High-sensitive analysis of DNA fragments by capillary gel electrophoresis using transient isotachophoresis preconcentration and fluorescence detection

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A B S T R A C T

In this report aimed on further development of a high-sensitivity capillary gel electrophoresis (CGE) method for analysis of DNA fragments, we firstly explored online transient isotachophoresis (tITP) preconcentration combined with fluorescence detection (FD). The fluorescence signal (excitation: 488 nm; emission: 590 nm) was generated using the intercalating dye of ethidium bromide (EB). It was found when the leading electrolyte (LE) was injected behind the sample zone, such a special tITP mode has significant advantages to solve the bubble formation issue and to improve the analytical performance stability. Two standard DNA samples, a 50 bp DNA step ladder and the φX174/HaeIII digest, were used to evaluate the qualitative and quantitative abilities of the tITP-FD approach. A highly diluted sample (10000-fold in the water, e.g. the φX174/HaeIII digest diluted from 500 μg/ml to the 50 ng/ml level) was enriched and detected; the LOD was down to 0.09 ng/ml for the 72 bp fragment, apparently improved more than thousand-fold in comparison with UV detection. Although the RSD of peak areas (n=3) was around 15.5% for the sample was electrokinetically injected, good linearity of peak area response showed that the proposed method is suitable for quantitative analysis.

Keywords:

Capillary gel electrophoresis

DNA fragments

Transient isotachophoresis

Fluorescence detection

Ethidium bromide

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1. Introduction

DNA fragments analysis by capillary gel electrophoresis (CGE) [1,2] has become the preferred instrumental technique because of its high resolving power, speed, and quantitative ability in comparison with conventional slab gel electrophoresis, though the latter is still an important tool in biochemistry [3]. To date in the DNA research field, CGE is a powerful method applied for screening genetic mutations and diagnosis of diseases [4]. Size-dependent separation of DNA fragments in CGE involves the use of noncrosslinked polymeric sieving medium, as linear poly(acrylamide), poly(ethyleneoxide), and hydroxyethylcellulose (HEC), which can be dissolved in buffer solution to obtain a replaceable gel after each run with acceptable repeatability [5]. Poor concentration sensitivity usually hinders many CGE applications when using normal UV detection as a result of small sample amount and small I.D. of capillaries (25-100 μm). To address this issue, online preconcentration strategies or the use of advanced sensitive detectors have been proposed in many reports for trance analysis of biosamples [6-10].

Transient isotachophoresis (tITP) is a familiar online preconcentration method applied not only for small ions but also biomolecules both in capillary and microchip electrophoresis [11-13]. The detection sensitivity could be enhanced by loading large volumes of the sample that are undergone preconcentration through a specifically devised sample/buffer introduction procedure. As well known, the traditional tITP process is that when the sample is loaded between a leading and a terminating electrolyte (LE and TE, respectively), which is then sharpened into a focused zone prior to CGE separation. Some special tITP modes have also been developed according to our studies [14, 15]. In one of these modes, LE was placed after the sample zone, and then the leading ions rapidly migrated and accumulated in front of the sample zone to achieve the tITP stacking. In spite of some limitations, such a mode was demonstrated to be an intrinsic tITP stacking process. The sensitivity enhancement strongly depends on different tITP modes. In general, the LOD could be improved several tens of times or even more.

In comparison with UV detection, fluorescence detection (FD) even laser-induced fluorescence (LIF) are more popular and sensitive techniques for the analysis of DNA when low levels of detection is required [16,17]. As described in references [17-19], many novel intercalating dyes have been developed to generate the fluorescent signal of DNA, ethidium bromide (EB) being the most commonly used dye in conventional DNA analysis [20]. It has been early reported that the intercalation kinetics of EB is viscosity-dependent and sensitive to ionic strength when EB binds to DNA by intercalating the ethidium ion between two successive DNA base pairs through a single-step bimolecular mechanism [21]. EB allows on-column intercalation along DNA double strand with a simple sample preparation protocol, so many applications to PCR samples and restriction digests have been proposed [22-24]. EB has been proved effective to improve fluorescence intensity when it interacts with dsDNA, additionally affecting DNA charge and mobility, separation resolution, baseline signal, and viscosity of the background electrolyte (BGE) [25, 26].

In the present study, we explored the combination of two sensitive approaches, FD and tITP preconcentration, for the CGE analysis of low levels (ng/ml)

of standard dsDNA. The EB dye was added not only to the BGE but also to the sample to produce fluorescent DNA-EB complexes for minimizing peak splitting as detailed later. In tITP preconcentration, a leading electrolyte (LE) is filled after a sample zone [14, 15]. This was especially important to obtain reproducible DNA peaks from an extremely diluted sample. With the proposed method, the average LODs were significantly improved and exceeded 20-fold those of LIF detection with electrokinetic injection (EKI) stacking (LOD reported at 2.0 ng/ml) [27]. This indicates that in conjunction with online tITP preconcentration and a sensitive detection technique CE has a great potential for the analysis of trace-level analytes.

2. Experimental

2.1. Chemicals and electrolytes

Two standard DNA samples, a 50 bp DNA step ladder (Promega, Madison, WI, USA) and the φX174/HaeIII digest (Wako, Osaka, Japan), were used for the method establishment. The 50 bp DNA step ladder at the final concentration of 340 μg/ml, consisted of 16 DNA fragments ranging from 50 to 800 bp with exactly 50 bp increments. The 800 bp band appeared 2-3 times more intense than all other bands, which are of approximately equal intensity. The φX174/HaeIII digest is more commonly used for DNA studies. It consisted of 11 fragments, from 72 to 1353 bp, at the total concentration of 500 μg/ml. Tris(hydroxymethyl)aminomethane (Tris), HEC, borate, and EDTA disodium salt were purchased from Sigma-Aldrich Japan (Tokyo, Japan). EB was the product of Nacalai Tesque (Kyoto, Japan). 2-(Cyclohexylamino)ethanesulfonic acid (CHES) was obtained from Tokyo Kasei (Tokyo, Japan).

For CGE separation, the developed BGE consisted of 1% (m/v) HEC, 2 mM EDTA, 10 μM EB, and 20 mM Tris (pH 8.3, adjusted by adding boric acid). LE (10 mM HCl, pH 8.3 adjusted by Tris) and TE (192 mM CHES, pH 8.1 adjusted by Tris) were prepared and used for tITP preconcentration. Low concentration DNA samples

were obtained by diluting the original standards. All solutions were prepared in Milli-Q water obtained from a Millipore purification system (Tokyo, Japan).

2.2. Apparatus and capillary

The whole lab-built system comprised a fluorescent detector, a high voltage power supply, and the computer interface for data recording. The ambient temperature was kept at 25°C. The fluorescent detector FP-1520S was purchased from JASCO (Tokyo, Japan) and installed with a long-life 150 W Xenon lamp from HAMAMATSU (Shizuoka, Japan). For generating DNA fluorescent intensity, the wavelengths for excitation and emission were 488 and 590 nm, respectively. The power supply 890-CE was also from JASCO. For data collection, the adopted computer interface NR-500 was from KEYENCE (Osaka, Japan). The pump for the introduction of viscosious BGEs was Air Cadet vacuum/pressure station made by Cole-Parmer (Illinois, USA). Separations were performed in a 80 cm long 75 μm I.D. fused-silica capillary from Otsuka Electronics, with an effective length of 45 cm. Before use, new capillaries were washed with 1 M sodium hydroxide, water, and the separation electrolyte for 10 min each. The capillary was flushed with water for 2 min and the BGE for 2 min prior to each run. The applied voltage was –15 KV for the EKI of sample and CGE separation (migration current, ca. 15 μA).

3. Results and discussion

3.1. To achieve high fluorescent intensity of DNA-EB complexes

The effect of EB on DNA fragments has been previously studied and explained in references (e.g. ref. 25): (i) After the intercalation of EB, the DNA chain becomes longer and stiffer. In addition, the net charge and hence mobility of the DNA fragments decrease due to the intercalation of the cationic dye. (ii) EB preferentially intercalates into G+C rich sequence, and the effect on DNA resolution is uneasy to

predict. (iii) The addition of EB should be optimized according to the amount of DNA. The ethidium ion can intercalate into DNA chains or out-side bind to phosphate groups, and only the intercalated EB exhibits the enhancement in emission intensity due to an energy transfer. Notably, the fluorescent intensity is determined by the ratio of DNA and EB amounts. Only before DNA is saturated by the intercalated ethidium ion, the increase in EB concentration results in an increase in S/N ratio.

When 10 μM EB was added only to the BGE, Fig. 1a demonstrated the fluorescence detection limit was not satisfactory for the 1000-fold diluted step ladder sample at EKI time 10 s. Some splitting peaks were observed for the 100-fold diluted sample (see Fig. 1b), but not found for 5-fold diluted sample (see Fig. 1c). The main reason of splitting peaks is the mobility difference between pre and post EB intercalation due to the changing of DNA charge and length. For the 5-fold diluted DNA sample, the splitting peaks are not obvious because the concentration of DNA-EB complexes after intercalation is much lower than the initial concentration of DNA. The splitting peaks must be avoided when samples with low concentration level are analyzed for accurate DNA size identification.

The total DNA concentration of the 100-fold diluted sample is 3.4 μg/ml, which is equivalent to 5.2 μM of the base concentration (each base pair is around 660 g/mol). The additional concentration of EB in BGE was 10 μM, which seems to be sufficient for building the DNA-EB complexes because the injected DNA amount was minute (several pmol level for 10 s EKI time). The occurred splitting peaks demonstrated that the intercalation process was not complete within the separation time. We tried to increase the EB concentration (up to 100 μM) for accelerating the intercalation, but the higher EB concentration in BGE caused lower S/N ratio. In order to achieve the successful intercalation in separation and preconcentration process, another approach is to incorporate the intercalating dye into the diluted sample. When EB was added to the sample at the concentration of 10 μM as well, the splitting peaks disappeared and the fluorescence intensity was enhanced in comparison with EB was only added in BGE (see electropherograms in Fig. 2 a and b). We compared lower (0.1 and 1.0 μM) and higher (100 μM) EB concentrations in sample, and obtained the strongest fluorescent intensity of DNA peaks when EB was 10 μM. Here, the optimal

condition for high fluorescent signal generated by the DNA-EB complexes was the addition of 10 μM EB not only to BGE but also to the sample, which was applied in the following tITP studies.

3.2. Special transient isotachophoresis mode for highly diluted DNA sample

In conventional tITP mode, the sample is sandwiched between LE and TE. In this study, when the highly diluted DNA sample (10000-fold in the water) was electrokinetically injected between LE and TE, it was found that the bubbles are usually formed that resulted in poor repeatability of preconcentration and separation. The main reason was assumed to be that the low-concentration sample zone, e.g. 34 ng/ml of 50 bp DNA step ladder, could be burdened with a very high electric field during tITP preconcentration because of extremely low conductivity of the sample. At long duration of tITP process, the generated Joule heat may cause the bubbles in the loaded sample zone. To address this issue, the LE (10 mM HCl/Tris, pH 8.3) could be introduced behind the sample zone. As explained in our previous papers [14, 15], the leading ion entered into the sample zone (high potential gradient part along the migration path), rapidly exceeded the sample components, and then performed its role in tITP preconcentration in front of the sample zone. Actually, when the leading ions pass through the low-conductivity sample zone, their movement will carry electric current and dispel the high potential gradient. Owing to the balance of the conductivity and the current, generation of excessive Joule heat might be partly suppressed because of the drop of potential field. In computer simulation of such tITP setting, we observed that the high potential gradient was created in the narrow part of the sample zone, which was dispelled after ITP started (data not shown).

Related to the tITP stacking performance, many important parameters should be optimized [11, 12]. For the tITP mode used in this work, the loaded amount of LE and TE will in the same way affect the eventual resolution and sensitivity. For the 34 ng/ml of 50 bp DNA step ladder, the sensitivity will not be improved by simply increasing the EKI time. In the case of the sample with 10000-fold dilution and EKI time was 10 s, almost all DNA fragments were beyond LOD of fluorescence detection,

unless tITP preconcentration is applied. By prolonging EKI time to 60 s, some broadened peaks were observed. This implies that simple field-amplified sample injection was not effective for such large amount of sample. Fig. 3 displays the electropherograms recorded at LE injection (10 and 60 s) after sample zone. The resolution is not good for some large DNA fragments (between 550 bp and 800 bp), which was concluded that their status were at tITP rather than at gel separation within the analytical time. Apparently, the longer injection time of LE, the better enrichment of DNA fragments were achieved by tITP. The injection times of LE and TE were optimized at 60 and 30 s, respectively, (applied voltage: -15 KV) for such highly diluted sample with due consideration of the resolution and sensitivity balance. With proposed tITP-FD-CGE method, the peak intensities for the ng/ml level sample seem to be large enough to perform qualitative and quantitative analysis of trace DNA sample (as shown in Fig. 3 b).

3.3. Precision and limit of detection of tITP-FD-CGE for DNA analysis

Determination of the size and amount of DNA fragments is a very significant work in the clinical DNA studies. The molecular weight marker, φX174/HaeIII digest, was adopted with the aim to evaluating the qualitative and quantitative abilities of the developed tITP-FD-CGE method. This marker includes the DNA size range from 72 to 1353 bp, and the ratio of each fragment is known as well. The standard DNA marker was 10000-fold diluted in water and analyzed for 3 times at the optimized conditions. The fragments from 72 to 603 bp were separated with good resolution within 18 min, as the electropherograms are displayed in Fig. 4. At such tITP stacking and later destacking conditions, the resolution of three big fragments of 872, 1078, and 1353 bp was not good. The sieving ability of 1% HEC was not suitable for 10 bp size difference, so that the fragments of 271 and 281 bp still kept co-migrating. The concentration, migration time, and peak area were calculated and listed in Table 1. An additional remark is that the concentration of each fragment was calculated by the total concentration after 10000-fold dilution (50 ng/ml) and the respective component ratio that described in the product leaflet. Excellent correlation was obtained between

the concentration (Y, ng/ml) and the peak area (X, a.u.×s), Y=0.0425*X with the high correlation coefficient R at 0.997, which illustrates that the method could enable quantification of unknown samples. The migration time had very good precision. But for the peak area, the RSD of each fragment was averagely around 15.5%. From our experiences, such a deviation was caused by poor repeatability of the sample EKI. If we can improve the repeatability of EKI, better precision for quantitative analysis could be expected. Here, with combined online tITP stacking and high-sensitive FD, the LOD was 0.09 ng/ml (for the weakest peak of 72 bp at S/N=3) or approximately 2.0 pM, which was significantly decreased more than thousand-fold than normal UV-Vis detection. To our best knowledge, the LOD of CGE analysis of dsDNA with UV-Vis detection is at μg/ml level, which could be improved up to a few ng/ml by online stacking techniques or by using LIF detection [27, 28].

4. Conclusions

The proposed method has great promise for carrying CGE analysis of DNA fragments with high sensitivity, due to the combination of online tITP stacking and FD. The traditional intercalating EB dye was both added to BGE and DNA sample, because an inadequate intercalation could lead to the splitting peaks troubling qualitative analysis. An uncommon tITP mode, with LE introduced after the sample zone, was found useful to address the bubble formation issue for the highly diluted standard sample. This can be elucidated as the result of the suppressed Joule heating. At the optimal tITP stacking and destacking conditions, the large amount of sample was preconcentrated, separated, and detected by emitting fluorescence at 590 nm. The resolution of large DNA fragments was not satisfied at the capillary effective length of 45 cm. For 50 ng/ml level DNA sample, the RSD (3 runs) of migration time and peak area was around at 1.1% and 15.5%, respectively. The LOD for 72 bp was 0.09 ng/ml, the value exceeding the sensitivity of LIF. The tITP-FD-CGE implies a promising strategy for DNA analysis by capillary or microchip electrophoresis.

5. References

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Table 1: RSD validation of tITP-FD-CGE analysis for 10000-diluted sample (φX174/HaeIII digest diluted from 500 μg/ml to the 50 ng/ml level)

DNA size (bp)	Concentration* (ng/ml)	Migration time (s)		Peak area (a.u.× s)	
		Average	RSD (%)	Average	RSD (%)
72	0.65	688	1.3	1.7E-02	9.8
118	1.10	748	1.0	3.4E-02	17.2
194	1.80	838	1.0	6.6E-02	17.3
234	2.15	877	1.2	8.7E-02	18.1
271 + 281	5.10	/	/	2.2E-01	16.0
310	2.90	951	1.1	1.3E-01	15.9

*Calculated based on the known respective band ratio of each fragment.

Captions of figures

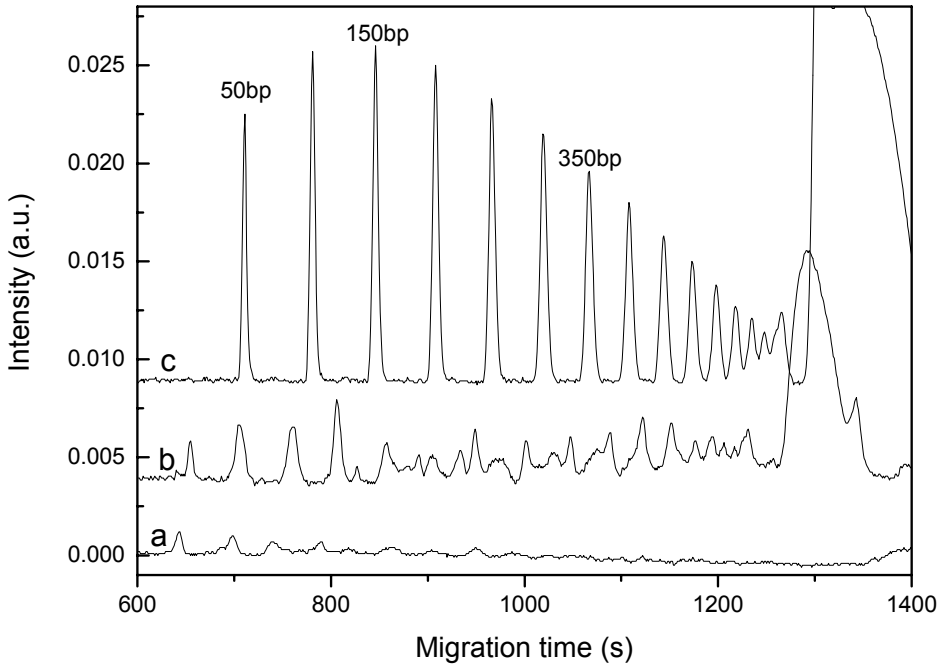
Fig. 1. Electropherograms of 50 bp DNA step ladder obtained at different concentration: (a) 1000-fold diluted; (b) 100-fold diluted; (c) 5-fold diluted. CGE conditions: capillary, fused-silica, 80 cm×75 μm I.D.; BGE, 1% (m/v) HEC, 2 mM EDTA, 10 μM EB, and 20 mM Tris-borate at pH of 8.3; sample injection, EKI at -15 KV for 10 s; separation voltage, -15 kV; fluorescence detection, λ_{ex}=488 nm, λ_{em}=590 nm.

Fig. 2. Electropherograms of 100-fold diluted 50 bp DNA step ladder (a) without and (b) with 10 μM EB addition. Other conditions as in Fig. 1.

Fig. 3. tITP preconcentration effect at varying LE injection time for (a) 10 s and (b) 60 s. Conditions: sample dilution, 10000-fold in water; LE, 10 mM HCl-Tris, pH 8.3; TE, 192 mM CHES-Tris, pH 8.1; sample injection, EKI at -15 kV for 60 s; TE injection, at -15 kV for 30 s. Other conditions as in Fig. 1.

Fig. 4. Electropherograms of 10000-diluted φX174/HaeIII digest sample obtained for continuous 3 runs. For tITP, sample, LE, and TE were in turn injected for the time of 60, 60, and 30 s, respectively. Other conditions as in Fig. 3.

Fig. 1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1200
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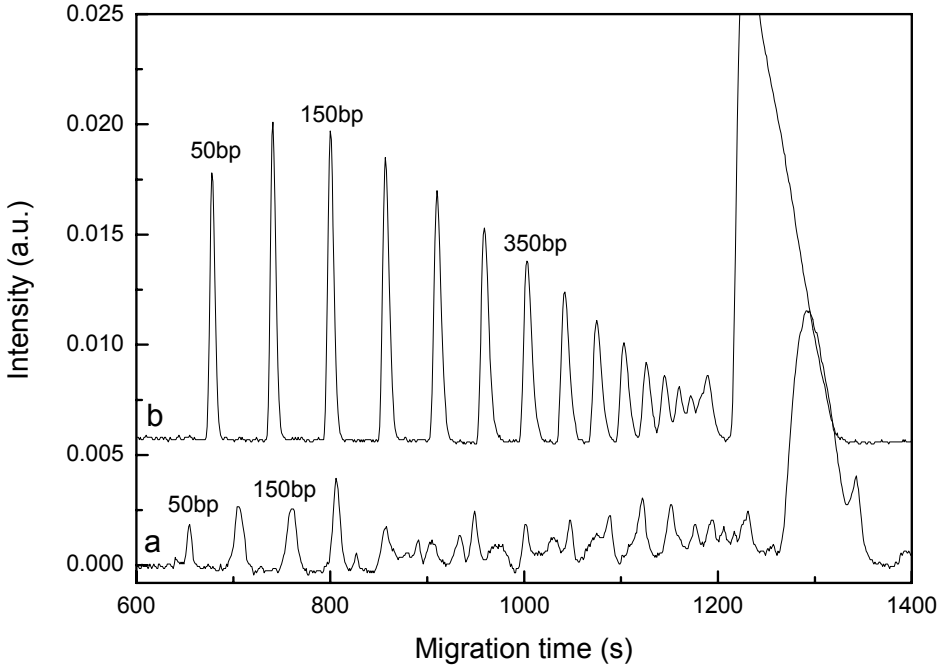
Migration time (s)

Fig. 2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Intensity (a.u.)
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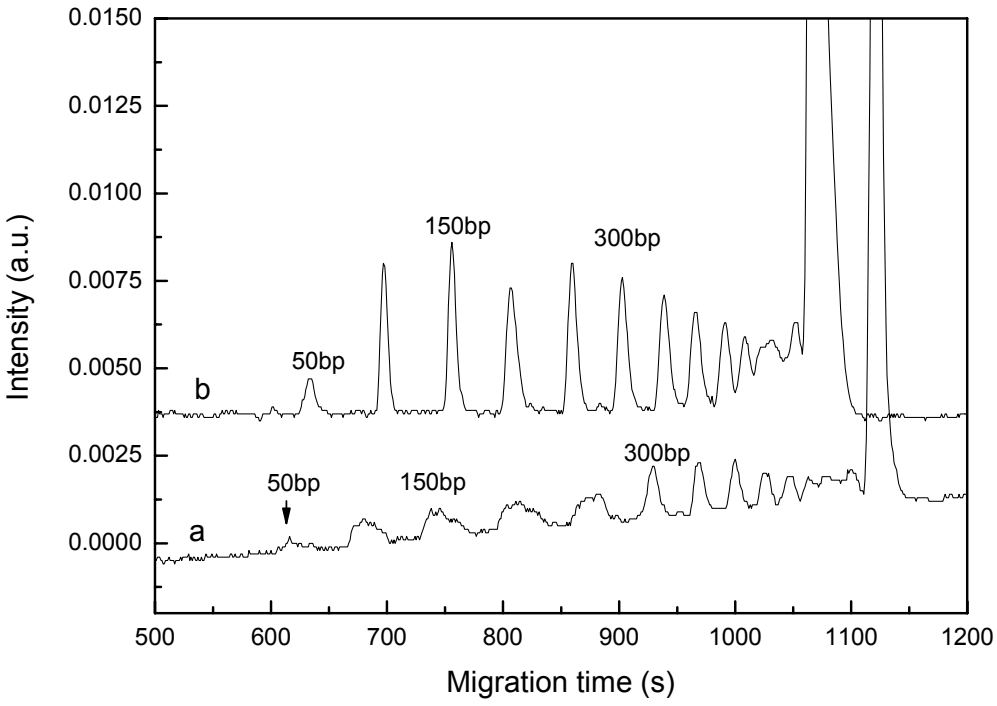
Migration time (s)

Fig. 3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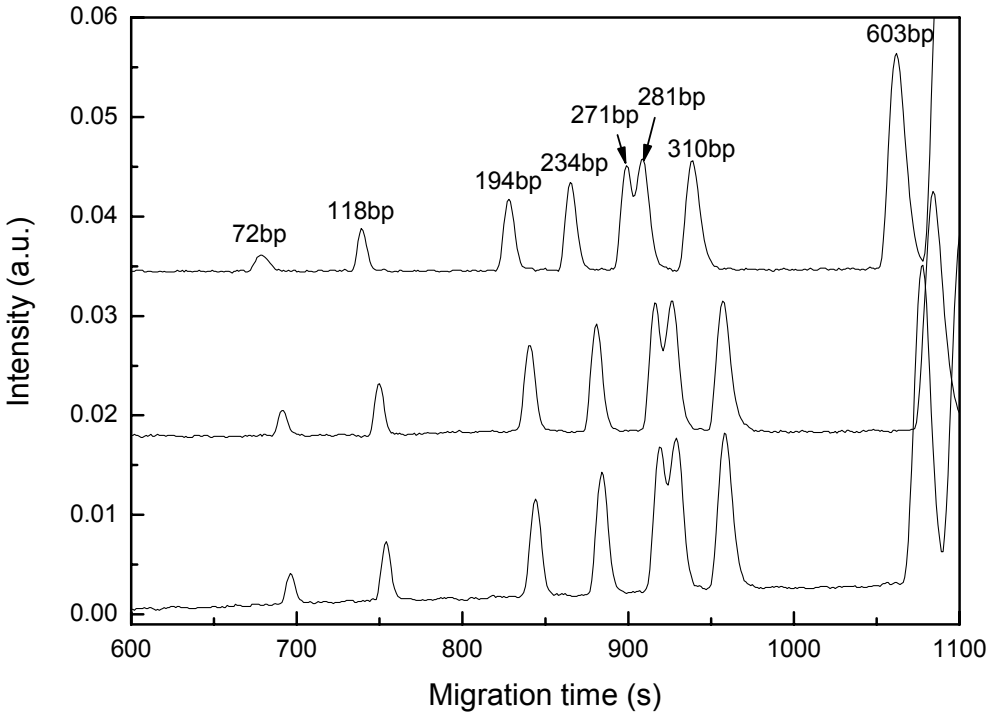
1100
1200
Migration time (s)

Fig. 4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