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We are still developing and optimizing this protocol

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Bacterial Genomic DNA Isolation

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ABSTRACT

Overview and Goals
Your bacterial isolate has been grown on agar plates. We now need to lyse open the bacteria and isolate only the genomic DNA for sequencing. For this, we need to gently lyse the bacteria with the enzyme lysozyme and then purify the genomic DNA. We will use the Monarch High Molecular Weight (HMW) DNA Extraction Kit (New England BioLabs) and protocol because we want long DNA strands to sequence with the Nanopore sequencer.

After completing this lab you will gain the following lab skills:

- Lab safety and proper personal protective equipment (PPE)
- Proper use of a centrifuge and pipettors
- Isolation and purification of high-molecular weight genomic DNA

GUIDELINES

Review the complete protocol before beginning. Several of the steps in this procedure are time and/or temperature sensitive, so it’s important that you know what to expect and how to manage your time.

MATERIALS

Bacterial Lysis:

- RNase A, 10 ul per sample (provided in kit), and Proteinase K (50 mg/ml), 20 ul per sample
- Stored on ice for the duration of the procedure.
- When in use, keep the vials in or near the surface of the ice as much as possible. Both substances are very temperature sensitive, and warming them will negatively affect your results.
- Cold PBS or 10 mM Tris buffer, 300 µl per sample
- HMW gDNA Tissue Lysis Buffer, 300 ul per sample
- Lysozyme solution, 10 ul per sample
- Protein Separation Solution, 300 ul per sample

HMW gDNA Binding and Elution:

- DNA Capture Beads
- Isopropanol, 550 µl per sample
- gDNA Wash Buffer, 1000 ul per sample
- Add ethanol (≥ 95%) as indicated on the bottle label. (This has most likely already been done for you. Ask your lab instructor if you are unsure.)
- gDNA Elution Buffer II, 100 ul per sample
- Bead Retainer, 1 per sample
- Collection Tube II, 1 per sample
- 1.5 ml DNase-free, low DNA binding microfuge tubes, 1 per sample
- 2 ml centrifuge tubes (2 per sample)
- Clean forceps (DNA Away and ethanol treated

Step 14 onwards:

- Monarch Collection Tube II (not labeled)
- 1 Monarch Bead Retainer inserted into the collection tube; this will be used to remove the wash buffer from the gDNA bound to the beads. (labeled)
- 2 Monarch 2 mL Tubes; one for phase separation and one for elution. (labeled)
- 1 1.5 mL microfuge tube; this will be used to collect the eluate. (labeled)

BEFORE START INSTRUCTIONS

Review the figures below to learn about how the New England BioLabs Monarch High Molecular Weight (HMW) DNA Extraction kit works.

Activity 1: Bacterial Lysis

1 Preheat two Eppendorf Thermal mixers with 1.5 mL blocks to 37 °C and 55 °C

2 Use a sterile cotton swab to obtain bacterial colonies and resuspend in 300 µL of cold PBS or Tris buffer.

3 Centrifuge each microtube of the sample at the maximum speed setting (> 12000 x g) for 00:01:00 1m

3.1 Balance the centrifuge with the placement of microtubes. Use empty tubes if necessary.

3.2 After centrifuging, there should be a “pellet” of bacteria at the bottom of the tube.

3.3 Depending on the concentration of bacteria in the solution, this may take a few cycles of centrifuging, removing supernatant, and adding more sample to get a sufficient pellet.

4 Discard the supernatant. There are two methods for this depending on the strength of the pellet.

4.1 If the pellet is tightly packed (small clump at the bottom, little movement when swirled), you can simply dump the supernatant into the biohazard bin. The pellet will remain at the bottom. This method is commonly called "decanting the supernatant."

4.2 If the pellet is not packed together at the bottom, first re-centrifuge up to two additional times. If it remains loose, remove the supernatant with a micropipette. This method is commonly called "aspirating the supernatant."

Note

Start removing supernatant from the top of the meniscus and lower the tip as the meniscus lowers so as to not remove any of the pellet. A small amount of remaining supernatant is acceptable.

5 Resuspend the bacterial pellet in 300 µL of cold PBS or Tris buffer. Add 10 µL Lysozyme (50 mg/ml) solution and mix by vortexing for 1-2 seconds 2s

Note

If the pellet remains at the bottom after vortexing, try flicking the tube. The cells will be lysed shortly, so you don't have to be too gentle. Use a micropipette to pull the pellet off the bottom if it continues to stick to the bottom of the tube.

6 Add 300 µL HMW gDNA Tissue Lysis Buffer to the sample and mix by inverting 5-10 times.

7  Incubate at 37 °C in a thermal mixer with agitation at 1400 rpm for 00:15:00, or until nearly clear (up to 00:20:00). 15m

Note

The samples may not become fully clear - that's ok. If samples haven't cleared up at all after incubation, ask the instructor for assistance.

8 Remove the tubes after finishing incubation, and set the thermal mixer to 56 °C (or use one already set to this temperature).

9 Add 20 µl of Proteinase K and mix by inverting 10–20 times.

Note

Keep the Proteinase K in or near the surface of the ice as much as possible; it is very temperature sensitive.

9.1 

The Proteinase K is thick and will stick to the outside of the pipette tip. When pipetting, just barely break the surface so as to not end up with excess on the outside of the pipette tip. Do the same when depositing into the sample tube.

10 

Incubate at 56 °C for 00:30:00 in a thermal mixer at 1400 rpm 30m

11 

Add 10 µL of RNase A and mix by inverting 5–10 times. Incubate for 00:10:00 at 56 °C with agitation in a thermal mixer at 1400 rpm 10m

12 Add 300 µL of Protein Separation Solution. Mix by inverting gently for 00:01:00 1m

13 

Centrifuge for 00:10:00 at maximum rpm. 10m

Note

The sample will separate into a large, clear upper phase (DNA) and a lower, clear phase (protein, usually on the bottom of the tube, but occasionally floating). There may also be a white precipitate at the bottom of the tube.

Safety information

It may take longer than 10 minutes for complete phase separation to occur. Centrifuge for no more than a total of 25 minutes.

14 If working with multiple samples, prepare and label the plastics for the upcoming steps. Each

sample will require:
-Monarch Collection Tube II (not labeled)

1. 1 Monarch Bead Retainer inserted into the collection tube; this will be used to remove the wash buffer from the gDNA bound to the beads. (labeled)
2. 2 Monarch 2 mL Tubes; one for phase separation and one for elution. (labeled)
3. 1 1.5 mL microfuge tube; this will be used to collect the eluate. (labeled)

15 Using a 1000 µl wide-bore pipette tip, transfer the clear upper phase (containing the DNA) of each sample to a corresponding labeled Monarch 2 ml Tube.

> **Note**
>
> Typically, the transferred volume is ~800 ul. If less than 700 ul, ask your instructor about adjusting the volumes of solutions added in the next steps.
>
> 1. It's important to remove as much of the upper phase as possible without removing the proteins collected at the bottom of the tube. If you're having trouble pulling only the upper layer, try using a 200 ul pipette. A small amount (1-2 ul) of protein will not greatly affect your results.
> 2. If there isn't a visible protein layer at the bottom of the tube, leave ~30 ul solution in the tube to be sure very little protein is transferred

Activity 2: HMW gDNA Binding and Elution

30s

16

> **Note**
>
> In all of the following steps, it is crucial not to let the gDNA dry out between steps and between buffers. Move quickly, keep caps closed, and take note of times where you'll need to have new solutions prepared before removing old ones.

Using clean forceps, add 2 DNA Capture Beads to each sample, which are now in Monarch 2 ml Tubes.

17 Add 550 µL isopropanol to each sample, close the cap, and invert slowly and gently 25–30 times.

Note

Each inversion should take 5–6 seconds. Slow and consistent inversions are critical for the DNA to bind to the beads. The beads may become stuck to the bottom of the tube as you go; lightly flick the tube to release them and continue.

After 2-3 inversions, the solution should start to become thicker and the DNA will start wrapping around the beads. As the beads bind to the beads, the solution will gradually thin out, and by the end of 30 inversions, it should no longer be thick.

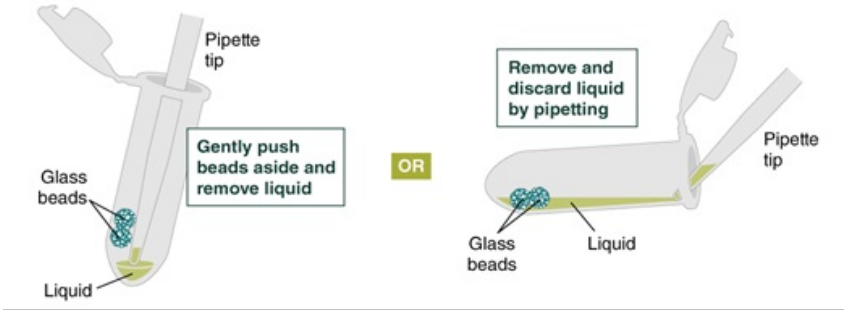
Invert as many times as is necessary to bind the DNA to the beads. It could take up to 50, but after that, not much will get done.

18 Remove and discard the excess liquid by pipetting.

Safety information

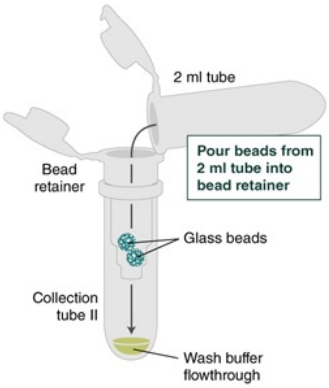
The DNA can dry out very quickly, so it's important to add the next buffer immediately after pipetting out the isopropanol.

18.1 To remove the liquid, you can use either of the methods pictured below. In the past, the right method has yielded more liquid and interfered less with the beads.



Images from NEB Monarch protocol.

19 Add 500 µL gDNA Wash Buffer, close the cap, and mix by inverting the tube 2–3 times. Then remove the gDNA Wash Buffer using the same method as the previous step.



Note

The gDNA complex should condense more tightly around the beads.

20 Repeat Step 4 and remove the gDNA Wash Buffer.

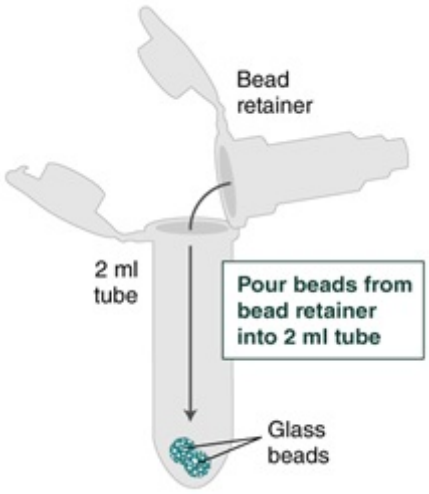
21 Place a labeled bead retainer into each Monarch Collection Tube II. Pour the beads from the 2 ml tube into the bead retainer and close the cap. You can discard the 2 ml tube.

Safety information

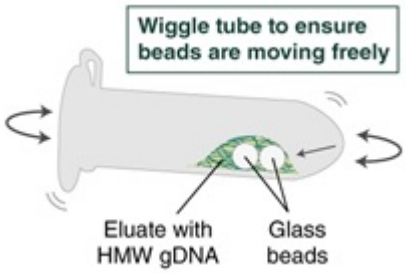
To prevent the gDNA from drying out, keep the cap closed after transferring.

22 Use a mini centrifuge to remove any residual wash buffer from the beads. Only spin out for a moment (≤1 second) so as not to dislodge the gDNA from the beads.

23 Pull out the bead retainer from the first collection tube, pour the beads into the other labeled 2 ml tube, and insert the used bead retainer into the labeled 1.5 ml microfuge tube for later use during elution. Discard the used collection tube.

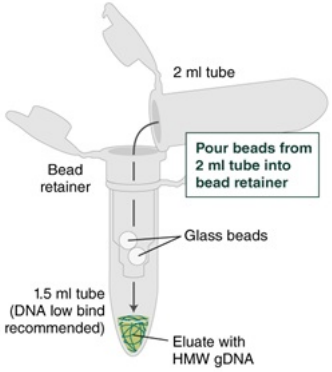


24 Immediately add 100 µL Elution Buffer II onto the glass beads and incubate for 00:10:00 at 56 °C in a thermal mixer with agitation at 300 rpm .



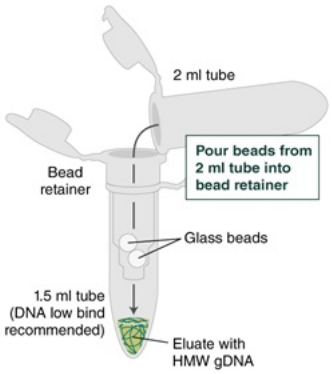
24.1

1. At 3 minutes, check to make sure the beads are not stuck to the bottom of the tube by tilting the tube almost horizontally and gently shaking. If they become stuck for too long, the DNA will not release properly from the beads.



24.2 Repeat the above process at 7 minutes.

25 After incubating, pour the eluate and beads into the bead retainer, which should already be inserted into a 1.5 ml microfuge tube.



> **Note**
>
> 1. Remember to close the cap after transfer.
> 2. Some of the eluate may stick to the walls of the 2 ml tube when you transfer. If so, spin down for 1 second in the centrifuge, and pour again. Pipette carefully if necessary.

26 Centrifuge each sample for 00:00:30 at 12000 x g to separate the eluate from the glass beads. After separating, discard the beads and retainer. 30s

27 Pipette the eluate up and down 5–10 times with a wide bore pipette tip and ensure any visible DNA aggregates are dispersed.

27.1 After pipetting, incubate at 37 °C for 30-60 minutes, Overnight at room temperature, or for > 24:00:00 at 4 °C. Pipette up and down 5-10 times again before analyzing or using the HMW DNA.

Note

Critical Thinking Questions for Bacterial Genomic DNA Isolation

1. What lab skills have you gained so far in this course that you can add to your resume? (HINT: These are listed in the Overview and Goals section of every lab protocol.)
2. Read Activity 1. Why do we resuspend the pellet of bacteria before we add Lysozyme to lyse open the bacteria?
3. Read Activity 1. Why do you think we keep Proteinase K on ice before we use it and then incubate it with our sample at 56°C?
4. In step 3 of Activity 2, you discard the excess liquid by pipetting. This is also called ... (HINT: read Activity 1).
5. What future protocols inform us to isolate high molecular weight (HMW) DNA? Compare the advantage of obtaining HMW DNA compared to short fragments.