IN SITU PCR

TECHNIQUES

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FOREWORD

Mullis's invention of the polymerase chain reaction (PCR) and its application to the detection and analysis of specific nucleic acid sequences revolutionized virtually all areas of molecular biology and has given us a wealth of new applications. Similarly, perfection of techniques for gene amplification in situ may be expected to fuel accelerated developments in the heightened understanding of embryogenesis and organogenesis as well as the pathogenesis of infectious, genetic, immunologic, neoplastic, and other disease processes. More than 300 papers describing the use of these techniques have already appeared, but the procedures remain cumbersome and difficult to reproduce, with many potential variables (as reviewed by Komminoth and Long). Bagasra and co-workers have provided a great service in giving us this comprehensive protocol. They have included hints and laboratory secrets that frequently get left out of scientific papers, making repetition of published experiments so problematic. This monograph should prove immensely useful not only for the novice but also for the experienced worker striving for maximum specificity or sensitivity in qualitative searches for cells containing low levels of genes or messenger as well as for those seeking greater sensitivity, precision, and reproducibility in enumeration of such cells.

As one of the true pioneers in the field, Bagasra has brought a wealth of experience to the preparation of this protocol. I recall our first discus-

sion of in situ amplification in his laboratory at the University of Medicine and Dentistry of New Jersey. We were preparing a collaborative protocol for study of the transmission of HIV-1 from mother to fetus using conventional PCR. In the course of the discussion, we realized that although PCR had great potential for early diagnosis of infant infection for studies of pathogenesis it would be incalculably valuable to be able to use the same amplification technique to detect individual HIV-1-infected cells, to distinguish between latently and productively infected cells, and to differentiate maternal from infant cells in tissue sections and in cytocentrifuged or cytofluorographed cell suspensions. I guessed that a number of large laboratories would be working on such applications and urged Bagasra—at the time a lone researcher—not to enter the race. Fortunately, he did not heed my advice and, in fact, spent much of his spare time developing his technique for in situ amplification. By the time he moved to Temple University and St. Christopher's Hospital for Children in 1990, he had already found at least preliminary solutions to the most vexing problems, and he made the system work for complementary HIV-1 DNA in presumed latently infected cells in which HIV-1 DNA and RNA were undetectable by direct hybridization. This was described in the Perkin Elmer Cetus periodical *Amplifications*. He and I filed half a dozen grant applications to develop the technique or use it in conjunction with other assays for study of the pathogenesis of perinatal AIDS infection—none of which was funded. Rather, most were returned with bitter criticism by experts, who claimed that it would be impossible to preserve cell integrity at the temperatures required to anneal DNA and impossible to prevent diffusion of the new amplification products from the cell—even after data and photomicrographs were presented to show that these problems had already been partially surmounted. Because we were unable to fund his work, by 1991 Bagasra had to move to Thomas Jefferson University. At that time, the assay had become sufficiently precise in recovery of HIV-1-infected cells added to populations of uninfected cells and reproducible to such a degree as to give us confidence in our findings that both adults and children had many more infected cells in their peripheral blood than had previously been suspected. At Jefferson, Bagasra refined the assay; definitively documented the relatively high proportion of CD4+ lymphocytes and monocytes containing HIV-1 DNA; and went on to initiate innovative applications of the technique to a variety of basic problems, particularly the pathogenesis of HIV-1 infection.

In 1990, Haase and co-workers, working independently of Bagasra, reported success in accomplishing in situ amplification with an interesting innovation: the use of a set of multiple overlapping primers to produce amplification products spanning more than 1000 base pairs, which are large enough to slow diffusion from their site of origin. However, the efficiency of amplification in this system is low, and Bagasra found that the large number of cycles required to get sufficient amplification frequently leads to more nonspecific labeling than with the use of single primer sets. Nuovo and co-workers implemented yet another innovation, that of incorporating digoxigenin-labeled nucleotides into the PCR amplicons so that the in situ PCR products might be detected directly by histochemical techniques. Komminoth and co-workers, however, have shown that such direct labeling is frequently associated with a false-positive labeling of cells. Patterson and Wolinsky developed a curious variation: amplifying and labeling cellular signals in tubes, then characterizing the cells through flow cytometry. Bagasra's approach to in situ amplification of gene sequences (described in this protocol) appears to be especially sensitive and specific. Specificity can be further increased by use of more than one set of primers for different genes from the same organism or from different regions of the same gene or message.

Even with the aid of this manual, today gene amplification in situ remains a difficult research procedure. Conceptually, however, it offers tremendous potential as an aid to clinical diagnosis through its ability to detect a single copy of a specific microbial, neoplastic, messenger, or mutated nucleic acid sequence in a cell smear, cell suspension, tissue section, or chromosome. A key challenge for the future will be the simplification of these techniques so they can be brought to the laboratory of the clinical pathologist.

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PREFACE

I know nothing except the fact of my ignorance.
—Socrates, as reported by Diogenes Laetius

Possibly we shall one day know a little more than we do now.
But the real nature of things, that we shall never know, never.
—Albert Einstein

Pass beyond forms, escape from names, flee titles and awards, walk towards meaning.
—Rumi

In situ polymerase chain reaction (PCR) is a powerful invention, allowing an investigator to visualize the presence of a single-copy gene, an individual virus, or a low abundance RNA signal in its original location inside a cell or tissue. Never before has a technique quite so sensitive been available to scientists who work in cytology or histology, and it is our hope that reproducible practice of the technique will serve as a wellspring from which will flow new understandings in biology and medicine.

Simultaneously, we wish to point out that in situ PCR is a relatively new technique that still exhibits characteristics of both science *and* art. DNA and RNA are, by themselves, each exceedingly complex phenom-

ena; but when these genetic molecules do their thing inside the complex milieu of whole cells and tissues, they sometimes exhibit characteristics that will probably always defy complete understanding. Empirical work has led to substantial knowledge of what works and what does not experimentally; and in this volume, we communicate this practical knowledge as best we can. But readers should realize that in situ PCR represents a sharp edge in research; sometimes it cuts through to greater understanding and sometimes it just cuts the self-confidence of those investigators who choose to play with it.

By no means do we wish to discourage any scientist from trying in situ PCR; rather we just wish to point out that ignorance still abounds in the field, and many promising pathways remain unexplored. This represents great opportunity for the imaginative scientist, and we encourage investigators to explore what might seem to be, at first glance, far-fetched ideas. But please practice a bit with simpler systems first, for much practical knowledge on the manipulation of signals, cells, and detection systems must be learned before proceeding onto the pioneering work.

Where has this protocol come from? For the past four years, one of us (OB) has offered numerous workshops and seminars on in situ PCR in an effort to communicate to as many investigators as possible the in situ techniques that were developed in his laboratory at Thomas Jefferson University. Simultaneously, the other author (JH) has worked in industry to develop instrumentation to drive the reaction with increasing ease and reproducibility. Between the two of us, we have listened to and tried to answer the myriad questions that constantly arise from the ever-curious seminar participants and from inquiring scientific colleagues. In doing so, we have learned much about what matters need special clarification, where technical problems often arise, and how to best present the subject matter in a manner that will be comprehensible to nonspecialists in molecular biology as well as to nonspecialists in tissue morphology.

We have tried to cover as many aspects of in situ PCR as possible, but we have included only those aspects with which we have personal experience. Thus this volume represents a specific viewpoint, and it is not intended to be a comprehensive textbook of all matters pertaining to in situ PCR. Rather it is intended to be a practical manual for use in the laboratory, and we hope the many photos will prove especially instructive.

We are especially indebted to Sashamma Thikavetapu, Lisa Bobroski, and Patricia Whittle, who assisted in numerous workshops and who gave us many excellent suggestions. We also wish to thank the entire staff of

the in situ laboratory at Thomas Jefferson University, all of whom have played an important role in the development of this protocol. We also wish to thank the Bagasra family—Theresa, Alexander, and Anisah—who cheerfully tolerated the two of us trying to iron out the various details while working in the basement of the Bagasra home in Laurel Springs.

Last, we wish to thank the many scientific colleagues who participated in the in situ seminars, for they provided the impetus for the development of this protocol. We have always been most impressed by the imagination of these individuals, who are constantly proposing new and exciting applications for in situ PCR. It is our fervent hope that you, the reader, will prove to be just as imaginative. In fact, it is our fervent hope that this very volume will become stained with the many colors that can now be used for multiplexed hybridization of PCR products—marks that you leave behind as you blaze new pathways to scientific discovery.

CONTENTS

CHAPTER 1

REVIEW OF THE PCR TECHNIQUE

This chapter is a primer for those investigators for whom molecular techniques are a relatively new experience. Many of those interested in performing in situ polymerase chain reaction (PCR) do not come from a molecular biology background; rather they are often pathologists, for example, skilled in careful tissue preparation and exacting histological analysis but not in the design of oligonucleotide primers and probes. Thus this chapter is an overview of the relevant molecular information. It reviews the history of the PCR technique, describes how it works, and details many of the technical points important for successful gene amplification. For those already familiar with these matters, perhaps the sections on the design of primers and probes might prove useful, particularly in regard to obtaining sequence information over the Internet. Otherwise, those investigators skilled in molecular biology might wish to skip to subsequent chapters, where the in situ methodologies are detailed.

KARY MULLIS'S INVENTION: THE POLYMERASE CHAIN REACTION

In the mid-1980s, Kary Mullis, a biochemist working for the Cetus Corporation, invented a method to identify a specific DNA sequence in an

aqueous solution that contains myriad sequences of DNA and then to geometrically amplify the targeted sequence millionsfold through a semi-automated procedure that takes just 1 or 2 h. This synthetic process makes available enough of the targeted DNA for ready analysis by conventional laboratory techniques, even if there was just one molecule of the DNA in the solution to start with.

According to Mullis, this invention originally sprang from a scheme he was devising to keep employed several technicians who worked in his laboratory. The lab specialized in synthesizing short chains of DNA called oligonucleotide primers (oligos), and apparently demand within the Cetus Corporation for these synthesized molecules was insufficient to justify the level of staffing Mullis was then enjoying. He was, therefore, trying to devise some sort of new application for the oligos that would consume large quantities of the lab's product. Following his famous drive one weekend up the Mendicino coast of California and his "Eureka!" experience at a highway rest area, the polymerase chain reaction (PCR) was born.

Since those salad days of PCR, methods have been found to simplify the procedure, particularly through the use of thermally stable polymerase enzyme, and to automate the process, through the adaptation of microprocessor-controlled thermal cyclers. Furthermore, reverse transcriptase (RT) reactions have been added, if one wishes to identify and amplify targeted RNA sequences by first converting them to cDNA templates; this is sometimes called RNA PCR.

Application of PCR has now spread far and wide throughout biotechnology. In particular, the reaction has found many uses in molecular biology laboratories for identifying and generating large quantities of DNA for routine assays, for example, as well as for cloning, gene mapping, and engineering new forms of DNA through in vitro mutagenesis. In medicine, PCR has proven useful not only for the identification of infectious agents but also in the diagnosis of genetic disease and in the understanding of the pathogenesis of disease processes. In forensic science, PCR has revolutionized the practice of DNA fingerprinting and HLA typing. In plant genetics, PCR has created new tools for analyzing and accelerating breeding experiments as well as easing the chore of precise taxonomic classification. The list goes on and on; and in 1993, the Nobel Prize Committee recognized Mullis's seminal contribution and awarded him the Nobel Prize in Chemistry for his invention.