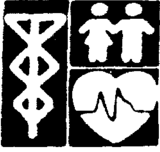
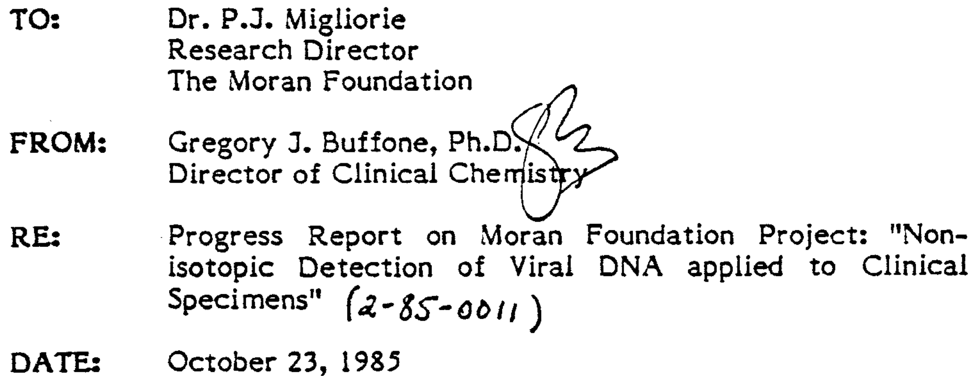
Memorandum Texas Children's Hospital - Department of Pathology





TO: Dr. P.J. Migliorie
Research Director
The Moran Foundation

FROM: Gregory J. Buffone, Ph.D.
Director of Clinical Chemistry

RE: Progress Report on Moran Foundation Project: "Non-isotopic Detection of Viral DNA applied to Clinical Specimens" (2-85-0011)

DATE: October 23, 1985

We have completed section B of the original proposal by subcloning an Xba I fragment of Towne CMV into a Riboprobe™ vector. Using this system we have evaluated the suitability of probes prepared by this method for application to in vitro viral detection. Attached is a letter to the editor of Clinical Chemistry which has been accepted for publication.

We are continuing to work on methods for urine preparation which will permit the use of non-isotopic probes. Attached is a letter to the editor describing a DNA extraction method which eliminates the use of phenol, a particularly undesireable reagent for the routine laboratory.

GJB/dee

Suitability of SP6 RNA Polymerase Transcipts for in Vitro Viral Diagnosis

To the Editor:

Application of DNA or RNA probes to microbiology and virology requires probes free of vector sequences, particularly vectors of bacterial origin, such as pBR322. Incomplete purification of inserts from vector sequences can produce a falsely positive result when nucleic acid probes are applied to tissue or body fluids that may be contaminated with bacteria.

A plasmid vector such as pBR322 provides a convenient system for production of large quantities of probe, but repeated preparative electrophoresis on agarose gel is required for it to be adequately pure. We find that large inserts ($\geq$ 10 kb) can be prepared so pure that up to 10 ng of pBR322 will not be detected in a dot blot assay, but smaller inserts (~4–6 kb) that are similar in size to pBR322 cannot be purified nearly as well. As little as 100 pg of pBR322 can be detected with inserts 4.9 kb in size, even after three preparative-electrophoretic steps.

We anticipated that if we used RNA transcripts prepared with use of DNA-directed SP6 RNA polymerase (EC 2.7.7.6) we could obtain reasonable quantities of probe but obviate the problem of vector sequence contamination. We cloned an Xba I fragment from the Towne strain of human cytomegalovirus (CMV) into pSP64 (Promega Biotec, Madison, WI), a 3.0 kb vector containing the SP6 RNA polymerase promoter. In theory the circular plasmid is then cut within the polylinker region which is downstream from the SP6 promoter relative to the direction of transcription. Subsequently, SP6 RNA polymerase is added, along with sufficient NTP substrates, and an RNA transcript of the DNA insert is produced. DNase (EC 3.1.21.1) is then added and the RNA transcript is purified by standard extraction with phenol and precipitation with ethanol.

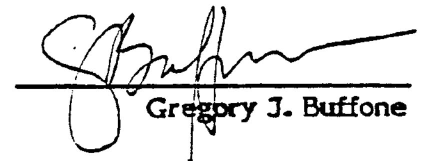
^{32}P-labeled RNA transcripts were prepared as described and hybridized with CMV DNA and pBR322 in a dot blot assay. The RNA probe was found to hybridize to CMV and pBR322 DNA.

We attributed this contamination of our probe with vector sequences to be due either to incomplete linearization of our plasmid, such that transcription continued into the vector region, or to additional polymerase initiation sites. We digested our clone with two different restriction endonucleases, Bam HI (EC 3.1.23.6) and Sma I (EC 3.1.23.4), to ensure complete linearization. Neither of these enzymes leaves a 3' overhang, a condition that apparently favors transcription of vector sequences. Each enzyme cuts downstream from our insert within the polylinker region. We again observed that the RNA probe hybridized to both CMV and vector sequences. The best results, in terms of the amount of cross-hybridization to vector DNA, occurred when the plasmid was digested with Eco RI (EC 3.1.23.13). This restriction endonuclease cuts both within the CMV DNA insert and within the polylinker region. The transcription reaction time was also reduced from 90 to 60 minutes. Although hybridization to pBR322 was not totally eliminated, the transcript produced after Eco RI digestion gave a negative reaction with up to 5-7 ng of pBR322 DNA. This is approximately equivalent to the specificity obtained with a Bam HI insert (14.3 kb) purified by three successive electrophoreses on agarose.

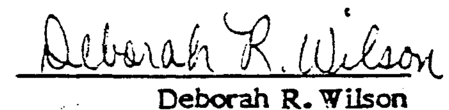
We believe we have eliminated the possibility of insufficient linearization of the cloned template as the major cause of contaminating vector sequences, which we believe is nonspecific initiation of SP6 RNA polymerase. The extent to which adventitious transcription occurs varies from reaction to reaction, perhaps reflecting slight differences in salt or nucleotide concentration, incubation temperature, or reaction time. Promega Biotec (Madison, WI 53711) has recently circulated a newsletter, Promega Notes (March 1985), in which they report a 0 to 1% "background" of adventitious transcription. This may be acceptable without further purification for

most applications, but it can be prohibitive in situations where there is significant bacterial contamination of experimental material.

Our experience indicates that only under carefully controlled conditions can RNA transcripts prepared in this manner produce probes that have as low a cross reactivity with bacterial plasmid DNA as do purified inserts. We did not find the system to be efficient or reliable as a routine alternative to standard purification of cloned inserts.


Gregory J. Buffone

Gretchen J. Darlington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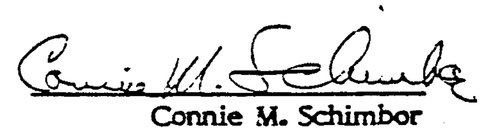
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FOR THE AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY
J. Stanton King, Executive Editor

Accepted and Approved by

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Isolation of DNA from Biological Specimens without Extraction with Phenol

To the Editor:

In isolating DNA from biological specimens (blood, leukocytes, tissue, urine and cultured cells), redistilled phenol is usually used to extract proteins and other material that may interfere with subsequent analysis for DNA. Distillation of phenol is not a practical procedure for most clinical laboratories, and although commercial supplies of relatively pure phenol are available, its shelf life is limited because of the formation of phenolic free radicals, which can cross-link DNA and cause anomalous results. More importantly, the use of such a noxious reagent, which has several associated safety hazards, is undesirable for a clinical laboratory setting.

DNA studies for genetic and infectious disease applications will become a valuable tool for the clinical laboratory in the foreseeable future. Two requirements for such studies are: (*a*) simple and rapid methods for isolation of genomic DNA, which can then be manipulated by using restriction endonucleases, and (*b*) nonisotopic labels for DNA and RNA probes.

We report here a modification of a standard DNA isolation procedure (*1*) that does not involve phenol extraction. We have applied this modified procedure to nucleated cells from peripheral blood and cultured cell lines; however, the procedure should be applicable to tissue pieces as well.

For peripheral blood leukocytes: Collect 20 mL of heparinized blood in a 50-mL conical tube. Separate plasma and cells by centrifugation (1800 × *g*, 20 min) at ambient temperature. Aspirate the plasma to approximately 5 mm above the buffy coat. Add the lysis buffer to the cell pellet to give a final volume of 50 mL (lysis buffer: 0.32 mol of sucrose, 10 mmol of Tris HCl, pH 7.5, 5 mmol of $MgCl_2$, and 10 mL of Triton X-100 per liter). Mix the suspension by inversion and allow it to stand in an ice bath for 30 min.

Centrifuge the suspension for 15 min at 1800 × *g* and remove 40 mL of the supernate (the nuclear pellet will not be visible, owing to the high concentration of hemoglobin in the supernate). Resuspend the pellet in lysis buffer to a final volume of 50 mL and recover the nuclear pellet by centrifugation.

For cultured cells: Wash the cells in Hank's balanced salt solution (GIBCO Laboratories, Grand Island, NY 14072), then prepare the cell pellet by centrifugation (1800 × *g*, 15 min). Add lysis buffer to a final volume of 50 mL, mix by inversion, and leave in an ice bath for 30 min. Recover the nuclear pellet by centrifugation at 1800 × *g* for 15 min.

Regardless of the sample type, remove and discard the final supernate. Resuspend the pellet from peripheral blood or cultured cells in 5 mL of a solution containing 200 mmol of Tris HCl, pH 8.5, 100 mmol of EDTA, and 35 mmol of sodium dodecyl sulfate per liter. Avoid vigorous mixing, which can break up the DNA. Add 100 μL of a 10 mg/mL solution of Proteinase K (Boehringer Mannheim, Indianapolis, IN 46250) dissolved in de-ionized water, then incubate the suspension for 4 h at 60 °C. Add an additional 100-μL aliquot of Proteinase K and continue the incubation overnight at 37 °C. The following morning, add 1 mL of 6 mol/L $NaClO_4$ and incubate for 2 h at 37 °C. Cool the suspension to ambient temperature and add 1.2 mL of 5 mol/L potassium acetate. Mix gently to obtain a homogeneous suspension and then let stand for 30 min in an ice bath. Centrifuge the suspension (27 000 × *g*, 15 min, 2 °C). Decant the supernatant fluid into a separate tube and discard the pellet. Precipitate the DNA by adding two volumes of ethanol. Let the solution stand for 15 to 30 min and, in the case of mammalian genomic DNA, harvest the DNA by "winding" the DNA onto a glass rod. Remove the ethanol by evaporation and resuspend the residual material in an appropriate buffer for subsequent studies.

RNA will be isolated along with DNA in this procedure. If this is a concern, RNase can be added now or at an earlier step in the procedure.

In our experience this procedure is somewhat less efficient than extraction with phenol in terms of recovery of DNA, although the quality of DNA is good for restriction analysis. In instances where the quantity of DNA is small, extraction with phenol may be preferable.

Although slower, this technique does allow for DNA to be isolated without the use of phenol. We have used this procedure to prepare mammalian genomic DNA for restriction enzyme analysis and have found it to yield high-M_r DNA that is susceptible to restriction enzyme activity.

This method provides a significant advantage to any laboratory interested in procedures for isolating DNA without the use of noxious chemicals.

Reference

1. Maniatis T, Fritsch EF, Sambrook J. *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, pp 458–460.

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