Simplified Agar Plate Method for Quantifying Viable Bacteria

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We have developed a simple and reproducible technique that we have termed "track dilution" for enumerating viable bacteria, which reduces time and materials required compared to standard agar plate colony counting methods. Many microbiological applications require an accurate determination of bacterial numbers prior to inoculation into, or subsequent to their isolation from, experimental models and systems. For example, our experimental infection models require injection of predetermined numbers of viable colony-forming units (cfu) (1,4). The model further requires careful monitoring of bacterial growth rates at specific time points during infection. In our models of rodent gastrointestinal ecology (3), bacterial concentration must be determined from extraordinarily large numbers of fecal samples.

The most commonly used technique for enumerating bacterial cfu involves serial dilution of samples followed by spreading 50–100 µL of each dilution onto agar media. Subsequent to incubation, colonies are counted and bacterial concentrations in the original sample estimated. This technique, though relatively accurate, has some drawbacks, including time required and a considerable expenditure of disposable plastics and media. To meet criteria for statistical accuracy of bacterial numbers in a given specimen samples are usually plated in triplicate, and cfu counted only from plates yielding between 20 and 200 visible colonies (2). Furthermore, since the actual number of bacteria present in a specimen at the completion of an experiment is unknown, samples must frequently be plated from dilutions ranging from 10^{-1} to 10^{-7}. The result of such exhaustive measures is that most plated samples will yield either too many colonies to count or no colonies at all. For example, an overnight bacterial broth culture containing 10^9 cfu/mL, serially diluted 10-fold over a range of 10^{-1} to 10^{-7} and plated (100 µL per plate) in triplicate, will yield only three agar plates with countable and statistically valid numbers of cfu; the remaining 18 plates must be discarded.

Table 1. Comparative Results of Track-Dilution vs. Spread-Plate Methods for Enumerating Viable *E. faecalis* from Broth Culture

	Spread-Plate	Track-Dilution
Time (includes plate drying)	31 min	5.5 min
Plates Required	18 (100-mm round)	3 (100-mm square)
Media Volume	450 mL	90 mL
Countable cfu at 10^{-7} Dilution (mean ± standard deviation)*	105.3 ± 10.6	102.0 ± 9.8
Statistical Difference		$P = 0.68$
Lower Detection Limit	>10 cfu/mL	>100 cfu/mL

*Representative data from *E. faecalis* enumeration performed in triplicate. Inter- and intra-test variations for *S. aureus, E. coli* and *B. subtilis* were not significantly different, and data are not shown.

To simplify bacterial enumeration and reduce time and material expenditures, we have developed a track-dilution technique whereby six 10-fold serial dilutions of a sample containing an unknown quantity of bacteria are plated onto a single agar plate. We compared track dilution with the standard spread-plate method by: *(i)* recording time required to process samples, *(ii)* recording materials expended and *(iii)* statistically analyzing the data for accuracy and reproducibility.

Enterococcus faecalis and *Staphylococcus aureus* were propagated in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) overnight at 37°C without aeration. *Bacillus subtilis* and *Escherichia coli* DH5α™ (Life Technologies, Gaithersburg, MD, USA) were propagated in LB overnight at 37°C with aeration. Cultures were serially diluted 10-fold by sequential transfer of 100 µL into 900 µL phosphate-buffered saline (PBS). Experiments were performed in triplicate to allow statistical comparisons between the two techniques. For conventional spread-plate counting, cultures were diluted by a factor of 10^{-7}, from which 100-µL samples were spread onto the surface of prewarmed BHI 1.5% agar plates (100- × 15-mm round plates; Fisher Scientific, Pittsburgh, PA, USA) using a sterile bent-glass rod and an inoculating turntable (Fisher Scientific). Plates were incubated upright for ap-

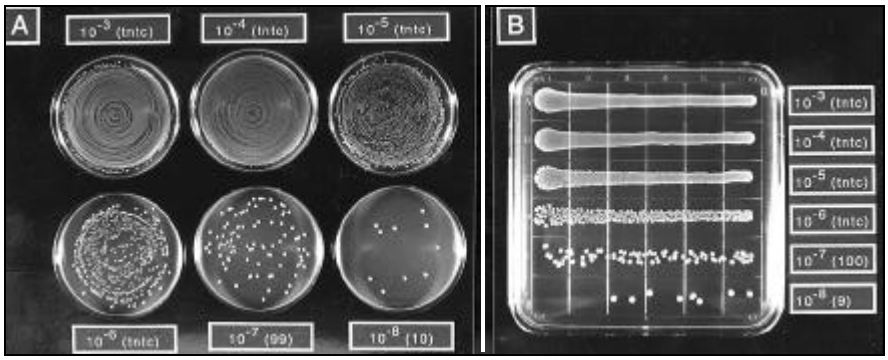

Figure 1. Representative results of spread-plate and track-dilution cfu enumeration techniques. Overnight broth culture of *E. faecalis* JH2SS was serially diluted 10-fold and plated onto BHI agar. (A) Conventional spread-plate method (100 µL per plate). (B) Track-dilution method (10 µL per track). For comparison, numbers represent the extent of dilution when the quantity plated is taken into consideration (e.g., 10^{-8} for a sample plated by conventional method represents 0.1 mL of a 10^{-7} dilution; whereas for the track-dilution plate, 10^{-8} represents 0.01 mL of a 10^{-6} dilution). Numbers in parentheses represent countable cfu. "tntc": too numerous to count.

proximately 15 min to allow absorption of excess moisture and then incubated inverted overnight. For the track-dilution technique, we used Falcon® Integrid™ 100- × 15-mm square plates with 13-mm grids (Becton Dickinson Labware, Bedford, MA, USA). Cultures were diluted by a factor of 10^{-6}, from which 10-µL samples were spotted in a column on the agar surface along one side of the square plate using a Pipetman™ micropipet (Rainin Instrument, Woburn, MA, USA). By spotting samples in ascending order of bacterial concentration, it is unnecessary to change micropipet tips between dilutions. The plate is then tipped onto its side (at a 45°–90° angle), and the spots are allowed to migrate in parallel tracks across the agar surface to the opposite side of the plate. A short time of 30 s to 1 min on the benchtop is required to allow the tracks to dry, after which the plate is incubated inverted overnight. For the conventional spread-plate technique, plates were selected that yielded the highest number of cfu that could be reasonably counted. For the track-dilution technique, tracks that yielded countable cfu were similarly selected.

Comparative results from the two techniques are presented in Figure 1 and Table 1. Track dilution resulted in a significant reduction in time and materials required compared to the standard spread-plate technique. On average, less than 5 min (including plate drying time) were required to perform track dilution in triplicate. In contrast, the spread-plate technique required approximately 15 min per dilution series and an additional 15 min for moisture absorption (30 min total). Three square plates and 75–90 mL of solid media were required for triplicate track dilution, whereas 18 round plates and 450 mL of media were expended for a single spread-plate dilution series in triplicate. Statistical analysis (Student's *t* test) of cfu data obtained by the two techniques revealed no significant differences ($P > 0.2$) in calculated cfu concentrations for any of the four organisms tested. No significant difference was observed when intra-test reproducibility was compared. For all organisms tested, spread-plate results demonstrated intra-test variances of 9.7 ± 2.7 cfu per 100 cfu counted, while track dilution intra-test variability was 10.7 ± 5.5 cfu per 100 cfu counted ($P > 0.5$).

The results demonstrated that this simplified bacterial colony enumeration technique provides an improvement over conventional spread-plate methods by conserving time, materials and labor required. Furthermore, although small sample volumes (10 µL) are used in track dilution, there were no significant differences in quantitation and reproducibility of the results compared to spread plating. Track

dilution is limited primarily in its sensitivity. However, this is of concern only if very small numbers of organisms are suspected in a particular specimen, because the lower limits of detection for a 10-µL aliquot of undiluted material would be approximately 100 cfu/mL. There exists the possibility of crossing of paths as the plates are tilted. We experienced this problem only: *(i)* on media that were either excessively dry or had gross irregularities on their surfaces or *(ii)* when more than 6 samples were placed on a single 100- × 15-mm plate. Use of media with a smooth surface and adequate spacing of samples minimizes erroneous track migration and mixing. Since detergents are often used for tissue homogenization (i.e., phagocytosis assays), we tested samples containing 10% (vol/vol) Triton® X-100 and observed no interference with proper track formation (data not shown). In conclusion, bacterial enumeration by track dilution should prove a simple and reproducible tool, applicable to any number of experimental systems including bacterial growth kinetic studies and time-kill determinations. Although not specifically tested in this study, track dilution may prove useful for rapid enumeration of yeasts as well.

REFERENCES

1. **Booth, M.C., R.V. Atkuri, S.K. Nanda, J.J. Iandolo and M.S. Gilmore.** 1995. Accessory gene regulator controls *Staphylococcus aureus* virulence in endophthalmitis. Invest. Ophthalmol. Vis. Sci. *36*:1828-1836.
2. **Greenberg, A., L. Clesceri and A. Eaton (Eds.).** 1992. Heterotrophic plate count. *In* Standard Methods for the Examination of Water and Wastewater, 18th ed. American Public Health Association, Washington, D.C.
3. **Huycke, M.M., M.S. Gilmore, B.D. Jett and J.L. Booth.** 1992. Transfer of pheromone-inducible plasmids between *Enterococcus faecalis* in the syrian hamster gastrointestinal tract. J. Infect. Dis. *166*:1188-1191.
4. **Jett, B.D., H.G. Jensen, R.E. Nordquist and M.S. Gilmore.** 1992. Contribution of the pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infect. Immun. *60*:2445-2452.

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Low-Voltage Electric-Discharge Biolistic Device

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Particle bombardment is a useful method for molecular biologists, allowing direct gene transfer to a broad range of cell and tissue types. This technique involves accelerating DNA-coated particles (microprojectiles) directly into cells. The basic system that has been widely adopted uses a macrocarrier to carry the microprojectiles, a mechanism for accelerating the macrocarrier and a means of stopping the macrocarrier (3).

In the first systems described, the macrocarrier is accelerated by a gunpowder discharge (4). In other systems, the macrocarrier is accelerated by shock waves created by sudden release of compressed air (8), nitrogen (10) or helium (9). Christou et al. have designed a device that uses an electric discharge through a drop of water (2). Although the electric discharge device is very simple, it needs a high-voltage power supply (25 kV) and a high-voltage capacitor.

We have lowered the voltage by discharging through a metal wire.

The new particle accelerator we developed is schematically illustrated in Figure 1. The motive force is generated in a spark discharge chamber containing a discharge electrode. A capacitor is discharged through the discharge electrode, which vaporizes instantly, creating a shock wave. A carrier sheet on which the DNA-coated particles are precipitated is inverted and weakly pasted to the opening side of the discharge chamber with glycerol. The function of the carrier sheet is to transfer the force of the shock wave from the spark discharge into acceleration of the DNA-coated particles. Located below the carrier sheet is a 100-mesh stainless steel screen that retains the carrier sheet, allowing the accelerated DNA-coated particles to continue through the screen and into target tissue.

Coronal slices of 300-µm thickness were cut from rat cortex and placed on 0.4-µm pore, 30-mm culture plate inserts (Millipore, Bedford, MA, USA) that were placed in 35-mm culture plates. Each plate contained 1 mL of conditioned medium. The slices were incubated at 37°C in 5% CO_2 atmosphere.

The cultured slices were transfected with a plasmid pCAGGS-*lacZ* carrying a fusion gene consisting of the human cytomegalovirus (CMV) immediate-early gene enhancer, chicken β-actin promoter and an *E. coli lacZ* reporter gene (7). The DNA was loaded onto 1.6-µm gold beads using a standard protocol (1,5,6): for each routine preparation (2–6 transfections), 5 µg of plasmid DNA, 50 µL of 2.5 M $CaCl_2$ and 10 µL of 1 M spermidine-free base were added to 50 µL of gold particles (30 mg/mL in water) and incubated for 20 min with occasional mixing, after which the particles were collected by brief centrifugation, washed with 70% ethanol and resuspended in 80 µL of absolute ethanol. Then, 10 µL of the gold suspension were applied to the center of the carrier sheet, and the ethanol was allowed to evaporate.

After transfection, the slices were returned to the incubator for 24 h and then fixed and stained for checking the activity of the exogenous β-galactosidase.

The slices were fixed for 30 min in

0.5% glutaraldehyde, washed three times in phosphate-buffered saline (PBS) and incubated overnight in a solution containing 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$, 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) and 1 mM $MgCl_2$.

To apply molecular approaches to complex tissues such as the central nervous system, a rapid and efficient assay system for gene expression is essential. Conventional transfection techniques have been of limited effectiveness in intact neural tissues. Viral vectors effectively transfect neurons both in vivo and in vitro but are labor-intensive to

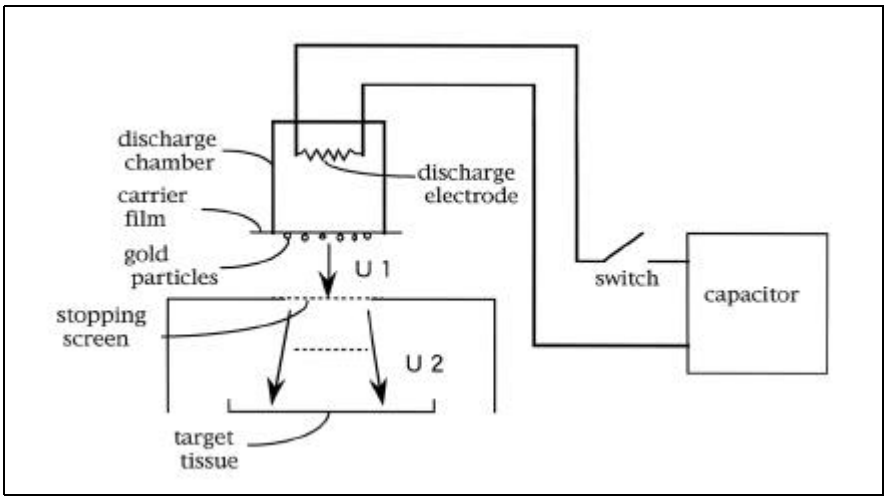

Figure 1. A schematic illustration of the low-voltage electric-discharge biolistic device. The motive force is generated in a spark discharge chamber containing a discharge electrode. A capacitor is discharged through the discharge electrode, which vaporizes instantly, creating a shock wave and accelerates a carrier sheet on which the DNA-coated particles are precipitated.

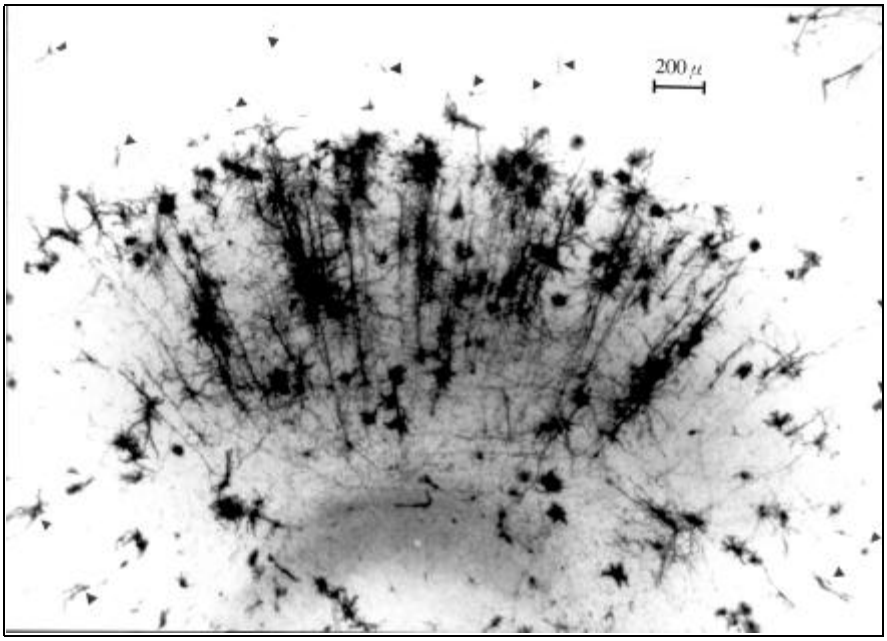

Figure 2. Neurons and glia transfected by biolistics and visualized by X-gal staining. Coronal slices (300 μm) cut from rat cortex at postnatal day 3 were grown for 4 days and then transfected with the *lacZ* fusion gene driven by cytomegalovirus enhancer and chicken β-actin gene promotor loaded onto 1.6-μm-diameter gold particles and incubated for 24 h before fixation and staining with X-gal. Positive cells are blue. The number of cells transfected in this slice illustrates the level of transfection achieved by biolistics. Some of the dissociated cells indicated by red arrowheads around the slice are also transformed by biolistics.

construct. The methodology that combines particle-bombardment transfection procedures and organotypic slice culture techniques might be a promising approach (1,5,6). Therefore, we focused on applying our biolistic device to neural tissues.

The dimensions of the apparatus and the intensity of the electric discharge have to be adjusted for each target tissue type. For rat cerebral cortex slices, we use the following values. The internal dimension of the discharge chamber is 13 mm in diameter and 15 mm in depth. The discharge electrode is a 2-mm wide, 35-μm thick and 10-mm long copper wire. A 200-V, 12 000-μF capacitor is used. The carrier sheet is a 20-mm square, 50-μm thick polyimide film. The screen locates 10 mm below the chamber. The target tissue locates 10 mm below the screen.

An example of the results of transfection experiments is shown in Figure 2. Cells that express β-galactosidase are stained blue. The number of cells transfected in the slice illustrates the level of transfection achieved by biolistics. Cell body and horizontally and/or vertically running long processes of each cell were clearly observed. When the slice was placed on the membrane, several dissociated cells were also placed on the membrane. Some of these dissociated cells were stained blue. This demonstrates that our biolistic device also works for dissociated cells.

Particle bombardment is used to transfect a broad range of cell and tissue types including plant meristems, insect embryos, fish embryos, bacteria, fungi, animal tissue slices and animal tissue cultured cells. In trying our biolistic device on these applications, the following adjustments or trade-offs should be considered. The diameter of the discharge chamber and the distance between the screen and the target tissue should be designed to cover the size of the target tissue. The diameter of the particles should be much smaller than the size of the target cells to avoid cell damage. The density of the particles on the target should be high enough that many cells receive particles, but low enough to avoid the situation where multiple particles converge to the same target cell and cause cell damage.

The velocity of the particles should be adjusted so that the particles penetrate the tissue surface and stop in the tissue. Velocity of the particles may be a complicated function of the dimension of the discharge chamber, distance to the target and the electric discharge power, and is difficult to estimate theoretically. It is practical to adjust the velocity empirically by varying the electric discharge power. The electric discharge power is proportional to the product of the capacity of the capacitor and the square of the applied voltage. Electric discharge power is easily and continuously controlled by changing the capacity of the capacitor and/or the applied voltage. The ease of the power control will be an advantage of the electric-discharge particle accelerator.

REFERENCES

1. **Arnold, D., L. Feng, J. Kim and N. Heintz.** 1994. A strategy for the analysis of gene expression during neural development. Proc. Natl. Acad. Sci. USA *91*:9970-9974.
2. **Christou, P., D.E. McCabe, B.J. Martinell and W.F. Swain.** 1990. Soybean genetic engineering—commercial production of transgenic plants. Trends Biotechnol. *8*:145-151.
3. **Klein, T.M., R. Arentzen, P.A. Lewis and S. Fitzpatrick-McElligott.** 1992. Transformation of microbes, plants and animals by particle bombardment. Bio/Technology *10*:286-291.
4. **Klein, T.M., E.D. Wolf, R. Wu and J.C. Sanford.** 1987. High-velocity microprojectiles for delivery of nucleic acids into living cells. Nature *327*:70-73.
5. **Lo, D.C., A.K. McAllister and L.C. Katz.** 1994. Neuronal transfection in brain slices using particle-mediated gene transfer. Neuron *13*:1263-1268.
6. **McAllister, A.K., D.C. Lo and L.C. Katz.** 1995. Neurotrophins regulates dendritic growth in developing visual cortex. Neuron *15*:791-803.
7. **Niwa, H., K. Yamamura and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene *108*:193-200.
8. **Oard, J.H., D.F. Paige, J.A. Simmonds and T.M. Gradziel.** 1990. Transient gene expression in maize rice and wheat cells using an air-gun apparatus. Plant Physiol. *92*:334-339.
9. **Sanford, J.C., M.J. Devit, J.A. Russell, F.D. Smith, P.R. Harpending, M.K. Roy and S.A. Johnston.** 1991. Am improved, helium-driven biolistic device. Technique *3*:3-16.
10. **Sautter, C., H. Waldner, G. Neuhaus-Url, A. Galli and I. Potrykus.** 1991. Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of microprojectiles. Bio/Technology *9*:1080-1085.

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Modification of an In Situ Renaturation Method for Analysis of Protein Kinase Activity with Multiple Substrates

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Renaturation of enzyme subunits in situ following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of protein preparations is a powerful tool for analyzing protein kinases (2,4). The technique allows for evaluating the efficacy of putative in vivo and model protein kinase substrates, divalent cation requirements and nucleotide specificity of several different protein kinases in a single preparation based on their subunit molecular weight (MW_{sub}). We have found this technique useful for examining changes in protein kinase activities in response to anaerobic stress in the rice weed, *Echinochloa phyllopogon* L. Beauv. More specifically, we are using it to study the role of phosphorylation of enolase during anaerobiosis.

One drawback with the current technique is the requirement for relatively large quantities of protein substrate (20–40 mg) to be incorporated into the

gel matrix (3). In our experience, it is preferable to run standard-size gels rather than minigels. The greater separation between the protein kinases in standard-size gels allows better resolution after autoradiography. With model substrates such as casein, phospholipase D, histone and protamine, larger quantities are less problematic because the substrates can be obtained commercially at reasonable cost. When evaluating putative in vivo substrates, however, obtaining the necessary amount of purified protein (20–40 mg) required for one standard-size PROTEAN® II xi Slab Gel (Bio-Rad, Hercules, CA, USA) may be difficult with respect to cost and time requirements when tissue amounts are limited. We encountered both kinds of problems when studying enolase phosphorylation in response to anaerobic stress in *E. phyllopogon*. Initially, we made use of commercially available sources of rabbit and yeast enolases. When we compared these sources with enolase purified from *E. phyllopogon*, however, we found different sets of protein kinases interacting with the different enolases (data not shown). Consequently, we prefer to use enolase purified from *E. phyllopogon* for our studies.

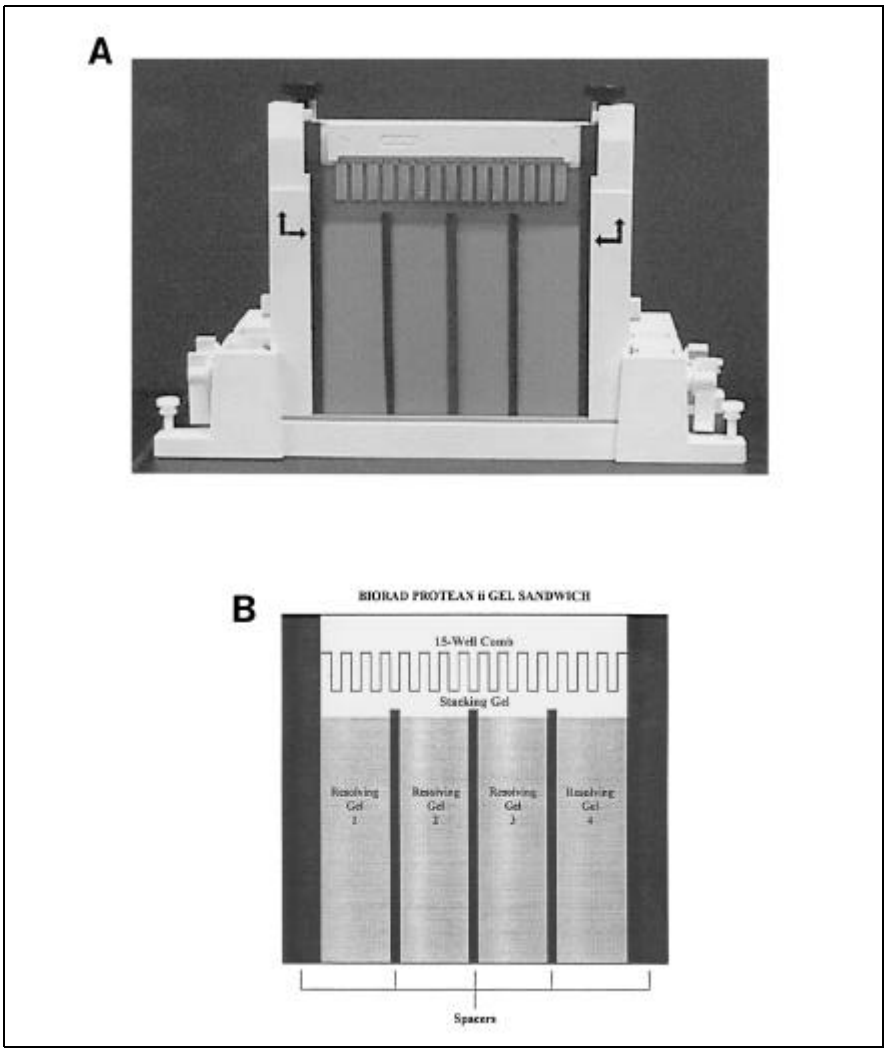

Figure 1. Modified in situ renaturation apparatus for simultaneous analysis of phosphorylation substrates. Photograph (A) and diagram (B) of a PROTEAN II xi Slab Gel System modified to run four modular gels in parallel. The resolving gel (0.75 mm thick) is divided into four modular gels by adding three spacers trimmed to 13.7 × 0.6 cm (slightly taller than the height of the resolving gel and the width of one well). The spacers are sealed in place with vacuum grease, allowing each minigel to be filled with an SDS-PAGE gel matrix (13.9% T, 2.7% C) containing 0.5 mg/mL of different protein substrates (e.g., enolase, casein, phospholipase D, histone, protamine) without cross-contamination.

Purification of enolase from *E. phyllopogon* is a four-step process involving ethanol precipitation, ammonium sulfate fractionation, Q Sepharose® (Pharmacia Biotech, Piscataway, NJ, USA) anion-exchange chromatography and gel filtration that requires three to four days to complete and yields only 0.25–0.50 mg protein from 10 g of shoot tissue (6). Large quantities of plant material are not readily available, and the low yields of purified enolase caused us to look for ways to reduce the amount of enolase protein required for in situ renaturation experiments.

We have modified our slab gel system (16 × 20 cm, 0.75 mm thick) to require less substrate while retaining the resolution of the longer gel (Figure 1). We divided the resolving gel into four modular gels by adding three spacers trimmed to 13.7 cm × 0.6 cm × 0.75 mm (i.e., slightly taller than the resolving gel and the width of one well). The spacers were sealed in place with vacuum grease, allowing each modular gel to be filled with SDS-PAGE gel matrix (13.9% T, 2.7% C) containing 0.5 mg/mL of different protein substrates (e.g., enolase, casein, phospholipase D, histone, protamine) without cross-contamination. By reducing both gel volume and the concentration of protein substrate to 0.5 mg/mL, only 3.5 mg of enolase per gel were required. Substrate concentrations ranging from 0.2 to 1.0 mg/mL were evaluated, and 0.5 mg/mL was judged as the minimum concentration to yield satisfactory detection of protein kinases. After polymerization of the resolving gel, a common stacking gel was poured, and proteins in crude extracts were resolved by standard SDS-PAGE. Following electrophoresis, SDS was removed from the gels by washing 5 times in 50 mM Tris-HCl (pH 7.6) for 15 min. In the examples shown here (Figure 2), phosphorylation was carried out by incubating individual gels in 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MnCl_2$ and 250 µCi [γ-^{32}P]ATP for 2 h at 25°C. Gels were washed three times with 40 mM Tris-HCl (pH 7.6), 1 mM EDTA, 2.5 mM Na^+ pyrophosphate and 5% (wt/vol) Dowex® 2 × 8-50 resin (Fluka, Hauppauge, NY, USA) for 1 h to remove unincorporated ^{32}P. The gels were briefly rinsed with deionized water and either fixed with three washes in 10% (wt/vol) trichloroacetic acid (TCA) at 70°C for 1 h or treated with 1 M KOH at 60°C for 1 h to dephosphorylate some ser/thr phosphoproteins and fixed with two washes in 10% (vol/vol) acetic acid at 60°C for 1 h (1). The gels were placed on plastic supports, sealed in plastic wrap and exposed directly to Fuji X-ray film at 4°C for 1–3 days.

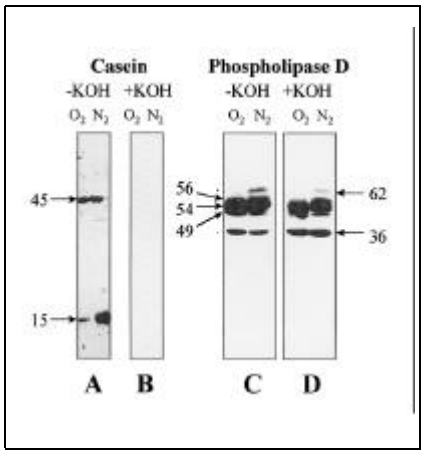

Figure 2. In situ renaturation of protein kinase activity. Protein extracts from shoots of *E. phyllopogon* seedlings grown aerobically (O_2) or anaerobically (N_2) were run in four modular gels in parallel. Panels A and B contained casein incorporated into the gel matrix, whereas Panels C and D contained phospholipase D. Following electrophoresis, SDS was removed from the gels and phosphorylation carried out in vitro. Parallel modular gels were treated with KOH following phosphorylation, and the resultant loss of ^{32}P in specific polypeptides suggests the presence of ser/thr kinases (Panels B and D).

Figure 2 shows the results of a typical in situ renaturation electrophoretic run in which the standard gel was divided into four modular gels run in parallel. Adjacent modular gels contained either casein (Figure 2, A and B) or phospholipase D (Figure 2, C and D). With casein as substrate, two polypeptides of 15 and 45 kDa were detected. Phosphorylation of the 15-kDa protein was enhanced in shoots of anaerobically grown seedlings, whereas the activity of the 45-kDa protein kinase was insensitive to the anaerobic treatment (Figure 2A). Treatment of the duplicate modular gel with potassium hydroxide dephosphorylated both proteins, indicating that phosphorylation occurs at ser/thr residues (Figure 2B). The size of the 45-kDa phosphoprotein is within the typical range for ser/thr protein kinases, and dephosphorylation with potassium hydroxide supports this conclusion. The 15-kDa phosphoprotein, however, is too small to contain the catalytic subunit typical of ser/thr protein kinases and may reflect the activity of nucleoside diphosphate kinase (5). With phospholipase D incorporated into the gel matrix, protein kinases with MW_{sub} of 56 and 62 kDa were stimulated by anoxia, those of 36 and 49 kDa were repressed by anaerobic treatment, and a protein kinase MW_{sub} of 54 kDa exhibited similar activities in both treatments (Figure 2C). Potassium hydroxide treatment did not dephosphorylate any of the labeled proteins, although the amount of phosphorylation of the 49- and 62-kDa polypeptides was reduced (Figure 2D). Since potassium hydroxide does not dephosphorylate all ser/thr phosphoproteins, we cannot definitively conclude whether these are ser/thr or tyr protein kinases from these results.

From a technical point of view, it is important to note that no cross-contamination between adjacent modular gels containing different substrates (Figure 2, B vs. C) was observed. The MW_{sub} of protein kinases acting on casein (Figure 2, A and B) was completely different from those acting on phospholipase D (Figure 2, C and D). Thus, we can survey up to four protein kinase substrates in one electrophoretic run with confidence that the protein kinases are specific to the intended substrate and not the result of protein substrates leaking from adjacent modular gels.

The technique of dividing the larger gel into modular gels is also advantageous when duplicate gels are required. Previously, we ran duplicate lanes and cut the gel for differential processing. The cut edges of the gels served as initiating points for tearing and necessitated very gentle handling of the gels during the high number of washes required in the protocol. The tendency of the gel to tear was exacerbated when SDS was removed from the gels and they became more fragile. The polymerized surface at the edges of the modular gels reduced the likelihood of tearing and made the gels easier to handle. Generation of multiple modular gels that contain the same protein substrate in one

electrophoretic run permits convenient side-by-side comparisons among several different kinase reaction conditions (e.g., the divalent cation [Ca^{2+}, Mg^{2+} or Mn^{2+}] requirements or various reaction pHs) following electrophoresis.

In situ renaturation detection of protein kinase activity is a time-consuming and fairly expensive process. The modifications presented here allow for the simultaneous processing of four different sets of protein kinases or generation of multiple gels to assess up to four different protein kinase reaction conditions in a single electrophoretic run. The resolving power of larger slab gels is retained while reducing the amount of protein substrates required by nearly 90%. Thus, the modular gel technique described here provides greater experimental flexibility and increased efficiency, leading to new levels of resolution compared to standard full-sized gels or minigels.

REFERENCES

1. **Cooper, J.A., B.M. Sefton and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. *99*:387-405.
2. **Fallon, K.M. and A.J. Trewavas.** 1994. Phosphorylation of a renatured protein from etiolated wheat leaf protoplasts is modulated by blue and red light. Plant Physiol. *105*:253-258.
3. **Geahlen, R.L., M. Anostario, Jr., P.S. Low and M.L. Harrison.** 1986. Detection of protein kinase activity in sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. *153*: 151-158.
4. **Lacks, S.A. and S.S. Springhorn.** 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J. Biol. Chem. *255*:7476-7484.
5. **Moisyadi, S., S. Dharmasiri, H.M. Harrington and T.J. Lukas.** 1994. Characterization of a low molecular mass autophosphorylating protein in cultured sugarcane cells and its identification as a nucleoside diphosphate kinase. Plant Physiol. *104*:1401-1409.
6. **Mujer, C.V., T.C. Fox, A.S. Williams, D.L. Andrews, R.A. Kennedy and M.E. Rumpho.** 1995. Purification, properties and phosphorylation of anaerobically induced enolase in *Echinochloa phyllopogon* and *E. crus-pavonis*. Plant Cell Physiol. *36*:1459-1470.

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