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Preparation and Restriction Digestion of *Escherichia coli* Chromosomal DNA in Agarose Plugs for Use in PFGE

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Introduction

Methods for preparing high molecular weight, intact DNA to be used in pulsed-field gel electrophoresis (PFGE) are numerous and sometimes contradictory. Cell numbers vary widely, as does the size of plug described, requiring different amounts to be inserted per well. Restriction digestion of DNAs imbedded in agarose plugs has become a necessity for analysis and mapping. The technique of restriction digestion is complicated by the need for the enzymes to reach the target DNA sequences.

Using a hemocytometer to determine the proper cell concentration, pre-equilibration of the plugs in the appropriate enzyme reaction buffer followed by an overnight digestion with the restriction enzyme, eliminates many of the short comings of previously described methods.

Methods

The most important task in preparing cells for imbedding in agarose is to obtain the proper cell concentration. Although optical density is frequently used, it is not reliable. Different strains, plasmid content and growth media all affect the actual cell number achieved at a particular optical density. Variation in this number will cause the amount of DNA per ml to vary, leading to over and/or under loading of sample. I have found that the use of a hemocytometer provides the most reproducible method for determining the proper cell concentration for different types of bacteria, yeast, and fungi. The hemocytometer eliminates the need to generate a growth curve for each individual strain.

Preparation of Genomic DNA Plugs

1. *E. coli* was inoculated (1:20) from an overnight culture into 50 ml of LB Broth. The culture was grown at 37 ˚C with shaking to an OD_{600} of 0.8 to 1. Optional: Add chloramphenicol to a final concentration of 180 µg/ml and continue to incubate for up to 1 hour. This prevents further initiation of chromosomal DNA synthesis, but allows ongoing replication to finish.
2. Immediately, make a twenty-fold dilution of the above bacterial suspension using; 1 ml bacteria, 1 ml Gram Crystal Violet (Difco), and 18 ml saline or PBS.
3. Place a small amount of the bacterial suspension on a hemocytometer and count at 400x power. Note: chloramphenicol will alter the morphology of the cells over time. (See note on hemocytometer use at end of this section.)
4. Remove 5 x 10^8 cells for each ml of plugs. Centrifuge the cells 5 min, at 1000 x g at 4 ˚C, and resuspend the cell pellet to half the final volume of plugs to be made (final cell concentration of 1 x 10^8 cells per ml) with cell suspension buffer (10 mM Tris pH 7.2, 20 mM NaCl, 50 mM EDTA). Equilibrate the cell suspension at 50 ˚C.
5. Pepare 2% Low Melting Point agarose (qualified for restriction digestion) in ultra pure water and equilibrate to 50 ˚C. Mix the cell suspension with an equal volume of LMP agarose (final concentration is 1% agarose at 5 x 10^8 cells per ml). Transfer to plug molds using sterile disposable transfer pipettes and allow the agarose to solidify. This step can be expedited by placing the molds at 4 ˚C for 10-15 minutes, and it adds strength to the agarose for removal from the mold.
6. Push the agarose plugs into lysozyme solution (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine with 1 mg/ml lysozyme), using a bent Pasteur pipette, and incubate for 2 hours at 37 ˚C. Note: this step has been performed overnight without detrimental effects to *E. coli.* Other bacteria have not been tested.
7. Remove the lysozyme solution and rinse the plugs with sterile water. Immerse the plugs in Proteinase K solution (100 mM EDTA, pH 8.0, 0.2% Na deoxycholate, 1% Na lauryl sarcosine, with 1 mg/ml Proteinase K) and incubate overnight at 50 ˚C. Note: various cells have been incubated, up to 4 days in Proteinase K, without detrimental effects.

8. Wash the plugs four times in wash buffer (20 mM Tris, pH 8.0, 50 mM EDTA) at least 1 hour each at room temperature with gentle agitation. If these plugs are to be used in subsequent enzyme reactions, it is advisable to wash the plugs in 1 mM PMSF (phenylmethane sulfonyl fluoride) during the second wash to inactivate any residual Proteinase K. PMSF can be made as a stock solution of 100 mM in isopropyl alcohol, and stored at -20 ˚C.

9. Wash once in 0.1x wash buffer and resuspend in the same buffer, this last step lowers the EDTA concentration allowing faster buffer equilibration with enzyme buffers.

Restriction Enzyme Digestion

1. Place one plug per enzyme in a sterile microfuge tube. Incubate the plug with 1 ml of the appropriate 1x restriction enzyme buffer for about 1 hour with mixing 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 optimal temperature; *i.e.* 37 ˚C for *Not* I and 50 ˚C for *Sfi* I.

2. After overnight incubation remove the buffer and incubate in 1 ml of 1x wash buffer for approximately 30 minutes. It is not necessary to incubate the restricted DNA with Proteinase K to digest away the restriction enzymes as has been reported in other procedures.

3. Remove the wash buffer and equilibrate the plug with the appropriate concentration of gel running buffer, *i.e.* 0.5x TBE, 1.0x TAE, etc. If the plugs are to be stored for any length of time, remove this buffer to prevent diffusion of potentially small DNA fragments of interest, and store at 4 ˚C.

4. Load 1/5 to 1/4 of a plug per well (approximately 500 ng of DNA/100 µl plug volume) and adjust the amount if necessary on subsequent gels. In addition, load appropriate size standards. Run conditions were generated by the Auto-Algorithm mode of the CHEF Mapper® pulsed-field electrophoresis system using a size range of 20-300 kb: 1% Pulsed Field Certified Agarose in 0.5x TBE at 14 ˚C for 26:40 hours at 6.0 V/cm (200 V) using a 120˚ included angle with a 2.77-26.32 second linear switch time ramp.

Results

Four strains of *E. coli* were grown as described above and the isolated DNA was cleaved with either *Not* I or *Sfi* I (Figure 1). Even among *E. coli* K-12 there are noticeable differences in the restriction patterns. It can be seen from these data that restriction analysis of chromosomal DNA from bacterial organisms can be a powerful tool in strain identification.

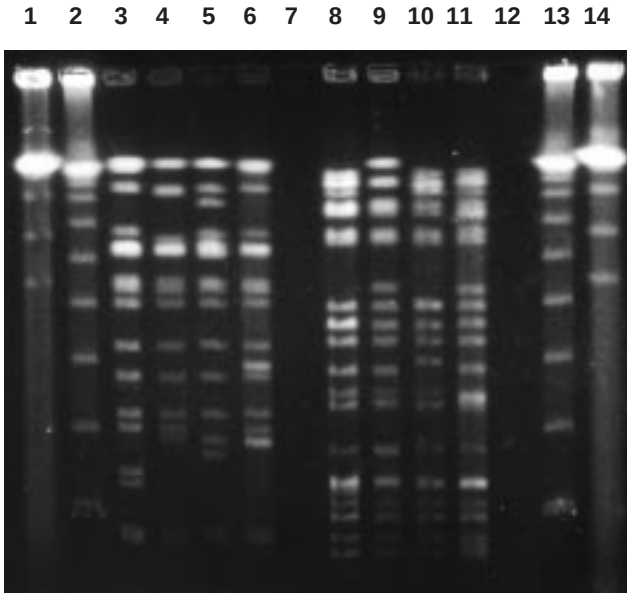

Fig. 1. Separation of *E. coli* chromosomal restriction fragments. 1 and 14) *S. cerevisiae* chromosomes as size standards. **2 and 13)** lambda ladder size standards. **3)** DH5αF⁺ cleaved with *Not* I. **4)** HB101 cleaved with *Not* I. **5)** MC1061 cleaved with *Not* I. **6)** MV1190 cleaved with *Not* I. **8)** DH5αF⁺ cleaved with *Sfi* I. **9)** HB101 cleaved with *Sfi* I. **10)** MC1061 cleaved with *Sfi* I. **11)** MV1190 cleaved with *Sfi* I. Fragments were separated on a 1% Pulsed Field Certified Agarose gel in 0.5x TBE at 14 ˚C for 26:40 hours at 6.0 V/cm (200 V) using a 120° included angle with a 2.77-26.32 second linear switch time ramp.

References

1. Smith, C. L., Klco, S. R., and Cantor, C. R., Genome Analysis: A Practical Approach, pp. 41-60, IRL Press, (1988).

2. Gunderson, K. and Chu, G., *Mol. Cell. Biol.*, **11**:3348-3354, (1991).

Appendix

Hemocytometer Usage

Refer to the accompanying figure of a hemocytometer (Figure 2). A hemocytometer is usually divided into nine large squares. Each large square is 1 x 10^{-4} cm^2 or 0.1 mm^3, one such square (A) is shown in the figure. The large circle (B) around the center square represents your field of view at 100x power. The center square is subdivided into 25 smaller squares. The small circle (C) in the center square represents your field of view at 400x power. The 25 center squares are further subdivided into 16 smaller squares.

Place a small drop of the bacterial suspension under the cover slip to both sides of the hemocytometer. Place the hemocytometer on the microscope and focus on one of the 25 center squares. Allow the suspension to sit for a few minutes, this allows the bacteria that are suspended and out of your field of focus to settle.

Bacteria 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 addition of Gram Crystal Violet greatly aids in the visualization of bacteria; any dye that will stain bacteria can be substituted.

Count 5 to 10 of the 25 center squares to get a representative sample of the bacterial suspension; you should have approximately 25 to 75 bacteria per square. If your suspension is too concentrated or dilute you will not get a accurate count.

Use the following equations to determine cell concentration and volume necessary to prepare 5 ml of agarose plugs:

$$\frac{\text{cells counted}}{\text{number of squares}} = \text{average cells per square}$$

$$\text{average cells per square} \times 25 \text{ squares} \times \text{dilution factor} \times 10^4 = \text{cells per ml}$$

$$\frac{5 \times 10^8 \text{ cells desired}}{\text{cells per ml}} \times \text{ml of plugs to be made} = \text{ml of cell suspension to use}$$

For example: 300 bacteria in 5 squares = average of 60 bacteria x 25 x 20 x 10^4 = 3 x 10^8 bacteria/ml. So for 5 ml of plugs you need 5 ml x 5 x 10^8 cells final concentration ÷ 3 x 10^8 actual cells concentration = 8.5 ml of suspension is required. This sample is centrifuged and resuspended in half the final plug volume (2.5 ml) in Cell Suspension Buffer as described in the methods section.

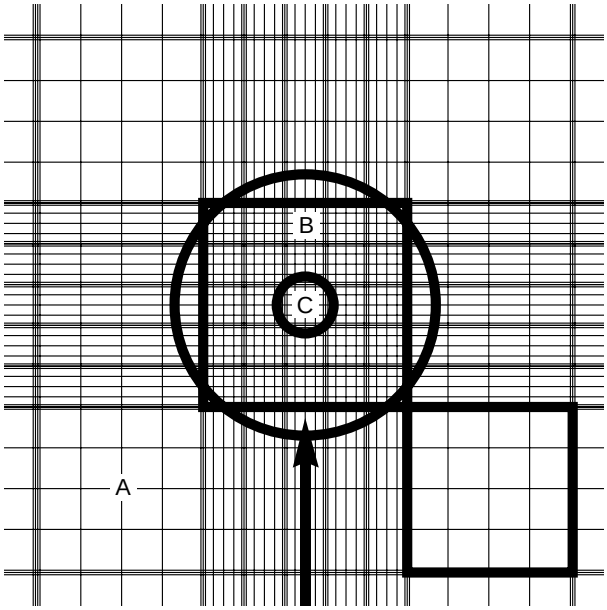

Fig. 2. Hemocytometer grid.

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