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LOCAL BIOREAGENT PRODUCTION IN DEVELOPING COUNTRIES*

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SUMMARY

The expansion of genetic engineering technology in most developing countries has brought forth evidence of a problem with reagents supply. The problem is not only a financial one of finding an adequate source of foreign currencies, but in many cases, the logistics for locally delivering deep-frozen biological products sent from abroad are not routine. This fact can be strong justification for local manufacture.

The overriding factor in the "make or buy" decision is to meet a growing need for products in teaching laboratories for students and technical trainees. Local production lends itself well to this particular need: few products, needed in relatively large quantity, and for which technical specifications may not be as exacting as for commercial research products.

Several aspects of local manufacturing are addressed in this report. Since Thailand has been one of the early players on the genetic engineering scene, Thai scientists were interviewed and the communication of their experience has been very valuable in the elaboration of this study.

The production of enzymes, oligonucleotides and biologicals were surveyed. Many products can be made in commercial quantities on a laboratory scale because they are used in very small quantities. In the case of enzymes, genetic engineers have applied their technology to their own needs; they have developed strains that overproduce enzymes by the millions of units at shake flask scale.

For enzymes and biologicals, a well-equipped laboratory for microbial production and biochemical purification is required. Acquisition of high-producing strains is an important step. For strains that are not available commercially, or which are under patent protection, an international

effort should be organized for the engineering of over-producing micro-organisms and their subsequent distribution to developing countries. Microbial strains are much easier to transfer than their products, and they could actually form the nucleus of a reagent commerce between developing nations.

For deoxyoligonucleotides, equipment available in most organic chemistry laboratories is needed. Reagents are not expensive. A kit, offered by several suppliers, (see Appendix 1) simplifies enormously the initial acquisition process and still gives the benefit of substantial savings in labour. A genetic engineering group reaching high productivity stages will find a need for many oligonucleotides and at that point the acquisition of an automated synthesizer becomes sensible. In the choice of a machine, availability of a local maintenance service should be of high consideration.

Production problems will be encountered mainly in the production of enzymes. They include the lack of an easy assay, which complicates the task of optimizing production and purification processes, and the exacting requirements for final product purity. Another problem may be a high turnover in technical labour, because initial efforts of this type are usually only temporarily funded. The high number of products in the line eventually creates inventory problems. However, these difficulties can be overcome and the local production of reagents then permits local expansion of genetic engineering technology, especially by making the mass-training of technical personnel possible.

INTRODUCTION

Genetic engineering is a relatively new discipline which has propelled biotechnology to the forefront among technologies capable of improving the economic well-being of the world. Its contemplated applications range from health to agriculture and to environmental control.

Among high technologies, genetic engineering is unusual by the speed with which it is spreading, both in developed and developing countries. Its basis in biological sciences has met a receptive audience among the healthcare infrastructure present at least to some extent in every country. The scope of its applications has caught the attention of every technocrat. Its reliance on renewable raw materials for large-scale production also makes it desirable. Compared to other high technologies, genetic engineering requires relatively little capital, in R&D as well as in manufacture. Finally, its reliance on fermentation as a major unit operation brings a familiarity justified by the centuries of experience many countries have developed in the preparation of fermented foods and beverages.

As a result, world-class research is already being conducted in a few developing countries and several international initiatives have been formed to promote the spread of this new technology. This report is a result of such an initiative, supported by the Department for Industrial Promotion, Consultations and Technology of the United Nations Industrial Development Organization (UNIDO).

The purpose of the work is to define the standard bioreagents supplies required to perform genetic engineering laboratory research, to analyze the financial and technical aspects of producing these bioreagents in a developing country and to outline the steps necessary to set up the production facilities. The rationale that prompted this study was the expense of the materials (in

foreign currency) and the great difficulty of ensuring proper shipment due to the short life of dry ice packages, necessary for many of the reagents.

The specific tasks were reviewed in Vienna before the start of the study and will be discussed in the following section of this report.

Budgetary limits have confined the study to one country but it is hoped that the results will be useful to other nations.

I. BACKGROUND

Scope

Typically, the annual cost of a genetic engineering researcher in developed countries is US$ 100,000, out of which US$ 15,000 is for reagents and disposable supplies. A telephone survey of laboratories and suppliers yielded a consensus approximate distribution as follows:

- Chemicals, radiochemicals: US$ 5,000
- Plastic-ware and disposables: US$ 5,000
- Biochemicals (enzymes, probes): US$ 5,000

Among these three major groups, only the third one is of relevance within the scope of this study for the following reasons:

a) The decision for local manufacture of supplies of the first two groups could not be made within the context of genetic engineering technology alone; and

b) Shipping problems are not as acute for the first two groups, even in the case of short-lived radiolabels. In any case, local production of radiochemicals is not often an economic option in developing countries.

For these reasons, the products of interest in this report will be limited to the third group, including:

- nucleic acid processing enzymes (restriction endonucleases, ligases, polymerases, nucleases, methylases, reverse transcriptases, etc.);
- host strains, vector plasmids and cosmids, biologically derived DNA and DNA packaging systems; and
- oligonucleotides, short DNA probes and synthetic genes.

The cost of bioreagents per unit of product can be expected to be higher in developing countries for several reasons: increased shipping distance, customs clearing costs, higher likelihood of losses and smaller size of individual orders. On the other hand, it is not expected that the yearly cost per researcher will be any higher than in developed countries as due to higher

budgetary constraints, better utilization of the products will be the rule and the strategy for experimental design will be to minimize the number of reagents involved in a given project, even if it results in a higher manpower requirement.

Regarding the choice of the locale for this study, the early start of Thailand (1981) and its national commitment to genetic engineering and biotechnology made this country a logical candidate. The fact that a group at Mahidol University in Bangkok had started local production and distribution of several of the products considered in this study was also a helpful consideration. The timing of the visit to Thailand coincided with a technical seminar organized by the National Center for Genetic Engineering and Biotechnology, in which a paper was presented by this group, relating their experience in making and distributing bioreagents.

Although Thailand may not be a representative case for all developing countries, problems and opportunities encountered there may be drawn upon by other nations in arriving at a "make of buy" decision for bioreagents.

Methodology

The data for this study were gathered in two distinct phases: a "field study" phase, consisting of a series of interviews with various groups in Bangkok, Thailand, and a technical phase, consisting mostly of telephone interviews with U.S. genetic engineering laboratories, bioreagents suppliers and technical experts.

In Thailand, the groups interviewed and their representatives are shown in Table 1. The interviews were centered on the genetic engineering component of individual projects, on the requirement and availability of bioreagents and on the "make or buy" issues concerning genetic engineering materials.

In the United States, major suppliers of bioreagents were contacted by telephone, at the level of sales managers. The purchasing agents of two large genetic engineering companies were also contacted. One technical expert on

restriction endonucleases, Dr. Roxanne Walder, of Iowa University and one technical expert on oligonucleotides, Dr. John Hachmann of Sigma Chemical, St. Louis, Missouri, were also interviewed by telephone. Products catalogues for bioreagents and equipment from North American, European and Japanese suppliers were consulted.

Table 1. Groups and personalities contacted in Thailand

Group	Persons visited
Thailand Institute of Scientific and Technological Research, Bangkok	Dr. Poonsook Atthasampunna Director, Biotechnology Department
National Center for Genetic Engineering and Biotechnology, Bangkok	Prof. Yongyuth Yuthavong Director
Mahidol University Department of Biochemistry	Prof. Sakol Panyim Head Prof. M.R. Jisnuson Svasti Project Leader
King Mongkut's Institute of Technology Dept. of Chemical Engineering	Prof. Sakarindr Bhumiratana Chairman
Mahidol University Department of Microbiology	Dr. Amaret Bhumiratana Head of Pilot Plant Prof. Pornchai Matangkasombut Chairman, Director Biotechnology Programme

II. NUCLEIC ACID PROCESSING ENZYMES

The tools of genetic engineering

Enzymes capable of synthesizing, cutting, protecting, and splicing nucleic acids truly constitute the tools of the genetic engineer. While this report is not intended to be a scholarly treatise on the subject, this short section will provide a few definitions for the layman.

The genetic material of most living organisms (found in chromosomes) is double stranded DNA, a linear polymer of four different nucleotides. The order of the sequence determines the genetic information. The two strands are complementary, like a mould and its casting, giving DNA an inherent capability for self-reproduction. Single stranded RNA is a working copy transcribed from DNA.

Restriction endonucleases cut double stranded DNA always at the same nucleotide sequence at a precise spot in the sequence, often leaving overhangs. It is then possible to mix DNA's from different species processed by the same restriction endonuclease and "splice" randomly new genetic sequences. Ligases are then used to covalently bind the rearranged genetic material. Reverse transcriptases are used to synthesize DNA from RNA. Various nucleases and polymerases are used to advantageously modify DNA pieces to achieve desired results.

Using these various tools, it is possible to modify a bacterium or a yeast so that it will make a human hormone, for example. The list of genetic engineering enzymes is growing with time, and it is difficult to keep track. A price list dated Winter 1986 for New England Biolabs, Inc., one of the most comprehensive suppliers, is included in Appendix 1, as a representive example. Since this price list was published, they have added an average of one new product per month. Although the product list is very long, it should be pointed out that genetic engineering experiments can be designed using only few products, even if additional labour is required. In developing countries

establishing new genetic engineering capabilities, a handful of enzymes, such as EcoRI, PstI, T4 ligase and a few others are sufficient at least for training purposes. Developing countries with a headstart, such as Thailand will have more complex requirements as time goes on, but their capabilities in self-supply will also grow more sophisticated. As will be shown in the economics section of this chapter, it is possible to manufacture commercial quantities of the more commonly used enzymes using only laboratory scale equipment, because genetic engineers have been very successful in improving the production of the tools of their trade.

If a decision to buy rather than to manufacture is made, a list of suppliers can be found in Table 2.

Table 2. Manufacturers of genetic engineering products

Company	Products	Address
Applied Biosystems Inc.	Oligo-nucleotides DNA Synthesis	850 Lincoln Centre Drive, Foster City, CA 94404 USA
Bethesda Research Laboratories	Enzymes Biologicals	8717 Grovemont Circle, Gaithersburg, MD 20877, USA
Biosearch	Oligo-nucleotides DNA Synthesis	2980 Kerner Blvd., San Rafael, CA 94901 USA
Boehringer Mannheim Biochemicals	Enzymes Biologicals	P.O. Box 50816 Indianapolis, IN 46250 USA
Boehringer Mannheim GmbH	" "	Sandhofferstrasse 116 6800 Mannheim 31 FDR
New England Biolabs Inc.	Enzymes Nucleotides Biologicals	32, Tozer Road, Beverly, MA 01915-9990 USA
Promega Biotech (Allied Signal)	Enzymes Biologicals	2800 S. Fish Hatchery Rd., Madison, WI 53711 USA
Sigma Chemical Company	Enzymes Nucleotides Biologicals	P.O. Box 14508 St. Louis, MO 63178 USA
Takara Co. Ltd.	Enzymes	Shimogyo-ku, Nissei Sijo, Kyoto, JAPAN
Vega Biotechnologies Inc.	Oligo-nucleotides DNA Synthesis	P.O. Box 11648 Tucson AZ 85734-1648 USA
Yamasa Biochemicals	Nucleotides Enzymes	10-1 Araoi-cho 2 chome, Choshi, Chiba-ken 288 JAPAN

Rationale for local production

In the process of interviewing various groups in Bangkok, the premises for this study were tested not only to validate initial perceptions, but also to uncover additional aspects, positive or negative, potentially affecting a "make or buy" decision for bioreagents used in genetic technology.

Cost

Through direct interviews and telephone calls with local importers, the total import volume for genetic engineering enzymes into Thailand during 1985 was estimated to be US$ 20,000. This volume met the need of three research groups. One of the groups (Mahidol) accounted for 50 per cent of this amount, and the other groups were estimated to account for 25 per cent each. While this is a significant sum in terms of overall research budgets, demand for these enzymes will have to increase significantly to justify local manufacture on cost alone. A large increase in demand can be projected. As an illustration of this growth, genetic engineering materials made at Mahidol have already been supplied to 21 scientists from eight universities and two research institutions during 1986. At the same time, availability of new suppliers will tend to lower the unit price due to competitive pressure. Consideration of a regional (multi-national), rather than national supply might make the concept of local production more feasible from an economic point of view.

Foreign currency availability

At least presently in Thailand, foreign currency availability does not seem to be a major problem in obtaining restriction endonucleases and other enzymes. Several of the projects are supported by foreign or international agencies' grants. Only for those locally originated projects does foreign currency seem to be a hindrance. As local needs are projected to increase in the future, the problem of currency may become significant.

Shipping difficulties

This is a very real problem for developing countries. According to the groups interviewed for this study, the problem has been solved in Thailand by placing an "insider" in the customs office to speed up the processing and make sure that frozen products are quickly transferred to adequate freezers. Other favourable factors in Bangkok include the proximity of a well-served airport and the availability of an excellent telecommunications network which permits the tracking of purchase orders. As a result, regular orders are filled in about three weeks, and rush orders in as little as one week. This situation is not typical of all developing countries. A real possibility could be that as the market develops in these nations, suppliers will attempt to develop more stable products, for example in lyophilized form. However for the present, shipping delays for deep-frozen reagents are a real incentive for local manufacture in most developing countries. Except for a few enzymes, such as Nuclease S7, Nuclease P1, or DNase I, most enzymes are available only in frozen solutions and are very unstable once thawed.

Teaching and training needs

The largest requirement for bioreagents will probably be for teaching and training students in genetic engineering techniques and to supply workshops designed for continuing education or retraining of technical personnel. University laboratories will need restriction endonucleases and other enzymes in sizeable quantities several times every year. Experiments for students can be designed around few products, but the usually large number of students in each class can run up the cost of materials, especially if these are imported and paid for in hard currencies. At the same time, problems associated with stringent quality control of products are not necessarily of major importance in a teaching environment (see next section on production). At least, initially, the supply of materials for teaching laboratories may be the strongest justification for local manufacturing.

Other motivations

Genetic engineering has elicited much enthusiasm in many countries and a strong motivation for local biochemical engineers to support home production of bioreagents is a genuine desire to be involved in that exciting technology. The choice of restriction enzymes as an initial product target is an excellent one since they are low volume and high value products, requiring little capital investment, often feasible with existing equipment.

The training aspect (i.e. acquiring new skills for biotechnicians) is of limited value due to the repetitive nature of the work, at least for the principals involved.

Overall, the "make or buy" decision for genetic engineering enzymes is the result of a multitude of factors. Local manufacture of some of the enzymes is a sound practice for many developing countries, especially if shipping delays are expected. One of the major benefits of local production will be a low cost supply for university laboratories and workshops.

Production

In this section, the project for production of genetic materials at Mahidol University will be described. Among several types of bioreagents, a selected number of enzymes has been or will be produced, including three restriction endonucleases (EcoR1, Pst1, and BamH1), and one DNA ligase (T4 ligase). Production equipment involves only shake flasks and shaking incubators. Purification equipment consists mainly of centrifuges and chromatography columns.

Status

Production goals and status (June 1986) are summarized in Table 3.

Table 3. Genetic engineering enzymes production

Enzyme	Projected (units)	Prepared (units)	Distributed (units)
EcoRI	400,000	274,000	66,900
PstI	120,000	5,500	-
BamHI	120,000	-	-
T4 ligase	15,000	-	-

Problems

One of the major problems encountered in the production of restriction endonucleases is the lack of a fast and reliable assay. Unlike most other enzymes for which spectrophoncmetric assays can be developed through the use of chromogenic substrates, restriction enzymes require time consuming and fastidious procedures. As a result, production problems can be difficult to track, and yield optimization is a painfully slow process.

Purification can also be a difficult problem, because other nucleic acid processing enzymes cannot be present without making the preparation useless. Contaminant proteases can also contribute to increased instability. The lack of an easy assay also contributes to difficulties in developing and optimizing purification processes.

Another difficulty encountered at Mahidol University has been a high labour turnover among technicians. This problem seems due in part to the short term funding of the project, contributing to job insecurity. After training, which takes about six months, technicians are tempted to seek more stable employment, especially with the government, which is viewed as a stable employer. A remedy

would be a long term commitment to this type of project. Another contributing cause may be the tight budget which limits the competitiveness of salaries for technicians.

The present and future (at least in the short term) market for bioreagents is not considered sufficiently large by the project principals to justify the creation of a new enterprise, even on a regional basis.

A long term problem for local production is the multiplicity of products involved, many of which are only rarely needed. Out of about 500 known nucleic acid processing enzymes, only 120, of different specificities, are commercially available and only a dozen or so are used frequently. It would be very costly to maintain inventories for a complete product line with only a local market to satisfy.

Finally, a problem common to all new endeavours is the long time needed to gain sufficient confidence in the products, for the manufacturers as well as the users. This is not a problem presently in Thailand, since the products made available so far have been distributed free of charge. It could be a problem if the group considered selling their products. Meanwhile, initial batches of products can be (and have been) used advantageously for teaching or training purposes.

Economics

In this section, the production economics of one restriction endonuclease, Pst I, will be examined. About a dozen or so genetic engineering enzymes have been cloned and over-expressed, and are therefore easy to manufacture in large quantities using laboratory equipment. They include enzymes used often in rDNA manipulations, such as Eco RI, Hae III, Hind III, Pst I, Taq I, T4 ligase and T4 polynucleotide kinase. Pst I is a good choice as a first candidate for two major reasons: it cuts the plasmid pBR322 only once, within the beta-lactamase gene, often marking an insertion, and cDNA molecules are frequently inserted in the Pst I site of a vector by G-C tailing because the procedure regenerates the

Pst I site on both sides of the insert, making recovery easy. The major source of information for this evaluation is the Ph.D. dissertation of Dr. Roxanne Y. Walder (available from University Microfilms, Ann Arbor, Michigan), presently in the Department of Biochemistry at the University of Iowa, Iowa City, Iowa. The strain of Providencia stuartii considered here is genetically engineered for overproduction of Pst I and is commercially available from Dr. Joseph Walder, Department of Biochemistry, University of Iowa, Iowa City, Iowa. The price of the strain will reflect the contemplated use, i.e. it will be higher for commercial resale of the enzyme than for in-house use. The highest price, for world-wide exploitation, is US$ 10,000. The process described here has not been tested at the scale contemplated. It was derived speculatively from a process described by Dr. Roxanne Walder for a batch size much too large to be of interest to a laboratory in a developing country (i.e. 7.5 million units per batch). However, it should be noted that even such large batches were made in laboratory-scale equipment by Dr. R. Walder. Assumptions for the process are listed in Table 4 and materials and methods are listed below.

Producing strain

P. stuartii pPst 304, derived from P. stuartii 164 by transformation with a plasmid made of pBR322 and two copies of the Pst I restriction-modification system (R. Walder, 1984).

Strain storage

Glycerol stabs: 2 ml of an overnight culture in LB broth from one single colony with 2 ml of sterile glycerol stored at -20°C.

Strain maintenance

Luria Broth (LB) agar plates grown at 37°C and kept at 4°C.

Table 4. Assumptions for the production of Pst I

Quantity per batch:	1,000,000 units
Weight:	0.3 mg
Overall purification yield:	20 per cent
Gross production:	1.5 mg
Cell activity:	150 u/mg paste
Quantity of cells required:	33 grams cell paste
Broth cell concentration:	10 g/l cell paste
Net broth required:	3.3 l
Assays, evaporation losses:	20 per cent
Total broth required for production:	41
Shake flask size:	2.0 l
Shake flask content:	400 ml
Number of shake flasks required:	10
Inoculum ratio:	5 per cent

Inoculum preparation

The same medium (LBA) is used for inoculum and production: sterile ampicillin (200 mg/l) is added to sterilized and cooled LB broth. A 250 ml shake flash containing 30 ml of LBA is inoculated from an LB agar plate and incubated overnight at 37°C in a shaking incubator. Three 500 ml shake flasks containing 100 ml of LBA are inoculated with 5 ml each from the previous flask and incubated overnight at 37°C in a shaking incubator. Two of the shake flasks are selected following microbiological examination for morphology and gross contamination.

Enzyme production

Fifteen one-litre shake flasks containing 200 ml LBA each are inoculated with 10 ml of inoculum from the previous operation. The shake flasks are

incubated at 37°C in a shaking incubator overnight or to an OD_{650} of 0.7-0.8 (late exponential phase). The cells are harvested by centrifugation at 2300 x g for 10 minutes. The pellets are collected and frozen and stored at -20°C. If desired, production can be done at a smaller scale, and cells can be accumulated at this stage from several smaller batches to about 25 grams of cell paste.

Pst I assay

One unit of Pst I is the amount required to produce a complete digest (28 cuts) of one microgram of lambda DNA in sixty minutes in a total reaction volume of 0.05 ml at 37°C in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 100 ug/ml Bovine Serum Albumin (BSA). Concentrated preparations are first diluted to about 1,000 u/ml using storage buffer (see below).

Purification

Purification steps are summarized in Table 5, showing the expected yield and purification factor for each step. Detailed steps, materials and equipment are shown in Appendix 2, as outlined in Dr. R. Walder's dissertation and can be appropriately scaled down by a factor of 7.5. After final assays, the product should be diluted to about 25,000 u/ml in storage buffer: 10 mM Tris-HCl (ph 7.4), 1 mM dithiothreitol, 0.1 mM EDTA, 200 mM NaCl, 50 per cent w/v glycerol and 0.15 per cent Triton X-100, and stored at -20°C, in reasonable aliquots for future use or distribution, for example 1,000 to 10,000 units per vial.

Cost

Estimates of the cost per unit of Pst I are difficult to assess in a meaningful way. Firstly, it is unlikely that one laboratory or even one whole developing country could utilize one million units in the expected one year of stable storage and the cost should be allocated on the actual units used.

Secondly, small quantities of reagents are required, many on-hand in most laboratories. However, if new reagents must be purchased especially for this preparation, it may be unfair to allocate their total cost to Pst I, while a large portion may be available for future use. The same holds true for labour. While the total time for this work is estimated to be one month, many time-consuming steps, such as dialysis, would require only little attention and labour. Suffice it to say that the purchase of 1,000,000 units of Pst I would be expected to cost about US$ 8,000 and depending on the particulars of a laboratory, could be made in-house for one tenth to one half of this amount. If a laboratory would choose to buy rather than to make enzymes, some suppliers are listed in Table 2.

Table 5. Purification of Pst I restriction endonuclease

Fraction	Step	Total activity 10^6 units	Specific activity 10^6 u/mg
0	Cell paste	4.9	0.00015
I	Supernatant	4.3	0.0003
II	Ammonium Sulfate	3.6	0.007
III	Phosphocellulose	2.5	0.2
IV	DEAE Cellulose	1.9	0.6
V	Sephadex G-200	1.5	0.7
VI	Heparin Agarose	1.0	2.0

III. OLIGONUCLEOTIDES AND SYNTHETIC GENES

Synthetic oligonucleotides and genes

These constitute another family of products necessary for genetic engineering. They are used for various tasks:

Primers

Primers are initiators of enzymatic synthesis, used in reverse transcription of RNA, in hybridization and in sequencing techniques such as the M13 system. They are short polymers of 10 to 15 nucleotides.

Linkers

Linkers are also short polynucleotides which incorporate sequences recognized by restriction endonucleases. They are used to incorporate DNA intc plasmids.

Adapters

Adapters are sequences which permit the splicing of DNA fragments cut by different restriction endonucleases. They incorporate the recognition sequences of two restriction enzymes.

Probes

Synthetic probes are short to medium sized sequences of nucleotides used to detect specific genetic sequences by hybridization to single stranded nucleic acids (long probes are made biologically by the genetic engineering of plasmids). They often incorporate radiolabels, such as 32_P. One of the more exciting uses of probes is for clinical diagnostics. Recently, probes have been used in Thailand for the detection of malarial parasites and the mosquito species which harbour them.

Synthetic genes

In many cases, it may be advantageous to synthesize a gene whose product is fully characterized, rather than attempting to obtain it out of a complex genome. While several companies offer custom synthesis of genes, recent advances in hardware as well as in chemistry have made gene synthesis a common operation in most genetic engineering laboratories. A common strategy is to make small overlapping oligonucleotide sections from both DNA strands, permitting self assembly and completed later on by ligase covalent linking.

Rationale for local production of oligonucleotides

Oligonucleotides are relatively stable and many suppliers ship by regular mail. Delays in shipping and loss of product do not constitute an important motivation for making oligonucleotides locally. However, there are several good reasons for establishing self-sufficiency in the supply of oligonucleotides.

Cost

The high price of oligonucleotides include a high labour cost component. Much of this labour cost is in purification and quality control. The cost of materials is often negligible. Depending on the length of the sequence, material cost ranges from US$ 5 to US$ 50 for most oligonucleotides, averaging about US$ 25, in quantities of 5-10 A_{260} units, which are sufficient for multiple genetic manipulations. A typical price for that quantity if ordered from a supplier will be US$ 500, assuming it is a catalogue item. Custom-made probes and genes would be much more expensive.

Time

A typical oligonucleotide takes about three days to be completed, assuming one day for synthesis and two days for purification. This is much shorter than the average delay between ordering and receiving.

Flexibility

While many linkers and primers are available commercially, many genetic constructions require special sequences which can be quickly made in a laboratory with in-house capability. Most probes and all synthetic genes must also be synthesized on-purpose.

For some applications, mixed position sequences are required and while not generally available from catalogues, they can be synthesized as easily as single sequences.

Large-scale manufacture

Typical applications require very little quantities of oligonucleotides. Most synthesis methods (discussed in the next section) are adequate for manufacturing commercial quantities, for example for diagnostic tests. The only variation involves the purification steps. While small quantities (one A_{260} unit or less) can be purified by gel electrophoresis, commercial quantities will require high performance liquid chromatography, a unit operation becoming common in most laboratories.

Production

There are three major approaches to the synthesis of oligonucleotides, regardless of the chemistry used: normal organic synthesis, using conventional equipment, the use of kits, supplied by several manufacturers and automated "gene machines". The first approach is the least expensive for capital and reagents, but has the highest labour cost. The use of kits minimizes the labour component, but has a higher material cost. The most productive approach is the use of an automated synthesizer. Although the initial capital outlay is high, it can be quickly recovered in a productive laboratory. It also minimizes human errors and often results in savings of labour and materials.

Several chemistries have been developed during the last decade but it seems that phosphoaramidite chemistry represents the state-of-the-art. It is used both in kits and in automated equipment.

The choice of a gene machine would be difficult on the basis of performance alone: most perform very satisfactorily. Depending on the model and the amount of optional equipment, prices range from US$ 55,000 to US$ 100,000. Availability of field service in a given area will probably be of prime consideration in choosing the apparatus.

Purification and quality control will retain the highest share of time and labour, although the job is easier if an automated synthesis machine is used, as yield per condensation reaction and consequently overall yield, are consistently higher than in manual synthesis.

Economics

As a model, the synthesis of five A_{260} units of a tridecamer probe with three multiple base sites, obtained in purified form, will be assumed. As a reference, a quote for custom synthesis of such a probe was obtained from a commercial supplier. The quoted price was US$ 5,625. For a list of suppliers see Table 2.

Manual synthesis using reagents purchased in bulk would have a low material cost of about US$ 25. (See detailed procedure in Appendix 3). Labour is estimated at one person-week, including two days for purification. At US$ 50 per person-day, the cost for the probe would be US$ 375.

If a commercial kit is used, it is assumed that it will be sufficient to perform 130 condensations for the manufacture of 10 different oligonucleotides. It is also assumed that standard laboratory equipment (scales, water bath, fume hood, vacuum source, microcentrifuge, *etc.*) is available. The cost of solvents required for the reactions (e.g. diethyl ether) is negligible. Although some manufacturers claim that only two hours would be needed for the synthesis, we

have assumed one person-day. Purification still will require two person days. At US$ 50 per person-day, and at US$ 80 for materials (10 per cent of kit purchase price), the cost of the probe comes to US$ 230.

If an automated machine were used, the largest cost component would be the amortization of capital, and the amount would depend essentially on the number of oligonucleotides synthesized during the useful life of the equipment. Assuming a useful life of five or ten years and a purchase price of US$ 75,000, Table 6 gives the amortization cost per oligonucleotide, based on straight line depreciation, in function of the number of oligonucleotides made each year.

Assuming a labour component of two days (mostly for purification) and a materials cost of US$ 25, the cost per oligonucleotide comes to US$ 275 at a production level of 100 per year for a 5-year depreciation. At a production level of 300/year, the cost drops to US$ 175. It is assumed that service costs are included in the purchase price, as is often the case for this type of equipment. For a 10-year depreciation schedule, a service component should be added.

Table 6. Amortization cost of automated synthesizers

(in US dollars)

Number/year	5-year depreciation	10-year depreciation
30	500	250
100	150	75
300	50	25
1000	15	8

IV. HOST STRAINS, PLASMIDS, DNA AND OTHER BIOLOGICAL MATERIALS

Biological Materials

Biological materials, which include host strains (bacteria, yeasts and mammalian cells), plasmids and cosmids, DNA of biological origin and other materials (such as lambda packaging kits), are yet another type of products in the arsenal of genetic engineers.

They share in common the fact that they are obtained by growing cells using classical microbiological techniques long widespread in developing countries. In theory, they need to be acquired only once. Only in rare instances are they needed after the first purchase (e.g. catastrophic failure of a freezer, or untractable contamination). Often they can be passed informally from laboratory to laboratory at no charge. As a rule they can be obtained from repositories in the USA (ATCC), Europe (EMBO) and in many other countries. In Thailand the organization having responsibility for culture collection services for bacteria and fungi is the Thailand Institute of Scientific and Technological Research (TISTR). Mahidol University acts as a service unit for the provision of mammalian cell lines.

Host strains are needed for the replication and expression of genetically engineered plasmids, cosmids and viruses. In turn, these can be isolated and purified and made available to other laboratories. Once purified, they can be further processed to generate, for example, marker DNA used in the calibration of gel electrophoresis plates.

As an example, requirements for sequencing DNA using the M13 system include one host strain (Escherichia coli K12 JM101), plasmids (M13 vectors), DNA (EcoR1 digests of M13 and Lambda DNA) and T4 ligase.

Specific bacterial strains are also needed for the production of enzymes such as restriction endonucleases.

Production

The production of host strains of bacteria and fungi for supplying laboratories is a simple matter, using classical microbiology techniques. For shipping short distances, or casual transfer from researcher to researcher, a simple Petri dish culture is all that is required. Lyophilized preparations have the advantage of long-term stability. Vectors and viruses can be transferred in the same way.

Mammalian cell cultures can also be easily transferred in T-flasks or in suspension cultures, depending on the cell line. However they are more fragile than bacteria or fungi, and must be quickly subcultured.

Purified DNA preparations require the use of an ultracentrifuge, an expensive piece of equipment, but found in many biochemistry laboratories.

Marker DNA can be obtained by digesting purified plasmids or viral chromosomes with restriction endonucleases. As an example, the restriction enzyme Eco R1 will cut the Lambda virus chromosome into six pieces of known and reproducible molecular weight.

Other preparations such as DNA packaging systems can be obtained by simple methods from lysed bacterial cultures.

Since the cost of preparation for the above biological materials is low, no economic evaluation will be made.

Biological materials made by the Mahidol University group and distributed to other scientists are shown in Table 7.

Table 7. Biological Materials Production at Mahidol University

Material	Projected	Prepared	Distributed
Host cells			
E. coli C-600	As needed	As needed	24 plates
E. coli JM107	As needed	As needed	19 plates
Plasmids			
pBR322 (ug)	2,000	1,000	50
pBR325 (ug)	1,500	400	-
pUN121 (ug)	500	-	-
Marker DNA			
(ug)	2,000	50	13

APPENDIX 1

PRICE LIST FOR GENETIC ENGINEERING PRODUCTS

Price List - Winter 1986

Restriction Endonucleases

Product	Number	Recognition Sequence	Price (small)	Price (large)
Ast II	#117	GACGT↑C	50 units/$44	250 units/$176
Acc I	#161	GT↑(AC)(GT)AC	60 units/$55	300 units/$220
Alw II	#121	GPu↑CGPyC	50 units/$100	250 units/$400
Alw I	#137	AG↑CT	400 units/$50	2,000 units/$200
Apa I	#116	GGGCC↑C	5,000 units/$55	25,000 units/$220
Asu I	#152	C↑PyCGPuG	200 units/$44	1,000 units/$176
Asu II	#153	G↑G(AT)CC	100 units/$44	500 units/$176
Aur II	#174	C↑CTAGG	20 units/$80	100 units/$320
Bal I	#150	TGG↑CCA	20 units/$80	100 units/$320
Bamh I	#136	G↑GATCC	2,500 units/$44	12,500 units/$176
Bca I	#118	G↑GPyPuCC	250 units/$50	1,000 units/$200
Bca II	#119	GPuGCPy↑C	1,000 units/$50	5,000 units/$200
Bbv I	#173	GCAGC(8/12)	10 units/$100	50 units/$560
Bcl I	#160	T↑GATCA	400 units/$44	2,000 units/$176
Bgl I	#143	GCCNNNN↑NGGC	1,000 units/$44	5,000 units/$176
Bgl II	#144	A↑GATCT	500 units/$44	2,500 units/$176
Bsm I	#134	GAATGC(1/-1)	100 units/$50	500 units/$200
Bsp 1286	#120	G(GAT)GC(CAT)↑C	200 units/$50	1,000 units/$200
• *Bsp*M I	#502	ACCTGC(4/8)	100 units/$50	500 units/$200
• *Bsp*M II	#504	T↑CCGGA	20 units/$80	100 units/$320
*Bsa*H I	#199	G↑CGCGC	100 units/$50	500 units/$200
*Bst*E II	#162	G↑GTNACC	2,000 units/$44	10,000 units/$176
*Bst*N I	#168	CC↑(AT)GG	1,000 units/$44	5,000 units/$176
*Bst*X I	#113	CCANNNNN↑NTGG	250 units/$50	1,250 units/$200
Cla I	#197	AT↑CGAT	500 units/$50	2,500 units/$200
Dde I	#175	C↑TNAG	200 units/$44	1,000 units/$176
Dpn I	#176	G^{m}A↑TC	200 units/$50	1,000 units/$200
Dra I	#129	TTT↑AAA	2,000 units/$50	10,000 units/$200
• *Eag* I (*Xma* III isoschizomer)	#505	C↑GGCCG	100 units/$50	500 units/$200
• *Eco*O 109 (*Dra* II isoschizomer)	#503	PuG↑GNCCPy	2,000 units/$50	10,000 units/$200
*Eco*R I	#101	G↑AATTC	10,000 units/$44	50,000 units/$176 250,000 units/$500
*Eco*R V	#195	GAT↑ATC	2,000 units/$44	10,000 units/$176
*Fau*D II	#164	CG↑CG	60 units/$55	300 units/$220
*Fau*4H I	#178	GC↑NGC	60 units/$55	300 units/$220
Fok I	#109	GGATG(9/13)	100 units/$50	500 units/$200
Fsp I	#135	TGC↑GCA	50 units/$50	250 units/$200
Hae II	#107	PuGCGC↑Py	1,000 units/$44	5,000 units/$176
Hae III	#108	GG↑CC	2,000 units/$44	10,000 units/$176
Hga I	#154	GACGC(5/10)	15 units/$80	75 units/$320
*Hgi*A I	#170	G(TA)GC(TA)↑C	100 units/$65	500 units/$260
Hha I	#139	GCG↑C	1,000 units/$44	5,000 units/$176
*Hin*c II	#103	GTPy↑PuAC	200 units/$44	1,000 units/$176
*Hin*d III	#104	A↑AGCTT	10,000 units/$44	50,000 units/$176

Msl I	#155	G↑AATC	1,000 units/$44	5,000 units/$176
MspP I	#124	G↑CGC	500 units/$50	2,500 units/$200
Nde I	#105	GTT↑AAC	100 units/$44	500 units/$176
Nde II	#171	C↑CGG	500 units/$44	2,500 units/$176
Nhe I	#158	GGTGA(8/7)	200 units/$44	1,000 units/$176
Kpn I	#142	GGTAC↑C	2,000 units/$44	10,000 units/$176
Nla I	#147	↑CATG	200 units/$55	1,000 units/$220
Nla II	#148	GAAGA(8/7)	400 units/$55	2,000 units/$220
Nla I	#198	A↑CGCGT	800 units/$50	4,000 units/$200
Nsi I	#163	CCTC(7/7)	45 units/$00	225 units/$240
Nsp I	#186	C↑CGG	2,000 units/$44	10,000 units/$176
Nst II	#179	CC↑TNAGG	40 units/$60	200 units/$240
Nco I	#190	GCC↑GGC	400 units/$44	2,000 units/$176
Nar I	#191	GG↑CGCC	100 units/$44	500 units/$176
Nci I	#196	CC↑(CG)GG	800 units/$50	4,000 units/$200
Nco I	#193	C↑CATGG	100 units/$44	500 units/$176
Nde I	#111	CA↑TATG	200 units/$50	1,000 units/$200
Nhe I	#131	G↑CTAGC	250 units/$55	1,250 units/$220
Nla III	#125	CATG↑	10 units/$00	50 units/$320
Nla IV	#126	GGN↑NCC	20 units/$60	100 units/$240
Not I	#189	GC↑GGCCGC	200 units/$55	1,000 units/$220
Nru I	#192	TCG↑CGA	200 units/$44	1,000 units/$176
Nsi I	#127	ATGCA↑T	250 units/$50	1,250 units/$200
PaeR7 I	#177	C↑TCGAG	2,000 units/$44	10,000 units/$176
• *Ppu*MI I	#506	RG↑G(AT)CCPy	50 units/$50	250 units/$200
Pst I	#140	CTGCA↑G	5,000 units/$55	25,000 units/$220
Pvu I	#150	CGAT↑CG	100 units/$55	500 units/$220
Pvu II	#151	CAG↑CTG	1,000 units/$44	5,000 units/$176
Rsa I	#167	GT↑AC	1,000 units/$44	5,000 units/$176
• *Rsr* II	#501	CG↑G(AT)CCG	25 units/$55	125 units/$220
Sac I	#156	GAGCT↑C	1,000 units/$44	5,000 units/$176
Sac II	#157	CCGC↑GG	1,000 units/$44	5,000 units/$176
Sal I	#138	G↑TCGAC	500 units/$44	2,500 units/$176
*Sau*3A I	#169	↑GATC	200 units/$44	1,000 units/$176
*Sau*96 I	#165	G↑GNCC	200 units/$44	1,000 units/$175
Sca I	#122	AGT↑ACT	1,000 units/$50	5,000 units/$200
*Scr*F I	#110	CC↑NGG	600 units/$50	3,000 units/$200
*Sfa*N I	#172	GCATC(5/9)	20 units/$100	100 units/$400
Sfi I	#123	GGCCNNNNN↑NGGCC	250 units/$50	1,250 units/$200
Sma I	#141	CCC↑GGG	400 units/$44	2,000 units/$176
*Sno*B I	#130	TAC↑GTA	100 units/$44	500 units/$176
Spe I	#133	A↑CTAGT	150 units/$50	750 units/$200
Sph I	#182	GCATG↑C	80 units/$50	400 units/$200
Ssp I	#132	AAT↑ATT	300 units/$55	1,500 units/$220
Stu I	#187	AGG↑CCT	400 units/$44	2,000 units/$176
Sty I	#500	C↑C(AT)(AT)GG	2,000 units/$55	10,000 units/$220
Taq I	#149	T↑CGA	2,000 units/$44	10,000 units/$176
*Tth*111 I	#185	GACN↑NNGTC	400 units/$44	2,000 units/$176
Xba I	#145	T↑CTAGA	2,000 units/$55	10,000 units/$220
Xho I	#146	C↑TCGAG	4,000 units/$55	20,000 units/$220
Xho II	#128	Pu↑GATCPy	25 units/$44	125 units/$176
Xma I	#180	C↑CCGGG	20 units/$50	100 units/$200
Xma I	#194	GAANN↑NNTTC	600 units/$44	3,000 units/$176

• New product

DNA Modification Methylases

M.*Alu* I	#220	AG^{m}CT	100 units/$50	500 units/$200
M.*BamH* I	#223	GGAT^{m}CC	100 units/$50	500 units/$200
M.*Cla* I	#218	ATCG^{m}AT	150 units/$50	750 units/$200
dam	#222	G^{m}ATC	500 units/$50	2,500 units/$200
M.*EcoR* I	#211	GA^{m}ATTC	10,000 units/$44	50,000 units/$176
M.*Hae* III	#224	GG^{m}CC	50 units/$50	250 units/$200
M.*Hha* I	#217	G^{m}CGC	1,000 units/$50	5,000 units/$200
M.*Hpa* II	#214	C^{m}CGG	30 units/$55	150 units/$220
M.*Hpa* I	#221	T^{m}CACC	10 units/$50	50 units/$200
M.*Msp* I	#215	^{m}CCGG	100 units/$50	500 units/$200
M.*Pst* I	#216	CTGC^{m}AG	100 units/$55	500 units/$220
M.*Taq* I	#219	TCG^{m}A	300 units/$50	1,500 units/$200

Enzymes Involved in Nucleic Acid Metabolism

T4 DNA Ligase	#202	20,000 units/$55	100,000 units/$220
T4 RNA Ligase	#204	100 units/$44	500 units/$176
DNA Ligase (E.coli.DPN)	#205	200 units/$44	1,000 units/$176
Polynucleotide Kinase	#201	500 units/$30	2,500 units/$100
DNA Polymerase I	#209	500 units/$50	2,500 units/$200
DNA Polymerase I Lg.Frag.	#210	125 units/$44	625 units/$176
RNA Polymerase (E.coli)	#208	100 units/$44	500 units/$176
T4 DNA Polymerase	#203	25 units/$50	125 units/$200
•T7 RNA Polymerase	#251	5,000 units/$50	25,000 units/$200
SP 6 RNA Polymerase	#207	200 units/$50	1,000 units/$200
Nuclease BAL-31	#213	50 units/$44	250 units/$176
Exonuclease III	#206	5,000 units/$44	25,000 units/$176
Lambda Exonuclease	#212	100 units/$44	500 units/$176
Mung Bean Nuclease	#250	1,500 units/$44	7,500 units/$176

M13 DNA Sequencing System

M13 Cloning Pack	#408	$150
Vector M13mp18 RF DNA	#408-18	10 µg/$60
Vector M13mp19 RF DNA	#408-19	10 µg/$60
M13 ^{32}P Dideoxy Sequencing Pack	#409	$185
M13 ^{35}S Dideoxy Sequencing Pack	#410	$185
M13 ^{32}P Sequencing Reagents	#405	$85
M13 ^{35}S Sequencing Reagents	#406	$85

Dephosphorylated pBR322 Cloning Vectors

BamH I cleaved pBR322	#320	5 µg/$75	25 µg/$300
EcoR I cleaved pBR322	#322	5 µg/$75	25 µg/$300
Hind III cleaved pBR322	#321	5 µg/$75	25 µg/$300
Pst I cleaved pBR322	#324	5 µg/$75	25 µg/$300

DNAs and DNA Molecular Weight Standards

Lambda DNA	#301-1	500 µg/$50	2,500 µg/$200
Lambda DNA (N^6 methyladenine-free)	#301-3	500 µg/$50	2,500 µg/$200
ϕX174 RF I DNA	#302-1	30 µg/$50	150 µg/$200
ϕX174 RF II DNA	#302-2	30 µg/$50	150 µg/$200
ϕX174 Virion DNA	#302-3	50 µg/$55	250 µg/$220
Lambda DNA *Mlu* III Digest	#301-2	250 µg/$50	1,250 µg/$200
Lambda DNA *Bst*E II Digest	#301-4	250 µg/$50	1,250 µg/$200
ϕX174 DNA *Hae* III Digest	#302-6	20 µg/$50	100 µg/$200
pBR322 DNA *Bst*N I Digest	#303-1	50 µg/$50	250 µg/$200
pBR322 DNA *Msp* I Digest	#303-2	50 µg/$50	250 µg/$200

Linkers (54 linkers currently available)

Non-Phosphorylated Linkers	8-mer or 10-mer	1.0 A_{260} unit/$60	5.0 A_{260} units/$240
	12-mer	1.0 A_{260} unit/$80	5.0 A_{260} units/$320
Phosphorylated Linkers	8-mer or 10-mer	1.0 A_{260} unit/$90	5.0 A_{260} units/$360

Primers (15 primers currently available)

Lambda gt11 Primers	2.5 µg/$95	12.5 µg/$380
M13 Primers	2.5 µg/$95	12.5 µg/$380
pBR322 Primers	2.5 µg/$95	12.5 µg/$380
mRNA Primers	2.5 µg/$30	any 5 pkgs./$100

Reagents and Matrices for mRNA Isolation

Oligo (dT)-Cellulose	#1401	1 gram/$80	5 grams/$320
Oligo (dT)-Cellulose (microcrystalline)	#1403	1 gram/$70	5 grams/$280
Oligo $d(T)_{12}$	#1316	5.0 A_{260} units/$60	
Ribonucleoside-Vanadyl Complex	#1402	10ml (200mM)/$50	
RNA Cap Structure Analog	#1404	25 A_{260} units/$75	

Other Organic Synthesis Products

Adapters		1.0 A_{260} unit/$60	5.0 A_{260} units/$240
Probes		2.5 µg/$45	12.5 µg/$180
DNA Synthesis Kit	#1500	(40-80 cond.)/$450	(80-160 cond.)/$650
DNA Synthesis Reagents			

• New product

APPENDIX II

PURIFICATION PROCEDURE FOR THE RESTRICTION ENZYME PST I

(from Dr. Roxanne Walder's Ph.D. dissertation)

Purification of Pst I restriction endonuclease

Frozen pPst304 cells, 148 g, were suspended in 120 ml of 20 mM Tris-HCl pH 7.0, 400 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5 per cent W/v glycerol containing 13 ug/ml phynylmethylsulfonylfluoride (PMSF) and allowed to thaw at 4° for 8 hours. The cells were treated with lysozyme at 100 ug/ml for 30 minutes on ice before sonication. Cells were disrupted using a Bioconik sonicator (Bronwill Scientific) in 30 g portions with 30 second pulses, 12 repetitions, with one minute of rest between each pulse. When all of the cells were disrupted, cell debris was removed by centrifugation at 15,000 x g for 20 minutes. The supernatant was pooled and centrifuged at 200,000 x g for 4 hours. The high speed supernatant (Fraction I) was precipitated with solid ammonium sulfate, added to 75 per cent saturation. The precipitated material was collected by centrifugation at 20,000 x g for 15 minutes. The precipitate was dissolved in 80 ml of phosphocellulose starting buffer, 10 mM sodium phosphate pH 7.0, 1 mM EDTA, 200 mM NaCl, 1 mM sodium azide, 5 per cent W/v glycerol, 13 ug/ml PMSF, and dialyzed against 1.5 liters, with four changes of the same buffer to yield Fraction II.

The dialysate (Fraction II) was applied to a phosphocellulose P-11 (Whatmann) column 2.5 x 30 cm and washed with 450 ml of phosphocellulose starting buffer. The column was developed with a 1200 ml linear gradient of 0.2 to 0.6 M NaCl. Ten ml fractions were collected and assayed for Pst I activity. Fractions containing enzymatic activity were pooled, concentrated by Amicon ultrafiltration to 40 ml and dialyzed against 4 liters of DEAE buffer: 10 mM potassium phosphate pH 7.8, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10 per cent W/v glycerol. The low tonic strength of this buffer caused precipitation of some proteins other than Pst I restriction enzyme. The dialysate was clarified by centrifugation at 15,000 x g for 30 minutes to yield Fraction III.

The supernatant of the dialyzed phosphocellulose pool (Fraction III) was applied to a 1.5 x 30 cm DEAE-52 column and washed with 300 ml of DEAE buffer. The column was developed with a 500 ml gradient of 0 to 0.4 M NaCl. Five ml fractions were collected and assayed for enzymatic activity. The pooled enzymatic fractions from the DEAE-cellulose column were concentrated by Amicon ultrafiltration to 6 ml, yielding Fraction IV and applied to a Sephadex G-200 column. The Sephadex G-200 column 2.5 x 30 cm, had been equilibrated with 10 mM sodium phosphate pH 7.0, 1 mM EDTA, 1 mM sodium azide, 7 mM 2-mercaptoethanol, and 10 per cent W/v glycerol. Fractions (2.5 ml) were collected and assayed for endonuclease activity. The pooled enzyme fractions were concentrated to 8.5 ml by ultrafiltration and dialyzed against heparin-agarose buffer which contains 20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 7 mM 2-mercaptoethanol and 10 per cent W/v glycerol.

The dialyzed enzyme (Fraction V) was applied to a 1 x 13 cm heparin-agarose column, washed with two column volumes of heparin agarose buffer, followed by a second wash with a 100 ml gradient of 0 to 0.2. M NaCl. Pst I restriction enzyme was eluted with a 200 ml gradient of heparin agarose buffer containing 0.2 to 0.8 M NaCl. The final pooled enzyme was concentrated by ultrafiltration to 5 ml (Fraction VI). A portion of Fraction VI was dialyzed against water for protein sequencing and antibody studies. The remainder of the enzyme was dialyzed against storage buffer which contained 10 mM Tris-HCl pH 7.4, 1 mM dithiothraitol, 0.1 mM EDTA, 200 mM NaCl, 50 per cent W/v glycerol and 0.15 per cent W/v Triton X-100, and stored at -20°C.

A summary of a purification of Pst I restriction enzyme from 148 g of the transformed Providencia stuartii 164 strain, pPst304, is presented in Table 1. From 148 g of cells 3.75 mg of Pst I restriction enzyme was purified. A total of 4 x 10^6 units or 2 mg Pst I was used for the Edman degradation studies and 2.2 x 10^6 units or 1.1. mg was used for antibody production. The remainder of the purified Pst I, 1.3 x 10^6 units, was used for restriction digests.

The method for purification of Pst I restriction enzyme was devised by Drs. Joseph Walder and Ranjit Chatterjee. This protocol was shared with me prior to publication, with their permission.

Amino acid analysis

Pst I restriction endonuclease was hydrolyzed under reduced pressure for 24 hours at 100° with 6 N HCl. Hydrolysates were analyzed on a Beckman 121 MB amino acid analyzer, equipped with a 2.8 x 350 mm AA-10 column. The analysis was accomplished in collaboration with Glen Wilson, director of the Protein Structure Facility.

APPENDIX III

A PROCEDURE FOR THE MANUAL SYNTHESIS OF DEOXYOLIGONUCLEOTIDES USING DIMETHOXYTRITYL NUCLEOSIDE PHOSPHORAMIDITES ON A SOLID SUPPORT

INTRODUCTION

The synthesis of DNA was first accomplished in 1955 by Michelson and Todd[1]. Since then, various laboratories around the world have contributed to the chemistry of making specific DNA's for biological research. Early methods required highly skilled nucleic acid chemists and specialized equipment. Before synthetic DNA became commonly available a number of criteria had to be met. These were:

Fast and efficient chemistry for addition of single nucleotides without affecting other functional groups on the DNA.

Stable and compatible reagents.

Very pure starting materials.

Easy separation of the product from residual reagents and by-products.

Low material and labor cost per cycle.

We feel that the 5'-DMTr nucleoside-3'-phosphoramidites developed by Beaucage and Caruthers[2] coupled with the solid phase methods developed by Matteucci and Caruthers[3,4] address these criteria better than any other synthesis method.

One of the most serious problems in DNA synthesis has been the time required and quantities of expensive reagents needed for efficient coupling. Diester procedures use very large excesses of reactants and coupling takes as long as six hours. The more recent triester approach requires reaction times of ½ to 1 hour and is inefficient to the extent that most

CAUTION!

The toxic, mutagenic and carcinogenic properties of dimethoxytrityl nucleoside phosphoramidites and some of the mixtures used in oligonucleotide synthesis have not yet been determined. All of the reagents should be considered dangerous and handled appropriately. Do not spill on skin or breathe powders or vapors.

triester procedures call for adding dimer or trimer blocks rather than single nucleotides. These dimers or trimers must be independently synthesized and purified prior to DNA synthesis. Nucleoside phosphoramidites, on the other hand, are highly reactive upon activation under mildly acidic conditions. Coupling reactions go to completion in less than three minutes. Products from minor side reactions which may occur, such as exocyclic base additions, are eliminated by hydrolysis in subsequent steps. The DMTr nucleoside phosphoramidites are very stable under neutral conditions. As is the case for all other types of DNA intermediates they should be stored desiccated.

With most chemical syntheses, a key to high product yield and easy purification is the use of pure starting materials. Our use of a ^{31}P FTNMR insures the quality of the dimethoxytrityl nucleoside phosphoramidites. The other important reagents and solvents are also the purest available and undergo quality control tests such as Karl Fisher titration, UV spectroscopy, and gas chromatography. Additionally, each lot is used in house to confirm performance before shipping.

The step-wise separation of excess reagents and reaction by-products from the growing oligonucleotide chain is facilitated by solid support synthesis. During the synthesis, all unused reagents and reaction by-products are simply washed out. After completion of the synthesis, the dimethoxytrityl group affords a means of purifying the final product from other oligomers by reverse-phase chromatography. After each addition step during the synthesis, all unreacted chains (with 5'-OH groups) are capped to prevent further reaction. When the synthesis is complete, only the desired product will bear the trityl group. This group is large and hydrophobic and significantly increases the retention of DNA's on reverse-phase columns.

Historically, DNA synthesis has been done only when the DNA was urgently required since many hours of time were required and the dimethoxytrityl base protected nucleosides precursors were quite expensive. Now due to the high coupling efficiency of the phosphoramidite method and the purity of the reagents offered by Applied Biosystems, DNA synthesis can be accomplished for as little as $4.00 per cycle for materials and a typical probe can be synthesized in less than 8 hours.

We would like to thank the many people, primarily from the laboratory of Dr. Marvin Caruthers at the University of Colorado, who have contributed to the evolution of the procedure described here. A more general description has previously been published.[5]

1 NUCLEOTIDES PART XXXII: SYNTHESIS OF A DITHYMIDINE DINUCLEOTIDE CONTAINING A 3':5'-INTERNUCLEOTIDIC LINKAGE. A.M. Michelson and A.R. Todd, J. Chem. Soc. p. 2632 (1955)

2 DEOXYNUCLEOSIDE PHOSPHORAMIDITES--A NEW CLASS OF KEY INTERMEDIATES FOR DEOXYPOLYNUCLEOTIDE SYNTHESIS. S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters, 22, #20. 1859-1862 (1981)

3 THE SYNTHESIS OF OLIGODEOXYPYRIMIDINES ON A POLYMER SUPPORT. M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 21, 719-722 (1980)

4 SYNTHESIS OF DEOXYOLIGONUCLEOTIDES ON A POLYMER SUPPORT. M.D. Matteucci and M.H. Caruthers, J. Am. Chem. Soc., 103, 3185-3191 (1981)

5 NEW METHODS FOR SYNTHESIZING DEOXYOLIGONUCLEOTIDES. M.H. Caruthers, et al, Genetic Engineering, Vol. 4, Edited by Setlow and Hollaender (Plenum Publishing Corporation, 1982)

THE CHEMISTRY

The Solid Support

The support is a fully porous silica bead which is covalently bonded to the 3' hydroxyl of one of the four dimethoxytrityl nucleosides through an aminopropyl-succinamide linkage. The 3' terminal nucleoside of the oligomer to be synthesized determines which of the four silicas is used.

The support is derivatized to yield approximately 40 μmoles of dimethoxytrityl nucleoside per gram of silica. An exact loading is indicated for each lot of silica. One μmole of starting material should yield enough DNA for most applications, therefore approximately 25 mg of support is used for a normal synthesis.

The dimethoxytrityl group attached to the 5'-OH of the support bound nucleoside (and the nucleoside phosphoramidites) serves several purposes: protection of the 5'-OH until an appropriate point in the reaction cycle, the means for quantitative determination of coupling efficiency, and a lipophilic terminus for reverse-phase HPLC purification.

The Synthesis Cycle

The first step of the cycle (Step # 1 in Figure 1) is the removal of the dimethoxytrityl group from the support bound nucleoside by treatment with acid (zinc bromide or trichloroacetic acid) to liberate the 5'-hydroxyl. The choice as to which detritylating solution is used depends upon how the final product is to be utilized. Trichloroacetic acid does cause minor

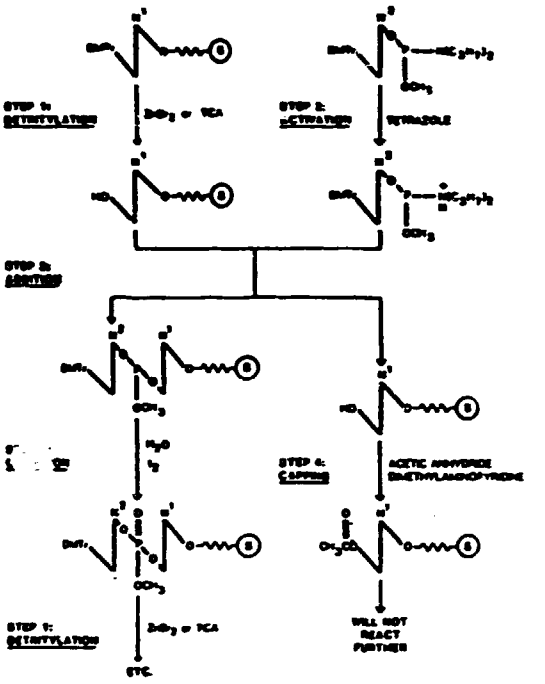

FIGURE 1.

depurination (especially on 3' terminal adenosine residues) but most of the product remains intact. For most purposes trichloroacetic acid is the detritylating solution of choice, though detritylation times should not exceed two minutes.

Zinc bromide, on the other hand, is a non-protic Lewis acid which does not cause depurination but is very slow to detritylate, especially on pyrimidines. The use of zinc bromide may add up to one half hour to each cycle. This protocol does not include instructions for $ZnBr_2$ detritylation.

After thorough washing, the support is treated with the proper dimethoxytrityl nucleoside phosphoramidite and tetrazole. The tetrazole acts as a mild acid and protonates the diisopropylamine on the phosphorous of the dimethoxytrityl nucleoside phosphoramidite

(Step #2). The protonated amine is a good leaving group and renders the phosphorous susceptible to nucleophilic addition. The support bound nucleoside 5'-hydroxyl in the presence of the activated dimethoxytrityl nucleoside phosphoramidite will then react to produce a nucleotide dimer (Step #3). The phosphorous at this stage is still in a trivalent state.

The addition reactions may not be quantitative. A finite amount of the support bound nucleoside 5'-hydroxyl may not react. If left as a free hydroxyl function, these unreacted chains would propagate in following addition steps. Large amounts of deletion sequences which contain one less base than the final product make purification more difficult. To avoid this problem, a capping reaction is utilized (Step #4). In this reaction, the unreacted support bound nucleoside 5'-hydroxyls are acetylated with acetic anhydride in the presence of dimethylaminopyridine and 2,6-lutidine. The 5'-acetylated support bound nucleosides are thus rendered unreactive and will not propagate in subsequent cycles.

After addition of the dimethoxytrityl nucleoside phosphoramidite, the phosphorus is in a trivalent state. The pentavalent state is achieved by treatment with iodine in the presence of water and base (Step #5).

SOME COMMENTS ABOUT THE CHEMISTRY AND REAGENTS

Repetitive Yields

Successful DNA synthesis requires high repetitive yields. Consider the synthesis of an oligomer 16 bases in length. The 3' terminal nucleoside is already bound to the support so a total of 15 nucleotide additions are required to achieve the proper length. Table 1 shows the overall yield as a function of various step yields.

TABLE 1.

STEP YIELDS	TOTAL YIELD
95%	46%
90%	21%
85%	9%
80%	4%
75%	1%

A high overall yield is beneficial in that a larger quantity of the desired DNA is produced. More importantly, the ease of purification is related to the purity of the crude product. In turn, the purity of the crude product is dependent on the quality of the reagents and solvents used throughout the synthesis, therefore it is desirable to take special care in the handling of all the reagents and solvents used.

Dimethoxytrityl Nucleoside Phosphoramidites

The inherent high reactivity of the dimethoxytrityl nucleoside phosphoramidites renders them somewhat unstable to oxygen and water.

Furthermore, any water associated with these compounds will, upon protic activation, act as a competitor with the 5'-hydroxyl of the DNA chain. Immediately upon delivery, place the dimethoxytrityl nucleoside phosphoramidites in a desiccator over $CaSO_4$ or other drying agent. Do not open them until all the apparatus has been assembled and all necessary reagents, solvents and equipment are ready.

Anhydrous Acetonitrile

Anhydrous acetonitrile is used during the course of the synthesis in two ways. First, it is the solvent in the dimethoxytrityl nucleoside phosphoramidite addition reactions. Second, it is used as an anhydrous wash to remove water and other nucleophiles from the reaction vessel. As with the phosphoramidites, the acetonitrile used in these steps must be anhydrous. After replacing the screw cap on the bottle with a septum, the anhydrous acetonitrile should only be accessed with a syringe and provided with an inert gas source to keep the bottle from developing a partial vacuum.

Capping Solutions

The capping solutions also need to be relatively anhydrous but not to the same degree as the acetonitrile. These bottles are also accessed through septa with a syringe. Accurate volumes of the capping reagents are required so the syringes used must be graduated. The syringes used for the anhydrous acetonitrile and capping reagents are not interchangable.

Other Reagents

The balance of the reagents and solvents need no special care other than being capped when not in use. They can all be delivered with Pasteur pipets. The pipets may be reused during the synthesis but only with the same reagents. Cross contamination may cause failures.

Throughout the protocol which follows, a number of steps call for delivery of 1.5ml of the solvents. This is an approximation and accurate measurements are not required.

SETUP OF THE APPARATUS

System Description

As shown in Figure 2, an integrated vacuum, inert gas and aspirator system is used for the synthesis. The oil pump is connected directly to a vacuum 3-way stopcock. One outlet is used to evacuate the vacuum desiccator. Another leads to the needle valve which is used to control the flow rate of inert gas into the evacuated desiccator. The outlet of the needle valve is connected to an inert gas 3-way stopcock. This stopcock provides the means for directing the inert gas towards either the vacuum desiccator system or the reaction vessel/water aspirator system. The second outlet of this 3-way stopcock leads to two plastic T-connectors terminated by 1 inch 20 gauge needles held in place by copper wire to prevent leaks. These lines are used to provide inert gas to the reaction vessel and reagent bottles. When not in use, these needles should be inserted into a rubber stopper to close the inert gas system to atmosphere.

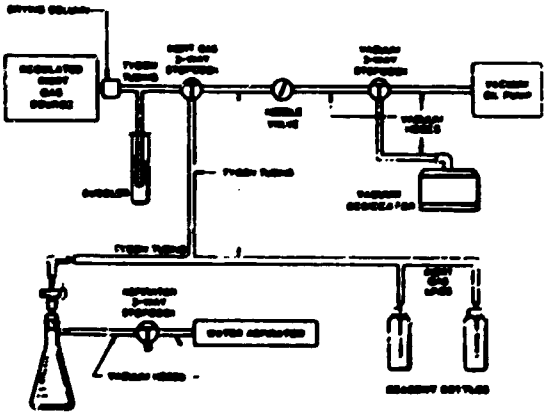

FIGURE 2.

The third line from the inert gas 3-way stopcock leads to a T-connector, one line of which terminates in a small tube containing mineral oil (bubbler). The bubbler provides a vent to prevent build up of a positive pressure in the gas system and serves as a monitor of vacuum/pressure fluctuations in the system. The other line from the T-connector leads through a drying tube to the regulated inert gas source.

The water aspirator is connected to a water aspirator 3-way stopcock. One outlet from this stopcock is open to air to relieve vacuum in the aspirator system and the other outlet is connected to a filter flask. A #8 stopper with #3 bore is used to cap the filter flask.

Reaction Vessel

The reaction vessel can be made in a glass shop or is available from Applied Biosystems[6]. It is a modified sintered funnel equipped with a stopcock to facilitate solvent and reagent removal from the support. The small port for adding reagents minimizes the chances of spills or loss of the silica. In various steps of the synthesis this port is capped with a rubber septum to maintain an anyhdrous environment. Inert gas is introduced to the reaction vessel by insertion of a hypodermic needle from the gas line through the septum.

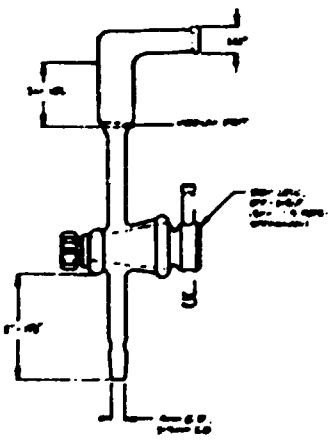

FIGURE 3. [6] Part #400055, Price $70.

MATERIALS AND REAGENTS LIST

1. Small test tubes or vials with tight fitting septa
2. Two ¼ml glass syringes with glass Luer hub (BD #2001)
3. One 1ml glass syringe with glass Luer hub (BD #2004)
4. Two ¼ml glass syringes with glass Luer hub (BD #2001)
5. Disposable hypodermic needles
6. Three 20 gauge one inch hypodermic needles with Luer hub (BD #1075)
7. Three 22 gauge five inch hypodermic needles with Luer hub (Hamilton 1/pkg N722, 5" 90022)
8. Rubber septa for the reaction vessel (Aldrich #Z10-072-2 or #Z10-073-0)
9. Rubber septa for the dimethoxytrityl nucleoside phosphoramidite bottles (Aldrich #Z10,074-9)
10. Rubber septa for the 250ml solvent and reagent bottles (Aldrich #Z10,076-5)
11. One liter filter flask
12. Tygon tubing
13. Vacuum hose
14. #8 rubber stopper with one #3 bore hole
15. #2 rubber stopper with one #3 bore hole
16. Test tube with side arm (Corning #9840)
17. Three 3-way stopcocks
18. Oil vacuum pump
19. Vacuum desiccator
20. Two desiccator jars
21. Drierite
22. Needle valve
23. Inert gas cylinder (Argon or Nitrogen)
24. Reguiator for inert gas cylinder
25. Pasteur pipets
26. Pasteur pipet bulbs
27. Wash bottle containing acetone

28. Wash bottle containing methanol
29. 100°C + oven
30. Visible spectrophotometer and cuvettes
31. Plastic T-connectors
32. 10 to 20 disposable hypodermic needles
33. Timer
34. Drying column
35. Applied Biosystems Reagents:
    Part #400026, anhydrous acetonitrile, or equivalent
    Part #400023, 3% trichloroacetic acid in dichloromethane, or equivalent
    Part #400041, support bound dimethoxytrityl-N-benzoyl-deoxyadenosine
    Part #400042, support bound dimethoxytrityl-N-benzoyl-deoxycytosine
    Part #400043, support bound dimethoxytrityl-N-isobutyryl-deoxyguanosine
    Part #400044, support bound dimethoxytrityl-deoxythymidine
    Part #400039, 5'-dimethoxytrityl-N-benzoyl-deoxyadenosine-3'-phosphoramidite
    Part #400013, 5'-dimethoxytrityl-N-benzoyl-deoxycytosine-3'-phosphoramidite
    Part #400012, 5'-dimethoxytrityl-N-isobutyrl-deoxyguanosine-3'-phosphoramidite
    Part #400014, 5'-dimethoxytrityl-thymidine-3'-phosphoramidite
    Part #400016, 500mg 1H-tetrazole
    Part #400018, cap B (6.5% dimethylaminopyridine in THF), or equivalent
    Part #400017, cap A (acetic anhydride/2,6-lutidine/THF 1:1:8), or equivalent
    Part #400022, oxididant (0.1M I_2 in water/2,6-lutidine/THF 1:10:40), or equivalent
36. Applied Biosystems Part #400055, reaction vessel, or equivalent
37. Reagent grade acetonitrile
38. Reagent grade methanol
39. Reagent grade nitromethane

SYNTHESIS PROTOCOL

Introduction

The protocol that follows is very detailed and intended as a guide for those who are inexperienced with organic chemical techniques in general and DNA synthesis in particular. It is important that successful syntheses are obtained with a minimum of initial failures. Therefore the "safest" procedure is presented although it is very tedious. Until some experience is gained, cycles may require up to one hour. After several syntheses, the cycle time will shorten to about 15–20 minutes.

Before beginning the synthesis, a number of photocopies of the protocol should be made. Each step can be checked off as it is completed so that no steps are missed. After using the cycle a number of times, the "Outline of Synthesis Protocol" should be sufficient as a guide.

Preparations Prior to Synthesis

The following preparation is required the day before synthesis begins:

1. Open the phosphoramidite stock bottles and place 20 µmoles of the appropriate dimethoxytrityl nucleoside phosphoramidites:
   1) A (16.8 mgs)
   2) C (15.9 mgs)
   3) G (16.2 mgs)
   4) T (14.1 mgs)

   into separate small tubes and seal with tight fitting septa.
2. Weigh 3.5 mg of tetrazole for each cycle planned in separate small test tubes and seal with septa.
3. Insert disposable hypodermic needles through septa of all stock bottles and measured aliquots.
4. Place all of the stock bottles, tubes and vials into the vacuum desiccator and connect the desiccator to a vacuum pump for at least 2 hours.
5. Wash one 1ml and two ¼ml glass syringes with 5 inch needles with acetone.
6. Place syringes and plungers separately into the oven.
7. Weigh out 1 µmole of the desired support bound 3' terminal nucleoside (about 25 mg).
8. Place the support in the reaction vessel.
9. Place a septum cap on the reaction vessel.
10. Insert the stem of the reaction vessel through the #2 rubber stopper with #3 bore.

THE ADDITION CYCLE

1. Detritylation (TCA)
   - 1.1 Remove the septum from the reaction vessel. (On subsequent cycles the septum will already be removed.)
   - 1.2 Close the reaction vessel stopcock.
   - 1.3 With a Pasteur pipet, add about 1.5ml of the 3% TCA solution.
   - 1.4 Replace the septum cap on the reaction vessel.
   - 1.5 Agitate carefully for 1 minute.
   - 1.6 Mount the reaction vessel (with #2 stopper) into the test tube with the side arm. (Use a separate test tube on each cycle if trityl yields are to be measured for each cycle.)
   - 1.7 Attach water aspirator 3-way outlet to the side arm of the test tube.
   - 1.8 Turn on the aspirator.
   - 1.9 Open the aspirator 3-way stopcock to vacuum.
   - 1.10 Remove the septum from the reaction vessel.
   - 1.11 Carefully open the reaction vessel stopcock and collect the solution
   - 1.12 Close the reaction vessel stopcock.
   - 1.13 With a Pasteur pipet, add about 1.5 ml of the 3% TCA solution.
   - 1.14 Let it stand for 15 seconds.
   - 1.15 Carefully open the reaction vessel stopcock and collect the solution.
   - 1.16 Close the reaction vessel stopcock.
   - 1.17 With a Pasteur pipet, add about 1.5 ml of nitromethane.
   - 1.18 Carefully open the reaction vessel stopcock and collect the solution.
   - 1.19 Close the reaction vessel stopcock.
   - 1.20 Open the aspirator 3-way stopcock to atmosphere.
   - 1.21 Remove the reaction vessel from the test tube and identify it with the cycle number if trityl yields are to be determined.
2. Removal of TCA
   - 2.1 Disconnect the aspirator line from the sidearm of the test tube.

2.2 Connect the aspirator line to the filter flask.
2.3 Mount the reaction vessel on the filter flask.
2.4 Open the aspirator 3-way stopcock to vacuum.
2.5 With a Pasteur pipet, add about 1.5ml of nitromethane to the reaction vessel.
2.6 Open the reaction vessel stopcock and drain the solvent.
2.7 Close the reaction vessel stopcock.
2.8 Open the aspirator 3-way stopcock to atmosphere.
2.9 With a Pasteur pipet, add about 1.5ml of nitromethane.
2.10 Replace the septum cap on the reaction vessel.
2.11 Remove the reaction vessel from the filter flask.
2.12 Agitate the reaction vessel to suspend the silica.
2.13 Remount the reaction vessel on the filter flask.
2.14 Open the aspirator 3-way stopcock to vacuum.
2.15 Remove the septum from the reaction vessel.
2.16 Open the reaction vessel stopcock and drain the solvent.
2.17 Close the reaction vessel stopcock.
2.18 Repeat steps 2.5 through 2.17.
2.19 Repeat steps 2.5 through 2.17 twice with methanol.
2.20 Repeat steps 2.5 through 2.17 twice with commercial reagent grade acetonitrile.

SAFE STOP

2.21 With a Pasteur pipet, add 1.5ml of commercial reagent grade acetonitrile.
2.22 Place a new septum cap on the reaction vessel.
2.23 Open the aspirator 3-way stopcock to atmosphere.
2.24 Turn off the aspirator.

3. Preparation for Addition
3.1 Remove the syringes and the plungers from the oven to cool.
3.2 Close the needle valve of the inert gas/vacuum aspirator system if it is open.
3.3 Adjust the inert gas 3-way stopcock so that the inert gas lines terminated with hypodermic needles are closed but the path leading to the vacuum desiccator is open.
3.4 Turn on the inert gas.
3.5 Adjust the vacuum 3-way stopcock so that the vacuum desiccator is not connected to the vacuum pump but the desiccator is connected to the needle valve inert gas system.
3.6 Very carefully open the needle valve while monitoring the bubbler, keeping a positive pressure in the inert gas system (bubbler must continue to bubble slightly at all times).
3.7 Continue to open the needle valve until the desiccator has come to ambient pressure.
3.8 Twist the collar of the desiccator.
3.9 Remove the vacuum hose from the desiccator.
3.10 Close the needle valve.
3.11 Adjust the vacuum 3-way stopcock to open the pump to atmosphere.
3.12 Turn off the vacuum pump.
3.13 Adjust the inert gas 3-way stopcock so that only the gas lines terminated with hypodermic needles are open to the inert gas.
3.14 Open the desiccator.
3.15 Quickly remove all of the needles from the reagent bottles and

tubes sealing them from atmosphere.

3.16 Remove the tube containing the dimethoxytrityl nucleoside phosphoramidites to be added and one tube of tetrazole. (Remember that the first dimethoxytrityl nucleoside phosphoramidite added is the second from the 3' end.)

3.17 Place stock bottles of phosphoramidites into the desiccator.

3.18 Remove the screw cap from the anhydrous acetonitrile and quickly recap with a rubber septum. Insert a needle from a gas line through the septum of the anhydrous acetonitrile bottle.

3.19 Insert the ¼ml syringe needle into the anhydrous acetonitrile, draw up to fill the syringe and dispose into waste.

3.20 Insert the ¼ml syringe needle into the anhydrous acetonitrile, draw up 100ul and add to the tetrazole.

3.21 Vortex the contents of the tetrazole tube until all of the tetrazole is dissolved.

3.22 Insert the ¼ml syringe needle into the anhydrous acetonitrile, draw up 100ul and add to the dimethoxytrityl nucleoside phosphoramidites.

3.23 Vortex the contents of the dimethoxytrityl nucleoside phosphoramidite tube until it is dissolved.

4. Addition Reaction

4.1 Insert a needle from a gas line through the septum of the reaction vessel.

4.2 Turn on the aspirator.

4.3 Open the aspirator 3-way stopcock to vacuum.

4.4 Carefully open the reaction vessel stopcock to drain the solvent, then close immediately. Monitor the bubbler making sure the gas pressure is set so that a vacuum is not created in the inert gas system.

4.5 Insert the 1ml syringe needle into the anhydrous acetonitrile and fill the syringe. Dispose into waste.

4.6 Insert the 1ml syringe needle into the anhydrous acetonitrile, draw up 1ml and add the solvent into the reaction vessel.

4.7 Carefully open the reaction vessel stopcock to drain the solvent, then close immediately.

4.8 Again, insert the 1ml syringe needle into the anhydrous acetonitrile, draw up 1ml and add the solvent into the reaction vessel.

4.9 Open the aspirator 3-way stopcock to atmosphere.

4.10 Remove the reaction vessel from the filter flask.

4.11 Agitate the reaction vessel to suspend all the silica.

4.12 Replace the reaction vessel onto the filter flask.

4.13 Open the aspirator 3-way stopcock to vacuum.

4.14 Carefully open the reaction vessel stopcock to drain the solvent, then close it immediately.

4.15 Repeat steps 4.6 through 4.14.

4.16 Insert the 1ml syringe needle into the anhydrous acetonitrile, draw up 1ml and add the solvent into the reaction vessel.

4.17 Carefully open the reaction vessel stopcock to drain the solvent, then close immediately.

4.18 Open the aspirator 3-way stopcock to atmosphere.

4.19 Turn off the aspirator.

4.20 Insert the ¼ml syringe into the anhydrous acetonitrile, fill and dispose into waste.

4.21 Remove the gas line from the anhydrous acetonitrile bottle and in-

sert the needle into the rubber stopper plug.

4.22 Insert the ¼ml syringe needle into the tetrazole solution, draw up all of the solution and add tc the reaction vessel.

4.23 Insert the ¼ml syringe needle into the dimethoxytrityl nucleoside phosphoramidite solution, draw up all of the solution and add it to the reaction vessel.

4.24 Remove the gas line from the reaction vessel and insert the needle into the rubber stopper.

4.25 Remove the reaction vessel from the filter flask.

4.26 Agitate the reaction vessel intermittently for 5 minutes to suspend all the silica.

4.27 Replace the reaction vessel onto the filter flask.

4.28 Reconnect the gas line to reaction vessel.

4.29 Turn on the aspirator.

4.30 Oper the aspirator 3-way stopcock to vacuum.

4.31 Carefully open the reaction vessel stopcock to drain the solution, then close it immediately.

4.32 Open the aspirator 3-way stopcock to atmosphere.

4.33 Turn off the aspirator.

5. Capping

5.1 Insert needles from the gas lines into both the Cap A and Cap B bottles.

5.2 Insert the ¼ml syringe needle into the Cap B solution (6.5% dimethylaminopyridine in THF). Draw up 0.15ml and add it to the reaction vessel.

5.3 Insert the ¼ml syringe needle into the Cap A solution (acetic anhydride/2,6-lutidine/THF 1:1:8). Draw up 0.15ml and add it to the reaction vessel.

5.4 Remove the gas needles from reaction vessel and Cap A and Cap B bottles and insert the needles into the rubber stopper plug.

5.5 Remove the reaction vessel from the filter flask.

5.6 Agitate the reaction vessel intermittently for 5 minutes to suspend the silica.

5.7 Replace the reaction vessel onto the filter flask.

5.8 Open the aspirator 3-way stopcock to vacuum.

5.9 Remove the gas line from the septum of the reaction vessel and plug it into the rubber stopper.

5.10 Turn on the aspirator.

5.11 Remove the septum from the reaction vessel.

5.12 Open the reaction vessel stopcock to drain the solvent.

5.13 Close the reaction vessel stopcock.

5.14 Open the aspirator 3-way stopcock to atmosphere.

5.15 Turn off the aspirator.

6. Oxidation

6.1 With a Pasteur pipet, add about 1ml of the oxidizing solution (0.1M I_2 in water/2,6,-lutidine/THF 1:10:40) to the reaction vessel.

6.2 Replace the septum cap onto the reaction vessel.

6.3 Remove the reaction vessel from the filter flask.

6.4 Agitate the reaction vessel to suspend the silica.

6.5 Replace the reaction vessel on the filter flask and let stand for 5 minutes.

6.6 Remove the septum from the reaction vessel.

6.7 Turn on the aspirator.

6.8 Open the aspirator 3-way stopcock to vacuum.

6.9 Open the reaction vessel stopcock to drain the solvent.

6.10 Close the reaction vessel stopcock.

7. Removal of Iodine

7.1 With a Pasteur pipet, add about 1.5ml of methanol to the reaction vessel.

7.2 Open the reaction vessel stopcock to drain the solvent.

7.3 Close the reaction vessel stopcock.

7.4 Open the aspirator 3-way stopcock to atmosphere.

7.5 With a Pasteur pipet add about 1.5ml of methanol to the reaction vessel.

7.6 Replace the septum cap on the reaction vessel.

7.7 Remove the reaction vessel from the filter flask.

7.8 Agitate the reaction vessel to suspend the silica.

7.9 Replace the reaction vessel onto the filter flask.

7.10 Open the aspirator 3-way stopcock to vacuum.

7.11 Remove the septum from the reaction vessel.

7.12 Open the reaction vessel stopcock to drain the solvent.

7.13 Close the reaction vessel stopcock.

7.14 Repeat steps 7.1 through 7.13 once.

7.15 Repeat steps 7.1 through 7.13 twice substituting nitromethane for methanol.

7.16 Open the aspirator 3-way stopcock to atmosphere.

7.17 Turn off the aspirator.

7.18 Disconnect the aspirator vacuum hose from the filter flask.

SAFE STOP

(If left overnight, cap the reaction vessel with a new septum.)

7.19 Clean the syringes as described under "Preparation" steps 10 and 11.

The cycle is now complete. Repeat the cycle beginning with Step 1.1 with the appropriate dimethoxytrityl nucleoside phosphoramidite until the desired length is achieved.

8. Trityl assay.

The high absorbance at 498nm ($E = 7.0 \times 10^4$) of the dimethoxytrityl cation which was liberated during detritylation affords an assay of the coupling efficiency of each nucleotide addition.

8.1 Determine the volume (V) of the trityl solution collected in the test tube with side arm.

8.2 Remove 0.1ml by syringe or pipet and dilute into 3ml of 0.1M toluenesulfonic acid in acetonitrile.

8.3 Read and record the absorbance (A) at 498nm.

8.4 Calculate the quantity (in µmoles) of trityl:

$$\frac{A \times V \times 31 \text{ (dilution factor)}}{70 \text{ ml } \mu\text{mol}^{-1}} = \mu\text{moles trityl} = \mu\text{moles oligonucleotide}$$

8.5 Rinse the test tube with side arm and the spectrophotometer cells extensively with methanol, then acetone.

8.6 Repeat 8.1 through 8.5 for each trityl solution if step yields are to be monitored.

OUTLINE OF SYNTHESIS PROTOCOL

I. Detritylation
   Treat for 1½ minutes with 3% trichloroacetic acid in dichloromethane
   Rinse with 3% trichloroacetic acid in dichloromethane
   Rinse the support with nitromethane.

II. Support Wash
   Wash and rinse* twice each with nitromethane
   Wash and rinse twice each with methanol
   Wash and rinse twice each with commercial grade acetonitrile
   Add acetonitrile to the reaction vessel.

III. Addition
   Seal the reaction vessel under inert gas, drain the acetonitrile and wash and rinse twice each with anhydrous acetonitrile
   Dissolve the dimethoxytrityl nucleoside phosphoramidite in anhydrous acetonitrile to give a 0.2M solution
   Dissolve the tetrazole in anhydrous acetonitrile to give a 0.5M solution
   Treat the support with tetrazole and the dimethoxytrityl nucleoside phosphoramidite for 2-5 minutes with intermittent agitation

IV. Capping
   Treat the support with 0.15ml of 6.5% DMAP in THF and 0.15ml 2,6-lutidine/acetic anhydride/THF (1:1:8) for five minutes

V. Oxidation
   Treat the support for 5 minutes with 0.1M I_2 in H_2O/2,6-lutidine/THF 1:10:40

VI. Washing the Support
   Wash and rinse the support twice each with methanol
   Wash and rinse the support twice each with nitromethane

*A wash includes an agitation step, a rinse does not

DEPROTECTION PROTOCOL

Discussion

Upon completion of the synthesis, four steps are necessary to yield biologically active DNA. These are:

1) Removal of the phosphate protecting groups (methyl) to transform the triester phosphates to diester phosphates.
2) Alkaline hydrolysis of the oligonucleotide from the support.
3) Alkaline hydrolysis of the base protecting acyl groups from the adenosine, cytosine, and guanasine residues.
4) Protic hydrolysis of the dimethoxytrityl group.

The chemistry of the deprotection is straightforward, however, a number of facts should be considered. Thiophenol, used in the removal of the methyl phosphate protecting groups, should always be handled very carefully. In addition to its corrosive and toxic properties, it produces a long lasting stench. Thiophenol should only be handled with gloves in a hood. The sulfur is readily oxidized by common household bleach. All contaminated glassware should be neutralized with bleach. It is also advisable to have bleach available in case of a spill.

Concentrated ammonium hydroxide is used to cleave the oligomer from the support (at room temperature) and to remove the base protecting groups (at 55°C). The concentration of the ammonium hydroxide is critical in these steps. The ammonium hydroxide should always be used in a hood and kept in the refrigerator when not in use. When in use, in either reaction, the container should be well capped to prevent any loss of ammonia.

MATERIALS AND REAGENTS LIST:

1) 15ml graduated conical test tubes
2) Pasteur pipets
3) Pasteur pipet bulbs
4) Rubber septa (Aldrich #Z10,076-5)
5) Copper wire
6) 55° oil bath
7) Evapomix or spin evaporator to lyopholize volatile solvents
8) 10-15ml screw cap test tubes
9) Applied Biosystems Part #400030 thiophenol/triethylamine/dioxane 1:2:2, or equivalent
10) Reagent grade dioxane
11) Reagent grade methanol
12) Reagent grade diethylether
13) Concentrated ammonium hydroxide
14) 80% acetic acid in water
15) Bleach
16) Solution of 0.01M TEAB (triethylammonium bicarbonate)
17) Sephadex G50-40

DEPROTECTION STEPS

1) Conversion of the triester to the diester
   - 1.1 With a Pasteur pipet, add 1.5ml diethylether to the reaction vessel.
   - 1.2 Turn on the aspirator.
   - 1.3 Open the aspirator 3-way stopcock to vacuum.
   - 1.4 Open the reaction vessel stopcock to drain the solvent and allow the support to dry.
   - 1.5 Close the reaction vessel stopcock.

1.6 Open the aspirator 3-way stopcock to atmosphere.
1.7 Turn off the aspirator.
1.8 Remove the reaction vessel from the filter flask.
1.9 Invert the reaction vessel into a 15ml conical test tube and collect the silica.
1.10 Rinse out the residual silica from reaction vessel with dioxane and add it to the conical test tube.
1.11 Centrifuge or allow the support to settle.
1.12 Carefully decant the dioxane.
1.13 With a Pasteur pipet, add 1ml of the thiophenol/triethylamine/dioxane 1:2:2 solution.
1.14 Quickly cap the conical test tube with a rubber septum.
1.15 Quickly cap the thiophenol/triethylamine/dioxane solution.
1.16 Quench the thiophenol on the Pasteur pipet with bleach.
1.17 Vortex the conical test tube and let it stand 45 minutes at room temperature.

2) Washing the Support
2.1 Remove the septum from the conical test tube and place it in the bleach.
2.2 Decant the solution into the bleach taking care to avoid loss of silica.
2.3 With a Pasteur pipet, add about 5ml of dioxane, vortex (or agitate) and centrifuge (or allow to settle).
2.4 Carefully decant the solvent.
2.5 Repeat steps 2.3 and 2.4 four times with methanol.
2.6 Repeat steps 2.3 and 2.4 once with diethylether.
2.7 Let the silica dry.

3) Hydrolysis of the Oligomer from the Support
3.1 Remove the concentrated ammonium hydroxide from the refrigerator.
3.2 With a Pasteur pipet, add 1ml of concentrated ammonium hydroxide to the conical test tube.
3.3 Place a septum on the test tube.
3.4 Fasten the septum cap with copper wire.
3.5 Replace the ammonium hydroxide in the refrigerator.
3.6 Allow the reaction to proceed at room temperature for 2½ hours with periodic agitation.

4) Hydrolysis of the Base Protection Acyl Group
4.1 Remove the concentrated ammonium hydroxide from the freezer.
4.2 Remove the copper wire to release the septum on the test tube.
4.3 Remove the septum and carefully decant the solution into a test tube with a tight fitting screw cap. Remember that the product is now in the solution and no longer bound to the silica support.
4.4 Cap the tube.
4.5 With a Pasteur pipet, add 1ml of ammonium hydroxide to the conical test tube containing the support, vortex (or agitate) and centrifuge (or allow to stand).
4.6 Carefully decant the solution into the test tube with the tight fitting screwcap and cap.
4.7 Cap the ammonium hydroxide and place it in the refrigerator.
4.8 Place the test tube containing the oligomer into the 55° bath for 8-18 hours.
4.9 Lyopholize to remove the ammonia. If trityl selection reverse-phase HPLC is to be used to purify the oligomer, a volatile base such as triethylamine should be added drop-wise periodically during lyopholization to maintain a basic environment.

4.10 The oligonucleotide is now in the proper form for purification by trityl selection on reverse-phase HPLC. After purification, continue with step 4.11. If other than reverse-phase purification is used, continue with step 4.12.

4.11 Lyopholize the purified fractions collected from the HPLC.

4.12 Treat the resultant dry pellet with 80% acetic acid in water for 45 minutes at room temperature.

4.13 Lyopholize to remove all the solvent.

4.14 Resuspend the pellet in ethanol, vortex and lyopholize.

4.15 Repeat step 4.14.

4.16 Take up the pellet in 0.2ml 0.01M TEAB.

4.17 Desalt on Sephadex G-50-40 using 0.01M TEAB as the mobile phase.

4.18 Combine the first 3ml which show absorbance at 260nm.

4.19 Lyopholize to remove all the solvent.

4.20 Resuspend the pellet in ethanol, vortex and lyopholize.

4.21 Repeat step 4.20.

The DNA should now be biologically active and ready for purification and characterization by gel electrophoresis.