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Journal of Chromatography A, 806 (1998) 141–147

JOURNAL OF CHROMATOGRAPHY A

Contamination-free and automated composition of a reaction mixture for nucleic acid amplification using a capillary electrophoresis apparatus

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Abstract

The acceptance of the polymerase chain reaction (PCR) as an amplification method in molecular diagnostics and the rapid development of capillary electrophoresis (CE) as an analysis method of those PCR products was a reason for us to investigate further integration of those two techniques. Using a fused-silica capillary as a pipette we were able to compose a PCR mixture in the CE apparatus. Because a capillary can be thoroughly rinsed and the CE apparatus is a closed system, the risk of contamination and therefore the occurrence of false positive results is minimized. The fact that a CE system can be fully automated contributes to a more reproducible and standardized PCR composition protocol. © 1998 Elsevier Science B.V.

Keywords: Polymerase chain reaction; Nucleic acids

1. Introduction

Over the years the polymerase chain reaction (PCR) [1] has become widely used in molecular diagnostics and biological research and accepted as a useful technique with many applications. However, it is difficult to introduce the PCR in routine diagnostic laboratories because of a major drawback of the technique: the occurrence of false positive results. These false positive results can be caused by sample to sample contamination or by the carry-over of previously amplified PCR products, known as amplicon contamination.

Several recommendations and guidelines have been described for the avoidance of those false positive results. These recommendations include physical measures like separate rooms for reagent preparation, template addition and PCR analysis, the use of disposable gloves and tips, aliquoting of reagents and avoiding re-amplification [2–4] as well as chemical measures like UV irradiation [5,6], Uracil DNA glycosylase treatment after dUTP incorporation [7], isopsoralen photochemistry [8], restriction enzyme digestion [9] or gamma irradiation [10].

Despite these recommendations the occurrence of false positives remains a fact. That is why in a routine diagnostic laboratory setting the use of a negative control (no template added) is obligatory. To exclude PCR artefacts, a double confirmation, based on length and nucleotide sequence, of the PCR products is strongly recommended. This confirmation can be performed by for example Southern blotting

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0021-9673/98/$19.00
PII S0021-9673(97)00856-X

or sequencing. Both confirmation methods are laborious and time consuming.

Since the introduction of capillary electrophoresis (CE) in the early 1980s [11,12] this fast, non-radioactive, reproducible analysis method has found applications in many fields. Especially the amount of applications in the molecular research is rapidly growing. The accuracy and the ease for automation of CE prompted us to investigate whether we were able to use a CE system not only to analyze PCR products but also to compose a PCR mixture using the capillary as a pipette. This has several advantages: the composition of a PCR mixture can be fully automated which should lead to a more reproducible and standardized PCR composition protocol. Because the capillary can be vigorously rinsed with chemical solutions it should be possible to eliminate DNA contamination from one sample to another. Another advantage is that a CE apparatus is a closed system so the risk of amplicon contamination is further reduced. Using the capillary of the CE system as a pipette is a step towards integration of methods and fully automated molecular diagnostics.

2. Experimental

2.1. Polymerase chain reaction

DNA was isolated [13] from the cell line SW480. A Ki-ras PCR was performed in an Eppendorf Mastercycler 5330 (Eppendorf, Hamburg, Germany) using the oligonucleotides Ki-ras 1A; 5′-GGC CTG CTG AAA ATG ACT GA-3′ and Ki-ras 1B; 5′-GTC CTG CAC CAG TAA TAT GC-3′ [oligonucleotides were synthesized on a 391A DNA synthesizer (Applied Biosystems, Warrington, UK)]. Dependent on the total reaction volume, 100 or 20 μl, each PCR contained 0.5 (0.1) μg SW480 DNA, 30 (6) pmol of oligonucleotides Ki-ras 1A and Ki-ras 1B, 250 μ*M* dNTPs and 2.5 (0.5) U Taq DNA polymerase (Life Technologies, Gaithersburg, MD, USA). The PCR mixture was overlaid with 80 (20) μl mineral oil. Amplification started with an initial denaturation step at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 60 s at 72°C. After an elongation step of 10 min at 72°C the PCR products were cooled to 15°C and analyzed on a 1.8% agarose (Life Technologies, Paisley, UK) gel stained with ethidiumbromide. The resulting PCR products had a length of 162 base pairs (bps).

2.2. Capillary electrophoresis

An uncoated fused-silica capillary (SGE, Ringwood, Victoria, Australia) (470 mm (effective length 323 mm)×0.075 mm I.D.) was placed in a PRINCE CE system (PRINCE Technologies, Emmen, Netherlands) with a laboratory-built LIF detection system. This CE system is suitable to perform this automated reaction mixture composition protocol. The system is known for its precise pressure application. This means that the injections but also the purge steps are substantially constant and thus very reproducible. Another advantage of the CE system we used is the capability for easily adapting hardware without disrupting the CE apparatus. Where other commercially available CE devices are perhaps more compact closed systems, suitable for routine applications, systems like the PRINCE are more suitable for adaptations and research work.

Before each run the capillary was rinsed with 2 *M* HCl for 5 min using 2000 mbar pressure. After this vigorously rinsing, the capillary was filled with a replaceable non-gel sieving buffer (Bio-Rad, Hercules, CA, USA). All samples were pressure injected at 500 mbar for 0.2 min. To avoid contamination of the sample with the non-gel sieving buffer, the inlet side of the capillary was dipped (10 mbar for 0.1 min) in a TBE (90 m*M* Tris–Borate, 0.2 m*M* EDTA, pH 8.3) buffer before injection. Samples were separated using a constant voltage of −350 V/cm in a TBE running buffer.

2.3. Composing the PCR mixture with the CE system

With a fused-silica capillary of 470 mm×0.075 mm I.D. a volume of 10 μl can be transferred from the inlet side to the outlet side of the capillary by applying a pressure of 1000 mbar for 1 min. The master mix (containing all the reagents for the PCR except the DNA) was prepared for ten reactions. To assure the reproducibility of the transferred volume, the concentrations of the PCR reagents were adjusted so that a minimum volume of 10 μl (1000 mbar

pressure for 1 min) of each reagent was transferred to obtain the right amount of that reagent in the master mix.

Fig. 1 shows a schematic drawing of the PCR composition procedure. All PCR reagents, in the appropriate concentrations, were placed in the tray of the PRINCE CE system. Vials containing SW480 DNA (1 μl) or water (1 μl), overlaid with 20 μl mineral oil were also placed in the tray. Before preparing the master mix the capillary was rinsed with 2 *M* HCl for 5 min using 2000 mbar pressure. The HCl depurinates and degenerates DNA molecules and so decontaminates the capillary. After that the capillary was rinsed with PCR buffer for 1 min to avoid inhibition of the PCR because of the HCl. The master mix was prepared by transferring the PCR

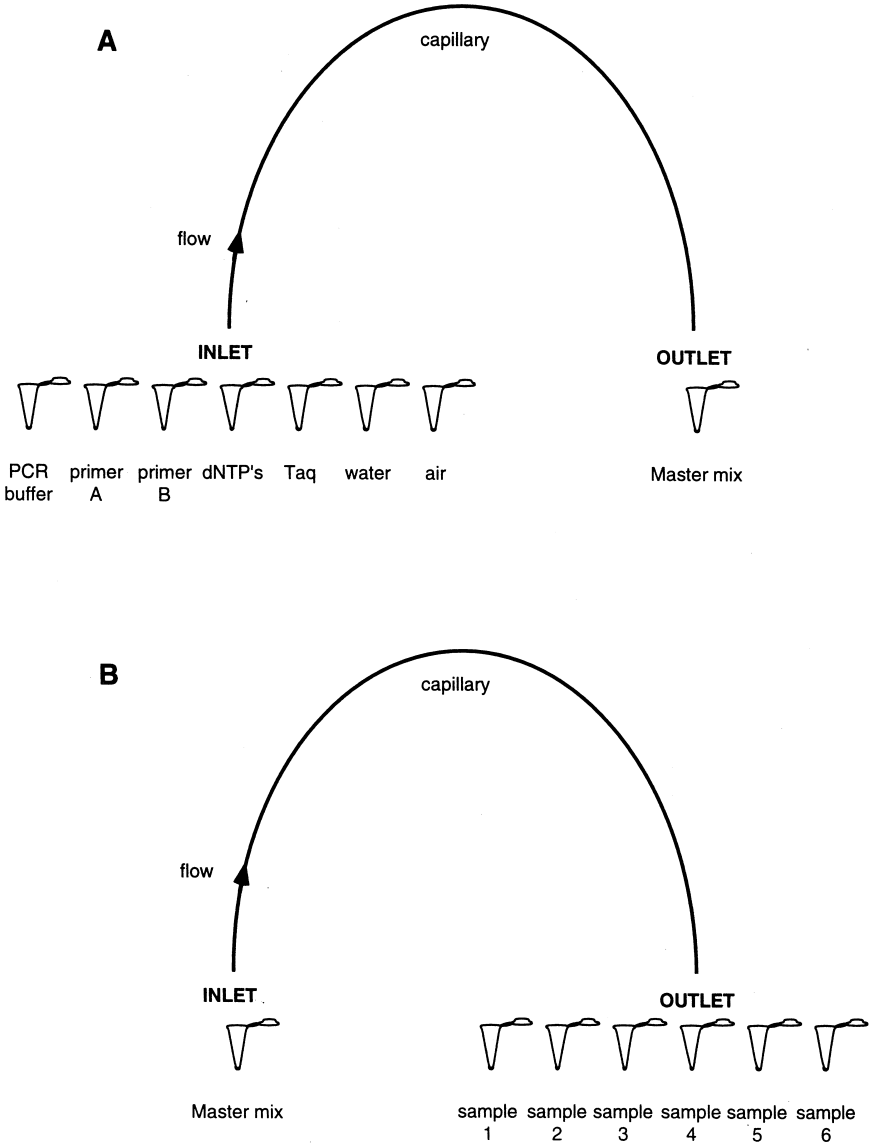

Fig. 1. Schematic drawing of the PCR composition procedure. (A) Composing a PCR master mix and (B) distributing the master mix over the samples.

Table 1
Concentration of the reagents and the transferring time and pressure applied for composing a PCR reaction mixture

Step	Reagents	Concentration	Pressure (mbar)	Time (min)
1	PCR buffer	2×	2000	5
2	Primer A	6 pmol/μl	1000	1
3	Primer B	6 pmol/μl	1000	1
4	dNTPs	2.5 n*M*	2000	1
5	Taq	0.5 U/μl	1000	1
6	Water		2000	2
7	Air		300	1

reagents one by one through the capillary by applying pressure for a certain time (Table 1). All reagents were collected in a vial at the outlet side of the capillary. Purging with air as final step is necessary to empty the capillary and to mix the contents of the master mix.

After preparing the master mix, it was distributed over the different sample vials by transferring it through the capillary, from inlet to outlet, using 2000 mbar pressure for 1 min (=20 μl). To eliminate cross contamination of the samples, the outlet side of the capillary was placed in 2 *M* HCl for 1 min and, to neutralize, dipped in TBE buffer between each sample.

3. Results and discussion

3.1. Rinsing procedure

We used a fused-silica capillary to compose a PCR mixture in the CE system. Because the capillary is normally used to analyze DNA fragments, which stick to fused-silica surfaces, we first had to develop a rinsing procedure to clean the capillary surface in order to prevent contamination of the PCR mixture with previously analyzed DNA fragments. From our experiments (data not shown) we knew that a Ki-ras PCR (in a total volume of 100 μl) performed on 20 μl of sterile TBE buffer, which was transferred through the capillary, showed a positive result if we did not rinse the capillary thoroughly after previously analyzing a Ki-ras PCR product. Fig. 2, lanes 1 and 2, shows that rinsing with 2 *M* HCl using 2000 mbar pressure for 5 min is efficacious to decontaminate the inner surface of the fused-silica capillary. By placing the inlet as well as the outlet side of the capillary in a vial containing 2 *M* HCl during rinsing, also the outer surface of the capillary ends is decontaminated. A precondition for this vigorously rinsing is the use of an uncoated capillary, since the 2 *M* HCl solution will affect any present coating resulting in poor electropherograms, when the capillary is used for analyzing again. For this reason we used a replaceable non-gel sieving buffer containing a dynamic coating in an uncoated capillary to analyze the PCR products.

Fig. 2 also shows that already after a single sample injection the inlet buffer is contaminated

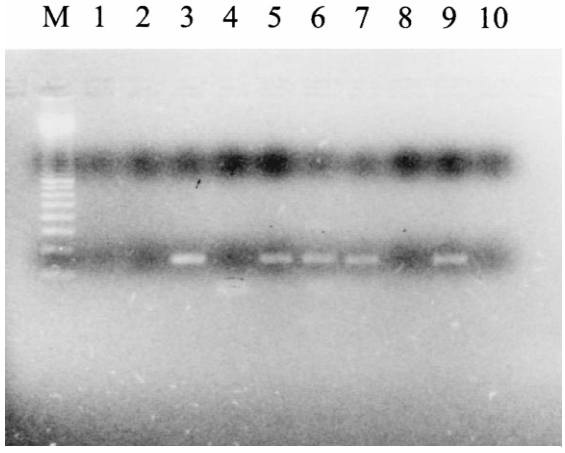

Fig. 2. Analysis of PCR products on an ethidiumbromide stained 1.8% agarose gel. (M) 100 bp molecular-mass marker, Ki-ras PCR on 20 μl 1×TBE (1) from the inlet after rinsing with HCl, no injection, (2) from the outlet after rinsing with HCl, no injection, (3) from the inlet after injection and 35 min separation, (4) from the outlet after injection and separation from 0 to 20 min, (5) from the outlet after injection and separation from 20 to 25 min, (6) from the outlet after injection and separation from 25–30 min, (7) from the outlet after injection and separation from 30–35 min, Ki-ras PCR on (8) 20 μl 1×TBE bulk, (9) 0.5 μg SW480 DNA and (10) water.

(lane 3), probably because the outer surface of the capillary is contaminated by placing the capillary in the sample during injection. The outlet buffer remains negative (''clean'') (lane 4) until the analyzed fragment reaches the outlet (±22 min, electropherogram not shown) (lane 5). Changing the outlet buffer every 5 min shows that the buffer stays positive after the fragment has reached the outlet (lanes 6 and 7). A possible explanation for this is that the outer surface of the capillary is contaminated by the fragment and thus every other buffer in which the capillary is placed afterwards becomes contaminated. Another explanation is, because the inlet buffer is contaminated after sample injection, there is a continuous flow of PCR molecules from the inlet buffer to the outlet buffer.

Fig. 3, lane 1, shows that after one sample injection a PCR performed on 20 μl of the inlet buffer is positive (see also Fig. 2, lane 3). Lanes 2 to 5 show that after more sample injections the inlet buffer becomes more and more contaminated. This means that a new inlet buffer should be used after each sample injection to avoid severe contamination. This is especially important when the peak has to be fractionated to, for example, reamplify the DNA fragment.

To determine the sensitivity of our PCR assay, we performed a Ki-ras PCR (total volume is 100 μl) on a known amount of DNA molecules. As can be seen in Fig. 4, the detection limit of the PCR is 100 molecules, so in our setting a capillary is called decontaminated if less then 100 DNA molecules are present.

Besides the development of a rinsing procedure we also had to make some adjustments to the CE system to prevent contamination. Normally the vials in the tray of the CE apparatus are sealed with a starburst cap, to prevent evaporation of the sample in the vials during analysis. While the capillary and the electrode are pushed through those caps, the outside of the capillary is in contact with the cap. This means that when the outer surface of the capillary is decontaminated by placing it in a vial containing 2 *M* HCl, the cap of that vial is contaminated. When transferring to another vial the capillary gets in contact with the cap and is contaminated again. We therefore removed the middle part of the cap to avoid contact of the capillary with the cap. Another adjustment we made was the relative position of the capillary and the electrodes. Because the capillary and the electrode diverge a little, to prevent that liquid is kept between them, the outside of the capillary is in direct contact with the wall of the vial.

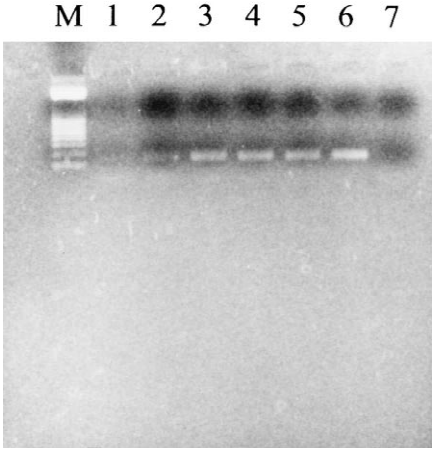

Fig. 3. Analysis of PCR products on an ethidiumbromide stained 1.8% agarose gel. (M) 100 bp molecular-mass marker, Ki-ras PCR on 20 μl 1×TBE from the inlet after (1) one injection, (2) two injections, (3) three injections, (4) four injections, (5) five injections, Ki-ras PCR on (6) 0.5 μg SW480 DNA and (7) 1×TBE bulk.

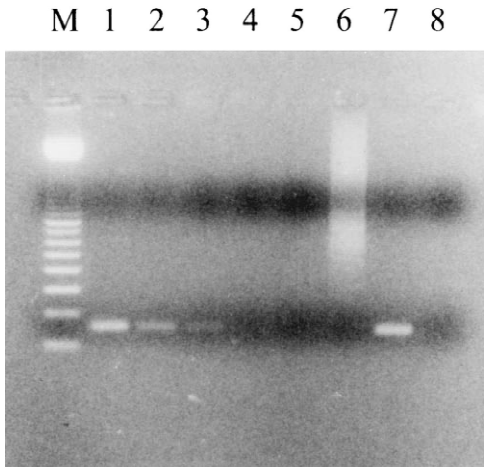

Fig. 4. Analysis of PCR products on an ethidiumbromide stained 1.8% agarose gel. (M) 100 bp molecular-mass marker, Ki-ras PCR on (1) 10^4 DNA molecules, (2) 10^3 DNA molecules, (3) 10^2 DNA molecules, (4) 10 DNA molecules, (5) 1 DNA molecule, (6) 10^{10} DNA molecules, (7) 0.5 μg SW480 DNA and (8) water.

This also can cause contamination of the previous decontaminated capillary. To avoid this kind of contamination we placed the capillary and the electrodes in a totally vertical position so the capillary and the electrodes do not get in contact with the wall of the vial.

3.2. Composing a PCR mixture

By using a pressure of 1000 mbar for 1 min a volume of 10 μl is transferred from the inlet side to the outlet side of a capillary with a length of 470 mm and an internal diameter of 75 μm. The standard deviation of this volume is 1% ($n=15$) and was determined by weighing the transferred amount of water. This standard deviation is comparable to the standard deviation of manual pipetting, which was determined by weighing ten samples of 10 μl pipetted by three persons ($n=30$).

To minimize the amount of time needed to compose a PCR mixture we downscaled the reaction volume from 100 μl to 20 μl. Because, using this smaller PCR volume, less reagents are needed using the regular concentrations, we adjusted the concentrations so that a minimum of 10 μl is required for preparing a mixture for ten reactions. This means that the concentration of the oligonucleotides was lowered from 10 pmol/μl to 6 pmol/μl, the dNTPs were diluted ten-times to 2.5 m*M* and the enzyme Taq DNA polymerase was diluted with PCR buffer to 0.5 U/μl (Table 1). Using these concentrations the minimal pressure used was 1000 mbar and the minimal time used was 1 min. These values contributed to a higher reproducibility of the transferred volume. Another reason to adjust the Taq concentration is the high viscosity of the original solution due to the presence of glycerol. By diluting the solution ten-times the viscosity was approximately the same as the other PCR reagents.

To test the composition of the master mix using a capillary as a pipette, the composed master mix was distributed, by transferring it through the capillary from the inlet to the outlet (Fig. 1), over alternating negative (water) and positive (SW480 DNA) samples. Fig. 5 shows that dipping the outlet side of the capillary in 2 *M* HCl results in the elimination of cross contamination between samples, because all water samples are negative (lanes 1, 3, 5, 7).

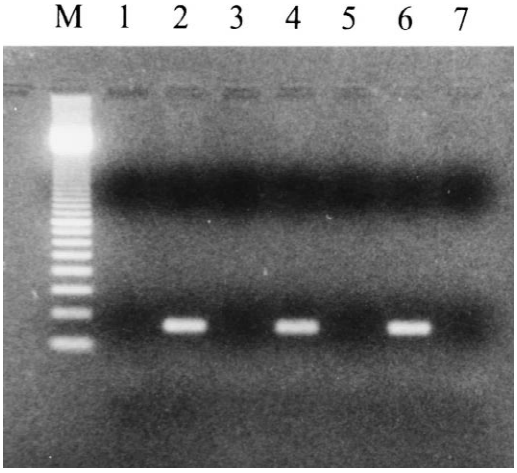

Fig. 5. Analysis of PCR products on an ethidiumbromide stained 1.8% agarose gel. (M) 100 bp molecular-mass marker, Ki-ras PCR on (1, 3, 5, 7) 20 μl water and (2, 4, 6) 0.5 μg SW480 DNA.

Fig. 6 shows another test for the master mix composition and distribution using a capillary. A serial dilution of SW480 DNA was placed in the CE tray. A Ki-ras PCR was performed on these samples after the master mix was added by the capillary. Again there is no cross contamination between the samples. The PCR on SW480 DNA where the

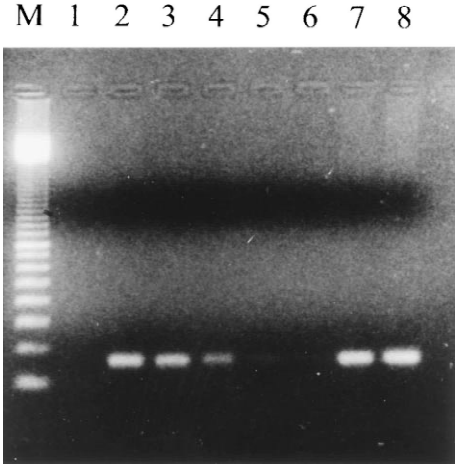

Fig. 6. Analysis of PCR products on an ethidiumbromide stained 1.8% agarose gel. (M) 100 bp molecular-mass marker, Ki-ras PCR on (1) water, (2) 10^{-1} μg DNA, (3) 10^{-2} μg DNA, (4) 10^{-3} μg DNA, (5) 10^{-4} μg DNA, (6) 10^{-5} μg DNA, (7) 10^{-1} μg DNA, with the mastermix added manually and (8) 10^{-1} μg DNA, with the mastermix composed and added manually.

master mix is composed and added manually (lane 8) and the PCR where the master mix is added by the capillary (lane 2) are equal in intensity. This means that composing and distributing a master mix in a CE apparatus is as good as composing and distributing it manually.

4. Conclusions

We investigated the possibility to automate the composition of a PCR master mix using a CE system as a pipette. An automated method of composing a PCR reaction mixture leads to a more reproducible and thus standardized PCR protocol.

Using a capillary in the CE system as an automated pipette has the advantage that the capillary can be vigorously rinsed. Our results show that rinsing with 2 *M* HCl is sufficient to clean the capillary to prevent DNA contamination from one sample to another. The fact that the capillary is placed in a closed CE system contributes to a contamination free PCR composition protocol. Our results also show that the accuracy of transferring a volume from inlet to outlet can be compared to manual pipetting.

Automation of composing a PCR master mix will, in the end, lead to a higher throughput of samples. It also leads to a higher reproducibility resulting in an overall quality increase. There are less ''drop-outs'' because of contaminations, which results in a more efficient use of time.

This paper describes a first step in the integration of a nucleic acid amplification method and a powerful analytical technique. This combination provides an important tool in automation of DNA analyses.

Acknowledgements

This work was supported by the STW foundation and the Maurits en Anna de Kock foundation.

References

[1] R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, N. Arnheim, Science 230 (1985) 1350.
[2] S. Kwok, R. Higuchi, Nature 339 (1989) 237.
[3] J.P. Clewley, J. Virol. Methods 25 (1989) 179.
[4] T. Victor, A. Jordaan, R. du Toit, P.D. Van Helden, Eur. J. Clin. Chem. Clin. Biochem. 32 (1993) 531.
[5] G. Sarkar, S.S. Sommer, Nature 343 (1990) 27.
[6] C.-Y. Ou, J.L. Moore, G. Schochetman, BioTechniques 10 (1991) 442.
[7] M.C. Longo, M.S. Berninger, J.L. Hartley, Gene 93 (1990) 125.
[8] G.D. Cimino, K.C. Metchette, J.W. Tessman, J.E. Hearst, S.T. Isaacs, Nucl. Acids Res. 19 (1990) 99.
[9] B. Furrer, U. Candrian, P. Wieland, J. Lüthy, Nature 346 (1990) 324.
[10] J.-M. Deragon, D. Sinnet, G. Mitchell, M. Potier, D. Labuda, Nucl. Acids Res. 18 (1990) 6149.
[11] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[12] F.E.P. Mikkers, F.M. Everaerts, T.P.E.M. Verheggen, J. Chromatogr. 169 (1979) 11.
[13] S.A. Miller, D.D. Dykes, H.F. Polesy, Nucl. Acids Res. 16 (1988) 1215.