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Genomic Fragment Library Generation (Coaux-Seq)

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We use this protocol and it's working

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Abstract

Protocols for creation of genomic fragment libraries for complementation assay Coaux-Seq.

Strain growth and preparation

20m

1 Streak out glycerol stock of desired strain on appropriate agar plate (typically LB or R2A, 1.5% agar). Grow at appropriate temperature (often 30°C or 37°C) and time (usually overnight) to obtain colonies. 10m

2 If colonies have formed, pick a colony and inoculate the strain into an appropriate liquid medium and scale volume of inoculation for necessary optical density (OD_{600}) equivalent to satisfy following genome extraction (typically 15-50 mL should suffice). Grow strains overnight at appropriate temperature conditions. 150 rpm, 30-37°C 10m

3 Measure OD_{600} the next morning to perform cell count calculation/approximation. 10m

NOTE: When working with non-model microbes (such as those isolated from the environment), their OD_{600} to cfu correlation is not inherently equal to model systems like *Escherichia coli*. Therefore, it can be valuable to separately run a dilution curve of OD_{600} and plate to perform cell counts to determine this relationship.

4 If the desired cell count has been achieved, pellet an appropriate volume of cell for subsequent steps. Centrifugation conditions - 4000 rpm, 4°C, 00:05:00 5m

Genome extraction

5 Use preferred Genomic DNA extraction kit, following the appropriate manufacturer instructions for gram-negative or gram-positive bacteria. Here, we used Thermo Scientific GeneJET Genomic DNA Extraction Kit (Thermo Scientific, K0721). 3h

NOTE: For subsequent steps, it is preferred to elute into nuclease free water at the end of the manufacturer protocol instead of the kit provided elution buffer.

6 Measure DNA concentration (Nanodrop, Qubit). 5m

NOTE: If your DNA concentration is low, you may need to run multiple extractions, pool, and concentrate to achieve the required 2 µg of genomic DNA in a <200 µL volume of nuclease free water for subsequent steps. This is why it is better to elute in nuclease free water than elution buffer, so as not to concentrate the salts when evaporating. Alternatively, one can do an additional DNA clean up step and load multiple clean up reactions onto a single column and elute into a smaller volume of nuclease free water. If

this option is chosen, be sure the column and protocol is suitable for high molecular weight DNA.

Genome Shearing

32m

7 Take 2-20 µg (2 µg is frequently used) of genomic DNA and combine with nuclease free water to a volume of 200 µL in a Covaris Blue miniTube (3 kb). 2m

8 Turn on Covaris 220 and chiller (add water to sonication components). Ensure that machine gets to appropriate temperature and all air is cleared from system (all system checks are passed). 15m

9 Add Blue miniTube containing genomic DNA to Covaris 220 using miniTube loading apparatus. Run 3 kb sonication protocol. (Covaris settings - SonoLab 7.2 software, temperature 4-25°C, Peak Power 3.0, Duty Factor 20.0, Cycles/Burst 1000, 600 seconds) 15m

NOTE: Make sure there are no air bubbles under or around the tube before starting the protocol!

10 Once sonication protocol is complete, remove Blue miniTube containing sheared genomic DNA (turn off machine, drain water thoroughly from sonication components) and utilize sheared genomic DNA for subsequent steps.

Genome fragment recovery and preparation for cloning

1h 5m

11 Add appropriate volume of DNA gel loading dye to Blue miniTube. 5m

NOTE: Most loading dyes, including the one provided with the GeneRuler 1 kb+ kit (Thermo Scientific, SM1333/4), are 6x and would suggest adding 40 µL of dye to the sheared DNA. However, using less loading dye (~20 µL) will be sufficient and prevent dilution of fragmented genomic DNA.

12 Cast a 1% agarose gel (in 1x TAE buffer) with appropriate amount of SybrSafe (Invitrogen, S33102) or equivalent DNA imaging reagent. Here, we use 50x TAE buffer and dilute (Omega, AC10089) and UltraPure Agarose (Invitrogen, 16500-500). 20m

13 Once gel is set, load ladder (Generuler 1 kb+) and genomic fragment material with loading dye added. 10m

14 Run gel at 100 v, 400 mA, for 25-30 minutes. 30m

15 Image gel with appropriate gel imaging equipment (e.g., Bio-RAD Gel Doc XR+). One should observe a smear of DNA with the strongest brightness centered at 3 kb. 5m

16 Cut desired segment of DNA from gel (recommend approximately 1-5 kb section). 10m

17 Store cut bands in 1.5 to 2 mL Eppendorf tubes. Use gel slices for following step. 1m

18 Use GeneJET Gel Extraction Kit (Thermo Scientific, K0691) or preferred gel extraction kit to extract genomic fragment DNA. 35m

NOTE: When in doubt, use greater volumes of binding buffer to dissolve gel. In addition, add binding buffer to column and do an extra spin before loading genomic fragment DNA (helps retention as column is better equilibrated). Perform an extra loading buffer wash before ethanol-based wash steps. Elute into desired volume of nuclease free water (30-50 µL). Measure DNA concentration (NanoDrop or Qubit).

19 Take gel extracted DNA and repairs ends and phosphorylate using Fast DNA End Repair Kit following manufacturer protocols (Thermo Fisher Scientific, FERK0771).

20 As a precaution, although the end repair kit should phosphorylate the ends of your fragmented genomic DNA, run one additional phosphorylation step, using manufacturer instructions with T4 Polynucleotide Kinase (PNK) kit (New England Biolabs, M0201S).

21 After phosphorylation, run a PCR clean up using GeneJET PCR Purification Kit (Thermo Scientific, K0701) or preferred alternative to prepare for subsequent ligation step. Measure DNA concentration (NanoDrop). **The genomic fragment library is now prepared for cloning into expression vector.**

Preparation of vector backbone for cloning 1h 25m

22 To generate the vector backbone for library cloning, use plasmid pBWB514 (Addgene #209326) as a PCR template, with: 45m
Forward primer BWB1471 (CTGTCTCTTATACACATCTGTCGACCTGCAGCGTACGNNNNNNNNNNNNNNNNNNNNAGAGACCTCGTGGACATCG)
Reverse primer BWB1472 (CTGTCTCTTATACACATCTTGGACTGAAGAGCttttctctatc).

Use PrimeSTAR Max 2x master mix (Takara, R045A) using manufacturers protocols. This PCR will both linearize the plasmid for blunt end cloning (conducted later) and add the barcode and other desired genetic parts (mosaic ends, U1, U2).

NOTE: Primers should not be phosphorylated (to prevent self-ligation in subsequent steps) and should be purified (such as by HPLC) when ordering to ensure full length primers are used during PCR.

NOTE: Use a low concentration of template plasmid DNA (~5 ng) to prevent false positives at the later transformation step (from DNA carryover).

NOTE: To prepare sufficient backbone for the creation of multiple genome libraries, run 4× 50 µL PCR reactions.

23 As a precaution, to remove remaining template plasmid DNA, run a dpnI digest with the PCR reaction. Simple approach: Add 1 µL of dpnI (Thermo Scientific, ER1701) to 50 µL PCR reaction and run at 37°C for 1 hour. Alternatively, one can run a PCR cleanup such as with a GeneJET PCR Purification Kit (Thermo Scientific, K0701), and following run a dpnI digest according to manufacturer protocols.

24 After the PCR and dpnI digest is complete, run a 1% agarose gel as described previously. 40m

25 Gel extract the linearized plasmid band with a GeneJET Gel Extraction Kit (Thermo Scientific, K0691). The desired band should be 2976 bp (approximately 3kb). Elute and measure DNA concentration (NanoDrop, Qubit).

26 As a precaution, run rSAP (Shrimp alkaline phosphatase) dephosphorylation reaction on backbone according to manufacturer protocols (New England Biolabs, M0371S).

27 Clean up DNA with GeneJET PCR Purification Kit (Thermo Scientific, K0701). Measure DNA concentration (NanoDrop). **The backbone is now prepared for fragment library cloning.**

Cloning fragment library 1d 18h

28 Using a high concentration T4 DNA ligase (New England Biolabs, M0202M), run a blunt end ligation reaction following manufacturer protocols. Utilize 2:1 or 3:1 insert to backbone molar ratio. As the backbone (2976 bp) and mean insert size (3 kb) are approximately the same, one can use a mass ratio for this step. When running ligation, utilize the 16°C overnight option. 16h

NOTE: Too high of insert amount will lead to a higher frequency of the insert ligating to insert. Too low of insert amount will lead to fewer successful ligation events.

29 Because of the desired library size, run 4 parallel ligations (20 µL each) and pool using a single PCR clean up step with a GeneJET PCR Purification Kit (Thermo Scientific, K0701). Elute into 18 µL of nuclease free water to prepare for electroporation. If desired, measure DNA concentration and quality (NanoDrop or Qubit). 45m

30 Use ~3 µL of pooled and purified ligation product to transform 10-β high-efficiency electrocompetent *E. coli* cells following manufacturer protocols (New England Biolabs, 15m

C3020K). It is recommended to run 3 or 4 transformations for each library.

31 After manufacturer specified recovery of transformed cells, create a dilution series in sterile 1x PBS and spot on LB+chloramphenicol plates (17 µg/mL chloramphenicol concentration) to determine transformation efficiency. Store recovered cells at 4°C overnight in recovery medium. Incubate dried, spotted plates at 37°C. 1h

32 The following morning, check the spotting plates to get colony forming unit (cfu) counts to determine transformation efficiencies for each transformation run. Based on the desired library size (calculated by total genome size, divided by 300 - 3 kb fragments, 10x coverage), combine volumes of the individual transformations to get to total desired cfu amount. 16h

NOTE: cfus often overestimate library size. Thus, to be conservative, it is best to have slightly more volume than calculated.

33 Transfer desired combination of individual transformation to 100 mL of liquid LB + chloramphenicol (17 µg/mL) in a non-baffled 500 mL shake flask. Grow for 8-10 hours. 8h

34 After outgrowth, make multiple (~10) glycerol stocks mixing cell culture and sterile 50% glycerol 1:1 (500 µL each) to make 1 mL stocks. Store glycerol stocks in appropriately labelled cryovials at -80°C.

35 Plasmid miniprep the entirety of the remaining culture with a GeneJET Plasmid Miniprep Kit (Thermo Scientific, K0502).

Mapping fragment libraries

36 Take cloned, plasmid miniprepped genomic fragment library and use as template to prepare for PacBio sequencing. Following manufacturer protocols, use SMRTbell prep kit 3.0 to prepare library for PacBio Sequencing.

37 For PCR initial step, use HPLC purified primers (Forward primer BWB1473 - /5Phos/GTCCAAGATGTGTATAAGAGACAG and Rv primer BWB1474 - /5Phos/GACGATGTCCACGAGGTCTC) and run a 10x cycle PCR with 100 ng template DNA using PrimeSTAR Max.

NOTE: Run 4× 50 µL PCR reactions to obtain sufficient material.

38 After PCR, run agarose gel as described previously and extract 1-5 kb band from gel with kit.

39 Run an additional dpnI digestion reaction on extracted DNA (37°C, 1 hour). Clean up DNA with a GeneJET PCR purification kit.

40 Follow manufacturer protocols for SMRTbell prep kit 3.0 steps, including rigorous clean up steps and DNA quantification.

41 Send for PacBio sequencing. Barcode and pool as needed with SMRTbell prep kit components.

42 Map libraries with https://github.com/OGalOz/Boba-seq. (https://www.biorxiv.org/content/10.1101/2022.10.10.511384v3.abstract). **Mapped libraries can now be used in Coaux-Seq complementation assays.**