

# Optimization of Assay Conditions in Pulsed Field Gel Electrophoresis

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**Pulsed field gel electrophoresis allows separation and visualization of very large DNA molecules. This powerful technique is predominantly used in many aspects in molecular biology and medical diagnostics. Since the procedure for the preparation of the samples involves several steps, many different parameters can be changed and subsequently influence the resolution of the results. Therefore, in the current work, different parameters were altered to optimize the assay to get good resolution of the *XbaI* digested DNA fragments isolated from *E.coli* MG1655. This study suggested that the type and concentration of agarose in the samples have a big effect on subsequent steps in DNA isolation and highly influenced their visualization. Slightly altered conditions in the sample preparation resulted in a clear resolution of the DNA fragments.**

Developed just 20 years ago, Pulsed Field Gel Electrophoresis (PFGE), nowadays represents a powerful tool in many aspects of medical diagnostics and molecular biology (7). While conventional electrophoretic techniques can be used to separate molecules up to 50 kb in size, PFGE allows separations of up to 10Mb (7). Conventional electrophoresis aligns DNA molecules with a constant one-directional electric field, so the DNA fragments travel horizontally, and separate according to size. However, large DNA molecules in one-directional electric field would end up trapped in agarose matrix, and predominantly stay in the wells (7). In PFGE the electric field occurs in pulses and the direction of the field periodically changes by switching the polarity of an electrode array, so DNA fragments are being reoriented. The separation based on size happens because larger DNA molecules need more time to respond to the fluctuating electric field by changing the direction, while shorter molecules move faster through the agarose gel (10).

The applications of PFGE are numerous and diverse. For example, this method can be used in identifying restriction fragment length polymorphisms (1), construction of physical maps (11), detecting *in vivo* chromosomal rearrangements (12), determining the number and size of chromosomes (6), and subtyping of pathogenic bacteria (2,8).

Several different procedures are used for the sample preparations in running this type of gel. The most common method for preparation of the high molecular weight DNA is isolation from cells embedded in agarose (2). This procedure involves several critical steps, namely, that the cells that contain DNA of interest are mixed with agarose, transferred into the plug molds and finally lysed. Then, cellular proteins (including nucleases) have to be inactivated and removed through a series of washes with solutions that contain detergents and high concentrations of inhibitors. After this treatment DNA is left behind as an

intact chromosome, protected in an agarose plug. An alternative method is to embed the cells in the agarose beads by vortexing the agarose / cell suspension with mineral oils, where the subsequent treatments are the same as described for agarose plugs (5,7). The agarose matrix is very convenient because it provides an easy way to manipulate very long DNA molecules and gives a good protection from mechanical forces. The standard DNA isolation procedures, such as phenol-chloroform (10) or kits (QIAGEN) are not recommended because they tend to shear DNA due to mechanical breakage and nuclease contamination, so the samples usually do not yield high molecular weight DNA (7). Once DNA has been isolated, it is subsequently digested with a rare-cutting restriction enzyme in order to compare the band patterns in the gel (in bacterial subtyping (2,8)).

Even though all of the protocols described in the literature generally involve all of the above described steps in preparation of the agarose embedded samples, many of these conditions can be varied. Prior to conducting an experiment that involves PFGE, it is necessary to optimize the assay conditions for the organism to be tested and the apparatus that is used (7). Therefore, the present study was performed to optimize the assay conditions in order to visualize and resolve large DNA fragments extracted from *E.coli* MG1655.

## MATERIALS AND METHODS

**Preparation of Growth Medium and Stock Solutions.** Luria broth, stock solution of chloramphenicol and PBS were all prepared as previously described (9).

**Bacterial Strains.** The strain used in this study was *Escherichia coli* MG1655 (*rphI*<sup>-</sup>), and it was provided by Dr William Ramey, Department of Microbiology and Immunology, University of British Columbia.

**Extraction of Genomic DNA.** Three different methods were used in isolation of genomic DNA: general (phenol/chloroform) DNA extraction protocol (10), DNeasy<sup>TM</sup> Tissue kit (QIAGEN, cat. # 69504) and extraction through preparation of agarose plugs.

**Preparation of Agarose Embedded Bacterial Cells for *in situ* DNA Isolation.** DNA was extracted from the cells embedded in the

agarose plugs using two different protocols. The first group of plugs were prepared as per manufacturer's instructions (3). The concentrations of DNA in the plugs were altered by adding the equal volumes of different cell suspension concentrations, and 2% pulsed field agarose (Bio-Rad, cat. # 62-0137XTU). Counting the cells was performed by using a hemocytometer (3); it was determined that a reading of 0.8 OD<sub>600</sub> represents approximately 5 x 10<sup>8</sup> cells/ml. The final plug concentrations were: 2.5 x 10<sup>8</sup> cells/ml (OD<sub>600</sub> = 0.4), 3.7 x 10<sup>8</sup> cells/ml (OD<sub>600</sub> = 0.6), 5.0 x 10<sup>8</sup> cells/ml (OD<sub>600</sub> = 0.8), and 6.3 x 10<sup>8</sup> cells/ml (OD<sub>600</sub> = 1.0).

The second group of plugs was prepared as previously described (2) with some modifications. In brief, when the cell culture reached 1.2 OD<sub>610</sub> cells were washed in 75mM NaCl - 25mM EDTA (pH 7.4), and resuspended in the original volume. Equal volumes of bacterial cell suspension and 1% chromosomal grade agarose (Bio-Rad, cat. #162-0135) were mixed and dispensed in 1.5 mm thick molds (Bio-Rad, cat.#170-3622XTU). After solidification, the plugs were incubated in lysis buffer (0.5M EDTA (pH 8.0), 1% N-laurylsarcosine, 1mg/ml ProteinaseK) overnight at 50° C. The plugs were first washed twice at room temperature in TE (10 mM Tris, 1mM EDTA (pH 8.0)) containing 1.5mM phenylmethylsulfonylflouride (PMSF) for 15 minutes and than four times in TE for 15 minutes, per wash.

**Restriction Digestion of Genomic DNA.** Genomic DNAs extracted using the kit and the general isolation protocol were cut with an infrequent cutting restriction enzyme *Xba* I (Invitrogen, cat. #15226-012), in 20  $\mu$ l reactions using 3 units of the enzyme and 0.25  $\mu$ g of DNA, for one hour at 37°C. Genomic DNA in the agarose plugs were digested for 2 hours, 4 hours or incubated overnight at 37°C in the reactions containing 30  $\mu$ l 1x enzyme buffer, 270  $\mu$ l distilled water and 50 units *Xba* I (Invitrogen, cat. #15226-012).

**Loading the Gels and PFGE.** Half plugs were loaded into 1% pulsed field agarose gel (Bio-Rad, cat. # 62-0137XTU) either as a solid plug or were first melted and then loaded into the wells. All of the gels were run in 0.5x TBE buffer (45 mM Tris-borate/1 mM EDTA), in a CHEF DR-II apparatus (Bio-Rad) for 22 hours at 14° C with an initial switch time of 5 seconds and final switch time 50 seconds and a voltage 6V/cm.

Plugs containing *Saccharomyces cerevisiae* chromosomes (BioLabs, cat. #N0345S) and plugs with lambda concatemers (BioLabs, cat. #N0350S) were used as molecular weight standards.

**Staining the Gels.** The gels were stained in 0.5  $\mu$ g/ml ethidium bromide for 30 minutes, photographed, and then destained in distilled water up to 4 hours and photographed again. The photographic image was captured digitally using a gel documentation system (AlphaImager, AlphaInnotech).

## RESULTS

Different DNA isolation methods and the final concentrations of DNA in the plugs affected visualization and resolution of the bands in the gel (Fig.1). In all lanes containing plugs (lanes 1-4) most of the DNA stayed in the wells. Only in lane 3 (plug concentration 5x10<sup>8</sup> cells/ml) a faint, but thick, band formed and a smear was detected in a high molecular weight region of the gel. DNA isolated by kit and phenol/ chloroform extracted DNA (lanes 6-9) also mostly stayed in the wells, however some portion of undigested DNA migrated to form the band just below the well. In the lane with *Xba*I digested, phenol/chloroform extracted DNA (lane 8), degradation was observed as a smear in the low molecular region of the gel.

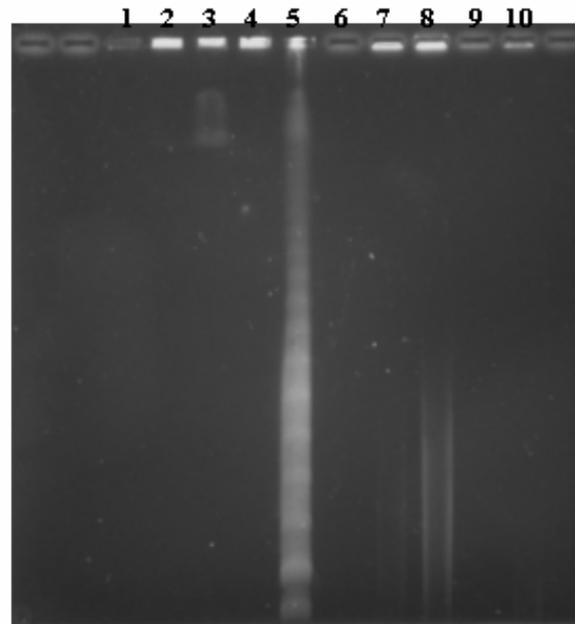


FIG.1 Pulsed field gel electrophoresis of *E. coli* MG1655 cultured in L Broth at 37°C. Lanes 1-4 represent DNA extracted from *E. coli* MG1655 embedded in the agarose plug. The cell concentration in each plug was 0.4, 0.6, 0.8 and 1.0 at OD<sub>600</sub> in lanes 1-4, respectively. Each plug was digested with *Xba*I for 4 hours at 37 °C. Additional lanes represent phenol-chloroform extracted DNA (lanes 7,8) and DNA extracted using QIAGEN kit (lanes 9,10). In lanes 8 and 10 DNA was digested with *Xba*I for 1 hour at 37°C, while in lanes 7 and 9 DNA was not treated. A low range PFG marker (lane 5) was also included.

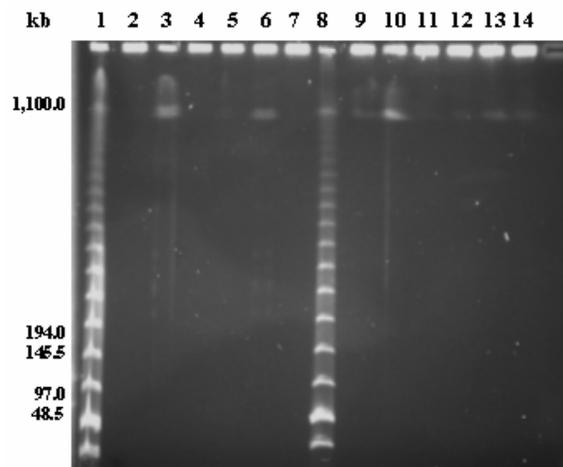


FIG. 2 Pulsed field gel electrophoresis of *E. coli* MG1655 cultured in Luria Broth at 37°C. Lanes 2-7 and 9-14 represent DNA extracted from *E. coli* MG1655 embedded in the agarose plug. The cell concentration in each plug was 0.4 OD<sub>600</sub> (lanes 2-4), 0.6 OD<sub>600</sub> (lanes 5-7), 0.8 OD<sub>600</sub> (lanes 9-11) or 1.0 OD<sub>600</sub> (lanes 12-14). DNA in each plug was digested with *Xba*I at 37 °C either for 2 hours (lanes 2,5,9,12), 4 hours (lanes 3,6,10,13) or overnight (lanes 4,7,11,14). A low range PFG marker (lanes 1 (15  $\mu$ l) and 8 (10  $\mu$ l)) was also included.

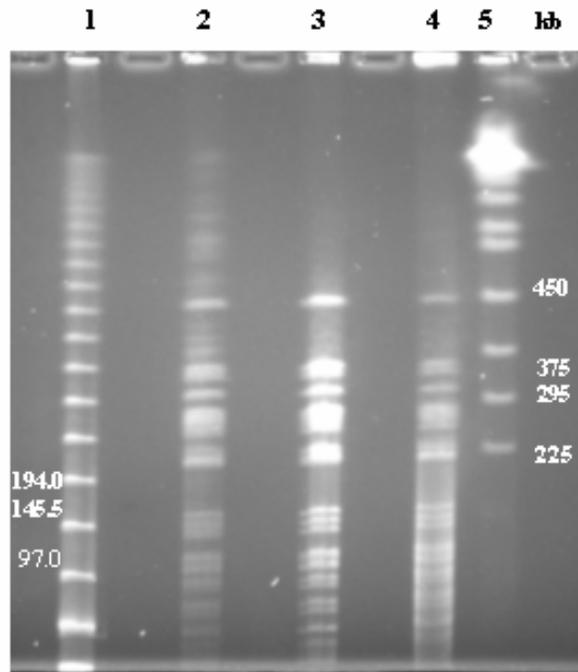


FIG. 3 Pulsed field gel electrophoresis of *E. coli* MG1655 cultured in Luria Broth at 37°C. Lanes 2-4 represent DNA extracted from *E. coli* MG1655 embedded in the agarose plug. The cell concentration in each agarose plug was 1.0 OD<sub>610</sub> (lanes 2 and 4) or 1.2 OD<sub>610</sub> (lane 3). DNA in each plug was digested with *Xba*I for 4 hours at 37 °C. Samples were applied either as a solid plugs (lanes 2,3) or melted and loaded (lane 4). A low range PFG marker (lane 1) and *S.cerevisiae* chromosomes were used as standards (lane 5).

Different DNA concentrations and restriction digest incubation times, showed to have the impact on the results (Fig. 2). Once again, the majority of the DNA stayed in the wells. However, in lanes 3, 6, 9, 10, 12, 13 and 14 (mostly obvious in plugs digested for four hours) a faint, but thick and distinct band formed in the region of 1.1 Mb in the gel. Also, in lanes 3 and 10 the smears are present throughout the whole lanes.

Finally, in the Figure 3, distinct bands are present in the gel. The resolution was significantly better so the number of bands in the gel could readily be counted. As we expected, 18 different fragments were observed (lanes 2, 3 and 4). To get these results, many parameters had to be changed in the process of preparation of the plugs. First, the new group of plugs contained 0.5 % chromosomal grade agarose instead of 1% pulsed field agarose (final concentration in the plug). Also, proteinase K was inactivated using PMSF which might have given better restriction results.

It seems that optimal DNA concentration was present in lanes 2 and 4, where the bands were most distinct. Loading methods showed little difference in results, however it seems that the resolution is slightly better when the plug was first melted and than loaded.

## DISCUSSION

This study was conducted to optimize a protocol for PFGE by altering different parameters that influence the visibility and the resolution of the results. The goal was to produce a protocol that would allow visualizing the bands in the gel using DNA isolated from *E.coli* MG1655. Genomic DNA was cut with *Xba*I, a restriction enzyme that cuts at rare sites. The number of *Xba*I restriction sites in *E.coli* genome was determined using the software available on the TIGR website (<http://www.tigr.org>). It was found that this enzyme was expected to cut the *E.coli* genome, yielding 38 fragments of different lengths, 18 of which would form the distinctive bands in the gel, considering the applied running parameters (Fig.3).

Different conditions were altered to optimize the assay in this study: methods of DNA extraction, final concentration and the type of the agarose in the plugs, lyses time, concentration of the DNA in the plugs, different methods for loading the sample, restriction digest incubation time and additional deactivation of ProteinaseK.

Though the results were not easy to interpret at the beginning, the optimization of this assay was in general successful. It is very hard to say with any reasonable accuracy what caused the initial problems in this study. It seems, however, that there was not just one, but rather a combination of problems.

First, it seems that the root of the most common problems was in the final concentration and the type of the agarose used in the plug preparation. The first gels (Fig.1 and Fig.2) contained the plugs that had a 1% final concentration of pulsed field agarose, and the last gel had 0.5% of chromosomal grade agarose. Since the diffusion process was much slower in the first group of plugs, this suggests that all of the reagents that were used (cell lysis, washes and restriction digestions) had short interaction times with the cell components, resulting in incomplete cell lysis. If most of the cells stayed intact, embedded in the agarose plug, most of the DNA wouldn't leave the wells (Fig.1and Fig.2). This would account for the absence of any band in the lanes where the plugs were digested overnight (Fig.2), because the DNA inside the cells would not be susceptible to the enzyme.

The smears, faint bands and lack of the corresponding bands in lanes with overnight digested plugs (in comparison with plugs digested over 4 hours), imply contamination with cellular nucleases. It is possible that due to the slower diffusion process, EDTA was not present in high enough concentration in the first group of prepared agarose plugs. In the protocols that involve DNA isolation, this reagent is used as a chelating agent to inactivate divalent cations necessary for activity of cellular nucleases (9,10). In

other words, EDTA, in the right concentrations, inhibits the activity of cellular nucleases. An inappropriate concentration of EDTA in the plug would cause several problems in different steps of the preparation. First if the concentration is not high enough in the phase of cell lysis and plug washes, the cellular nucleases will stay active in the plug. Alternatively, if this agent is still present in the plug while digesting DNA with the enzyme of interest, the activity of above-mentioned restriction enzyme will be much lower than expected (7,10). However, incomplete digestion of DNA in the cells does not account for all of the observations.

Phenol/chloroform isolated DNA and kit isolated were also cut using the restriction enzyme from the same vial (Fig.1 lanes 8 and 10). These liquid samples were 'naked' (agarose free) and the EDTA concentration could be highly controlled in the solution. Nevertheless, most of DNA stayed or migrated just below the wells forming the thin bands. Also, a degraded DNA was detected as a faint smear in lane 8, and it is not clear if this was the result of *Xba*I activity or the activity of other cellular nucleases.

An alternative explanation is that Proteinase K was not effective as it should be. The role of this enzyme is to inhibit cellular nucleases and to dissociate proteins bound to DNA molecule, leaving the 'naked' molecule in the plug (free of nucleases) (7).

In trying to solve all of these above-described problems a slightly different protocol for preparation of the next group of the plugs was used. First, a different agarose type, that has much better diffusion properties (chromosomal gradient), and final concentration were used (0.5%). This allowed better diffusion of reagents in the plug and more complete cell lysis and restriction digestion. Also, reagents that were used in cell lysis and plug washes had slightly different composition and a fresh vial of restriction enzyme was used. The combination of changes resulted in significantly improved visibility and good resolution, meaning all of the expected fragments could be detected in the gel (Fig. 3).

Different loading techniques were also tested to see if they affect resolution; it seems that even though the difference is not big, the resolution was better when the plug was first melted and then loaded. Different DNA concentrations in the plugs showed that the optimal value is probably when plugs were made with cells that reached 1.0 at OD<sub>610</sub> (Fig3. lanes 2-4). Higher concentrations showed poorer resolution where the bands were not very distinct (Fig.3, lane 3). Destaining the gel for up to 4 hours (instead of looking at them immediately after staining) further improved resolution and gave more distinct and clearer bands (data not shown).

In conclusion, it seems that a very precisely defined protocol is needed to achieve good visibility and

reasonable resolution of the bands when running the PFGE gel. Though it was not easy to interpret some of the results, this study showed that the agarose type and concentration probably highly influenced the subsequent steps in DNA isolation and digestion.

## FUTURE EXPERIMENTS

To get the better results further studies could be performed in condition optimization. Since it seems that most of the problems were caused by inadequate agarose type and its final concentration in the plug, it would be useful to test other kind of agarose, (for example, low melting agarose) at a range of concentrations. It is also important to test the effect of different concentrations and types of protein inhibitors on the presence of endonucleases in the samples. In addition, to test whether the digestion is diffusion impacted the digest could be done with DNA isolated in different sizes of small agarose beads.

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