CHEF Genomic DNA Plug Kits

Instruction Manual

Catalog Numbers
170-3591
170-3592
170-3593

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Section 1
Introduction

Pulsed-Field Gel Electrophoresis (PFGE) allows the separation of DNA ranging in size from a few kilobase pairs to 10 megabase pairs. Because of the large size of these molecules, simple pipetting mechanically shears the DNA resulting in unacceptable quality for PFGE separations. This has necessitated procedures for lysis of whole cells embedded in agarose, allowing purification of chromosome-sized DNA without shearing.

The most important and difficult task in preparing cells for embedding in agarose is to obtain the proper cell concentration. Although optical density is frequently used to determine cell concentration, it is not reliable. Different strains, plasmid content, and growth media all contribute to the actual cell number achieved for a particular optical density. Variation in cell number will cause the amount of DNA per agarose plug to vary, leading to over- and or under-loading of the sample. To eliminate the need to generate a growth curve for each strain, a hemocytometer is the most reproducible method for achieving the proper cell concentration for different types of cells, bacteria, yeast, and fungi. Detailed instructions for the use of a hemocytometer are given in the Appendix.

1.1 Kit Components

The CHEF Genomic DNA Plug Kits are designed to produce 100 sample plugs of agarose embedded DNA with the quality necessary for PFGE separations. All kits consist of a core module, which contains all the buffers and proteinase solutions. This core module is the only component supplied with the mammalian DNA kit. Other kits contain additional modules with lysis buffers specific to that cell type. For example, the bacterial kit contains lysozyme and its reaction buffer necessary to digest the outer cell membrane of lysozyme-sensitive bacteria; the yeast module contains lyticase and its reaction buffer necessary to digest the cell walls of most yeast. Each of the disposable plug molds provided contains 50 wells which are 1.5 mm thick and 5 mm wide and hold 85 µl of volume. It is also possible to use the 10 well reusable sample plug mold (170-3622). Each of the wells in the reusable mold are 1 cm wide and 1.5 cm thick and hold 300 µl of volume.

Catalog Number	Product Description
170-3591	**CHEF Mammalian Genomic DNA Plug Kit,** contains Cell Suspension Buffer, 12 ml; >600 U/ml Proteinase K, 1.3 ml; Proteinase K Reaction Buffer, 30 ml; 2% CleanCut™ Agarose, 12 ml; 10x Wash Buffer, 60 ml, 50 well disposable plug mold, 2; Screened Cap, 1
170-3592	**CHEF Bacterial Genomic DNA Plug Kit,** contains Cell Suspension Buffer, 12 ml; >600 U/ml Proteinase K, 1.3 ml; Proteinase K Reaction Buffer, 30 ml; 2% CleanCut Agarose, 12 ml; 10x Wash Buffer, 60 ml; 25 mg/ml Lysozyme, 1.6 ml; Lysozyme Buffer, 30 ml, 50 well disposable plug mold, 2; Screened Cap, 1

170-3593	**CHEF Yeast Genomic DNA Plug Kit,** contains Cell Suspension Buffer, 12 ml; >600 U/ml Proteinase K, 1.3 ml; Proteinase K Reaction Buffer, 30 ml; 2% CleanCut Agarose, 12 ml; 10x Wash Buffer, 60 ml; 5000 U/ml Lyticase, 1.6 ml; Lyticase Buffer, 30 ml, 50 well disposable plug mold, 2; Screened Cap, 1

Section 2
Preparation of Agarose Embedded Mammalian DNA

Reagents and Equipment Needed

Sterile transfer pipettes
50 °C water bath
PMSF stock solution *see Appendix*
Hemocytometer *see Appendix*
Microscope
2, 5, 50 ml sterile plastic tubes

1. Prepare a cell suspension in isotonic saline or tissue culture medium without fetal bovine serum. Count the cells and remove 5 x 10^7 cells for each ml of agarose plugs to be made (use 100 µl/plug for disposable mold or 300 µl/plug for reusable mold) and place on ice. *See Appendix for hemocytometer usage.*
2. Melt the 2% CleanCut agarose solution using a microwave and equilibrate the solution to 50 °C in a water bath.

3. Calculate the amount of Cell Suspension Buffer and CleanCut agarose necessary (see Section 9.2). For a final concentration of 0.75% agarose use 0.63 ml of Cell Suspension Buffer per ml of agarose plugs. Use 0.37 ml of 2% CleanCut agarose per ml of agarose plugs.
4. Centrifuge the cell suspension at 1,000 x g for 5 minutes at 4 °C. Resuspend the cells in the volume of Cell Suspension Buffer calculated above and equilibrate the cell suspension to 50 °C.
5. Combine the calculated volume of 2% CleanCut agarose with the cell suspension and mix gently, but thoroughly. Keeping the cell/agarose mixture at 50 °C, transfer the mixture to plug molds using sterile transfer pipettes (Bio-Rad's disposable transfer pipettes, catalog 223-9524, are recommended). Allow the agarose to solidify. This step can be expedited by placing the molds at 4 °C for 10–15 minutes, which also adds strength to the agarose for removal from the mold.
6. Using a 50 ml conical centrifuge tube, add 100 µl of Proteinase K stock to 2.5 ml of Proteinase K Reaction Buffer for each ml of agarose plugs. Push the solidified agarose plugs into the 50 ml centrifuge tube containing the Proteinase K solution. Incubate the plugs overnight at 50 °C without agitation.

   Note: Various cell lines have been incubated up to 4 days in Proteinase K without detrimental effects to the quality of DNA.

   Note: For processing a few plugs, use a 2 or 5 ml tube.

7. Wash the plugs four times in 1x Wash Buffer, 1 hour each at room temperature with gentle agitation (for each plug, use 1 ml of 1x Wash Buffer). Prepare the Wash Buffer by diluting the 10x stock (1:10) with sterile ddH_2O. If the plugs are to be used in subsequent enzyme reactions, it is advisable to wash the plugs in 1 mM PMSF during the second or third wash to inactivate residual Proteinase K. *See Appendix for PMSF stock solution.*

   Note: For washing a few plugs, use a 2 or 5 ml sterile tube.
8. Store the plugs at 4 °C in 1x Wash Buffer. The plugs should be stable for 3 months.

Section 3
Preparation of Agarose Embedded Bacterial DNA

Reagents and Equipment Needed

Sterile transfer pipettes
50 °C water bath
Grams Crystal Violet (Difco)
Microscope
PMSF stock solution *see Appendix*
Hemocytometer *see Appendix*
2, 5, 50 ml sterile plastic tubes

1. Inoculate a bacterial culture into 50 ml of LB Broth or appropriate media and grow with agitation to an $O.D._{600}$ of 0.8–1.0 at the appropriate temperature. See appendix for LB Broth.

2. When the desired $O.D._{600}$ is reached, add chloramphenicol to a final concentration of 180 µg/ml and continue incubation up to 1 hour while performing step 3.

   Note: Chloramphenicol is used to synchronize ongoing rounds of chromosomal replication and inhibit further rounds of replication. This step is optional, but regions near the replication terminus might be under-represented. In addition, chloramphenicol will alter the morphology of the cells over time causing the appearance of a mixed culture; therefore proceed as quickly as possible with step 3.

3. Make a twenty-fold dilution of the above bacterial suspension using 1 ml bacteria, 1 ml Gram Crystal Violet, and 18 ml saline or PBS. Place a small amount of the bacterial suspension on a hemocytometer and count at 400x power. *See Appendix on hemocytometer usage.*

4. Melt the 2% CleanCut agarose solution using a microwave or hot water bath and equilibrate the solution to 50 °C in a water bath.

5. Calculate the amount of Cell Suspension Buffer and CleanCut agarose necessary (see Section 9.2). For a final concentration of 1% agarose use 0.5 ml of Cell Suspension Buffer per ml of agarose plugs (use 100 µl/plug for disposable mold or 300 µl/plug for reusable mold). Use 0.5 ml of 2% CleanCut agarose per ml of agarose plugs.

6. Remove 5 x 10^8 cells for each ml of agarose plugs to be made. Centrifuge for 3 minutes in a microcentrifuge. If the volume is too large, spin at 10,000 x g for 5 min at 4 °C in an appropriate size tube. Resuspend the cells in the volume of Cell Suspension Buffer calculated above and equilibrate the cell suspension to 50 °C.

7. Combine the calculated volume of 2% CleanCut agarose with the cell suspension and mix gently, but thoroughly. Keeping the cell/agarose mixture at 50 °C, transfer the mixture to plug molds using sterile transfer pipettes (Bio-Rad's disposable transfer pipettes, catalog 223-9524, are recommended). Allow the agarose to solidify. This step can be expedited by placing the molds at 4 °C for 10–15 minutes, which also adds strength to the agarose for removal from the mold.

8. Push the solidified agarose plugs into a 50 ml conical centrifuge tube containing lysozyme solution. Prepare lysozyme solution by adding 100 µl of Lysozyme stock to 2.5 ml of Lysozyme Buffer for each 1 ml of agarose plugs. Incubate the plugs for 2 hours at 37 °C.

   Note: For processing a few plugs, use a 2 or 5 ml tube.

9. Remove the lysozyme solution and rinse the plugs with sterile water. Add 2.5 ml of Proteinase K Reaction Buffer for each ml of agarose plugs, followed by 100 µl of Proteinase K stock. Incubate the plugs overnight at 50 °C without agitation.

   Note: Various cell lines have been incubated up to 4 days in Proteinase K without detrimental effects to the quality of DNA.

10. Wash the plugs four times in 1x Wash Buffer, 1 hour each at room temperature with gentle agitation (for each plug, use 1 ml of 1x Wash Buffer). Prepare the Wash Buffer by diluting the 10x stock (1:10) with sterile ddH_2O. If the plugs are to be used in subsequent enzyme reactions, it is advisable to wash the plugs in 1 mM PMSF during the second or third wash to inactivate residual Proteinase K. *See Appendix for PMSF stock solution.*

    Note: For washing a few plugs, use a 2 or 5 ml sterile tube.

11. Store the plugs at 4 °C in 1x Wash Buffer. The plugs should be stable for 3 months.

Section 4
Preparation of Agarose Embedded Yeast DNA

Reagents and Equipment Needed

50 mM EDTA, pH 8
Sterile transfer pipettes
50 °C water bath
Microscope
PMSF stock solution *see Appendix*
Hemocytometer *see Appendix*
2, 5, 50 ml sterile plastic tubes

1. Inoculate a single colony into 50 to 100 ml YPD broth or appropriate media. Grow with agitation to an $O.D._{600}$ of >1.0 at the appropriate temperature for your strain. *See Appendix for YPD broth.*
2. When the desired $O.D._{600}$ is reached, centrifuge the cells at 5,000 x g, 10 min, 4 °C. Pour off the supernatant and resuspend in 10 ml cold 50 mM EDTA, pH 8.
3. Determine the cell concentration by adding 10 µl of cells to 990 µl of water. Place the yeast suspension on a hemocytometer and count at 400x power. *See Appendix on hemocytometer usage.*
4. Heat the 2% CleanCut agarose solution using a microwave and equilibrate the solution to 50 °C in a water bath.
5. Calculate the amount of Cell Suspension Buffer and CleanCut agarose necessary (see Section 9.2). For a final concentration of 0.75% agarose use 0.63 ml of Cell Suspension Buffer per ml of agarose plugs (use 100 µl/plug for disposable mold or 300 µl/plug for reusable mold). Use 0.37 ml of 2% CleanCut agarose per ml of agarose plugs to be made.
6. Remove 6 x 10^8 cells for each ml of plugs to be made. Centrifuge in a microfuge for 3 minutes if volumes are small, otherwise centrifuge the cells at 5,000 x g, for 10 minutes at 4 °C. Resuspend the cells in the volume of Cell Suspension Buffer calculated above and equilibrate to 50 °C.

7. Just prior to mixing the cells with agarose, add 30 µl of the Lyticase stock, for each ml of plugs to be made, to the cell suspension.

   Note: It is recommended that Lyticase be added immediately prior to imbedding the cells in agarose. It has been found that certain strains do not give acceptable DNA when Lyticase is allowed to diffuse into the agarose plug.

8. Combine the calculated volume of 2% CleanCut agarose with the cell suspension and mix gently, but thoroughly. Keeping the cell/agarose mixture at 50 °C, transfer the mixture to plug molds using sterile transfer pipettes (Bio-Rad's disposable transfer pipettes, catalog 223-9524, are recommended). Allow the agarose to solidify. This step can be expedited by placing the molds at 4 °C for 10–15 minutes, which also adds strength to the agarose for removal from the mold.

9. Push the solidified agarose plugs into a 50 ml conical centrifuge tube containing Lyticase solution. Prepare Lyticase solution by adding 85 µl of Lyticase stock to 2.5 ml of Lyticase Buffer for each 1 ml of plugs. Incubate the plugs for 2 hours at 37 °C.

   Note: For processing a few plugs, use a 2 or 5 ml tube.

10. Remove the lyticase solution and rinse the plugs with sterile water. Add 2.5 ml of Proteinase K Reaction Buffer for each ml of agarose plugs, followed by 100 µl of Proteinase K stock. Incubate the plugs overnight at 50 °C without agitation.

   Note: Various cell lines have been incubated up to 4 days in Proteinase K without detrimental effects to the quality of DNA.

11. Wash the plugs four times in 1x Wash Buffer, 1 hour each at room temperature with gentle agitation (for each plug, use 1 ml of 1x Wash Buffer). Prepare the Wash Buffer by diluting the 10x stock (1:10) with sterile ddH_2O. If the plugs are to be used in subsequent enzyme reactions, it is advisable to wash the plugs in 1 mM PMSF during the second or third wash to inactivate residual Proteinase K. *See Appendix for PMSF stock solution.*

    Note: For washing a few plugs, use a 2 or 5 ml sterile tube.

12. Store the plugs at 4 °C. The plugs should be stable for 3 months.

Section 5
Restriction Enzyme Digestion of Plugs

1. Place one plug per digest in a sterile 1.5 ml microcentrifuge tube. Wash once for 1 hour in 1 ml 0.1x Wash Buffer (1:100 dilution of 10x stock Wash Buffer) Use 1 ml 0.1x Wash Buffer per plug. Decant and resuspend in a sufficient amount of fresh 0.1x Wash Buffer to cover the plugs. This last wash reduces the EDTA concentration, allowing faster buffer equilibration with restriction enzyme buffers.

2. Aspirate the Wash Buffer and add 1 ml of the appropriate 1x restriction enzyme buffer for about 1 hour with gentle agitation at room temperature. Aspirate off the buffer and add 0.3 ml of fresh 1x enzyme buffer. Add the restriction enzyme (30–50 U per 100 µl plug) and incubate overnight at the appropriate temperature.
3. After overnight digestion, remove the buffer and incubate in 1 ml of 1x Wash Buffer for approximately 30 minutes with gentle agitation.
4. **Optional:** Remove the Wash Buffer and equilibrate the plug in the appropriate concentration of gel running buffer, *i.e.* 0.5x TBE, 1.0x TAE, etc.
5. Load ¼ to ⅓ of a plug per well (this is approximately 50 µg DNA) and adjust the volume if necessary on subsequent gels. In addition, always load appropriate size standards.

Section 6
References

1. Smith, C. L., Klco, S. R. and Cantor, C. R., *Genome Analysis*, Chapter 3, K. Davis ed., IRL Press Ltd., Oxford, England (1988).
2. Smith, C. L. and Cantor, C. R., *Methods in Enzymology*, **155,** 449–467 (1988).

Section 7
Additional Reagents for Pulsed Field Electrophoresis

Catalog Number	Product Description
162-0137	**Pulsed Field Certified Agarose,** 100 g
162-0135	**Chromosomal Grade Agarose,** 25 g
170-3594	**CleanCut Agarose,** 2%, 12 ml
170-3624	**DNA Size Standards,** 5 kb ladder
170-3605	**DNA Size Standards,** Yeast chromosomal
170-3633	**DNA Size Standards,** *S. pombe* chromosomes
170-3635	**DNA Size Standards,** lambda ladder
162-0196	**Zeta-Probe® GT Nylon Membrane,** roll, 30 cm x 3.3 m
161-0733	**10x TBE Buffers,** 1 liter

Section 8
Instruments for Pulsed Field Electrophoresis

Catalog Number	Product Description
170-3612	**CHEF-DR® II System**
170-3695	**CHEF-DR III System**
170-3670	**CHEF Mapper® XA Chiller System**
170-3654	**Cooling Module**
165-5031	**GS GeneLinker® UV Chamber**

Section 9
Appendix

9.1 Solutions

PMSF, 100 mM (phenylmethanesulfonyl fluoride)

Add 0.174 g to 10 ml of 100% isopropanol. Store at room temperature.

A stock solution of 100 mM in 100% isopropanol is stable at room temp for 1 year. PMSF is not stable in aqueous solutions. Activity lasts approximately 30 to 50 min at room temperature at pH 7.5-8.

LB

Per liter:
10 g Bacto Tryptone
5 g Bacto Yeast Extract
10 g NaCl
pH 7

YPD

Per liter:
20 g Bacto Peptone
10 g Bacto Yeast Extract
20 g dextrose (glucose)

9.2 Agarose Concentrations

Various agarose plug percentages are reported in the literature. The most common final concentration is 0.5%, but the softness of these plugs makes them difficult to keep intact and handle. We have found that 0.75% to 1% agarose greatly enhances the strength of the plug without interfering with the migration of the DNA out of the plug. These kits contain a 2% CleanCut agarose solution, which when mixed with differing amounts of Cell Suspension Buffer, will give agarose concentrations ranging from 0.5 to 1%. This allows the option of using a different final concentration of agarose if desired.

Desired Agarose Concentration	CleanCut Agarose 2%*	Cell Suspension Buffer*
1%	(0.5)vol. = ____	(0.5)vol. = ____
0.75%	(0.375)vol. = ____	(0.625)vol. = ____
0.5%	(0.25)vol. = ____	(0.75)vol. = ____

* Vol. is the total volume of plugs to be made at 75 µl/plug, *i.e.* for 5 ml of plugs at 0.75% final agarose concentration, add (0.375)5 ml = 1.9 ml of 2% agarose solution to (0.625)5 ml = 3.4 ml of cell suspension.

9.3 Hemocytometer Usage

A hemocytometer is usually divided into nine large squares (Figure 1). Each large square is 1 x 10^{-4} cm^2 or 0.1 mm^3, two such squares are shown the figure with darkened borders (A&B). The large circle around the center square (B) represents your field of view at 100x power (10x objective lens, 10x eye piece). The center square is subdivided into 25 smaller squares. The smaller circle in the center square (C) represents your field of view at 400x power (40x objective lens, 10x eye piece). These 25 center squares are further divided into 16 squares.

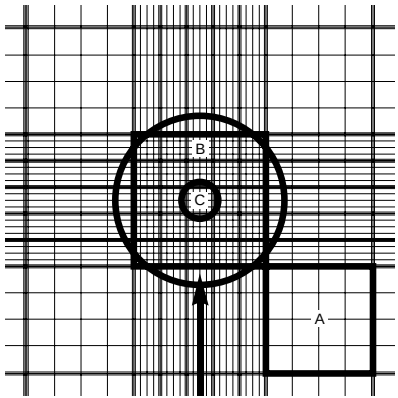

Fig. 1. Hemocytometer grid.

A. Mammalian or Tissue Culture Cells:

Because of the large size, tissue culture cells can be counted at 100x power. Count 10 of the large squares, five on each side of the hemocytometer. Determine the average cells per square using the equations below:

$$\frac{\text{Cells Counted}}{\text{Number of Center Squares}} = \text{Average Cells per Square}$$

$$\text{Average Cells per square} \times \text{Dilution Factor} \times 10^4 = \text{Cells per ml.}$$

Use the following ratio to determine how many ml of cell suspension to use to achieve the desired cell concentration for the plugs.

$$\frac{5 \times 10^7 \text{ cells desired}}{\text{actual cell concentration}} \times \text{ml of plugs to be made} = \text{ml of cell suspension to use.}$$

For Example: 500 cells in 10 squares = average of 50 cells /square x 5 (dilution factor) x 10^4 = 2.5 x 10^6 cells per ml. For 1 ml of plugs you need 1 ml x (5 x 10^7) cells final concentration divided by 2.5 x 10^6 actual cells concentration = 20 ml of cell suspension is required to make 1 ml of agarose plugs.

B. Bacteria and Yeast Cells:

Count five to ten of the 25 center squares, at 400x power, to get a representative sample of your cell suspension. You should have approximately 25 to 75 cells per square. The cells should be relatively free of clumps. Bacteria which naturally chain or grow in clusters are relatively easy to count

and do not have to be dispersed by chemical or enzymatic methods. The Grams Crystal Violet aids in the visualization of bacteria.

Use the equations below to determine the cell concentration:

$$\frac{\text{Cells Counted}}{\text{Number of Squares}} = \text{Average Cells per Square}$$

$$\text{Average Cells per square} \times 25 \text{ Squares} \times \text{Dilution Factor} \times 10^4 = \text{Cells per ml}$$

$$\frac{\text{desired cell concentration}}{\text{actual cell concentration}} \times \text{ml of plugs to be made} = \text{ml of cell suspension to use.}$$

For example: 300 bacteria in 5 squares = average of 60 bacteria/square x 25 (squares) x 20 (dilution factor, yeast use 100 for dilution factor) x 10^4 = 3 x 10^8 bacteria per ml. For 5 ml of plugs you need 5 ml x (5 x 10^8) cells final concentration ÷ (3 x 10^8) actual cells concentration = 8.33 ml of cell suspension is required.

For yeast use 6 x 10^8 cells per ml for the final concentration (see page 9).

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LIT510 Rev E