DIONEX

Application Note 162

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Determination of Nucleotides by Ion Chromatography with UV Absorbance Detection

INTRODUCTION

Nucleotides are negatively charged ionic molecules consisting of a nitrogenous organic base, a 5-carbon pentose sugar, and phosphoric acid. Figure 1 shows the five common bases and two pentose sugars that are building blocks of common nucleotides. Also shown in Figure 1 is the chemical structure of the nucleotide 2'-deoxyadenosine 5'–triphosphate (dATP), showing the ionic charge at pH >7. Four of the bases (A, T, C, and G), substituted with 2-deoxy-α-D-ribose, polymerize to form deoxyribonucleic acid (DNA) and three of these plus uracil (A, U, C, and G), substituted with α-D-ribose, combine to form RNA.

The successive nucleotides of both RNA and DNA are covalently linked to each other when an enzyme (RNA or DNA polymerase) catalyzes formation of a phosphodiester bond between the 3'-hydroxyl group of one pentose and the 5'-hydroxyl of the next pentose. The hydrolysis of phosphate bonds in the activated precursors—that is, the nucleotide triphosphates—drives the DNA and RNA polymerase reactions.

Polymerase reactions can be performed in the laboratory to produce pure sequences of DNA or RNA for research, diagnostic, or forensic purposes. In the polymerase chain reaction (PCR),[1] now carried out routinely by automated thermal cyclers, a target DNA sequence (template) is amplified by adding primers, deoxynucleoside triphosphates (dNTPs), and a thermostable DNA polymerase. For a PCR reaction to succeed, each of the dNTPs must be present at the proper concentration. Furthermore, too high a concentration of deoxynucleoside diphosphates (dNDPs) or deoxynucleoside monophosphates (dNMPs) can also cause the PCR reaction to fail.

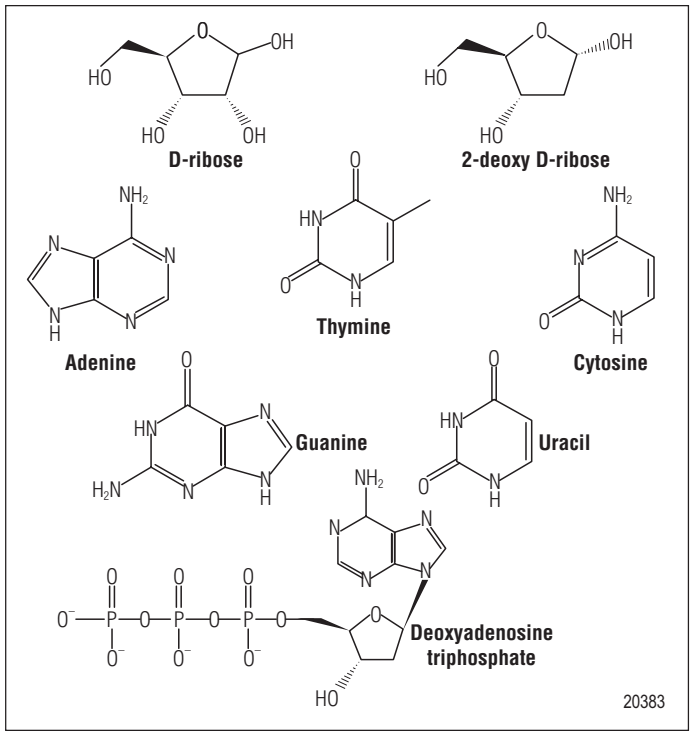

***Figure 1**. Chemical structures of the five bases and two pentoses found in common nucleotides; chemical structure of the nucleotide 2'-deoxyadenosine 5'-triphosphate (dATP) showing the ionic charge at pH ≥7.*

The purpose of this application note is to provide a simple and direct assay of nucleotides and deoxynucleotides in amplification cocktails used for PCR, rolling circle amplifications, DNA and RNA polymerase reactions, and reverse-transcriptase reactions. Nucleotides are separated by anion exchange on the DNAPac® PA100 column using gradient elution and detected by absorbance at 260 nm. Resolution of these components provides a means to assay their quality and help identify causes of amplification failure.

EQUIPMENT

Dionex BioLC® system consisting of:

- GP50 Gradient Pump
- AD25 Absorbance Detector
- AS50 Autosampler, PEEK, with sample cooling and thermal compartment
- Chromeleon® 6 Chromatography Workstation

Miscellaneous:

- Syringe filters (Gelman IC Acrodisk® 0.2-µm, PN 4483)
- Disposable syringes, 5 mL (Dionex P/N 16640)
- Vortex mixer
- Autosampler vials, 0.3 mL polypropylene with caps and septa (Dionex P/N 055428)
- Thermal cycler (Progene model FPR0G05Y, Techne)

CONDITIONS

Columns:	DNAPac PA100, 4 × 250 mm (P/N 43010)
Temperature:	20 °C
Injection:	10 µL
Detection:	Absorbance at 260 nm
Backpressure:	1100 psi
Noise and Drift:	100–200 µAU
Run Time:	15 min
Flow Rate:	1.5 mL/min
Eluent A:	Deionized water
Eluent B:	34 mM trisodium phosphate/100 mM sodium hydroxide
Eluent C:	270 mM sodium perchlorate
Eluent D:	100 mM sodium perchlorate

Time (min)	*%A*	*%B*	*%C*	*%D*	*Comment*
0.00	79	17	0	4	Inject
8.50	79	17	0	24	
8.51	3	17	80	0	Begin column cleanup
9.00	3	17	80	0	End column cleanup
9.10	79	17	0	4	Return to initial conditions
15.0	79	17	0	4	End analysis

```
Pressure.LowerLimit =     0
Pressure.UpperLimit =     3000
%A.Equate =               "H2O"
%B.Equate =               "34 mM Na3PO4, 100 mM
                           NaOH"
%C.Equate =               "270 mM NaClO4"
%D.Equate =               "100 mM NaClO4"
Flush Volume =            200
Wait FlushState
NeedleHeight =            1
CutSegmentVolume =        0
SyringeSpeed =            3
ColumnTemperature =       20
TrayTemperature =         8
CycleTime =               0
Data_Collection_Rate =    5.00
Rise_Time =               2.0
Wavelength =              260
UV_Lamp =                 On
Visible_Lamp =            Off
WaitForTemperature =      False
Wait                      SampleReady
0.000  UV.Autozero
       Flow =             1.50
       %B =               17
       %C =               0.0
       %D =               4.0
       Curve =            5
       Load
       Wait               CycleTimeState
       Inject
       Wait               InjectState
       UV_VIS_1.AcqOn

8.500  Flow =             1.50
       %B =               17
       %C =               0.0
       %D =               24.0
       Curve =            5

8.510  Flow =             1.50
       %B =               17
       %C =               80.0
       %D =               0.0
       Curve =            5
```

9.000	Flow =	1.50
	%B =	17
	%C =	80.0
	%D =	0.0
	Curve =	5
9.100	Flow =	1.50
	%B =	17
	%C =	0.0
	%D =	4.0
	Curve =	5
15.000		UV_VIS_1.AcqOff
	Flow =	1.50
	%B =	17
	%C =	0.0
	%D =	4.0
	Curve =	5
	End	

REAGENTS AND STANDARDS

Sodium hydroxide solution 50% w/w (Fisher SS254)

Sodium perchlorate monohydrate, HPLC-grade (Fisher S490)

Sodium phosphate tribasic dodecahydrate ($Na_3PO_4 \cdot 12\ H_2O$), (J. T. Baker 3836-01)

Trizma pH 8.0 (Sigma T-4753)

Disodium ethylenediaminetetracetate dihydrate (Sigma E-4884)

dNTP mix (Bioline PCR BIO-39028)

dATP sodium salt (Sigma D 4788)

dCTP sodium salt (Sigma D 4913)

dGTP sodium salt (Sigma D 5038)

dTTP sodium salt (Sigma T 9656)

ATP disodium salt (Sigma A 6559)

CTP sodium salt (Sigma C 8552)

GTP sodium salt (Sigma G 3776)

UTP trisodium salt (Sigma U 1006)

PREPARATION OF SOLUTIONS AND REAGENTS

Prepare all solutions from ACS reagent-grade chemicals, or better, and Type IA[2] deionized water with a specific resistance of 18.0 MΩ-cm or greater. Filter through a 0.2-µm filter immediately before use to minimize biological contamination.

ELUENT PREPARATION

Eluent 1 (Deionized Water)

Use deionized water as described above. Use an on-line degasser or degas by sparging with helium or sonicating under vacuum for 10–20 min. Keep this and all other eluents blanketed under helium at 34–55 kPa (5–8 psi) after preparation. If maintained under helium, the following eluents can be used for approximately one week.

Eluent 2 (100 mM Sodium Hydroxide/34 mM Trisodium Phosphate)

Always prepare sodium hydroxide eluents with low-carbonate 50% (w/w) sodium hydroxide solution. Do not use sodium hydroxide pellets. These pellets are covered with a thin layer of sodium carbonate that will contaminate the eluent.

To prepare 2 L of 100 mM NaOH/34 mM Na_3PO_4, place 25.84 g trisodium phosphate dodecahydrate into a 2-L volumetric flask containing 1.9 L of deionized water and degas for 20 min by sonicating under vacuum. Use a plastic pipette to deliver 10.3 mL (or 15.9 g) of 50% (w/w) sodium hydroxide. Bring to volume with degassed deionized water, cap, and gently invert the flask eight times to mix. Transfer to a plastic eluent reservoir and blanket with helium as described above.

Eluent 3 (270 mM Sodium Perchlorate)

Place 75.85 g HPLC-grade sodium perchlorate monohydrate ($NaClO_4 \cdot H_2O$) into a 2-L volumetric flask containing about 1 L of degassed deionized water. Dissolve, bring to volume with degassed deionized water, and mix. Transfer to a plastic eluent reservoir and blanket with helium as described above.

Eluent 4 (100 mM Sodium Perchlorate)

Place 28.09 g HPLC-grade sodium perchlorate monohydrate ($NaClO_4 \cdot H_2O$) into a 2-L volumetric flask containing about 1 L of degassed deionized water. Dissolve, bring to volume with degassed deionized water, and mix. Transfer to a plastic eluent reservoir and blanket with helium as described above.

BUFFER PREPARATION

10 mM Tris/1 mM EDTA Buffer (TE Buffer)

Place 1.418 g Trizma pH 8 and 0.372 g disodium EDTA dihydrate in a 1-L volumetric flask containing about 800 mL deionized water. Dissolve, bring to volume with deionized water, and mix.

STANDARD PREPARATION

Prepare stock standards containing 200 µM of each of the dNTPs by diluting the dNTPs with TE buffer. For example, dilute 80 µL of the 25 mM dNTP mix (dA, dC, dG, and dT) to a final volume of 10 mL with TE buffer. Likewise, prepare stock standards containing 200 µM of each of the NTPs by diluting the NTP mix obtained from Bioline (25 mM each A, C, G, and U). Prepare a 100 µM mixed calibration standard by mixing equal volumes of the 200 µM dNTP stock and the 200 µM NTP stock. Prepare daily calibration standards by diluting the mixed standard with TE buffer as needed on the day of use.

SAMPLE PREPARATION

Filter all samples through a 0.22-µm ion chromatography-certified syringe filter before injecting.

A dNTP mix (dATP, dCTP, dGTP, and dTTP, 25 mM each) was a gift from Bioline USA, Inc. It was diluted as described with TE buffer.

Thermally degraded XTPs were prepared by heating 0.5-mL aliquots of the individual nucleoside or deoxynucleoside triphosphates (XTPs) (10 mM concentration) in a 100 °C heating block (Pierce) for varying lengths of time. Chromatography of the resulting solutions revealed that the bulk of the deoxynucleoside triphosphates were converted to the di- and monophosphate forms within 1 h. The dGTP solution required 2 h for conversion of most of the triphosphate to mono- and diphosphate forms.

In the PCR reaction, primers specific for the human DYS271 allele (Accession #S76940) yielding a 209 base pair (bp) product were obtained from SigmaGenosys, and used at 20 µM in a PCR mixture containing three intact dNTPs and one thermally degraded dNTP. The process was performed in a Progene model FPR0G05Y thermal cycler (Techne), and used 40 cycles of:

- Denaturing: 95 °C, 35 s
- Annealing: 60 °C, 45 s
- Extension: 72 °C, 80 s

The resulting products were assayed for full-length (dsDNA) products on the DNAPac column with a gradient of 106–198 mM sodium perchlorate, at pH 10.9 flowing at 1.5 mL/min. The residual dNTPs were evaluated by the method described in this application note.

SYSTEM PREPARATION AND SETUP

Assemble and configure the chromatography system modules. Verify that the pump flow rate is within specifications and recalibrate if necessary. Use a GM-5 gradient mixer between the pump and injection valve. Verify that the AD25 Absorbance Detector wavelength accuracy is within specifications and recalibrate if necessary. (Both the pump flow rate and detector wavelength accuracy can be verified by performing the Instrument OQ/PQ per Document No. 031726). To aid future troubleshooting, periodically record the visible lamp output (i.e., the reference cell current in nA) and elapsed time. Consult the pump or detector manuals for procedural details. For best results, use a 250-µL sample syringe with the AS50; confirm that the correct sample syringe volume information is entered in the AS50 Plumbing Configuration screen.

Install a 10-µL PEEK sample loop and confirm that the correct loop volume information is entered in the AS50 Plumbing Configuration screen. The most accurate and precise injections are made with a calibrated sample loop rinsed thoroughly with the sample, as in the AS50 full-loop injection mode that we used for this work. To conserve sample, use one of the partial-loop injection modes. Refer to the *AutoSelect™ AS50 Autosampler Operator's Manual* (Document No. 31169) for details. Fill the AS50 sample reservoir daily with fresh deionized water and expel any air bubbles from the sample syringe and tubing.

Install a 4 × 250 mm DNAPac PA100 column. Rinse the column with the ending eluent composition (3:17:80:0) for 30 min. Equilibrate the column with the initial eluent composition (79:17:0:4) for 10 min before analyzing a system blank of deionized water. In an equilibrated system, the background shift during the gradient run should be less than 200 µAU. The peak-to-peak noise and drift should not exceed 20 µAU/min. No significant peaks should elute within the retention time windows of the analyte anions.

Inject a mid-level standard (e.g., 10 μmol/L). The column is equilibrated when two consecutive injections of the standard produce the same retention time for the analyte anions. Confirm that the resulting chromatogram resembles the chromatogram of the standard shown in Figure 2.

Calibrate the system by injecting one blank and at least five standards for every two decades of the calibration range. Plot the peak area for each XTP versus the concentration injected and use a linear regression to fit the data.

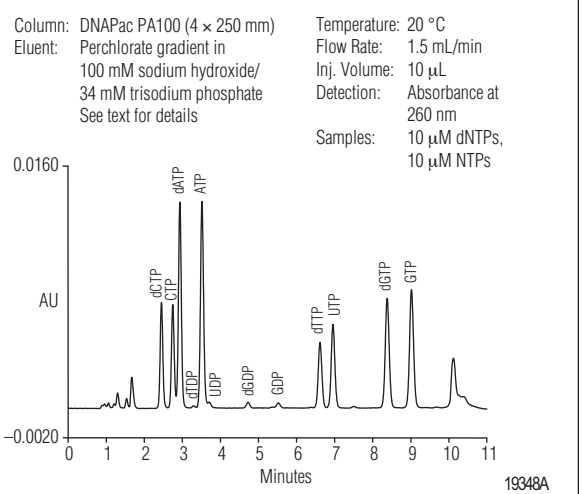

***Figure 2**. Anion-exchange separation with absorbance detection at 260 nm of 100 pmol XTP standards on DNAPac PA100. See text for conditions.*

RESULTS AND DISCUSSION

Linearity

The calibration curve is linear from 0.1 to 150 μmol/L ($r^2 \geq 0.995$) for each of the XTPs. Table 1 summarizes the calibration data obtained by injecting standards in the ranges shown. Figure 2 shows that baseline resolution of an XTP standard is obtained even at 100 pmol each. At concentrations higher than 100 pmol, CTP/dATP can coelute and cause a deviation from linearity. If coelution occurs, it can be resolved by diluting the sample. The chromatogram in Figure 2 demonstrates a very minor contamination by mono- and diphosphonucleotides, typical for XTP mixtures that have not undergone significant degradation.

Sensitivity

This method is sensitive enough to detect each of the analytes at concentrations below 0.075 μmol/L (i.e., 75 nmol/L). To determine minimum detection limits (MDLs), we analyzed seven injections of TE buffer fortified with the analytes at concentrations yielding peak heights approximately five times higher than the background noise. Column 4 of Table 1 lists the concentrations of the nucleotides analyzed. The minimum detection limits in column 5 were calculated from the precision of these measurements by using the formula footnoted below Table 1. Greater sensitivity is possible if more than 10 μL of sample is injected.

Table 1. Linearity and Detection Limits for Nucleotides and Deoxynucleotides

Analyte	Range (μmol/L)	r^2*	MDL Standard (μmol/L)	MDL** μmol/L
dCTP	0.1–150	0.9998	0.100	0.039
CTP	0.1–150	0.9998	0.100	0.039
dATP	0.1–150	0.9999	0.100	0.021
ATP	0.1–150	0.9998	0.100	0.012
dTTP	0.1–150	0.9999	0.100	0.040
UTP	0.1–150	0.9998	0.100	0.025
GTP	0.1–150	0.9998	0.100	0.051

*r^2 = degrees-of-freedom-adjusted coefficient of determination
**MDL = $t_{99} \times S$ (for n = 7) where t_{99} = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom (t = 3.14 for n = 7), and S = standard deviation of the replicate analysis.

Control of Selectivity by [NaOH] and Temperature

A combination of ionic character (net charge) and the intrinsic base selectivity designed into the DNAPac column govern retention of the mono-, di-, and triphosphate components. Because net charge on the analytes depends on both eluent pH and temperature, users can modify the eluent gradient program to optimize the separation for a particular analysis. For example, when the eluent pH is varied (by varying the percentage of Eluent B from 15 to 23%), the retention times of all the analytes vary as shown in Figure 3. Likewise, Figure 4 shows the effect of temperature on retention time. Figures 3 and 4 provide guidance for fine-tuning the separation for a particular application.

Figure 5 shows a completed PCR reaction mixture containing a mixture of XTPs, mono- and diphosphate degradation products, and a 483 bp PCR reaction product eluting in the column wash at the end of the separation. Column washing with 200–220 mM sodium perchlorate prevents column fouling by the reaction products in such mixtures. The DNAPac PA100 can withstand repeated cycles of column washing with no adverse effects.

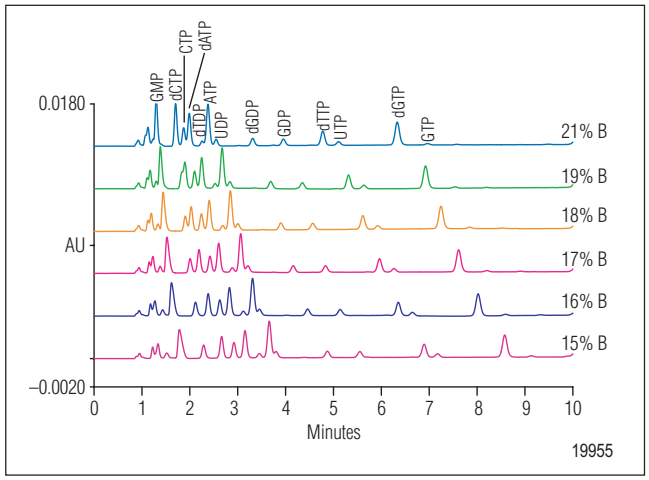

***Figure 3**. Effect of eluent pH (i.e., percent eluent B) on retention of XTPs, XDPs, and XMPs on the DNAPac PA100 analytical column. Conditions: as in Figure 2, except the percentage of eluent B was varied as shown.*

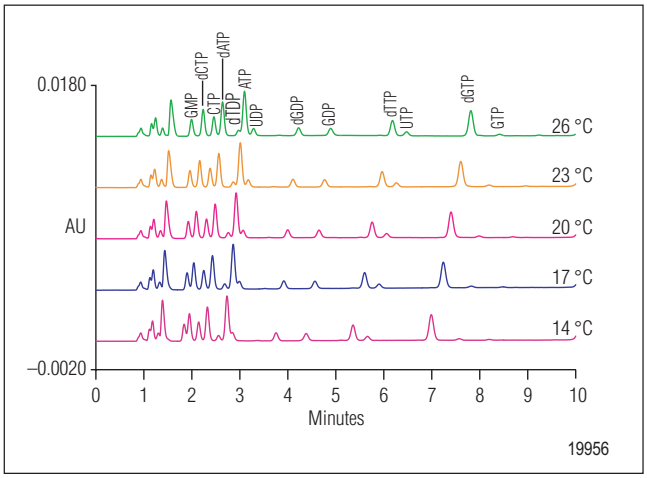

***Figure 4**. Effect of temperature on retention pf XTPs, XDPs, and XMPs on the DNAPac PA100 analytical column. Conditions: as in Figure 2, except the column temperature was varied as shown.*

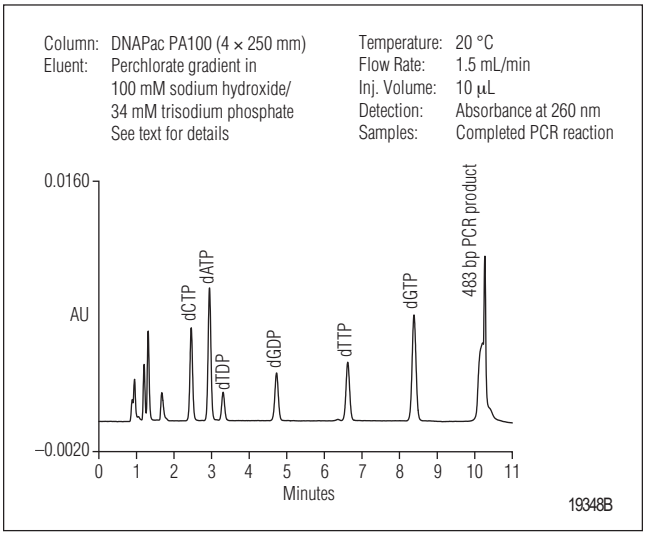

***Figure 5**. Determination of XTPs in a successful PCR reaction mixture. Conditions: as in Figure 2.*

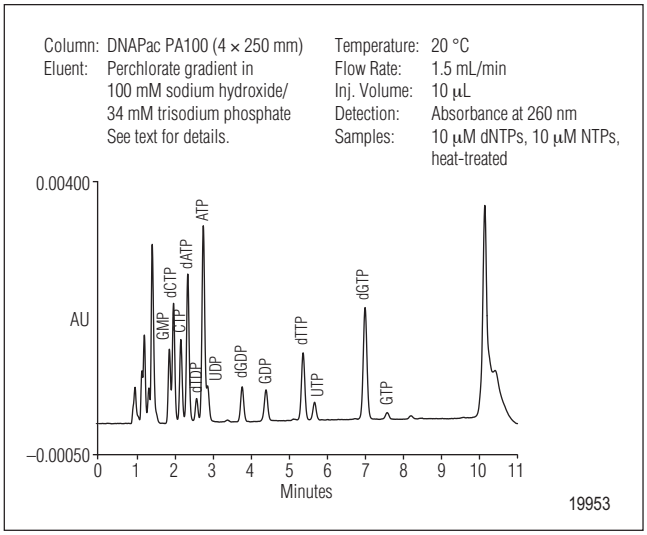

***Figure 6**. Determination of XTPs, XDPs, and XMPs in a thermally degraded XTP mixture. Conditions: as in Figure 2.*

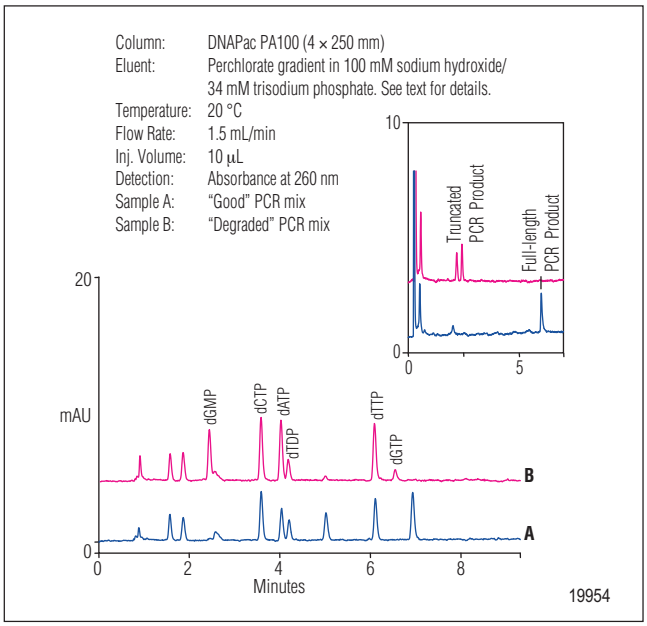

***Figure 7**. Determination of XTPs in a failed PCR reaction mixture. Chromatogram 7A is of a PCR cocktail containing four intact dNTPs; chromatogram 7B is of a PCR cocktail in which the dGTP has been thermally degraded. Inset: The bottom trace shows the full-length PCR product resulting from PCR with the cocktail in 7A; upper trace shows the truncated product tesulting from PCR using the cocktail in 7B (both assays performed as described in the text).*

Identification of Contaminants

Figure 6 shows a mixture of XTPs that were heated to 100 °C for 1–2 h to hydrolyze the XTPs to their mono- and diphosphate degradation products. The mono-, di, and triphosphates all appear in the chromatogram. Although resolution among all of the analyte peaks is still good, higher concentrations of dTDP and UDP than shown in this figure could coelute with ATP, rendering integration of the analyte peaks less precise and accurate. Dilute samples that contain high levels of XDPs and XMPs, if necessary, to restore baseline resolution.

Figure 7A demonstrates the chromatogram of a typical PCR cocktail after PCR; it still contains excess dATP, dCTP, dTTP, and dGTP. Figure 7B shows a similar PCR cocktail in which the dGTP was thermally degraded before adding it to the cocktail; dGMP is seen in the chromatogram but dGTP is absent. The insert to Figure 7 shows the separations of the PCR products generated with these two different cocktails. The upper trace in the inset shows the truncated product resulting from failed PCR with the thermally degraded PCR cocktail. The bottom trace shows the full-length PCR product (209 bp) resulting from PCR with the intact PCR cocktail. These assays clearly show that the lack of a full-length PCR product correlates to the absence of dGTP in the thermally degraded PCR cocktail.

Ruggedness

Even though eluent pH and column temperature influence the selectivity (as shown in Figures 3 and 4), different analysts working over several days can run the method with consistent results. The retention time precision obtained when three analysts separately prepared eluents and ran the method over 5 days was better than 5% RSD for all analytes.

PRECAUTIONS

Store standards and archived samples at –20 °C. Minimize freeze-thaw cycles; do not store in a frost-free freezer.

Minimize contamination by wearing latex-free gloves when handling samples and by keeping samples covered whenever possible. Contaminating DNAase or RNAase enzymes can degrade contaminating DNA or RNA and introduce positive interferences. Use TE buffer for dilutions; it contains EDTA to sequester the metal cations required by the enzymes.

Filter samples and solutions through 0.22-µm filters to remove particles and microbes. Cool thawed samples to 8 °C and analyze quickly to minimize degradation of the XTPs.

Strongly retained compounds (e.g., RNA, DNA, or other reaction products from injected samples) can accumulate on the column and degrade its performance. Signs of a contaminated column include loss of capacity, loss of resolution, shortened retention times, higher noise and background, spurious peaks, and peak tailing. The method cleanup step (216 mM sodium perchlorate for 0.5 min) should elute most contaminants. Use more time for column cleanup if necessary. For more information on column troubleshooting and cleanup, see the *Installation Instructions and Troubleshooting Guide for the DNAPac PA100 Analytical Column* (Document No. 034411).

CONCLUSION

Anion-exchange chromatography on the DNAPac PA100 readily differentiates good quality PCR reagents from those containing nucleoside mono- and diphosphate degradation products that can ruin PCR cocktails. PCR cocktail composition analysis identified the cause of failure in an example PCR reaction that occurred due to unacceptably high levels of degradation products

UV absorbance detection provides ample sensitivity to directly evaluate nucleotide triphosphates and their degradation products commonly found in poor-quality PCR cocktails or failed PCR reaction mixtures. Sensitivity at the nmol/L level and a linear range of 0.1–150 µmol/L ensure that very little sample is sacrificed.

The DNAPac PA100 withstands the column cleanup step necessary to prevent reaction products from fouling the column. The method performs robustly with multiple operators over several days. The retention time stability of nucleotide phosphate standards injected over a period of five days is less than 5% RSD for over 150 injections.

REFERENCES

1. The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.
2. ASTM D 1193. Standard Specification for Reagent Water. *Annu. Book ASTM Stand.* **2002**, Vol. 11.01.

SUPPLIERS

Bioline, PMB 311, 28 South Main Street, Randolph, MA 02368-4800 USA, Tel: 781-830-0360, www.bioline.com.

Fisher Scientific, 2000 Park Lane, Pittsburgh, PA 15275-1126 USA, Tel: 800-766-7000, www.fishersci.com.

Sigma-Aldrich Chemical Company, P.O. Box 14508, St. Louis, MO 63178 USA, Tel: 1-800-325-3010, www.sigma-aldrich.com.

Sigma-Genosys, 1442 Lake Front Circle, The Woodlands, TX 77380 USA, Tel: 800-234-5362, www.sigma-genosys.com.

Techne Inc., 3 Terri Lane, Suite 10, Burlington, N.J. 08016 USA, Tel: 800-225-9243, www.techne.com.

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