“In gel patch electrophoresis”: a new method for environmental DNA purification

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Abstract

Most of the microorganism species are largely untapped and could represent an interesting reservoir of genes useful for biotechnological applications. Unfortunately, a major difficulty associated with the methods used to isolate environmental DNA is related to the contamination of the extracted material with humic substances. These polyphenolic compounds inhibit the DNA processing reactions and severely impede cloning procedures. In this work, we describe a rapid, simple and efficient method for the purification of genomic DNA from environmental samples: we added a chromatography step directly embedded into an agarose gel electrophoresis. This strategy enabled the DNA extraction from various environmental samples and it appeared that the purity grade was compatible with digestion by restriction enzymes and PCR amplifications.

1. Introduction

With the discovery over the past three decades of a vast diversity of previously unsuspected and largely uncultivated microorganisms inhabiting diverse natural environments throughout the biosphere, biotechnology has undergone a revolution. However, current estimations indicate that less than 1% of the total microbial community observed under the microscope can be cultivated with current cultivation techniques [1-5]. Thus, most of the microorganism diversity is largely untapped and these species could represent an interesting reservoir of genes useful for biotechnological applications [6, 7] such as the production of antibiotics, agrochemicals, cosmetics, fine chemicals, flavors or pharmaceuticals [8, 9].

The extraction of total genomic DNA from environmental samples enables microbiologists to obtain biological material without the need to isolate microorganisms by cultivation. A major difficulty associated with the used methods is related to the contamination of the extracted DNA with humic substances: polyphenolic compounds that are co-purified with the DNA. These compounds are difficult to remove and it is well known that polyphenols also interfere with enzymatic modifications of isolated DNA [10, 11]. These compounds severely inhibit amplification reactions by PCR, hydrolysis by restriction enzymes, as well as ligation and cloning procedures.

Numerous attempts have focused on methods of genomic DNA extraction from a variety of environmental samples [11-13]. Laborious and time-consuming protocols involving DNA purification (gradient centrifugation, glass bead extraction, chromatography column, long-termed gel electrophoresis, spin column, agarose plug, two phase system) or extensive sample dilution prior to PCR have been necessary to obtain a PCR amplification from an environmental template. These available methods to extract DNA directly without any cultivation step are very time consuming. Additionally, they often result in significant losses of extracted DNA and the recovery rates are rather poor.

In this work, we describe a rapid, simple and efficient method for the purification of genomic DNA from environmental samples called *"In gel patch electrophoresis"*. We added a chromatography step directly into an agarose gel electrophoresis in order to increase significantly the purity grade of the extracted genomic DNA and to speed up the whole purification process.

2. Materials and methods

Sampling

Soil was collected in Stuttgart at the Institute of Technical Biochemistry (University of Stuttgart, Germany). Sediment and moss were collected from a settling pond in the lake Bärensee in Stuttgart (Germany). Activated sludge was collected from a sewage plant at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management of the University of Suttgart (Germany). All samples were expressed as wet weights.

Purification of genomic DNA from Environmental samples

Environmental samples (soil, sediment, activated sludge, moss, 0.5 g wet weight) were suspended in 1 mL of Tris buffer (100 mM, 10 mM EDTA, 100 mM NaCl, 1 % (w/v) CTAB, 20% (w/v) SDS, pH 8.0). The samples were then frozen in liquid nitrogen for 5 min and subsequently thawed at 65°C for 5min. After centrifugation at 14000 rpm for 5 min, the resulting supernatant was transferred to a fresh sterile tube. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the combined supernatant and mixed by inversion. The sample was then centrifuged and the aqueous upper layer was transferred to a fresh sterile tube. The supernatant was collected and 1 mL ethanol (70%) was added before centrifugation at 14000 rpm for 5 min. The supernatant was

discarded and the resulting pellets were dark brown colored. Crude DNA solutions were obtained by resuspending these pellets in 50 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). To eliminate humic substances and other coextracted substances, the precipitated brownish pellets and genomic DNA were loaded onto an 0.5% agarose gel. The *in gel patch electrophoresis* method was used to remove humic substances in two distinct procedures (figure 1B). A volume of 40 mL melted 0.5% low melting point (LMP) agarose was gently mixed with 2g of hydroxyapatite resin (Fluka, Buchs, Ch). The mixture was poured into sterile rectangular molds and rotated with ROTAMIX RM1 (ELMI) to ensure a uniform distribution of the resin in the agarose gel prior to solidification. Then the gel patch was extracted from the mold. After fixing the gel patch in the electrophoresis rack, melted 0.5 % LMP agarose was poured in the rack and then electrophoresis was carried out at 120V for 20min.

Microwave-based method for purification of genomic DNA from environmental samples, according to Orsini and Romano-Spica [14]

Environmental samples (0.1 g) were collected and suspended in 1 mL of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 0.1% (w/v) SDS, 0.1% (w/v) polyvinylpyrrolidone (PVP), pH 8.0). Samples were centrifuged at 8000 rpm for 1 min

and resuspended in 500 μL of a lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 3% (w/v) SDS, 1% (w/v) PVP, pH 8.0). Eppendorf tubes were heated in a microwave oven at 600-700 W for 1 min. And 500 μL volume of extraction solution of 65°C (10 mM Tris-HCl, 1 mM EDTA, 300 mM sodium acetate, 1% (w/v) PVP) was added to the sample. Phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed by inversion. DNA precipitation was performed with 70% ethanol and resuspended in 100 μL of TE buffer (pH 8.0).

Used Primers

Primers used in this paper include specific prokaryotic small subunit rRNA of bacteria : FORB 5'AGAGTTTGATCCTGGCTCAG, REVB 5'AGAAAGGAGGTGATCCAGCC [15], and the internal transcribed spacer (ITS) region for lichen fungi : LICHITSF 5'GCGGAAGGATCATTACTGAG, LICHITSR 5'GGGTATCCCTACCTGATCCG [16], and eukaryotic rRNA internal transcribed spacer (ITS) region : ITS1F 5' TCCGTAGGTGAACCTGCGG, ITS4R 5'TCCTCCGCTTATTGATATGC [17].

Moreover, degenerate primers designed for the detection of cytochrome P450 monooxygenase : For 5'CTACTGGGTSGTCACSCGSTACGA, Rev 5'GCAYTCCTCG AYGGCSTTGGGGAT, specific primer of human cytochrome b_5 : For

5’CCGGAATTCGAGATGGCAGAGCAGTCG, Rev 5’CCGCTCGAGTTATCAGTCC TCTGCCATGTATAG and specific primer of β-lactamase : For 5’CATGATCCATGAGTATTCAACATTTCCGT, Rev 5’CGCGGATTCTTACCAATGC TTAATCAGT.

Restriction endonuclease treatment and PCR

Restriction enzyme digestions were performed with approximately 5 μg of DNA and 10 U of endonuclease (*Bam* HI, *Eco* RI, *Hind* III, *Sma* I, *Xho* I) in 20 μL of the appropriate buffer as provided by the manufacturer. After incubation for 3 h, the DNA fragments were resolved in a 1% agarose gel. Purified genomic DNA extracted from environmental samples was used as template in the PCR. PCR was conducted with a Perkin-Elmer thermal cycler as follows: 2 min of denaturation at 94°C, followed by 35 amplification cycles (1 min at 94°C, 1 min at the optimized annealing temperature, 1 min at 72°C (extension), with a final 10 min at 72°C extension step after cycling was complete).

Determination of purity and amount of genomic DNA

To evaluate the purity of the extracted DNA, absorbance ratios at 260nm/230nm and

260nm/280nm were determined according to Sambrook *et al* [18]. The concentration of DNA was determined by assuming that an O.D. of 1 at 260 nm corresponds to 50 μg/ml for double-stranded DNA.

DNA sequencing

The DNA sequencing reaction was carried out on both strands of double-stranded templates using the BigDye Terminator Cycle Sequencing Kit RR-100 (Applied Biosystems, Weiterstadt, Germany). The sequencing product was analyzed on a ABI Prism^{TM} 377 DNA Sequencer (Perkin Elmer, Shelton, USA).

3. Results and discussion

In order to increase the purification efficiency of available DNA extraction methods, we added a chromatography step in an agarose gel electrophoresis. First, a piece of gel was packed with chromatography material (Hydroxyapatite, HA). After solidification, this "patch gel" piece was packed in a standard agarose gel (Figure 1). This combined system was used to remove the humic substances in two steps. Figure 1A shows that humic substances were filtered through the *In gel patch*. Figure 1B shows the principle of the method: in step 1, the sample is loaded in a well located in the front of the *in gel*

patch. Then, during step 2, the genomic DNA with humic substances initially moves toward the farthest patch edge and penetrates into the *in gel patch*. During the step 3, the polarity of the current is inverted and the humic substances of large molecular weight remain in the in-gel patch while the DNA and humic substances of small molecular weight migrate. Compared to genomic DNA (Figure 1A, (b)), humic substances of small molecular weight migrate more quickly in the agarose gel. These small molecular weight humic substances were observed by brownish color at the apposite side of *in gel patch* (Figure 1A, (a)). The high molecular weight humic substances filtered in the resin caused a dark-brown color of the *in gel patch* (Figure 1A, (c)).

To determine the purity of the genomic DNA extracted according to the "*In gel patch electrophoresis*" method, environmental DNA was purified and compared to a negative control (without patch in the agarose gel). First, gel slices with genomic DNA were cut from the gel after the electrophoresis and the DNA was extracted using an extraction kit (QIAEX II (150), QIAGEN). Then, the A_{260}/A_{280} and A_{260}/A_{230} ratios of each extract was determined by a spectrophotometric method. The different treatments (with or without *in gel patch*) led to significant differences in DNA yield and purity (Table 1). Extraction of DNA with the "*In gel patch electrophoresis"* resulted in significant higher concentrations and had higher A_{260}/A_{280} and A_{260}/A_{230} ratios than the solutions extracted

without the "*In gel patch electrophoresis"*. Moreover, it appeared that both ratios calculated with DNA extracted with the "*In gel patch electrophoresis"* were very close to the ratios determined with pure cultures, thus demonstrating the efficiency of the purification and the humic substances removal. Additionally, the DNA purity was verified by restriction enzyme analysis. While all enzymes digested the genomic DNA, which was extracted with the "*In gel patch method*", genomic DNA obtained without "*In gel patch electrophoresis*" could be only partially or not all digested (Table 2).

In order to test the compatibility of the purity grade of the extracted DNA with processing reactions, we assayed the possibility of PCR amplification of various genome regions. First, to detect phylogenetic groups from the extracted genomic DNA, a PCR-based rRNA survey using a primer set specific for the prokaryotic small subunit rRNA of the internal transcribed spacer (ITS) region for lichen fungi and the eukaryotic rRNA internal transcribed spacer (ITS) region was performed. Successful amplifications were achieved using genomic DNA extracted according the "*In gel patch*" technique as a PCR template. All amplification products from respective environmental genomic DNA were found when the phylogenetic primers for rRNA survey were used (Figure 2). Second, various specific primers were designed for the detection of genes encoding for different kinds of enzymes. Using primers designed to be specific for monooxygenases,

cytochrome b_5 and β-lactamases, a clear fragment was amplified from genomic DNA of environmental samples (Figure 3). After sequencing, a BLAST search was performed against the NCBI database and it appeared that the sequenced fragments showed an identity of more than 80% towards the corresponding enzyme class (monooxygenase, cytochrome b_5). While PCR products were obtained from the genomic DNA extracted with the "*In gel patch electrophoresis*", no PCR products were found under the same experimental conditions when genomic DNA was used without the "*In gel patch electrophoresis*".

To evaluate the potential of the developed method, we performed a comparison with a recently published method, using the same environmental samples. The extraction procedure described by Orsini and Romano-Spica avoids the co-purification of humic substances by applying a microwave thermal shock and the addition of polyvinylpyrrolidone at high concentrations [14]. As can be seen in table 1 the values concerning the DNA yield and purity obtained by the microwave-based method lay in between the "*in gel patch*" method and the electrophoresis performed without the in gel patch. Only in the case of sediment samples the microwave-based method yielded in a higher amount of extracted DNA. The digestion of extracted genomic DNA was only partially possible with *Sma* I and *Xho* I and was not possible with the other three tested

restriction enzymes. In contrast to the *in gel patch* method no PCR amplification products were obtained.

Many workers have attempted to increase genomic DNA purity and yield from environmental samples by using various kinds of treatments. The "*In gel patch electrophoresis"* method is more simple, faster and more efficient than other methods usually reported. Under standard electrophoretic conditions, humic substances with phenolic groups co-migrate with nucleic acids [11, 12]. The addition of a hydroxyapatite patch to the agarose gel eliminates co-migration by retarding the electrophoretic mobility of humic substances. Hydroxyapatite resin has been used for many years as a high-performance liquid chromatography column matrix to bind and separate nucleic acids and proteins [19, 20]. The observation that humic substances, especially those of large molecular weight, tightly bind to hydroxyapatite is in agreement with former studies. Katsumata *et al.* tested the applicability of bone char to remove humic substances from natural water to improve the water quality [21]. Hydroxyapatite is the major inorganic constituent of bone char. This crystalline calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$) possess to have ion exchange ability with positive charges coming from the Ca^{2+} ions [22]. The optimal pH value for the adsorption of humic acids to bone char was 8, giving it a negative charge. This correlates with the pH value of the

extraction buffer used in this study. In agreement with the results obtained in this work Katsumata *et al.* also found a size dependent binding of humic substances, which have a wide range of different molecular weight [23-26], to hydroxyapatite with an almost quantitative removal of large molecular weight humic substances. Another important parameter for efficient binding of humic substances is the pore size of the resin. It was reported that 50-200 μm pore size of the chromatographic material was suitable for their adsorption [27]. The pore size of the HA resin used in this study was 80-350 μm, showing to be effective as an adsorbent for the binding of the humic substances.

4. Concluding Remark

In conclusion, the "*In gel patch electrophoresis"* procedure enables a rapid genomic DNA extraction with a purity grade compatible with digestion by restriction enzymes and PCR amplification. This approach could speed-up and facilitate nucleic acids isolation for metagenome study purposes or to complete genome sequences of uncultivated microorganisms.

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Table 1. Analysis of the genomic DNA extracted from environmental samples with or without the *in gel-patch electrophoresis* and with the microwave-based method [14]:

Samples	**DNA yield**	**µg/g [wet wt]**[a]	**µg/g [wet wt]**[a]	**A_{260}/A_{280}**	**A_{260}/A_{280}**	**A_{260}/A_{280}**	**A_{260}/A_{230}**	**A_{260}/A_{230}**	**A_{260}/A_{230}**
	with "In gel patch"	**without "In gel patch"**	**microwave based method**	**with "In gel patch"**	**without "In gel patch"**	**microwave based method**	**with "In gel patch"**	**without "In gel patch"**	**microwave based method**
Soil	8.3 ± 1.2	3.8 ± 0.6	4.5 ± 1.1	1.79 ± 0.03	0.99 ± 0.07	1.68 ± 0.05	1.86 ± 0.03	1.02 ± 0.06	1.61 ± 0.07
Sediment	4.5 ± 0.9	2.8 ± 0.8	5.1 ± 0.8	1.89 ± 0.02	1.08 ± 0.06	1.71± 0.05	2.00 ± 0.02	1.21 ± 0.05	1.68 ± 0.07
Activated Sludge	5.3 ± 0.6	3.2 ± 0.8	4.5 ± 0.7	1.78 ± 0.03	1.05 ± 0.04	1.77 ± 0.05	1.78 ± 0.03	1.14 ± 0.04	1.73 ± 0.05
Moss	5.3 ± 0.6	3.2 ± 0.8	-	1.71 ± 0.02	1.08 ± 0.05	-	1.82 ± 0.02	1.10 ± 0.05	-
Pure Culture				1.87 ± 0.02			1.93 ± 0.03		

[a] DNA yields were determined by fluorometry. Values are mean value of four independently purified samples with standard errors.

The ratios were calculated from spectrophotometric measurements.

Ratio of A_{260} to A_{230}: a high ratio (> 2) is indicative of pure DNA, a low ratio indicates humic substances contamination.

Table 2. Restriction enzyme analysis of the purified gDNA from environmental samples

Type of purification		Digestion by [a]				
		Bam HI	*Eco* RI	*Hind* III	*Sma* I	*Xho* I
with "In gel patch"	Soil	+	+	+	+	+
	Sediment	+	+	+	+	+
	Activated sludge	+	+	+	+	+
	Moss	+	+	+	+	+
without "In gel patch"	Soil	-	±	-	+	-
	Sediment	-	±	-	+	-
	Activated sludge	-	-	-	-	±
	Moss	-	±	-	±	-
microwave based method	Soil	-	-	-	±	±
	Sediment	-	-	-	±	±
	Activated sludge	-	-	-	±	±

+ complete digestion, ± partial digestion, - no digestion

[a] Restriction enzyme treatment was carried out by four independently purified environmental samples.

Legends to figures

Figure 1. (A) *In gel patch electrophoresis,* a) In gel patch electrophoresis consists largely of two step. In the first step the genomic DNA of environmental samples with humic substances moves into the gel patch. In the second step the applied potential is inverted causing a reversion of the moving direction of the DNA. b) Agarose gel image by UV irradiation. c) Humic substances filtered in HA patch. d) Negative control, showing a sliced fragment without penetration of sample. (B) Principle of the *In gel patch electrophoresis* method.

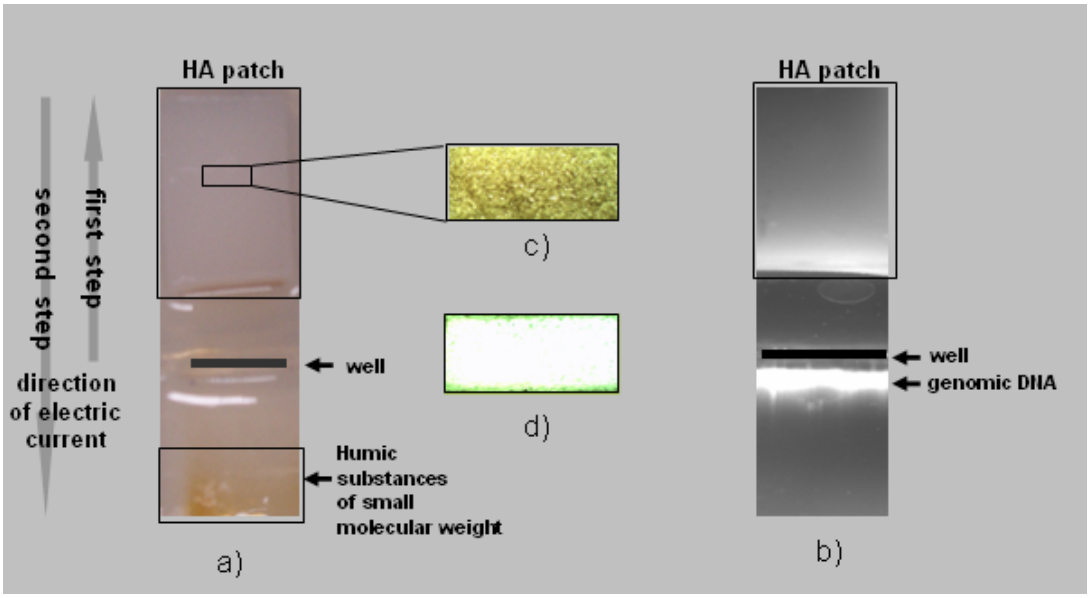
Figure 2. PCR amplification products of DNA extracted from environmental samples. lane 1 and 14: DNA marker; lane 2: prokaryotic small subunit rRNA of soil; lane 15: negative control of lane 2; lane 3: internal transcribed spacer (ITS) region for lichen fungi of soil; lane 16: negative control of lane 3; lane 4: eukaryotic rRNA internal transcribed spacer (ITS) region of soil; lane 17: negative control of lane 4; lane 5; prokaryotic small subunit rRNA of sediment; lane 18: negative control of lane 5; lane 6: internal transcribed spacer (ITS) region for lichen fungi of sediment; lane 19: negative

control of lane 6; lane 7: eukaryotic rRNA internal transcribed spacer (ITS) region of sediment; lane 20: negative control of lane 7; lane 8: prokaryotic small subunit rRNA of activated sludge; lane 21: negative control of lane 8; lane 9: internal transcribed spacer (ITS) region for lichen fungi of activated sludge; lane 22: negative control of lane 9; lane 10: eukaryotic rRNA internal transcribed spacer (ITS) region of activated sludge; lane 23: negative control of lane 10; lane 11: prokaryotic small subunit rRNA of moss; lane 24: negative control of lane 11; lane 12: internal transcribed spacer (ITS) region for lichen fungi of moss; lane 25: negative control of lane 12; lane 13: eukaryotic rRNA internal transcribed spacer (ITS) region of moss; lane 26: negative control of lane 13. As negative control the PCR was performed with genomic DNA of environmental samples extracted without in gel patch as template.

Figure 3. PCR amplification products of DNA extracted from environmental samples. Various kinds of gene products from DNA of environmental samples were obtained. lane 1 and 14: DNA marker; lane 2: monooxygenase of soil; lane 15: negative control of lane 2; lane 3: monooxygenase of sediment; lane 16: negative control of lane 3; lane 4: monooxygenase of activated sludge; lane 17: negative control of lane 4; lane 5: monooxygenase of moss; lane 18: negative control of lane 5; lane 6: cytochrome b_5 of

soil; lane 19: negative control of lane 6; lane 7: cytochrome b_5 of sediment; lane 20: negative control of lane 7; lane 8: cytochrome b_5 of activated sludge; lane 21: negative control of lane 8; lane 9: cytochrome b_5 of moss; lane 22: negative control of lane 9; lane 10, β-lactamase of soil; lane 23: negative control of lane 10; lane 11: β-lactamase of sediment, lane 24, negative control of lane 11; lane 12: β-lactamase of activated sludge; lane 25: negative control of lane 12; lane 13: β-lactamase of moss; lane 26: negative control of lane 13. As negative control the PCR was performed with genomic DNA of environmental samples extracted without in gel patch as template.

A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B

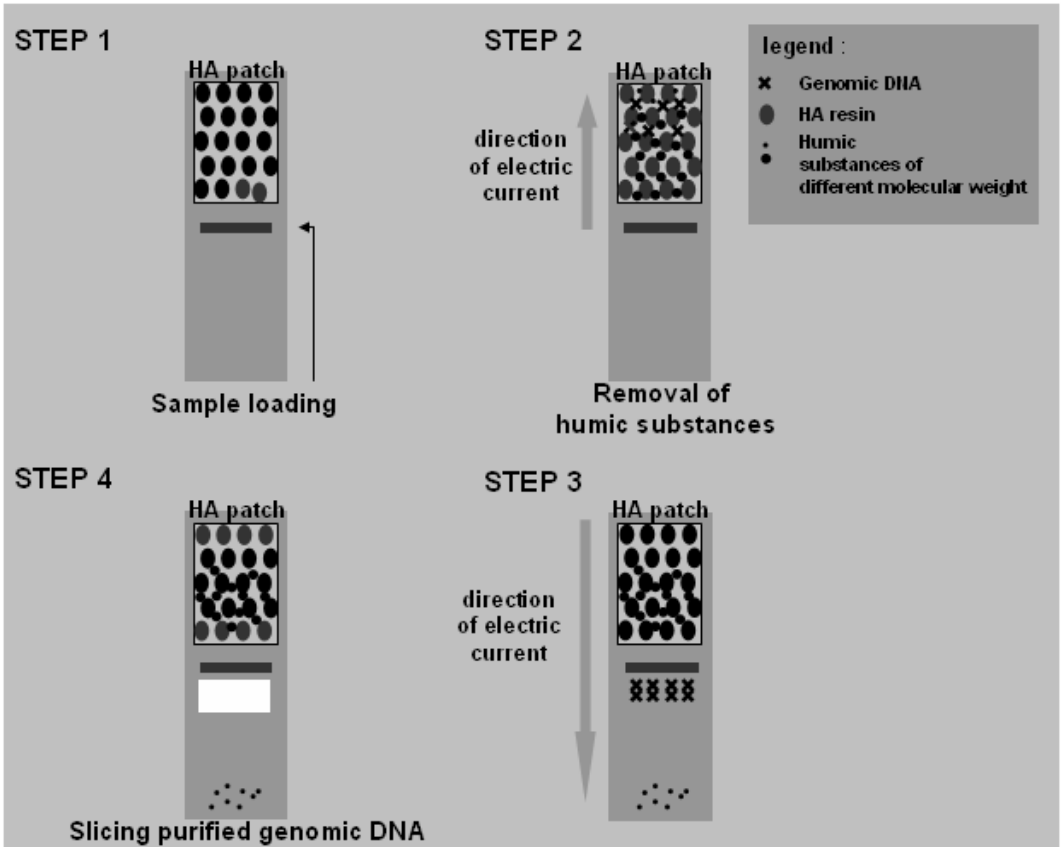
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Figure 1.

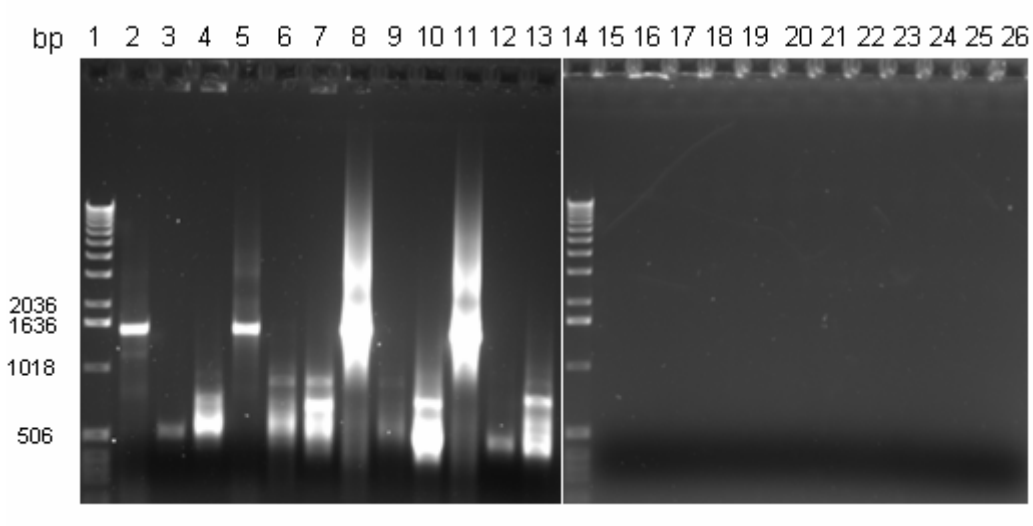
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Figure 2.

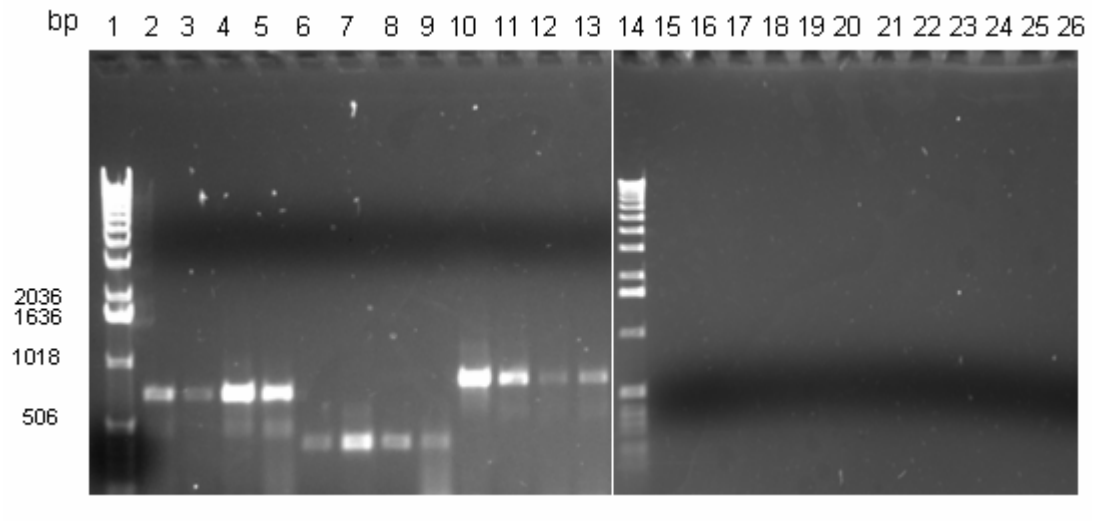
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Figure 3.