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NÁRODNÍ
PLÁN OBNOVY

KUPNÍ SMLOUVA

dle § 2079 a násl. zákona č. 89/2012 Sb., občanský zákoník, (dále jen „OZ“)

I.
Smluvní strany

1) Kupující

Název: **Masarykova univerzita, Středoevropský technologický institut**
Sídlo: Žerotínovo nám. 617/9, 601 77 Brno
Kontaktní adresa: Kamenice 753/5, 625 00 Brno
IČ: 00216224
DIČ: CZ00216224
Zastoupen: doc. Mgr. Pavlem Plevkou, Ph.D., ředitelem
Kontaktní osoby:

Masarykova univerzita je veřejná vysoká škola dle zákona č. 111/1998 Sb., zákon o vysokých školách, v platném znění, nezapsaná v obchodním rejstříku
(dále jen „kupující“)

2) Prodávající

Obchodní firma/název/jméno: I.T.A. – Intertact s.r.o.
Sídlo: Pařížská 67/11, Josefov, 110 00 Praha1
IČ: 65408781
DIČ/VAT ID: CZ65408781
Zastoupen: Ing. Dan Němec, jednatel
Zápis v obchodním rejstříku: Městský soud v Praze sp.zn. C 44200
Bankovní spojení:
IBAN:
Korespondenční adresa: Černokostelecká 616/143, Malešice, 108 00 Praha 10
Kontaktní osoby:

(dále jen „prodávající“; prodávající společně s kupujícím také jen „smluvní strany“)

II.

Účel smlouvy

1) Kupující, jakožto zadavatel veřejné zakázky s názvem „**High-throughput sekvenátor pro dlouhé čtení**“ **(dále jen „veřejná zakázka“)**, rozhodl o výběru nabídky prodávajícího jako nejvhodnější ke splnění veřejné zakázky. Smluvní strany tak ke splnění předmětu veřejné zakázky uzavírají níže uvedeného dne, měsíce a roku tuto kupní smlouvu **(dále také jen „smlouva“)**.

2) Účelem této smlouvy je dodávka high-throughput sekvenátoru pro dlouhé čtení. Předmět smlouvy a jeho instalace tak, aby mohl spolehlivě plnit svůj účel, jsou blíže specifikovány v příloze č. 1 – Technická specifikace (**dále jen „příloha č. 1“**) této smlouvy **(dále také jen „věc“; je-li na základě této smlouvy pořizováno více věcí, vztahují se ustanovení pojednávající o „věci“ na všechny věci, jež mají být na základě této smlouvy pořízeny, není-li výslovně uvedeno jinak).**

3) Věc bude sloužit k plnění úkolů kupujícího, obzvláště úkolů vyplývajících ze zákona č. 111/1998 Sb., o vysokých školách a o změně a doplnění dalších zákonů, ve znění pozdějších předpisů, zejména pro zajištění vědecké a výzkumné činnosti kupujícího. Pracoviště kupujícího je interdisciplinárním vědeckým pracovištěm a centrem vědecké excelence v oblasti věd o živé přírodě a pokročilých materiálů a technologií, jehož hlavním posláním je budování významného evropského centra vědy a vzdělanosti se špičkovým zázemím a podmínkami pro nejlepší vědecké pracovníky.

4) Prodávající zajistí v rámci plnění Smlouvy legální zaměstnávání osob a zajistí pracovníkům podílejícím se na plnění Smlouvy férové a důstojné pracovní podmínky. Férovými a důstojnými pracovními podmínkami se rozumí takové pracovní podmínky, které splňují alespoň minimální standardy stanovené pracovněprávními a mzdovými předpisy. Prodávající je povinen zajistit splnění požadavků tohoto ustanovení Smlouvy i u svých poddodavatelů. Nesplnění povinností Prodávajícího dle tohoto ustanovení Smlouvy se považuje za podstatné porušení Smlouvy.

5) Prodávající dále zajistí řádné a včasné plnění finančních závazků svým poddodavatelům, kdy za řádné a včasné plnění se považuje plné uhrazení poddodavatelem vystavených faktur za plnění poskytnutá Prodávajícímu ke splnění této Smlouvy, a to vždy nejpozději do 10 dnů od obdržení platby ze strany Kupujícího za konkrétní plnění (pokud již splatnost poddodavatelem vystavené faktury nenastala dříve). Prodávající se zavazuje přenést totožnou povinnost do dalších úrovní dodavatelského řetězce a zavázat své poddodavatele k plnění a šíření této povinnosti též do nižších úrovní dodavatelského řetězce. Kupující je oprávněn požadovat předložení dokladů o provedených platbách poddodavatelům a smlouvy uzavřené mezi Prodávajícím a poddodavateli a Prodávající je povinen je bezodkladně poskytnout. Nesplnění povinností Prodávajícího dle tohoto ustanovení Smlouvy se považuje za podstatné porušení Smlouvy.

6) Prodávající dále zajistí, aby byl při plnění této Smlouvy minimalizován dopad na životní prostředí, a to zejména tříděním odpadu, úsporou energií, a respektována udržitelnost či možnosti cirkulární ekonomiky.

7) Prodávající výslovně utvrzuje, že není ve střetu zájmů dle § 4b zákona č. 159/2006 Sb., o střetu zájmů, ve znění pozdějších předpisů, splnění uvedeného zajistil i u svých poddodavatelů.

8) Prodávající výslovně utvrzuje, že splňuje požadavky stanovené v Nařízení Rady (EU) 2022/576 ze dne 8. dubna 2022, kterým se mění nařízení (EU) č. 833/2014 o omezujících opatřeních vzhledem k činnostem Ruska destabilizujícím situaci na Ukrajině, v Rozhodnutí Rady (SZBP) 2022/578 ze dne 8. dubna 2022, kterým se mění rozhodnutí 2014/512/SZBP o omezujících opatřeních vzhledem k činnostem Ruska destabilizujícím situaci na Ukrajině, v Prováděcím nařízení Rady (EU) 2022/581 ze dne 8. dubna 2022, kterým se provádí nařízení (EU) č. 269/2014 o omezujících opatřeních vzhledem k činnostem narušujícím nebo ohrožujícím územní celistvost, svrchovanost a nezávislost Ukrajiny, a v Rozhodnutí Rady (SZBP) 2022/582 ze dne 8. dubna 2022, kterým se mění rozhodnutí 2014/145/SZBP o omezujících opatřeních vzhledem k činnostem narušujícím nebo ohrožujícím územní celistvost, svrchovanost a nezávislost Ukrajiny. Splnění uvedeného zajistil i u svých poddodavatelů.

9) Kupující pořizuje plnění z dotace na realizaci veřejné zakázky; název programu: Národní plán obnovy (NPO): "MUNI BioPharma Hub", reg. č. Z332802000004, financován Evropskou unií, financovaným Evropskou unií (dále též „projekt“). Smluvní strany berou na vědomí, že jakékoli, byť jen částečné, neplnění povinností vyplývajících ze smlouvy, ať už na straně kupujícího či prodávajícího, může ohrozit čerpání dotace, příp. může vést k udělení sankcí kupujícímu ze strany orgánů oprávněných k výkonu kontroly projektu. Škoda, která může kupujícímu neplněním povinností smluvních stran stanovených smlouvou vzniknout, tak může i přesáhnout cenu věci.

III.

Předmět smlouvy

1) Prodávající se zavazuje, že kupujícímu odevzdá věc, která je předmětem koupě, a umožní mu nabýt vlastnické právo k této věci, a že splní další s tím související závazky uvedené ve smlouvě. Kupující se zavazuje, že věc převezme a zaplatí prodávajícímu kupní cenu.
2) Množství, jakost a provedení, jakož i další vlastnosti věci jsou ujednány touto smlouvou, zejména pak v příloze č. 1 této smlouvy.
3) Prodávající prohlašuje, že:
   a) je výlučným vlastníkem věci, kterou kupujícímu odevzdá,
   b) věc odpovídá této smlouvě; tzn., že má vlastnosti, které si strany ujednaly, a chybí-li ujednání, takové vlastnosti, které prodávající nebo výrobce popsal nebo které kupující očekával s ohledem na povahu věci a na základě reklamy jimi prováděné, že se hodí k účelu, který vyplývá zejména z této smlouvy, že vyhovuje požadavkům právních předpisů, že je bez jakýchkoli jiných vad, a to i právních,
   c) věc je nová, tzn. nikoli dříve použitá, a to ani repasovaná.
4) Pokud jsou k řádnému a včasnému splnění požadavků kupujícího uvedených v této smlouvě potřebné i další dodávky či služby ve smlouvě výslovně neuvedené, je prodávající povinen tyto dodávky či služby na své náklady obstarat či provést jako součást závazku odevzdat věc bez dopadu na kupní cenu.

IV.

Podmínky plnění předmětu smlouvy

1) Smluvní strany prohlašují, že svoje závazky budou plnit řádně a včas. Prodávající odevzdá věc s potřebnou odbornou péčí v souladu s touto smlouvou, příslušnými právními předpisy a technickými i jinými normami, které se na odevzdání věci přímo či nepřímo vztahují.
2) Závazek prodávajícího odevzdat věc zahrnuje i:
   a) dopravu věci na místo jejich odevzdání, jejich vybalení a kontrola,
   b) provedení instalace věci tak, aby mohla spolehlivě plnit svůj účel,
   c) odzkoušení a ověření správné funkčnosti věci;

d) zaškolení obsluhy věci,
e) bezplatné provedení výrobcem věci či zákonem předepsaných preventivních prohlídek, kontrol včetně bezpečnostních, revizí, validace a kalibrace a preventivních údržbových prací (jestliže je výrobce věci nebo právní předpisy ČR vyžadují, nebo jimi prodávající podmiňuje platnost záruky),
f) předání dokladů, které jsou nutné k užívání věcí, zejména návodů k použití, a příp. které se k věcem jinak vztahují (**dále jen „doklady k věci“**) a
g) předání dodacího listu kupujícímu.

3) Požadavky na instalaci věci

a) Prodávající se zavazuje provést instalaci věci, tj. její usazení v místě odevzdání věci, napojení na zdroje a dále vzájemné funkční propojení s dalšími věcmi či dalším vybavením kupujícího, je-li plný provoz věci podmíněn takovým napojením nebo propojením tak, aby věc mohla spolehlivě plnit svůj účel.

b) Prodávající se zavazuje s kupujícím konzultovat návrh napojení věci na zdroje, jakož i návrh na vzájemné funkční propojení věci s dalšími věcmi či dalším vybavením kupujícího ve smyslu předchozího písmene **(dále také jen „návrh napojení“)**. Návrh napojení předloží prodávající kupujícímu v termínu umožňujícím včasné splnění závazku odevzdat věc. Prodávající nesmí před schválením návrhu napojení kupujícím plnit ty závazky vyplývající ze smlouvy, pokud by tím vznikl nebo mohl vzniknout rozpor se schváleným návrhem napojení.

4) Odzkoušení a ověření správné funkčnosti věci

Prodávající se zavazuje provést odzkoušení a ověření správné funkčnosti věci, případně její seřízení, revizi včetně předložení dokladů o odborné způsobilosti osoby, která seřízení či revizi prováděla, jakož i jiné úkony a činnosti nutné pro to, aby věc mohla spolehlivě plnit svůj účel.

5) Zaškolení obsluhy věci

a) Prodávající se zavazuje provést zaškolení obsluhy věci. Zaškolením obsluhy věci se pro účely této smlouvy rozumí seznámení pracovníků kupujícího s obsluhou věci, zejména s technickými a provozními podmínkami, všeobecnými pokyny pro bezpečnost a ochranu zdraví při práci a požární ochranu a veškerými dalšími náležitostmi vyplývajícími z příslušných právních předpisů.

6) Předávané dokumenty a doklady k věci

Veškeré dokumenty a doklady k věci, které se prodávající dle této smlouvy zavazuje předat kupujícímu, budou zpracovány v českém jazyce a předány kupujícímu v listinném nebo elektronickém vyhotovení (např. na DVD).

7) Atesty, certifikáty a prohlášení o shodě věci

Prodávající se zavazuje obstarat a předat kupujícímu ke dni odevzdání věci veškeré atesty, certifikáty a prohlášení o shodě věci s požadavky příslušných právních předpisů či technických norem.

8) Licence

a) Prodávající poskytuje kupujícímu podpisem této smlouvy nevýhradní oprávnění k výkonu práva duševního vlastnictví ve smyslu § 2358 a násl. OZ ve spojení s příslušnými ustanoveními zákona č. 121/2000 Sb., o právu autorském, o právech souvisejících s právem autorským a o změně některých zákonů (autorský zákon), ve znění pozdějších předpisů, (dále jen „licence“), a to k jakémukoli plnění, k němuž se zavázal podle této smlouvy a které je nebo bude chráněno autorským právem.

b) Licence je poskytnuta na zařízení, které je předmětem smlouvy, na dobu trvání majetkových práv autorských k předmětnému plnění a ke všem způsobům užití. Prodávající prohlašuje, že předmětné plnění je vytvořeno jejím autorem či autory jakožto dílo zaměstnanecké, případně že

je oprávněn poskytnout kupujícímu licenci na základě smluvního ujednání s jejím autorem či autory, a to v plném rozsahu dle této smlouvy.

c) Kupující není povinen licenci využít.

9) Pokyny kupujícího

a) Při plnění závazků dle této smlouvy postupuje prodávající samostatně, není-li uvedeno jinak.

b) Prodávající se zavazuje respektovat pokyny kupujícího, kterými jej kupující upozorňuje na možné porušení jeho smluvních či jiných povinností.

c) Prodávající upozorní kupujícího bez zbytečného odkladu na nevhodnou povahu věci, kterou mu ke splnění závazků dle této smlouvy předal, nebo pokynu, který mu kupující dal. To neplatí, nemohl-li nevhodnost zjistit ani při vynaložení potřebné péče.

10) Odborná způsobilost pracovníků prodávajícího

a) Veškeré odborné práce musí vykonávat pracovníci prodávajícího nebo jeho poddodavatelů mající příslušnou odbornou způsobilost.

b) Doklad o odborné způsobilosti pracovníků je prodávající povinen na požádání kupujícímu předložit.

c) Kupující je oprávněn po prodávajícím požadovat, aby odvolal z plnění závazků dle této smlouvy pracovníka, který nemá příslušnou odbornou způsobilost, který si počíná tak, že to ohrožuje bezpečnost a zdraví jeho, jiných pracovníků či třetích osob, příp. je-li jeho chování hrubě nemravné. Neodvolá-li prodávající takového pracovníka, je kupující zejména oprávněn takového pracovníka vykázat z místa odevzdání věci. Uvedené platí přiměřeně i ve vztahu k pracovníkům poddodavatele prodávajícího.

11) Poddodavatelé prodávajícího

a) Na žádost kupujícího se prodávající zavazuje bezodkladně, nejpozději však do 3 (slovy: tří) pracovních dnů po sdělení takové žádosti, předložit písemný seznam poddodavatelů, které hodlá pověřit plněním části závazků dle této smlouvy.

b) Kupující je oprávněn zamítnout účast těch poddodavatelů prodávajícího, u nichž by byl dán důvod k vyloučení podle ustanovení § 48 odst. 5 a 6 ZZVZ. Prodávající však odpovídá za plnění takových závazků poddodavateli, jako by je plnil sám.

c) Prodávající se zavazuje, že ve smlouvách s případnými poddodavateli zaváže poddodavatele k plnění těch závazků, k jejichž splnění se zavázal v této smlouvě, a to v rozsahu, v jakém budou poddodavatelem tyto závazky plněny.

d) Nesplnění povinností prodávajícího dle tohoto odstavce se považuje za podstatné porušení smlouvy.

12) Škody

a) Pokud v souvislosti s plněním závazků dle této smlouvy prodávajícím dojde ke vzniku škody kupujícímu nebo třetím osobám z důvodu opomenutí, nedbalosti, neplnění povinností vyplývajících z příslušných právních předpisů, technických či jiných norem, z této smlouvy nebo i z jiných důvodů, je prodávající povinen bez zbytečného odkladu tuto škodu nahradit uvedením v předešlý stav, a není-li to možné, tak nahradit v penězích. Veškeré náklady s tím spojené nese prodávající.

b) Prodávající odpovídá i za škodu způsobenou činností těch, kteří pro něj závazky dle této smlouvy plní jako jeho pracovníci, poddodavatelé nebo jinak.

13) Odvoz a likvidace odpadů; závěrečný úklid

Prodávající se zavazuje

a) odvést a zlikvidovat veškerý odpad, který vznikne při plnění závazků odevzdat věc, v souladu s příslušnými ustanoveními zákona č. 541/2020 Sb., o odpadech, ve znění pozdějších předpisů, příslušnou vyhláškou Statutárního města Brna a dalšími právními předpisy; doklady o likvidaci odpadů je prodávající povinen na požádání kupujícímu předložit,
b) provést závěrečný úklid včetně uvedení všech povrchů dotčených plnění závazků odevzdat věc dle této smlouvy do původního stavu.

14) Prohlášení prodávajícího

Prodávající prohlašuje, že dodávka bude vyhovovat všem technickým specifikacím uvedeným v příloze č. 1. Pokud by se v průběhu přípravy a realizace dodávky ukázalo, že ke splnění požadavků Kupujícího dle této přílohy je nezbytné dodání dalších zařízení, součástí či příslušenství nebo provedení dalších služeb či prací, zavazuje se Prodávající dodat tato zařízení a provést tyto práce či služby jako součást plnění dodávky dle smlouvy bez zvýšení Kupní ceny (zmíněné dodávky, práce či služby nebudou mít charakter vícedodávek či víceprací).

V.

Odevzdání a převzetí věci

1) Čas, místo a způsob odevzdání věci prodávajícím

a) Prodávající odevzdá věc kupujícímu

1. do dvanácti týdnů ode dne objednání věci – výzvy ke splnění závazku,
2. na adrese prostor pavilonu G61 Univerzitního kampusu Bohunice, 625 00 Brno, 2. až 4. nadzemní podlaží
3. najednou, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak,
4. prodávající však bere podpisem této smlouvy na vědomí, že kupující nemá zájem na opožděném plnění po datu 1. 12. 2026 a to z důvodu nedostatku finančních prostředků (vzhledem k podmínkám, za kterých je oprávněn čerpat poskytnutou dotaci). Smluvní strany sjednávají, že pokud nejpozději do 1. 12. 2026 nedojde k dodání a instalaci kompletního předmětu plnění ve smyslu čl. IV. odst. 2) této smlouvy, smlouva se od počátku ruší a smluvní strany jsou povinny vrátit si veškeré poskytnuté plnění. Ukončení právního vztahu založeného touto smlouvou dle předchozí věty se nedotýká již zaplacených sankcí dle této smlouvy, ani nároků na jejich zaplacení.

b) Prodávající je povinen písemně informovat kontaktní osobu kupujícího uvedenou v čl. I. odst. 1) této smlouvy o přesném termínu, ve kterém věc odevzdá, a to alespoň 2 (slovy: dva) pracovní dny před jejím odevzdáním, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak. Nesplní-li prodávající tuto povinnost, je kupující oprávněn odevzdání věci odmítnout.

c) Připadne-li poslední den lhůty pro odevzdání věci na sobotu, neděli nebo svátek, je posledním dnem lhůty pracovní den nejblíže následující. Nebude-li mezi prodávajícím a kupujícím dohodnuto jinak, platí, že odevzdání věci proběhne v době od 9:00 do 17:00. Kupující je oprávněn v případě svých provozních potřeb dobu, po kterou je prodávajícímu umožněn přístup na místo odevzdání věci, upravit písemným pokynem prodávajícímu.

d) Kupující v souvislosti s umožněním přístupu na místo pro odevzdání věci seznámí prodávajícího s

1. přístupovými cestami pro dopravu věci na místo odevzdání věci,
2. přípojnými body pro napojení věci na zdroje a pro vzájemné funkční propojení s dalšími věcmi či dalším vybavením kupujícího,

3. provozním řádem místa odevzdání věci.

e) Prodlení s odevzdáním věci se považuje za podstatné porušení smlouvy.

2) Prodloužení lhůty pro odevzdání věci

Lhůta pro odevzdání věci může být přiměřeně prodloužena

a) jestliže dojde k přerušení plnění závazků dle této smlouvy na základě písemného pokynu kupujícího,

b) jestliže dojde k přerušení plnění závazků dle této smlouvy z důvodu prodlení na straně kupujícího,

c) zjistí-li prodávající při plnění závazků dle této smlouvy skryté překážky týkající se místa odevzdání věci znemožňující odevzdat věc dohodnutým způsobem,

d) jestliže dojde k přerušení plnění závazků dle této smlouvy vlivem mimořádných nepředvídatelných a nepřekonatelných překážek vzniklých nezávisle na vůli prodávajícího ve smyslu § 2913 odst. 2) OZ; smluvní strany jsou povinny se bezprostředně vzájemně informovat o vzniku takových překážek, jinak se jich nemohou dovolávat.

Prodloužená lhůta pro odevzdání věci se určí adekvátně podle délky trvání překážky s přihlédnutím k době nezbytné pro splnění závazku odevzdat věc za podmínky, že prodávající učinil veškerá rozumně očekávatelná opatření k tomu, aby předešel či alespoň zkrátil dobu trvání takové překážky. Prodloužená lhůta pro odevzdání věci ve smyslu tohoto odstavce musí být smluvními stranami sjednána či stvrzena dodatkem k této smlouvě.

Kupující výslovně deklaruje, že se nejedná o vyhrazené změny závazku ve smyslu § 100 odst. 1 ZZVZ, všechny změny budou klasifikovány dle § 222 ZZVZ.

3) Převzetí věci kupujícím

a) Převzetí věcí bude Kupujícím potvrzeno na dodacím listu, který bude pro účely této Smlouvy plnit i funkci předávacího protokolu.

b) Předávací protokol (dodací list) musí obsahovat:

1. Název a sídlo Prodávajícího a Kupujícího;
2. Identifikaci kupní smlouvy;
3. Označení dodaných věcí včetně výrobního čísla;
4. Datum podpisu předávacího protokolu, které je dnem zdanitelného plnění;
5. Stav věcí v okamžiku jejich předání a převzetí;
6. Seznam předaných dokladů a dokumentace;
7. Informace o provedení zaškolení obsluhy věci;
8. Ověření, zda bylo splněno řádně.

c) Převzetím věci přechází na kupujícího vlastnické právo k věcem, jakož i nebezpečí vzniku škody na věcech.

4) Kontrola zjevných vad věci a její převzetí kupujícím

a) Kupující po odevzdání věci provede kontrolu zjevných vad věci, zejména co do jejich provedení. Kupující neprovádí kontrolu zjevných vad věci při jejich odevzdání; přesto zjistí-li ještě před jejím převzetím od prodávajícího, že věc trpí jakýmikoli vadami, je oprávněn její odevzdání rovnou odmítnout.

b) Zjistí-li kupující, že věc vykazuje vady, oznámí to nejpozději do 5 (slovy: pěti) pracovních dnů ode dne převzetí věci prodávajícímu. Kupující pak postupuje buď dle ust. odst. 4) písm. c), nebo odst. 4) písm. d) tohoto článku smlouvu.

c) Závazek odevzdat věc kupující nepovažuje za splněný

1. Kupující oznámí prodávajícímu, že jeho závazek odevzdat věc, byť pro ojedinělé drobné vady, které by samy o sobě ani ve spojení s jinými nebránily řádnému užívání věci, nebyl splněn. Na věc se hledí, jako by prodávajícím nebyly odevzdány ani kupujícím převzaty. Pokud již lhůta pro odevzdání věcí uplynula, je prodávající v prodlení s odevzdáním věcí se všemi důsledky, které se s tím pojí.
2. Prodávající je povinen odevzdanou věc na své náklady od kupujícího vzít zpět, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak.

d) Závazek odevzdat věc kupující považuje za splněný s vadami bez následku prodlení

1. Kupující oznámí prodávajícímu, že splnil závazek odevzdat věc s vadami. Smluvní strany výslovně utvrzují, že prodávající se v tomto případě nemůže dostat do prodlení.
2. Při řešení práv z vadného plnění smluvní strany postupují přiměřeně v souladu s ustanoveními o reklamaci vad věc v záruční době. Práva z takto oznámených vad se prodávající zavazuje uspokojit v souladu s uplatněným právem kupujícího bezodkladně, nejpozději však do 10 (slovy: deseti) dnů ode dne jejich oznámení, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak.

e) Neoznámení vad věci dle ust. odst. 4) tohoto článku smlouvy nevylučuje uplatnění práv z vadného plnění z důvodu těchto vad v záruční době.

f) Převzetím věci přechází na kupujícího vlastnické právo k věci, jakož i nebezpečí vzniku škody na věci.

VI.

Kupní cena a platební podmínky

1) Kupní cena za splnění závazků prodávajícího je stanovena na základě nabídky prodávajícího podané do výběrového řízení k veřejné zakázce „ **High-throughput sekvenátor pro dlouhé čtení**“ a činí:

10.499.990,- Kč

bez daně z přidané hodnoty (**dále jen „DPH“**).

2) Prodávající je oprávněn ke kupní ceně připočíst DPH ve výši stanovené v souladu se zákonem č. 235/2004 Sb., o dani z přidané hodnoty, ve znění pozdějších předpisů, **(dále jen „ZDPH“)**, a to ke dni uskutečnění zdanitelného plnění **(dále jen „DUZP“)**. DUZP je den převzetí věci.

3) Kupní cena je cenou nejvýše přípustnou. Prodávající prohlašuje, že kupní cena obsahuje jeho veškeré nutné náklady na dodávky a služby nezbytné pro řádné a včasné splnění předmětu smlouvy včetně všech nákladů souvisejících při zohlednění veškerých rizik a vlivů, o nichž lze uvažovat během plnění dle této smlouvy. Smluvní strany výslovně utvrzují, že podaří-li se prodávajícímu zpeněžit odpad, připadá mu i celá částka, kterou tím získá. Smluvní strany mají za to, že prodávající částku, kterou uvažoval ze zpeněžení odpadu získat, zohlednil při stanovení ceny předmětu smlouvy.

4) Prodávající přebírá nebezpečí změny okolností ve smyslu § 1765 odst. 2 OZ.

5) Právo na zaplacení kupní ceny

a) Právo na zaplacení kupní ceny věci vzniká jejím převzetím kupujícím.

b) Kupující neposkytne prodávajícímu žádné zálohy.

6) Úhrada kupní ceny

a) Kupní cena bude uhrazena na základě řádně vystaveného daňového dokladu **(dále také jen „faktura“)**. Fakturačně musí být na faktuře jednoznačně oddělena výše plnění investičního charakteru, včetně k němu se vztahujícímu příslušenství, a výše plnění neinvestičního charakteru nemajícího povahu příslušenství. Prodávající je povinen před vystavením faktury rozdělení na investiční a neinvestiční plnění projednat s kupujícím.

b) Faktura bude doručena kupujícímu nejpozději do 3 (slovy: tří) pracovních dní ode dne převzetí věci. Prodávající se zavazuje zaslat kupujícímu bezodkladně po odeslání listinného vyhotovení faktury její elektronickou kopii, a to na e-mailovou adresu: fakturace@ceitec.muni.cz.

c) Splatnost faktury je 30 (slovy: třicet) dní ode dne jejího doručení kupujícímu.

d) Kupní cena bude kupujícím uhrazena bezhotovostním převodem na bankovní účet prodávajícího uvedený v čl. I. odst. 2) smlouvy. Uvede-li prodávající na faktuře bankovní účet odlišný, má se za to, že požaduje provedení úhrady na bankovní účet uvedený na faktuře. Peněžitý závazek kupujícího se považuje za splněný v den, kdy je dlužná částka odepsána z bankovního účtu kupujícího ve prospěch bankovního účtu prodávajícího.

7) Náležitosti faktury

Faktura bude splňovat veškeré zákonné a smluvené náležitosti, zejména

a) náležitosti daňového dokladu dle § 26 a násl. ZDPH,

b) náležitosti daňového dokladu stanovené v zákoně č. 563/1991 Sb., o účetnictví, ve znění pozdějších předpisů,

c) uvedení lhůty splatnosti,

d) uvedení údajů bankovního spojení prodávajícího,

e) uvedení názvu a registračního čísla projektu uvedeného v ustanovení II. odst. 9) této smlouvy

Kupující si vyhrazuje právo vrátit fakturu prodávajícímu bez úhrady, jestliže tato nebude splňovat požadované náležitosti. V tomto případě bude lhůta splatnosti faktury přerušena a nová 30denní (slovy: třicetidenní) lhůta splatnosti bude započata po doručení faktury opravené. V tomto případě není kupující v prodlení s úhradou příslušné částky, na kterou faktura zní.

8) V případě, že faktura nebude obsahovat předepsané náležitosti a tuto skutečnost zjistí až příslušný správce daně či jiný orgán oprávněný k výkonu kontroly u prodávajícího nebo kupujícího, nese veškeré následky z tohoto plynoucí prodávající.

9) V případě, že

a) úhrada kupní ceny má být provedena zcela nebo zčásti bezhotovostním převodem na účet vedený poskytovatelem platebních služeb mimo tuzemsko ve smyslu § 109 odst. 2 písm. b) ZDPH nebo že

b) číslo bankovního účtu prodávajícího uvedené v této smlouvě či na faktuře nebude uveřejněno způsobem umožňujícím dálkový přístup ve smyslu § 109 odst. 2 písm. c) ZDPH,

je kupující oprávněn uhradit prodávajícímu pouze tu část peněžitého závazku vyplývajícího z faktury, jež odpovídá výši základu DPH, a zbylou část pak ve smyslu § 109a ZDPH uhradit přímo správci daně. Stane-li se prodávající nespolehlivým plátcem ve smyslu § 106a ZDPH, použije se tohoto odstavce obdobně.

VII.

Práva z vadného plnění; záruka za jakost

1) Věc je vadná, neodpovídá-li smlouvě. Smluvní strany sjednávají, že věc bude smlouvě odpovídat a že práva z vadného plnění lze uplatňovat i po smluvenou záruční dobu. Smluvní strany výslovně utvrzují, že v záruční době lze uplatnit jakékoli vady, které věc má, mj. tedy zcela bez ohledu na to, zda vznikly před či po převzetí věcí kupujícím, nebo kdy je kupující měl či mohl zjistit, nebo kdy je zjistil, a to i v případě vad zjevných.

2) **Záruka za jakost**

   a) Záruční doba činí 24 (slovy: dvacetčtyři) měsíců; je-li pro věc nebo její části v záručním listu nebo jiném prohlášení o záruce (včetně přílohy č. 1) uvedena záruční doba delší, platí tato delší záruční doba. Prodávající má povinnosti z vadného plnění nejméně v takovém rozsahu, v jakém trvají povinnosti z vadného plnění výrobce věcí.

   b) Záruční doba věci začíná běžet ode dne jejího převzetí kupujícím. Je-li věc kupujícím převzata s alespoň jednou vadou, počíná záruční doba běžet až dnem odstranění poslední vady. Podobně byla-li věc kupujícím převzata i přes to, že prodávající neodevzdal některou z věcí ve smluvené lhůtě, počíná záruční doba běžet až dnem odevzdání chybějící věci.

   c) Neodpovídá-li věc smlouvě, má kupující právo zejména na

      1. odstranění vady dodáním nové věci bez vad, pokud to není vzhledem k povaze vady nepřiměřené; pokud se vada týká pouze součásti věci, může kupující požadovat jen výměnu součásti,
      2. odstranění vady opravou věci, je-li vada opravou odstranitelná,
      3. odstraněním vady dodáním chybějící věci nebo její součásti,
      4. přiměřenou slevu z kupní ceny,
      5. odstoupení od smlouvy.

      Kupující je oprávněn zvolit si a uplatnit kterékoli z uvedených práv dle svého uvážení, případně zvolit a uplatnit kombinaci těchto práv.

3) **Reklamace vad věci v záruční době**

   a) Práva z vadného plnění v záruční době uplatní kupující oznámením prodávajícímu (**dále jen „reklamace“**), a to kdykoli po zjištění vady. I reklamace odeslaná kupujícím poslední den záruční doby se považuje za včas uplatněnou. Smluvní strany sjednávají, že § 2111 OZ a § 2112 OZ se nepoužijí.

   b) V reklamaci kupující uvede alespoň:

      1. popis vady věci nebo informaci o tom, jak se vada projevuje, a
      2. jaká práva v souvislosti s vadou věci uplatňuje.

      Neuvede-li kupující, jaká práva v souvislosti s vadou věci uplatňuje, má se za to, že požaduje provedení opravy věci, příp. dodání jiné věci v souladu s čl. III. odst. 3) smlouvy bez vad, není-li vada věci opravou odstranitelná.

   c) Uplatnění práv z vadného plnění kupujícím, jakož i plnění jim odpovídajících povinností prodávajícího není podmíněno ani jinak spojeno s poskytnutím jakékoli další úplaty kupujícího prodávajícímu, příp. jiné osobě.

d) Kupujícímu náleží i náhrada nákladů účelně vynaložených při uplatnění práv z vadného plnění.

e) Prodávající se zavazuje zahájit diagnostické úkony (jako např. testy, kontrolní měření nebo analýzu dat) směřující k odstranění vady nejpozději do 2 (slovy: dvou) pracovních dnů od doručení reklamace. Prodávající se zavazuje prověřit reklamaci, oznámit kupujícímu, zda reklamaci uznává, a dohodnout termín odstranění závady (termín pro odstranění vady bude vždy dohodnut písemně) do 5 (slovy: pěti) pracovních dnů od doručení reklamace. Pokud tak prodávající v uvedené lhůtě neučiní, má se zato, že reklamaci uznává a odstraní ji nejpozději ve lhůtě dle písm. f) tohoto odstavce smlouvy.

f) Uplatněná práva kupujícího z vadného plnění se prodávající zavazuje plně uspokojit bezodkladně, nejpozději však do 15 (slovy: patnácti) pracovních dnů ode dne doručení reklamace, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak.

g) Pokud prodávající reklamaci neuzná, může být její oprávněnost ověřena znaleckým posudkem, který obstará kupující. V případě, že reklamace bude tímto znaleckým posudkem označena jako oprávněná, ponese prodávající i náklady na vyhodnocení znaleckého posudku. Právo z vadného plnění vzniká i v tomto případě doručením reklamace prodávajícímu. Prokáže-li se, že kupující reklamoval neoprávněně, je povinen uhradit prodávajícímu prokazatelně a účelně vynaložené náklady na odstranění vady.

4) Při odstraňování vad se prodávající zavazuje poskytovat kupujícímu veškerou potřebnou součinnost. Nebude-li mezi prodávajícím a kupujícím dohodnuto jinak, pak je prodávající povinen zejména:

a) věc, jejíž vada má být odstraněna opravou, převzít k opravě v místě, kde byla kupujícímu odevzdána, a po provedení opravy opravenou věc opět v tomto místě předat kupujícímu, a

b) v případě odstranění vady dodáním jiné věci v souladu s čl. III. odst. 3) smlouvy dodat jinou věc v souladu s čl. III. odst. 3) smlouvy na tutéž adresu, kde byla kupujícímu odevzdána nahrazovaná věc.

Převzetí věci k odstranění vad a následně předání věci po odstranění vad proběhne vždy v pracovní dny v době od 9:00 do 17:00, nebude-li mezi prodávajícím a kupujícím dohodnuto jinak.

5) Záruční servis

a) Prodávající je povinen v průběhu záruční doby provádět bezplatně veškeré servisní úkony, jejichž provedením podmiňuje platnost záruky. Termíny servisních úkonů budou stanoveny dle provozních možností kupujícího.

b) Prodávající je povinen v průběhu záruční doby provést alespoň jednou ročně bezplatnou servisní prohlídku věci. Servisní prohlídka bude zahrnovat základní servisní úkony předepsané výrobcem, zejména seřízení, odzkoušení a ověření správné funkčnosti věci tak, aby věc mohla nadále spolehlivě plnit svůj účel.

6) Smluvní strany sjednávají, že záruku za jakost nijak neovlivňují běžné servisní úkony prováděné přímo kupujícím bez přítomnosti prodávajícího, pokud jsou prováděny v souladu s doklady k věci.

7) Stavení záruční doby

Záruční doba věci neběží od okamžiku reklamace až do dne odstranění vady, příp. do dne uhrazení přiměřené slevy z kupní ceny.

8) Práva Kupujícího z vad po konci záruční doby; pozáruční servis

a) Prodávající je povinen minimálně po dobu 5 let ode dne uplynutí posledního dne záruční doby zabezpečit na písemnou výzvu Kupujícího za úplatu v místě a čase obvyklou pozáruční servis. Prodávající se zavazuje poskytovat pozáruční servis za stejných podmínek, jaké jsou Smlouvou sjednány pro záruční servis.

b) Prodávající garantuje dostupnost náhradních dílů do 30 dnů ode dne doručení výzvy Kupujícího pro všechny části věcí po dobu minimálně 5 let ode dne uplynutí posledního dne záruční doby.

c) Poskytování pozáručního servisu Prodávajícím není sjednáno jako výhradní. Kupující si vyhrazuje právo zajistit pozáruční servis i od třetích osob bez jakékoliv sankce ze strany Prodávajícího.

VIII.

Smluvní pokuty a náhrada škody

1) V případě prodlení prodávajícího oproti lhůtě pro odevzdání věcí dle ust. čl. V. odst. 1) písm. a) bod 1. smlouvy se prodávající zavazuje kupujícímu zaplatit za každý započatý den prodlení smluvní pokutu ve výši 0,1 (slovy: nulacelájednadesetina) % z kupní ceny bez DPH.
2) V případě prodlení prodávajícího oproti lhůtě dle ust. čl. V. odst. 4) písm. d) bod 2. smlouvy se prodávající zavazuje kupujícímu zaplatit za každý započatý den prodlení smluvní pokutu ve výši 0,05 (slovy: nulacelápětsetin) % z kupní ceny bez DPH, a to za každou vadu či chybějící věc, ve vztahu k nimž je v prodlení, celkem však za všechny takové případy nejvýše 5 (slovy: pět) % z kupní ceny bez DPH.
3) Pokud prodávající ve sjednané lhůtě neuspokojí práva kupujícího z vadného plnění v záruční době, zejména ve sjednané lhůtě nezaplatí částku odpovídající požadované slevě z kupní ceny či neodstraní reklamovanou vadu, zavazuje se kupujícímu zaplatit smluvní pokutu ve výši 0,05 % (slovy: nulacelápětsetin procenta) z kupní ceny včetně DPH, a to za každou vadu, ve vztahu k níž je s uspokojením práv kupujícího z vadného plnění v prodlení, a za každý započatý den prodlení.
4) Pokud je kupující v prodlení s úhradou faktury proti sjednanému termínu, je prodávající oprávněn požadovat po kupujícím zaplacení úroků z prodlení ve výši 0,05 % (slovy: nulacelápětsetin procenta) z dlužné částky za každý i započatý den prodlení.
5) V případě porušení povinnosti zajistit legální zaměstnávání a férové a důstojné pracovní podmínky dle ust. II. 4) Smlouvy se Prodávající zavazuje Kupujícímu zaplatit smluvní pokutu ve výši 25 000 Kč za každé porušení.
6) V případě porušení povinnosti řádného a včasného plnění finančních závazků poddodavatelům Prodávajícího nebo nepřenesení této povinnosti Prodávajícím do nižších úrovní dodavatelského řetězce dle ust. II. 5) Smlouvy se Prodávající zavazuje Kupujícímu zaplatit smluvní pokutu ve výši 5 000 Kč za každé jednotlivé porušení.
7) Smluvní pokuty se stávají splatnými dnem následujícím po dni, ve kterém na ně vzniklo právo. Kupující si vyhrazuje právo započíst smluvní pokuty vůči pohledávkám prodávajícího za kupujícím.
8) Zaplacením smluvní pokuty není dotčen nárok kupujícího na náhradu škody způsobené mu porušením povinnosti prodávajícího, ke které se vztahuje smluvní pokuta. To platí i tehdy, bude-li smluvní pokuta snížena rozhodnutím soudu.

IX.

Odstoupení od smlouvy

1) Prodávající je oprávněn od smlouvy odstoupit v případě podstatného porušení povinností kupujícího.
2) Kupující je oprávněn od smlouvy odstoupit

   a) v případě byť nepodstatného porušení smlouvy prodávajícím,

b) bez zbytečného odkladu poté, co z chování prodávajícího nepochybně vyplyne, že poruší smlouvu podstatným způsobem, a nedá-li na výzvu kupujícího přiměřenou jistotu,

c) v případě vydání rozhodnutí o úpadku prodávajícího dle § 136 zákona č. 182/2006 Sb., o úpadku a způsobech jeho řešení (insolvenční zákon), ve znění pozdějších předpisů,

d) v případě, že prodávající v nabídce podané do zadávacího řízení k veřejné zakázce uvedl informace nebo předložil doklady, které neodpovídají skutečnosti a měly nebo mohly mít vliv na výsledek tohoto zadávacího řízení,

e) v případě, že bude pozastaveno nebo ukončeno poskytování dotačních prostředků čerpaných na realizaci předmětu smlouvy z projektu,

f) v případě, že výdaje, které by mu na základě smlouvy měly vzniknout, budou poskytovatelem dotačních prostředků, případně jiným oprávněným správním orgánem označeny za nezpůsobilé k proplacení z dotačních prostředků projektu.

3) Smluvní strany sjednávají, že za podstatné porušení smlouvy se mimo výslovně uvedených případů považuje rovněž takové porušení povinnosti smluvní strany, o němž již při uzavření smlouvy věděla nebo musela vědět, že by druhá smluvní strana smlouvu neuzavřela, pokud by toto porušení předvídala.

4) Odstoupení od smlouvy musí být provedeno písemně, jinak je neplatné. Odstoupení od smlouvy je účinné doručením písemného oznámení o odstoupení od smlouvy druhé smluvní straně.

X.

Dodatky a změny smlouvy; kontaktní osoby

1) Tuto smlouvu lze měnit nebo doplnit pouze písemnými průběžně číslovanými dodatky podepsanými oběma smluvními stranami. Předloží-li některá ze smluvních stran návrh dodatku, je druhá smluvní strana povinna se k takovému návrhu vyjádřit do 15 (slovy: patnácti) dnů ode dne následujícího po doručení návrhu dodatku.

2) Pouze to, co se uvozuje nebo k čemu se dodává „nebude-li mezi prodávajícím a kupujícím dohodnuto jinak“, může být smluvními stranami dohodnuto i ústně. Má se za to, že osobami oprávněnými k takové dohodě za smluvní strany jsou i jejich kontaktní osoby.

3) Kontaktní osoby smluvních stran

Kontaktní osoby smluvních stran uvedené v této smlouvě jsou oprávněny

a) vést vzájemnou komunikaci smluvních stran, zejména odesílat a přijímat oznámení a jiná sdělení na základě této smlouvy, a

b) jednat za smluvní strany v záležitostech, které jsou jim touto smlouvou výslovně svěřeny.

Jako kontaktní osoba může za smluvní stranu v rozsahu tohoto odstavce jednat i jiná či další osoba, a to na základě písemného oznámení smluvní strany o jiné či další kontaktní osobě doručeného druhé smluvní straně.

XI.
Závěrečná ujednání

1) Není-li ve smlouvě dohodnuto jinak, řídí se práva a povinnosti smlouvou neupravené či výslovně nevyloučené příslušnými ustanoveními OZ a dalšími právními předpisy účinnými ke dni uzavření smlouvy.

2) Prodávající se zavazuje strpět uveřejnění kopie smlouvy ve znění, v jakém byla uzavřena, a to včetně případných dodatků.

3) Pokud se stane některé ustanovení smlouvy neplatné nebo neúčinné, nedotýká se to ostatních ustanovení smlouvy, která zůstávají platná a účinná. Smluvní strany se v takovém případě zavazují nahradit dohodou ustanovení neplatné nebo neúčinné ustanovením platným a účinným, které nejlépe odpovídá původně zamýšlenému účelu ustanovení neplatného nebo neúčinného.

4) Prodávající není oprávněn převést svoje práva a povinnosti ze smlouvy na třetí osobu. § 1879 OZ se nepoužije. Kupující je oprávněn převést svoje práva a povinnosti ze smlouvy na třetí osobu.

5) Případné rozpory se smluvní strany zavazují řešit dohodou. Teprve nebude-li dosažení dohody mezi nimi možné, bude věc řešena u věcně příslušného soudu dle zákona č. 99/1963 Sb., občanský soudní řád, ve znění pozdějších předpisů, a to u místně příslušného soudu, v jehož obvodu má sídlo kupující.

6) Prodávající se za podmínek stanovených touto smlouvou v souladu s pokyny kupujícího a při vynaložení veškeré potřebné péče zavazuje:

   a) archivovat veškeré písemnosti zhotovené pro plnění zakázky podle této smlouvy a kdykoli po dobu uvedenou v tomto ustanovení Kupujícímu umožnit přístup k těmto archivovaným písemnostem, a to po dobu stanovenou příslušnými právními předpisy a pravidly projektu. Po uplynutí této doby je Kupující oprávněn od Prodávajícího výše uvedené dokumenty bezplatně převzít;

   b) jako osoba povinná dle § 2 písm. e) zákona č. 320/2001 Sb., o finanční kontrole ve veřejné správě, ve znění pozdějších předpisů, spolupůsobit při výkonu finanční kontroly, mj. umožnit řídícímu orgánu, Ministerstvu školství, mládeže a tělovýchovy, Ministerstvu financí jako auditnímu orgánu a platebnímu a certifikačnímu orgánu, pověřeným auditním subjektům, finančním úřadům, orgánům Evropské komise, Evropského účetního dvora a Evropského úřadu pro potírání podvodného jednání, státním zastupitelství, Nejvyššímu kontrolnímu úřadu, Úřadu pro ochranu hospodářské soutěže a dalším orgánům, které ke kontrole opravňují příslušné právní předpisy, vstup na místa, kde budou závazky dle této smlouvy plněny, a přístup k informacím a dokumentům vyhotoveným v souvislosti s plněním závazků dle této smlouvy včetně přístupu i k těm informacím a dokumentům, které podléhají ochraně podle zvláštních právních předpisů (např. obchodní tajemství, utajované skutečnosti), a to za předpokladu, že budou splněny požadavky kladené příslušnými právními předpisy. Prodávající je povinen poskytnout výše uvedeným orgánům součinnost při prováděných kontrolách;

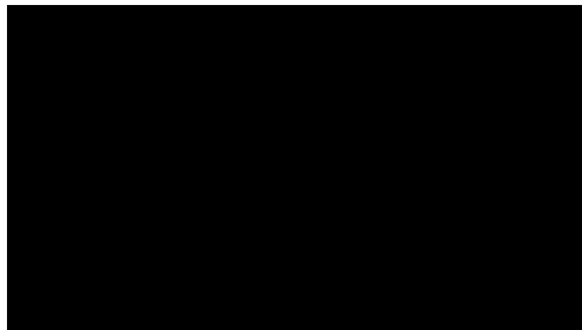
   c) ve smlouvách se svými poddodavateli umožnit kontrolním orgánům uvedeným v předchozím písmenu kontrolu poddodavatelů prodávajícího v rozsahu dle předchozího písmena.

7) Nedílnou součástí smlouvy je příloha č. 1 – Technická specifikace. V případě jakýchkoli rozporů nebo nesrovnalostí mezi smlouvou a přílohou č. 1 - Technická specifikace má znění přílohy č. 1 - Technická specifikace aplikační přednost.

8) Smlouva je uzavřena dnem podpisu poslední ze smluvních stran. Smlouva nabývá účinnosti jejím zveřejněním v registru smluv v souladu se zákonem č. 340/2015 Sb., o zvláštních podmínkách účinnosti některých smluv, uveřejňování těchto smluv o registru smluv (zákon o registru smluv), v platném znění.

9) Smlouva je vyhotovena ve 4 (slovy: čtyřech) originálech. Každá smluvní strana obdrží po 2 (slovy: dvou) z nich. V případě, že je smlouva uzavřena elektronicky, je uzavřena v jednom originále podepsaném prostřednictvím elektronického podpisu smluvních stran.

10) Smluvní strany potvrzují, že si smlouvu před jejím podpisem přečetly a s jejím obsahem souhlasí. Na důkaz toho připojují své podpisy.



příloha č. 1 – Technická specifikace

Příloha č. 1 - Technické podmínky		
Typové označení přístroje		
PromethION 24 - PRM24CapEx (PRO-SEQ024 + PRO-PRCAMP)		
Základní požadavky zadavatele		
High-throughput sekvenátor pro dlouhé čtení		
Požadované technické a funkční vlastnosti (nabídky uchazečů musí splňovat všechny níže uvedené parametry. U hodnocených parametrů musí nabídka vyhovět alespoň stanovené požadované úrovni)	**Požadovaná hodnota**	**Nabídka uchazeče** (uchazeči uvedou splnění požadovaného parametru ověřitelným způsobem, např. konkrétním odkazem na technické listy, výkresy apod.)
Sekvenování dlouhých čtení (long read)	ano	*ANO (ULK-Prom.pdf , str.1)*
Počet nezávislých pozic pro sekvenaci	Alespoň 8	*ANO - 24 (promethion-24-Specification.pdf, str.3)*
Sekvenování velmi dlouhých čtení (long read) delších než 100 kb	ano	*ANO (T2T.pdf, str. 5)*
Analýza DNA modifikací na nativní DNA – 5mC, 5hmC, 4mC a 6mA	ano	*ANO (https://nanoporetech.com/platform/accuracy#base-modifications)*
Přímá sekvenace RNA včetně analýzy modifikací RNA bází	ano	*ANO (https://nanoporetech.com/platform/accuracy#base-modifications)*
Generování sekvencí v reálném čase	ano	*ANO (Adaptive.pdf)*
Řízení sekvenace jednotlivých molekul v reálném čase	ano	*ANO (Adaptive.pdf)*
Kapacita jedné reagenční sady	> 100 Gb	*ANO, >100 Gb (LSK114.pdf, str.3)*
Řídící jednotka pro basecalling v reálném čase na všech pozicích	ano	*ANO - 24 (promethion-24-Specification.pdf, str.4)*
záruční doba	Alespoň 24 měsíců	*24 měsíců*

PROMETHION: PROTOCOL

Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114)

V ULK_9177_v114_revQ_06Nov2025

This protocol is for the extraction and sequencing of ultra-high molecular weight (uHMW) genomic DNA.

The protocol:

- Will allow the reliable generation and sequencing of ultra-long read length N50s (>50 kb)
- Is optimised for high yield
- Is compatible with R10.4.1 flow cells

For Research Use Only

FOR RESEARCH USE ONLY

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Library preparation

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8. Clean-up
9. Priming and loading the PromethION Flow Cell
10. Reloading ultra-long DNA library on a PromethION Flow Cell

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1. Overview of the protocol

This is an Early Access product.

For more information about our Early Access programmes, please see this article on product release phases.

Please ensure you always use the most recent version of the protocol.

Introduction to the Ultra-Long DNA Sequencing Kit protocol (SQK-ULK114)

This protocol describes the complete workflow from extracting gDNA from frozen tissue or purified cells from whole blood to the sequencing of ultra-high molecular weight (uHMW) gDNA using the Ultra-Long DNA Sequencing Kit (SQK-ULK114). We have also included the procedure to isolate white blood cells (WBC) from whole blood and how to quantify gDNA developed by Paul A 'Giron' Koetsier & Eric J Cantor, 2021.

We have used the Monarch® HMW DNA Extraction Kit for Tissue (NEB, T3060) to extract the uHMW gDNA for both input types when developing this protocol. Alternative kits are available from NEB which are specifically designed for the extraction from blood and cells. However, they have not been validated by Oxford Nanopore Technologies.

For each reaction, there is sufficient library generated for multiple consecutive loads onto a flow cell to increase output. To load a library three times on a PromethION Flow Cell, a flow cell wash is required to recover channels.

Steps in the sequencing workflow

Prepare for your experiment

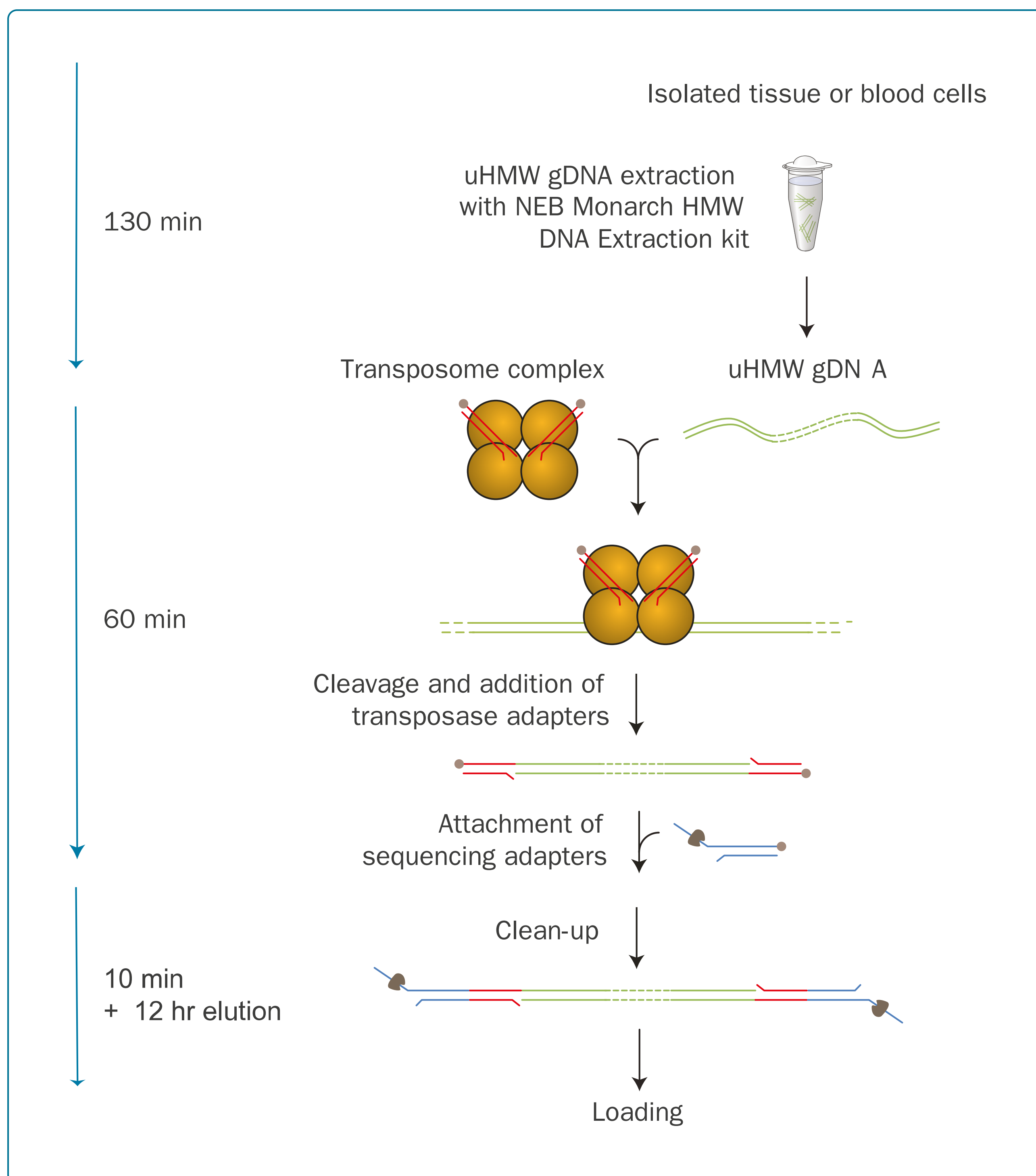
You will need to:

- If working with whole blood, isolate white blood cells. If working with frozen tissue, isolate cells from the tissue.
- Extract your uHMW gDNA.
- Quantify your sample.
- Ensure you have your sequencing kit, the correct equipment and third-party reagents.
- If not already installed, download the software for acquiring and analysing your data.
- Check your flow cell(s) to ensure it has enough pores for a good sequencing run.

Library preparation

You will need to:

- Tagment your DNA using a diluted fragmentation mix.
- Attach Rapid Adapters to the DNA ends.
- Clean-up your sample by precipitating your DNA and eluting overnight.
- Prime the flow cell and load your DNA library into the flow cell.



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software, which will collect raw data from the device and convert it into basecalled reads.

- **Optional:** Start the EPI2ME software and select a workflow for further analysis.

Flow cell loading and flushing

The Ultra-Long DNA Sequencing Kit (SQK-ULK114) protocol generates viscous DNA which can affect flow cell loading. To account for this, we have modified the flow cell loading steps. Please take care and follow the steps carefully to avoid damaging the flow cell.

To increase output, we recommend loading an ultra-long library three times per flow cell. A flow cell wash using the Flow Cell Wash Kit (EXP-WSH004) is required between each subsequent library load to recover channels. To run a second library straight away, please follow the modified method in this protocol: Reloading ultra-long DNA library on a PromethION Flow Cell.

Best practice for handling uHMW gDNA

When mixing, we recommend using wide-bore pipette tips to mix the full volume of a sample to ensure thorough mixing whilst minimising mechanical shearing of long fragments.

To preserve longer DNA, mix slower and more gently. Vortexing on low speeds may also be used at the expense of very long fragments.

While precautions should be taken to ensure that DNA fragment lengths are preserved, there should be no compromise to ensuring that reagents are thoroughly mixed with DNA. Insufficient mixing will lead to reduced read length and output.

For further information, please refer to the troubleshooting section.

Compatibility of this protocol

This protocol should only be used in combination with:

- Ultra-Long DNA Sequencing Kit (SQK-ULK114)
- Flow Cell Wash Kit (EXP-WSH004)
- R10.4.1 flow cells (FLO-PRO114M)
- EEB Expansion (EXP-EEB001)
- Ultra-Long Auxiliary Vials (EXP-ULA001)
- PromethION 24/48 device - PromethION IT requirements document
- PromethION 2 Solo device - PromethION 2 Solo IT requirements document

2. Equipment and consumables

Materials Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114)

Monarch® HMW DNA Extraction Kit for Tissue (New England Biolabs, T3060)
Flow Cell Wash Kit (EXP-WSH004)

Consumables

1.5 ml Eppendorf DNA LoBind tubes
2 ml Eppendorf DNA LoBind tubes
5 ml Eppendorf DNA LoBind tubes
15 ml Falcon tubes
Isopropanol, 100% (Fisher Scientific, 10723124)
Ethanol, 100% (e.g. Fisher, 16606002)
Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

Thermal cycler or heat block
Thermomixer set at 56°C (suitable for 1.5 ml, 2 ml and 5 ml tubes)
Vortex mixer
Microfuge
Wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
Qubit fluorometer (or equivalent)
Ice bucket with ice
Timer

The above list of materials, consumables, and equipment is for the library preparation section of the protocol. Depending on the sample type, additional reagents will be needed for sample processing and DNA extraction. These are listed in the "Sample preparation" section of the protocol.

For this protocol, you will need to extract gDNA from 6 million cells in 40 µl PBS before starting the library preparation.

This protocol has been developed using the [Monarch® HMW DNA Extraction Kit for Tissue (NEB, T3060)](). Alternative kits are available from NEB which are specifically designed for the extraction from blood and cells. However, they have not been validated by Oxford Nanopore Technologies.

This method has been validated for use on the following inputs:

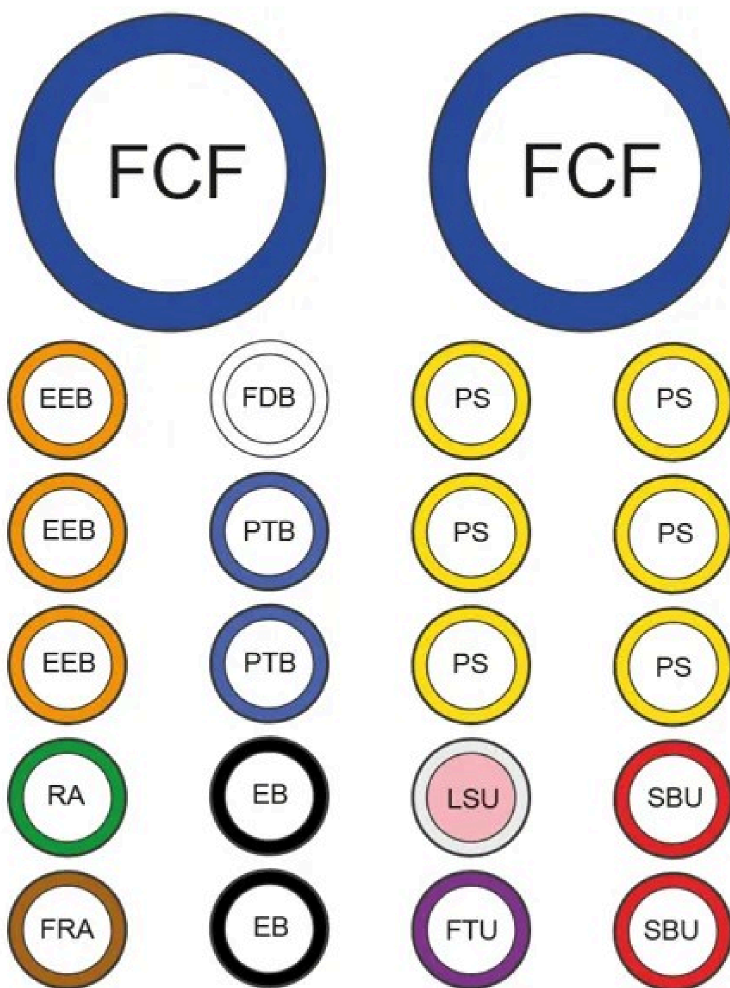
- 6 million white blood cells isolated from 1.6 ml blood (bovine), using RBC Lysis Solution (QIAGEN, 158904)
- 6 million cells isolated from 1 g frozen tissue, using pluriSelect cell straining equipment.

Check your flow cell

We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within 12 weeks of purchasing your MinION/GridION/PromethION Flow Cells. Oxford Nanopore Technologies will replace any unused flow cell with fewer than the number of pores listed in the Table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

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

Ultra-Long DNA Sequencing Kit (SQK-ULK114) contents

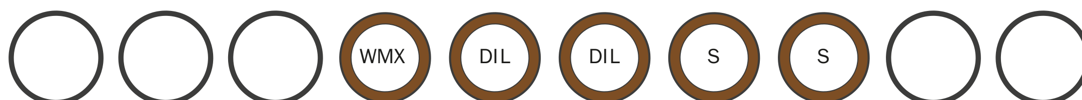


EEB : Extraction EB
RA : Rapid Adapter
FRA : Fragmentation Mix
FDB : FRA Dilution Buffer
PTB : Precipitation Buffer
EB : Elution Buffer

SBU : Sequencing Buffer UL
LSU : Loading Solution UL
FCF : Flow Cell Flush
FTU : Flush Tether UL
PS : Precipitation Star

Name	Acronym	Cap colour	Number of vials	Fill volume per vial (µl)
Rapid Adapter	RA	Green	1	40
Fragmentation Mix	FRA	Amber	1	50
FRA Dilution Buffer	FDB	Clear	1	1,600
Elution Buffer	EB	Black	2	1,500
Extraction EB	EEB	Orange	3	1,700
Sequencing Buffer UL	SBU	Red	2	1,000
Loading Solution UL	LSU	White cap, pink label	1	200
Flush Tether UL	FTU	Purple	1	600
Flow Cell Flush	FCF	Blue	2	15,500
Precipitation Buffer	PTB	Blue	2	1,700
Precipitation Star	PS	Yellow	6	1 star

Flow Cell Wash Kit (EXP-WSH004) contents



WMX : Wash Mix
DIL : Wash Diluent
S : Storage Buffer

Name	Volume (µl)	No. of tubes	No. of uses
Wash Mix (WMX)	15	1	6
Wash Diluent (DIL)	1,300	2	6
Storage Buffer (S)	1,600	2	6

- Wash Mix (WMX) contains DNase I.
- Wash Diluent (DIL) contains the exonuclease buffer that maximises activity of the DNase I.
- The Storage Buffer allows flow cells to be stored for extended periods of time.

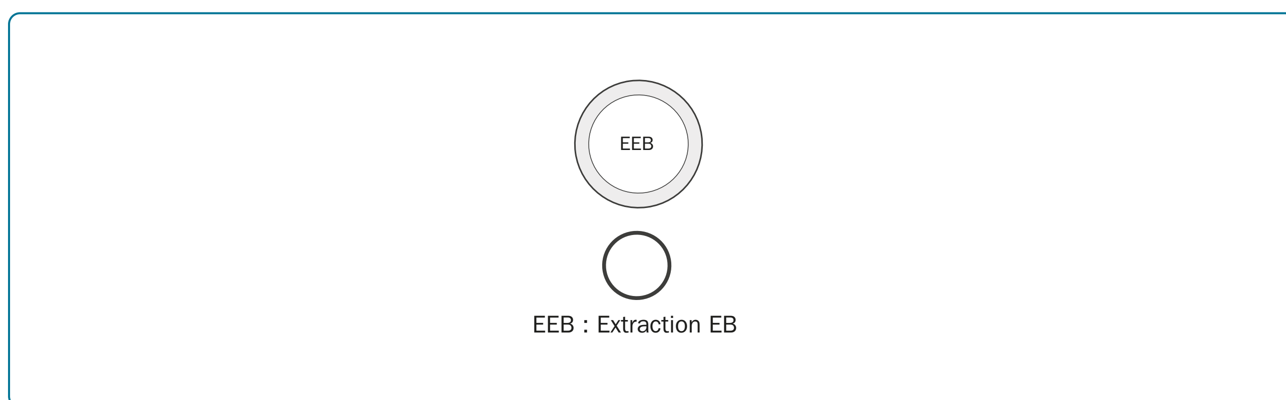
To maximise the use of the Ultra-Long DNA Sequencing Kit V14, the EEB Expansion (EXP-EEB001) and the Ultra-Long Auxiliary Vials (EXP-ULA001) expansion packs are available.

These expansions provide extra library preparation and flow cell priming reagents to allow users to maximise the use of their Ultra-Long DNA Sequencing Kit V14.

The EEB Expansion (EXP-EEB001) contains enough reagents for at least six standard extraction elution steps.

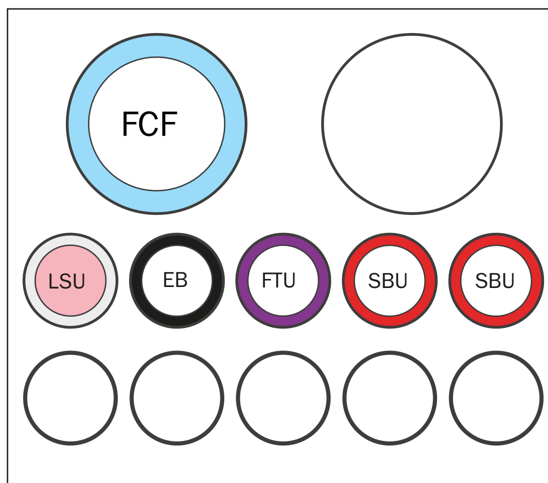
The Ultra-Long Auxiliary Vials (EXP-ULA001) provides enough reagents to carry out twelve additional flow cell loads on MinION or PromethION Flow Cells.

EEB Expansion (EXP-EEB001) contents:



Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Extraction EB	EEB	White	1	6,000

Ultra-Long Auxiliary Vials (EXP-ULA001) contents:



LSU: Loading Solution UL
EB: Elution Buffer
FTU: Flush Tether UL
SBU: Sequencing Buffer UL
FCF: Flow Cell Flush

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
Elution Buffer	EB	Black	1	1,500
Sequencing Buffer UL	SBU	Red	2	1,000
Loading Solution UL	LSU	White cap, pink label	1	200
Flush Tether UL	FTU	Purple	1	600
Flow Cell Flush	FCF	Clear cap, light blue label	1	15,500

3. Isolation of white blood cells (WBCs) from whole blood

Materials 1.6 ml of whole blood

Consumables

RBC Lysis Solution (QIAGEN, 158106)
Phosphate buffered saline (PBS), pH 7.4 (ThermoFisher, 10010023)
15 ml Falcon tubes

Equipment

Microfuge
P1000 pipette and tips
P200 pipette and tips
P20 pipette and tips

White blood cell sample preparation for the Ultra-long DNA experiment

Approximately 6 million isolated white blood cells must be prepared from 1.6 ml of whole blood to use as input in the Ultra-long DNA experiment.

Users may isolate white blood cells by any means they feel are most appropriate for the whole blood sample to be used. If 6 million cells have been isolated, users can start from the uHMW gDNA extraction step.

1. **Add 4.8 ml of RBC Lysis Solution to 1.6 ml of whole blood in a 15 ml Falcon tube.**

2. **Gently invert the tube ten times to mix.**

3. **Incubate for 5 minutes at room temperature and gently invert twice during the incubation.**

4. **Centrifuge at 2,000 x g for 2 minutes at 4°C to pellet the white blood cells.**

5. **Discard the supernatant by pouring. There will be ~200 µl supernatant remaining in the tube.**

6. **Resuspend the cells in the residual supernatant by gently flicking the tube.**

7. **Make up the volume to 1.6 ml with 1x PBS.**

8. **Repeat steps 1-7 twice more to complete three washes in total.**

If any red colouration persists, repeat the wash step until the cell pellet is white.

9. **After the final spin, remove the entire supernatant by pouring and aspirating any remaining supernatant.**

10. **Resuspend the cell pellet in 40 µl 1x PBS. There will be approximately 6 million cells in the suspension.**

Take the cell pellet forward into the "uHMW gDNA extraction" step.

4. Preparation of tissue samples for gDNA extraction

Materials

Cell Suspension Buffer (CSB): 0.35 M sucrose, 100 mM EDTA, 50 mM Tris.HCl pH 8
Frozen tissue sample

Consumables

Phosphate buffered saline (PBS), pH 7.4 (ThermoFisher, 10010023)
1 M Tris-HCl pH 8.0 (Thermo Scientific, 15893661)
0.5 M EDTA, pH 8 (Thermo Scientific, R1021)
2.5 M sucrose
Nuclease-free water (e.g. ThermoFisher, AM9937)
50 ml Falcon tubes
5 ml Eppendorf DNA LoBind tubes

Equipment

Centrifuge suitable for 5 ml Eppendorf tubes (Eppendorf centrifuge 5804/5804 R or equivalent)
Eppendorf tube rack suitable for 5 ml Eppendorf tubes
Scalpel
TissueRuptor II (QIAGEN, 9002755)
TissueRuptor Disposable Probes (QIAGEN, 990890)
Florescent microscope with functionality to quantify nuclei (Logos CELENA S Digital Imaging System or equivalent)
Heat block equipped with thermoblock suitable for 5 ml Eppendorf tubes
200 µm PluriStrainer® (pluriSelect, 43-50200-03)
100 µm PluriStrainer® (pluriSelect, 43-50100-51)

50 µm PluriStrainer® (pluriSelect, 43-50050-03)
30 µm PluriStrainer® (pluriSelect, 43-50030-03)
PluriStrainer® Connector Ring (pluriSelect, 41-50000-03)
PluriStrainer® Funnel (pluriSelect, 42-50000)
P1000 pipette and tips
10 ml syringe

1 Prepare the Cell Suspension Buffer (CSB) as follows:

Reagent	Stock	Final conc.	Volume
Tris.HCl, pH 8	1 M	0.05 M	50 ml
EDTA	0.5 M	0.1 M	200 ml
Sucrose	2.5 M	0.35 M	140 ml
Nuclease-free water	-	-	610 ml
Total	-	-	**1,000 ml**

2 Add 1 g of the frozen tissue sample to a weighing boat.

3 Using the scalpel, slice the tissue into thin strips and then dice the sample.

4 Transfer the tissue sample to a fresh 50 ml Falcon tube.

5 Add 10 ml of the Cell Suspension Buffer (CSB) into the 50 ml Falcon tube.

6 Using the QIAGEN TissueRuptor, gently homogenise the tissue sample.

1. Insert the probe and pulse at minimum speed for one second. Stir the homogenate between each pulse.
2. Repeat this five times.

During homogenisation, only apply as much force as is required to gently break up the tissue. Excessive force will damage the nuclei and make them difficult to quantify. It is not a problem if there is intact material remaining at the end of this step, as it will be re-processed in later steps.

7 Assemble the pluriStrainer apparatus with a 200 μm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions.

8 Pass the full volume of the tissue sample homogenate through the 200 μm PluriStrainer®.

The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer.

9 Disassemble the pluriStrainer® apparatus according to the manufacturer's instructions, setting aside the strained homogenate in the 50 ml Falcon tube for later use.

10 Repeat the homogenisation process on any intact tissue caught by the pluriStrainer®:

1. Transfer any intact tissue caught by the 200 μm pluriStrainer® into a fresh 50 ml Falcon tube by inverting the strainer and tapping out the intact tissue.
   Tip: A spatula can be used to help remove the intact tissue from the strainer.
2. Add 10 ml of the Cell Suspension Buffer (CSB) into the 50 ml Falcon tube.

11 Repeat steps 6-10 two more times to perform a total of three rounds of tissue homogenisation.

12 Combine the contents of the 50 ml Falcon tube with the original strained homogenate set aside in step 10.

The combined volume of 200 µm strained homogenate is ready for further processing.

13 Strain the 200 µm strained homogenate through the 100 µm pluriStrainer®:

1. Assemble the pluriStrainer apparatus with a 100 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions.
2. Pass the full volume of the 200 µm strained homogenate through the 100 µm PluriStrainer®. **Tip:** The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer.
3. Disassemble the pluriStrainer® and retain the 100 µm strained homogenate in the 50 ml Falcon tube.

14 Strain the 100 µm strained homogenate through the 50 µm pluriStrainer®:

1. Assemble the pluriStrainer apparatus with a 50 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions.
2. Pass the full volume of the 100 µm strained homogenate through the 50 µm PluriStrainer®. **Tip:** The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer.
3. Disassemble the pluriStrainer® and retain the 50 µm strained homogenate in the 50 ml Falcon tube.

15 Strain the 50 µm strained homogenate through the 30 µm pluriStrainer®:

1. Assemble the pluriStrainer apparatus with a 30 µm strainer, connector ring, funnel and 50 ml Falcon tube according to the manufacturer's instructions.
2. Pass the full volume of the 50 µm strained homogenate through the 30 µm PluriStrainer®. **Tip:** The homogenate can be gently agitated, and a small amount of negative pressure can be applied with the syringe to help pass the homogenate through the strainer.
3. Disassemble the pluriStrainer® and retain the 30 µm strained homogenate in the 50 ml Falcon tube.

16 Determine the concentration of the nuclei in the purified homogenate using a fluorescent microscope and a stain appropriate for the nuclei in the sample.

17 **Take forward a volume corresponding to 6 million nuclei and add this to a 5 ml Eppendorf DNA LoBind tube.**

18 **Centrifuge the 5 ml Eppendorf tube at 16,000 x g for 5 minutes to pellet the nuclei/cells.**

19 **Pipette off all the supernatant and discard, taking care not to disturb the pellet.**

20 **Add 40 µl of PBS to the 5 ml Eppendorf DNA LoBind tube.**

21 **Thoroughly mix the tube by repeatedly flicking. Ensure the pellet breaks up and no clumps remain in the nuclei/cell suspension.**

Note: You may need to flick quite hard and thoroughly to ensure the pellet breaks up and no clumps remain.

Take the nuclei/cell suspension forward into the "uHMW gDNA extraction" step.

5. uHMW gDNA extraction

Materials

6 million cells/nuclei isolated from frozen tissue or white blood cells isolated from whole blood
Extraction EB (EEB)
Monarch® HMW DNA Extraction Kit for Tissue (New England Biolabs, T3060)

Consumables

5 ml Eppendorf DNA LoBind tubes
Phosphate buffered saline (PBS), pH 7.4 (ThermoFisher, 10010023)
Isopropanol, 100% (Fisher Scientific, 10723124)
Ethanol, 100% (e.g. Fisher, 16606002)
2 ml Eppendorf DNA LoBind tubes

Equipment

Heat block set at 56°C
Thermomixer set at 56°C (suitable for 1.5 ml, 2 ml and 5 ml tubes)

Hula mixer (gentle rotator mixer)
Microfuge
P1000 pipette and wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
P20 pipette and tips
Eppendorf 5424 centrifuge (or equivalent)

This method does NOT use the Monarch Elution Buffer II from the Monarch® HMW DNA Extraction Kit.

This method has been optimised using the Extraction EB (EEB) from the Oxford Nanopore sequencing kit.

Ensure ethanol is added to the Monarch gDNA Wash Buffer as per kit guidance.

1. **Thaw the Extraction EB (EEB) at room temperature, mix by vortexing and place on ice.**

2. **Add 6 million cells resuspended in 40 µl PBS to a fresh 5 ml tube. Cells can be isolated from cell culture, white blood cells from blood, or tissue according to the above methods.**

Thorough but gentle resuspension of cells is required to ensure efficient lysis and to prevent heterogeneity in the subsequent steps.

3. **In a separate 2 ml Eppendorf DNA LoBind tube, combine the following reagents:**

Reagent	Volume
Monarch HMW gDNA Tissue Lysis Buffer	1,800 µl
Proteinase K	60 µl
Total	**1860 µl**

4. **Add 1.8 ml of mixed Monarch HMW gDNA Tissue Lysis Buffer and Proteinase K to the resuspended cells.**

5. **Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip.**

When using cell lines, we have found that the incubation step below can be omitted.

6. **Incubate the reaction at 56°C for 10 minutes.**

7. **Using a regular pipette tip, add 15 µl of Monarch RNase A.**

8. **Gently mix by slowly pipetting the reaction five times using a 1 ml wide-bore pipette tip.**

9. **Incubate the reaction at 56°C for 10 minutes on a thermomixer at 650 rpm.**

When using cell lines, we have found the protein removal steps can be omitted. If using cell lines, proceed directly to step 13.

10. **Using a regular pipette tip, add 900 µl of the Monarch Protein Separation Solution to the reaction and mix using a Hula Mixer (rotator mixer) for 10**

minutes, rotating at 3 rpm.

11 **Centrifuge the reaction at 16,000 x g for 10 minutes at 4°C to separate the protein from the DNA.**

DNA will be present in the upper phase, whereas protein and other contaminants will be in the lower phase.

12 **Using a wide-bore pipette tip, carefully aspirate the upper phase containing the DNA and transfer to a fresh 5 ml tube without disturbing the phase below.**

The DNA in the upper phase should be extremely viscous and should only be possible to aspirate using a wide-bore pipette tip.

If the protein phase is disturbed, the tube can be centrifuged again at 16,000 x g for 10 minutes at 4°C.

13 **Add three Monarch DNA Capture Beads to the collected DNA phase (or to the lysis reaction if proceeded directly from Step 9).**

Note: The first bead is a sacrificial bead and will remain at the bottom of the tube throughout the remainder of the process.

14 **Add 2.5 ml isopropanol to the tube and mix using a Hula Mixer (rotator mixer) for 20 minutes rotating at 3 rpm. Ensure the DNA has fully precipitated around the glass beads.**

Check the DNA is binding to the beads by looking for a viscous mass around the beads. The mixing step can be extended if the DNA is not obviously condensing around the beads.

15 **Leave the tube to stand for 1 minute, without rotating, at room temperature.**

16. **Aspirate the supernatant from the tube, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any supernatant remaining in the lid of the tube.**

Note: If ~100 µl of supernatant is remaining in the tube, perfomance will not be affected.

17. **Add 2 ml of Monarch gDNA Wash Buffer to the tube containing DNA bound to the beads and invert the tube to mix.**

Ensure ethanol is added to the Monarch gDNA Wash Buffer as per kit guidance.

18. **Aspirate the Wash Buffer, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any Wash Buffer remaining in the lid of the tube.**

19. **Add 2 ml of Monarch gDNA Wash Buffer to the tube containing the DNA bound to the beads.**

20. **To a fresh 2 ml Eppendorf tube, add 560 µl of Extraction EB (EEB).**

21. **Aspirate the Wash Buffer, being careful not to aspirate the DNA that is bound to the beads. Check for and remove any Wash Buffer remaining in the lid of the tube.**

22. **Transfer the beads to a Monarch Bead Retainer inserted in a Monarch Collection Tube II.**

23. **Briefly spin the tube using a microfuge to remove any remaining Wash Buffer from the beads. Dispose of the collection tube containing residual wash buffer.**

Do NOT use the Monarch Elution Buffer II in the Monarch® HMW DNA Extraction Kit for Tissue.

24 **Immediately transfer the beads from the bead retainer into the 2 ml tube containing 560 µl of Extraction EB (EEB).**

Beads should be transferred immediately to ensure that they do not over-dry, which could lead to increased solubilisation times.

25 **Incubate the tube for 10 minutes at 56°C.**

26 **Pour the eluate and beads into a clean bead retainer inserted in a collection tube. Spin the tube at 1,000 x g for 1 minute to separate eluate from the beads. Dispose of beads and bead retainer.**

27 **Add 200 µl of Extraction EB (EEB) to the collection tube to bring the total elution volume to 760 µl.**

28 **Transfer the eluate to a fresh 2 ml Eppendorf DNA LoBind tube.**

29 **Incubate the eluate for 10 minutes at 56°C.**

30 **Gently mix the eluate by slowly pipetting 10 times using a 1 ml wide-bore pipette tip.**

Thorough but gentle resuspension of DNA is required to prevent heterogeneity in the sample.

Take forward the resuspended DNA into the quantification step. However, at this point it is possible to store the sample at room temperature overnight.

6. (Optional) gDNA quantification

Materials

Monarch® DNA Capture Beads
Monarch® Bead Retainer

	Monarch® Collection Tubes II
Consumables	2 ml Eppendorf DNA LoBind tubes Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
Equipment	Vortex mixer Centrifuge Qubit fluorometer (or equivalent) P200 pipette and tips

Quantification of uHMW gDNA

The method to quantify uHMW gDNA was developed by Paul A 'Giron' Koetsier & Eric J Cantor, 2021, which recommends the use of a regular P200 pipette and tip.

This optional uHMW gDNA quantification step has also been included in the protocol for user QC. However, this step can be omitted and 750 µl of DNA in Extraction EB (EEB) can be taken straight into the tagmentation step of the protocol.

1. **Use a regular P200 pipette tip to aspirate 10 µl of gDNA.**

> **If the DNA is particularly viscous, the aspirated DNA can be separated from the sample by forcing the sample against the side of the tube to break the DNA off. It is critical that the DNA is completely homogenous, so that the 10 µl of sample that is removed is representative of the entire sample.**

2. **Dispense the aspirated gDNA into a fresh 2 ml Eppendorf DNA LoBind tube.**

3. **Add a Monarch DNA Capture Bead to the 10 µl of gDNA and vortex aggressively for 1 minute to shear the gDNA.**

4. **Transfer the gDNA and beads into a clean Monarch Bead Retainer inserted in a Monarch Collection Tube II. Spin the tube at 1,000 x g for 1 minute to separate gDNA from the beads. Dispose of beads and bead retainer.**

5 Using a wide-bore pipette tip, transfer the gDNA into a clean 1.5 ml Eppendorf DNA LoBind tube.

Quantify the sample using a Qubit fluorometer. The expected yield is 30-40 µg of DNA.

Take forward 750 µl DNA into the tagmentation step.

7. Tagmentation

Materials	750 µl of extracted uHMW gDNA in EEB Rapid Adapter (RA) Fragmentation Mix (FRA) FRA Dilution Buffer (FDB)
Consumables	1.5 ml Eppendorf DNA LoBind tubes
Equipment	Thermal cycler or heat block Microfuge P1000 pipette and wide-bore pipette tips P1000 pipette and tips P20 pipette and tips Ice bucket with ice

Best practice for handling uHMW gDNA

When mixing, we recommend using wide-bore pipette tips to mix the full volume of a sample to ensure thorough mixing whilst minimising mechanical shearing of long fragments.

To preserve longer DNA, mix slower and more gently. Vortexing on low speeds may also be used at the expense of very long fragments.

While precautions should be taken to ensure that DNA fragment lengths are preserved, there should be no compromise to ensuring that reagents are thoroughly mixed with DNA. Insufficient mixing will lead to reduced read length and output.

For further information, please refer to the troubleshooting section.

1. **Thaw the the kit components at room temperature, spin down briefly using a microfuge and mix by pipetting as indicated by the Table below:**

Once thawed, keep all the kit components on ice.

Reagent	Thaw at room temperature	Briefly spin down	Mix well by pipetting
Fragmentation Mix (FRA)	Not frozen	✓	✓
FRA dilution buffer (FDB)	Not frozen	✓	✓
Rapid Adapter (RA)	Not frozen	✓	✓

2. **In a 1.5 ml Eppendorf DNA LoBind tube, dilute the Fragmentation Mix (FRA) with FRA Dilution Buffer (FDB) as follows:**

Reagent	Volume
Fragmentation Mix (FRA)	6 µl
FRA dilution buffer (FDB)	244 µl
Total	**250 µl**

3. **Mix the diluted Fragmentation Mix (FRA) by pipetting.**

4. **Using a regular pipette tip, add 250 µl of diluted Fragmentation Mix (FRA) to the 750 µl of extracted DNA. Stir the reaction with the pipette tip whilst expelling the diluted Fragmentation Mix (FRA) to ensure an even distribution.**

5. **Immediately mix the reaction by slowly pipetting 10 times with a wide-bore pipette tip.**

Visually check the reagents are thoroughly mixed. It is important to immediately mix the diluted Fragmentation Mix (FRA) with the DNA thoroughly.

6 Incubate the reaction as follows:

Temperature	Time
Room temperature	10 minutes
75°C	10 minutes
On ice	Cool on ice for a **minimum** of 10 minutes

Note: the reaction must be cooled on ice before adding Rapid Adapter (RA) to prevent denaturing the enzyme.

7 Add 5 µl Rapid Adapter (RA) to the reaction using a regular pipette tip.

8 Gently mix the reaction by slowly pipetting five times using a 1 ml wide-bore pipette tip.

Note: visually check to ensure the reaction is thoroughly mixed.

9 Incubate the reaction for 30 minutes at room temperature.

8. Clean-up

Materials	Elution Buffer from the Oxford Nanopore kit (EB) Precipitation Buffer (PTB) Precipitation Star (PS)
Consumables	1.5 ml Eppendorf DNA LoBind tubes
Equipment	Centrifuge Microfuge Hula mixer (gentle rotator mixer) P200 pipette and tips P1000 pipette and tips

P1000 pipette and wide-bore pipette tips

1. **Thaw the kit components at room temperature, spin down briefly using a microfuge and mix by vortexing as indicated by the Table below:**

Reagent	Thaw at room temperature	Briefly spin down	Mix well by pipetting
Precipitation buffer (PTB)	✓	✓	✓
Elution Buffer (EB)	✓	✓	✓

Once thawed, keep all the kit components on ice.

We strongly recommend using a 1.5 ml Eppendorf DNA LoBind tube for the clean-up method when using the Precipitation Star (PS)

2. **Add a Precipitation Star (PS) to the sample.**

3. **Using a regular pipette tip, add 500 µl of Precipitation Buffer (PTB) to the sample.**

4. **Mix the sample by rotating on a Hula Mixer (rotator mixer) for 20 minutes at 3 rpm.**

5. **Visually inspect to check the DNA has precipitated around the Precipitation Star (PS).**

6. **Using a regular pipette tip, carefully remove the supernatant from the tube, taking care not to aspirate the DNA.**

In the 1.5 ml Eppendorf LoBind DNA tube, the Precipitation Star (PS) should be suspended mid-way in the tube, allowing the supernatant beneath the star to be removed.

7. **Briefly spin down the tube and remove any residual supernatant using a regular pipette tip, taking care not to aspirate the DNA.**

8. **Using a regular pipette tip, add 300 µl of Elution Buffer (EB) to the tube containing the Precipitation Star (PS) and DNA. Incubate overnight at room temperature, for a minimum of 12 hours.**

9. **Using a wide-bore pipette tip, remove and retain eluate containing the DNA library into a clean 1.5 ml Eppendorf LoBind DNA tube.**

10. **Briefly spin down the tube containing the Precipitation Star (PS) and remove any remaining eluate with a wide-bore pipette tip, ensuring there is no liquid remaining on the Precipitation Star (PS).**

Dispose of the tube containing the Precipitation Star (PS).

11. **Gently mix the DNA library by slowly pipetting 10 times with a wide-bore pipette tip.**

Thorough but gentle resuspension of DNA is required to prevent heterogeneity in the sample.

Take the DNA library forwards for loading into the flow cell. Store the library on ice until ready to load.

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short-term** storage or repeated use, for example, re-loading flow cells between washes. For single use and **long-term storage** of more than 3 months, we recommend storing libraries at **-80°C** in Eppendorf DNA LoBind tubes.

9. Priming and loading the PromethION Flow Cell

Materials

Flow Cell Flush (FCF)
Flush Tether UL (FTU)

	Loading Solution UL (LSU) Sequencing Buffer UL (SBU)
Consumables	PromethION Flow Cells 1.5 ml Eppendorf DNA LoBind tubes
Equipment	PromethION device PromethION 2 Solo or PromethION 24/48 device PromethION Flow Cell Light Shield P1000 pipette and wide-bore pipette tips P200 pipette and wide-bore pipette tips P1000 pipette and tips P200 pipette and tips P20 pipette and tips

Please note, this kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).

Only use the reagents provided with the SQK-ULK114 kit for priming and loading the flow cell. Reagents from other kits are not compatible with this protocol.

After taking the flow cell out of the fridge, wait 20 minutes for the flow cell to reach room temperature, before inserting it into the PromethION. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.

1. **Thaw the Sequencing Buffer UL (SBU), Loading Solution UL (LSU), Flush Tether UL (FTU) and one tube of Flow Cell Flush (FCF) at room temperature and mix by vortexing. Then spin down and place on ice.**

2. **In a fresh 1.5 ml Eppendorf DNA LoBind tube, prepare the DNA library for loading as follows using a wide-bore pipette tip for the addition of the DNA library:**

Reagent	Volume per flow cell
Sequencing Buffer UL (SBU)	100 µl
Loading Solution UL (LSU)	10 µl
DNA library	90 µl
Total	**200 µl**

Note: ensure the Sequencing Buffer UL (SBU) and Loading Solution UL (LSU) are thoroughly mixed by pipetting before the addition of the DNA library.

3. **Gently mix the prepared DNA library by slowly pipetting ten times using a wide-bore pipette tip.**

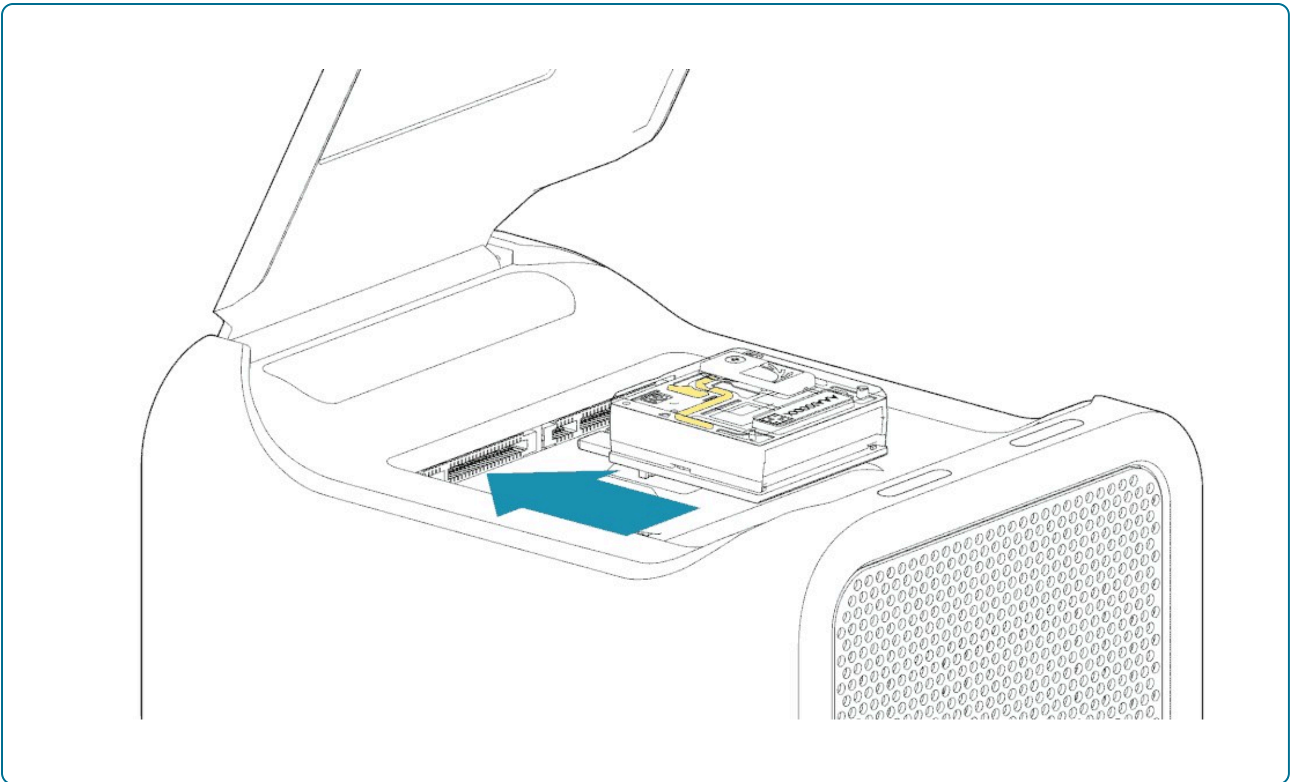
4. **Incubate at room temperature for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous.**

5. **Prepare the flow cell priming mix in a 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing at room temperature.**

Reagent	Volume
Flush Tether UL (FTU)	30 µl
Flow Cell Flush (FCF)	1,170 µl
Total	**1,200 µl**

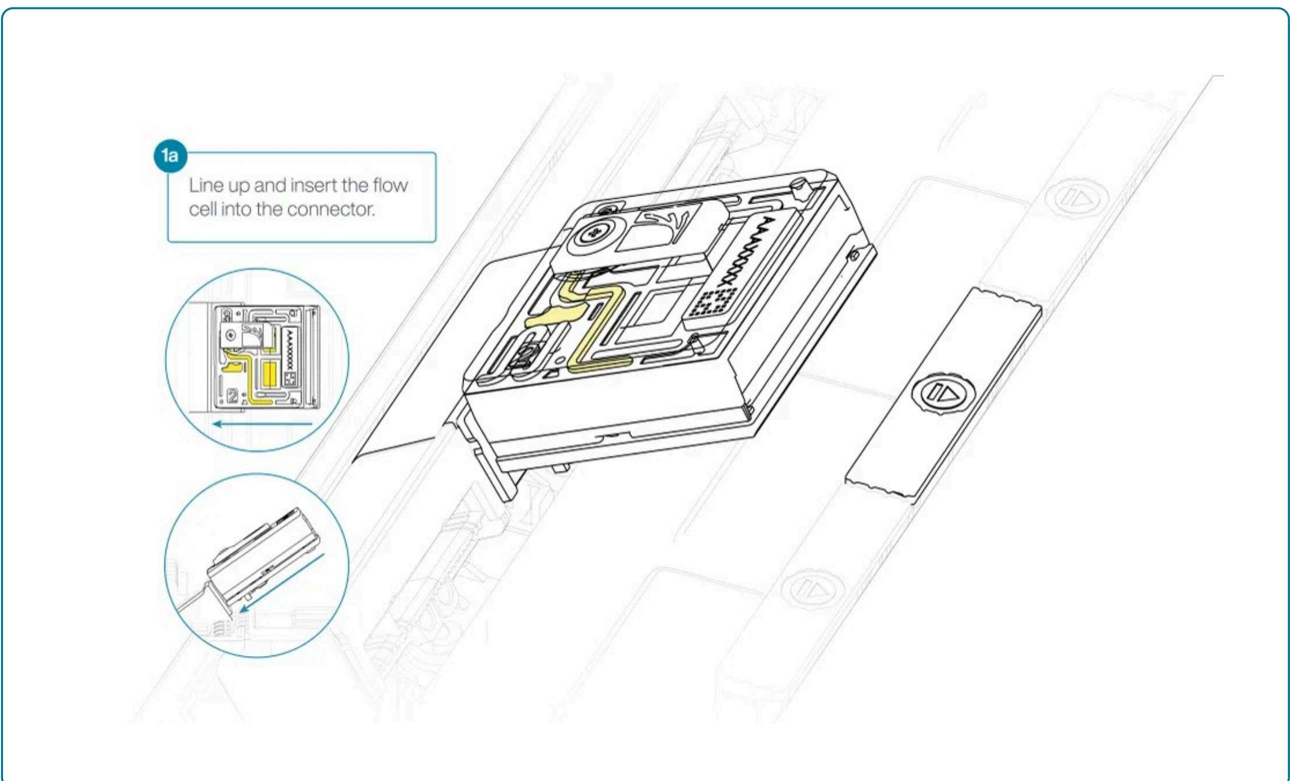
6. **For PromethION 2 Solo, load the flow cell(s) as follows:**

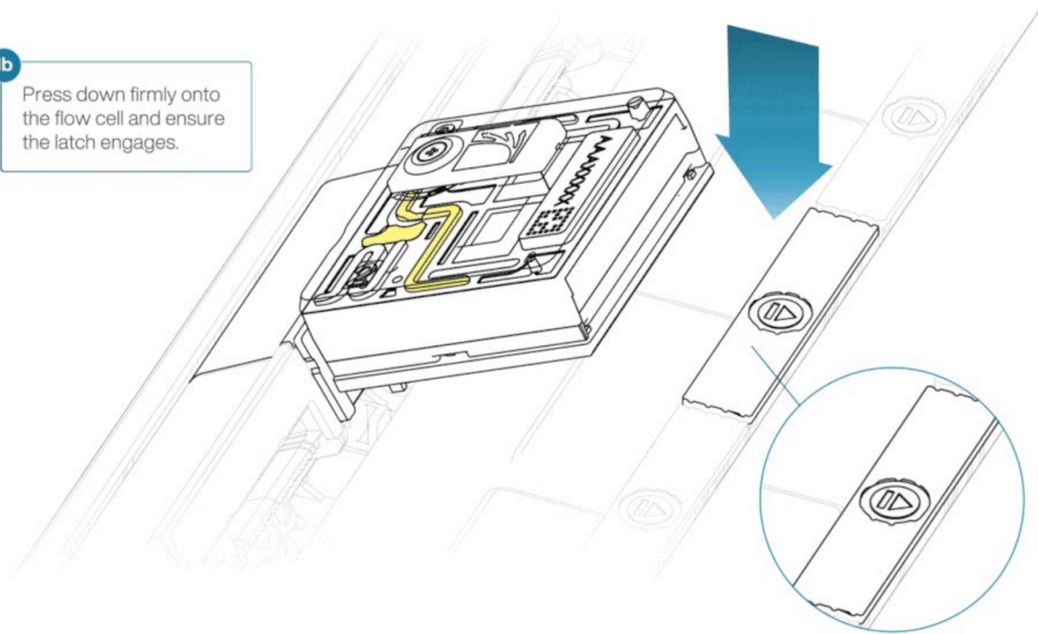
   1. Place the flow cell flat on the metal plate.
   2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.



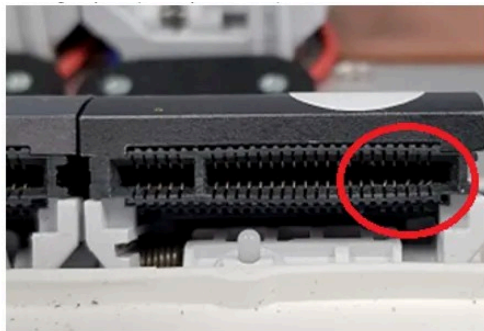
7 For the PromethION 24/48, load the flow cell(s) into the docking ports:

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.





Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.

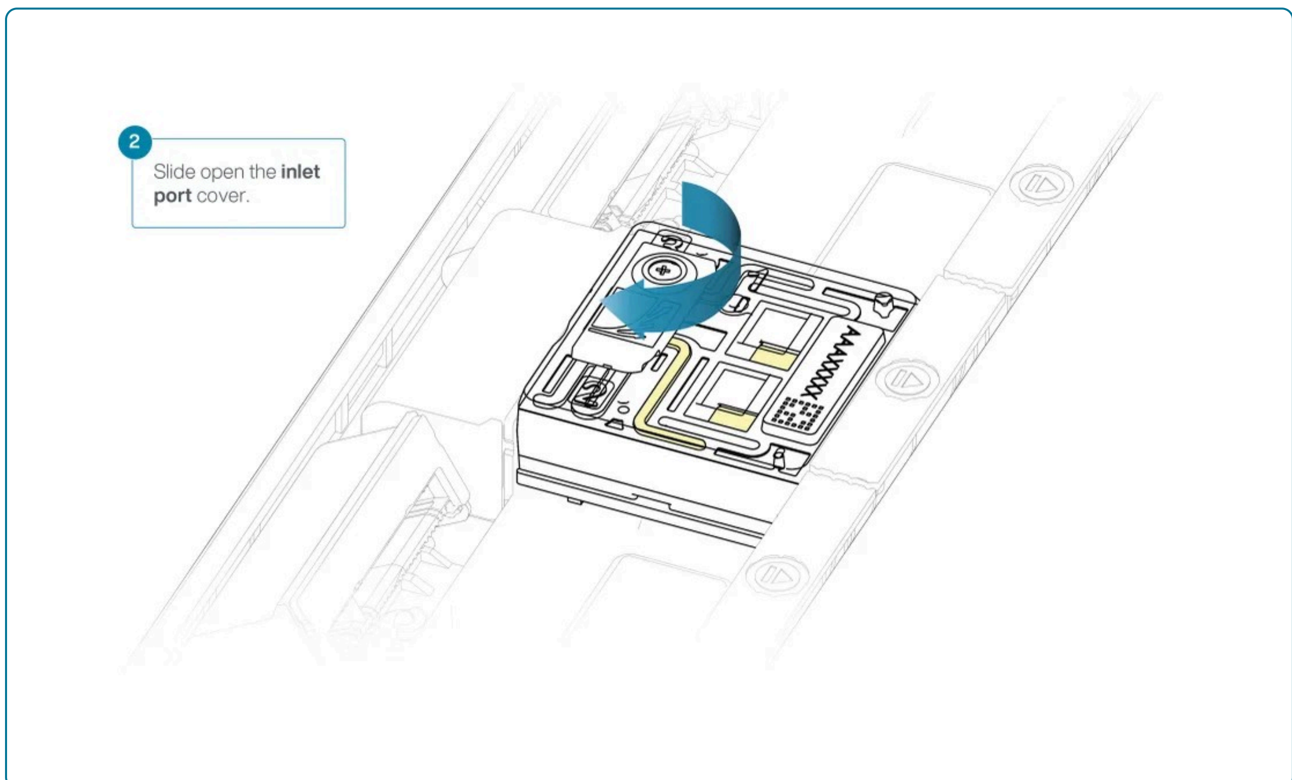


Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check document for more information.

8 Slide the inlet port cover clockwise to open.

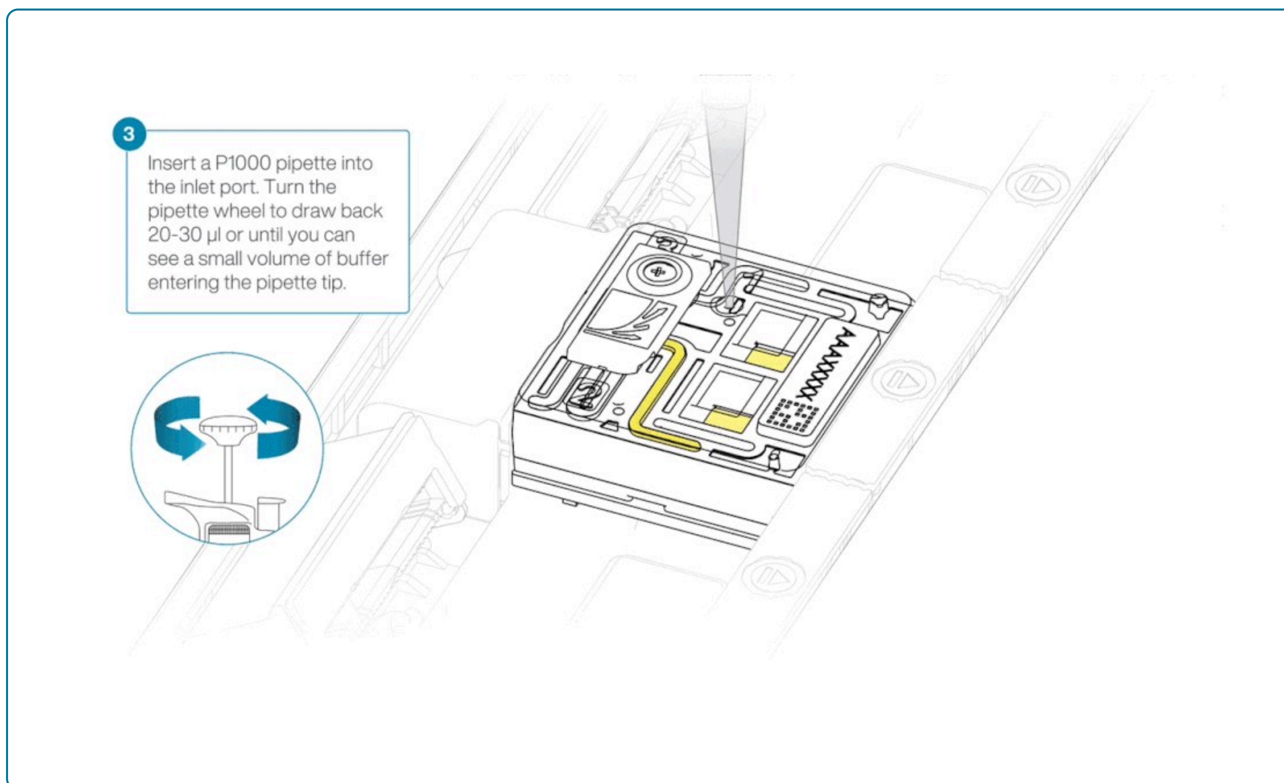


Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

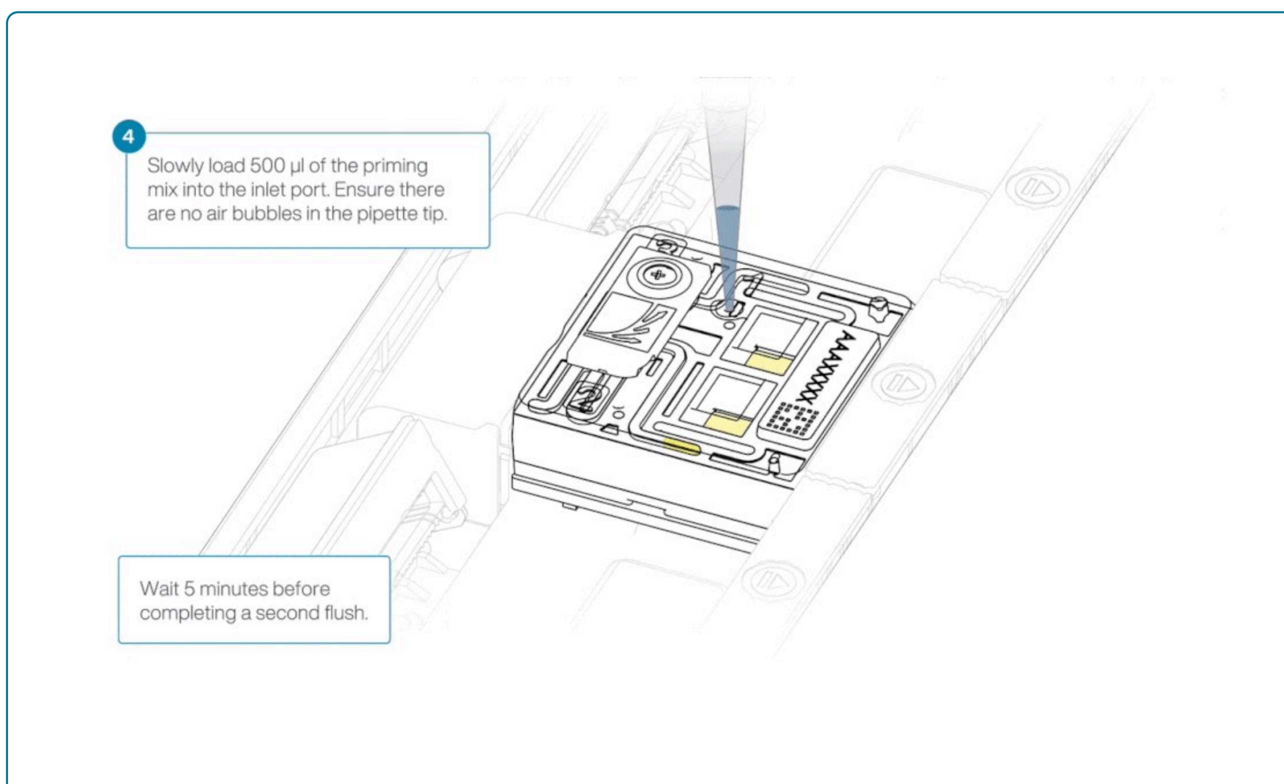
9 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.

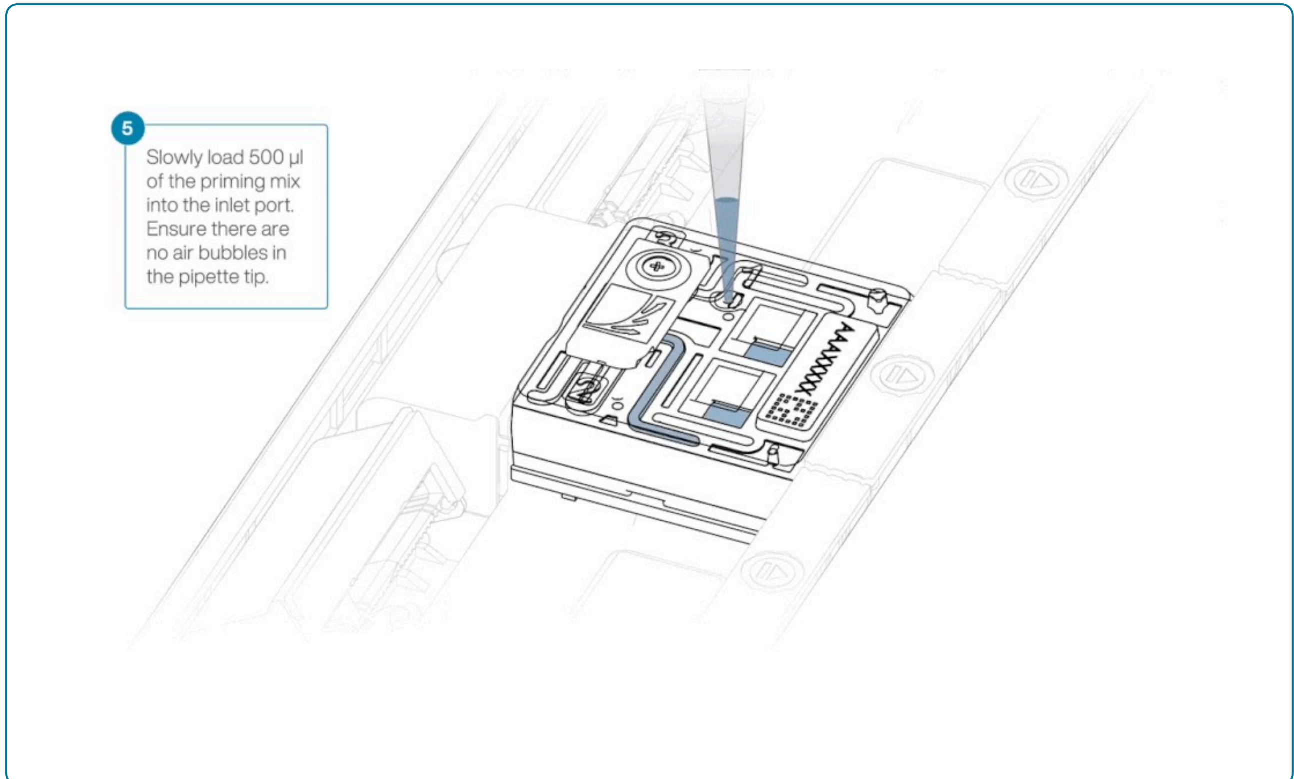
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



10 **Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes.**

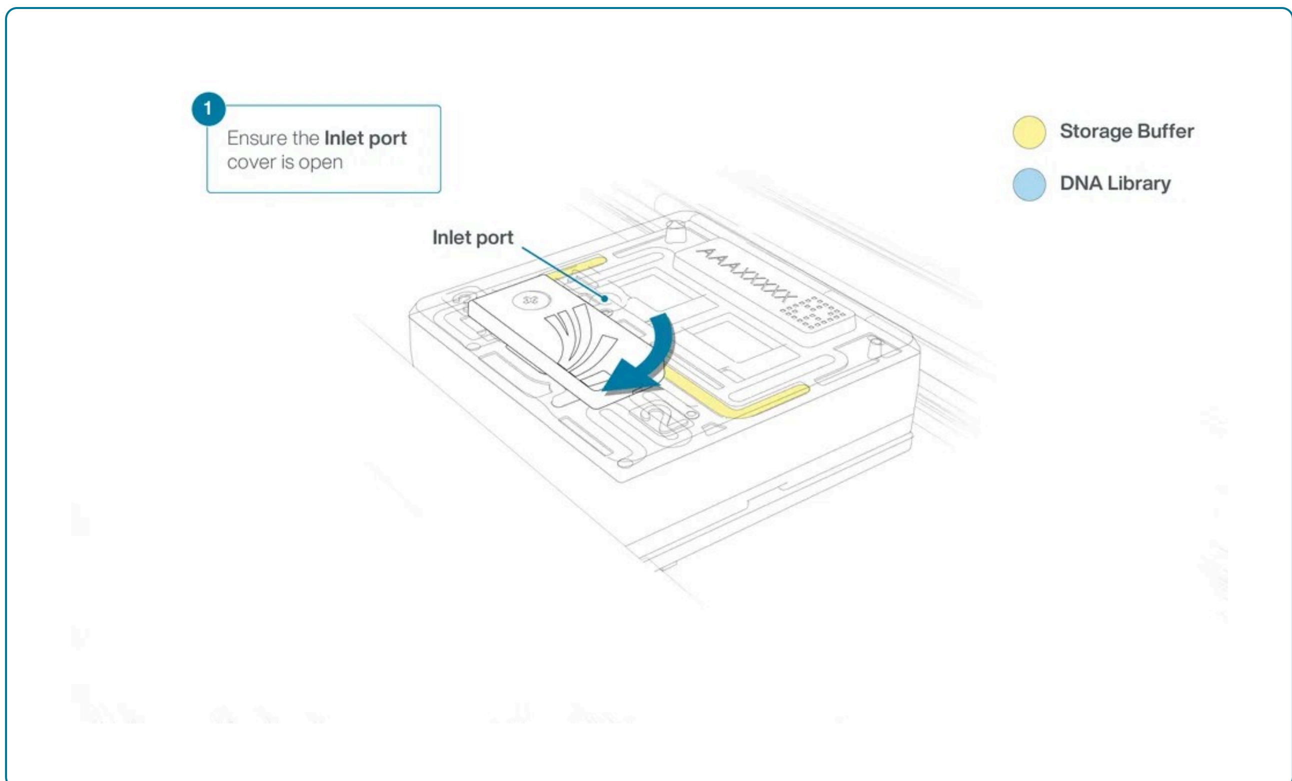


11 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.



12 Ensure the inlet port cover of the flow cell is still open in preparation for loading.

Check that no air bubbles have been introduced to the inlet port during flow cell priming. If air is present, draw back a small volume to remove any air bubbles by using a P1000 pipette set to 200 µl and turning the pipette wheel (as per the instructions above).

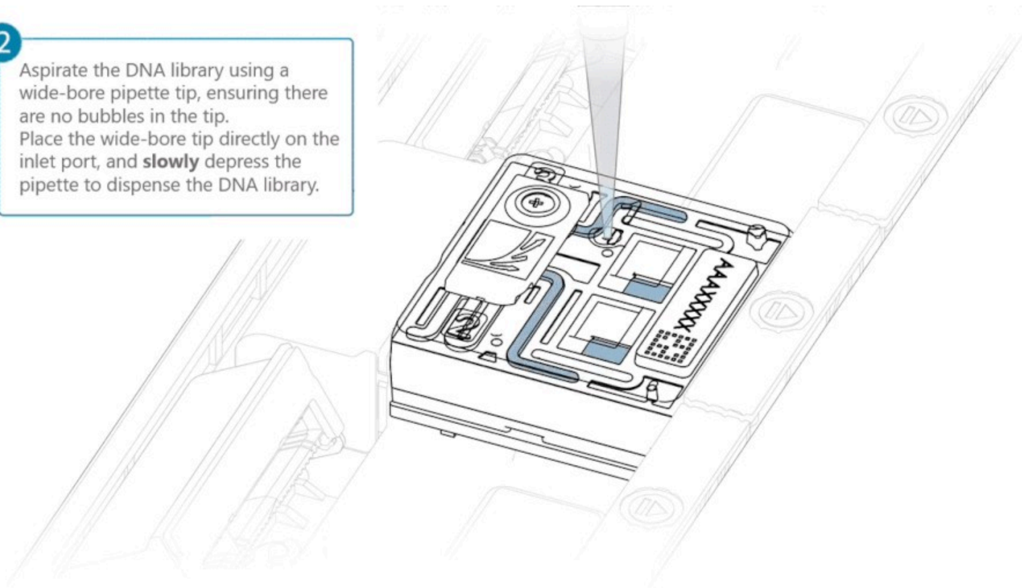


13 Aspirate the DNA library with a wide-bore pipette tip. Ensure there are no air bubbles in the pipette tip. Place the wide-bore pipette tip directly on the inlet port. Slowly depress the pipette to dispense the library into the inlet port.

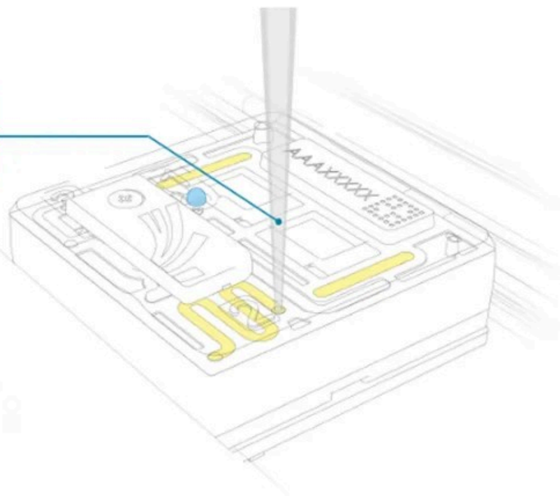
There can be a delay between depressing the pipette and the library dispensing from the pipette tip. Dispense the library **slowly**, allowing the library to dispense from the pipette tip before depressing the pipette further.

It is important to dispense the library **slowly** to prevent air being introduced onto the flow cell.

Note: The DNA library loaded in this step is viscous and may not readily flow through the inlet port into the flow cell. In this case, we recommend applying negative pressure in the flow cell as explained in the steps below.



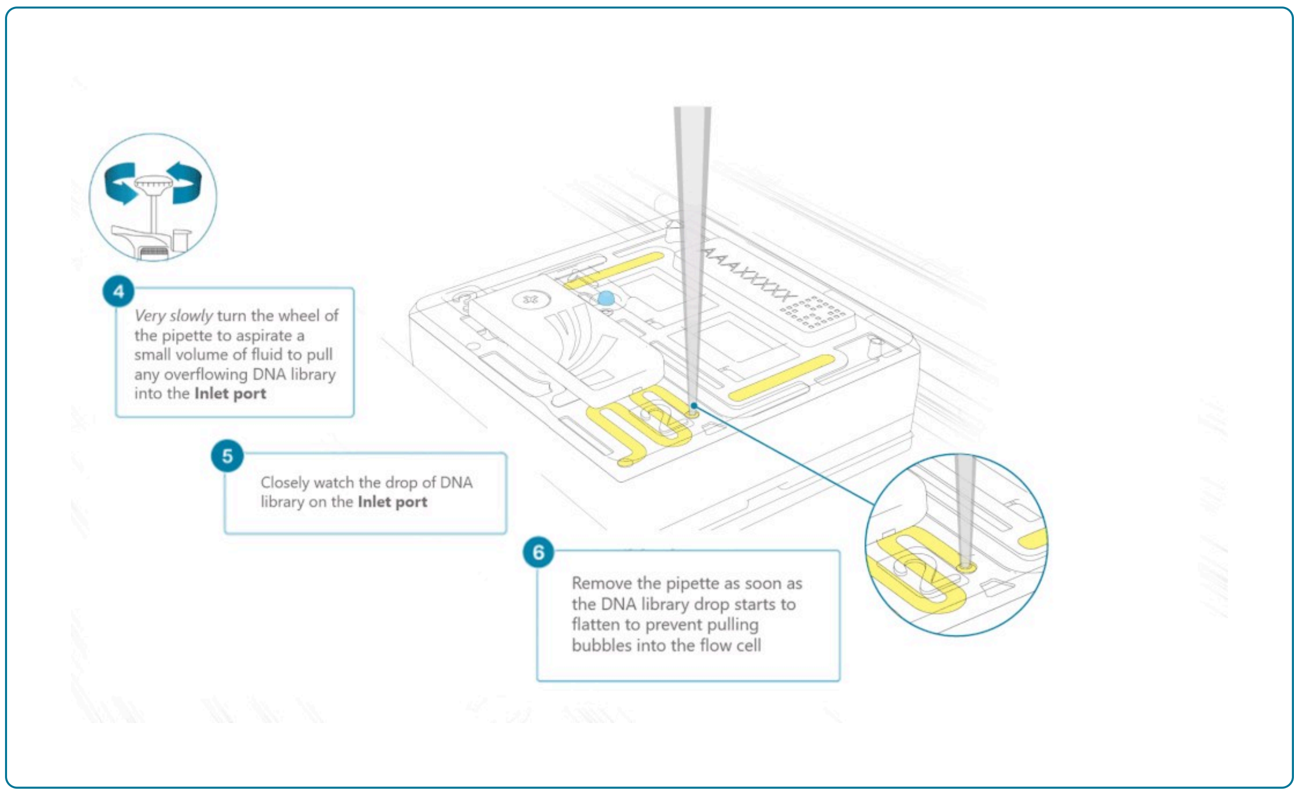
14. **Using a P200 pipette, set the pipette to 50 µl and insert the tip into Port 2.**



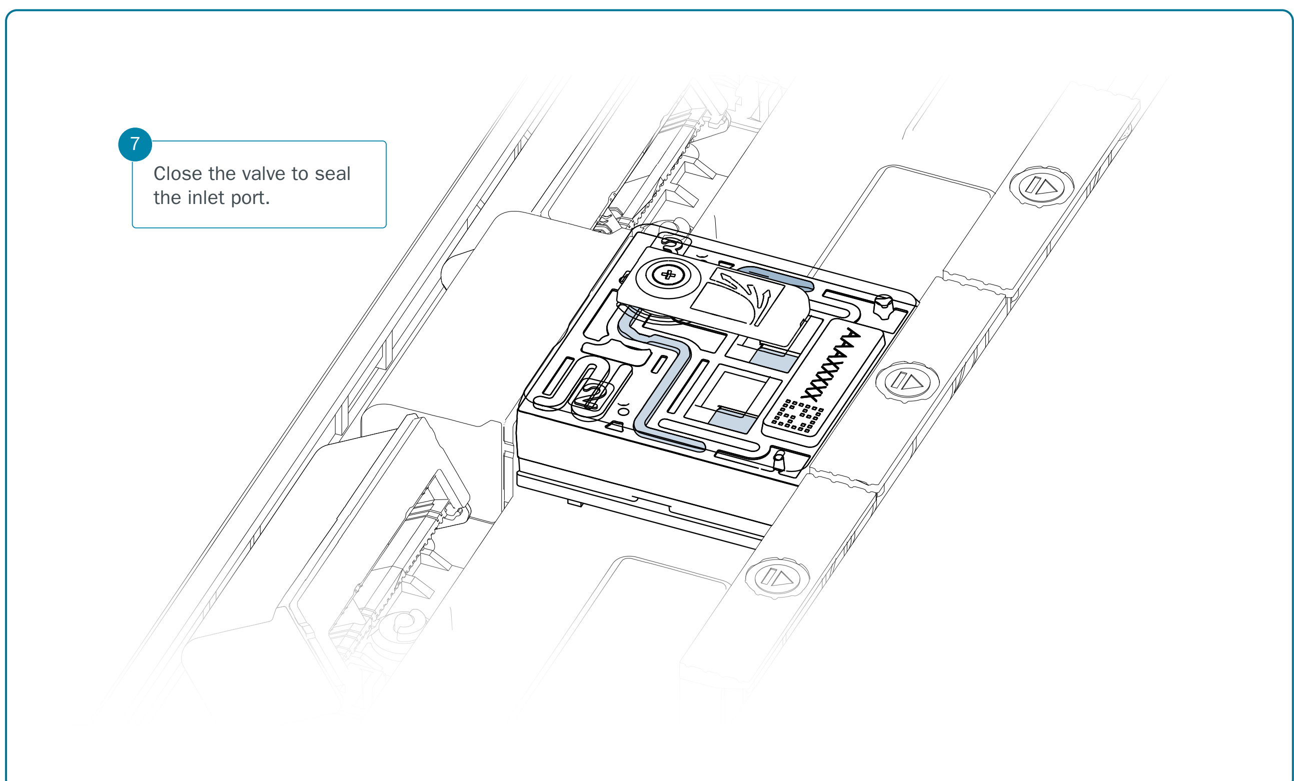
15. **Very slowly turn the wheel of the pipette to pull the DNA library into the inlet port. Closely watch the DNA library on the inlet port and completely remove the pipette as soon as the library starts to be pulled into the port.**

This step is required if the DNA library has not been fully absorbed into the inlet port.

Note: Take care to not apply too much negative pressure too quickly to avoid bringing air bubbles into the flow cell. Air bubbles will cause irreversible damage to the flow cell.



16 Close the valve to seal the inlet port.

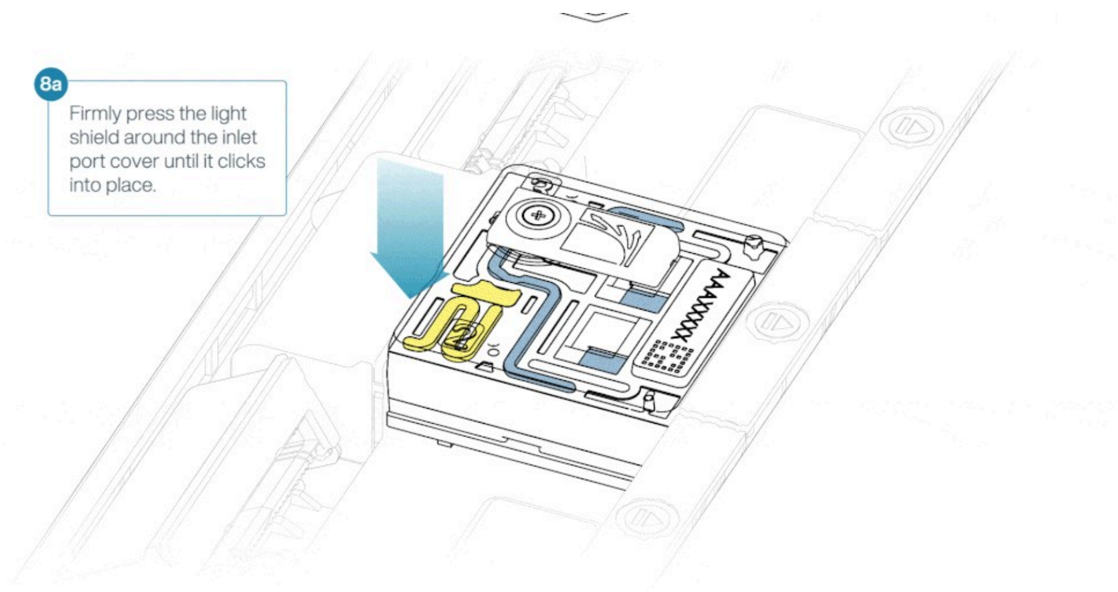


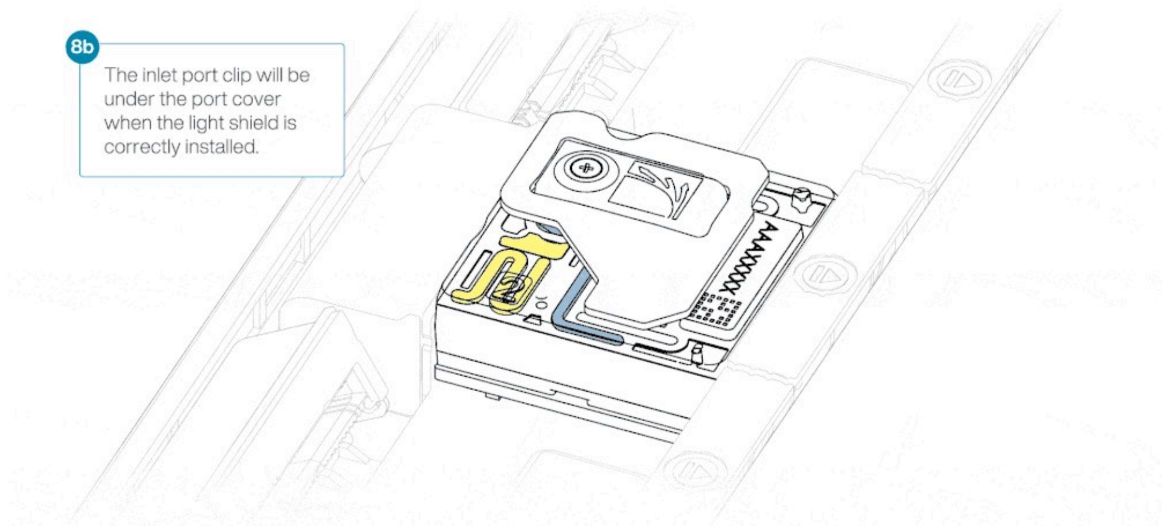
For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

17 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.





Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.

We recommend loading an ultra-long DNA library three times per flow cell to increase output.

A nuclease wash using the Flow Cell Wash Kit (EXP-WSH004) is required between each subsequent library load to recover channels and maximise sequencing output.

For PromethION flow cells, there is enough ultra-long DNA library generated for three consecutive loads per reaction, using 90 µl of fresh library combined with 100 µl of Sequencing Buffer UL (SBU) and 10 µl of Loading Solution UL (LSU) before re-loading for further sequencing.

Please follow Flushing a PromethION Flow Cell in the Flow Cell Wash Kit protocol for the nuclease wash instructions. To run another ultra-long library straight away, follow the instructions below.

10. Reloading ultra-long DNA library on a PromethION Flow Cell

Materials

Flow Cell Wash Kit (EXP-WSH004)
Flush Tether UL (FTU)
Flow Cell Flush (FCF)

Loading Solution UL (LSU)
Sequencing Buffer UL (SBU)

Consumables 1.5 ml Eppendorf DNA LoBind tubes

Equipment
P1000 pipette and wide-bore pipette tips
P200 pipette and wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
P20 pipette and tips

Before reloading your library or loading a new library, please ensure you wash the flow cell using the Flow Cell Wash Kit (EXP-WSH004).

Follow the instructions in the Flow Cell Wash Kit (EXP-WSH004) for PromethION protocol.

- This washing procedure aims to remove most of the initial library and prepare the flow cell for loading of a subsequent library.
- Data acquisition in MinKNOW should be paused during the wash procedure and library loading.
- After the flow cell has been washed, another library can be loaded.

We recommend keeping the light shield on the flow cell during washing if a second library will be loaded straight away.

If the flow cell is to be washed and stored, the light shield can be removed.

To run a second library of ultra-long DNA straight after flushing a flow cell, we recommend removing all fluid from the waste channel after each priming step.

1. **Thaw the Sequencing Buffer UL (SBU), Loading Solution UL (LSU), Flush Tether UL (FTU) and one tube of Flow Cell Flush (FCF) at room temperature and mix by vortexing. Then spin down and place on ice.**

2. **In a fresh 1.5 ml Eppendorf DNA LoBind tube, prepare the DNA library for loading as follows using a wide-bore pipette tip for the addition of the DNA library:**

Reagent	Volume per flow cell
Sequencing Buffer UL (SBU)	100 µl
Loading Solution UL (LSU)	10 µl
DNA library	90 µl
Total	**200 µl**

Note: ensure the Sequencing Buffer UL (SBU) and Loading Solution UL (LSU) are thoroughly mixed by pipetting before the addition of the DNA library.

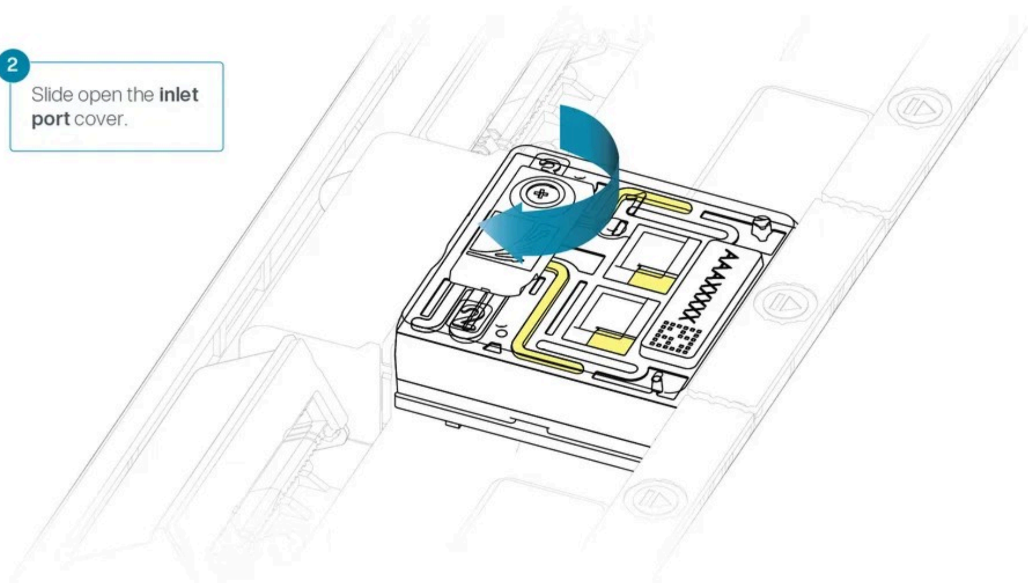
3. **Gently mix the prepared DNA library by slowly pipetting ten times using a wide-bore pipette tip.**

4. **Incubate at room temperature for 30 minutes then gently mix by slowly pipetting with a wide-bore tip. Visually inspect to ensure the sample is homogenous.**

5. **Prepare the flow cell priming mix in a 1.5 ml Eppendorf DNA LoBind tube and mix by vortexing at room temperature.**

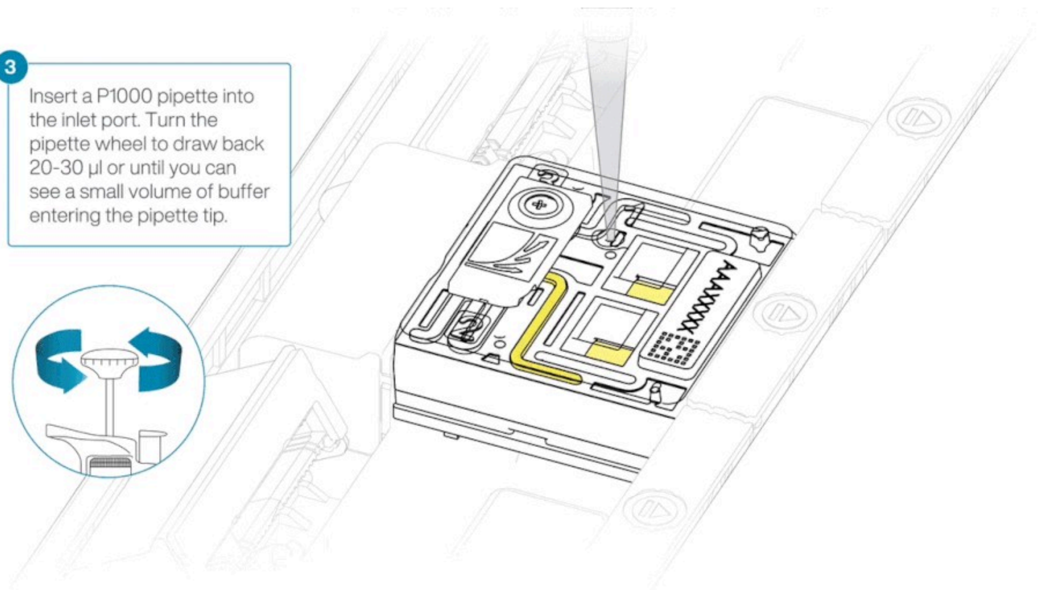
Reagent	Volume
Flush Tether UL (FTU)	30 µl
Flow Cell Flush (FCF)	1,170 µl
Total	**1,200 µl**

6. **Turn the valve to expose the inlet port.**

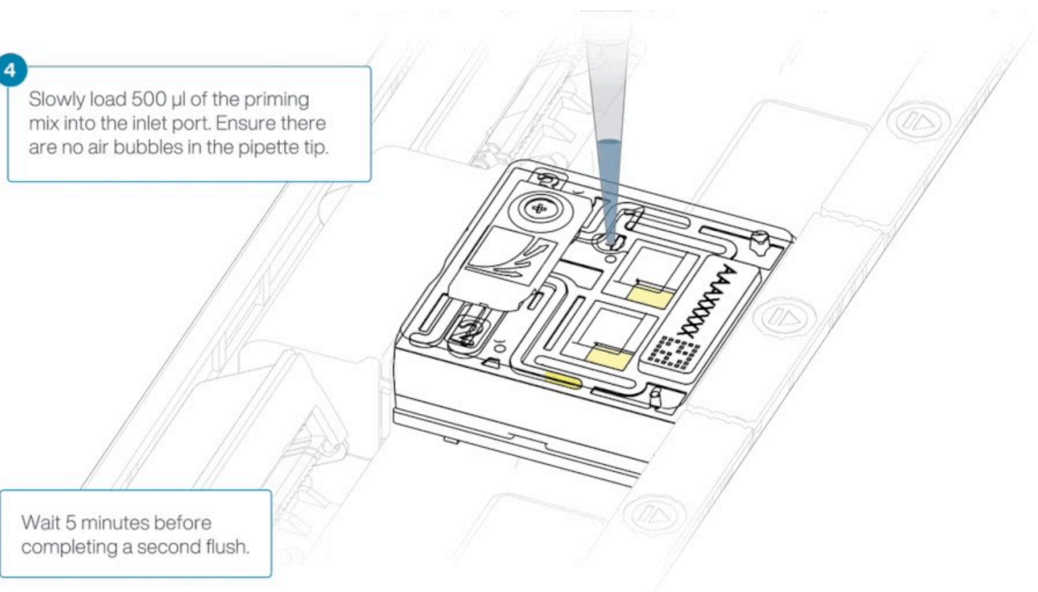


7 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



8 Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes.



It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.

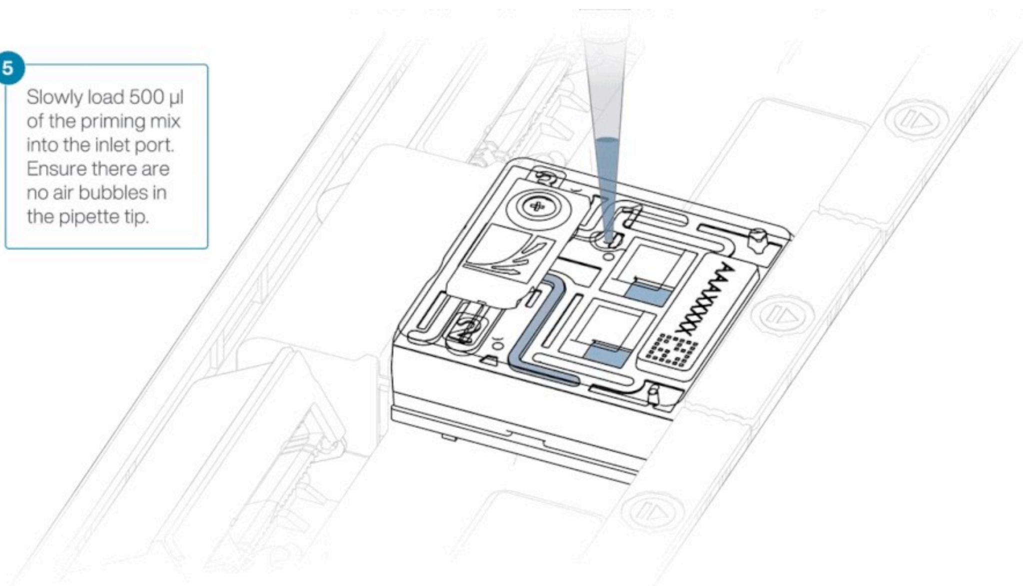
It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

9 Remove waste buffer, as follows:

1. Close the inlet port.
2. Insert a P1000 pipette into a waste port and remove the waste buffer.

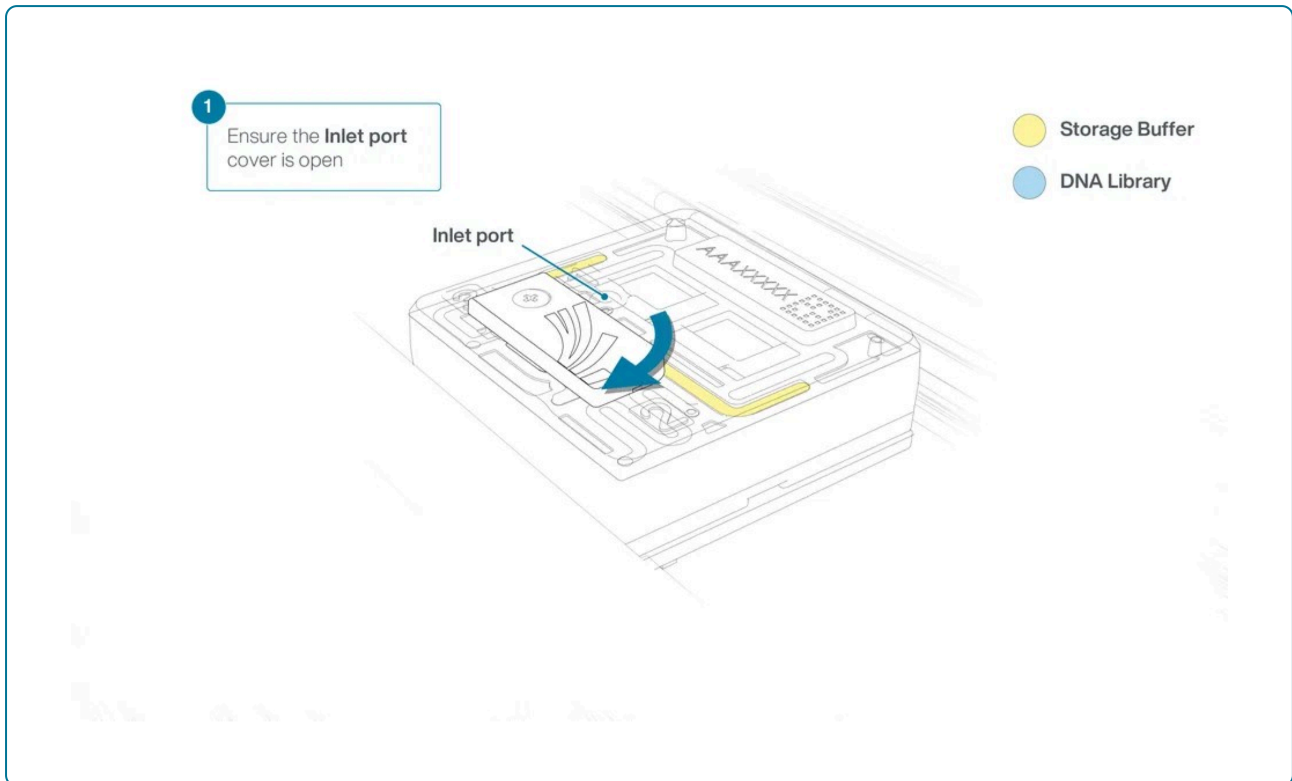
Note: As both the inlet port is closed, no fluid should leave the sensor array area.

10 Slide open the inlet port and load 500 µl of the priming mix into the flow cell via the inlet port to complete a second flow cell flush, avoiding the introduction of air bubbles.



11 Close the inlet port and use a P1000 to remove all fluid from the waste channel through a waste port again.

12 Open the inlet port cover of the flow cell in preparation for loading.

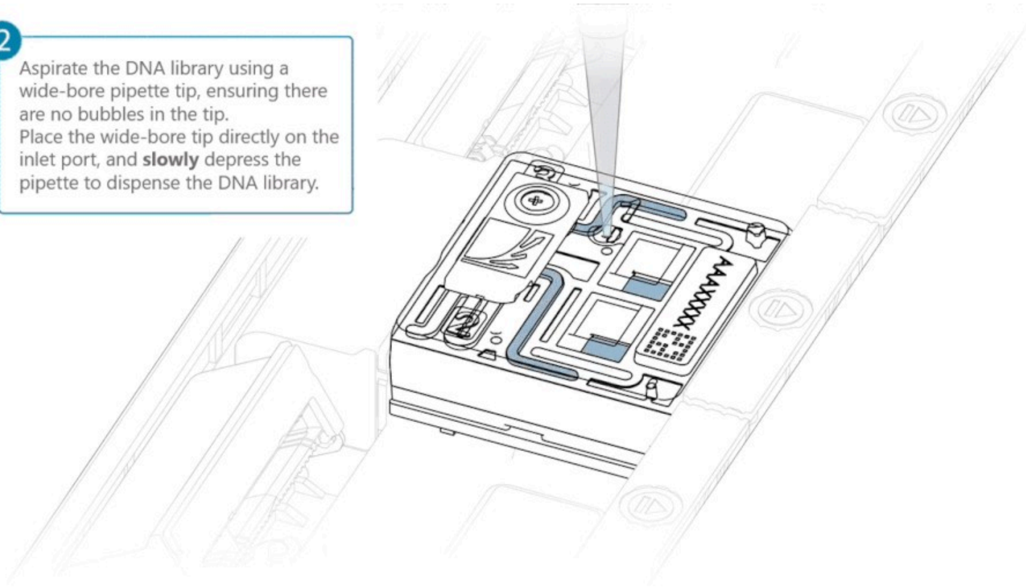


13 Aspirate the DNA library with a wide-bore pipette tip. Ensure there are no air bubbles in the pipette tip. Place the wide-bore pipette tip directly on the inlet port. Slowly depress the pipette to dispense the library into the inlet port.

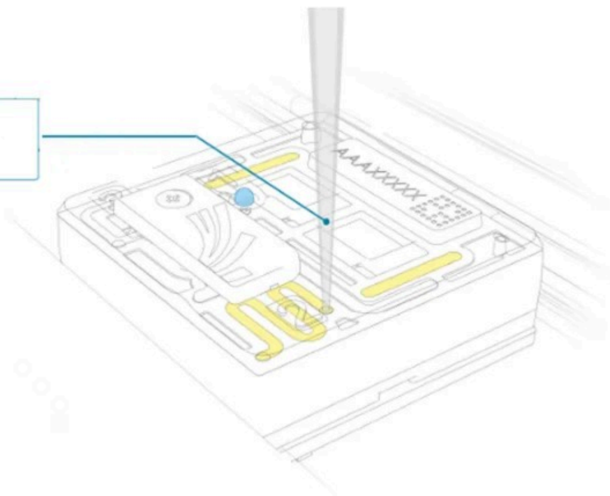
There can be a delay between depressing the pipette and the library dispensing from the pipette tip. Dispense the library **slowly**, allowing the library to dispense from the pipette tip before depressing the pipette further.

It is important to dispense the library **slowly** to prevent air being introduced onto the flow cell.

Note: The DNA library loaded in this step is viscous and may not readily flow through the inlet port into the flow cell. In this case, we recommend applying negative pressure in the flow cell as explained in the steps below.



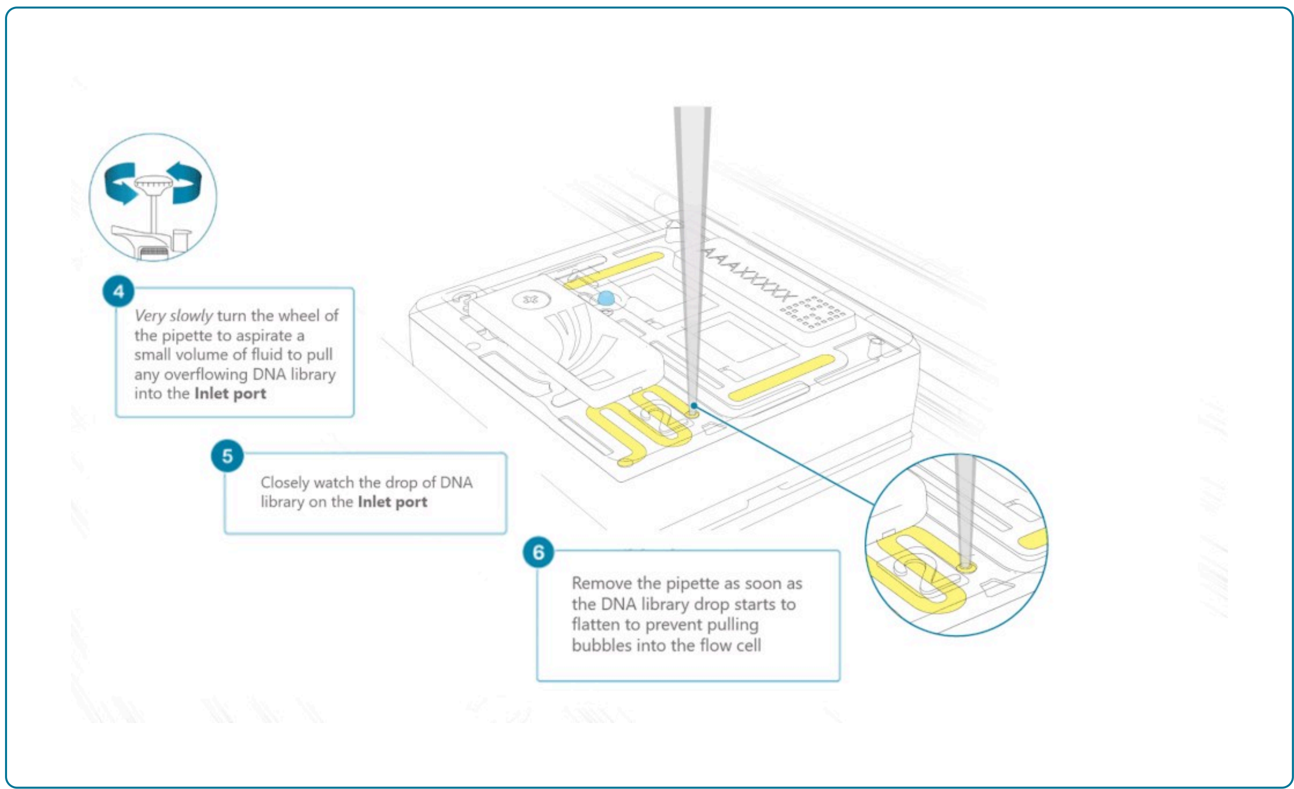
14 Using a P200 pipette, set the pipette to 50 µl and insert the tip into Port 2.



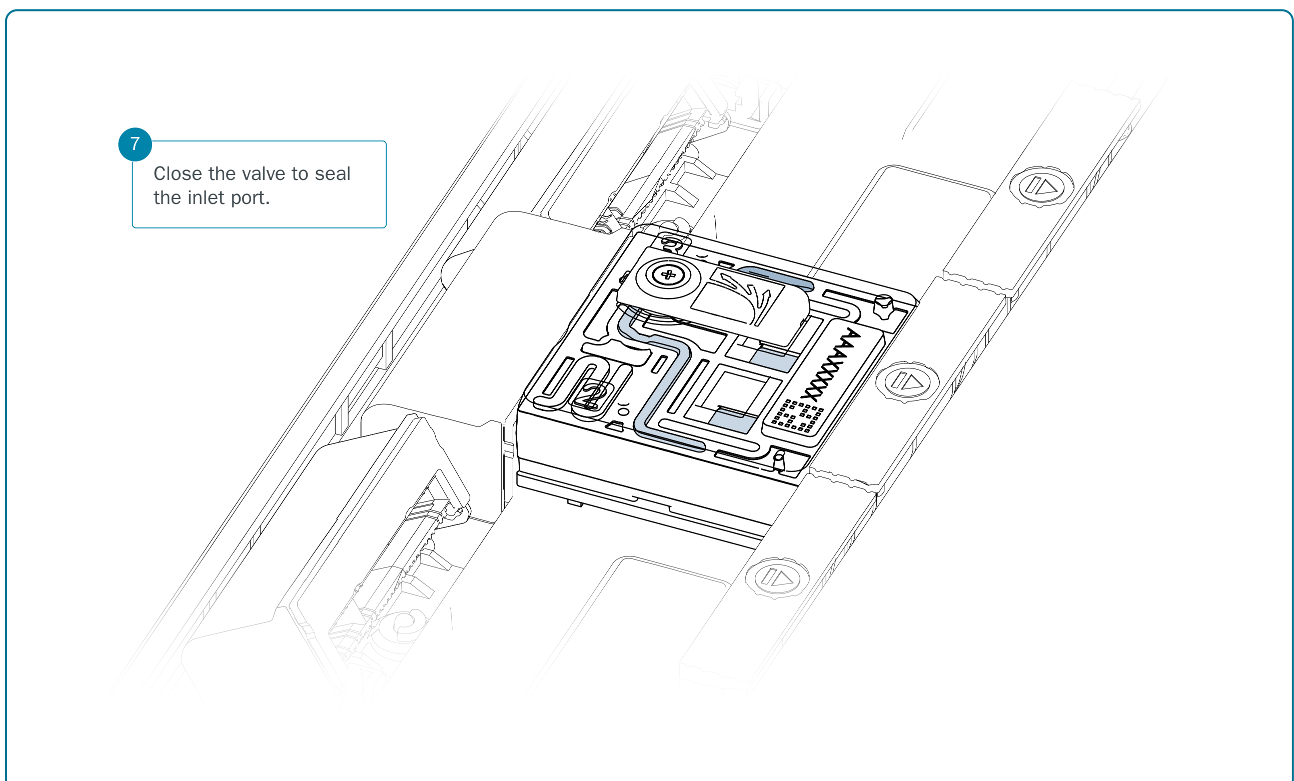
15 Very slowly turn the wheel of the pipette to pull the DNA library into the inlet port. Closely watch the DNA library on the inlet port and completely remove the pipette as soon as the library starts to be pulled into the port.

This step is required if the DNA library has not been fully absorbed into the inlet port.

Note: Take care to not apply too much negative pressure too quickly to avoid bringing air bubbles into the flow cell. Air bubbles will cause irreversible damage to the flow cell.



16 Close the valve to seal the inlet port.



For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

17 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
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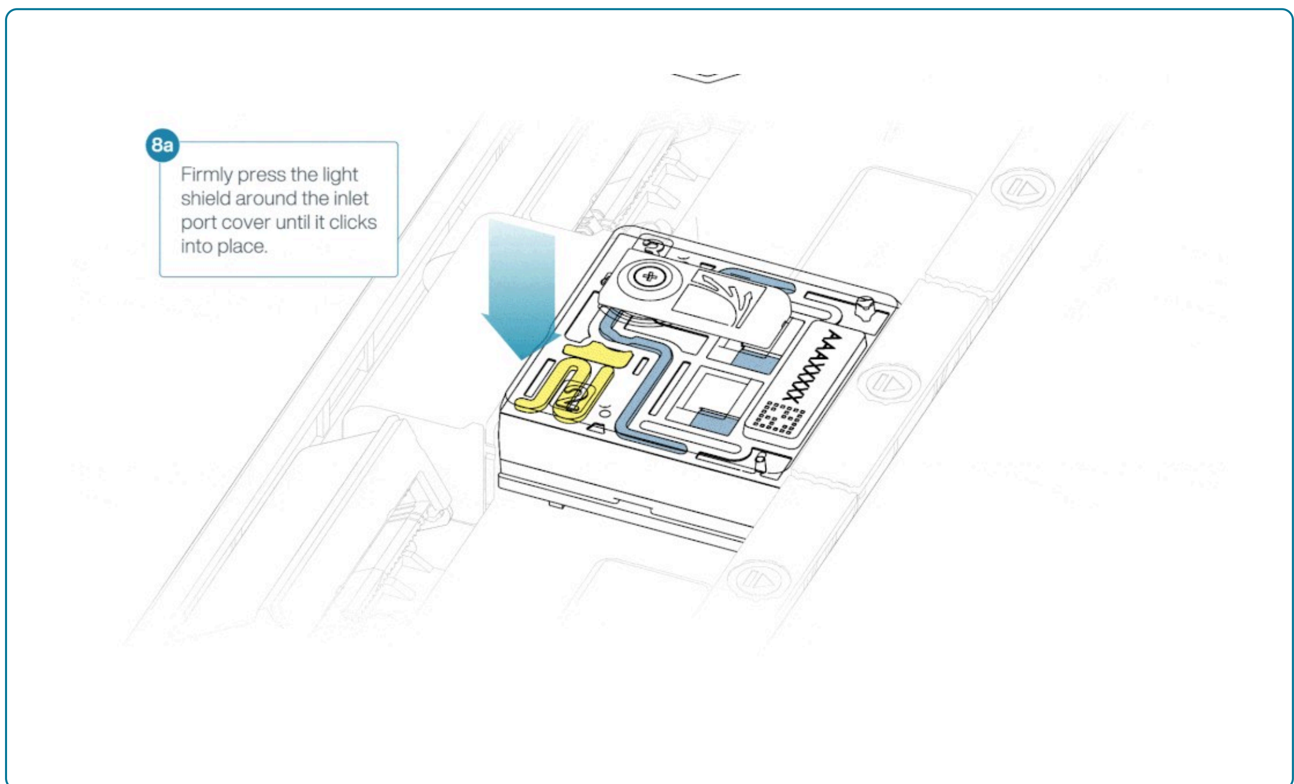
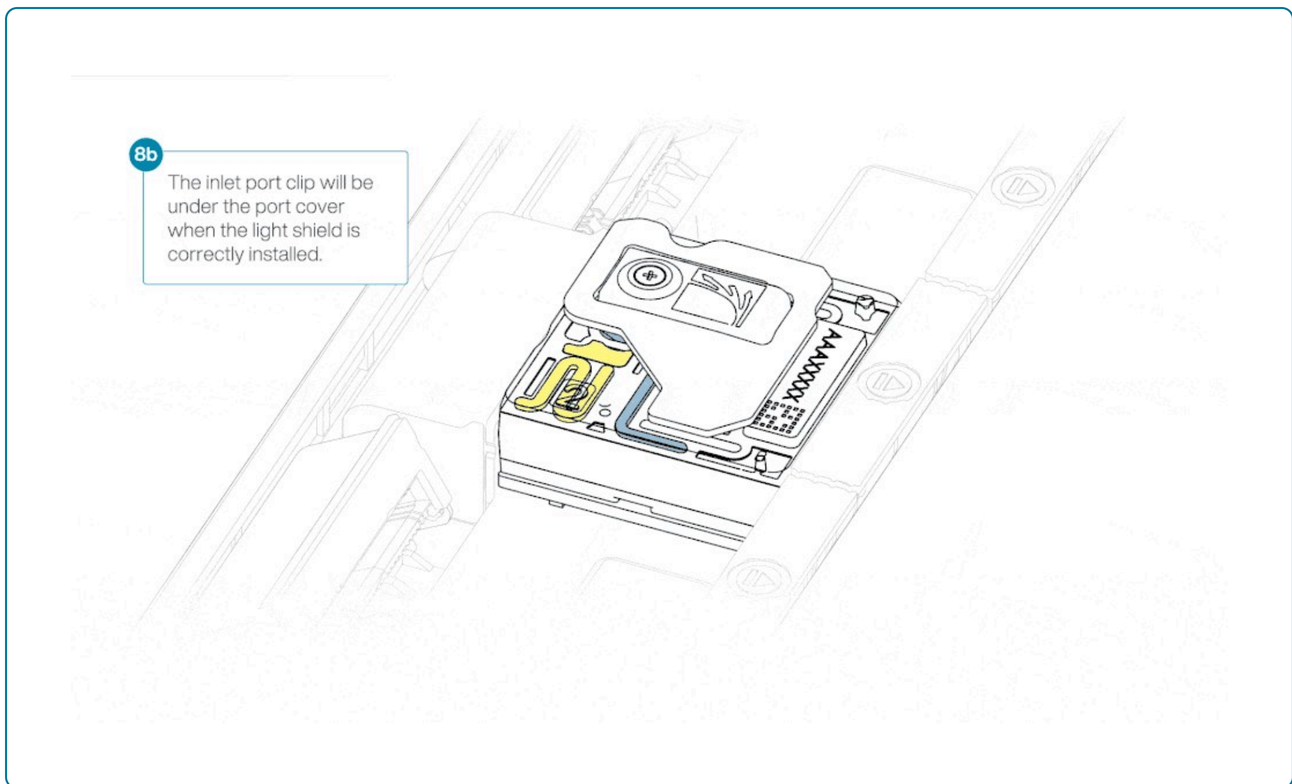




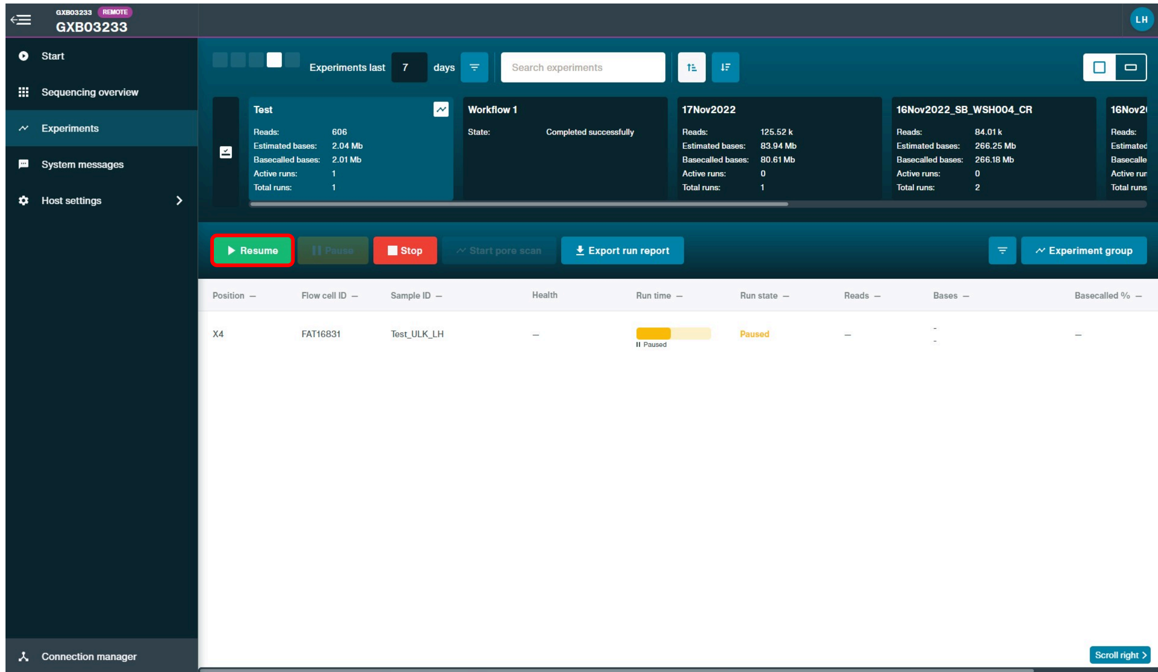
18 Once the flow cell is reloaded, resume the sequencing run on MinKNOW and trigger a pore scan.

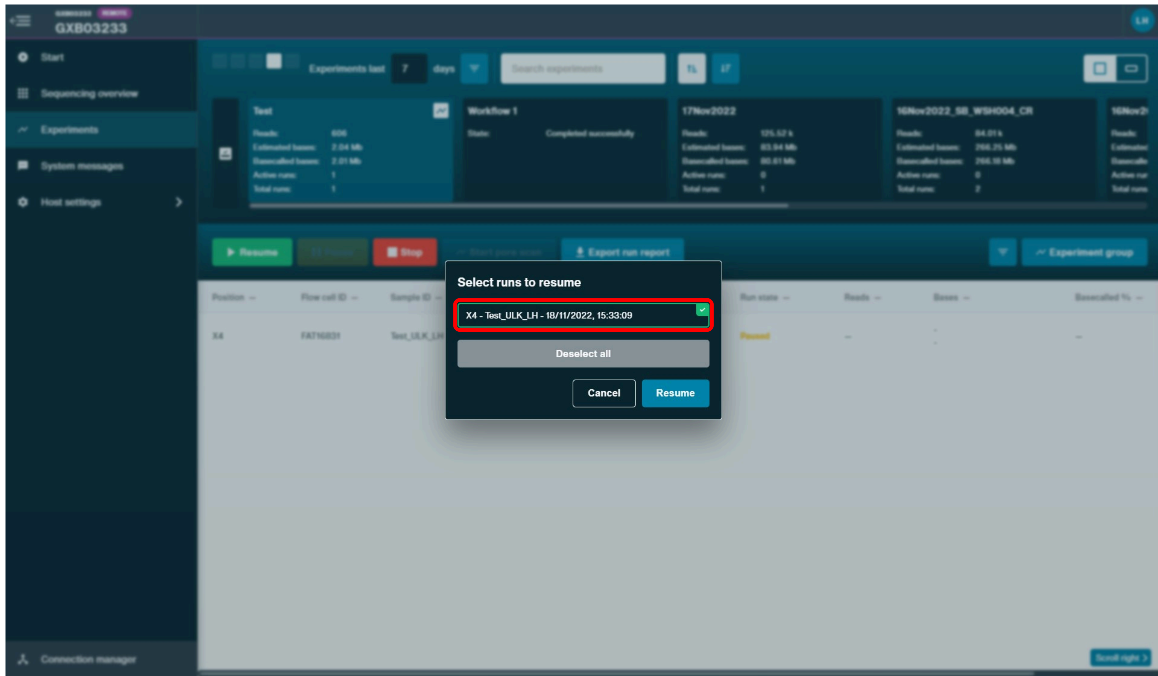
To resume sequencing run, navigate to the Experiments page, click 'Resume' and select flow cell position.

To manually trigger a channel scan, click 'Start pore scan' and select flow cell position.

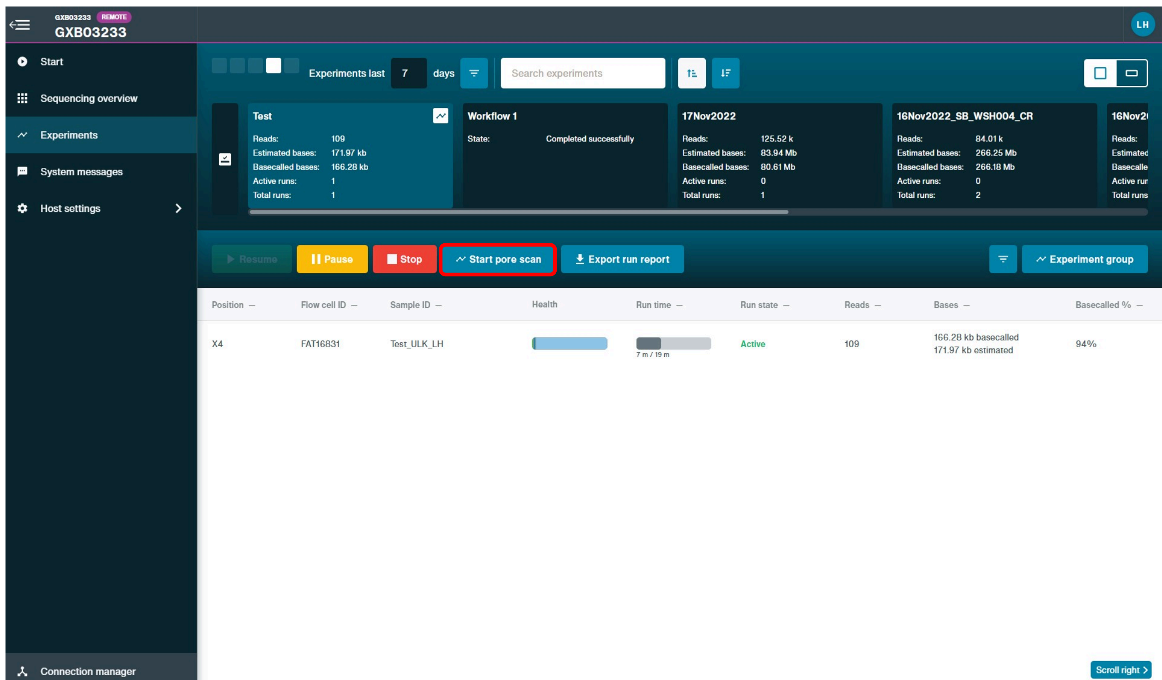
For further information, please see the MinKNOW protocol.

Resume run:

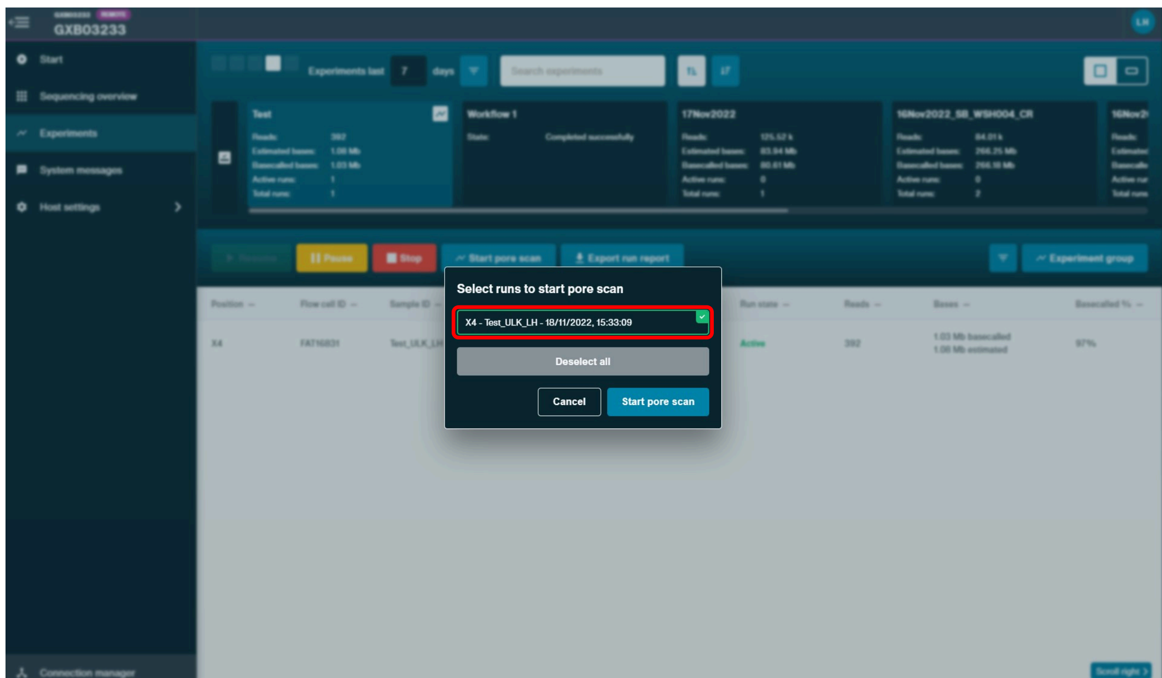




Pore scan:



pore scan



pore scan

11. Data acquisition and basecalling

How to start sequencing

Once you have loaded your flow cell, the sequencing run can be started on MinKNOW, our sequencing software that controls the device, data acquisition and real-time basecalling. For more detailed information on setting up and using MinKNOW, please see the MinKNOW protocol.

MinKNOW can be used and set up to sequence in multiple ways:

- On a computer either direcly or remotely connected to a sequencing device.
- Directly on a GridION or PromethION 24/48 sequencing device.

For more information on using MinKNOW on a sequencing device, please see the device user manuals:

- PromethION 24/48 user manual
- PromethION 2 Solo user manual

To start a sequencing run on MinKNOW:

1. Navigate to the start page and click **Start sequencing.**

2. Fill in your experiment details, such as name and flow cell position and sample ID.

3. Select the **Ultra-Long DNA Sequencing Kit (SQK-ULK114)** on the Kit page.

4. Configure the sequencing and output parameters for your sequencing run or keep to the default settings on the Run configuration tab.

Note: If basecalling was turned off when a sequencing run was set up, basecalling can be performed post-run on MinKNOW. For more information, please see the MinKNOW protocol.

5. Click **Start** to initiate the sequencing run.

Data analysis after sequencing

After sequencing has completed on MinKNOW, the flow cell can be reused or returned, as outlined in the Flow cell reuse and returns section.

After sequencing and basecalling, the data can be analysed. For further information about options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

In the Downstream analysis section, we outline further options for analysing your data.

12. Flow cell reuse and returns

Materials Flow Cell Wash Kit (EXP-WSH004)

1. **After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Flow Cell Wash Kit protocol and store the washed flow cell at +2°C to +8°C.**

The Flow Cell Wash Kit protocol is available on the Nanopore Community.

We recommend you to wash the flow cell as soon as possible after you stop the run. However, if this is not possible, leave the flow cell on the device and wash it the next day.

2. **Alternatively, follow the returns procedure to send the flow cell back to Oxford Nanopore.**

Instructions for returning flow cells can be found here.

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

13. Downstream analysis

Post-basecalling analysis

There are several options for further analysing your basecalled data:

EPI2ME workflows

For in-depth data analysis, Oxford Nanopore Technologies offers a range of bioinformatics tutorials and workflows available in EPI2ME. The platform provides a vehicle where workflows deposited in GitHub by our Research and Applications teams can be showcased with descriptive texts, functional bioinformatics code and example data.

Research analysis tools

Oxford Nanopore Technologies' Research division has created a number of analysis tools, which are available in the Oxford Nanopore GitHub repository. The tools are aimed at advanced users,

and contain instructions for how to install and run the software. They are provided as-is, with minimal support.

Community-developed analysis tools

If a data analysis method for your research question is not provided in any of the resources above, please refer to the resource centre and search for bioinformatics tools for your application. Numerous members of the Nanopore Community have developed their own tools and pipelines for analysing nanopore sequencing data, most of which are available on GitHub. Please be aware that these tools are not supported by Oxford Nanopore Technologies, and are not guaranteed to be compatible with the latest chemistry/software configuration.

14. Issues during library preparation

Below is a list of the most commonly encountered issues, with some suggested causes and solutions.

We also have an FAQ section available on the Nanopore Community Support section.

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Troubleshooting

Observation	Comments and actions
Low throughput	1. Vortex gently after adding the diluted Fragmentation Mix (FRA) to break up the largest fragments. 2. Ensure the diluted Fragmentation Mix (FRA) is thoroughly mixed with the gDNA. 3. Use less input material if the DNA library was too viscous to load onto the flow cell.
DNA is too viscous and will not load onto a flow cell	1. Lower the input material to reduce the amount of gDNA going into the library preparation and reduce viscosity. 2. If DNA library will not load using the method outlined in this protocol, slowly pipette mix 5 times with a standard P200 pipette set to the full volume of the library and reload the flow cell.
Read lengths are not long enough	1. Increase input material. **Note:** Library viscosity increases with more gDNA. 2. Reduce volume of Fragmentation Mix (FRA) added to FRA Dilution Buffer (FDB) to avoid over-fragmentation of gDNA. **Note:** We do not recommend diluting less than 2 µl Fragmentation Mix (FRA). 3. We recommend using PFGE to check the extracted gDNA is of ultra-high molecular weight (uHMW), thus capable of generating ultra-long read lengths.
No sequencing output	1. Check gDNA has been recovered in library preparation using a NanoDrop spectrophotometer. 2. Check viscosity of the sample. The library should be viscous if it contains uHMW gDNA in this protocol.
Aspirating supernatant when the DNA has precipitated	Take care to not aspirate the DNA. Remove smaller volumes of supernatant incrementally to reduce the risk of aspirating the DNA.
Mixing	Mix slowly and carefully to prevent DNA shearing. Low vortexing can be used to mix at the expense of ultra-long reads. With vortexing, long read lengths of ~90 kb N50 can still be generated with improved outputs.
No DNA recovered from the library	If the DNA is no longer viscous or the NanoDrop reading is low, DNA may have been lost during the clean-up step of the library

Observation	Comments and actions
preparation clean-up	preparation. 1. Ensure uHMW DNA is used or users risk DNA loss. 2. Take care to not aspirate the precipitated DNA during the clean-up step. To avoid this, remove smaller volumes of supernatant incrementally. Ensure as much supernatant is removed as possible.

REQUIREMENTS

PromethION 24/48 A-Series – device and IT specifications

FOR RESEARCH USE ONLY

Contents

Device models

Device and IT specifications

1. Overview
2. Technical specifications
3. Site planning (pre-delivery)
4. Package contents
5. Physical installation
6. Data formats and analysis
7. Data handling and storage
8. Safety and compliance

Frequently asked questions

Appendix A: Shipment and logistics

Appendix B: Compatibility

Change log

Device models

PromethION A-Series Data Acquisition Unit (PRO-PRCAMP; PRO-PRCA100)
PromethION 24 Sequencing Unit (PRO-SEQ024)
PromethION 48 Sequencing Unit (PRO-SEQ048)
PromethION 24 Combined (PRO-CMB024)*

*available in selected countries only

Device and IT specifications

Overview

The PromethION™ 24/48 A-Series is a high-throughput nanopore sequencing platform that enables parallel sequencing on up to 24 or 48 flow cells. It is ideal for labs with multiple projects that need the advantages of nanopore sequencing:

- Simple library preparation
- Real-time analysis
- Biological insights from long reads

The PromethION features onboard compute, enabling device control, data acquisition, basecalling, and data streaming without placing an additional burden on existing infrastructure.

Further information is available for the PromethION 24 device.

Technical specifications

Component	Specification
Operating system	Ubuntu Focal (20.04) / Ubuntu Noble (24.04)
Storage	60 TB internal SSD (56 TB available)
GPU	Data Acquisition Unit: 4 × NVIDIA Ampere-series GPUs + 1 × Turing-series GPU (display output only)
Memory	512 GB RAM
Size and weight	Sequencing Unit: H 190 × W 590 × D 430 mm; PromethION 24: 23 Kg PromethION 48: 31 Kg Data Acquisition Unit: H 440 × W 178 × D 470 mm; 26 kg
Environmental range	Designed to sequence at: +18°C to +22°C*
Heat output	Sequencing Unit: 4,129 BTU/h maximum Data Acquisition Unit: 9,008 BTU/h maximum
Power	Sequencing Unit: 100–240 VAC (50/60 Hz); Maximum power consumption: 1,210 W Plug type: 1 × C13 input Data Acquisition Unit: 200–240 VAC (50/60 Hz); Maximum power consumption: 2640 W Plug type: 2 × C13 inputs

*Functional range of electronics: +5°C to +40°C

Site planning (pre-delivery)

Installation checklist

This checklist outlines the minimum IT and infrastructure requirements for installing the PromethION™ 24/48 A-Series at your institution. For a complete explanation of each item, refer to the sections that follow.

Notes:

- The system supports either fibre or copper connectivity.
- The PromethION 24 Combined model has the same IT requirements as the PromethION 24 A-Series, and is only available in selected countries.

Item/setup required	Purpose
2 x SFP+ modules supporting 10 GBASE-SR or 10 GBASE-LR (it is not necessary if using copper)	Connection to the IT infrastructure and the internet
Appropriate 10 Gbps cabling for chosen SFP+ modules, **or** 10 Gbps Ethernet cabling	Connection to the IT infrastructure and the internet
2 x 10 Gbps fibre ports with DHCP enabled, **or** 2 x 10 Gbps Ethernet ports with DHCP or static IP	Connection to the IT infrastructure and the internet
Wired USB mouse and keyboard	Device control
DisplayPort (recommended) or VGA-compatible monitor Recommended screen resolution: 1920x1080	Device control
2 X 200–240 VAC (50/60 Hz) outlets 1 X 100–240 VAC (50/60 Hz) outlet See below for full details	Device power
Uninterruptible power supply (optional: see section 7.1 for details)	Power stability
Sufficient local infrastructure to support storage requirements	Real-time and long-term data storage

Power and UPS requirements

Power requirements

The PromethION 24/48 consists of two components with separate power requirements: the Sequencing Unit (maximum power consumption 1,210 W) and the Data Acquisition Unit (2,640 W).

Before installation, ensure your local infrastructure can support the combined power and voltage requirements, and that suitable power sockets are available at the intended location. We recommend consulting your facilities team and/or a qualified electrician to determine the most appropriate configuration for your environment.

Power reliability

Power reliability is essential for protecting sequencing runs and ensuring stable operation. We strongly recommend using Uninterruptible Power Supplies (UPS) to protect both the Sequencing Unit and Data Acquisition Unit.

Why use a UPS?

A UPS provides backup power and voltage regulation. In the event of a power cut or fluctuation, it can:

- Keep the system running for several minutes
- Trigger alerts so you can pause or safely shut down sequencing runs
- Help prevent issues by stabilising or improving poor-quality mains power

Recommended UPS specifications

You can choose to use either:

- One larger UPS to power both the Sequencing Unit and the Data Acquisition Unit
- Two separate UPS units, one for each device

The best option will depend on your site setup, including space, power access, and budget. Either approach is acceptable as long as the UPS units meet the total power and redundancy requirements. We recommend consulting your facilities team and/or a qualified electrician to determine the most suitable configuration for your environment.

Parameter	Minimum requirement
Power rating	Sequencing unit: 1.5 kVA Data Acquisition Unit: 3 kVA
UPS type	Online/double conversion
Transition time	<5 min
Battery runtime	≥5 min (to allow safe shutdown or generator switch-over)
Output type	Pure sine-wave
Output voltage	200–240 VAC @ 50/60 Hz
Power cables	Ensure cables are rated to a minimum of 10 A 250 VAC, and are compatible with the C13 inputs of the PromethION 24/48

You will need to provide your own UPS. Oxford Nanopore Technologies™ does not validate or supply specific models. Selection, installation and maintenance are your responsibility.

Installation readiness

- Install the UPS ahead of system delivery to avoid delays.
- Schedule regular UPS maintenance to ensure continued protection.
- Train relevant staff on safe shutdown procedures to minimize risk during power interruptions.

Network and connectivity requirements

Network access requirements

The system uses outbound-only connections over TCP ports 80 and 443 and does not require any inbound access.

Oxford Nanopore Technologies does not have remote access to your system.

Required domains

The table below outlines the domains that must be accessible for specific system functions:

Access type	Purpose	Required domains
Telemetry	Enables MinKNOW to run and communicate telemetry	`ping.oxfordnanoportal.com`

Software and OS updates	Access to MinKNOW updates, OS packages, and GPU drivers	`cdn.oxfordnanoportal.com`
		`*.ubuntu.com`
		`*.nvidia.com`
		`*.canonical.com`

EPI2ME™	Access for container-based analysis workflows	`*.github.com` `hub.docker.com`
Nanopore account login	To log in to your Nanopore account to access cloud services	`id.nanoporetech.com` `*.okta.com`

If your institution uses a proxy or firewall, configure it to allow outbound access to these domains to ensure software functionality, updates, and user authentication.

Telemetry

MinKNOW and EPI2ME collect telemetry data during use as outlined in our Terms and Conditions. This helps monitor device performance, supports troubleshooting, and enables flow cell warranty replacement where applicable.

Privacy note: Some telemetry fields allow free-text entry. Avoid entering personally identifiable information in these fields. We do not collect sequence data.

Package contents

PromethION 24/48 Sequencing Unit

Quantity (P24/P48)	Item	Function
1	PromethION 24/48 Sequencing Unit	DNA/RNA sequencing instrument
2/3	USB-A to USB Mini-B cable	Used for Sequencing Unit firmware updates
1	USB-B to USB-A cable	Used for Sequencing Unit firmware updates
24/48	Configuration test cell	Used to verify sequencing hardware functionality (1 per flow cell position)
1	Quick start guide	Overview of system setup
1	Safety and regulatory document	Safety information and regulatory compliance details
1	UK (Type G → C13) 2 m 10 A 250 VAC mains power cord	Region-specific power supply cables for the sequencing unit
1	EU (Schuko Type C → C13) 2 m 10 A 250 VAC mains power cord	
1	USA (Type B → C13) 2 m 15 A 125 VAC mains power cord	
1	China (Type I → C13) 2 m 10 A 250 VAC mains power cord	
1	Australia (Type I → C13) 2 m 10 A 250 VAC mains power cord	

Data Acquisition Unit

Quantity (P24/P48)	Item	Function
1	Data Acquisition Unit	Provides device control, data acquisition, basecalling, and data streaming
2 / 3	2 m PCIe cable	Establishes a high-speed data connection from the Data Acquisition Unit to the Sequencing Unit
1	2 m Mini DisplayPort to DisplayPort cable	Connects the Data Acquisition Unit to a monitor
1	USB-A to USB-C adapter	Connects peripherals to the Data Acquisition Unit USB-C ports
1	64 GB factory reset USB drive	Enables system recovery and factory settings
2	UK (Type G → C13), 2 m 10 A 250 VAC mains power cord	Region-specific power supply cables for the data acquisition unit
2	EU (Schuko Type C → C13) 2 m 10 A 250 VAC mains power cord	
2	USA (NEMA L6-20P → C13), 2 m 15 A 250 VAC mains power cord	
2	China (Type I → C13) 2 m 10 A 250 VAC mains power cord	
2	Australia (Type I → C13) 2 m 10 A 250 VAC mains power cord	

Physical installation

- The bench space required for a PromethION installation is 167 × 75 cm.
- Install the Sequencing Unit and the Data Acquisition Unit on a stable laboratory bench.
- Allow at least 30 cm clearance at the front, rear and sides of each unit to ensure adequate ventilation and access.
- Place the devices in a controlled environment: away from direct sunlight and heat sources to avoid issues with temperature regulation, and in a dust-free

area, away from open windows to minimise contamination and airflow issues.
- The Data Acquisition Unit can be placed underneath the laboratory bench if required. Please consult Oxford Nanopore if you wish to install the PromethION Combined in this way.

Data formats and analysis

File types

The system stores nanopore sequencing data in the following file types:

- FASTQ - A text-based format that stores DNA/RNA sequences and quality scores
- BAM - A format for aligned reads, including modified base calls (e.g., methylation)
- `sequencing_summary.txt` - Contains metadata for all basecalled reads from a sequencing run. This includes details such as read ID, sequence length, per-read Q-score, and read duration. The size of a sequence summary file will depend on the number of reads sequenced

Optional:

- POD5 - The primary raw data format, replacing the legacy .fast5. It is more efficient in both storage and processing

The table below provides estimated storage requirements based on different sequencing throughputs from a single flow cell. These values assume a run that saves POD5, FASTQ, and BAM files, with a read N50 of 23 kb.

Flow cell output (Gbases)	FASTQ.gz storage (Gbytes)	Unaligned BAM with modifications (Gbytes)	(Optional) POD5 storage (Gbytes)
100	65	60	700
200	130	120	1,400

Note: When basecalling is turned on, the system will create FASTQ and/or BAM files, and will require additional temporary storage while the sequencing run is active. If you elect to turn on POD5 output, the system will generate POD5 files continuously during the experiment.

EPI2ME analysis

The EPI2ME Desktop Application provides user-defined local or cloud-based analysis solutions:

- Local analysis runs directly on the user's computer, using available compute resources

- The cloud-based analysis runs on Amazon Web Services (AWS) and requires an internet connection

Find out more about how EPI2ME can support your data analysis needs here.

Data upload formats: EPI2ME receives FASTQ, BAM, and other relevant workflow formats, processes data via custom Nextflow pipelines, and provides interactive HTML reports.

Software updates

You can download updates via the MinKNOW interface or terminal (using `apt`). The system only requires outbound access. We share notifications about software updates through the Nanopore Community and provide full update instructions in each release note.

Data handling and storage

Real-time data transfer

During sequencing, the device uses its internal storage as a temporary cache. When operating at full throughput, this cache can quickly reach capacity. You must stream data in real-time to an external storage system to avoid interruption or failure of the sequencing run.

The PromethION 24/48 A-Series includes two 10 Gbps network ports (Fibre or Ethernet) for high-speed data transfer. Use these to connect to SSD-based networked storage. Don't use USB ports for data transfer, as they cannot support real-time streaming at high data volumes.

If real-time streaming fails or is insufficient, sequencing may stop unexpectedly.

Estimated transfer times

The table below provides approximate transfer times for different flow cell configurations using 1 Gbps and 10 Gbps connections.

Flow cell configuration	1 Gbps transfer time	10 Gbps transfer time
1 × 200 Gbase flow cell	~7 h	~1 h
24 × 200 Gbase flow cells	~160 h	~16 h

Use this information to assess your site's bandwidth requirements and plan infrastructure accordingly.

Storage recommendations

To ensure stable data handling and long-term accessibility, consider the following storage strategies:

- Short-term storage Use SSD-based networked storage (e.g., NFS or CIFS) for high-speed streaming during active sequencing runs
- Long-term archiving After sequencing, you can transfer the data to slower, cost-effective archival storage solutions
- File format considerations
    - Store POD5 files if you plan to re-basecall the data later or use tools requiring raw signal data access
    - Store FASTQ files for downstream analysis if you don't need to reprocess raw data

Oxford Nanopore Technologies does not prescribe specific storage volumes or architectures, as these requirements vary by site. We recommend working with your local IT team to define:

- Real-time data streaming infrastructure
- Short-term high-speed storage
- Long-term archival capacity

Safety and compliance

Device indentification

Device part numbers:

PRO-PRCAMP (PromethION A-Series Data Acquisition Unit)
PRO-SEQ024 (PromethION 24 Sequencing Unit)
PRO-SEQ048 (PromethION 48 Sequencing Unit)
PRO-CMB024 (PromethION 24 Combined*)

*available in selected countries only

Intended use

The PromethION A-Series is an electronic analysis platform designed for use in scientific research. Its core technology uses a nanopore to detect single-molecule events, including nucleic acids (DNA/RNA), proteins, and small molecules.

This product is for research use only.

Safety information

Before use, review the following safety guidelines:

- Ensure that only an Oxford Nanopore Technologies field service engineer unpacks and installs the PromethION 24 Combined

- Install the system on a stable surface with adequate ventilation
- Allow 30 cm clearance to the front, rear and sides of the device
- Ensure the environment is dust-free and located away from open windows

Emergency procedures

In the event of an emergency:

1. Switch off the PromethION A-Series device using the power switch
2. Unplug all power cables from the rear of the device

Declaration of conformity

The PromethION A-Series complies with relevant EMC and Electrical Safety directives, as outlined in the EC Declarations of Conformity below.

Declaration of Conformity for the PromethION 24 and 48 Sequencing Units
Declaration of Conformity for the PromethION A-Series Data Acquisition Unit
Declaration of Conformity for the PromethION 24 Combined

Compliance labels

PromethION 24 compliance label
PromethION 48 compliance label
PromethION 48 Sequencing Unit

License and warranty

The license and warranty contract ensure your instrument performs optimally by providing the latest up-to-date hardware and software. Oxford Nanopore Technologies guarantees the delivery of its support obligations during the contract period, as laid out in sections 4 and 7 of the Nanopore Product Terms and Conditions.

More information on the device warranty is available in the Oxford Nanopore Store.

Frequently asked questions

How can I transfer data off the PromethION during a run?

The software includes scripts for real-time transfer. For assistance, contact support@nanoporetech.com.

Which SFP+ modules are compatible with the PromethION?

Any standard SFP+ modules will work. Select one that is compatible with your existing infrastructure.

What storage options can I connect?

The PromethION can connect to any NAS. Using the guidance provided above, discuss suitable solutions with your IT team.

Appendix A: Shipment and logistics

Oxford Nanopore Technologies ships PromethION devices in protective packages, using one or two boxes depending on the model. Each package includes a pallet base for easy transport and is fitted with data trackers to monitor G-forces, orientation, temperature and humidity during shipping.

If you notice any damage to the boxes or signs of mishandling, refuse the delivery and notify Customer Service at support@nanoporetech.com.

Shipping configuration

- **PromethION 24/48 A-Series** These systems ship to most countries worldwide in two boxes: one for the Data Acquisition Unit and one for the Sequencing Unit.
- **PromethION 24 Combined** This model ships to selected countries only and arrives in a single box.

Important: Only an Oxford Nanopore Technologies field service engineer must unpack and install the PromethION 24 Combined unit.

We store and ship all systems at ambient temperature (+15˚C to +25˚C)

Shipping container specifications

Component	Weight (kg)	Dimensions (H × W × D, cm)
PromethION A-Series Data Acquisition Unit	37	45 × 68 × 60
PromethION 24 Sequencing Unit	33	59 × 70 × 54
PromethION 48 Sequencing Unit	44	59 × 70 × 54
PromethION 24 Combined	120	90 × 120 × 100

Appendix B: Compatibility

The PromethION 24/48 A-Series is compatible with all the latest versions of chemistry for PromethION Flow Cells, sequencing kits, and expansions.

This includes compatibility with:

- Flow Cells: PromethION R10 Series Flow Cell (FLO-PRO114M), RNA004 PromethION Flow Cell (FLO-PRO004RA)
- Sequencing kits: the PromethION 24/48 is compatible with all V14 chemistry kits (ligation, rapid, barcoding, PCR, cDNA, and Direct RNA kits). We have not tested the following:
    - 16S Barcoding Kit 1-24 (SQK-16S114.24)
- Latest sequencing software: MinKNOW, Dorado Basecall Server
- Downstream analysis tools: EPI2ME (and included workflows), Oxford Nanopore pipelines (MinKNOW-compatible workflows), and custom-developed tools (Nanopore Community developed tools)

Change log

Date	Version	Changes made
3 Feb 2026	V2	In the table under Section 3, 'Site planning (pre-delivery)', 'Network access requirements', cannonical.com domain was included as a required domain for Software and OS updates.
7 Jan 2026	V1	This document consolidates the PromethION 24/48 Technical Specifications and the PromethION 24/48 IT Requirements into a single document. The previous documents are now in legacy. What's new: - Combined the two documents and reorganised the overall structure. - Made the compatibility section easier to read: instead of lists of kit codes, it now provides a simple statement about which kits and software are compatible, along with notes on any exceptions. - Added guidance on UPS selection and suitability - Added details of package contents

REQUIREMENTS

Telomere-to-telomere sequencing (T2T) know-how document

FOR RESEARCH USE ONLY

Contents

Introduction

Data and results

1. Depth vs performance: ULK
2. Depth vs performance: Pore-C
3. Depth vs performance: Assembly polishing
4. Analysis

Change log

Introduction

This know-how document provides supplementary information for the end-to-end workflow for telomere-to-telomere sequencing of the human genome using the Oxford Nanopore PromethION platform.

The Telomere-to-telomere sequencing (T2T) from blood and cells using SQK-APK114, SQK-LSK114, and SQK-ULK114 protocol includes three separate sequencing experiments: Ultra-Long DNA Sequencing Kit V14 (SQK-ULK114), Assembly Polishing Kit V14 (SQK-APK114), and the Pore-C protocol using the Ligation Sequencing Kit V14 (SQK-LSK114).

The telomere-to-telomere (T2T) workflow combines ultra-long reads, Pore-C and our new assembly-polishing chemistry to completely resolve haplotypes and achieve a state-of-the-art Q50 human assembly.

Our recommendation is that four PromethION Flow Cells are used for telomere-to-telomere sequencing of a single human sample, and are allocated accordingly:

Preparation	Kit	R10.4.1 PromethION Flow Cells	Input (whole blood)
Ultra-long DNA sequencing	SQK-ULK114	2	2 x 1.6 ml (2 x ~6 million cells)
Pore-C	SQK-LSK114	1	5–10 ml (~10 million cells)
Assembly polishing	SQK-APK114	1	1 ml (for 5 µg gDNA) (~5 million cells)

Data and results

Depth vs performance: ULK

We see that 130 Gb of pass data (equating to ~43X depth of the human genome) provides good assembly contiguity with a high number of T2T contigs and scaffolds (when combined with pore-C).

Figure 1 shows how performance (the number of T2T contigs/scaffolds) differs with varying amounts of ULK data, shown as both depth of coverage and total output (Gb). The figure represents data phased with the Verkko native Pore-C phaser. Also shown is the expected output/depth from ULK experiments.

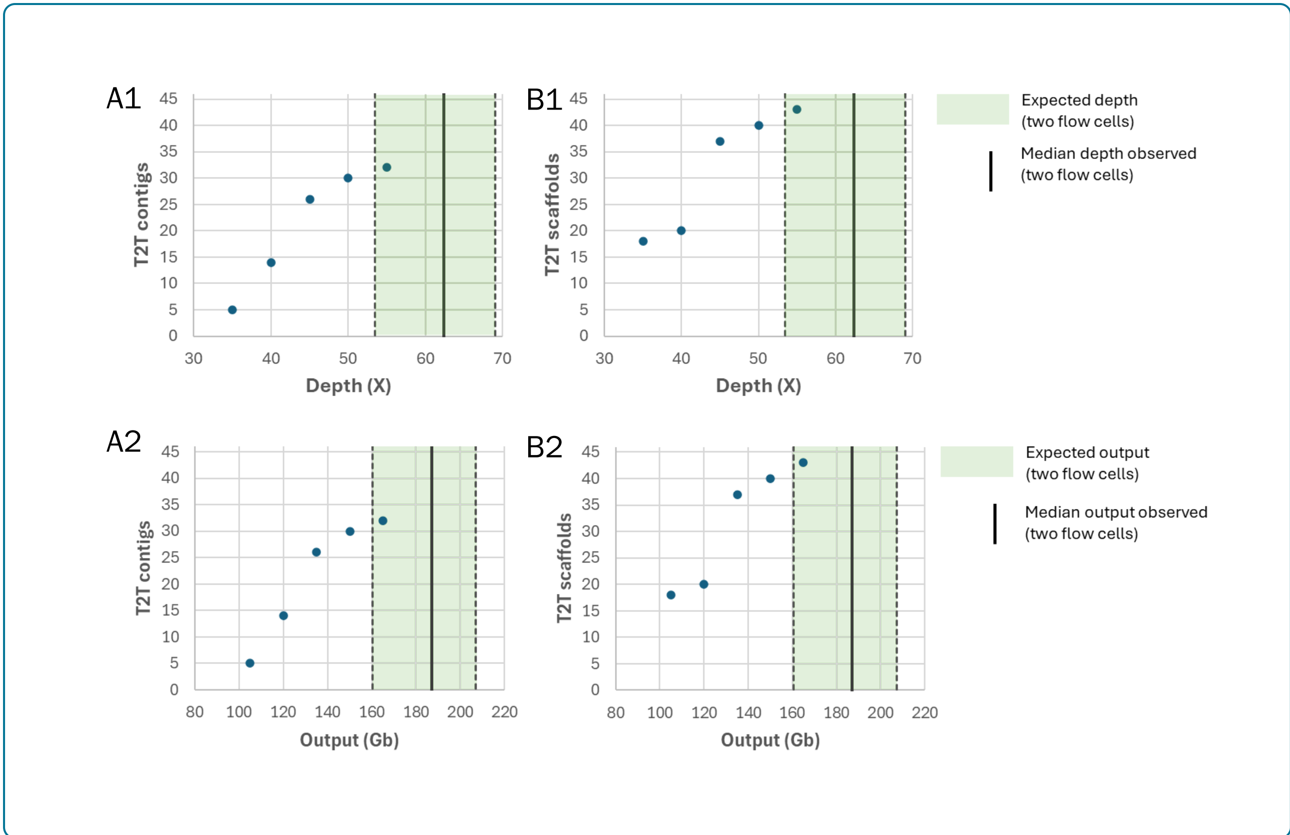


Figure 1: T2T contig and scaffold assembly performance across ULK data coverage depth. Data is phased with the Verkko native Pore-C phaser. Solid vertical lines represent median coverage depths obtained (in total over two flow cells) from a number of ULK experiments, with the shaded green box representing the interquartile range of the dataset (which we equate to expected depth).

It is important to consider how the assembly quality is affected by the N50 yielded from the ULK preparation. Our data shows an N50 of 100 kbp is on trend with that of 120 kbp, but an N50 of 75 kbp shows some deterioration in performance (Figure 2).

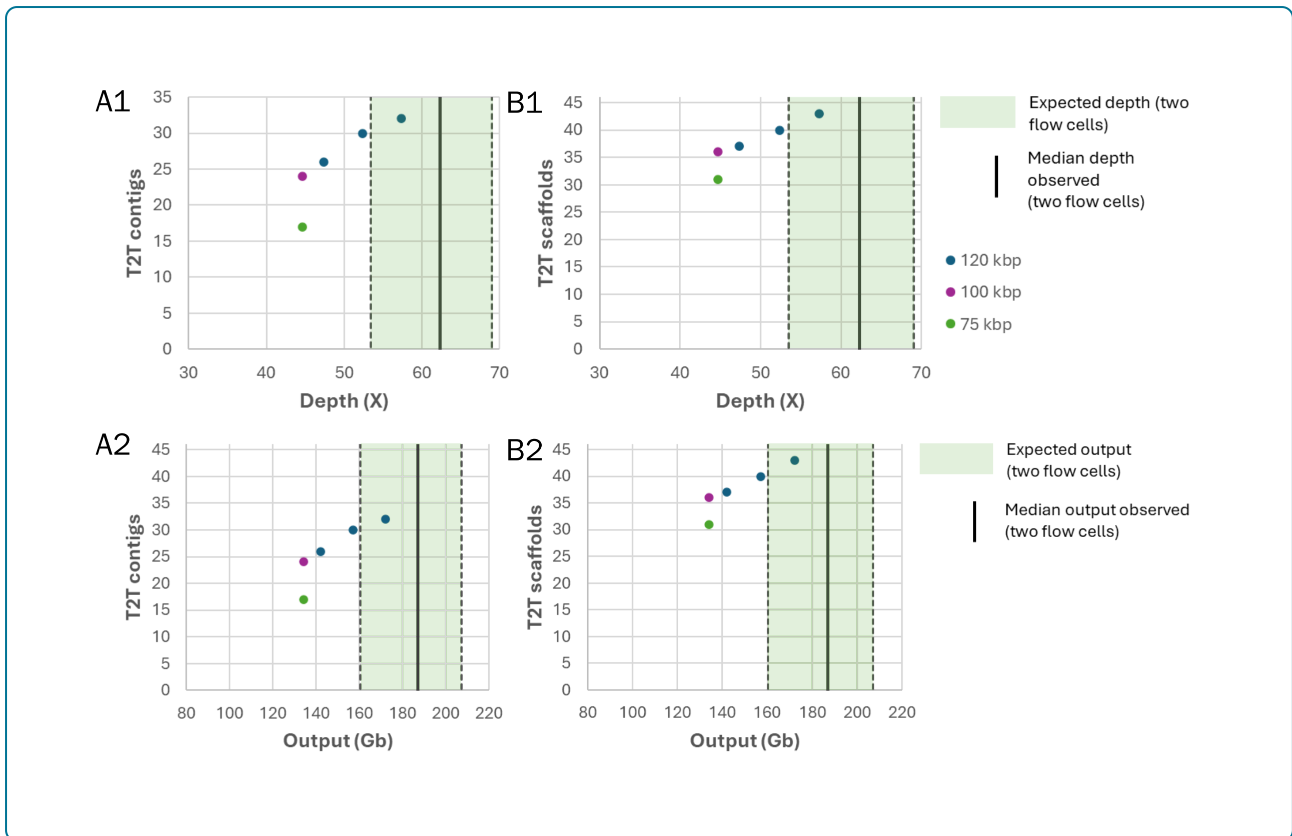


Figure 2: Initial data on the dependence of N50 on the performance of ULK data. At 45X depth (filtered data) the number of T2T configs and T2T scaffolds produced with an N50 of 100 kbp (purple •) appears on trend with 120 kbp data (blue •). A noticeable drop in performance is observed at 75 kbp (green •). As in Figure 1, solid vertical lines represent median read depths obtained (in total over two flow cells) from a number of ULK experiments, with the shaded green box representing the interquartile range of the dataset (which we equate to expected depth).

Depth vs performance: Pore-C

Pore-C provides long-range data; longer range than even Ultra-long data, and is required for genome phasing. Data supports that 25X depth of the human genome from Pore-C data gives us good contig and scaffold assembly performance (Figure 3). In our experiments we saw no benefit to increasing Pore-C coverage depth above 25x.

Without the Pore-C data you will obtain no information for T2T contigs and/or scaffolds. If the Pore-C experiment is omitted, T2T contigs will be broken at un-spanned homozygous regions, as Verkko won't know which haplotypes on either side belong together.

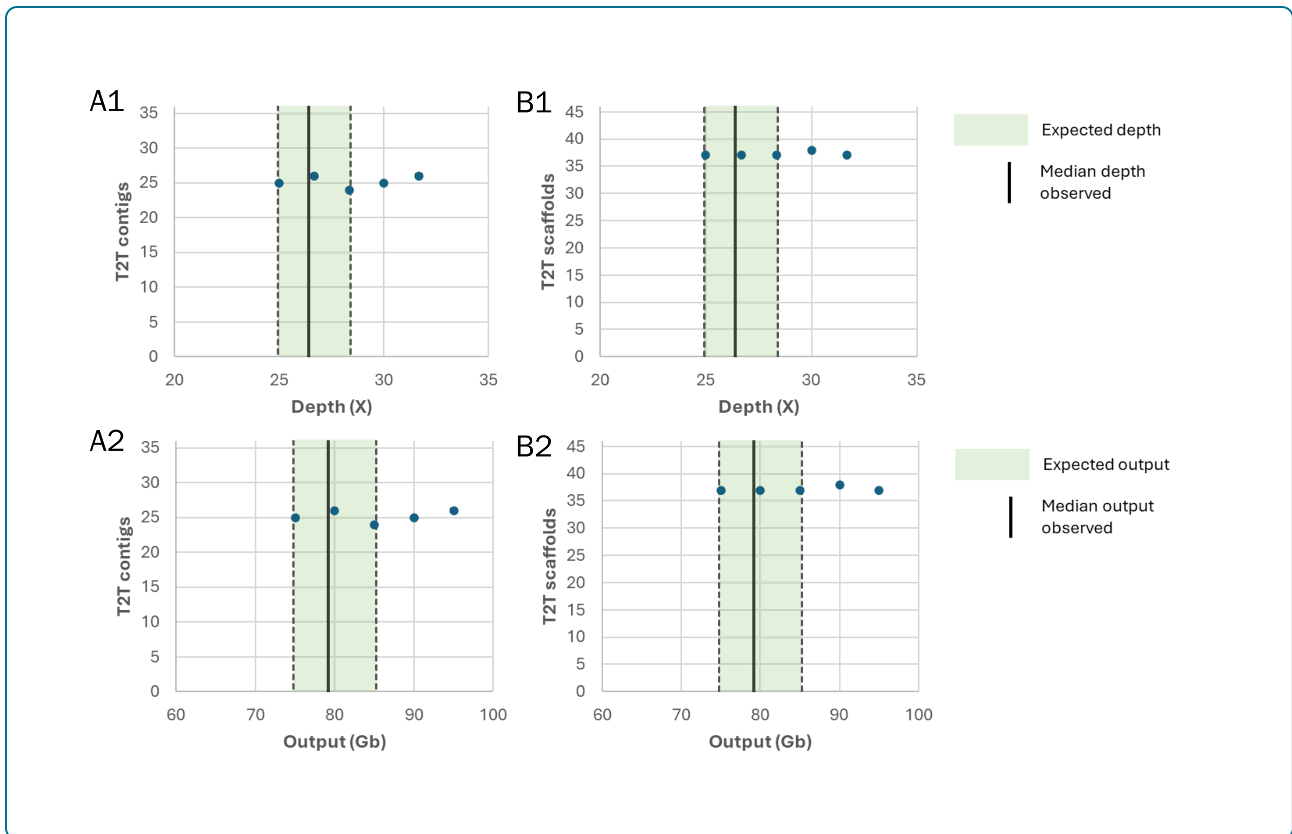


Figure 3: Dependence of phasing performance on Pore-C human genome depth. Solid vertical line represents median depth obtained from a number of Pore-C experiments, with the shaded blue box representing the interquartile range of the dataset (which we equate to expected depth).

For Pore-C data generation, ultimate performance is dependent on how well DNA cross-linking occurred during the sample preparation, and not solely on the outputs/depths achieved during sequencing.

Depth vs performance: Assembly polishing

Assembly polishing represents one of the final steps in the T2T workflow and uses the data generated with the Assembly Polishing Kit (SQK-APK114) with the aim to attain Q50 accuracies. The graph below (Figure 4) details the relationship between the amount of APK data and Q-accuracy.

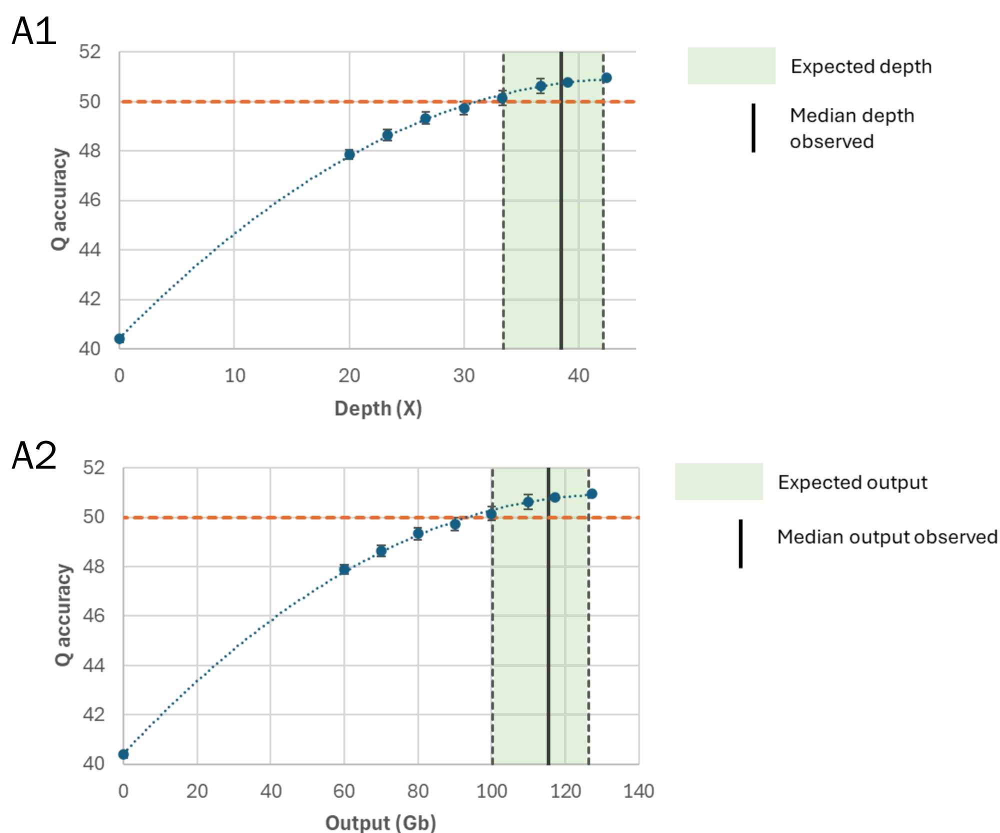


Figure 4: Dependence of Q-accuracy on APK output (depth). The solid vertical line represents median depth obtained from a number of APK experiments, with the shaded green box representing the interquartile range of the dataset (which we equate to expected depth).

Read lengths achieved in APK sequencing runs are expected to be ~6-8 kb. If assembly polishing is not used during the workflow assemblies are expected to be in the range of ~Q40 rather than ~Q50.

Analysis

Current data processing requirements dictate that experienced bioinformaticians are needed, with an appropriate level of compute power as described in the community protocol. Details of how to undertake the analysis are also contained within the community protocol with appropriate Github links. An example Nanopore-only T2T dataset is also linked via this EPI2ME blog post.

Change log

Date	Version	Changes made
February 2025	V1	Document release

Oxford Nanopore Technologies

PROTOCOL

Adaptive sampling

V ADS_S1016_v1_revN_05Sep2025

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Advanced guide

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1. Introduction

Adaptive sampling introduction

In some sequencing applications, the focus of study — a single gene, or a selection of genomic regions — makes up a small fraction of the genome or sample. In these cases, whole-genome sequencing can be inefficient and costly. Targeted sequencing is a term used to describe strategies that reduce the time spent sequencing regions that are not of interest, which significantly reduces the amount of data required to achieve the desired depth of the regions of interest. This reduces sequencing costs and the data analysis burden, and enables a quicker workflow. Targeted sequencing using nanopore technology can be achieved in several ways:

- amplicon sequencing
- pull-down
- adaptive sampling

Oxford Nanopore sequencing allows real-time decoding of the region of the genome being sequenced. This characteristic allows decisions to be made in real time on whether a particular strand is of interest or not. This called adaptive sampling, and it can perform real-time selection of reads when the sequencing software (MinKNOW) is supplied with a .bed file containing the regions of interest (ROI) and a FASTA reference file.

Adaptive sampling offers a fast and flexible method to enrich regions of interest by rejecting off-target regions: target selection takes place during sequencing itself, with no requirement for upfront sample manipulation. Prepare and load the library as normal and select "adaptive sampling" in MinKNOW (you will need to upload a FASTA file with the reference as well as a .bed file detailing the regions of interest). Once sequencing begins, due to the real-time nature of nanopore sequencing, MinKNOW identifies whether the strand that is being sequenced is within the ROI. If the read does not map to the ROI, MinKNOW reverses the polarity of the applied potential, ejecting the strand from the pore so that it is able to accept a new strand. Off-target strands are continually rejected until a strand from the ROI is detected and sequencing is allowed to proceed.

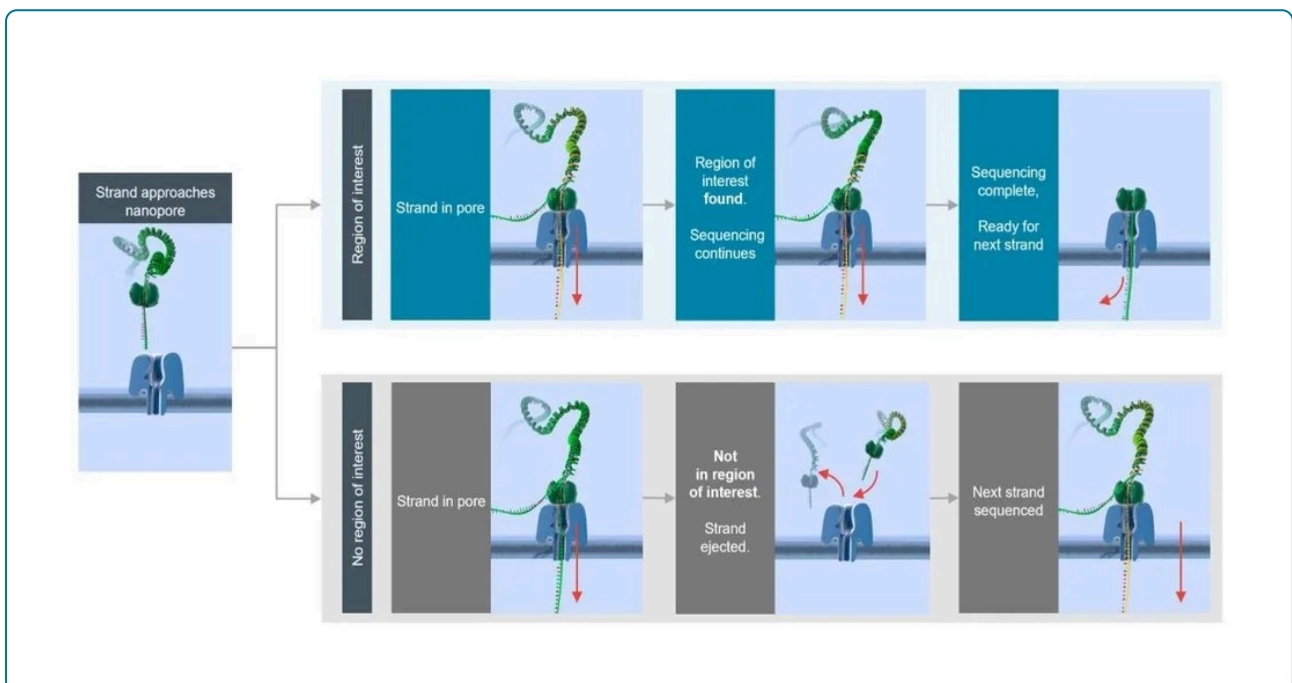

Figure 1. Overview of an adaptive sampling experiment

Adaptive sampling can run in two different modes: enrichment and depletion. In enrichment mode you would upload ROIs to MinKNOW, which then rejects strands that fall outside of these regions. In depletion mode, upload targets that are not of interest (e.g. host DNA in a host : microbiome metagenomic analysis) to MinKNOW, which then rejects strands that fall within these regions. We observe an enrichment for ROI of ~5-10-fold when using adaptive sampling, and we outline our advice on how this can be achieved below. When targeting regions within human genomes, we find this level of enrichment to be robust if the total fraction that is being targeted is <10% of the total genome. This allows you to obtain a mean depth of >20-40x of ROI on a MinION Flow Cell.

For a visual overview of adaptive sampling, refer to this video: Adaptive sampling on nanopore technology.

2. Sample preparation and analysis

Although adaptive sampling does not require any particular sample preparation, there are some aspects of library preparation which benefit an adaptive sampling experiment.

There are two main aspects to consider when trying to maximise output: pore occupancy and library fragmentation.

Pore occupancy

The adaptive sampling methodology is based on rejecting unwanted DNA strands to free up the pore, ready to capture a new strand. This can cause a significant reduction in pore occupancy, as the constant rejection of strands reduces the amount of time that pores are occupied with a strand. Therefore, maintaining high pore occupancy is one of the most important aspects in adaptive sampling. To achieve this, we recommend loading a higher amount of sample than you would normally use for a sequencing run. The right amount of DNA to load into the flow cell needs to be calculated from the point of view of molarity instead of mass (explained in more detail below).

Library fragmentation

This is important for two reasons: firstly, the fragment length affects the molarity, which is the main measure of DNA to be loaded in an adaptive sampling run. Secondly, adaptive sampling runs are more likely to block pores due to the high amount of strand rejection. Using a library made up of shorter fragments increases flow cell longevity and therefore data output, since the library causes less blocking and gives a higher molarity with lower amounts of total DNA. Not only does shearing reduce blocking, but it can also increase enrichment depending on the size of your individual ROIs. If most of your ROIs are a few kb long (e.g. 2–5 kb), then using a library with an N50 in the 30 kb range is going to be wasteful. This is because every time a strand is accepted for sequencing, the pore will be occupied sequencing 30 kb of data to extract 2–5 kb of on-target sequence. This is a potential waste of 25–28 kb, when the pore could be sampling more reads during this timeframe instead of sequencing off-target.

Another method to increase output from an adaptive sampling run is to perform multiple flow cell washes throughout the run and reload the library. However, by reducing the library fragment size you can reduce the number of flow cell washes needed to maximise the output from a sequencing experiment.

Figure 2 shows the difference in pore blocking in adaptive sampling mode over time with two different fragment size libraries: 5 kb and 25 kb. Each bar represents the number of pores available for sequencing every 1.5 hours throughout the run. Attrition of channels due to blocked pores occurs at a faster rate in the library with the longer fragments. Although flow cell washing can recover some lost pores, it is a hands-on process which adds hands-on time to the run. For this reason, it is important to give consideration the size of your library when designing adaptive sampling experiments to keep blocking to a minimum and reduce the need to interact with the flow cell.

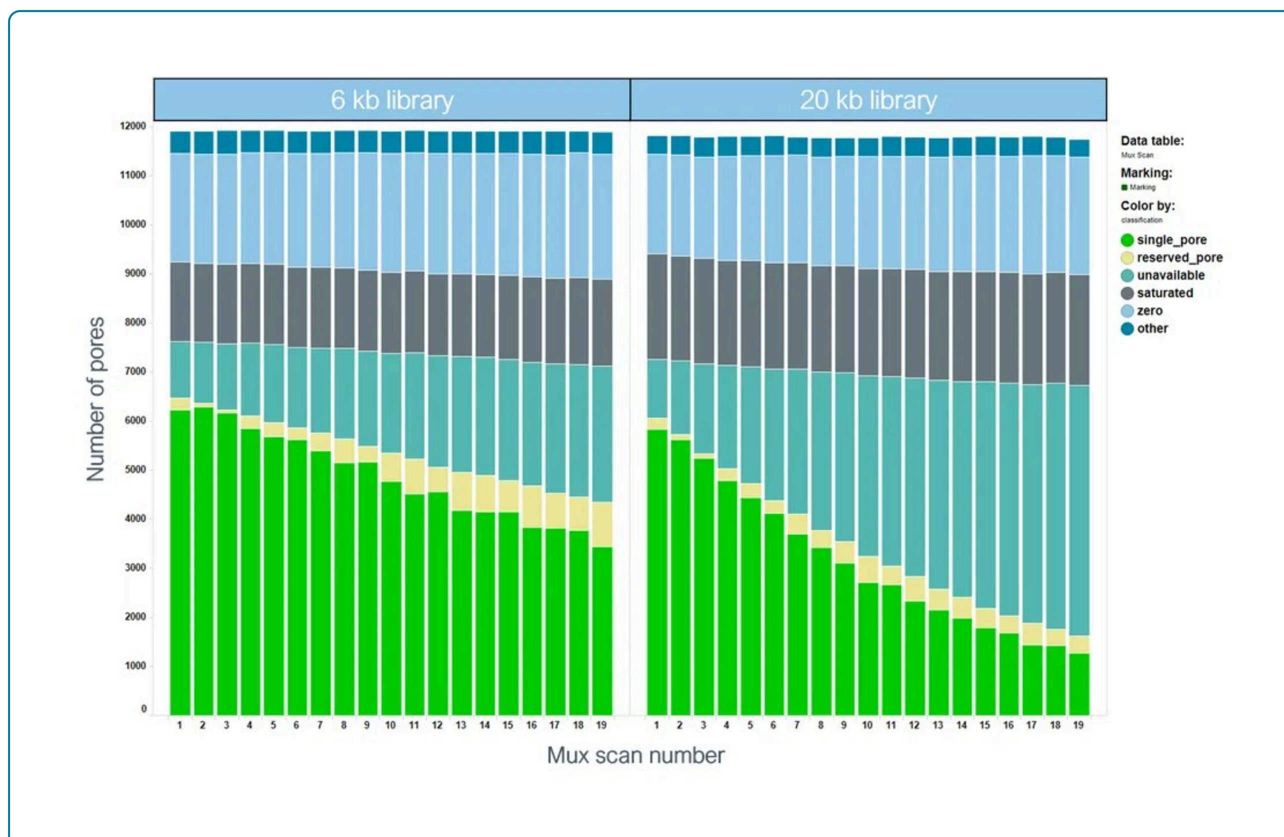

Figure 2. Pore scans for 6 kb (left) and 20 kb (right) libraries in adaptive sampling runs without flow cell washes.

The fragment size will also affect the molarity of your sample if you are using a Qubit or other mass-related measurement to calculate the amount of DNA loaded into the flow cell. Qubit is the recommended method for evaluating your DNA library concentration, but this should be converted into a molarity concentration which can be done based on your average fragment length. You can evaluate fragment lengths using the Agilent Femto Pulse (for fragments >10 kb), or the Agilent Bioanalyzer (for fragments <10 kb).

Using the average molecular weight of a base pair (660 g/mol), you can easily calculate the molarity of the sample. This will make the mass of DNA needed for short and long libraries quite different when normalising for the same molarity. Molarity is an important property to consider, since the number of DNA ends available to be captured by the pore is the main factor in improving pore occupancy. The ideal molarity when using the latest V14 chemistry is 50–65 fmol per load.

With a library which has a normal read length distribution centred at 6.5 kb (Figure 3), 50 fmol would correspond to approximately 200 ng, according to the following calculations:

- Total mass of a mol of 6.5 kb fragments: 6500 base pairs x 660 (g/mol) = 4,290,000 g in 1 mole
- Multiply this by the number of femtomoles needed: 4,290,000 x 50 x 10^-15 = 2.145 x 10^-7
- Convert grams to ng: 2.145 x 10^-7 x 1,000,000,000 = 214.5 ng

To facilitates these calculations, you can use a biomath calculator such as the following: Biomath Calculators | DNA Calculator | Vector Insert Ratio

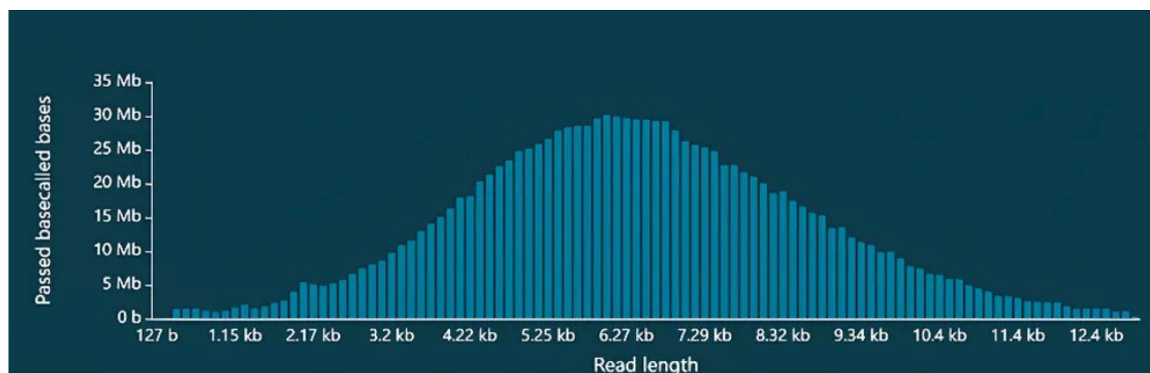

Figure 3. Read length distribution of a library with an N50 of 6.5 kb.

This is a rough approximation, based only on the N50 of the library. The real molarity calculation is more intricate to calculate, as you need to consider the range of the distribution. Nevertheless, this is a good approximation to understand the amount of DNA you would need for an adaptive sampling run. It is also worth noting that these calculations and values are assuming optimal ligation efficiencies. If for any reason a library is not ligating as efficiently, we advise adding more sample to the sequencing run. Note that a higher DNA input has not been shown to affect the run negatively when using V14 chemistry up to a maximum of 600 ng.

For more detailed information about ideal sample metrics for adaptive sampling runs, refer to the Adaptive sampling advanced guide below.

3. Targeting and buffering a .bed file

1 Targeting and buffering a .bed file

Disclaimer: *This section was shortened for the purpose of providing the most useful, quick-use information. The cases described are ideal adaptive sampling use cases. For detailed information of targeting (how it works) and buffering, please visit the "Targeting and buffering page" in the Advanced guide section. For information on depletion mode, refer to the corresponding section of the advanced guide.*

Adaptive sampling ideally requires a reference (.fasta) and a bed file (.bed) to know which strands to select for sequencing. The reference contains a representation of the whole sample, and the

.bed file serves as a mask to subset the reference and inform MinKNOW which regions of the reference are of interest.

To provide maximum benefit to enrichment, the .bed files should ideally target less than 5% of the sample. However, you can target as much as 10% for reasonable returns of enrichment (for instance the RRMS panel available in the Adaptive Sampling Catalogue). Targeting more than 10% will reduce the enrichment values obtained.

As an ideal targeting example, we will use the hereditary cancer panel also available in the Adaptive Sampling Catalogue. This example panel targets ~0.54% of the human genome. This means that in a sample composed exclusively of human DNA, this panel would instruct MinKNOW to accept and sequence ~0.54% of all the reads captured by pores in the flow cell.

The decision to accept or reject a read is made on the first chunk of a read, and this is the only information that MinKNOW will have to make a decision. This causes MinKNOW to reject strands which start (first chunk) in the flanking regions of a target, but does not actually hit the target within the first chunk. To account for this behaviour we use a “buffer” which is added to the side of the regions of interest (ROI). This will allow MinKNOW to accept a strand which starts in a flanking region and that will likely extend into the ROI.

To define the buffer, you need to know the read length distribution of the library being sequenced. Ideally the amount of buffer to add should be equal to ~N10 of the read length distribution of the library. The buffer value does not need to be very precise due to the nature of a library red lengths being a normal distribution. This means that as long as the library preparation method stays consistent, a characterisation of the read length distribution is not necessary for every single library made.

As a rule of thumb, for a library with a normal distribution with an N50 of ~8 kb, aim for adding 20 kb of buffer.

There are several caveats and exceptions to this, mostly around how much the buffer with modify the total amount targeted. For instance, for the hereditary panel mentioned above (which targets 0.54% of the human genome) a 20 kb buffer will make it target ~0.67% if added site-specifically. For more information about the details of buffering, refer to the “Targeting and buffering page” in the advanced guide section.

Lastly, Oxford Nanopore provides a page for checking your .bed file, which should be done before and after buffering:

Bed file checker - Bed Bugs

This page will catch errors with the .bed files and prevent runs from crashing. You will need to provide the same reference file which you used to acquire the .bed file coordinates and use this same reference during the run setup. If Bed Bugs is reporting issues with your .bed file (besides overlapping), these will need to be fixed, before using the bufferer.py script. Furthermore, Bed Bugs has now a functionality to automatically buffer your .bed file with a directionally-aware 10 kb buffer. If your .bed file shows no errors besides a “self overlap warning, a new options will be available to download an “buffered” version of your bed file (Figure 4). The buffer size is currently set to 10 kb and cannot be changed. It will not fit all .bed files and applications, but it is a good start for most ideal size .bed files. for more information of .bed file buffer sizes, please visit the “Targeting and buffering page” in the advanced guide section.

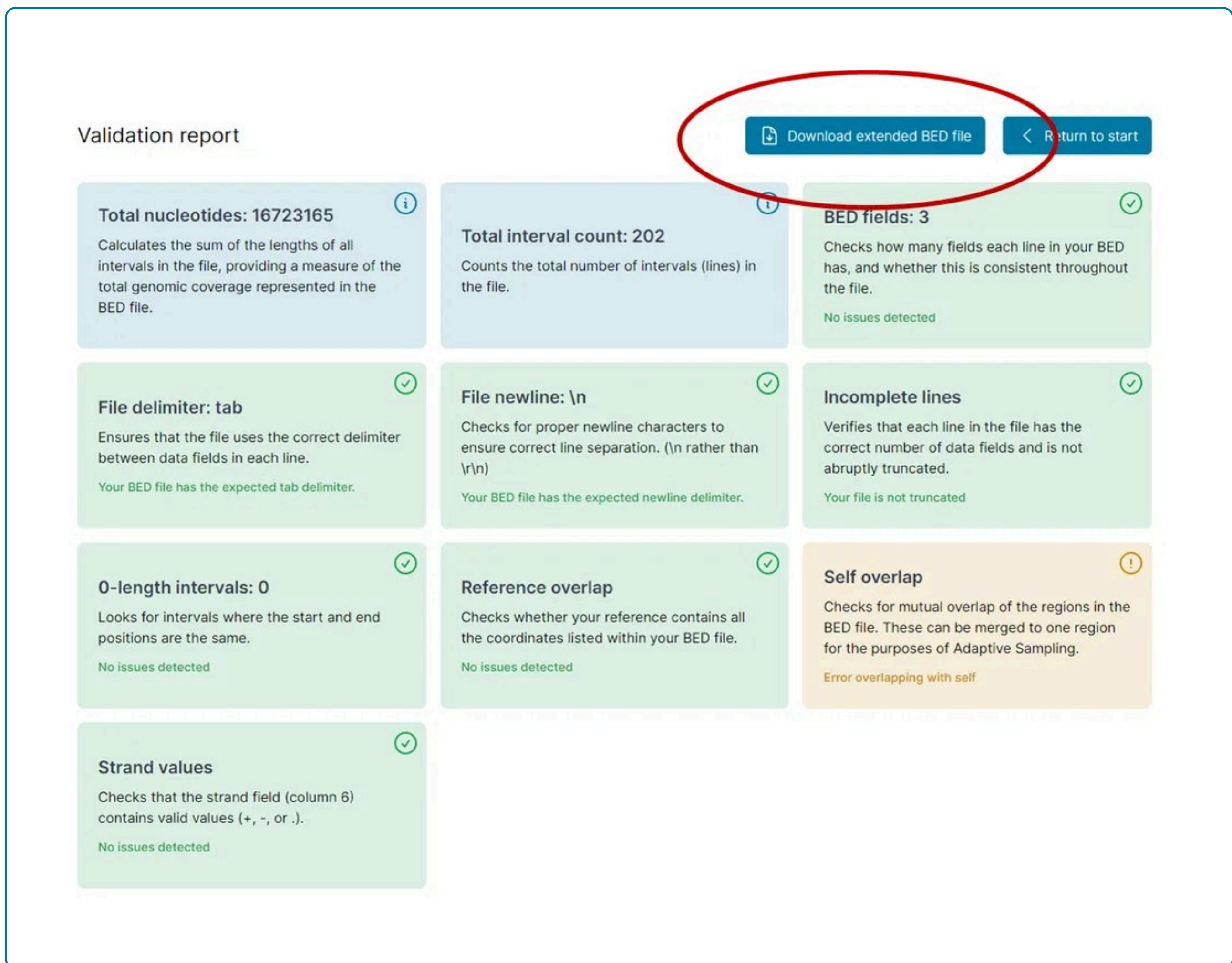

Figure 4. Screenshot of Bed Bugs after validation of a .bed file containing no run threatening errors.

Note: Sometimes, you may observe a “self overlap” issue reported by Bed Bugs. The reason for this is that after using the buffering script, the new .bed file will have overlapping region defined. However these overlapping regions are necessary as they are different depending on the strand (coding or non-coding). If you see this error reported by Bed Bugs, it will not affect MinKNOW performance and you can ignore the error.

4. MinKNOW UI and dialogs

During an adaptive sampling experiment, MinKNOW carries out basecalling and alignment with adaptive sampling in parallel with live basecalling. The MinKNOW user interface shows dialogs with information for both processes. Here, we explain where you can find each type of information.

Firstly, there are separate sections in MinKNOW for uploading a live sequencing alignment reference and .bed file, and an adaptive sampling alignment reference and .bed file. Both the reference FASTA file and the .bed file can be the same in both sections (hence the Alignment section is pre-populated with the files uploaded in the Adaptive Sampling section). Nevertheless, it is important to understand the function of each file as you can get a better ongoing view of

coverage obtained throughout the run, by loading a different .bed file in the live alignment section.

The adaptive sampling files are loaded in the **3. Run Options** section under the section named "Adaptive Sampling" - Figure 5, top section. These will be used for targeting your sample and will affect the reads which get selected by MinKNOW for sequencing. The .bed file loaded in this section should also contain a buffer region, when applicable. For more information on this, refer to previous guide sections about buffers.

The live alignment files are loaded in the **4. Analysis** section of the run setup in MinKNOW. The purpose of the FASTA reference is to align the reads after live basecalling and therefore should be the same file as the one loaded for the adaptive sampling decision. This will allow to output BAM files containing the basecalled and aligned sequence in real time.

The .bed file in the **4. Analysis** section is used for two different processes: Firstly, it provides an identifier in the sequencing summary reporting whether the complete read hit the regions described in the .bed file loaded in **4. Analysis**. This is shown in the sequencing_summary.txt file under the column bed_alignment and populated with a 0 or a 1, for whether it hits or does not hit the .bed file. Secondly, this .bed file is used to check for the coverage obtained at each of the regions described in the same .bed file. You can follow this live during the run in the **Alignment hits** tab of MinKNOW.

To make the most of the live alignment feature and the coverage tracking feature, load the buffered .bed file (bed file containing the ROI + buffer) in the **3. Run options** Adaptive sampling section, and the .bed file containing only ROI (unbuffered) in the **4. Analysis** section. This guarantees that you only track coverage on the targets of interest, and will give a more accurate description of coverage on the ROIs. The .bed file provided in the alignment section does not modify the run output and is not strictly necessary. Nevertheless, depending on the amount of buffer added to each region in the adaptive sampling .bed, having a targets only (unbuffered) .bed in the alignment section may provide a more accurate coverage report. Importantly, the coverage tracking (and therefore the files provided in **4. Analysis**) does not modify the sequencing run in any way. It is a tool for real-time alignment and for checking during a run how much coverage has already been obtained for each bed region. Lastly, note that the coverage reported is referring to the percentage of sequence that has been basecalled. This means that if the live basecalling is not keeping up, the coverage reported is only relative to the percentage already basecalled.

Live alignment is a computationally demanding process which can easily affect the adaptive sampling decision time. Therefore, please refer the tables of advised metrics on how many flow cells you can run with this feature per device to avoid affecting enrichment rates. To prevent live alignment being used, remove the reference sequence denoted "1" in Figure 5.

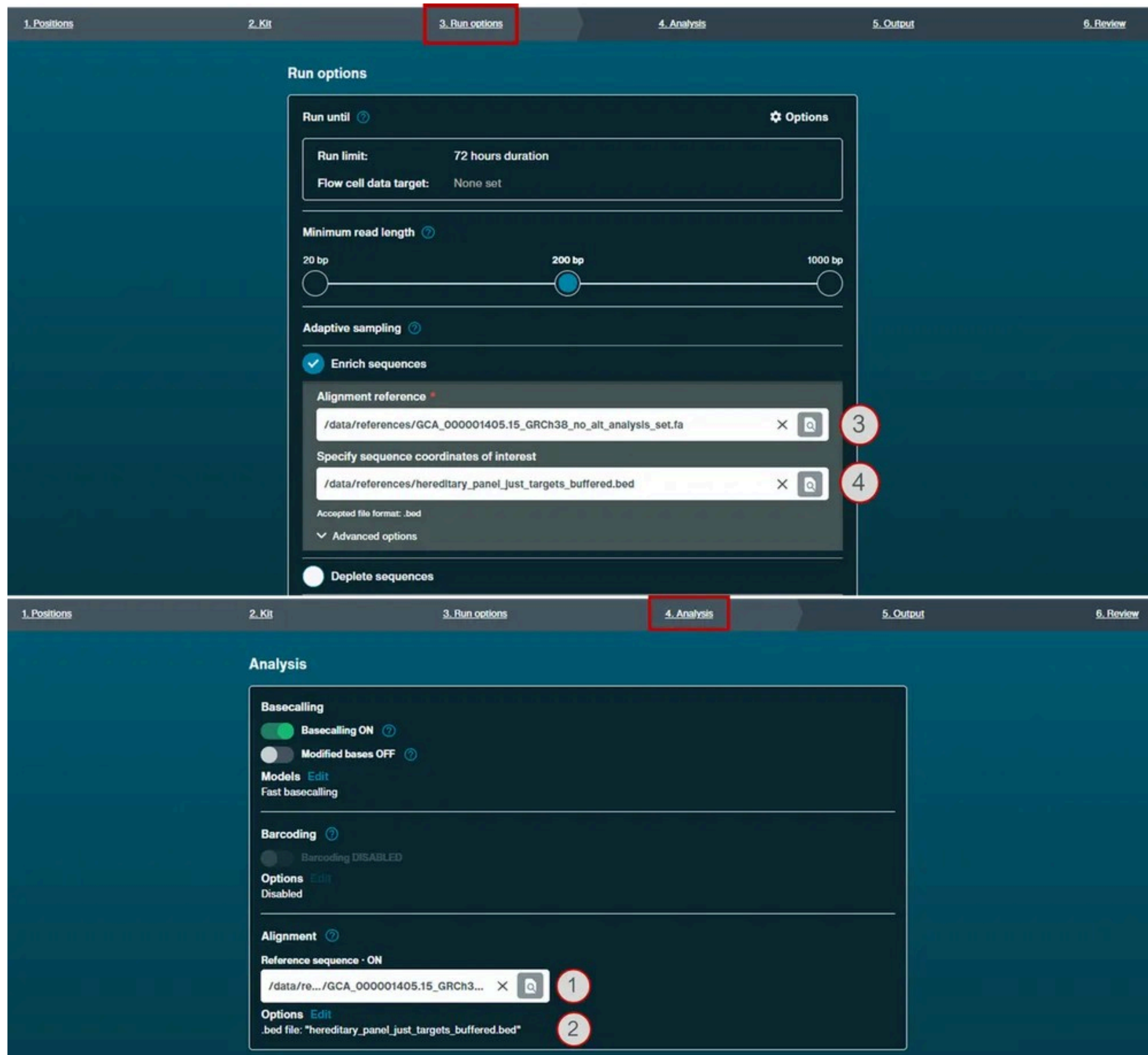

Figure 5. MinKNOW dialogs for uploading the FASTA reference file and .bed file for the adaptive sampling panel (top, sections 3 and 4) and the live basecalling panel (bottom, sections 1 and 2).

You can see which files are being used in each section in the first page of the run summary once the run is started. An example of this panel is shown in Figure 6.

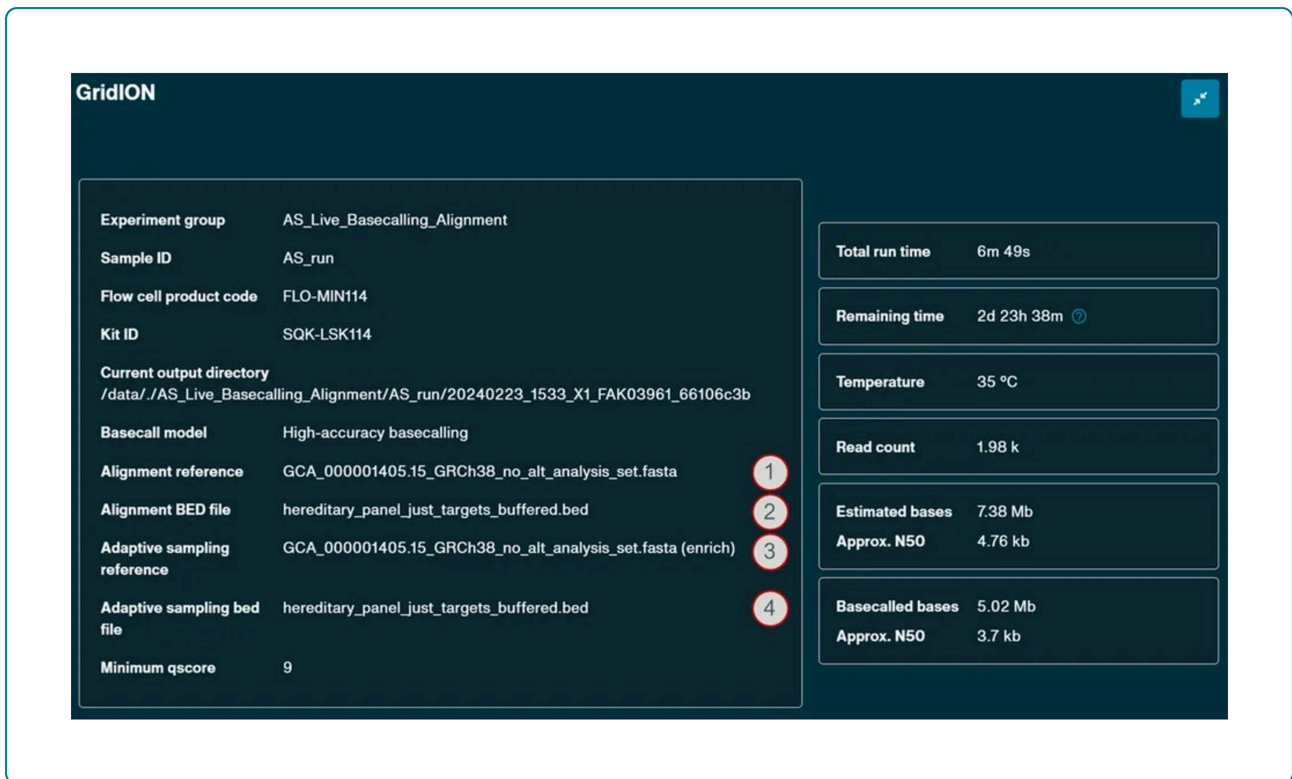

Figure 6. MinKNOW front panel containing run summary details. Alignment information has been labelled accordingly.

5. Final notes

Final notes

The information provided in this quick start guide is a starting point for learning how to use adaptive sampling. Caveats and exceptions apply to most sections of the quick start guide. Moreover, our sequencing devices have limits on the amount of real-time analysis they can perform, and adaptive sampling is a real-time analysis tool with minimal latency. Adaptive sampling therefore demands a considerable amount of resources from the machines. You can find recommendations on the limits of adaptive sampling in the “Advanced guide” pages.

We also provide a troubleshooting guide, along with more in-depth explanation of how adaptive sampling works. We welcome all feedback on the new guide and would like to hear what details you would like to see explained in more detail. More information will be added to this guide over time, in particular regarding features surrounding the adaptive sampling capabilities: coverage tracking, multiplexing, barcode balancing, etc.

6. Introduction

Adaptive sampling introduction

In some sequencing applications, the focus of study — a single gene, or a selection of genomic regions — makes up a small fraction of the genome or sample. In these cases, whole-genome sequencing can be inefficient and costly. Targeted sequencing is a term used to describe

strategies that reduce the time spent sequencing regions that are not of interest, which significantly reduces the amount of data required to achieve the desired depth of the regions of interest. This reduces sequencing costs and the data analysis burden, and enables a quicker workflow. Targeted sequencing using nanopore technology can be achieved in several ways:

- amplicon sequencing
- pull-down
- adaptive sampling

Oxford Nanopore sequencing allows real-time decoding of the region of the genome being sequenced. This characteristic allows decisions to be made in real time on whether a particular strand is of interest or not. This called adaptive sampling, and it can perform real-time selection of reads when the sequencing software (MinKNOW) is supplied with a .bed file containing the regions of interest (ROI) and a FASTA reference file.

Adaptive sampling offers a fast and flexible method to enrich regions of interest by rejecting off-target regions: target selection takes place during sequencing itself, with no requirement for upfront sample manipulation. Prepare and load the library as normal and select "adaptive sampling" in MinKNOW (you will need to upload a FASTA file with the reference as well as a .bed file detailing the regions of interest). Once sequencing begins, due to the real-time nature of nanopore sequencing, MinKNOW identifies whether the strand that is being sequenced is within the ROI. If the read does not map to the ROI, MinKNOW reverses the polarity of the applied potential, ejecting the strand from the pore so that it is able to accept a new strand. Off-target strands are continually rejected until a strand from the ROI is detected and sequencing is allowed to proceed.

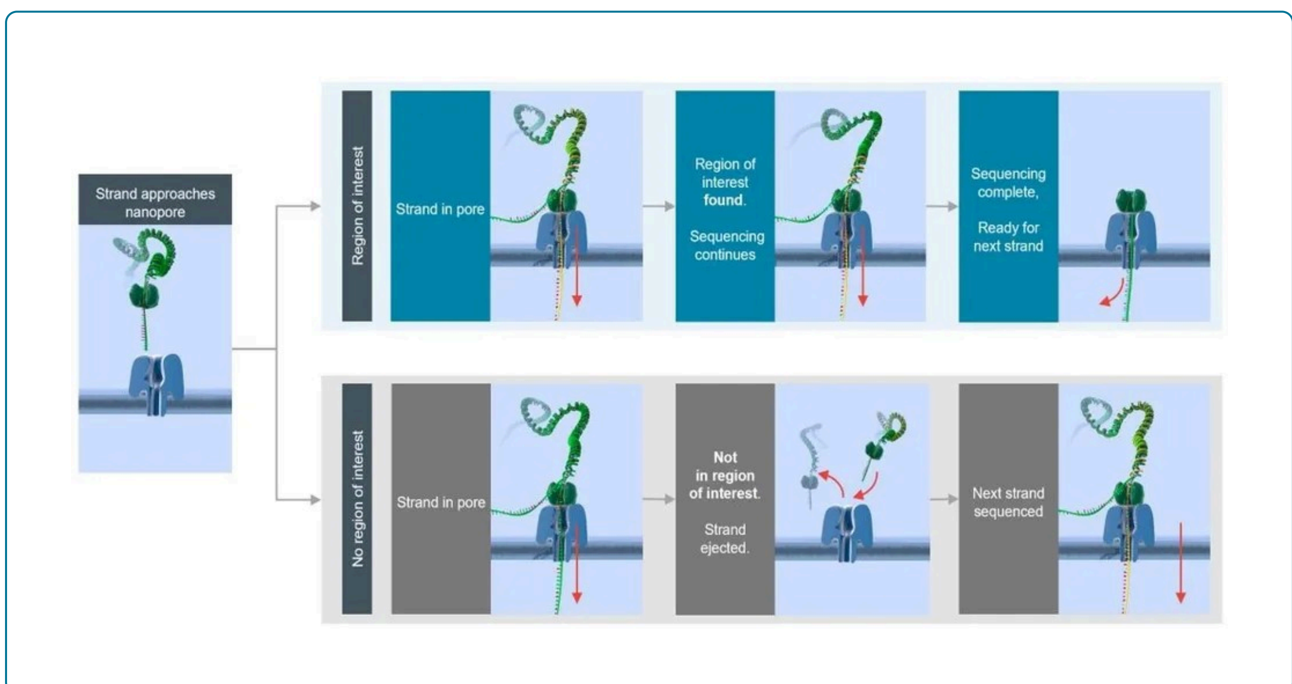

Figure 1. Overview of an adaptive sampling experiment

Adaptive sampling can run in two different modes: enrichment and depletion. In enrichment mode you would upload ROIs to MinKNOW, which then rejects strands that fall outside of these regions. In depletion mode, upload targets that are not of interest (e.g. host DNA in a host : microbiome metagenomic analysis) to MinKNOW, which then rejects strands that fall within these regions. We observe an enrichment for ROI of ~5-10-fold when using adaptive sampling, and we outline our advice on how this can be achieved below. When targeting regions within human

genomes, we find this level of enrichment to be robust if the total fraction that is being targeted is <10% of the total genome. This allows you to obtain a mean depth of >20-40x of ROI on a MinION Flow Cell.

For a visual overview of adaptive sampling, refer to this video: [Adaptive sampling on nanopore technology].

How adaptive sampling works

Adaptive sampling is used to target ROIs in the genome by ejecting strands from the pore that do not map in these regions. As strands are captured by the pore the start of the reads are basecalled and aligned to a reference. If the strand maps to the ROI provided, the software allows the strand to continue through the pore and be sequenced. If the strand is off target, it is ejected from the pore by reversing the applied potential at the electrode and released back to the top side of the membrane. Rejection of strands reduces the amount of time that pores are occupied with a strand, freeing the pore to capture another strand which could be of interest.

Adaptive sampling can be run in two modes:

Enrichment: Sequences present in the target file are accepted and sequenced. In this mode, decisions follow the logic:

- If sequence aligns in .bed region: Accept
- If sequence aligns outside of .bed region: Reject
- If sequence does not align: Reject

Depletion: Sequences present in the target file are rejected back to the cis side of the membrane.

- If sequence aligns in .bed region: Reject
- If sequence aligns outside of .bed region: Accept
- If sequence does not align: Accept

Notes:

- Reads which cannot be aligned have different destination in enrichment and depletion modes, according to the description above.
- If only a FASTA file is uploaded then the previous rules would apply equally to this file instead of the .bed file.

Target sequences are defined by use of the .bed format file. This file specifies the start and end coordinates of the target sequences based on a reference (FASTA) file which contains the actual sequence of the sample. If you do not provide a .bed file, then the reference (FASTA) is not subset, and all the sequences present in the reference will be used as a target. This means that if no .bed file is provided, in enrichment mode, everything present in the reference will be accepted, and in depletion mode, everything will be rejected.

The best practice, regardless of whether you are using adaptive sampling in enrichment or depletion mode, is to provide a FASTA reference containing as much information about the sample as possible, and then use a .bed file to subset the ROIs. This is because when a particular sequence in the sample is not present in the reference, it is more likely to be 'force-aligned' to the wrong reference. For example, if you target the complete sequence of chromosome 7 from a human sample and provide a FASTA reference containing only chromosome 7, sequences from

other chromosomes will be force-aligned to the chromosome 7 reference. This will cause for a large amount of off-target sequences to be accepted, affecting the enrichment ability of adaptive sampling.

Defining concepts and vocabulary

ROI (Region of Interest)

The ROI is your region of interest, and in this guide, it will be used to refer to your actual target of interest excluding the buffer regions. For an adaptive sampling experiment, submit a .bed file containing the coordinates for the ROIs + buffer region. This file is uploaded under the reference input in the “Adaptive sampling” section in the **Run Options** tab the run setup in MinKNOW.

You can upload another .bed file in the **Analysis** tab in MinKNOW UI under the alignment options. MinKNOW uses this file to check whether a strand falls within the targets set in this .bed uploaded in the **Analysis** tab. This is a separate process to the adaptive sampling alignment and is used to calculate coverage of targets in real-time whenever live alignment is turned on. The presence of this second .bed file also populates the column `alignment_bed_hits` in the final `sequencing_summary.txt`. This alignment .bed file is optional and not necessary for the normal functioning of adaptive sampling. Because the alignment .bed file is used for real-time coverage calculation, you can make this calculation more accurate by providing a .bed file containing ROIs only, as opposed to the .bed loaded in the **Adaptive Sampling** section where the .bed contains ROI + buffer. More information on how and where to load each one of these files is shown in the “UI information and dialogs” section of this guide.

Buffer region

Buffer regions are flanking regions added to the side of every ROI. Since adaptive sampling only aligns the beginning of each captured strand, these regions allow the software to accept reads which begin with a sequence that may not map to the ROI but may extend into it as the strand continues being sequenced. By accepting reads which map to these flanking regions, you increase the number of accepted reads that hit your target.

Target

“Target” refers to the sequence targeted by the adaptive sampling process. This includes ROI + buffer region and accounts for the total sequence that adaptive sampling uses to decide whether to accept or reject a strand.

The calculation of the percentage of sample targeted by AS needs to consider the prevalence of the targeted regions in the sample. It is important to consider the relative abundance of the targeted sequences in your sample to calculate the total amount of genomic sequence targeted from that same sample. For instance, if targeting 50% of the *E. coli* genome spiked into a human sample, but the *E. coli* genome representation only makes up 10% of the total amount of DNA present in the sample, this means you are targeting 5% of the genomic sequences present in the sample. This is important, as the percentage of sequence targeted from the overall sample affects the performance of the adaptive sampling enrichment.

Note: there is no wrong targeting range, and adaptive sampling will work with any target range and distribution of ROIs. However, the larger the amount you target, the lower your potential enrichment. You can read more details on this in the “Defining buffer regions” section.

Reference

The reference is a FASTA file which contains the sequences present in a sample for a particular sequencing experiment. This should, whenever possible, represent the complete sample. So if your sample contains three genomes, your reference should also contain the sequence for these same three genomes to prevent possible false positives in the adaptive sampling decision. Whenever you provide an incomplete reference (a reference that does not represent the totality of your sample), the software will try to force-align the reads that are not represented in the reference. In the case of similar sequences, this could cause miss-alignments due to the lack of a full reference. A common example of this, is providing the reference for a single chromosome, which will cause similar/repetitive sequences in other chromosomes to be force-aligned to the single chromosome reference provided.

In the case of environmental samples, you may not know all the genomes contained within the sample. An example of adaptive sampling use in this case is a depletion approach to reject all the known genomes present in the sample to enrich for unknown genomes. In this case, a reference accounting for all the sequences in the sample cannot be used.

FASTA files can be obtained from NCBI in full for a particular genome, or subset to contain only particular parts of the genome (this use case will be discussed in more detail for the MinION Mk1C in the "Devices" section of this guide).

Choosing the correct reference FASTA file depends on the ROIs you are planning to target. If the ROIs were obtained from an annotation file, you will need to use the reference FASTA that is coupled with this annotation file. To avoid misalignments, you will need to use the full reference genome when using a target .bed file.

The UCSC Table browser is a good option to look for reference FASTA files and associated annotations files. The NCBI RefSeq database is another good option for accessing reference FASTA files with stable genome annotation for a wide range of organisms. However, these are only two examples of databases that can be useful, and other options are available. The most important point to consider is to use the same reference for the adaptive sampling sequencing run as the reference used to make the annotation.

.bed files

The .bed file is a text file with a minimum of three columns up to 12 (https://samtools.github.io/hts-specs/BEDv1.pdf, but currently only the first three are mandatory for adaptive sampling). .bed files are used to subset the provided reference. The first column is the sequence name identifier from the provided FASTA reference. The second column is the coordinate at which the target region starts, and the third column is the final coordinate of the target region. .bed files function as a mask for the FASTA file, which should ideally contain the sequences for the whole sample, allowing you to define your targets for enrichment.

Advanced options: The 6th column (referring the direction of a sequence) is usable if present. This is not mandatory and targeting will work without it. However by providing the 6th column with the directionality, you are telling MinKNOW you are only interested in that particular sequence from one of the strands only. By duplicating a region and having opposing signs in the 6th column, you can target the sequence in both strands. Additionally you can also use a "." (dot) in the 6th column to express that you are interested in both strands. You can read more details on this in the "Strand directionality" section.

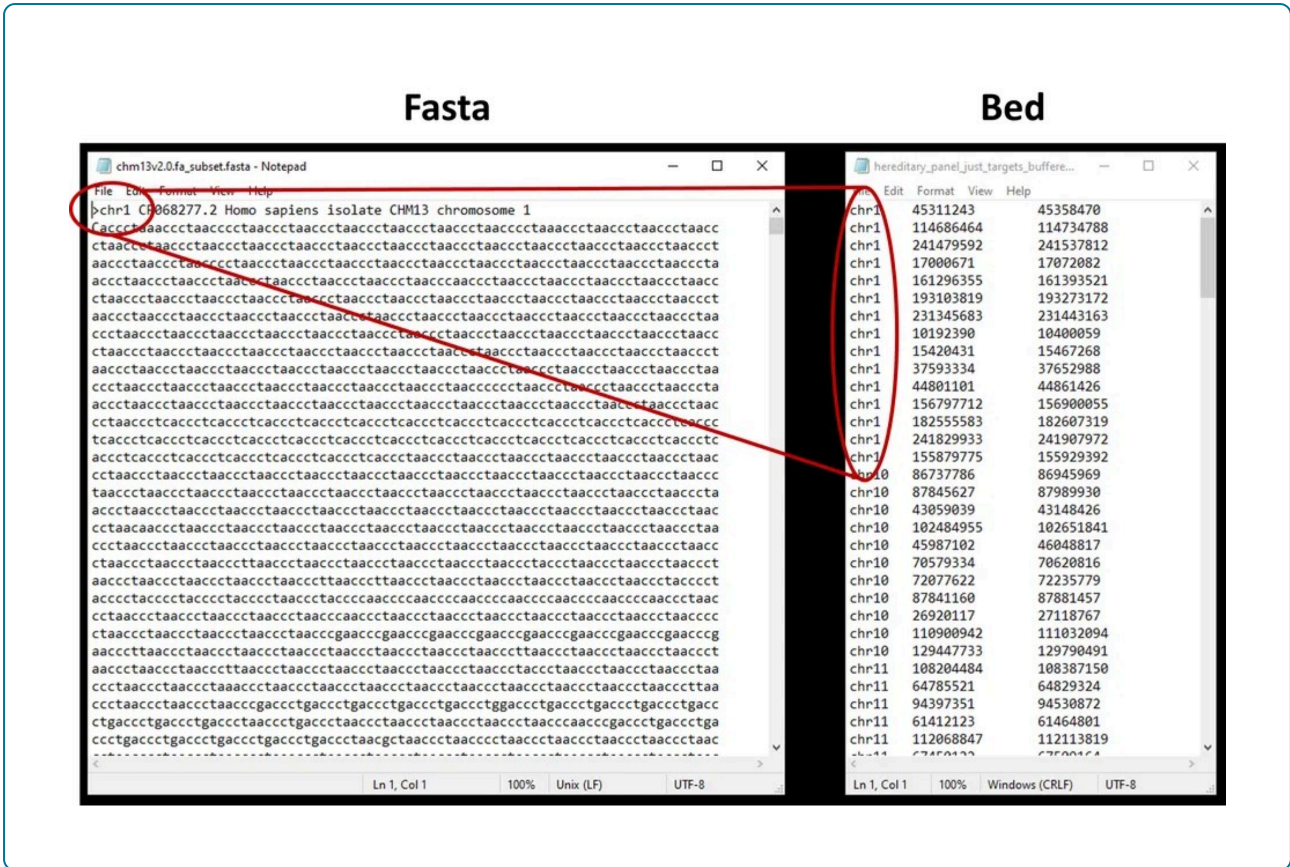

Figure 2. Conjugation of a .bed file sequence identifier with a FASTA identifier (circled in red).

Enrichment

Enrichment is the metric used for evaluating the performance of adapting sampling. This metric compares the on-target output from an adaptive sampling run vs the on-target output from a normal sequencing run. Since the output of a flow cell depends mostly on the number of available pores, output is normalised per number of pores used during the sequencing and then compared with the same normalised output from the non-adaptive sampling run. The calculation is made as follows:

$$Enrichment = \frac{\frac{Condition\ output\ on-target}{\#\ of\ pores}}{\frac{Control\ output\ on-target}{\#\ of\ pores}}$$

7. Sample preparation and analysis

Although adaptive sampling does not require any particular sample preparation, there are some aspects of library preparation which benefit an adaptive sampling experiment.

There are two main aspects to consider when trying to maximise output: pore occupancy and library fragmentation.

Pore occupancy

The adaptive sampling methodology is based on rejecting unwanted DNA strands to free up the pore, ready to capture a new strand. This can cause a significant reduction in pore occupancy, as the constant rejection of strands reduces the amount of time that pores are occupied with a strand. Therefore, maintaining high pore occupancy is one of the most important aspects in adaptive sampling. To achieve this, we recommend loading a higher amount of sample than you would normally use for a sequencing run. The right amount of DNA to load into the flow cell needs to be calculated from the point of view of molarity instead of mass (explained in more detail below).

Library fragmentation

This is important for two reasons: firstly, the fragment length affects the molarity, which is the main measure of DNA to be loaded in an adaptive sampling run. Secondly, adaptive sampling runs are more likely to block pores due to the high amount of strand rejection. Using a library made up of shorter fragments increases flow cell longevity and therefore data output, since the library causes less blocking and gives a higher molarity with lower amounts of total DNA. Not only does shearing reduce blocking, but it can also increase enrichment depending on the size of your individual ROIs. If most of your ROIs are a few kb long (e.g. 2–5 kb), then using a library with an N50 in the 30 kb range is going to be wasteful. This is because every time a strand is accepted for sequencing, the pore will be occupied sequencing 30 kb of data to extract 2–5 kb of on-target sequence. This is a potential waste of 23–25 kb, when the pore could be sampling more reads during this timeframe instead of sequencing off-target. Lastly, the use of longer fragments may cause the flow cell to block longer, requiring flow cell washes to be performed more frequently to extract maximum output from the flow cell.

Another method to increase output from an adaptive sampling run is to perform multiple flow cell washes throughout the run and reload the library. However, by reducing the library fragment size you can reduce the number of flow cell washes needed to maximise the output from a sequencing experiment.

Figure 3 shows the difference in pore blocking in adaptive sampling mode over time with two different fragment size libraries: 5 kb and 25 kb. Each bar represents the number of pores available for sequencing every 1.5 hours throughout the run. Attrition of channels due to blocked pores occurs at a faster rate in the library with the longer fragments. Although flow cell washing can recover some lost pores, it is a hands-on process which adds hands-on time to the run. For this reason, it is important to give consideration the size of your library when designing adaptive sampling experiments to keep blocking to a minimum and reduce the need to interact with the flow cell.

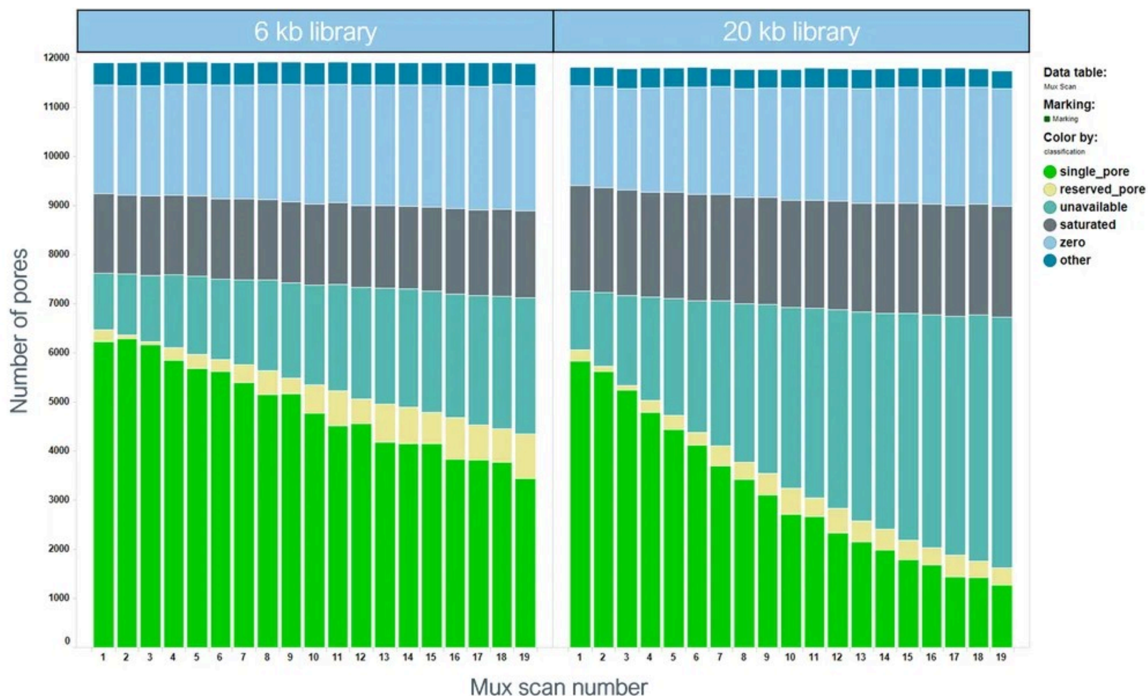

Figure 3. Pore scans for 6 kb (left) and 20 kb (right) libraries in adaptive sampling runs without flow cell washes.

The fragment size will also affect the molarity of your sample if you are using a Qubit or other mass-related measurement to calculate the amount of DNA loaded into the flow cell. Qubit is the recommended method for evaluating your DNA library concentration, but this should be converted into a molarity concentration which can be done based on your average fragment length. You can evaluate fragment lengths using the Agilent Femto Pulse (for fragments >10 kb), or the Agilent Bioanalyzer (for fragments <10 kb).

Using the average molecular weight of a base pair (660 g/mol), you can easily calculate the molarity of the sample. This will make the mass of DNA needed for short and long libraries quite different when normalising for the same molarity. Molarity is an important property to consider, since the number of DNA ends available to be captured by the pore is the main factor in improving pore occupancy. The ideal molarity when using the latest V14 chemistry is 50–65 fmol per load.

With a library which has a normal read length distribution centred at 6.5 kb (Figure 4), 50 fmol would correspond to approximately 200 ng, according to the following calculations:

- Total mass of a mole of 6.5 kb fragments: 6500 base pairs x 660 (g/mol) = 4,290,000 g in 1 mole
- Multiply this by the number of femtomoles needed: 4,290,000 x 50 x 10^-15 = 2.145 x 10^-7
- Convert grams to ng: 2.145 x 10^-7 x 1,000,000,000 = 214.5 ng

To facilitates these calculations, you can use a biomath calculator such as the following: https://www.promega.co.uk/resources/tools/biomath/

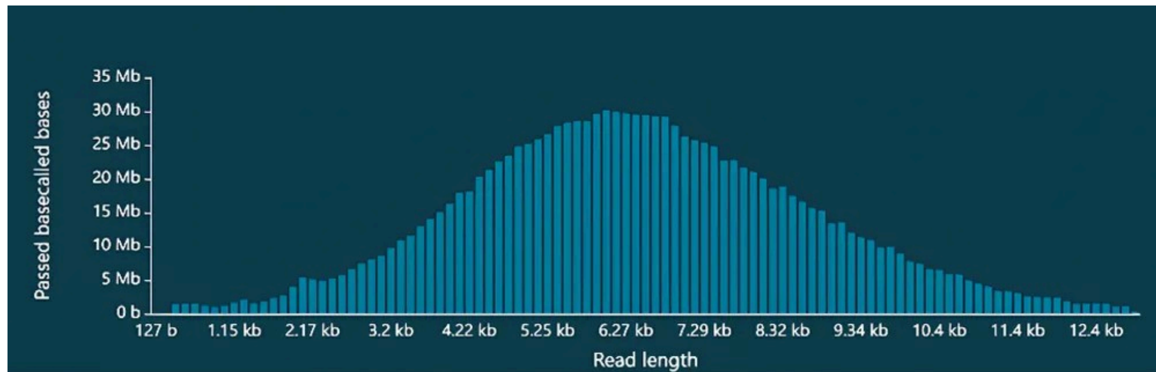

Figure 4. Read length distribution of a library with an N50 of 6.5 kb.

This is a rough approximation, based only on the N50 of the library. The real molarity calculation is more intricate to calculate, as you need to consider the range of the distribution. Nevertheless, this is a good approximation to understand the amount of DNA you would need for an adaptive sampling run. It is also worth noting that these calculations and values are assuming optimal ligation efficiencies. If for any reason it is suspected or shown that a library may not be ligating as efficiently, adding an extra amount of sample is advised. It should be noted that a higher DNA input has not been shown to affect the run negatively when using Kit 14 up to a maximum of 600 ng.

8. Targeting and buffering

How an adaptive sampling decision is made

Disclaimer: *examples and schematics shown in most of this section are for adaptive sampling run in enrichment mode. Please read this section even if you plan to use depletion mode. The end of this section will build on the knowledge presented for the enrichment case, to provide advice for depletion mode use cases.*

When creating .bed files for adaptive sampling experiments, it is important to have a general idea of the mechanisms behind the decision process. Adaptive sampling is a tool for real-time basecalling and alignment of the reads that are inside the pore at any moment. This means that as soon as a strand enters a pore, the software starts trying to make a decision on whether this strand is of interest or not. For this, the following steps are executed in this order:

1. The software acquires one second of data (letting the strand go through the pore for one second to acquire the first 400 bases – this is called “the adaptive sampling (AS) chunk”).
2. The adaptive sampling (AS) chunk is sent to the basecaller*.
3. The basecalled sequence is aligned to the reference you provided to MinKNOW.
4. The location where the sequence aligned to the reference is checked against the .bed file (or reference only if .bed not provided) that you provided to MinKNOW.

5. A decision is made based on whether the strand aligned inside or outside the sequences defined in the .bed file.
6. The strand is left to continue sequencing or ejected from the pore and not sequenced.

*The basecalling and alignment process in adaptive sampling is independent of the live basecalling and alignment shown in the running options of MinKNOW. Adaptive sampling has its own basecaller and aligner; this is set in the background and cannot be modified or turned off.

There are also cases where strands do not align to the reference at all. There are multiple reasons for this, but the two most common are low strand quality or an incomplete reference. For this reason, a reference should always represent the entirety of the sample. If there are genomes or sequences present in the sample which are not present in the reference, or the reference only contains partial genomes, this will cause some strands to not get aligned as well as increase the likelihood of false positive alignments. If you are using a complete reference and still see a lack of alignments, this can come from long pore blocks or low q-scores from the library being sampled.

Adaptive sampling uses the Fast basecalling model (independently from the live basecalling function in MinKNOW) to basecall the first chunk of acquired data as quickly as possible, and then uses a special set of alignment parameters (different from live basecalling alignment) to quickly align these short sequences to the provided FASTA reference. The alignment makes use of minimap2 short read mode preset (-sr) with some modifications to the default parameters. After the alignment, the region to which the chunk was mapped is checked against the provided .bed file, which carries the target regions for adaptive sampling. This is shown in Figure 5.

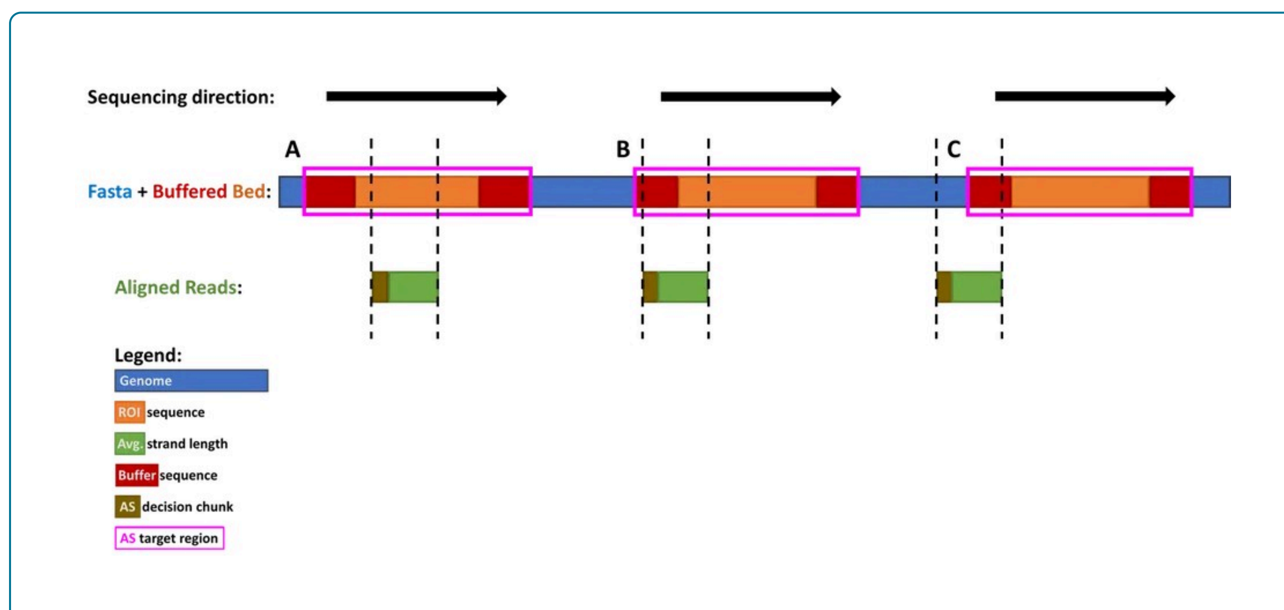

Figure 5. Schematic of all the important components for the adaptive sampling alignment and decision process. The top strand is the reference sequence (blue, red and orange), and in green is the actual length of the DNA strands, with brown marking the beginning of each strand that is read by adaptive sampling to make a decision. The first example (A) is where the strand's first adaptive sampling chunk (brown) maps within the target region defined by the .bed file, and this strand will be accepted for sequencing. In the second example (B) the adaptive sampling chunk falls within the buffer region (in red) and therefore the strand is accepted for sequencing. In the third example (C), the first chunk falls outside the target region and the buffer region; this leads to a rejection of this strand from the pore, meaning it will not be sequenced.

Defining buffer regions

Defining correct buffer regions is one of the most important tasks before starting an adaptive sampling run. Buffer regions allows you to capture strands which may align to flanking regions of your ROI but as they continue being sequenced, will eventually reach the ROI. This allows you to offset possible coverage drops at the edges of your targets.

Figure 6 shows a similar schematic to Figure 5, but using buffer regions.

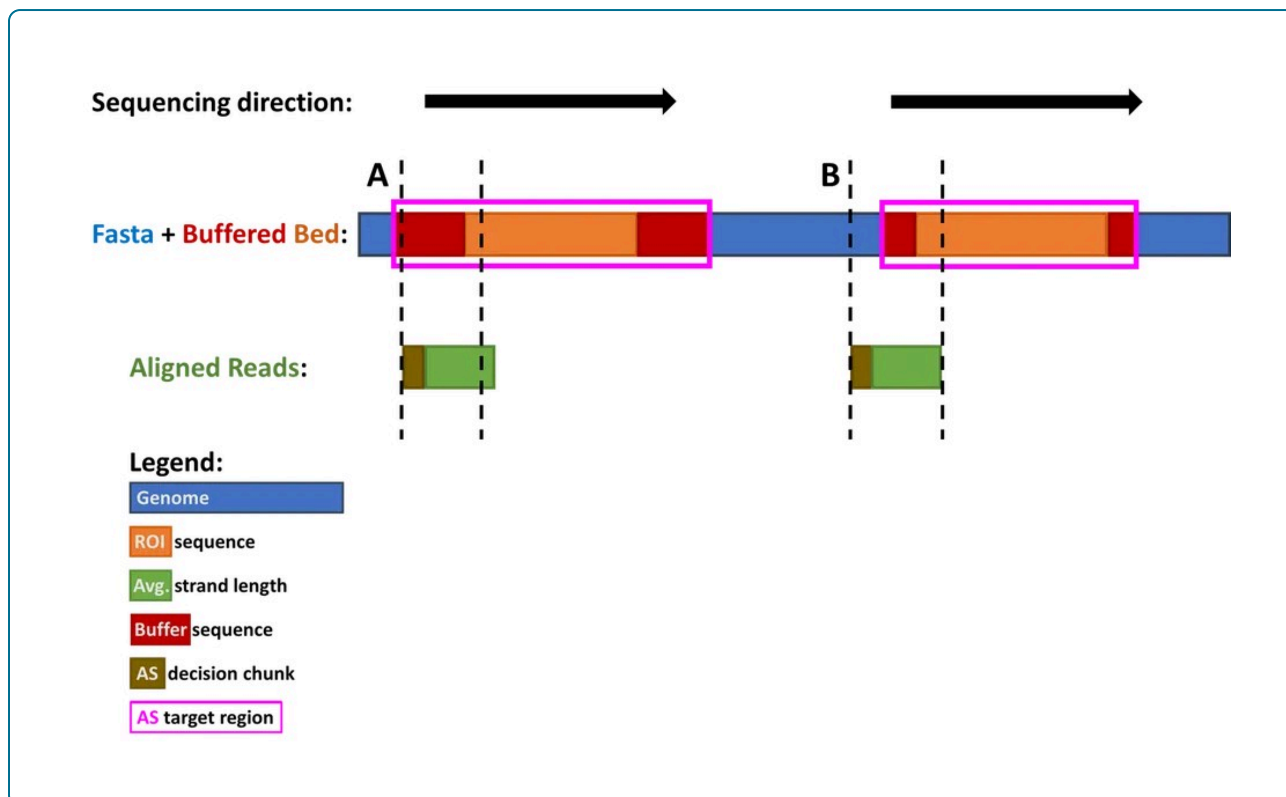

Figure 6. Examples of A: a well-defined buffer region, and B: a badly-defined buffer region.

In example A, given the AS target region (pink), the software would accept any strand which contains a sequence with any regions shown in pink (ROI and buffer). On the other hand, in example B, the buffer was not defined correctly according to the size of the strand. This results in a strand that contains some of the target region being rejected, because the first ~400 bases that are used for the adaptive sampling decision lie outside the buffer region. This example demonstrates the importance of picking the right length of buffer to add to the flanking regions of the ROIs. It is not possible to accurately know the length of a strand until after the entire strand is sequenced. Nevertheless, you can estimate its length based on the read length distribution of the library as shown in Figure 4. By doing this, you can define the buffer regions for the individual ROIs. Table 1 below summarises potential outcomes when defining buffer regions.

Diagram	System description	Comments
Fasta + Buffered Bed: Aligned Reads:	Short read length library; small buffer size	Ideal strand acceptance rate; maximum enrichment
Fasta + Buffered Bed: Aligned Reads:	Short read length library; large buffer size	High acceptance rate; diminished enrichment
Fasta + Buffered Bed: Aligned Reads:	Long read length library; large buffer size	Ideal strand acceptance rate; maximum enrichment
Fasta + Buffered Bed: Aligned Reads:	Long read length library; small buffer size	Low acceptance rate; diminished enrichment

Legend:
Genome
ROI sequence
Avg strand length
Buffer sequence
AS decision chunk
AS target region

Table 1. Summary of how different buffer sizes and fragment lengths affect the adaptive sampling outcomes.

How to define the right amount of buffer

The buffer size should be defined based on the library's read length distribution. Figure 7 shows the read length distribution of a library fragmented with a Covaris g-TUBE. Different Nxx values are marked for this library.

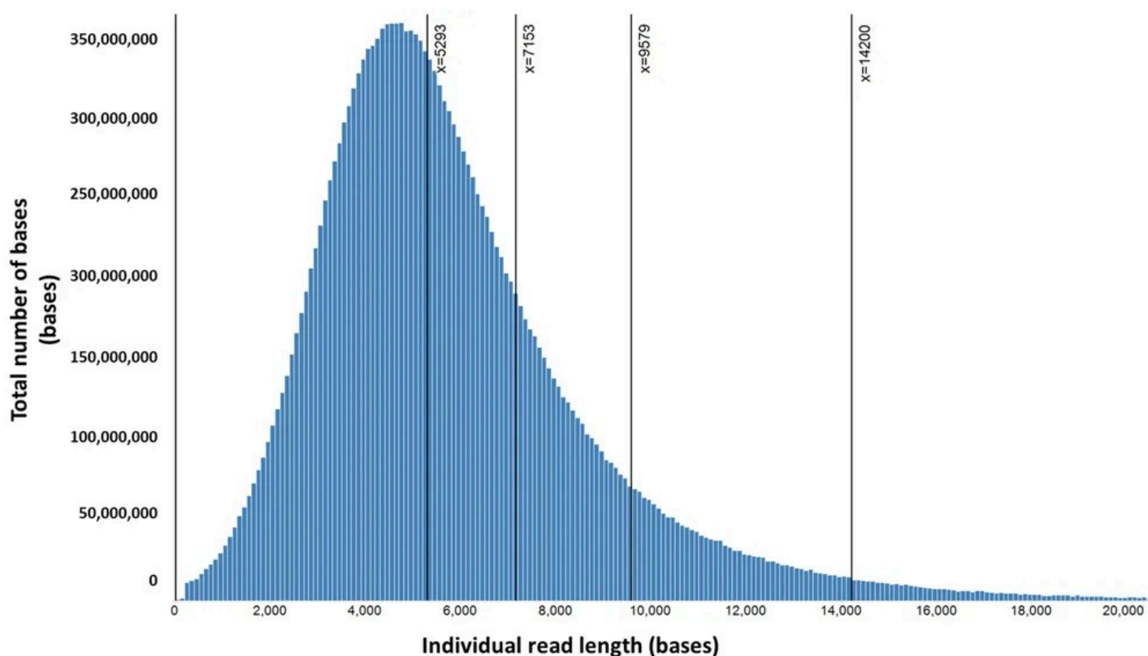

Figure 7. Read length distribution of a 5 kb library fragmented with a Covaris g-TUBE. The vertical lines show the N50, N25, N10 and N01 of the read length distribution from left to right.

As a rule of thumb, an N25 to N10 of the read length distribution is a good measure of buffer to be added to each side of each individual target. Figure 8 shows the results of an enrichment experiment from two distinct sized libraries enriched using different buffer sizes.

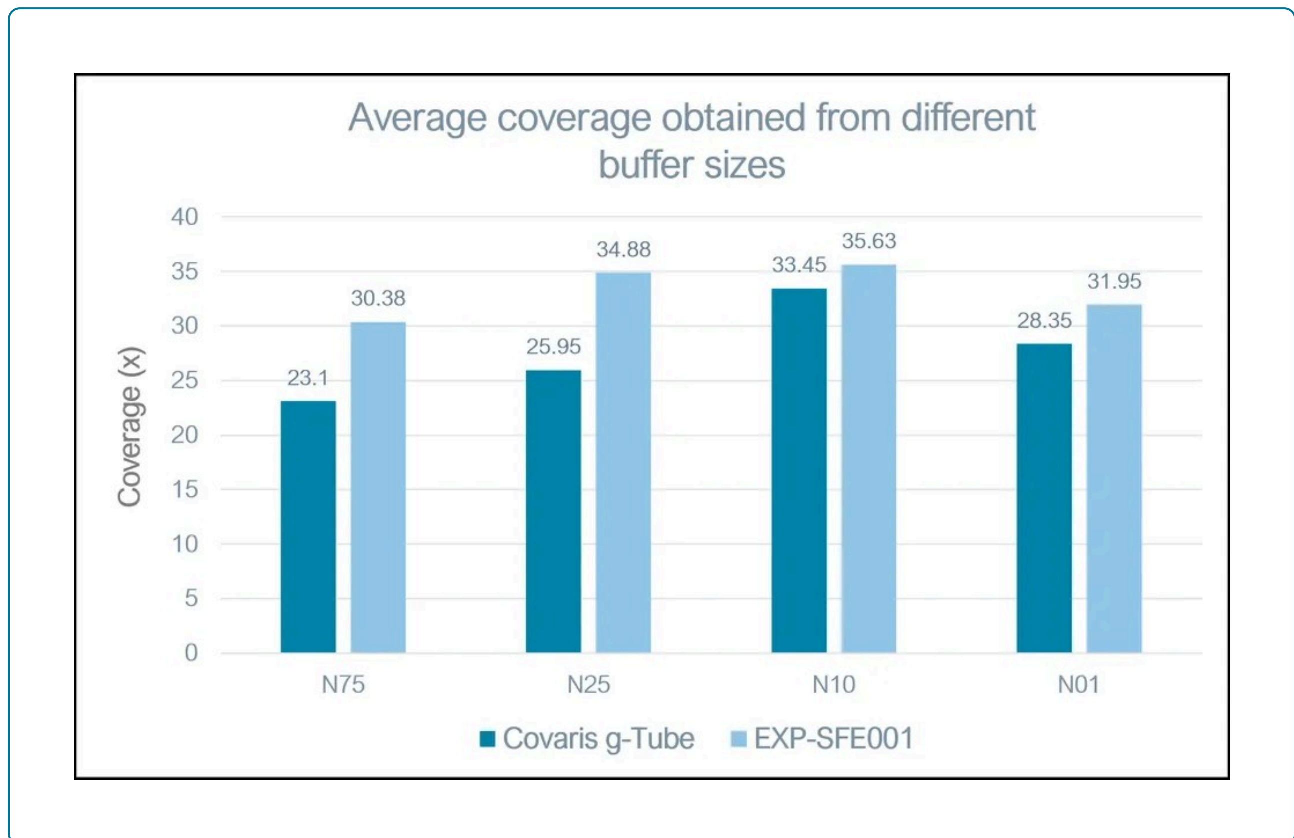

Figure 8. Enrichments obtained from libraries with different buffer sizes, prepared using the Short Fragment Eliminator Expansion (EXP-SFE001) and the Covaris g-TUBE.

However, the buffer size also depends on the details of your ROI. Because the buffer is added on a per-individual region basis, the total number of individual regions becomes an important measure when deciding the amount of buffer to add. As mentioned in the beginning of this guide, the total amount of target given to adaptive sampling to make a decision (ROI + buffer) will determine the degree of enrichment. Because of this, it is important to consider the amount of buffer added to the target .bed file.

Notes:

- Overlapping buffers from closely-located individual targets will not cause any issues. However, in the case of an overlap, adaptive sampling will treat the overlapping regions as a single region rather than two separate ones.
- Consider also the minimum coverage per individual ROI. The purpose of the buffer is to help with drop-offs in coverage at the edges of your ROIs. Therefore you may consider a slightly lower amount of average coverage in favour of a more homogeneous coverage intra-ROI (meaning a higher minimum coverage). A simulation ran over 60 different conditions depicts the impact of the buffer size on the minimum coverage obtained per ROI. As

shown in Figure 9. below, increasing the amount of buffer reduces the difference between the minimum coverage and the average coverage of a given region. This is understandable, as increasing the buffer allows you the chance to capture longer reads which will only overlap with the ROIs at their very end, adding coverage to the edges of the ROI.

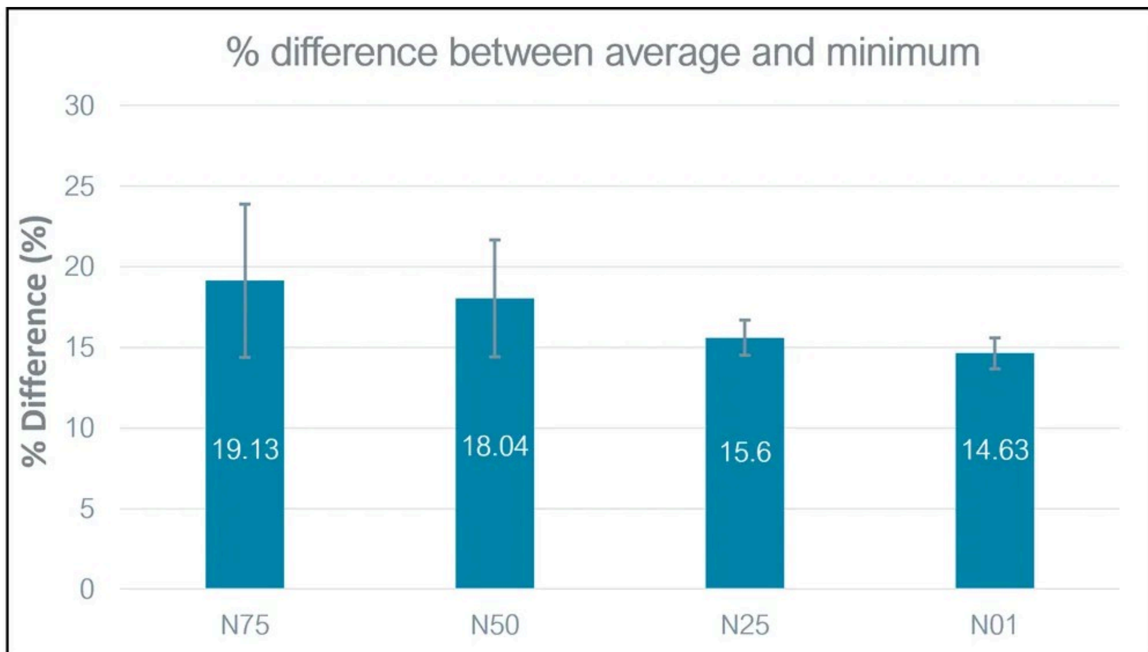

Figure 9. Simulated data showing the difference between average coverage and minimum coverage as a percentage of average coverage (%).

Buffer calculations

The examples below illustrate how adding buffer may affect your adaptive sampling run. Table 2 shows examples of two different ROIs relative to the human genome (3.2 Gbases). The following equation is used to calculate the final target amount for an adaptive sampling decision:

Selection target (bases) = Number of targets ∗ (Nxx ∗ 2) + Total % of genome targeted ∗ genome size

Variable	Example 1	Example 2
# of targets	200	18000
Total % of genome targeted	0.5%	4.5%
Genome size	3.2 Gb or 3200000000 bases	

Table 2. Example ROIs for calculating the total target.

As seen in Table 2, there is a large difference in the number of targets between examples 1 and 2. According to the equation above, this will result in a significant difference in terms of total target added from buffer alone. Table 3 contains two columns for each of the previous examples. The first column contains the total amount of target (ROI + buffer) in bases, and the second column (%) depicts the value of the first column as a percentage of the human genome. All of the values shown for each Nxx are based on the read length distribution shown in Figure 7 above. The second column is effectively the percentage of sample targeted by adaptive sampling assuming sample homogeneity.

Nxx\Sample	Example 1	Example 1 (%)	Example 2	Example 2 (%)
N50	18117200	0.57	33454800	10.45
N25	18861200	0.59	401508000	12.55
N10	19831600	0.62	488844000	15.28
N01	21680000	0.68	655200000	20.475

Table 3. Total number of bases targeted for each condition in Table 2 using various buffer sizes. The amount targeted is also expressed as a percentage of the human genome in the second column of each example (%).

As seen from Example 2 using N01, adding a buffer increases the adaptive sampling target from an initial 4.5% of the sample (ROI only) to 20.5%. However, in Example 1, adding the highest amount of buffer results in an adaptive sampling target of only 0.68% of the total sequence present in the sample. These are two opposite cases. In Example 1, adding an N01 of the read length distribution maximises the chances of sequencing a strand that may still extend into the target without significantly increasing the total sequence target. However, in Example 2, using the largest buffer size increases the target four-fold, leading you to target over 20% of the sample. Such a high amount of target decreases the adaptive sampling enrichment power by keeping the pores occupied for too long sequencing off-target reads.

One way to avoid this is to reduce the Nxx used for adding buffer. In Example 2, an N50 would be a more reasonable amount of buffer to add to the ROI. Another way of reducing the buffer is to reduce the average length of the library fragments. If your library has a read length distribution of >15 kb, shearing the fragments to make them shorter would reduce the values of the Nxx values picked from the read length distribution.

9. Library length considerations

Library length considerations

As shown in the previous section, the ideal buffer size to be added depends on several factors, the most important being the read length distribution of your library. The average length of your library will be important when considering how much you want to get out of your run. If you are sequencing a library with very short read lengths (~1000 bases), this results in minimal blocking and also provides the least benefit per flick. Assuming correctly defined buffers, the “benefit per flick” is the amount of sample that you sequence through when you reject a read. For instance, if

your library has strands averaging 1000 bases, then every time a read is rejected, the software "sampled" 1000 bases of the sample. On the other hand, if your library has an average read length of 30 kb, every time a read is rejected, the software sampled 30 kb. Since the time to to make a decision and reject is the same for short and long libraries, you can sample the full genome much faster if you are using longer fragments.

However, there are some downsides to sequencing long libraries. Although they provide faster sampling power and the ability to phase out regions that you are targeting, they will lead to a higher rate of flow cell blocking as well as require a higher amount of input to load the necessary molarity to keep the pore occupancy high. Long libraries will also require more frequent flow cell washes, and may generate a lower total amount of data from the flow cell due to the higher chance of terminal pore blocking.

Lastly, it is important to think about the distribution of your targets and their average size. If your .bed file contains 20,000 targets with an average size per target of 3 kb, using a 30 kb library could prove detrimental. Aside from a higher rate of pore blocking, every time the pore accepts a strand for sequencing, it will sequence an average of 30 kb (which at 400 b/s takes 75 seconds), yielding only 3 kb of useful sequence. With a decision time of ~2 seconds, this gives 75/2 = ~38 reads that the software could have sampled through. In this example, the total target is 60 Mb (without buffer), which corresponds to ~2% of the human genome. This translates to a probability of finding your target in 1 out of 50 reads sequenced. Therefore, sequencing a single 30 kb strand prevents the sampling of 38 reads out of the necessary 50 reads you need to find the next on-target read. This will detrimentally affect enrichment and the total coverage obtained from using adaptive sampling.

A better use case of adaptive sampling would be to target e.g., 200 individual regions, with an average of 60 kb per target. A library sheared with the Covaris g-TUBE (N50 of ~6-8 kb) would be ideal for maximising output as well as minimising pore blocking and wasted sequencing. Adding a ~20 kb buffer, corresponding to ~N01 of the library, would minimise the difference between average and minimum coverage, giving the ideal output.

10. Strand directionality

Strand directionality

Another factor to consider when designing your buffer regions is strand targeting with respect to directionality. Side-specific buffering allows reads to be correctly accepted on either side of the target depending on the strand of origin (see the IGV plot in Figure 10). **Note that this is an advanced feature and not necessary for the correct functioning of adaptive sampling.** Use discretion if you are adding the directionality function for buffering.

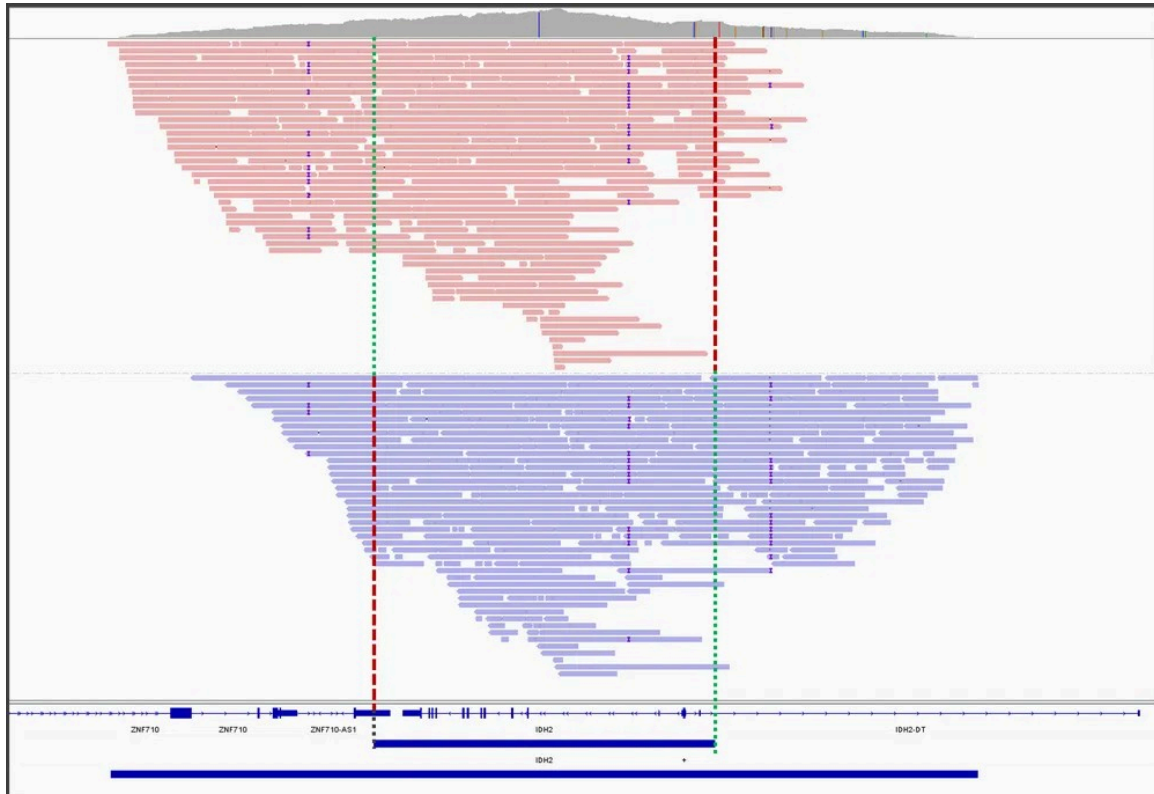

Figure 10. IGV plot, coloured by strand direction, showing the effect of strand-specific targeting using the 6th column of the .bed file. Red lines represent the cut-off at each strand, where the sequence is not accepted anymore for sequencing, and they match the end of the ROI (no buffer) in each direction.

Setting the buffer to be side-specific prevents the acquisition of reads which start downstream of the ROI. This can be seen in Figure 11, where there are no reads starting after the red lines in each direction. However, reads which start within the ROI can still extend beyond the end of the ROI. Directionality can be specified by using the 6th column of a .bed file (Figure 11). More information is available from the following links:

https://samtools.github.io/hts-specs/BEDv1.pdfhttps://en.wikipedia.org/wiki/BED_(file_format)

You can add a side-specific buffer to your ROIs, for example, only adding buffer upstream of the target sequence. To do this, include two lines in the .bed file for the same target, one for each strand direction, and add the buffer amount upstream of the targeted sequence. For example:

```
Chr1		“start-buffer”	“end”			“name”	“score”
+

Chr1		“start”			“end + buffer”	“name”	“score”
-
```

```
chr5	6569183	6643142	NSUN2	0	+
chr9	36802927	37044609	PAX5	0	+
chr2	189753776	189887937	PMS1#	0	+
chr3	12553459	12674293	RAF1	0	+
chr3	141456862	141625673	RASA2	0	+
chr4	56877857	56945868	REST	0	+
chr1	155867775	155921392	RIT1	0	+
chr8	56037271	56085307	RPS20	0	+
chr19	49605282	49650167	RRAS	0	+
chr10	110888942	111024094	SHOC2	0	+
chr2	38951050	39131378	SOS1	0	+
chr14	50086835	50242156	SOS2	0	+
chr4	56437476	56513950	SRP72	0	+
chr3	169734604	169775083	TERC	0	+
chr14	24209626	24252698	TINF2	0	+
chr10	129435733	129782491	MGMT	0	+
chr2	208206243	208266317	IDH1	0	+
chr15	90052993	90143663	IDH2	0	+
chr6	31545564	31588353	TNF	0	+
chr9	21936939	22131191	CDKN2A	0	+
chr2	47339311	47644501	EPCAM	0	+
chr16	2009821	2099634	NTHL1	0	+
chr10	87829160	87981930	PTEN	0	+
chr11	502230	545598	HRAS	0	+
chr7	93069475	93158598	SAMD9	0	+
chr5	112697503	112876242	APC	0	-
chr11	108212484	108399150	ATM	0	-
chr17	65518565	65591652	AXIN2	0	-
chr2	214715644	214839682	BARD1	0	-
chr3	52391025	52440060	BAP1	0	-
chr10	86745786	86957969	BMPR1A	0	-
chr17	43034294	43155363	BRCA1	0	-
chr13	32305479	32429671	BRCA2	0	-
chr17	61669142	61894117	BRIP1	0	-
chr16	68727291	68865536	CDH1	0	-
chr12	57737731	57782317	CDK4	0	-
chr22	28677743	28771873	CHEK2	0	-
chr14	95076229	95188275	DICER1	0	-
chr15	32708007	32775106	GREM1	0	-
chr17	48714763	48758749	HOXB13	0	-
chr3	36983352	37080850	MLH1	0	-
chr5	80644653	80906815	MSH3	0	-
chr2	47773082	47836954	MSH6	0	-
chr1	45319243	45370470	MUTYH	0	-
chr8	89923341	90014724	NBN	0	-
chr17	31084930	31407700	NF1	0	-
chr16	23593164	23671340	PALB2	0	-
chr7	5960927	6039104	PMS2	0	-
chr19	50374291	50448018	POLD1	0	-
```

Figure 11. Example of a .bed file containing six columns. From left to right: chromosome; starting coordinate; end coordinate; region name; score; direction of strand.

Including a side-specific buffer halves the amount of buffer sequence added, since you are only adding the buffer to a single side of the target in each strand. In contrast, when you are not defining a strand direction, you are adding buffer regions indiscriminately to both sides of the targets.

11. Depletion mode

Adaptive sampling depletion mode

In contrast to running adaptive sampling in enrichment mode, in depletion mode you are choosing which sequences to reject rather than accept. This requires a different approach to enrichment mode. Firstly, the advised total targeted amount is the inverse of what you would use for the enrichment strategy. The amount of sample you specify to be rejected needs to be large enough that the pores have the maximum time available to sequence the regions of interest. You can still reach a similar level of enrichment as with the enrichment strategy if your sequences of interest comprise a small amount of your sample (1-5%). This means you would need to create a .bed file which targets/rejects 95-99% of the sample.

The second consideration is that, when depleting unwanted regions from a single genome sample, the buffer size needs to work as a negative value. Therefore, instead of adding buffer to your target file (which defines the sequences to be depleted), you will need to subtract it. Below is a schematic of the logic used to transform an enrichment strategy into a rejection strategy.

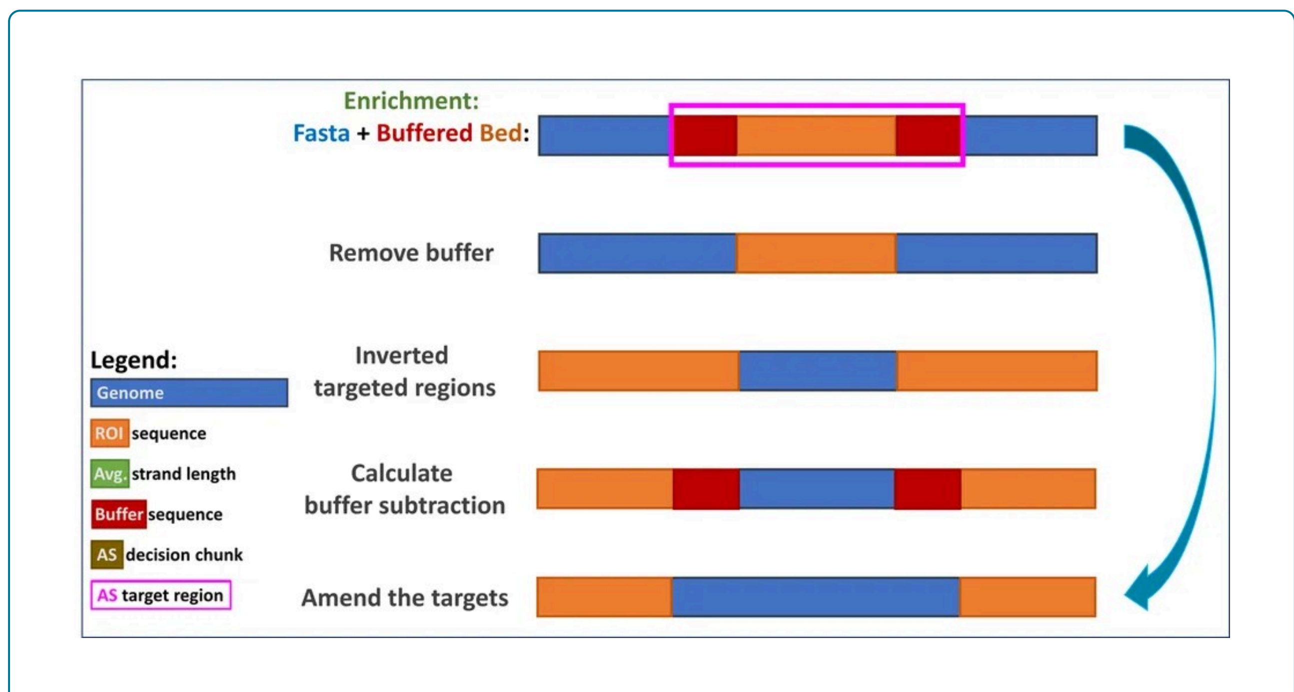

Figure 12. Schematic of how to convert an enrichment .bed file into a depletion .bed file.

The schematic in Figure 12 goes through all the steps from taking an enrichment buffered .bed with known buffers and creating a buffered depletion .bed file. You can easily go from the first to the last step in a situation where the buffer size remains the same. With such an approach, you need to know the total size of a chromosome so that you can target for depletion the entire

region from the end of the ROI to the end of the chromosome. These values may vary depending on the reference used. Figure 13 shows an example of how this would look like in a .bed file targeting two regions of the human chromosome 1:

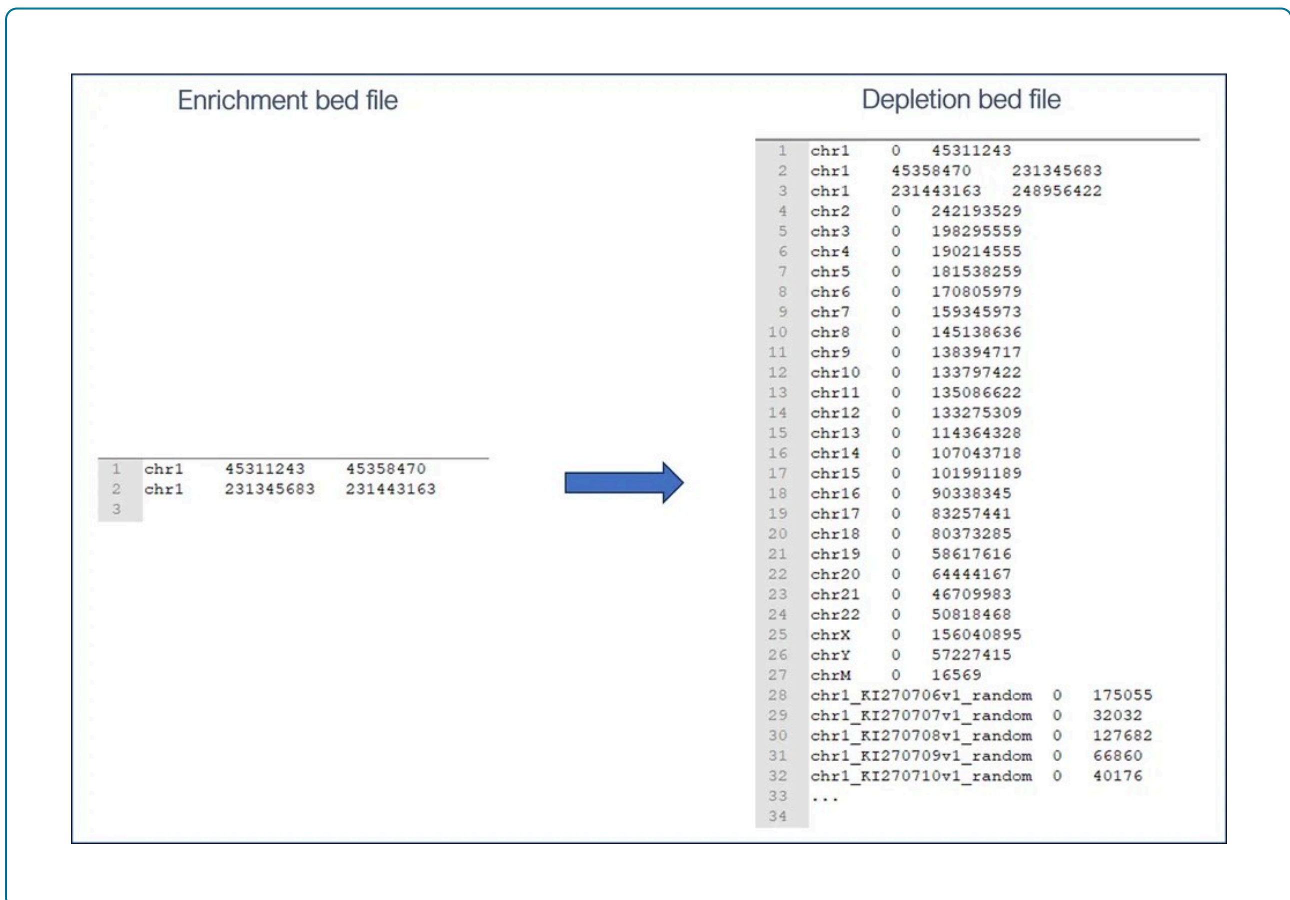

Figure 13. Example of a .bed file converted from an enrichment to a depletion strategy.

If you are trying to enrich for specific regions in your genomes by depleting the rest of the sample, it is easier to set up an enrichment strategy instead. Depletion mode is best used when you want to enrich for unknown sequences or genomes. This is most common in cases where the sample contains a mixture of organisms, and you want to deplete everything already known to be in the sample so that adaptive sampling can enrich for rare or unknown genomes present in the sample.

12. Device specifications

Device specifications for adaptive sampling

Adaptive sampling requires a lot of computing power because of its need to basecall, align, and make a decision on all the strands captured in real-time. We currently recommend turning off live basecalling when running adaptive sampling on more than one flow cell. Running live basecalling during an adaptive sampling run may lead to reduced enrichment (reduction in on-target coverage obtained) due to the lack of resources to handle both basecallers.

For this reason we recommend that nothing else is running on the device. This includes offline basecalling, basecalling from WGS runs or any other processes which may consume CPU/GPU

usage. Once adaptive sampling runs are started, you can check the performance of adaptive sampling by using the "Read length distribution" panel in the MinKNOW UI.

As long as the "Adaptive sampling voltage reversal" value is below 1 kb, the runs should be performing ideally to obtain enrichment of the selected targets. If the value is above 1 kb, consider the following:

- Check that there are no other flow cells running on the device that are sequencing with basecalling, and that there are no flow cells running super accurate (SUP) basecalling models.
- Check there are no other background processes consuming resources (analysis tools, etc.)
- Reduce the number of flow cells being used for adaptive sampling.

Once the changes have been applied, restart the run. The accumulated data during the initial slow period will average out the new data (after turning off features/flow cells) and may mask the effect on the run for a considerable time. For this reason it is best, when identifying an issue, to stop the run, perform the necessary check/modifications, and restart the runs, to quickly observe the effect on adaptive sampling performance.

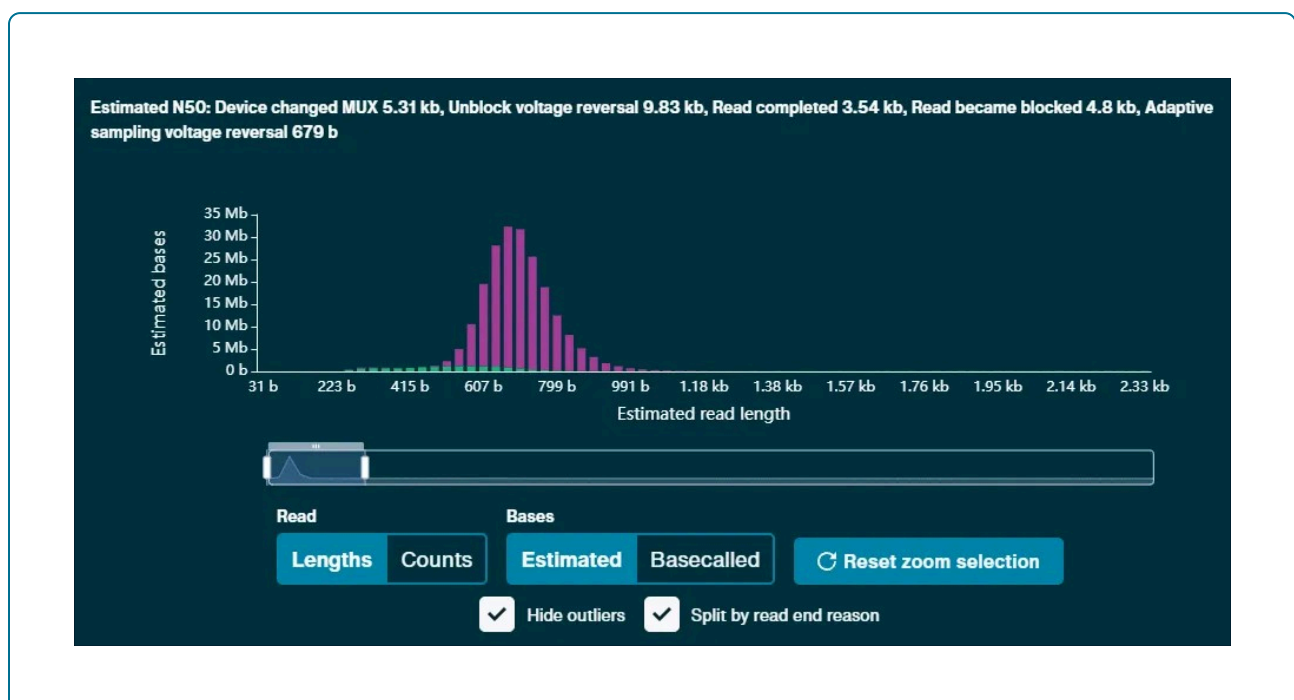

Figure 14. Read length histogram in MinKNOW, showing the estimated N50s.

Alignment and memory limit on the MinION Mk1C

Due to insufficient RAM, the MinION Mk1C cannot hold a fully indexed human reference in memory, and going over the memory limit will crash the adaptive sampling process. Alignment time is another metric to bear in mind when considering the maximum reference file size for the MinION Mk1C. The larger the reference provided, the longer it takes the adaptive sampling process to run through the indexed reference to find the alignment. For this reason, we advise uploading no more than 125 Mb of non-indexed reference (FASTA). Above this size, the MinION Mk1C will experience heavy delays in the adaptive sampling decision.

Final notes

In devices with larger amounts of memory, you can align your sequences to larger reference files if you are not using adaptive sampling (for example, by performing regular sequencing with live alignment). Nevertheless, you can expect the speed of the live basecalling to decline if the references size exceeds the suggested values.

Do not pre-index your reference files before uploading them to MinKNOW to use with adaptive sampling. In the latest version of MinKNOW, the software automatically indexes the reference at the beginning of the run, and the run cannot start until indexing is completed. This allows Oxford Nanopore to keep the alignment parameters flexible and to optimise for the fastest and most accurate alignment for adaptive sampling. If you pre-index your reference, you will most likely not use the same indexing parameters that MinKNOW uses, and this can affect the adaptive sampling decisions, leading to false positives and false negatives.

13. Where to find, create, and modify FASTA and .bed files

Where to find, create, and modify FASTA and .bed files

Adaptive sampling requires the following inputs:

- A genomic reference (FASTA or .mmi file) containing the sequences present in the sample. An .mmi file is also called an indexed reference, and you can obtain this by indexing the reference file (FASTA) before starting the run either through MinKNOW (start page) or by using minimap2. Nevertheless, it is not advised that references are pre-indexed, as the parameters for indexing are subject to change by Oxford Nanopore to maximise adaptive sampling performance.
- The coordinates for the regions of interest, provided in a .bed file. Coordinates should be based on the reference provided, meaning that chromosome names should match with chromosome names used in the reference.

We recommend that the reference you provide represents the complete sample being used. For example, if your sample contains three different organisms, then the reference should contain the complete genome of all three organisms. This will reduce possible false positive alignments that arise from using an incomplete reference.

You can download reference files from trustworthy databases such as the UCSC Table browser and the NCBI RefSeq database. These files will represent the template to align the reads captured during the sequencing run. If more than one organism is present in the sample, download multiple references, one for each organism, and concatenate the files to create a single file with all the references. Check the names of the individual chromosomes to make sure that there is no duplication of chromosome names (e.g. avoid “Chr1” in cat.fasta and also “Chr1” in human.fasta).

Once you have downloaded the correct references, build the .bed file using the existing annotations for your references. In this guide, we will use the USCS Table browser as an example of how to obtain a .bed file to target regions of structural variation in the human genome, as shown in Figure 15. First, pick the clade, genome and assembly corresponding to human, choose

a group. This group corresponds to the type of sequenced you are targeting (e.g. Genes and Genes predictions, Regulation sequences, variation of repetition regions, etc.). This will modify the options available in the track with all the different pieces of annotation work developed for the chosen group. The table will give the available list of regions provided by each track from various combinations. For the current example of Structural variation regions, it is possible to obtain common SV regions for different sets of the human population (drop-down menu shown in Figure 16). This will give a complete list of regions for the whole genome.

Table Browser

Use this tool to retrieve and export data from the Genome Browser annotation track database. You can limit retrieval based on data attributes

Select dataset

clade: Mammal genome: Human assembly: Dec. 2013 (GRCh38/hg38)
group: Variation track: dbVar Common SV
table: dbVar Curated 1000 Genomes SVs (dbVar_common_1000g) describe table schema

Define region of interest

region: genome position chr1:15,000 -15,000,000 lookup define regions
identifiers (names/accessions): paste list upload list

Optional: Subset, combine, compare with another track

filter: create
subtrack merge: create
intersection: create

Retrieve and display data

output format: BED - browser extensible data Send output to Galaxy GREAT
output filename: chr1_SV_human_subset (leave blank to keep output in browser)
file type returned: plain text gzip compressed

get output summary/statistics

Figure 15. Example of settings to download a .bed file for common structural variation regions in the human X chromosome.

dbVar Curated 1000 Genomes SVs (dbVar_common_1000g)
dbVar Curated 1000 Genomes SVs (dbVar_common_1000g)
dbVar Curated African SVs (dbVar_common_african)
dbVar Curated All Populations (dbVar_common_global)
dbVar Curated DECIPHER SVs (dbVar_common_decipher)
dbVar Curated European SVs (dbVar_common_european)
dbVar Curated gnomAD SVs (dbVar_common_gnomad)

Figure 16. List of tables from the dbVar Common SV Track.

You can further subset the list of genes in each of these tables if you want to target regions in a single chromosome. You can do this in the following section of the Table Browser, “Define region of interest”. You can choose the genome or provide an interval of coordinates, as shown in Figure

15. Click **lookup** to activate the selection. If targeting the whole genome, select the "genome" option, and no sub-setting will be performed. You can select further complex options in the "Optional: Subset, combine, compare with another track", however these will not be explained in this guide.

Lastly, in the final section "Retrieve and display data", set the "output format" to **BED – browser extensible data** and a file name given in the input box below. Set "file type returned" to **plain text**, as MinKNOW does not accepted compressed files. Click **get output** to be redirected to a new page.

In this page (Figure 17), ensure that "include custom track header" is **not** selected. Instead, in "Create one BED record per:", select **Whole Gene** to get the exact regions defined in the track. You can also select other options if you are interested in upstream, downstream, or specific strand sequences only. Click **get BED** to download a file with the name previously specified but without an extension. Rename the file to "name_chosen".bed and your .bed file is ready to be used.

By following all the settings in this guide, you will download a file with 62 lines, all of which are from chromosome 1 represented by "chr1" in the first column of every line. You can visualise this by opening the .bed file with any text file editor.

Figure 17. The last page in Table Browser before downloading the final .bed file.

Subsetting a large FASTA file for use with the MinION Mk1C

The MinION Mk1C does not have sufficient memory for large references sizes (>125 Mb). This means that if you are working with a large reference, you will need to subset the FASTA file. In the

case of subsetting a large FASTA where only the regions of interest are present, there is no need for a .bed file, as the reference has already been subset.

Below are instructions for making a subset FASTA file. You will need samtools and bedtools installed on your computer. This may require some previous knowledge of how to use Conda and samtools/bedtools.

1. Open a Terminal window.
2. Install samtools and bedtools using the following commands:
   ```
   wget https://repo.anaconda.com/miniconda/Miniconda3-latest-Linux-x86_64.sh bash
   Miniconda3-latest-Linux-x86_64.sh -b -f -p source ~/miniconda3/bin/activate
   conda create -name adasamp -c bioconda samtools bedtools conda activate adasamp
   ```
3. Navigate to the folder containing your FASTA and .bed files. If they are in different folders, create a link to the location of the FASTA file. If you are already in the folder containing the .bed file:
   ```
   ln -s /long/path/to/my/reference/in/different/folder/myref.fasta myref.fasta
   REF=myref.fasta
   ```
   If you are already in the folder containing the FASTA file:
   ```
   ln -s /long/path/to/my/bed/in/different/folder/mybed.bed mybed.bed
   BED=mybed.bed
   ```
4. Edit the settings for your reference and your .bed with target regions: `BASES_TO_EXPAND_PER_SIDE` = this is the size of the buffer you calculated for each side. `REF=your_reference.fasta` `BED=your_bed_with_target_regions.bed` (The subsetted FASTA file will be called `your_reference-your_bed_with_target_regions.fasta`)
5. These are the intermediate files that will be created: `CHROM_SIZES=${REF}.chrom.sizes` `SLOPPED_BED=${BED%.*}_slop-${BASES_TO_EXPAND_PER_SIDE}.bed` These will be saved in the same location where the commands are being run, and will remain until they are deleted. This is the final file which you will upload into MinKNOW: `SUBSETTED_FASTA=${REF%.}-${SLOPPED_BED%.}.fasta`. This is also saved in the same location where the commands are being run.
6. Index the reference and get chromosome sizes:
   ```
   samtools faidx ${REF} cut -f1,2 ${REF}.fai > ${CHROM_SIZES}
   ```
7. Expand the .bed and extract the FASTA from the expanded .bed:
   ```
   bedtools slop -l ${BASES_TO_EXPAND_PER_SIDE} -r ${BASES_TO_EXPAND_PER_SIDE} -i
   ${BED} -g ${CHROM_SIZES} > ${SLOPPED_BED} bedtools getfasta -fi ${REF} -bed
   ${BED} -fo ${SUBSETTED_FASTA} -name
   ```
   These commands take the .bed file that contains the regions of interest and add a number of bases on either side of the ROI. This is your buffer region for the adaptive sampling selection.
8. Copy the subset FASTA file to your sequencing device.

14. Adaptive sampling catalogue

Adaptive Sampling Catalogue

The Nanopore Community has a page where you can share .bed file the whole community. You may also find other users' .bed files that are relevant to your work. To browse adaptive sampling .bed files submitted by other Community members, or to submit your own, visit the Adaptive Sampling Catalogue. Instructions are provided on the page for how to add a .bed file to the catalogue.

Figure 18. Adaptive Sampling Catalogue page on the Nanopore Community website.

15. Troubleshooting

How to diagnose performance issues

High variability between samples and runs

Before comparing the performance of two runs, take note if both experiments were run under similar conditions. This includes the run settings (number of flow cells, basecalling models, alignment), references and .bed files, sequencing kit and sample preparation protocols.

If you tick the option **Split by read end reason** below the read length histogram, you will see above the graph the mean value for the adaptive sampling rejection peaks as "Adaptive sampling voltage reversal". This values can be used as a proxy of the adaptive sampling decision speed. There may be some variation between runs and samples. However, a variation above 30% may indicate an issue with the run. The most probable cause of the increase in length of distributions of the rejection peak is a delay in the decision. This can be due to slow basecalling or slow alignment. Check that the device is not running any other background processes that are occupying compute resources. If in doubt, reboot the device. If after the reboot the adaptive sampling is still showing a delay in decision time, make sure you are complying with the limits established for the correct performance of each device. This can be revisited in the "Devices" section of this guide.

If after all the checks the adaptive sampling performance is still low, check the metrics for sequencing quality (Q-scores, sequencing speed and device temperature). Issues with sequencing speed could cause an increase in decision time by taking longer to acquire the necessary amount of data.

High percentage of failed reads

The cumulative output, which you can see in the MinKNOW graphs if you are running live basecalling, provides information about the amount of failed reads. This value should typically be below 10%. If this value is >20%, this suggests an issue with the sample, which may then affect basecalling performance and prevent the software from making an adaptive sampling decision on

time. This issue usually also coincides with the presence of a bimodal rejection peak in the read length histogram.

Bimodal rejection peak

A bimodal rejection peak is a clear indicator of poor adaptive sampling performance. Sometimes, a small peak can appear after the main peak. This means that a small proportion of reads are taking longer to get a decision made up. There a couple of reasons which could lead to this:

- Low quality strands can prevent the a decision to be made quickly by requiring more strand to be sequenced until we are confident with the alignment.
- An overloaded system which can be caused by running to many instances of adaptive sampling and/or live basecalling with heavy models.

A smaller peak appearing after the first main peak is not usually a cause for concern. However, when the height of the second peaks reaches ~50% of the first peak, or there is a long tail behind the first peak (as shown in Figure 19), this indicates reduced adaptive sampling performance. The most common cause is too many MinKNOW features or flow cells running simultaneously. To resolve this, either reduce the number of flow cells or switch off live basecalling.

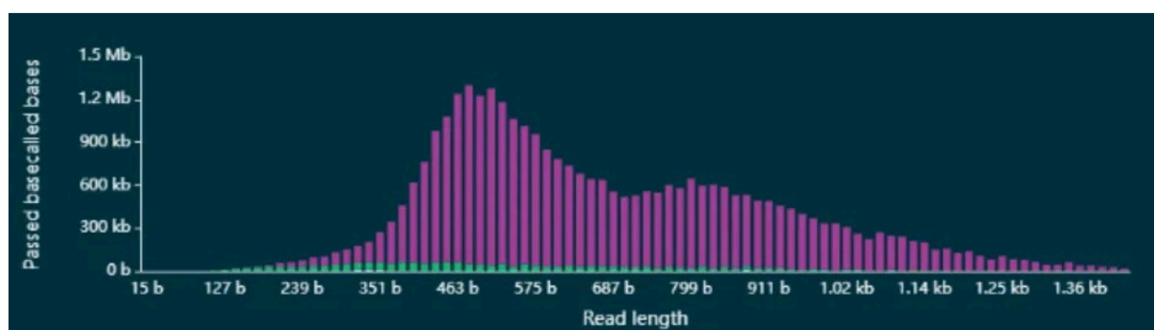

Figure 19. An example of a bimodal rejection peak from the Read Length Histogram plot in MinKNOW for a single flow cell running adaptive sampling.

How to interpret output files

The files that MinKNOW outputs during a sequencing experiment are described in the Data analysis technical document.

For adaptive sampling experiments, there are two additional CSV files named `AS_decisions_x_x_x.csv` and `AS_timmings_x_x_x.csv` that are saved in `other_reports` in the run folder, which you can use for troubleshooting. These files can be "matched" with the sequencing summary by `read_id` to concatenate adaptive sampling information and sequencing summary information. This allows you to see the metrics of each read along side the decision performed by adaptive sampling.

- The `AS_decisions_x_x_x.csv` file containing three columns - `read_id`, `action`, and `action_response`

- The `AS_timmings_x_x_x.csv` containing nine columns - `channel`, `read_id`, `batch_time`, `samples`, `bases`, `barcode_arrangement`, `mean_qscore`, `time_to_package_and_send`, and `time_in_basecaller`

Field	Description
read_id	An individual ID for each read, matching the read_ids provided by the sequencing summary
action	Whether a read was accepted (sequence) or rejected (unblock)
action_response	Whether an action was successfully executed by MinKNOW (`SUCCESS` / `FAILED_READ_FINISHED` / `FAILED_READ_TOO_LONG`)
channel	The channel in which the read was sequenced
batch_time	Batch time of when the read was processed by the Adaptive Sampling script
samples	Number of samples received by the Adaptive Sampling script for each read
bases	Number of bases basecalled from each read for the Adaptive Sampling decision
barcode_arrangement	Barcode arrangement detected on first chunk (this will be empty if not using a barcoding kit)
mean_qscore	Mean Q-score of the Adaptive Sampling first chunk basecalling
time_to_package_and_send	Time between the Adaptive Sampling script getting a read and sending it to the basecaller
time_in_basecaller	Time between sending a read to the basecaller and getting it back from the basecaller

How to verify that you are targeting the correct regions

Using the metrics available in the `adaptive_sampling.csv` in the “other reports” folder and the `sequencing_summary.txt`, you can get an idea of the adaptive sampling performance and to

check whether you are targeting all the regions you intended with your .bed file.

If you know the size of all your regions of interest, you can sum these together to calculate your total sequence target. Divide this value by the size of the genomes present in the sample (if you have multiple genomes in the sample, take into account the prevalence of each genome) to estimate the % of reads that should be getting the decision `stop_receiving`. There will always be a variation of 10-20% depending on the prevalence of the regions targeted, efficiency of ligation for different genome regions, q-score of particular regions of the genome, etc. However, you can use this calculation as a first approximation for whether you are accepting the right number of reads.

This check is worth doing if by looking at the read length histogram, you believe that the number of reads being accepted is too high or too low based on the amount of the sample you believe you are targeting.

If you are getting fewer `stop_receiving reads` than expected, the possible causes are:

- Your buffer regions are too small
- Incorrectly set lines in the .bed file. Incorrect lines are ignored by adaptive sampling
- Sequences are not correctly targeted from the reference
- Sequences are not present in the sample
- There are hard to sequence regions (e.g. repetitive regions) where the aligner cannot decide on the position of the read. This is a very rare case.
- Low q-scores for the reads

If you are getting more `stop_receiving` reads than expected, the possible causes are:

- Your buffer regions are too large
- You have used an incomplete FASTA reference that does not represent the whole sample
- You provided an invalid .bed file. In this case, the system defaults to targeting the complete FASTA reference provided
- There is an error in the .bed file coordinates of a particular region

16. MinKNOW UI and dialogs

During an adaptive sampling experiment, MinKNOW carries out basecalling and alignment with adaptive sampling in parallel with live basecalling. The MinKNOW user interface shows dialogs with information for both processes. Here, we explain where you can find each type of information.

Firstly, there are separate sections in MinKNOW for uploading a live sequencing alignment reference and .bed file, and an adaptive sampling alignment reference and .bed file. Both the reference FASTA file and the .bed file can be the same in both sections (hence the Alignment section is pre-populated with the files uploaded in the Adaptive Sampling section). Nevertheless,

it is important to understand the function of each file as you can get a better ongoing view of coverage obtained throughout the run, by loading a different .bed file in the live alignment section.

The adaptive sampling files are loaded in the **3. Run Options** section under the section named "Adaptive Sampling" - Figure 20, top section. These will be used for targeting your sample and will affect the reads which get selected by MinKNOW for sequencing. The .bed file loaded in this section should also contain a buffer region, when applicable. For more information on this, refer to previous guide sections about buffers.

The live alignment files are loaded in the **4. Analysis** section of the run setup in MinKNOW. The purpose of the FASTA reference is to align the reads after live basecalling and therefore should be the same file as the one loaded for the adaptive sampling decision. This will allow to output BAM files containing the basecalled and aligned sequence in real time.

The .bed file in the **4. Analysis** section is used for two different processes: Firstly, it provides an identifier in the sequencing summary reporting whether the complete read hit the regions described in the .bed file loaded in **4. Analysis**. This is shown in the sequencing_summary.txt file under the column bed_alignment and populated with a 0 or a 1, for whether it hits or does not hit the .bed file. Secondly, this .bed file is used to check for the coverage obtained at each of the regions described in the same .bed file. You can follow this live during the run in the **Alignment hits** tab of MinKNOW.

To make the most of the live alignment feature and the coverage tracking feature, load the buffered .bed file (bed file containing the ROI + buffer) in the **3. Run options** Adaptive sampling section, and the .bed file containing only ROI (unbuffered) in the **4. Analysis** section. This guarantees that you only track coverage on the targets of interest, and will give a more accurate description of coverage on the ROIs. The .bed file provided in the alignment section does not modify the run output and is not strictly necessary. Nevertheless, depending on the amount of buffer added to each region in the adaptive sampling .bed, having a targets only (unbuffered) .bed in the alignment section may provide a more accurate coverage report. Importantly, the coverage tracking (and therefore the files provided in **4. Analysis**) does not modify the sequencing run in any way. It is a tool for real-time alignment and for checking during a run how much coverage has already been obtained for each bed region. Lastly, note that the coverage reported is referring to the percentage of sequence that has been basecalled. This means that if the live basecalling is not keeping up, the coverage reported is only relative to the percentage already basecalled.

Live alignment is a computationally demanding process which can easily affect the adaptive sampling decision time. Therefore, please refer the tables of advised metrics on how many flow cells you can run with this feature per device to avoid affecting enrichment rates. To prevent live alignment being used, remove the reference sequence denoted "1" in Figure 20.

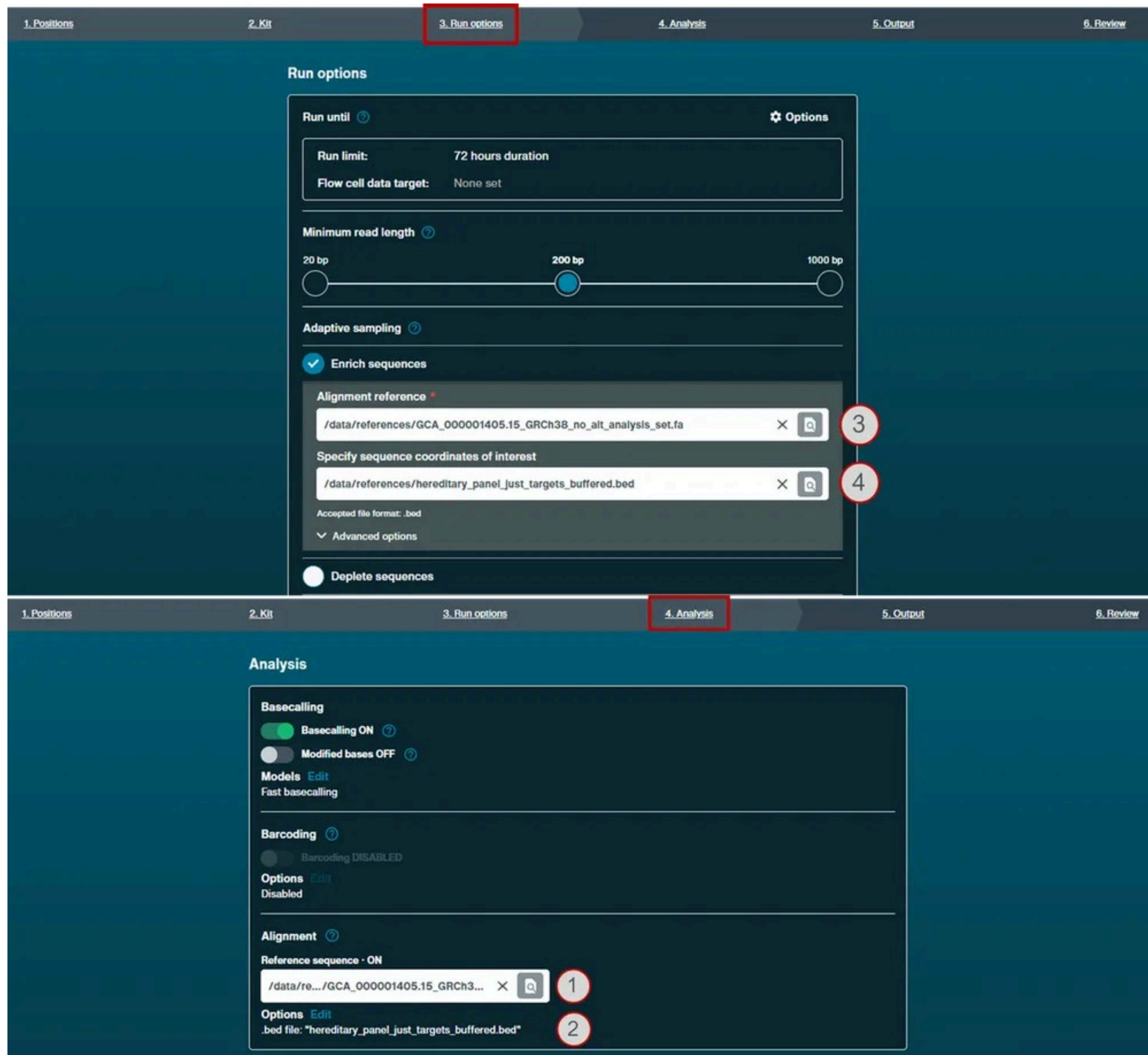

Figure 20. MinKNOW dialogs for uploading the FASTA reference file and .bed file for the adaptive sampling panel (top, sections 3 and 4) and the live basecalling panel (bottom, sections 1 and 2).

You can see which files are being used in each section in the first page of the run summary once the run is started. An example of this panel is shown in Figure 21.

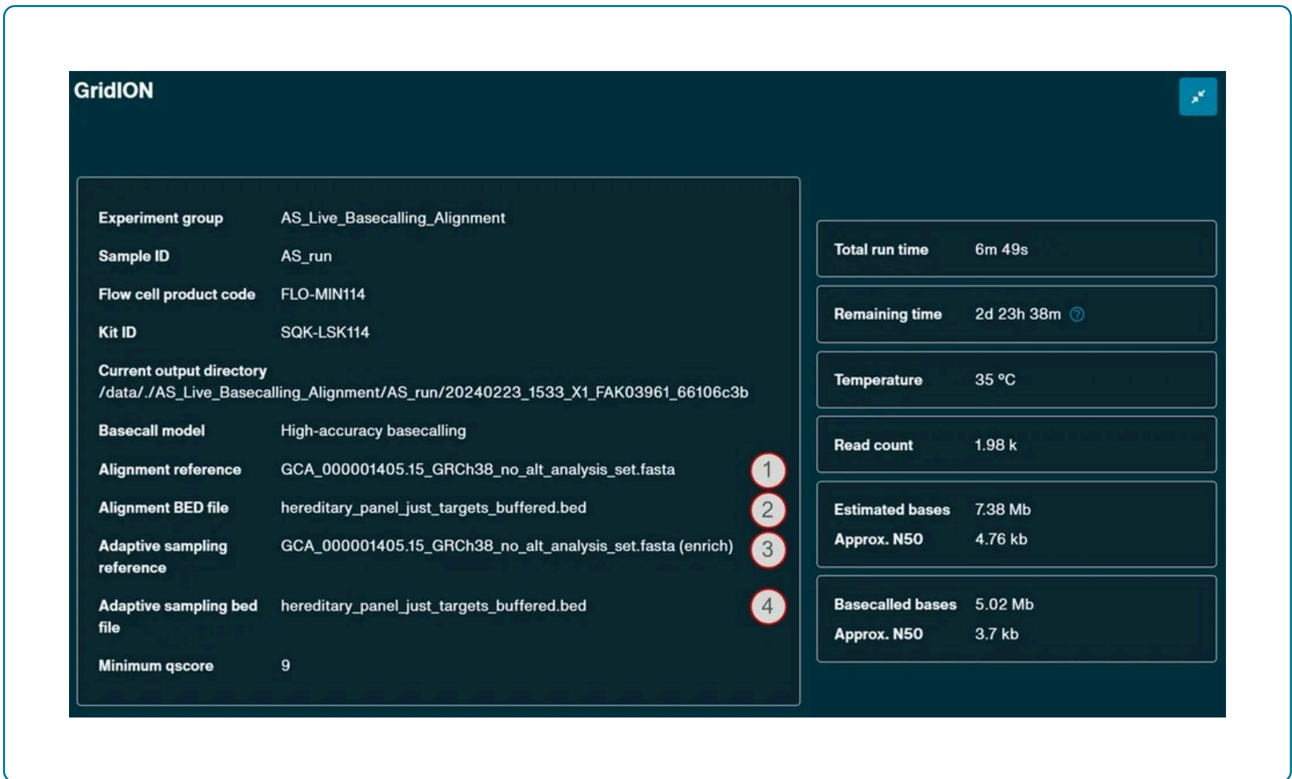

Figure 21. MinKNOW front panel containing run summary details. Alignment information has been labelled accordingly.

Oxford Nanopore Technologies

PROMETHION: PROTOCOL

Human variation sequencing from 15kb extracted blood samples using SQK-LSK114

V HVSB_9230_v114_revA_24Oct2025

This is an end-to-end method outlining sample extraction, library preparation, sequencing and data analysis.

This protocol:

- Uses genomic DNA extracted from human blood samples and sheared to 15kb length
- Takes ~240 minutes for sample preparation and ~90 minutes for library preparation
- Requires no PCR
- Is compatible with R10.4.1 flow cells

For Research Use Only

FOR RESEARCH USE ONLY

Contents

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1. Overview of the protocol

Introduction to the human variation sequencing from 15 kb extracted blood samples using SQK-LSK114 protocol

This protocol describes an end-to-end process to prepare and sequence gDNA from human blood samples, and analyse the data using the wf-human-variation workflow in EPI2ME.

The identification of structural variants (SVs) and single nucleotide variants (SNVs) play a pivotal role in our understanding of genetic diversity, disease mechanisms, and evolutionary biology. This protocol aims to produce libraries with a read N50 ~15 kb and generate ~40–50x coverage of the genome, to provide robust calling of small and large variants, as well as methylation and phasing information.

Briefly, genomic DNA is extracted from 1 ml of whole blood using the QIAGEN Puregene Blood Kit. DNA is then sheared with Megaruptor® 3 (Diagenode).

Note: Users who do not have access to the Megaruptor and wish to omit this step are likely to observe a drop in coverage.

The extracted DNA input is then prepared using our Ligation Sequencing Kit V14 (SQK-LSK114) and sequenced on a PromethION device. Detailed instructions for washing and reloading the flow cell once to maximise data output to reach 120 - 170 Gb of sequencing data using the Flow Cell Wash Kit (EXP-WSH004) and Sequencing Auxiliary Vials V14 (EXP-AUX003).

Your sequencing data is basecalled and aligned by MinKNOW, and the BAM output data is aligned and analysed using the wf-human-variation workflow which uses Sniffles2, Clair3 and modbam2bed software to call structural variants (SVs), single nucleotide polymorphisms (SNPs) and for reporting DNA methylation.

Steps in the workflow

Prepare for your experiment

You will need to:

- Extract your input sample (human blood).
- Ensure you have your sequencing kit, the correct equipment, and third-party reagents.
- Download the software for acquiring and analysing your data.
- Check your flow cell to ensure it has enough pores for a good sequencing run.

Sample preparation

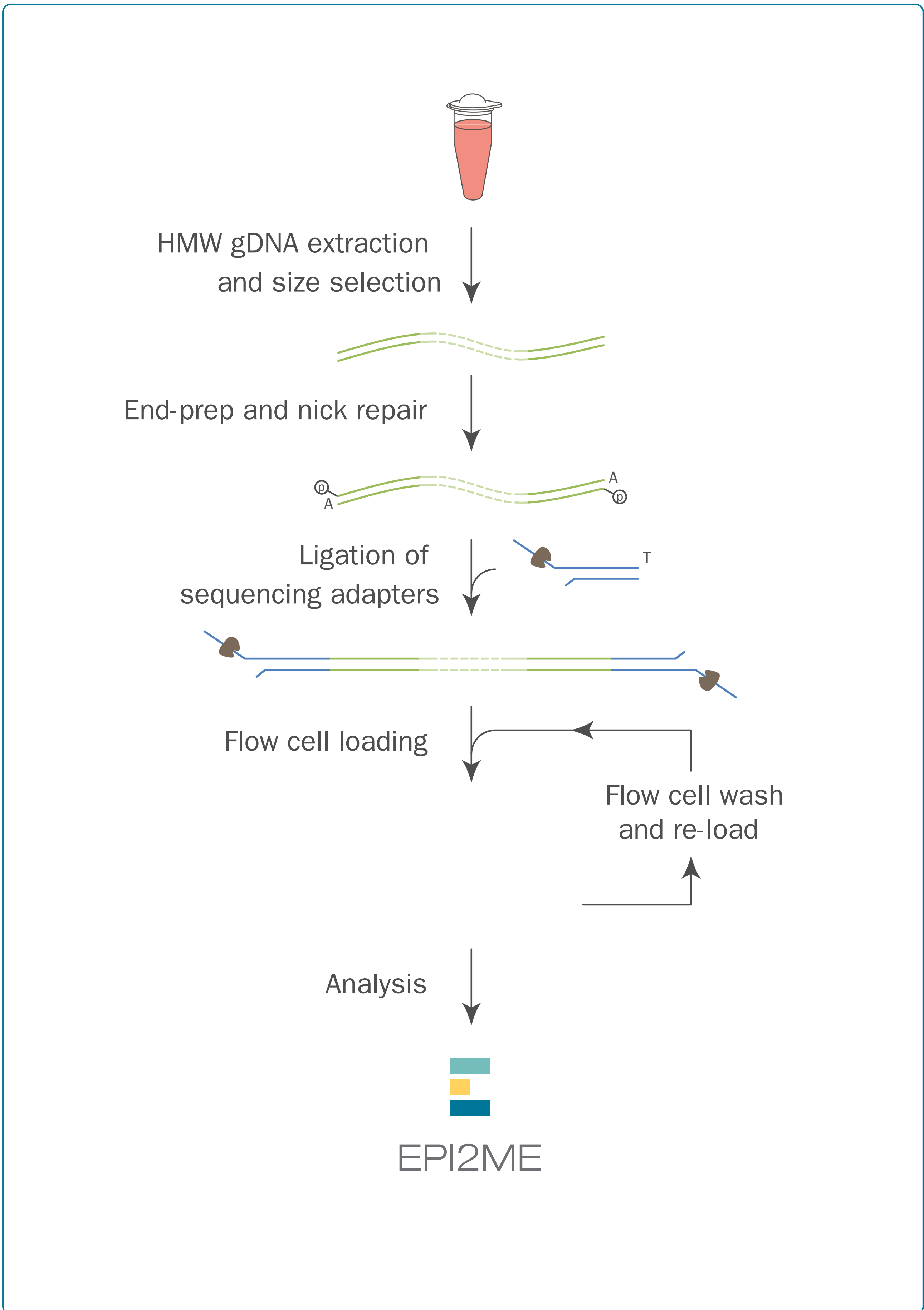
Using the outlined extraction method, extract the gDNA from your human blood sample and fragment your gDNA using the Megaruptor.

Check the length, quantity and purity of your extracted material. **The quality checks performed during the protocol are essential in ensuring experimental success.**

Library preparation and sequencing

The Table below is an overview of the steps required in the library preparation, including timings and optional stopping points.

Library preparation	Process	Time	Stop option
DNA repair and end-prep	Repair and prepare the DNA ends for adapter attachment.	35 minutes	4°C overnight
Adapter ligation and clean-up	Attach the sequencing adapters to the DNA ends.	55 minutes	4°C short-term storage or for repeated use, such as re-loading your flow cell. -80°C for single-use, long-term storage. We strongly recommend sequencing your library as soon as it is adapted.
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing.	10 minutes	
Washing and reloading the flow cell	Pause your sequencing run. Wash your flow cell with nuclease to remove the previous library load and unblock pores. Prime the flow cell and reload the prepared library to continue sequencing.	60 minutes	



Sequencing and analysis

You will need to:

- Start a sequencing run using the MinKNOW software which will collect raw data from the device and basecall reads.
- Start the EPI2ME software and select the wf-human-variation bioinformatics workflow to analyse your data.
- (*Optional*) Alternatively, external tools can be used to further analyse and explore your data.

Compatibility of this protocol

This protocol should only be used in combination with:

- Ligation Sequencing Kit V14 (SQK-LSK114)
- R10.4.1 PromethION Flow Cells (FLO-PRO114M)
- Flow Cell Wash Kit (EXP-WSH004)
- Sequencing Auxiliary Vials V14 (EXP-AUX003)
- PromethION 24/48 device - PromethION IT requirements document
- PromethION 2 Solo device - PromethION 2 Solo IT requirements document

2. Equipment and consumables

Materials

(FOR EXTRACTION) ≥ 1 ml of human blood in EDTA K2 vacuum tube
(FOR LIBRARY PREPARATION) 3 µg of Megaruptor fragmented gDNA
Ligation Sequencing Kit V14 (SQK-LSK114)
Flow Cell Wash Kit (EXP-WSH004)
Sequencing Auxiliary Vials V14 (EXP-AUX003)

Consumables

PromethION Flow Cells
Puregene Blood Kit (QIAGEN, 158023)
Megaruptor 3 Shearing Kit (Diagenode, E07010003)
Agencourt AMPure XP beads (Beckman Coulter™, A63881)
NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L). Contains the 4 reagents listed below:
NEBNext FFPE Repair Mix (NEB, M6630)
NEBNext® FFPE DNA Repair v2 Module (NEB, E7360)
NEBNext Ultra II End repair/dA-tailing Module (NEB, E7546)
Salt-T4® DNA Ligase (NEB, M0467)
Nuclease-free water (e.g. Thermo Scientific, AM9937)

Freshly prepared 80% ethanol in nuclease-free water
Isopropanol, 100% (Fisher Scientific, 10723124)
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Fisher scientific, 10224683)
Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
Qubit™ dsDNA HS Assay Kit (ThermoFisher, Q32851)
Agilent Genomic DNA 165 kb Analysis Kit (Agilent, FP-1002-0275)
Qubit™ Assay Tubes (Invitrogen, Q32856)
15 ml Falcon tubes
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes

Equipment

PromethION device
PromethION Flow Cell Light Shield
Hula mixer (gentle rotator mixer)
Magnetic separation rack, suitable for 1.5 ml Eppendorf tubes
Heating block
Incubator or water bath set at 37°C and 50°C
Microfuge
Vortex mixer
Thermal cycler
Centrifuge and rotor suitable for 15 ml Falcon tubes
Megaruptor 3 (Diagenode, B06010003)
Wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips
Ice bucket with ice
Timer
Qubit™ fluorometer (or equivalent for QC check)
Agilent Femto Pulse System (or equivalent for read length QC)

The above list of materials, consumables, and equipment is for the extraction method in the sample preparation section, as well as the library preparation section of the protocol. If you have pre-extracted sample(s), you will only require the materials for the library preparation section of this protocol.

For this protocol, the following inputs are required:

Input requirements per sample for the extraction method:

- ≥ 1 ml of human blood in EDTA K2 vacuum tube

Input requirements per sample for the library preparation:

- 3 µg of Megaruptor fragmented gDNA

Input DNA

How to QC your input DNA

It is important that the input DNA meets the quantity and quality requirements. Using too little or too much DNA, or DNA of poor quality (e.g. highly fragmented or containing RNA or chemical contaminants) can affect your library preparation.

For instructions on how to perform quality control of your DNA sample, please read the Input DNA/RNA QC protocol.

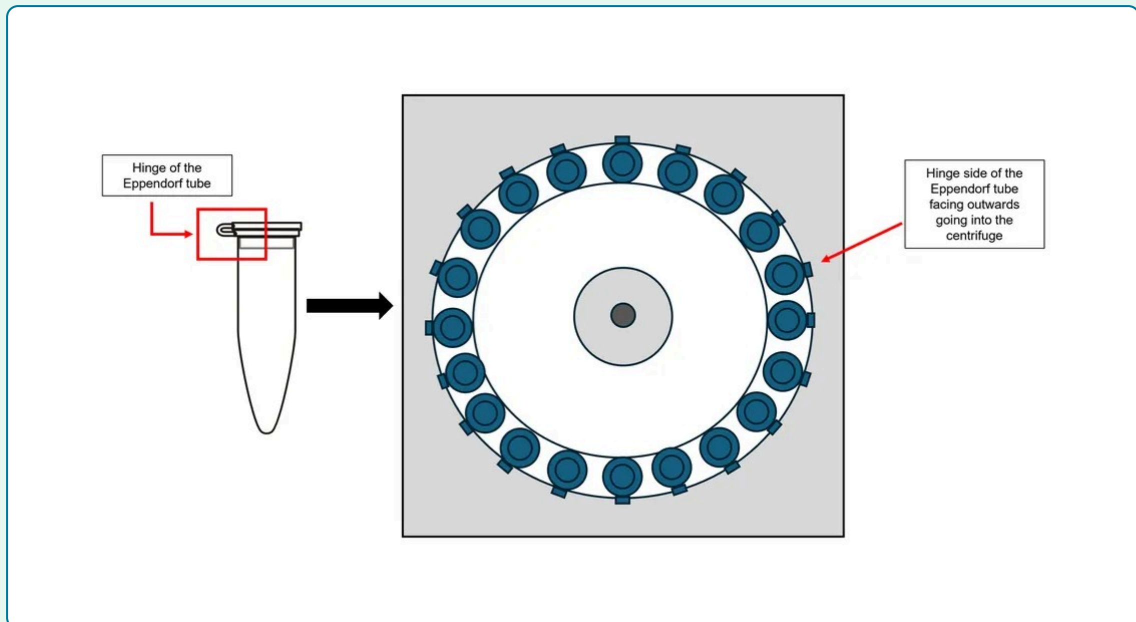
Chemical contaminants

Depending on how the DNA is extracted from the raw sample, certain chemical contaminants may remain in the purified DNA, which can affect library preparation efficiency and sequencing quality. Read more about contaminants on the Contaminants page of the Community.

Eppendorf tube orientation in centrifuge

For all centrifugation steps, ensure that tubes are loaded into the centrifuge with the hinge side of the tube facing outwards. This will assist in visual identification of the pellet.

Ensure gentle handling when removing the tubes from the centrifuge to avoid dislodging the pellet.



We recommend using the NEBNext® Companion Module v2 for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7672S or E7672L), which contains all the NEB reagents needed for use with the Ligation Sequencing Kit.

The previous version, NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (NEB, E7180S or E7180L) is also compatible, but the recommended v2 module offers more efficient dA-tailing and ligation.

Third-party reagents

We have validated and recommend the use of all the third-party reagents used in this protocol. Alternatives have not been tested by Oxford Nanopore Technologies.

For all third-party reagents, we recommend following the manufacturer's instructions to prepare the reagents for use.

Check your flow cell

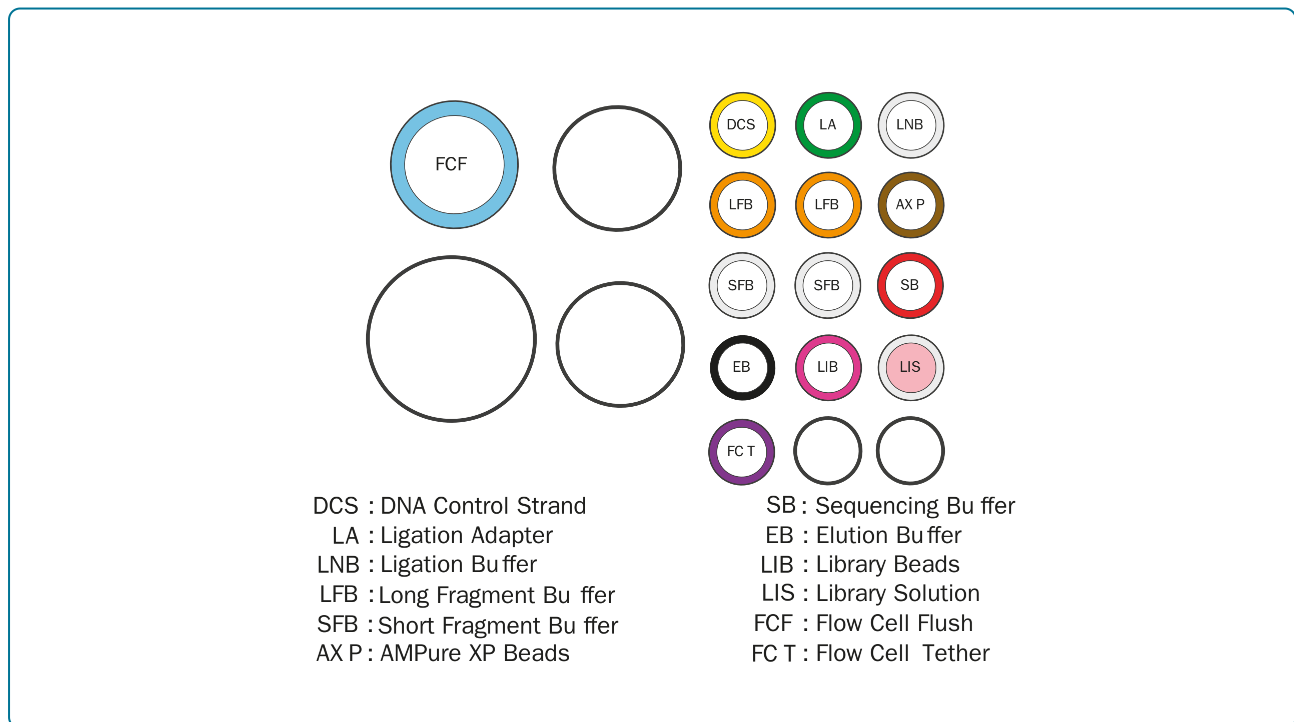
We highly recommend that you check the number of pores in your flow cell prior to starting a sequencing experiment. This should be done within 12 weeks of purchasing your PromethION Flow Cells. Oxford Nanopore Technologies will replace any unused flow cell with fewer than the number of pores listed in the Table below, when the result is reported within two days of performing the flow cell check, and when the storage recommendations have been followed. To do the flow cell check, please follow the instructions in the Flow Cell Check document.

Flow cell	Minimum number of active pores covered by warranty
PromethION Flow Cell	5000

We strongly recommend using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit V14 rather than any third-party ligase buffers to ensure high ligation efficiency of the Ligation Adapter (LA).

Ligation Adapter (LA) included in this kit and protocol is not interchangeable with other sequencing adapters.

Ligation Sequencing Kit V14 (SQK-LSK114) contents



Note: This product contains AMPure XP reagent manufactured by Beckman Coulter, Inc. and can be stored at -20°C with the kit without detriment to reagent stability.

Note: The DNA Control Sample (DCS) is a 3.6 kb standard amplicon mapping the 3' end of the Lambda genome.

3. Purification of gDNA from 1 ml of human blood

Materials

≥ 1 ml of human blood in EDTA K2 vacuum tube

Consumables

Puregene Blood Kit (QIAGEN, 158023)
Absorbent material e.g. paper towel or tissues
Freshly prepared 80% ethanol in nuclease-free water
TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Fisher scientific, 10224683)
Nuclease-free water (e.g. Thermo Scientific, AM9937)
Qubit dsDNA BR Assay Kit (Invitrogen, Q32850)
Qubit™ Assay Tubes (Invitrogen, Q32856)
15 ml Falcon tubes
1.5 ml Eppendorf DNA LoBind tubes

Equipment

Centrifuge and rotor suitable for 15 ml Falcon tubes
Incubator or water bath set at 37°C and 50°C
Vortex mixer
Microfuge

Qubit™ fluorometer (or equivalent for QC check)
Ice bucket with ice
Timer
Wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips

Optional equipment

Agilent Femto Pulse System (or equivalent for read length QC)

1. **Dispense 3 ml RBC Lysis Solution into a 15 ml centrifuge tube.**

2. **Ensure the blood is mixed well in the EDTA K2 tube, then transfer 1 ml of the blood sample into the tube containing the RBC lysis solution.**

3. **Mix by inverting the tube 10 times.**

4. **Incubate for 5 minutes at room temperature (15–25°C). Invert at least once during the incubation.**

5. **Centrifuge for 2 minutes at 2000 x g to pellet the white blood cells.**

6. **Carefully discard the supernatant, ensuring your leave approximately 200 µl of the residual liquid and the white blood cell pellet.**

Note: The supernatant can be removed by pipetting or by pouring the volume out on to an absorbent material.

7. **Gently flick the tube and/or pipette mix using a wide bore tip to resuspend the pellet in the residual liquid.**

Note: The pellet should be completely dispersed, this greatly facilitates the cell lysis in the next step.

8. **Add 3 ml of Cell Lysis Solution.**

Note: Pipette mix gently 10-15 times to lyse the cells and homogenise the solution until no clumps remain. Ensure that the solution is homogenous.

9 Incubate the reaction at 37°C for 30 minutes.

Note: Ensure the solution is homogenous by the end of the incubation, and no clumps should remain.
If necessary, you can mix the reaction by pipette mixing with a wide bore pipette tip or gently inverting the tube to assist with homogenisation.

10 Add 15 µl of RNase A solution and incubate the reaction for 15 minutes at 37°C.

11 Transfer the reaction to ice bucket with ice, and incubate for 3 min to quickly cool the sample.

12 Add 1 ml of Protein Precipitation Solution to your sample. Pulse vortex the tube twice for 5 seconds.

13 Centrifuge your sample for 5 minutes at 2000 x g.

Note: The precipitated protein should form a tight, reddish-brown pellet. If the protein pellet is not tight, incubate the tube on ice for 5 minutes and repeat the centrifugation.

14 Pipette 3 ml of isopropanol into a clean 15 ml falcon tube.

15 Carefully pour the supernatant from the sample tube into the 15 ml falcon tube containing the isopropanol.

Ensure that the protein pellet is not dislodged during pouring.

Alternatively, the supernatant can also be transferred by pipetting. Please ensure the protein pellet is not disturbed and remains intact when transferring the supernatant.

Note: If at any point the protein pellet is disturbed, repeat the 10min at 2000 x g centrifugation step. Ensure only the clear supernatant is transferred to avoid protein contamination in the final elute.

16 Gently mix the tube by inverting 50 times until the DNA is visible as threads or a clump.

17 **Centrifuge the tube for 3 minutes at 2000 x g.**

Note: Your DNA should be visible as a small white pellet at the bottom of the tube.

18 **Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper. Ensure the DNA pellet is undisturbed and remains in the tube.**

Note: The supernatant can be removed by pipetting or by pouring the volume out on to an absorbent material.
Take care as the pellet might be loose and easily dislodged.

19 **Prepare 300 µl of fresh 80% ethanol in nuclease-free water, and place on ice.**

20 **Add 300 µl of ice-cold freshly-prepared 80% ethanol to the sample tube. Gently invert the tube several times to wash the DNA pellet.**

21 **Transfer the pellet and the full 300 µl volume of ethanol into a new 1.5 ml Eppendorf tube.**

22 **Centrifuge the sample tube for 1 minute at 2000 x g.**

23 **Carefully discard the supernatant and drain the tube by inverting on a clean piece of absorbent paper. Ensure the DNA pellet is undisturbed and remains in the tube.**

Note: The supernatant can be removed by pipetting or by pouring the volume out on to an absorbent material.
Take care as the pellet might be loose and easily dislodged.

24 **Leave the lid off the sample tube and air dry the pellet for 1 min.**

Note: Avoid over-drying the pellet, ensure it is not dried to the point of cracking.

25 **Add 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to the tube containing the sample pellet. Gently resuspend the pellet by flicking.**

26 Incubate the tube for 2 hours at 50°C, occasionally pipette mixing the whole volume tube contents (100 μl) with a wide-bore pipette tip.

Note: The DNA pellet may take some time to solubilise. Please ensure the solution is homogenous before quantifying.

Optional: Alternatively, this incubation can be performed at room temperature overnight.

27 Quantify your sample three times using the Qubit dsDNA BR Assay Kit. Ensure the replicate Qubit measurements are consistent before continuing to the next step.

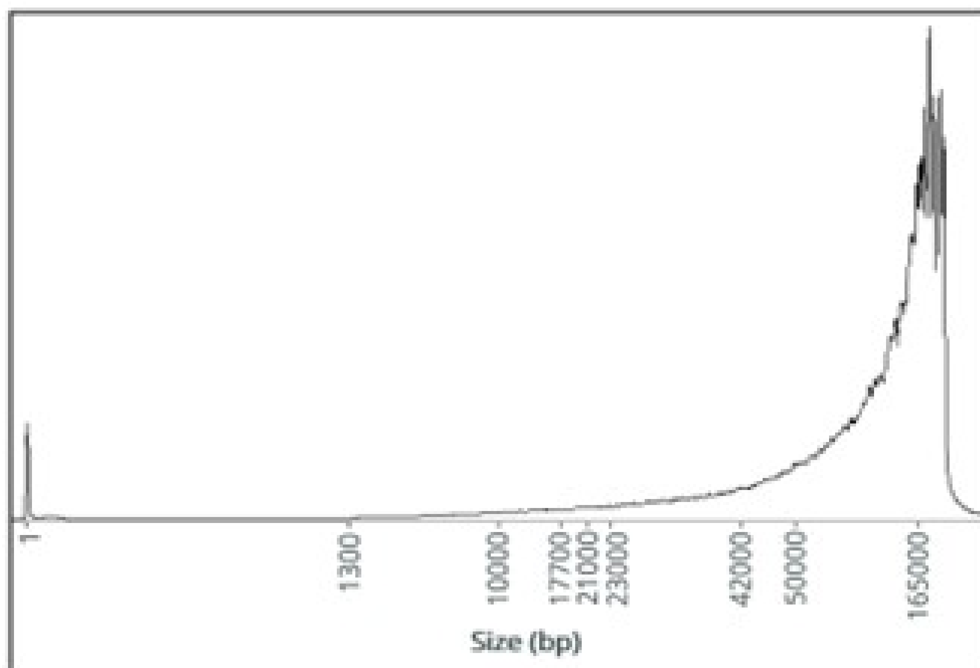
Note: Approximately 10–30 μg of gDNA is expected following sample extraction.
Expected Qubit measurements of 100–300 ng/μl.

If your Qubit measurements are not consistent, this could indicate that the DNA has not been homogeneously resuspended.

If this occurs, we recommend increasing the incubation time, allowing more time for the DNA pellet to solubilise.

Note: The elution can be aided by incubating at 50°C on a thermomixer with gentle agitation 300 RPM. Alternatively an end over end rotating mixer can be used.

Your extracted gDNA can also be analysed using Femto Pulse (Agilent) to check the size and quality.



Example fragment length profile of gDNA extracted from human blood using the Puregene Blood Kit.

Take your extracted gDNA forward into the size selection of gDNA step of this protocol. Alternatively, your sample can be stored at 4°C overnight.

4. gDNA fragmentation using the Megaruptor

Materials	2 µg of extracted gDNA
Consumables	Megaruptor 3 Shearing Kit (Diagenode, E07010003) TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (Fisher scientific, 10224683) Agilent Genomic DNA 165 kb Analysis Kit (Agilent, FP-1002-0275) 1.5 ml Eppendorf DNA LoBind tubes

Equipment

Megaruptor 3 (Diagenode, B06010003)
Microfuge
Agilent Femto Pulse System (or equivalent for read length QC)
Ice bucket with ice
Timer
Wide-bore pipette tips
P1000 pipette and tips
P200 pipette and tips
P20 pipette and tips
P10 pipette and tips
P2 pipette and tips

1 Prepare the DNA in nuclease-free water:

1. Transfer 2 µg of extracted gDNA into a clean Megaruptor 3 shearing tube.
2. Adjust the volume to 90 µl with with 10mM Tris 1mM EDTA pH8.
3. Mix thoroughly by pipetting up and down using a wide-bore pipette tip.
4. Spin down briefly in a microfuge.

2 Transfer the sample tube to the Megaruptor 3, ensuring the instrument is appropriately balanced according to the manufacturers instructions.

Note: Ensure the Megaruptor 3 Hydropore-Syringes are screwed tight before inserting into the Megaruptor.

Ensure no bubbles are present in the sample, and visually confirm that the syringe is immersed 2/3rd of the way into the sample volume.

3 Setup the shearing parameters on the Megaruptor 3 device as follows:

Megaruptor 3 setting	
Shearing speed	31
Volume	90 µl
Concentration	23 ng/ul

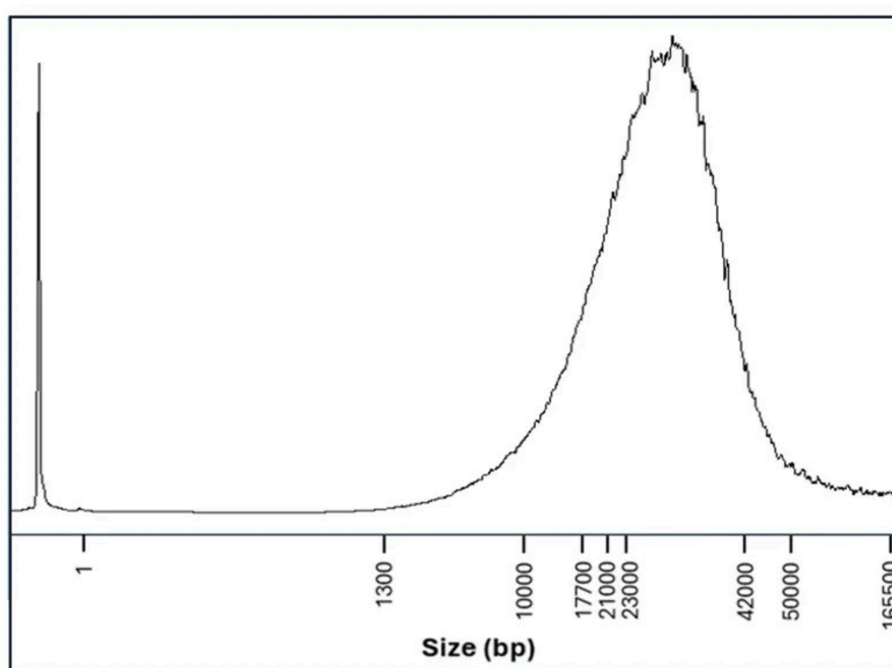
4 Begin the shearing of DNA using the Megaruptor 3.

5 Quantify your sample using the Qubit dsDNA BR Assay Kit.

Note: Approximately 2 µg of gDNA is expected following shearing.
Expected Qubit measurements of ~23.33 ng/µl.

Note: No more than 10 µl volume loss should be observed post-shearing.

6 Assess the fragmented gDNA for fragment size using Femto Pulse (Agilent).



Example fragment length profile of gDNA extracted, size selected and fragmented using Megaruptor 3.

The Megaruptor fragmentation reduces the fragment length profile to a size

range between 3 kb and 40 kb, centred around approximately 15 kb.

Overlay of fragment length profiles of a) gDNA extracted using the Puregene Blood Kit, b) gDNA extracted and fragmented using Megaruptor 3 (MR).

Take your SFE size selected and Megaruptor fragmented gDNA forward into the library preparation section of this protocol. Alternatively, your sample can be stored at 4°C overnight.

5. DNA repair and end-prep

Materials	2 µg of Megaruptor fragmented gDNA AMPure XP Beads (AXP)
Consumables	NEBNext® FFPE DNA Repair Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L) NEBNext® FFPE DNA Repair Buffer v2 from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)

NEBNext® Ultra II End Prep Enzyme Mix from the NEBNext® Companion Module v2 (NEB, E7672S or E7672L)
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
Nuclease-free water (e.g. Thermo Scientific, AM9937)
Freshly prepared 80% ethanol in nuclease-free water
Qubit™ Assay Tubes (Invitrogen, Q32856)
0.2 ml thin-walled PCR tubes
1.5 ml Eppendorf DNA LoBind tubes

Equipment

P1000 pipette and tips
P100 pipette and tips
P10 pipette and tips
Microfuge
Thermal cycler
Hula mixer (gentle rotator mixer)
Magnetic separation rack
Ice bucket with ice

Optional equipment

Qubit™ fluorometer (or equivalent for QC check)

Check your flow cell.

We recommend performing a flow cell check before starting your library prep to ensure you have a flow cell with enough pores for a good sequencing run.

See the flow cell check document for more information.

1 Prepare the NEB reagents in accordance with manufacturer's instructions, and place on ice.

For optimal performance, NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert the reagent tubes to ensure they are well mixed.
   Note: Do not vortex the FFPE DNA Repair Mix or Ultra II End Prep Enzyme Mix.
3. Always spin down tubes before opening for the first time each day.
4. Vortex the FFPE DNA Repair Buffer v2 to ensure it is well mixed.
   Note: This buffer may contain a white precipitate. If this occurs, allow the mixture to come to room temperature and pipette the buffer several times to break up the precipitate, followed by a quick vortex to mix.

5. The FFPE DNA Repair Buffer v2 may have a yellow tinge and is fine to use if yellow.

2 Prepare the DNA in nuclease-free water:

1. Transfer 2 µg of Megaruptor fragmented gDNA from the sample extraction into a 0.2 ml thin-walled PCR tube.
2. Adjust the volume to 80 µl with nuclease-free water.
3. Mix thoroughly by pipetting up and down, or by flicking the tube.
4. Spin down briefly in a microfuge.

3 In the 0.2 ml thin-walled PCR tube containing your gDNA, mix the following:

Reagent	Volume
DNA from the previous step	80 µl
NEBNext FFPE DNA Repair Buffer v2	11.7 µl
NEBNext FFPE DNA Repair Mix	3.3 µl
Ultra II End-prep Enzyme Mix	5 µl
Total	**100 µl**

4 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

5 Using a thermal cycler, incubate the reaction at 20°C for 5 minutes, then 65°C for 5 minutes and hold at 4°C.

6 Resuspend the AMPure XP Beads (AXP) by vortexing.

7 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

8 Add 100 µl of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.

9. **Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.**

10. **Prepare 600 µl of fresh 80% ethanol in nuclease-free water.**

11. **Spin down the sample and pellet on a magnet for 10 minutes until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.**

12. **Keep the tube on the magnet and wash the beads with 250 µl of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.**

13. **Repeat the previous step.**

14. **Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

15. **Remove the tube from the magnetic rack and resuspend the pellet in 61 µl nuclease-free water by gently pipetting up and down or by flicking the tube. Incubate for 2 minutes at room temperature.**

16. **Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute.**

17. **Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Quantify 1 µl of eluted sample using a Qubit fluorometer.

Note: You should expect to recover between 1000–1500 ng.

Take forward the repaired and end-prepped DNA into the adapter ligation step. However, at this point it is also possible to store the sample at 4°C overnight.

6. Adapter ligation and clean-up

Materials

Ligation Adapter (LA)
Ligation Buffer (LNB)
Long Fragment Buffer (LFB)
AMPure XP Beads (AXP)
Elution Buffer (EB)

Consumables

Salt-T4® DNA Ligase (NEB, M0467)
1.5 ml Eppendorf DNA LoBind tubes
Qubit dsDNA HS Assay Kit (Invitrogen, Q32851)
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

Magnetic separation rack
Microfuge
Vortex mixer
P1000 pipette and tips
P100 pipette and tips
P20 pipette and tips
P10 pipette and tips
Qubit™ fluorometer (or equivalent for QC check)

Although third-party ligase products may be supplied with their own buffer, the ligation efficiency of the Ligation Adapter (LA) is higher when using the Ligation Buffer (LNB) supplied in the Ligation Sequencing Kit.

1. **Spin down the Ligation Adapter (LA) and Salt-T4 DNA Ligase, and place on ice.**

2. **Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.**

3. **Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then spin down and place on ice.**

4. **Thaw the Long Fragment Buffer (LFB) at room temperature and mix by vortexing. Then spin down and place on ice.**

5. **In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:**

Between each addition, pipette mix 10–20 times.

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Buffer (LNB)	25 µl
Salt-T4 DNA Ligase	10 µl
Ligation Adapter (LA)	5 µl
Total	**100 µl**

6. **Thoroughly mix the reaction by gently pipetting and briefly spinning down.**

7. **Incubate the reaction for 10 minutes at room temperature.**

8. **Resuspend the AMPure XP Beads (AXP) by vortexing.**

9. **Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.**

10. **Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.**

11. **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colourless.**

12. **Wash the beads by adding 250 µl Long Fragment Buffer (LFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet for at least 5 minutes. Remove the supernatant using a pipette and discard.**

Note: Take care when removing the supernatant, the viscosity of the buffer can contribute to loss of beads from the pellet.

13. **Repeat the previous step.**

14. **Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.**

15. **Remove the tube from the magnetic rack and resuspend the pellet in 65 µl Elution Buffer (EB). Spin down and incubate for 10 minutes at 37°C.**

16. **Pellet the beads on a magnet for 10 minutes, until the eluate is clear and colourless.**

17. **Remove and retain 65 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

Note: You should expect to recover 350–1000 ng of adapter ligated library in a volume of 64 µl.

The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.

Library storage recommendations

We recommend storing libraries in Eppendorf DNA LoBind tubes at **4°C for short-term** storage or repeated use, for example, re-loading flow cells between washes. For single use and **long-term storage** of more than 3 months, we recommend storing libraries at **-80°C** in Eppendorf DNA LoBind tubes.

7. Priming and loading the PromethION Flow Cell

Materials	Sequencing Buffer (SB) Library Beads (LIB) Flow Cell Tether (FCT) Flow Cell Flush (FCF)
Consumables	PromethION Flow Cells 1.5 ml Eppendorf DNA LoBind tubes
Equipment	PromethION device PromethION Flow Cell Light Shield P1000 pipette and tips P200 pipette and tips P20 pipette and tips

This kit is only compatible with R10.4.1 flow cells (FLO-PRO114M).

After taking the flow cell out of the fridge, wait 20 minutes for the flow cell to reach room temperature before inserting it into the PromethION. Condensation can form on the flow cell in humid environments. Inspect the gold connector pins on the top and underside of the flow cell for condensation and wipe off with a lint-free wipe if any is observed. Ensure the heat pad (black pad) is present on the underside of the flow cell.

1 Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

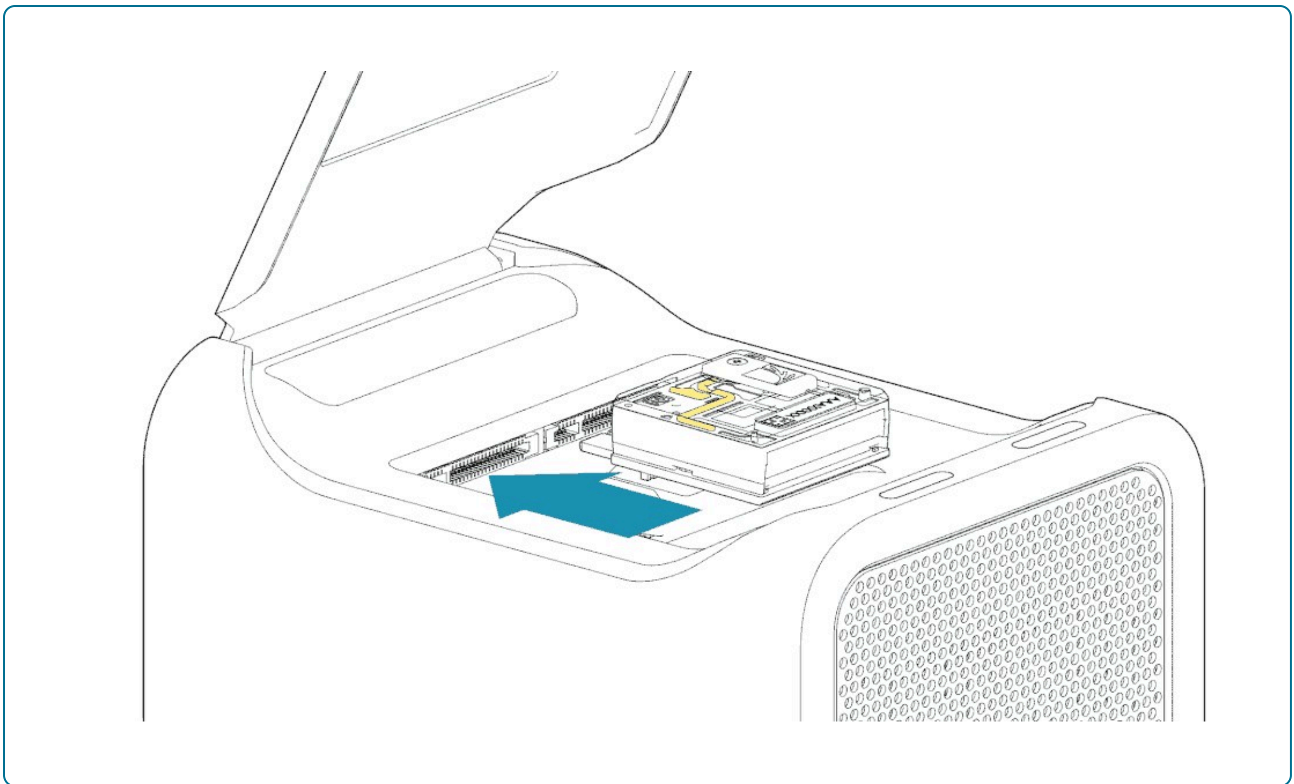
2 To prepare the flow cell priming mix, combine Flow Cell Tether (FCT) and Flow Cell Flush (FCF), as directed below. Mix by vortexing at room temperature.

In a clean suitable tube for the number of flow cells, combine the following reagents:

Reagent	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,200 µl**

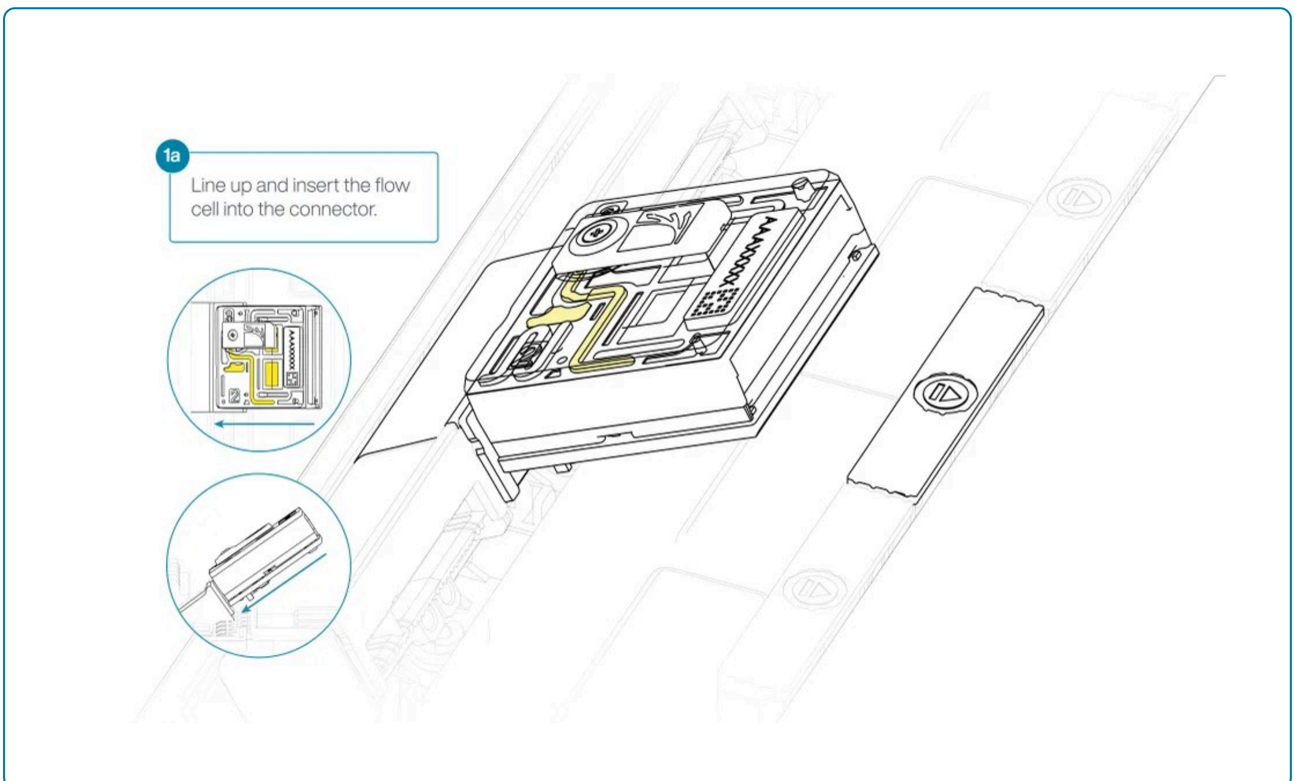
3 For PromethION 2 Solo, load the flow cell(s) as follows:

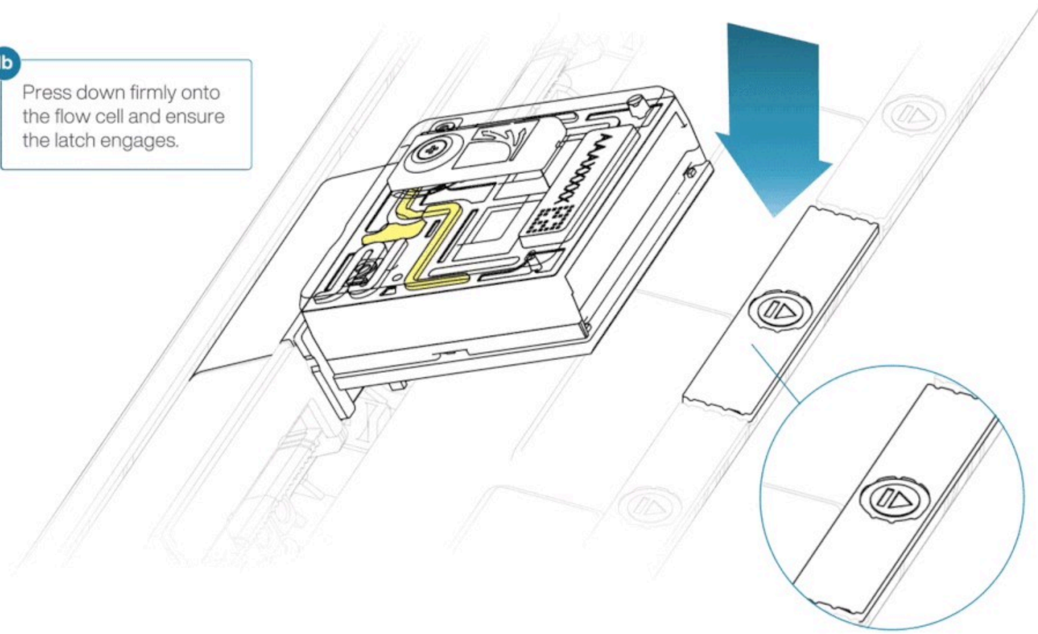
1. Place the flow cell flat on the metal plate.
2. Slide the flow cell into the docking port until the gold pins or green board cannot be seen.



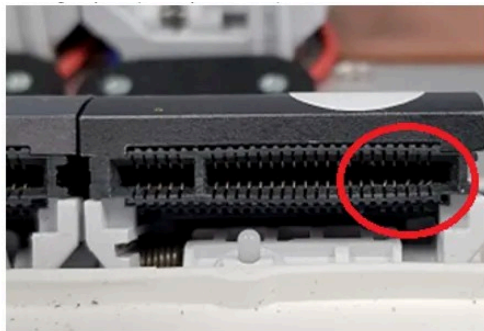
4 For the PromethION 24/48, load the flow cell(s) into the docking ports:

1. Line up the flow cell with the connector horizontally and vertically before smoothly inserting into position.
2. Press down firmly onto the flow cell and ensure the latch engages and clicks into place.





Insertion of the flow cells at the wrong angle can cause damage to the pins on the PromethION and affect your sequencing results. If you find the pins on a PromethION position are damaged, please contact support@nanoporetech.com for assistance.

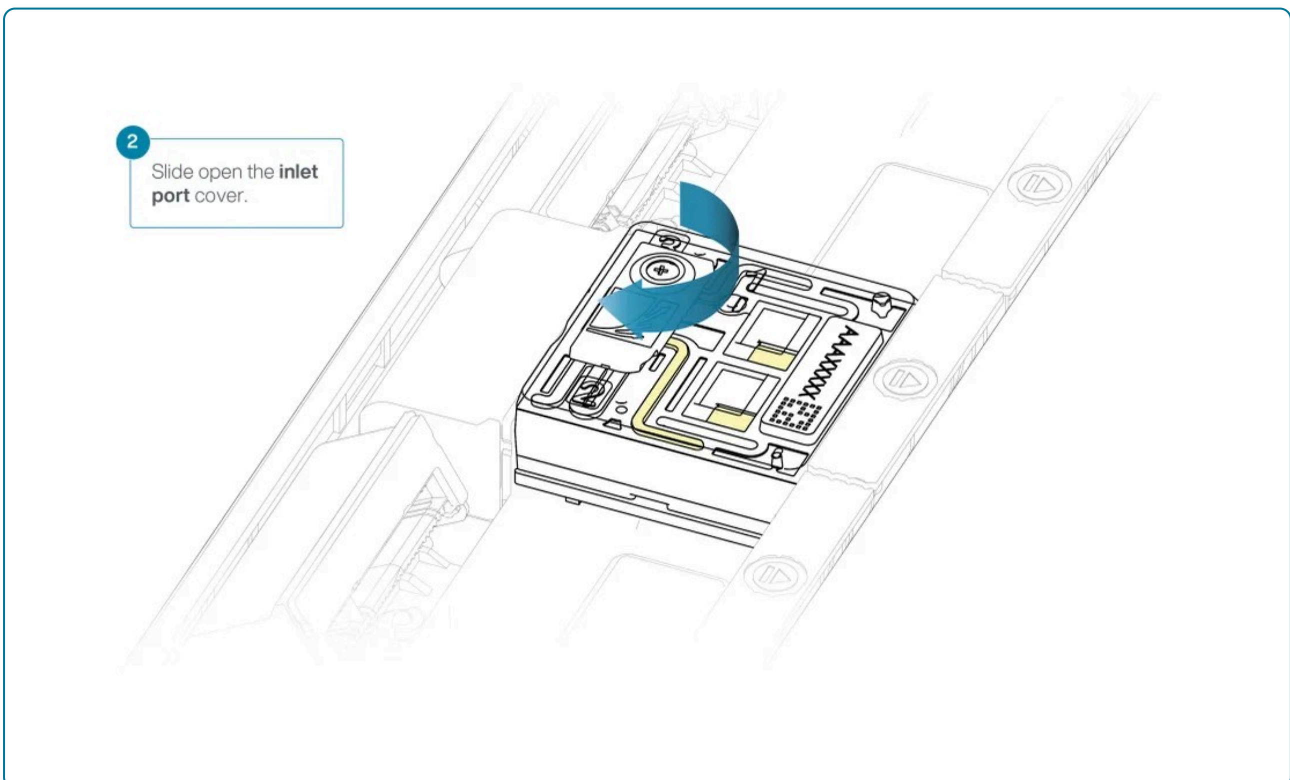


Complete a flow cell check to assess the number of pores available before loading the library.

This step can be omitted if the flow cell has been checked previously.

See the flow cell check document for more information.

5 Slide the inlet port cover clockwise to open.

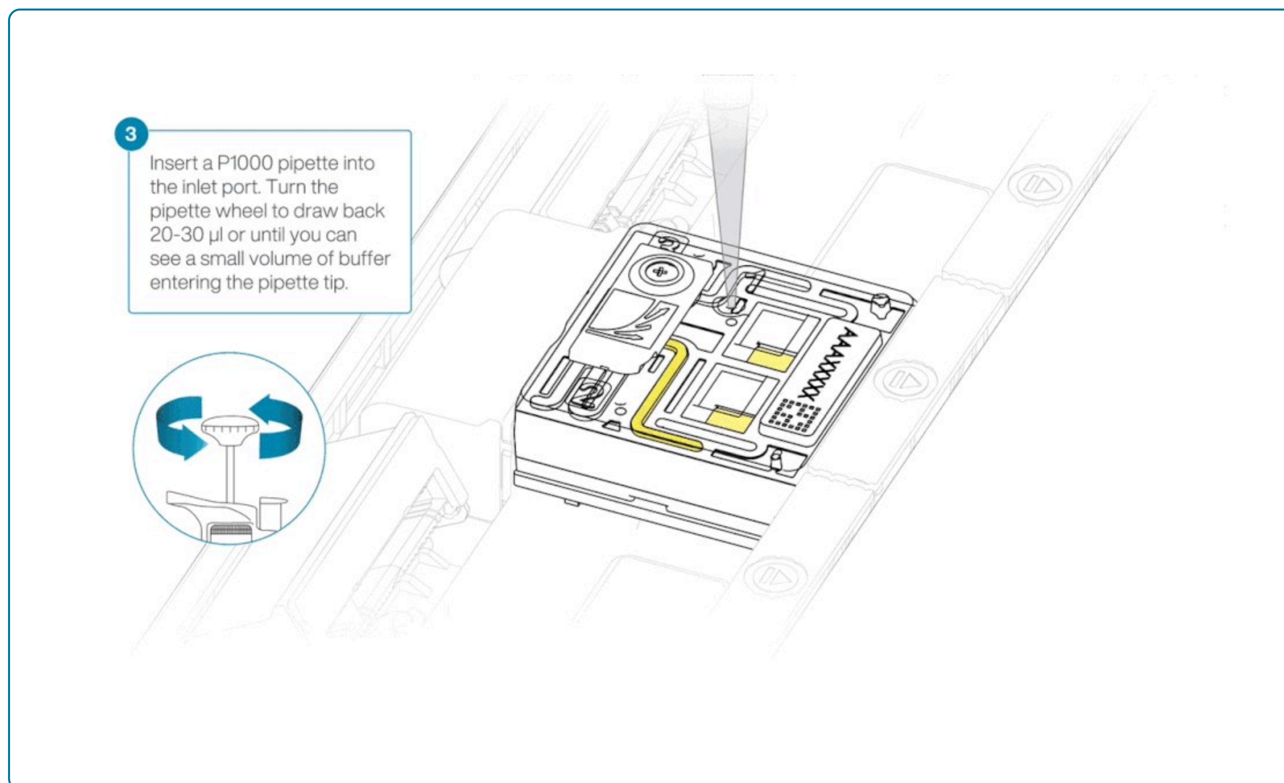


Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

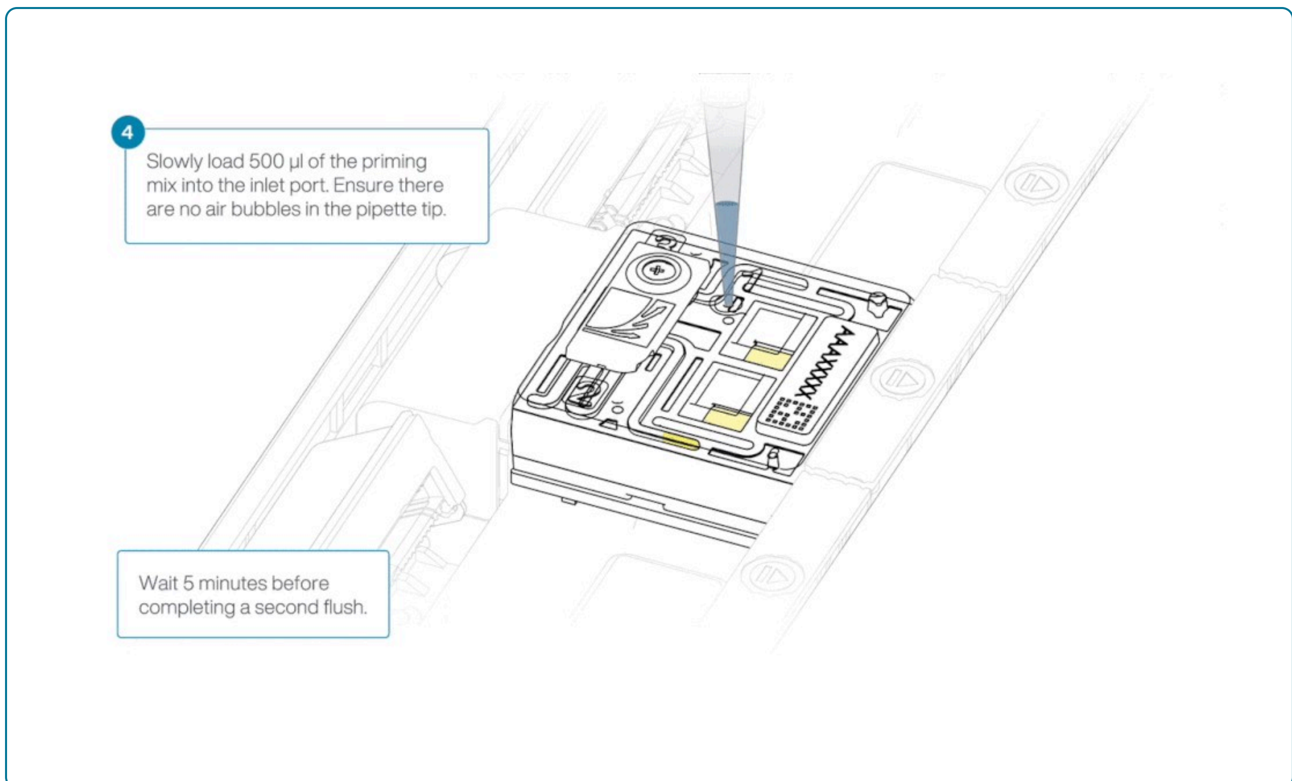
6 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.

3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



7 **Load 500 µl of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait five minutes. During this time, prepare the library for loading using the next steps in the protocol.**



8 **Thoroughly mix the contents of the Library Beads (LIB) by pipetting.**

The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

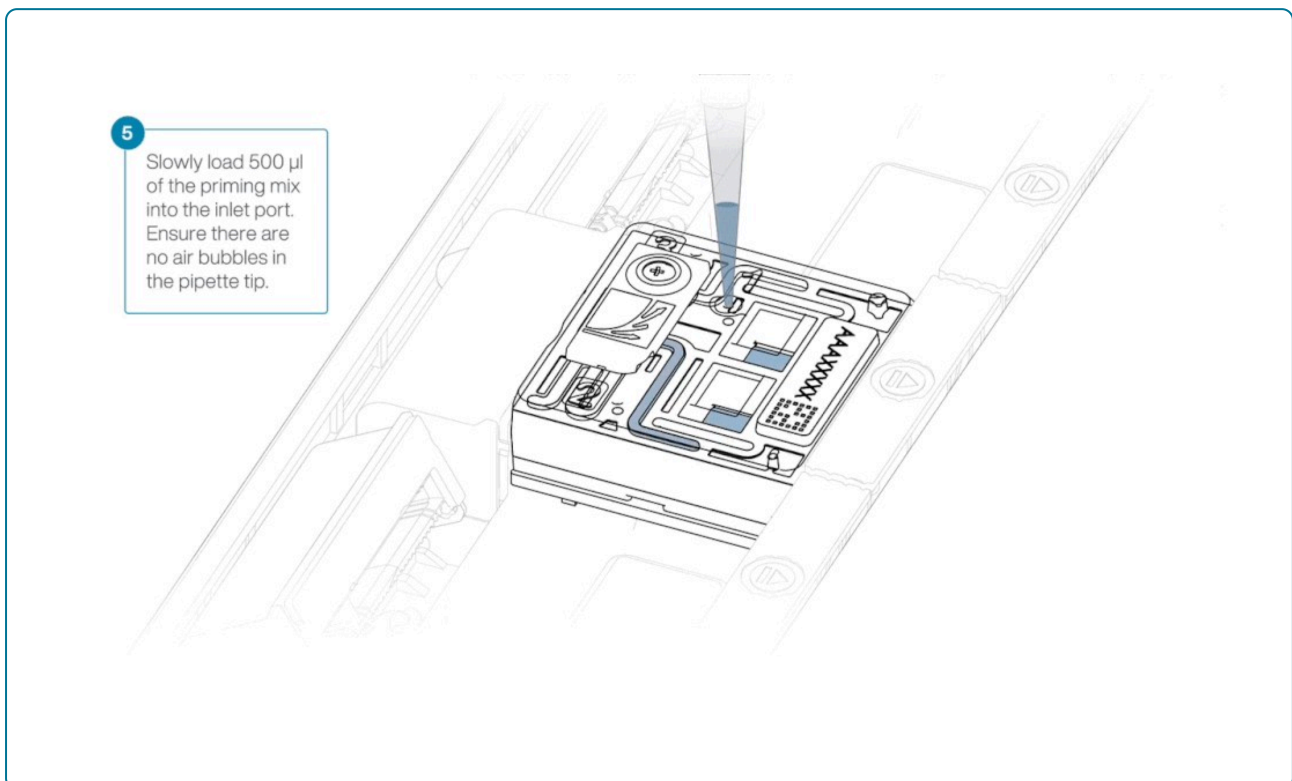
We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

9 **In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:**

Reagent	Volume per flow cell
Sequencing Buffer (SB)	100 µl
Library Beads (LIB) thoroughly mixed before use	68 µl
DNA library	32 µl
Total	**200 µl**

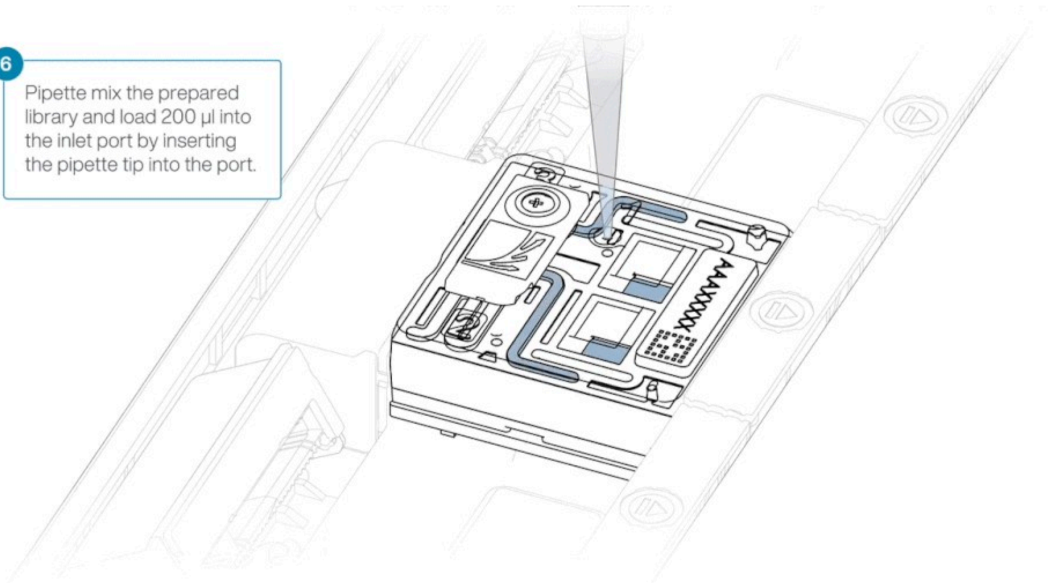
Note: The prepared library is used for loading into the flow cell. Store the library on ice or at 4°C until ready to load.

10 Complete the flow cell priming by slowly loading 500 µl of the priming mix into the inlet port.

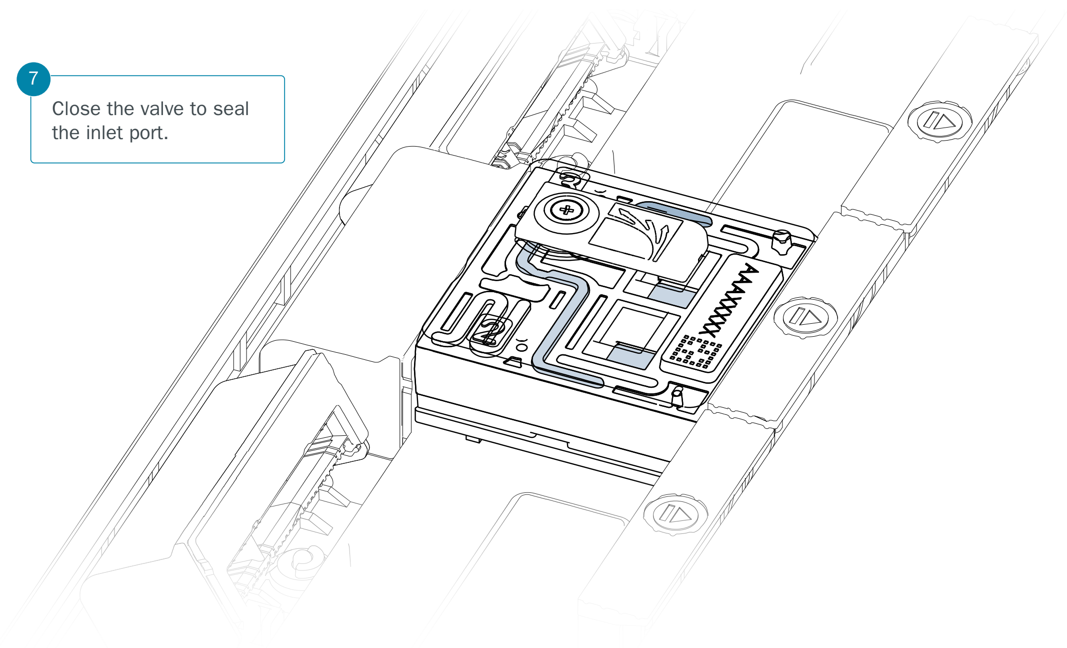


11 Mix the prepared library gently by pipetting up and down just prior to loading.

12 Load 200 µl of library into the inlet port using a P1000 pipette.



13 Close the valve to seal the inlet port.

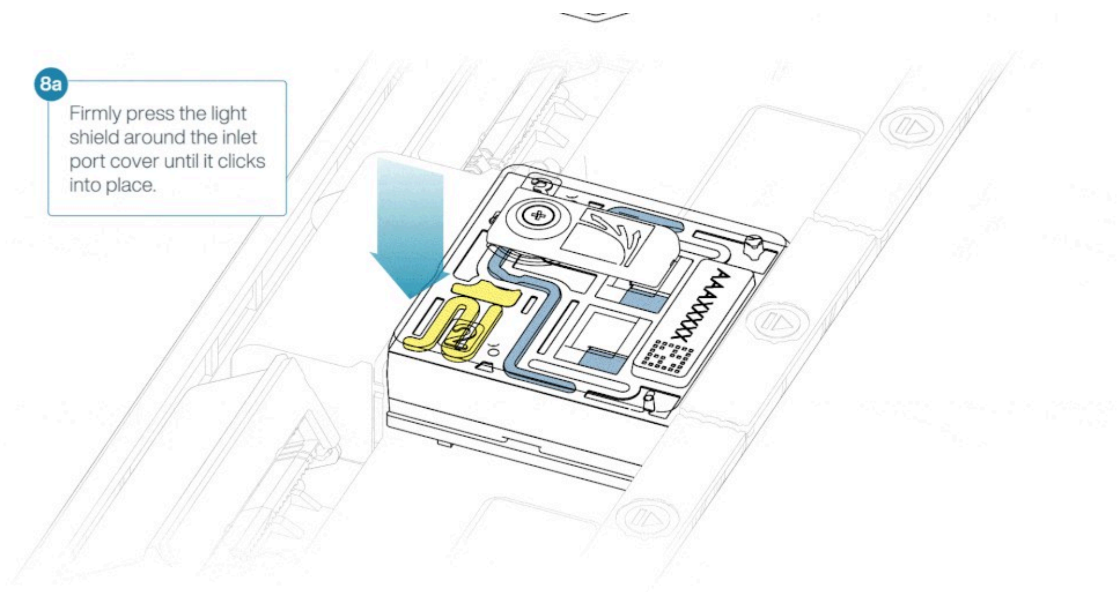


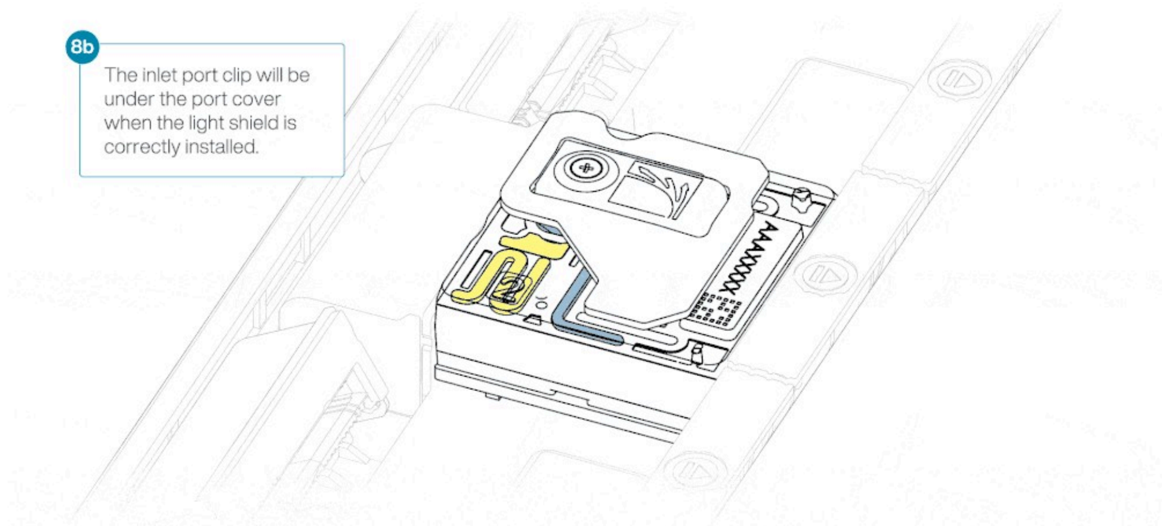
For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

14 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.





Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.

For instructions on setting up your sequencing run please visit the data acquisition and basecalling section of this protocol.

Reminder: For this protocol, we recommend washing and reloading your flow cell with fresh library to maintain high data acquisition after ~30 hours of sequencing.

Follow the instructions in the washing and reloading a PromethION Flow Cell section of this protocol.

8. Washing and reloading a PromethION Flow Cell

Materials

Adapter ligated DNA library (from previous section)
Flow Cell Wash Kit (EXP-WSH004)
Sequencing Auxiliary Vials V14 (EXP-AUX003)

Consumables

1.5 ml Eppendorf DNA LoBind tubes

Equipment

P1000 pipette and tips
P20 pipette and tips
Ice bucket with ice
Vortex mixer

We recommend washing and reloading the flow cell after ~30 hours of sequencing.

For this method, the flow cell is washed after ~30 hours of sequencing to restore pores to ensure efficient data acquisition.
For this reason, enough library was generated for 2 flow cell loads in the adapter ligation step of the protocol.

- This washing procedure aims to remove most of the initial library and unblock the pores to prepare the flow cell for the loading of a subsequent library.
- Data acquisition in MinKNOW should be **paused** during the wash procedure and library loading.
- After the flow cell has been washed, the next library can be loaded.

You can navigate to the Pore Activity or the Pore Scan Results plot to see pore availability.

Below you can find example data for pore states observed on a flow cell before and after wash steps are performed. Additionally, you can observe an example for the cummulative sequencing data output, including the wash and reload steps. The red asterisks indicate the flow cell wash and reloads.

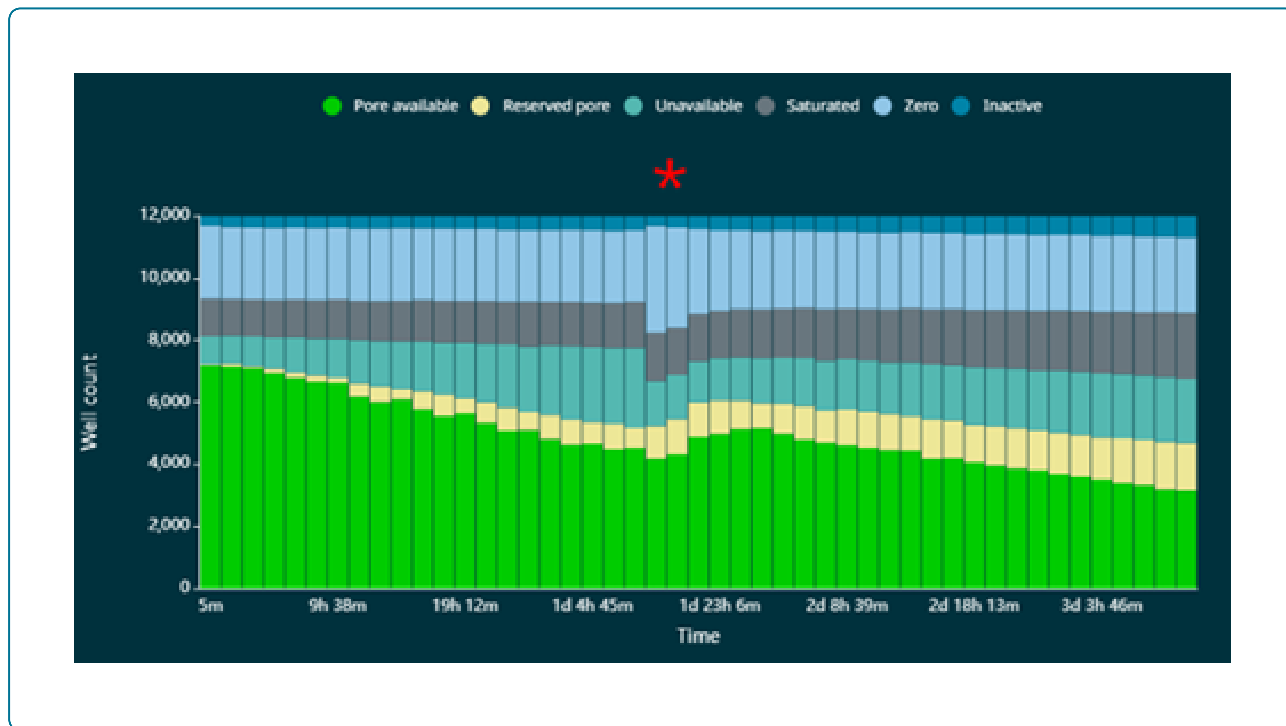


Figure 1. Channel state over a 72 hour run. The flow cell washes are incorporated into the method to restore blocked pores, to allow continuous data acquisition. Red asterisks denote

when a flush was performed.

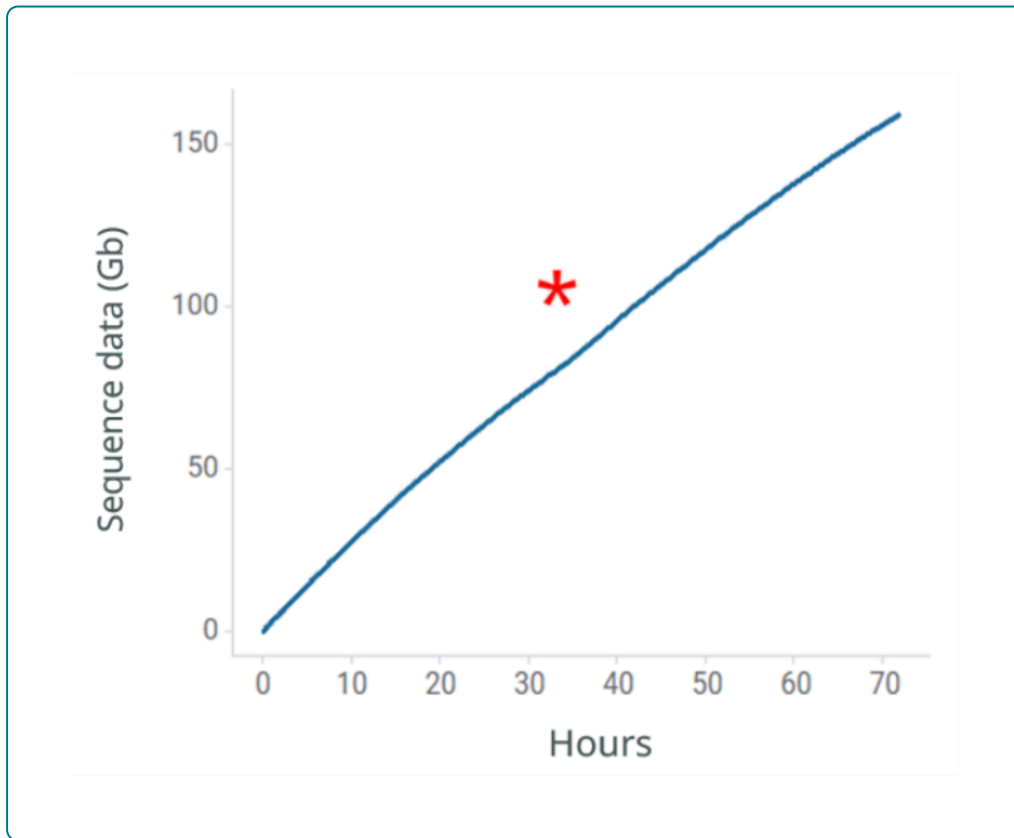


Figure 2. Cumulative sequencing data output, over a 72 hour run. Red asterisks denote when a flush was performed.

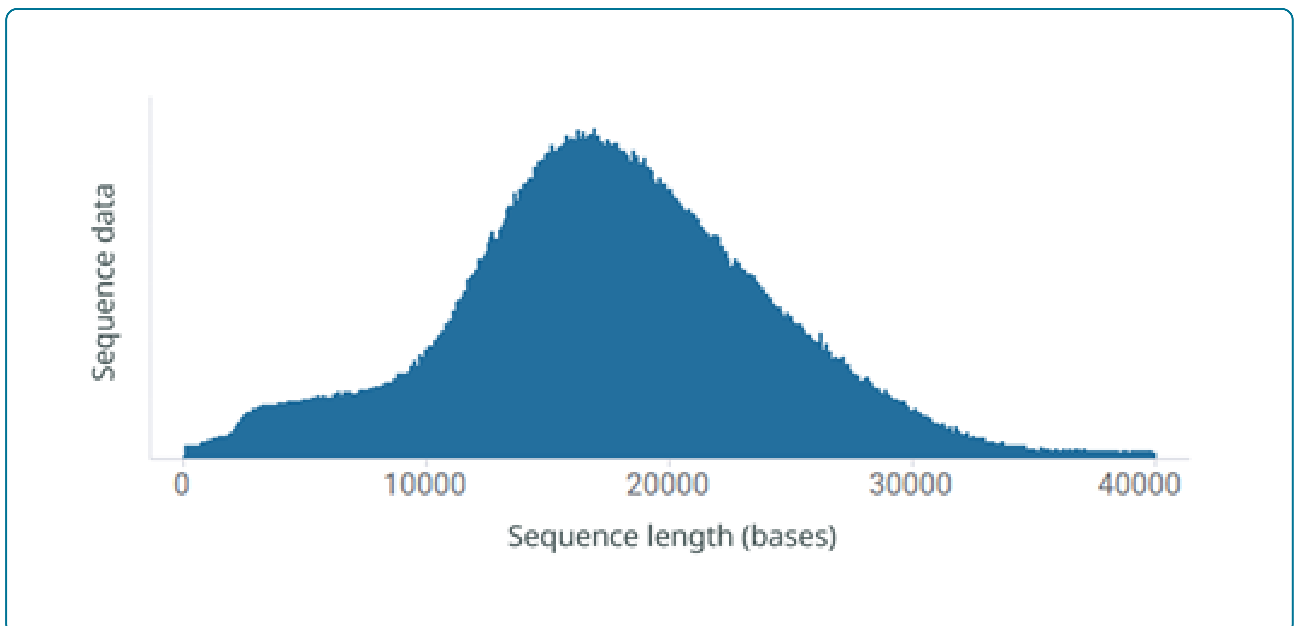


Figure 3. Read length profile for a 15 kb N50 library. The approximately gaussian shape is characteristic of gDNA that has undergone Megaruptor shearing.

Washing and reloading a PromethION Flow Cell video

This video will show you how to wash a flow cell after a sequencing run and how to load a new library.

We recommend keeping the light shield on the flow cell during washing if a second library will be loaded straight away.

If the flow cell is to be washed and stored, the light shield can be removed.

1. **Place the tube of Wash Mix (WMX) on ice. Do not vortex the tube.**

2. **Thaw one tube of Wash Diluent (DIL) at room temperature.**

3. **Mix the contents of Wash Diluent (DIL) thoroughly by vortexing, then spin down briefly and place on ice.**

4. **In a fresh 1.5 ml Eppendorf DNA LoBind tube, prepare the following Flow Cell Wash Mix:**

Reagent	Volume per flow cell
Wash Mix (WMX)	2 µl
Wash Diluent (DIL)	398 µl
Total	**400 µl**

5 Mix well by pipetting, and place on ice. Do not vortex the tube.

6 Pause the sequencing experiment in MinKNOW, and leave the flow cell in the device.

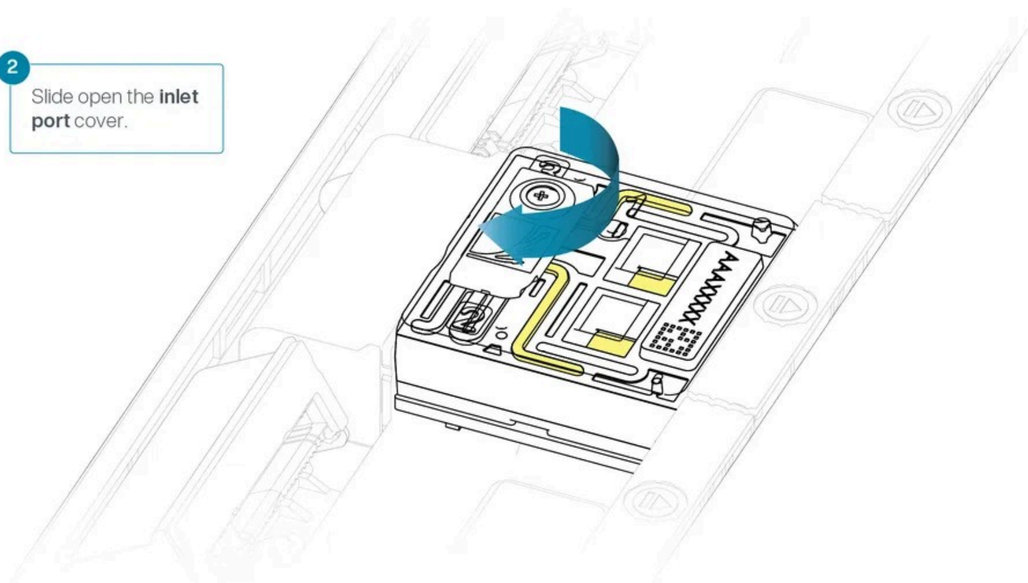
It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

7 Remove waste buffer, as follows:

1. Close the inlet port.
2. Insert a P1000 pipette into a waste port and remove the waste buffer.

Note: As both the inlet port is closed, no fluid should leave the sensor array area.

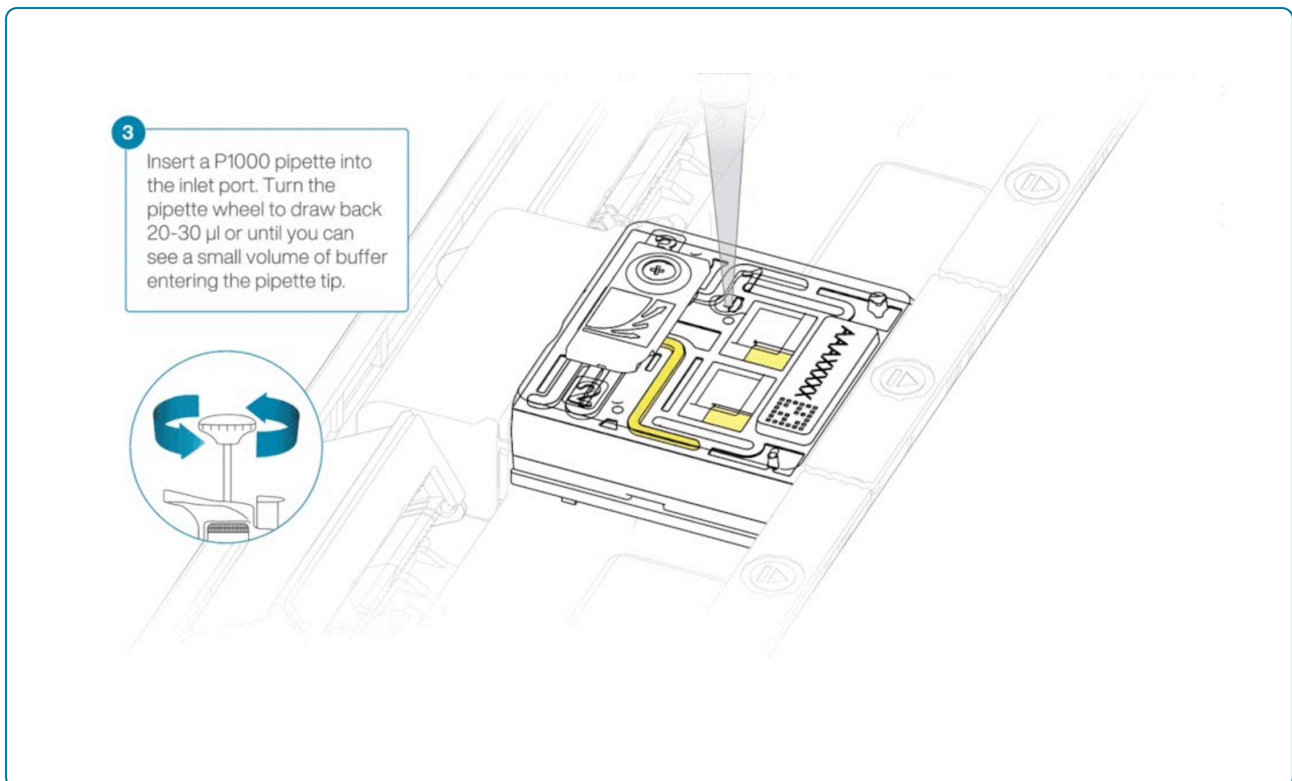
8 Slide the inlet port cover clockwise to open the inlet port.



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.

9 After opening the inlet port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

1. Set a P1000 pipette to 200 µl
2. Insert the tip into the inlet port
3. Turn the wheel until the dial shows 220-230 µl, or until you can see a small volume of buffer entering the pipette tip.



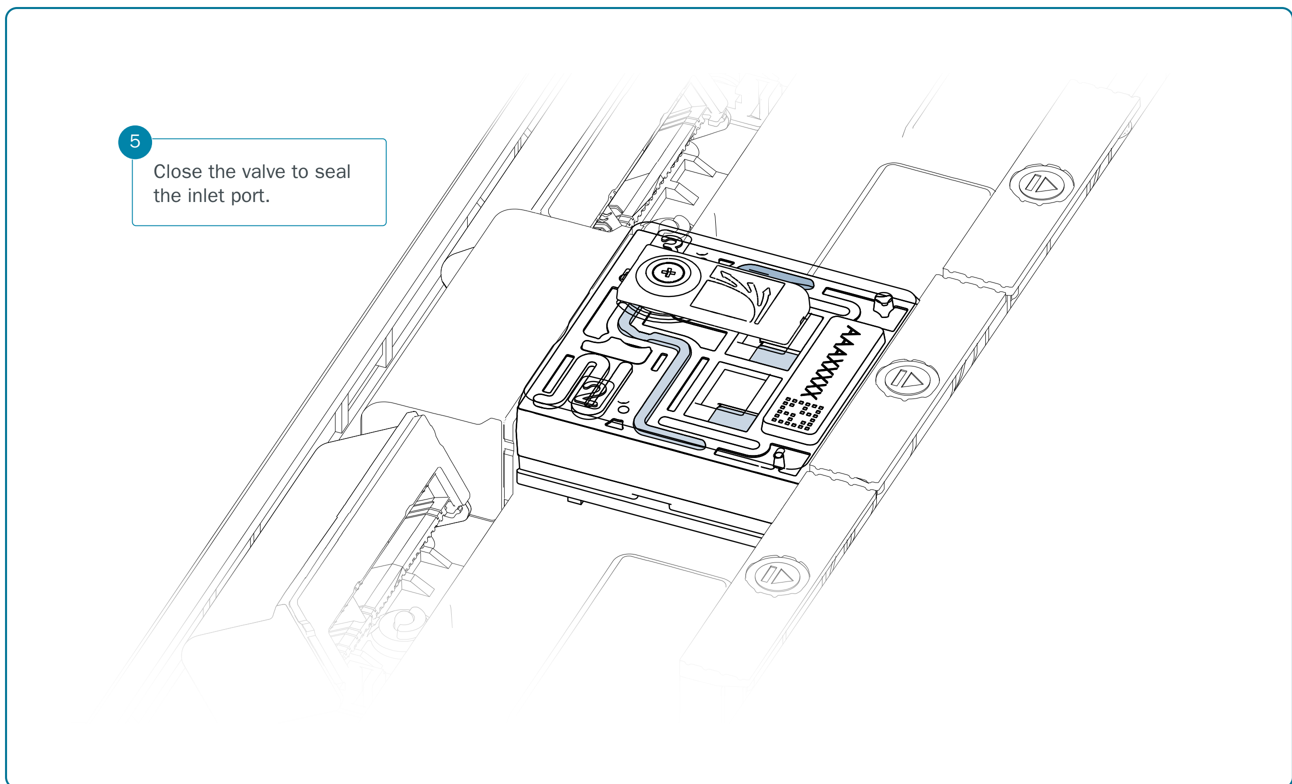
10 Slowly load 200 µl of the prepared flow cell wash mix into the inlet port, as follows:

1. Using a P1000 pipette, take 200 µl of the flow cell wash mix
2. Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip
3. Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger **very slowly**, leaving a small volume of buffer in the pipette tip.
4. Set a timer for a 5 minute incubation.

11 Once the 5 minute incubation time is complete, carefully load the remaining 200 µl of the prepared flow cell wash mix into the inlet port, as follows:

1. Using a P1000 pipette, take 200 µl of the flow cell wash mix
2. Insert the pipette tip into the inlet port, ensuring there are no bubbles in the tip
3. Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger **very slowly**, leaving a small volume of buffer in the pipette tip.

12 Close the inlet port and wait for 1 hour.



It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

13 Remove the waste buffer, as follows:

1. Ensure the inlet port is closed.
2. Insert a P1000 pipette into a waste port and remove the waste buffer

Note: As the inlet port is closed, no fluid should leave the sensor array area.

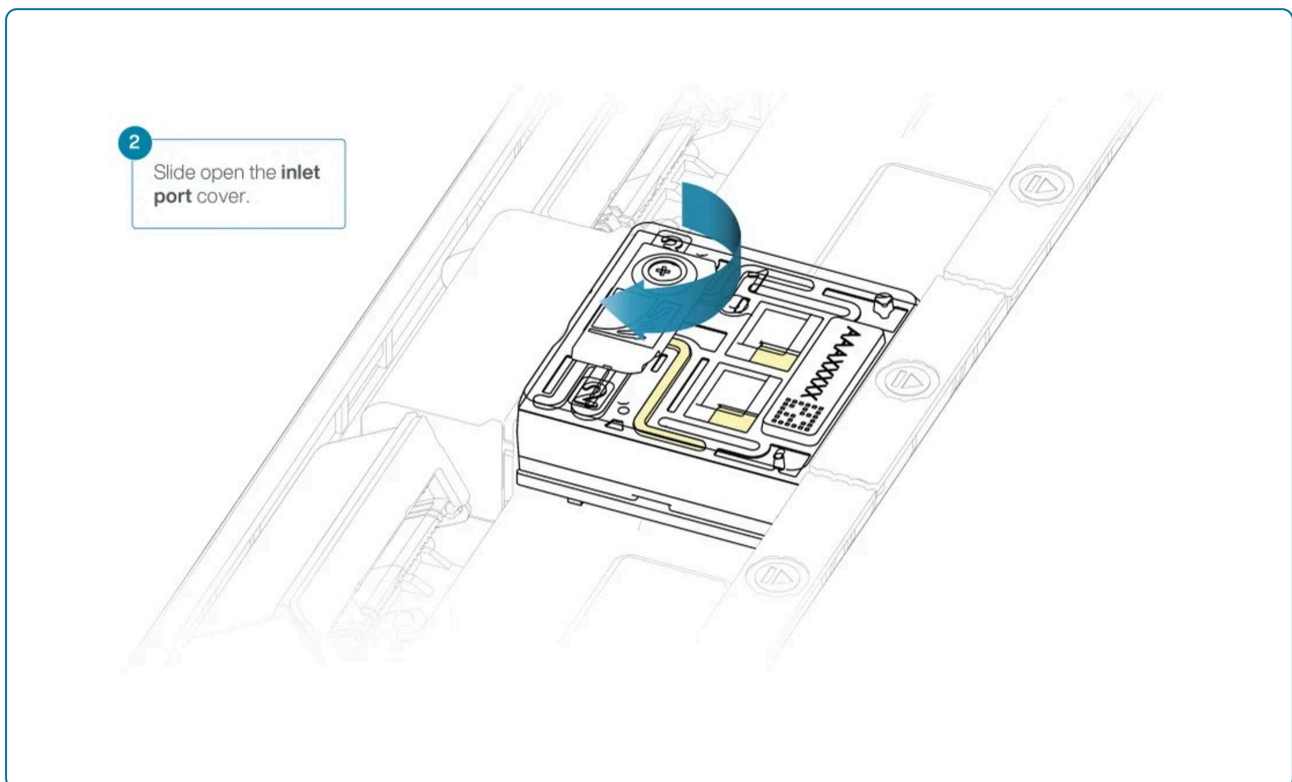
The buffers used in this process are incompatible with conducting a Flow Cell Check step prior to loading the subsequent library. However, number of available pores will be reported after the next pore scan.

14 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature, before mixing by vortexing. Then spin down before storing on ice.

15 **Prepare the flow cell priming mix in a suitable tube for the number of flow cells to flush. Once combined, mix well by briefly vortexing.**

Reagents	Volume per flow cell
Flow Cell Flush (FCF)	1,170 µl
Flow Cell Tether (FCT)	30 µl
Total volume	**1,200 µl**

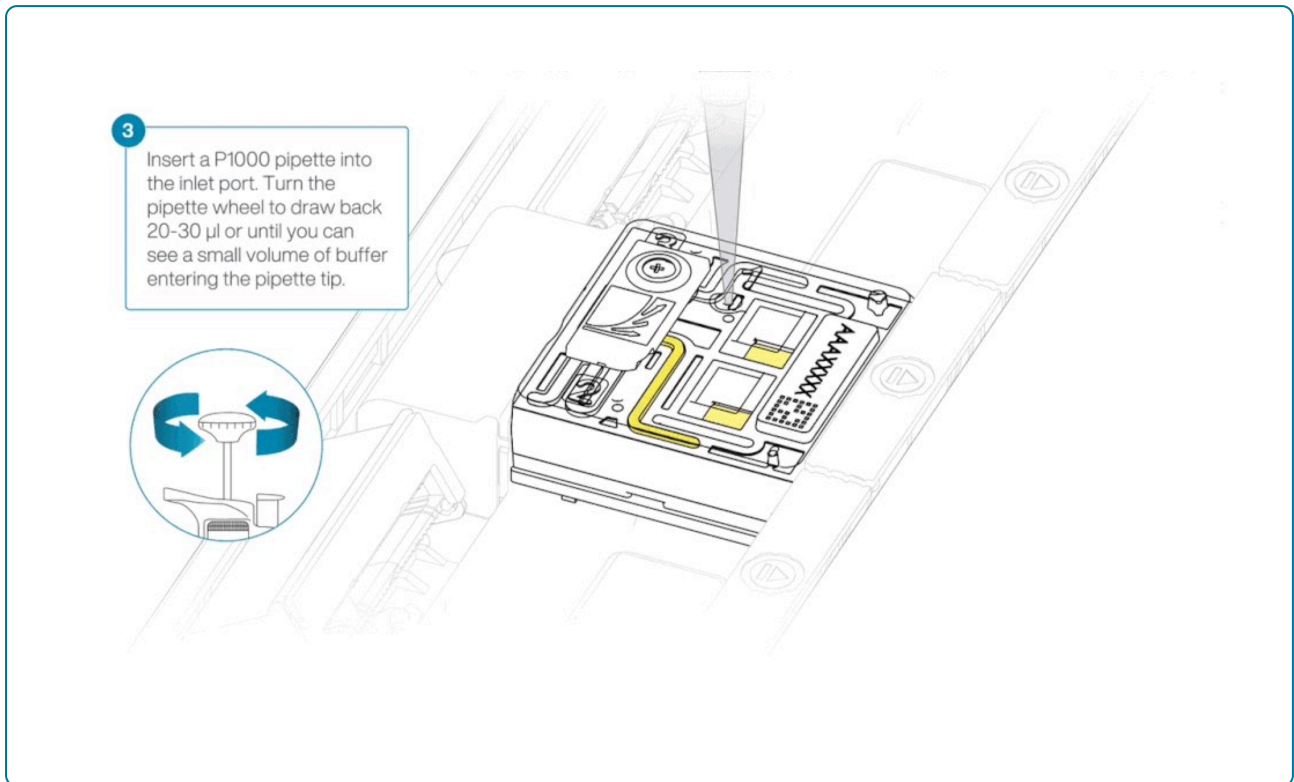
16 **Slide the inlet port cover clockwise to open.**



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.

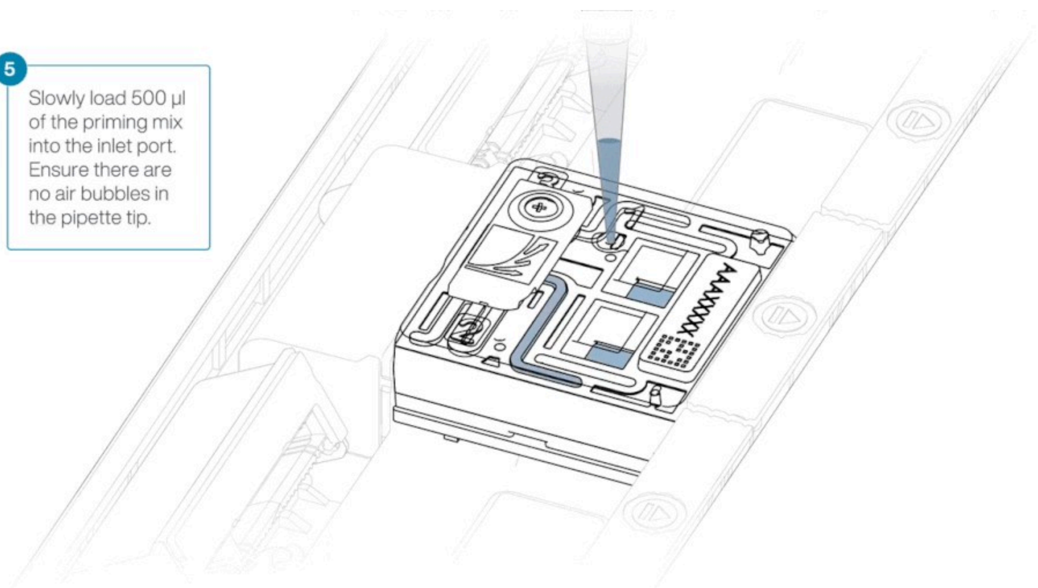
17 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



18 Slowly load 500 µl of the priming mix into the inlet port, as follows:

2. Using a P1000 pipette, take 500 µl of the priming mix
3. Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip
4. Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger **very slowly**, leaving a small volume of buffer in the pipette tip.



It is vital to wait five minutes between the priming mix flushes to ensure effective removal of the nuclease.

19 Close the inlet port and wait five minutes.

During this time, prepare the library for loading using the next steps in the protocol.

20 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

The Library Beads (LIB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

We recommend using the Library Beads (LIB) for most sequencing experiments. However, the Library Solution (LIS) is available for more viscous libraries.

21 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

Reagent	Volume per flow cell
Sequencing Buffer (SB)	100 µl
Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS)	68 µl
DNA library	32 µl
Total	**200 µl**

Note: Library loading volume has been increased to improve array coverage.

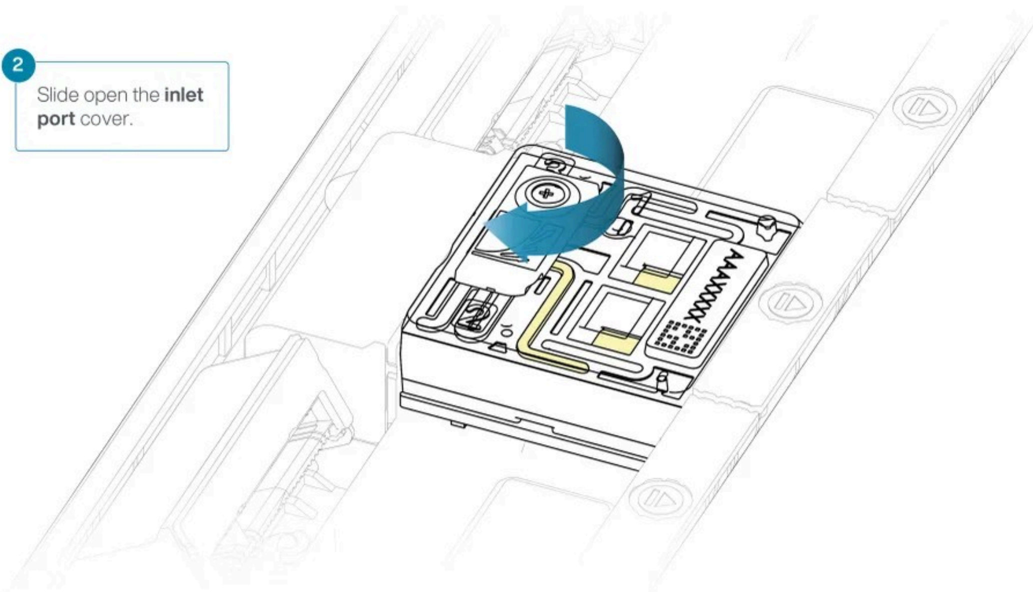
It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

22 Remove the waste buffer, as follows:

1. Ensure the inlet port is closed.
2. Insert a P1000 pipette into a waste port and remove the waste buffer

Note: As the inlet port is closed, no fluid should leave the sensor array area.

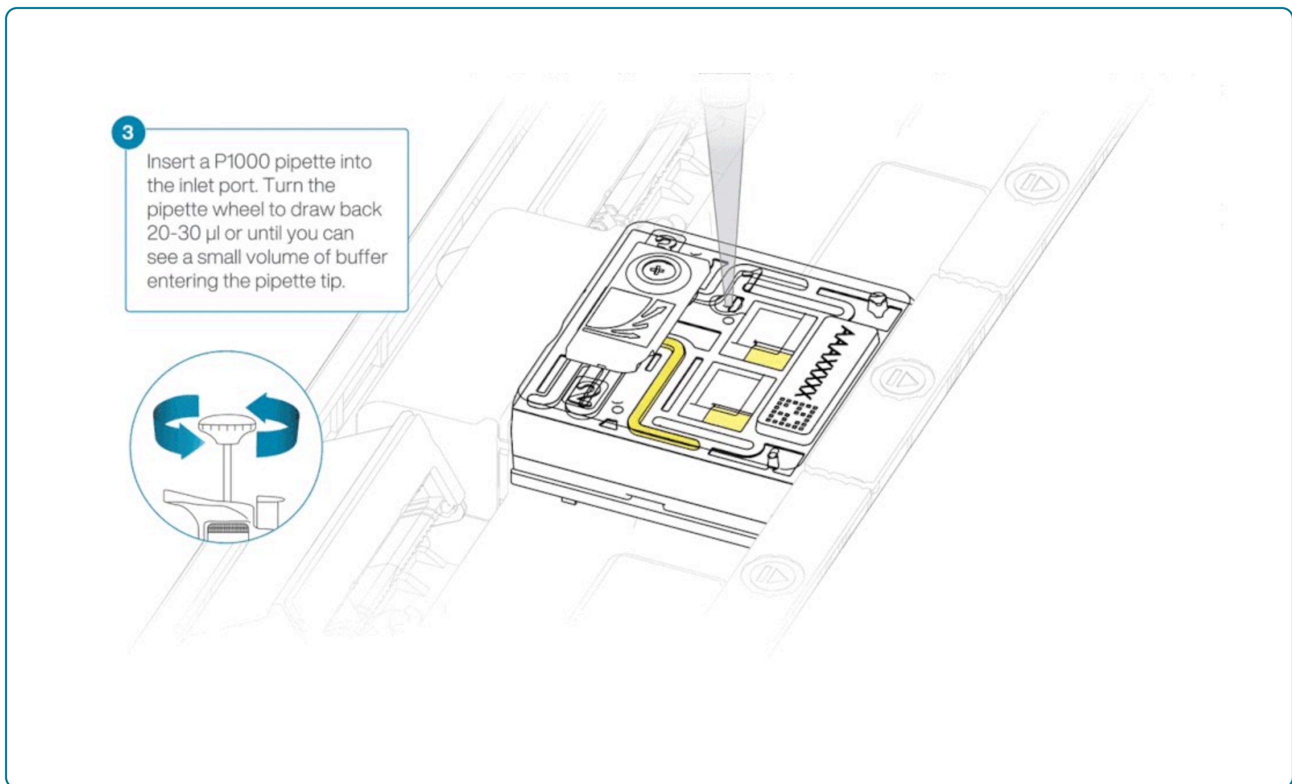
23 Slide the inlet port cover clockwise to open.



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.

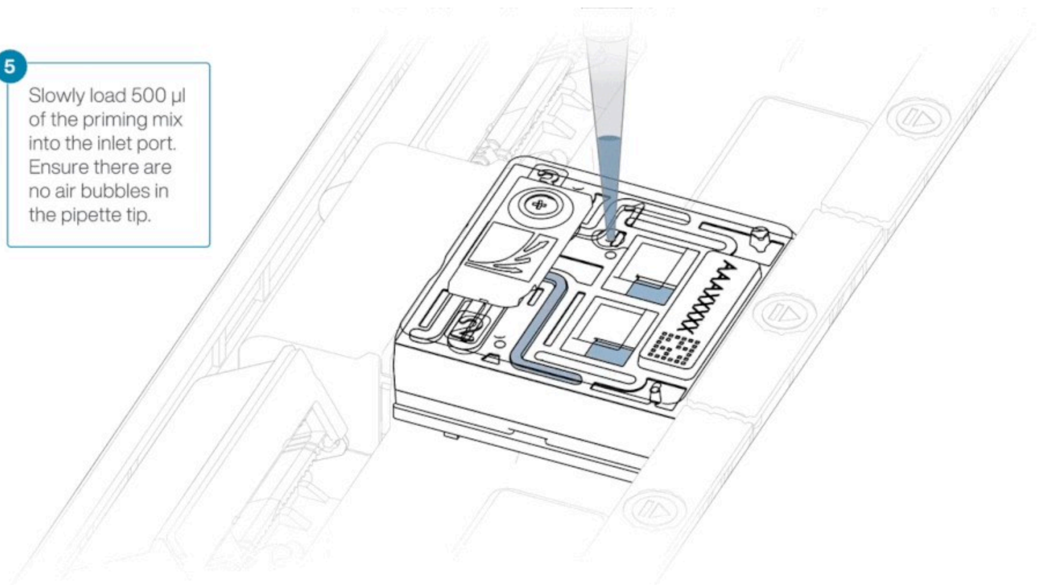
24 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.



25 Slowly load 500 µl of the priming mix into the inlet port, as follows:

2. Using a P1000 pipette, take 500 µl of the priming mix
3. Insert the pipette tip into the priming port, ensuring there are no bubbles in the tip
4. Slowly twist the pipette wheel down to load the flow cell (if possible with your pipette) or push down the plunger **very slowly**, leaving a small volume of buffer in the pipette tip.



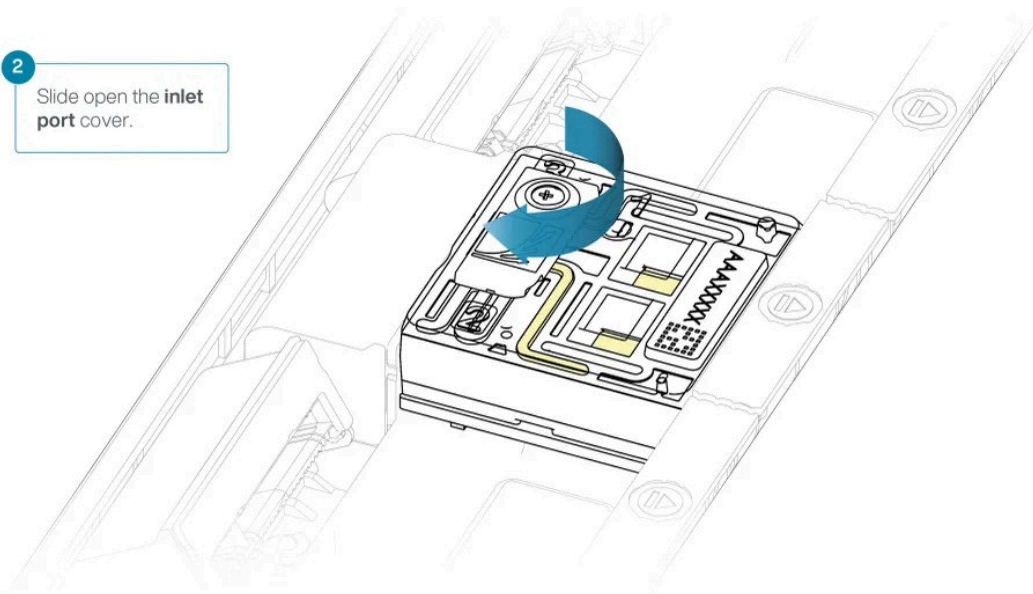
It is vital that the inlet port is closed before removing waste to prevent air from being drawn across the sensor array area, which would lead to a significant loss of sequencing channels.

26 Remove waste buffer, as follows:

1. Close the inlet port.
2. Insert a P1000 pipette into a waste port and remove the waste buffer.

Note: As both the inlet port is closed, no fluid should leave the sensor array area.

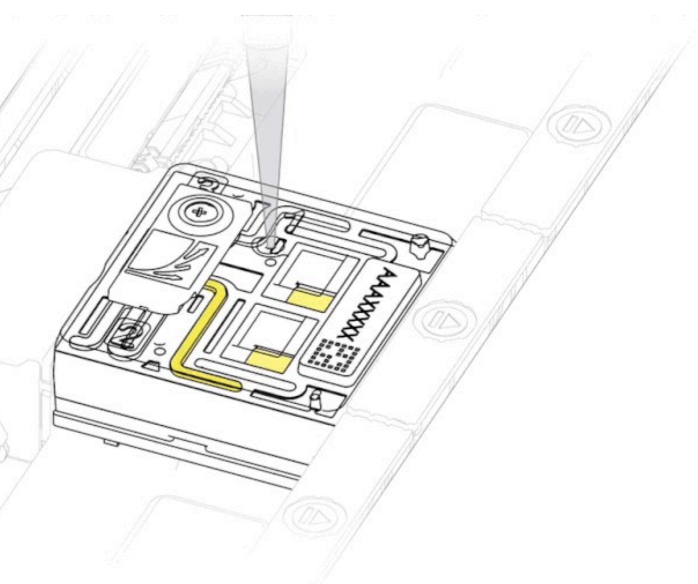
27 Slide the inlet port cover clockwise to open.



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.

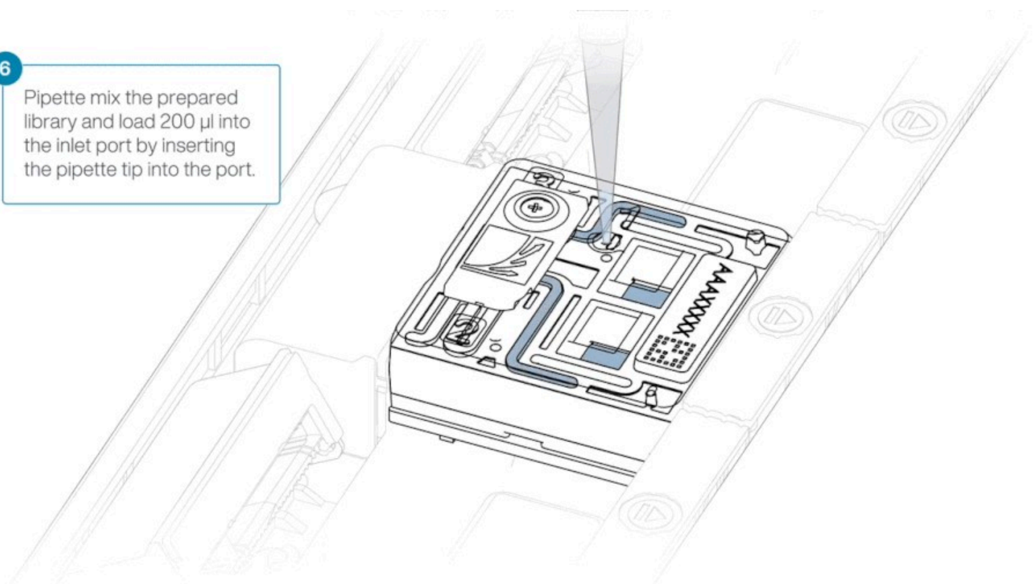
28 After opening the inlet port, draw back a small volume to remove any air bubbles:

1. Set a P1000 pipette tip to 200 µl.
2. Insert the tip into the inlet port.
3. Turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.

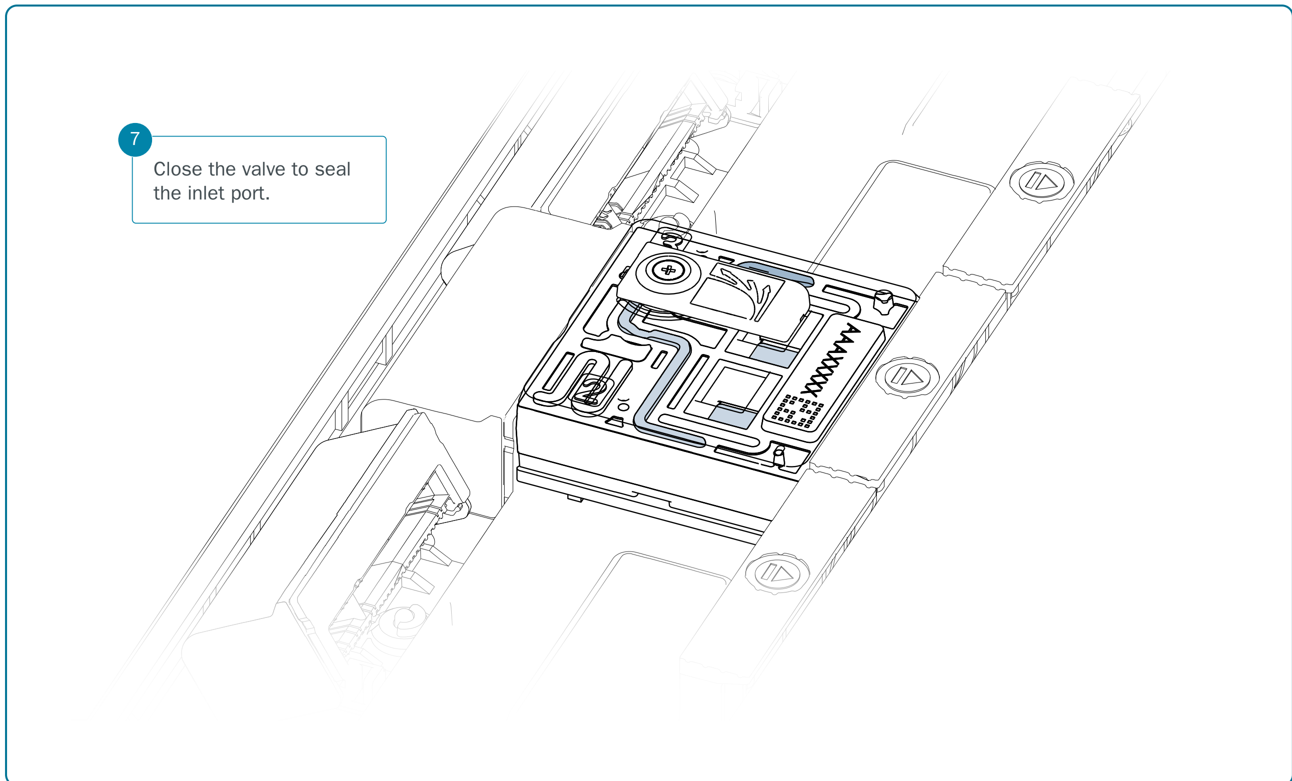


29 **Mix the prepared library gently by pipetting up and down just prior to loading.**

30 **Load 200 µl of library into the inlet port using a P1000 pipette.**



31 Close the valve to seal the inlet port.

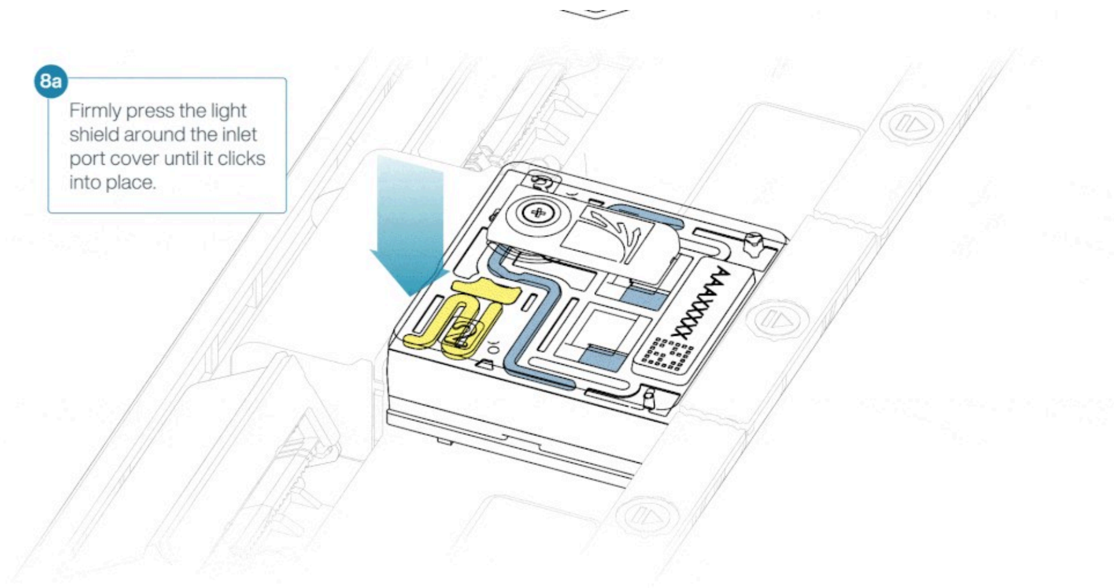


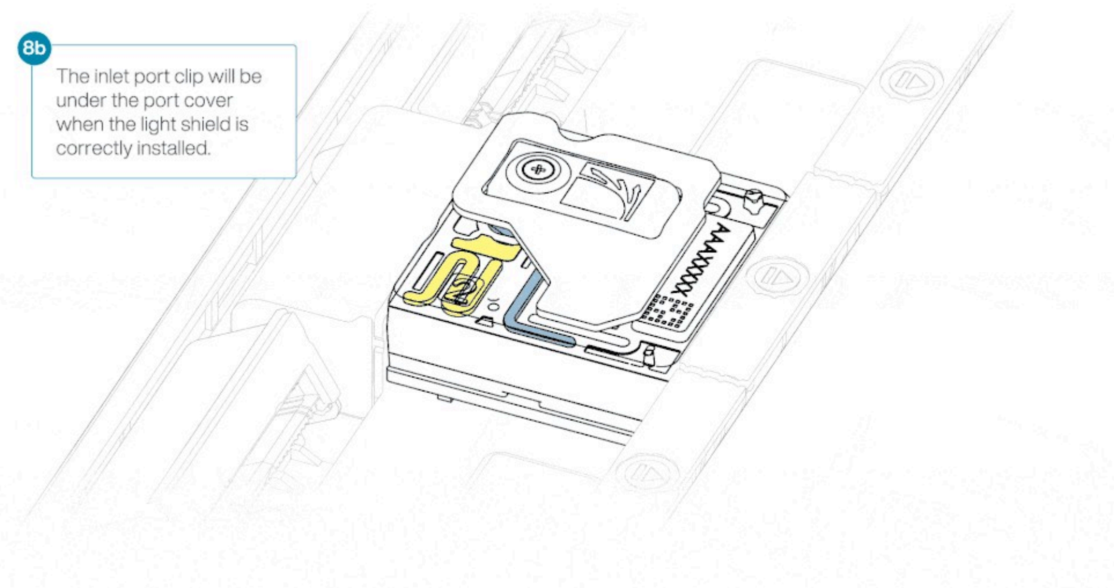
For optimal sequencing output, install the light shield on your flow cell as soon as the library has been loaded.

We recommend leaving the light shield on the flow cell when library is loaded, including during any washing and reloading steps. The shield can be removed when the library has been removed from the flow cell.

32 If the light shield has been removed from the flow cell, install the light shield as follows:

1. Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.
2. Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.





33 Close the PromethION lid when ready to start a sequencing run on MinKNOW.

Wait a minimum of 10 minutes after loading the flow cells onto the PromethION before initiating any experiments. This will help to increase the sequencing output.

9. Data acquisition and basecalling

How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software.

We recommend basecalling with the high accuracy (HAC) basecaller in real-time with BAM selected as output type using the P2 Solo or P24/P48 device.

You must generate a BAM file from your sequening run, as this is required for input into the wf-human-variation workflow.

Refer to the links below containing the detailed instructions for setting up the device and sequencing run:

- PromethION 24 and 48: "Starting a sequencing run with PromethION 24 and 48"
- PromethION 2 Solo: "Starting a sequencing run on PromethION 2 Solo"

Below are the recommended sequencing parameters for MinKNOW.

MinKNOW settings for 15 kb extracted human blood sample variant workflow on PromethION

We recommend setting the run time to 72 hours to accommodate for the flow cell wash, using the modified bases option for basecalling and ensuring a BAM output is selected. All other sequencing parameters can be kept to their default settings. Below are our current recommendations:

Positions

Flow cell position: [user defined]

Experiment name: [user defined]

Flow cell type: FLO-PRO114M

Sample ID: [user defined]

Kit

Kit selection: Ligation Sequencing Kit (SQK-LSK114)

Run configuration

Sequencing and analysis

Basecalling: On [default] **Modified bases**: On with '5mC & 5hmC CG contexts' selected **Model**: High-accuracy basecalling (HAC) [default]

Barcoding: Disabled [default]

Alignment: Off [default]
We do not currently recommend live alignment during sequencing, as it can slow down system processing.

Adaptive sampling: Off [default]

Advanced options **Active channel selection**: On [default] **Time between pore scans**: 1.5 [default] **Reserve pores**: On [default]

Data targets

Run limit: 72 hours [default]

Output

Output format **.POD5**: On [default] **.FASTQ**: On [default] **.BAM**: On

Filtering: On [default] **Qscore**: 9 [default] **Minimum read length**: 200 bp [default]

We do not recommend live alignment during sequencing, as it can slow down system processing.

You can align your BAM file post-sequencing by following one of the methods below:

Aligning the BAM file in MinKNOW	Aligning the BAM file during the wf-human-variation workflow
Align the BAM output after live basecalling in MinKNOW. This will prevent slowing down your sytems processing. The aligned BAM file can be used as your file input in the wf-human-variation workflow. Using mapped BAM as input, the workflow will take 1-2 hours.	You can provide a reference genome along with the unaligned BAM file during the wf-human-variation workflow set-up. Using an unmapped BAM is used as input, the workflow will take approximately 5-8 hours.

Further information is available in the Downstream analysis section of this protocol.

10. Downstream analysis

Analysis of human blood DNA sequence data

For the analysis of human blood DNA sequence data, we recommend the wf-human-variation workflow. This end-to-end software pipeline is implemented using the Nextflow workflow language and implements methods for the calling of single nucleotide polymorphisms (SNPs), structural variants (SVs), and for reporting DNA methylation information.

The wf-human-variation workflow is best run from the BAM file produced by MinKNOW when the modified base model for basecalling is selected. If sequence read mapping to the reference genome is not performed by MinKNOW, we recommend to perform the basecalling using the wf-basecalling workflow. Ensure you save the outputs in BAM format by providing the **--output_bam** option.

The tools below are used in the analysis workflow and can be run in isolation or together:

1. Sniffles2 calls SVs and file output include an HTML report of QC metrics and VCF format list of variants and their quality scores.
2. Clair3 calls SNPs and file output includes an HTML report of QC metrics and VCF format list of variants and their quality scores.
3. modkit extracts methylation information from the provided BAM file which is summarised in a BEDmethyl format file.

The wf-human-variation workflow is preconfigured using appropriate parameters and requires tuning only for the choice of reference genome and Clair3 model. Please see the project's documentation for further details.

The results from the wf-human-variation workflow can be further explored by viewing in a track-based genome browser such as IGV can be assessed for known pathogenicity through tertiary analysis software.

EPI2ME analysis workflow

The wf-human-variation workflow is intended to be run using the Nextflow software.

We recommend performing downstream analysis using EPI2ME which facilitates bioinformatic analyses by allowing users to run Nextflow workflows in a Graphical User Interfaces (GUI). EPI2ME maintains a collection of bioinformatic workflows which are curated and actively maintained by experts in long-read sequence analysis. The collection of all our available EPI2ME workflows can be found here.

For new users, the quick start guide can be found here outlining how to use this interface.

Compute requirements for the wf-human-variation workflow on EPI2ME

Recommended requirements	Minimum requirements
CPUs = 32	CPUs = 16
Memory = 128GB	Memory = 32GB

Approximate run time: Variable depending on whether it is targeted sequencing or whole genome sequencing, as well as coverage and the individual analyses requested. For instance, a 90X human sample run (options: --snp --sv --mod --str --cnv --phased --sex male) takes less than 8h with recommended resources.

ARM processor support: False

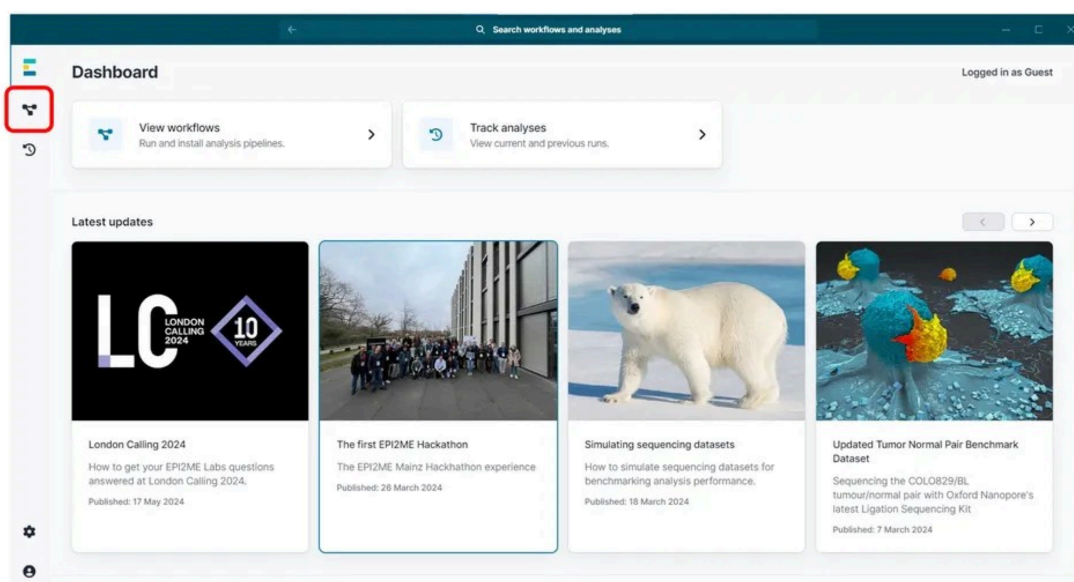
The wf-human-variation workflow can also be run using the command line interface (CLI)

Please see the Github page for further details.

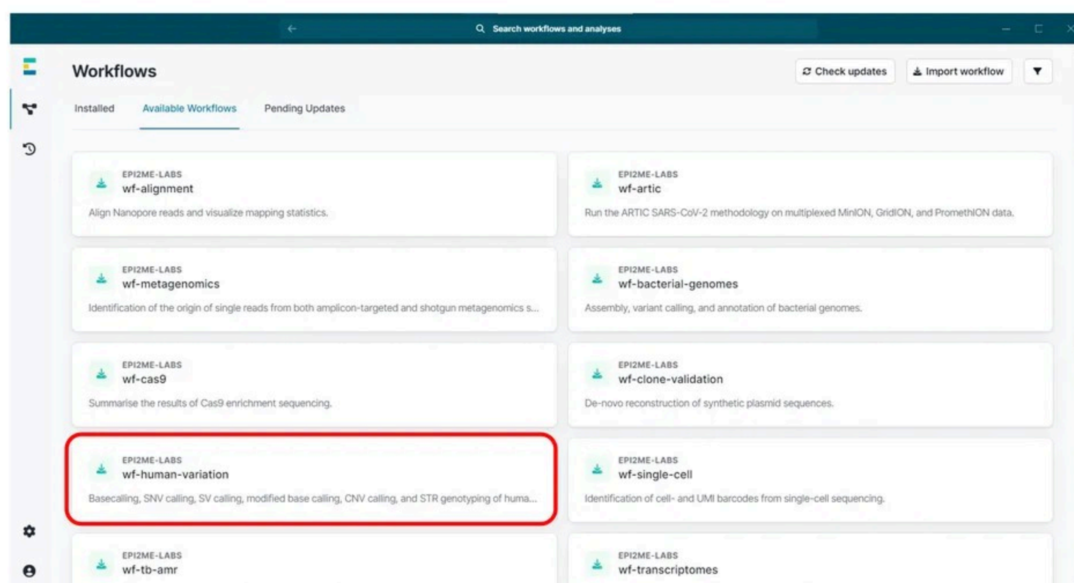
Note: We only recommend the command line interface (CLI) for experienced users.

1. **Open the EPI2ME app using the desktop shortcut.**

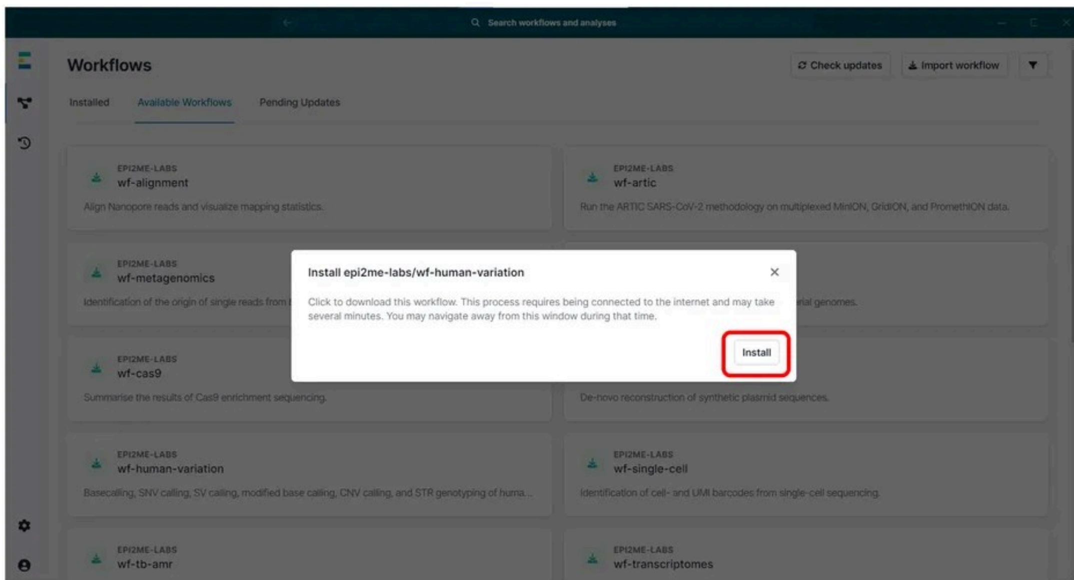
2. **On the landing page, open the workflow tab on the left-hand sidebar.**



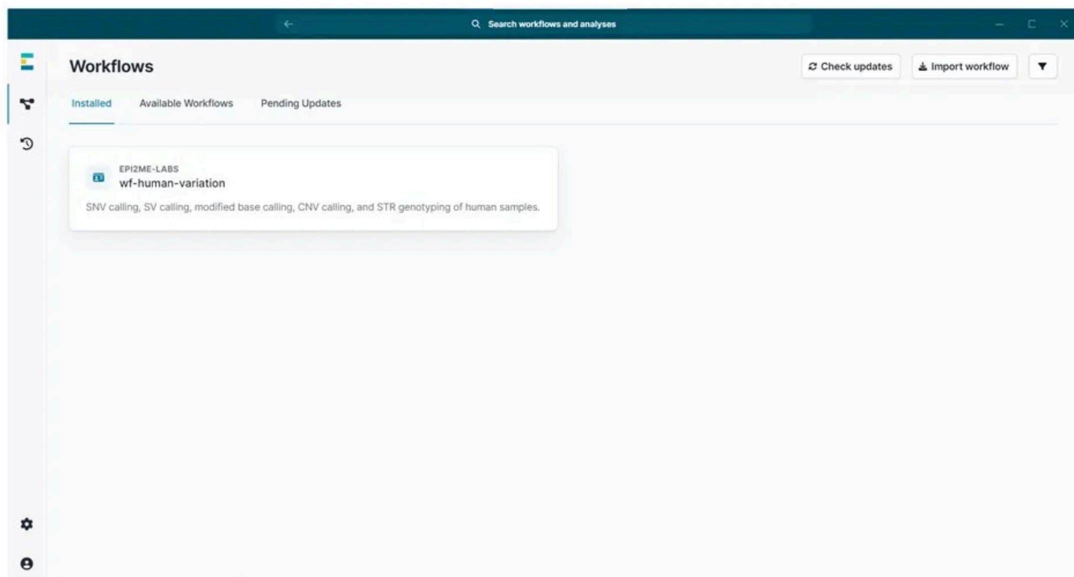
3. **Navigate to the Available workflows tab and click on wf-human-variation option.**



4. **Click install.**

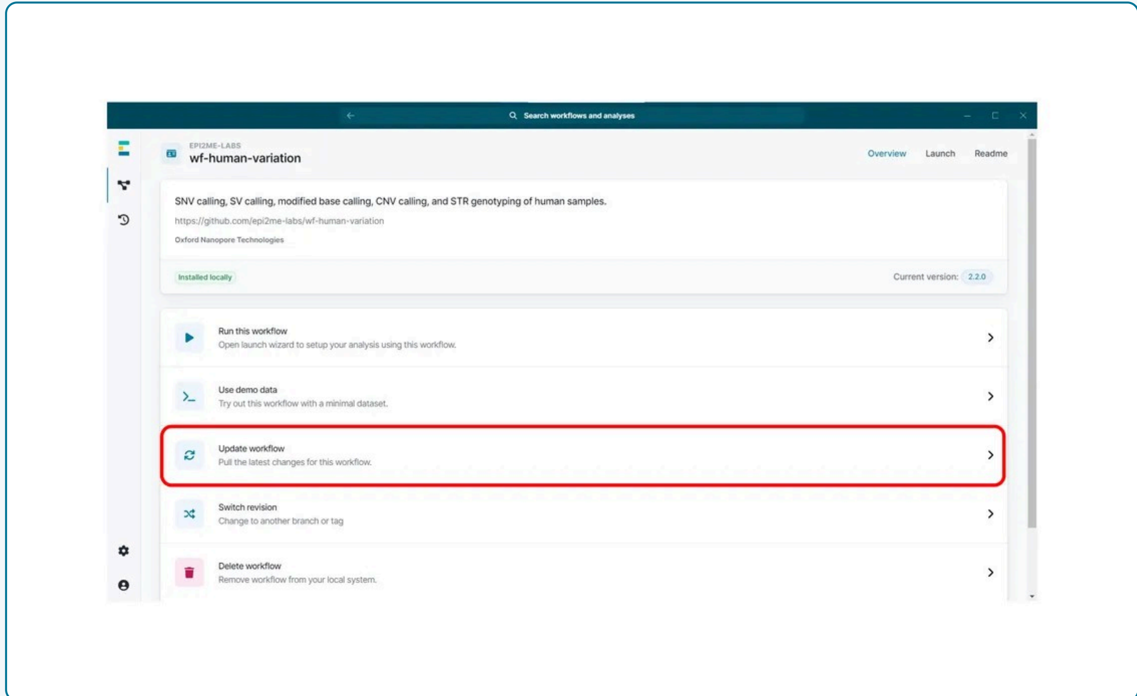


5 **Navigate to the Installed tab and click on the installed wf-human-variation workflow.**

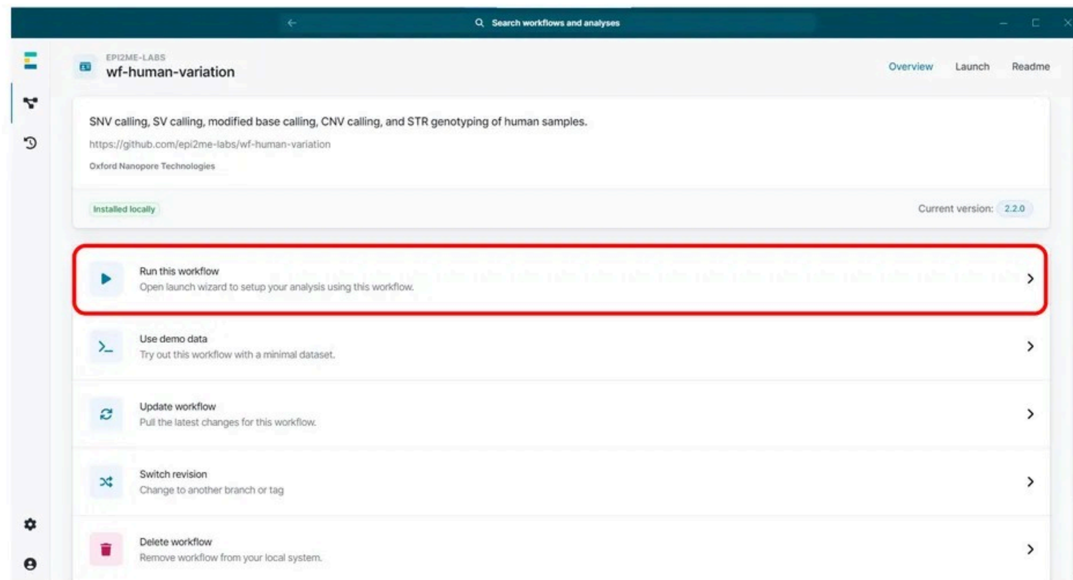


If the workflow was already installed, check for updates by clicking 'Update workflow'.

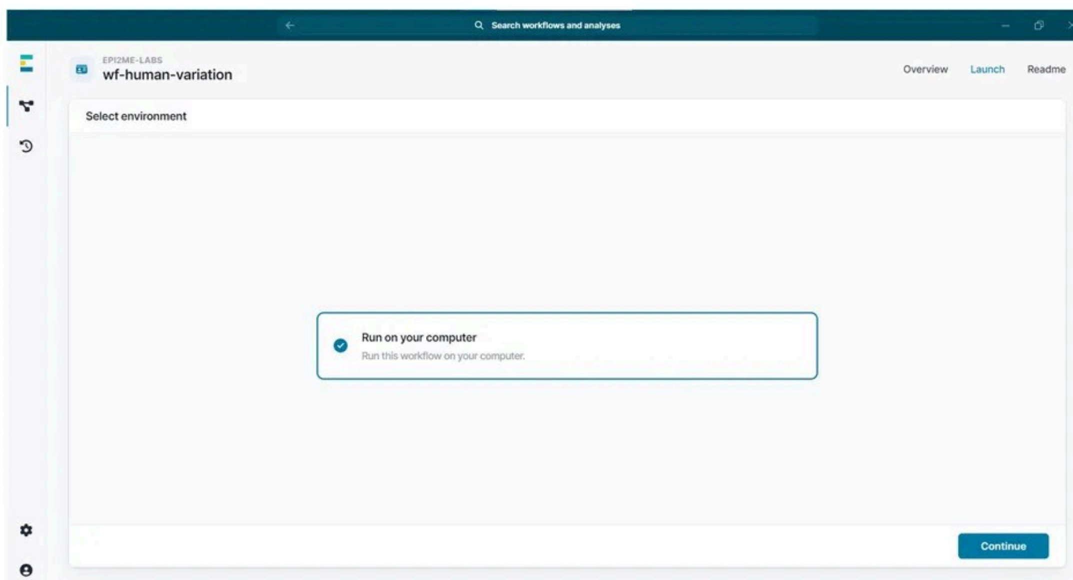
We recommend running the latest version of our workflows for the best results.



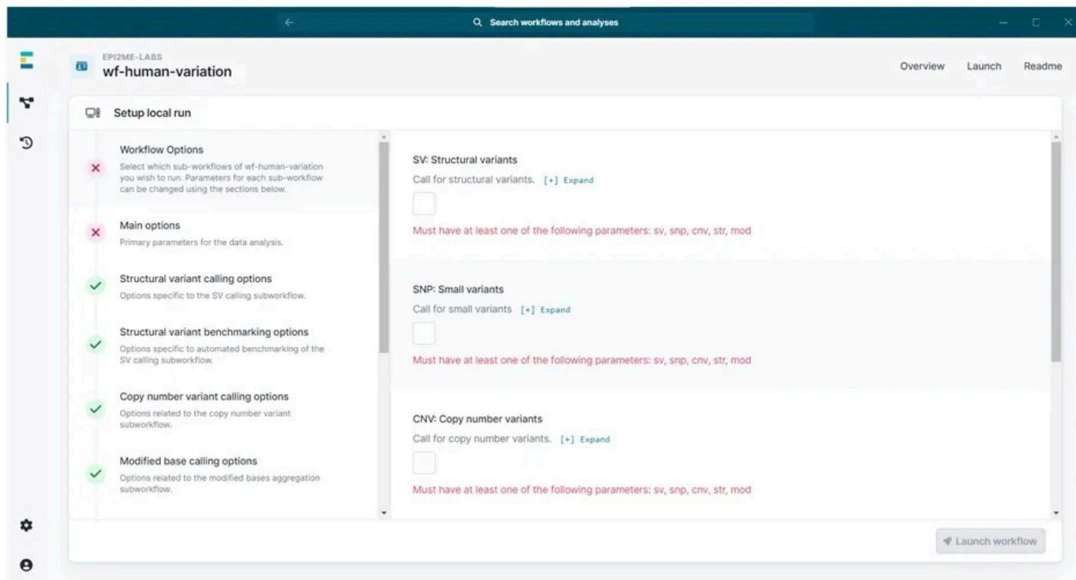
6 Click on Run this workflow to open the launch wizard.



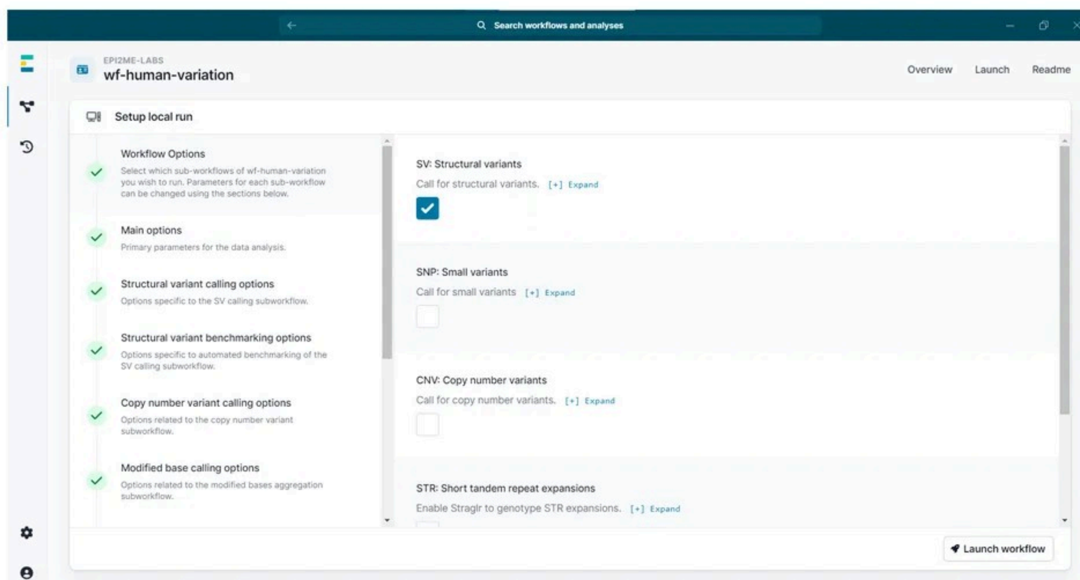
Select the environment you are running the workflow from:



7 Click on the sub-workflow(s) you want to run in the wf-human-variation analysis.

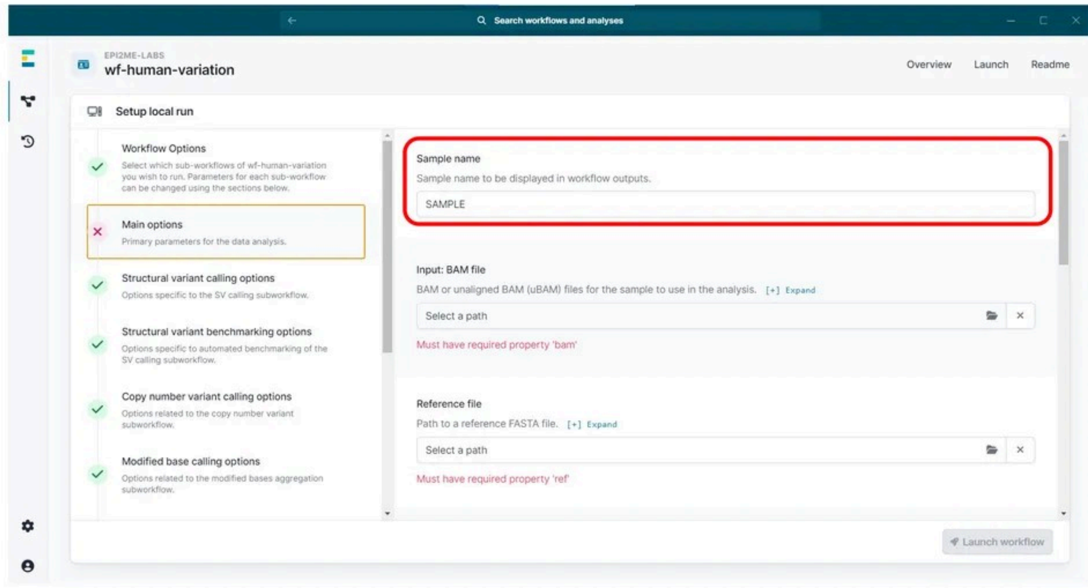


You must have at least one of the sub-workflows selected to proceed with analysis.



Note: For more information on the sub-workflows click on the "Expand" option in the platform, or visit our online EPI2ME documentation.

8 Navigate to the 'Main options' tab to assign a 'Sample name' as an identifier in workflow outputs.

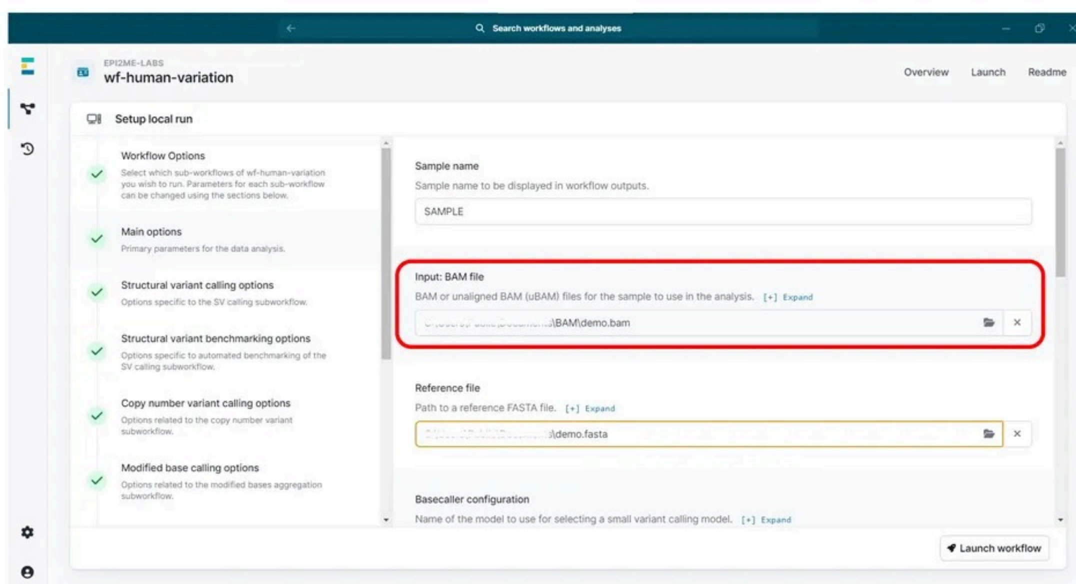

Search workflows and analyses
EPI2ME-LABS
wf-human-variation
Overview
Launch
Readme
Setup local run
Workflow Options
Select which sub-workflows of wf-human-variation you wish to run. Parameters for each sub-workflow can be changed using the sections below.
Main options
Primary parameters for the data analysis.
Structural variant calling options
Options specific to the SV calling subworkflow.
Structural variant benchmarking options
Options specific to automated benchmarking of the SV calling subworkflow.
Copy number variant calling options
Options related to the copy number variant subworkflow.
Modified base calling options
Options related to the modified bases aggregation subworkflow.
Sample name
Sample name to be displayed in workflow outputs.
SAMPLE
Input: BAM file
BAM or unaligned BAM (uBAM) files for the sample to use in the analysis. [+] Expand
Select a path
Must have required property 'bam'
Reference file
Path to a reference FASTA file. [+] Expand
Select a path
Must have required property 'ref'
Launch workflow

The wf-human-variation workflow uses sequencing data in the form of a single BAM file or a folder of BAM files.

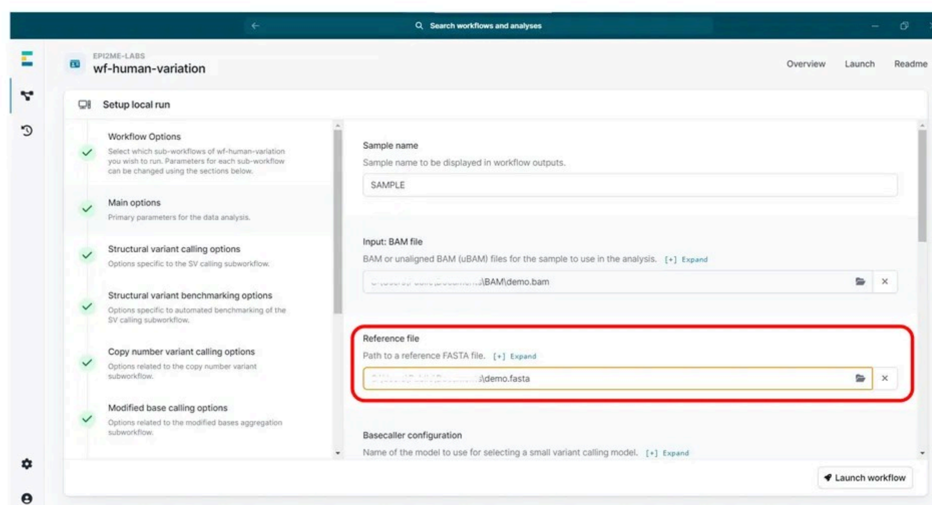
The BAM files used as an input can be aligned or unaligned:

Aligning the BAM file in MinKNOW (prior to the wf-human-variation workflow)	Aligning the BAM file during the wf-human-variation workflow (during the wf-human-variation workflow)
Align the BAM output after live basecalling in MinKNOW. This will prevent slowing down your sytems processing. The aligned BAM file can be used as your file input in the wf-human-variation workflow. For more information on post-run alignment in MinKNOW please visit our MinKNOW protocol. Using mapped BAM as input, the workflow will take 1-2 hours.	You can provide a reference genome along with the unaligned BAM file during the wf-human-variation workflow set-up. Using an unmapped BAM is used as input, the workflow will take approximately 5-8 hours.

9. **In the 'Main options' upload your sequencing data in the form of a single BAM file or a folder of BAM files.**

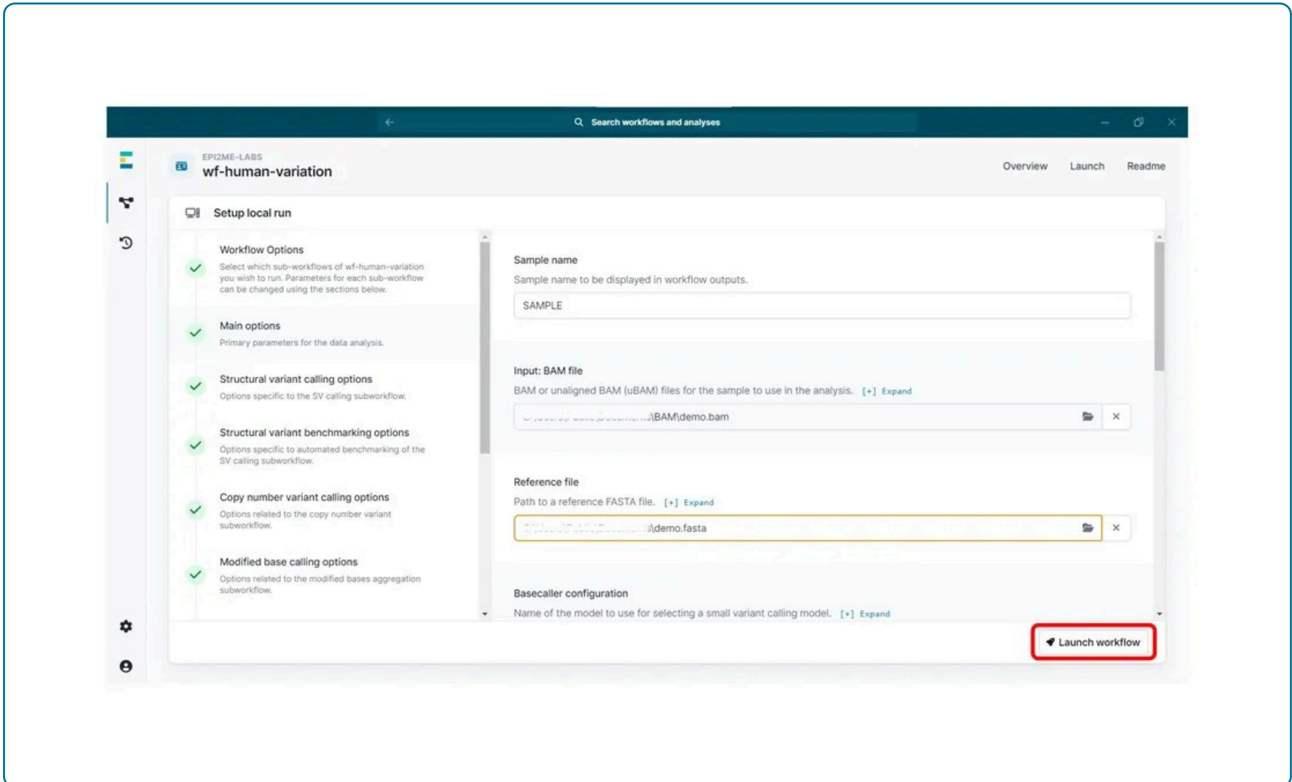


If you have an unaligned BAM file as input, in the 'Main options' upload your reference genome in FASTA format.

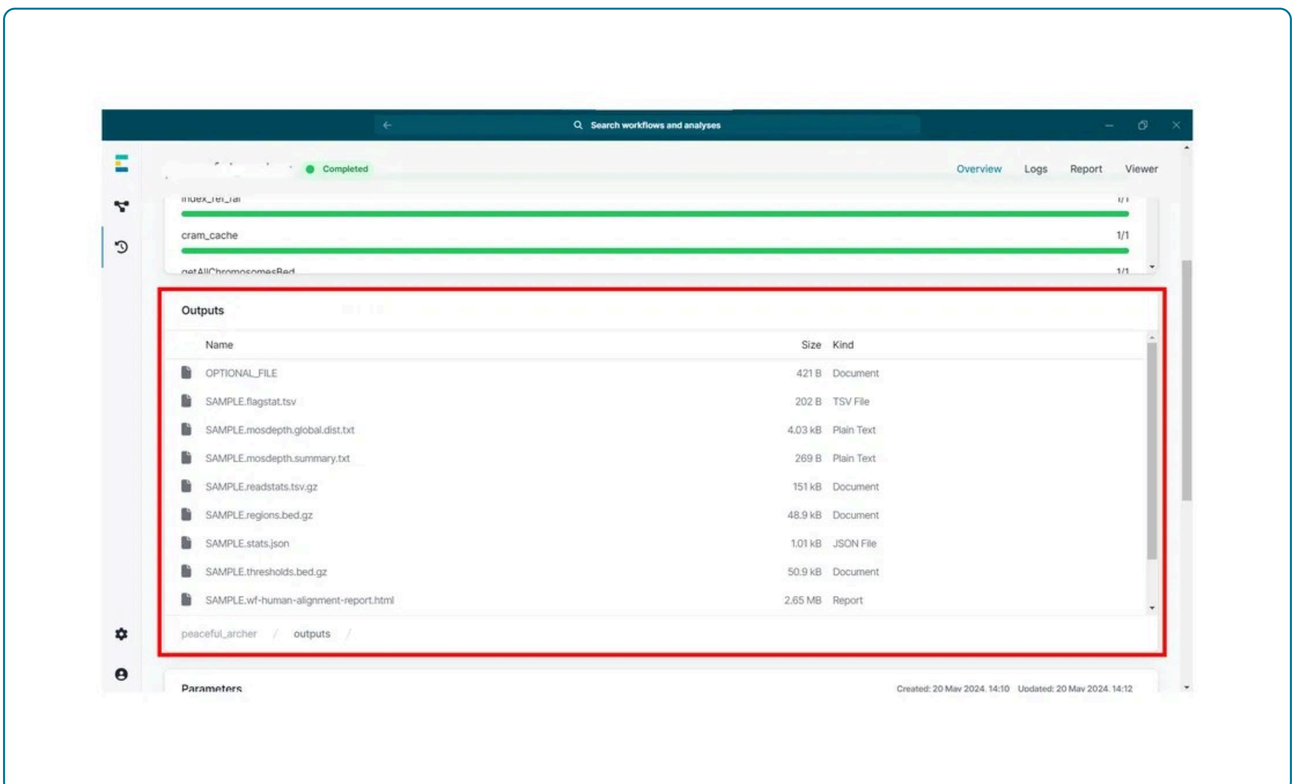


10 Click Launch workflow.

Ensure all parameter options have green ticks.



11 Once the wf-human-variation workflow finishes, a report will be produced alongside output files.



wf-human-variation workflow outputs

The **primary workflow** outputs include:

- gzipped VCF file containing the SNPs in the dataset from `--snp`
- gzipped VCF file containing the SVs in the dataset from `--sv`
- gzipped bedMethyl file aggregating modified base counts from `--mod`
- HTML report detailing the primary findings of the workflow for QC metrics, and SNP and SV calling
- If an unaligned BAM file was provided, the workflow will ouput a CRAM file containing the alignments used to make the downstream variant calls.

The **secondary workflow** outputs:

- `mosdepth` ouputs include:
    - `{sample_name}.mosdepth.global.dist.txt` : a cumulative distribution indicating the proportion of total bases for each and all reference sequences
    - `{sample_name}.regions.bed.gz` : the mean coverage for each region in the provided BED file
    - `{sample_name}.thresholds.bed.gz` : the number of bases in each region that are covered at or above each threshold value (1, 10, 20, 30X)

- bamstats ouputs include:
    - `{sample_name}.readstats.tsv.gz` : a gzipped TSV summarising per-alignment statistics produced by bamstats
    - `{sample_name}.ftagstat.tsv` : a text file with summary alignment statistics for each reference sequence

wf-human-variation workflow tips

It is possible to phase SNPs, SVs and modified bases by providing the `--phased` option.

To improve the accuracy of SV calling, specify a suitable tandem repeat BED for your reference with `--tr_bed` .

Aggregation of methylation calls with `--mod` requires data to be basecalled with a model that includes base modifications, providing the `MM` and `ML` BAM tags. To do so on MinKNOW,

ensure 'Modified bases' option is selected during basecalling set up, with the '5mC' model selected.

Ensure to retain the input reference when basecalling or alignment is performed as CRAM files cannot be read without the corresponding input reference.

For a full list of available basecalling models, refer to the Dorado documentation.

11. Flow cell reuse and returns

We do not recommend washing and reusing your flow cells for this method.

Due to the extended sequencing time, and the multiple flow cell washes and library reloads, we do not recommend re-using the flow cells used in this method.

Re-using these flow cells for subsequent sequencing experiments may result in insufficient data generation for analysis.

1 **Follow the returns procedure to send back flow cells to Oxford Nanopore for recycling.**

Instructions for returning flow cells can be found here.

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.

12. Issues during DNA extraction and library preparation

Below is a list of the most commonly encountered issues, with some suggested causes and solutions.

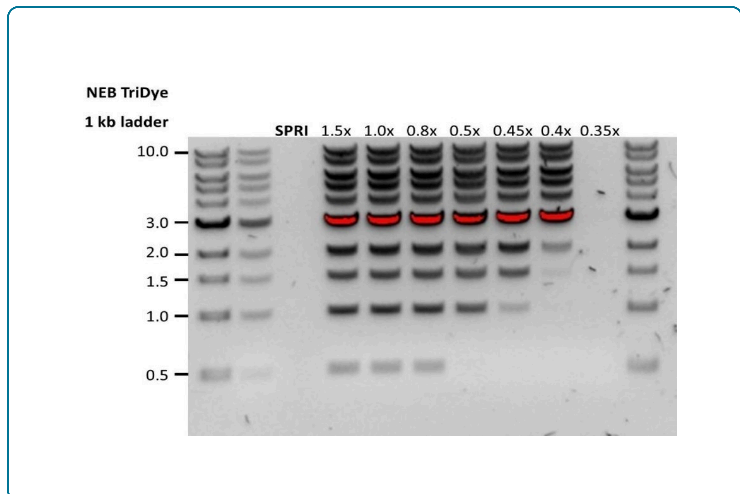
We also have an FAQ section available on the Nanopore Community Support section.

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Low sample quality

Observation	Possible cause	Comments and actions
Low DNA purity (Nanodrop reading for DNA OD 260/280 is <1.8 and OD 260/230 is <2.0–2.2)	The DNA extraction method does not provide the required purity	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover. Consider performing an additional AMPure bead clean-up step.

Low DNA recovery after AMPure bead clean-up

Observation	Possible cause	Comments and actions
Low recovery	DNA loss due to a lower than intended AMPure beads-to-sample ratio	1. AMPure beads settle quickly, so ensure they are well resuspended before adding them to the sample. 2. When the AMPure beads-to-sample ratio is lower than 0.4:1, DNA fragments of any size will be lost during the clean-up.
Low recovery	DNA fragments are shorter than expected	The lower the AMPure beads-to-sample ratio, the more stringent the selection against short fragments. Please always determine the input DNA length on an agarose gel (or other gel electrophoresis methods) and then calculate the appropriate amount of AMPure beads to use. 
Low recovery after end-prep	The wash step used ethanol <70%	DNA will be eluted from the beads when using ethanol <70%. Make sure to use the correct percentage.

13. Issues during the sequencing run

Below is a list of the most commonly encountered issues, with some suggested causes and solutions.

We also have an FAQ section available on the Nanopore Community Support section.

If you have tried our suggested solutions and the issue still persists, please contact Technical Support via email (support@nanoporetech.com) or via LiveChat in the Nanopore Community.

Fewer pores at the start of sequencing than after Flow Cell Check

Observation	Possible cause	Comments and actions
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	An air bubble was introduced into the nanopore array	After the Flow Cell Check it is essential to remove any air bubbles near the priming port before priming the flow cell. If not removed, the air bubble can travel to the nanopore array and irreversibly damage the nanopores that have been exposed to air. The best practice to prevent this from happening is demonstrated in this video for how to load a PromethION Flow Cell.
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	The flow cell is not correctly inserted into the device	Stop the sequencing run, remove the flow cell from the sequencing device and insert it again, checking that the flow cell is firmly seated in the device and that it has reached the target temperature. If applicable, try a different position on the device (GridION/PromethION).
MinKNOW reported a lower number of pores at the start of sequencing than the number reported by the Flow Cell Check	Contaminations in the library damaged or blocked the pores	The pore count during the Flow Cell Check is performed using the QC DNA molecules present in the flow cell storage buffer. At the start of sequencing, the library itself is used to estimate the number of active pores. Because of this, variability of about 10% in the number of pores is expected. A significantly lower pore count reported at the start of sequencing can be due to contaminants in the library that have damaged the membranes or blocked the pores. Alternative DNA/RNA extraction or purification methods may be needed to improve the purity of the input material. The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

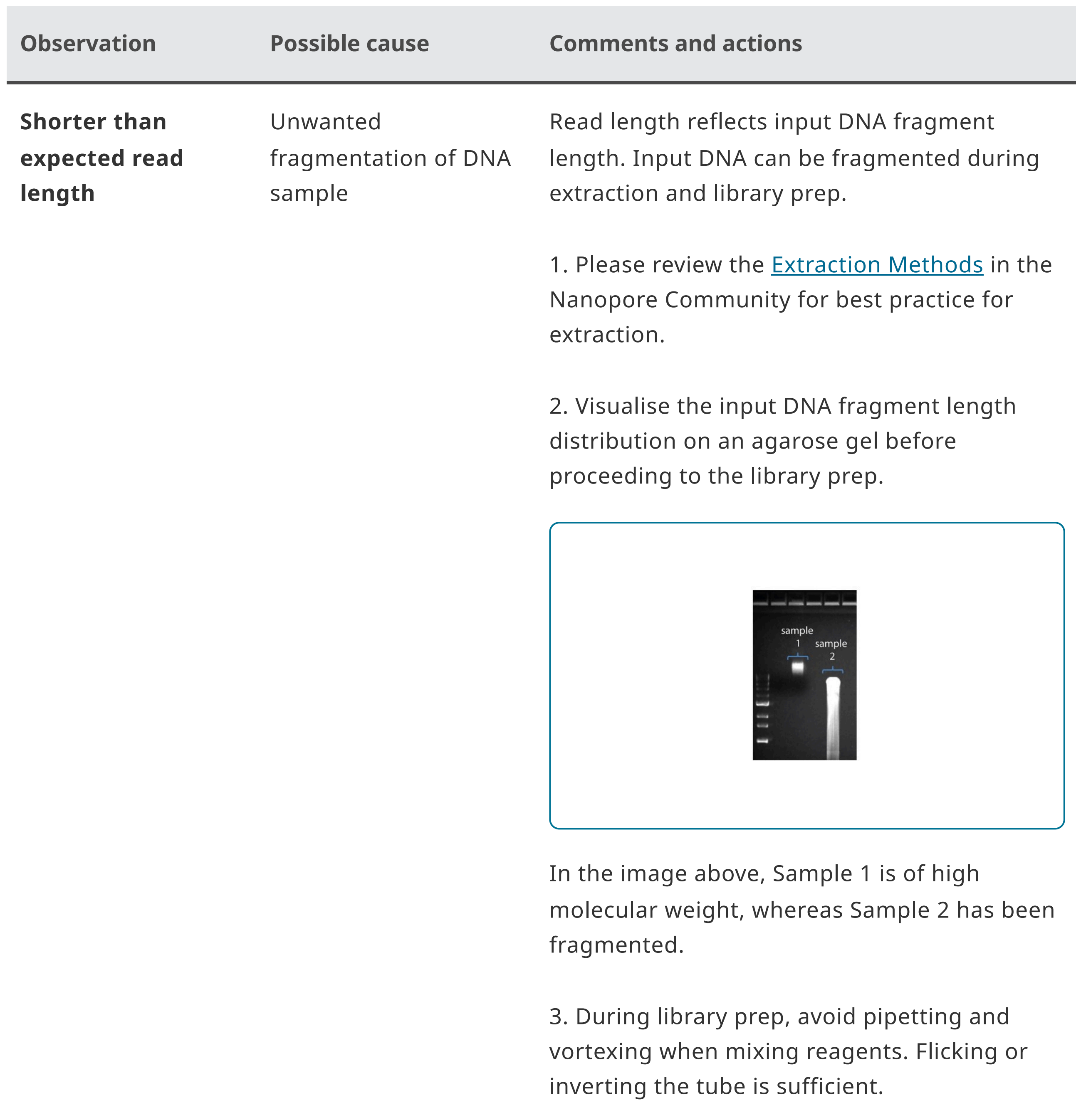
MinKNOW script failed

Observation	Possible cause	Comments and actions
MinKNOW shows "Script failed"		Restart the computer and then restart MinKNOW. If the issue persists, please collect the MinKNOW log files and contact Technical Support. If you do not have another sequencing device available, we recommend storing the flow cell and the loaded library at 4°C and contact Technical Support for further storage guidance.

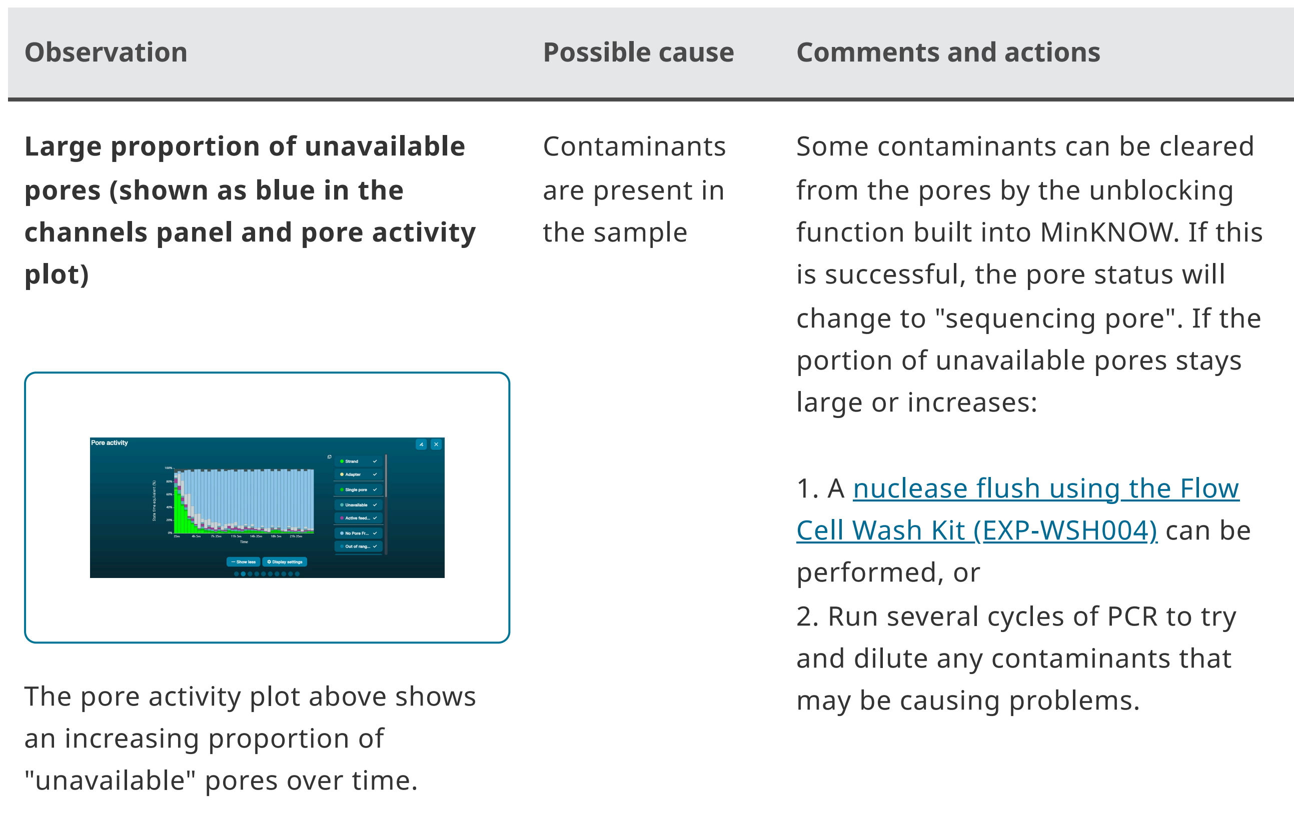
Pore occupancy below 40%

Observation	Possible cause	Comments and actions
Pore occupancy <40%	Not enough library was loaded on the flow cell	For the human genome sequencing protocols, 200-300 ng of good quality library should be loaded on to an R10.4.1 flow cell to keep pore occupancy high.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and sequencing adapters did not ligate to the DNA	Make sure to use the NEBNext Quick Ligation Module (E6056) and Oxford Nanopore Technologies Ligation Buffer (LNB, provided in the SQK-LSK114 kit) at the sequencing adapter ligation step, and use the correct amount of each reagent. A Lambda control library can be prepared to test the integrity of the third-party reagents.
Pore occupancy close to 0	The Ligation Sequencing Kit was used, and ethanol was used instead of LFB or SFB at the wash step after sequencing adapter ligation	Ethanol can denature the motor protein on the sequencing adapters. Make sure the LFB or SFB buffer was used after ligation of sequencing adapters.
Pore occupancy close to 0	No tether on the flow cell	Tethers are adding during flow cell priming (FCT tube). Make sure FCT was added to FCF before priming.

Shorter than expected read length

Observation	Possible cause	Comments and actions
Shorter than expected read length	Unwanted fragmentation of DNA sample	Read length reflects input DNA fragment length. Input DNA can be fragmented during extraction and library prep. 1. Please review the Extraction Methods in the Nanopore Community for best practice for extraction. 2. Visualise the input DNA fragment length distribution on an agarose gel before proceeding to the library prep.  In the image above, Sample 1 is of high molecular weight, whereas Sample 2 has been fragmented. 3. During library prep, avoid pipetting and vortexing when mixing reagents. Flicking or inverting the tube is sufficient.

Large proportion of unavailable pores

Observation	Possible cause	Comments and actions
Large proportion of unavailable pores (shown as blue in the channels panel and pore activity plot)  The pore activity plot above shows an increasing proportion of "unavailable" pores over time.	Contaminants are present in the sample	Some contaminants can be cleared from the pores by the unblocking function built into MinKNOW. If this is successful, the pore status will change to "sequencing pore". If the portion of unavailable pores stays large or increases: 1. A nuclease flush using the Flow Cell Wash Kit (EXP-WSH004) can be performed, or 2. Run several cycles of PCR to try and dilute any contaminants that may be causing problems.

Large proportion of inactive pores

Observation	Possible cause	Comments and actions
Large proportion of inactive/unavailable pores (shown as light blue in the channels panel and pore activity plot. Pores or membranes are irreversibly damaged)	Air bubbles have been introduced into the flow cell	Air bubbles introduced through flow cell priming and library loading can irreversibly damage the pores. Watch the how to load a PromethION Flow Cell video for best practice.
Large proportion of inactive/unavailable pores	Certain compounds co-purified with DNA	Known compounds, include polysaccharides. 1. Clean-up using the QIAGEN PowerClean Pro kit. 2. Perform a whole genome amplification with the original gDNA sample using the QIAGEN REPLI-g kit.
Large proportion of inactive/unavailable pores	Contaminants are present in the sample	The effects of contaminants are shown in the Contaminants Know-how piece. Please try an alternative extraction method that does not result in contaminant carryover.

Temperature fluctuation

Observation	Possible cause	Comments and actions
Temperature fluctuation	The flow cell has lost contact with the device	Check that there is a heat pad covering the metal plate on the back of the flow cell. Re-insert the flow cell and press it down to make sure the connector pins are firmly in contact with the device. If the problem persists, please contact Technical Services.

Failed to reach target temperature

Observation	Possible cause	Comments and actions
MinKNOW shows "Failed to reach target temperature"	The instrument was placed in a location that is colder than normal room temperature, or a location with poor ventilation (which leads to the flow cells overheating)	MinKNOW has a default timeframe for the flow cell to reach the target temperature. Once the timeframe is exceeded, an error message will appear and the sequencing experiment will continue. However, sequencing at an incorrect temperature may lead to a decrease in throughput and lower q-scores. Please adjust the location of the sequencing device to ensure that it is placed at room temperature with good ventilation, then re-start the process in MinKNOW.