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REVIEW

CHROMATOGRAPHIC SEPARATION OF DNA RESTRICTION FRAGMENTS

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CONTENTS

1. INTRODUCTION

DNA restriction fragments are of increasing interest in molecular biology, gene technology and biophysical chemistry. This interest is not restricted to studies in basic research but is also appearing in the field of applied sciences, e.g. medical diagnosis. Thus the studies of restriction fragments in different scientific areas should not be seen as isolated, but as interdependent. For example, biophysical studies on the structure and dynamics of double-stranded DNA (ds DNA) of defined length and sequence became possible only when new techniques in molec-

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ular biology and preparative biochemistry made available those DNA species in the form of purified restriction fragments. Another example is the use of DNA and RNA fragments as hybridization probes. Their application in basic research, as well as in diagnosis of infectious diseases or genetic disorders, is derived from our present knowledge of the physical chemistry of base pairing. A particularly intimate relationship exists between work on restriction fragments and gene technology. On the one hand it is possible, owing to modern gene technology, to "construct" restriction fragments in a wide variety of lengths and sequences; on the other hand the separation of restriction fragments by high-performance liquid chromatography (HPLC) is particularly useful during the different steps of cloning and sequencing. These few examples indicate the need for highly purified restriction fragments in different areas of life science. Purity alone may not be sufficient as a specification for the fragments; additionally, they are supposed to be highly active in a variety of enzymic reactions and biological transformations.

The recent progress in synthesizing in vitro large amounts of RNA fragments from double- or single-stranded DNA as the template has made RNA and DNA fragments available in comparable amounts and by comparable effort. For this reason we have included in this chapter the preparation of RNA fragments transcribed from DNA.

Interactions of nucleic acids with a chromatographic resin are very dependent on the structure of the nucleic acids. Because we concentrate in this chapter on DNA restriction fragments, we are dealing with nucleic acids of almost only one type of structure, i.e. negatively charged double strands of different length. This implies that the only chromatographic techniques applied are those that separate either according to charge or to size. This restriction limits this chapter to the description of anion-exchange, mixed-mode and size-exclusion chromatography. We will not describe chromatographic principles in general, but will concentrate on their application to the separation of restriction fragments. After discussing the available methods with a series of current examples in Section 2, we will outline in Section 3 special chromatographic applications, such as scaling-up and preparation of RNA fragments. Section 4 describes ancillary procedures for HPLC, such as sample preparation and recovery and a particularly simple form of nucleic acid chromatography with disposable columns. We regard this order as most convenient for the reader, because the relevance of special applications and sample handling may best be understood after the basic features of nucleic acid chromatography have been explained. In the final section, we discuss the increasing importance of nucleic acid preparation in medicine.

2. CHROMATOGRAPHIC METHODS FOR SEPARATION OF DNA RESTRICTION FRAGMENTS

2.1. Mixed-mode chromatography

Although RPC is the abbreviation for reversed-phase liquid chromatography, the chromatographic material RPC-5 is actually a material for mixed-mode chromatography. RPC-5 was the first chromatographic technique for nucleic acids,

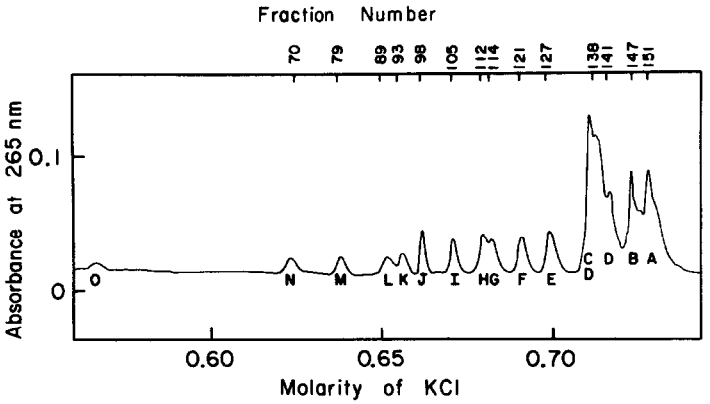

Fig. 1. RPC-5 of DNA restriction fragments. Column: RPC-5 (150×1.5 mm I.D.). Sample: 50 μg of DNA restriction fragments from the plasmid pRZ2 cleaved with restriction endonuclease HaeIII. The fragment sizes are (in base pairs): A = 850; B = 575; C = 465; D = 425 (three fragments); E = 255; F = 203; G = 180; H = 169; I = 135; J = 117; K = 102; L = 98; M = 85; N = 69, O = 43. Chromatographic conditions: linear gradient from 0.55 to 0.75 *M* potassium chloride in 180 min, in 10 m*M* Tris-HCl (pH 6.8), 2 m*M* sodium thiosulphate, 0.1 m*M* EDTA; flow-rate; 0.2 ml/min; temperature, 43°C. (Reproduced with permission from ref. 5.)

having been introduced more than twenty years ago [1]. The separation is based on ionic as well as on hydrophobic interactions. The resin applied originally consists of a charged reversed-phase matrix with a quaternary ammonium derivative (such as trioctylmethylammonium chloride, Adogen 464) being adsorbed on a non-porous polymer support, such as polychlorotrifluoroethylene beads (Plaskon 2300). In contrast to other chromatographic resins, the surface-forming groups of RPC-5 are physically adsorbed to the polymeric support and are not covalently bound. More recently, resins with a covalently bound mixed-mode surface have been developed by Bischoff and co-workers [2,3], who have used partially modified aminopropylsilica with different alkyl chains and aryl groups.

Because ionic interactions are prevalent in mixed-mode resins of this kind, elution of the adsorbed nucleic acid is achieved by a salt gradient. The chromatographic parameters for the separation of dsDNA fragments have been optimized systematically by Wells et al. [4]. General guidance on how to apply RPC-5 to DNA restriction fragment separation is given by these authors [4–6]. An example of the fractionation of DNA fragments under optimum conditions is shown in Fig. 1. In this example, fragments up to 850 base pairs (bp) could be fractionated with a remarkable resolution. Furthermore, one must remember that these studies were carried out at a time before modern HPLC instrumental techniques were available. In another publication, RPC-5 was combined with particular cloning procedures and fractionated precipitations, not so much to increase the resolution as to scale up the preparation of particular fragments to milligram amounts [7]. As another special application, strand separation of the single strands from dsDNA was obtained under alkaline conditions [8].

In spite of the good results reported by Wells et al. [4] and of the low-cost equipment needed, very few other authors reported on the application of RPC-5

TABLE I

SIZE-EXCLUSION HPLC COLUMNS FOR DNA FRAGMENT SEPARATION

Name	Support material	Particle size (μm)	Pore size (Å)	Ref.	Supplier
Superose 6	Cross-linked agarose	13±2		11	Pharmacia (Uppsala, Sweden)
TSK G 5000 PW	Hydrophilic polyether	10±2	1000	10	Toyo Soda (Kyoto, Japan)
TSK G 2000 SW	Hydrophilic polymer-coated silica	10±2	125	9	Toyo Soda
TSK G 3000 SW		10±2	250	9	
TSK G 4000 SW		13±3	400	9	

for DNA fragment preparation. This may be because the RPC-5 material was not commercially available in a constantly high quality. Furthermore, bleeding of the stationary phase at salt concentrations lower than 0.2 *M* and destruction by traces of organic solvents, are disadvantages for users who are not experts in chromatography. Finally, RPC-5 is not in the recent trend to shorten separation times with HPLC instrumentation.

2.2. Size-exclusion high-performance liquid chromatography

Size-exclusion (SEC), or gel-permeation (GPC), chromatography is a widely used chromatographic method in biochemistry. The chromatographic matix consists of neutral and hydrophilic porous particles. Large molecules are excluded from the pores and eluted first, whereas small molecules totally invade the pores and are eluted last. DNA fragments are separated by partitioning between mobile phase and stationary phase within the pores of the support.

Since penetration into the pores is dependent on shape, generally applicable calibration curves of the molecular mass versus the retention volume do not exist. Calibration curves determined with polyethylene glycol, dextran or proteins cannot be transferred to nucleic acids. The most common SEC columns, however, have been evaluated particularly for DNA fragments [9,10]. As mentioned above, the retention of a nucleic acid on a size-exclusion column is dependent not only on the molecular mass but also on the shape. This dependence does not play a major role with DNA fragments because they all have the solution structure of a stiff rod, where the residual flexibility is characterized by a uniform persistence length. Exceptions from the homogeneous structure have been reported for peculiar sequences, which lead to a non-random bending of short double helices. This bending effect seems to affect the retention more in ion-exchange chromatography (IEC) (see below) than in SEC.

The chromatographic matrices used in high-performance SEC are based on resins that are completely organic in nature, or on silica coated with a hydrophilic surface. The materials currently available are listed in Table I. Although these resins may exhibit slightly hydrophobic interactions with proteins, particularly

at high ionic strength, such interactions are not detectable with nucleic acids. This is because DNA fragments are highly hydrophilic polyanions with a strong hydration shell, which protects them from hydrophilic contact.

Two examples of SEC of DNA fragments are given in Figs. 2 and 3. Of these, only the elution profile from the TSK G 4000 SW column yielded satisfactory resolution. Although the resolution is not excellent, particularly in comparison with IEC (see below), there is the advantage that SEC can be carried out under almost any solvent conditions. In a particular separation problem, such as separating small linkers from large plasmids, SEC is appropriate [13]. However, SEC has considerable restrictions, which cannot be circumvented. All SEC materials have an upper limit in the size of the restriction fragments that can be fractionated. If the DNA fragment cannot invade the pores, it will be eluted in the void volume. For comparison of the fractionation range, the upper limit is ca. 800 bp for TSK G 5000 PW (1000-Å pores) and only 200 bp for TSK G 3000 SW (250-Å pores) [9,14]. For proteins the exclusion limits are remarkably higher than for DNA fragments, reflecting the compact, globular conformation of proteins in contrast to the extended structure of DNA fragments.

Flow-rate and sample volume represent restrictions for scaling up. Large amounts of DNA material need large sample volumes and, in consequence, large columns if the resolution is to be maintained. That means that a compromise between costs and resolution is necessary. The examples in the literature were always on an analytical scale. The low linear flow-rate, typically 3–20 cm/h, is mainly a restriction on the separation time. Typically, the times of the whole elution process as reported were between 4 and 6 h. Higher flow-rates diminish the resolution.

2.3. Anion-exchange high-performance liquid chromatography

In order to fractionate for polyanions, such as nucleic acids, anion-exchange chromatography is the most obvious method. It was many years before modern HPLC techniques were developed, that anion-exchange chromatography was first applied to nucleic acids. The introduction of high-performance anion-exchange matrices has led to an enormous increase in resolution and made it possible to tackle the problem of high-molecular-mass DNA purification.

The negatively charged nucleic acids are adsorbed on positively charged groups of the anion-exchange resin. They are displaced from the resin by the ions of an increasing salt gradient, in a sequence corresponding to the number of their interacting charges. As with SEC, two types of support material are in use for anion-exchange HPLC. Completely organic resins as well as surface-modified silica gel resins are available; they are listed in Table II. TSK-DEAE 5PW [15] and Mono Q [16] are organic polymers with surface charges. TSK-DEAE 3SW [15] is a polymer-coated silica gel, whereas Nucleogen-DEAE [18] is a brush-type coated silica gel. All anion-exchange resins are based on porous supports. The pores have to meet two requirements: they have to enlarge the interaction surface, and they have to allow for a free penetration of the nucleic acids in order to avoid size-

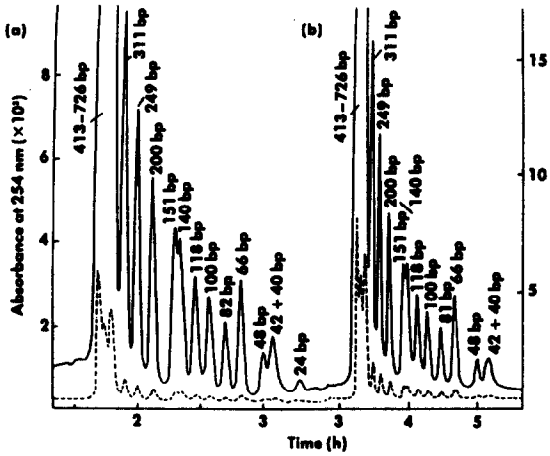

Fig. 2. High-performance SEC of DNA restriction fragments. Column: (a) TSK-G 4000 SW (600×7.5 mm I.D.); (b) tandem TSK-G 4000 SW (600×7.5 mm I.D.) and TSK-G 3000 SW (600×7.5 mm I.D.). Sample: (a) 5 μg of DNA restriction fragments from the phage ΦX 174 cleaved with restriction endonuclease HinfI. The fragment sizes are indicated in base pairs; (b) 10 μg of sample a. Chromatographic conditions: 50 m*M* triethylammonium acetate (pH 7.0); flow-rate, 0.1 ml/min; temperature, ambient. (Reproduced with permission from ref. 12.)

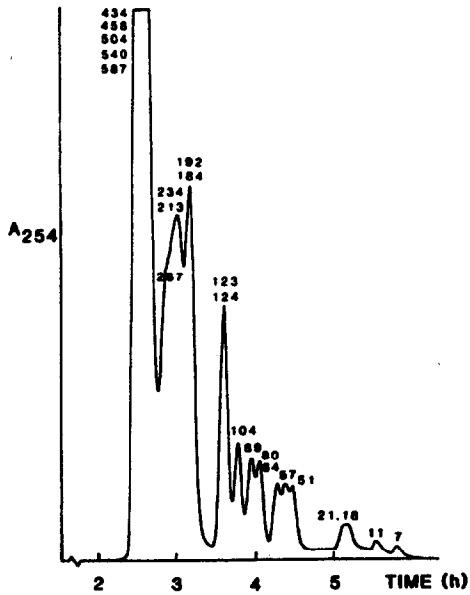

Fig. 3. High-performance SEC of DNA restriction fragments. Column: tandem Superose 6 HR 10/30 (300×10 mm I.D.). Sample: 10 μg (100 μl) of DNA restriction fragments from the plasmid pBR322 cleaved with restriction endonuclease HaeIII. The fragment sizes are indicated in base pairs. Chromatographic conditions: 50 m*M* Tris–HCl, 1 m*M* EDTA (pH 8.0); flow-rate, 0.1 ml/min; temperature, ambient. (Reproduced with permission from ref. 11.)

TABLE II

ANION-EXCHANGE HPLC COLUMNS FOR DNA FRAGMENT SEPARATION

Name	Support material	Functional group	Particle size (μm)	Pore size (Å)	Ref.	Supplier
TSK-DEAE 5PW*	Hydroxylated polyether	Diethylamino	10	1000	15	Toyo Soda
Mono Q	Hydrophilic acrylic polymer	Trimethylamino (quarternary amine)	10	700	16	Pharmacia
TSK-DEAE 3SW**	Coated silica	Diethylamino	10	250	17	Toyo Soda
Nucleogen-DEAE 4000	Coated silica	Diethylamino	7	4000	19	Macherey-Nagel (Düren, F.R.G.); Diagen (Düsseldorf, F.R.G.)

*TSK-DEAE 5PW is analogous to IEX-645 DEAE.
**TSK-DEAE 3SW is analogous to IEX-545 DEAE.

exclusion effects. Consequently, large-pore resins (4000 Å) are to be preferred for DNA restriction fragments of high molecular mass.

Several mobile phases have been used for the elution of nucleic acids from anion-exchange columns. No systematic interpretation of the influence of the buffer–salt combination on the resolution has been given so far, but some of the empirical results are of practical importance. The most widely used combination, Tris–sodium chloride, is not always the best choice. Detailed investigations with Nucleogen columns have shown that phosphate buffer exhibits superior resolution with sodium chloride or potassium chloride as the eluting salt [19]. Addition of 4–6 *M* urea has been shown to achieve a further increase in resolution and to contribute significantly to the prevention of cross-contamination. Under normal chromatographic conditions, the addition of urea does not denature the double helical structure of the DNA restriction fragments, but eliminates residual interactions between different nucleic acid molecules as well as between nucleic acid and the resin. Hydrophobic interactions and hydrogen bonds are considered to contribute to these unspecific interactions.

In contrast to SEC, the influence of the flow-rate on the resolution is fairly minor. It is obvious that a higher flow-rate and a shallower gradient will increase the peak volume and therefore decrease the detection limit. Fig. 4 shows chromatograms obtained at different gradients (10, 5 and 2.5 m*M*/min) but at constant flow-rate. The absolute amounts of DNA fragments in the chromatograms were the same. The steepest gradient resulted in the highest peak amplitudes and nearly the same resolution as with the shallower gradients. Therefore, steep gradients (10 m*M*/min) are most appropriate for fast analysis, i.e. they give all the information needed in a very short time. For preparative applications, however, shallower gradients in some cases lead to a better baseline separation (cf. peaks f′ and g′ in Fig. 4) and facilitate collection of the different peaks without cross-contamination.

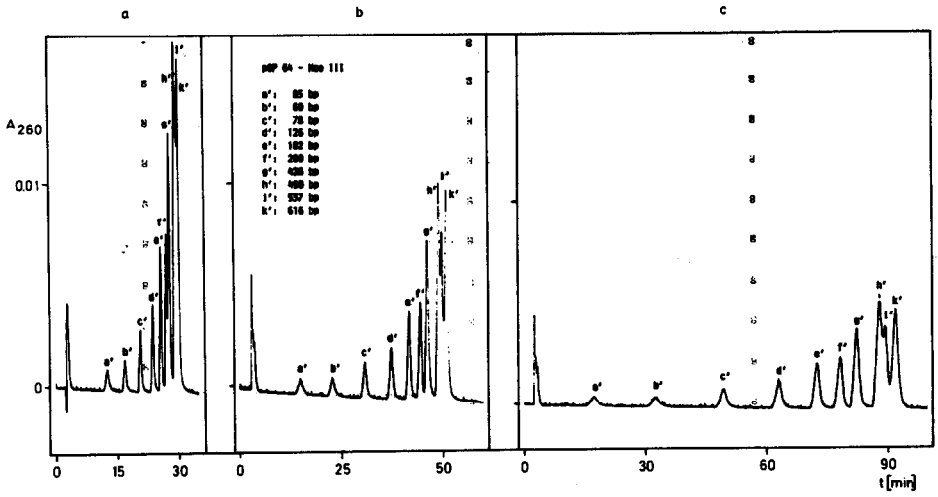

Fig. 4. Influence of the gradient slope in anion-exchange HPLC. Column: Nucleogen-DEAE 4000-10 (125×6 mm I.D.). Sample: 5 μg of DNA restriction fragments from the plasmid pSP64 cleaved with restriction endonuclease HaeIII. The fragment sizes are indicated in the figure. Chromatographic conditions: (a) linear gradient from 0.7 to 1.2 *M* sodium chloride 50 min (10 m*M*/min), (b) linear gradient from 0.7 to 1.2 *M* sodium chloride in 100 min (5 m*M*/min), (c) linear gradient from 0.7 to 1.2 *M* sodium chloride in 200 min (2.5 m*M*/min), in 6 *M* urea, 30 m*M* sodium phosphate (pH 5.5); flow-rate, 1.0 ml/min; temperature, 22°C. (Reproduced with permission from ref. 19.)

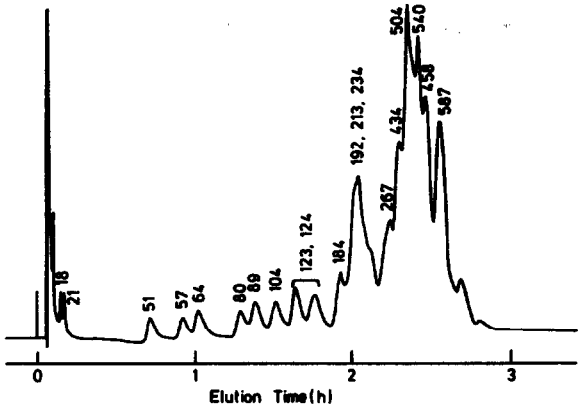

Fig. 5. Anion-exchange HPLC of DNA restriction fragments. Column: TSK DEAE 5PW (150×6 mm I.D.). Sample: 27 μg of DNA restriction fragments from the plasmid pBR322 cleaved with restriction endonuclease HaeIII. The fragment sizes are indicated in base pairs. Chromatographic conditions: linear gradient from 0.35 to 0.5 *M* sodium chloride in 300 min, in 0.1 *M* Tris-HCl (pH 7.6); flow-rate, 1.0 ml/min; temperature, 25°C. (Reproduced with permission from ref. 15.)

The effect of an increase in the flow-rate is similar to that caused by a decrease in the gradient, but the flow-rate is limited by the increasing pressure. For the preparation of milligram amounts of restriction fragments, flow-rates of 2–3 ml/min and gradients of 1–2 m*M*/min may be recommended (see below).

Variation of the temperature between room temperature and 60°C does not show a marked effect on the resolution. With Nucleogen-DEAE columns no

change in resolution but only a slight shift to higher ionic strength (20 m*M* per 10°C) was observed [18]. It has been reported that Mono Q columns, however, show best resolution and reproducibility at 60°C [16].

As reported in the literature, large DNA restriction fragments have been fractionated on the following anion-exchange columns: TSK DEAE 5PW [15], Nucleogen-DEAE 4000 [19] and Mono Q [16]. Chromatograms are available where the same set of restriction fragments is eluted from the three different columns. The cleavage of the plasmid pBR322 with the restriction endonuclease HaeIII releases 22 DNA restriction fragments, ranging in size fom 7 up to 587 bp. These chromatograms are depicted in Figs. 5–7. As expected for porous anion exchangers, the order of elution of the fragments is the same on all three columns. Generally it is in the order of size, but deviations from this are most obvious from the position of the fragment with 458 bp. It elutes at higher ionic strength than the fragment with 540 bp. This deviation is the same on all three columns; therefore, it has to be due to a peculiarity of the sequence of this particular DNA fragment. The fragment contains a stretch of 51 bp with 86% AT. It has been proposed that the unusually high AT content influences the elution behaviour either by an anomalous bending of the fragment [19] or by the difference in counter-ion binding between AT and GC base pairs [16].

When comparing these chromatograms, Mono Q and Nucleogen-DEAE 4000 show superior resolution over TSK-DEAE 5PW. One has to keep in mind, however, that the chromatogram with TSK is from a very early publication about HPLC of DNA fragments; more recent reports are not known to us. The quality of the chromatograms with Mono Q and Nucleogen-DEAE 4000 seems comparable; the comparison cannot be exact, because the elution with Nucleogen was seven times faster but the amount of DNA injected was only 20% of that with Mono Q. The failure in one report [16] to obtain high-quality chromatograms with Nucleogen-DEAE 4000 is hard to explain; possibly it was an isolated instance of a destroyed column, which should have been recognized, however, with some experience in chromatography. Evidently, it was not due to the higher loading applied [16], because a similar chromatogram found in another report on fragment resolution on Nucleogen [19] showed the same resolution when the loading was raised by a factor of 200.

3. SPECIAL CHROMATOGRAPHIC APPLICATIONS

3.1. Large-scale purification of DNA restriction fragments

Large amounts of DNA restriction fragments of high purity are needed for biophysical studies and for molecular hybridization in medical diagnosis. A general strategy for large-scale preparation does not consist of a mere scaling up of chromatographic runs, as in Figs. 5–7, but involves an optimum combination of molecular biology and chromatography. The main idea is to reduce the purification of the wanted DNA restriction fragment from a complex mixture to a simple two-fragment separation. The general scheme is depicted in Fig. 8. The DNA fragment of interest may first be obtained in analytical amounts (typically 10–100

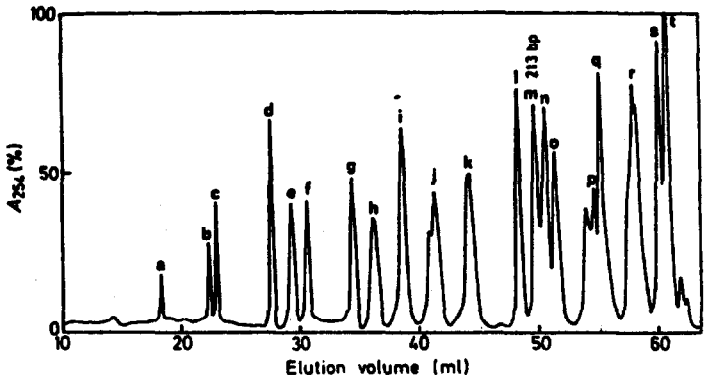

Fig. 6. Anion-exchange HPLC of DNA restriction fragments. Column: Mono Q (50×5 mm I.D.). Sample: 55 μg of DNA restriction fragments from the plasmid pBR322 cleaved with restriction endonuclease HaeIII. The fragment sizes are (in base pairs): a=7; b=11; c=18,21; d=51; e=57; f=64; g=80; h=89; i=104; j+k=123, 124; l=184; m=213; n=234; o=192; p=267; q=434; r=504,540; s=458; t=587. Chromatographic conditions: 0.2 *M* sodium chloride, 20 m*M* Tris-HCl, pH 8.2 for 33 min; linear gradient from 0.2 to 0.4 *M* sodium chloride in 33 min; from 0.4 to 0.65 *M* sodium chloride in 100 min; from 0.65 to 0.9 *M* sodium chloride in 666 min, in 20 m*M* Tris-HCl (pH 8.2); monitor sensitivity, 0–48 ml (0.05 a.u.f.s.), 48–55 ml (0.1 a.u.f.s.), 55–125 ml (0.2 a.u.f.s.); flow-rate, 0.15 ml/min; temperature, 21°C. (Reproduced with permission from ref. 16.)

ng) from a complex mixture by chromatography or by gel electrophoresis and gel elution. It may also be obtained by DNA synthesis or reverse transcription from a specific RNA. The DNA fragment is then sub-cloned in a high-copynumber plasmid, such as pUC 18 or pWH 802 [20–22]. Under special conditions multiple inserts can be obtained [23]. The newly constructed plasmid is now used for large-scale preparation of the specific piece of DNA in the form of a cloned DNA restriction fragment. For plasmid isolation a method is used that is fast and avoids cesium chloride gradient centrifugation [7,24]. The plasmid is cut with the appropriate restriction endonuclease. This results in two fragments: the plasmid vector and the cloned DNA restriction fragment. If multiple insertions of the DNA fragment are made, the yield is higher. The vector and the inserted DNA fragment may be separated easily and rapidly by anion-exchange HPLC. An example of the purification of 1.1 mg of DNA restriction fragment from 7 mg of plasmid DNA is shown in Fig. 9.

3.2. Preparation and purification of unlabelled and labelled RNA fragments transcribed from DNA

Until recently, DNA restriction fragments were of particular interest because these were the only nucleic acids that could be prepared in high purity and large amounts, and the sequence of which could be selected in advance. This situation has changed markedly, owing to the combination of molecular biology and chromatography. Therefore, in a chapter on DNA restriction fragments, the preparation of RNA fragments of a defined sequence in high purity and large amounts may be of interest too. These RNA fragments are used today for biophysical studies, and particularly as hybridization probes, for the following reasons: (i) they are synthesized as single strands, i.e. they are strand-specific; (ii) they may be

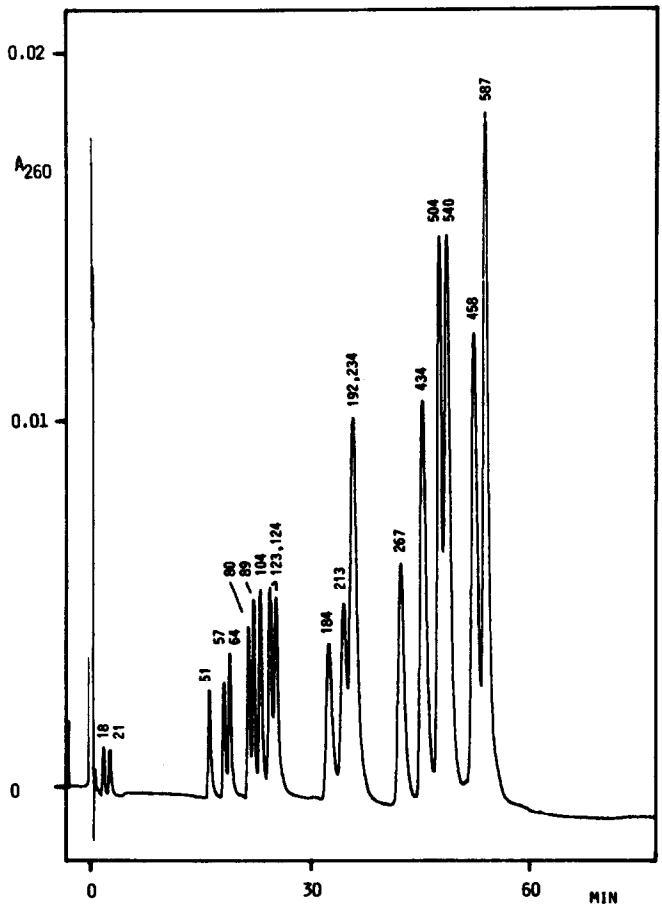

Fig. 7. Anion-exchange HPLC of DNA restriction fragments. Column: Nucleogen-DEAE 4000-7 (125×6 mm I.D.). Sample: 10 μg of DNA restriction fragments from the plasmid pBR322 cleaved with restriction endonuclease HaeIII. The fragment sizes are indicated in base pairs. Chromatographic conditions: linear gradient from 0.5 to 0.8 *M* sodium chloride in 30 min, from 0.8 to 0.9 *M* sodium chloride in 50 min, in 30 *M* sodium phosphate (pH 6.0), 6 *M* urea; flow-rate, 1.0 ml/min; temperature, 23°C.

synthesized containing radioactive labels as well as with non-radioactive biotin-labels; (iii) hybridization with RNA probes yields more stable hybridization complexes. In principle, the RNA is transcribed from its DNA template by an in vitro transcription system derived from the bacteriophages SP6 or T7 [25,26]. Depending on the length of the synthesized RNA two different strategies are possible (Fig. 10). For long RNA sequences the corresponding DNA sequence is cloned into a plasmid behind the promotor specific for the RNA polymerase [25,26]. For short RNA sequences, i.e. if the DNA template may be synthesized in an automatic DNA synthesizer, two single-stranded DNA fragments have to be synthesized: the template for the RNA transcript (including the promotor sequence) and a second strand consisting only of the promotor sequence. After hybridization of both fragments a DNA template is obtained for transcription, which is double-stranded only in the promotor region [31]. After synthesis the

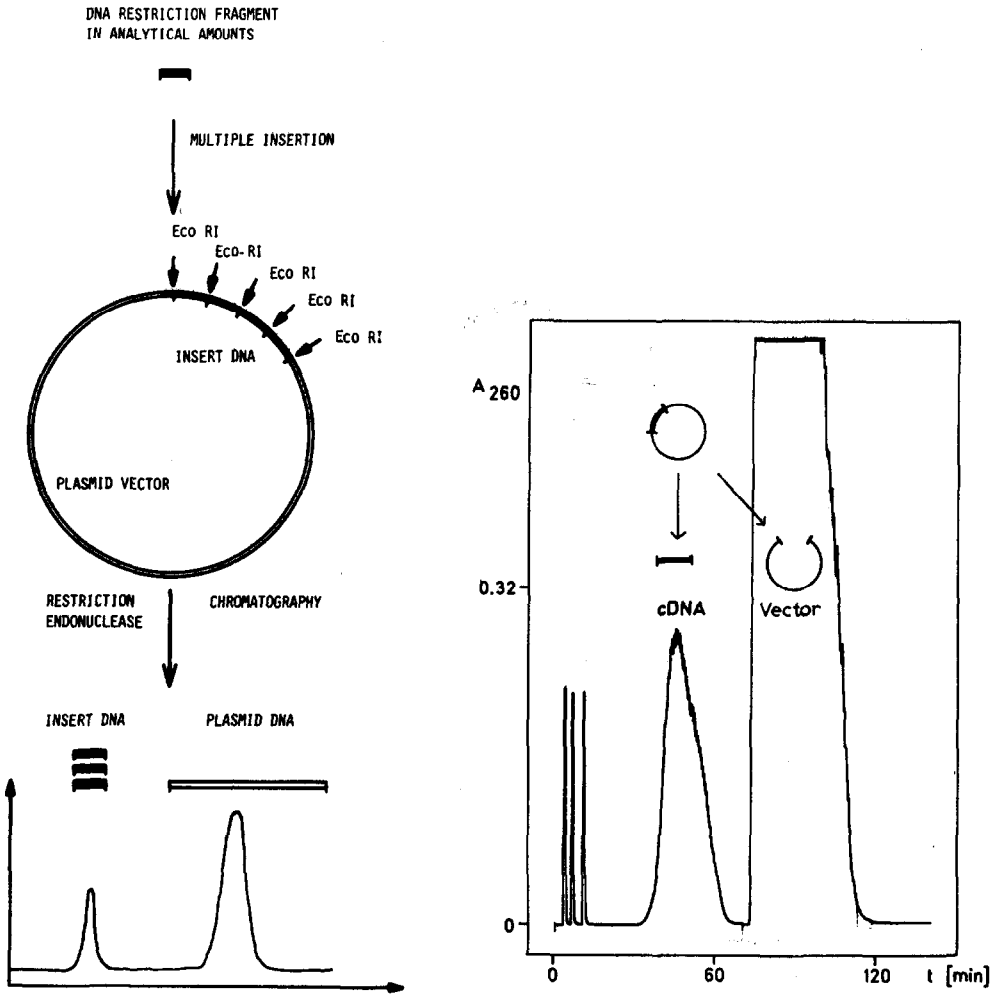

Fig. 8. Large-scale preparation of DNA restriction fragments. The wanted DNA restriction fragment is cloned into a high-copynumber plasmid, if possible as a multiple insert. The plasmid carrying the DNA restriction fragment is prepared in large amounts. The inserted DNA fragment is released by cleavage with the appropriate restriction endonuclease (in this scheme: Eco RI). The DNA restriction fragment is isolated in a single run with anion-exchange HPLC.

Fig. 9. Large-scale preparation of a DNA restriction fragment by anion-exchange HPLC. Column: Nucleogen-DEAE 4000-10 (125×10 mm I.D.). Sample: 7 mg of plasmid pRH101 cleaved with restriction endonuclease BamHI to release a DNA restriction fragment with 359 base pairs in size. The sample was applied by three injections. Chromatographic conditions: linear gradient from 0.84 to 1.2 *M* potassium chloride in 360 min, in 6 *M* urea, 20 m*M* potassium phosphate (pH 6.7); flow-rate, 2.25 ml/min; temperature, 23 °C. (Reproduced with permission from ref. 19.)

RNA has to be separated from the DNA template, the T7-RNA polymerase and the nucleoside-triphosphates. This may be done in a single HPLC run. An example of a chromatogram is shown in Fig. 11. From the peak of the newly synthesized RNA an amplification of 100–500 RNA transcripts per DNA template can be

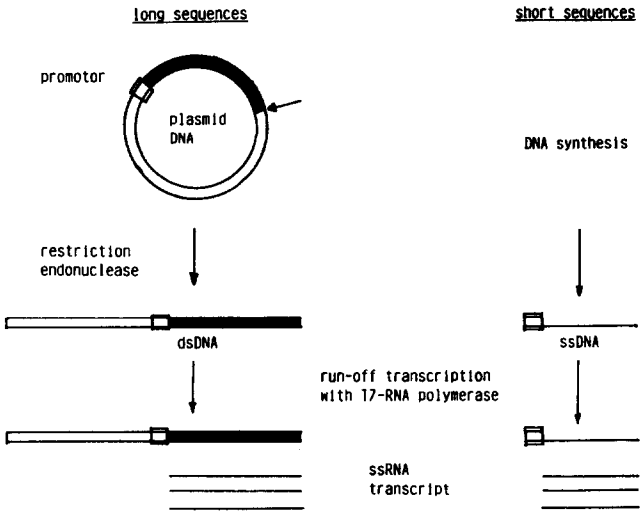

Fig. 10. Preparation of RNA fragments. Depending on the length of the wanted RNA sequence, two strategies are possible. For long sequences, the DNA sequence of the wanted RNA fragment is cloned in a plasmid vector behind the promotor for the T7-RNA polymerase. The recombined plasmid is linearized behind the insert DNA with a restriction endonuclease. The RNA is synthesized by in vitro transcription from the plasmid template with the T7-RNA polymerase. The RNA fragment is isolated from the in vitro transcription reaction by anion-exchange HPLC. For short sequences, the DNA sequence complementary to the wanted RNA sequence, including the T7 promotor sequence, is synthesized in an automatic DNA synthesizer. The active T7 promotor region is obtained by hybridization with its complementary DNA oligonucleotide. The RNA is synthesized with the T7-RNA polymerase by in vitro transcription as for long sequences. The RNA fragment is isolated from the synthesis mixture by anion-exchange HPLC.

calculated. The RNA was desalted by absorption on hydroxy apatite, elution with 0.5 *M* potassium phosphate and precipitation with cetyltrimethylammonium bromide [27]. The DNA template can be reisolated from the corresponding chromatographic peak by polyethylene glycol precipitation [19] used again for RNA synthesis.

4. SAMPLE HANDLING

4.1. Sample preparation and recovery in chromatography

The preparation of the sample prior to chromatography and the recovery of the DNA from a particular elution peak may be done by standard procedures. These techniques are described in the laboratory guide for molecular cloning by Maniatis et al. [28]. After cutting a plasmid or a large DNA with a restriction endonuclease, the DNA fragments are extracted with phenol, precipitated with ethanol and redissolved in a low-salt buffer. Prior to chromatography the samples are adjusted to the starting conditions by adding buffer from concentrated stock solutions. In SEC the sample volume has to be kept as small as possible, whereas in IEC the ionic strength of the sample must not be higher than in the starting buffer.

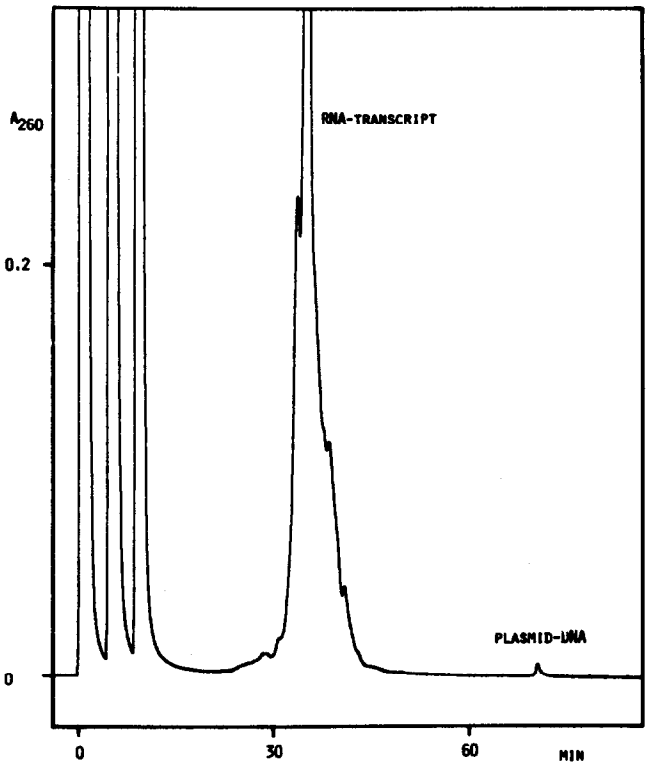

Fig. 11. Anion-exchange HPLC of a preparative in vitro transcription for RNA synthesis. Column: Nucleogen-DEAE 4000-7 (125×6 mm I.D.). Sample: total in vitro transcription mixture containing 2 μg of linear plasmid pRH717/EcoRI as template. The amount of RNA fragment synthesized is 160 μg; the length is 725 nucleotides. It corresponds to a 500-fold transcription from each template DNA. The sample was applied by three injections. Chromatographic conditions: 0.5 *M* sodium chloride, 6 *M* urea, 25 m*M* sodium phosphate (pH 6.0) for 15 min; linear gradient from 0.5 to 0.7 *M* sodium chloride in 40 min, from 0.7 to 1.2 *M* sodium chloride in 25 min, in 6 *M* urea, 25 m*M* sodium phosphate (pH 6.0); flow-rate, 2 ml/min; temperature, 22°C. The shoulders in the peak of the RNA transcript are due to different conformers. In gel electrophoresis under denaturing conditions only one RNA species was detectable.

After chromatography the peak fractions are combined. The combined fractions have to be analysed by gel electrophoresis for purity and identification of the DNA restriction fragment. In SEC and RPC-5 the DNA may be precipitated directly by adding two volumes of ethanol to the pooled fractions. After IEC, however, ethanol precipitation cannot be carried out because of the high salt content in the eluted fractions, which would co-precipitate, particularly with phosphate as anion. The salt may be removed by dialysis, but such a procedure is time-consuming and may cause degradation of the DNA and loss of the sample. The following procedures are recommended as reliable and rapid for recovering and concentrating DNA from eluted fractions. The DNA may be precipitated by either polyethylene glycol [19] or 2-propanol [29]. Solid polyethylene glycol (M_r 6000) is added and dissolved up to a final concentration of 10% (w/v); the DNA is allowed to precipitate on ice for 2 h or longer. With 2-propanol, samples

TABLE III

DISPOSABLE SMALL-SCALE COLUMNS FOR PRE-PURIFICATION AND RECOVERY

Name	Mode of separation	Recommended application	Supplier
RDP	Not described	Recovery of DNA after gel elution	Bio-Rad (Richmond, CA, U.S.A.)
Elutip d	Not described	Recovery of DNA after gel elution	Schleicher and Schüll (Dassel, F.R.G.)
NACS.52 PREPAC	RPC-5	Separation of large DNA from nucleotides and linkers, concentration of DNA, removal of DNA after gel elution	Bethesda Research Labs. (Eggenstein, F.R.G.)
NAP	Sephadex G-25 SEC	Desalting, buffer exchange	Pharmacia
NENSORB	C_8 reversed phase	Separation of DNA or RNA from protein, salt and nucleotides	New England Nuclear, Du Pont de Nemours (Dreieich, F.R.G.)
DIASORB-AX	DEAE anion exchange	Concentration of DNA and RNA, purification of DNA and RNA from salts, metabolites and proteins, fractionation of DNA and RNA	Diagen

are first diluted with the same volume of water, and the DNA is then precipitated by adding one volume of 2-propanol and keeping at $-20°C$ for 2 h or longer. The precipitates are collected by centrifugation and washed once with 75% ethanol in water. Usually the DNA is dissolved in a low-salt buffer, such as 10 m*M* Tris–HCl, 0.1 m*M* EDTA (pH 7.0), and stored at $-20°C$.

4.2. Disposable small-scale columns for prepurification and recovery

For fast preparation of DNA fragments, as well as of other nucleic acids, several chromatographic techniques are applicable in disposable columns. They are appropriate for processing many samples on a small scale. These include RPC-5, SEC, RPC and IEC. A list of commercially available columns is given in Table III. These minicolumns are manufactured as attachments for simple syringes, and so they may be handled without any instrumental effort. They may be used for the following procedures on DNA: desalting, concentration, recovery after gel elution, separation from other components, extraction from cellular extracts and others. The DIASORB-AX system is claimed even to allow a size fractionation, as for example separation of double-stranded M13 DNA from the single-stranded M13 DNA [30].

5. PROSPECTS FOR MEDICAL APPLICATION

In this chapter we have attempted to demonstrate that the present state of chromatography (and particularly HPLC) allows most analytical and prepara-

tive separation problems with DNA restriction fragments to be solved. This, however, does not imply that chromatography (including HPLC) is the technique most recommended for all purposes.

Advantages of one or the other method cannot be discussed per se but have to be seen in the light of a user's particular requirements. Most examples in this chapter were taken from molecular biology or physical biochemistry. Application of HPLC to restriction fragments in medicine is not different in principle from those in the other fields, but it is at a much earlier stage. It will be used in future together with other molecular biological techniques entering medicine. DNA and RNA fragments will be needed as diagnostic tools in different medical disciplines, for example in medical microbiology for detection of infectious disease, in human genetics for identification of genetic disorders or in forensic medicine for affiliation and other cases. Without explaining the details of the methodology one has to differentiate between two different types of diagnosis: (i) testing for the presence of an infectious agent by hybridizing its genome or part of its genome to a labelled complementary DNA or RNA probe and (ii) analysing the structure of the genome of an individual for a genetic disorder or for genetic identity with a sample of unknown origin.

The first set of analyses requires the solution of preparative problems. The nucleic acid of the infectious agent, which is present in a sample from a patient in very small amounts, has to be pre-purified, i.e. separated from non-nucleic acid components, which otherwise would impede the hybridization reaction. Possibly a simple batch chromatography, as described in Section 4.2, may be helpful in this step. Furthermore, the complementary DNA or RNA probe has to be prepared in amounts sufficient for many series of tests. These nucleic acid probes are prepared as cloned DNA fragments or as RNA transcripts, as described in Section 3. In all cases chromatography is advantageous for the preparation. It is fast and can be applied batch-wise. The "biological quality" of samples prepared by anion-exchange chromatography, is evidently an additional advantage. It has been reported recently [30] that these samples show high template activity for restriction and other enzymes. For example, high template activity of a DNA sample is important for a restriction polymorphism analysis. Anion-exchange HPLC is particularly potent in preparing the large amounts of DNA and RNA fragments needed as hybridization probes. In summary, for preparative purposes chromatography and particularly HPLC is superior to gel electrophoresis, cesium chloride density centrifugation, etc., in respect to time consumption, capacity, quality and recovery of the sample.

In the second series of tests mentioned above, a small DNA sample of an individual has first to be prepared (see above) and has then to be analysed. In many applications, restriction fragments of the DNA are produced and their sizes have to be determined. For these purely analytical purposes, gel electrophoresis seems to have advantages over chromatographic methods. Many samples may be analysed in the same electrophoretic run. The resolution power of gel electrophoresis is still superior to chromatography. Although HPLC requires at present more effort for a similar analysis, this may be changed by new developments. One may think of small, inexpensive and disposable columns that do not need any prepa-

ration work before an analysis or storage after the analysis, but yield high resolution. Progress may be achieved also in instrumental automatization of HPLC applied to nucleic acids and reach the same level as it is presently the case in other applications of HPLC to medical problems.

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7. SUMMARY

Reversed-phase liquid chromatography (RPC-5) was the first chromatographic technique to be successfully applied to DNA restriction fragments. Size-exclusion high-performance liquid chromatography (HPLC) has the advantage of wide variability in buffer conditions but is restricted to an upper size limit at 800 base pairs. Anion-exchange HPLC is most versatile and may best be scaled up for the preparation of milligram amounts of specific DNA fragments. The preparation of the sample before and after chromatography is described. Because several applications of DNA fragments will be complemented by RNA fragments, their preparation and purification is included. Disposable small-scale columns are available for processing many samples. Future medical applications of HPLC of DNA fragments concern medical microbiology, human genetics and forensic medicine.

REFERENCES

1 A.D. Kelmers, D.G. Novelli and M.P. Stulberg, J. Biol. Chem., 240 (1965) 3979-3991.
2 R. Bischoff and L.W. McLaughlin, J. Chromatogr., 270 (1983) 117-126.
3 R. Bischoff, E. Graeser and L.W. McLaughlin, J. Chromatogr., 257 (1983) 305-315.
4 R.D. Wells, S.C. Hardies, G.T. Horn, B. Klein, J.E. Larson, S.K. Neuendorf, N. Panayotatos, R.K. Patient and E. Selsing, Methods Enzymol., 65 (1980) 327-347.
5 J.E. Larson, S.C. Hardies, R.K. Patient and R.D. Wells, J. Biol. Chem., 254 (1979) 5535-5541.
6 R.K. Patient, S.C. Hardies, J.E. Larson, R.B. Inman, L.E. Maquat and R.D. Wells, J. Biol. Chem., 254 (1979) 5548-5554.
7 W. Hillen, R.D. Klein and R.D. Wells, Biochemistry, 20 (1981) 3748-3756.
8 H. Eshaghpour and D.M. Crothers, Nucleic Acids Res., 5 (1978) 13-21.
9 Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, J. Chromatogr., 266 (1983) 341-349.
10 Y. Kato, Y. Yamasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, J. Chromatogr., 320 (1985) 440-444.
11 T. Andersson, M. Carlsson, L. Hagel, P.-A. Pernemalm and J.-C. Janson, J. Chromatogr., 326 (1985) 33-44.
12 J. Kruppa, L. Graeve, A. Banche and P. Földi, L.C. Liq. Chromatogr. HPLC Mag., 2 (1984) 848-853.
13 M.E. Himmel, P.J. Perna and M.W. McDonnell, J. Chromatogr., 240 (1982) 155-163.

14 Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, J. Biochem. (Tokyo), 95 (1984) 83-86.
15 Y. Kato, K. Nakamura, and T. Hashimoto, J. Chromatogr., 266 (1983) 385-394.
16 W. Müller, Eur. J. Biochem., 155 (1986) 203-212.
17 Y. Kato, M. Sasaki, T. Hashimoto, T. Musotsu, S. Fukushige and K. Matsubara, J. Chromatogr., 265 (1983) 342-346.
18 M. Colpan and D. Riesner, J. Chromatogr., 296 (1984) 339-353.
19 R. Hecker, M. Colpan and D. Riesner, J. Chromatogr., 326 (1985) 251-261.
20 C. Yanisch-Perron, J. Vieira and J. Messing, Gene, 22 (1985) 103-119.
21 J. Vieira and J. Messing, Gene, 19 (1982) 259-269.
22 B. Unger, G. Klock and W. Hillen, Nucleic Acids Res., 12 (1984) 7693-7703.
23 A. Dugaiczyk, H.W. Boyer and H.M. Goodman, J. Mol. Biol., 96 (1975) 171-184.
24 D.B. Clewell and D.R. Helinski, Biochemistry, 9 (1970) 4428-4436.
25 D.A. Melton, P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn and M.R. Green, Nucleic Acids Res., 12 (1984) 7035-7056.
26 S. van der Werf, J. Bradley, E. Wimmer, F.W. Studier and J.J. Dunn, Proc. Natl. Acad. Sci. U.S.A., 83 (1986) 2330-2334.
27 M. Colpan, J. Schumacher, W. Brüggemann, H.L. Sänger and D. Riesner, Anal. Biochem., 131 (1983) 257-265.
28 T. Maniatis, E.P. Fritsch and J. Sambrook, in Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
29 H. Lehrach and A.M. Frischauf, EMBL Lett. Manual, EMBL, Heidelberg, 1982.
30 DIASORB-AX, Diagen, Düsseldorf, 1987.
31 P. Lowary, J. Sampson, J. Milligan, D. Groebe and O.C. Uhlenbeck, in P.H. van Knippenberg and C.W. Hilbers (Editors), Structure and Dynamics of RNA, Plenum Press, New York, 1986, pp. 69-76.