# Preparation of YAC DNA for oocyte microinjection

CURRENT PROTOCOL *Lluís Montoliu*
Last update: 2003

Based on Andreas Schedl's and Clare Huxley's original protocols with modifications by Thorsten Umland and Lluis Montoliu.

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## Part I: Large scale preparation of agarose plugs of yeast DNA

1) Inoculate 200 ml of selectium medium for YACs (i.e. AHC or drop out medium, SD-W-U) with 1 ml of an O/N culture. Use 1 liter flasks. Let it grow at 30°C with shaking (180-250 rpm) for 1-2 days until saturation.
NOTE: Saturated cultures in AHC medium would have about $1x10^8$ cells/ml whereas in drop out medium the concentration achieved might be somewhat lower. Alternatively, inoculation can be done with a single colony. In this case, let the cells grow for 3-5 days until saturation.

2) Count the number of yeast cells with an hemocytometer. Use a 1/20 dilution (i.e. 50 µl of the saturated culture plus 950 µl of sterile water or medium). The total number of cells is obtained as follows:

Number of cells present within the 16 central squares x $10^4$ x dilution factor

3) Spin down the cells at 600 g for 5 minutes at RT. (=3000 rpm in an Heraeus midi centrifuge with the 50 ml Falcon tubes adaptor and swinging buckets). Discard the medium.
NOTE: From now on, work with this cells is best done in 50 ml Falcon tubes.

4) Wash the cells by resuspending them in 50 mM EDTA pH=8.0. Use 40 ml of this solution per 100 ml of original media. Spin down the cells as in step 2. Repeat this washing step one more time with 10-20 ml of in 50 mM EDTA pH=8.0. Discard the medium.
NOTE: This washing steps can be used to pool all cells into one single pellet.

5) Weigh the pellet of cells and assume a density of 1 g/ml.

6) Warm briefly the pellet at 37-40ºC (about 30 seconds) and thereafter add enough prewarmed **Solution I** to give a final concentration of $8x10^9$ yeast cells / ml. Carefully resuspend the cells.
NOTE: Take into account the volume of the pellet before adding the solution I. Normally, yeast cells should be more than 50% of the final volume. As a rule of thumb, the volume of liquid added should be kept as small as possible, the cell suspension must be thick. The lower the volume the higher the concentration of DNA in the agarose plugs. Sometimes, counting yeast cells and estimating total cell numbers can be unaccurated. In this cases, it is reccommended to resuspend the cells with a volume of buffer equal or similar to the volume of cells.

7) Immediately add an equal volume of prewarmed (37-40ºC) **Solution II**. Mix quickly and pipette 80 µl aliquotes (LKB-Pharmacia system, Gene Navigator Unit) into block formers (plug mold) previously placed on ice and bottom-sealed with tape.This will make a final concentrarion of $4x10^9$ yeast cells / ml of agarose plug.
NOTE: Use a cut off yellow tip to distribute the agarose solution into the plug molds. Proceed as fast as possible and avoid trapping air-bubbles in the plugs. Every 20 seconds or so shake the tube with cells and agarose in the water bath (37-40ºC) to prevent for too early solidification. 80 µl aliquotes represent about $3.2x10^8$ cells / agarose plug.

8) Chill on ice for 10 minutes until the agarose plugs solidify.

9) Transfer agarose plugs into **Solution III**. Use 8 ml of solution per ml of plug. Incubate at 37ºC for 2-3 hours with gentle agitation (less than 100 rpm). Alternatively, incubate in a water bath inverting occasionally.
NOTE: This is one of the most important steps and will determine the quality and yield of your DNA. An unefficient spheroplasting step will result in very poor yield. Correspondingly, overdigesting the sample or using enzymatic batches of lower quality might result in DNA degradation and/or bad electrophoretic mobilities.

10) Decant the solution III and replace it with identical volume of **Solution IV** (8 ml of solution per ml of plug). Incubate at 37ºC with gentle agitation for at least 1 hour. Then, replace buffer with fresh Solution IV and continue incubating O/N at 37ºC with gentle agitation.
NOTE: This step can also be performed at higher temperatures (37-50ºC).

11) Next day, decant buffer and wash the agarose plugs with 20% **NDS buffer**. Use 8 ml of buffer per ml of plug. Proceed for 2 hours with gentle agitation at RT. Repeat wash one or two times. Agarose plugs can be loaded directly onto PFGE gels or stored in this buffer at 4ºC.
NOTE: For better resolution and PFGE running conditions it is reccommended to equilibrate the agarose plugs with running buffer or TE pH=8.0 before loading the gels. Equilibration is performed with at least 4 consecutive washes of 30 minutes each in excess of buffer. Alternatively, plugs can also be stored in sterile 0.5 M EDTA pH=8.0 for longer periods (months) at 4ºC.

Solutions:

**Solution I**

| | For 10 ml: |
|---|---|
| ~1 M Sorbitol | 10 ml of Sorbitol 1 M (Merck, autoclaved) |
| 20 mM EDTA pH=8.0 (autoclaved) | 400 µl of EDTA 0.5 M pH=8.0 |
| 14 mM β-mercaptoethanol | 10 µl from stock (=14 M, Merck) |
| 2 mg/ml Zymolyase-20T | 20 mg (Zymolyase-20T, ICN #320921) |

NOTE: Prepare always fresh. Do not store it. Zymolyase might not get easily into solution. Then, try to use an uniform suspension.

**Solution II**

| | For 10 ml: |
|---|---|
| ~1 M Sorbitol | 10 ml of Sorbitol 1 M (Merck, autoclaved) |
| 20 mM EDTA pH=8.0 (autoclaved) | 400 µl of EDTA 0.5 M pH=8.0 |
| 2% SeaPlaque GTG agarose (FMC #50112) | 0.2 g |
| 14 mM β-mercaptoethanol | 10 µl from stock (=14 M, Merck) |

NOTE: Prepare fresh. Melt the Sorbitol plus EDTA and agarose in the microwave. Avoid boiling over. Equilibrate in a water bath down to 37-40°C. Then add the β-mercaptoethanol and old the solution at 37-40°C until needed.

**Solution III**

| | For 100 ml: |
|---|---|
| ~1 M Sorbitol | 100 ml of Sorbitol 1 M (Merck, autoclaved) |
| 20 mM EDTA pH=8.0 | 4 ml of EDTA 0.5 M pH=8.0 (autoclaved) |
| 10 mM Tris-HCl pH=7.5 (autoclaved) | 1 ml of Tris-HCl 1 M pH=7.5 |
| 14 mM β-mercaptoethanol | 100 µl from stock (=14 M, Merck) |
| 2 mg/ml Zymolyase-20T | 200 mg (Zymolyase-20T, ICN #320921) |

NOTE: Prepare fresh and only the required amount of it.

**Solution IV**

| | For 500 ml: |
|---|---|
| 1% dodecyl lithium sulfate | 5 g (Sigma #L-4632) |
| 100 mM EDTA pH=8.0 (autoclaved) | 100 ml of EDTA 0.5 M pH=8.0 |
| 10 mM Tris-HCl pH=8.0 (autoclaved) | 5 ml of Tris-HCl 1 M pH=8.0 |
| Sterile $H_2O$ | up to 500 ml |

NOTE: Filter sterilize (0.22 µm). Store at RT.

**100% NDS Buffer**

Mix 350 ml $H_2O$ with 93 g of EDTA and 0.6 g of TRIS base. Equilibrate pH to > 8.0 with pellets of solide NaOH (about 100-200 pellets). Then, add 5 g of N-laurylsarcosine (Sigma) pre-dissolved in 50 ml of $H_2O$. Equilibrate the pH to 9.0 with 10 M NaOH and bring the final volume to 500 ml with $H_2O$. Filter sterilize (0.22 µm). Store at 4°C.

Part II: **Purification of YAC-DNA for microinjection**

1) Pour a 1% SeaPlaque GTG (FMC) LMP PFGE gel using a comb with a preparative slot in the center. This can be achieved joining consecutive wells with tape in order to cover 4-5 cm.
NOTE: During all steps throughout the protocol you should work with gloves, using always sterile solutions, reagents, glass- and plastic-ware. When possible, use material only dedicated for the preparation of YAC DNAs.

2) Load the agarose plugs vertical and consecutively into preparative slot (6-9 blocks, depending on the size).Include on either side marker lanes with a very small slice of the same batch of agarose plugs. Include as well, on either side, additional marker lanes with lambda DNA multimers (NEB or Boehringer Mannheim) and/or endogenous chromosomes from standard yeast strains (NEB or Boehringer Mannheim).
NOTE: Make sure that you have not enclosed air bubbles within the slots.

3) Cover all slots with 1% SeaPlaque GTG (FMC) LMP, let it solidify and start the gel with appropiate running conditions to ensure optimal resolution in the desired size range.
NOTE: There are many PFGE running conditions. For example (LKB-Pharmacia system, Gene Navigator Unit): to isolate a 250 kb YAC we have used 0.25x TAE buffer (1x TAE contains 40 mM Tris-acetate pH=8.0, 2 mM EDTA pH=8.0) at 10°C, running at 250 V with a pulse of 9 seconds for 10 hours followed by a pulse of 15 seconds during 6 hours. To cover a wide range of chromosomal sizes (50-2000 kb) we have used 0.5x TAE buffer at 10°C, running at 180 V with a pulse of 30 seconds for 12 hours followed by a pulse of 60 seconds during 15 hours. Avoid using too much running buffer within the PFGE tank (not more than 2 mm over the surface of the gel) to improve the quality of the chromosomal separation.

4) After the gel run cut off marker lanes plus a small part of the preparative lane on either side and stain these two external parts with Ethidium Bromide for 30 minutes with very gentle agitation at RT using the very same running buffer. Add 50 µl of 10 mg/ml Ethidium Bromide stock solution per 1 liter of running buffer.
NOTE: See enclosed graphic to better understand the whole process described in the next steps of the protocol. The central part of the gel containing most of the preparative slot remains unstained in running buffer,

5) Mark under UV-light the precise location of the YAC chromosomal band on either side of the gel. Use a razor-blade and indicate with nicks on the agarose gel the position of the desired DNA bands. Mark the position of two additional chromosomal bands too (any of the endogenous yeast chromosomes) to be used as a marker lanes in the second electrophoresis.
NOTE: Usually, the most proximal endogenous chromosomal bands, above and below the YAC, are selected for markers.

6) Put the stained parts of the PFGE gel next to the preparative central lane and carefully cut out the YAC containing agarose slice with a sterile razor-blade using the marked nicks and a ruler as a guide. Thereafter, remove equivalent slices corresponding to the two endogenous marker lanes for the second gel run.

NOTE: Try to produce an agarose slice not thicker that 5-6 mm. The slices will be 4-5 cm long. Take into account any smiling effect or abnormal running artifact to precisely follow the expected position of the YAC chromosomal band.

7) Equilibrate the agarose slice containing YAC DNA in the following equilibration buffer for a minimum of 2 hours (can be left O/N at +4°C):

10 mM **Bis**-Tris-HCl pH=6.5
0.1 mM EDTA
100 mM NaCl
0.030 mM Spermine
0.070 mM Spermidine

NOTE: Prepare it always fresh. Do not store it.Use polyamines x1000.

8) Decant last buffer and transfer the gel slice onto a sterile surface (i.e. the inner side of a cell-culture plate). Carefully remove all drops of buffer by sucking with a tissue paper.

9) Weigh a sterile Eppendorf tube. Carefully transfer the gel slice ino this tube and weigh again to calculate the weigh (and hence, the volume) of the slice.

10) Melt the slice for 10 min at 65°C.

11) Spin tube for 5 seconds to bring down all drops.

12) Add 4-8 units of Gelase (Epicentre, or Agarase from NEB) per 100 mg of gel slice with a cut off yellow tip.
NOTE: Briefly prewarm the yellow tip with the enzyme (i.e. prepare the volume of enzyme to add in advance and leave it at RT for a few minutes). This is important to avoid immediate solidification of the gel upon contacting a solution normally stored at -20°C.
We don't use any special buffer for the Gelase digestion in order to minimize the reagents which get in contact with the YAC DNA and to reduce the presence of additional toxics which might impair the survival ratio of microinjected oocytes. Gelase does work, albeit less efficiently, in Equilibration buffer.
(NOTE: It is possible, and works equally well, to substitute the Agarase from Epicentre by the equivalent enzyme from NEB, #392, 1 u/µl)

13) Immediately place the tube in a water bath at +40°C. After 5 minutes, take the tube and with a cut off yellow tip gently pipette up and down 2 or 3 times. Immediately return the tube to the water bath and proceed with the agarose digestion for 2-3 hours at +40°C.
NOTE: Every hour, the sample can be carefully mixed by pipetting up and down 2 or 3 times with cut off tips.

14) Chill the tube on ice for 5-10 minutes and check for the completeness of the agarose-gel digest. The appearance of pale brown or opaque clouds within the tube clearly indicates that the digestion has not been complete. In this case, go back to step 10 and perform a second incubation with additional enzyme. Normally, after two consecutive digestions there should not be any rest of agarose.
NOTE: This is, again, a very important step. From now on it will be very difficult to get rid of any agarose piece left over undigested. They can cause troubles clogging the microinjection needle and eventually result toxic for the embryos. Thus, make sure that you go ahead only if you are absolutely convinced that the agarose has been totally digested.

15) Spin at Vmax for 20 min (to get rid of undigested agarose bits)

16) Transfer up to 400 µl of the digested agarose with YAC-DNA (carefully, with a cut-off blue tip) into the upper reservoir of a Millipore ultrafiltration Unit (either use Ultrafree. MC 30,000 NMWL #UFC3 TTK 00 from Millipore, or Microcon-30 #42409 from Amicon, Inc.)

17) Spin for 2 min at 6000 rpm. Check the volume that has passed through the membrane and, if necessary, continue with the centrifugation step (in rounds of 2 min.) until about 320 µl have already passed through the membrane.

NOTE: Most of the required volume should pass within the first 10 min (5 rounds of centrifugation).

18) Let the tubes stand at +4ºC for a few hours (best left O/N). Resuspend the YAC-DNA (possibly attached to the surface of the membrane) by pipetting up and down with a cut-off yellow tip (max. 2-3 times) very carefully (slowly!).
NOTE: This is the most risky step where you might shear/brake your YAC-DNA prep.

19) Prepare a Petri dish with 40 ml of **Microinjection Buffer**. Carefully place a Millipore dialysis filter (Millipore #VMWP02500, pore size 0.05 µm) floating onto the surface of the buffer (place glossy side up of the filter). Use Millipore plain forceps to manipulate the filter.

20) Carefully spot the YAC DNA liquid solution into the center of the filter by transferring with a yellow cut off tip. Dialyze for 2-3 hours.

NOTE: Up to 200 µl can be place per filter without soaking it into the buffer. Leave the dialysis to proceed quietly without any shaking or movement. Do not let the dialysis to go more than 3 hours, otherwise the drop might begin to evaporate.

21) Pipette off the solution with a cut off yellow tip. Place the tip in the middle of the YAC DNA droplet and carefully aspirate as much as possible without stopping. Transfer the YAC DNA solution into an sterile Eppendorf. This is your stock YAC DNA solution. Store it from now on at +4ºC. This YAC DNA sample CANNOT be frozen. It can only be briefly centrifuged (less than 30 seconds) prior the loading of the microinjection needle.
NOTE: Recoveries between 50-70% of the original volume are normal. The rest remains attached as a very thin liquid layer onto the surface of the filter and it is difficult to pipette it off. Final volumes obtained oscillate between 100-400 µl.

22) Check concentration of YAC DNA by fluorimetry and by comparing to a marker lane of lambda/BAC/YAC DNA with known concentration (or any other DNA band from which the concentration is known). Check 1,5 and 10 µl of the sample in a 1% standard agarose gel with Ethidium Bromide. Do not let the samples enter more than 2 cm into the gel matrix. Then, it is much easier to assess and compare quantities of DNA bands.
NOTE: Yield is highly variable. However, we should expect the YAC DNA sample to be mostly between 2-20 ng/µl.

23) It is reccommended to dilute the sample with Microinjection Buffer down to 0.5-2 ng/µl because it guarantees the dilution of other possible contaminants which might have co-purified along with the YAC-DNA and might result toxic for the embryos.

NOTE: There is no general rule in this last step. If a sample, when microinjected, does not give rise to any newborn or transgenic it must be considered the dilution step or the preparation of a new batch.

24) The integrity and quality of the YAC DNA preparation can also be studied with two different techniques. An aliquote of the liquid can be spotted onto a electron microscope grid to direct visualization or, alternatively, an aliquote (100-200 ng) can be loaded (adding some glycerol or Ficoll containing standard loading buffer) into a well of a PFGE gel, next to a lane with all chromosomal bands plus the YAC, to evaluate if the isolated YAC co-migrates with the YAC DNA band in the original agarose plug.
NOTE: It has been reported different mobilities of isolated YACs compared to their corresponding parental bands within the chromosomal array. In this sense, it is not strange to observe isolated DNA apparently running above the parental band. When loading the liquid YAC DNA solution into the PFGE gel the well must be pre-loaded with a coating layer of molten 1% agarose, then DNA and then more agarose to cover the well.

Solutions:

**1000x Polyamines mix**

30 mM Spermine (Sigma, tetrahydrochloride, #S-1141)
70 mM Spermidine (Sigma, trihydrochloride, #S-2501)

NOTE: 1x Polyamines are 30 µM Spermine and 70 µM Spermidine, also called as 100 µM polyamines. Both reagents are solved together in sterile $H_2O$, filter sterilized (0.22 µm), and stored at -20°C. The two polyamines are highly hygroscopic. Therefore, it is reccommended to order pre-weighted small bottles (1 g), do the required calculations and prepare the whole content at once.

**Microinjection Buffer**

| | For 50 ml: |
|---|---|
| 10 mM Tris-HCl pH=7.5 | 0.5 ml of 1 M Tris-HCl pH=7.5 (autoclaved) |
| 0.1 mM EDTA pH=8.0 | 10 µl of 0.5 M EDTA pH=8.0 (autoclaved) |
| 100 mM NaCl | 1 ml of 5 M NaCl (autoclaved) |
| 1x Polyamines mix | 50 µl **1000x Polyamines mix** |
| Sterile $H_2O$ | up to 50 ml |

NOTE: Prepare it fresh. Do not store it.