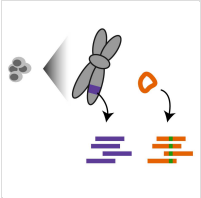

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Targeted isolation of circular extrachromosomal DNA by CRISPR-CATCH

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ABSTRACT

This protocol enables targeted isolation of circular extrachromosomal DNA using CRISPR-Cas9-mediated linearization followed by pulsed field gel electrophoresis. Isolated DNA products can be subsequently used for short-read sequencing, single-molecule long-read sequencing or other assays.

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KEYWORDS

extrachromosomal DNA, ecDNA, pulsed field gel electrophoresis, oncogene amplification, CRISPR-CATCH

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GUIDELINES

Note 1 - PFGE parameters:

Settings for DNA 0.22-2.2Mb, 0.5X TAE, 14C, 1% LMP agarose, linear ramping.
Two-state mode
Run time duration: 16:39
Initial switch time: 0:00:20.16
Final switch time: 0:02:55.12
Gradient: 6V/cm
Included angle: 120
Ramping: 0.0 (linear)

Settings for DNA 1-6Mb, 0.5X TAE, 14C, 0.8% LMP agarose, linear ramping.
Multi-state mode
Block 1:
Duration: 59:02
initial switch time: 0:20:00
final switch time: 0:40:24
voltage gradient: 2V/cm
ramping: 0.0 (linear)
angle: -53 (state 1), +53 (state 2)
Block 2:
Duration: 9:15
initial switch time: 0:01:10.2
final switch time: 0:02:09.92
voltage gradient: 6V/cm
ramping: 0.0 (linear)
angle: -60 (state 1), +60 (state 2)

Electro-depletion for removal of short linear DNA from agarose plugs.
Block 1:
duration: 3:00:00
initial switch time: 3:00:00
final switch time: 3:00:00
voltage gradient: 5.2V/cm
ramping: 0.0 (linear)
angle: 0 (state 1)
Block 2:
duration: 0:02:00
initial switch time: 0:02:00
final switch time: 0:02:00
voltage gradient: 5.2V/cm
ramping: 0.0 (linear)
angle: 180 (state 1)

MATERIALS TEXT

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Reagents used

A	B	C
Reagent	**Manufacturer**	**Catalog number**
Certified Low Melt Agarose	Bio-rad	1613112
50-Well Disposable Plug Molds	Bio-rad	1703713
CHEF DNA Size Marker, 1-3.1MB, H.wingei ladder	Bio-rad	1703667
CHEF DNA Size Marker, 0.2-2.2MB, S. cerevisiae Ladder	Bio-rad	1703605
20% N-lauroylsarcosine sodium salt solution	Sigma-Aldrich	L7414-10ML
0.5M EDTA pH 8.0	Invitrogen	AM9260G
proteinase K (20mg/ml)	Amresco	E195-5ML
10% SDS	Thermo Fisher Scientific	15553-027
PMSF	Millipore Sigma	10837091001
RNase A	Sigma-Aldrich	R6148-25ML
Bis-Tris-HCl	Sigma-Aldrich	B6032-25G
CRISPRevolution sgRNA EZ Kit	Synthego	custom
Cas9 Nuclease, S. pyogenes	New England Biolabs (NEB)	M0386T
10X NEBuffer 3.1	New England Biolabs (NEB)	B7203
3M Sodium acetate, pH5.5	Thermo Fisher Scientific	AM9740
Beta agarase I	New England Biolabs (NEB)	M0392

Reagent preparation

sgRNA: Reconstitute sgRNA at 30uM in nuclease-free water. Store aliquots at -80C. Dilute stock 1:100 to make a 300nM working stock before use. Aliquot remaining solution and store at -80C.

ES buffer: 1% N-laurolsarcosine sodium salt solution, 25mM EDTA (pH8.0), 50ug/ml proteinase K.
to make 20ml ES buffer:

A	B
20% N-lauroylsarcosine sodium salt solution	1ml
0.5M EDTA pH 8.0	1ml
proteinase K (20mg/ml)	50ul
H2O	18ml

SDE: 1% SDS, 25mM EDTA (pH8.0)
to make 20ml SDE:

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A	B
10% SDS	2ml
0.5M EDTA pH 8.0	1ml
H2O	17ml

25mM EDTA:

A	B
0.5M EDTA pH 8.0	1ml
H2O	19ml

PMSF: make 100mM PMSF stock in isopropanol (stable at -20C for 9 months, add to aqueous solution right before use)

A	B
PMSF powder	0.174g
isopropanol	10ml

Dilute to 1mM right before use using 25mM EDTA:

A	B
25mM	10ml
100mM PMSF	100ul

10X β-Agarase I Reaction Buffer: 100 mM Bis-Tris-HCl, 10 mM EDTA (pH 6.5 @ 25°C)
To make 50ml:

A	B
Bis-Tris-HCl	1.228g
0.5M EDTA	1ml
H2O	40ml
adjust pH to 6.5	
Bring volume up to 50ml with H2O	

aliquot and store at -20C.

Embedding cells in agarose plugs

1 Melt 1% Certified Low Melt Agarose solution in PBS, place in 45C water bath.

2 Pellet 1 million cells per agarose plug at 300g for 5 minutes. (for example, spin down 10 million cells to make 10 agarose plugs)

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3 Two cold PBS washes (30ml) with gentle pipet mixing. Pellet cells after each wash at 300g for 5 minutes.

4 Resuspend cells at 1 million cells per 30 ul PBS using a wide-bore pipet tip. Warm to 37C.

5 Place plug molds on ice.

6 Mix with equal volume 1% Low Melt Agarose (pre-cooled to 37-45C) using a wide-bore pipet tip. Immediately pipet into plug mold without introducing bubbles.

7 Cool samples at 4C for 10 minutes.

8 Transfer each agarose plug into an eppendorf tube. Add 1ml SDE and incubate at room temperature for 10min with gentle shaking.

9 Remove supernatant without breaking the agarose plug. Add 1ml ES and incubate at 50C for 1-2 days with gentle shaking (~400rpm).

10 Cool briefly on ice.

11 Remove supernatant, add 1ml of 25mM EDTA containing 1mM PMSF (dilute PMSF right before use) to inactivate proteinase K. Incubate at RT for 1 hour on a shaker.

12 Remove supernatant, add 1ml of 25mM EDTA with 1mg/ml RNase A, incubate at 37C for 30min.

13 Remove supernatant, wash with 1ml of 25mM EDTA with a 5min incubation.

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14 Remove supernatant, add 1ml of 25mM EDTA and store at 4C (2-12 months).

Removal of degraded DNA fragments (optional)

15 For samples with high dead-cell fractions, samples that were previously frozen, or tumor samples with degraded DNA due to the sample preparation process, this optional section allows background depletion for a cleaner gel and improved ecDNA enrichment. This is usually not necessary for cell lines and high-quality cell samples, but may be necessary for primary tumor samples.

16 Make a PFGE gel using 1% Certified Low Melt Agarose in 0.5X TAE buffer. Do not add stain. Gently remove the gel comb to avoid destroying the integrity of the wells.

17 Clean electrophoresis chamber with 2 liters of 10% bleach. Turn on power module, power outlet to pump, turn pump knob to 100 to allow bleach to circulate for 30 minutes.

18 Remove and completely drain bleach from the system. Add 2 liters of DI water and turn on pump to allow water to circulate. Drain water and repeat for a total of 3 washes.

19 Remove and drain water from the system. Add 2 liters of running buffer and turn on pump to allow buffer to circulate. Drain buffer.

20 Refill electrophoresis chamber with 2.2 liters of running buffer. Turn pump knob to 70, and turn on cooling module, in this order. Set temperature to proper temperature (typically 14C; see Note 1 in Guidelines) on cooling module.

21 Load DNA samples in agarose plugs into wells. Do NOT seal wells with agarose. Carefully place gel in the electrophoresis chamber.

22 Start electrophoresis using the electro-depletion settings. (See Note 1 in Guidelines, "Electro-depletion for removal of short linear DNA from agarose plugs"; if very large >1Mb DNA fragments are observed, the settings for DNA 0.22-2.2Mb may also be used for electro-depletion)

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23 Transfer the gel carefully to a container. Make two incisions in each upper corner of the well to remove the part of the gel above the well, exposing the plug sitting inside. Carefully take out the plug without damaging it. Place the plug in an eppendorf tube, add 1ml of 25mM EDTA and store at 4C (2-12 months). This plug contains intact genomic DNA from the original sample but is depleted of degrade DNA fragments. This plug can be used subsequently for CRISPR-CATCH.

ecDNA linearization by CRISPR-Cas9

24 Remove supernatant from agarose plug, 3 washes with 300ul 1X NEBuffer 3.1 with 5min incubations.

25 Set up a **reaction mix** in the following order:

A	B
H2O	161ul
NEBuffer 3.1 (10X)	21ul
300nM sgRNA	21ul (30nM final)
1uM Cas9 (pre-dilute if needed)	7ul (~30nM final)
TOTAL: 210ul	

Incubate **reaction mix** at room temperature for 10 minutes.

26 Remove supernatant from agarose plug. Add **reaction mix** to agarose plug.

27 Incubate at 37C for 4 hours on a shaker with gentle shaking (~400-500 rpm).

28 Remove supernatant. Add 200ul 25mM EDTA with 3ul proteinase K, mix, incubate at RT overnight with gentle shaking (~400-500 rpm).

29 Remove supernatant. Add 1ml 25mM EDTA with 1mM PMSF, incubate at RT for 1 hour with gentle shaking.

30 (for storage) Remove supernatant, do two washes in 25mM EDTA, resuspend in 25mM EDTA and store at 4C. (Skip to next step if running PFGE immediately)

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ecDNA separation by pulsed field gel electrophoresis (PFGE)

31 Make a PFGE gel using 1% Certified Low Melt Agarose in 0.5X TAE buffer. Do not add stain yet. Gently remove the gel comb to avoid destroying the integrity of the wells.
Note: agarose type, percentage, and buffer type all depend on the objective. See Note 1 under Guidelines for recommendations.

32 Clean electrophoresis chamber with 2 liters of 10% bleach. Turn on power module, power outlet to pump, turn pump knob to 100 to allow bleach to circulate for 30 minutes.

33 Remove and completely drain bleach from the system. Add 2 liters of DI water and turn on pump to allow water to circulate. Drain water and repeat for a total of 3 washes.

34 Remove and drain water from the system. Add 2 liters of running buffer (composition depends on PFGE parameters; typically 0.5X TAE. See Note 1 in Guidelines) and turn on pump to allow buffer to circulate. Drain buffer.

35 Refill electrophoresis chamber with 2.2 liters of running buffer. Turn pump knob to 70, and turn on cooling module, in this order. Set temperature to proper temperature (typically 14C; see Note 1 in Guidelines) on cooling module.

36 Equilibrate DNA ladder agarose plugs and samples in running buffer (2X washes with 5min incubations).

37 Load DNA ladders into wells using a microscope cover slip. Push plug all the way to the bottom of the well to make sure there is no space.

38 Load DNA samples in agarose plugs into wells. Seal all wells (including empty ones) with molten 1X Low Melt Agarose. Wait ~10min for agarose to solidify. Carefully place gel in the electrophoresis chamber.

39 Start electrophoresis using appropriate settings based on size range, agarose type, percentage, and buffer type. (See Note 1 in Guidelines)

40 Make notches on the side of the gel using glass cover slips to mark distances.

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41 Transfer the gel carefully to a container. Stain gel in 3X gelred with 0.1 M NaCl on rocker for 30min. Cover with aluminum foil to protect from light.

42 Rinse gel with water.

43 Take an image of the gel.

44 Excise bands using a clean cover glass based on relative distances to notches on the side.

Extraction of high molecular weight DNA molecules after PFGE

45 Wash agarose twice with 2 volumes or 500ul of 1X agarase buffer on ice for 10-30min each.

46 Remove buffer, melt agarose at 65C for 10min.

47 Incubate agarose at 43C for 10min.

48 Add 1U agarase per 200ul agarose, incubate at 43C for 1 hour with shaking (400rpm).

49 Add 0.1 volume 3M sodium acetate. Chill on ice for 15 minutes.

50 Centrifuge at 15,000g for 15 minutes at 4C to pellet any remaining undigested carbohydrates.

51 Remove the DNA-containing supernatant. Add 2 volumes of isopropanol.

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52 Mix, chill at 4C for 10min, centrifuge at 15,000g for 15 minutes at 4C.

53 Remove supernatant, wash the pellet with 500ul ice-cold 70% ethanol. Pipet up and down to solubilize salt in the pellet. Pellet DNA at max speed for 5min at 4C.

54 Remove supernatant. Do a second 70% ethanol wash if pellet looks like it has excessive salt.

55 Re-pellet, use a small pipet tip to remove residual ethanol.

56 Air-dry for 5-10min. Do not over-dry. Resuspend in EB buffer.

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