PlasmidSeq with Recombinant Tn5

Introduction

Approach for nanopore sequencing of plasmids using recombinant Tn5

Materials

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- Nanopore LSK-110 kit
- NEB Quick Ligase
- Recombinant Tn5 (at 40ng/ul in 50% glycerol)
- 10x EzTn5 buffer
- 1M DTT
- NEB End Repair kit
- NEB FFPE kit
- Ampure beads
- 70% ethanol
- Nanopore Flongle
- Annealed oligo barcodes at 2.5uM

Procedure

Tagmentation reaction

This step differs depending on how many samples are being used. In general you want 2-5ug of plasmid (total) so if you are sequencing 20 plasmids you want to put more plasmid and Tn5 per sample than if you were doing 96 plasmids

1. Set up the tagmentation reaction in the following order:

   1 - Plasmid
   2 - Barcode
   3 - Master mix (Tn5, EzTn5 buffer, DTT, water)

Table1

	A	B	C	D
1		<48 plasmids	>48 plasmids	
2	In each well			
3	Plasmid (@40ng/ul)	2.5ul	1.25ul	
4	Barcode (@2.5uM)	2ul	1ul	
5	Master mix, multiply vol by # of samples + 1			
6	Tn5 (@40ng/ul)	1ul	0.5ul	
7	10x EzTn5 Buffer	1ul	0.5ul	
8	1M DTT	0.01ul	0.005ul	
9	H2O	3.5ul	1.75ul	
10	Vol of master mix per well	5.5ul	2.75ul	
11				
12	Final vol	10ul	5ul	

2. Tagmentation reaction in a PCR machine (skirted plates dont fit in our normal PCR machines, you can only use the RT if you are using a plate)

   10min at 23C (this is to let Tn5 load) - Do this incubation on a plate shaker instead of PCR
   10min at 37C (this is to let Tn5 tagment)
   5min at 55C (this is unclear, protocols for library preps skip the 37C step and go straight to 55C, but doing 55C only doesnt work as well)

3. Stop the reaction by adding 1 volume of 0.2% SDS. Mix well and incubate at RT for >5 mins

4. Combine all samples, and 0.5x ampure beads. Follow the normal ampure bead clean up. Be careful not to let the beads dry because the longer fragments of DNA will never elute.

5. Elute in 25ul of H2O

LSK-110 library prep

This is basically the standard nanopore protocol

6. FFPE + End-repair

Table2

	A	B
1	Tagmented plasmids	24ul
2	NEBNext FFPE DNA Repair Buffer	1.75ul
3	NEBNext FFPE DNA Repair Enzyme	1ul
4	NEBNext End-Repair Buffer	1.75ul
5	NEBNext End-Repair Enzyme	1.5ul
6	Total vol	30ul

7. Mix well and run the following PCR protocol

    7.5min at 20C
    7.5min at 65C

8. Add 30ul of H2O, 30ul of ampure beads (0.5x), follow a standard ampure clean up protocol

9. Elute in 30ul H2O

10. LSK-110 ligation, set this reaction up on ice, adding the reagents in the following order quickly, and then immediately mix well

Table3

	A	B
1	Repairs plasmids	30ul
2	Ligation buffer (LNB)	12.5ul
3	Quick Ligase	5ul
4	Adapter Mix (AMX)	2.5ul
5	Total	50ul

11. Allow the reaction to proceed at RT for 10min (this is a good time to do a flow cell check, see "Loading the flongle")

12. Add 50ul of H2O and 50ul of ampure beads (0.5x)

13. **Wash 2x with 125ul ONT Long Fragment Wash Buffer**

14. Elute in 6-8ul of ONT Elution buffer

Loading the flongle

15. Thaw the FLT buffer from the LSK-110 kit and Flush buffer, Sequencing buffer, and Loading beads from a flongle-expansion kit

16. Take a flongle out of the 4C

17. Put it into the minion + flongle adaptor and run a flow cell check

18. Gently peel back the sticker that is sealing the sample loading port (this is pulled back parallel to the flongle, away from the flongle ID)

19. Dab away any remaining flongle storage solution (yellow stuff) from the sticker (but not from the port because you dont want to capillary action it away from the flow cell)

20. Prepare flush buffer (117ul of flush buffer + 3ul of flush tether)

21. Using a P200 pipet (yellow tips work better than filter tips since they are sturdier) place the tip in the sample port and increase the volume of the pipet using the knob to gently remove 2-3ul from the flow cell (This should remove any bubbles, I have never found any in a flongle)

22. Using a P200 set at 119ul add the flush buffer by turning the knob, slow and steady is best, to the sample port without introducing bubbles

23. Set up the sequencing reaction, NB: shake the loadng beads well immediatly prior to loading because they settle very quickly

Table4

	A	B
1	Sequencing buffer	15ul
2	Loading beads	10ul
3	Plasmid library	5ul
4	Total vol	30ul

24. Load the library and sequence for 24-36 hours by turning the knob, adding it slowly