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Short Communication

Development of a simple and effective silver staining protocol for detection of DNA fragments

Silver staining is one of the widely used methods for DNA fragment detection in biological research. Silver staining protocols have been steadily optimized to improve detection efficiency. This research reports a continuous effort to simplify the existing silver staining protocols, lower experiment cost, and improve DNA detection sensitivity and image clarity. The new method only requires three reagents (silver nitrate, sodium hydroxide, and formaldehyde) and 6–7 min with high detection sensitivity to visualize as low as 14.6 pg (3.3 pg/mm^2) of DNA in a non-denaturing polyacrylamide gel. In comparison to previous reported protocols, the new one has the highest resolution, is the easiest to operate, takes the shortest time, and uses the fewest chemical reagents. Therefore, the new method can be used for quick generation of high quality molecular marker data in genetic analysis.

Keywords:
DNA / Non-denaturing polyacrylamide gel / Silver staining
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Silver staining of DNA in polyacrylamide gels has the highest sensitivity compared with other staining methods [1, 2], and has been widely used to detect DNA fragments such as SSR and other types of DNA fragments as molecular markers [3]. Like many other biological laboratory techniques, silver staining of gels has been steadily improved since its first report as a fragment visualization technique in 1979 [4]. Bassam and coworkers [2] made the first modification in 1991, and Sanguinetti et al. have made further improvement 3 years later [5]. Then, continuous optimizations of the staining protocol have been reported in the past several decades [6–10]. However, most of these updated protocols still have some drawbacks because they involve high technical demand and time-consuming processes in fixation and mounting steps [2]. These drawbacks make application of the silver staining more difficult and resource consuming [3, 6]. Further optimization of the protocols by reducing cost and improving efficiency of fragment detection will facilitate routine application of the technique in biological research, especially in large-scale genotyping.

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Abbreviations: **EBT**, eriochrome black T; **HCHO**, formaldehyde; **SSR**, simple sequence repeat

Silver staining can be used in either denaturing or non-denaturing polyacrylamide gels. It has been proved that the resolution for detection of DNA fragments did not have significant differences between denaturing and non-denaturing polyacrylamide gels, but non-denaturing polyacrylamide gels have the advantage of being simpler to apply [11]. This research aimed to improve the existing silver staining protocols for application in non-denaturing polyacrylamide gels by reducing cost, simplifying process, and enhancing detection sensitivity.

The reagents used in this study were of electrophoresis grade and bought from Sigma-Aldrich Corporation (St. Louis, MO, USA). Electrophoresis was conducted using 6% non-denaturing polyacrylamide gels (mix of acrylamide and N,N′-methylene-bis-acrylamide at a ratio of 29:1) made using glass plates of 33 cm × 16 cm and a two-sided vertical electrophoresis tank (C.B.S. Scientific Inc., USA). Each of the gel combs were 3 mm wide and the gel thickness was 1.5 mm which makes a lane base area of 4.5 mm^2 (3 mm × 1.5 mm). The electrophoresis was run with 1× TBE buffer (100 mM Tris-HCl, 83 mM boric acid, and 1 mM EDTA-Na_2 at pH 8.0) at 120 V for 120 min.

The new protocol involves two steps: impregnation and development. After electrophoresis, gels were impregnated into a $AgNO_3$ solution (0.15%) for 3–4 min with shaking at

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Table 1. Reagents, process time, and minimum amount of DNA used in different silver staining protocols

	Bassam et al. [2]	Sanguinetti et al. [5]	Qu et al. [6]	Byun et al. [9]	An et al. [8]	Liang et al. [3]	Kumar et al. [10]	New procedure
Fix	10% acetic acid (20 min)	10% ethanol, 0.5% acetic acid (3 min)	—	—	—	—	—	—
Wash	Water (2 min, 3 times)	—	—	—	—	—	Water (5 s 2 times)	—
Stain	0.1% $AgNO_3$, 0.0555% HCHO (30 min)	0.2% $AgNO_3$ (5 min)	0.1%$AgNO_3$, 25% ethanol, 1% HNO_3, (5–10 min)	0.2%$AgNO_3$, 10% ethanol, 0.5% acetic acid (3~20 min)	0.1% $AgNO_3$, 5% ethanol, 1% nitric acid, (5 min)	0.2% $AgNO_3$, 10% ethanol, 2% HNO_3 (4 min)	10% ethanol 0.7% HNO_3 0.22% $AgNO_3$ (5–30 min)	0.1–0.15% $AgNO_3$ (3–4 min)
Wash	Water (20 s, optional)	Water (20 s and 2 min)	Water (3 min)	Water (rinse,1 time)	Water (10 s)	Water (5 s)	—	Water (5 s)
Develop	3% Na_2CO_3, 0.0555% HCHO, 0.0002%$Na_2S_2O_3.5H_2O$ (10°C, 2–5 min)	3% NaOH, 0.5% HCHO (5 min)	3%Na_2CO_3, 0.074%HCHO (2–5 min)	3% NaOH, 0.037%HCHO (55°C, 5–10 min)	1.3% NaOH, 0.65% $NaCO_3$, 0.148% HCHO; (2–3 min)	3% NaOH, 0.148% HCHO, 0.005% EBT (3 min)	2.29% Na_2CO_3 0.046% HCHO, 0.0002%$Na_2S_2O_3.5H2O$ (4°C, 3–5 min)	1% NaOH, 0.037% HCHO, (3 min)
Stop	10% acetic acid (4°C, 5 min)	Fixing solution (5 min)	10% acetic acid (2–5 min)	10% ethanol, 0.5% acetic acid (1 min)	5% ethanol, 1% nitric acid (1 min)	—	3% acetic acid 10% ethanol (4°C, 5 min)	—
Wash	—	Water (10 min)	—	—	—	—	—	—
Run time (min)	63–66	30	12–25	9–31	8–9	7	13–42	6–7
Reagents	5	5	6	5	6	6	7	3
Minimum detection	5–10 pg/mm^2	—	3.5 ng	16 pg (4–10 pg/mm^2)	27.8 pg (5.6 pg/mm^2)	97 pg	—	14.6 pg (3.3 pg/mm^2)

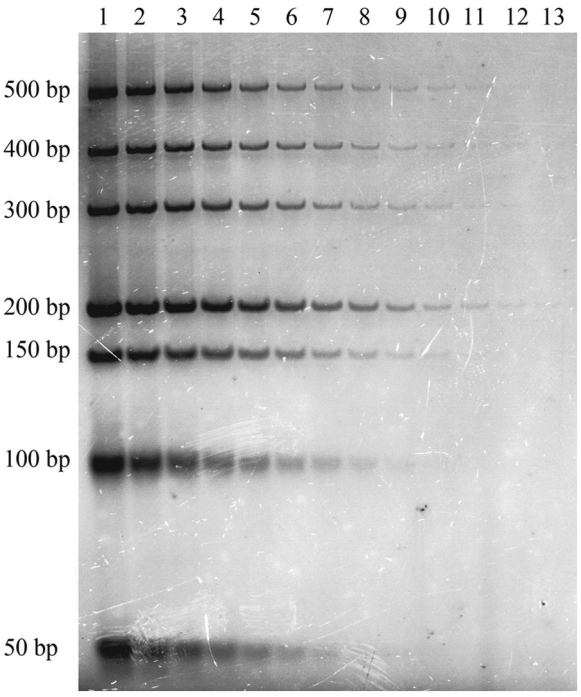

Figure 1. Sensitivity of the improved protocol as measured by DL500 DNA marker (Takara Bio Inc., Japan) on a non-denaturing polyacrylamide gel. The first lane was loaded with 1 μL DL500 DNA marker with fragment sizes of 50, 100, 150, 200, 300, 400, and 500 bp at a concentration of 10 ng/μL except for 200 bp standard at 30 ng/μL. The remaining samples were loaded with twofold dilutions of each sample in preceding lanes in the series. The minimum detectable amount for the new protocol was 14.6 pg in the 12th lane (3.3 pg/mm^2 in a 4.5 mm^2 well size).

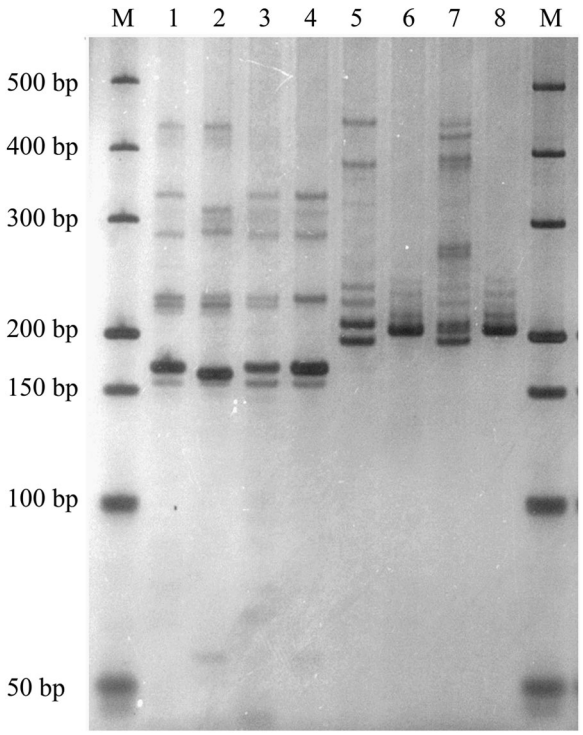

Figure 2. Detection of PCR products of SSR markers in tobacco and flowering Chinese cabbage genotypes on a non-denaturing polyacrylamide gel using the improved protocol. Lane M is DL500 DNA size marker (Takara Bio Inc., Japan). Lanes 1 to 4 represent the amplicons of SSR primer pairs of TME0580 (forward TGGAAACGTTTGCTTAAGGGTA and reverse GTGCAACGTGGACATTTGAA) in tobacco genotypes "Yanyan 97", "Honghua dajinyuan", "118-3" and "Yueyan 98", respectively. Lanes 5 to 8 indicate the amplicons of SSR primer pairs of CX-3 (forward ACTCGAATTCCGGTGAGTTG and reverse CATTGCACCTGCTCATGTTT) in flowering Chinese cabbage genotypes "Youlv 501", "Liuye 50", "49-19" and "3T6", respectively.

60 rpm, then rinsed with deionized water for 5 s and soaked in a developing solution (1% NaOH, 0.037% HCHO) for 3 min without shaking or agitation. The gel was rinsed with water, air-dried, and then scanned to generate an image using a BenQ M800 scanner (BenQ, China). All the processes were carried out at room temperature.

The entire staining process takes only 6–7 min, which is the shortest time compared to other existing staining protocols (Table 1). The new protocol shortens the processing time by removing prefixing and several washing steps that were required in other protocols, which significantly simplifies staining process, but does change staining quality. The next fast staining protocol took us an average of 10 min to get the similar results as our protocol in repeated experiments although it was reported to be 7 min [3].

The number of reagents used in different protocols ranged from three (the new method in this study) to seven [10] (Table 1). The new protocol is obviously more economical and less safety concern on chemical hazards because it does not use chemicals such as HNO_3, ethanol, acetic acid, EBT, $Na_2S_2O_3$, and Na_2CO_3 that are required in other protocols [5, 6, 8, 10]. The optimized protocol not only reduces reagent cost, but also avoids the hazardous handling of these chemicals in the laboratory and reduces the risk of environmental pollution.

DNA detection sensitivity is another major concern in selecting staining protocol for laboratory use. Our new protocol can efficiently detect DNA at a minimum concentration of 14.6 pg DNA (Fig. 1). The base area of each well is 4.5 mm^2 that gives a detection sensitivity of 3.3 pg/mm^2. The new protocol exhibited a higher detection sensitivity than that of An et al. [8] and Byun et al. [9] who reported to detect minimum concentrations of 27.8 pg (5.6 pg/mm^2) and 16–39 pg/μl (4-10 pg/mm^2), respectively. The protocol by Liang et al. [3] was even less sensitive than that of An et al. [8] (Table 1).

The improved protocol clearly detected PCR products from tobacco and flowering Chinese cabbage (Fig. 2) and generated the picture with a high contrast and low background effect. The new protocol provides an effective method for easy detection of SSR markers in tobacco and flowering Chinese cabbage.

In summary, our new protocol reduces the number of reagents down to three, and the processing time to 6 min without losing detection sensitivity as compared to the previously reported protocols. Though recent advances in detecting DNA using fluorescence-labeling technologies enable

multiplexing of several primers in one gel run to save time and gel cost, these techniques require specialized equipment and more expensive chemicals that may not be accessible to many laboratories, especially in developing countries. Thus our improved protocol is an important alternative for these laboratories to conduct routine DNA analysis. The new protocol not only shortens the staining processing time, but also eliminates several chemical reagents that reduce reagent cost and environmental contamination. In addition, the high repeatability of the new staining protocol has been confirmed by repeated tests of the new protocol. This protocol has been routinely used for DNA marker analysis in tobacco and flowering Chinese cabbage in our laboratory.

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