

DNA sequencing with chain-terminating inhibitors

[Frederick Sanger](#), S. Nicklen, and A.R. Coulson, (Proc. Natl. Acad. Sci. 1977)

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In accordance with Biology 4241, instructed by Dr. Steven M. Carr

Introduction

[Frederick Sanger](#), the father of modern Genomics.

The term DNA sequencing refers to sequencing methods for determining the order of the nucleotide bases—adenine, guanine, cytosine, and thymine—in a molecule of DNA.

Some [background](#) information which Sanger was able to use to aid his discovery of DNA sequencing.

Before Sanger published his paper on chain-terminating inhibitors, he had devised the '[plus-minus](#)' method to sequence DNA.

Maxam and Gilbert [method](#) of sequencing.

Principle's of Sanger's Method

Atkinson *et al.* in 1975 showed that 2',3'-dideoxythymidine triphosphate (**ddTTP**) causes inhibition of DNA polymerase when incorporated into the growing oligonucleotide in the place of thymidylic acid (**dT**).

Sanger's Method:

- The same primer, template DNA, DNA polymerase, **ddTTP**, dNTP's (**dATP**, **dTTP**, **dCTP**, **dGTP** with one having a ^{32}P label) are incubated together produces DNA
 - Have the same 5' end due to same primer sequence
 - Have the same 3' end due to the **ddTTP** inhibition
 - Oligonucleotide fragment length will be **DIFFERENT!**
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Materials and Methods

Dideoxynucleotides, or ddNTPs, are nucleotides that are lacking a [3'-hydroxyl](#) (-OH) group on their deoxyribose sugar.

Since the deoxyribose already lacks a 2'-OH, dideoxyribose lacks hydroxyl groups at both its 2' and 3' carbons.

Without the hydroxyl group, no further nucleotides can be added by a DNA polymerase to a growing nucleotide chain.

This is due to the fact that a phosphodiester bond can not be created since deoxyribonucleoside triphosphates allow DNA chain synthesis to occur through a condensation reaction between the 5' phosphate of the current nucleotide with the 3' hydroxyl group of the previous nucleotide.

For this reason, dideoxynucleotides are sometimes called chain terminators.

[Sanger's preparation of the Triphosphate Analogues](#)

Sequencing Procedure

1. A DNA template.
2. A DNA primer.
3. Deoxynucleotides (**dATP**, **dCTP**, **dGTP**, **dTTP**).
4. Dideoxynucleotides.
5. DNA polymerase.

- Restriction enzyme fragments were obtained from Φ -X174 replicative form and separated by electrophoresis.
- Fragments from 5 μ g of Φ -X174 in 5 μ l of water mixed with 1 μ l of viral or complementary strand Φ -X174 DNA (0.6 μ g) and 1 μ l of H x 10 buffer, heated to 100 °C for 3 minutes, and then incubated at 67°C for 30 minutes.
- This solution was diluted to 20 μ l with H buffer. Samples of 2 μ l taken for each incubation and mixed with 1 μ l DNA polymerase (Klenow, Boehringer, Mannheim) and 2 μ l of “mix” consisting of 1 μ Ci [32 P]dATP, 1 μ l 1.5 X H buffer and the following reactions:

ddT: 0.1 mM dGTP, 0.1 mM dCTP, 0.005 mM dTTP, 0.5 mM ddTTP

ddA: 0.1 mM dGTP, 0.1 mM dCTP, 0.1 mM dTTP, 0.5 mM ddATP

ddG: 0.1 mM dCTP, 0.1 mM dTTP, 0.005 mM dGTP, 0.5 mM ddGTP

ddC: 0.1 mM dGTP, 0.1 mM dTTP, 0.005 mM dCTP, approximately 0.25 mM ddCTP

(Low yield of ddC caused Sanger to use an araC derivative)

araC: 0.1 mM dGTP, 0.1 mM dTTP, 0.005 mM dCTP, 12.5 mM araCTP

- These samples were then incubated at room temperature for 15 minutes, and 1 μ l of 0.5 mM dATP was added and incubation continued for another 15 minutes, which is known as the 'chase' step. (If the chase is omitted then termination at A residues occurred in samples due to low concentration of [32 P]dATP).
- For small primers, when subsequent splitting was not needed, the various reaction mixtures directly denatured.
- Long primers were split using 1 μ l of appropriate restriction enzyme and added after the 'chase' and incubated at 37°C

Samples were then denatured and separated on 12% acrylamide gel at 40 mA for 14 hours.

Sanger also used a single-site ribo-substitution method which produced similar results.

What is an [Autoradiogram](#)?

[Sanger Sequencing](#)

Results

The Sequences obtained in the gel results are representative of the viral strand of the DNA with the exception of **Figure 3** where the primer was annealed to the viral strand, creating a complement. The results were compared to those from Sanger's previous experiment with Φ X174 where the genome was sequenced using the plus and minus methods.

[Figure 1](#)

[Figure 2](#)

[Figure 3](#)

Discussion

The Dideoxy inhibition of DNA elongation method has a number of advantages over the plus and minus methods:

1. The dideoxy method is much simpler; requires no preliminary extension, cutting out an incubation and purification step on a sephadex column.
2. Requires only the commercially available DNA polymerase I.
3. The results are clearer with fewer artefact bands.

4. The sequences can be read further; more sequence can be analyzed at a time. Gels can be read from 15-200 nucleotides from priming site, and upwards of 300 in quality samples.
5. Intermediate nucleotides in the 'runs' show up as bands, whereas estimation of the number of bands was sometimes required in the plus & minus methods. Bands would be theoretically expected to be the same, but is not the case experimentally. Bands sometimes run together, but it is often possible to estimate the number of nucleotides from the strength and width of the band.
6. Inhibitor method can be used on smaller scale. Better incorporation of ^{32}P -labeled triphosphates.

But there are also a number of disadvantages:

1. In longer 'runs' minor artefacts bands still occur, but are easily identified.
2. "[Pile-ups](#)" of bands can occur. These are caused by DNA forming base-paired loops under the conditions of the acrylamide gel electrophoresis.
3. [Difficulty in obtaining all the inhibitors](#) - particularly ddGTP, which was not commercially available.

Improvements:

1. Accurate determination of sequence requires multiple methods; should not rely on this method alone, but in conjunction with plus and minus.
2. Priming both the forward and reverse strands.

How Sequencing has [Changed](#)

Chain-termination methods have greatly simplified DNA sequencing. For example, the chain-termination-based "Sequenase" kit from USB Biochemicals contains most of the reagents needed for sequencing, which are pre-aliquoted and ready to use.

Here at Memorial University scientists are able to take advantage of high-throughput DNA sequencing technology through the [CREAIT Network](#).