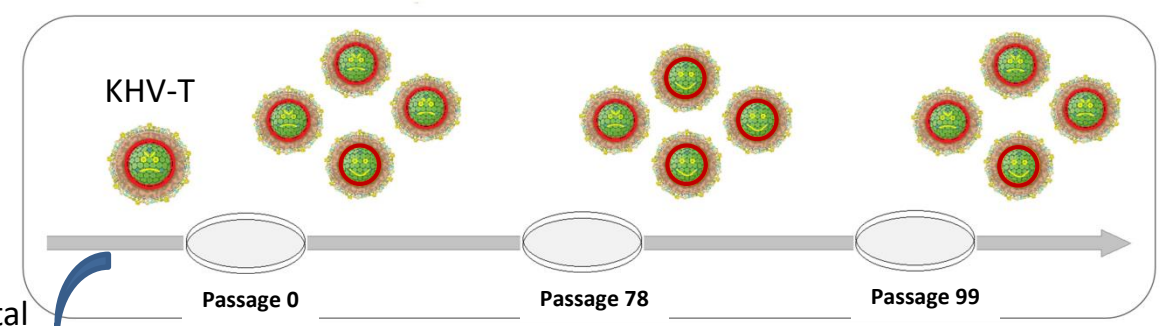
Objectifs spécifiques:

Réaliser une extraction d'ADN

Réaliser la construction de banques pour séquençage 3G

Comprendre comment les séquences sont filtrées et assemblées en génomes (par alignement sur une référence et par assemblage *de novo*)

Objectifs scientifiques du TP



99 consecutive passages on CCB cells

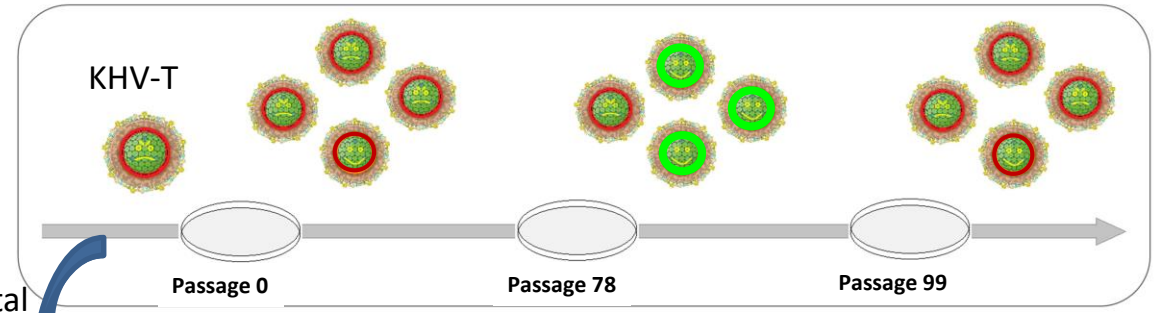
Experimental infections

90% morbidity
90% mortality

0% morbidity
18% mortality

90% morbidity
30% mortality

Objectifs scientifiques du TP



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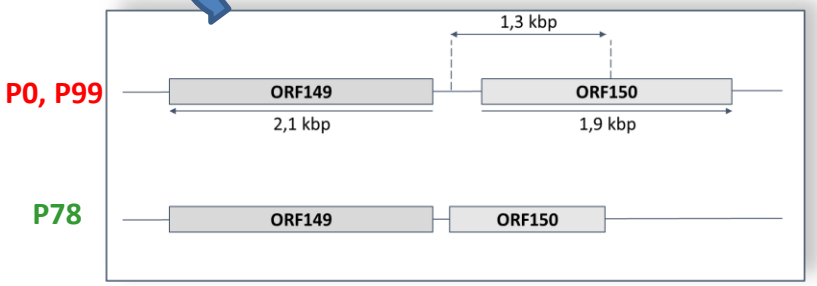
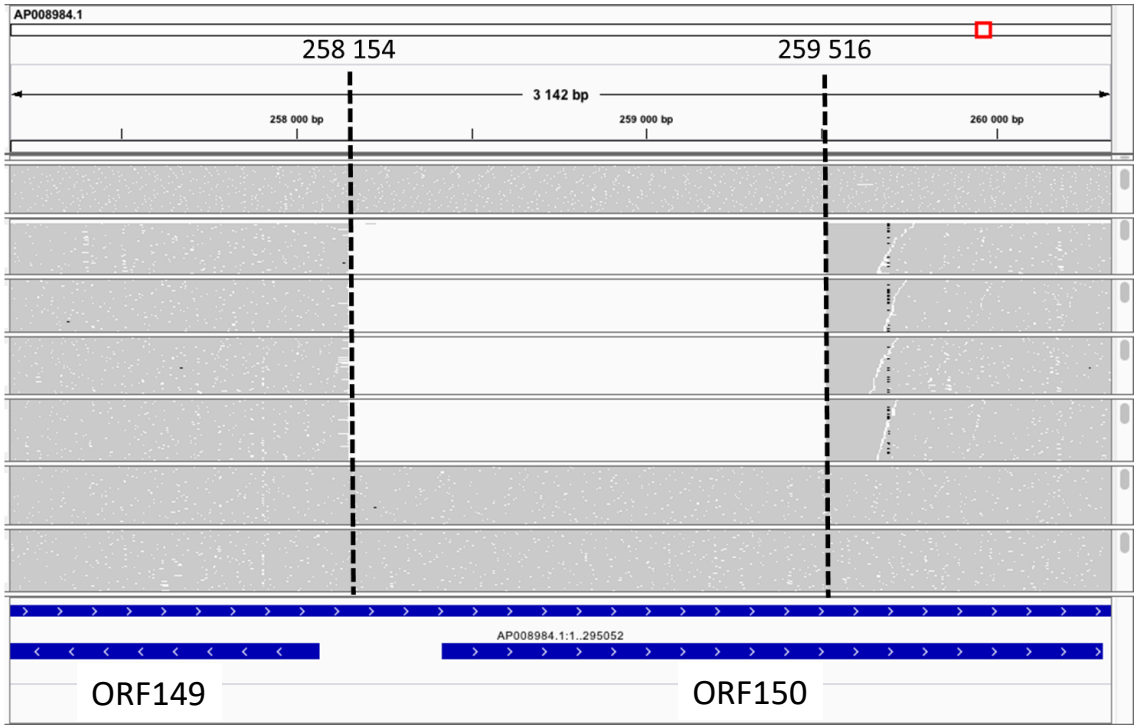
30% mortality

Sequencing



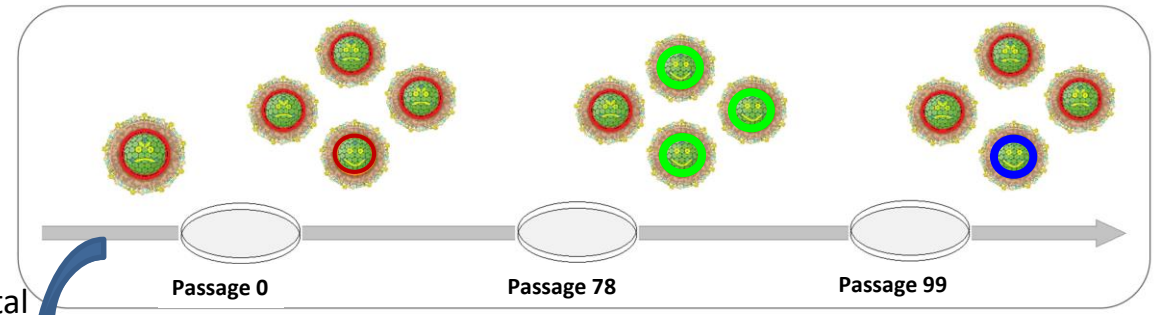
Objectifs scientifiques du TP

Sample	Mean Coverage
P0	7802
P78-1	262
P78-2	418
P78-3	356
P78-4	568
P99-1	559
P99-2	395



Integrated Genomics Viewer

Objectifs scientifiques du TP



99 consecutive passages on CCB cells

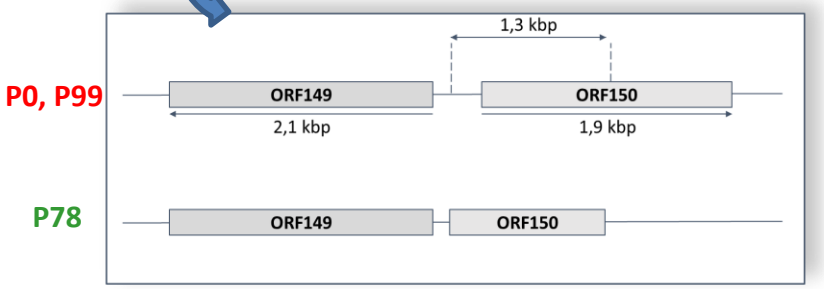
Experimental infections

90% morbidity
90% mortality

0% morbidity
18% mortality

90% morbidity
30% mortality

Séquençage



⇨ Though at extremely low amount, the undeleted haplotype was still present in P78

⇨ It again became dominant in P99

Objectifs scientifiques du TP

viruses

MDPI

Article

Cyprinid Herpesvirus 3 Evolves in vitro through an Assemblage of Haplotypes that Alternatively become Dominant or Under-Represented

Sandro Klafack [1], Anna-Sophie Fiston-Lavier [2], Sven M. Bergmann [1,*], Saliha Hammoumi [2], Lars Schröder [3], Walter Fuchs [3], Angela Lusiastuti [4], Pei-Yu Lee [5], Sarahi Vega Heredia [2], Master student consortium [6], Anne-Sophie Gosselin-Grenet [7] and Jean-Christophe Avarre [2,*]

1 Institute of Infectology, Friedrich-Loeffer-Institut, Federal Research Institute for Animal Health, 17493 Greifswald-Insel Riems, Germany
2 ISEM, IRD, CNRS, EPHE, University of Montpellier, 34095 Montpellier, France
3 Institute of Molecular Virology and Cell Biology, Friedrich Loeffer Institut, Federal Research Institute for Animal Health, 17493 Greifswald-Insel Riems, Germany
4 Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor 16129, Indonesia
5 GenReach Biotechnology, Taichung City 407, Taiwan
6 Faculty of Sciences, University of Montpellier, 34095 Montpellier, France
7 DGIMI, University of Montpellier, INRA, 34095 Montpellier, France
* Correspondence: sven.bergmann@fli.de (S.M.B.); Jean-christophe.avarre@ird.fr (J.-C.A.)

Objectifs scientifiques du TP

Peer Community Journal

Section: Infections

RESEARCH ARTICLE

Published
2022-07-29

Cite as
Nurul Novelia Fuandila, Anne-Sophie Gosselin-Grenet, Marie-Ka Tilak, Sven M Bergmann, Jean-Michel Escoubas, Sandro Klafack, Angela Mariana Lusiastuti, Munti Yuhana, Anna-Sophie Fiston-Lavier, Jean-Christophe Avarre and Emira Cherif (2022) *Structural variation turnovers and defective genomes: key drivers for the in vitro evolution of the large double-stranded DNA koi herpesvirus (KHV)*, Peer Community Journal, 2: e44.

Correspondence
jean-christophe.avarre@umontpellier.fr
emira.cherif@ird.fr

Peer-review
Peer reviewed and recommended by PCI Infections,

Structural variation turnovers and defective genomes: key drivers for the in vitro evolution of the large double-stranded DNA koi herpesvirus (KHV)

Nurul Novelia Fuandila[1], Anne-Sophie Gosselin-Grenet[2], Marie-Ka Tilak[1], Sven M Bergmann[3], Jean-Michel Escoubas[4], Sandro Klafack[5], Angela Mariana Lusiastuti[6], Munti Yuhana[7], Anna-Sophie Fiston-Lavier[8], Jean-Christophe Avarre[1], and Emira Cherif[1]

Volume **2** (2022), article e44

https://doi.org/10.24072/pcjournal.154

Objectifs scientifiques du TP

Peer Community Journal

Section: Infections

RESEARCH ARTICLE

Published
2022-07-29

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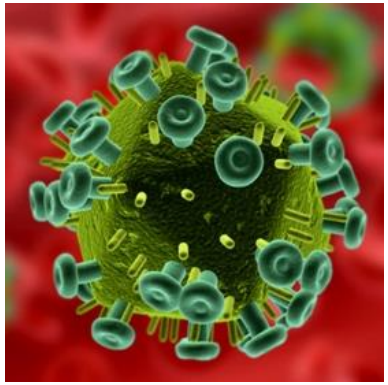
Volume **2** (2022), article e44

https://doi.org/10.24072/pcjournal.154

⇨ **Poursuivre la compréhension de l'évolution de ce virus *in vitro***



2. Préparation des échantillons et extraction des ADN viraux

Les différentes étapes

Echantillons

↓ 1 par binôme ⇨ 20 x 2 extractions
1 par trinôme ⇨ 2 x 3 extractions

Extraction d'ADN

↓ Pool de 2 extractions ⇨ 22 tubes

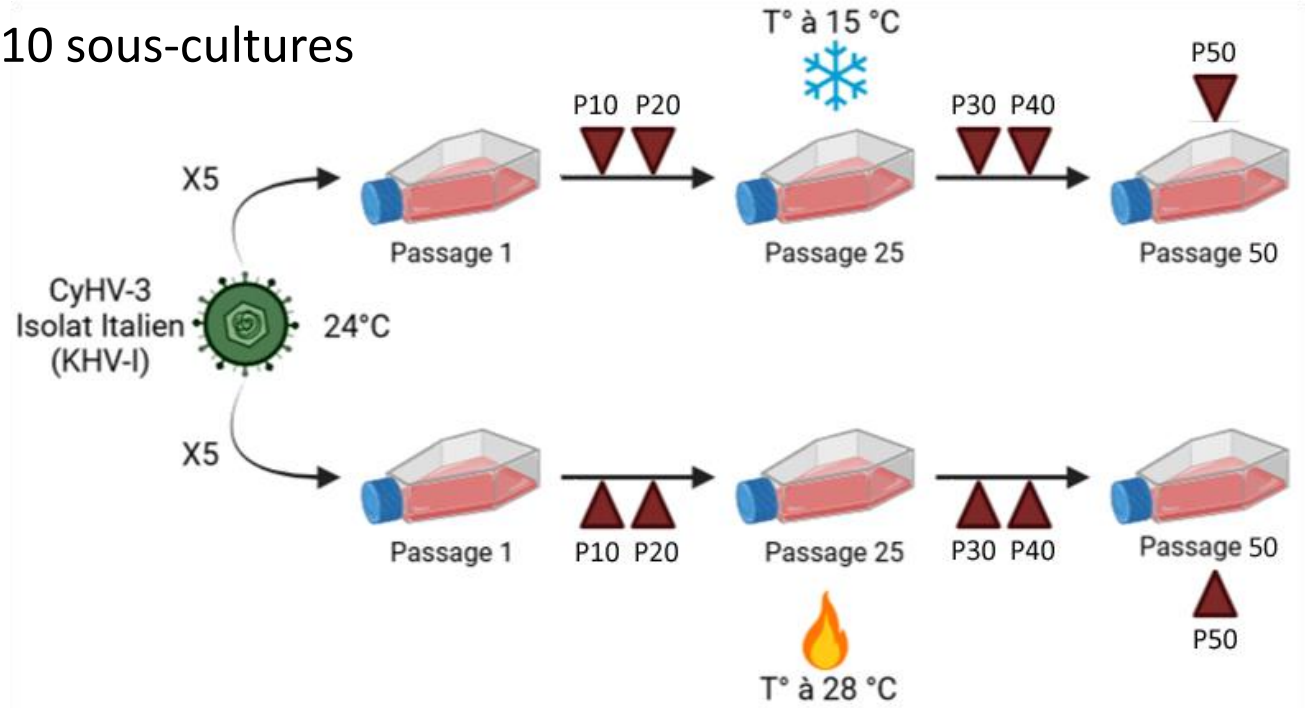
Préparation des librairies

↓ Pool de 5-6 librairies ⇨ 4 séquençages

Séquençage

Préparation des échantillons

✓ Echantillons: 10 sous-cultures



• Surnageants de cultures cellulaires infectées :

- Sous-culture initiale P1 (X7)
- 2 sous-cultures 15°C (C1, C5) à 10, 20, 30, 40 et 50 passages (en double)
- 2 sous-cultures 28°C (C6, C10), à 10, 20, 30, 40 et 50 passages (en double)

Préparation des échantillons

- ✓ Echantillons:

  1 passage (x7)
  10 passages : sous cultures C1 et C5 (15°C) ; sous-cultures C6 et C10 (28°C) (x2)
  20 passages : sous cultures C1 et C5 (15°C) ; sous-cultures C6 et C10 (28°C) (x2)
  30 passages : sous cultures C1 et C5 (15°C) ; sous-cultures C6 et C10 (28°C) (x2)
  40 passages : sous cultures C1 et C5 (15°C) ; sous-cultures C6 et C10 (28°C) (x2)
  50 passages : sous cultures C1 et C5 (15°C) ; sous-cultures C6 et C10 (28°C) (x2)

- ✓ Marquage des tubes (2 tubes / binôme):

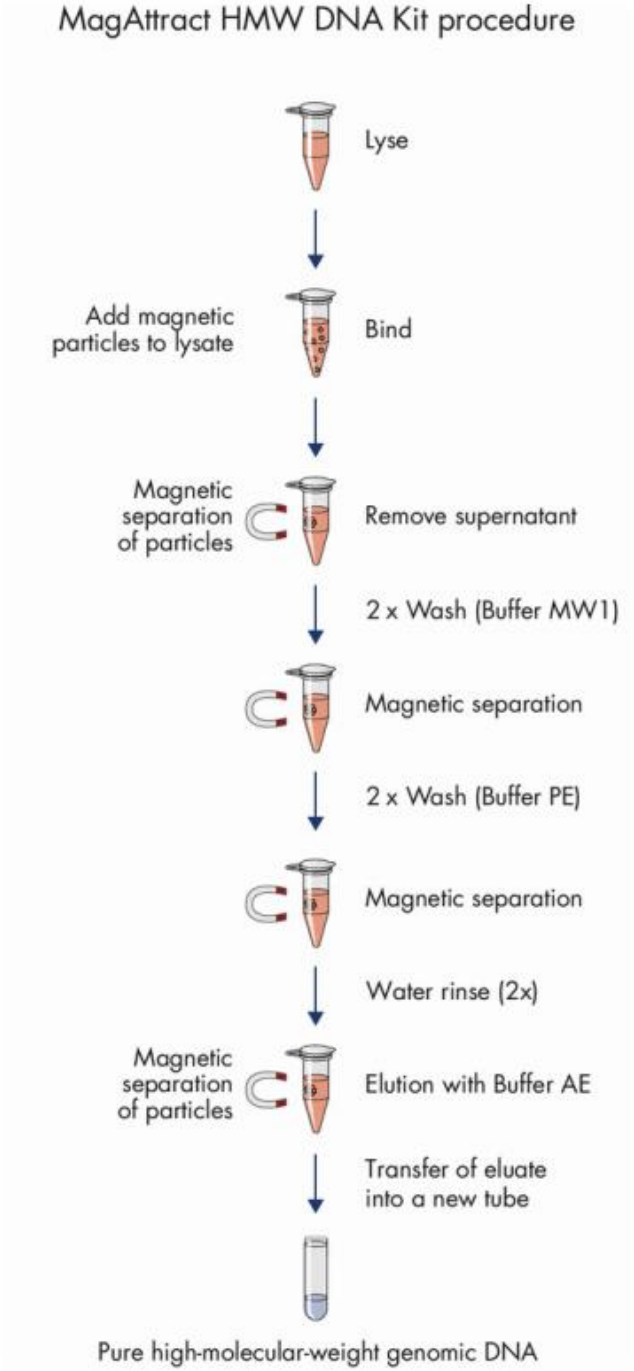
  P1.1 à P1.7
  P10.C1.1 et P10.C1.2 ; P10.C5.1 et P10.C5.2
  P10.C6.1 et P20.C6.2 ; P20.C10.1 et P20.C10.2
  ….

- ✓ Distribution de 2 aliquots de 220 µL dans des tubes de 1,5 mL

Contenu du kit d'extraction (MagAttract HMW DNA, Qiagen)

MagAttract HMW DNA Kit	**(48)**
Catalog no.	**67563**
Number of samples	**48**
MagAttract Suspension G	2 x 1 ml
Buffer ATL	11.2 ml
Buffer AL	12 ml
Buffer MB	15 ml
Buffer MW1 (concentrate)	77 ml
Buffer PE (concentrate)	20 ml
Buffer AE	22 ml
Proteinase K	1.25 ml
RNase A (100 mg/ml)	25 mg
Nuclease-Free Water	2 x 50 ml
Quick-Start Protocol	1

Principe


MagAttract HMW DNA Kit procedure
Lyse
Add magnetic particles to lysate
Bind
Magnetic separation of particles
Remove supernatant
2 x Wash (Buffer MW1)
Magnetic separation
2 x Wash (Buffer PE)
Magnetic separation
Water rinse (2x)
Magnetic separation of particles
Elution with Buffer AE
Transfer of eluate into a new tube
Pure high-molecular-weight genomic DNA

Extraction: recommandations

Important points before starting

- To ensure that the extracted DNA is not fragmented, avoid shearing stress such as fast or unnecessary pipetting steps.

Extraction: recommandations

Important points before starting

- To ensure that the extracted DNA is not fragmented, avoid shearing stress such as fast or unnecessary pipetting steps.

☹ Pas de vortex, pas de pipetage rapide

Extraction: recommandations

Important points before starting

- To ensure that the extracted DNA is not fragmented, avoid shearing stress such as fast or unnecessary pipetting steps.

☹ Pas de vortex, pas de pipetage rapide

Things to do before starting

- If using frozen blood samples, thaw and equilibrate to room temperature.
- ~~If using fresh blood samples in primary tubes, mix the blood samples carefully (e.g., by inverting the tubes several times)~~.
- Check that Buffers AL and MB do not contain a precipitate. If necessary, dissolve by incubating at 37°C with occasional shaking.
- Ensure that Buffers MW1 and PE have been prepared according to the instructions on page 13.

Buffer MW1 is supplied as a concentrate. Before using for the first time, be sure to add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

Buffer PE is supplied as a concentrate. Before using for the first time, be sure to add the appropriate amount of ethanol (96–100%) as indicated on the bottle.

Extraction: recommandations

Important points before starting

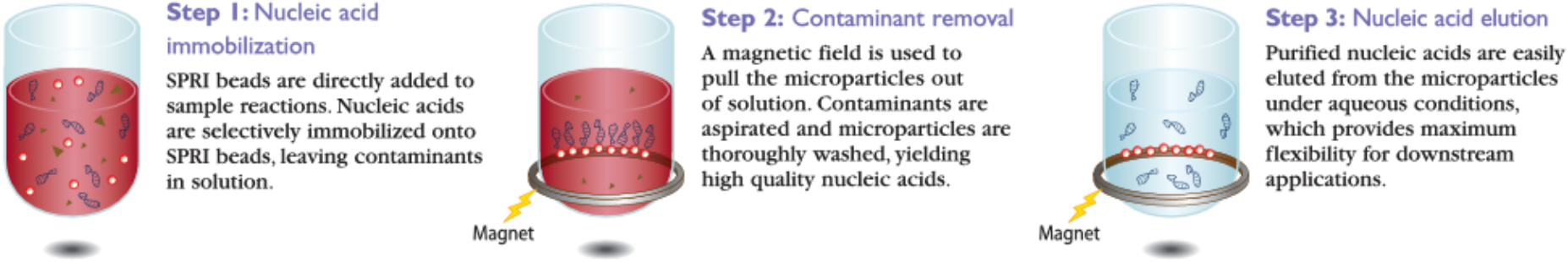
- To ensure that the extracted DNA is not fragmented, avoid shearing stress such as fast or unnecessary pipetting steps.

☹ Pas de vortex, pas de pipetage rapide

Things to do before starting

- If using frozen blood samples, thaw and equilibrate to room temperature.
- ~~If using fresh blood samples in primary tubes, mix the blood samples carefully (e.g., by inverting the tubes several times)~~.
- Check that Buffers AL and MB do not contain a precipitate. If necessary, dissolve by incubating at 37°C with occasional shaking.
- Ensure that Buffers MW1 and PE have been prepared according to the instructions on page 13.
- Before starting, ensure that the magnetic particles are fully resuspended. Vortex the vessel containing the magnetic particles vigorously for at least 3 min before first use.

Note: purification sur billes magnétiques



SPRI: Solid Phase Reversible Immobilization.

SPRI beads are paramagnetic (magnetic only in a magnetic field) and this prevents them from clumping and falling out of solution. Each bead is made of polystyrene surrounded by a layer of magnetite and coated with carboxyl molecules.

Carboxyl molecules reversibly bind DNA in the presence of a “crowding agent” (such as polyethylene glycol) and salts.

Extraction : lyse et digestion

1. Pipet 20 µl Proteinase K into the bottom of a 2 ml sample tube.
2. Add 200 µl of total blood to the sample tube.
3. Add 4 µl RNase A solution and 150 µl Buffer AL to the sample. Mix carefully by vortexing.

   Note: Do not add Proteinase K directly to Buffer AL.

   Note: To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed to yield a homogeneous solution.
4. Incubate at room temperature for 30 min.

   Note: During lysis, the color of the solution will change to dark brown or black.

   Note: To shorten the lysis time, it is possible to incubate for 10 min at 65°C.
5. Briefly centrifuge the 2 ml sample tube to remove drops of liquid from inside the lid.

Extraction : attachement et 1ers lavages

6. Add 15 µl MagAttract Suspension G to the sample.

   Note: Ensure that the magnetic particles are fully resuspended.

7. Add 280 µl Buffer MB to the sample ~~and place the microcentrifuge tube with the sample in the tube holder of the MagAttract Magnetic Rack~~.

8. Place the tube ~~holder of the MagAttract Magnetic Rack~~ onto the mixer and incubate at room temperature for 3 min at 1400 rpm.

   8a. Place the tube ~~holder of the MagAttract Magnetic Rack~~ on its magnetic base, wait until bead separation has been completed (~1 min), and remove the supernatant.
   Note: Due to the dark color of the solution, the bead pellet is not easily visible. While aspirating the supernatant, avoid disturbing the magnetic bead pellet. Remove the supernatant completely.

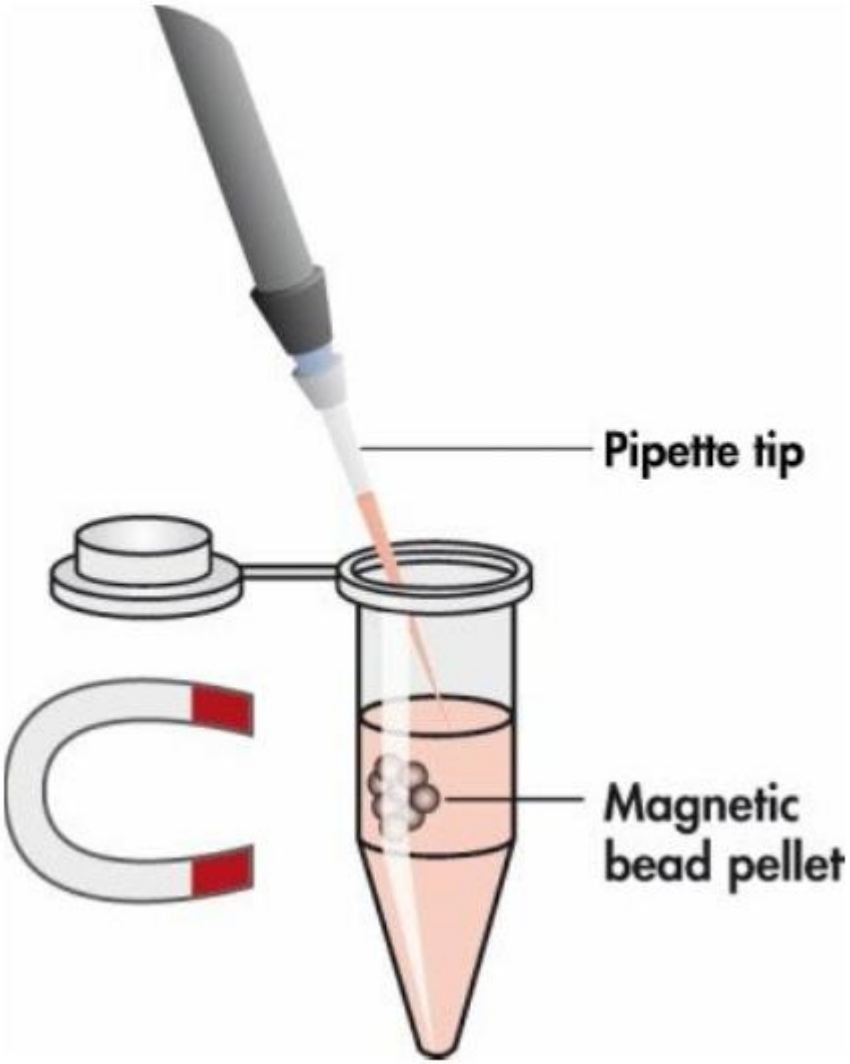
9. Add 700 µl Buffer MW1 to the sample and place the tube ~~holder of the MagAttract Magnetic Rack~~ onto the mixer. Incubate at room temperature for 1 min at 1400 rpm.

   Note: To increase the efficiency of the wash step, remove the tube ~~holder~~ from magnetic base before adding the wash buffer. Add the wash buffer directly onto the magnetic bead pellet.

   9a. Place the tube ~~holder of the MagAttract Magnetic Rack~~ on its magnetic base, wait until bead separation has been completed (~1 min), and remove the supernatant.

10. Repeat steps 9 and 9a.

Astuce pour retirer le surnageant



Extraction : 2èmes lavages

11. Add 700 µl Buffer PE to the sample and place the tube ~~holder of the MagAttract Magnetic Rack~~ onto the mixer. Incubate at room temperature for 1 min at 1400 rpm.

    Note: To increase the efficiency of the wash step, remove the tube ~~holder~~ from the magnetic base before adding the wash buffer. Add the wash buffer directly onto the magnetic bead pellet.

    11a. Place the tube ~~holder of the MagAttract Magnetic Rack~~ on its magnetic base, wait until bead separation has been completed (~1 min), and remove the supernatant.

12. Repeat steps 11 and 11a.

    Note: Remove all the supernatant. Use a small pipette tip to remove any traces of Buffer PE.

13. Rinse the particles with 700 µl distilled water while the tube ~~holder~~ is on the magnetic base and the beads are fixed to the wall of the sample tube. Incubate for 1 min at room temperature, and remove the supernatant completely.

    Important: Do not pipet water directly onto the bead pellet – pipet it into the sample tube against the side facing away from the bead pellet. All pipetting steps must be performed carefully to avoid disturbing the fixed bead pellet (see **Figure 2**, page 10).

14. Repeat step 13.

Extraction : rinçage et élution

15. Remove the tube ~~holder of the MagAttract Magnetic Rack~~ from its magnetic base and add 60 µL of H20 ~~volume of Buffer AE (100–200 µl)~~. Place the tube ~~holder~~ onto the mixer and incubate at room temperature for 3 min at 1400 rpm.

    Optional: Elute the DNA with distilled pure water if the DNA will be used in enzymatic downstream applications.

16. Place the tube ~~holder of the MagAttract Magnetic Rack~~ on its magnetic base, wait until bead separation has been completed (~1 min), and transfer the supernatant with the high-molecular-weight DNA to a new sample tube.

    Note: The yield of genomic DNA depends on the sample type and the number of cells in the sample. Typically, a 200 µl sample of whole blood from a healthy individual will yield 5–6 µg of DNA. For most whole blood samples, a single elution with 200 µl Buffer AE is sufficient.

Concentration des ADN (SpeedVac)

- ✓ Evaporation pendant 1h30 à 37°C
- ✓ Vérification du volume final (5 µL)
- ✓ Pool des 2 réplicas (20 µL)

Dosage et vérification des ADN

- ✓ Absorbance (Nanodrop) ⇨ pureté

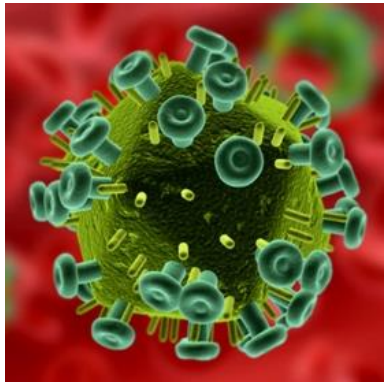
  Faire un blanc avec le tampon d'élution
  Mesurer avec 1,5 µL d'échantillon

- ✓ Fluorescence (Qbit, kit High Sensitivity) ⇨ quantité

  Ajouter 10 µL de marqueur à 190 µL de tampon
  Ajouter 2 µL d'échantillons à 198 µL de tampon

- ✓ Electrophorèse ⇨ taille

  Charger 1-5 µL d'ADN par puits (5-20 ng) mélangés au tampon de charge 5X



3. Quantification relative des ADN viraux

Dosage et vérification des ADN

- ✓ Absorbance (Nanodrop) ⇨ pureté

  Faire un blanc avec le tampon d'élution
  Mesurer avec 1,5 µL d'échantillon

- ✓ Fluorescence (Qbit, kit High Sensitivity) ⇨ quantité

  Ajouter 10 µL de marqueur à 190 µL de tampon
  Ajouter 2 µL d'échantillons à 198 µL de tampon

- ✓ Electrophorèse ⇨ taille

  Charger 1-5 µL d'ADN par puits (5-20 ng) mélangés au tampon de charge 5X

Bilan des dosages (1) Nanodrop - Qbit

Résultat des gels

Echantillon

ng/µL

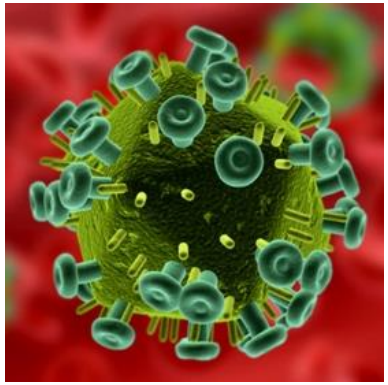
Résultat des gels

Echantillon

ng/µL	**7,3***	**27***	**68***	**>100***	**58**		**140**

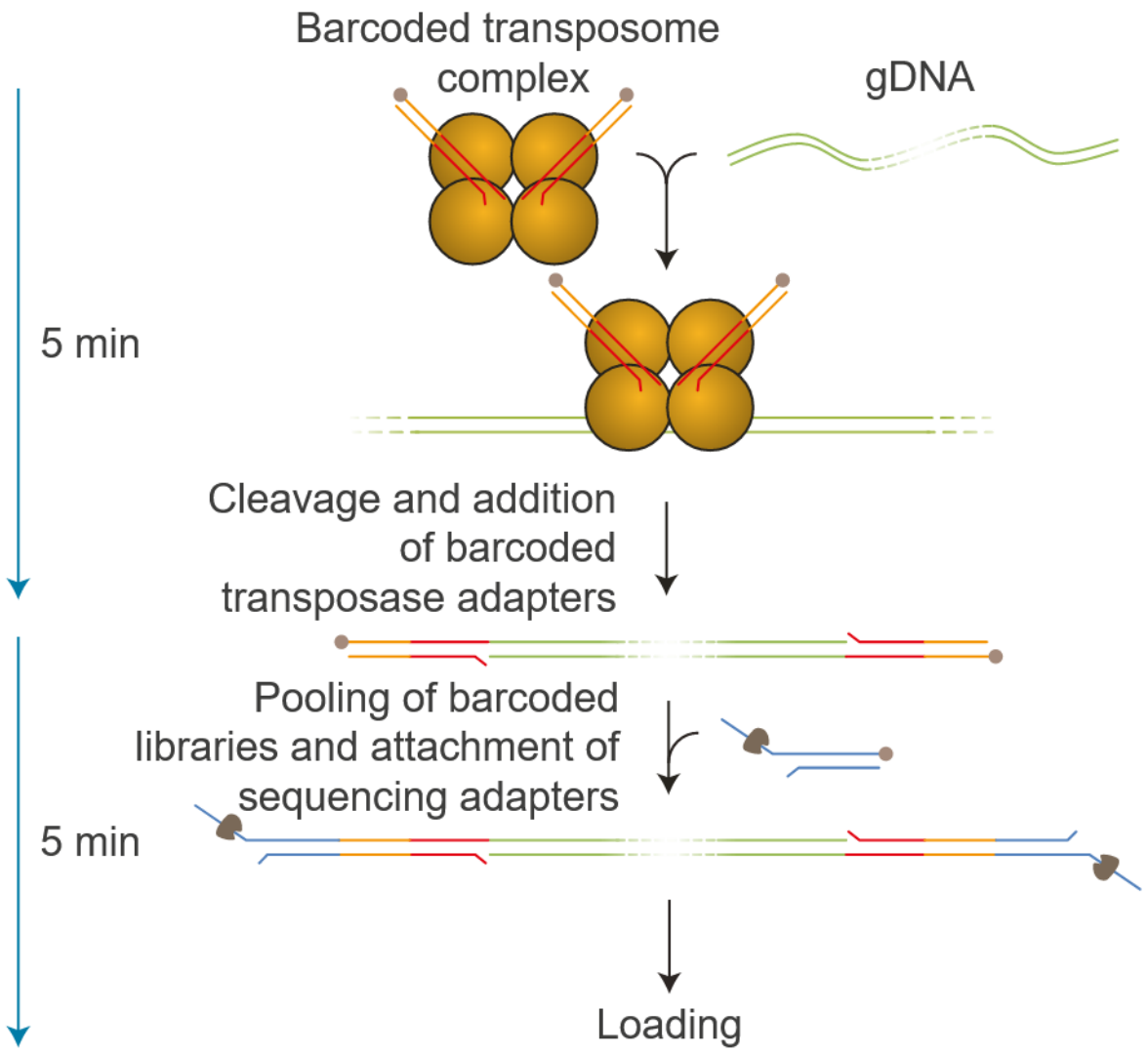
Bilan des dosages (2) Nanodrop - Qbit

	Qubit	Nanodrop					
Sample ID	ng/µl	ng/µl	A260	A280	260/280	260/230	**volume**
KR1	7,3	8,3	0,229	0,137	2,13	2	**7,5**
KR2	27	24,8	0,484	0,299	2,01	2,1	**7,5**
KR3	140	114,2	2,376	1,298	1,88	0,52	**2,9**
KR4	68	69,4	0,771	0,417	1,9	2,01	**5,9**
KR5	>100	131,1	1,154	0,647	1,9	2,24	**4,5**
KR6	58	71,2	1,68	0,997	1,89	0,13	**7,5**
KC1	16	13,6	0,325	0,194	2,24	3,1	**7,5**
KC2	44	62,3	2,171	1,202	1,91	2,14	**7,5**
KC3	102	103,3	0,954	0,537	1,9	2,3	**3,9**
KC4	90	114,4	0,877	0,516	1,89	0,84	**4,4**
KC5	51	53,7	1,135	0,631	1,86	0,26	**7,5**
KC6	96	181	3,274	1,816	1,86	1,32	**4,2**



3. Préparation des banques d’ADN pour séquençage

Principe



https://store.nanoporetech.com/eu/catalog/product/view/id/226/s/rapid-barcoding-kit/category/28/

Rapid Barcoding Sequencing kit (SQK-RBK004, Oxford Nanopore)

Rapid Barcoding Sequencing (SQK-RBK004)

Version: RBK_9054_v2_revP_14Aug2019
Last update: 09/12/2020

Oxford NANOPORE Technologies

Flow Cell Number: ..... DNA Samples: .....

Before start checklist

Materials

- [ ] ~400 ng high molecular weight genomic DNA
- [ ] Rapid Barcoding Sequencing Kit (SQK-RBK004)
- [ ] Flow Cell Priming Kit (EXP-FLP002)

Consumables

- [ ] 1.5 ml Eppendorf DNA LoBind tubes
- [ ] 0.2 ml thin-walled PCR tubes
- [ ] Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
- [ ] Agencourt AMPure XP beads (optional)
- [ ] Freshly-prepared 70% ethanol in nuclease-free water (optional)
- [ ] 10 mM Tris-HCl pH 8.0 with 50 mM NaCl (optional)

Equipment

- [ ] Ice bucket with ice
- [ ] Microfuge
- [ ] Timer
- [ ] Thermal cycler or heat block at 30°C and 80°C
- [ ] Pipettes and pipette tips P2, P20, P100, P200, P1000

Rapid Barcoding Sequencing kit (SQK-RBK004, Oxford Nanopore)

The Rapid Barcoding Kit contains twelve unique barcodes and sufficient reagents to generate six sequencing libraries.

RAP : Rapid adapter
SQT : Sequencing tether
LB : Loading beads
SQB : Sequencing buffer
RB01 : Fragmentation Mix RB 1
RB02 : Fragmentation Mix RB 2
RB03 : Fragmentation Mix RB 3
RB04 : Fragmentation Mix RB 4
RB05 : Fragmentation Mix RB 5
RB06 : Fragmentation Mix RB 6
RB07 : Fragmentation Mix RB 7
RB08 : Fragmentation Mix RB 8
RB09 : Fragmentation Mix RB 9
RB10 : Fragmentation Mix RB 10
RB11 : Fragmentation Mix RB 11
RB12 : Fragmentation Mix RB 12

The Flow Cell Priming Kit Expansion Pack is also supplied.

FLB : Flush buffer
FLT : Flush tether

Protocole (SQK-RBK004, Oxford Nanopore)

INSTRUCTIONS	NOTES/OBSERVATIONS
Library preparation	
Thaw kit components at RT, spin down briefly using a microfuge and mix by pipetting as indicated by the table below: ☐ Fragmentation Mix RB01-12: not frozen, briefly spin down, mix well by pipetting ☐ Rapid Adapter (RAP): not frozen, briefly spin down, mix well by pipetting ☐ Sequencing Buffer (SQB): thaw at RT, briefly spin down, mix well by pipetting* ☐ Loading Beads (LB): thaw at RT, briefly spin down, mix by pipetting or vortexing immediately before use ☐ Flush Buffer (FLB) - 1 tube: thaw at RT, briefly spin down, mix well by pipetting* ☐ Flush Tether (FLT): thaw at RT, briefly spin down, mix well by pipetting Prepare the DNA in Nuclease-free water. ☐ Transfer ~400 ng genomic DNA into a DNA LoBind tube ☐ Adjust the volume to 7.5 µl with Nuclease-free water ☐ Mix by flicking the tube to avoid unwanted shearing ☐ Spin down briefly in a microfuge In a 0.2 ml thin-walled PCR tube, mix the following: ☐ 7.5 µl 400 ng template DNA ☐ 2.5 µl Fragmentation Mix RB01-12 (one for each sample) ☐ Mix gently by flicking the tube, and spin down. ☐ Incubate the tube at 30° C for 1 minute and then at 80° C for 1 minute. Briefly put the tube on ice to cool it down. ☐ Pool all barcoded samples in your desired ratio, noting the total volume.	

Protocole (SQK-RBK004, Oxford Nanopore)

INSTRUCTIONS	NOTES/OBSERVATIONS
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Protocole

- [ ] If barcoding four or more samples, increased throughput can be achieved through cleaning up and concentrating the pooled material using AMPure XP beads as outlined in Steps 6-15. Otherwise, for a more rapid sample preparation, transfer 10 µl of pooled sample from Step 5 into a clean 1.5 ml Eppendorf DNA LoBind tube, and proceed directly to Step 16.

- [ ] Resuspend the AMPure XP beads by vortexing.
- [ ] To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube.
- [ ] Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
- [ ] Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.
- [ ] Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
- [ ] Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- [ ] Repeat the previous step.
- [ ] Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.
- [ ] Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at RT.
- [ ] Pellet the beads on a magnet until the eluate is clear and colourless.

Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

- [ ] Remove and retain the eluate which contains the DNA in a clean 1.5 ml Eppendorf DNA LoBind tube
- [ ] Dispose of the pelleted beads

End of optional steps.

- [ ] Add 1 µl of RAP to 10 µl of barcoded DNA.
- [ ] Mix gently by flicking the tube, and spin down.
- [ ] Incubate the reaction for 5 minutes at RT.

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

Protocole

- [ ] If barcoding four or more samples, increased throughput can be achieved through cleaning up and concentrating the pooled material using AMPure XP beads as outlined in Steps 6-15. Otherwise, for a more rapid sample preparation, transfer 10 µl of pooled sample from Step 5 into a clean 1.5 ml Eppendorf DNA LoBind tube, and proceed directly to Step 16.

- [ ] Resuspend the AMPure XP beads by vortexing. ☹ Vortexer régulièrement
- [ ] To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube.
- [ ] Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
- [ ] Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.
- [ ] Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant. ☹ Bien laisser le tube sur le portoir
- [ ] Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.
- [ ] Repeat the previous step. ☹ Bien laisser le tube sur le portoir
- [ ] Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry. ☹ Bien attendre la sédimentation
- [ ] Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at RT.
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End of optional steps.

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- [ ] Mix gently by flicking the tube, and spin down.
- [ ] Incubate the reaction for 5 minutes at RT.

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

Préparation de la flow cell

- [ ] Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at RT.

- [ ] Mix the Sequencing Buffer (SQB), Flush Tether (FLT) and Flush Buffer (FB) tubes by vortexing and spin down at RT.

- [ ] Open the MinION Mk1B lid and slide the flow cell under the clip.

- [ ] Slide the priming port cover clockwise to open the priming port.

IMPORTANT

- [ ] Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):

- [ ] Set a P1000 pipette to 200 µl
- [ ] Insert the tip into the priming port
- [ ] Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip

- [ ] To 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 (FB), and mix by vortexing at RT.

- [ ] Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.

Chargement de la flow cell

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

- [ ] 34 µl Sequencing Buffer (SQB)
- [ ] 25.5 µl Loading Beads (LB), mixed immediately before use
- [ ] 4.5 µl Nuclease-free water
- [ ] 11 µl DNA library

IMPORTANT

- [ ] 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.

Complete the flow cell priming:

- [ ] Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
- [ ] Load 200 µl of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

- [ ] Mix the prepared library gently by pipetting up and down just prior to loading.

- [ ] 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.

- [ ] Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

Chargement de la flow cell

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

- ☐ 34 µl Sequencing Buffer (SQB)
- ☐ 25.5 µl Loading Beads (LB), mixed immediately before use ☹ Bien vortexer juste avant
- ☐ 4.5 µl Nuclease-free water
- ☐ 11 µl DNA library

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☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION Mk1B lid.

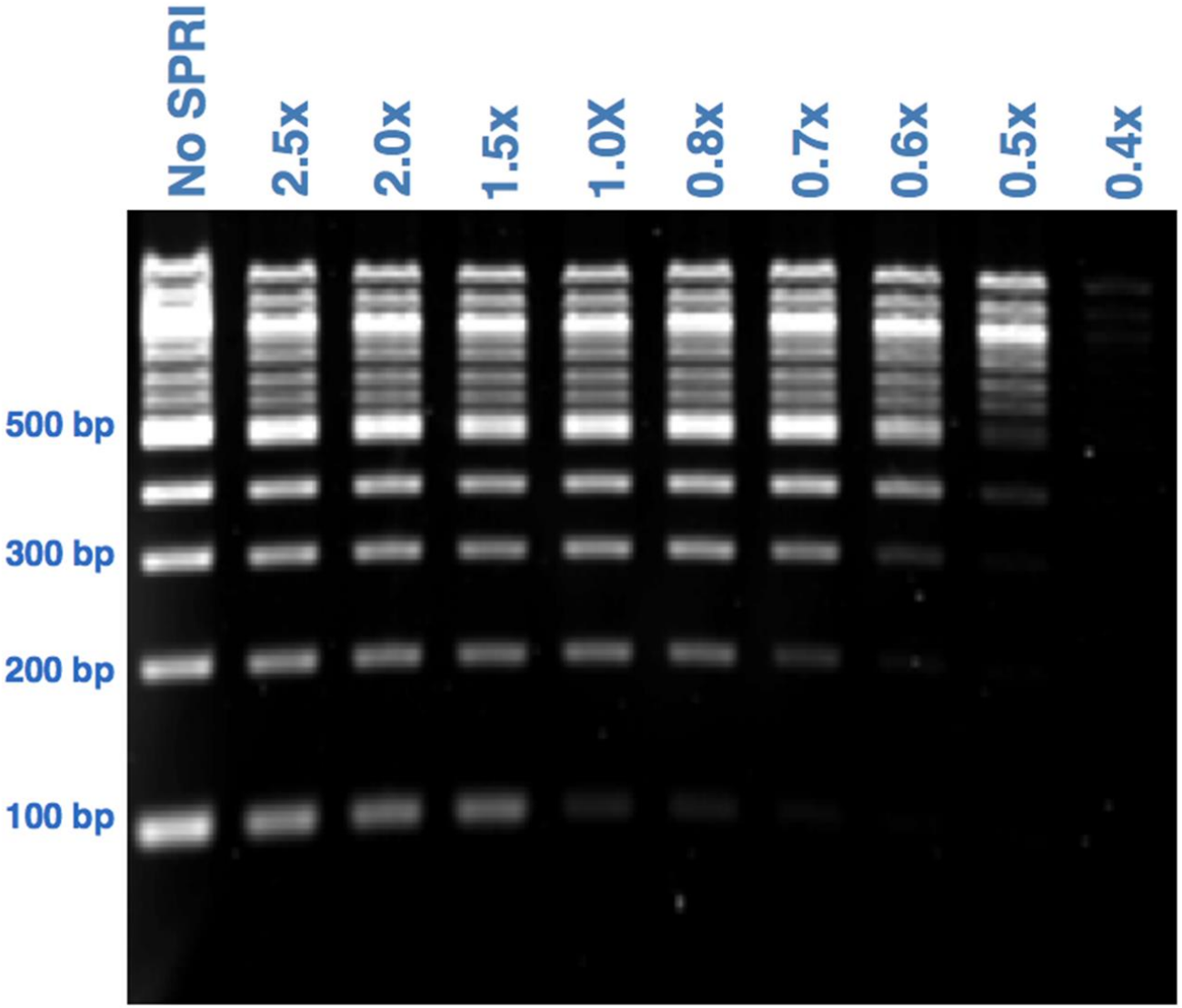
Fin du séquençage

Ending the experiment

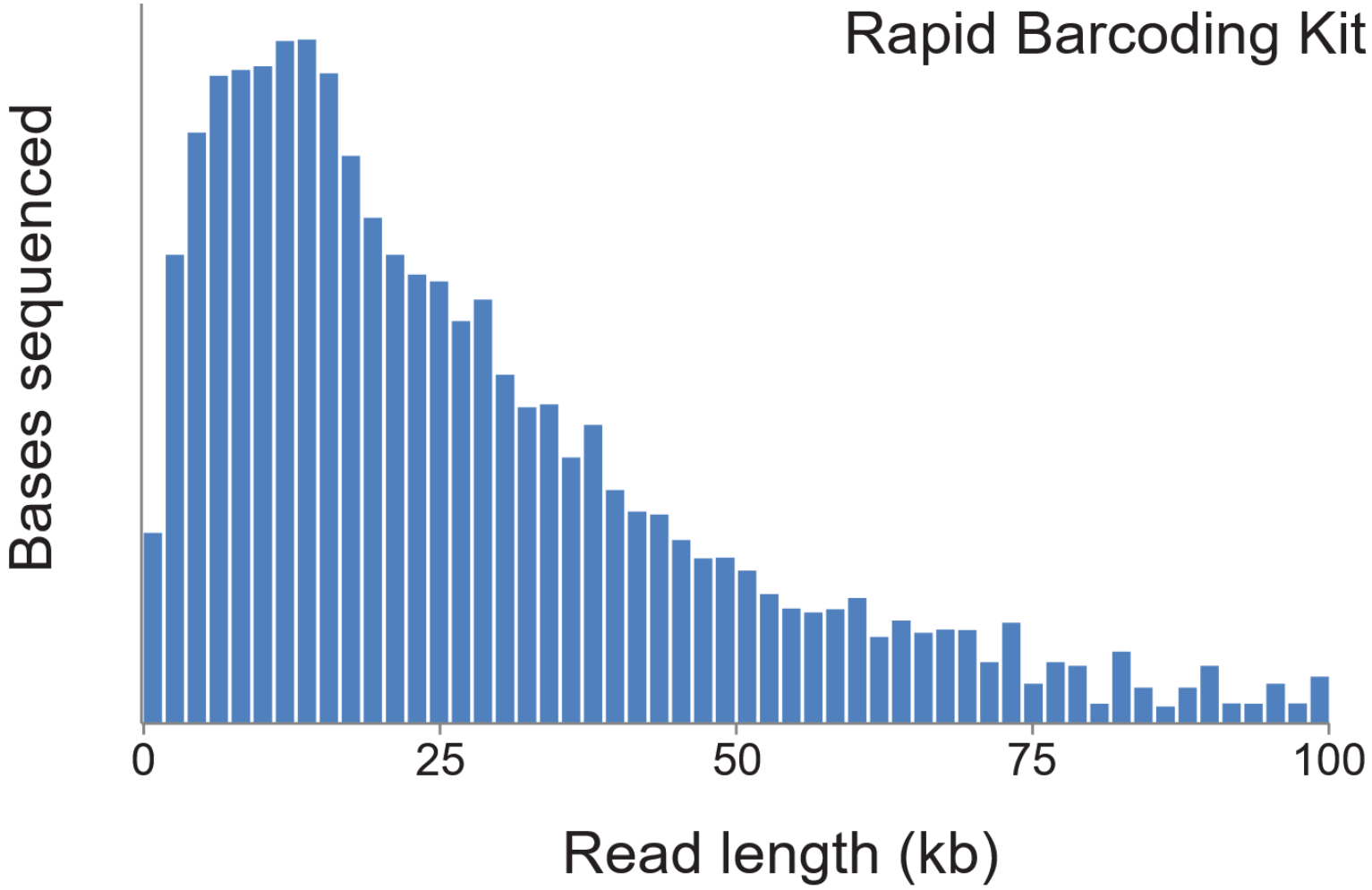
- [ ] After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR
- [ ] Follow the returns procedure by washing out the flow cell ready to send back to Oxford Nanopore.

IMPORTANT

- [ ] If you encounter issues or have questions about your sequencing experiment, please refer to the Troubleshooting Guide that can be found in the online version of this protocol.


No SPRI
2.5x
2.0x
1.5x
1.0X
0.8x
0.7x
0.6x
0.5x
0.4x
500 bp
300 bp
200 bp
100 bp

Distribution de tailles de lectures



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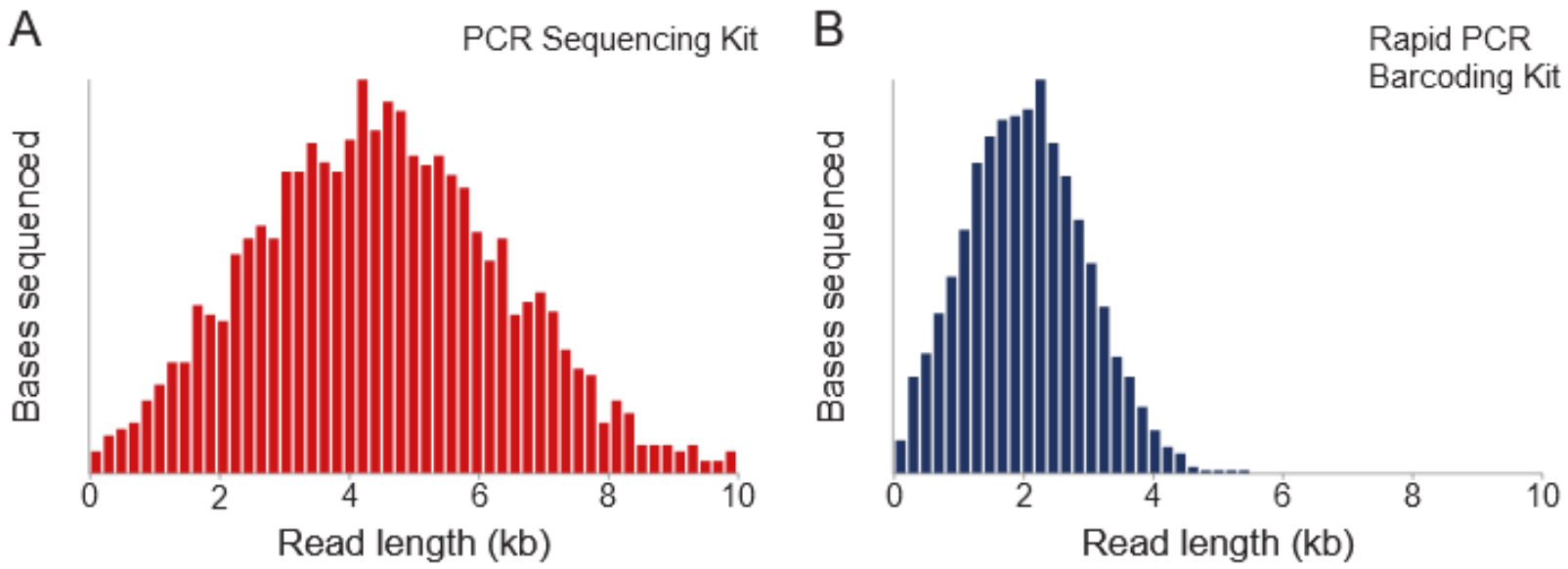

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