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Methylene blue: an alternative, multi-purpose stain for detection, analysis and isolation of nucleic acids

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A series of experiments was performed utilizing Methylene Blue (MeB) in place of the intercalating dyes ethidium bromide (EtBr) and acridine orange (AO) to stain, visualize, and isolate DNA and RNA. MeB proved to be superior to the other dyes for several purposes: 1) visualization of glyoxalated (chemically denatured) RNA in agarose gels, 2) staining of nucleic acids that are to be used in subsequent hybridization experiments, and 3) isolation and purification of plasmid DNA by CsCl ultracentrifugation. MeB was found to perform at least as well as EtBr or AO for visualization of DNA in agarose of acrylamide gels, and DNA stained with MeB can be purified from agarose gel slices by the Gene Clean protocol. These results indicate that MeB is a very effective nucleic acid stain. Its safety versus conventional intercalating dyes will be discussed.

Introduction. Detection and analysis of nucleic acids is an integral part of molecular biology, and several dyes are commonly used for this purpose, including ethidium bromide (EtBr) and acridine orange (AO). The ideal stain is rapid, sensitive, stable, non-toxic, required no special equipment for use, is useful for double and single stranded nucleic acids, and does not interfere with subsequent hybridization experiments. We have investigated the use of methylene blue (MeB) as an alternative to EtBr and AO, and found it to perform as well as or better than these intercalating dyes in many experimental procedures. Additional advantages to the use of MeB are that exposure to ultra-violet (UV) light is not necessary to visualize DNA or RNA, thus eliminating undesirable UV cross linking of samples and exposure of laboratory personnel to the potentially harmful effects of UV light.

Evidence also indicates that MeB is less toxic than either EtBr or AO, making it safer to use and easier to dispose of than standard intercalating dyes. The lowest reported lethal dose (LDLo) for intraperitoneal administration in mice is 20 mg/kg for EtBr [1], and 64 mg/kg for AO [2]. The LDLo of MeB for subcutaneous administration in guinea pig is 300 mg/kg, and 1000 mg/kg and 500 mg/kg in rabbit and dog, respectively, for oral administration [3].

Materials and Methods. *Methylene blue staining of denatured RNA.* Duplicate sets of samples, consisting of 15 μg total soybean leaf RNA, were electrophoresed in identical 1.0 % (w/v) agarose gels in 10 mM sodium phosphate buffer, pH 7.0. RNA was visualized by one of two methods: 1) stained in 10 μg/ml MeB in DEPC-treated, sterile water for 15 min, then destained in 3 changes of deionized water over approximately 1 hr, or 2) stained in 1 μg/ml AO in sterile water, then destained.

RNA was then transferred by capillary blotting to Schleicher and Schuell Nytran nylon membranes in 10·SSC (1·SSC = 0.15 M NaCl, 0.015 M Na-Citrate) overnight. Nylon membranes were baked at 67 °C for approximately 1.5 hr, then pre-hybridized in Solution 1 (5 % (w/v) SDS, 4·SSC, 5·Denhardt's, 50 mM $NaPO_4$, pH 6.8, 100 μg/ml denatured salmon sperm DNA) at 42 °C for at least 6 hr. Hybridization to a ^{32}P-labeled (random priming; specific activity of at least $1 \cdot 10^8$ cpm) cDNA probe was performed in Solution 2 (50 % (v/v) formamide, 0.5 % (w/v) SDS,

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4·SSC, 5·Denhardt's, 50 mM $NaPO_4$, pH 6.8, 50 μg/ml denatured salmon sperm DNA) at 42 °C for at least 12 hr. Blots were washed in 0.1·SSC plus 0.1% (w/v) SDS, and exposed to film (Kodak X-AR).

Densitometer scans of autoradiographs were performed on an LKB. Ultroscan XXL Laser Densitometer with LKB 2400 Gelscan XL software version 1.0. Peak areas were calculated as:

$$\text{peak area} = \text{mm} \cdot AU_{633\ nm}.$$

The valley-to-valley method was used to calculate baselines, and data were normalized to the strongest signal.

Methylene blue staining of DNA in agarose and polyacrylamide gels. Duplicate samples of 500, 100, 50, and 10 ng of lambda DNA digested with *HindIII* were electrophoresed in identical 1.0 % (w/v) agarose gels in 1·TAE (40 mM Tris acetate, 1 mM EDTA). Similarly, duplicate sets of samples were separated in 7 % acrylamide gels (prepared from a 40 % (w/v) solution of acrylamide:bisacrylamide, 19:1) in 1·TBE (90 mM Tris borate, 2 mM EDTA; 4) Gels were stained with either 1 μg/ml EtBr or 10 μg/mM MeB in water, then destained.

Use of methylene blue in DNA isolation and recovery. Standard large scale preparations of *pUC118/119* plasmid DNA were prepared by the alkaline lysis method [4]. The density of the crude plasmid DNA solutions was adjusted to 1.55 g/ml with solid CsCl, and equal volumes (6.2 ml) of this solution were transferred to 2 15-ml Corex tubes. 300 μl of 10 mg/ml EtBr or 300 μl of 2 mg/ml MeB was added, the density readjusted to 1.55 g CsCl/ml, and tubes were centrifuged for 5 min at 7000×*g* to pellet remaining precipitated proteins and cell debris. The supernatant fractions were transferred to 5-ml Beckman quick-seal tubes (the pellet in the MeB tube was looser and more flaky than that in the EtBr tube, making it more difficult to avoid transferring some solid to the quick-seal tube).

Ultracentrifugation was performed at 50,000×*g* for about 15 hr at 20 °C. Plasmid DNA bands were clearly visible in each tube, and were extracted with a 16 gauge needle attached to a 3-ml syringe. 100 μl more of each stain was added to the appropriate sample, and a second round of ultracentrifugation was performed as above. Distinct plasmid bands were visible in both tubes (Fig. 4); note that MeB-stained plasmid DNA migrates further down the CsCl gradient due to its increased density. Plasmid DNA bands were extracted from the tubes as above. *n*-Butanol was found to extract MeB and EtBr equally well from the samples (data not shown). Following dialysis in TE buffer pH 8.0 (TE = 10 mM Tris, 1 mM EDTA) to remove remaining CsCl [4], DNA samples were precipitated with 95 % (v/v) ethanol, dried under vacuum, and resuspended in sterile TE. Concentrations were determined spectrophotometrically on duplicate samples.

Results and Discussion. Although methylene blue is commonly used as a stain for the detection of RNA on Northern blots [5, 6], it has not been tested as a general purpose nucleic acid stain. Thus, we substituted MeB for traditional intercalating dyes, ethidium bromide and acridine orange, in several molecular biology procedures. Use of MeB was found to be preferable to intercalating dyes for staining glyoxalated RNA in agarose gels (Fig. 1). Glyoxal chemically denatures RNA by covalently modifying guanine residues, and glyoxalated RNA does not stain well with EtBr or AO unless the glyoxal is first removed from the gel by brief treatment with 0.05 M NaOH and neutralization [7], which we found to have undesirable effects on RNA stability and its transfer

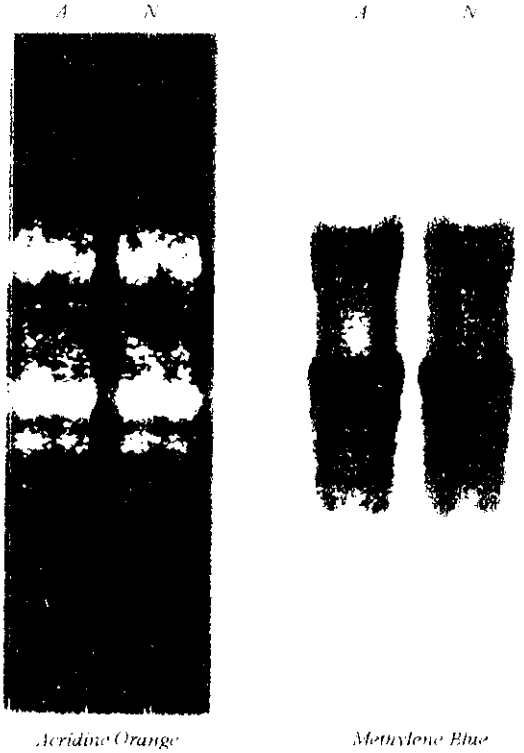

Fig. 1. Visualization of total RNA isolated from the soybean cultivars Acme (*A*) and Norchief (*N*). Fifteen μg samples of glyoxalated (denatured) RNA were electrophoresed as described in «Materials and Methods» and stained with 1 μg/ml AO or 10 μg/ml MeB. MeB was more effective than intercalating dyes for staining chemically denatured RNA

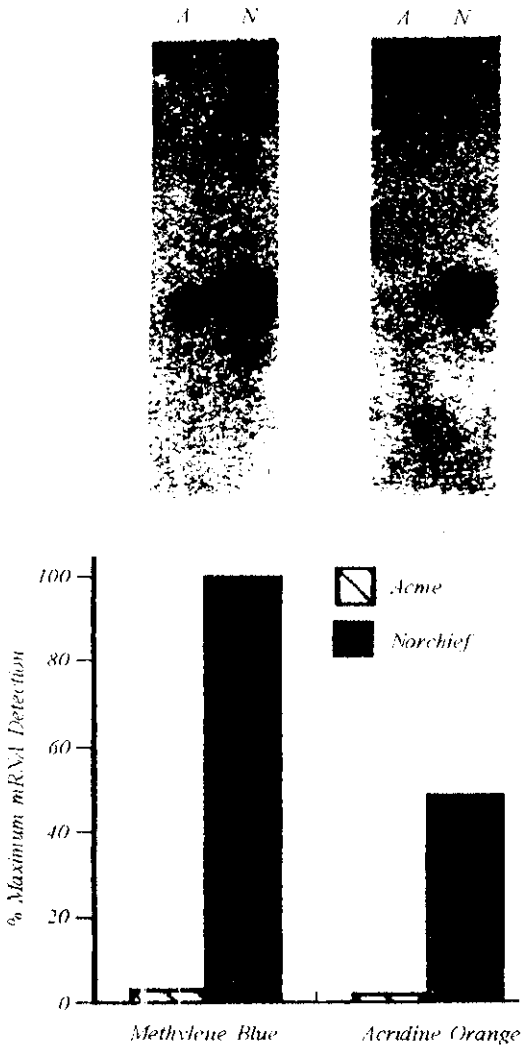

Fig. 2. Northern blot analysis of total RNA isolated from the soybean cultivars Acme (*A*) and Norchief (*N*). After electrophoresis and staining of RNA samples with either AO or MeB, RNA was transferred to nylon membranes by blotting and hybridized with a ^{32}P labeled DNA probe specific for an mRNA transcript found in N, but not A (A was included as a negative control). Visual inspection of the autoradiograph revealed that MeB-stained RNA bound the probe more effectively (top panel). Densitometric analysis of the film showed that the increase in hybridization efficiency over AO-stained RNA was approximately 100 % (bottom panel)

to nylon membranes. In addition, our results indicate that binding of a ^{32}P-labeled cDNA probe to AO-stained RNA samples was inhibited about two-fold in comparison to MeB-stained samples (Fig. 2), demonstrating that MeB-stained RNA is also superior for hybridization to DNA probes. Because visualization of RNA by MeB staining does not require prior removal of glyoxal from the gel, denatured RNA can be processed as the glyoxalated adduct throughout electrophoresis and transfer, thus minimizing the risk of RNA degradation [8].

For visualizing DNA after electrophoresis, MeB provides comparable sensitivity to EtBr in agarose gels (Fig. 3), and in acrylamide gels (data not shown). MeB-stained gels can be viewed in ambient room light and photographed on a white light box; they require no exposure of the investigator or DNA samples to UV light. In contrast to EtBr, however, MeB can not be used in the gel to stain nucleic acids during electrophoresis, as this was found to cause distortions in the migration of DNA fragments.

MeB can also be substituted for traditional intercalating dyes in the isolation and purification of plasmid DNA by CsCl density ultracentrifugation (Fig. 4). Surprisingly, plasmid yield after CsCl den-

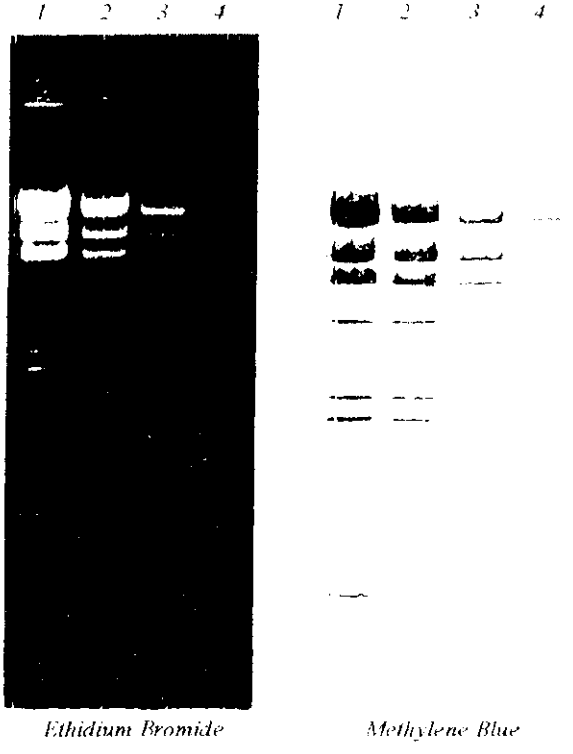

Fig. 3. Visualization of *HindIII* restriction endonuclease-digested lambda DNA after electrophoresis. Duplicate sets of 500, 100, 50 and 10 ng samples (lanes *1* through *4*, respectively) were electrophoresed and stained with either 1 μg/ml EtBr or 10 μg/ml MeB. MeB stained the DNA at least as effectively as the more commonly used dye, EtBr

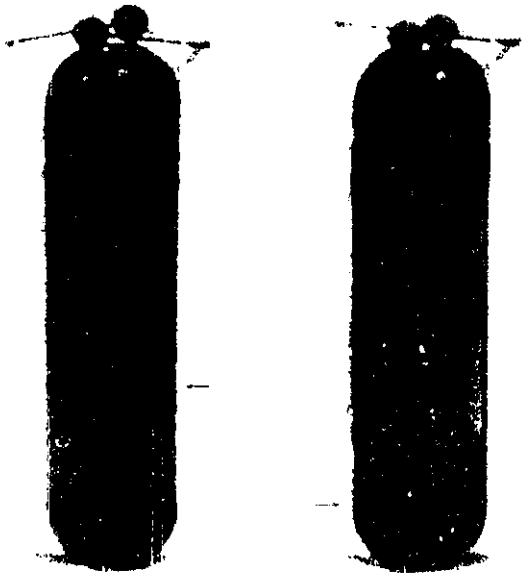

Fig. 4. Cesium chloride density gradient purification of plasmid DNA. Crude plasmid DNA was prepared by a standard alkaline lysis protocol and then purified by ultracentrifugation in a CsCl gradient, utilizing either EtBr or MeB for visualization of the plasmid band (see «Materials and Methods» for details). Arrows indicate the position of each band; note that MeB increases the density of the DNA, resulting in its migration further down the tube

sity ultracentrifugation with MeB staining was up to 10-fold higher than with EtBr staining. While we cannot explain this result, the increased yield with MeB was consistent and repeatable. Both MeB and EtBr plasmid preparations yielded very pure DNA that was easily digestible with restriction endonucleases.

Finally, we determined that agarose gel slices containing MeB-stained DNA bands could be processed by the Gene Clean protocol (Bio101, La Jolla, CA) to recover DNA fragments for cloning. DNA fragments stained with either EtBr or MeB are equally recoverable by this procedure (data not shown).

In this safety-conscious age, it is desirable to develop molecular biology protocols that are less hazardous to the scientist and have fewer negative environmental side effects than conventional methods. This series of experiments was carried out to demonstrate that MeB can be used in place of the more hazardous intercalating dyes EtBr or AO in many applications involving DNA or RNA.

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Метиленовий синій: альтернативний багатоцільовий барвник для виявлення, аналізу та виділення нуклеїнових кислот

Резюме

Здійснено серію експериментів з використання метиленового синього (МС) замість інтеркалюючих барвників бромистого етидію (ЕБ) та акридинового оранжевого (АО) для забарвлення, візуалізації та виділення ДНК і РНК. Показано, що МС переважає інші барвники у використанні для декількох цілей: 1) візуалізації гліоксильованої (хімічно денатурованої) РНК в агарозних гелях; 2) забарвлювання нуклеїнових кислот, які в подальшому будуть використані в експериментах з гібридизації; 3) виділення та очистки плазмідної ДНК ультрацентрифугуванням в CsCl. Знайдено, що за допомогою МС ДНК так само добре виявляється в гелях агарози або акриламіду, як і з використанням ЕБ і АО, а ДНК, забарвлена МС, може бути очищена із зрізів гелей агарози по протоколу «Gene Clean». Ці результати вказують на те, що МС є дуже ефективним барвником нуклеїнових кислот. Обговорюється його безпека порівняно з загальноприйнятними інтеркалюючими барвниками.

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Метиленовый синий: альтернативный многоцелевой краситель для обнаружения, анализа и выделения нуклеиновых кислот

Резюме

Проведена серия экспериментов по использованию метиленового синего (МС) вместо интеркалирующих красителей бромистого этидия (ЭБ) и акридинового оранжевого (АО) для окрашивания, визуализации и выделения ДНК и РНК. Показано, что МС превосходит другие красители в использовании для нескольких целей: 1) визуализации глиоксилированной (химически денатурированной) РНК в агарозных гелях; 2) окрашивания нуклеиновых кислот, которые впоследствии будут использоваться в экспериментах по гибридизации; 3) выделения и очистки плазмидной ДНК ультрацентрифугированием в CsCl. Обнаружено, что МС так же пригоден для визуализации ДНК в гелях агарозы или акриламида, как ЭБ или АО, а ДНК, окрашенная МС, может быть очищена из срезов гелей агарозы по протоколу «Gene Clean». Эти результаты указывают на то, что МС — очень эффективный краситель нуклеиновых кислот. Обсуждается его безопасность по сравнению с общепринятыми интеркалирующими красителями.

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