

# **EMBO COURSE**

## **Practical Course on Genetic and Molecular Analysis of *Arabidopsis***

### **Module 7**

#### **PHYSICAL MAPPING IN *ARABIDOPSIS***

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## 1. INTRODUCTION

Physical mapping of the five chromosomes was one of the top priorities in Arabidopsis research. Coverage of the entire genome with overlapping DNA clones (contig) represents a major transition step from gene- to genome-orientated biology. The impact of even a partial physical map of Arabidopsis is already palpable in many areas of research today. For example, many mutations based on phenotypic analyses can be mapped genetically relative to anchored molecular markers (for example, RFLPs) and thus allowing rapid access to existing contigs without chromosome walking. Conversely, genes or any DNA fragments can also be assigned a genetic position by virtue of hybridization to particular clones in a mapped contig. Furthermore, a complete physical map is necessary as the template for whole genome sequencing, which in turn, would undoubtedly accelerate the discovery of new genes and revolutionize the scope of biological questions that can be addressed. For those interested, some of these timely issues of genome analyses (Arabidopsis and other model organisms) and ensuing implications have been discussed in the journal *Science*, the Genome Issue (Vol. 282, Oct 23, 1998).

For physical mapping of the Arabidopsis genome, two different types of genomic banks are most frequently used: YACs (Yeast Artificial Chromosomes) and BACs (Bacterial Artificial Chromosomes).

The purpose of using YACs is to provide a rapid coverage of the entire Arabidopsis genome. Several YAC banks are available. The CIC bank has been particularly useful by virtue of the large insert sizes (450 kb) and very low content of chimeric clones. Furthermore, a large number of these YACs are anchored with molecular markers and their approximate genetic positions are known, which allows a virtually complete coverage of the Arabidopsis genome by anchored YAC contigs. These features make them as attractive tools to map new genes by simple hybridization or PCR. References of partial to nearly complete coverage of the five chromosomes are given at the end of this chapter.

YACs are, however, time-consuming to prepare as templates for many facets of sequencing projects mainly because they are difficult to purify in large amounts (for example, needed for shot-gun cloning). To circumvent these problems, genomic banks constructed in BACs (Bacterial Artificial Chromosomes) have been favored since BACs are propagated in standard *E. coli* hosts and can be recovered by routine "mini-prep" methods. Also, the cloned inserts are smaller, about 100 kb, and are thus more manageable for sequencing purposes. Participants in Arabidopsis Genome Initiative program from Europe, Japan and the United States have agreed on a strategy that combined BAC end sequencing, fingerprinting, hybridization with anchored YACs and molecular markers as starting points to spread across the genome to begin sequencing contiguous clusters of BACs with minimal overlaps. Thus, we would expect a progressive shift from YACs to BACs in the future as the units of reference of the physical maps. Another group at the Kazusa Institute in Japan has opted for sequencing with P1 Artificial Chromosomes (PACs) (<http://www.kazusa.or.jp/arabi/>). How sequences and map positions from BACs and P1 are eventually cross-referenced remains to be resolved.

For general information on high capacity cloning vectors consult Monaco, AP, Larin, Z (1994). YACs, BACs, PACs and MACs: Artificial Chromosomes as Research Tools. *Trends Biotech.* 12: 280-286.

## 2. EXPERIMENTS

The goal of the experiments are to learn basic techniques in manipulation and screening of large-insert genomic libraries in YACs and BACs.

Three experiments are planned : PCR-screening of the Arabidopsis CIC YAC library ; hybridization screening of the IGF BAC library ; analysis of large-insert clones by pulsed-field gel electrophoresis (PFGE).

Protocols used in the lab courses are grouped at the end of this chapter, together with other useful protocols , references and web resources.

## 2.1. Experiment 1 : Screening of Arabidopsis YAC and BAC libraries

Half of the students will work on PCR screening of the CIC YAC library, and the other half on hybridization screening of IGF BAC filters.

### 2.1.1. PCR Screening of the CIC YAC library on 3-D Pools

The construction of the CIC YAC library has been described in Creusot et al. (1995). This library is comprised of 12 microtiter plates (1152 clones). Clones in this library have been grouped in pools according to a 3-dimensional matrix arrangement that allows rapid screening of the entire library by a PCR approach (see 3.1.1).

DAY 1 (Monday June 7<sup>th</sup>)

Materials provided :

- PCR 96-well plate with DNA templates (3.1.1.2)
- PCR reagents
- PCR oligonucleotide primers (10  $\mu$ M)
- ddH<sub>2</sub>O

- Keep all reagents on ice. Put the PCR plate in a rack kept on ice.
- On ice, prepare a master mix with all reagents except Taq Polymerase (3.1.1.2). You have 87 templates. Prepare a master mix for 90 reactions to allow for pipetting errors.
- Switch on the PCR machine, select the appropriate program according to the primers to be used. Turn the heated lid on, then put the machine on “ pause ” at 94°C.
- Once the machine is ready, add Taq polymerase to the mix, mix well, centrifuge.
- Distribute 20  $\mu$ l reaction mix in each well using an electronic distributor. Put the silicone rubber cap firmly. Place the plate in the machine and start the program.
- While the PCR is running, prepare a 2% agarose gel in TBE 1x (10x25 cm<sup>2</sup>), with two 51-teeth combs). You will need 120 ml of gel solution. Each gel can accomodate an entire 96 well plate.
- Once the program is finished (~2h), remove the plate, carefully remove the cap and add 5  $\mu$ l loading dye per well using a multichannel distributor.
- Load the gel with a multichannel pipettor, according to the arrangement shown in 3.1.1.3.

- Run the gel and take a picture.

### 2.1.2. Hybridization Screening of the IGF BAC library

The construction of the IGF BAC library has been described in Mozo et al. (1998). This library is comprised of 28 384-well microtiter plates (10,752 clones). Hybridization filters for the entire library are available upon request from Arabidopsis stock centers (see 3.2).

DAY 1 (Monday June 7<sup>th</sup>)

Materials provided :

- IGF BAC filter (purchased from RZPD)
- Probe DNA (10 ng/ $\mu$ l)
- Labelling reagents (3.2.1.1)
- Hybridization buffer (3.2.1.2)

- Probe labelling reactions : Clearly mark a screw-cap Eppendorf tube. Prepare 20 ng probe in 29  $\mu$ l (3.2.1.1). Denature in boiling water 3' and keep on ice.
- Add buffer and nucleotide mix (3.2.1.1).
- We'll add the label and Klenow enzyme.
  
- Put the membrane in a hybridization tube, add 50 ml hybridization buffer, and incubate at 42°C.
  
- At the end of the day, remove 30 ml from the prehybridization, denature probe (3' in boiling water, 3' on ice) and add the probe to the tube. Incubate at 42°C O/N.

DAY 2 (Tuesday June 8<sup>th</sup>)

- Wash filters according to 3.2.1.2.
  
- Expose for a few hours on a PhosphorImager screen, or O/N on a classical X-Ray film.

### 2.2. Experiment 2 : PFGE Analysis of BAC clones (all the students)

The goal is to establish the size of the insert in a few selected BAC clones. The first step is to release the inserted fragment (which is flanked by NotI sites in the vector pBeloBAC-Kan) and to run the digest on a PFGE gel (BioRad CHEF system). Regular digests of BAC clones will also be performed.

DAY 1 (Tuesday June 8<sup>th</sup>)

Materials provided :

- BAC DNA
- Restriction enzyme buffers (10x)
- Restriction enzymes

The BAC clones are digested with NotI (to release the insert) and with several restriction enzymes to fingerprint the insert.

- Digest 1 µg BAC DNA in 12 µl final volume. Use BglIII, ClaI, DraI, EcoRI, HindIII, NotI, XbaI. Incubate 1h30 at 37°C (Use this gap to wash the BAC blots).
- In the mean time, prepare the agarose gels :
  - one regular, 25 cm-long agarose gel (1% in 0.5x TBE)
  - one CHEF gel (1% agarose in 0.5x TBE)
- Add 2 µl loading dye to the digests.
- Load BglIII, ClaI, DraI, EcoRI, HindIII, XbaI digests along with size standards on the regular gel and run the electrophoresis (40V, O/N).
- Load the NotI digest along with undigested BAC DNA, and size markers (lambda concatemers, Yeast chromosomes, 1 kb DNA marker (BRL)) on the CHEF gel. Run in TBE 0.5x with buffer circulation and cooling at 150V, 16h, 5 seconds switch time.

DAY 2 (Wednesday June 9<sup>th</sup>)

- Stop the gels, incubate in Ethidium Bromide solution for 15 min, rinse in water, take picture.

### 2.3. Experiment 3 : Physical Mapping data on the web

(Wednesday June 9<sup>th</sup>)

The results of YAC and BAC mapping experiments will be analyzed and discussed, and compared to data available on internet databases (see part 4).

## 3. PROTOCOLS

### 3.1. YAC PROTOCOLS

#### 3.1.1. YAC library screening by PCR on three-dimensional DNA pools

##### 3.1.1.1. Preparation of DNA Pools

- Grow 3 replicas of the CIC YAC library in 150 µl super-AHC medium in microtitre plates at 28°C without agitation. After 24h of culture, reinoculate poorly growing clones from the glycerol master stock.

Super-AHC selective medium

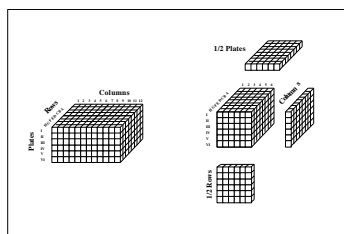
Yeast Nitrogen Base w/o amino acids	0.255%
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.75%
Glucose	3%
Casamino acids	1.65%

Drop-out powder w/ adenine, w/o tryptophan and uracil 0.195%

- After 1 additional day of culture, mix clone cultures according to the following scheme : the library is divided into two parts, plates I-VI and plates VII-XII ; then each pile is theoretically divided again in two (columns 1-6 and columns 7-12) to give four super-pools ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) in matrix arrangement. Each super-pool contains six half-microtiter plates (288 clones) and represents roughly one genome-equivalent (see Figure).

Using the three replicas, clones are mixed along each dimension, resulting in twenty 3-D pools for each super-pool (6 pools of half-plates (48 clones each), 8 pools of half-rows (48 clones each), 6 pools of columns (36 clones each)). The whole library is therefore represented in a total of 80 pools (see Figure).

Each mixture of clone cultures (about 5 ml) is extracted using a standard yeast DNA miniprep procedure (Ausubel et al., 1995). Final DNA concentrations are 100-200 ng/ $\mu$ l. For PCR screening, DNA stocks are diluted 50-fold.



#### • DNA Pools

##### Pool $\alpha$ :

Plates	(6x8)	$\alpha$ I	$\alpha$ II	$\alpha$ III	$\alpha$ IV	$\alpha$ V	$\alpha$ VI		
Rows	(6x6)	$\alpha$ A	$\alpha$ B	$\alpha$ C	$\alpha$ D	$\alpha$ E	$\alpha$ F	$\alpha$ G	$\alpha$ H
Columns	(6x8)	$\alpha$ 1	$\alpha$ 2	$\alpha$ 3	$\alpha$ 4	$\alpha$ 5	$\alpha$ 6		

##### Pool $\beta$ :

Plates	(6x8)	$\beta$ I	$\beta$ II	$\beta$ III	$\beta$ IV	$\beta$ V	$\beta$ VI		
Rows	(6x6)	$\beta$ A	$\beta$ B	$\beta$ C	$\beta$ D	$\beta$ E	$\beta$ F	$\beta$ G	$\beta$ H
Columns	(6x8)	$\beta$ 7	$\beta$ 8	$\beta$ 9	$\beta$ 10	$\beta$ 11	$\beta$ 12		

##### Pool $\gamma$ :

Plates	(6x8)	$\gamma$ VII	$\gamma$ VIII	$\gamma$ IX	$\gamma$ X	$\gamma$ XI	$\gamma$ XII		
Rows	(6x6)	$\gamma$ A	$\gamma$ B	$\gamma$ C	$\gamma$ D	$\gamma$ E	$\gamma$ F	$\gamma$ G	$\gamma$ H
Columns	(6x8)	$\gamma$ 1	$\gamma$ 2	$\gamma$ 3	$\gamma$ 4	$\gamma$ 5	$\gamma$ 6		

##### Pool $\delta$ :

Plates	(6x8)	$\delta$ VII	$\delta$ VIII	$\delta$ IX	$\delta$ X	$\delta$ XI	$\delta$ XII		
Rows	(6x6)	$\delta$ A	$\delta$ B	$\delta$ C	$\delta$ D	$\delta$ E	$\delta$ F	$\delta$ G	$\delta$ H
Columns	(6x8)	$\delta$ 7	$\delta$ 8	$\delta$ 9	$\delta$ 10	$\delta$ 11	$\delta$ 12		

## 3.1.1.2. PCR Screening of 3-D YAC Pools

## • DNA templates

For each primer pair, several DNAs are tested :

negative controls : TE, Yeast DNA (4 ng/ $\mu$ l),

positive control : Columbia genomic DNA (5 ng/ $\mu$ l)

Pools  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  total DNA (5 ng/ $\mu$ l)

80 3-D Pool DNAs (20 for each super-pool)

All the DNAs (5  $\mu$ l) are prepared in a 96-well PCR plate in the following arrangement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TE	$\alpha$ I	$\alpha$ II	$\alpha$ 3	$\alpha$ 4	$\beta$ G	$\beta$ H	$\gamma$ C	$\gamma$ D	$\delta$ XI	$\delta$ XII	
B	Col-0	$\alpha$ III	$\alpha$ IV	$\alpha$ 5	$\alpha$ 6	$\beta$ 7	$\beta$ 8	$\gamma$ E	$\gamma$ F	$\delta$ A	$\delta$ B	
C	Yeast	$\alpha$ V	$\alpha$ VI	$\beta$ I	$\beta$ II	$\beta$ 9	$\beta$ 10	$\gamma$ G	$\gamma$ H	$\delta$ C	$\delta$ D	
D	$\alpha$	$\alpha$ A	$\alpha$ B	$\beta$ III	$\beta$ IV	$\beta$ 11	$\beta$ 12	$\gamma$ 1	$\gamma$ 2	$\delta$ E	$\delta$ F	
E	$\beta$	$\alpha$ C	$\alpha$ D	$\beta$ V	$\beta$ VI	$\gamma$ VII	$\gamma$ VIII	$\gamma$ 3	$\gamma$ 4	$\delta$ G	$\delta$ H	
F	$\gamma$	$\alpha$ E	$\alpha$ F	$\beta$ A	$\beta$ B	$\gamma$ IX	$\gamma$ X	$\gamma$ 5	$\gamma$ 6	$\delta$ 7	$\delta$ 8	
G	$\delta$	$\alpha$ G	$\alpha$ H	$\beta$ C	$\beta$ D	$\gamma$ XI	$\gamma$ XII	$\delta$ VII	$\delta$ VIII	$\delta$ 9	$\delta$ 10	
H		$\alpha$ 1	$\alpha$ 2	$\beta$ E	$\beta$ F	$\gamma$ A	$\gamma$ B	$\delta$ IX	$\delta$ X	$\delta$ 11	$\delta$ 12	

Keep the plate on ice while preparing reaction mixes in a 2-ml Ependorf tube (kept on ice also).

## • PCR Reaction mix

Diluted DNA stock	5 $\mu$ l	(10-20 ng)	x90	x100
10x PCR Buffer	2.5 $\mu$ l	(1x final)	225 $\mu$ l	250 $\mu$ l
25 mM MgCl <sub>2</sub>	2.5 $\mu$ l	(2.5 mM final)	225 $\mu$ l	250 $\mu$ l
dNTPs (10 mM each)	0.5 $\mu$ l	(200 $\mu$ M final)	45 $\mu$ l	50 $\mu$ l
Primer 1 (10 $\mu$ M)	1.0 $\mu$ l	(10 pmoles)	90 $\mu$ l	100 $\mu$ l
Primer 2 (10 $\mu$ M)	1.0 $\mu$ l	(10 pmoles)	90 $\mu$ l	100 $\mu$ l
Taq DNA polymerase	1.0 $\mu$ l	(1 unit)		90 $\mu$ l
$\mu$ l				100
H <sub>2</sub> O	11.5 $\mu$ l	(qsp 25 $\mu$ l)	1035 $\mu$ l	1150 $\mu$ l

[PCR buffer 10x : 100 mM Tris-HCl pH 9 at 25°C, 500 mM KCl, 1% Triton X-100]

PCR reactions are performed in a PTC100-96 thermal cycler (MJ Research) in polycarbonate 96-well PCR plates sealed with a silicone-rubber cap and heated-lid on.



Cycle conditions :

	2 min at 94°C
40 cycles	15 sec at 94°C
	15 sec at 50°C
	30 sec at 72°C
	2 min at 72°C.

### 3.1.1.3. Gel electrophoresis

- Add 5 µl of loading dye to each sample (using an electronic multichannel distributor). Prepare a 2% agarose gel sufficient to accommodate all the samples (we use a Biorad 25x10 cm<sup>2</sup> gel, with two 51-teeth combs).

- Load the samples (10 µl each) using a multichannel pipet. The samples should be organized as follows (L=ladder ; we use the “ 1 kb ladder ” from BRL) :

Comb#1 : L • TE • Col • Yeast • α β γ δ • αI-αVI • αA-αH • α1-α6 • βI-βVI • βA-βF • L  
 Comb#2 : L • βG,βH • β7-β12 • γVII-γXII • γA-γH • γ1-γ6 • δVII-δXII • δA-δH • δ7-δ12 • L

Positive clones are determined by the intersection of three positive planes ; for example αIII + αG + α5 means clone CIC3G5 is positive with the tested marker.

### 3.1.1.4. Confirmation

Individual clones are checked using a 1/200 dilution of the individual clone DNA isolated as described below. When multiple positive clones were detected in the same super-pool, all the possible intersections need to be tested individually.

## 3.1.2. YAC library screening by hybridization

### 3.1.2.1. Preparation of YAC DNA in MT plates

The 1152 clones of the CIC YAC library were each grown individually in 1 ml of selective medium (super-AHC) in 1.5 ml microtube racks (96-well format) at 28°C without agitation. After 24h of culture, clones growing poorly were inoculated again from the glycerol master stock. After 1 additional day of culture, 800 µl of medium were aseptically removed without disturbing the yeast pellet using a home-made 96-channel pipettor, and replaced with fresh medium. The cultures were further incubated overnight at 28°C. The following day, most of the culture supernatant was removed without centrifugation, and the yeast cells were resuspended with a multi-channel pipetman in the remaining medium (ca. 200 µl) and transferred to regular MT plates. Following centrifugation in a swing-out rotor (1200 rpm, 3 min), the medium was completely removed using the 96-channel pipettor. The yeast pellets were then resuspended in 120 µl TES (100 mM Tris-HCl pH 8.0, 100 mM EDTA, 0.9 M Sorbitol, 0.2% β-mercaptoethanol, 2 mg/ml Zymolyase 20-T (Seikagaku)) using a multi-channel pipetman, and incubated for 2 h at room temperature. Following spheroplasting, cells were pelleted by centrifugation (1200 rpm, 5 min) and the supernatants removed using the 96-channel pipettor. The spheroplasts were then lysed by resuspension in 70 µl TNLE (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1% Lithium Dodecyl Sulfate, 5 mM EDTA) and incubation 1h at room temperature. DNA was then precipitated by addition of 70 µl

isopropanol. The plates were centrifuged (4000 rpm, 10 min), and the pellets rinsed with 100  $\mu$ l 70% ethanol, and dried on the bench. The final pellets were resuspended in 50  $\mu$ l TE + RNase A (0.01 mg/ml), incubated for 1 h at 50°C, and then stored frozen at -20°C.

### 3.1.2.2. Preparation of DNA filters

For preparation of hybridization filters, 20  $\mu$ l of these DNA stocks were transferred to 384-well plates containing 5  $\mu$ l of 5x denaturing solution (0.05 % xylene-cyanol, 2N NaOH, 0.05% SDS). Denatured DNAs (ca. 0.2  $\mu$ l) were simultaneously transferred to 12 x 8 cm<sup>2</sup> Hybond N+ membranes (Amersham) using a disposable 384-pin inoculator. The amounts indicated here were enough for at least 80-90 membranes.

Membranes were prehybridized for a few hours in Church hybridization buffer (250 mM Na<sub>2</sub>HPO<sub>4</sub> pH 7.4, 7% SDS, 2 mM EDTA, 0.2 mg/ml heparin, 0.1 mg/ml denaturated salmon-sperm DNA) at 65°C in an hybridization oven. The probe was added and hybridization performed for 24 h at 65°C. Following hybridization, the membranes were washed 15 min in 2xSSC, 0.5% SDS, 0.2% Na pyrophosphate at 55°C and 5-10 min in 0.2xSSC, 0.5% SDS, 0.2% Na pyrophosphate at 55°C. Membranes were autoradiographed against regular X-ray films or the BAS-1500 system (Fuji).

### 3.1.2.3. Preparation of YAC end probes

YAC end probes from yUP and CIC clones were either prepared using vectorette-PCR as described by (Matallana et al., 1992), or by right- and left-arm rescue in *Escherichia coli*.

#### 3.1.2.3.1. Left-arm rescue

For left-arm rescue, the YAC clone was grown in 5 ml super-AHC medium at 28°C. A standard yeast DNA miniprep was performed (Ausubel et al., 1995), and 500 ng were digested to completion either by XhoI or NdeI. The DNA was then precipitated, and self-ligated in 400  $\mu$ l total volume with 10 units of T4 DNA ligase. After precipitation, the DNA was resuspended in 10  $\mu$ l H<sub>2</sub>O, and 2  $\mu$ l were electroporated into competent *E. coli* DH5 $\alpha$  cells, which were plated on LB medium containing 200 mg/l ampicillin. The end probe was isolated from the vector sequences using EcoRI and XhoI or NdeI.

#### 3.1.2.3.2. Right-arm rescue

For right-arm rescue, we used the ability of the URA3 gene to complement pyrF mutations in *E. coli* (Rose et al., 1984). We used strain SØ5009, a pyrF derivative of MC1061 (a gift from K. Schnorr, Denmark) (araD139, ?(ara-leu)7647, ?lac74, galU, hsdR, rpsL, pyrF30). DNA was extracted from YAC clones as above and 500 ng were digested with XhoI, and co-precipitated with 500 ng pBluescript II-SK+ that had been cut by XhoI and dephosphorylated. Ligation was performed in a total volume of 20  $\mu$ l with 1 unit T4 DNA ligase. The ligation mixture was then digested by BamHI or XbaI and precipitated again. A second ligation was performed in diluted conditions (400  $\mu$ l total volume with 10 units T4 DNA ligase) to favour circularisation. After precipitation, the DNA was resuspended in 10  $\mu$ l H<sub>2</sub>O, and 4  $\mu$ l were electroporated into competent *E. coli* SØ5009 cells, which were plated on M9 selective medium (Sambrook et al., 1989), supplemented with 200 mg/l ampicillin. Clones appeared after 2 days incubation at 37°C. This two-step cloning procedure dramatically improved the number of complementing clones recovered. The clones obtained were then analysed by restriction. The end probe was isolated from the vector sequences using EcoRI and BamHI or XbaI.

### 3.2. BAC PROTOCOLS

Arabidopsis BAC membranes are available from the Ohio Stock center (ABRC) (<http://aims.cps.msu.edu/aims/>) or RZPD (<http://www.rzpd.de>), the German DNA stock center which provides excellent IGF BAC membranes. RZPD membranes will be used in this EMBO course.

#### 3.2.1 BAC library screening by hybridization to a radioactive probe

BAC filters can be hybridized with labelled probes according to the following protocol:

##### 3.2.1.1. Labelling reactions

- In a screw-cap Eppendorf tube, mix :

DNA	5-20 ng
H <sub>2</sub> O	qsp 29 µl

Heat denature (3 min, 100°C), put on ice for 3 min, then add :

OLB 5x	10 µl
dNTPs 10x	5 µl
<sup>32</sup> P-dCTP	5 µl
Klenow (6 u/µl)	1 µl

- mix, spin down, incubate at 37°C for 5h-Overnight.
- Heat denature (3 min, 100°C), put on ice for 3 min, add to prehybridization buffer.

OLB 5x : PIPES pH 6.6 500 mM ; MgCl<sub>2</sub> 25 mM ; DTT 50 mM ; random primers 1.25 µg/µl (Pharmacia 27-2166-01) ; BSA 1 µg/µl (BRL)  
dNTPs 10x : dATP, dGTP, dTTP 200 µM each in Tris-HCl pH 7.5 10 mM

##### 3.2.1.2. Hybridization

Hybridization buffer:

200 mM Na-phosphate pH 7.2
1mM EDTA
15% formamide
1% BSA
7% SDS

- For prehybridization, incubate the filter for two hours in at least 50 ml hybridization buffer at 42°C.
- For hybridization, exchange the hybridization buffer (reduce the volume to 20 ml), add the freshly denatured radiactively labelled probe and incubate o/n at 42°C.
- Wash the filter twice with 2x SSC, 0.1% SDS at 60°C for 20 to 30 min.

- Rinse with 2x SSC and expose x-ray film. If washing at higher stringency is required, reduce the SSC concentration in the washing solution down to 0.2x SSC and incubate twice at 60°C for 20 min.
- For removal of the probe (stripping) pour boiling 0.1% SDS onto the filter and leave it shaking for 30 min at 60°C. Filters can usually be reused for more than 10 times.

### 3.2.2 BAC end-probes (T. Altmann protocol)

[http://www.mpimp-golm.mpg.de/101/mpi\\_mp\\_map/bac\\_end\\_prep.html](http://www.mpimp-golm.mpg.de/101/mpi_mp_map/bac_end_prep.html)

Preparation of BAC ends by IPCR:

IPCR protocol for the amplification of pBelo-BAC ends (pBeloBAC-Kan which we used to generate the IGF-BACs is a pBeloBAC derivative):

For the IPCR use 1/10 of a standard plasmid miniprep from 2 ml o/n culture.

- Restriction digest 1

for the T7 end with HhaI or EcoRV or HincII or RsaI  
for the Sp6 end with HhaI or HaeIII or EcoRV

As the (unpredictable) size of the product is determined by the digest, it is recommended to test all these enzymes in parallel.

Phenol/chloroform/isoamylalcohol extract and ethanol precipitate after the digest.

- Ligation

in 100 µl reaction volume, >3 h RT  
then 15 min. 70°C (heat inactivation) and ethanol precipitation.

- Restriction digest 2

In 10 µl reaction volume, >1 h,  
for the T7 end with PvuI  
for the Sp6 end with BsrBI

- PCR:

take 5 µl of the restriction digest 2 for a 100 µl reaction volume (2.5 mM MgCl<sub>2</sub>); perform 25 cycles (1 min. 94°C, 1.5 min. 56 °C, 2 min. 72°C); conditions may need modification depending on the PCR-machine.

primers for the T7 end :      5'-TTC CCA ACA GTT GCG CAG C-3'  
   5'-TCT TCG CTA TTA CGC CAG CT-3'

primers for the Sp6 end:      5'-TCA CAC AGG AAA CAG CTA T-3'  
   5'-ACA CAA CAT ACG AGC CGG AA-3'

#### 4. ARABIDOPSIS THALIANA PHYSICAL MAPPING RESOURCES

**Most web sites and services are accessible from the Stanford Arabidopsis website :**

**<http://genome-www.stanford.edu/Arabidopsis/>**

**The Arabidopsis genomic view (a resource for viewing and querying integrated genetic and physical maps) : <http://genome-www.stanford.edu/Arabidopsis/maps.html>**

#### **YAC Libraries**

EW (150 kb) *Ward E.R. and Jen G.C. (1990) Isolation of single-copy-sequence clones from a yeast artificial chromosome library of randomly-sheared Arabidopsis thaliana DNA. Plant Mol. Biol. 14, 561-568.*

EG (150 kb) *Grill E. and Somerville C. (1991) Construction and Characterization of a Yeast Artificial Chromosome Library of Arabidopsis Which is Suitable for Chromosome Walking. MGG 226, 484-490.*

YUP (250 kb) *Ecker J.R. (1990) PFGE and YAC Analysis of the Arabidopsis Genome. Methods 1, 186-194.*

CIC (480 kb) *Creusot F., Fouilloux E., Dron M., Lafleuriel J., Picard G., Billault A., Le Paslier D., Cohen D., Chabouté M.-E., Durr A., Fleck J., Gigot C., Camilleri C., Bellini C., Caboche M. and Bouchez D. (1995) The CIC library : a large insert YAC library for genome mapping in Arabidopsis thaliana. Plant J. 8, 763-770.*

#### **YAC contig maps**

Chromosome 1 :

<http://genome.bio.upenn.edu/physical-mapping/ch1-WWW.html>

Chromosome 2 :

<http://weeds.mgh.harvard.edu/goodman/Pmap/c2.shtm>

*Zachgo, Wang, Dewdney, Bouchez, Camilleri, Belmonte, Huang, Dolan & Goodman (1996) A Physical Map of Chromosome 2 of Arabidopsis thaliana. Genome Research 6 : 19-25*

Chromosome 3 :

<http://genome-www.stanford.edu/Arabidopsis/Chr3-INRA/>

*Camilleri, Lafleuriel, Macadré, Varoquaux, Parmentier, Picard, Caboche & Bouchez (1998). A YAC Contig Map of Arabidopsis thaliana chromosome 3. Plant Journal 14 : 633-642*

Chromosome 4 :

<http://genome-www.stanford.edu/Arabidopsis/JIC-contigs.html>

*Schmidt, West, Love, Lenehan, Lister, Thompson, Bouchez & Dean (1995) Physical map and organization of Arabidopsis thaliana chromosome 4. Science, 270, 480-483*

Chromosome 5 :

<http://genome-www.stanford.edu/Arabidopsis/JIC-contigs.html>

*Schmidt, Love, West, Lenehan & Dean (1997) Detailed description of 31 YAC contigs spanning the majority of Arabidopsis thaliana chromosome 5. Plant Journal 11, 563-573.*

### **BAC Protocols on the Web**

[http://www.mpimp-golm.mpg.de/101/mpi\\_mp\\_map/bac.html](http://www.mpimp-golm.mpg.de/101/mpi_mp_map/bac.html) (T. Altmann pages)  
<http://www.tamu.edu :8000/~creel/TOC.html> (Texas A&M BAC training manual)  
<http://www.tree.caltech.edu/> (Caltech site)  
<http://hubcap.clemson.edu/~schoi/BAC.html> (Clemson University BAC Center)  
<http://www.bio.cornell.edu/biotech/BIBAC/BIBACHomePage> (the BIBAC Page at Cornell)

### **BAC Libraries**

IGF Library (7x coverage, 10,752 clones, 100 kb)

[http://194.94.225.1/101/mpi\\_mp\\_map/bac.html](http://194.94.225.1/101/mpi_mp_map/bac.html)

*Mozo et al. (1998) Construction and characterization of the IGF Arabidopsis BAC library. Mol. Gen. Genet. 258, 562-570*

TAMU Library

*Choi S., Creelman R.A., Mullet J.E. and Wing R.A. (1996) Construction and characterization of a bacterial artificial chromosome library of Arabidopsis thaliana. Weeds World 2, 17-20 & Plant Mol Biol Rep 13, 124-128, 199*

### **BAC Physical Map**

FPC Assembly

<http://genome.wustl.edu/gsc/arab/arabsearch.shtml>

11,440 TAMU clones, 11,145 IGF clones fingerprinted

396 contigs produced, (45 clones per contig, 300 kb average contig size)

IGF Assembly

[http://194.94.225.1/101/mpi\\_mp\\_map/bac.html](http://194.94.225.1/101/mpi_mp_map/bac.html)

*Mozo et al. (1998) Use of the IGF BAC library for physical mapping of the Arabidopsis thaliana genome. Plant J. 16, 377-384*

27 contigs, 8285 IGF-BAC clones and 141 connecting TAMU-BAC clones

## **5. REFERENCES**

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995). Current Protocols in Molecular Biology, K. Janssen, ed.: John Wiley & Sons, Inc.).

Matallana, E., Bell, C. J., Dunn, P. J., Lu, M., and Ecker, J. R. (1992). Genetic and physical linkage of the *Arabidopsis* genome: methods for anchoring yeast artificial chromosomes. In *Methods in Arabidopsis research*, C. Koncz, N. H. Chua and J. Schell, eds. (Singapore: World scientific), pp. 144-169.

Rose, M., Grisafi, P., and Botstein, D. (1984). Structure and function of the yeast URA3 gene : expression in Escherichia coli. *Gene* 29, 113-124.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning : a laboratory manual*, C. S. H. L. Press, ed. (Cold Spring Harbor, New-York).