Journal of Chromatography, 508 (1990) 279–287
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 360

High-performance affinity chromatography of DNA

THOMAS A. GOSS and MARTIN BARD
Department of Biology, Purdue University School of Science, 1125 East 38th Street, Indianapolis, IN 46205 (U.S.A.)
and
HARRY W. JARRETT*
Department of Biochemistry, 800 Madison Avenue, University of Tennessee, Memphis, TN 38168 (U.S.A.)
(Received January 3rd, 1990)

SUMMARY

The octadecamer of thymidylic acid, $(dT)_{18}$, was synthesized with a primary amino group on the 5'-terminal phosphate and this was covalently coupled to 300 Å pore macroporous silica. Coupling was performed inside a prepacked column to an activated N-hydroxysuccinimidyl ester silica. The $(dT)_{18}$-silica column succesfully separates mixtures of adenine oligomers differing in length by one nucleotide. The dependence upon salt concentration, temperature and length for elution of oligonucleotides was determined. Methods were also developed to selectively elute such columns using either salt or temperature gradients.

INTRODUCTION

Affinity chromatography has proven to be a powerful technique for the separation of many types of biologically important molecules. While the technique has most often been used for the separation of proteins, the specific base pairing of nucleic acids has also been exploited for affinity-based isolation of DNA and RNA.

Conceptually, polynucleotide affinity chromatography is simple. Polynucleotides naturally form double-stranded duplexes between two strands of complementary nucleotide sequence. In DNA duplexes, adenine (A) specifically base pairs with thymidine (T) while cytosine specifically pairs with guanidine. By attaching one strand of a potential duplex to a chromatographic support, a column can be made which is highly selective for the complementary strand. In mixtures of polynucleotides, this complementary strand alone, of all other possible sequences, is capable of forming the greatest number of base pairs with the attached strand and the duplex formed is the most stable one possible. Such a column could be eluted in several ways: column temperature can be raised until thermal motion dissociates the duplexes. Since DNA is a polyanion, columns can also be eluted by lowering the salt concentration in the mobile phase to the point at which charge repulsion between strands exceeds the

0021-9673/90/$03.50

stability of the base pairs formed. Formamide and other organic solvents can also be used for elution.

Polynucleotide affinity chromatography at low pressure has been in use for over 25 years[1]. The specificity of base pairing allows highly selective chromatography even with the poor resolution which accompanies many low-pressure chromatographic techniques. The superior mass transfer characteristics and higher efficiency of the macroporous silica supports should improve resolution and make possible truly high-performance affinity chromatography (HPAC) of nucleic acids. This type of HPAC has remained virtually unexplored and this is unfortunate since the high resolution of HPAC has potentially significant advantages in both the preparation and the analysis of polynucleotides.

One impediment to the wide-spread use of HPAC arises from the fact that most users of high-performance liquid chromatography (HPLC) do not pack their own columns. Therefore, methods which allow the coupling of nucleic acids inside prepacked columns could speed the investigation of polynucleotide HPAC. Here, we report on such a coupling methodology, using the octadecamer of thymidylic acid [$(dT)_{18}$], applicable to synthetic oligonucleotides and characterize some of the parameters governing polynucleotide HPAC separations.

EXPERIMENTAL

Materials

N-Hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC) and oligonucleotides A_3, A_6 and A_9 were from Sigma (St. Louis, MO, U.S.A.). Aminolink I and other reagents for oligonucleotide synthesis were from Applied Biosystems (San Jose, CA, U.S.A.). 5′-Aminoethyl-$(dT)_{18}$ was synthesized by Dr. Steven Larsen, Department of Microbiology and Immunology, Indiana University School of Medicine (Indianapolis, IN, U.S.A.) and was deblocked using the protocol provided with the Aminolink I reagent and then purified to apparent homogeneity using a modification of the method of Ashman *et al.*[2]. Briefly, a 250 mm × 4.6 mm I.D. Macrosphere C_4 reversed-phase column (Alltech, Deerfield, IL, U.S.A.) was used with 0.1 *M* triethylamine–acetic acid, pH 6.5 (solvent A) and acetonitrile (solvent B) with a linear gradient of 5 to 25% solvent B in 10 min. Oligonucleotides A_{12}, A_{14}, A_{16} and A_{18} as well as mixtures containing A_{12-18} and A_{19-24} were from Pharmacia (Piscataway, NJ, U.S.A.).

Chromatography

The chromatograph was a Varian 5000 ternary gradient instrument outfitted with a Varichrome UV–VIS detector. Column temperature was controlled by immersing the column in a Lauda refrigerated circulating water bath (Brinkman Instruments, Westbury, NY, U.S.A.). Between the injector and the column was inserted a 0.5-ml rheodyne sample loop (also immersed in the water bath) to act as a buffer preheater. To detect temperature, a Simpson Model 383 dual-channel digital thermometer (Simpson Electric, Elgin, IL, U.S.A.) was used with two J-type thermocouples (0.5 mm diameter, Cole-Parmer Instrument, Chicago, IL, U.S.A.). The thermocouples were inserted into three-way unions and one union was coupled to the inlet of the column and the other to the column outlet. The temperatures reported are those recorded at the outlet. At heating rates below 0.5°C/min and flow-rates below 0.2 ml/min, the inlet, outlet and bath temperatures were all the same.

Column preparation

The coupling scheme is depicted in Fig. 1. Macrosphere-WCX, 300 Å pore size, 7-μm beads packed in 30 mm × 4.6 mm I.D. cartridge columns were supplied by Alltech. The columns were activated to the N-hydroxysuccinimidyl ester using the procedure and apparatus previously described[3]. After activation, the columns were washed sequentially with 10 ml of 2-propanol, 1 ml of methanol, 1 ml of water and finally 1 ml of 0.5 *M* sodium phosphate, pH 7.5. Immediately, 1 ml of this last buffer containing 5′-aminoethyl-$(dT)_{18}$ was recirculated through the column for 30 min at room temperature. The flow-rate was 1 ml/min throughout. The column was then thoroughly washed with buffer and left in buffer overnight to allow the N-hydroxysuccinimidyl esters to spontaneously hydrolyze back to the parent, unreactive support[3]. The columns were then washed extensively with buffer to remove the hydrolyzed N-hydroxysuccinimide prior to use.

Alternative forms of the procedure in which various other concentrations of sodium phosphate and other column sizes were used are also reported. The coupling time was lengthened to 60 min in some experiments and gave similar results. For one experiment, a 23 mm × 2 mm I.D. column was prepared. The column used was packed from an aqueous slurry of 60 Å pore Adsorbosphere-WCX using a reservoir specially prepared to allow slurry packing of the Alltech direct-connect refillable guard column. The column was packed from a slurry containing 0.1 g silica in 1 ml under a constant flow-rate of 1 ml/min. These columns were found to contain 0.033 ± 0.001 g ($n = 3$) of silica in a total column volume of 0.072 ml. In other experiments, columns were packed

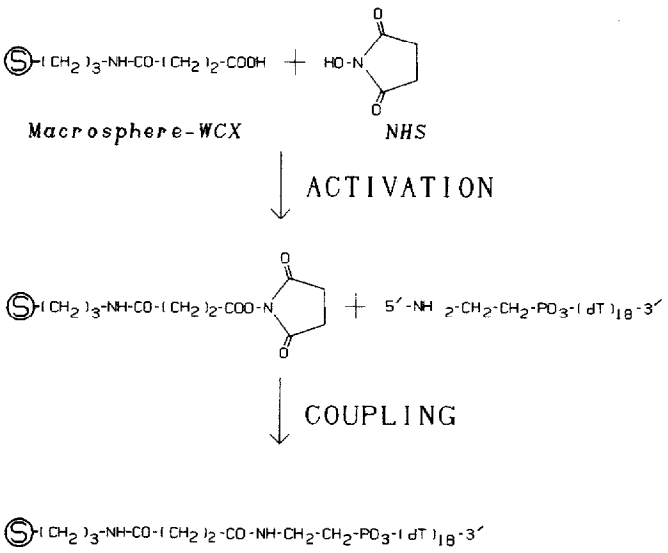

Fig. 1. Synthetic DNA coupled to derivatized silica by an amide bond. Macrosphere-WCX cation-exchange silica in a prepacked cartridge column was activated using N-hydroxysuccinimide (NHS) and a carbodiimide (EDAC) as described previously[3]. 5′-Aminoethyl-$(dT)_{18}$ was then recirculated through the column and coupled via an amide linkage.

at flow-rates as high as 5 ml/min and these columns also gave good chromatographic performance.

Determination of the amount of DNA coupled

During coupling, N-hydroxysuccinimide is released from the column by two processes: as a by-product of the reaction which couples DNA and as a result of spontaneous hydrolysis in aqueous buffers. N-Hydroxysuccinimide and DNA both absorb strongly at 260 nm making it difficult to quantitate the amount of DNA coupled from absorption data at pH 7–8. However, it was found that by titrating solutions to pH 2, the absorption of N-hydroxysuccinimide in the 260-nm range can be greatly reduced while DNA's absorption is barely affected. Thus, after coupling, the recycled DNA solutions were titrated to pH 2 with hydrochloric acid and the absorption at 266 nm was measured and compared to the absorption of starting sample also at pH 2. The amount coupled is expressed in terms of absorption units coupled. In some experiments, the amount coupled was also determined by repurifying and quantitating the 5'-aminoethyl-$(dT)_{18}$ remaining uncoupled by reversed-phase chromatography[2] and these results were similar to the simpler method described above.

RESULTS

Initially, other column coupling protocols were investigated. In model experiments, 5'-TMP was coupled to 3-aminopropyl-silica (Adsorbosphere-Amino, Alltech) using EDAC in 0.25 *M* imidazole, pH 6.1. While TMP was found to couple efficiently, it was latter discovered that DNA binds tightly to unmodified 3-aminopropyl-silica and is only eluted at ionic strengths above *ca.* 0.3 *M*. Since DNA is a highly anionic molecule, this was probably due to anion exchange with the cationic aminopropyl-silica. Thus, DNA-silica prepared by this approach would of necessity be mixed-mode chromatography, and this approach was abandoned for the approach depicted in Fig. 1.

As shown in Fig. 1, DNA which had been synthesized to contain a 5'-amino moiety, 5'-aminoethyl-$(dT)_{18}$, was coupled by way of an amide bond to carboxylic acid-based silica. The chemistry used allows a stable, unreactive silica (Adsorbosphere-WCX) to be activated to the reactive N-hydroxysuccinimidyl ester and reacted to couple DNA all inside a prepacked column. Column preparation only requires a few hours. Unreacted N-hydroxysuccinimidyl ester silica spontaneously hydrolyzes in aqueous buffers[3] and the column is available for use the next day. After *ca.* six months of use, no loss of coupled DNA or column capacity has been detected suggesting that the linkage is stable during routine usage.

The salt concentration used during coupling has an effect on coupling efficiency as shown in Table I. In 0.1 *M* sodium phosphate, pH 7.5, only 18–40% of the applied DNA couples. Increasing the salt concentration increases the fraction of DNA that couples, with 80% coupling efficiency observed at 0.5 *M*. The failure to couple efficiently in low salt may be due to charge repulsion between the DNA and unreacted groups on the Macrosphere-WCX.

Table I also shows that columns containing 72 units of DNA per g of silica can be made by this coupling chemistry. This corresponds to about 2.7 mg of $(dT)_{18}$ per g of

TABLE I

EFFICIENCY OF DNA COUPLING

Experiment No.	*Ionic strength in total Na^+ (M)*	*Column dimensions (mm × mm I.D.)*	*DNA reacted (units)*	*DNA coupled (%)*	*Load[a] (units/g)*
1	0.1	30 × 4.6	1.3	18	0.78
2	0.1	30 × 4.6	1.8	40	2.5
3	0.14	30 × 4.6	19.2	59	38
4	0.5	30 × 4.6	2.0	80	5.3
5	0.42	23 × 2.0	3.0	78	72

[a] The units of 5'-aminoethyl-$(dT)_{18}$ coupled per gram of silica assuming 0.3 g silica for 30 mm × 4.6 mm I.D. columns and 0.033 g for the 23 mm × 2 mm I.D. column.

silica or about 87 μg of DNA coupled inside the small 23 mm × 2 mm I.D. column. Similarly, the highest amount coupled inside a 30 mm × 4.6 mm I.D. column (37 units/g) corresponds to *ca.* 400 μg of DNA coupled to the 0.3 g of silica these columns were found to contain. If a significant fraction of this DNA can participate in specific hybridization during chromatography, adequate capacity for many types of genetic engineering experiments is feasible.

In Fig. 2 is shown the separation of a mixture of A_{12}–A_{18} using temperature-dependent elution. At 8°C, the mixture was loaded onto the column and the column was slowly raised to 44°C over the next 2 h. Essentially all of the 0.3 U of oligoadenines applied was retained by the column. At the flow-rate and column size used, the loaded sample volume would traverse the column in about 1 min and this time was evidently sufficient for complete hybridization at 8°C. The high resolution is apparent from the seven separations shown in Fig. 2 which result from a difference in length of a single base pair.

When adenine oligomers longer than 18 were applied to the column, they could

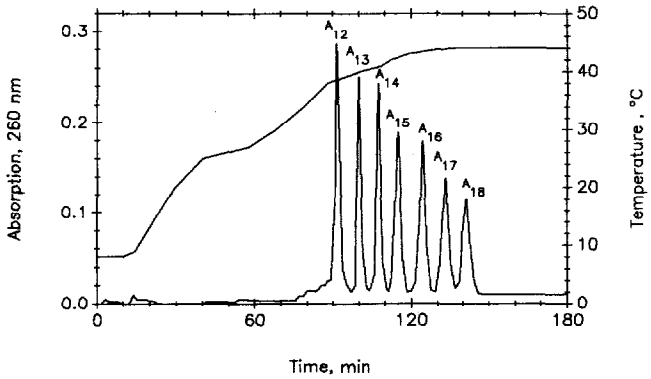

Fig. 2. Mixture containing seven different oligomers of adenine resolved using a temperature gradient. The 30 mm × 4.6 mm I.D. $(dT)_{18}$-silica column used for this experiment contained 11 U of oligo-(dT). The column was immersed in an 8°C water bath and at time 0, 0.3 U of an A_{12}–A_{18} mixture was loaded onto the column. The mobile phase was 0.49 *M* sodium chloride, 0.01 *M* sodium phosphate, pH 6.5, and the flow-rate was 0.2 ml/min throughout. Temperature was controlled by manually adjusting the temperature of the water bath and is shown on the right hand ordinate.

not be separated from one another but rather all elutd near where A_{18} would elute (data not shown). Thus, a column can separate based on length only up to the length of the coupled DNA, in this case, octadecamers. This was not necessarily a predictable result. Longer adenine oligomers should have more alternative ways of forming hybrids with the column-coupled octadecamer of thymidine. The larger degrees of freedom should contribute to make such hybrids more thermally stable but the effect is apparently too small to affect the chromatography.

In a separation analogous to that shown in Fig. 2, the A_{12}–A_{18} mixture was loaded onto the column at 8°C and the temperature rapidly raised to 30°C followed by rapid gradient to 47°C in 30 min. Using this protocol, all seven of the oligomers were also separated within 35 min, albeit with some decrease in resolution (data not shown). Thus, the long gradient shown in Fig. 2 is not necessary to obtain separation but does demonstrate the resolution obtainable.

To further characterize the temperature dependence of elution, single adenine oligomers were applied to the column individually and the column was heated at a very low rate (1°C per 10 min or less) and the position of the peak more precisely measured. Control experiments showed that the rate of heating was low enough that thermal equilibrium across the column was obtained and reproducible melting temperatures were measured. This is a more stringent requirement than that needed to simply separate mixtures. The results are in Fig. 3.

Elution temperatures were measured at both 0.1 and 0.5 *M*, as shown in Fig. 3. As expected[4,5], decreasing salt concentration lowers the temperature at which duplex hybrids melt and elution occurs. At either salt concentration, the data over this range of lengths fit a straight line. The slope of the lines show that increasing oligoadenine length by one base increases the melting temperature by 2.5–3.5°C at 0.1–0.5 *M* salt, respectively. Other studies of DNA hybrids in solution or immobilized to nitrocel-

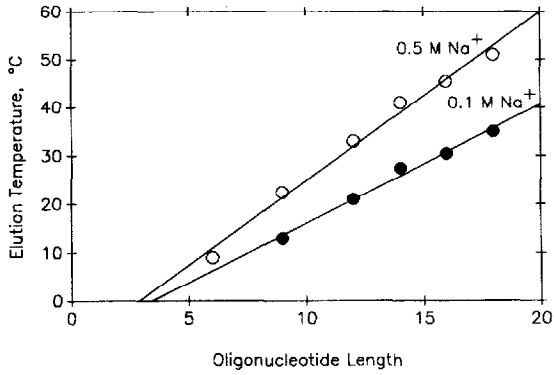

Fig. 3. Dependence of temperature on oligomer length and ionic strength at which an oligomer elutes. For this experiment, the 30 mm × 4.6 mm I.D. column containing 1.6 U of DNA was used and the various oligomers of adenine used had been previously kinased using γ-$^{32}PO_4$-ATP and polynucleotide kinase[8]. The flow-rate was 0.2 ml/min throughout and the mobile phase was 0.01 *M* sodium phosphate, pH 6.5, containing either 0.49 *M* sodium chloride (0.5 *M* Na^+) or 0.09 *M* sodium chloride (0.1 *M* Na^+). The individual oligoadenines were applied to the column at a temperature at least 4°C below the anticipated temperature of hybrid melting, and the temperature was slowly raised while collecting fractions. The absorption at 260 nm of the fractions was then measured, and a portion was mixed with scintillation fluid and counted for ^{32}P. The temperature at which each oligomer eluted in either mobile phase is shown.

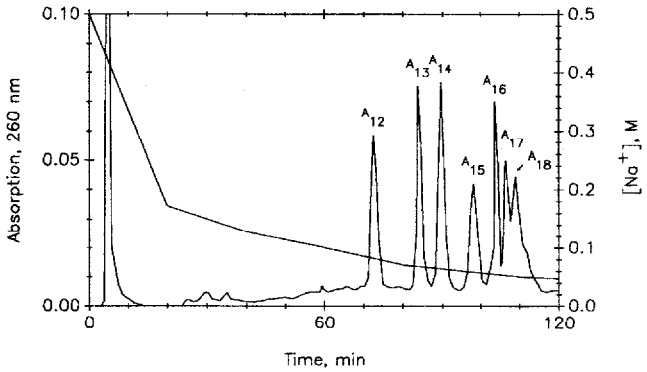

Fig. 4. Oligoadenine mixtures resolved using salt gradients. The 30 mm × 4.6 mm I.D. column containing 11 U of DNA was immersed in the water bath which was maintained at 35°C throughout. At time 0, 0.3 U of the oligoadenine mixture A_{12}–A_{18} was injected and eluted using a ternary salt gradient. Buffer A (0.5 *M* Na^+) and buffer B (0.1 *M* Na^+) were the buffers described in Fig. 3; buffer C was water. The gradient, shown on the right ordinate, was a linear rate of change between the following times and percentages: 0 min, 100% A; 20 min, 18% A, 82% B; 40 min, 7% A, 93% B; 60 min, 100% B; 80 min, 70% B, 30% C; 100 min, 60% B, 40% C; 140 min, 50% B, 50% C.

lulose have shown a 2°C change per base pair in 0.9–1 *M* salt[4,5]. The higher incremental temperature found here is probably due to the lower salt concentrations used but may suggest an influence of the stationary phase silica on the hybrid melting temperature.

For aqueous-based chromatography, a practical limit is imposed by the fact that water freezes at 0°C. As Fig. 3 shows, at either salt concentration used, the column could potentially be used to separate hybrids of lengths down to about a trimer at temperatures above 0°C, and that raising salt concentration can be used to extend the range of useful temperatures somewhat. Columns could thus be made to specifically bind even the hexamers typical of many restriction endonuclease cleavage sites.

Salt gradients can also be used for highly selective elution, as shown in Fig. 4. For this experiment, the sample was loaded at 35°C which is close to the upper limit of temperature at which hybridization with A_{12}, the smallest oligomer present in the sample, can still occur (see Fig. 3). Under these conditions, not all of the oligoadenine applied is retained by the column as indicated by the absorption eluting early in the chromatogram. This is in contrast to the results obtained in Fig. 2 where the sample was loaded at 8°C. However, the oligoadenines that are retained by the column are selectively eluted by the salt gradient used, and hybrid lengths differing by a single nucleotide base are readily resolved. Since many HPLC systems are not outfitted with programmable temperature controllers, salt gradient elution could be more widely used with existing instruments.

DISCUSSION

Polynucleotide affinity chromatography at low pressures has proven to be a valuable technique for over 20 years. Most commonly it has been used where low resolution is inconsequential such as in the purification of polyadenylated mRNA. For

these uses, the mRNA is loaded onto an oligo-(dT) or oligo-(uracil) column in a high-ionic-strength buffer and eluted by an abrupt change to low ionic strengths. For such uses, resolving mRNAs which differ in a single base pair is not necessary or desirable.

Although the coupling chemistry used should work with any nucleotide sequence, we have used oligo-(dT) columns as a model to investigate the limits and potential of the method. The results show that resolution based upon a single base difference can be obtained by either temperature-dependent or salt-dependent elution. When injection occurs at temperatures well below the hybrid melting temperature, all of the DNA injected was able to hybridize with the column in 1 min or less (Fig. 2); however, this is not necessarily the case for higher-temperature injections (Fig. 4). Lengths of 3–4 are the minimum which will hybridize at temperatures above 0°C, but increasing the salt concentration in the mobile phase increases the temperature of melting (Fig. 3) and this, or the inclusion of trimethylamine in the mobile phase[6], could probably be used to extend the method to shorter-length hybrids. However, even with a length limit as high as six bases, columns specific to average length restriction endonuclease cleavage sites could be made. However, since at random, any sequence length L will occur once in every 4^L nucleotides, such short sequence columns would not be very selective.

In these model studies, we have not tried to optimize either speed or column capacity. We have used very long gradients to carefully observe where elution occurs. With a little effort, the column and equipment could be changed to obtain more rapid temperature equilibration. Higher salt concentrations or trimethylamine can be used to increase the temperature at which loaded samples will rapidly hybridize with the column. These and other improvements would speed the separations obtained.

Capacity can also be increased. The Macrosphere-WCX silica used has about 500 μmol of free carboxylate per g of silica while the columns we have prepared contain at most 72 absorbance units of 5′-aminoethyl-$(dT)_{18}$ per g. This amount represents only about 0.5 μmol of DNA per g of silica, and thus little of the potential coupling capacity of the silica was used in the experiments presented here. Coupling of larger amounts of DNA is thus probably feasible but not necessarily desirable. Most experiments in molecular biology typically require smaller amounts of DNA than the capacity of the columns already made.

The high resolution possible with polynucleotide HPAC may allow different uses than the preparative role the low-pressure version of the technique has typically served. Analytical and clinical uses of polynucleotide HPAC may also be important. Polynucleotide hybridization is certainly capable of the required selectivity as demonstrated by the common use of Southern[7] and "Northern" hybridizations to specifically detect a single nucleotide sequence in the presence of other polynucleotides. However, detection may need to be improved. For example, a simple calculation shows that the peak of A_{12} in Fig. 2 resulted from about 0.2 nmol of oligomer chain or about 10^{14} chains. A peak one hundredth the size would easily be detectable and thus about 10^{12} chains of A_{12} should be detectable. This would be considered to be sensitive detection for most chromatographic methods but probably would not be sensitive enough for work with low-copy-number polynucleotides obtained from tissue. Radioisotope labeling with ^{32}P can, however, be used to increase sensitivity in these cases, and polymerase chain reaction technology[9] could be used to amplify low-abundance polynucleotides.

Polynucleotide HPAC is thus a sensitive, selective, high-resolution chromatographic technique which has remained relatively unexplored but is potentially useful.

ACKNOWLEDGEMENTS

This work was supported by grants from the NIH (GM 43609) and NSF (DMB 8996229). We thank William Foster of Alltech/Applied Science for the kind gift of the Macrosphere-WCX cartridges and Professor Steve Larsen for synthesizing the 5′-aminoethyl-$(dT)_{18}$ and for much helpful discussion. One of us (T.A.G.) is a recipient of the Alltech Fellowship in Chromatography, the support of which is gratefully acknowledged.

REFERENCES

1 P. T. Gilham, *J. Am. Chem. Soc.*, 86 (1964) 4982–4989.
2 K. Ashman, A. Bosserhoff and R. Frank, *J. Chromatogr.*, 397 (1987) 137–140.
3 H. W. Jarrett, *J. Chromatogr.*, 405 (1987) 179–189.
4 R. B. Wallace and C. G. Miyada, *Methods Enzymol.*, 152 (1987) 432–442.
5 G. M. Wahl, S. L. Berger and A. R. Kimmel, *Methods Enzymol.*, 152 (1987) 399–407.
6 A. G. DiLella and S. L. C. Woo, *Methods Enzymol.*, 152 (1987) 447–451.
7 E. M. Southern, *J. Mol. Biol.*, 98 (1975) 503–517.
8 T. Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning*, Cold Spring Harbor Laboratory, New York, 1982, pp. 122–127.
9 R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich, *Science (Washington, D.C.)*, 239 (1988) 487–491.