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研究成果の証明に役立つ研究記録ノート

RESEARCH LAB NOTEBOOK

Genome Engineering Laboratory

ノートNo.	01
氏　名	黄 沐陽
所属名	慶應義塾大学 ~~理工学部~~ 政メ・BI
使用期間	2021 年 10 月 20 日～　　年　　月　　日
備　考	

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2021年10月20日

プロジェクト：ゲノム工学実験 01

<目的>

・長鎖DNA抽出 (Genomic-Tip, PHGE, nanodrop, Qubit).

<対象>

・慶應義塾大学先端生命科学研究所（以下同研究所），荒川グループ学部4年の石川創良氏のご好意によって提供された *Enterococcus faecalis* (サンガー)と思われるバクテリアの長鎖DNA ~~を抽~~ 黄沐陽 訂正日10/20/21

黄沐陽 訂正日10/20/21

・以下、単離してきたまでの経緯を述べる。

メタ細菌叢の入手:

・トゲアリのコロニーは同研究所荒川グループの岩井氏と栗原氏によって山形県小国町（N 37°55'13", E 139°40'54"; N 37°55'24", E 139°40'27"）にて採取された（Iwai *et al.* 2021）

・飼育手法は Iwai *et al.* 2021 と同じである

・解剖に用いたトゲアリワーカーは宿主であるムネアカオオアリが全て死んだ後のコロニーから採取した

・トゲアリワーカーは3匹用い，4°C にて約3分静置することで動きを抑えたのち，RNase Zap (invitrogen) で除染したハサミを用いて腹部を除去した

・腹部は1 × PBS で満たした時計皿の中でピンセットを用いて解剖し，midgut のみを摘出した

・midgut は形態学的特徴に基づいて確認した

・解剖後，midgut は1 × PBS にて洗浄し，バイオマッシャーII (funakoshi) を用いて破砕し，遠心（4°C, 14000 rpm）

・上清を抽出し，LB で（1,10^-1,10^-2,10^-3,10^-4,10^-5）の希釈を作成

・それぞれ 90 mm シャーレに作成した LB 寒天培地に植菌し，30°C で培養

・翌日，培地に変化がなかった為，37°C に移動し培養を継続

・その翌日，複数のコロニーを確認

・内大きさや形質の異なる 10 コロニーを希釈なし（1x）のシャーレから選択し，別の 90 mm シャーレに作成した LB 寒天培地に滅菌爪楊枝を用いて薄く伸ばし，再び培養

・10 個のシングルコロニーを入手

サンガー

・Tks Gflex PCR kit プロトコルに従い，16S-27f と 16S-1492R を用いて全 10 サンプルに PCR を行った（コロニーPCR）

・AMpure DNA 精製プロトコルに従い，PCR 産物を精製

・Nanodrop で DNA の純度を確認した後，tape station D5000 で電気泳動を行った

・うち 8 サンプルにおいて標的の長さにバンドが現れた（1.5k ぐらい）

・これらのサンプルをサンガーシーケンシングに発注

・解析結果を NCBI BLAST で検索し，*Enterococcus faecalis* にヒットしたサンプルを最終的に実験に用いた

記入者 黄沐陽　確認者　日付　年　月　日

02
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培養

・選択されたサンプルは 150 ml の LB 液体培地の中で震盪培養した（overnight, 37°C）

・*Enterococcus* は OD1 以上を計測した時点で，40 ml をファルコンチューブに移し，遠心（11000rpm, 10min）

・上清を除去し，-20°C で保管

<手法>

・QIAGEN Genomic DNA Handbook (June 2015) 参照以下 黄沐陽 訂正日10/20/21

・実行した手順をハイライトまたは"✓"チェックで示した。

Protocol: Preparation Gram-negative and some Gram-Positive Bacterial Samples

This protocol is designed for the rapid, easy, and nontoxic preparation of up to 20 µg (Genomic-tip 20/G), 90 µg (Genomic-tip 100/G), or 450 µg (Genomic-tip 500/G) of genomic DNA from Gram-negative and some Gram-positive (*B. subtilis*, *Staphylococcus* spp., *Lactobacillus* spp.) bacteria. The purified genomic DNA ranges in size from 20 to 160 kb. Text marked with a ■ denotes values for mini-preps using the Genomic-tip 20/G; text marked with a ▲ denotes values for midi-preps using the Genomic-tip 100/G; and text marked with a ● denotes values for maxi-preps using the Genomic-tip 500/G.

Recommended culture volumes

QIAGEN protocols are optimized for use with fixed cell densities corresponding to the capacity of the QIAGEN Genomic-tip used. Overloading tips with DNA from an excessive number of cells (too much culture volume) will lead to reduced performance of the system.

Yield of DNA and flow characteristics of the QIAGEN Genomic-tips depend on the number of bacteria in culture and on the size of the bacterial genome.

Bacterial growth is usually measured using a spectrophotometer. However, it is very difficult to give specific and reliable recommendations for the relations between OD values and cell number in cultures. Cell density is influenced by a variety of factors (e.g., species, media, incubation time, and shaker speed), and OD readings of cultures measure light scattering rather than absorption. Measurements of light scattering are highly dependent on the distance between the sample and the detector. Therefore readings vary between different types of spectrophotometers. In addition, different bacterial species show different OD values at the same wavelength. QIAGEN Genomic DNA Handbook 06/2015 p.43

・従って、グラム陽性菌である *Enterococcus faecalis* ペレットに、40mL の LB 培地を加え、OD ≈1 の状態から 1mL 採り、以下の手順を行った。 黄沐陽，訂正日10/20/21

・その後、再び遠心し、培地を除去してから -80℃で保管した。

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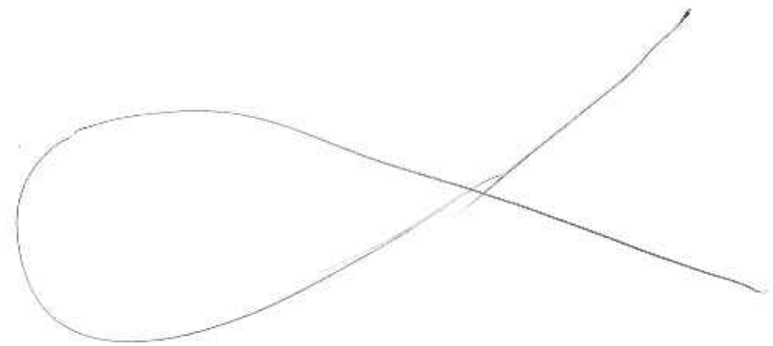
Note: Do not use more than ■ 4.5 x 10^9, ▲ 2.2 x 10^{10}, or ● 1 x 10^{11} cells for each Genomic-tip.

Table 9. Recommended bacterial culture volumes

	QIAGEN Genomic-tip		
	20/G	**100/G**	**500/G**
DNA	5–20 µg	25–90 µg	200–400 µg
Culture volume			
E. coli	0.6–1.2 ml	3.0–6.0 ml	15.0–30.0 ml
E. coli + plasmid	0.4–0.8 ml	2.0–4.0 ml	10.0–20.0 ml
Xanthomonas spp.	0.3–0.6 ml	1.5–3.0 ml	7.5–15.0 ml
Bacillus subtilis	1.2–1.8 ml	6.0–9.0 ml	30.0–45.0 ml
Number of bacteria	4.5 x 10^9	2.2 x 10^{10}	1 x 10^{11}

Volumes given are for bacterial cultures grown in LB medium to an OD_{600} value of 2.0 on a Beckman DU-40 spectrophotometer or an OD_{600} value of 3.6 on a Beckman DU-7400 spectrophotometer. OD_{600} values depend on the length of the light path and therefore differ between spectrophotometers.

Note: Culture volumes for other bacterial species may differ.



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Sample preparation and lysis of bacteria

This is the standard sample preparation and lysis protocol for Gram-negative and some Gram-positive bacteria (*B. subtilis*, *Staphylococcus* spp., *Lactobacillus* spp.), prior to genomic DNA isolation. The Genomic-tip protocol for nucleic acid purification begins on page 49. Text marked with a ■ denotes values for mini-preps using the Genomic-tip 20/G, text marked with a ▲ denotes values for midi-preps using the Genomic-tip 100/G, and text marked with a ● denotes values for maxi-preps using the Genomic-tip 500/G.

Important notes before starting

- Read the recommended culture volumes section on pages 43–46 to determine how to prepare the sample and the appropriate amount to use. Note that culture volumes may differ for other species of bacteria.
- Required reagent volumes are provided in Table 10 (page 46).
- To obtain maximum purity and optimal flow rates, it is very important not to overload the QIAGEN Genomic-tip. Refer to the section on culture volumes (pages 43–46).
- Equilibrate buffers to room temperature (15–25°C). The required buffers may be stored at either 2–8°C or at room temperature. Long-term storage at 2–8°C is recommended.
- Before using this protocol for the first time, stock solutions of lysozyme and QIAGEN Protease should be carefully prepared. See step 2 for details of preparation. RNase A and QIAGEN Proteinase K stock solutions may be used directly. See Appendix A (page 65) for ordering information for lysozyme.
- Steps 4–6 of the protocol are carried out in standard 10 ml, 50 ml, or 50 ml screw-cap tubes.
- **Optional**: remove aliquots at the indicated steps to monitor the procedure on an analytical gel (page 53).

Table 10. Reagent volumes required per bacterial prep

Reagent	Mini (tip 20/G)	Midi (tip 100/G)	Maxi (tip 500/G)
B1	1 ml	3.5 ml	11 ml
B2	0.35 ml	1.2 ml	4 ml
QBT	1 ml	4 ml	10 ml
QC	3 ml	15 ml	30 ml
QF	2 ml	5 ml	15 ml
Isopropanol	1.4 ml	3.5 ml	10.5 ml
70% ethanol	1 ml	2 ml	4 ml
RNase A stock solution	2 µl	7 µl	22 µl
Lysozyme stock solution (100 mg/ml)	20 µl	80 µl	300 µl
QIAGEN Protease or QIAGEN Proteinase K stock solution	45 µl	100 µl	500 µl

1. **Prepare Buffers B1, B2, QBT, QC, and QF according to the instruction in Appendix A (page 62), or use the Genomic DNA Buffer Set (for ordering information see page 69).**

Appendix A: Buffers, media and stock solutions

Table 11. Buffer composition and storage

Buffer	Composition	Storage
Buffer B1 (bacterial lysis buffer)	50 mM Tris·Cl, pH 8.0; 50 mM EDTA, pH 8.0; 0.5% Tween®-20; 0.5% Triton X-100	2–8°C or RT
Buffer B2 (bacterial lysis buffer)	3 M guanidine HCl; 20% Tween-20	2–8°C or RT
Buffer C1 (cell lysis buffer)	1.28 M sucrose; 40 mM Tris·Cl, pH 7.5; 20 mM $MgCl_2$; 4% Triton X-100	2–8°C
Buffer G2* (digestion buffer)	800 mM guanidine HCl; 30 mM Tris·Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween-20; 0.5% Triton X-100	2–8°C or RT
Buffer Y1 (yeast lysis buffer)	1 M sorbitol; 100 mM EDTA; 14 mM β-mercaptoethanol	2–8°C
Buffer QBT (equilibration buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol, 0.15% Triton X-100	2–8°C or RT
Buffer QC (wash buffer)	1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol	2–8°C or RT
Buffer QF (elution buffer)	1.25 M NaCl; 50 mM Tris·Cl, pH 8.5; 15% isopropanol	2–8°C or RT
PBS	Standard phosphate-buffered saline, pH 7.4	RT
TE	10 mM Tris·Cl, pH 8.0; 1 mM EDTA, pH 8.0	RT
Culture media		
LB (bacterial culture medium)	10 g/liter tryptone; 5 g/liter yeast extract; 1 g/liter NaCl; pH 7.0	RT
YPD (yeast culture medium)	10 g/liter yeast extract; 20 g/liter peptone; 20 g/liter dextrose	RT

* Not compatible with disinfection reagents containing bleach. Contains guanidine HCl, which is an irritant. Take appropriate safety measures, and wear gloves when handling.

RT: room temperature (15–25°C).

Table 12. Enzyme information

Enzyme	Provider	Storage
QIAGEN Protease	See ordering information on page 69	15°–25°C
QIAGEN Proteinase K	See ordering information on page 69	2–8°C or room temperature (15–25°C)
Lysozyme or lysostaphin	Sigma Chemicals, SERVA/Boehringer Ingelheim Bioproducts*	–20°C
Lyticase or zymolase	Sigma Chemicals*	–20°C

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Preparation of buffers

Buffer compositions are given per liter of solution. Do not autoclave buffers; sterilize by filtration instead.

Buffer C1 and Buffer Y1 should always be stored at 2–8°C. All other buffers may be stored at either 2–8°C or at room temperature (15–25°C). Long-term storage at 2–8°C is recommended. All buffers except Buffer C1 must be equilibrated to room temperature before use.

B1: Dissolve 18.61 g $Na_2EDTA{\cdot}2H_2O$ and 6.06 g Tris base in 800 ml distilled water. Add 50 ml 10% Tween-20 solution and 50 ml 10% Triton X-100 solution. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water.

B2: Dissolve 286.59 g guanidine HCl in 700 ml distilled water. Add 200 ml of 100% Tween-20. Adjust the volume to 1 liter with distilled water. pH does not need to be adjusted.

C1: Dissolve 438.14 g sucrose, 4.06 g $MgCl_2{\cdot}6H_2O$, and 4.84 g Tris base in 680 ml distilled water. Add 42 g Triton X-100 (100%). Adjust the pH to 7.5 with HCl. Adjust the volume to 1 liter with distilled water.

G2: Dissolve 76.42 g guanidine HCl, 11.17 g $Na_2EDTA{\cdot}2H_2O$, and 3.63 g Tris base in 600 ml distilled water. Add 250 ml 20% Tween-20 solution and 50 ml 10% Triton X-100 solution. Adjust the pH to 8.0 with NaOH. Adjust the volume to 1 liter with distilled water.

Y1: Dissolve 182.2 g sorbitol in 600 ml distilled water. Add 200 ml of a 0.5 M Na_2EDTA (pH 8.0) solution. Add 1 ml of β-mercaptoethanol (14.3 M). Adjust the volume to 1 liter with distilled water. pH does not need to be adjusted.

QBT: Dissolve 43.83 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol and 15 ml 10% Triton X-100 solution. Adjust the volume to 1 liter with distilled water.

QC: Dissolve 58.44 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

QF: Dissolve 73.05 g NaCl and 6.06 g Tris base in 800 ml distilled water. Adjust the pH to 8.5 with HCl. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

Preparation of culture media

YPD: Dissolve 10 g yeast extract and 20 g peptone in 800 ml distilled water. Adjust the volume to 900 ml with distilled water. Dissolve 20 g dextrose in 80 ml distilled water, and adjust the volume to 100 ml. Sterilize both solutions promptly by autoclaving. Mix the two solutions before use. (**Note**: Although the dextrose may be added before autoclaving, it is recommended to prepare a separate solution which is added to the other ingredients after autoclaving. This will prevent darkening of the media and will promote optimal growth.)

LB: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 ml distilled water. Adjust the pH to 7.0 with 1 M NaOH. Adjust the volume to 1 liter with distilled water. Sterilize promptly by autoclaving.

Preparation of enzyme stock solutions

QIAGEN Protease provided in the Blood & Cell Culture DNA Kit should be carefully dissolved in 1.4 ml of distilled water per vial. QIAGEN Protease may also be purchased separately (cat. nos. 19155 and 19157). QIAGEN Protease in solution must be stored at 2–8°C and is stable for 2–3 months. Storage at –20°C will prolong the lifetime of the QIAGEN Protease to one year, but repeated freezing and thawing should be avoided.

QIAGEN Proteinase K may be purchased from QIAGEN in 2 ml or 10 ml solutions (cat. nos. 19131 and 19133). QIAGEN Proteinase K solution is at the proper concentration to be used directly in the QIAGEN Genomic DNA protocols and is stable at room temperature (15–25°C) for at least one year. For longer-term storage, we recommend storing at 2–8°C. If using proteinase K from another supplier, a 20 mg/ml stock solution in distilled water is generally recommended.

Before use, RNase A should be added to Buffer G2 (or Buffer B1 for bacterial DNA isolation) to a concentration of 200 μg/ml and should then be stable for 6 months when stored at 2–8°C.

Lyticase should be dissolved in distilled water to a concentration of 1000 U/ml and stored at –20°C. For efficient lysis of some yeast species, zymolase is the enzyme of choice. Use the appropriate enzyme for the particular species. Dissolved lyticase or zymolase must be stored at –20°C, but repeated freezing and thawing should be avoided. Dividing the enzyme solutions into aliquots is recommended. Lyticase or zymolase can be purchased from Sigma Chemicals, Medac Diagnostika, or Seikagaku Corporation*.

Lysozyme should be dissolved in distilled water to a concentration of 100 mg/ml and stored at –20°C. For efficient lysis of some bacterial species, enzymes other than lysozyme may be necessary. Lysis of *Staphylococcus* spp., for example, is much more efficient with lysostaphin. Use the appropriate enzyme for the particular species.

Dissolved lysozyme or lysostaphin must be stored at –20°C, but repeated freezing and thawing should be avoided. Dividing the enzyme solutions into aliquots is recommended. Lysozyme or lysostaphin can be purchased from Sigma Chemicals, or SERVA/Boehringer Ingelheim Bioproducts.*

* This is not a complete list of suppliers and does not include many important vendors of biological supplies.

14:27 start

2. **For each prep, add ■ 2 µl, ▲ 7 µl, or ● 22 µl of RNase A solution (100 mg/ml) to a ■ 1 ml, ▲ 3.5 ml, or ● 11 ml aliquot of Buffer B1.** (**Dissolve lysozyme in distilled water to a concentration of 100 mg/ml. Prepare QIAGEN Protease stock solution in distilled water, or use QIAGEN Proteinase K stock solution.**)

If using a kit, dissolve each vial of lyophilized QIAGEN Protease provided in 1.4 ml of distilled water, as indicated on the label. Alternatively, QIAGEN Proteinase K solution may be used directly. See "Preparation of enzyme stock solutions" (page 64) for further details and page 69 for ordering information.

Storage conditions for QIAGEN Protease and QIAGEN Proteinase K are described on page 5.

Lysozyme should be dissolved in distilled water to a concentration of 100 mg/ml and should be stored at –20°C. For efficient lysis of some bacterial species, enzymes other than lysozyme may be necessary. Lysis of *Staphylococcus* spp., for example, is much more efficient with lysostaphin. Use the appropriate enzyme for the particular species. See Appendix A (page 65) for ordering information for lysozyme.

RNase A should be added to Buffer B1 to a concentration of 200 µg/ml and will be stable for 6 months when stored at 2–8°C.

3. **Pellet bacteria from an appropriate volume of culture by centrifugation at ~~3000–5000 x g for 5–10 min~~. Discard the supernatant, ensuring that all liquid is completely removed.** 13,000 rpm 2min 黄沐陽 訂正日 10/20/21

See pages 43–44 for the appropriate starting volume.

Because of the different growth characteristics of bacterial species, performing a preliminary experiment to determine the optimal starting volume is recommended.

4. **Resuspend the bacteria pellet from step 3 in ■ 1 ml, ▲ 3.5 ml, or ● 11 ml of Buffer B1 (with RNase A) by vortexing at top speed.**

Add the RNase A to Buffer B1 according to step 2 before adding the buffer to the bacteria.

Resuspend the bacterial pellet as thoroughly as possible by vortexing.

For efficient lysis of the bacteria, it is important to have a homogeneous suspension.

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14:34

5. **Add ■ 20 µl, ▲ 80 µl, or ● 300 µl of lysozyme stock solution (100 mg/ml), and ■ 45 µl, ▲ 100 µl, or ● 500 µl of QIAGEN Protease or QIAGEN Proteinase K stock solution. Incubate at 37°C for at least 30 min.** 14:43 ~ 15:13

Use the stock solutions from step 2.

The length of incubation depends on how well the bacteria were resuspended in step 4.

If the suspension is not homogeneous after vortexing, a longer incubation is recommended to avoid clogging the QIAGEN Genomic-tip.

During incubation, the lysozyme enzymatically breaks down the bacterial cell wall, while the detergents in Buffer B1 ensure complete lysis of the bacteria.

Degradation of RNA by RNase A ensures complete removal during column procedure.

15:20 S 16:20

6. **Add ■ 0.35 ml, ▲ 1.2 ml, or ● 4 ml of Buffer B2, and mix by inverting the tube several times or by vortexing for a few seconds. Incubate at 50°C for ~~30~~ min.** 60 黄沐陽 訂正日 10/20/21

Mix the lysate and Buffer B2 very thoroughly. This step is important for efficient deproteinization.

Buffer B2 denatures proteins such as nucleases and DNA-binding proteins. The excess QIAGEN Protease digests the denatured proteins into smaller fragments, facilitating efficient removal during purification.

Buffer B2 and QIAGEN Protease, in combination, strip the genomic DNA of all bound proteins. It is important that the lysate becomes clear at this stage. ~~If necessary,~~ extend the incubation time, or pellet the particulate matter by centrifugation for ~~10~~ min at ~~5000 x g~~, 4°C. 15min 10000 g

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Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 1).

7. **Proceed with the Genomic-tip protocol on page 49.**

The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

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・まず、黄いアダプターをカラムにかける

Protocol: Isolation of Genomic DNA from Blood, Cultured Cells, Tissue, Yeast, or Bacteria using Genomic-tips

All samples must first be prepared according to the relevant sample preparation and lysis protocol (pages 18–48).

Text with a ■ denotes values for mini-preps using the Genomic-tip 20/G; text with a ▲ denotes values for midi-preps using the Genomic-tip 100/G, and ● denotes values for maxi-preps using the Genomic-tip 500/G.

1. **Equilibrate a QIAGEN Genomic-tip 20/G, Genomic-tip 100/G, or Genomic-tip 500/G with ■ 1 ml, ▲ 4 ml, or ● 10 ml of Buffer QBT, and allow the QIAGEN Genomic-tip to empty by gravity flow.**

   Place a QIAGEN Genomic-tip over a tube using a tip holder or into a QIArack over the waste tray. Equilibrate the QIAGEN Genomic-tip with the volume of buffer described in the protocol. Flow begins automatically by reduction in surface tension due to the presence of detergent (0.15% Triton® X-100) in the equilibration buffer. Allow the QIAGEN Genomic-tip to drain completely. The flow of buffer will stop when the meniscus reaches the upper frit. The frit prevents the QIAGEN Genomic-tip from running dry, allowing it to be left unattended. Do not force out the remaining buffer, as this will necessitate restarting the flow with a syringe and adapter.

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2. **Vortex the sample (from the last step of the specific sample preparation and lysis protocol) for 10 s at maximum speed and apply it to the equilibrated QIAGEN Genomic-tip. Allow it to enter the resin by gravity flow.** 軽くボルテックス、この段階で壊すぎると詰まる。

   Vortexing the genomic DNA has a minimal effect on the size of the DNA, and it accelerates the QIAGEN procedure by eliminating poor flow rates associated with clogging. The average size of genomic DNA is reduced by only 10 kb when vortexed for up to 20 s (see Figure 3, page 10).

   The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

   Once the QIAGEN Genomic-tip is loaded with the clear and particle-free sample, flow will begin unassisted. Allow gravity to determine the flow rate. The flow rate will depend on the sample source, the number of cells from which the DNA sample was prepared, and on genome size.

   Particularly concentrated genomic DNA lysates may exhibit diminished flow rates due to increased viscosity. Flow can be assisted by the application of gentle positive pressure using a disposable syringe and the appropriate adapter. It may also be helpful to dilute the lysate with an equal volume of Buffer QBT prior to loading.

   When using positive pressure, do not allow the flow rate to exceed ■ 4–10 drops/min, ▲ 10–20 drops/min, or ● 20–40 drops/min.

   Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 2).

17:03

3. **Wash the QIAGEN Genomic-tip with ■ 3 x 1 ml, ▲ 2 x 7.5 ml, or ● 2 x 15 ml of Buffer QC.**

   Allow Buffer QC to move through the QIAGEN Genomic-tip by gravity flow. Two washes are sufficient to remove all contaminants in the majority of DNA preparations.

   An additional wash is occasionally necessary if large culture volumes or bacterial strains containing large amounts of carbohydrate are used.

   It is particularly important not to force out residual Buffer QC. Traces of Buffer QC will not affect the elution step.

   Optional: Take a ■ 120 µl, ▲ 600 µl, or ● 300 µl aliquot and save it for an analytical gel (aliquot 3).

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18:04

4. Elute the genomic DNA with ■ 2 x 1 ml, ▲ 1 x 5 ml, or ● 1 x 15 ml of Buffer QF.

Place the QIAGEN Genomic-tip over a clean ■ 10 ml, ▲ 10 ml, or ● 30 ml collection tube. Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. Elute with the appropriate volume of Buffer QF, and collect the eluate. Flow begins automatically. Allow the QIAGEN Genomic-tip to drain by gravity flow.

Use of Buffer QF prewarmed to 50°C will increase yields. 予め50℃で暖める

Optional: Take a ■ 120 µl, ▲ 300 µl, or ● 600 µl aliquot and save it for an analytical gel (aliquot 4).

プロトコルが A と色付けされる →

5. Precipitate the DNA by adding ■ 1.4 ml, ▲ 3.5 ml, or ● 10.5 ml (0.7 volumes) room-temperature (15–25°C) isopropanol to the eluted DNA. Recover the precipitated DNA as described in step 5A or step 5B. 黄沐陽 訂正日 10/20/21

小分なDNAがあると、白いDNAのもやもやがでる

5A. Precipitate the DNA by inverting the tube 10 to 20 times, and spool the DNA using a glass rod. Proceed with step 6A.

18:19

5B. Alternatively, mix and centrifuge immediately at >5000 x g for at least 15 min at 4°C. Carefully remove the supernatant. Proceed with step 6B. 残液白から見えない場合が多い → 遠心 (6B)

All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 5000 x g is the minimal force required for efficient precipitation. Higher g-force is recommended where possible. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

6. Transfer material according to steps 6A and 6B.

6A. Immediately transfer the spooled DNA to a microcentrifuge tube containing 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.

10/20/21
記入者 黄沐陽

続き 15

エタノール 18:45

Air-dry: 19:00 s

19:04 乾燥のDNA溶解

6B. Wash the centrifuged DNA pellet with ■ 1 ml, ▲ 2 ml, or ● 4 ml of cold 70% ethanol. Vortex briefly and centrifuge at >5000 x g for 10 min at 4°C. Carefully remove the supernatant without disturbing the pellet. Air-dry for 5–10 min, and resuspend the DNA in 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.

4 黄沐陽 訂正日 10/20/21

EB buffer 40µL

The 70% ethanol removes precipitated salt and replaces the isopropanol with the more volatile ethanol, making the DNA easier to redissolve. A second wash with cold 70% ethanol may improve results in more sensitive applications. 見ているDNAの量に依存、小さい場合は40µL、大きいペレットが見える場合は100µL(上限)

After careful and complete removal of the ethanol supernatant with a pipet, the pellet should be air-dried briefly before resuspending in a small volume of suitable buffer. Overdrying the pellet will make the DNA difficult to redissolve. Resuspend the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. DNA dissolves best under slightly alkaline conditions (pH 8.0–8.5) and does not dissolve easily in acidic buffers.

Determination of yield, purity, and length of the DNA

DNAの量 長さ 電気泳動

Yields of genomic DNA will depend on the number of cells and the capacity of the QIAGEN Genomic-tip used. DNA yield is usually determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. To ensure accuracy, make sure the absorbance readings fall into the linear range of your method (e.g., between 0.1 and 1.0 for spectrophotometric OD). Sample dilution should be adjusted accordingly: for example, an eluate containing 25–50 ng of DNA/µl (A_{260} = 0.5–1) should not be diluted with more than 4 volumes of buffer. Use elution buffer or water (as appropriate) to dilute samples and to calibrate the spectrophotometer. Measure the absorbance at 260 nm and 280 nm, or scan absorbance from 220–320 nm. (A scan will show whether there are other factors affecting absorbance at 260 nm.) Readings on a spectrophotometer are not always accurate, particularly if a single wavelength measurement is taken rather than a scan, and should always be verified by visual examination of the DNA on an agarose gel. Fluorimetric measurements are more accurate and should be used if precise concentrations are needed.

Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9.

The precise length of genomic DNA is determined by pulsed-field gel electrophoresis (PFGE) through an agarose gel. Standard PFGE conditions are as follows: 1.5% agarose gel in 0.5x TBE electrophoresis buffer; switch intervals, 1–10 s; run time, 16 h; voltage, 170 V.

10/20/21
記入者 黄沐陽

<結果>

① nanodrop

Sample ID	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
ab	10/20/2021 8:01:02 PM	401.9 ng/µl		13.439	8.013	1.68	1.17	DNA	50

~~純粋な~~ 黄沐陽 訂正日:10/20/21

- DNAが非常によくとれている。
- 純粋なDNAはA260/A280の比が1.7~1.9であると考えられている。
- エタノールが残っていると考えられるため、もう少しとばすことにした。

② DNA ScreenTapeR (右頁 p.17, p.18)

- 薄くバンドが見えるが、先程のnanodropの結果と比べると、低すぎる
- 機械の方からもサンプル濃度が範囲外と出ている。

~~DNAが~~ 黄沐陽 訂正日 10/20/21

①と②を合わせると、DNAが溶け切れていないことが考えられるため、シェーカーで一晩溶かし、明日再チェックすることにした。なお、値が不安定なため、泳動はしなかった。

以下、空白

10/20/21

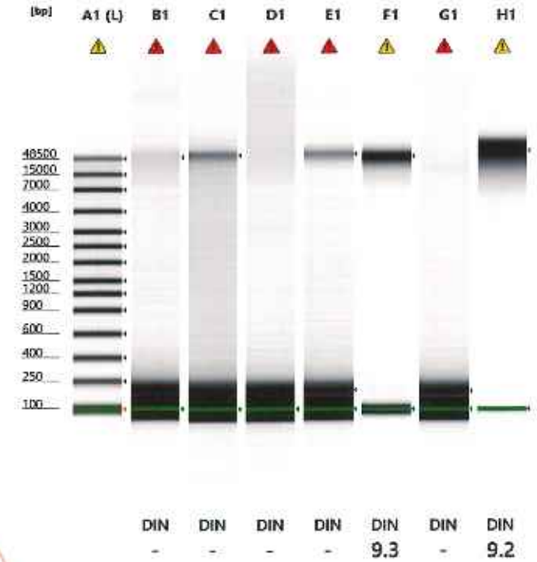
記入者 黄沐陽

確認者

日付 年 月 日

Genomic DNA ScreenTapeR

Filename: 2021-10-20-03.gDNA



Default Image (Contrast 50%, Image is Scaled to Sample

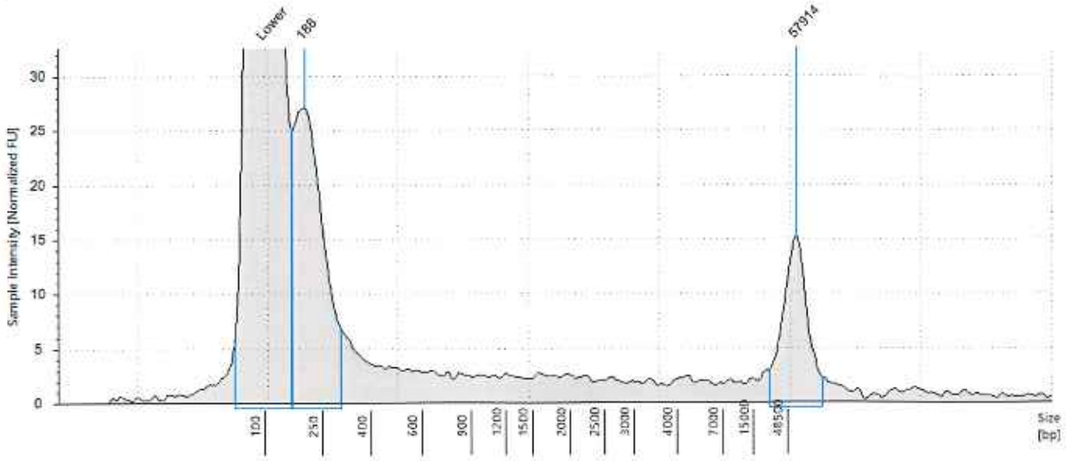
Sample Info

Well	DIN	Conc. (ng/µl)	Sample Description	Alert	Observations
A1	-	66.6	Ladder	⚠	Caution! Expired ScreenTape device; Ladder
B1	-	1.99	ray	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
C1	-	2.14	watanabe	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
D1	-	1.71	uri	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
E1	-	2.22	mh	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
F1	9.3	14.9	yoyoyo	⚠	Caution! Expired ScreenTape device
G1	-	1.26	mitsu	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
H1	9.2	165	takada	⚠	Caution! Expired ScreenTape device; Sample concentration outside recommended range

10/20/21

記入者

E1: nh



Sample Table

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
E1	-	2.22	nh	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN

Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	% Integrated Area	From [bp]	To [bp]	Peak Comment	Observations
100	8.50	8.50	-	63	152		Lower Marker
188	0.733	-	54.46	155	299		
57914	0.329	-	24.47	21700	>60000		
>60000	0.171	-	12.72	>60000	>60000		
-	-	-	-	-	-		Sample Well

10/20/21
記入者

c Agilent Technologies, Inc. 2017

10/21/21 プロジェクト：ゲノム工学実習

<目的>

・前日に抽出し、1時シューカーで壊かした DNA の変化の観察、→ 変化なしのため

・最鎖 DNA を再抽出

<対象> ※ 黄沐陽 訂正日 10/21/21

・研究ノート p.01~p.02 を参照

<手法>

・前日の実験では、DNA は抽出できたが、濃度が足りないことが分かった。

(nanodrop は何かのコンタミの可能性がある)

・そのため、今回は実験中に細心の注意を払うと同時に、前回の ~~DDT~~, /ml, OD1 黄沐陽 訂正日 10/21/21

のサンプルを ~~1ml, OD1 x3~~ 1.5mL, OD.1 x3 で用意し、リスクの回避を行った。(前で失敗しても もう片方ある。

ので、濃度は下がるが、成功する可能性を高めた。なお、カプセルは再び遠心し 赤色

ある程度 を除去してから on ice で一時保管した。

・試薬の用意は、研究ノート p.06~p.07 を参照。

・QIAGEN Genome DNA Handbook (June 2015) 以下参照。

・実行した手順を ハイライト 或は "V" チェックで示した。

以下空白。

10/21/21
記入者 黄沐陽
確認者
日付 年 月 日

13:12 start

Table 10. Reagent volumes required per bacterial prep 必要な試薬量

Reagent	Mini (tip 20/G)	Midi (tip 100/G)	Maxi (tip 500/G)
B1	1 ml ×3 = 2ml	3.5 ml	11 ml
B2	0.35 ml ×3 = ~~0.7~~ml 1.05	1.2 ml	4 ml
QBT	1 ml ×3 = 2ml	4 ml	10 ml
QC	3 ml ×3 = ~~6~~ml 9	15 ml	30 ml
QF	2 ml ×3 = ~~4~~ml 6	5 ml	15 ml
Isopropanol	1.4 ml ×3 = ~~2.8~~ml 4.2	3.5 ml	10.5 ml
70% ethanol	1 ml ×3 = 2ml	2 ml	4 ml
RNase A stock solution	2 µl ×3 = ~~4~~µl 6	7 µl	22 µl
Lysozyme stock solution (100 mg/ml)	20 µl ×3 = ~~40~~µl 60	80 µl	300 µl
QIAGEN Protease or QIAGEN Proteinase K stock solution	45 µl ×3 = ~~90~~µl 135	100 µl	500 µl

黄沐陽 訂正日 10/21/21

1. **Prepare Buffers B1, B2, QBT, QC, and QF according to the instruction in Appendix A (page 62), or use the Genomic DNA Buffer Set (for ordering information see page 69).**

研究ノート p.46~p.47を参照

10/21/21 配入者 黄沐陽

13:31

2. **For each prep, add ■ ~~2~~ µl, ▲ 7 µl, or ● 22 µl of RNase A solution (100 mg/ml) to a ■ ~~1~~ ml, ▲ 3.5 ml, or ● 11 ml aliquot of Buffer B1. Dissolve lysozyme in distilled water to a concentration of 100 mg/ml. Prepare QIAGEN Protease stock solution in distilled water, or use QIAGEN Proteinase K stock solution.**

#6 黄沐陽 訂正日 10/21/21

If using a kit, dissolve each vial of lyophilized QIAGEN Protease provided in 1.4 ml of distilled water, as indicated on the label. Alternatively, QIAGEN Proteinase K solution may be used directly. See "Preparation of enzyme stock solutions" (page 64) for further details and page 69 for ordering information.

Storage conditions for QIAGEN Protease and QIAGEN Proteinase K are described on page 5.

Lysozyme should be dissolved in distilled water to a concentration of 100 mg/ml and should be stored at –20°C. For efficient lysis of some bacterial species, enzymes other than lysozyme may be necessary. Lysis of *Staphylococcus* spp., for example, is much more efficient with lysostaphin. Use the appropriate enzyme for the particular species. See Appendix A (page 65) for ordering information for lysozyme.

RNase A should be added to Buffer B1 to a concentration of 200 µg/ml and will be stable for 6 months when stored at 2–8°C.

13:22

3. **Pellet bacteria from an appropriate volume of culture by centrifugation at 3000–5000 x g for 5–10 min. Discard the supernatant, ensuring that all liquid is completely removed.**

1.5ml×3

See pages 43–44 for the appropriate starting volume.

Because of the different growth characteristics of bacterial species, performing a preliminary experiment to determine the optimal starting volume is recommended.

4. **Resuspend the bacteria pellet from step 3 in ■ ~~1~~ ml, ▲ 3.5 ml, or ● 11 ml of Buffer B1 (with RNase A) by vortexing at top speed.**

2 黄沐陽 訂正日 10/21/21

Add the RNase A to Buffer B1 according to step 2 before adding the buffer to the bacteria.

Resuspend the bacterial pellet as thoroughly as possible by vortexing.

For efficient lysis of the bacteria, it is important to have a homogeneous suspension.

10/21/21 配入者 黄沐陽

22
続き.

lysozyme 13:41
黄沐陽訂正日. 10/21/21
~~protease~~ 13:44
proteinase K 53

Incubate: 13:46
5
14:16

5. **Add ■ 20 µl, ▲ 80 µl, or ● 300 µl of lysozyme stock solution (100 mg/ml), and ■ 45 µl, ▲ 100 µl, or ● 500 µl of QIAGEN Protease or QIAGEN Proteinase K stock solution. Incubate at 37°C for at least 30 min.** 黄沐陽 訂正日. 10/21/21
Use the stock solutions from step 2.

The length of incubation depends on how well the bacteria were resuspended in step 4.

If the suspension is not homogeneous after vortexing, a longer incubation is recommended to avoid clogging the QIAGEN Genomic-tip.

During incubation, the lysozyme enzymatically breaks down the bacterial cell wall, while the detergents in Buffer B1 ensure complete lysis of the bacteria.

Degradation of RNA by RNase A ensures complete removal during column procedure. 黄沐陽 訂正日. 10/21/21

Incubate: 14:20
5
15:20

6. **Add ■ 0.35 ml, ▲ 1.2 ml, or ● 4 ml of Buffer B2, and mix by inverting the tube several times or by vortexing for a few seconds. Incubate at 50°C for ~~30~~ min.** 黄沐陽 訂正日. 10/21/21

Mix the lysate and Buffer B2 very thoroughly. This step is important for efficient deproteinization.

Buffer B2 denatures proteins such as nucleases and DNA-binding proteins. The excess QIAGEN Protease digests the denatured proteins into smaller fragments, facilitating efficient removal during purification.

Buffer B2 and QIAGEN Protease, in combination, strip the genomic DNA of all bound proteins. It is important that the lysate becomes clear at this stage. ~~If necessary,~~ extend the incubation time, or pellet the particulate matter by centrifugation for ~~10 min at 5000~~ x g, 4°C. 必ずやるように
15 min at 10,000 訂正日: 10/21/21 黄沐陽

Centrifuge 15:24
5
15:39

黄沐陽
訂正日
~~15:24~~
10/21/21

Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 1).

7. **Proceed with the Genomic-tip protocol on page 49.**

The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

10/21/21
記入者 黄沐陽

続き.

23

Protocol: Isolation of Genomic DNA from Blood, Cultured Cells, Tissue, Yeast, or Bacteria using Genomic-tips

All samples must first be prepared according to the relevant sample preparation and lysis protocol (pages 18–48).

Text with a ■ denotes values for mini-preps using the Genomic-tip 20/G; text with a ▲ denotes values for midi-preps using the Genomic-tip 100/G, and ● denotes values for maxi-preps using the Genomic-tip 500/G.

15:41

1. **Equilibrate a QIAGEN Genomic-tip 20/G, Genomic-tip 100/G, or Genomic-tip 500/G with ■ 1 ml, ▲ 4 ml, or ● 10 ml of Buffer QBT, and allow the QIAGEN Genomic-tip to empty by gravity flow.** 黄沐陽 訂正日 10/21/21

Place a QIAGEN Genomic-tip over a tube using a tip holder or into a QIArack over the waste tray. Equilibrate the QIAGEN Genomic-tip with the volume of buffer described in the protocol. Flow begins automatically by reduction in surface tension due to the presence of detergent (0.15% Triton® X-100) in the equilibration buffer. Allow the QIAGEN Genomic-tip to drain completely. The flow of buffer will stop when the meniscus reaches the upper frit. The frit prevents the QIAGEN Genomic-tip from running dry, allowing it to be left unattended. Do not force out the remaining buffer, as this will necessitate restarting the flow with a syringe and adapter.

10/21/21
記入者 黄沐陽

24 終了

2. **Vortex the sample (from the last step of the specific sample preparation and lysis protocol) for 10 s at maximum speed and apply it to the equilibrated QIAGEN Genomic-tip. Allow it to enter the resin by gravity flow.** 勢くボルテックス

Vortexing the genomic DNA has a minimal effect on the size of the DNA, and it accelerates the QIAGEN procedure by eliminating poor flow rates associated with clogging. The average size of genomic DNA is reduced by only 10 kb when vortexed for up to 20 s (see Figure 3, page 10).

The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

Once the QIAGEN Genomic-tip is loaded with the clear and particle-free sample, flow will begin unassisted. Allow gravity to determine the flow rate. The flow rate will depend on the sample source, the number of cells from which the DNA sample was prepared, and on genome size.

Particularly concentrated genomic DNA lysates may exhibit diminished flow rates due to increased viscosity. Flow can be assisted by the application of gentle positive pressure using a disposable syringe and the appropriate adapter. It may also be helpful to dilute the lysate with an equal volume of Buffer QBT prior to loading.

When using positive pressure, do not allow the flow rate to exceed ■ 4–10 drops/min, ▲ 10–20 drops/min, or ● 20–40 drops/min.

Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 2).

3×3= 9 mL 黄沐陽 訂正日: 10/21/21

15:54

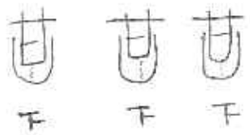
3. **Wash the QIAGEN Genomic-tip with ■ 3 x 1 ml, ▲ 2 x 7.5 ml, or ● 2 x 15 ml of Buffer QC.**

Allow Buffer QC to move through the QIAGEN Genomic-tip by gravity flow. Two washes are sufficient to remove all contaminants in the majority of DNA preparations.

An additional wash is occasionally necessary if large culture volumes or bacterial strains containing large amounts of carbohydrate are used.

It is particularly important not to force out residual Buffer QC. Traces of Buffer QC will not affect the elution step.

Optional: Take a ■ 120 µl, ▲ 600 µl, or ● 300 µl aliquot and save it for an analytical gel (aliquot 3).



10/21/21 黄沐陽

終了 25

17:10

4. **Elute the genomic DNA with ■ 2 x 1 ml, ▲ 1 x 5 ml, or ● 1 x 15 ml of Buffer QF.** 2×3=6 黄沐陽 訂正日: 10/21/21

Place the QIAGEN Genomic-tip over a clean ■ 10 ml, ▲ 10 ml, or ● 30 ml collection tube. Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. Elute with the appropriate volume of Buffer QF, and collect the eluate. Flow begins automatically. Allow the QIAGEN Genomic-tip to drain by gravity flow.

Use of Buffer QF prewarmed to 50°C will increase yields. 予め暖めた(50℃)

Optional: Take a ■ 120 µl, ▲ 300 µl, or ● 600 µl aliquot and save it for an analytical gel (aliquot 4). 黄沐陽 訂正日 10/21/21 #2

とアルコールが入っていると 凝出するので 速くつけ直す → 水溶 している状態だと 凝固しない。

5. **Precipitate the DNA by adding ■ 1.4 ml, ▲ 3.5 ml, or ● 10.5 ml (0.7 volumes) room-temperature (15–25°C) isopropanol to the eluted DNA. Recover the precipitated DNA as described in step 5A or step 5B.** 十分なDNAがあると 白いDNAのもやもや が出る

17:57

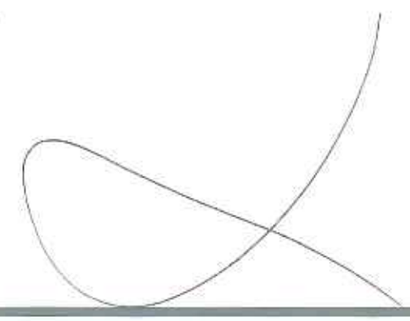
5A. **Precipitate the DNA by inverting the tube 10 to 20 times, and spool the DNA using a glass rod. Proceed with step 6A.**

5B. **Alternatively, mix and centrifuge immediately at >5000 x g for at least 15 min at 4°C. Carefully remove the supernatant. Proceed with step 6B.** 残念ながら見えない場合が多い → 遠心(6B)

All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 5000 x g is the minimal force required for efficient precipitation. Higher g-force is recommended where possible. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

6. **Transfer material according to steps 6A and 6B.**

6A. **Immediately transfer the spooled DNA to a microcentrifuge tube containing 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.**



10/21/21 黄沐陽

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続き
コタ洗：18:27
Air-dry：18:40
〜
18:45

6B. Wash the centrifuged DNA pellet with ■ 1 ml, ▲ 2 ml, or ● 4 ml of cold 70% ethanol. Vortex briefly and centrifuge at >5000 x g for 10 min at 4°C. Carefully remove the supernatant without disturbing the pellet. Air-dry for 5–10 min, and resuspend the DNA in 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.

3 黄沐陽 訂正日 10/21/21

EB buffer 40μl

糸状のDNAを拾う

下限3μl

すくんだら、50〜100μl

返ったら 100μl下

The 70% ethanol removes precipitated salt and replaces the isopropanol with the more volatile ethanol, making the DNA easier to redissolve. A second wash with cold 70% ethanol may improve results in more sensitive applications.

After careful and complete removal of the ethanol supernatant with a pipet, the pellet should be air-dried briefly before resuspending in a small volume of suitable buffer. Overdrying the pellet will make the DNA difficult to redissolve. Resuspend the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. DNA dissolves best under slightly alkaline conditions (pH 8.0–8.5) and does not dissolve easily in acidic buffers.

Determination of yield, purity, and length of the DNA

Yields of genomic DNA will depend on the number of cells and the capacity of the QIAGEN Genomic-tip used. DNA yield is usually determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. To ensure accuracy, make sure the absorbance readings fall into the linear range of your method (e.g., between 0.1 and 1.0 for spectrophotometric OD). Sample dilution should be adjusted accordingly: for example, an eluate containing 25–50 ng of DNA/μl (A_{260} = 0.5–1) should not be diluted with more than 4 volumes of buffer. Use elution buffer or water (as appropriate) to dilute samples and to calibrate the spectrophotometer. Measure the absorbance at 260 nm and 280 nm, or scan absorbance from 220–320 nm. (A scan will show whether there are other factors affecting absorbance at 260 nm.) Readings on a spectrophotometer are not always accurate, particularly if a single wavelength measurement is taken rather than a scan, and should always be verified by visual examination of the DNA on an agarose gel. Fluorimetric measurements are more accurate and should be used if precise concentrations are needed.

Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9.

The precise length of genomic DNA is determined by pulsed-field gel electrophoresis (PFGE) through an agarose gel. Standard PFGE conditions are as follows: 1.5% agarose gel in 0.5x TBE electrophoresis buffer; switch intervals, 1–10 s; run time, 16 h; voltage, 170 V.

10/21/21
記入者 黄沐陽

続き

~~<結果>~~ 黄沐陽. 訂正日：10/21/21

27

・また、DNAの収量、純度、長さの測定は、nanodrop, Qubit, Tapestation, PFGEを行いました。

・Qubitは、dsDNA BRを2μlサンプル量で測った。
黄沐陽, 訂正日：10/21/21

・TapeStationでの泳動はGenomic ScreenTapeを使った。
黄沐陽, 訂正日 10/21/21

・PFGE (pulsed-field gel electrophoresis)は、pippin Pulseという機械で、FastGene Agarose 0.75%, 0.5×KBBで75V 16時間泳動した。マーカーはCHEF DNAサイズスタンダード#1703707.
黄沐陽, 訂正日：10/21/21

<結果>

① nanodrop

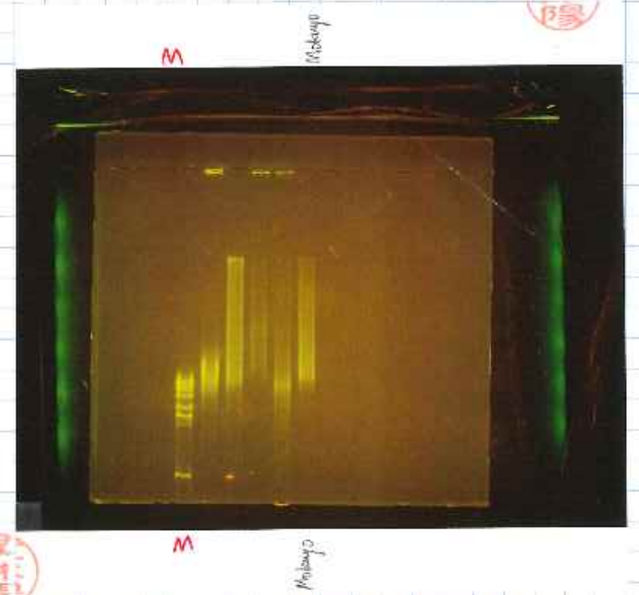
Sample ID	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
kan	10/21/2021 10:24:17 PM	[illegible] ng/μl		0.029	0.02	1.49	[illegible] DNA		50
mikaze	10/21/2021 2:11:55 PM	62 ng/μl		0.125	0.06	2.07	46.42 DNA		50

・前日が上で（シェーカーで1晩反応させた後）、今回行った抽出の結果は最終的に1本にまとめ、その濃度と純度を示したのが下段のデータである。

・比較的に量と濃度が向上していることが分かる。

② Qubit, 5.52 ng/μl 黄沐陽 訂正日 10/21/21

③ ~~Tap~~ PFGE
黄沐陽
訂正日
10/21/21

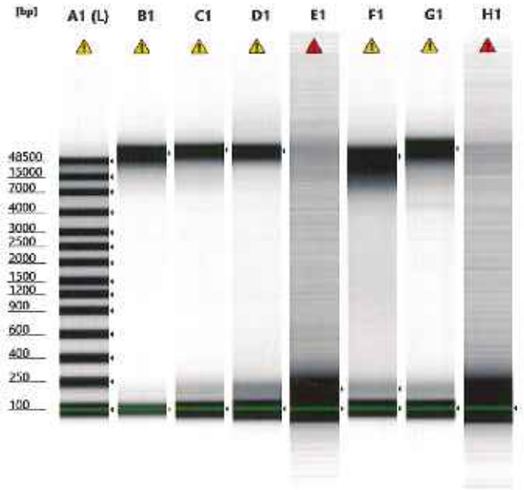


色んな長さのDNAが見られるが、長いものもしっかりと長さ確認ある。
↓
DNAは取れているが濃度がそれほど高くない。黄沐陽. ~~[illegible]~~ 訂正日 10/21/21
解した結晶も薄くなっており、サンプルの濃さが結果を影響しているのではと。
↓
再実験

④ TapeStation. 長鎖DNAがある程度きれいに取れている (p.28, p.29)

10/21/21
記入者 黄沐陽

Filename: 2021-10-20-04gew.gDNA



DIN	DIN	DIN	DIN	DIN	DIN	DIN
9.5	9.8	9.7	-	7.7	9.7	-

Default image (Contrast: 100%), Image is Scaled to Sample

Sample Info

Well	DIN	Conc. [ng/ul]	Sample Description	Alert	Observations
A1	-	61.0	Ladder	⚠	Caution! Expired ScreenTape device; Ladder
B1	9.5	38.7	way	⚠	Caution! Expired ScreenTape device
C1	9.8	7.05	tanaka	⚠	Caution! Expired ScreenTape device; Sample concentration outside recommended range
D1	9.7	4.35	hori	⚠	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
E1	-	2.67	watanabe	▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN
F1	7.7	13.6	hirasaka	⚠	Caution! Expired ScreenTape device
G1	9.7	7.03	mokuyo	⚠	Caution! Expired ScreenTape device; Sample concentration outside recommended range
H1	-	1.41		▲	Caution! Expired ScreenTape device; Sample concentration outside functional range for DIN

10/21/21

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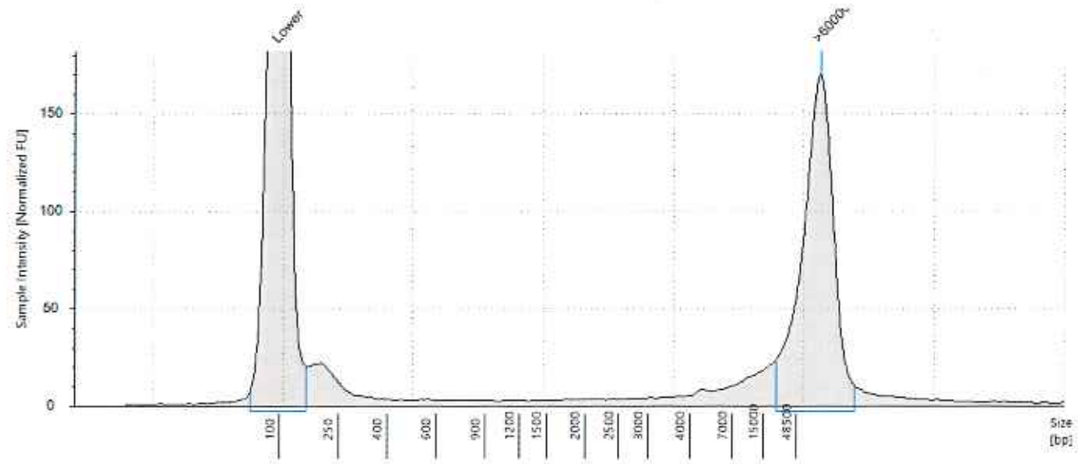
G1: mokuyo



Sample Table

Well	DIN	Conc. [ng/ul]	Sample Description	Alert	Observations
G1	9.7	7.03	mokuyo	⚠	Caution! Expired ScreenTape device; Sample concentration outside recommended range

Peak Table

Size [bp]	Calibrated Conc. [ng/ul]	Assigned Conc. [ng/ul]	% Integrated Area	From [bp]	To [bp]	Peak Comment	Observations
100	8.50	8.50	-	62	150		Lower Marker
>60000	4.81	-	100.00	20183	>60000		

10/21/21

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30
続き

<目的>

・長鎖DNAの再再抽出…

<対象>

・研究ノート p.01～p.02を参照

<手法>

・p.19～行ったDNA抽出の結果がp.01で行った実験より向上したことが分かった。

・濃度も上がり、長鎖DNAも取れていることが分かった。

・実験手順や操作ミスがないことが分かり、問題がサンプル濃度であると考えた。

・O.D.の再計測をする時間もないので ペレットのロスと細菌の死滅がないと仮定した状態で、O.D 1の菌体を2mL×4採取した。

・試薬の用意は、研究ノートp.06～p.09を参照

・QIAGEN Genome DNA Handbook (June 2015) UKT参照

・実行した手順をハイライトまたは"✓"チェックでメモした

UKT 空白

10/21/21

記入者 黄沐陽

確認者

日付 年 月 日

続き

31

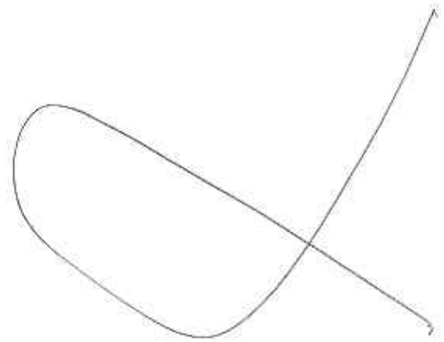
20:14
start

Table 10. Reagent volumes required per bacterial prep 必要な試薬量

Reagent	Mini (tip 20/G)	Midi (tip 100/G)	Maxi (tip 500/G)
B1	1 ml ×4= 4mL	3.5 ml	11 ml
B2	0.35 ml ×4= 1.4mL	1.2 ml	4 ml
QBT	1 ml ×4= 4mL	4 ml	10 ml
QC	3 ml ×4= 12mL	15 ml	30 ml
QF	2 ml ×4= 8mL	5 ml	15 ml
Isopropanol	1.4 ml ×4= 5.6mL	3.5 ml	10.5 ml
70% ethanol	1 ml ×4= 4mL	2 ml	4 ml
RNase A stock solution	2 µl ×4= 8µL	7 µl	22 µl
Lysozyme stock solution (100 mg/ml)	20 µl ×4= 80µL	80 µl	300 µl
QIAGEN Protease or QIAGEN Proteinase K stock solution	45 µl ×4= 180µL	100 µl	500 µl

1. **Prepare Buffers** B1, B2, QBT, QC, and QF according to the instruction in **Appendix A** (page 62), or use the Genomic DNA Buffer Set (for ordering information see page 69).

研究ノートp.04～p.09を参照



10/21/21

記入者 黄沐陽

確認者

日付 年 月 日

20:20

2. **For each prep, add ■ ~~2~~ 8 µl, ▲ 7 µl, or ● 22 µl of RNase A solution (100 mg/ml) to a ■ ~~1~~ 1.4 ml, ▲ 3.5 ml, or ● 11 ml aliquot of Buffer B1. Dissolve lysozyme in distilled water to a concentration of 100 mg/ml. Prepare QIAGEN Protease stock solution in distilled water, or use QIAGEN Proteinase K stock solution.**

黄沐陽 訂正日 10/21/21

If using a kit, dissolve each vial of lyophilized QIAGEN Protease provided in 1.4 ml of distilled water, as indicated on the label. Alternatively, QIAGEN Proteinase K solution may be used directly. See "Preparation of enzyme stock solutions" (page 64) for further details and page 69 for ordering information.

Storage conditions for QIAGEN Protease and QIAGEN Proteinase K are described on page 5.

Lysozyme should be dissolved in distilled water to a concentration of 100 mg/ml and should be stored at –20°C. For efficient lysis of some bacterial species, enzymes other than lysozyme may be necessary. Lysis of *Staphylococcus* spp., for example, is much more efficient with lysostaphin. Use the appropriate enzyme for the particular species. See Appendix A (page 65) for ordering information for lysozyme.

RNase A should be added to Buffer B1 to a concentration of 200 µg/ml and will be stable for 6 months when stored at 2–8°C.

20:15

3. **Pellet bacteria from an appropriate volume of culture by centrifugation at 3000–5000 x g for 5–10 min. Discard the supernatant, ensuring that all liquid is completely removed.**

200ml×4

See pages 43–44 for the appropriate starting volume.

Because of the different growth characteristics of bacterial species, performing a preliminary experiment to determine the optimal starting volume is recommended.

4. **Resuspend the bacteria pellet from step 3 in ■ ~~1~~ 4 ml, ▲ 3.5 ml, or ● 11 ml of Buffer B1 (with RNase A) by vortexing at top speed.**

黄沐陽 訂正日 10/21/21

Add the RNase A to Buffer B1 according to step 2 before adding the buffer to the bacteria.

Resuspend the bacterial pellet as thoroughly as possible by vortexing.

For efficient lysis of the bacteria, it is important to have a homogeneous suspension.

10/21/21 黄沐陽

Lysozyme 20:09

Incubate 20:33 S 21:03

5. **Add ■ ~~20~~ 80 µl, ▲ 80 µl, or ● 300 µl of lysozyme stock solution (100 mg/ml), and ■ ~~45~~ 180 µl, ▲ 100 µl, or ● 500 µl of QIAGEN Protease or QIAGEN Proteinase K stock solution. Incubate at 37°C for at least 30 min.**

黄沐陽 訂正日 10/21/21

10分から30分にボルテックス

Use the stock solutions from step 2.

The length of incubation depends on how well the bacteria were resuspended in step 4.

If the suspension is not homogeneous after vortexing, a longer incubation is recommended to avoid clogging the QIAGEN Genomic-tip.

During incubation, the lysozyme enzymatically breaks down the bacterial cell wall, while the detergents in Buffer B1 ensure complete lysis of the bacteria.

Degradation of RNA by RNase A ensures complete removal during column procedure.

Incubate 21:07 S 22:07

6. **Add ■ ~~0.35~~ 1.4 ml, ▲ 1.2 ml, or ● 4 ml of Buffer B2, and mix by inverting the tube several times or by vortexing for a few seconds. Incubate at 50°C for ~~30~~ 10 min.**

黄沐陽, 訂正日: 10/21/21

Mix the lysate and Buffer B2 very thoroughly. This step is important for efficient deproteinization.

Buffer B2 denatures proteins such as nucleases and DNA-binding proteins. The excess QIAGEN Protease digests the denatured proteins into smaller fragments, facilitating efficient removal during purification.

Buffer B2 and QIAGEN Protease, in combination, strip the genomic DNA of all bound proteins. It is important that the lysate becomes clear at this stage. ~~If necessary,~~ extend the incubation time, or pellet the particulate matter by centrifugation for ~~10 min at 5000~~ x g, 4°C.

Centrifuge 22:10 S 22:25

黄沐陽 訂正日 10/21/21

少なくするように

15 min at 10,000 黄沐陽 10/21/21

Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 1).

7. **Proceed with the Genomic-tip protocol on page 49.**

The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

10/21/21 黄沐陽

Protocol: Isolation of Genomic DNA from Blood, Cultured Cells, Tissue, Yeast, or Bacteria using Genomic-tips

All samples must first be prepared according to the relevant sample preparation and lysis protocol (pages 18–48).

Text with a ■ denotes values for mini-preps using the Genomic-tip 20/G; text with a ▲ denotes values for midi-preps using the Genomic-tip 100/G, and ● denotes values for maxi-preps using the Genomic-tip 500/G.

1. Equilibrate a QIAGEN Genomic-tip 20/G, Genomic-tip 100/G, or Genomic-tip 500/G with ■ 1 ml, ▲ 4 ml, or ● 10 ml of Buffer QBT, and allow the QIAGEN Genomic-tip to empty by gravity flow.

Place a QIAGEN Genomic-tip over a tube using a tip holder or into a QIArack over the waste tray. Equilibrate the QIAGEN Genomic-tip with the volume of buffer described in the protocol. Flow begins automatically by reduction in surface tension due to the presence of detergent (0.15% Triton® X-100) in the equilibration buffer. Allow the QIAGEN Genomic-tip to drain completely. The flow of buffer will stop when the meniscus reaches the upper frit. The frit prevents the QIAGEN Genomic-tip from running dry, allowing it to be left unattended. Do not force out the remaining buffer, as this will necessitate restarting the flow with a syringe and adapter.

2. Vortex the sample (from the last step of the specific sample preparation and lysis protocol) for 10 s at maximum speed and apply it to the equilibrated QIAGEN Genomic-tip. Allow it to enter the resin by gravity flow.

Vortexing the genomic DNA has a minimal effect on the size of the DNA, and it accelerates the QIAGEN procedure by eliminating poor flow rates associated with clogging. The average size of genomic DNA is reduced by only 10 kb when vortexed for up to 20 s (see Figure 3, page 10).

The sample should be loaded onto the QIAGEN Genomic-tip promptly. If left too long, it may become cloudy due to further precipitation of protein. The sample must then be centrifuged or filtered before loading to prevent clogging of the QIAGEN Genomic-tip.

Once the QIAGEN Genomic-tip is loaded with the clear and particle-free sample, flow will begin unassisted. Allow gravity to determine the flow rate. The flow rate will depend on the sample source, the number of cells from which the DNA sample was prepared, and on genome size.

Particularly concentrated genomic DNA lysates may exhibit diminished flow rates due to increased viscosity. Flow can be assisted by the application of gentle positive pressure using a disposable syringe and the appropriate adapter. It may also be helpful to dilute the lysate with an equal volume of Buffer QBT prior to loading.

When using positive pressure, do not allow the flow rate to exceed ■ 4–10 drops/min, ▲ 10–20 drops/min, or ● 20–40 drops/min.

Optional: Take a ■ 300 µl, ▲ 300 µl, or ● 150 µl aliquot and save it for an analytical gel (aliquot 2).

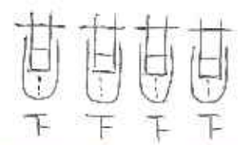
3. Wash the QIAGEN Genomic-tip with ■ 3 x 1 ml, ▲ 2 x 7.5 ml, or ● 2 x 15 ml of Buffer QC.

Allow Buffer QC to move through the QIAGEN Genomic-tip by gravity flow. Two washes are sufficient to remove all contaminants in the majority of DNA preparations.

An additional wash is occasionally necessary if large culture volumes or bacterial strains containing large amounts of carbohydrate are used.

It is particularly important not to force out residual Buffer QC. Traces of Buffer QC will not affect the elution step.

Optional: Take a ■ 120 µl, ▲ 600 µl, or ● 300 µl aliquot and save it for an analytical gel (aliquot 3).



22:57 **4. Elute the genomic DNA with ■ 2 x ~~1~~ ml, ▲ 1 x 5 ml, or ● 1 x 15 ml of Buffer QF.** 4 = 8 黄沐陽 訂正日 10/21/21

Place the QIAGEN Genomic-tip over a clean ■ 10 ml, ▲ 10 ml, or ● 30 ml collection tube. Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. Elute with the appropriate volume of Buffer QF, and collect the eluate. Flow begins automatically. Allow the QIAGEN Genomic-tip to drain by gravity flow.

Use of Buffer QF prewarmed to 50°C will increase yields. 予め50℃で暖める

Optional: Take a ■ 120 µl, ▲ 300 µl, or ● 600 µl aliquot and save it for an analytical gel (aliquot 4). 黄沐陽 訂正日10/21/21

アルコールが入っていると、流出するので、逆に作業する → 利用している状態だと安定する。

5. Precipitate the DNA by adding ■ ~~1.4~~ 5.6 ml, ▲ 3.5 ml, or ● 10.5 ml (0.7 volumes) room-temperature (15–25°C) isopropanol to the eluted DNA. Recover the precipitated DNA as described in step 5A or step 5B. 十分なDNAがあれば、白いDNAのモヤモヤが出る

5A. Precipitate the DNA by inverting the tube 10 to 20 times, and spool the DNA using a glass rod. Proceed with step 6A. 白いDNAのモヤをピペットで吸いとり

23:27 **5B. Alternatively, mix and centrifuge immediately at >5000 x g for at least 15 min at 4°C. Carefully remove the supernatant. Proceed with step 6B.**

沈殿は長いため、5Bの処理を行った。

All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. A centrifugal force of 5000 x g is the minimal force required for efficient precipitation. Higher g-force is recommended where possible. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

6. Transfer material according to steps 6A and 6B.

23:31 **6A. Immediately transfer the spooled DNA to a microcentrifuge tube containing 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.** ~~buffer~~ EB buffer

黄沐陽 訂正日 10/21/21

10/21/21 記入者 黄沐陽

エタノール 23:59 **6B. Wash the centrifuged DNA pellet with ■ ~~1~~ 4 ml, ▲ 2 ml, or ● 4 ml of cold 70% ethanol. Vortex briefly and centrifuge at >5000 x g for 10 min at 4°C. Carefully remove the supernatant without disturbing the pellet. Air-dry for 5–10 min, and resuspend the DNA in 0.1–2 ml of a suitable buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Dissolve the DNA overnight on a shaker or at 55°C for 1–2 h.**

黄沐陽 訂正日 10/21/21

残ったエタノールを捨てる

Air-dry 24:09 ~ 24:14

EB buffer

40µL 下限30µL 少ないと、30µLで 遠心したら 50µLで

The 70% ethanol removes precipitated salt and replaces the isopropanol with the more volatile ethanol, making the DNA easier to redissolve. A second wash with cold 70% ethanol may improve results in more sensitive applications.

After careful and complete removal of the ethanol supernatant with a pipet, the pellet should be air-dried briefly before resuspending in a small volume of suitable buffer. Overdrying the pellet will make the DNA difficult to redissolve. Resuspend the DNA pellet by rinsing the walls to recover all the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. DNA dissolves best under slightly alkaline conditions (pH 8.0–8.5) and does not dissolve easily in acidic buffers.

Determination of yield, purity, and length of the DNA

Yields of genomic DNA will depend on the number of cells and the capacity of the QIAGEN Genomic-tip used. DNA yield is usually determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. To ensure accuracy, make sure the absorbance readings fall into the linear range of your method (e.g., between 0.1 and 1.0 for spectrophotometric OD). Sample dilution should be adjusted accordingly: for example, an eluate containing 25–50 ng of DNA/µl (A_{260} = 0.5–1) should not be diluted with more than 4 volumes of buffer. Use elution buffer or water (as appropriate) to dilute samples and to calibrate the spectrophotometer. Measure the absorbance at 260 nm and 280 nm, or scan absorbance from 220–320 nm. (A scan will show whether there are other factors affecting absorbance at 260 nm.) Readings on a spectrophotometer are not always accurate, particularly if a single wavelength measurement is taken rather than a scan, and should always be verified by visual examination of the DNA on an agarose gel. Fluorimetric measurements are more accurate and should be used if precise concentrations are needed.

Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9.

The precise length of genomic DNA is determined by pulsed-field gel electrophoresis (PFGE) through an agarose gel. Standard PFGE conditions are as follows: 1.5% agarose gel in 0.5x TBE electrophoresis buffer; switch intervals, 1–10 s; run time, 16 h; voltage, 170 V.

10/21/21 記入者 黄沐陽

・また、DNAの収量、純度の測定は、nanodrop、Qubitを用いて行った。

・Qubitは、dsDNA BRを、2μLサンプル量で測った。

<結果>

① nanodrop

Sample ID	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
A_Rob_DNA_1		0.8 ng/µl	0.016	0.004	3.52	0.47	DNA	50
A_Rob_DNA_2		10.3 ng/µl	0.207	0.105	1.96	2.03	DNA	50
B_ethanol_DNA		172.3 ng/µl	2.047	1.548	1.21	1.85	DNA	50

黄沐陽 修

・1行、2行目は、p.36の5A、6Aで拾ってきた純化のDNAを測定した。

・3行目は、目視でDNAを確認できなかったが、5B、6B(p.36、p.37)で処理を行い、最終的に1本にまとめたサンプル~~ごとの~~の測定である。
黄沐陽 訂正日 10/21/21

・3回目の測定で用いられたサンプルのDNA濃度、量が共に飛躍的に向上し、純粋なDNAが示すA260/A280の比(1.7~1.9)の間にもあるため、確実に純度も高いと考えられる。

② Qubit

・純化DNA: 9.18 ng/μL

・エタノールDNA: 7000ng/μL

・どちらでもやはりエタノールの手順を行ったサンプルの方が高い濃度を示すことが分かった。

このサンプルもシェーカーで1晩回した。

以下空白

10/21/21
記入者 黄沐陽

<目的>

黄沐陽 訂正日 10/22/21

・~~3回の~~前日に抽出し、1晩シェーカーで溶かしたDNAの変化の観察。

・一番良い結果を出せたサンプルのナノポアシーケンシング(1D-Rapid、minION)

<対象>

・研究ノート p.26、p.36、p.37で得られた3サンプル。

<手法1>

・Qubitを用いて、dsDNA BRを、2μLサンプル量で測った。

<結果1>

・p.26 10/21/21 昼サンプル: 17.30 ng/μL

・p.36 10/21/21 夜の純化DNAサンプル: 5.68 ng/μL

・p.37 10/21/21 夜のエタノールDNAサンプル: 71.40 ng/μL

黄沐陽 訂正日 10/22/21

研究ノート p.50式の手法で行った実験で、p.36で遠心し、p.37でエタノールで得られたサンプルが、最も高い濃度を示した。そのため、このサンプルをナノポアシーケンスに用いた。

1晩のシェーカーで量があまり変わらないことが考えられたため、今後は、

「Dissolve the DNA overnight on a shaker ~~or~~ at 55°C for 1~2h」

こっちでDNAを解凍の方を試したらいいかと思われる。

あるいは、p.36の6Aで加えたEB bufferが多過ぎたため、濃度を影響していると考えるのが妥当と思われる。

<手法2>

・ナノポアシーケンスを行うにあたり、Nanopore Protocol Rapid Barcoding Sequencing (SQK-RBK004) version: RBK_9054_v1_revM_14Aug2019を参照した。

・なお、実行した手順をハイライトまたは"V"チェックで示した。 以下空白

10/22/21
記入者 黄沐陽

Rapid Adapter Top Up Kit

The Rapid Adapter Top Up Kit (EXP-RAP001) supplies additional tubes of Rapid Adapter, Sequencing Buffer, Sequencing Tether, and Loading Beads. The Rapid Barcoding Sequencing Kit contains sufficient reagents for six pooled libraries using all 12 barcodes. The Top Up kit can be used for cases where several experiments are run with fewer than 12 barcodes and the Rapid Barcoding kit no longer has sufficient Rapid Adapter and Sequencing Buffer.

IMPORTANT

Compatibility of this protocol

This protocol should only be used in combination with:

- Rapid Barcoding Sequencing Kit SQK-RBK004
- FLO-MIN106 flow cells (FLO-MIN107 not advised)
- EPI2ME FASTQ Barcoding workflow, or
- Barcode demultiplexing in Guppy
- Wash Kit (EXP-WSH003)

Equipment and consumables

必要なもの：

Materials

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

Consumables

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

Equipment

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

Optional Equipment

- Standard gel electrophoresis equipment

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以下空白

10/22/21

記入者 黄沐陽

確認者

日付 年 月 日

- Agilent Bioanalyzer (or equivalent)
- Qubit fluorometer (or equivalent for QC check)
- Eppendorf 5424 centrifuge (or equivalent)
- Magnetic rack
- Hula mixer (gentle rotator mixer)

For this protocol, you will need ~400 ng high molecular weight genomic DNA

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.

Rapid Barcoding Kit contents

キットの内容

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

Contents	Description	No. of tubes
RB01-12	Fragmentation Mix RB01-12	12
RAP (green cap)	Rapid adapter Contains leader adapters with loaded motor protein	1
SQT (purple cap)	Sequencing tether	1
LB (pink cap)	Loading beads	1
SQB (red cap)	Sequencing buffer	1

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確認者

日付 年 月 日

IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

Flow Cell Priming Kit contents (EXP-FLP002)

FLB Flush buffer
FLT Flush tether

Computer requirements and software

コンピューター 計算機の条件.

MinION Mk1B IT requirements

Unless you are using a MinIT device, sequencing on a MinION Mk1B requires a high-spec computer or laptop to keep up with the rate of data acquisition. Read more in the MinION IT Requirements document.

Software for nanopore sequencing

MinKNOW

The MinKNOW software controls the nanopore sequencing device, collects sequencing data in real time and processes it into basecalls. You will be using MinKNOW for every sequencing experiment. MinKNOW can also demultiplex reads by barcode, and basecall/demultiplex data after a sequencing run has completed.

MinKNOW use

For instructions on how to run the MinKNOW software, please refer to the relevant section in the MinKNOW protocol.

EPI2ME (optional)

The EPI2ME cloud-based platform performs further analysis of basecalled data, for example alignment to the Lambda genome, barcoding, or taxonomic classification. You will use the EPI2ME platform *only* if you would like further analysis of your data post-basecalling.

EPI2ME installation and use

For instructions on how to create an EPI2ME account and install the EPI2ME Desktop Agent, please refer to the EPI2ME Platform protocol.

Guppy (optional)

The Guppy command-line software can be used for basecalling and demultiplexing reads by barcode instead of MinKNOW. You can use it if you would like to re-analyse old data, or integrate basecalling into your analysis pipeline.

Guppy installation and use

If you would like to use the Guppy software, please refer to the Guppy protocol.

Check your flow cell

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記入者 黄沐陽 確認者 日付 年 月 日

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 three months of purchasing for MinION/GridION/PromethION flow cells, or within four weeks of purchasing for Flongle flow cells. Oxford Nanopore Technologies will replace any flow cell with fewer than the number of pores 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
Flongle Flow Cell	60
MinION/GridION Flow Cell	800
PromethION Flow Cell	5000

Library preparation

~10 minutes

Materials
- 400 ng high molecular weight genomic DNA
- Fragmentation Mix FBC1-12
- Rapid Adaptor (RAP)

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

Equipment
- Thermal cycler or heat block at 30°C and 80°C
- Microfuge
- P10 pipette and tips

Optional Equipment
- Magnetic rack
- Tube mixer (gentle rotator mixer)

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

Reagent 試薬	1. Thaw at room temperature	2. Briefly spin down	3. Mix well by pipetting
Fragmentation Mix RB01-12	Not frozen	✓	✓
Rapid Adapter (RAP)	Not frozen	✓	✓
Sequencing Buffer (SQB)	✓	✓	✓*
Loading Beads (LB)	✓	✓	Mix by pipetting or vortexing immediately before use
Flush Buffer (FLB) - 1 tube	✓	✓	✓*
Flush Tether (FLT)	✓	✓	✓

*Vortexing, followed by a brief spin in a microfuge, is recommended for Sequencing Buffer (SQB) and Flush Buffer (FLB).

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

2 **Prepare the DNA in nuclease-free water.**

- Transfer ~400 ng genomic DNA into a DNA LoBind tube
- Adjust the volume to 7.5 µl with nuclease-free water
- Mix by flicking the tube to avoid unwanted shearing
- Spin down briefly in a microfuge

14:04 Start

3 **In a 0.2 ml thin-walled PCR tube, mix the following:**

Reagent	Volume
400 ng template DNA	7.5 µl
Fragmentation Mix RB01-12 (one for each sample)	2.5 µl
Total	**10 µl**

4 **Mix gently by flicking the tube, and spin down.**

5 **Incubate the tube at 30° C for 1 minute and then at 80° C for 1 minute. Briefly put the tube on ice to cool it down.** 時間厳守!! 黄沐陽, 訂正日: 10/22/21 ~~バーコード B11~~

30℃で基本的に失活するけど、少しくらいどうしても生きているやつがいるのでそれ以降はオンアイスで。

6 **Pool all barcoded samples in your desired ratio, noting the total volume.** → on ice

バーコード、B11

IMPORTANT

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

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7 **Resuspend the AMPure XP beads by vortexing.**

8 **To the entire pooled barcoded sample from Step 6, add an equal volume of resuspended AMPure XP beads, and mix by flicking the tube.**

9 **Incubate on a Hula mixer (rotator mixer) for 5 minutes at room temperature.**

10 **Prepare 500 µl of fresh 70% ethanol in nuclease-free water.**

11 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.**

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

13 **Repeat the previous step.**

14 **Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.**

15 **Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 7.5-8.0 with 50 mM NaCl. Incubate for 2 minutes at room temperature.**

16 **Pellet the beads on a magnet until the eluate is clear and colourless.**

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

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

End of optional steps.

18 **Add 1 µl of RAP to 10 µl of barcoded DNA.**

19 **Mix gently by flicking the tube, and spin down.**

20 **Incubate the reaction for 5 minutes at room temperature.** これでライブラリーができる。

END OF STEP

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

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Priming and loading the SpotON Flow Cell

~10 minutes

必要なもの：

Materials
- Flow Cell Priming Kit (EXP-FLP002)
- Sequencing Buffer (SQB)
- Loading Beads (LB)

Consumables
- 1.6 ml Eppendorf DNA LoBind tubes
- Nuclease-free water (e.g. ThermoFisher, cat. # AM9937)

Equipment
- MinION Mk1B
- SpotON Flow Cell
- P1000 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips

IMPORTANT

Please note that the Sequencing Tether (SQT) tube will NOT be used in this protocol. It is provided in the kit for potential future product compatibility.

1 **Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature.**

2 **Mix the Sequencing Buffer (SQB), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at room temperature.**

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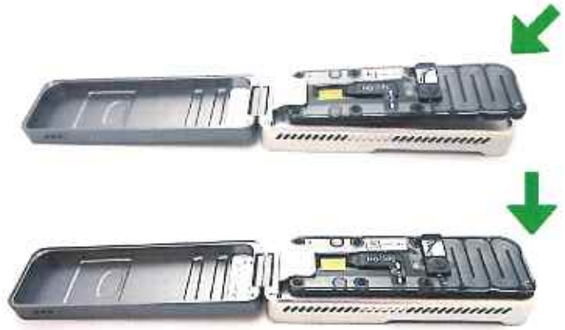
3 **Open the MinION Mk1B lid and slide the flow cell under the clip.**

Press down firmly on the flow cell to ensure correct thermal and electrical contact.



4 **Slide the priming port cover clockwise to open the priming port.**

How to prime and load the SpotON Flow Cell

Priming and loading: The steps for priming and loading the SpotON Flow Cell. Written instructions are given below. The library is loaded dropwise without putting the pipette tip firmly into the port.

Take care to avoid introducing any air during pipetting.

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記入者 黄沐陽
確認者
日付 年 月 日

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Priming and loading the SpotON Flow Cell

Version: RBK_9054_v2_revN_14Aug2019

IMPORTANT

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

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

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

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

6 **To prepare the flow cell priming mix, add 30 µl of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.** 黄沐陽 記入日 10/22/21

タンパク質を保存する溶液を 洗浄 Buffer.

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

8 **Thoroughly mix the contents of the Sequencing Buffer (SQB) and Loading Beads (LB) tubes by vortexing.**

TIP

Using the Loading Beads

Demo of how to use the Loading Beads.

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

Reagent	Volume
Sequencing Buffer (SQB)	34 µl 固定
Loading Beads (LB), mixed immediately before use	25.5 µl beads = 75−34−(8+8+7) = 18
Nuclease-free water	~~16 µl~~ 0
DNA library	11 µl 7+8+8
Total	**75 µl** 固定

この2つの量を調整

IMPORTANT

The Loading Beads (LB) tube contains a suspension of beads. These beads settle very quickly. It is vital that they are mixed immediately before use.

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10/22/21
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Sequencing and data analysis

Version: RBK_9054_v2_revN_14Aug2019

10 **Complete the flow cell priming:**

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

11 **Mix the prepared library gently by pipetting up and down just prior to loading.**

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

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

*from pore exposure in closed position

Data acquisition and basecalling

pore: 1,025

Overview of nanopore data analysis

For a full overview of nanopore data analysis, which includes options for basecalling and post-basecalling analysis, please refer to the Data Analysis document.

How to start sequencing

The sequencing device control, data acquisition and real-time basecalling are carried out by the MinKNOW software. It is assumed you have already installed MinKNOW on your computer, or that you are using the MinIT device for data acquisition and basecalling. There are three options for how to carry out sequencing:

1. Data acquisition and basecalling in real-time using MinKNOW on a computer

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run"

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Rapid Barcoding Sequencing (SQK-RBK004)
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Downstream analysis

Version: RBK_9054_v2_revN_14Aug2019

section.

2. Data acquisition and basecalling in real-time using the MinION Mk1C device

Follow the instructions in the MinION Mk1C protocol

3. Data acquisition and basecalling in real-time using the MinIT device

Follow the instructions in the MinIT protocol.

4. Data acquisition using MinKNOW on a computer and basecalling at a later time using Guppy

Follow the instructions in the MinKNOW protocol beginning from the "Starting a sequencing run" section until the end of the "Completing a MinKNOW run" section. **When setting your experiment parameters, set the *Basecalling* tab to OFF.** After the sequencing experiment has completed, follow the instructions in the Guppy protocol starting from the "Quick Start Guide for Guppy" section.

Nanopore Protocol
Rapid Barcoding Sequencing (SQK-RBK004)
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Ending the experiment

Version: RBK_9054_v2_revN_14Aug2019

Ending the experiment

Materials • Flow Cell Wash Kit (EXP-WSH003)

1 **After your sequencing experiment is complete, if you would like to reuse the flow cell, please follow the Wash Kit instructions and store the washed flow cell at 2-8°C, OR**

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

<結果>

・シーケンスは無事に完了し、BC11.fastq と名付けた。

以下空白

10/22/21
記入者 黄沐陽

<目的>

・ナノポアでシーケンスしたリードデータのゲノムアセンブリーを行い，1 本の環状 DNA にする．

<対象>

・研究ノート p. 50 で得られたナノポアシーケンスリード生データを処理したもの（BC11.fastq）．

・なお BC11.fastq は guppy ソフトウェアでベースコール後，バーコードの demultiplex を行なってある．

<手順>

・冨田研 king サーバーで解析を行った．

・以下，行頭が "$" から始まる黒背景の白字は実際に実行したコードで，灰字白背景の文字はその出力とし，結果は赤字で記述した．

・なお，dry 解析は以下の web ページを参考にした．

http://web.sfc.keio.ac.jp/%7Egaou/wiki/wiki.cgi?page=%A5%B2%A5%CE%A5%E0%B9%A9%B3%D8%BC%C2%BD%AC

 # dry 解析の準備

ゲートウェイサーバーにアクセスし，king サーバーに入った．

```
$ ssh king
```

クラスターサーバーの空いているノードに入った．

```
$ qsub -I -l nodes=1:ppn=32
```

解析ディレクトリーを作成し，生データ（BC11.fastq）等をコピーして ~~した~~ また

黄沐陽 訂正日：11/09/21

```
$ mkdir 2021_gew
$ cd 2021_gew/
$ cp /home/gaou/gew/2021/BC11.fastq.gz .
$ cp /home/gaou/gew/2021/report_FAP20986_20211022_1525_282fadcf-BC08-10.pdf .
$ mkdir Illumina
$ cd Illumina/
$ cp /home/gaou/gew/2021/illumina/GEW-BC11_S5_merged_R1.fq.gz .
$ cd ..
```

11/09/21
記入者 黄沐陽

```
$ mkdir NanoPlot
$ cd NanoPlot/
$ cp -r /home/gaou/gew/2021/NanoPlot/BC11/ .
$ cp /home/gaou/gew/2021/NanoPlot/BC12NanoPlot_20211026_1109.log .
$ cd ..
```

データの解凍.

```
$ gunzip BC11.fastq.gz
```

 # リードのフィルタリング

シーケンスできているかを確認するために，リードをフィルタリングした.

```
$ /home/gaou/kumamushi/software_smith/bbmap/stats.sh BC11.fastq
```

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.3097	0.1886	0.1921	0.3096	0.0000	0.0000	0.0000	0.3807	0.0508

Main genome scaffold total:	70022	
Main genome contig total:	70022	
Main genome scaffold sequence total:	297.995 MB	
Main genome contig sequence total:	297.995 MB	0.000% gap
Main genome scaffold N/L50:	10118/8.417 KB	
Main genome contig N/L50:	10118/8.417 KB	
Main genome scaffold N/L90:	35530/2.471 KB	
Main genome contig N/L90:	35530/2.471 KB	
Max scaffold length:	201.273 KB	
Max contig length:	201.273 KB	
Number of scaffolds > 50 KB:	36	
% main genome in scaffolds > 50 KB:	0.80%	

Minimum Scaffold Length	Number of Scaffolds	Number of Contigs	Total Scaffold Length	Total Contig Length
Coverage				
All	70,022	70,022	297,994,916	297,994,916
100.00%				
50	67,120	67,120	297,891,233	297,891,233
100.00%				
100	61,668	61,668	297,515,439	297,515,439
100.00%				
250	58,312	58,312	296,968,871	296,968,871
100.00%				
500	55,050	55,050	295,761,781	295,761,781
100.00%				
1 KB	49,179	49,179	291,412,781	291,412,781
100.00%				
2.5 KB	35,275	35,275	267,572,629	267,572,629
100.00%				
5 KB	19,265	19,265	208,580,101	208,580,101
100.00%				
10 KB	7,682	7,682	126,660,297	126,660,297
100.00%				
25 KB	756	756	25,373,452	25,373,452
100.00%				
50 KB	36	36	2,373,125	2,373,125
100.00%				
100 KB	1	1	201,273	201,273
100.00%				

Scaffold total で 70022 本のリードを読めていることが分かった.
Scaffold sequence total で実際に 297.995 MB 読まれていることが分かった.
Max scaffold length で最長の配列は 201.273 KB で，Illumina 等と比べると大分違うことが分かった.
N50 は 8.417 KB.
50 KB 以上の配列は 36 本あることが分かった.
10 KB は約 40×.
短い配列はあっても無駄.
場合によっては捨てた方が良い.

Illumina の場合は 100×くらいないとダメ.
Nanopore は最初からリードが長い.
その為 1 K 以下は捨てた方が良い（量的にも誤差）.

また，Histogram of read lengths, Read length vs Average read quality plot から，Q10 は 97~99%であることが分かった.

NCBI で enterococcus faecalis の genome を検索:
https://www.ncbi.nlm.nih.gov/genome/?term=enterococcus%20faecalis
Enterococcus faecalis EnGen0336
Statistics: median total length (Mb): 2.97338
50×の場合:
2. 97338 ×50 ~ 2.87 ×50 = 148.669 ~ 143.5 M
結果から見ると，10KB は 126,660,297 あるので，これはおよそ 40×
基本 20× ~ 50×にあれば良いので十分と判断.

現状ではリードが多過ぎるので，大体 20× ~ 50×になるようにリードを調整した.
この時，長いリードは残した.

```
$ /home/gaou/kumamushi/software_smith/bbmap/reformat.sh in=BC11.fastq out=BC11-filter10k.fq minlength=10000 qin=33
```

```
Input is being processed as unpaired
Input:                  70022 reads        297994916 bases
Short Read Discards:    62340 reads (89.03%)  171334619 bases (57.50%)
Output:                  7682 reads (10.97%)  126660297 bases (42.50%)

Time:                   1.899 seconds.
Reads Processed:    70022   36.86k reads/sec
Bases Processed:    297m    156.88m bases/sec
```

BBMap で統計値を算出して，カバレッジを合わせた.

```
$ /home/gaou/kumamushi/software_smith/bbmap/stats.sh BC11-filter10k.fq
A       C       G       T       N       IUPAC   Other   GC      GC_stdev
0.3093  0.1883  0.1920  0.3104  0.0000  0.0000  0.0000  0.3802  0.0244

Main genome scaffold total:     7682
Main genome contig total:       7682
```

11/09/21
記入者 黄沐陽


```
Main genome scaffold sequence total:  126.660 MB
Main genome contig sequence total:    126.660 MB    0.000% gap
Main genome scaffold N/L50:           2675/16.482 KB
Main genome contig N/L50:             2675/16.482 KB
Main genome scaffold N/L90:           6476/11.02 KB
Main genome contig N/L90:             6476/11.02 KB
Max scaffold length:                  201.273 KB
Max contig length:                    201.273 KB
Number of scaffolds > 50 KB:          36
% main genome in scaffolds > 50 KB:   1.87%
```

Minimum Scaffold Length	Number of Scaffolds	Number of Contigs	Total Scaffold Length	Total Contig Length	Coverage
All	7,682	7,682	126,660,297	126,660,297	100.00%
5 KB	7,682	7,682	126,660,297	126,660,297	100.00%
10 KB	7,682	7,682	126,660,297	126,660,297	100.00%
25 KB	756	756	25,373,452	25,373,452	100.00%
50 KB	36	36	2,373,125	2,373,125	100.00%
100 KB	1	1	201,273	201,273	100.00%

Scaffold total が 7682 本のリードになったことが分かった.
Scaffold sequence total で実際に 126.660 MB になったことが分かった.
Max scaffold length で最長の配列は変わらず 201.273 KB で，N50 は 16.482 KB.
50 KB 以上の配列も変わらなかった.

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記入者 黄沐陽

更に，5 KB 以下の配列は全て切り捨てたことを確認した．

 # Canu でのアセンブリー

毎回バージョンが変わるけど，最新版を使う必要があるので，ダウンロードした．
genomeSize オプション（頭にハイフンをつけない点に注意）に推定（予想）ゲノムサイズより少し大きめに設定した（バクテリアの場合大抵 4m で良い）．
genomeSize は最終的に何×で計算しているのかと，結果の目安を持つのと，計算に用いる計算領域を確保する為のものなのでそれ程重要ではない．
maxThreads は，使用するサーバに合わせて設定した（king は 32）．
d はディレクトリ名の設定で p はファイル名の設定．
異なるファイル名を指定してあっても，同じディレクトリー名の場合は並列して処理しない方が良い．

```
$ /home/gaou/kumamushi/software_smith/canu-2.2/bin/canu -nanopore-raw BC11-filter10k.fq -d BC11_1 -p BC11_1 -fast useGrid=false genomeSize=3m maxThreads=32
$ /home/gaou/kumamushi/software_smith/bbmap/stats.sh BC11_1/BC11_1.contigs.fasta
```

統計値の算出

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.3103	0.1878	0.1866	0.3154	0.0000	0.0000	0.0000	0.3744	0.0135

Main genome scaffold total:	2	
Main genome contig total:	2	
Main genome scaffold sequence total:	2.975 MB	
Main genome contig sequence total:	2.975 MB	0.000% gap
Main genome scaffold N/L50:	1/2.885 MB	
Main genome contig N/L50:	1/2.885 MB	
Main genome scaffold N/L90:	1/2.885 MB	
Main genome contig N/L90:	1/2.885 MB	
Max scaffold length:	2.885 MB	
Max contig length:	2.885 MB	
Number of scaffolds > 50 KB:	2	
% main genome in scaffolds > 50 KB:	100.00%	

Minimum Scaffold	Number of Scaffolds	Number of Contigs	Total Scaffold Length	Total Contig Length	Contig Coverage
All	2	2	2,974,837	2,974,837	100.00%
50 KB	2	2	2,974,837	2,974,837	100.00%
100 KB	1	1	2,884,939	2,884,939	100.00%
250 KB	1	1	2,884,939	2,884,939	100.00%
500 KB	1	1	2,884,939	2,884,939	100.00%
1 MB	1	1	2,884,939	2,884,939	100.00%
2.5 MB	1	1	2,884,939	2,884,939	100.00%

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記入者 黄沐陽

リードは 2 本の配列にアセンブルされ，より長い配列は 2,884,939 B あることが分かった．

-fast オプションは精度を犠牲にして実行速度を上げるオプションで，時間がかかっても構わないので外した．

```
$ /home/gaou/kumamushi/software_smith/canu-2.2/bin/canu -nanopore-raw BC11-filter10k.fq -d BC11_1 -p BC11_2 useGrid=false genomeSize=3m maxThreads=32
$ /home/gaou/kumamushi/software_smith/bbmap/stats.sh BC11_2/BC11_2.contigs.fasta
```

統計値算出

A	C	G	T	N	IUPAC	Other	GC	GC_stdev
0.3103	0.1878	0.1866	0.3154	0.0000	0.0000	0.0000	0.3744	0.0135

Main genome scaffold total:	2	
Main genome contig total:	2	
Main genome scaffold sequence total:	2.975 MB	
Main genome contig sequence total:	2.975 MB	0.000% gap
Main genome scaffold N/L50:	1/2.885 MB	
Main genome contig N/L50:	1/2.885 MB	

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Main genome scaffold N/L90: 1/2.885 MB
Main genome contig N/L90: 1/2.885 MB
Max scaffold length: 2.885 MB
Max contig length: 2.885 MB
Number of scaffolds > 50 KB: 2
% main genome in scaffolds > 50 KB: 100.00%

Minimum Scaffold Length	Number of Scaffolds	Number of Contigs	Total Scaffold Length	Total Contig Length	Coverage
All	2	2	2,974,947	2,974,947	100.00%
50 KB	2	2	2,974,947	2,974,947	100.00%
100 KB	1	1	2,884,967	2,884,967	100.00%
250 KB	1	1	2,884,967	2,884,967	100.00%
500 KB	1	1	2,884,967	2,884,967	100.00%
1 MB	1	1	2,884,967	2,884,967	100.00%
2.5 MB	1	1	2,884,967	2,884,967	100.00%

同じくリードは 2 本の配列にアセンブルされたが，最も長い配列は 28 B 長くなったことが分かった．

 # 末端処理

11/09/21
記入者 黄沐陽 確認者 日付 年 月 日

環状化ができているかを確認した．

各 contig の FASTA ヘッダーで suggestCircular が yes のものは，染色体かプラスミドの可能性が高い．

```
$ grep ">" BC11_2.contigs.fasta
```

>tig00000001 len=2884967 reads=7034 class=contig suggestRepeat=no suggestBubble=no suggestCircular=yes trim=7174-2864934
>tig00000002 len=89980 reads=138 class=contig suggestRepeat=no suggestBubble=no suggestCircular=yes trim=10444-77950

長さが 1Mbp に満たないものはプラスミドの可能性が高いので，適当に数 kbp 分をコピーして NCBI BLAST に投げた．

プラスミド配列にヒットしたので，高い確率でプラスミドであると考えられた．

Enterococcus faecalis, plasmid.

suggestCircular が yes になっていても，環状であることを確認しているだけで環状化されているわけではない．

最初の 50 文字程度で自身の配列内を検索して，末端部分に該当する場所を見つけた．

末端部位の検索:

染色体は AAGTCGCCATTAACACTTTATCCAGGTACATCTAATAGTGTCCAAT で一致．

プラスミドは，

GCCCACCTAAAACTATGACCACAAAAAGTCCACCAATGCTTAAAAACAGTCGTATTTTTCGTTTGTCTTGCATACTTTACTCTCCTTTTACATA で一致．

その後に続く配列が先と部分と末端の該当部分で一致することを確認したため，末端の一致部分を削除した（元ファイルをコピーし，新しいファイル名を BC11-2.contigs-removed.fasta にした）．

11/09/21
記入者 黄沐陽 確認者 日付 年 月 日

<目的>

・環状化したDNAのエラーコレクションを行い，アノテーションの付与する．

<対象>

・研究ノートp. 59で得られたナノポア環状化DNA (BC11-2.contigs-removed.fasta).

<手順>

・冨田研kingサーバーで解析を行った．

・以下，行頭が"$"から始まる黒背景の白字は実際に実行したコードで，灰字白背景の文字はその出力とし，結果は赤字で記述した．

・なお，dry解析は以下のwebページを参考にした．

http://web.sfc.keio.ac.jp/%7Egaou/wiki/wiki.cgi?page=%A5%B2%A5%CE%A5%E0%B9%A9%B3%D8%BC%C2%BD%AC

 # dry解析の準備

ゲートウェイサーバーにアクセスし，kingサーバーに入った．

```
$ ssh king
```

クラスターサーバーの空いているノードに入った．

```
$ qsub -I -l nodes=1:ppn=32
```

 # アセンブリークオリティの検証

アセンブリーのクオリティ確認はN50以外に，ゲノムとして全遺伝情報がカバーできているかを確認する必要があった．

その為にはCEGMAやBUSCOといった手法を用いた．

http://kazumaxneo.hatenablog.com/entry/2017/07/19/145640

BUSCOスコアは理研が開発しているgVolanteというウェブサーバを使用した(https://gvolante.riken.jp).

BUSCOはv.1がバクテリアに対応しているため，指定した．

Job Title: 01st-gVolante:

---

gVolante Analysis: ver.2.0.0

Summary of the submitted job:
JOB_ID: 202112290053-MUFDD3U2A91DL1UL
PROJECT_NAME: 01st-gVolante
FASTA_FILE: BC11-2.contigs-removed.fasta
MIN_LENGTH_OF_SEQ_STATS: 1
ASSEMBLY_TYPE: genome
PROGRAM: BUSCO_v1
SELECTED_REFERENCE_GENE_SET: Bacteria

Result:

Total length (nt)	2925260
Longest sequence (nt)	2857754
Shortest sequence (nt)	67506

Core gene set: 39 (97.50%)
https://gvolante.riken.jp/script/detail.cgi?202112290053-MUFDD3U2A91DL1UL
Detected as Missing: COG0184
COG0184: RpsO
Ribosomal protein S15P/S13E [Translation, ribosomal structure and biogenesis]
COG0184 is a member of the superfamily cl00349.

更に，バクテリア用にはCheckMというソフトウェアの方がより詳細にcompletenessを計算できる．

CheckMはDFAST Quality Controlツール(https://dfast.ddbj.nig.ac.jp/dqc/submit/)で検証した．

Job Title: 01st-DFAST:
Rank: species; Taxon: Enterococcus faecalis (20 genomes, 298 marker sets).
JobID: 62e9a4f0-2039-455d-b6ba-9b35c8d4a6ba
https://dfast.ddbj.nig.ac.jp/dqc/62e9a4f0-2039-455d-b6ba-9b35c8d4a6ba
Result: Completeness: 98.41%; Contamination: 0.14%.

 # エラーコレクション

エラー補正には，ナノポアリードを使う場合と，Illumina リードを使う場合の 2 通りがある．
ただし，Illumina リードを用いた方が確実に良い結果が得られる．
今回は Illumina シーケンスを用意したので，これを使ってエラーコレクションを行った．
新規で読んでいて Illumina リードがない場合には nanopore オンリーのエラーコレクションを行う（非推奨）．

1. Illumina での補正は Pilon を使った．
基本的には BWA で mapping 後 pilon をかけるだけ．

まずは mapping.

```
$ gunzip Illumina/GEW-BC11_S5_merged_R1.fq.gz
$ mkdir error_correction_1
$ /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa index BC11-2.contigs-removed.fasta
```

```
[bwa_index] Pack FASTA... 0.04 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 0.77 seconds elapse.
[bwa_index] Update BWT... 0.03 sec
[bwa_index] Pack forward-only FASTA... 0.03 sec
[bwa_index] Construct SA from BWT and Occ... 0.28 sec
[main] Version: 0.7.11-r1034
[main] CMD: /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa index BC11-2.contigs-removed.fasta
[main] Real time: 1.238 sec; CPU: 1.160 sec
```

```
$ /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa mem -t 32 BC11-2.contigs-removed.fasta Illumina/GEW-BC11_S5_merged_R1.fq | /home/gaou/kumamushi/software_smith/samtools-1.9/samtools view -@ 32 -b -o error_correction_1/aln.bam -
```

```
[M::bwa_idx_load_from_disk] read 0 ALT contigs
[M::process] read 4267406 sequences (320000053 bp)...
[M::mem_process_seqs] Processed 4267406 reads in 197.896 CPU sec, 11.828 real sec
[M::process] read 4267886 sequences (320000013 bp)...
[M::mem_process_seqs] Processed 4267886 reads in 172.051 CPU sec, 5.880 real sec
[M::process] read 4267162 sequences (320000101 bp)...
```

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記入者 黄沐陽


```
[M::mem_process_seqs] Processed 4267162 reads in 186.243 CPU sec, 6.062 real sec
[M::process] read 4267700 sequences (320000058 bp)...
[M::mem_process_seqs] Processed 4267700 reads in 162.162 CPU sec, 5.517 real sec
[M::process] read 4267440 sequences (320000027 bp)...
[M::mem_process_seqs] Processed 4267440 reads in 170.300 CPU sec, 5.569 real sec
[M::process] read 4267756 sequences (320000064 bp)...
[M::mem_process_seqs] Processed 4267756 reads in 166.643 CPU sec, 5.739 real sec
[M::process] read 4267658 sequences (320000013 bp)...
[M::process] read 2254447 sequences (169011728 bp)...
[M::mem_process_seqs] Processed 4267658 reads in 153.991 CPU sec, 5.151 real sec
[M::mem_process_seqs] Processed 2254447 reads in 90.172 CPU sec, 3.728 real sec
[main] Version: 0.7.11-r1034
[main] CMD: /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa mem -t 32 BC11-2.contigs-removed.fasta Illumina/GEW-BC11_S5_merged_R1.fq
[main] Real time: 81.399 sec; CPU: 1342.168 sec
```

```
$ /home/gaou/kumamushi/software_smith/samtools-1.9/samtools sort -T sort.tmp -o error_correction_1/aln.sorted.bam -@ 32 error_correction_1/aln.bam
```

```
[bam_sort_core] merging from 0 files and 16 in-memory blocks...
```

```
$ /home/gaou/kumamushi/software_smith/samtools-1.9/samtools index error_correction_1/aln.sorted.bam
```

次に pilon.

```
$ java -Xms8g -jar /home/gaou/kumamushi/software_smith/pilon-1.23.jar --genome BC11-2.contigs-removed.fasta --bam error_correction_1/aln.sorted.bam --threads 32 --output pilon1
```

```
Pilon version 1.23 Mon Nov 26 16:04:05 2018 -0500
Genome: BC11-2.contigs-removed.fasta
Fixing snps, indels, gaps, local
Input genome size: 2925260
Scanning BAMs
aln.sorted.bam: 32230986 reads, 0 filtered, 31390418 mapped, 0 proper, 0 stray, Unpaired 100% 75+/-4, max 86 unpaired
Processing tig00000002:1-67506
Processing tig00000001:1-2857734
tig00000002:1-67506 log:
unpaired aln.sorted.bam: coverage 638
```

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記入者 黄沐陽

Total Reads: 786779, Coverage: 638, minDepth: 64

Confirmed 67432 of 67506 bases (99.89%)

Corrected 1 snps; 0 ambiguous bases; corrected 51 small insertions totaling 64 bases, 2 small deletions totaling 2 bases

Attempting to fix local continuity breaks

fix break: tig00000002:22971-22991 22980 -7 +12 BreakFix

fix break: tig00000002:38000-38045 0 -0 +0 NoSolution

fix break: tig00000002:38606-38624 0 -0 +0 NoSolution

fix break: tig00000002:48397-48633 0 -0 +0 NoSolution

Finished processing tig00000002:1-67506

tig00000001:1-2857754 log:

unpaired aln.sorted.bam: coverage 588

Total Reads: 30603639, Coverage: 588, minDepth: 59

Confirmed 2856516 of 2857754 bases (99.96%)

Corrected 5 snps; 0 ambiguous bases; corrected 1156 small insertions totaling 1406 bases, 4 small deletions totaling 4 bases

Large collapsed region: tig00000001:2799301-2838323 size 39023

Attempting to fix local continuity breaks

fix break: tig00000001:14927-14931 14927 -30 +34 BreakFix

fix break: tig00000001:496246 496319 -11 +14 BreakFix

fix break: tig00000001:1287986-1288034 0 -0 +0 NoSolution

fix break: tig00000001:1310793-1310801 0 -0 +0 NoSolution

fix break: tig00000001:1361621 0 -0 +0 NoSolution

fix break: tig00000001:2188198-2188219 0 -0 +0 NoSolution

fix break: tig00000001:2254232-2254277 0 -0 +0 NoSolution

Finished processing tig00000001:1-2857754

Writing updated tig00000001_pilon to pilon1.fasta

Writing updated tig00000002_pilon to pilon1.fasta

Mean unpaired coverage: 589

Mean total coverage: 589

BUSCO スコアと CheckM を再度算出.

Job Title: 02nd-gVolante:

---

gVolante Analysis: ver.2.0.0

12/29/21

記入者 黄沐陽

確認者

日付 年 月 日

Summary of the submitted job:

JOB_ID: 202112290249-DJRHPP61D9TPCVW7

PROJECT_NAME: 02nd-gVolante

FASTA_FILE: pilon1.fasta

MIN_LENGTH_OF_SEQ_STATS: 1

ASSEMBLY_TYPE: genome

PROGRAM: BUSCO_v1

SELECTED_REFERENCE_GENE_SET: Bacteria

Result:

Total length (nt)	2926729
Longest sequence (nt)	2859161
Shortest sequence (nt)	67568

Core gene set: 39 (97.50%)

Base composition (%)	A: 31.07
	T: 31.48
	G: 18.71
	C: 18.74
	N: 0.00
	Other: 0.00
GC-content (%)	37.45

Job Title: 02nd-DFAST:

JobID: d7452594-564c-452a-988b-ab1333133964

https://dfast.ddbj.nig.ac.jp/dqc/d7452594-564c-452a-988b-ab1333133964

Result: Completeness: 99.66%; Contamination: 0.1%.

pilon 後に再度 CheckM を算出すると，研究ノート p. 61 の CheckM と比較して大幅に向上していることが確認できた.

ただし，1 回では不十分なことが多いので，ここで作成したエラーコレクション後のファ

12/29/21

記入者 黄沐陽

確認者

日付 年 月 日

イル（pilon1.fasta）に再度 Illumina リードをマッピングし，pilon をかけ直し，精度が向上する限りこれを繰り返した．

 # エラーコレクション２回目

```
$ mkdir error_correction_2
$ /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa index pilon1.fasta
[bwa_index] Pack FASTA... 0.05 sec
[bwa_index] Construct BWT for the packed sequence...
[bwa_index] 0.81 seconds elapse.
[bwa_index] Update BWT... 0.03 sec
[bwa_index] Pack forward-only FASTA... 0.02 sec
[bwa_index] Construct SA from BWT and Occ... 0.26 sec
[main] Version: 0.7.11-r1034
[main] CMD: /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa index pilon1.fasta
[main] Real time: 1.232 sec; CPU: 1.186 sec
$ /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa mem -t 32 pilon1.fasta
Illumina/GEW-BC11_S5_merged_R1.fq |
/home/gaou/kumamushi/software_smith/samtools-1.9/samtools view -@ 32 -b -o
error_correction_2/aln.bam -
[M::bwa_idx_load_from_disk] read 0 ALT contigs
[M::process] read 4267406 sequences (320000053 bp)...
[M::mem_process_seqs] Processed 4267406 reads in 185.212 CPU sec, 11.269 real sec
[M::process] read 4267886 sequences (320000013 bp)...
[M::mem_process_seqs] Processed 4267886 reads in 184.892 CPU sec, 9.736 real sec
[M::process] read 4267162 sequences (320000101 bp)...
[M::process] read 4267700 sequences (320000058 bp)...
[M::mem_process_seqs] Processed 4267162 reads in 142.942 CPU sec, 4.781 real sec
[M::mem_process_seqs] Processed 4267700 reads in 193.344 CPU sec, 10.418 real sec
[M::process] read 4267440 sequences (320000027 bp)...
[M::process] read 4267756 sequences (320000064 bp)...
[M::mem_process_seqs] Processed 4267440 reads in 140.470 CPU sec, 4.620 real sec
[M::mem_process_seqs] Processed 4267756 reads in 197.813 CPU sec, 10.372 real sec
[M::process] read 4267658 sequences (320000013 bp)...
[M::process] read 2254447 sequences (169011728 bp)...
```

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記入者 黄沐陽


```
[M::mem_process_seqs] Processed 4267658 reads in 143.899 CPU sec, 4.856 real sec
[M::mem_process_seqs] Processed 2254447 reads in 89.071 CPU sec, 4.079 real sec
[main] Version: 0.7.11-r1034
[main] CMD: /home/gaou/kumamushi/software_smith/bwa-0.7.11/bwa mem -t 32
pilon1.fasta Illumina/GEW-BC11_S5_merged_R1.fq
[main] Real time: 98.571 sec; CPU: 1327.346 sec
$ /home/gaou/kumamushi/software_smith/samtools-1.9/samtools sort -T sort.tmp -o
error_correction_2/aln.sorted.bam -@ 32 error_correction_2/aln.bam
[bam_sort_core] merging from 0 files and 32 in-memory blocks...
$ /home/gaou/kumamushi/software_smith/samtools-1.9/samtools index
error_correction_2/aln.sorted.bam

$ java -Xms8g -jar /home/gaou/kumamushi/software_smith/pilon-1.23.jar --genome
pilon1.fasta --bam error_correction_2/aln.sorted.bam --threads 32 --output pilon2
Pilon version 1.23 Mon Nov 26 16:04:05 2018 -0500
Genome: pilon1.fasta
Fixing snps, indels, gaps, local
Input genome size: 2926729
Scanning BAMs
error_correction_2/aln.sorted.bam: 32231856 reads, 0 filtered, 31393952 mapped, 0 proper,
0 stray, Unpaired 100% 75 1/-4, max 86 unpaired
Processing tig00000001_pilon:1-2859161
Processing tig00000002_pilon:1-67568
tig00000002_pilon:1-67568 log:
unpaired error_correction_2/aln.sorted.bam: coverage 639
Total Reads: 789524, Coverage: 639, minDepth: 64
Confirmed 67548 of 67568 bases (99.97%)
Corrected 0 snps; 0 ambiguous bases; corrected 0 small insertions totaling 0 bases, 0 small
deletions totaling 0 bases
# Attempting to fix local continuity breaks
# fix break: tig00000002_pilon:38040-38085 0 -0 1 0 NoSolution
Finished processing tig00000002_pilon:1-67568
tig00000001_pilon:1-2859161 log:
unpaired error_correction_2/aln.sorted.bam: coverage 588
Total Reads: 30604428, Coverage: 588, minDepth: 59
Confirmed 2859092 of 2859161 bases (100.00%)
```

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記入者 黄沐陽

Corrected 3 snps; 0 ambiguous bases; corrected 0 small insertions totaling 0 bases, 4 small deletions totaling 5 bases
Large collapsed region: tig00000001_pilon:2800691-2839722 size 39032
Attempting to fix local continuity breaks
fix break: tig00000001_pilon:496465-496552 496539 -14 +11 BreakFix
fix break: tig00000001_pilon:1288612-1288660 0 -0 +0 NoSolution
fix break: tig00000001_pilon:2255360-2255405 0 -0 +0 NoSolution
Finished processing tig00000001_pilon:1-2859161
Writing updated tig00000001_pilon_pilon to pilon2.fasta
Writing updated tig00000002_pilon_pilon to pilon2.fasta
Mean unpaired coverage: 589
Mean total coverage: 589

BUSCO スコアと CheckM を再度算出.

Job Title: 03rd-gVolante:

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gVolante Analysis: ver.2.0.0

Summary of the submitted job:
JOB_ID: 202112290312-266JUL572S77KK1U
PROJECT_NAME: 03rd-gVolante
FASTA_FILE: pilon2.fasta
MIN_LENGTH_OF_SEQ_STATS: 1
ASSEMBLY_TYPE: genome
PROGRAM: BUSCO_v1
SELECTED_REFERENCE_GENE_SET: Bacteria

Result:

Total length (nt)	2926723
Longest sequence (nt)	2859155
Shortest sequence (nt)	67568

Core gene set: 39 (97.50%)

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Job Title: 03rd-DFAST:
JobID: 36482103-b657-413c-ac7a-ca8196e83920
https://dfast.ddbj.nig.ac.jp/dqc/36482103-b657-413c-ac7a-ca8196e83920
Result: Completeness: 99.66%; Contamination: 0.1%.

結果より，2 回目のエラーコレクションで CheckM の Completeness と Contamination は変化しなくなっただけでなく，全長が 6 nt 短くなることが分かったので，1 回目のエラーコレクションをアノテーションに用いることにした.

 # アノテーション

アノテーションには，遺伝子予測，機能予測，ゲノム開始位置の dnaA への調整，等々非常に手間のかかる作業が沢山あるのだが，今は DDBJ DFAST というオンラインツールで，全自動でやってくれる.
https://dfast.ddbj.nig.ac.jp/dfc/
TIGR, COG, pseudo/framshifted gene のアノテーション結果は，アノテーションエディタに表示されない.
これらの結果を確認するには，GenBank-または GFF-形式のファイルを参照する.

Perform Taxonomy Check.
Perform Completeness Check:
Select a Taxonomic Group for CheckM. (Default: automatically inferred):
Rank: species.
Taxon: Enterococcus faecalis (20 genomes, 298 marker sets).
General Settings:
Locus tag prefix: LOCUS.
Minimum sequence length: 200
Genetic code: 11 (Standard bacterial/archaeal).
Check Rotate/flip the chromosome so that the dnaA gene comes first.
Offset from the start codon of the dnaA gene: 100 bp.
Structural Annotation:
CDS: MetaGeneAnnotator (default).
rRNA: Barrnap (default).
tRNA: tRNAscan-SE (Bacteria).
CRISPR: CRT (default)

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Thresholds for Default Database Search:

E-value: 1e-6 (default)

Identity (%): 0

Query coverage (%): 75

Subject coverage (%): 75

TIGRFAM and COG:

These options may take time and cause timeout error, when you enable them for a large genome.

Check Enable HMM scan against TIGRFAM

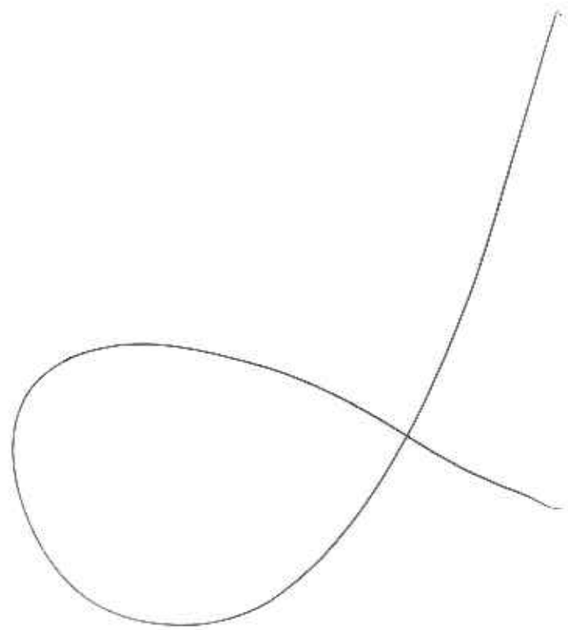
Check Enable RPSBLAST against COG

Job Title: Annotation-01:

JobID: 5bc3f036-9964-48ae-a6aa-fe88de07759c

https://dfast.ddbj.nig.ac.jp/analysis/annotation/5bc3f036-9964-48ae-a6aa-fe88de07759c

Download files では全てをまとめた anotation.zip と pseudogene_summary.tsv をダウンロードした.



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確認者

日付 年 月 日