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28006 Madrid



SERIE UNIVERSITARIA

Fundación Juan March

# Lecture Course on The Polymerase Chain Reaction

Organized by

M. Perucho
E. Martínez-Salas

D. Gelfand
K. Hayashi
H. H. Kazazian
E. Martínez-Salas
M. McClelland
K. B. Mullis
C. Oste
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J. Sninsky



263 Lecture Course on The Polymerase Chain Reaction

Fundación Juan March

Serie Universitaria

263



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# I N D E X

## FUNDACION JUAN MARCH

## COURSE ON THE POLYMERASE CHAIN REACTION.

CUENCA, MAY 13-15, 1991.
*PROGRAM*

MONDAY 13. MORNING.

9.00 - **M. PERUCHO.** INTRODUCTION.
9.15 - **K. MULLIS.** OVERVIEW. MECHANISMS. APPLICATIONS.

11.00 - **D. GELFAND.** THE ENZYMOLOGY BENEATH.
12.00 - **C. OSTE.** THE ENGINERING BENEATH.

AFTERNOON.

*GENETIC ANALYSIS.*
3.00 - **H. KAZAZIAN.** DIAGNOSIS OF HEREDITARY DISEASES.
4.00 - **K. HAYASHI.** DETECTION OF POINT MUTATIONS BY SSCP.
5.00 - **M. MCCLELLAND.** FINGERPRINTING BY APPCR.

TUESDAY 14. MORNING.

*INFECTIOUS DISEASES.*
9.00 - **E. MARTINEZ-SALAS.** GENETICS OF RNA VIRUSES.
10.00 - **J. SNINSKY.** DIAGNOSIS OF HUMAN VIRUSES. HIV GENETIC VARIABILITY.

*CANCER DIAGNOSIS.*
11.30 - **M. PERUCHO.** RAS MUTATIONS. TUMOR SUPPRESSOR GENES.
12.30 - SHORT COMMUNICATIONS. I.

AFTERNOON.

3.00 - *ROUND TABLE. GENERAL DISCUSSION.*
SHORT COMMUNICATIONS. II.

WEDNESDAY 15. MORNING.

RECAPITULATION. THE TECHNIQUE IN BIOLOGICAL RESEARCH.

9.00 - **D. GELFAND.** TAQ POLYMERASE.
9.40 - **H. KAZAZIAN.** MONOGENIC DISEASES.
10.20 - **K. HAYASHI.** SSCP AND GENETIC ANALYSIS.

11.30 - **M. MCCLELAND.** GENE MAPPING.
12.10 - **M. PERUCHO.** GENETIC DAMAGE IN TUMORIGENESIS
12.50 - **K. MULLIS.** PERSPECTIVES & CLOSING REMARKS.

# INTRODUCTION

MANUEL PERUCHO

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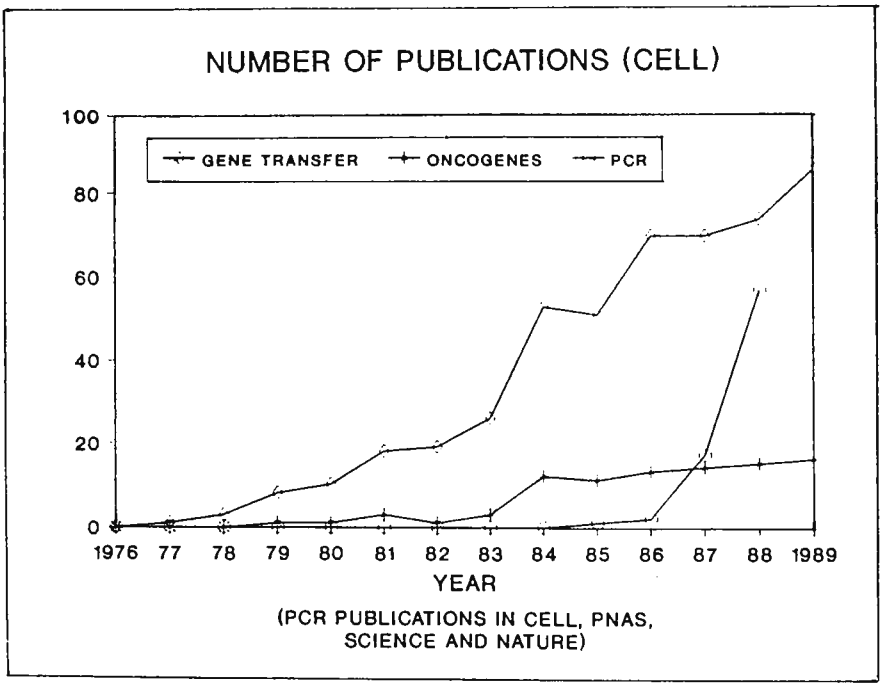
This book contains a heterogeneous collection of papers with only one common denominator: all deal one way or another with the *in vitro* amplification of gene sequences by the Polymerase Chain Reaction (PCR). There are methodological papers providing experimental details useful at the bench, and general discussions on the principles and properties of various PCR-related techniques. These papers were written by most of the lecturers of a Course on the Polymerase Chain Reaction held in Cuenca on May 1991, sponsored by the Fundacion Juan March. It is my hope that the contents of this book will be useful for the researchers that attended the Course in particular and for the interested reader in general, because most of these techniques are not described in any of the currently available books on PCR protocols.

The idea of organizing a Lecture Course on the PCR originated in another Course from the Fundacion Juan March, "Gene Transfer and Oncogenes", that took place on July 1990 in the Parador Nacional of Sigüenza. The topic of that course was the development of the techniques of gene transfer in animal cells and their contribution to the rapid progress achieved during the last decade in the study of the molecular genetics of higher eukaryotes, as well as their application to the detection, isolation and characterization of oncogenes. I had the curiosity of estimating the number of papers that utilized DNA-mediated gene transfer techniques, published since the initial reports describing the possibility of achieving genetic transformation in cultured animal cells, for both biochemical (Wigler *et al*, Cell *14*, 725, 1978) and oncogenic (Shih *et al*, PNAS *76*, 5714, 1979) phenotypes. Because of lack of time, I concentrated only on papers published in Cell.

As I expected, this search revealed a continued rise in the number of gene transfer and oncogene publications during the eighties, reaching near 50% of all Cell papers studying mammalian systems at the end of the decade. However, my surprise was great when, also for curiosity, I counted the papers published on the PCR in the three years following the initial publication (Saiki *et al*, Science, *230*, 1350, 1985). In contrast to the gene transfer papers, which showed a linear increase in number, when the computer displayed the curve of the PCR publications, there it was, the beginning of a perfect exponential growth curve! (See Figure).




Then, I thought that a Lecture Course on the PCR would be timely and interesting if I could get some of the PCR experts to come to Spain. The justification was simple: it is easier to learn from what you hear and see than from what you read. I knew that from personal experience, when I attended at Cold Spring Harbor a Banbury Meeting on the PCR in December of 1988. The basic and most advanced aspects of the technique were discussed by its main contributors and I learned a lot in a short time and with little effort.

As I was succesful previously in convincing some of the gene transfer and oncogene pioneers to come to Sigüenza (Mike Wigler, Angel Pellicer, Jim Feramisco and Frank McCormick, in addition to the local organizer of the course, Juan Ortin), I was also lucky to obtain the acceptance to our invitation to come to Cuenca by scientists that have played a major role in making possible the PCR revolution, and by the inventors of some of its most interesting applications.

The organization of the Course then took shape easily and nicely. In the first Session, Kary Mullis gave an introduction on the mechanisms and properties of the technique (who could be better for the task than its own inventor?). This was followed by David Gelfand who reviewed the properties of the Taq polymerase, which allowed the

automatization of the technique and therefore, the exponential growth of the field. The luckily coincidental availability of Christian Oste provided the perfect ending for the first introductory session, with a talk on the important aspects of the PCR instrumentation.

Because of obvious limitations, I had to select only a few of the multiple applications of the PCR. Thus, three topics were contemplated: genetic analysis, infectious diseases and cancer diagnosis. The applications to genetic analysis included the diagnosis of hereditary diseases, covered by Haig Kazazian, and the development of two of the most recent and innovative adaptations of the PCR: the Single Stranded Conformation Polymorphysms (SSCP) for mutation detection, which was dealt with by its inventor, Kenshi Hayashi, and the Arbitrary Priming PCR (AP-PCR) for various types of genetic analyses, also presented by its discoverer, Mike McClelland.

In the area of diagnosis of infectious diseases, John Sninsky gave a general overview on the topic with special emphasis in what may be considered the quintessence of the PCR: the diagnostic detection of HIV sequences, which are present in only one cell in every many thousand uninfected cells. He also described his analysis of the genetic variability of the virus, a topic that was previously reviewed by Encarna Martinez, using the foot and mouth disease virus as a model system.

Finally, on the topic of cancer diagnosis, I did my best to cover the gap left by Frank McCormick's absence, and concentrated in the detection of point mutations in *ras* oncogenes and in the p53 tumor suppressor gene. I described a new mutation detection and sequencing method based on a cycling reaction with Taq polymerase and also discussed the application of the AP-PCR for the analysis of tumor progression.

These three main topics were reiterated in a series of short communications by some of the participants and in a final, recapitulating session with talks by all the lecturers. In addition, a round table, where various theoretical and technical aspects of the PCR were discussed among the participants, completed the Course.

All in all, the Course was a pleasant and rewarding experience. Besides the scientific aspects, which as expected, offered a lot of new and useful information for me and I hope for the rest of the participants, there were other aspects that contributed to its success. The main factor was the choice of place, with the unique blend of natural beauty and quiet and relaxing atmosphere that Cuenca offers to the visitor. As usual, the other factor was the excellent organization of the logistic, gastronomic and artistic details of the event by Andres Gonzalez, from the Fundacion Juan March. There was a visit to the Museum of Contemporary Art in the Casas Colgadas, followed by a dinner in the Meson overlooking the Huecar's cannyon. That combination of spiritual and corporeal pleasures is difficult to surpass. If we add the high level of interaction between speakers and participants that the schedule of the Course favoured, specially at the end of the otherwise intensive working hours, it is reasonable to predict that we all will have pleasant memories of an event where hard work blended with leisure, and where science harmonized with art. I believe I speak for all participants in conveying our gratitude to the Fundacion Juan March.

My thanks also to the speakers, who made room in their busy schedules to come to Cuenca and made possible the scientific success of the Course. I really appreciate their professionalism and enthusiasm.

I also want to thank Encarna Martinez Salas, who took care of the most important details in the organization of the scientific aspects, including the task of selecting the over thirty participants (about 10 more than initially contemplated) from the near 150 applications, and therefore of the unpleasant task of turning down many of them. I wish to give her the credit for selecting the succesful applications and take upon myself the responsibility and blame for those who did not make it (mainly, because I feel safer from the distance of Southern California).

I also wish to thank C. Taylor, who has taken care of retyping the manuscripts and normalizing and editing their formats to increase the esthetics of the publication.

Finally, my gratitude to Andres Gonzalez, who not only enthusiastically supported the idea of the Course, but also encouraged the editorial task of putting together this book.

La Jolla, August 1991.

# RAPID PURIFICATION OF DNA FROM BLOOD

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## INTRODUCTION

We have developed a rapid and simple method for obtaining highly purified DNA from blood. The procedure relies on the fact that carefully lysed whole blood contains DNA of very high molecular weight which can be trapped physically on a membrane filter.

The explanation which we have developed for this phenomenon is that although the pores in the filter--we are currently using a cellulose acetate filter with 0.45 micron pores--are much larger than the diameter of the DNA molecules, the pores are much closer together than the contour length of very high molecular weight DNA (perhaps hundreds of microns). Thus, somewhat like spaghetti in a colander the DNA is physically retained by the filter. Somewhat unlike spaghetti in a colander the DNA becomes very tightly attached to the filter. This is probably due to the fact that many little protrusions of the DNA molecules become trapped in multiple pores of the membrane, and this many-point attachment is difficult to break. This has two consequences for the procedure.

One, the DNA, once attached to the filter can be subjected to exhaustive washing without noticeable loss; washing can be done very conveniently with generous and unmeasured volumes of buffer dispensed from a squeeze bottle.

Two, the DNA is hard to elute. However, we have arrived at several methods whereby this can be accomplished. All of these relay on strand breakage. Everything we have tried which will cleave DNA will release it, which firms our confidence in the fact that the DNA is retained physically rather than absorbed. The most easily controlled and simplest elution is with heat. Magnesium accelerates the process significantly. For instance, the washed filters can be removed to a buffer containing magnesium in a tightly closed tube which is placed for fifteen minutes in a boiling water bath and from 50 to

100% of the DNA originally present in the blood can be recovered in a highly purified form. A ratio of the absorbance at 260 nm to that at 280 nm averages 1.9 indicating very pure DNA. Other agents have been utilized to remove the DNA. These were developed while we were working with a membrane containing nitrocellulose which could not be heated to 100 C without discoloring the solution, and are not now of any usefulness besides helping to illuminate the nature of the bonding. Ethidium bromide and ultraviolet light, even the low levels issuing from fluorescent overhead lighting is very effective. The restriction enzyme Taq I was able to slowly release DNA, but not quickly in economical amounts. Microwaves have been employed, and rather strangely seem to release the DNA independent of heating the surrounding solution. That is, a one minute exposure in a microwave oven to the DEFROST level, whatever level that is, of a filter in a tube with a magnesium containing buffer, raises the temperature to 65 C, but releases most of the DNA. Heating the tube to 65 C in a water bath for up to an hour does not release a comparable amount of DNA. However, it is very difficult to accurately control the level of irradiation delivered, and after experimenting extensively and blowing up quite a few tubes, this approach although intriguing has been abandoned for practical reasons.

The procedure described in detail in this report has been thoroughly tested. It is simple and always works. Only high MW chromosomal DNA is retained on the filter. RNA and DNA of less than about a million base pairs is not retained.

It would be very convenient if a device were available wherein the filtration, washing and heat elution could be performed without removing the filters from the apparatus. This would solve two minor problems. Firstly, it is awkward when dealing with clinical samples, where cross-contamination must be prevented, to remove the filters one by one from the apparatus. We are currently employing a set of tweezers which can be decontaminated between runs. Secondly, and more importantly, removing the filters from

the apparatus exposes the narrow rim of the filter which has been under an O-ring during the filtration to the elution buffer. In order to make accurate optical density measurements, this rim, which sometimes contains reddish material, must be removed. We are doing this with a cork borer, but it is a little troublesome and constitutes the weakest point in the procedure. A great deal of thought is being put into finding or designing a better device.

## MATERIALS AND METHODS

Lysis Buffer:

Dulbecco's 1X PBS:

| | |
|---|---|
| Sodium chloride | 138 mM |
| Disodium hydrogen phosphate | 8.1 mM |
| Potassium dihydrogen phosphate | 1.1 mM |
| Magnesium chloride | 0.5 mM |
| Potassium chloride | 2.7 mM |
| Sodium dodecyl sulfate | 35 mM |
| Polyvinyl alcohol 30-70k MW | 2% W/V |

Wash Buffer I:

As above but without the polyvinyl alcohol

Wash Buffer II:

| | |
|---|---|
| Tris hydrochloride pH 8 | 50 mM |

Elution Buffer:

| | |
|---|---|
| Tris hydrochloride pH 8 | 50 mM |
| Magnesium chloride | 10 mM |

Blood is drawn into tubes prepared with any of the common anticoagulants and kept refrigerated until use. One ml aliquots of whole blood are pipetted into standard size scintillation vials containing 20 ml of the lysis buffer and gently mixed by inversion. The lysis reaction is allowed to stand at room temperature for ten minutes without further agitation.

## FILTRATION

Filtration is performed by suction on a 12-position Millipore filter holder using 2.5 cm, 0.45 micron cellulose acetate filters, MSI, Inc. Cat # E04WPO. The initial filtration usually requires 1-2 minutes, but for reasons not understood an occasional sample will filter very slowly taking up to 10 minutes. No effect of the filtration rate on the outcome of the procedure has been noticed. A high capacity vacuum system is employed to prevent a significant decrease in the vacuum when some but not all of the samples have filtered to dryness.

## WASHING

The filters are rinsed twice with Wash Buffer I. This buffer is dispensed from a plastic squeeze bottle without regard for exact volume although generally about five mls are employed. The pink residue on the filters turns white. SDS is removed from the residue by two rinses with Wash Buffer II dispensed in the same way.

## ELUTION

The filters are removed from the filtration apparatus and placed in 1.7 ml screw cap vials containing 1 ml of Elution Buffer. There is often a ring of reddish material on the margin of the filter where the O-ring in the filter plate pressed against it during the washing steps. This can be conveniently removed by placing the filter between two sheets of parafilm and employing a 22mm core borer. The (trimmed) filters are eluted by placing the tightly closed tubes into a boiling water bath for 15 minutes. The tubes are then vortexed and centrifuged for five minutes to removed fine particulate material, and the eluted DNA is quantitated directly by optical density readings at 260 and 280 nm. In the experiment described here elution times from 5 minutes to 25 minutes were compared with the result that we now employ 15 minutes in boiling water as a standard.

## RESULTS

DNA from twelve 1-ml aliquots of whole blood collected in acid citrate tubes was prepared as above. A white blood cell count indicated 7,800 nucleated cells per uL equivalent to 51.5 uG DNA per ml. The elution was performed in two stages.

| Stage I | Time @100 C | OD260 | OD280 | 260/280 | uG/ml |
|---|---|---|---|---|---|
| 1 | 5 min | .371 | .201 | 1.84 | 18.5 |
| 2 | " " | .321 | .172 | 1.86 | 16.0 |
| 3 | " " | .227 | .125 | 1.81 | 11.3 |
| 4 | " " | .357 | .190 | 1.87 | 17.8 |
| 5 | 10 min | .567 | .295 | 1.92 | 28.3 |
| 6 | " " | .640 | .336 | 1.90 | 30.0 |
| 7 | " " | .538 | .289 | 1.86 | 26.9 |
| 8 | " " | .564 | .299 | 1.88 | 28.2 |
| 9 | 15 min | .935 | .482 | 1.94 | 46.7 |
| 10 | " " | .640 | .336 | 1.90 | 32.0 |
| 11 | " " | .611 | .323 | 1.89 | 30.5 |
| 12 | " " | .744 | .391 | 1.90 | 37.2 |

In the second stage of elution, which was done for the purpose of exploring various elution times, the elution buffer was returned to the filter and 100 C for a further ten minutes, centrifuged and optical densities redetermined.

| Stage II | Time @100 C | OD260 | OD280 | 260/280 | uG/ml |
|---|---|---|---|---|---|
| 1 | 15 min | .904 | .438 | 2.06 | 45.2 |
| 2 | " " | .784 | .412 | 1.90 | 39.2 |
| 3 | " " | .639 | .340 | 1.88 | 31.2 |
| 4 | " " | .677 | .357 | 1.90 | 33.8 |
| 5 | 20 min | .816 | .432 | 1.89 | 40.8 |
| 6 | " " | .827 | .431 | 1.92 | 41.3 |
| 7 | " " | .742 | .397 | 1.87 | 37.1 |
| 8 | " " | .699 | .367 | 1.90 | 34.9 |
| 9 | 25 min | .974 | .505 | 1.93 | 48.7 |
| 10 | " " | .696 | .370 | 1.88 | 34.8 |
| 11 | " " | .656 | .353 | 1.86 | 32.8 |
| 12 | " " | .779 | .416 | 1.87 | 38.9 |

## DISCUSSION

This method is rapid and simple. Although identical aliquots do not result in exactly the same amount of DNA, the fact that the DNA content of the final elution buffer can be easily determined and the quality of the DNA verified by UV spectrometry, makes this a near ideal method for preparing chromosomal DNA for PCR. No precise measurements of volume or time are required throughout the procedure. Sufficient DNA is recovered such that only a small fraction need be used in most PCR applications. Thus, an aliquot is taken out for analysis prior to the UV determination so as not to cross-contaminate clinical samples from the non-disposable quartz tubes.

In order to be trapped on the filters the DNA must be of a very high MW. Shaking the lysis tubes vigorously for five minutes is sufficient to very seriously reduce the yield. The use of polyvinyl alcohol (PVA) in the lysis buffer is to maintain a high viscosity. In early experiments without PVA it was determined that dilutions of blood greater than 1 to 10 resulted in a solution from which DNA could not be filtered. Dilutions less than 1 to 10 were too viscous to filter at all. It was reasoned that more flexibility could be introduced into the system by using a lysis agent with a substantial viscosity of its own, thus separating the level of dilution from the final viscosity. Polyvinyl alcohol was chosen for this purpose. It is cheap, non-toxic and easy to handle. It allows greater dilution of the samples and thus more uniformity from sample to sample in spite of the differences in viscosity among different blood samples.

# PCR RELATED INSTRUMENTATION

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## INTRODUCTION

The advent of the PCR technology has greatly facilitated the detection and characterization of specific nucleic acids sequences, both at the level of genome analysis and in establishing the presence in a sample of viral or other extraneous sequences. Significant advances have also been made in the domain of the instrumentation related to the use of the PCR technique and the characterization of the amplified product. In that regard, three main areas should be considered, to review the existing instrumentation and help defining what type of instrumentation remains to be designed:

*Pre-PCR *PCR reaction *per se* *Post-PCR

## I. PRE-PCR

Isolating a purified PCR-able DNA sample still remains nowadays a tedious and time consuming process. Although various protocols are available for the simple extraction of DNA from, for instance, minicultures, in the majority of the cases, accessing the DNA to proceed with the PCR reaction still requires a large number of steps, some of which requiring the use of noxious reagents. Several DNA extractors are commercially available; they allow for the processing of samples of various types (blood, tissues, etc.) in an automated fashion. However, while reducing significantly the amount of "hands-on" work, they still present some limitations at the level of the minimum amount of starting samples required.

Alternatively, for instance, in the case of whole blood samples, simple techniques exist for the isolation of PBMC fractions (lymphocytes), from which DNA can be readily prepared. Recently, even simpler protocols have been proposed, (see the method described by Kary Mullis before) allowing to proceed directly from the blood sample to the PCR reaction in a single tube. The time saving and the easiness of the protocol should guarantee the success of this protocol, in particular in the diagnostics laboratory.

occasionally crease during the centrifugation steps, thereby increasing the risks of post-PCR contamination.

An alternative format, that is currently becoming more popular, is the microtiter plate configuration. Several variants of this format are currently being used, from the classic microtiter plate, molded with a warp-resistant polymer, to the strips configuration, which provides more flexibility in adjusting to the actual number of samples. The major concern associated with the use of this format is the possible sample-to-sample contamination. With the classic microtiter plate, the plate obviously needs to remain covered during the whole PCR reaction, while at the same time, providing an adequate seal for each individual well. Condensation may result on the lid, thereby requiring to briefly centrifuge the plate, post-PCR, prior to lifting the lid. With the strip format, each well is more efficiently isolated from its neighbors, since each sample features its own cap. However, the caps themselves come in strips, requiring the researcher to cut between each cap, in order to prevent possible well-to-well carry-over at the moment of opening the tubes, resulting in a more complicated handling of the samples, post-PCR.

The newest alternative is the capillary tube format, recently described. In addition to permitting to use much lower reaction volumes than with microcentrifuge tubes, thereby reducing the cost of reagents, this format also allows for much faster PCR reactions. Typically, the total per cycle time can be reduced from possibly several minutes to approximately 20 seconds. This format requires the use of tube sealing equipment and a different type of thermocycler. In my opinion, the only significant disadvantage of this format is that the whole sample needs to be "sacrificed: in the "one-shot," post-PCR analysis. If several, distinct, product characterization methods are to be used, the researcher would have to run replicates of the same sample, which, in some cases ("drop-in, drop-out"), could lead to inconclusive results.

Finally, although not currently a routine technique, *in situ* PCR represents the solution to a number of hitherto unanswered questions. In particular, this technique could substantially facilitate the analysis by PCR of tissue sections, for the precise localization of viral or oncogene sequences. Although some protocols have been tentatively described, the main problem remains the anchoring of the amplified product in the actual location of the target sequence. Undoubtedly, more efforts will be devoted to optimizing this technique.

B. Thermocylers

Since the introduction, in late 1987, of the first two commercially available instruments, the offering has expanded considerably, to the point of creating some confusion in the mind of the researcher. Various heating, as well as cooling, configurations have been designed and are currently offered in different combinations.

Heating of the sample is most usually performed by transfer from a block to the sample. Other designs use a fluid (water or air) for the same purpose. Cooling can be performed by circulating liquid through the block, either from an external or an integrated source. Alternatively, semi-conductors or fluid (water or air) can be used to lower the sample temperature. The requirement for sub-ambient capability still remains an open debate: Most currently used thermostable DNA Polymerases lack 3' to 5' exonuclease activity, but posses synthesis-dependent 5' to 3' exonuclease activity. Therefore, no degradation of the amplified product should be expected, even at ambient temperature, provided that the Polymerase preparation is devoid of other nuclease activity.

The most important parameter of all is the sample-to-sample reproducibility, with respect to the actual sample temperature over the whole range of temperatures covered by the PCR process. It has been extensively and emphatically documented that, in that

regard, all commercially available thermocyclers are not equal. Block edge effects have been noticed with some instruments, cross-block temperature gradients with others. Obviously, the ideal situation, from the researcher standpoint, would be not having to preoccupy oneself with those instrument-dependent inherent idiosyncracies. My conclusion at this point is that, although significant improvements have recently been implemented in some commercially available instruments, it behooves the researcher to exercise adequate judgement at the time of committing to one particular instrument. Indeed, temperature uniformity and reproducibility are two of the main elements in guaranteeing the success of any PCR reaction.

## III. POST-PCR

### A. Post-PCR product handling

Prior to proceeding with the characterization of the amplified product, it is critical to ensure oneself that no carry-over occurs, which, logically, could lead to inconclusive results in the next round of amplification. As mentioned in the first section of this review paper, pre-PCR UV irradiation of the complete sample reaction mix, devoid of target DNA but containing all the other components of the reaction, has been proposed as a simple technique to inactivate any carried-over DNA that might serve as template. However, this technique does not seem to be completely reliable, in the sense that there are substantial variations in its efficiency, depending on the type of template and the irradiation conditions. Incorporation of dUTP during the PCR reaction, followed by treatment of the next round complete reaction mix with uracil *N*- glycosylase, appears to be a safer and simpler method for inactivating carried-over DNA. One minor disadvantage of this technique is that the original template, for example, in a series of successive PCR reactions, will not be modified during the first PCR reaction. One can assume that, since the original template will probably be present at a relatively low copy number, even fewer

copies would be accidently carried-over, thereby resulting into a "vanishing" problem over the course of the series of amplifications.

One aspect of this approach, which, to my knowledge, has not been answered yet, is whether the uracil *N* - glycosylase will efficiently cleave at modified U sites (i.e. biotin or digoxigenin derivatized). If not, amplified DNA containing modified U might not be inactivated, unless a dUTP/modified dUTP mix is used during the PCR reaction. If the enzyme recognizes modified U, even on single strand DNA, this approach might preclude the use of primers containing modified U. A more promising approach, perhaps, consists into modifying the amplified product, post-PCR, by covalently attaching derivatized psoralens to each strand, upon UV irradiation. The resulting modified product can still be characterized by gel electrophoresis, hybridization and sequencing. However, it is inert with respect to further amplification with thermostable DNA Polymerases. The advantage of the system is that the psoralen derivative can be added to the PCR reaction mix from the beginning, since it does not interfere with the PCR reaction, as long as the reaction mix is not UV irradiated. Therefore, the tubes are transferred after the PCR reaction to the photoreaction chamber and UV irradiated in order to inactivate the amplified product. The major advantage of this procedure is that there is no requirement for opening the sample tubes between the PCR reaction and the UV irradiation, thereby also eliminating a possible source of contamination. In all cases, it is recommended to use positive displacement pipets at all times, in order to minimize the extent of potential carry-over of any of the reaction components.

B. PCR product characterization

Entering in details about this whole area clearly goes beyond the scope of this review paper.

At this point, gel electrophoresis is still the most commonly used method for characterizing a PCR product. Both agarose and polyacrylamide gels can be used, depending on the size of the amplified product and the resolution level required. The commercial availability of pre-cast polyacrylamide gels will significantly facilitate the routine analysis of PCR reaction products, in particular in the diagnostics laboratory. When fluorescently labelled primers are used in the reaction, the amplified product can be detected directly by fluorescence, eliminating the need for staining the gel with toxic, polyaromatic compounds such as ethidium bromide or acridine. I should also mention that metal-based direct staining reagents kits, offering the same sensitivity as the above mentioned conventional staining reagents, have also recently become available. Alternatively, fluorescently labelled PCR products can be detected on more sophisticated instruments such as automated DNA sequencers.

Protocols for readily eliminating the excess labelled primers, such as by spin-dialysis, have been extensively described in the literature. Detecting by gel electrophoresis specific hybrids between a PCR product and the corresponding, fluorescently labelled probe appears somewhat more difficult, possibly due to sensitivity problems which remain to be solved. Hybridization with a specific probe offers one additional level of specificity in the characterization of the PCR product, the highest level of sensitivity still being provided by the use of radioactively labelled probes. The hybridization step can be performed directly in the gel, although a transfer to a membrane is still the preferred method. The transfer can be accomplished either by capillarity or by using an electrical field: Small instruments have been developed for either variant. Also, hybridization ovens have recently appeared on the market: They should definitely improve the reproducibility of the blot hybridization process.

Dot blot hybridization is also quite commonly used as an alternative to Southern blot hybridization. The advent of the reverse dot blot format is substantially simplifying the characterization of a single PCR product with multiple probes of different specificity. However, the set-up of reverse dot blot strips is somewhat complex, in particular at the level of designing probes that would tolerate "shared" stringency conditions. Alternatively, the liquid hybridization technique ("Reverse Southern") offers considerable advantages in the sense that the tedious steps of transferring the sample and probing the blot are eliminated. The only problem remains at the level of detecting the specific hybrid in the gel, in particular if non-isotopic detection methods are used.

Finally, chromatography in various forms also offers distinct possibilities for rapid detection and characterization of PCR products. Recently, it was demonstrated that HPLC could resolve quickly and cleanly a complex mixture of primers and PCR products. Isotachophoresis, a somewhat related technique, although promising at one point, turned out not to be conveniently usable for this application. Gel filtration and ion-exchange chromatography have also been used for the rapid separation of PCR product from excess primers. Affinity chromatography, using modified supports, permits to selectively separate derivatized PCR products. However, one should remember that the column will also bind the excess primers, if they carry the reporter group. Introducing a protein binding region into the PCR product, via the primers, should remedy the problem in the sense that a column modified with the DNA binding protein will most likely retain only the PCR product and not the single strand primers. Although not directly related to chromatography, the use of derivatized beads, either inert or magnetic, provides a convenient alternative to setting up an affinity column, while possibly reducing the volumes to be analyzed subsequently.

## IV. CONCLUSION

One of the conclusions that can be drawn from this report is that, if anything, the PCR technology has forced all of us to rethink a number of technologies that we had grown accustomed to. Indeed, due to the recent advances in performing the PCR reaction *per se*, including the reduction in total reaction time, it appears that the sample preparation and product characterization have become the bottlenecks. It is therefore comforting to notice that, in these areas as well, a lot of efforts are being dedicated to simplifying the protocols routinely used. This, in turn, results in the development and the commercialization of new instruments, apparatuses, accessories and reagents, whose availability will eventually allow the researcher to fully benefit from the advantages offered by the PCR technology. We should only hope that "flexible automation" will finally come in the picture and free more of our time, which we could therefore use more efficiently to think about new lines of experimentation.

# USE OF DENATURING GRADIENT GEL ELECTROPHORESIS AFTER PCR IN THE ANALYSIS OF MUTATIONS IN THE FACTOR VIII GENE

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## ABSTRACT

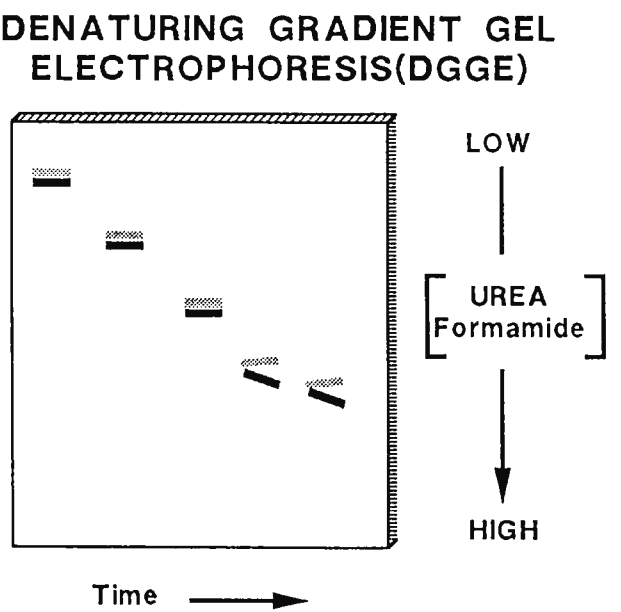
To date, it has been difficult to characterize completely a genetic disorder, such as hemophilia A, in which the involved gene is large and unrelated affected individuals have different mutations, most of which are point mutations. Towards this end, we analyzed the DNA of 29 patients with mild-to-moderate hemophilia A, in which the causative mutation is likely to be a missense mutation. Using computer analysis, we determined the melting properties of factor VIII gene sequences to design primer sets for PCR and subsequent denaturing gradient gel electrophoresis (DGGE). A total of 45 primer sets were chosen to amplify 99% of the coding region of the gene and 41 of 50 splice junctions. To facilitate detection of point mutations, DNA from two male patients was mixed and both homoduplexes and heteroduplexes were analyzed. Using these 45 primer sets, 26 DNAs containing previously identified point mutations in the factor VIII gene were studied and all 26 mutations were easily distinguishable from normal. After analyzing the 29 patients with unknown mutations, we identified the disease-producing mutation in 25 (86%). Two polymorphisms and two rare normal variants were also found. Therefore, DGGE after computer analysis is a powerful method for nearly complete characterization of disease-producing mutations and polymorphisms in large genes such as factor VIII.


## INTRODUCTION

Hemophilia A, an X-linked recessive bleeding disorder which affects approximately 1 in 10,000 males in all population groups, is due to deficiency of factor VIII procoagulant activity (1,2). A large number of mutations in the factor VIII gene causing hemophilia A have been reported (see 3 for review). Gross gene rearrangements, such as deletions, insertions, and duplications, account for only 5% of the molecular defects in hemophilia A patients, while point mutations are thought to account for the remaining 95% of defects.

Detection of all possible point mutations in the factor VIII gene is hampered by its large size, its many exons, and the high frequency of *de novo* mutations which result in different mutations in unrelated affected individuals. These factors make hemophilia A a model for the development of methods for the complete characterization of disease-producing mutations in a single gene disorder caused by an almost unlimited variety of point mutations.

DGGE separates DNA fragments according to their melting properties in a gel system that contains a linear gradient of DNA denaturants. Partially melted DNA fragments are required for separation of normal and mutant DNA, where mutations fall in the melted region of the fragments (Figure 1). For example, base changes in all but




A) A double-stranded DNA molecule is shown migrating in denaturing gradient gel electrophoresis over time. When the DNA molecule reaches the denaturant concentration, at which its lowest melting region denatures, a branched structure forms and the mobility of the molecule declines greatly. 7 M urea and 40% formamide represent 100% denaturant concentration.

the highest temperature melting domain are usually detected by DGGE (4,5). Attachment of a highest melting domain, called a "GC-clamp," to fragments of interest improves the gel system such that base changes in the highest temperature melting domain become accessible to DGGE analysis (6,7). In addition, the use of heteroduplexes between normal and mutant DNA which contain a mismatch increases the resolution by DGGE so that virtually all possible base changes can be detected, even when a base change does not produce a shift in the mobility of the mutant homoduplex (8,9).

In this report, we demonstrate the usefulness of DGGE analysis to detect all possible mutations in the coding region of the factor VIII gene. We analyzed 29 patients with mild-to-moderate hemophilia A, which accounts for 30-40% of the disease. We found 20 novel disease-producing mutations in 25 of these patients.

## MATERIALS AND METHODS

### Subjects

A total of 29 patients with mild-to-moderate hemophilia A were analyzed, including 15 German patients (10) and 14 Japanese patients (11). When genomic DNA was analyzed by Southern blot analysis using several restriction endonucleases including Taq I and three cDNA fragments as hybridization probes (12), no molecular defects were found. Factor VIII activity in these 29 patients ranged from 2.4% to 38% of normal. Computer analysis was carried out using the program MELT87 and SQHTX, provided by L. Lerman (MIT), to aid in the placement of PCR primers (13).

### Amplification of genomic DNA

High molecular weight leukocyte DNA was amplified using Taq DNA polymerase (Cetus). PCR was performed in volumes to 40 $\mu$l for DGGE analysis and 100 $\mu$l for sequence analysis containing 200-400 ng genomic DNA, 400 nM of each PCR primer, 200 $\mu$M of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KC1, 1.5 mM $MgCl_2$, 0.02% gelatin and 2 units/100 $\mu$l reaction mixture of Taq DNA polymerase for 35 cycles consisting of

94° C for 20 sec., thermal transition from 95° C to annealing temperature over 2 min., 52 - 60° C for 45° sec., 72° C for 30 - 60 sec. followed by extension at 72° C for 10 min. For amplification of exon 14 regions a first amplification of 30 cycles was performed using a stepcycle program (94° C for 20 sec., 55° C for 30 sec., 72° C for 3 min.) in a total volume of 100 $\mu$l. The 3.2 kb PCR product containing the entire exon 14 was used as template DNA for amplification of appropriate exon 14 subfragments. For a list of oligonucleotides used see (14).

DGGE analysis

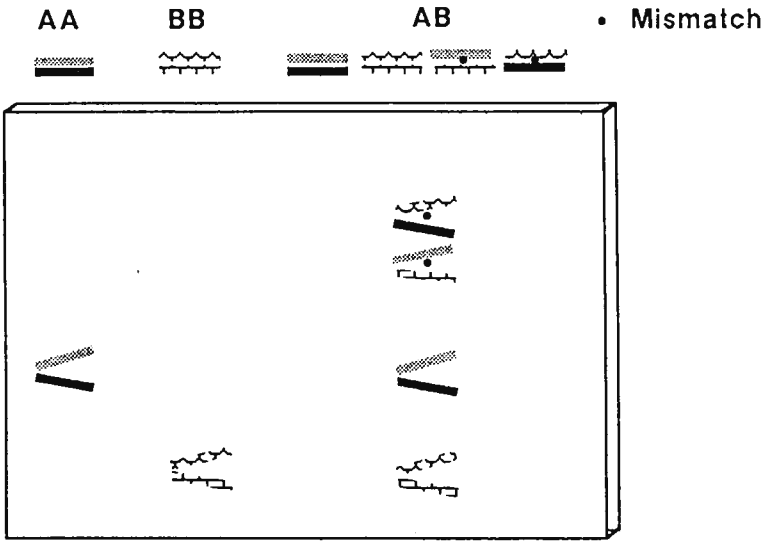
Approximately 80 ng of each PCR product from 2 patients were combined to form heteroduplexes. After heat denaturation at 95° C for 5 min., the DNA solution was slowly cooled to room temperature (over 30 mins.) and subjected to DGGE under conditions determined empirically for each PCR product (5,9). DNA was loaded onto a 6.5% polyacrylamide gel (14 cm x 19 cm, 0.75 mm thick) containing a linear gradient of denaturants and electrophoresed at 2-4 V/cm for 16-23 hrs. The gradient difference in denaturants used was 20% (100% denaturants = 7M urea/40 (vol/vol)% formamide). Gels were then stained in ethidium bromide and photographed using a UV transilluminator. PCR products containing mutations were directly sequenced using internal primers.

## RESULTS

Screening for mutations

The majority of mutations causing mild-to-moderate hemophilia A should reside within the coding region or splice consensus sequences of the factor VIII gene. After analyzing all 45 regions, we identified disease-producing mutations in 25 of 29 patients studied (14). In addition, 2 rare variants and 2 polymorphisms were also detected. All of these nucleotide alterations were detected by a typical "4-band-pattern" on denaturing gradient gels, which consists of two upper bands representing the heteroduplexes and two lower bands representing the homoduplexes of mutant and normal DNA (Figure 2).

MIGRATION OF MISMATCHED FRAGMENTS ON DGGE




B) Migration in denaturing gradient gel electrophoresis of an AA molecule derived from an AA homozygote, a BB molecule of a BB homozygote containing a point mutation which allows its separation from the AA molecule, and an AB mixture from an AB heterozygote. In the AB mixture, there are AA molecules, BB molecules, and AB and BA heteroduplex molecules. The heteroduplexes denature more readily because they each contain a mismatch shown by the dot.

Although functional studies are needed to determine the consequences of the mutations (14), we concluded that these mutations were likely to be disease-producing for two reasons. First, 24 of 25 patients had only one sequence change within the coding region of the factor VIII gene while one patient (JH-138) had two changes. Second, the frequency of neutral substitutions within the factor VIII gene is extremely low (15 and present study). The 25 defined mutations occurred at 20 different sites. These included one novel putative splicing defect and 29 novel missense mutations, of which 4 were found in more than one individual. All missense mutations led to nonconservative amino acid substitutions.

## DISCUSSION

DGGE of amplified PCR products with a 40 nt GC-clamp is a powerful tool for mutation screening of large genes, (16,17). In this study, in order to increase sensitivity and to facilitate screening of a large number of patients we modified our previous procedures. First, genomic PCR primers were chosen after computer analysis to assure that the regions of interest reside within the lowest melting domains (5,13). Second, we determined the optimal conditions for each test fragment by varying denaturant concentrations and time of electrophoresis in order to maximize the difference in mobility between homoduplexes of normal and mutant DNA (9). However, certain nucleotide substitutions do not change the melting properties of the test fragment. Third, to overcome this problem we combined PCR products from 2 randomly selected patients to observe heteroduplexes. Mutations can be detected by heteroduplexes even when mutant DNA is not distinguishable from normal DNA on denaturing gradient gels.

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# PCR-SSCP: A SIMPLE AND SENSITIVE METHOD FOR DETECTION OF MUTATIONS

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## WHAT IS PCR-SSCP?

In PCR-single strand conformation polymorphism (PCR-SSCP) analysis, the target sequence is first labeled and amplified simultaneously by the PCR of the genomic DNA (1) or cDNA (2) using labeled substrates. The PCR product is then denatured and resolved in polyacrylamide gel electrophoresis. Mutations are detected as altered mobility of separated single strands in the autoradiogram. Electrophoretic mobility of a molecule in gel is sensitive to both its size and shape. In non-denaturing conditions, single stranded DNA has folded structure which is determined by intramolecular interactions, therefore, by its sequence. Most conformational changes caused by such subtle sequence differences as one base substitution in a fragment of several hundred bases can be detected by this analysis because of the high resolving power of polyacrylamide gel electrophoresis. Fig. 1 illustrates how mutations are detected in PCR-SSCP. At present, it is not possible to predict how mutations cause shift of electrophoretic mobility from sequences.

## SOME BACKGROUND CONSIDERATIONS

In PCR-SSCP analysis, any sequence change in the whole amplified region can cause mobility shift. This is in contrast to some other techniques, such as allele-specific oligonucleotide hybridization of the PCR products, in which possible changes in relatively short, a few to 20 base sequences are concerned. Therefore, PCR-SSCP analysis is sensitive to replication errors that may occur during the PCR. However, theoretical calculations based on the recent estimation of error rate of *Taq* DNA polymerase indicated that fragments with incorrect sequences contribute to at most a few percent of the final amplified fragment of several hundred bases (3). Also, since the errors can be assumed to occur randomly, any particular erroneous sequence will never become significant subpopulation if the PCR is started from concentrations of DNA usually adopted for amplifying genomic single-copy sequences (more than $10^4$ copies per

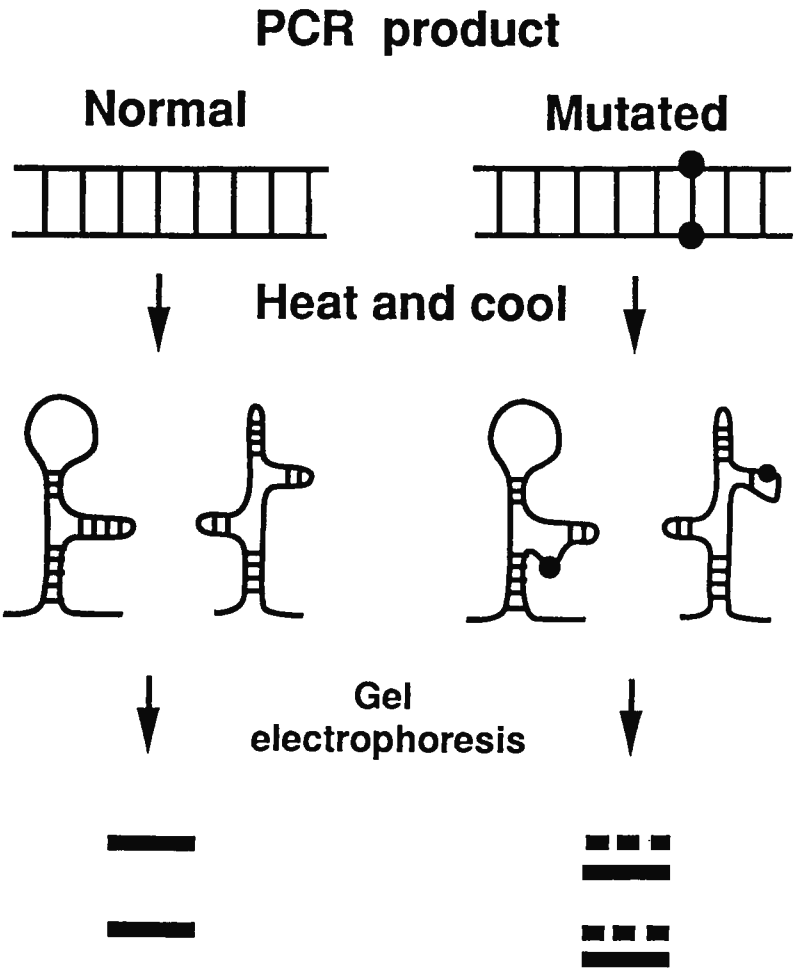


Fig. 1. A schematic diagram of detection of mutations by SSCP.

reaction). If, however, the reaction was started from much smaller number (e.g. 10) of molecules, errors in the early cycles of amplification can produce erroneous bands in the PCR-SSCP analysis (4). In such cases, results may have to be confirmed by independent reactions.

## CONDITIONS OF PCR FOR SSCP

The amount of products required for the detection in PCR-SSCP is much less than that in some other PCR-based techniques where products are detected by staining or by other indirect methods. This means that volumes of the reaction and concentrations of substrates can be reduced for lower running cost and less radioactive hazard. We found that the PCR in 5 $\mu$l using primers at 25 nM (when using labeled primers) or deoxynucleotide at 10 $\mu$M (when using labeled deoxynucleotide) gives satisfactory results for most sequences (5). Perhaps small volume is advantageous for strict temperature control, and low concentrations of primers and deoxynucleotides increase the specificity of annealing of primers to templates and reduce rate of misincorporation by the *Taq* DNA polymerase (6). Shorter fragments are better suited for detection of mutations in SSCP gel. Therefore, usually, fragments of less than 400 bp are amplified. Alternatively, longer fragments can be amplified using labeled radioactive deoxynucleotides, and the uniformly labeled PCR products are then digested by restriction enzymes. Samples are then denatured and applied to non-denaturing polyacrylamide gel.

## ELECTROPHORESIS

Detection of mutations depends on the conformational charges of the single-stranded molecule induced by the mutation, and therefore, sensitive to physical environment in the gel, e.g. temperature, concentration of ions and solvents. Temperature rise during electrophoresis is especially hazardous for reproducible results. For this reason, little Ohmic heating and efficient cooling is important, and use of thin gel

has obvious advantage. Vigorous air cooling and attachment of aluminum plate on one side of gel (1), or, use of water-jacketed electrophoretic apparatus (personal observation) are effective for keeping temperature even throughout the gel, and constant during the run. It should be noted that the mobility of single stranded DNA and the shift caused by mutation can change dramatically if temperature of electrophoresis is different (4° C versus room temperature which is mostly 20 to 26 ° C, ref. 1).

Extent of cross-link of polyacrylamide gel is expressed by %C, a ratio in percent of concentration of N,N'-methylenbisacrylamide to concentration of total acrylamide monomer. At 5 %C, the gel is the most rigid and has minimal pore size at any given total acrylamide concentration. Gel with lower %C is more soft, remarkably increased pore size, and seem to be more sensitive to conformation. Increased freedom of acrylamide fiber may explain these effects. In SSCP analysis, gels at 1 to 2 %C and 5 to 6 % total acrylamide are commonly used (5). Presence of low concentrations (5 to 10 %) of glycerol in gel frequently improves separation of mutated sequences (1). The reason for this is unknown. Perhaps, glycerol, because of its weak denaturing action to nucleic acid, partially opens folded structure of single-stranded nucleic acid so that more surface area of the molecule is exposed, and more chance for the acrylamide fibers to sense locally confined structural difference caused by mutation. Glycerol, however, reduces mobility especially when electrophoresis are run at 4 ° C, probably because of its viscosity. It should be noted that in rare cases, mutated sequences show mobility shift only in gels without glycerol.

In summary, by electrophoresis at room temperature using 5 % gels having 1 %C and containing 5 % gycerol, the majority of mutations will be detected. Additional mutations may be found by electrophoresis in gels without glycerol at room temperature

or at 4 ° C. Whether to try multiple conditions of electrophoresis depends on the purpose of one's experiments.

## SENSITIVITY

Estimation of sensitivity of PCR-SSCP is meaningful only when the estimation is based on the observation of mutations in various sequence contexts. Here, mutations are regarded to be detected if one strand of mutated sequence shows mobility different from that of reference sequence in at least one electrophoretic condition.

The first example is *RAS* family genes (7). Ten point-mutated sequences (4 in c-Ki-*RAS* exon 1, two in N-*RAS* exon 2, and one each in c-Ki-*RAS* exon 2, c-Ha-*ras* exon 1, c-Ha-*RAS* exon 2, and N-*RAS* exon 1) were examined after amplifying 100 to 200 bp fragments, and all mutations were detected as significant mobility shifts in at least one of the separated strands.

The next example is $O^6$-methylguanine alkyltransferase gene (*ada A*) of *B. subtilis* (8). Bacterium was mutagenized and mutant clones sensitive to nitrosoguanidine (i.e. phenotype of *ada A*$^-$) were selected. DNA from six clones were examined by PCR-SSCP using sets of primers, each amplifying about 350 bp fragments which cover the gene. One clone turned out to have large deletion in the gene and amplification products were absent in some of the PCR. Each of the five mutant revealed significant mobility shift in one of the amplification products. Subsequent sequencing revealed that all mutants had sequence alterations of single-base substitutions or small deletions in the regions where the shifts were observed.

A different approach was tried to estimate the sensitivity of PCR-SSCP analysis. The probability of shift of a mutated strand (x) can be estimated from the observed ratio

(R) of cases where both strands are shifted ($x^2$) to cases where only one strand is shifted (2x(1-x)). This estimation assumes that whether one mutated strand shifts or not is independent of chance of shift of its complementary strand, but does not require prior knowledge of whether the examined sequences have mutations or not. Various regions of the genomic and cDNA sequences of the p53 gene and the retinoblastoma susceptibility gene from DNA or RNA of surgically obtained hepatoma tissues were examined by PCR-SSCP. The number of mutations that induced mobility shift in both strands as well as only in one strand were then counted and compared to the number of mutations that were found by subsequent sequencing. From observations of 30 such cases, it was concluded that using gel with 5 to 10 % glycerol, 97 % of mutations in strands of 100 to 300 bases long caused shift, while this value droped to 67 % for strands of 300 to 450 bases long. These estimations are translated to sensitivity of PCR-SSCP analysis (probability of detecting at least one strand shifted) of more than 99 % and 89 % for 100 to 300, and 300 to 450 bp fragments, respectively. It was also found that the sensitivity is much lower in gels without glycerol.

## SOME EXAMPLES

Development of cancer is a multi-step, multi-pathway event, in which various genetic changes seem to be involved. Various somatic mutations of *RAS* oncogene families (7), p53 gene (9, 10, 11, 12) and the gene responsible for retinoblastoma (2) in cancer cell lines and in surgically obtained cancer tissues have been detected by PCR-SSCP analysis. Search for mutations in clinically obtained cancer cells by the conventional method were difficult because such specimens are often heavily contaminated with surrounding normal tissues and inflammatory cells. However, by our recently published technique, mutated sequences that constitute only small portion in the majority of normal sequences can still be unambiguously characterized (13). Further study of genetic changes in tumors of various cell origins, histological types, and other

characters such as drug-resistance, may lead to establishment of DNA-based diagnosis of cancer.

PCR-SSCP is successfully used in the detection of DNA polymorphisms. Polymorphisms in certain repetitive sequences can be detected by PCR-SSCP if primer sequences for the PCR are chosen in the neighboring single copy region. Using PCR-SSCP, we (14) have detected polymorphisms in about half of examined *Alu* repeats because of their ubiquitous distribution, *Alu* repeats should be useful as a rich source of polymorphic DNA marker in construction of high resolution linkage map of human genome.

## CONCLUSIONS

An advantage of PCR-SSCP analysis over other PCR-based techniques for detection of mutations is its simplicity. In this technique, mutations are detected by the presence of shifted bands rather than by the absence of signal which is the case in some other detection methods. Therefore, failure in the PCR does not lead to false positive results. Most mutations in various sequence contexts seem to be detected, although further accumulation of data may be necessary to accurately estimate the rate of detection of this technique.

PCR-SSCP analysis has the potential use in field works such as clinical DNA diagnosis. To transfer this laboratory technique to clinical test system, use of non-radioactive detection method is important. Ainsworth *et al.* have shown that the bands of SSCP analysis can be detected by silver staining (15). We have preliminary results that by using fluorescence-labeled primers in the PCR (16), and analyzing the products with a fluorescence-based automated sequencing machine, mutations can be detected at a sensitivity equal to that of the radioactivity-based PCR-SSCP analysis.

Since PCR-SSCP analysis is a relatively new technique, only a limited number of published results are available. Because of the features described above, we believe PCR-SSCP will be used in various fields of basic and applied biology.

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# APPLICATIONS OF PCR-BASED GENOMIC FINGERPRINTING TO GENETIC MAPPING, POPULATION BIOLOGY AND EPIDEMIOLOGY

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## INTRODUCTION

### Arbitrarily primed PCR for genetic mapping and phylogeny

Several approaches to genetic mapping and phylogenetic analysis using PCR have been developed over the past two years. The ability to examine specific sequences of DNA from many organisms simultaneously and without the need of standard cloning has streamlined several types of analysis that were already commonly used but rather labor intensive and has also made possible several types of analysis that were previously not possible. For example, RFLP analysis using Southern blotting has been the principle tool for chromosomal mapping in mammals and plants and, in some situations, has been replaced by PCR-based methods (e.g. Litt and Luty, 1989; Nelson et al., 1989; Orita et al., 1990; Sinnet et al., 1990; Ledbetter et al., 1990). Methods based on PCR have also been developed for studies in phylogenetics and population biology. Specific sequences of phylogenetic significance, such as ribosomal DNA or DNA from surface antigen genes, can be sequenced to derive phylogenetic and population genetics information (e.g. see Woese 1987). Prior to the invention of PCR, these analyses required cloning and were correspondingly labor intensive. PCR allows for the direct amplification and sequencing of interesting sequences without the need for cloning.

In addition to these obvious extensions of PCR, novel applications of PCR have also evolved. For example, we have developed a method for genomic fingerprinting based on PCR termed Arbitrarily Primed PCR (AP-PCR). Genomic fingerprinting reveals sequence polymorphisms which can be used similarly to RFLPs in genetic mapping, and differences between fingerprints for closely related organisms can be used to determine their relatedness in phylogenetic and population biology experiments. The purpose of this brief review is to explore these and several other of the new possibilities that are facilitated by PCR.

### Mapping polymorphisms in the mouse

There are two high through-put methods based on PCR for mapping sequence polymorphisms in the mouse. Jeffreys et al., (1985) recognized that the level of polymorphism in VNTRs (Variable Number Tandem Repeats) between different

members of a population make them particularly suited as polymorphic markers in genetic analysis. Rapid variation in the length of a VNTR results from the instability of simple repeats. By following the segregation of VNTRs in families or in a breeding population, recombination frequencies, and therefore genetic distances, can be determined. These experiments first used Southern analysis, and in some situations, this is still the method of choice. However, as the sequence data base for the mouse expands, sequences flanking VNTRs are being identified. This allows primers that bracket VNTRs to be constructed and used to detect VNTR length polymorphisms in the mapping population. Because there are over 13,000 potential VNTRs in the mouse genome, the possible resolution of a genetic map based on this approach is restricted only by the nature of the mapping population. This method does, however, have some practical limitations. First, oligonucleotide primers are expensive, so each polymorphism is obtained at a cost of several hundred dollars in reagents. Second, these primers are designed for the genetic mapping of polymorphisms in a *particular* organism and cannot be used to map other organisms. Also, there is some concern that the non-random distribution of VNTRs in the genome may lead to the incompleteness of maps generated by this method (Moyzis et al., 1989; McClelland and Ivarie, 1982). An advantage of this approach is that, once a polymorphism is mapped, the primers used for each polymorphism are immediately available for further experiments.

Another approach to genetic mapping uses the often observed but seldom appreciated property of PCR that, if the stringency of the annealing step is not properly adjusted, products other than the desired sequence result. By intentionally reducing the stringency of the annealing step and choosing a single primer that is unlikely to match *anything* in the genome very well, an information-rich genomic fingerprint can be obtained reproducibly **(Figure 1)** (Welsh and McClelland, 1990; Welsh et al., 1991a,b; Martin et al., 1991; Williams et al 1990). In Figure 1a, the strategy for AP-PCR is outlined. Two cycles are performed at low stringency during the annealing step (at which time some synthesis occurs, thereby stabilizing the template-primer interaction). Following these two low stringency steps, subsequent cycles are performed under standard, high stringency PCR conditions. This procedure is

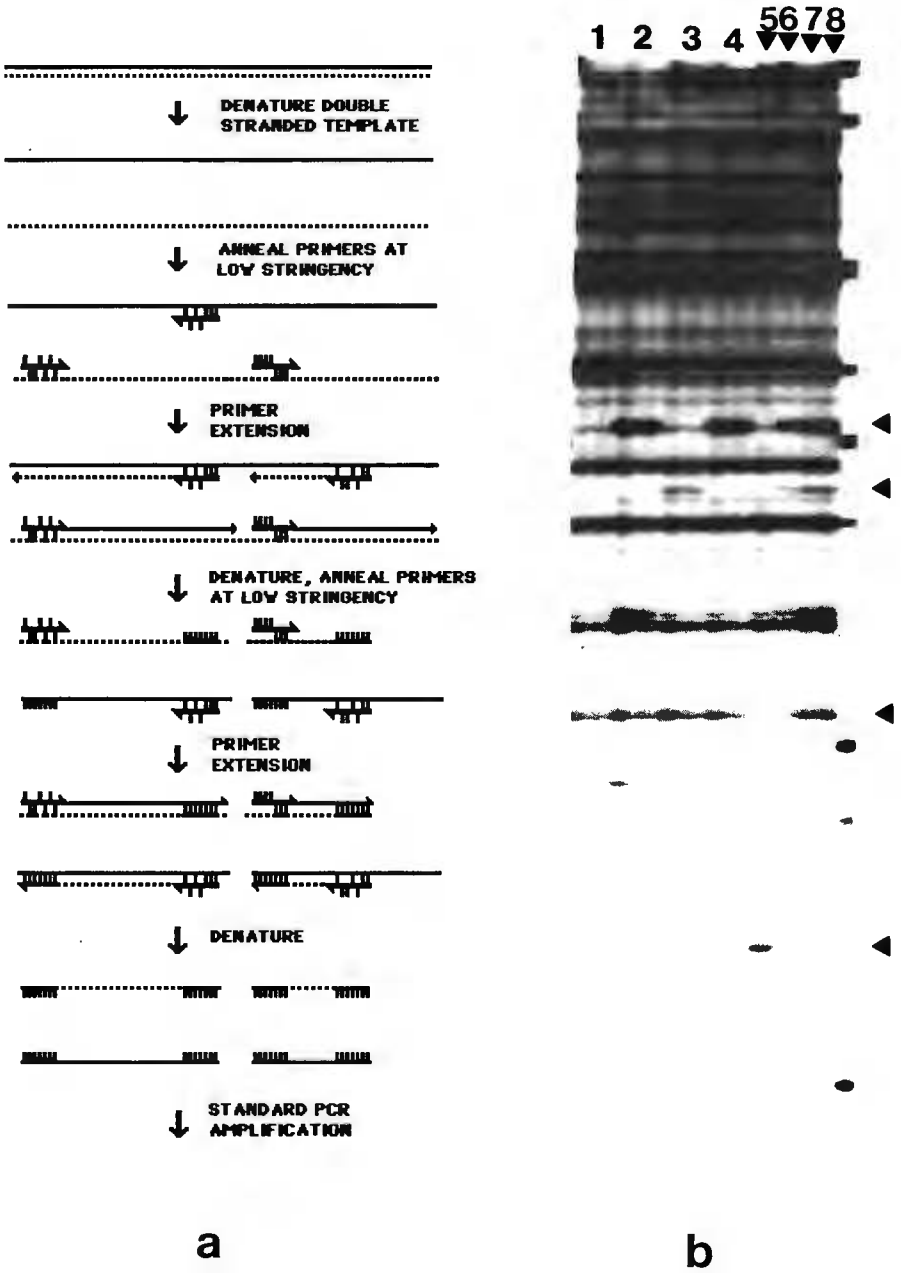



Figure 1

sensitive to sequence polymorphisms in the target genome. Thus, the segregation of AP-PCR polymorphisms in a recombinant inbred or other mapping population can be used to infer genetic distances and to place the polymorphisms on the genetic map. In Figure 1b, the results of AP-PCR applied to several genomic DNAs from recombinant inbred mice is shown. As can be seen, the patterns are non-identical. The two parents of the recombinant inbred population, as well as an F1, are included on the autoradiogram. Polymorphisms are found in one parent or the other but not both, and all polymorphic bands appear in the F1, as expected.

In a typical experiment in the mouse, we have observed three to seven sequence polymorphisms between two parental lines, with an average slightly higher than four. AP-PCR requires no prior knowledge of sequence and apparently samples the genome randomly. However, if desired, a sequence bias can be engineered into the primer. For example, GC-rich regions can be mapped preferentially if desired by constructing a GC-rich primer. Since primers can be chosen arbitrarily, any organism can be mapped with the same set of primers. In addition to these advantages, new AP-PCR patterns can be generated by the application of primers in pairwise combinations. Thus, 50 primers can be used to generate $(50)^2/2 = 1250$ different fingerprints (Welsh and McClelland, in press). Due to the complex kinetics of AP-PCR, the fingerprints produced by AP-PCR are more than 50% unique in a band-by-band comparison with the patterns produced by either primer alone. Given that about four polymorphisms can be detected in genomes as related as mouse strains C57BL6 and DBA with each new primer or primer pair, the cost of mapping is correspondingly low. A disadvantage of AP-PCR is that each new polymorphism must be purified to be of further use, but this procedure is very straightforward. Also, it is difficult to score heterozygotes using this method.

**The use of AP-PCR fingerprinting to characterize individuals**

Polymorphisms in fingerprint patterns generated by AP-PCR can be used to distinguish between even very closely related individuals. AP-PCR fingerprints are quite complex or "information rich", and because this information is easily and inexpensively obtainable, the resolution of AP-PCR for taxonomic comparison is very

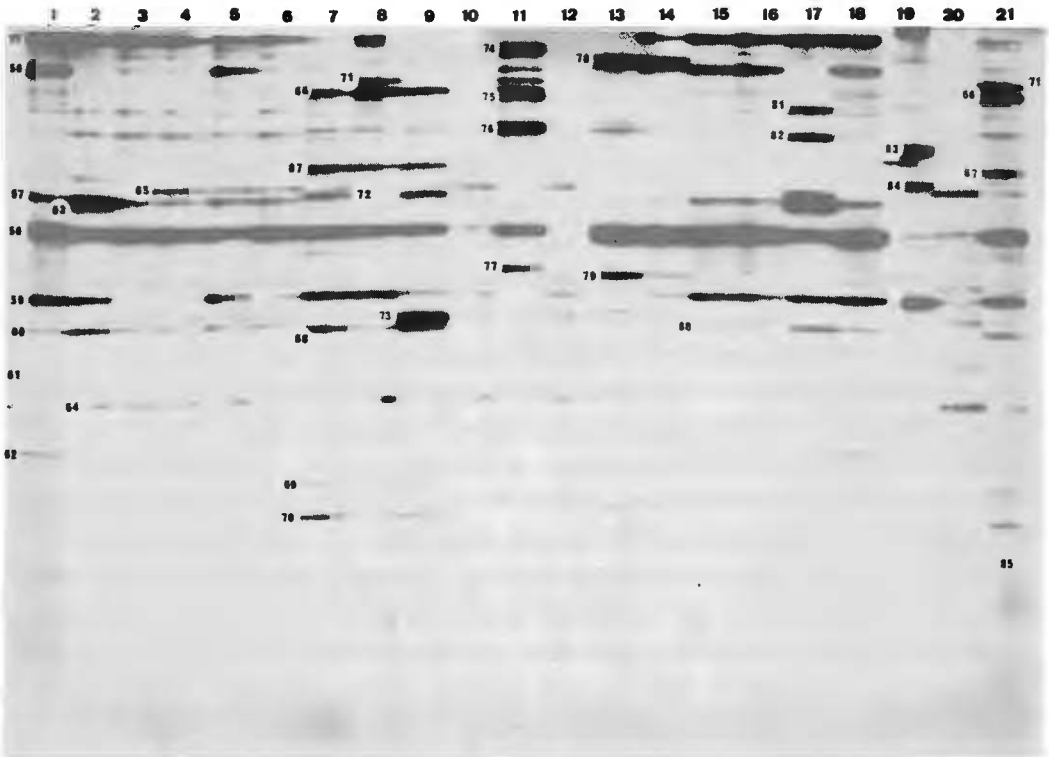
high. Thus, for example, we have been able to distinguish between isolates of the same species of bacteria including *Staphylococcus haemolyticus* (Welsh and McClelland, 1990), *Streptococcus pyogenes* and *Borrelia burgdorferi.*

Polymorphisms in fingerprints generated by AP-PCR can be treated as apomorphic characters in phylogenetic analysis and population biology. Using this approach, *Streptococcus pyogenes* strains can be resolved by AP-PCR into groups that correspond to their surface antigen types. This formally demonstrates that AP-PCR fingerprints can be phylogenetically informative (**FIGURE 2**). Bacterial phylogenetic information can be useful in population genetics. In **Figure 2a**, a sample of fingerprinting data for a collection of *Streptococcus pyogenes* is presented, along with the resultant dendrogram displaying the resolution of several of the isolates according to their surface antigen type. In principle, genotypes characterized by fingerprinting can be used to study a number of problems in population biology. For example, linkage disequilibrium of various sorts, such as fixed heterozygosity in diploids can shed light on such problems as clonality. By reconstructing the phylogeny of a bacterial pathogen and identifying polymorphisms for individual phylogenetic groups, genetic and ecological interactions between populations can be studied.

**PCR between tRNA gene repeats**

PCR primers can be designed to recognize specific sequences that are interspersed throughout the genome. Amplification of sequences between interspersed sequence elements results in a genetic fingerprint. Amplification of sequences between *Alu* repeats in somatic cell hybrids containing fragments of human chromosomes in rodent backgrounds was one of the first demonstrations of this principle (Nelson et al, 1989; Ledbetter et al., 1990). Our own work has focused on the amplification of sequences between tDNA genes in bacteria (Welsh et al., 1991a). In bacterial genomes there are about 100 tRNA genes, generally clustered head to tail with short intergenic spacers (Jinks-Robertson and Nomura, 1987; Vold, 1985). Because tDNA sequences evolve slowly, distinctions between genera of bacteria on the basis of inter-tDNA sequence length polymorphism and tDNA cluster

a.




b.

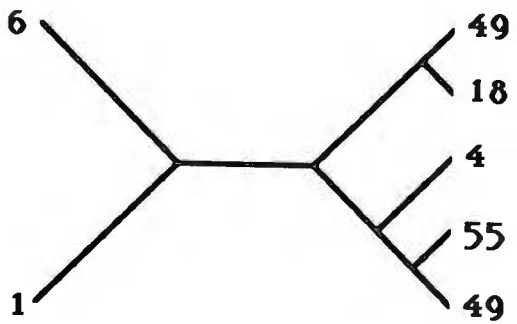



Figure 2

rearrangement can be achieved. The method works as follows: first, consensus tDNA primers are constructed that amplify tDNA spacer regions for any eubacteria. These primers point out of the consensus gene and are slightly recessed from the ends of the tDNA sequence, for reasons that will become clear later. PCR amplification is performed at moderate rather than high stringency and a pattern of bands is obtained. The primers anneal to the best matches available and some pairs of matches are close enough on opposite strands to allow PCR. The resulting pattern displays differences in spacer length and tDNA cluster organization between genera and species that are phylogenetically useful (see **Figure 3**).

The tDNA-PCR experiment described above used weak consensus primers at moderate stringency. Such primers will not amplify the desired tDNA spacer regions if the bacterial DNA is heavily contaminated with DNA from another source, as is the case with medical specimens, for example. However, because the primers are slightly recessed from the ends of the tDNA sequences, primers that can be used at *high* stringency can now be derived from the tDNA-PCR fingerprint. The tDNA-PCR length polymorphism that most clearly distinguishes between the set of related test species is purified, reamplified and sequenced from both ends. The sequences immediately 3' to the original primers are still within conserved tDNA genes and can be used to design perfectly homologous primers. These new primers can be used at high stringency and can therefore be used for medical diagnostics where samples are contaminated with human DNA and in epidemiology where culturing various organisms of interest may be cumbersome. tDNA gene sequences and the order of the genes in the clusters evolve rather slowly, so PCR primers homologous to two particular adjacent tDNA genes give a positive signal for almost any species within the same genus.

Using this approach, we have developed a pair of primers for high stringency PCR that identify most, if not all, *Staphylococci* and define species based on the lengths of the products. Similarly, we have developed a pair of primers that identify and distinguish between members of the *Streptococcus pyogenes* cluster of species.

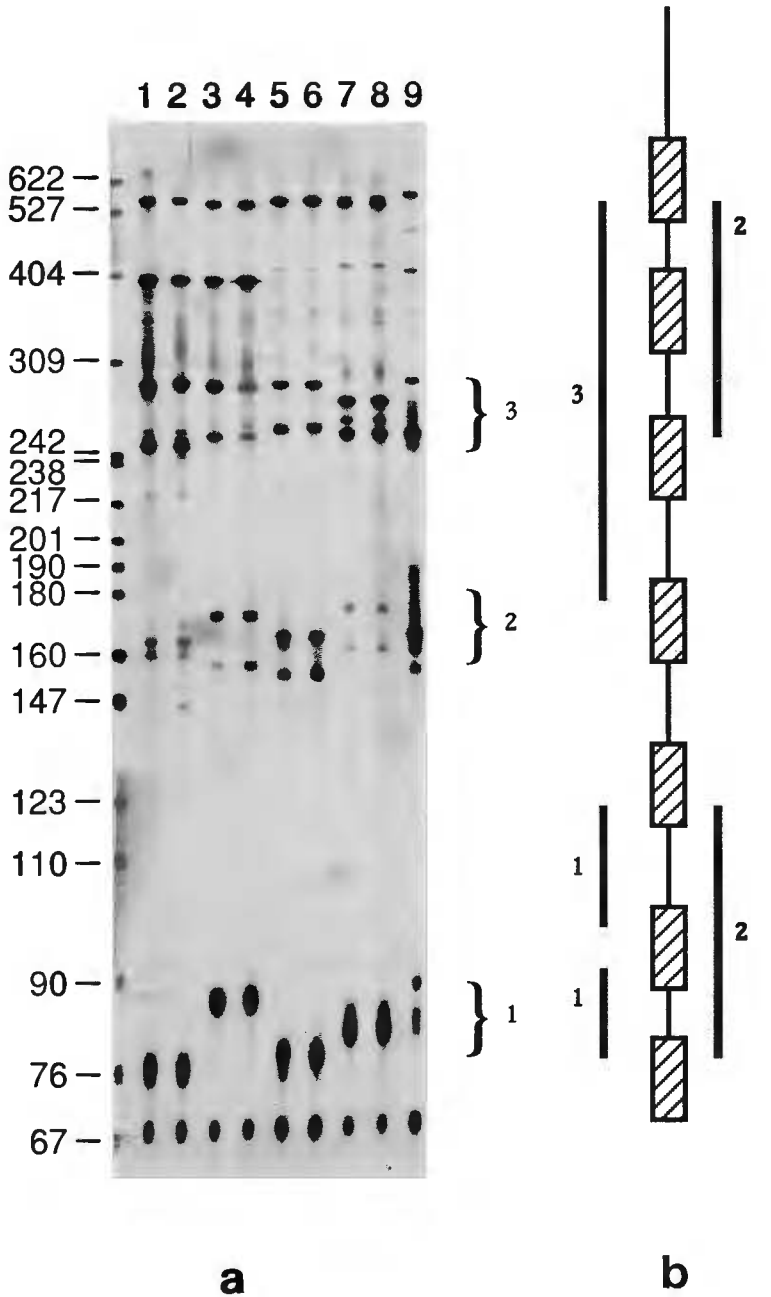



Figure 3

## SUMMARY

The versatility of PCR as an analytical tool seems to increase with each new twist on the basic methodology. Taking advantage of the ability of oligonucleotide primers to prime DNA synthesis at imperfect matches, we and others have extended PCR to genomic fingerprinting. Genomic fingerprinting is useful in genetic mapping, strain identification and population characterization. Due to relative simplicity and speed, molecular methods of comparison based on PCR have made possible a number of experiments that might otherwise be rather impractical.

## FIGURE LEGENDS

Figure 1. AP-PCR can be used to generate polymorphic fingerprints for genetic mapping. (a.) AP-PCR is performed as described in this figure. Oligonucleotide primers are annealed at low stringency to the template. This reaction depends on the sequence of the template and is influenced by sequence polymorphism. Two such low stringency cycles are performed, followed by 40 high stringency cycles. (b.) AP-PCR applied to mouse recombinant inbreds: lanes 1-4, 8 are recombinant inbreds. The parentals of this population are lane 5, C57BL/6J and lane 7, DBA/2J. Lane 6 is the F1 progeny of parents in lanes 5 and 7. Note that the 5 polymorphisms visible in this gel are each present only in one of the two parents, and all are present in the F1 (DNA from recombinant inbreds was kindly supplied by Benjamin Taylor, Jackson Labs).

Figure 2. In panel a., lanes 1-10 and 12-21 are strains *S. pyogenes*. Each strain has a characteristic antigenic type. Lane 11 is *E. faecalis*. Genomic fingerprinting was performed as described in the text. Polymorphic bands, indicated by small numbered circles, were scored as either present (1) or absent (0). This gel represents one of three experiments from which polymorphisms were scored. Panel b. summarizes the most parsimonious Wagner networks generated from this data, placing antigenic types 1, 6, 49, 55, 18 and 4 in their positions relative to one another. Outgroup analysis suggests that antigen type 49 is ancestral to types 4, 18 and 55.

Figure 3. Panel a. Lanes 1, 2 are *Staphylococcus haemolyticus*; lanes 3, 4 are *S. hominis*; lanes 5, 6 are *S. warneri*; lanes 7, 8 are *S. aureus* and lane 9 is *S. cohnii*. tDNA-PCR was performed as described in the text. Polymorphisms can result from three sources (1) polymorphic inter-tDNA spacer lengths (2) selection of different tDNA sequences by the primer and (3) tDNA cluster rearrangement. Since these species are closely related and since tDNA sequences and clusters evolve only very slowly, most of the polymorphisms here reflect spacer length differences. Panel b. schematically illustrates the regions of a tDNA cluster that might be amplified by the consensus primers, and correlates these targets with the experimental result.

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# PCR AMPLIFICATION OF FMDV RNA FRAGMENTS: APPLICATION TO THE STUDY OF GENE EXPRESSION IN EUKARYOTIC CELLS AND OF FMDV GENETIC VARIABILITY

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## INTRODUCTION

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes a systemic, acute infection, characterized by fever, followed by vesicular lesions, mainly in mouth and feet. It affects cattle, swine, sheep, goats and several wild species. In ruminants the acute phase may be followed by an inapparent infection involving limited viral replication in the upper respiratory tract. Persistent infection constitutes an important reservoir of FMDV in nature.

FMDV is characterized by two important features: 1) its ability to persist, both in vitro and in vivo, 2) the genetic heterogeneity, shared with other RNA viruses whose genomes are described as quasispecies.

The FMDV genome consists of a single stranded RNA molecule, of positive polarity, about 8500 nucleotides in length (see fig. 1 A). The genomic RNA is infectious and it has a Vpg molecule linked to the 5' end. The first 1500 nt from the 5' end of the molecule correspond to a noncoding sequence. It contains a segment probably involved in replication (S), a poly(C) tract of about 150 nt, and a sequence involved in the regulation of viral protein synthesis termed internal ribosome entry site (IRES). This region increases the efficiency of translation of viral RNA by allowing the entry of ribosomes, rending RNA translation independent of the presence of cap structure. A single open reading frame gives rise to a polyprotein which is quickly and efficiently processed by viral encoded proteases (L, 2A, 3C). In addition to the capsid proteins (VP4, VP2, VP3, VP1), the viral replicase and the Vpg proteins, there are other nonstructural proteins whose functions are unknown.

Persistent FMDV infections can be established in cell culture (de la Torre et al, 1985). With the aim of understanding if there is a particular component of the virus

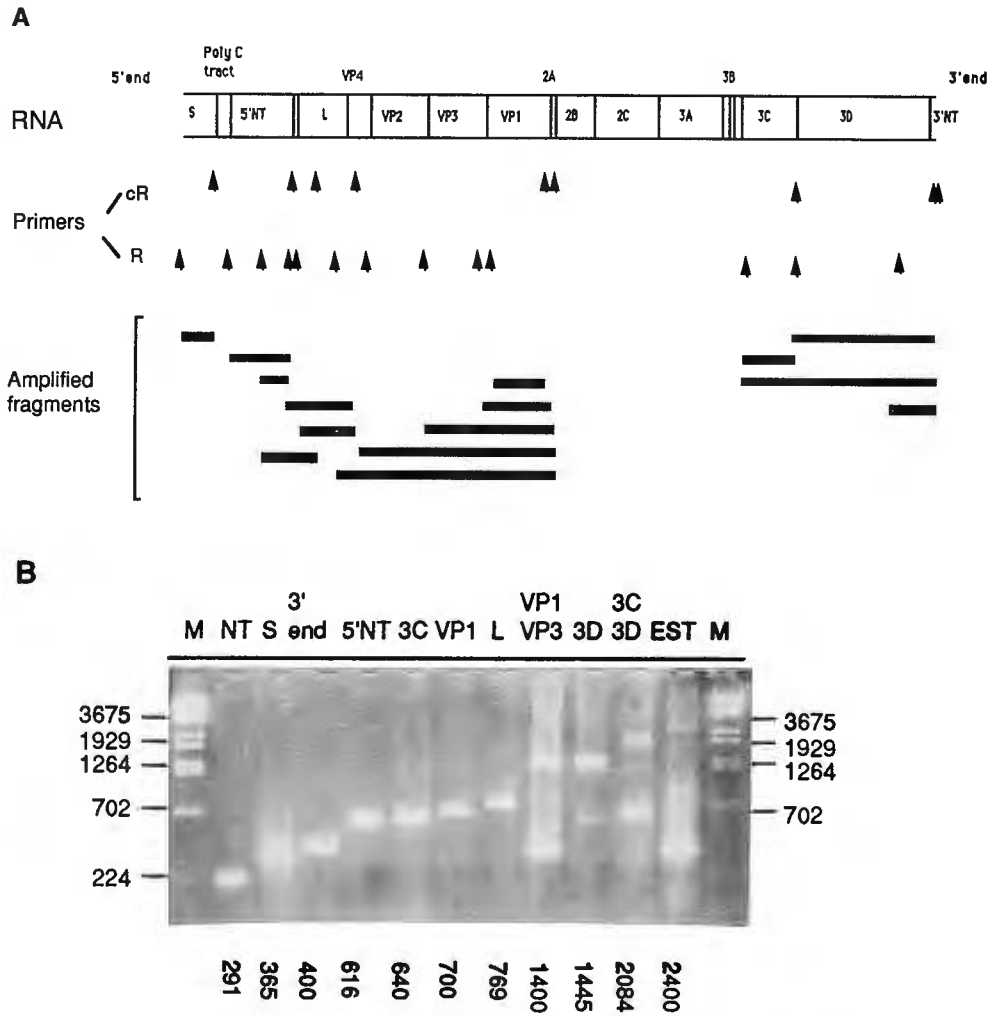


Fig. 1. PCR on FMDV C-S8 RNA. **A.** Top bar: Scheme of the FMDV RNA with indication of the main genomic segments. S stands for S fragment. 5'NT, 5'non-translated region. 3'NT, 3'non-translated region. L, VP4, etc. stand for the different proteins encoded in the genome. The position of primers used in the PCR are shown as filled arrows.cR correspond to primers whose sequence is complementary to the RNA. R, primers that have the same sequence and polarity than the RNA. Black bars drawed at the bottom indicate the position and length of the amplified fragments. **B.** 1.5% agarose gel, ethidium-bromide stained, of 1/20th PCR reaction products. M correspond to λ BstEII fragments, used as Mw markers. NT correspond to a portion of 5'NT. The expected nucleotide size is indicated at the bottom of each lane.

involved in the establishment of persistence in BHK cells, we have undertaken the expression of discrete components of viral RNA or proteins in eukaryotic cells. Infection of cell lines constitutively expressing those viral components will allow us to determine if any of them may mediate or help in the state of persistence of FMDV in cultured cells.

## EXPRESSION OF FMDV GENES

To facilitate the cloning of specific fragments of the viral genome we have applied the PCR method to the amplification of defined segments of FMDV type C (see fig. 1 B). The standard protocol for RNA amplification consisted in the synthesis of a first cDNA strand using reverse transcriptase and a primer complementary to a sequence at the 3' side of the stretch to be amplified. Then the complementary band of the cDNA strand was synthesized using Taq DNA polymerase and a primer of the same sequence and polarity than a defined region of the initial RNA template. By appropriate cycles of thermal denaturation and reannealing of the oligonucleotide primers, exponential amplification of the nucleic acid of interest was achieved. The efficiency of amplification was at least 2.5 X $10^7$ per initial molecule. We have been able to detect in ethidium bromide stained agarose gels DNA amplified from RNA extracted from 4 x $10^3$ cells, containing an average of one copy of FMDV RNA per cell.

PCR products have been cloned in pUC plasmid vectors by blunt end ligation of repaired PCR products. The inserts of pUC plasmids were transferred to eucaryotic expression vectors based on the Herpes simplex *tk* promoter and the firefly luciferase as reporter gene. T7 and SP6 promoters from pGEM vectors are also present in the vector to facilitate in vitro transcription of RNA. Cotransfection of the plasmids coding for FMDV sequences with pSV2PAC, that confers puromycin resistance, resulted in constitutive expression, determined at the level of transcription, in BHK cells of the 3C protease gene, the 3D polymerase gene, and the 400 nucleotide fragment corresponding to the 3' end

of the FMDV RNA. A moderate delay in the time of full detachment of the monolayer was observed in the population of transfected cells constitutively expressing 3C. Experiments are in progress to determine the possible influence of other regions of the genome in modulating the infection.

## GENETIC VARIABILITY OF FMDV

One of the main features of FMDV is its intrinsic genetic variability (Domingo et al., 1980; for a review, see Domingo et al., 1991). Genetic heterogeneity is observed all over the genome, in the antigenic regions (Sobrino et al., 1986) in the replicase gene (Martinez-Salas et al., 1985) and in non-coding regions (unpublished results of our laboratory). Moreover, genetic variability is observed both in nature (Martinez et al, 1988) and in viruses propagated in the laboratory (Diez et al, 1989), and in acute as well as in persistent infections (Diez et al., 1990).

Phenotypic variability is very often reflected in the antigenic diversity of FMDV, both in field and in laboratory isolates, PCR products have been used also to compare sequences of the VP1 gene (that encodes the main antigenic determinant of the virus) of field isolates (Martinez et al., submitted for publication). In that case, the double stranded band was sequenced by the dideoxi method, using primers labelled with $p^{32}$ at their 5' end (see Wong et al., 1987), and Jansen et al., 1990 for details of the method). Direct PCR sequencing offers the possibility of determining the genomic sequence of virus without the need to purify large amounts of RNA. Sequences obtained by this method have been identical to those found by direct sequence of the viral RNA. Extension of this methodology to other genomic segments and to different isolates will help us in determining the appearance of new variant genomes and to better approach the important problem of virus variation in nature.

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# TAQ POLYMERASE CYCLING SEQUENCING OF PCR AMPLIFIED DNA

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## INTRODUCTION

We have developed a simple and rapid method for the direct sequencing of DNA fragments amplified *in vitro* by the Polymerase Chain Reaction (PCR). After the target DNA substrate has been generated by a standard PCR, sequencing is performed by a cycling reaction with Taq polymerase in the presence of dideoxynucleotides (ddNTPs). If both PCR primers are used, the presence of ddNTPs during the cycling reaction generates a ladder corresponding to the interrupted extension products from both DNA strands. While this procedure does not determine the nucleotide sequence of the DNA fragments analyzed, it is useful to determine their relative sequence differences, including point mutations. The nucleotide sequence of any of the DNA strands can be determined by omitting one of the primers during the sequencing reaction. Since this reaction is performed directly with an aliquot of the initial PCR product in the presence of a radioactive deoxynucleotide, purification of the PCR product or labeling of the primer is not necessary. These are significant improvements over currently available sequencing methods, both in speed and simplicity.

As an example of the applications of this method, we will describe here the experimental conditions for the detection of mutations and sequencing of the human p53 tumor suppressor gene using reverse transcribed total cellular RNA.

## EXPERIMENTAL DETAILS

### PCR of p53 gene cDNA.

Total cellular RNA was prepared from human colo-rectal tumors or from tumor cell lines as described (Forrester *et al*, 1987). p53 cDNA was synthesized using Reverse Transcriptase (RT) and an antisense primer located at the 3' end of the last coding exon (codons 387-394, Lamb and Crawford, 1986). The reaction was carried out as described (Kawasaki, 1990), in 20 $\mu$l of PCR buffer (see Figure 1) containing 20 units of RNAsin (Promega) and 2mM DTT, with 50 units of M-MuLV RT (Stratagene) and 0.1-1 $\mu$g of RNA, for 10 min at room temperature, 1 hour at 42ºC and 5 minutes at 95ºC.

Amplification of p53 DNA sequences was performed by two PCR experiments. In the first, a fragment of 1163 bp spanning all coding exons of the p53 gene was obtained.

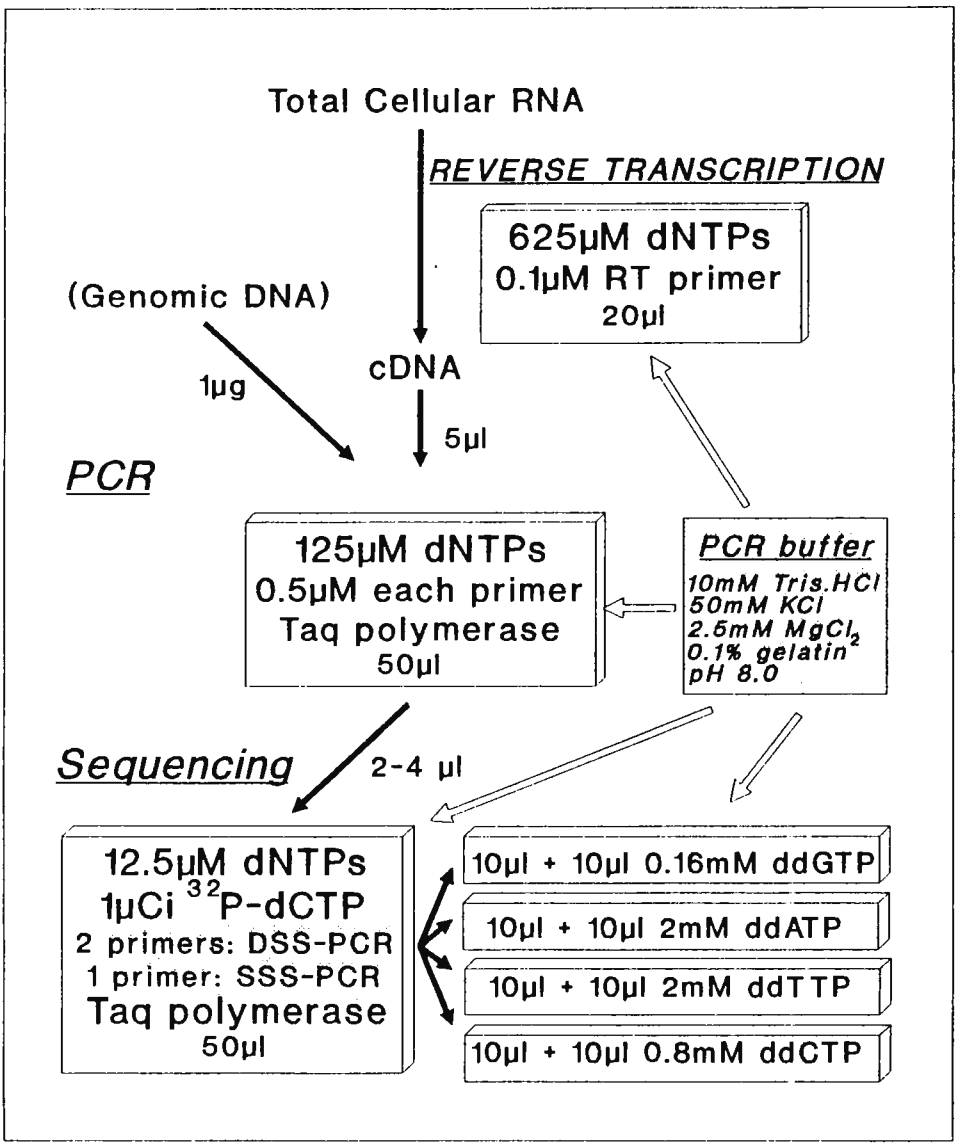


Figure 1.- Direct sequencing of PCR products.

Five $\mu$l of the RT reaction were added to a tube containing 1 unit of Taq polymerase (Cetus-Perkin Elmer), 0.1 $\mu$M of upstream (positions -23/-3) and downstream (codons 374-380) primers, and 125 $\mu$M each dNTP, in a final volume of 50 $\mu$l of PCR buffer. The reaction was carried out after 3 min at 95ºC, for 25 cycles in a thermal cycler (Perkin Elmer) at 95ºC 1 min; 53ºC 35 sec; 72ºC 2 min 15 sec. The second PCR was carried out with 0.1-0.5 $\mu$l of the previous reaction, 0.5 $\mu$M of nested upstream (codons 32-37, 53-59 or 129-135) and downstream (codons 325-331) primers, for 35 cycles at 95ºC 45 sec; 55ºC 35 sec and 72ºC 1 min 15 sec. The final PCR product included exons 5 to 9, or 4 to 9, depending on the upstream primer used. More than 95% of the reported mutations in the p53 occur between exons 5 and 9 (Hollstein *et al*, 1991).

**Sequencing of the PCR product.**

Two to four ul of the nested PCR product were added to a mix containing 12.5 $\mu$M each dNTP, 1$\mu$Ci of $\alpha$-$^{32}$P-dCTP and 2 units of Taq polymerase, in a final volume of 50 $\mu$l of PCR buffer, with either 0.5 $\mu$M each of the same nested primers (DSS-PCR), or 1$\mu$M of only one of these primers or of another internal sequencing primer (SSS-PCR). Ten $\mu$l of this solution were added to 10 $\mu$l of each of the four ddNTPs (Pharmacia) in PCR buffer. The dNTP:ddNTP ratios were approximately 1:10 for G, 1:120 for A and T and 1:50 for C (see Figure 1). The reaction was carried out for 30 additional cycles in identical conditions as the previous nested PCR. The final product was analyzed in a 6 % polyacrylamide denaturing sequencing gel.

## RESULTS

**Double Strand Sequencing by PCR (DSS-PCR)**

Figure 2 shows the DSS-PCR ladders obtained for the p53 gene from 3 human colon tumor cell lines. Single point mutations are reflected in the appearance and the dissapearance of single bands in the gel (marked with arrows). DLD-1 cell line shows a C to T transversion (lane 1, botton panel) at codon 241 (TCC-->TTC; SER-->PHE), while SW480 cell line shows a G to A transversion (lane 3, top panel) at codon 273 (CGT-->CAT; ARG-->HIS). The position of the mutations was determined by sequencing with only one primer (SSS-PCR, see below).

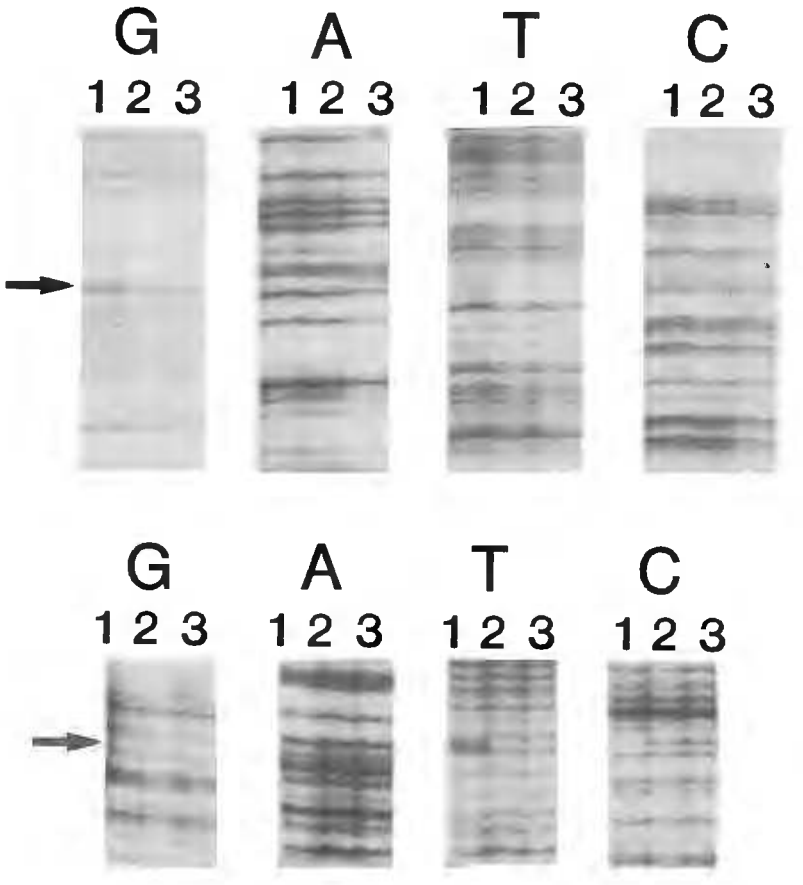


Figure 2.- DSS-PCR sequencing ladders of the p53 gene from human colon tumor cell lines.

Sometimes the bands diagnostic of the mutation can be coincident with bands corresponding to the other DNA strand. In that case, the mutation will be reflected only in a relative increase and/or decrease of the intensity of the band. However, each mutation is represented twice in complementary regions of the gel, therefore increasing the probabilities that it will be evidenced as a new band. The specificity of the approach is increased by the fact that a *bona fide* mutation has to fullfill two requirements: the appearance (or increased intensity) and the dissapearance (or decreased intensity) of a band in the same position in the gel. This helps to avoid false positive mutations due to artifacts of the PCR or sequencing reactions yielding spurious bands.

**Single Strand Sequencing by PCR (SSS-PCR).**

Because both DNA strands are used as templates in the extension reaction, there is an ambiguity in the localization of the mutations. This is the main disadvantage of the DSS-PCR approach. We found that to obtain a readable sequence of any of the DNA strands, it is sufficient to carry out the last radioactive cycling reaction with only one of the primers already used for the previous nested amplification, or with a single internal primer. In these conditions, essentially only one of the DNA strands is extended, incorporating consequently the radioactive deoxynucleotide. The concentration of the primers carried over with the aliquot of the nested PCR is very low relative to the newly added primer, and do not interfere with the generation of the sequencing ladder if the reaction is carried out for a sufficient number of cycles.

Figure 3 shows the sequencing ladders corresponding to the DSS-PCR and to the SSS-PCR for each of the DNA strands of the p53 gene from a human colon carcinoma. As expected, the DSS-PCR ladder is the composite of the two SSS-PCR ladders. One reaction yield sufficient sequence information of more than 500 bp of the p53 gene using indistinctively upstream or downstream primers. But at least two loadings are required to cover a reading frame of 500-600 bp. Due to the use of labeled dCTP of low specific activity, the region of the first 50-80 bases closer to the sequencing primer is difficult to read. Mutation detection is facilitated by loading the samples ordered by the ddNTP (Figure 2) rather than all the four ddNTPS one next to the other (Figure 3).

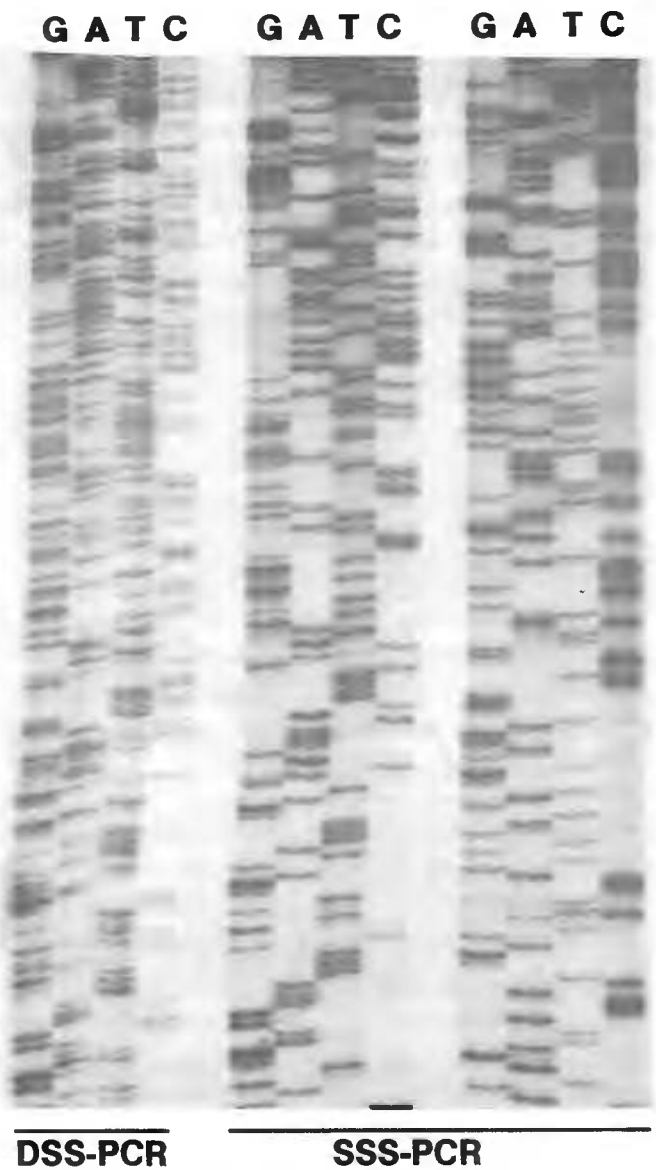


Figure 3.- DSS-PCR and SSS-PCR sequencing ladders of the p53 gene.

## DISCUSSION

There are a number of factors that are critical for the success of the method. Like for all PCR-based sequencing methods (Brow, 1990; Gyllensen, 1989), one of the most critical parameters is the quality of the PCR product. In our hands, the easiest way to achieve a clean amplified DNA product was the use of nested PCR. This is a common practice in our laboratory in order to eliminate artifacts and equalize the degree of amplification from sample to sample. However, this is not an essential prerequisite, and when the PCR product is not a unique band, an internal primer can be used for the sequencing, to minimize background and possible artifacts.

The amount of template used for the sequencing reaction is also an important factor. Since many of the synthesized strands are not completely extended due to the incorporation of a ddNTP, they can not be used as template in next cycles. Therefore, the target DNA used at the beginning of the cycling reaction is essentially the only template available. Less than 0.1-0.2 pmole of target DNA usually rendered faint sequencing bands. Whether one or two primers are used, the incomplete extension products from one or both DNA strands, accumulate essentially in a linear manner during the cycling reaction. Therefore, the sequencing reaction is very dependent on the number of cycles. We find 25-30 cycles to be optimum to achieve sufficient accumulation of the linearly amplified radioactive products. The radioactive bands are fainter with less cycles, while the background increases after more cycles.

The ddNTP:dNTP ratio is also important, but the main determining factor is the dNTPs concentration. If the dNTPs are at concentrations of 20 $\mu$M or more, even with the highest ddNTPs/dNTPs ratios used, only a very small proportion of the elongated DNA strands are stopped by incorporation of the ddNTP under our assay conditions. Thus, for optimal results, the dNTPs concentration must be about 10 $\mu$M or less. The ddNTPs concentration should be adjusted according to the length of the fragment.

Our method is similar to that recently reported (Ruano and Kidd, 1991) for the direct sequencing of PCR products, by adding dideoxynucleotides and an end-labeled primer after 15 cycles of an standard PCR and continuing the cycling reaction for another 15 cycles (Coupled Amplification and Sequencing, CAS). Both strands are

generated during the PCR amplification and the subsequent sequencing cycles, but only one of them is visualized by the incorporation of the labeled primer. This is the simplest method reported to date for direct sequencing of PCR products. However, our method offers the advantage over CAS and other similar methods (Richterich *et al*, 1991; Tracy and Mulcahy, 1991), of eliminating the previous labeling of the primer. Moreover, the conditions for the first amplification step(s) (mainly dNTPs and primer concentrations) are not restricted by the last sequencing step of the method and consequently, the performance of the PCR amplification is optimum.

In summary, the sequencing method described here is simple and fast, it is a true direct sequencing of PCR products and do not require labeled primers. In addition to the intrinsic advantages of the use of the Taq polymerase (high processivity, generation of large amounts of product, activity over a broad range of temperatures, etc.), we believe that the method should have wide applications for sequencing many other genes because the assay conditions for the sequencing are practically identical (except for minor changes) to the conditions for the PCR amplification.

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# ABSTRACTS

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## GENETIC CHARACTERIZATION OF HIV ISOLATES BY THE RNase A MISMATCH METHOD.

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The RNase A mismatch method is a simple technique that allows the primary genetic characterization of viral isolates. It is based in the ability of RNase A to cut single mismatches in RNA heterohybrids. The technique was used for the detection of activated *ras* oncogenes in human tumors using RNA:RNA hybrids (1) and of mutant globin genes using RNA:DNA (2) hybrids.

RNA viruses are characterized by a great genetic variability that is exemplified by the occurrence of numerous mutations in each strain. As a consequence, when viruses are compared to a reference strain, each strain yields a complex pattern of bands, after the gel separation of the fragments resistant to the RNase A digestion. However, this pattern is specific for each strain as a fingerprint. Furthermore, despite the presence of many mutations accumulating in each virus, the occurrence of common bands in the digestion pattern allows the establishment of relatedness among strains. Therefore, the technique is useful in molecular epidemiology and variation studies of viruses. This was demonstrated in RNA viruses with influenza virus as a model (3).

We have also used this method for studying different aspects of the genetics of the human immunodeficiency virus (HIV): comparison of sequential isolates from infected individuals, molecular epidemiology studies and detection of phenotypic variants such as AZT resistant viruses.

We have analyzed field isolates from San Diego (California, USA) and Madrid (Spain). By analysis of the ENV gene, we have detected the presence of two common genotypes circulating in the San Diego area: one related to the III-B/LAV reference strain and another to the SF-2 virus. Spanish isolates appear more homogeneous and closer to the III-B/LAV virus.

When the RT gene was similarly analyzed, we detected among the isolates the presence of a set of bands that could represent the result of cleavage at the mutations responsible for the AZT resistant phenotype. The resistance to AZT has been ascribed to a series of four mutations at positions 67, 69, 215 and 219 of the RT gene. The mutation at codon 215 is a double substitution that is recognized and cleaved by RNase A very efficiently. Therefore the method is useful for the screening of AZT resistant viruses because its relative simplicity.

These studies were performed using total cellular RNA from infected cultures. To avoid the need of the culture process that disturb the viral population and also is very time consuming we undertook the characterization of strains by amplification of viral RNA with the PCR. This was performed first from infected cultured cells and later by direct amplification from peripheral blood lymphocytes from infected individuals.

In summary, the RNase A mismatch method offers an easy, rapid and convenient approach to the primary characterization of isolates of HIV and other viruses and to the detection of phenotypic variants. The PCR avoids the need of cultivation, it can be used in cases when very small viral load is present and it increases the scope and possibilities of the technique.

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## GENERATION OF DOMINANT-NEGATIVE MUTATIONS OF THE c-*erbB-2/neu* ENCODED ONCOPROTEIN

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The proto-oncogene c-*erbB-2/neu* encodes an EGF receptor-like transmembrane protein of 185 kDa (gp 185). It is amplified and overexpressed in human breast cancer and can act as a dominant oncogene when overexpressed in mouse fibroblasts. In the nervous system the *neu*-oncogene is activated in experimental neurogenic tumors of the rat by a single point mutation in the transmembrane domain. In both instances the transformed phenotype is associated with a constitutive phosphorylation of the receptor molecule. Introduction of a mutated form of the c-*erbB-2* gene product with a defect or deletion in the tyrosine kinase domain interferes with receptor oligomerization and phosphorylation. This may result in a reversion of the transformed phenotype and tumor growth inhibition.

To test this effect we have generated two tyrosine-kinase negative c-*erbB-2* constructs using the polymerase chain reaction (PCR): One construct contains a point mutation in the ATP-binding site and the second an insertion of two STOP in position 814/815. This converts a valine to an alanine (KA753) and deletes the carboxy terminal 440 amino acids (CD440), respectively. The strategy for site directed mutagenesis during PCR was this:

In the first PCR round oligonucleotides containing the mutated sequence and a restriction site flanking the mutated site were used as primers in one reaction. In a separate second reaction oligonucleotides containing the sequence complementary to

the mutated site and an downstream flanking restriction site were used as primers. The c-*erbB-2* cDNA was used as template. In the second PCR round the oligonucleotides containing the flanking restriction site were used as primers and the PCR products of the reactions of the first PCR round were used as template. The resulting PCR fragment containing the mutated sequence was then cloned in an expression vector.

NIH 3T3 fibroblasts were transformed by the activated c-*erbB-2* which contains the same point mutation in the transmembrane domain as the rat *neu* oncogene. Clones were transfected with the kinase-negative constructs. We observed a growth inhibition in those cell populations in which the activated oncogene and the kinase-negative construct were expressed at almost the same level. This growth inhibition could be due to dominant-negative effect by heterodimer formation of the mutated construct with its wild-type counterpart. Neurinoma cell lines derived from ENU-induced rat tumors which contain the point mutation in the *neu* oncogene were also stably transfected with the kinase-negative constructs. In these experiments we observed a growth inhibition by the insertion of the kinase-negative c-*erbB-2* construct but also in some instances by transfection with the normal rat *neu* proto-oncogene.

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## PCR GENOTYPING OF FORMALIN-FIXED AUTOPSIED BRAIN TISSUE FROM ALZHEIMER'S DISEASE PATIENTS

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Recent studies on the genetic localization of the gene defect responsible for familial Alzheimer's Disease (AD) suggested the existence of at least two loci responsible for the presenile form of AD (1). In two AD families a mutation has been detected in the Amyloid Precursor Protein (APP) gene located in 21q21 (2). However, in other families DNA markers located in the centromeric region of 21q have also shown suggestive linkage (3). Due to this genetic heterogeneity it is essential to be able to perform linkage analysis in a single pedigree. To overcome the problems raised by the low information content of most AD pedigrees we followed two strategies.

First, using the Polymerase Chain Reaction (PCR) we genetically typed deceased patients from two Belgian AD pedigrees using formalin-fixed autopsied brain tissue and/or paraffin embedded formalin-fixed brain tissue. The DNA of these fixed tissues is severely degraded and therefore not analyzable by the Southern blot technique. However, previous reports proved the feasibility to use this DNA as template for PCR analysis (4). Therefore we mapped, cloned and sequenced 2 RFLP's associated with locus D21S13, located in 21q11.2, and designed PCR primers and allele specific oligonucleotides for the analysis of the polymorphic sites (5,6). We designed extra primer-sets in order to amplify DNA segments with a maximal length of 200-300 bp. Locus D21S13 has shown suggestive linkage to AD in the Belgian FAD families (3). In addition we used the PCR technique to prove that the patients' DNA's do not contain the FAD APP mutation (2).

Second, probes showing linkage to FAD were made more informative by isolating highly informative $(CA)_n$ repeat polymorphism. We recently identified a polymorphic $(CA)_{14}$ repeat polymorphism in a cosmid corresponding to locus D21S13 (7).

The information gained by these two strategies can now be used to obtain more conclusive linkage results.

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## PCR AS A DIAGNOSTIC TOOL: A TALE OF THREE VIRUSES

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It is a truism that different viruses interact with their hosts in different ways. Classically, bacteriophages are thought of as having either a lytic or a lysogenic life cycle. Many human viruses can be viewed in an analogous way. Human immunodeficiency virus (HIV) is, in this view, a lysogenic virus. It can integrate in the cellular genome and subsequently reactivate leading to full blown infection. The presence of even one complete provirus can therefore be life threatening. Although serological tests for viral antigen and induced host antibody are sufficient to diagnose most HIV infections, there are few situations where only PCR will detect the presence of the viral genome in the infected individual.

Hepatitis C virus HIV), the recently characterized agent of most parenterally transmitted non-A, non-B hepatitis can also be thought of as a lysogenic virus. Although the mechanism of its persistence is unclear, it is apparent that patients can be chronically infected and frequently develope cirrhosis and sometimes hepatocellular carcinoma. At present the only serological tests that are available are based on recombinant expressed antigen and detect, with varying degrees of specificity, the host's antibody response. PCR is the only method currently available for detecting circulating virus and confirming the accuracy of the antibody tests.

In contrast to HIV and HCV, parvovirus B19 is a lytic virus. Viraemia is brief, lasting perhaps five days, and intense, and often precedes the onset of symptoms (usually a feverish illness with a rash). Diagnosis can be best achieved through a combination of tests for specific IgM, IgG and viral antigen. A dot hybridization test for the presence of

viral DNA is useful a adjunct to these serological assays. In a laboratory that has reagents necessary for these tests, PCR is not very useful as it merely extends the window through which virus is detectable into the recovery period of the infected individual. It is just too sensitive for a routine diagnostic test. However, because B19 cannot be easily cultivated and supplies of antigen for development of tests are confined to a few laboratories, PCR allows the detection of B19 in laboratories where otherwise it would be impossible.

Therefore, as a diagnostic tool PCR needs to be considered in the light of the life cycle of the virus being detected; the significance of the presence of a few copies of the viral genome; and the availability of other, complementary tests.

## TRANSCRIPTIONAL STIMULATION OF THYROGLOBULIN GENE BY INSULIN AND IGF-1 REQUIRES CIS AND TRANS ACTING SIGNALS

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The regulation of gene expression by hormones is a key phenomenon in the physiology of multicellular organism. Hormone action on gene transcription was widely studied in the last years. Respect to insulin, is known that this hormone regulates the expression of several genes (1) however very little is known about the intrinsic mechanism involved in that regulation and in particular the transcriptional events. The regulation of the Thyroglobulin (Tg) mRNA synthesis in the FRTL-5 cell line (2) represents a good model system to study the phenomenon. Our previous data demonstrated that insulin and IGF-1 increase 3 fold the Tg mRNA levels. Nuclear elongation assays indicate that the effect is due to an increase at transcriptional level (3). The Tg promoter has been well characterized: It consists of a 170 base pairs long DNA fragment that promotes transcription only in the biochemical environment provided by the differentiated thyroid follicular cells (4). This cell type specific expression is amenable to experimental manipulation because of the availability of the differentiated rat thyroid cell line, the FRTL-5. Using nuclear extracts from the FRTL-5 cells and from control cells unable to express the differentiated thyroid phenotype it has been demonstrated that two FRTL-5 specific (TTF-1 and TTG-2) and an ubiquitous factor (UFA) bind to the Tg promoter (5). TTF-1 binds at three sites in the promoter. Mutation to two of the three sites abolish promoter activity, suggesting that TTF-1 is an essential factor for thyroglobulin transcription and probably is the main mediator of the observed cell-type specific expression. Mutations at the TTF-2 binding site only decrease transcription by 50% while mutations at the UFA binding site also have a drastic effect on promoter function (5).

The thyroid-specific transcription factor TTF-1 has been recently cloned (6). The protein encoded by the cDNA shows bindings properties indistinguishable from those of TTF-1 present in nuclear extracts of differentiated rat thyroid cells. The DNA binding domain of TTF-1 is a novel mammalian homeodomain that show considerable sequence homology to the drosophila NK-2 homeodomain. To express TTF-1 and its homeodomain alone in bacteria, cDNA inserts were cloned into the expression vector pT7,7. The cDNA corresponding to homeobox was amplified by PCR using the oligos 5'-CCCGGGCATATGCGCCGGAAGCGTCGGGTGCTCTTCTCCCAG-3'and 5' CGCTGTCCTGCTGCAGTTACTGCTGCGCCGCC-3'. The amplified DNA was cut with NdeI and PstI and cloned in the homologous sites of pT7,7. The homeodomain alone is able to determine the binding specificity of TTF-1 (6).

The availability of the above described reagents makes it feasible to study in further detail the biochemical mechanisms responsible for the regulation of Tg transcription by insulin and IGF-1.

We transiently transfect to FRTL-5 cells the construction Tg promoter fused to the Choramphenicol Acetyltransferase (CAT) gene. Insulin and IGF-1 stimulate Tg promoter activity 4 fold. This response is similar to that found with the endogenous Tg gene, suggesting that the minimal promoter element contains the DNA signals necessary for insulin and IGF-1 regulation. Experiments of protein-DNA interaction indicate that a thyroid specific transcription factor TTF-1 from cells cultured with and without insulin has different mobility in the gel retardation assay. Another thyroid transcription factor TTF-2 is absent in nuclear extracts from insulin depleted cells. Readdition of insulin restores the TTF-2 concentration to normal levels. Cycloeximide blocked this effect suggesting that protein synthesis is required for TTF-2 induction by insulin, but did not block the effect on TTF-1. In these experiments the insulin effects were maximal at 24 hours and at a

concentration of 1$\mu$g/ml. The same effect were observed with doses 10 fold lower of IGF-1. These results suggest that insulin (probably through the IGF-1 receptor) stimulates, in FRTL-5, cells the activity of the Thyroglobulin promoter by modulating the levels of the trans-acting protein TTF-2 and postranslational modifying the trans-acting factor TTF-1. At the postranslational level the most studied mechanism, on transcription factor activation, is phosphorylation (6). Our hypothesis of work is that insulin/IGF phosphorylate the TTF-1 transcription factor.

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# SUMMARY

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The Polymerase Chain Reaction (PCR) is a methodology recently incorporated as a routine in any Molecular Biology laboratory. Nevertheless, what all of us attending the Lecture Course on The Polymerase Chain Reaction, held at Cuenca, have learnt is that doing a good PCR may not be as easy as it looks and that there are may subtleties both in the technique as in its diverse applications. I will try here to summarize a number of ideas and give some hints helpful at the bench that were developed during the course.

## PCR EFFICIENCY

K. Mullis, D. Gelfand and J. Sninsky told us how important it is to know the efficiency of the exponential amplification. Knowing the number of initial template molecules, it is possible to calculate the efficiency of amplification as follows:

$$Y = (1 + X)^N$$

where Y is the extent of amplification, X the mean efficiency, and N the number of cycles.

About $10^{12}$ bp are required to visualize a band in an ethidium bromide stained agarose gel. Thus, in optimal conditions, a single molecule of 100 nucleotides will produce a nice band after 39 cycles. However, $10^5$ molecules will produce the same amount of DNA after only 21 cycles. Yet, small differences in efficiency make a big difference in the final yield:

A 100% efficiency will produce 1.048.576 molecules after 20 cycles while with 80% efficiency only 127.482 molecules will be produced after the same number of cycles. That is, 12% of the expected yield. In this situation, whenever we have 10 copies of the template per reaction we expect to have 100% of positives cases, while 5 copies will produce 99% and 1 copy, only 63% of positive cases.

**PARAMETERS AFFECTING PCR**

*Annealing temperature and time: The highest possible temperature increases the specificity and the level of detection. Primer hybridization happens in 1/10 of a second.

*Denaturing temperature and time. It should be high enough to denature the template without compromising the half life of the enzyme. Half life of Taq at 97° C is 11 minutes while at 95° C is 40 minutes.

*Taq addition at elevated temperature enhances specificity. Addition of single-stranded binding proteins is equivalent.

*Enzyme concentration. Too much enzyme results in amplification of undesired products.

*$MgCl_2$ and dNTPs concentration. Each dNTP optima needs different $MgCl_2$ concentrations. 0.7 mM each dNTP needs 1.5 mM $MgCl_2$. Km for dNTPs is 10-15 $\mu$M.

*Primer concentration. Km for primers is 10-20 mM. Too much primer will result in the amplification of undesired products. For example, to increase specificity in the detection of retroviruses while keeping low background noise due to the presence of unknown endogenous elements similar to LTRs of retroviruses, one should go from 2.5 units of Taq, 2.5 mM $MgCl_2$, and 1 $\mu$M primers to 0.6 units of Taq, 1.25 mM $MgCl_2$, and 0.12$\mu$M primers.

*Number of cycles. Extra cycles decrease the quality of the final product and interfere with most of the quantitative PCR experiments. If one of the components, such as the number of primed templates or the number of Taq molecules becomes limiting,

the reaction will go linearly instead of exponentially. Product reannealing may lead to a decrease in both extension rate and processivity as well as to branch migration. Preferential amplification of "non-specific sequences" initially at low concentration can also occur during the PCR-late cycles.

*Cosolvents in the PCR buffer (helix-destabilizers such as glycerol, formamide, etc.) affect Tm, enzyme thermoresistance and thermoactivity.

Changes in Tm with different cosolvents:

| Cosolvent | Tm | Taq half life | Activity |
|---|---|---|---|
| nothing | 90° C | | |
| 10% glycerol | 87° C | 3 fold increase | |
| 10% DMSO | 87° C | | inhibition |
| 10% formamide | 83° C | decrease | inhibition |
| 10% glycerol +5% formamide | 84° C | 2 fold decrease | |

## PCR FIDELITY

To achieve the highest fidelity during PCR, dNTPs should be balanced, 40-50 $\mu$M each; $MgCl_2$, 1-1.5mM; annealing temperature, the highest possible; enzyme concentration the lowest possible; extension time, the shortest possible and the number of cycles, the fewest possible. The 61 kD fragment of the Taq polymerase (94kD), called Stoffel fragment lacks the 5' to 3' exonuclease activity associated to the polymerization activity. Therefore its fidelity is higher than Taq.

## PCR TROUBLE SHOOTING

The plateau effect is due to the properties of reactants: Competition (primer-dimer), utilization (dNTPs, primers), and stability (dNTPs, enzyme). Nested-PCR helps to increase the specificity of PCR particularly when the initial template is of low abundance

as from formalin-fixed material. Under particular conditions of reaction, thermostable polymerases, such as Tth show a reverse transcriptase activity. This activity requires $MnCl_2$ and 70° C, for 1 minute to produce full length products.

Cloning of PCR products often represents a difficulty, due to the addition of an A to the 3'-end of the product at the very late cycles when plenty of substrate has been accumulated. D. Gelfand gave several suggestions: 1) include restriction sites in the primers to clone cohesive ends, 2) when forced to clone blunt ends, phosphorylate the 5' end with kinase and repair the 3-'ends using the exonuclease activity of Klenow or T4 DNA polymerase in the presence of dNTPs, 3) use non-phosphorylated adapters, with phosphorylated primers.

## EQUIPMENT PERFORMANCE

The performance of the equipment is a critical step in the PCR reaction. The better time and temperature are achieved in every step, and the fastest it goes from one stop to the next, the better yield and quality of the product will be obtained. C. Oste recommended what to use and what to discard, too.

## CARRY OVER CONTAMINATION

Carryover was addressed by C. Oste and J. Sninsky, $10^{-7}$ $\mu$l of a PCR reaction contains 1 copy of the amplified PCR product so it could produce 63% positives cases in repetitive reactions. Use of positive displacement pipettes was recommended. Addition of uracil glycosidase together with substitution of T by U will inactivate the PCR material when desired. Only protein binding studies or certain endonuclease cleavages will be compromised by the substitutions of T by U in the PCR product.

## PCR APPLICATIONS

PCR products can be subjected to many different analysis. The patterns of restriction enzyme digestion are used in many cases to detect polymorphisms due to point mutations. H. Kazazian, K. Hayashi and M. Perucho gave us very good examples of them. Hybridization to specific oligonucleotides also indicates the presence of mutations in the sequence that was amplified. But the contrary can be done too, i.e. to fix the deoxyoligonucleotide(s) to the nylon membrane and use the PCR product to hybridize at the same time to different deoxyoligonucleotide(s). That is called blot-dot in Kazazian's lab. Haig Kazazian also described the application of denaturing gradient gel electrophoresis (DGGE) of PCR products to the diagnostic detection of point mutations in the factor VIII gene in hemophliacs. SSCP-PCR (single stranded conformational polymorphism) represents a way of exploiting the folding properties of single-stranded DNA molecules. In that case, denatured PCR products will reveal the presence of mutations by looking at the different mobilities of those molecules in a nondenaturing gel (K. Hayashi).

M. McClelland showed how to take advantage of the reproducibility of mispriming in the AP-PCR (arbitrarily primed-PCR) to map polymorphisms. A primer of 20 nucleotides will be unique in the human genome. Thus, by doing the first 2 cycles at low annealing temperatures, many imperfect matches are amplified. Then, a conventional PCR will amplify those rare but good matches. In that way, 3 to 6 polymorphisms can be obtained from each primer between two moderately divergent strains. The technique can also be used to detect polymorphisms in the human population as well as sequential alterations in the genomes occurring during tumor progression (M. Perucho). Mike McClelland also described another neat PCR adaptation, by amplifying the intergenic regions of tRNA genes with conserved primers, useful for bacterial taxonomic and diagnostic applications.

Direct sequencing of the product is done when the actual nucleotide sequence is required, as in many cases of diagnosis of genetic diseases (Kazazian), cancer diagnosis (M. Perucho, K. Hayashi), genetic variability of viruses (E. Martinez-Salas), etc. Both symmetric and asymmetric PCR was sequenced in different labs, with no consensus on why one method works better than the other. Presence of dideoxynucleotides during the PCR reaction is used to detect point mutations in amplified p53 tumor suppressor genes and to obtain their sequence during the PCR reaction (M. Perucho).

Sequences of independently cloned PCR products was shown by J. Sninsky to present evidence of genetic heterogeneity of HIV. C. Lopez-Galindez described the use of the RNase A mismatch cleavage method for detecting genetic variability in HIV and AZT resistant mutations of the virus by amplifying directly viral sequences from peripheral blood lymphocyte from infected individuals.

Obviously, primers are the most adequate target to modify the amplified sequence, such as incorporation of restriction sites or ATG and stop codons when required, as in the detection of point mutations in *ras* oncogenes (M. Perucho) and the cloning of FMDV genes for expression purposes (E. Martinez-Salas), respectively.

There are other cases in which tolerance for priming events is needed. RNA viruses such as HIV (J. Sninsky) or FMDV (E. Martinez-Salas), are characterized by a high degree of genetic heterogeneity, a property shared with all the genomes described as quasispecies. In that case amplification done in very stringent conditions will eliminate from the final PCR population those molecules which because of the genetic heterogeneity are not efficiently primed. In fact, J. Sninsky gave some rules to obtain specificity or mismatch tolerance.

| mismatch tolerance | sequence specificity |
|---|---|
| 25-36 nt oligo | 14-18 nt oligo |
| high dNTP | low dNTP |
| low anneal. temp. (25° C) | High anneal. temp. (60° C) |
| more cycles | Less cycles |
| internal mismatches | 3' terminal mismatch |
| 3'T | 3'C (avoid G:T) |

Another consideration to have in mind is that a G-T mismatch will be equally amplified than a fully matched template. T-T, T-C, A-A, OR G-G mismatches will be amplified less than 1% efficiently under stringent conditions. C-C mismatches are among the less tolerated.

In conclusion, several applications of PCR to different aspects of Biology were heard at Cuenca. Diagnosis of genetic diseases, such as $\beta$-Thalassemia, cystic fibrosis, Duchene muscular dystrophy, hemophilia, can be detected prior to birth. Molecular diagnosis of cancer can be facilitated by the PCR. Most of the infectious diseases, including HIV infection, can be detected quickly and with accuracy to discriminate between serotypes when it is needed. Efficient detection of new variants naturally occurring as well as induced by drug resistance will help in the treatment of the disease as well as in our understanding of the mechanism of viral evolution. Mapping of polymorphisms among related strains or among different individuals from the same species can be done with just a few nanograms of DNA and one primer. Those are cases of application to the day by day life. Nevertheless, the uses of PCR is countless, as shown by the different scientific background of the participants. Their participation helped all of us to have interactive and profitable discussions.

# LIST OF INVITED SPEAKERS

Lecture Course on
THE POLYMERASE CHAIN REACTION

List of Invited Speakers

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THE POLYMERASE CHAIN REACTION

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Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon and F. Jacob.

244 **Course on Genome Evolution.**
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246 **Workshop on Tolerance: Mechanisms and implications.**
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247 **Workshop on Pathogenesis-related Proteins in Plants.**
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248 Beato, M.:
**Course on DNA - Protein Interaction.**

249 **Workshop on Molecular Diagnosis of Cancer.**
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251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
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253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
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254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P. Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Camillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop on The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

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