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Purification of Plasmid and BAC Transgenic DNA Constructs

Chengyu Liu, Yubin Du, Wen Xie, and Changyun Gui

Abstract

Pronuclear microinjection is the most used method for generating transgenic mice. The quality of DNA to be microinjected is a key determinant of the success rate of this method. DNA purity is a critical factor because trace amounts of many substances, when microinjected into the pronucleus of the fertilized egg, can kill or prevent the further development of the embryo. Avoiding all contaminants is not a trivial issue, because most transgenic fragments need to be purified from agarose gels. Small particles and viscous materials in the DNA solution can also dramatically reduce the efficiency of microinjection because they tend to clog the injection needles. DNA shearing or breakage during purification and microinjection is also a potential problem, particularly when linearized bacterial artificial chromosomes (BAC) DNAs are used. The overall quantity and the final DNA concentration are also important considerations, because egg pronuclei are very sensitive to the amount of foreign DNA. In this chapter, we first discuss the general guidelines and cautions for preparing microinjection-quality DNA, and then describe in detail two protocols, one for gel purification of transgenic fragments from plasmid vectors and the other for isolating high-quality BAC DNA from bacteria.

Keywords

Transgenic; BAC; Plasmid; DNA purification; Microinjection

1 Introduction

Transgenic and knockout mouse models have been extensively used in lipoprotein and atherosclerosis research [1-3], just as in many other fields of biomedical research. A great majority of these transgenic lines were generated using the so-called pronuclear microinjection method. Presently, the most commonly used vectors for cloning transgenes are plasmids and bacterial artificial chromosomes (BACs). Plasmids are versatile and convenient for precise DNA recombination, and most biomedical scientists are already familiar with the use of plasmids. The main disadvantage of plasmids is that they can accept only relatively small DNA fragments. Although there is not a specific size limit for plasmid vectors, cloning efficiency decreases dramatically when the DNA insert is larger than 20–30 kb. Small transgenes are more prone to chromosomal position effects, which often lead to uncontrolled transgene expression.

In recent years, BACs have gained in popularity in making transgenic mice for the following reasons. First, the Genome Sequencing Project has made it very easy to obtain BAC clones for almost any mouse or human gene from repositories such as the BACPAC Resource Center at Oakland Children's Hospital (<http://bacpac.chori.org/>). Second, the large DNA fragment contained in the BAC (100–300 kb) is more resistant to chromosomal position effects and is more likely to contain all the cis genomic elements required for conferring

authentic gene expression patterns. Third, the recently developed recombineering method [4-6] has enabled precise modification of BAC DNA molecules.

No matter which type of vector is used, the purity and integrity of the transgene are critical factors in determining the efficiency of microinjection and transgenic mouse production. Both BACs and transgenic inserts from plasmid vectors can be purified using essentially the same methods as those used in conventional molecular biology experiments, but the requirements for purity, integrity, and quantity are much higher for transgenic applications. Extreme caution must be exercised throughout the entire purification procedures, so that the highest quality of DNA is obtained to maximize the efficiency of the laborious and expensive micromanipulation and surgical procedures involved in transgenic mouse production.

2 Materials

2.1 Commercial Kits

1. QIAquick Gel Extraction Spin Column Kit (Qiagen Inc., Valencia, CA, Cat: 29706), including the following components:
 - a. QIAquick spin columns.
 - b. Buffer QG.
 - c. Buffer PE. Before use, add the appropriate volume of ethanol to the PE buffer as instructed on the bottle label.
 - d. Collection tubes (2 ml).
2. Qiagen Large-Construct Kit (Qiagen, Cat: 12462), including the following components:
 - a. QIAGEN-tip 500.
 - b. Buffer P1. Before use, add one vial of supplied RNase A to each bottle of Buffer P1 to give a final concentration of 100 µg/ml. Store Buffer P1 containing RNase at 4 °C.
 - c. Buffer P2. Under low temperature storage conditions, SDS may precipitate. If this occurs, heat the P2 buffer in a 37 °C water bath to redissolve the SDS.
 - d. Buffer P3. Prechill Buffer P3 on ice before using and store at 4 °C.
 - e. Buffer QBT.
 - f. Buffer QC.
 - g. Buffer QF. Prewarm Buffer QF to 65 °C before eluting DNA to increase DNA recovery.
 - h. ATP-dependent exonuclease. Completely resuspend each vial of exonuclease (80 µg) in 225 µl of supplied exonuclease solvent before use.
 - i. Buffer EX.
 - j. Buffer QS.
 - k. Folded paper filters.
 - l. QIAGEN-tip 500 holders.

2.2 Equipment and Supplies

1. Beckman Allegra 64R centrifuge (Beckman Coulter, Inc., Fullerton, CA), Beckman Conical C0650 rotor, and Beckman F0650 rotor.
2. Beckman Microfuge 18 microcentrifuge.
3. PowerPac 1000 electrophoresis power supply (Bio-Rad Laboratories, Hercules, CA).
4. Gel boxes for electrophoresis.
5. UV spectrophotometer (Bio-Rad, SmartSpec 3000) with 100 μ l cuvette.
6. Gel documentation system (Bio-Rad, GelDoc 2000).
7. Water bath.
8. Shaker incubator for bacteria culture.
9. 2-l flask.
10. 50-ml disposable conical tubes.
11. 15-ml disposable conical tubes.
12. 50-ml round bottom superspeed centrifuge tubes (Sorvall, Newtown, CT, Cat: 03530).
13. 5-ml Falcon culture tube (Becton Dickinson, Franklin Lakes, NJ, Cat: 5-2058-0).
14. 1.5-ml microcentrifuge tubes.

2.3 Chemicals

1. Hydro PicoPure-2 UV Plus distilled water (Hydro, Durham, NC).
2. UltraPure agarose (Invitrogen, Carlsbad, CA, Cat: 15510-027).
3. 10 $\times$ TAE (400 mM Tris acetate, 2 mM EDTA) (Advanced Biotechnology Inc., Columbia, MD).
4. DNA gel loading buffer (10 $\times$ BlueJuice, Invitrogen, Cat: 10816015).
5. Ethidium bromide (10 mg/ml solution, Invitrogen, Cat: 15585011).
6. 3 M sodium acetate (pH 5.2) (Quality Biologicals Inc., Gaithersburg, MD, Cat: 351-035-060).
7. Isopropanol.
8. TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA; Quality Biologicals Inc., Cat: 351-011-131).
9. Phenol:Chloroform:Isoamyl Alcohol (25:24:1 v/v), saturated with 0.1 M Tris-HCl buffer (pH 8.0) (Invitrogen, Cat: 15593031). Store at 2–8 $^{\circ}$ C.
10. Chloroform (Sigma, St. Louis, MO, Cat: C2432).
11. Ethanol.
12. Microinjection buffer (5 mM Tris-HCl, pH 7.4, 0.2 mM EDTA).
13. LB bacterial culture medium (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter).
14. Spermidine (Sigma, Cat: S2501).

15. Spermine (Sigma, Cat: S1141).
16. ATP (Sigma, Cat: A3377). Dissolve 2.75 g ATP (dehydrated disodium salt) in 50 ml distilled water. Adjust the pH to 7.5 and store at -20°C .
17. BAC microinjection buffer (5 mM Tris-HCl, pH 7.4; 0.2 mM EDTA, 100 mM NaCl, 30 μM spermine, and 70 μM spermidine).

3 Methods

3.1 General Guidelines and Cautions for Purifying Microinjection-Quality DNA

3.1.1 DNA Integrity—DNA samples stored at 4°C for extended periods of time or those that have been frozen and thawed multiple times may contain extensive nicks and breakages. Therefore, it is important to use freshly prepared plasmids or BACs, or ones that have been stored in alcohol in precipitated form. For BACs, DNA shearing is a potential problem. Avoid vigorous shaking and repetitive pipetting. Wide-bore pipette tips should be used. When commercial kits are used for BAC preparation, make sure the kits are specifically designed for processing large DNA molecules. Inclusion of salt and polyamines in the solution may help make the long DNA strands more compact and hence reduce shearing. When microinjecting linearized BACs, slightly larger needle openings and lower injection pressures are preferable for minimizing shearing when the DNA passes through the needle tip.

3.1.2 DNA Purity—When microinjected into the pronuclei of fertilized eggs, trace amount of many substances, including agarose, phenol, chloroform, alcohol, and detergents, can kill the embryos or inhibit their further development. Always use the highest grade of water and chemical reagents, and avoid carrying any residual unintended materials into the final DNA solution. Prewash glass beads and columns with elution buffer before loading the DNA for binding. Perform extra extraction and washing steps and use a longer drying time to evaporate residual alcohol.

3.1.3 Particles—The tip of a microinjection needle is usually $\sim 1\ \mu\text{m}$ in diameter, and hence it is fairly easy to become clogged by tiny particles such as dust, agarose particles, powder from gloves, and microorganisms. Special attention must be paid to avoid including any particles in the final DNA solution. All solutions used for dissolving DNA should be filtered through a $0.22\ \mu\text{m}$ filter. All test tubes and pipette tips should be rinsed with filtered water. Avoid wearing powdered gloves during the last several steps of transgenic DNA purification. Immediately before microinjection, the DNA solution should be centrifuged at a high speed to pellet any particles.

3.1.4 DNA Quantity and Concentration—Although microinjection needs only a couple of micrograms of DNA, this amount is still at least one order of magnitude more than the amount required for most molecular biological experiments, such as DNA cloning. For BAC clones, DNA quantity is usually not a problem, because no gel purification is involved. However, for transgenes cloned in plasmids, purifying a substantial amount of the transgenic insert is often a challenge for beginners, especially when the extra precautions have to be taken to avoid any contamination. It is recommended to start with 20–60 μg of plasmid DNA, depending on the size of the transgenic insert relative to the cloning vector. When separating this large amount of DNA on an agarose gel, it is important to avoid overloading by using a sufficiently wide loading well in a large-size gel.

The optimal DNA concentration for pronuclear microinjection is 1–2 $\mu\text{g}/\text{ml}$, but a higher DNA concentration is preferable for storage. For measuring DNA concentration, in addition

to UV absorbance, an aliquot of the final DNA solution should be run on an agarose gel in parallel with a known concentration of DNA marker to confirm the concentration and integrity of the transgene DNA.

3.2 Isolation of the Transgenic Fragment from the Plasmid Vector

Plasmid preparation (Maxi-prep) for a transgenic project is the same as a plasmid preparation for most molecular biology experiments. For most plasmid extraction kits, simply following the vendor-supplied protocols should suffice. Some researchers suggest using an endotoxin-free kit to prepare plasmid DNA, but this is probably not necessary because the many subsequent steps, particularly the electrophoresis and gel extraction procedures, make the endotoxin status of the initial DNA samples irrelevant. The plasmid backbone needs to be removed from the transgenic insert before microinjection, because the prokaryote-derived cloning vector sequence often interferes with transgene expression after integrating into the mouse genome.

Sucrose gradient centrifugation is an old-fashioned and very reliable method for separating the transgene from the cloning vector [7]. It is still used by some transgenic laboratories for preparing microinjection-quality DNA, but most laboratories currently use agarose gel electrophoresis to separate the DNA pieces. Most of the methods used in routine molecular biology experiments can be adapted for recovering transgenes from agarose gel slices, including electroelution, absorption to glass beads, DNA affinity column chromatography, and low-melting-point agarose gel electrophoresis followed by GELase (Epicentre Biotechnologies, Madison, WI) digestion. A convenient method using the QIAquick Gel Extraction Spin Column Kit is described in detail below. This protocol is adapted from the QIAquick® Spin Handbook (Qiagen, March 2008).

3.2.1 Procedure

1. Digest 20–60 µg of freshly prepared plasmid DNA using appropriate restriction enzymes. The exact amount of plasmid varies depending on the size of the insert relative to the vector backbone. Ideally, 5–10 µg of transgene insert should be released from the vector. We usually digest DNA for 1–3 h only. Extended incubation at 37 °C can increase DNA nicking. Completion of digestion can be checked by running an aliquot of the digestion mixture (0.2 µg DNA) on an agarose gel (*see* Note 1).
2. Load the digested DNA sample into a wide slot (formed by taping multiple teeth of the comb together) in an agarose gel. Run the gel at a relatively low voltage of 2–4 V/cm. Low voltages can increase the gel's resolution.
3. Stain the gel for 15 min with 0.5 µg/ml ethidium bromide in distilled water. Under long-wavelength UV light, cut out the gel slice containing the transgenic insert (*see* Note 2).
4. Weigh the gel slice in a 15-ml conical tube. Add 3 volumes of Buffer QG from the QIAquick Gel Extraction Spin Column Kit to 1 volume of gel (3 ml per gram of gel). For concentrated agarose gels (>1.5 %), double the volume of Buffer QG (6 ml per gram of gel).
5. Incubate the tube in a 50 °C water bath and mix by inverting the tube every 2 min. When the gel is completely solubilized, the color of the solution should be yellow.

¹If the vector backbone and the transgenic insert are similar in size, additional restriction enzymes should be used to cut the vector into smaller pieces.

²To minimize the size of the gel slice, trim off the part that contains little DNA.

If the color of the solution is orange or violet, slowly add drops of 3 M sodium acetate (pH 5.2) into the tube until the color turns to yellow. The yellow color indicates that the pH of the solution is below 7.5, which is required for efficient DNA binding in the following steps.

6. If the DNA fragment is >4 kb or <0.5 kb, add 1 gel volume of isopropanol to the sample and mix.
7. Determine the number of columns needed, assuming that each QIAquick spin column can process 400 mg of agarose gel. Place each spin column into a manufacturer-provided 2-ml collection tube and put the assembly into a microcentrifuge.
8. Prewash the columns by adding 0.8 ml of TE (pH 7.4) into each QIAquick column, and centrifuge at $13,000 \times g$ in a microcentrifuge for 1 min. This prewash step will wash out any contaminants that cannot be washed out by the sample loading buffer (QG) and washing buffer (PE), because TE's ionic strength is much less than that of these buffers.
9. Discard the flow-through and place the washed QIAquick columns back into the same collection tubes. Load 0.8 ml of DNA mixture into each QIAquick column, and centrifuge for 1 min at $13,000 \times g$. Discard the flow-through and place the QIAquick columns back into the same collection tube. More DNA mixture can be loaded into the same spin column until the loading capacity of 400 mg gel per column is reached.
10. Add 0.8 ml of Buffer QG to each QIAquick column and centrifuge at $13,000 \times g$ for 1 min to wash out residual agarose.
11. Add 0.8 ml of Buffer PE to each QIAquick column and leave the wash buffer in the column for 2–3 min, and then centrifuge at $13,000 \times g$ for 1 min. Discard the flow-through and repeat the PE wash step.
12. Centrifuge the QIAquick column for an additional 1 min at $13,000 \times g$ to remove residual PE buffer.
13. Place the QIAquick column into a clean 1.5-ml microcentrifuge tube, and add 50 μ l of TE buffer (pH 7.4) to the center of the QIAquick membrane. Wait 2 min and then centrifuge the column for 1 min. Add another 30 μ l of TE buffer to the center of the column membrane. Wait for another 2 min and then centrifuge to recover the second wash in the same microcentrifuge tube.
14. Combine the eluted DNA solutions from all QIAquick spin columns. Add a tenth volume of 3 M sodium acetate (pH 5.2).
15. Extract the combined DNA solution twice with an equal volume of equilibrated phenol/chloroform/isoamyl alcohol, and then once with chloroform alone.
16. After the final extraction, carefully transfer the aqueous phase into a rinsed microcentrifuge tube using a rinsed pipette tip. Both the tube and tip are rinsed with filtered (0.22 μ m) TE buffer to eliminate any particles. In all subsequent steps, tips and tubes should be rinsed with filtered TE. All buffers or water used to dissolve or dilute DNA should be filtered through 0.22 μ m filters.
17. Precipitate the DNA by adding 2 volumes of ethanol, and then store at -20°C for at least 30 min.

18. Recover the DNA by centrifuging at $13,000 \times g$ for 10 min at room temperature. At this stage, the DNA pellet should be a small, slightly yellowish (see Note 3) translucent mass at the bottom of the tube.
19. Discard the supernatant. Wash the DNA pellet with 1 ml of 70 % ethanol. Centrifuge at $13,000 \times g$ for 3 min. Discard the supernatant, and then briefly spin all the liquid to the bottom of the tube. Carefully remove as much liquid as possible using a pipette tip.
20. Air-dry the DNA pellet for 20 min, and then redissolve in 50 μ l of TE buffer (pH 7.4).
21. Remove a small aliquot for DNA quantification by UV spectrophotometry. Ideally, the OD_{260}/OD_{280} should be 1.8.
22. Confirm the DNA concentration, purity, and integrity by running another small aliquot of the DNA on an agarose gel in parallel with a known concentration marker.
23. Store the DNA solution at 4 °C. Before microinjection, remove an aliquot and dilute to 1.5 μ g/ml using microinjection buffer (5 mM Tris-HCl, pH 7.4, 0.2 mM EDTA). Immediately before loading the needles, the diluted DNA should be centrifuged at $13,000 \times g$ for 5 min to pellet any particles.

3.3 Purification of BAC DNA for Microinjection

One controversy about microinjecting BAC DNA is whether the BAC should be linearized before microinjection. Early studies on plasmid DNA have clearly demonstrated that linear DNA molecules are more efficient for producing positive founders. Saunders and colleagues reported that linear and circular BAC DNAs are equally efficient in giving rise to transgenic mice [8], but in our hands, linear BAC appears to be more efficient (unpublished observation). We normally do not eliminate the BAC vector sequence from the transgene to be microinjected because of the difficulty of separating and purifying large BAC DNA fragments from gels and the fact that the influence of the prokaryote-derived vector sequence is often diminished by the enormous piece of mammalian DNA contained in the BAC.

BACs can be extracted from bacteria using the same alkaline lysis method that is often used for plasmid extraction. However, precautions to avoid DNA shearing should be practiced throughout the purification and microinjection procedures. Unlike plasmid-derived transgenes, BACs will not be further gel-purified. Therefore, greater caution is required for minimizing inclusion of impurities and particles in the DNA solution when extracting BACs from bacteria.

After supercoiled BACs are extracted by alkaline lysis, an additional purification step is required. Cesium chloride gradient centrifugation followed by dialysis is a reliable method for purifying a large amount of supercoiled DNA [9]. However, for researchers who do not have the necessary ultracentrifuge and rotor or who prefer columns and kits, Sepharose 4B-CL chromatography [10] and the Nucleobond column method (see University of Michigan Transgenic Animal Model Core website <http://www.med.umich.edu/tamc/BACDNA.html> for details) are also excellent. Below we describe a protocol using QIAGEN's Large-Construct Kit for BAC DNA purification. The protocol is adapted from the QIAGEN® Large-Construct Kit Handbook (Qiagen, May 2002).

³If the pellet is white, it is likely that the DNA is contaminated with other substances; repeating the column binding and phenol/chloroform extraction (steps 7–17) may improve DNA purity.

3.3.1 Procedure

1. Inoculate a single *E. coli* colony containing the desired BAC into a tube containing 2 ml LB medium with the appropriate antibiotic selection. Incubate with vigorous shaking (~300 rpm) at 37 °C for ~8 h.
2. Transfer the entire 2-ml starter culture into a 2-l flask containing 500 ml LB medium with antibiotic selection. Grow with vigorous shaking (~300 rpm) at 37 °C for another 12-16 h.
3. Divide the culture into six disposable 50-ml conical tubes. Centrifuge the tubes in a Beckman Conical C0650 rotor (*see Note 4*) at $6,000 \times g$ for 10 min. Decant the supernatant and drain out as much medium as possible by inverting the tube on paper towels for several minutes.
4. Add 10 ml of P1 buffer (without RNase A) from the Qiagen Large-Construct Kit into each tube. Vortex to resuspend the bacteria. Combine the bacterial suspensions from three tubes into one tube, so that only two tubes are left, each containing 30 ml bacterial suspension in P1 buffer. This step washes out the compounds present in the culture medium, which maybe toxic to embryos when microinjected.
5. Centrifuge the two tubes at $6,000 \times g$ for 10 min. Discard the supernatant and completely resuspend the bacterial pellet in 10 ml Buffer P1 containing RNase A.
6. Slowly add 10 ml Buffer P2, and mix gently by inverting the tubes 5-10 times (*see Note 5*). Incubate the tubes at room temperature for 5 min. The lysate should appear viscous and free of clumps of bacteria.
7. Add 10 ml ice-cold Buffer P3 and mix gently by inverting the tube repeatedly until a fluffy white material forms and the lysate is no longer viscous.
8. Transfer the entire 30 ml contents from each conical tube into a 50-ml Sorvall superspeed centrifuge tube (*see Note 6*).
9. Centrifuge at $20,000 \times g$ for 30 min at 4 °C. Filter the supernatant through a pre-wetted folded filter (supplied in the kit) to eliminate the white material floating at the top or the solid precipitate at the bottom of the tube.
10. Collect the filtered lysate into a new Sorvall centrifuge tube. Add 0.6 volumes of room-temperature isopropanol. Mix and centrifuge immediately at $20,000 \times g$ for 15 min at 4 °C.
11. Discard the supernatant, wash the DNA pellet with 10 ml room-temperature 70 % ethanol, and centrifuge at $20,000 \times g$ for 5 min.
12. Discard the supernatant. Briefly centrifuge the tube to spin down all liquid. Aspirate as much liquid as possible without disturbing the DNA pellet.
13. Allow the DNA pellet to air-dry for 20 min.
14. Redissolve DNA pellet in 5 ml Buffer EX. Extended incubation at 4 °C and gentle shaking help dissolve the DNA, but avoid repeated pipetting and vortexing.

⁴The Beckman Conical C0650 rotor nicely fits standard 50-ml conical tubes.

⁵Do not vortex or shake vigorously as this may lead to shearing of DNA.

⁶50-ml Sorvall superspeed centrifuge tubes fit nicely into a Beckman F0650 rotor and tolerate much higher centrifugal forces than conical tubes.

15. Combine the DNA solutions from the two tubes. Add 200 μ l ATP-dependent exonuclease and 300 μ l ATP solution. Incubate at 37 °C for about 1 h to digest away *E. coli* genomic DNA and nicked BAC DNA.
16. Add 10 ml Buffer QBT to wet the QIAGEN-tip 500. Then add 15 ml of TE (pH 7.4) buffer to prewash the column (see Note 7).
17. Add 5 ml Buffer QBT to re-equilibrate the QIAGEN-tip 500.
18. Dilute the DNA sample from **step 15** with 10 ml Buffer QS, and then apply the whole sample to the equilibrated QIAGEN-tip 500.
19. Wash the QIAGEN-tip twice, each with 30 ml Buffer QC.
20. Elute the BAC DNA with 15 ml pre-warmed (65 °C) Buffer QF into a new 50-ml tube.
21. Add 10.5 ml of room-temperature isopropanol to precipitate the DNA and then centrifuge at $20,000 \times g$ for 30 min at 4 °C.
22. Discard the supernatant. Briefly centrifuge the tube to spin down any liquid. Aspirate as much liquid as possible without disturbing the DNA pellet.
23. Allow the DNA pellet to air-dry for 20 min.
24. Redissolve the DNA in 500 μ l of TE buffer. If the DNA does not readily dissolve, the tube can be left at 4 °C overnight.
25. Add a tenth volume of 3 M sodium acetate (pH 5.2) to the solution containing the dissolved DNA. Extract twice with equilibrated phenol/chloroform/isoamyl alcohol, and once with chloroform alone. Remember to use wide-bore pipette tips during this and all subsequent steps when transferring BAC DNA solution.
26. After the final extraction, carefully transfer the aqueous phase into a rinsed microcentrifuge tube using a rinsed pipette tip. Both the tube and tip are rinsed with filtered (0.22 μ m) TE buffer to eliminate any particles. In all subsequent steps, tips and tubes should be rinsed with filtered TE, and the buffers or water used to dissolve or dilute the DNA should also be filtered through 0.22 μ m filters.
27. Precipitate the DNA by adding 2 volumes of ethanol. Recover the DNA by centrifuging for 10 min at $13,000 \times g$ in a microcentrifuge.
28. Discard the supernatant. Wash the DNA pellet with 1 ml of 70 % ethanol. Centrifuge at $13,000 \times g$ for 3 min. Discard the supernatant, and then briefly spin all the liquid to the bottom of the tube. Carefully remove as much liquid as possible using a pipette tip.
29. Air-dry the DNA pellet for 15 min, and then redissolve the DNA pellet in 100 μ l of TE buffer (pH 7.4). After the BAC DNA is completely redissolved, NaCl, spermine, and spermidine should be added to a final concentration of 100 mM, 30 μ M, and 70 μ M, respectively, to compact the large DNA molecule and hence preserve its integrity.
30. Remove a small aliquot for DNA quantification by UV spectrophotometry. Ideally, the OD_{260}/OD_{280} should be 1.8.

⁷This step helps to wash out any contaminants that cannot be washed out by the sample loading (QS) and washing (GC) buffers, because TE has a much lower ionic strength than these buffers.

31. Confirm the DNA concentration, purity, and integrity by running another small aliquot of the DNA on an agarose gel in parallel with a known concentration of DNA marker. Pulsed-field gel electrophoresis should be used for analyzing intact BAC DNA molecules (*see* Note 8).
32. The supercoiled BAC DNA can be microinjected directly after diluting to 1.5 µg/ml using BAC microinjection buffer (5 mM Tris-HCl, pH 7.4; 0.2 mM EDTA, 100 mM NaCl, 30 µM spermine, and 70 µM spermidine). However, if appropriate restriction enzymes exist for linearization, we recommend linearizing the BAC before microinjection.
33. For linearizing BAC DNA, 10 µg of supercoiled DNA can be digested with the chosen restriction enzyme (usually NotI) for 1–3 h. Avoid an extended period of digestion, which may increase DNA nicking.
34. After digestion is completed, the DNA needs to be purified by phenol/chloroform/isomyl alcohol extraction as described in **steps 25–31**.
35. The linearized BAC DNA can be stored in 10 mM Tris (pH 7.4), 1 mM EDTA, 100 mM NaCl, 30 µM spermine, and 70 µM spermidine at 4 °C. Before each microinjection, take out an aliquot and dilute to 1.5 µg/ml using BAC microinjection buffer (5 mM Tris-HCl, pH 7.4; 0.2 mM EDTA, 100 mM NaCl, 30 µM spermine, 70 µM spermidine). Immediately before loading the needles, the diluted DNA should be centrifuged at 13,000 × *g* for 5 min to pellet any particles.

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⁸Because regular agarose gel electrophoresis has very limited resolution for separating large DNA molecules (>20 Kb), even partially degraded BAC DNA will run as a sharp band at the top of the gel. Therefore, it cannot be used to judge the integrity of BAC DNA preparations. If pulsed-field gel electrophoresis is not available in the laboratory, the BAC DNA can be digested with a common restriction enzyme that recognizes six-base-pair cutting sites. The smaller DNA bands generated from this digestion can be analyzed using conventional agarose gel electrophoresis. The sharpness of these bands can indicate the integrity of the BAC, and the intensity of these bands in comparison with known concentrations of DNA marker can confirm the DNA concentration.

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