

## Construction of Yeast Artificial Chromosome Libraries From Pathogens and Nonmodel Organisms

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### Summary

Many infectious diseases of global impact are caused by parasites. This includes diseases with protozoan etiology, such as malaria, African sleeping sickness, Chagas disease, toxoplasmosis, and amoebiasis, as well as diseases caused by metazoa, such as river blindness, schistosomiasis, echinococcosis, and ascariasis. Combined, parasitic diseases affect more than half the world's human population and are responsible for decreased gross national products and billions of dollars in lost earnings. Although the magnitude of the problem precludes quick solutions, there is reasonable hope that a better understanding of these organisms, especially the host–parasite interactions that underpin virulence and pathogenicity mechanisms, will provide new opportunities for rational intervention strategies. Yeast artificial chromosomes (YAC) have substantially aided in this endeavor by providing an unlimited access to defined parts of a parasite's genome, which, in turn, has facilitated a broad range of molecular studies. For example, YACs have facilitated positional cloning strategies to identify genes involved in antigenic variation and drug resistance mechanisms. Moreover, YACs have been invaluable tools for the many genome sequencing projects examining parasites. In this chapter, we provide a detailed protocol of how to generate representative YAC libraries from parasite genomes. This protocol can be applied to both protozoa and metazoa, and can even be used for YAC library construction of parasite material isolated from a single infected host.

**Key Words:** Parasite; YAC library; patient material; novel species.

### 1. Introduction

The discovery that yeast can accept large fragments of heterologous DNA as yeast artificial chromosomes (YAC) marked a breakthrough in the analysis of complex genomes (*1*). Although standard prokaryotic cloning systems, such as plasmids and cosmids, have a limited cloning capacity, YACs are able to incorporate large fragments of DNA ranging from between 30 kb and several

megabases (2–4). Access to large pieces of cloned DNA has facilitated a number of applications, including positional cloning of genes of interest, transcriptional mapping of chromosomal domains, and the physical mapping of chromosomes and entire genomes (5). Last but not least, the numerous genome sequencing projects would not have progressed as rapidly if it were not for YACs to assemble the short reads into long stretches of contiguous sequence information, identify gaps in the sequence, and help close them.

The basis of a YAC are the vector arms, which contain all functions necessary for mitotic segregation in yeast, including a centromeric sequence (CEN), an autonomous replication sequence (ARS), and telomere sequences (TEL). In addition, the vector arms harbor selectable markers (*TRP1* and *URA3*, mediating tryptophan and uracil autotrophy in suitable yeast hosts) and an interruptible marker containing the *EcoRI* cloning site (*SUP4-o*, mediating a red/white color selection for DNA insertion events). The most frequently used YAC vector is pYAC4 (1), which, besides the elements previously mentioned, contains the *Col EI* replication origin and the ampicillin selectable marker for propagation in *Escherichia coli*.

YAC libraries have been generated for numerous organisms, including human (2,3), mouse (2), bovine (6), *Arabidopsis* (7), rice (8,9), zebra fish (10), and several human pathogens, such as the human malarial parasites *Plasmodium falciparum* (11,12) and *Plasmodium vivax* (13,14), the etiological cause of Chagas disease, *Trypanosome cruzi* (15), and *Schistosoma mansoni*, an agent of schistosomiasis (16). In the case of *P. falciparum*, YAC technology has provided, for the first time, a stable source of DNA from this organism. *P. falciparum* DNA exhibits an unusually high A+T content that averages 82% and approaches 90–95% in intergenic regions. As a consequence of its high A+T content, *P. falciparum* DNA is unstable in *E. coli* and is subject to frequent recombination and deletion events. In addition to DNA fragments with a high A/T content, long inverted repeats or Z-structures also tend to be quite unstable in *E. coli* (17–19), but can be stably maintained in yeast as YACs (20–22). Generally, YACs appear to be more tolerant of DNA fragments with unusual properties than are other cloning systems, including plasmids, cosmids, and bacterial artificial chromosomes. Thus, in cases in which DNA stability causes concerns, YACs offer a viable method to generate representative libraries. An additional advantage of using a yeast cloning system is the opportunity to selectively clone a specific chromosomal region from a complex genome by methods termed transformation-associated recombination (TAR) cloning and radical TAR cloning (23,24). TAR cloning allows a specific region of interest, such as a large gap in a sequence, to be captured from a complex genome and transferred, by recombination, to a specific yeast cloning vector.

YAC technology has substantially aided in the analysis of pathogens and their disease-causing interactions with their respective hosts. A prominent example is the dissection of a 400-kb locus on the *P. falciparum* chromosome 7 containing a determinant of resistance to the formerly first line antimalarial drug, chloroquine (25). Other examples include the identification and characterization of multicopy gene families, such as the *P. vivax* *vir* (14) and the *P. falciparum* *var* gene family (26), which are involved in antigenic variation and other immune evasion mechanisms. The physical mapping of entire chromosomes by overlapping YAC clones and the subsequent genome project have revealed that these variant genes comprise large gene families with their members being predominantly located at subtelomeric domains (14,26,27) where they are subjected to frequent recombination events. Since its first description, YAC technology has been improved and simplified (4,28), rendering it almost a routine technique for laboratories with a background in molecular biology. The construction of a YAC library consists of the following steps:

1. Isolation of high quality genomic DNA.
2. Partial digestion of the genomic DNA.
3. Preparation of the YAC vector arms.
4. Ligation of the vector arms with the partially digested DNA.
5. Yeast transformation.
6. Library organization and maintenance.

The protocols detailed in this chapter were originally developed for the generation of YAC libraries from malarial parasites; however, the protocols can be adapted to any other pathogen, and may even be used to generate YAC libraries from scarce pathogen material isolated from a single patient or a single infected animal.

## 2. Materials

### 2.1. Embedding Chromosomal DNA in Agarose

1. 0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8.0. Autoclave and store at room temperature.
2. TSE buffer: 100 mM NaCl, 50 mM EDTA, and 20 mM Tris-HCl, pH 8.0. Autoclave and store at room temperature.
3. TE buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Autoclave and store at room temperature.
4. 1.25% InCert agarose (Biozym, Oldendorf, Germany) dissolved in TE buffer. Store at 4°C. Melt at 68°C and equilibrate at 42°C prior to use.
5. 2 mg/mL Proteinase K (Roche Applied Science, Mannheim, Germany) dissolved in 0.5 M EDTA, 1% *N*-lauryl-sarcosinate, pH 8.0. Prepare fresh.
6. T<sub>10</sub>E<sub>50</sub> buffer: 10 mM Tris-HCl and 50 mM EDTA, pH 8.0. Autoclave and store at room temperature.

7. 10X TBE buffer for pulse-field gel electrophoresis (PFGE): 890 mM Tris-base, 890 mM boric acid, and 20 mM EDTA, pH 8.0.
8. SeaKem LE agarose (Biozym).
9. Casting molds (Bio-Rad, Hercules, CA).
10. PFGE system (Bio-Rad).

## 2.2. Partial Digestion of Genomic DNA

1. 100 mM Phenylmethylsulfonyl fluoride (PMSF) in 2-propanol. Dissolve at 68°C and store at -20°C. (**Caution: very toxic.**) Very unstable in aqueous solution. Prepare fresh shortly before use.
2. T<sub>10</sub>E<sub>50</sub> buffer: 10 mM Tris-HCl and 50 mM EDTA, pH 8.0. Autoclave and store at room temperature.
3. TE buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Autoclave and store at room temperature.
4. 10 mg/mL Bovine serum albumin (Fraction V, Sigma, St. Louis, MO).
5. 10X *Eco*RI methylase buffer: 0.8 mM *S*-adenosyl-methionine, 20 mM MgCl<sub>2</sub>, 1 M NaCl, 10 mM dithiothreitol (DTT), and 0.5 mM Tris-HCl, pH 7.6. Store at -20°C.
6. 100 mM Spermidine. Store at -20°C.
7. 20 U/μL *Eco*RI (New England Biolabs, Ipswich, MA).
8. 40 U/μL *Eco*RI methylase (New England Biolabs).
9. 0.5 M EDTA, pH 8.0. Autoclave and store at room temperature.
10. 10 mg/mL Proteinase K (Roche Applied Science) dissolved in TE buffer. Prepare fresh.

## 2.3. Vector Preparation

1. pYAC4 vector (American Type Culture Collection, registration number: 67379).
2. 20 U/μL *Eco*RI (New England Biolabs).
3. 20 U/μL *Bam*HI (New England Biolabs).
4. 1 U/μL Calf intestinal phosphatase (New England Biolabs).
5. 10X Calf intestinal phosphatase buffer (New England Biolabs).
6. TE-saturated phenol/chloroform (1:1 v/v). Prepare fresh. Do not use red phenol.
7. 100% Ethanol.
8. 75% Ethanol.
9. TE buffer: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.
10. 10 U/μL T<sub>4</sub> polynucleotide kinase (New England Biolabs).

## 2.4. Ligation

1. 1X ligation buffer: 30 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.75 mM spermidine, 0.3 mM spermine, and 50 mM Tris-HCl, pH 7.6. Store at -20°C.
2. 10 U/μL T<sub>4</sub> polynucleotide kinase (New England Biolabs).
3. 100 mM Adenosine triphosphate, pH 7.5. Store at -20°C.
4. 1 M DTT.
5. 400 U/μL T<sub>4</sub> DNA ligase (New England Biolabs).

## 2.5. Size Fractionation

1. SeaKem low gelling agarose, molecular biology grade (Biozym).
2. 10X TBE buffer for PFGE: 890 mM Tris-base, 890 mM boric acid, 20 mM EDTA, pH 8.0.
3. Yeast chromosome size marker (New England Biolabs).
4. 10 mg/mL Ethidium bromide solution (**Caution: mutagenic**).
5. 1 U/ $\mu$ L  $\beta$ -agarase I (New England Biolabs).
6.  $\beta$ -agarase I buffer (New England Biolabs).
7. PGFE system (Bio-Rad).

## 2.6. Yeast Spheroplast Preparation and Transformation

1. *Saccharomyces cerevisiae* strain AB1380 (American Type Culture Collection, Palo Alto, CA; reference number: 20843).
2. YPD medium: 1% Bacto-yeast extract (BD Biosciences), 2% Bacto-peptone (BD Biosciences), pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
3. YPD agar: 1% Bacto-yeast extract (BD Biosciences), 2% Bacto-peptone (BD Biosciences), 2% Bacto-agar (BD Biosciences), pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
4. 0.5% Adenine hemisulfate (Sigma). Sterile filter the solution.
5. 1 M Sorbitol. Autoclave and store at 4°C.
6. Na-phosphate buffer, pH 7.6: 84.5 mL 1 M Na<sub>2</sub>HPO<sub>4</sub> and 15.5 mL 1 M NaH<sub>2</sub>PO<sub>4</sub> in 100 mL H<sub>2</sub>O.
7. SPEM buffer: 1 M sorbitol, 0.1 M Na-phosphate buffer, pH 7.6, and 10 mM EDTA. Sterile filter. Add 30 mM of  $\beta$ -mercaptoethanol prior to use.
8. 10 mg/mL of 20T Zymolase (MP Biomedicals, Irvine, CA) dissolved in 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5. Store in 100  $\mu$ L aliquots at -70°C. Use a fresh aliquot for each transformation.
9. STC solution: 1 M sorbitol, 10 mM CaCl<sub>2</sub>, and 10 mM Tris-HCl, pH 7.5. Sterile filter and store at 4°C.
10. PEG solution: 20% polyethylene glycol (molecular weight 8000), 10 mM CaCl<sub>2</sub>, and 10 mM Tris-HCl, pH 7.5. After autoclaving, the appropriate amount of a sterile 1 M CaCl<sub>2</sub> solution is added. A white precipitate may form in the solution on the addition CaCl<sub>2</sub>. However, this does not seem to interfere with the transformation efficiency.
11. SOS medium: 1 M sorbitol, 6.5 mM CaCl<sub>2</sub>, 0.25% Bacto-yeast extract (BD Biosciences), and 0.5% Bacto-peptone (BD Biosciences). Filter-sterilize and store at room temperature. Before use, add sterile-filtered 20  $\mu$ g/mL adenine, 20  $\mu$ g/mL histidine, 30  $\mu$ g/mL leucine, 30  $\mu$ g/mL lysine, 20  $\mu$ g/mL tryptophan, and 20  $\mu$ g/mL uracil.
12. AHC medium: 1 M sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 20  $\mu$ g/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.

13. AHC-TOP agar: 1 M sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 5% Bacto-agar (BD Biosciences), and 20 µg/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
14. AHC agar: 1 M sorbitol, 0.67% Bacto-yeast nitrogen base without amino acids (BD Biosciences), 1% casamino acids (BD Biosciences), 2% Bacto-agar (BD Biosciences), and 20 µg/mL adenine hemisulfate (Sigma). After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
15. 96- or 384-Well microtiter plates (Greiner, Frickenhausen, Germany).
16. Hematocytometer (Fisher Scientific, Nidderau, Germany).

### **2.7. Replication and Long-Term Storage of YAC Library**

1. YPD medium: 1% Bacto-yeast extract (BD Biosciences) and 2% Bacto-peptone (BD Biosciences). Adjust pH to 5.8. After autoclaving, add glucose to a final concentration of 2% from a stock of filter-sterilized 40% glucose.
2. YPD medium/glycerol (2:3).
3. 96 or 384 replicator (Sigma).

## **3. Methods**

### **3.1. Preparation of High-Quality DNA**

DNA is a fragile polymer that easily breaks when subjected to mechanical stress. It is therefore imperative to handle chromosomal DNA with the utmost care. Shearing is to be avoided, as is contamination with DNA-digesting nucleases, or exposure to X-rays or ultraviolet (UV) light (*see Note 1*). The preparation of high-quality chromosomal DNA begins with enriched or purified pathogens of interest (*see Note 2*) being embedded in agarose blocks. All subsequent manipulation steps are carried out using these agarose blocks. For protocols to grow, enrich, and purify the organism of interest the reader is referred to the specialized literature (*see Note 2*).

1. Resuspend cells in TSE buffer at a concentration of approx  $5 \times 10^8$  cells/mL. Use a 50-mL tube and place the cells at 37°C.
2. Add an equal volume of melted 1.25% InCert agarose equilibrated at 37°C and mix gently by tapping against the tube.
3. Using a 1-mL tip, slowly suck up the cell/agarose suspension and fill each casting mold with 100 µL of suspension. Keep the molds on ice for 5 min until the agarose hardens.
4. Push the hardened agarose blocks out of the molds with a flat object, such as a Pasteur pipet bent into an L-shape.
5. Place agarose blocks in proteinase K solution and incubate at 50°C for 48 h. Use 0.5 mL of solution for each block.

6. Wash blocks twice in  $T_{10}E_{50}$  buffer for 15 min at room temperature while gently shaking.
7. Store blocks in  $T_{10}E_{50}$  buffer at 4°C until use.
8. Check the quality of the DNA by PFGE analysis (0.4% SeaKem LE agarose gel; 0.5X TBE buffer; voltage, 2.7 V/cm; running time, 36 h; initial switching time, 120 s; final switching time, 720 s; temperature, 18°C). Each block should contain between 5 and 10  $\mu$ g of genomic DNA. (See **Subheading 3.5.** for more details on the preparation of the gel.)

### 3.2. Partial Digestion of Genomic DNA

Prior to digestion with restriction endonuclease, the blocks containing the DNA of interest need to be treated with PMSF in order to inactivate any residual proteinase K from the DNA preparation. Partially digested genomic DNA for YAC cloning is then generated using a mixture of the restriction endonuclease *Eco*RI and *Eco*RI methylase. The proper conditions need to be determined experimentally for each DNA preparation by titrating both enzymes against each other. The ratio of both enzymes determines the average DNA size, which, for YAC cloning, should range between 200 and 500 kb. It is recommended to start initially with 1 U of *Eco*RI and 30 U of *Eco*RI methylase per agarose block (5–10  $\mu$ g of DNA), and then stepwise increase the concentration of the methylase until a ratio of 1:320 is reached. In some cases it may be also necessary to titrate the concentration of *Eco*RI in order to generate DNA fragments of the desired average size. The size of the digested DNA is monitored by PFGE (see **Subheading 3.5.** for details on gel preparation and running conditions).

1. Wash blocks twice in  $T_{10}E_{50}$  buffer containing 1 mM of fresh PMSF (1 mL per block) for 30 min. Shake gently.
2. Wash blocks three times in  $T_{10}E_{50}$  buffer (1 mL per block) for 30 min. Shake gently.
3. Wash blocks three times in  $T_{10}E_1$  buffer (1 mL per block) for 30 min. Shake gently.
4. Transfer each block into a sterile 1.5-mL Eppendorf tube and add:
  - a. 50  $\mu$ L of bovine serum albumin (10 mg/mL).
  - b. 50  $\mu$ L of 10X *Eco*RI methylase buffer.
  - c. 13  $\mu$ L of spermidine (100 mM).
  - d. *Eco*RI restriction endonuclease.
  - e. *Eco*RI methylase.
  - f. Sterile  $H_2O$  to a final volume of 500  $\mu$ L.
5. Incubate at 37°C for 4 h.
6. Stop the reaction by adding:
  - a. 55  $\mu$ L of 0.5 M EDTA, pH 8.0.
  - b. 62  $\mu$ L of proteinase K solution (10 mg/mL).
7. Incubate at 50°C for 30 min.
8. Wash the blocks once in TE buffer at 50°C for 30 min. Shake gently.

9. Incubate the blocks in TE buffer containing 1 mM PMSF at 50°C for 30 min to inactivate the proteinase K (1 mL of solution per block). Shake gently.
10. Wash the blocks twice in TE buffer at 50°C for 30 min.
11. Check the size of the partially digested DNA by PFGE (see **Subheading 3.5.** for details on gel preparation and running conditions).

The genomic DNA is now ready for ligation.

### 3.3. YAC Vector Preparation

The left and right YAC cloning vector arms are liberated from the plasmid pYAC4 by digestion with the restriction endonucleases *Eco*RI and *Bam*HI. pYAC4 can be propagated in suitable *E. coli* strains, such as DH5 $\alpha$ .

1. Cleave purified pYAC4 plasmid DNA with *Eco*RI and *Bam*HI to completion.
2. Denature restriction endonucleases by heat treatment at 68°C for 10 min.
3. Dephosphorylate free 5' ends using a phosphatase (0.05 U/ $\mu$ g DNA) in 1X phosphatase buffer. Incubate at 37°C for 30 min. According to our experience, phosphatases from calf intestine or shrimp work equally well.
4. Add EDTA, pH 8.0 to a final concentration of 5 mM and incubate the reaction at 68°C for 10 min to inactivate the phosphatase.
5. Purify the DNA by phenol/chloroform extraction followed by ethanol precipitation.
6. Resuspend the DNA in TE buffer at a final concentration of 2  $\mu$ g/ $\mu$ L.

Digestion of the vector arms is monitored electrophoretically. Dephosphorylation is verified by ligation in the presence and absence of T<sub>4</sub> polynucleotide kinase. The ligation assays are then transformed into *E. coli*. In the case of the dephosphorylated vector arms, only a few, if any, colonies should grow, whereas a large number of colonies should appear when the vector arms are ligated in the presence of T<sub>4</sub> polynucleotide kinase.

### 3.4. Ligation

1. Wash agarose blocks three times in 1X ligation buffer at room temperature for 30 min.
2. Remove all liquid and place six agarose blocks into a 1.5-mL Eppendorf tube.
3. Add an equal amount of prepared vector arm DNA. Assuming that each of the six agarose blocks contains 5–10  $\mu$ g of genomic DNA, approx 60  $\mu$ g of prepared vector arm DNA are required. The mass ratio of 1:1 between genomic and vector arm DNA translates approximately into a 500-fold molar excess of vector arms vs genomic DNA (see **Note 3**).
4. Melt agarose blocks at 68°C for 10 min.
5. Allow genomic and vector arm DNA to equilibrate at 37°C for 2 h (see **Note 3**).
6. Add 1/10 vol of premade ligation mix (22 U/ $\mu$ L T<sub>4</sub> DNA ligase, 5 mM adenosine triphosphate, and 100 mM DTT in 1X ligation buffer). Pipet into the middle of the molten agar. Do not mix (see **Note 3**).

7. Remove 10  $\mu\text{L}$  of the sample and add 10 U of  $T_4$  polynucleotide kinase to it in order to verify ligation conditions. Incubate at 20°C for 12 h. Self-ligation of the vector arms will occur and is monitored electrophoretically using a conventional 1% agarose gel. As a control, run unligated vector arms on the same gel.
8. Allow the solution to equilibrate at 37°C for 1 h (**Note 3**).
9. Incubate the ligation reaction at 20°C for 24 h. The agarose will solidify during this time.

### 3.5. Size Fractionation

A size fractionation of the ligation reaction is highly recommended to remove the excess vector arms, as well as small YACs, which would otherwise be over-represented in the YAC library because of their higher transformation efficiency. Size selection is carried out using PFGE.

1. Prepare a 1% agarose gel in 0.5X TBE using a low gelling agarose, such as SeaKem molecular grade (**Note 4**). Tape up several teeth of the gel comb in order to generate a well large enough for the ligation sample. Load yeast chromosome size markers in the outside wells and seal all wells with molten agarose (1% in TBE buffer).
2. Run PFGE (voltage, 4.5 V/cm; running time, 18 h; initial switching time, 5 s; final switching time, 25 s; temperature, 14°C; field angle 120°, running buffer, 0.5X TBE).
3. After electrophoresis, cut off the gel lanes containing the size markers and a very small part of the lane containing the sample. Stain these pieces in 200 mL of running buffer containing 80  $\mu\text{L}$  of a 10- $\mu\text{g}/\text{mL}$  ethidium bromide solution for 30 min and examine them under UV light.
4. Mark the area of the gel that holds DNA fragments ranging from between 100 and 500 kb.
5. Reassemble the gel pieces with the rest of the gel which was kept in running buffer during this time to avoid drying out.
6. Excise the region containing the high molecular weight DNA using a sterile glass cover slip.
7. Place this gel slice into a 15-mL tube. Store at 4°C until use.
8. Stain the entire gel as described in **step 3**, examine by UV light, and take a picture for documentation.
9. Wash the gel slice containing the size-fractionated DNA three times for 20 min each in  $\beta$ -agarase buffer.
10. Melt the gel slice containing the size-fractionated DNA at 68°C for 10 min.
11. Allow the sample to cool to 42°C, add  $\beta$ -agarase I to a final concentration of 50 U/mL, and incubate at 42°C for 3 h. The DNA is now ready for transformation into yeast spheroplasts.

### 3.6. Yeast Spheroplast Preparation and Transformation

*S. cerevisiae* strain AB1380 is used as the host in YAC (1). As a selective medium we routinely use AHC-medium. The AHC medium is as selective as synthetic minimal media, allows for red/white color selection of positive transformants, but is much easier to prepare (Note 5).

1. For high transformation efficiency, plate AB1380 yeast cells from a frozen glycerol stock onto a YPD plate, and incubate the plate at 30°C for 48 h.
2. Take a single red colony and prepare an overnight culture in YPD medium supplemented with 0.002% adenine hemisulfate.
3. Inoculate 50 mL of YPD medium supplemented with 0.002% adenine hemisulfate with the fresh overnight culture to obtain an OD<sub>600</sub> of 1.0. Use a 500-mL Erlenmeyer flask with bumps. Incubate the culture at 30°C in a shaking incubator (180 rpm) until an OD<sub>600</sub> of 4.0 is reached. This corresponds to approx  $3 \times 10^7$  cells/mL. The cell density should double every 90 min.
4. Collect the cells by centrifugation at 400–600g for 10 min at room temperature.
5. Wash the cells once with 20 mL sterile water and centrifuge as described in step 4.
6. Wash once with 20 mL of 1 M sorbitol and centrifuge as described in step 4.
7. Resuspend the cells in 20 mL of 30°C prewarmed SPEM buffer containing fresh  $\beta$ -mercaptoethanol. The cell density should be close to  $7.5 \times 10^7$  cells/mL. Remove sample, dilute it 10-fold in water, and determine the OD<sub>600</sub>. The OD<sub>600</sub> value obtained serves as the prespheroplast reference.
8. Add 45  $\mu$ L of 20T Zymolase solution. Incubate cells by slowly shaking in a 30°C water bath for about 20 min. The extent of spheroplast formation is determined spectrophotometrically. Every 5 min a 50- $\mu$ L aliquot is removed, diluted 10-fold in water, and the OD<sub>600</sub> value determined. As spheroplasts lyse in water owing to the lack of a cell wall, the reduction in the OD<sub>600</sub> value is directly proportional to the amount of spheroplasts formed. Eighty to 90% of the cells must be spheroplasts within 20 min to achieve optimal transformation efficiency. If not, it is strongly recommended to repeat the preparation.
9. Collect the spheroplasts by centrifugation at 200–300g for 4 min at room temperature.
10. Gently resuspend the cells in 20 mL of 1 M sorbitol, and centrifuge as described in step 9.
11. Wash the spheroplasts once in 20 mL of STC, centrifuge as in step 9, and finally resuspend them in 2 mL of STC. At this point, the spheroplasts are stable at room temperature for at least an hour. The cell density is now determined microscopically using a hemacytometer. Adjust the volume to a final cell concentration of  $6.5 \times 10^8$  cells/mL.
12. To 150  $\mu$ L of spheroplasts, add 50  $\mu$ L of  $\beta$ -agarase I treated size fractionated DNA (approx 80 ng) in a 15-mL tube (use a 1-mL tip with a widened opening). Incubate at 20°C for 10 min. Scaling up the reaction significantly reduces transformation efficiency (Note 6). It is, therefore, recommended to run parallel assays.

13. Add 1.5 mL of PEG solution and mix by gently inverting the tube. Incubate at 20°C for 10 min.
14. Immediately centrifuge at 200–300g for 4 min at room temperature.
15. Carefully decant the supernatant and resuspend cells in 225  $\mu$ L of SOS solution. Incubate at 30°C for 30 min.
16. Gently resuspend the settled spheroplasts. Add 8 mL of molten AHC-TOP agar (prewarmed to 50°C) and gently invert to mix. Quickly pour onto an AHC plate that has been prewarmed to 37°C (**Note 5**). For yeast transformation, AHC-Top agar and AHC plates need to contain 1 M sorbitol to osmotically buffer the spheroplasts. Allow plates to sit for 10 min at room temperature.
17. Incubate plates at 30°C for 7 d. The first yeast clones should be visible after 2–3 d. Those yeast clones that contain artificial chromosomes turn red on AHC-plates, whereas revertants and clones harboring pYAC4 remain white.

Red colonies are picked and transferred to 96- or 384-well microtiter plates containing 150 or 80  $\mu$ L of YPD medium, respectively. Cells are grown for 2–3 d at 30°C.

### 3.7. Replication and Long-Term Storage of YAC Library

1. Using a 96- or 384-replicator, transfer YAC clones to a new set of microtiter plates containing an appropriate amount of YPD media in each well. Use a sterile replicator for each microtiter plate to avoid cross-contamination of YAC clones. Metal replicators can be sterilized as follows: dip the pins of the replicator in 80% ethanol, flame, and then cool for several seconds on a sterile YPD agar plate.
2. Grow YAC clones for 2–3 d at 30°C. During incubation approximately one-third of the media will evaporate. The YAC library can be stored at 4°C for up to 3 mo or can be used to make frozen glycerol stocks.
3. To freeze down the YAC clones, add an appropriate volume of YPD media/glycerol (2:3 v/v) to each well to yield a final glycerol concentration of 20%. In the case of 96-well plates approx 50  $\mu$ L YPD media/glycerol is usually added. Resuspend the cells in the medium, using a multichannel pipet.
4. Cells can now be stored at –80°C.

## 4. Notes

1. Success greatly depends on the quality and purity of the starting DNA material. DNA that is partially degraded, or enzymatically or chemically modified, will never produce a YAC library. Therefore, shearing and exposure of the DNA to UV light or X-rays are to be avoided. UV light and X-rays can introduce strand breakages, depurination, and cross-linking events into the DNA. Exposure to X-rays becomes a problem when DNA is shipped by air freight as all packages, not only hand luggage and carry-ons, are routinely X-rayed for safety inspections. It is, therefore, best to deliver chromosomal DNA in person and, if a flight is involved in the travel, to keep the DNA on your person.

2. In some cases it may be necessary to collect pathogen material from patients or infected animals, for instance when an in vitro culture system is not available. An example is the malarial parasite *P. vivax*, which propagates within reticulocytes. As reticulocytes cannot be readily obtained in large quantities, in vitro culture conditions for *P. vivax* are difficult to establish. Access to parasite DNA, therefore, relies on material obtained from *P. vivax*-infected patients or monkeys. If pathogen material for library construction is collected from host organisms, great care needs to be taken in order to remove any contaminating nucleated cells from the host prior to DNA preparation. For the construction of a *P. vivax* YAC library, we devised a two-step purification protocol (13). Host leukocytes were initially removed from the erythrocytes using Plasmodipur filters (Organon Teknika). Erythrocytes infected with *P. vivax* were then concentrated using a single step 16% Nycodenz (Sigma) gradient. Nycodenz is resuspended in phosphate buffered saline and centrifugation is carried out at 900g for 30 min at 15°C. The resulting material was found to be free of contaminating human DNA, as verified by both Southern blotting and polymerase chain reaction analyses (13). Recently, we use a strong magnetic field to purify erythrocytes infected with malarial parasites (29). Malaria parasites degrade the erythrocyte's hemoglobin, thereby liberating large amounts of iron-containing heme. In the oxidative environment of the parasite's food vacuole, where hemoglobin degradation occurs, ferrous iron ( $\text{Fe}^{2+}$ ) is oxidized to ferric iron ( $\text{Fe}^{3+}$ ), which because of its magnetic properties allows infected erythrocytes to be retained in a magnetic field, whereas uninfected erythrocytes freely pass through this field. Magnetic devices and columns can be purchased from Miltenyi Biotec, Germany.
3. Do not speed up the process by pipetting or vortexing. Sheared DNA will never produce a high-quality YAC library.
4. Be careful when handling low gelling agarose gels, as they are slippery and prone to breakage.
5. It has been observed that YAC transformants often do not grow on synthetic minimal medium selective for both tryptophan and uracil autotrophy. This phenomenon has been attributed to the weak *TRP* promoter, which limits expression of the *TRP* gene product. To overcome this problem selective pressure can be applied sequentially, first for uracil and subsequently for tryptophan autotrophy. Alternatively, transformants can be selected on AHC-medium as described herein. AHC medium is initially rich in amino acids. However, autoclaving breaks down most of the tryptophan. The residual amounts of tryptophan do not support permissive growth of tryptophan auxotrophic clones, yet they are sufficient to allow the cells to recuperate after transformation and express the *TRP* gene encoded by the YAC vector arm.
6. It is tempting to scale up the yeast transformation reaction. However, there is ample evidence that scaling up the reaction significantly reduces transformation efficiency. Although processing and handling many reaction tubes at the same time is cumbersome and time consuming, it is still faster than repeating the experiment.

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