Genetic Engineering

Principles and Methods

Volume 5

Edited by

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and

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PREFACE TO VOLUME 1

This volume is the first of a series concerning a new technology which is revolutionizing the study of biology, perhaps as profoundly as the discovery of the gene. As pointed out in the introductory chapter, we look forward to the future impact of the technology, but we cannot see where it might take us. The purpose of these volumes is to follow closely the explosion of new techniques and information that is occurring as a result of the newly-acquired ability to make particular kinds of precise cuts in DNA molecules. Thus we are particularly committed to rapid publication.

Jane K. Setlow

Alexander Hollaender

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MICROCLONING OF MICRODISSECTED CHROMOSOME FRAGMENTS

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INTRODUCTION

An average mammalian chromosome contains around 0.1 pg of DNA while an average Drosophila chromosome is about five times smaller. In dipteran insects, however, salivary gland chromosomes are highly polytenized, in the case of Drosophila containing up to 2000 copies of the euchromatic arms of the chromosomes (for giant mutants). These copies are arranged in parallel bundles of chromatin which, in ways not yet understood, undergo local condensation to give rise to a characteristic band-interband pattern visible in the phase microscope. Genetic mapping using visible rearrangements or deletions frequently allows the localization of Drosophila genes to within a single band-interband interval.

An average band is 0.1 to 0.2 µm thick and contains some 20 to 40 kb of genomic DNA or, assuming a 2000 fold polytenization, about 0.04 to 0.1 pg of DNA. With micromanipulator techniques it is possible to isolate chromosome segments approaching these dimensions or equivalent to 100 to 200 kb of genomic DNA, depending on the size and density of bands in the region of interest. The amount of DNA obtained is sufficient starting material for molecular cloning although when a suitable probe is available for clone selection it might be more convenient to work with larger segments.

The DNA extracted is cut with an enzyme like EcoRI or HindIII. This produces a number of independently clonable fragments 3 to 4 kb in average length with ends that can be

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conveniently ligated to a vector molecule. We use bacteriophage lambda DNA as a cloning vector because of the high efficiency with which it can be converted into infectious phage particles by the in vitro packaging technique (2). Furthermore, a series of λ vectors constructed by Murray et al. (3) permits easy identification of recombinant clones.

While the in vitro packaging reaction is independent of phage DNA concentration and can be performed in a volume of 10 to 20 µl, enzymatic reactions and, in particular the ligation of genomic fragments to vector DNA, are critically dependent on the concentration of the reactants. The very low concentration of genomic fragments could be compensated by using a very large excess of vector DNA, but in practice this leads to unacceptably high backgrounds of vector molecules containing no inserts. Cloning of picogram amounts of DNA obtained from microdissected fragments therefore requires a technique to perform reactions in nanoliter volumes to keep the concentrations of the reactants in an efficient range. Whereas no doubt several approaches are possible for this goal, the micromanipulation technique of de Fonbrune (4) in oil-filled chambers has the advantage that the same instrumentation can be used for both microdissection and for microchemistry (5). It is also well suited for intranuclear injections of DNA (6). However, microcloning (7) requires the addition of new technologies: volumetry in the nanoliter range and a micro-procedure for phenol extraction of the DNA. Finally, improvements in the in vitro packaging technique (8) now permit us to convert 1 pg of intact lambda DNA into 500 to 1000 phage plaques.

THE OIL CHAMBER

The oil chamber is a thick glass slide with a central slot bridged by a narrow glass coverslip (Figure 1). The space between the slide and the coverslip can be filled with paraffin oil and remains accessible from the front with a micromanipulator. The underside of the coverslip, in contact with the oil, is the working surface for micromanipulation. It will carry the chromosome preparation for microdissection or small aqueous droplets hanging from the coverslip for biochemical manipulation. Oil chambers cut from a single block of glass are obtainable from Bischoff Glastechnik, Alexanderstrasse 2, D-7518 Bretten, F.R. Germany.

The paraffin oil (Merck, no. 7161, liquid paraffin, spectroscopic grade) prevents evaporation of the aqueous droplets, but does not interfere with the biochemical reactions required for microcloning. It is kept in a bottle over a 1 to 2 cm layer of R buffer (50mM KCl, 10mM Tris pH 7.5, 10mM $MgCl_2$ and 10mM mercaptoethanol). The operations are followed through a phase contrast microscope of the fixed stage variety, focused by moving

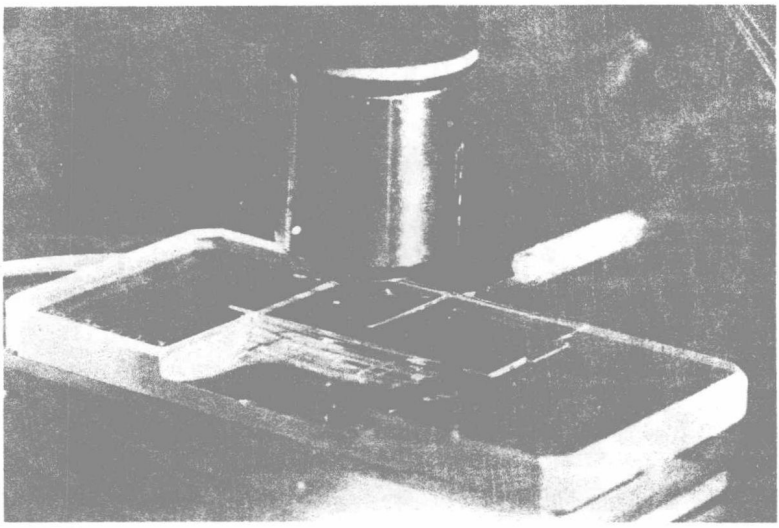

Figure 1. An oil chamber on the microscope stage with inserted micropipette.

the objective and equipped with a high focal length phase contrast condenser. The coverslips used in most of the work are glass, 6x32x0.17 mm, narrow enough to allow different coverslips containing samples, reagents, etc. to be introduced or removed from the oil chamber independently. They are obtainable from AB Termoglas, Box 14137, S-40020 Goeteborg, Sweden. Both oil chamber and coverslips are hydrochloric acid washed before use. Coverslips, which are used for extracting DNA from microdissected components and for the further processing of the extract, are then siliconized by dipping in a 1% solution of dimethyl dichlorosilane in CCl_4 for 1 min followed by rinsing in 1 mM EDTA. The siliconization should be such that nanoliter drops applied to the surface in the oil chamber leave a wetting angle of about 90°C.

THE MICROMANIPULATOR

A de Fonbrune micromanipulator holds the instruments which enter the oil chamber from the open side away from the experimenter. The micromanipulator is placed on an elevated platform in front of the microscope (Figure 2).

The micromanipulator achieves movement reducti n pneumatically through a separate control stick whose movements apply pressure to three orthogonal syringes which in turn communicate with the tool holder or receiver part by means of rubber tubing.

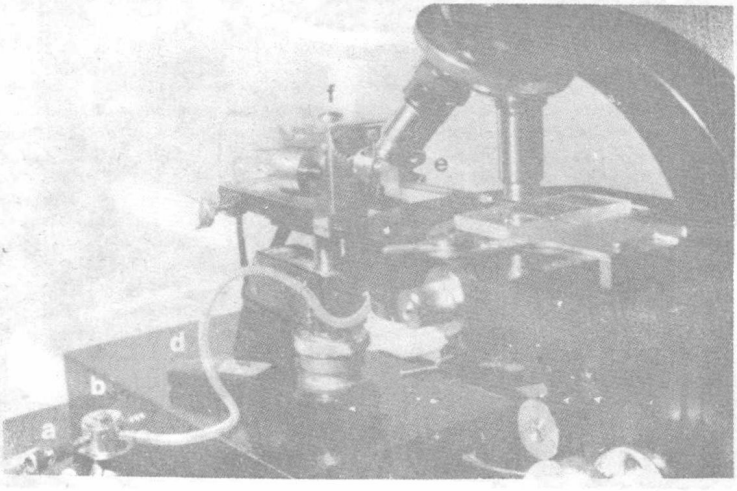

Figure 2. Setup for handling nanoliter volumes in the oil chamber. The syringe (a) is fastened to a three-way stopcock (b) and this in turn onto a wooden plate with a small metal stand (c). It connects with teflon tubing (d) to the holder of the micropipette (e) which is attached to the receptor part of the micromanipulator (f). From Timm et al. (6).

In addition to the movements controlled by the pneumatic system, the receiver part of the micromanipulator can also be adjusted by coarser screw controls. These are useful for proper positioning of the instruments before introduction into the oil chamber but, in addition, permit the use of a second instrument holder attached to the side of the receiver. Such a second instrument is not controlled pneumatically but can be accurately placed in the oil chamber using the adjusting screws and can be useful in dissection as a fixed needle in combination with a pneumatically controlled needle. Both the micromanipulator and the microforge are obtainable from Bachofer Laboratorium Geraete, Postfach 7089, D-7410 Reutlingen, F.R. Germany.

PREPARATION OF TOOLS

Micropipettes

These are used to transfer liquids in the oil chamber. They are constructed in a de Fonbrune microforge, an instrument which permits the controlled melting of glass capillaries by means of

an electrically heated platinum filament under a low power microscope. Commercially available capillaries can be used, but optimal pipettes are made from high melting point glass capillaries (Pyrex or Duran) with relatively thin walls and about 1 mm diameter. Such capillaries may be produced by pulling Pyrex or Duran tubing 10 mm outer diameter, 1 mm thick, in such a way that the size relations between the diameter and the wall thickness are kept unchanged. A length of 10 cm is cut, heated over a small flame to give a local thinning of 0.2 to 0.3 mm diameter, about 2 cm from one end which is then bent to a hook (Figure 3). The long end is introduced into the long arm of a holder made of 3 mm glass tubing bent to right angle arms of 2 and 4 cm length. The capillary is held in place and the holder sealed with melted paraffin. The holder is then mounted on the microforge with the short arm pointing right and the hook of the capillary pointing down and right (Figure 4). A weight of 1 to 2 g is attached to the hook to pull the capillary while it is heated. The thin part of the capillary is first bent to an angle of about 30^{o} from the axis, then heated further to produce a constriction. The platinum wire is then lowered slightly and heat is applied again to make a second constriction below the first (the distance depending on the planned size of the pipette bulb). As the glass melts and stretches, the heat must be decreased and the filament brought as near as possible to the glass. Minimal heat is applied until the glass finally breaks forming an open pipette (Figure 3). The heavier the weight, the wider the opening.

In use, the micropipettes are connected with a rubber tube to a 2 ml "Inaltera" glass syringe connected with a Luerlock

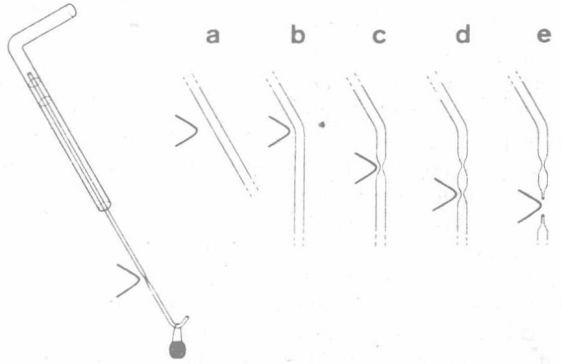

Figure 3. Diagram of construction of a nanoliter volume pipette with the aid of an electrically heated platinum wire in the de Fonbrune microforge.

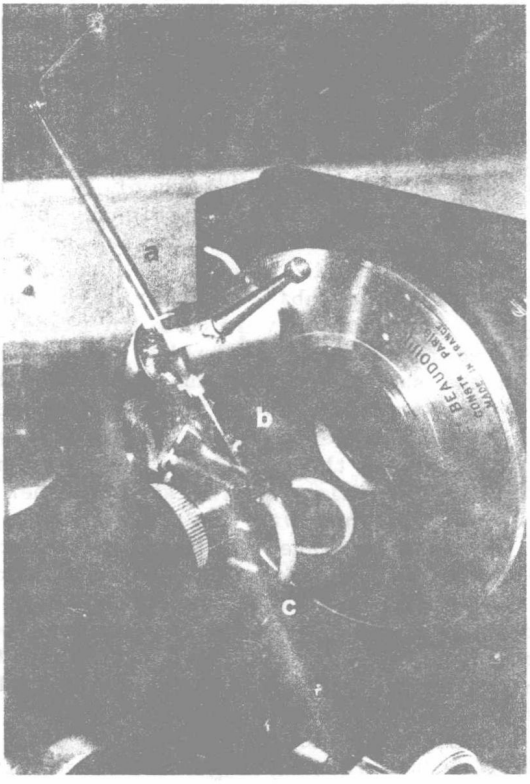

Figure 4. The pipette holder (a) with capillary (b) and weight (c) mounted in the de Fonbrune microforge.

attachment to a three-way stopcock, the plug of which is fastened to a cone-shaped brass stand which in turn is attached to the platform on which both microscope and micromanipulator are placed (Figure 2). By sideways movement, the syringe can be connected to the micropipette or opened towards the exterior for intake or expulsion of air. Both syringe and stopcock are obtainable from Henke-Sass Wolf GmbH, Kronenstrasse 16, D-7200 Tuttlingen, F.R. Germany. After the micropipette has been connected to the syringe, it is connected to the axis of the micromanipulator, introduced into the oil chamber, and calibrated by filling the bulb with tritiated uridine from a supply drop placed on a coverslip in the oil chamber. The contents are deposited on another coverslip and the radioactivity transferred is counted by putting the whole coverslip in a vial with scintillation cocktail. Pipettes in the range 0.1 to 5 nl are useful in the microcloning procedure (Figure 5). After calibration, a micropipette should be siliconized and as a rule used for only one purpose. For

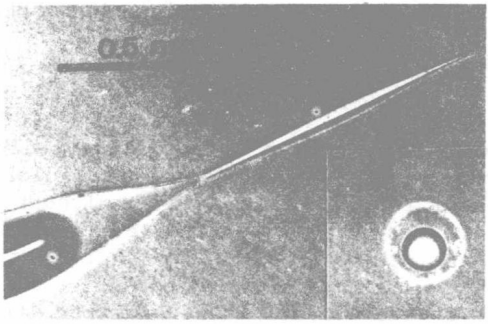

Figure 5. Side-view of a volumetric micropipette. The aqueous sample fills the bulb and is followed by a column of oil and air with which it communicates with the syringe. The insert shows a 1 nl proteinase K-SDS extract surrounded by 4 nl of buffer-saturated phenol at the same magnification.

siliconization the micropipette tip is immersed in 1% dimethyl dichlorosilane and enough solution sucked into the pipette to fill the working area. It is left immersed for one minute after which it is removed and the contained solution aspirated away. The treatment is followed by a short rinse in 1 mM EDTA, introduced and removed in the same way.

Micropipettes need no special cleaning, except filling and emptying a couple of times with the solution to be pipetted. Pipettes which have accumulated denatured protein (uneven movement of the meniscus) may be cleaned by rinsing with buffer-saturated phenol in the oil chamber.

Dissecting Needles

These can be prepared from 2 mm diameter rods of soft glass (soda glass) pulled so that a length of 5 cm is thinned to a diameter of about 0.5 mm. The end of the thin part is then heated in the microforge by contact with the filament until the glass melts and pulled at 40° to 50° from the axis to a cone 1 to 2 mm long. A sharp tip can be obtained by reducing the heat when the tip is formed. Finer needles for high resolution microdissection can also be made by pulling glass rods of 1 mm diameter in a standard electrode puller adjusted to low heat to give a tip which tapers sharply to a fine point. The last 1 to 2 mm of the tip are bent in the microforge to an angle of 40° to 50° from the axis by approaching the hot filament to the side of the needle which then bends towards the heat (hot glass contracts).

MICRODISSECTION OF POLYTENE CHROMOSOMES

Large Chromosomes

Puffs, etc. from large chromosomes like those of Chironomus or Sciara salivary glands can be isolated from glands fixed in 70% ethanol for 50-60 min with or without prior fixation for one minute or less in cold ethanol:acetic acid (3:1). The gland is transferred to glycerol:ethanol (1:1) for 1 hr, then placed on a narrow coverslip with enough liquid to keep it moist. When most of the ethanol has evaporated, the coverslip is placed on an oil chamber for dissection. Chromosomes are released from the glands with two needles connected to the micromanipulator and puffs isolated by stretching the chromosome between the two needles and using the movable needle to pinch off the puff. A segment corresponding to 10-15 bands can be isolated this way (Figure 6).

Chromosome Squashes

This method allows greater resolution and is suitable for all kinds of dipteran salivary gland chromosomes, e.g., Drosophila. The glands are dissected in insect Ringer solution and transferred to a drop of 45% acetic acid on a coverslip long enough to bridge the sides of the oil chamber. The squash is performed with a second, smaller and well siliconized coverslip placed over the first. Care should be taken to minimize the exposure to acetic acid (1 to 2 min at room temperature) to avoid depurination of the DNA. The coverslip sandwich is then frozen in liquid nitrogen and the smaller coverslip flipped off with a razor blade. The lower coverslip carrying the chromosomes is washed with 70% ethanol followed by 95% and absolute ethanol and then air dried. Dried squashes can be stored before use for at least several weeks in a dry, dust-free box.

For microdissection, the dry squash is placed over the oil chamber. The chromosome region to be cut can be wetted by applying to it a microdrop of GP buffer (4 volumes 87% glycerol: 1 volume 0.05 M Na-K phosphate buffer pH 6.8). The chromosome is then scratched with the needle and the fragment removed and deposited into a small microdrop of GP buffer for pooling and storage.

Alternatively, dried chromosome squashes can be dissected directly in the oil chamber. In this condition, the chromosomes are more brittle and suitable for high resolution cutting using a fine needle. The upwards pointing tip of the needle is used to scrape its way into and across the chromosome (Figure 7). The scraped material will stick to the tip or can be made to adhere to it after cutting. With a well placed chromosome, perpen-

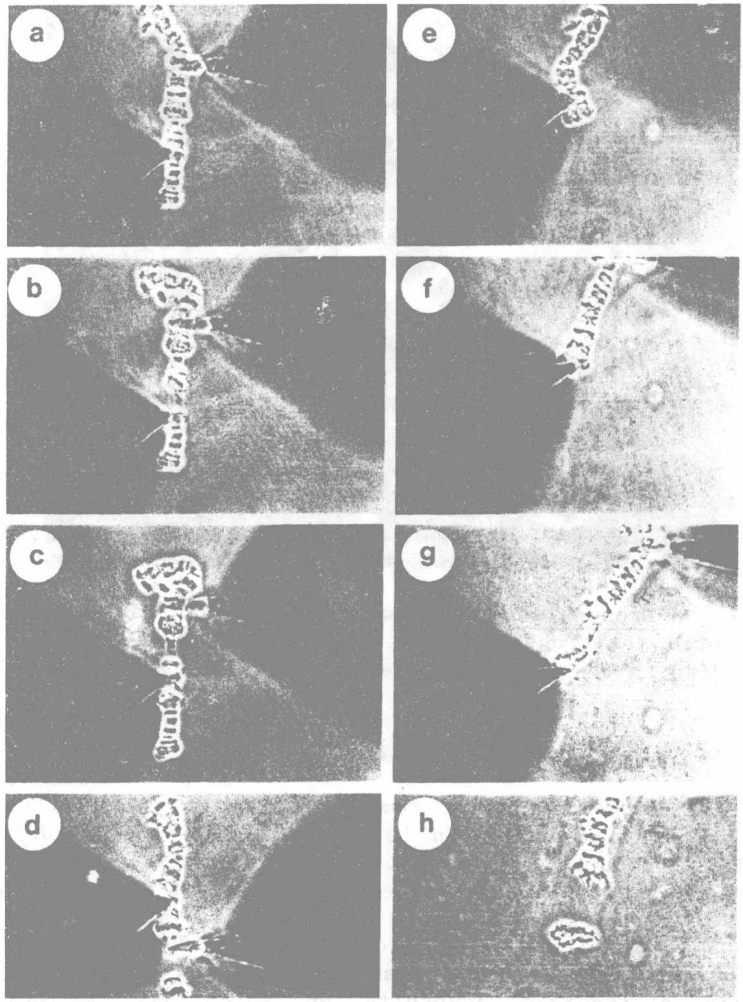

Figure 6. Microdissection of a large puff (BR6) from Chironomus chromosomes with the aid of two glass microneedles.

dicular to the needle, segments down to about 0.5 µm can be scraped off. The fragment on the tip of the needle is transferred to a microdrop. Several fragments can be cut and collected in the same microdrop for cloning.

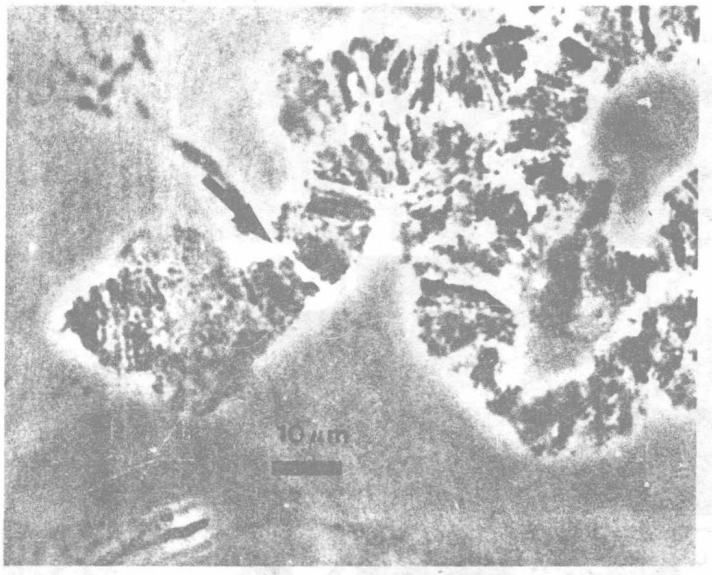

Figure 7. A Drosophila chromosome from gt/gt^{x11} larvae after the removal with a microneedle of a segment in the 3C region. The arrow indicates the motion of the needle.

DNA EXTRACTION

DNA is extracted from chromosome fragments with a solution of 0.5 mg/ml proteinase K in 10 mM Tris pH 7.5, 10 mM NaCl and 0.1% SDS. A supply drop of this solution is deposited on a narrow coverslip which is placed drop down on the oil chamber after which paraffin is added to fill the space between oil chamber and coverslip. With a calibrated micropipette 0.5 to 1 nl of the solution is taken up and deposited on the underside of a second siliconized coverslip on which the microcloning reactions will take place. Before taking up a sample, a micropipette should be filled with paraffin oil so that the aqueous phase is always against an oil and never against an air interphase. The chromosome fragments can be introduced into the proteinase microdrop as they are being dissected (brief dissection time) or they are transferred from the GP drop in which they have first been collected. Chromosomes are instantaneously dissolved and DNA probably released in a few minutes since it has been possible to proceed with the subsequent step within 5 to 10 min. However, incubation can be continued for 90 to 120 min at 37°C with the oil chamber placed in a petri dish containing filter paper moistened with the extraction buffer. This is in turn placed in a larger petri dish also

containing a moistened filter paper. For protracted incubations, a small piece of moist filter paper may be placed on the bottom of the oil chamber to keep the volume of the droplet from altering.

Phenol Extraction

Distilled phenol is shaken with R buffer and a small buffer-saturated supply drop placed on a coverslip which is then placed on the oil chamber. It is advisable to include some of the aqueous phase with the phenol in the oil chamber so as to maintain maximal saturation. Since phenol in this state dissolves slowly in the oil the phenol volume should be kept as small as possible and the phenol supply removed from the oil chamber immediately after use. The proteinase drop is extracted three times with four volumes of phenol which is removed after 3 to 4 min with the micropipette and discarded by depositing it on the phenol supply coverslip. The phenol phase surrounds the aqueous droplet, often detaching it from the coverslip so that it forms a sphere hanging in the phenol drop (Figure 5). When the phenol is removed the aqueous phase will reattach to the coverslip. The DNA drop is then extracted with chloroform. This is done by filling a micropipette outside of the oil chamber with a large quantity of chloroform (1 to 2 µl) which is then ejected over the drop, extracting the remaining traces of phenol. The chloroform, being heavier than the paraffin oil, will sink as it dissolves in the oil. After extraction, the aqueous drop is considered to be equilibrated with R buffer exchanged in from the phenol.

ENZYMATIC DIGESTION OF THE DNA

We have found it prudent to use a large excess of the restriction enzyme and to minimize the possibility of its inactivation. After phenol extraction, it is advisable to transfer the coverslip carrying the DNA to a new oil chamber containing fresh paraffin oil. This is done by preparing a second chamber bridged by a coverslip and filled with fresh oil. The new chamber is placed in front of the old one and the coverslip with the microdrop is slid rapidly across and onto the new chamber. This procedure may be repeated if necessary.

EcoRI restriction enzyme from a stock of 200 U/µl in 50% glycerol is diluted with an appropriate amount of concentrated R buffer, and placed as a supply drop in the oil chamber. Efficient restriction is obtained at final EcoRI concentrations in the range of 20 to 40 U/µl and final glycerol concentrations of 5 to 10%. To avoid going below 20 U enzyme/µl, or above 10% glycerol, the volume of the microdrop, which may have changed