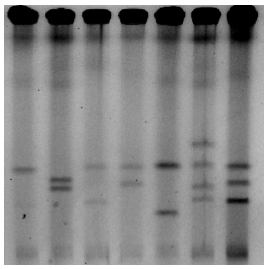
Dec 17, 2019

Isolation of supernumerary mini-chromosomes from fungi for enrichment sequencing



PLOS Genetics

DOI
dx.doi.org/10.17504/protocols.io.9t7h6rn

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.9t7h6rn

External link: https://doi.org/10.1371/journal.pgen.1009386

Protocol Citation: Thorsten Langner, Adeline Harant, Sophien Kamoun 2019. Isolation of supernumerary mini-chromosomes from fungi for enrichment sequencing. **protocols.io https://dx.doi.org/10.17504/protocols.io.9t7h6rn**

Manuscript citation:
Langner T, Harant A, Gomez-Luciano LB, Shrestha RK, Malmgren A, Latorre SM, Burbano HA, Win J, Kamoun S (2021) Genomic rearrangements generate hypervariable mini-chromosomes in host-specific isolates of the blast fungus. PLoS Genet 17(2): e1009386. doi: **10.1371/journal.pgen.1009386**

Protocol status: Working
We use this protocol and it's working

Created: November 27, 2019

Last Modified: December 17, 2019

Protocol Integer ID: 30303

Keywords: Supernumerary chromosomes, Mini-chromosomes, DNA extraction, Genome sequencing, sequencing fungal genome, fungal genome, fungi for enrichment, comparative genomics analysis, whole genome assembly, fungi, dynamic genome rearrangement, many genomic project, genome, chromosome

Abstract

Fungal genomes are highly dynamic and often contain supernumerary mini-chromosomes. However, our knowledge about the role of mini-chromosomes in dynamic genome rearrangements and evolution is scarce. Mini-chromosomes are usually smaller then core-chromosomes and are variable in size and numbers between individuals of a species. Mini-chromosome occurence in fungi is well documented since decades, but many genomic projects have neglected them, mainly because it was technically challenging to identify them in whole genome assemblies.

Here we present a protocol for isolation of mini-chromosomes for enrichment sequencing. This allows identification of mini-chromosomes in whole genome assemblies and thus comparative genomics analyses of core- and mini-chromosomes.

Guidelines

The protocol requires protoplast preparation of fungal cells and is therefore most suitable for culturable species. Separation of chromosomes involves contour-clamped homogenous electric field (CHEF) gel electrophoresis.

This protocol has been developed for the blast fungus *Magnaporthe oryzae*. We tested this protocol by isolating mini-chromosomes from >10 individual isolates and identified corresponding contigs in whole genome assemblies.

The protocol is optimized for isolation of chromosome sized fragments between 400 kb and 3 Mb.

Materials

Required reagents:

YG medium:
5 g of yeast extract
20 g of glucose
Add ddH_2O to 1l
Autoclave

Lysing enzyme solution:
10 mg/ml lysing enzymes (*Trichoderma harzianum*, Sigma #L1412)
15 ml 1M sorbitol
Filter-sterilize

1M Sorbitol:
182.17 g sorbitol
ddH_2O to 1 l
Autoclave

1M Sorbitol/50 mM EDTA:
91.086 g sorbitol
50 ml EDTA 0.5 M
ddH_2O to 500 ml
Autoclave

NDS buffer:
1 g laurylsarcosine
100 mM Tris-HCL pH9.5
500 mM EDTA
ddH_2O to 100 ml
Filter-sterilize.
Add proteinase K freshly to a concentration of 200 µg/ml

Complete medium:
50 ml 20X Nitrate Salts (See Below)
1 ml Trace Elements (See Below)
10 g D-Glucose
2 g Peptone
1 g Yeast Extract
1 g Casamino Acids
1 ml Vitamin Solution (See Below)

15 g Agar
ddH_2O to 1 l
Adjust pH to 6.5 with NaOH
Autoclave

20 X Nitrate Salts:
120 g $NaNO_3$
10.4 g KCl
10.4 g $MgSO_4*7H_2O$ (5.2 g if anhydrous)
30.4 g KH_2PO_4
ddHOH to 1 L
Autoclave
Store at 4°C.

1000 X Trace Elements:
Add the compounds in order!
80 ml ddH_2O
2.2 g $ZnSO_4*7H_2O$
1.1 g H_3BO_3
0.5 g $MnCl_2*4H_2O$
0.5 g $FeSO_4*7H_2O$
0.17 g $CoCl_2*6H_2O$
0.16 g $CuSO_4*5H_2O$
0.15 g $Na_2MoO_4*2H_2O$
5 g Na_4EDTA
Boil briefly and let cool to 60°C
Adjust pH to 6.5 with KOH
Cool to room temperature
ddH_2O to 100 ml
Store at 4°C

1000x Vitamin Solution:
0.01 g Biotin
0.01 g Pyridoxin
0.01 g Thiamine
0.01 g Riboflavin
0.01 g PABA (p-aminobenzoic acid)
0.01 g Nicotinic Acid
100 ml ddH_2O
Store in a dark glass bottle at 4°C

Megabase agarose:
Certified Megabase Agarose (Biorad, #1613108)

TAE buffer 10x:
48.4 g Tris
3.72 Na_2EDTA
ddH_2O to 1 l
adjust pH to 8.0 with acetic acid

TBE buffer 10x
108 g Tris
55 g Boric acid
9.3 g Na_2EDTA
ddH_2O to 1 l

0.5 M EDTA
93.05 g Na_2EDTA
Add 400 ml of ddH_2O
Adjust pH to 8.0
ddH_2O to 500 ml

Additional material required:

D-Tube Dialyzer Midi 3.5 kDa MWCO (Merck, #71506)

D-Tube™ Electroelution Accessory Kit (Merck, #71511)

Contour-clamped homogenous electric field (CHEF) gel electrophoresis system (e.g. Biorad, CHEF-DR II)

Troubleshooting

Preparation of the protoplasts

1 **Prepare a fresh culture of *M. oryzae* on complete medium agar plates.**

2 **Incubate at 24°C for 7 days.**

3 **Inoculate 150 ml of YG medium in a 500 ml Erlenmeyer flask with 8 pieces (~0.5 × 0.5 cm) of 7 days old *M. oryzae* mycelium.**

4 **Shake at 120 rpm at 24°C for 3 days.**

5 **Prepare lysing enzymes solution and filter sterilise it.**

6 **Filter culture through 2 layers of miracloth and transfer mycelia to a 50 ml falcon tube or 100 ml beaker.**

Note

Put the miracloth in a funnel on top of a bottle to collect the media.
Remove as much media residues as possible by squeezing gently.

7 **Add 15mL of lysing enzyme solution to the mycelia.**

8 **Place the tube on a horizontal shaker at room temperature for 2 to 4 h. Shake at 30 rpm.**

Note

Check the progress of protoplast formation under the microscope.

9 **Filter protoplasts through 2 layers of miracloth and collect the flow through.**

Note

Place the miracloth in a funnel on top of a 50mL falcon.

10 **Centrifuge flow through at 1500 rpm at room temperature for 10 min. Discard the supernatant.**

Note

Be careful not to dislodge the pellet. Use a pipette to remove supernatant.

11 **Add 25 mL of Sorbitol 1 M and centrifuge again. Discard the Supernatant and repeat (2 wash steps in total).**

12 **After the washing steps, remove as much of the supernatant as possible using a pipette. Do not disrupt the pellet.**

13 **Add 100-200 µl of 1M Sorbitol/50mM EDTA and resuspend the protoplast with a cut 1mL tip.**

Note

The protoplasts are very fragile! Pipette very slowly up and down or shake very gently to not damage the protoplasts.

14 **Check quality and concentration of protoplast under the microscope using a hemocytometer.**

Note

To visualize chromosome size fragments after CHEF gel electrophoresis and for subsequent DNA extraction, high protoplast concentration is required. Concentration of protoplasts should be ~ 3 x 10^9 protoplasts/ml.

Preparation of agarose gel plugs for contour-clamped homogenous electric field (CHEF) gel electrophoresis

15 **Mix 1/3 volume of protoplasts with 2/3 volume of 1% megabase agarose in 1M Sorbitol/50mM EDTA at 45°C.**

Note

Prepare the gel in advance and let it cool down in a water bath at 55°C.

16 **Immediately after mixing, pipette protoplast mix into Bio-Rad plug mould (#1703713) and let solidify on ice for at least 30 min.**

Note

Make sure to proceed quickly before the gel can solidify.
Tip: Pipette the liquid gel while keeping the tube in the water bath. Mix briefly by pipetting up and down twice and directly transfer the solution in the plug mould.
Alternatively, prepare protoplast/agarose gel mix in 1.5 ml Eppendorf tube and cut to size after the mix has solidified. For DNA extraction more than one plug might be required.

17 **Remove the protoplast-agarose plug from the mould. Transfer to a 1.5 ml Eppendorf tube and add 1 ml NDS buffer containing 200 µg/ml proteinase K.**

18 **Incubate at 50°C for 48 h.**

19 **Remove the NDS buffer, add 1 ml of fresh 50mM EDTA and shake slowly at room temperature for 30 min to 1 h. Repeat 3 times.**

Note

Washing steps can be carried out on a thermomixer set to 22°C and 200 rpm.

20 **Carefully remove EDTA solution and store the plugs in NDS buffer (without proteinase K) at 4°C until separation by CHEF gel electrophoresis.**

CHEF gel electrophoretic separation of mini-chromosomes

21 **Prepare 1% megabase agarose gel in 0.5% buffer (TAE or TBE buffer) and let solidify.**

Note

Use TBE buffer for better resolution during long runs and TAE buffer for extraction of DNA for library preparation. The borate in the TBE buffer can inhibit downstream enzymatic reactions.

22 **Place the agarose plugs in the wells. Place the size markers.**

Note

Saccharomyces cerevisiae and *Schizosaccharomyces pombe* chromosomes are commercially available and can be used as size markers (Biorad, #1703633, #1703605).

23 1. **Run the gel with following settings:**

a.Initial switch interval	120s
b.End switch interval	3600s
c.Voltage	1.8 - 2V/cm
d.Run time	96 h
e.Temperature	14°C

Note

Prepare the system early and let the circulating buffer cool down to 14°C for at least 30 min. Do not move the system during the run, constant buffer flow and temperature are important for good resolution. We developed this protocol on a Biorad CHEF DRII system. The settings specified above are optimized to separate chromosomes between 400 kb and 3 MB.

24 **To visualize chromosome sized fragments, place gel into ethidium bromide solution (1 µg/ml in 0.5% buffer) for 30 min and wash in 0.5% buffer (TAE or TBE) for 10 min and observe on a UV transilluminator.**

Extraction of chromosome sized fragments from agarose gel by electroelution

25 **Cut bands out of the gel using a scalpel and store gel slices in Eppendorf tubes at 4°C.**

Note

Remove as much of the gel as possible. The gel slice should have clean edges to prevent contamination of the buffer with agarose in the next step.

26 **Moisturize a D-Tube Dialyzer Midi 3.5 kDa MWCO (Merck, #71506) with 800 µl ddH_2O for 1 min. Check membrane for leaks.**

27 **Remove water with a pipette and place agarose gel slice into the chamber.**

Note

Do not touch the membrane with the pipette tip.

28 **Fill the chamber of the D-Tube with 0.5% TAE buffer and close the lid tightly. Place tube in electrophoresis rack (D-Tube Electroelution Accessory Kit, Merck, #71511). Place in electrophoresis chamber.**

29 **Run electroelution in 0.5% TAE buffer at 90 V for 3 h at room temperature.**

Note

Membranes should face in running direction. The chromosomal DNA will migrate out of the gel and gets collected in the buffer. Some DNA might stick to the membrane.

30 **Carefully resuspend DNA by pipetting up and down close to the membrane.**

Note

Do not touch the membrane with the pipette tip to prevent sample loss. Do not touch the gel slice with the pipette tip as this can lead to agarose contamination which can decrease efficiency during downstream enzymatic reactions or library preparation.

31 **Transfer DNA solution to a 1.5 ml eppendorf tube and concentrate in a speedvac concentrator.**

Note

Concentrate DNA solution to a volume of 20-50 µl. For library preparation adjust concentration following manufacturer's instructions. DNA concentration of 0.5 ng/µl is sufficient for low input library preparations. We recommend to use fluorometric methods to measure DNA concentration.

32 Proceed with Library preparation according to manufacturer’s instructions. We tested this protocol using a Nextera low input library preparation kit (Illumina) with custom barcoding primers and sequenced mini-chromosome derived libraries on a Nextseq 500 system.