ONT Ligation Sequencing Kit LSK-109

Merideth Freiheit

Merideth Freiheit

COMMENTS 0

WORKS FOR ME 1

PROTOCOL INFO

Merideth Freiheit . ONT Ligation Sequencing Kit LSK-109. **protocols.io**
https://protocols.io/view/ont-ligation-sequencing-kit-lsk-109-chn2t5ge

CREATED

Oct 10, 2022

LAST MODIFIED

Nov 28, 2022

PROTOCOL INTEGER ID

71098

GUIDELINES

Tips for Successful Lab Research

Keep these in mind throughout your research to help prevent mistakes and ensure your work goes smoothly.

- Keep your workstation clean and be careful to not contaminate your samples and solutions
- Carefully and clearly label **all** your tubes
- Create a naming system for tubes and samples in your group to prevent confusion
- Always double check that you have the correct pipette with the correct amount BEFORE drawing up all solutions
- In fact, always double check that you are drawing up the correct solution - using the wrong solution can ruin the whole experiment!
- Use your non-dominant hand, such as a pinky above the tip, to help balance your pipette (ask a demonstrator if unsure about this technique)
- As you progress through a protocol, check off completed steps
- Take thorough lab notes throughout the lab work, including notes on naming systems, issues encountered, and all results - even take photos if you need to!
- Always ask questions if unsure of anything!

MATERIALS TEXT

Stock solutions

1 M Tris HCl (pH 8):
Add 15.7 g of Tris HCl to bottle. Add 75 mL ddH2O. Add 5 mL ***5 M NaOH*** to adjust to pH 8. Bring to 100 mL final volume with ddH2O. Place on HulaMixer until all solids dissolve.

0.5 M EDTA (pH 8):

Add 18.6 g of disodium EDTA dihydrate to bottle. Add 75 mL ddH2O and 10.2 mL ***5 M NaOH*** to adjust to pH 8. Bring to 100 mL final volume with ddH2O. Place on HulaMixer until all solids dissolve.

5 M NaCl:
Dissolve 29.2 g sodium chloride in 75 mL ddH2O. Bring to 100 mL final volume with ddH20.

50% PEG 8000:
To 50 g polyethylene glycol 8000 add ddH20 to a final volume of 100 mL. Place on HulaMixer until all solids dissolve.

10% Tween 20:
To 900 µL ddH2O add 100 µL Tween 20. Invert repeatedly to mix.

Note

With the exception of 10% Tween 20 all stock solutions can be UV sterilised. Stock solutions can be stored at room temperature. Store 10% Tween 20 in the dark.

Working solutions

Oxford Nanopore Technologies (ONT) Ligation Sequencing Kit (LSK-109):

ONT DNA CS (DCS)

ONT Adapter Mix (***AMX***)

ONT Ligation Buffer (***LNB***)

ONT Elution Buffer (***EB***)

ONT Sequencing Buffer (***SQB***)

ONT Loading Beads (***LB***)

ONT Flow Cell Priming Kit:

ONT Flush Tether (***FLT***)

ONT Flush Buffer (***FB***)

NEBNext Companion Module for ONT Ligation Sequencing:

NEBNext FFPE DNA Repair Mix

NEBNext FFPE DNA Repair Buffer

NEBNext Ultra II End-prep Reaction Buffer

NEBNext Ultra II End-prep Enzyme Mix

NEBNext Quick T4 DNA Ligase

Wash Solution:
To 80 mL 100% ethanol add 20 mL ddH2O. Invert to mix.

Library Prep Bead Solution (for final 10 mL vol):
Mix 100 µL ***1 M Tris HCl (pH 8)***, 20 µL ***0.5 M EDTA (pH 8)*** and 3.2 mL ***5 M NaCl***. Add 4 mL ***50% PEG 8000***. Add 2.43 mL ddH20. Invert to mix thoroughly.

Note

Working solutions should be stored on ice, except for ***Library Prep Bead Solution*** which can be stored at room temperature.

Bead suspension

It is simplest to take aliquots of beads from the Sera Mag SpeedBead bottle at adequate amounts for use, e.g. 200 µL in the case of 10 mL ***Library Prep Bead Solution***. Allow Sera-Mag SpeedBeads bottle to reach room temperature. Vortex the bottle until the beads are completely resuspended - this may take some time but it is essential they are fully resuspended (i.e. they are not clumped on the base of the bottle). Immediately after resuspension transfer the desired volume of Sera-Mag SpeedBeads to a 1.5 ml tube. Store aliquots in the fridge ready for preparation.

Bead suspension preparation for 10 mL ***Library Prep Bead Solution***:

1. Allow Sera-Mag SpeedBeads aliquot to reach room temperature.
2. Add 200 µL ddH2O. Vortex thoroughly to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
3. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately 00:02:00 but can take longer.
4. While on the stand carefully remove and discard supernatant without disturbing beads.
5. Add 500 µL ddH2O. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.
6. Place on magnetic stand until supernatant is completely clear and beads are bound towards magnet. This should take approximately 00:10:00 but can take longer.
7. While on the stand carefully remove and discard supernatant without disturbing beads.
8. Repeat steps 5 to 7 three more times.
9. Add 200 µL ***Elution Buffer*** to match the starting volume of aliquot. Vortex tube to resuspend beads. Centrifuge briefly to remove droplets from tube lid.

Add 50 µL of ***10% Tween 20*** to ***Library Prep Bead Solution*** (10 mL) then add the prepared 200 µL aliquot of ***Bead suspension.*** Place on HulaMixer until mixed thoroughly.

Note

Once ***10% Tween 20*** and Bead Suspension are added to ***Library Prep Bead Solution*** it should be refrigerated, avoid prolonged exposure to light.

BEFORE STARTING

Thaw ***ONT DNA CS (DCS)*** at room temperature, spin down, mix by pipetting, and place on ice.

Prepare the ***NEBNext FFPE DNA Repair Mix*** and ***NEBNext Ultra II End repair / dA-tailing Module*** reagents in accordance with manufacturer's instructions, and place on ice. NEB recommend the following:

1. Thaw all reagents on ice.
2. Flick and/or invert reagent tube to ensure they are well mixed.
3. Always spin down tubes before opening for the first time each day. The ***NEBNext Ultra II End-prep Reaction Buffer*** and ***NEBNext FFPE DNA Repair Buffer*** may have a little precipitate. Allow the mixture to come to room temperature and pipette the buffer up and down several times to break up the precipitate, followed by vortexing the tube for several seconds to ensure the reagent is thoroughly mixed.
4. The ***NEBNext FFPE DNA Repair Buffer*** may have a yellow tinge and is fine to use if yellow.

Spin down the ***ONT Adapter Mix*** (***AMX***) and ***NEBNext Quick T4 DNA Ligase***, and place on ice.

Thaw ***ONT Ligation Buffer*** (***LNB***) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.

Thaw the ***ONT Elution Buffer*** (***EB***) at room temperature, mix by vortexing, spin down and place on ice.

For flow cell loading:
Thaw the ***ONT Sequencing Buffer*** (***SQB***), ***ONT Loading Beads*** (***LB***), ***ONT Flush Tether*** (***FLT***) and one tube of ***ONT Flush Buffer*** (***FB***) at room temperature before mixing the reagents by vortexing, and spin down at room temperature.

Perform hardware check on MinION device then retrieve a MinION flow cell, allowing it to reach room temperature. Once it has reached room temperature, place the flow cell in the device and perform a flow cell check (minimum number of pores is 800).

DNA Repair and End-prep

1 Prepare DNA in molecular grade water

1.1 Transfer 1 µg of extracted DNA to a 1.5 ml LoBind tube and adjust volume to 47 µL with molecular grade water

Note

Example calculation for determining amounts of extracted DNA and molecular grade water to add:

Average amount of DNA was determined to be 38.1 ng/µL and a total of 1000 ng (or 1 µg) is required.

1000 ng / 38.1 ng/µL = 26.2 µL of extracted DNA

47 µL - 26.2 µL = 20.8 µL of molecular grade water

1.2 Mix thoroughly by flicking tube

1.3 Centrifuge tube for 00:00:01

1s

2 Add 1 µL of ***ONT DCS*** to a PCR strip tube

3 Add 47 µL of prepared DNA in molecular grade water and pipette mix 10-20 times

4 Add 3.5 µL of ***NEBNext FFPE DNA Repair Buffer*** and pipette mix 10-20 times

5 Add 2 µL of ***NEBNext FFPE DNA Repair Mix*** and pipette mix 10-20 times

6 Add 3.5 µL ***NEBNext Ultra II End-prep Reaction Buffer*** and pipette mix 10-20 times

7 Add 3 µL ***NEBNext Ultra II End-prep Enzyme Mix*** and pipette mix 10-20 times

8 Centrifuge for 00:00:05

5s

9 Use a Thermal Cycler to incubate at 20 °C for 00:05:00 then at 65 °C for 00:05:00 10m

Note

Take ***Library Prep Bead Solution*** from the fridge and allow it to reach room temperature while in the Thermal Cycler

10 Vortex ***Library Prep Bead Solution***

11 Transfer 60 µL of the DNA sample to a 1.5 ml LoBind tube

12 Add 60 µL ***Library Prep Bead Solution*** and gently flick to resuspend beads (ensure all beads are resuspended with no clumps)

13 Place on HulaMixer (continual rotation, 30 01 orbital rpm, reciprocal and vibro off) for 00:05:00 5m

14 Centrifuge for 00:00:05 5s

15 Place on magnetic stand until supernatant is clear and beads are bound towards magnet

16 While on the stand carefully remove and discard supernatant without disturbing beads

17 Add 200 µL ***Wash Solution***

17.1 Incubate at room temperature for 00:00:30 30s

18 While on the stand carefully remove and discard supernatant without disturbing beads

19 Repeat steps 17 to 18 a further time

20 Centrifuge for 00:00:05 and place back on magnetic stand ensuring beads are bound towards magnet 5s

20.1 Remove all remaining ***Wash Solution*** with a 10 µL pipette

20.2 Air dry tube with cap open until beads are completely dry (i.e. no longer shiny), but not long enough to allow the pellet to crack

21 Remove from the magnetic rack and add 62 µL molecular grade water and gently flick to resuspend beads (ensure all beads are resuspended with no clumps)

22 Centrifuge for 00:00:05 5s

23 Incubate 00:02:00 Room temperature 2m

24 Place on magnetic stand until supernatant is clear and beads are bound towards magnet

25 Carefully transfer eluate to a fresh 1.5 ml LoBind tube without disturbing beads

Adapter Ligation and Clean-up

26 Add 25 µL ***ONT LNB*** to the DNA sample and pipette mix 10-20 times

27 Add 10 µL ***NEBNext Quick T4 DNA Ligase*** and pipette mix 10-20 times

28 Add 5 µL ***ONT AMX*** and pipette mix 10-20 times

29 Centrifuge for 00:00:05 5s

30 Incubate for 00:20:00 Room temperature 20m

31 Add 40 µL ***Library Prep Bead Solution*** and and gently flick to resuspend beads (ensure all beads are resuspended with no clumps)

32 Place on HulaMixer (continual rotation, 30 01 orbital rpm, reciprocal and vibro off) for 00:05:00 5m

33 Centrifuge for 00:00:05 5s

34 Place on magnetic stand until supernatant is clear and beads are bound towards magnet

35 While on the stand carefully remove and discard supernatant without disturbing beads

36 Add 200 µL ***Wash Solution***

36.1 Incubate at room temperature for 00:00:30 30s

37 While on the stand carefully remove and discard supernatant without disturbing beads

38 Repeat steps 36 to 37 a further time

39 Centrifuge for 00:00:05 and place back on magnetic stand ensuring beads are bound towards magnet 5s

39.1 Remove all remaining ***Wash Solution*** with a 10 µL pipette

39.2 Air dry tube with cap open until beads are completely dry (i.e. no longer shiny), but not long enough to allow the pellet to crack

40 Add 15 µL ***ONT EB*** and gently flick to resuspend beads (ensure all beads are resuspended with no clumps)

41 Centrifuge tube for 00:00:05 5s

10m

42 Place in Thermomixer 550 rpm, 37°C, 00:10:00

43 Place on magnetic stand until supernatant is clear and beads are bound towards magnet

44 Carefully transfer eluate to a fresh 1.5 ml LoBind tube without disturbing beads

Note

Quantify 2 µL of eluted sample using a Qubit fluorometer and store DNA sample in 4 °C fridge overnight

Priming and Loading Flow Cell

45 Ensure there is the appropriate amount of DNA in the final library based on previous Qubit results - ONT requires 5-50 fmol in 12 µL for sequencing

Note

The next steps will all be performed by a demonstrator for multiple groups.

46 Prepare the ***Priming Mix*** by adding 30 µL of ***ONT FLT*** directly to the tube of ***ONT FB***

46.1 Vortex ***Priming Mix***

47 Slide the priming port cover clockwise to open the priming port on the MinION flow cell

48 Set a P1000 pipette to 200 µL and insert the tip into the priming port

49 Turn the wheel until the dial shows 230 µL, to draw back 30 µL, or until you can see a small volume of buffer entering the pipette tip

> Note
>
> Visually check that there is continuous buffer from the priming port across the sensor array and do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times

50 Load 800 µL of the ***Priming Mix*** into the flow cell via the priming port, avoiding the introduction of air bubbles

51 Thoroughly mix the contents of the ***ONT LB*** by pipetting

> Note
>
> These beads settle very quickly. It is vital that they are mixed immediately before use.

52 To your 12 µL of final library, add 37.5 µL of ***ONT SQB***

53 Add 25.5 µL of ***ONT LB***

54 Gently lift the SpotON sample port cover on the MinION flow cell to make the SpotON sample port accessible

55 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

56 Gently mix the final library by pipetting

57 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)

57.1 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port

58 Close the priming port and replace the MinION device lid