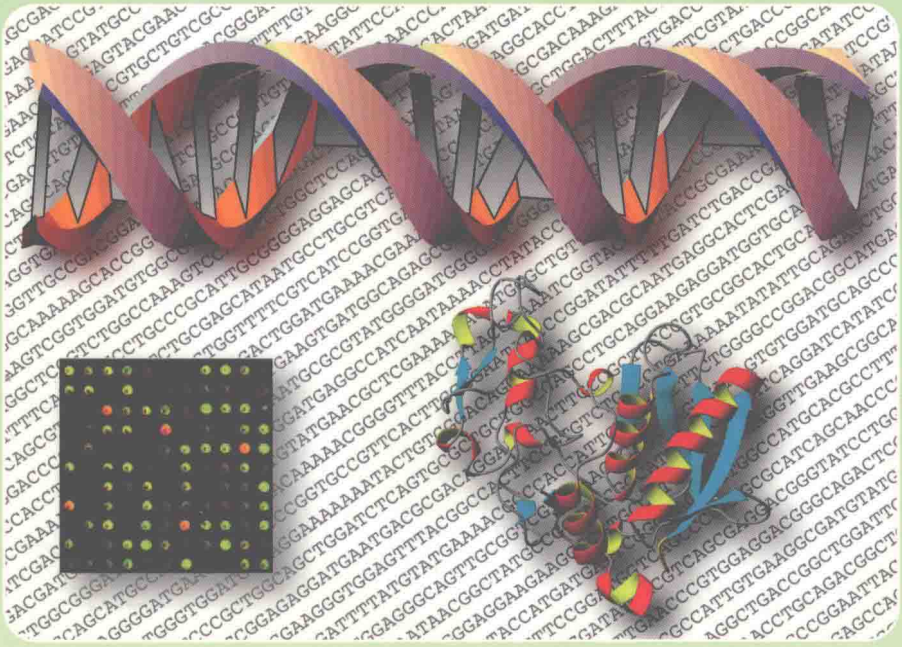
Frédéric Dardel and François Képès

Bioinformatics

Genomics and post-genomics

Translated by Noah Hardy



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Bioinformatics

Preface to the French edition

This book is directly based on a course that we teach at the *École Polytechnique*. We thank all our colleagues and friends there who have made its existence and publication possible:

Sylvain Blanquet, Chairman of the Biology Department, who first thought of creating this interdisciplinary course some ten years ago, when such a project was highly innovative. He has followed and supported the development of the project in the context of the genomic revolution.

Jean-Marc Steyaert, our colleague at the Computer Science Department, where he initiated the teaching of bioinformatics, and in which he remains active. His critical attention, constant theoretical and methodological contributions, and increasing involvement in biological problematics, have contributed in an essential way to bringing this book about, as well as influencing its contents.

Philippe Dessen, who participated in some of the very early stages in the teaching of bioinformatics at the *École Polytechnique* while he was there; our contacts with him over the years have been invaluable.

Finally, going from the stage of course-notes to a published book would not have been possible without the decisive contributions of Jean-Paul Coard, of *Éditions de l'École Polytechnique*, as well of those of Jean-Claude Mathieu, Véronique Lecointe, Martine Maguer, and Frédéric Zantonio, involved in the technical production.

Frédéric Dardel
François Képès

Preface to the English edition

At the suggestion of Vincent Schachter, to whom we are very grateful, we decided to produce an English version of our book. We have worked closely with Noah Hardy, to ensure the accuracy of the translation, and have updated all chapters with new material where necessary. Chapter 8 was rewritten entirely in English. We hope that this edition will enable many more readers to enjoy our book. We would like to thank Joan Marsh and her colleagues at Wiley for their help in producing this edition.

Frédéric Dardel
François Képès

Contents

1

Genome sequencing

1.1 Automatic sequencing

The dideoxyribonucleotide method, developed during the early 1980s in England, at the Cambridge University laboratory of Fred Sanger, is today universally employed to sequence DNA fragments. It is based on the use of DNA polymerase to elongate a single strand of DNA, starting from a primer, utilizing another DNA strand as the template. The DNA polymerase elongates the strand in the presence of four deoxyribonucleotide monomers (dATP, dTTP, dGTP, and dCTP) and a dideoxyribonucleotide analog (ddNTP), which acts as the chain terminator (Figure 1.1). Specific incorporation of the analog by DNA polymerase yields a mixture of fragments that selectively terminate at positions corresponding to each nucleotide (*A*s, in the example below).

The principle of the dideoxyribonucleotide ('dideoxy') method is illustrated in Figure 1.2. Four parallel reactions are carried out, one with each ddNTP, the DNA fragments obtained being separated by electrophoresis. A fluorescent tracer is used to identify fragments synthesized by the polymerase so as to distinguish them from template DNA. The tracer is attached to one extremity of the fragment, either at the 5′-end of the sequencing primer or at the 3′-end of the dideoxynucleotide terminator. Modern automatic sequencers utilize an *in situ* detection system during electrophoresis, in which a laser beam emitting in the fluorophore absorption spectrum is passed through the gel (Figure 1.3). A migrating DNA fragment in the path of the laser beam then emits a fluorescent signal detected by a photodiode on the other side of the gel. The signal is amplified and transmitted to a computer programmed with special software for analyzing it.

Under favorable conditions, this technique permits reading up to 1,000 nucleotides per sequenced fragment, and an average of **500 to 800 nucleotides during routine experiments.** Two dideoxy methodologies coexist at present: one employs a single photophore, and the other uses four, each with a distinct emission spectrum. In the first system, the four mixtures, corresponding to the four ddNTPs, are introduced into different electrophoresis gel wells. Analysis is

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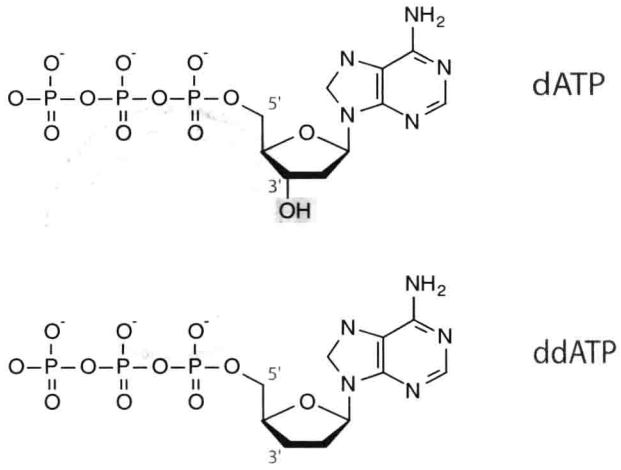

Figure 1.1 Dideoxynucleotide structure
Replacement of the 3′-OH group in the dideoxynucleotide (ddNTP) by a 3′-H group prevents formation of a phosphodiester link at its 3′-end. The modified nucleotides have a normal 5′-triphosphate side, thus may be incorporated into the chain by DNA polymerase. Since A-T and G-C pairing rules are followed during ddNTP incorporation, the ddATP will be incorporated wherever there is a T facing it on the template strand.

accomplished by comparing the migration rates of the fragments in the four resulting lanes.

In the second system, each of the four sequencing reactions uses a different fluorophore that modifies the corresponding ddNTP. After the four polymerization reactions have taken place, the resulting DNA fragments are mixed and introduced into the same gel well. Constituent nucleotides are identified according to the emission properties of the fluorescent tracers exposed to the laser beam using selective color filters, after which a single gel lane is analyzed.

The four-fluorophore technique is a bit more expensive, since it requires somewhat more varied chemistry. However, it has the advantage of being better adapted to high-throughput systems, since more samples are analyzed on the same gel. In the latest generation of automatic sequencers, the classical rectangular polyacrylamide gel is replaced by a reusable capillary tube, whereas the separation and detection principles remain unchanged. This technique reduces the time required for an experiment from several hours to a few minutes, also minimizing preparation time. In principle, the highest-performance multicapillary machines can process up to 1,000 samples per day, equivalent to 0.5 Mbases of **raw** sequence per day per machine.

Massive high-throughput sequencing centers today often use several dozen such machines with robots that control the sequencing reactions automatically executing pipetting, mixing, and incubation steps, thereby minimizing the risk

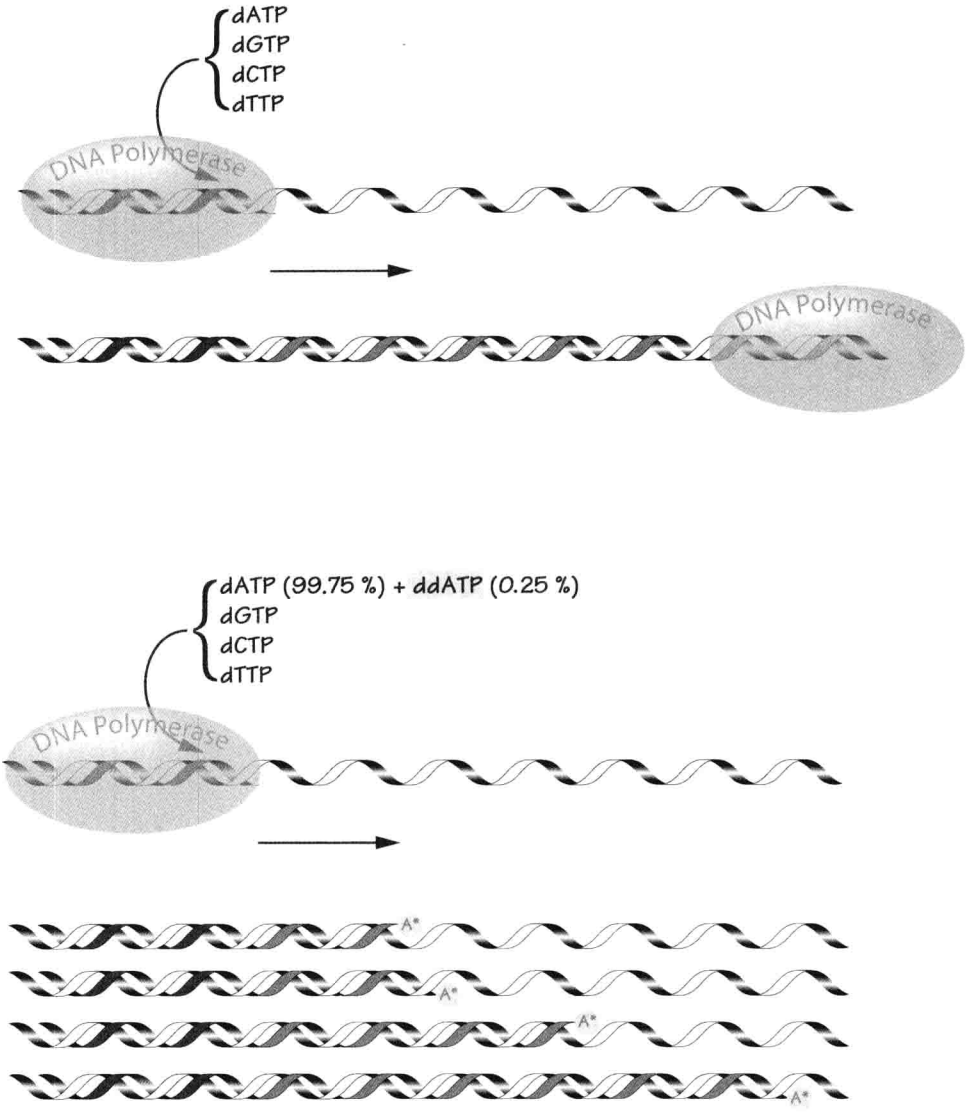

Figure 1.2 Principle of the Sanger sequencing method
In the presence of a template DNA strand and the four dNTPs, DNA polymerase can elongate a complementary DNA strand starting from an **oligonucleotide primer**, which hybridizes to the template strand. When a dideoxynucleotide is incorporated by the polymerase, it acts as a chain terminator, blocking further elongation. This incorporation is entirely random, proceeding at a rate that is a function of the ratio of the dideoxynucleotide concentration to that of the corresponding deoxynucleotide (here it is [ddATP] / [dATP] = 1 / 400).

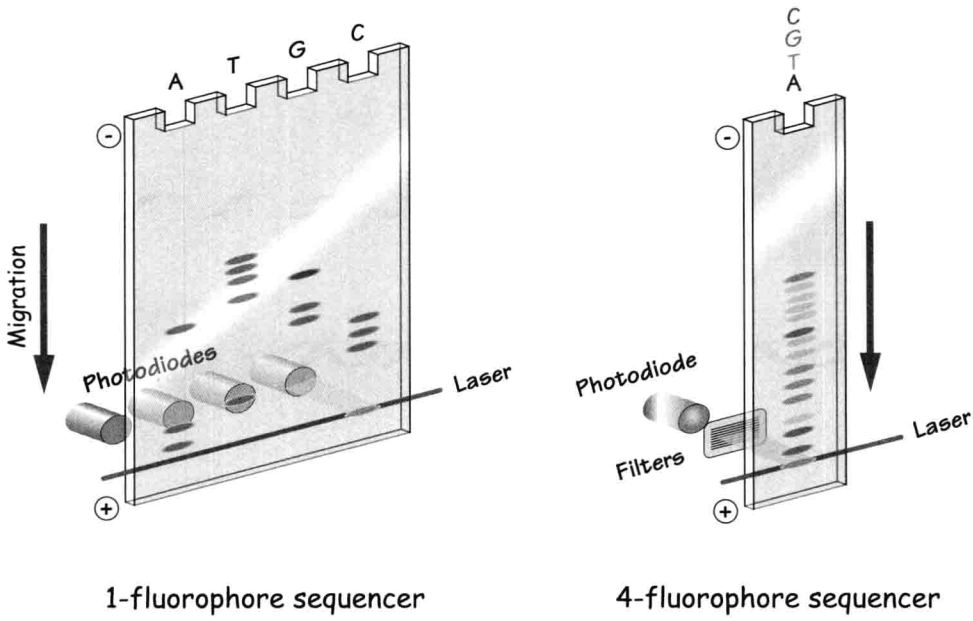

Figure 1.3 Automatic sequencing using 1- and 4-fluorophore sequencers
Samples introduced into the wells (top) are separated by electrophoresis on a polyacrylamide-urea gel. The 5′-CAATCCCGGATGTTT sequence is read from bottom to top.

of human error (see Figure 1.4). The preparation of DNA templates remains the most difficult step to automate, although significant progress has been made in this respect.

1.2 Sequencing strategies

The sequencing methodologies described above fail to address major difficulties that need to be considered when operating a large-scale sequencing program:

- Only DNA fragments of between 500 and 1,000 nucleotides may be sequenced;
- A sequencing primer that is complementary to the template is required for the DNA polymerase to begin synthesizing.

Fortunately, these two obstacles may be simultaneously overcome by fragmenting the DNA that is to be sequenced into segments of size compatible to

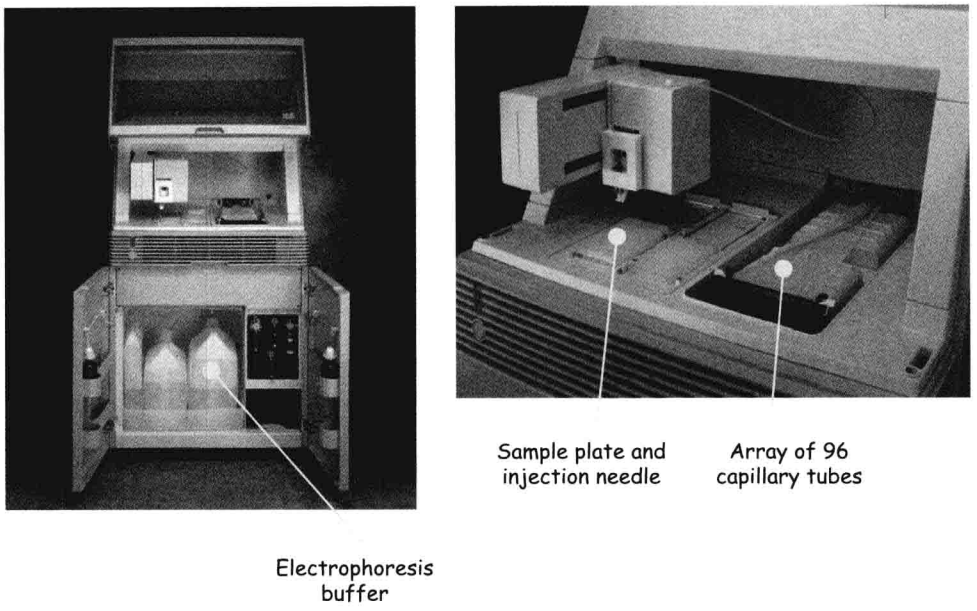

Figure 1.4 Advanced automatic multicapillary DNA sequencer for simultaneous sequencing of 96 samples. An automatic injection system executes several consecutive separations without manual intervention (®Applied Biosystems).

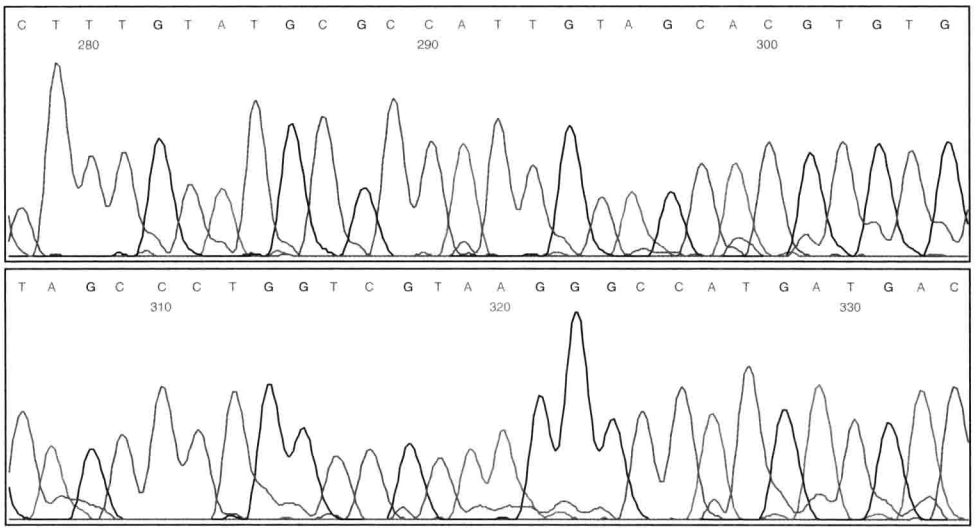

Figure 1.5 Example of a sequencing profile
Intensity of the signal detected by the photodiodes as a function of separation time. Each color is associated with one of the four separation reactions (A, G, C, and T).

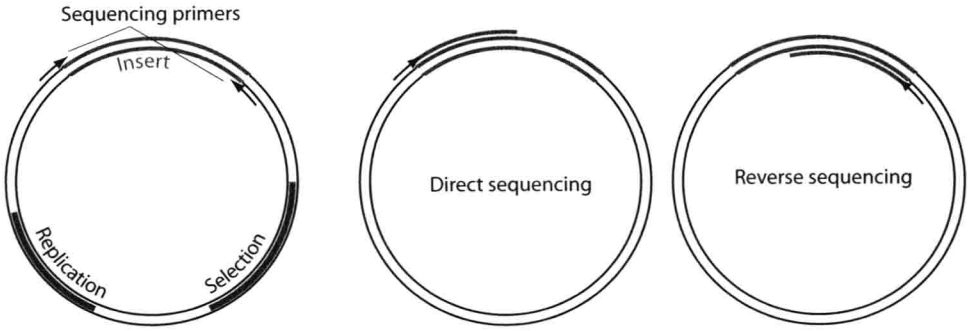

Figure 1.6 Sequencing in a vector starting with universal primers.

that of the sequencing system yield (~10^3 base pairs) and by inserting them into an appropriate vector (plasmid or virus). The vector is selected according to several criteria:

- It must be able to replicate autonomously in a convenient host cell (usually *Escherichia coli*);
- It must bear one or several gene markers that permit selection of cells that contain it (antibiotic resistance, for example);
- Its nucleotide sequence must be known;
- It must contain restriction endonuclease sites that permit cloning by insertion of foreign DNA fragments.

In practice, small bacterial plasmids are generally used. The DNA to be sequenced is fragmented and ligated into the vector, which is then propagated in host cells. The clone cell lines (derived from a single initial cell by successive division), each containing a different recombinant vector with the same inserted DNA fragment are then isolated. A library of DNA fragments may thus be constituted by collecting a large number of these clone cell lines, and used for further study (see Figure 1.7).

In order to determine the DNA sequence of such a fragment, the corresponding cell line is cultured and its DNA extracted for sequencing by the dideoxynucleotide method. Since the nucleotide sequences located on each side of the vector clone site are known, they are used as the primer (see Figure 1.6). These primers are independent of the DNA inserted into the vector and may be used to sequence any fragment; they are therefore called *universal primers*. Because

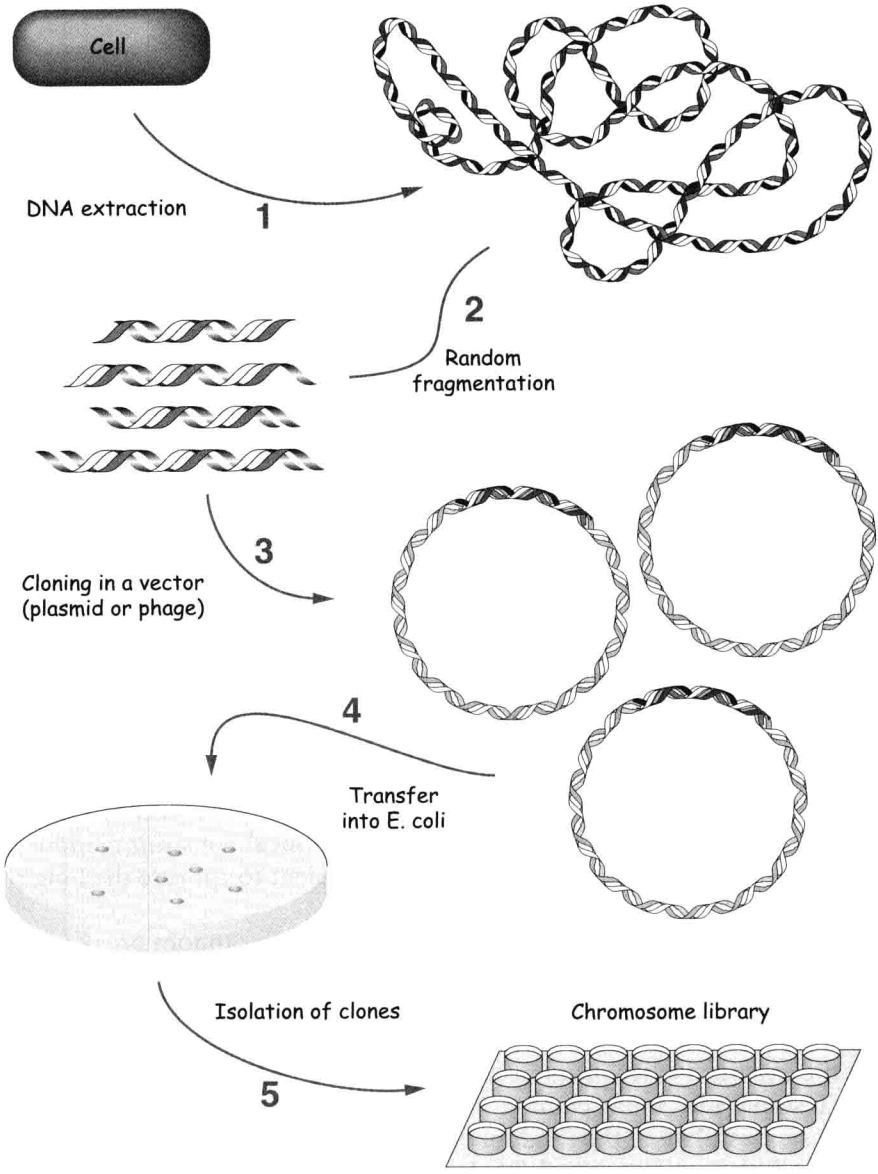

Figure 1.7 Strategy for constructing a DNA library.

such primers are constant, it is very easy to incorporate fluorescent tracers needed during oligonucleotide synthesis into them. The fluorescent primers thus produced may be used in most sequencing procedures.

1.3 Fragmentation strategies

In sequencing a long stretch of DNA – especially a complete genome – it is essential that it be cut into fragments of a size compatible with the sequencing technology. This poses two additional questions:

- Which cutting strategy should be employed?
- How can the complete sequence be reconstituted from the pieces?

These two questions are intricately related, since the reassembly method is sensitively dependent on how the fragmentation is accomplished. Two approaches have mainly been used: **random fragmentation** and **segmentation after mapping.**

Random fragmentation

In random fragmentation, the full length of the DNA to be sequenced is cut into small pieces of optimal sequencing size (~1,000 base pairs). A high cutting frequency (one site per 200–250 bp) restriction enzyme may be used for this purpose, under conditions of partial digestion (10–20 percent) in order to generate 1,000- to 2,000-bp fragments. Alternatively, ultrasound may be used to break the DNA into small pieces, since the mechanical constraints induced by ultrasonic vibrations in DNA in solution are sufficient to rupture the long phosphodiester chain.

The mechanical (ultrasound) method results in more random breaks than the enzymatic method but necessitates an additional step to repair the extremities of the DNA fragments produced, since breaks produced by ultrasound treatment do not occur at the same level in the two DNA strands. This may require paring the extended extremities of single strands, so that the resulting fragments may be inserted into blunt cloning sites in the sequencing vector.

The basic postulate of the random or *shotgun* method is that if enough clones are analyzed, the entire original DNA sequence will be covered. Assuming that fragmentation and cloning are really random processes and that the DNA sequence is sufficiently large compared with that of individual clones (which is generally the case for a full genome), the probability that a given DNA nucleotide studied **not be covered** by random sequencing is a Poisson distribution:

$$p_0 = e^{-N/L},$$

where N is the total number of nucleotides sequenced in the set of clones and L the total length of the DNA studied. N/L is the coverage rate, which is the rate of data redundancy. In order to obtain a 99 percent sequencing rate, that is, $p_0 = 0.01$, it is necessary to sequence a number of clones equal to 4.6 times ($\log 0.01 \approx -4.6$) the length of the DNA studied.

In the case of a genome or a very long DNA fragment, it is thus practically inevitable that gaps remain in the sequence, which must be filled using some approach other than the random *shotgun* method. It is also possible to statistically evaluate the length and average number of such gaps:

$$\text{Total length of gaps} = Le^{-N/L}$$

$$\text{Average length of each gap} = Ln/N$$

$$\text{Number of gaps} = N/ne^{-N/L},$$

where n is the average length of each fragment sequenced (~500 nucleotides). The following is an example of the results for a bacterial genome ($L \approx 10^6$ bp) and for the genome of a higher organism, such as a mammal or a plant ($L \approx 10^9$ bp) with a coverage rate of factor 6 (an average value for this type of project), which yields 99.75 percent of the sequenced nucleotides:

The random approach raises two important points:

- It is impossible to cover the entire genome without greatly increasing the number of clones to be sequenced; to be nearly certain of covering the entire bacterial genome in the above example would require that $p_0 \ll 10^{-6}$, at least 14 times the coverage rate. From the practical point of view, it is more economical to accept a coverage rate of between 4 and 6 and then fill the few dozen remaining gaps *ad hoc* (see Table 1.1 below).

- Assembly of the puzzle of the set of fragments may require systematic side-by-side comparison of all the sequences obtained. For k sequences, this

Table 1.1

	Bacteria (1 Mbp)	Mammals (1 Gbp)
Number of sequences	12,000	12,000,000
Number of remaining gaps	30	29,750
Average gap size	200	200