BACRB - Parallel Sequencing of Barcoded BAC Clones by random tagging PCR

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Abstract

A genome is assembled by connecting large scaffolds via a collection of thousands of mate-paired ends of various sizes (3-20 kbp). Terminal ends of long DNA fragments can be obtained by sequencing large insert clones by Sanger sequencing, a labour-intensive and expensive approach. Here we describe a high-throughput protocol to tag 11,520 BAC plasmids randomly with barcoded oligonucleotides at the end of a random hexamer by PCR.This method assisted the kiwifruit (*Actinidia chinensis*) genome assembly in a similar way as mate paired end datasets. Although not fully sequenced, each randomly tagged BAC clone provides a collection of short sequence tags mapped to a genome across one or multiple scaffolds under three different scenarios: validate a single scaffold, join two scaffolds, or group several scaffolds by proximity only. Our method provides a simple way of cataloging BAC libraries to assist genome assembly projects.

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Guidelines

A BAC library from kiwifruit (*Actinidia chinensis*) was constructed by Bio S&T, Québec, Canada. The nuclear genomic DNA was partially digested with *Hind* III, cloned in the plant transformable binary vector pCLD04541, maintained in *Escherichia coli* DHB10 and arrayed in 180 382-well plates. The BAC library represents ~ 6.7 times the haploid genome; the average insert size is 71.3 kbp (n = 309), and 0.6% of the library contain organellar DNA clones.

The process of oligonucleotide design will be reported in a separate protocol. But here is an overview:

Conditions for generating BAC Random Barcoded oligonucleotides (BACRB)

From all possible combinations (4^{14} = 268,435,456) select 384 unique barcodes with the following characteristics:

- GC = 0.5 ± 0.03
- No palindromes
- No repeats > 3 bases long
- Should not hybridize to plasmid vector or *Escherichia coli* genome
- 14 b long

An example...

Amplification step	Oligonucleotide set	Sequence
PEP-PCR	BACRB_Q1_A1	ACTTACACGTCGAGNNNNNN
TD-PCR	t_BACRB_Q1_A1	GGCGTAACTTACACGTCGAG

Before start

NOTES

1. When **pipetting dense solutions**, like the PCR master mix, prime the pipette tips by drawing and expelling the liquid slowly, one time. Multichannel pipettes (and robotic stations) allow you to select the speed of this operation. Also, if the required volume is 47.5 µL, set the pipette to 46.7 µL, and don't dip the tips too far into the solution. The amount of carry over solution outside the tip will compensate for the "missing" µL in the settings.
2. This method was developed in 2011/2012, and since then the library preparation and sequencing technologies have changed rapidly. You can select any approach as long as the amplicon ends are recovered during the library preparation. Do not use a tagmentation based protocol to prepare the libraries. And we recommend to obtain a pair end read data set to increase the read mapping rate.
3. We are aware that a small amount of *Escherichia coli* genomic DNA co-purifies with the BAC plasmid and is tagged and amplified along with the target BAC DNA. The *E. coli* DNA can be digested with enzyme blends such as PlasmidSafe but given the number of BAC plasmid preps needed, it becomes too expensive. An option is to remove the contaminant by genomic subtractive hybridization. A subtracter is prepared with purified *E. coli* DNA which is sheared, end repaired and biotinylated at the 3'-end with terminal deoxynucleotidyl transferase. The subtracter is hybridized with the plate pool amplicons and the double stranded *E. coli* molecules (subtracter+random tagged *E. coli* DNA) are removed with streptavidin magnetic beads. After 3 rounds of subtraction, the remaining amplicon sample should be fairly clean of host DNA and

ready for library preparation.

Consumables

- ABgene 96 well polypropylene storage microplate, conical bottom (cat# AB1058)
- 2 mL deep well plate (NUNC cat# 278752)
- 96-well Filter plate (NUNC cat# 278011)
- Conical polypropylene sterile tubes, 15 mL
- Disposable sterile reservoirs
- Breathable tape (rayon acrylate NUNC cat# 241205)
- Silicone mat that fits the 2 mL deep well plate or a piece of silicone rubber sheet (1.5 mm thickness)
- Clear packaging tape (48 mm wide) for temporary plate sealing
- Cutter with retractable blade
- Microcentrifugues, 1.5 and 2 mL, sterile, and racks
- PCR plates (Thermo-Fast AB-0600)
- Aluminium tape for PCR reactions and long term storage (NUNC cat# 232698, or similar)
- Paper towels, low lint
- Rectangular plastic box
- Waste containers for biological material and isopropanol

Solutions

- LB medium
- 12.5 mg/mL tetracycline hydrochloride, dissolved in absolute ethanol
- RNase A 100 µg/µL (Qiagen cat# 19101)
- Resuspension buffer (25 mM Tris-HCl pH 8, 10 mM EDTA, sterile). Add RNase A to a final concentration of 50 µg/mL before resuspending the bacterial cells
- 0.2 N NaOH prepared in sterile deionized water and stored in a plastic bottle
- 10% SDS, sterile
- Lysis solution (Use sterile deionized water to prepare 0.2 M NaOH, 0.1% SDS. This solution is freshly made from stocks described above)
- Neutralizing solution (3 M potassium acetate, 2 M acetic acid, pH 5.6). To prepare this solution dissolve 295.5 g potassium acetate (MW 98.5 g/mol) in ~400 mL sterile deionized water. Adjust the volume to 600 mL and store in a sterile 1 L glass bottle. Add 115 mL glacial acetic acid and 285 mL sterile deionized water. Do not autoclave and do not adjust the pH, which should be around 5.6. Split the solutions in 2 or 3 sterile glass bottles and store at 4°C
- Isopropanol
- 70% ethanol, in sterile deionized water
- Plasmid resuspension buffer (28 mM Tris-HCl pH 8, 1 mM EDTA, 0.6 mM cresol red)
- Deionized water, sterile
- AccuPrime Taq DNA polymerase High Fidelity 5 U/µL and 10X AccuPrime High Fidelity Buffer I and II (Invitrogen 12346094)
- Mineral oil (Sigma-Aldrich cat # M5904-500ML)
- 2% xylene cyanole FF solution prepared in sterile deionized water
- Platinum *Pfx* DNA polymerase 2.5 U/µL (Invitrogen 11708039)

- 1.2 M Trehalose, dissolved in sterile deionized water and filtered through 0.2 or 0.4 µm
- 10X *Pfx* reaction buffer homemade: 200 mM Tris-HCl, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8, sterile. ***Note****: I have not tried the 10X buffer and enhancer solutions supplied with the Platinum Pfx DNA polymerase (Invitrogen 11708039). It should work, but do a preliminary test*
- 50 mM $MgSO_4$ (use the stock provided with either DNA polymerase)
- 2 mM dNTP (dilution prepared in deionized sterile water from 100 mM stocks)
- 2% agarose gels in 1X TAE buffer and 1X TAE running buffer
- 1 kb+ DNA ladder (Invitrogen) or similar
- SybrSafe (Invitrogen)
- 3 M sodium acetate pH 5.2. To prepare this solution dissolve 24.6 g sodium acetate anhydrous (MW 82.03 g/mol) in ~ 80 mL deionized water. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 100 mL, split into two glass bottles, and autoclave
- TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), sterile

Equipment and kits

- Cell culture incubator
- Bench top centrifuge with plate rotor (for example, Eppendorf SKU 5810000084 Rotor A-4-62, with 4 MTP buckets; Cat. # 5810711002)
- Bench top microcentrifuge for 1.5 mL tubes
- Vacuum manifold (for example Supleco PlatePrep 96-well Vacuum Manifold SKU:575650-U)
- Vortex with plate adaptor
- Qubit 2.0 or above and High Sensitivity dsDNA kit
- Horizontal gel electrophoresis unit with power supply
- Image capture equipment (for example, GelDoc, LSA3000, etc)
- Multichannel pipettes (1-10 µL, 5-200 µL, 50-1000 µL) or liquid handling robotic station with appropriate multichannel tools and tips, for example Biomek series, Hamilton, Integra's Viaflo, etc.
- PCR machine
- Timer

Protocol

BAC clones cultures

Step 1.

Transfer an aliquot of the BAC library glycerol stock stored in the 384-well library plate using the 96-pin HDRT of the robotic unit into the 96-well conical bottom working plate which contains 150 µL of LB medium, 12.5 µg/mL tetracycline per well.

Cover the plate with its corresponding lid and incubate at 37°C overnight.

TEMPERATURE

37 °C Additional info: cell culture incubator

NOTES

Elena Hilario 22 May 2018

Thirty 384-well plates from the kiwifruit (*Actinidia chinensis*) BAC library were selected and represent 1X coverage of the genome (~750 Mbp). Each 384-well plate was divided in 4 quadrants to be transferred to 96-well working plates: A1 = quadrant 1; A2 = quadrant 2; B1 = quadrant 3; B2 = quadrant 4. A total of 120 quadrant plates were generated.

Here we describe how to process one 384-well library plate (4 quadrants) only. You can scale it up to the desired number of library plates according to the level of coverage required.

Elena Hilario 22 May 2018

Use a liquid handling robotic unit to manipulate the BAC library plates. The robot should include a 96-pin high density replicating tool (HDRT).

BAC clones cultures

Step 2.

Transfer 20 µL of each well of the 96-well working plate to its corresponding address in a 2 mL 96-deep well plate filled with 1.2 mL LB medium and 12.5 µg/mL tetracycline per well. Seal the plate with breathable tape and incubate at 37°C in a cell culture shaker at 180 rpm for 16 h.

TEMPERATURE

37 °C Additional info: cell culture shaker incubator

NOTES

Elena Hilario 23 May 2018

To increase the BAC plasmid yield, the plates can be stored in a cold room for ~ 6 h or overnight before harvesting the bacterial cells.

BAC clones cultures

Step 3.

Collect the bacterial cells by centrifugation at 3000 rpm at room temperature for 25 min in a benchtop microtitre plate centrifuge. Remove the tape and pour out the medium by inverting the plate over a rectangular plastic box. Blot briefly over paper towels and seal with packaging tape.

Store the plates containing the bacterial cells at -20°C until all the plates have been processed.

SAFETY INFORMATION

Load the plates according to their matching weights. Use rectangular pieces of paper the same size as the plates to match the weight values and place it under the plates.

BAC plasmid preps

Step 4.

Remove the BAC cell culture plates from the freezer and let the pellets defrost at room temperature for 10-20 min.

BAC plasmid preps

Step 5.

Add 100 µL of resuspension buffer per well. Seal the plate with packaging tape and vortex for 10-20 seconds. Spin down briefly.

NOTES

Elena Hilario 25 May 2018

The bacterial cell pellet should be completely dissolved.

BAC plasmid preps

Step 6.

Add 200 µL of lysis solution per well and gently swirl the plate to ensure complete lysis. Incubate without shaking for 5 min at room temperature.

BAC plasmid preps

Step 7.

Neutralize the lysate by adding 150 µL of ice cold potassium acetate solution per well. Seal the plate with packaging tape and gently mix by vortexing at the lowest speed, 3-4 pulses of 5 s each. Keep the plates on ice for 10 min.

TEMPERATURE

0 °C Additional info: ice box

BAC plasmid preps

Step 8.

Centrifuge the plates for 15 min at 3000 rpm, room temperature.

GOTO

Centrifugation safety warning -> go to step #3

NOTES

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Most of the floculent material is collected at the bottom of the well. Don't worry if some is transferred to the fritted plate in the next step.

BAC plasmid preps

Step 9.

Transfer the lysate supernatant to a NUNC fritted plate assembled over a vacuum manifold containing its corresponding 2 mL 96-deep well receiving plate, in the same orientation. Cover the fritted plate with a silicone mat and apply vacuum to -10 in Hg for 2 min or until all liquid has been filtrated. Discard the fritted plate.

BAC plasmid preps

Step 10.

Remove the receiving plate containing the BAC plasmid and add 0.7 volumes of isopropanol per well at room temperature (approximately 260 µL) to each well. Seal the plate with packaging tape and vortex gently for 2-3 seconds, 3 times. Incubate at room temperature for 1 h.

BAC plasmid preps

Step 11.

Collect the BAC plasmid DNA by centrifugation at 3000 rpm, 25 min at room temperature.

GOTO

Centrifugation safety warning -> go to step #3

BAC plasmid preps

Step 12.

Pour out the supernatant into a rectangular waste tray. While the plate is still upside down, remove any traces of liquid by blotting it over paper towels for 2-3 seconds only.

SAFETY INFORMATION

Dispose the isopropanol waste according to your local environmental regulations.

BAC plasmid preps

Step 13.

Add 100 or 200 µL of 70% ethanol to each well. Seal the plate with packaging tape and swirl it gently. Let it stand at room temperature for 10-20 min. Collect the BAC DNA by centrifugation at 3000 rpm, 25 min at room temperature.

GOTO

Centrifugation warning -> go to step #3

BAC plasmid preps

Step 14.

Pour out the ethanol supernatant over a rectangular tray and briefly blot the plate over paper towels.

The ethanol waste can be poured down the drain and flushed with water for 30 sec.

Air dry the BAC pellet for 10 min at room temperature. Add 150 µL of plasmid resuspension buffer per well, seal with packaging tape, spin down briefly and store at 4°C.

NOTES

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The purpose of adding cresol red to the BAC plasmid DNA solution is to ensure the pH of the DNA solution is >7.5, and to visually confirm that the template transfer was successful in the following PCR reaction. The BAC plasmid solution should be red/pink, not yellow, which means it still contains sodium acetate and the pH is below 7.5.

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Weight the plate at t = 0 and after 5 and 10 min or until you see no change in weight. But if you prefer not to over dry the BAC DNA prep, add the buffer, seal the plate with packaging tape, vortex at medium speed and spin down briefly. Remove the tape and let any remaining ethanol evaporate at room temperature for 10-20 min. Cover the plate with a tissue paper (lint free) to prevent foreign objects fall into the wells.

BAC plasmid preps

Step 15.

Quantify at least 12 BAC plasmid preps from each quadrant plate (48 BAC preps per library plate). Use 1 µL of BAC plasmid for the Qubit HS dsDNA kit. Take the average value of those 48 reactions and if needed adjust the concentration to 3 ng/uL by adding more plasmid resuspension buffer.

Random tagging BAC clones - PEP-PCR

Step 16.

Make a master mix according to the following table, with at least 16 extra reactions:

PEP-PCR

	One reaction, µL	400 reactions, µL
Deionised sterile water	38.8	15520
10X AccuPrime High Fidelity Buffer II	5	2000
BACRB oligonucleotide, 10 pmol/µL	1	
AccuPrime Taq DNA polymerase High Fidelity 5 U/µL	0.2	80
BAC plasmid prep (3 ng/µL)	5	
Total volume	50	

Read note regarding pipetting dense solutions. Aliquot 44 µL of master mix per well into a PCR plate.

Random tagging BAC clones - PEP-PCR

Step 17.

Add 1 µL of its corresponding BACRB oligonucleotide to each well. Add 5 µL of BAC plasmid solution to its corresponding well. Seal the plate with packaging tape and vortex vigorously. Spin down briefly.

Add 20 µL of sterile mineral oil to each well with a multichannel pipette, seal the plate with aluminium tape.

NOTES

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The addition of mineral oil should prevent evaporation but check all wells, especially the ones at the edge of the plate. If less than half of the total volume of the reaction has evaporated, add enough 1X TE pH 7.5 buffer to bring back the volume to 50 µL. Repeat the PEP-PCR reaction if more than half of the liquid has evaporated.

Avoid touching the rim of the well with mineral oil because it will prevent the aluminium tape stick properly.

Random tagging BAC clones - PEP-PCR

Step 18.

Amplify as follows:

94°C, 2 min → (94°C, 40 sec → 30°C, 2 min → 48°C, 4 min (set ramp at +0.1°C/sec) → 68°C, 1 min) x 50 cycles → 68°C, 7 min → stop

and leave at room temperature (running time: 10 h)

NOTES

Elena Hilario 23 May 2018

The annealing step is done at two temperatures: 30 and 48°C, with a very gentle ramp that will increase 0.1°C per second.

Random tagging BAC clones - PEP-PCR

Step 19.

For short term storage, keep the plate at 4°C, otherwise store it at -20°C.

NOTES

Elena Hilario 23 May 2018

The PEP-PCR plates are kept at 4°C until all the BAC plasmid preps have been processed.

One microlitre of 2% xylene cyanole FF solution can be added to each PEP-PCR reaction before the next round of amplification to visually confirm the addition of template.

Enrich rt-BACs by touch down PCR

Step 20.

Make a master mix according to the following table, with at least 16 extra reactions:

TD-PCR

	One reaction, µL	400 reactions, µL	Final concentration
Sterile deionized water	0.7	280	
1.2 M Trehalose	10	4000	0.6 M
10X *Pfx* Reaction Buffer (homemade)	4	1600	2X
50 mM $MgSO_4$	0.2	80	0.5 mM
2 mM dNTP	2	800	0.2 mM
t-BACRB oligonucleotide, 4 pmol/µL	1		4 pmol

Platinum *Pfx*, 2.5 U/µL	0.1	40	0.25 units
PEP-PCR reaction, undiluted	2		
Total volume	20		

This is a dense solution. Aliquot 17 µL of master mix per well with a multichannel pipette into its corresponding PCR plate.

NOTES

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No need to add BSA, it has no effect on the amplification and it only adds to the cost.

Platinum Pfx has been discontinued (April 2018) and replaced by another enzyme blend called Superfi (Invitrogen cat# 12351010). If you prefer to use another enzyme mix, just make sure is a proofreading type.

Enrich rt-BACs by touch down PCR

Step 21.

Add 1 µL of its corresponding t-BACRB oligonucleotide to each well. Add 2 µL of PEP-PCR solution to its corresponding well. Seal the plate with aluminium tape and vortex vigorously. Spin down briefly.

Enrich rt-BACs by touch down PCR

Step 22.

Amplify as follows:

94°C, 2 min → (94°C, 30 sec → Touchdown 68°C-58°C, 30 sec at -0.5°C/cycle → 68°C, 1 min) x 20 cycles → (94°C, 30 sec → 58°C, 30 sec → 68°C, 1 min) x 10 cycles → 68°C, 7 min → stop

and leave at room temperature.

Enrich rt-BACs by touch down PCR

Step 23.

Store the PCR plates at 4°C until all plates have been processed and you are ready to prepare the plate pools.

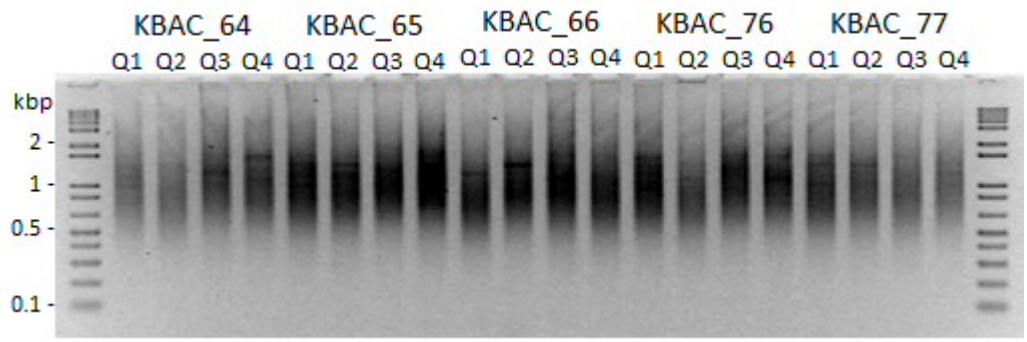
Prepare plate pools

Step 24.

Transfer the contents of each 96-well plate (one quadrant) into a 2 mL microcentrifuge tube (final volume 1.8 mL) and analyze 20 µL by agarose gel electrophoresis (2% agarose in 1X TAE buffer, 1 kb+ ladder, 140 V, constant current, 30 min. Stain with SybrSafe 10 min (3 µL SybrSafe in 50 mL 1X

TAE).

EXPECTED RESULTS



Prepare plate pools

Step 25.

Transfer 700 µL of each quadrant pool into a 15 mL conical tube (final volume 2.8 mL). Add 280 µL of 3 M sodium acetate pH 5.2 to each tube and vortex briefly. Add 1.96 mL isopropanol per tube at room temperature and vortex until completely mixed. Incubate at room temperature for 1 h.

GOTO

Isopropanol waste disposal warning -> go to step #12

Prepare plate pools

Step 26.

Centrifuge the sample at 3000 rpm for 25 min. Discard the isopropanol supernatant and add 1 mL 70% ethanol per tube. Make sure the pellet is detached from the tube and keep at room temperature for 10 min. Centrifuge again at the same speed and time.

GOTO

Centrifugation safety warning -> go to step #3

Prepare plate pools

Step 27.

Remove the ethanol supernatant and blot the tube on paper towels without losing the pellet. Air dry the pellet 10 min and add 300 µL TE buffer per tube.

GOTO

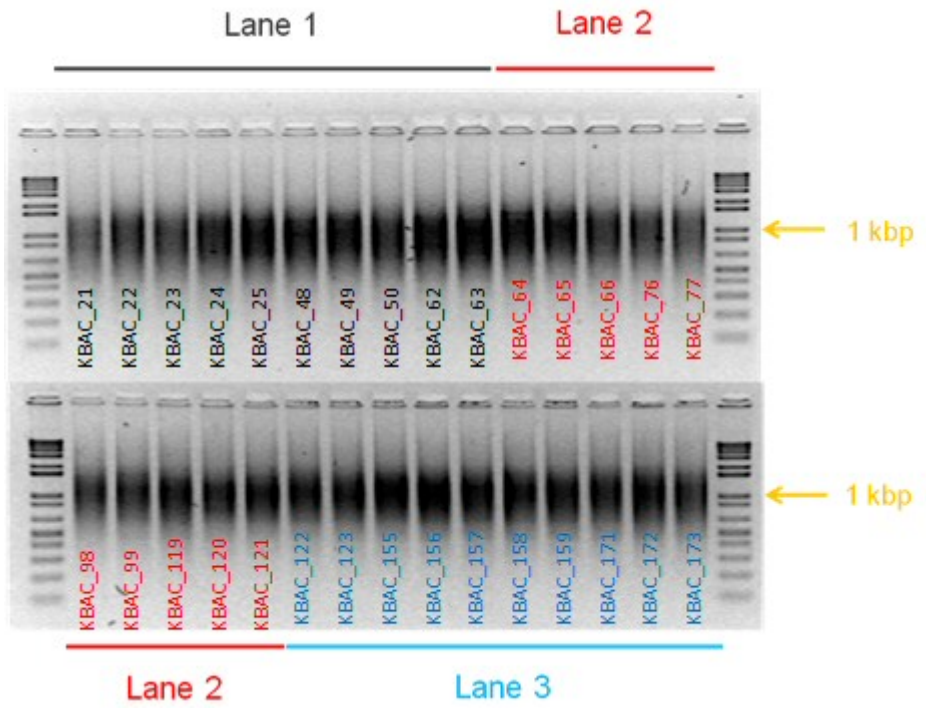
Note on how to resuspend DNA -> go to step #14

Prepare plate pools

Step 28.

Run 2 µL of each plate pool in a 2% agarose gel in 1X TAE buffer.

EXPECTED RESULTS



GOTO

Agarose gel running and staining conditions -> go to step #24

Illumina library prep and sequencing

Step 29.

The dataset reported in our paper was obtained by preparing TruSeq Illumina libraries. Each plate pool was fragmented, end repaired, dA tailed and barcoded with Illumina adaptors. Ten plate libraries were pooled per lane and sequenced in the HiSeq2000 platform, 100 cycles, single end mode.

See 'Guidelines and Warnings' section for comments on library and sequencing platform improvements developed since this method was created.

Warnings

Please read the safety warnings for the use of plate bench centrifuge and for bacterial and isopropanol waste disposal noted in the protocol.