**Lab. Work 13.**

**A Protocol Guide for the N. crassa Yeast Artificial Chromosome Library**

1. **Construction of Yeast Artificial Chromosomes**
2. **Preparation of a YAC library filter set**

A yeast artificial chromosome (YAC) library of Neurospora crassa strain 74-OR23-1A has been constructed.

This library has been used to clone 750 kb of contiguous DNA sequences from the centromere region of linkage group VII.

The purpose of this article is explicitly to outline procedures that have been developed for library screening and chromosome walking. The library was constructed in the YAC vector pYAC4.

This vector contains *Tetrahymena telomere*,

*Saccharomyces cerevisiae CEN4, and ARS1 DNA sequences* that specify in cis full telomeric, centromeric and replication functions in *S. cerevisiae*.

YAC clones are maintained in approximately single copy as highly stable linear "artificial chromosomes". pYAC4 also encodes the yeast TRP1 and URA3 for selection of YAC clones in the yeast host strain AB1380 MATa ade2-1 can1-100 lys2-1 trp1 ura3 his5 {psi+}.

A model of a YAC clone is shown in **Fig. 1.**

The N. crassa YAC library contains 2204 clones, with an average insert size of 170 kb. Inserts from 40 clones have been characterized and range in size from 75-260 kb.



**Figure 1.** A schematic of a typical clone from the N. crassa YAC library is shown. The markers shown are described in the text.

The following wells in the library contain cultures that will not grow or grow slowly upon inoculation: 1:6A l:llH 2:8F 2:11E 5:9G 6:5F 6:11E 10:1F 10:6A 10:9B l l:lH 11:2G 11:6A 12:7D 12:9C 14:3D 14:3E 14:9E 17:3G 17:6F 17:9H 17:10H 18:3E 19:7G 19:9F 20:2D 20:5E 20:7H 21:4F 22:3D 22:4A 22:9C 23:11D 24:4A 24:4B 24:4C.

There are no clones in columns 5 to 12 on dish 24. Additionally, well 21:3E was contaminated with cells from well 21:3D. At least 5% of the clones besides those listed did not grow or grew poorly upon inoculation from a third generation copy of the YAC library distributed by the Fungal Genetics Stock Center (C. Yamashiro, unpublished observations).

**Chromosome Walking**
1. A protocol is outlined for hybridization screening of the YAC library. Positive YAC clones obtained from screening the library must be verified by *Southern analysis*. Mixed cultures within an individual well can occur; therefore, streak out cultures for single colonies on selective plates.

Prepare genomic DNA from one or more individual colonies, and perform genomic Southern blot hybridizations with these DNAs as outlined below.

2. Several of the YAC clones obtained from a given library screening should be physically mapped with restriction endonucleases and the resultant maps compared. They should fit into a single set of contiguously overlapping sequences referred to as a "contig". Construction of a contig map assures the continuity of individual clones and avoids possible confusion resulting from multiple insert clones, whose restriction maps will not fit into the consensus maps generated from the bona fide majority of clones.

3. DNA fragments containing the terminal ends of the inserts of YAC clones which map to the periphery of the contig can be subcloned into E. coli or amplified directly (as described below). These fragments can then be used as hybridization probes for chromosome walking, or RFLP mapping.

***Hybridization screening of the YAC library***.

1. **Preparation of a YAC library filter set.**

1. Positively charged nylon membranes (MSI Inc, Westboro, MA) are layered onto the surface of large petri plates containing SD medium with casamino acids and adenine, "SD + CAA + A" (a supplemented minimal medium).

Cultures from microtiter wells are replicated onto marked membranes using a multi-pin replicator. Each microtiter plate should be replicated onto a single membrane. Growth will take 3 to 4 days at 30oC. Fast growing colonies can be allowed to grow quite large (0.5 mm or larger) to allow the slow growing colonies to be well established (other protocols prefer smaller colonies so that cells are actively growing but lysis of old colonies works well here).

**2. Prepare spheroplasting buffer CES/DTT/YLE (CDY).** For 40 ml: Add 80 mg yeast lytic enzyme, 70,000 units/gram (ICN Biochemicals Irvine, CA) and 300 ml of 2 M dithiothreitol (DTT) to 40 ml CES solution (1 M sorbitol 100 mM Sodium citrate pH 7.0, 50 mM EDTA pH 8.0).

**3. Place Whatman 3 MM** or equivalent in lid of 150 mm Petri dish. Add approximately 7.0 ml CDY to Whatman and remove any bubbles (only a small amount of excess liquid should be present in lid). Carefully place a membrane containing YAC colonies onto the CDY saturated paper. Carefully remove bubbles from under the membrane.

**4. Place the b**ottom of the Petri dish over the membrane and seal dish well with parafilm. Incubate 2 days 30-32oC.

**5. Colony lysis** and DNA denaturation is done by placing the membranes sequentially on Whatman 3MM paper saturated with the following solutions at room temperature for the times shown:

a. 10% SDS/100 ug/ml proteinase K for 10 min.
b. 0.5 N NaOH for 10 min.
c. Three washes of 200 mM Tris pH 7.5/2X SSC for 5 min each.

**6. Air dry the membranes** on 3 MM paper for 2 h minimum (can go overnight). Bake for 2 h in vacuum oven at 80oC. Wrap membranes in plastic wrap (do not stack membranes, arrange them side to side). Store at -20oC.

After plasmid DNA purification, two distinct digestions are performed:

the first with BamHI that cuts twice adjacent to the two telomeric DNA sequences flanking the HIS3 gene, which therefore is excised from the plasmid and lost (Figure 2a). This first digestion generates a long linear fragment carrying telomeric sequences at each end.

The excision of the HIS3 gene is used as negative selective marker for uncut pYAC molecules.

The second digestion consists of the opening of the cloning site within the SUP4 gene (Figure 2a). As a result of this second digestion, two linear fragments are produced as left and right arms of the future linear YAC (Figure 2b).

The selective markers are thus separated: TRP1 adjacent to ARS1 and CEN4 on the left arm and URA3 on the right arm. Large DNA fragments with ends compatible to the cloning site, obtained from the desired genome source by digestion with an appropriate restriction endonuclease, are ligated with phosphatase-treated YAC arms, to create a single yeast-transforming DNA molecule (Figure 2c). Primary transformants can be selected for complementation of the ura3 mutation in the host, and successively for complementation of the host trp1 mutation, thereby ensuring the presence of both chromosomal arms.

Transformant colonies containing the exogenous DNA insert within the SUP4 gene are detected by their *red colour*, due to the inactivation of the suppressor activity and the consequent accumulation of a red metabolic precursor in ade host cells.

***Biological Features of YACs.***

The stability of YAC vectors in yeast per se is similar to that of natural chromosomes (1025/1026) provided that all three structural elements (ARS,CENand TEL) are present and functional and, in addition, that the minimal required size is reached by the insertion of enough exogenous DNA. However, the genetic and biochemical background of the host cell also plays an important role in determining the stability of YACs. Indeed, several mutations are known to affect YAC stability and segregation together with natural chromosomes.

 For example, alterations in the expressions of genes such as BUB1, BUB2 and BUB3, MAD1, MAD2 and MAD3, CDC6, CDC20, PDS1 and others lead to chromosome losses, including YACs. Another important consideration is that faithful duplication of YACs is guaranteed only if other DNA sequences incompatible with ARS do not exist on the construct. This point is particularly relevant when unknown DNA inserts are cloned in the YAC vector, as is the case for genomic libraries, in which there could be cryptic or otherwise unknown ARS-like sequences able to interfere with the ARS function.

***Modifying YACs by Homologous Recombination***

*Depending on the experimental systems and the yeast strains, different selectable markers and restriction sites are appropriate on th eYAC vectors. These can be constructed in vitro by standard techniques and then used for subcloning DNA fragments.*

 *Additionally, existing YAC clones can be modified by homologous recombination in yeast, a process called ‘retrofitting’ (Figure 3). Accordingly, genetic markers can be modified by simply transforming YAC-containing yeast cells with a disruption cassette carrying the desired genetic marker flanked by short DNA sequences homologous to one of the markers present on the artificial chromosome. The same result can be obtained by using one arm of the artificial chromosome carrying the new genetic marker as the transforming molecule. As shown in Figure3a, the introduction of the yeastLYS2 gene, together with the mammalian selectable marker Neo, into the URA3 gene on the right arm of a YAC is achieved by using recombination techniques. Neo is the usual marker gene for subsequent selection of mammalian cells after YAC transfection. Moreover, disruption of this marker provides a selectable replacement marker for introducing specific genetic changes within the exogenous DNA insert (Figure 3).*

*A yeast integrative plasmid (YIp) carrying the mutated copy of an exogenous DNA segment and the functional copy of a previously disrupted marker can be used for this purpose. After plasmid integration, the target DNA is duplicated in tandem via homologous recombination, generating a YAC with one wild-type and one mutant copy of insert DNA (‘pop-in’). At this stage, YACs with only the mutated copy of the marker can be obtained after spontaneous recombination within duplicated DNA regions (‘pop-out’) by growing the transformed cells in the absence of selection pressure for several generations (Figure 3b). However, the frequency is not very high (from 1024 to 1025 per generation) and there is approximately the same probability for both the mutated and wild-type copy of the marker to remain within the cell on the YAC vector. In the latter case it is necessary to perform additional molecular tests of the obtained ‘popouts’.*