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Genetic analysis of Gastric B-cell lymphoma: Application of cycle sequencing technique with fluorescent dyes

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DNA sequencing now becomes a basic technique to study the molecular mechanism of diseases including lymphomas. Depending on a several evolutionary techniques, for example enzymatic chain termination sequencing technique, PCR (polymerase chain reaction) method and an automated sequencer with non-RI materials, DNA sequencing becomes easier and accurate. Nevertheless, DNA sequencing still has some difficulties according to the techniques and evaluation. We here present examples of cycle sequencing technique for medical materials including gastric B-cell lymphoma, and discuss the usefulness and limitation.

DNA sample preparation

Extraction DNA from human blood and tissues are one of the critical points to obtain accurate results of DNA sequence. Phenol-chloroform method has been used to extract DNA from tissues, but other methods without phenol are to be widely used. In our laboratory, we select the methods according to sample condition; NaI method for fresh blood sample and Phenol-chloroform method for frozen or paraffin fixed tissues.

PCR amplification and Purification of DNA

Using DNA extracted from samples, PCR is performed by specific primers that are designed to amplify the sequence region wanted to know; immunoglobulin heavy chain gene variable region. After PCR, the PCR products are purified by PCR product purification kit; GENECLEAN™, QIAGEN DNA Isolation™ etc.

DNA labeling and Sequencing reactions with fluorescent dyes

There are two DNA labeling methods with four different dyes identified the A, C, G, and T extension reactions; 5'-dye labeled primers (dye primers) and 3'-dye labeled dideoxynucleotide triphosphates (dye terminators) (Figure 1).

Dye Terminator Labeling : With dye terminator labeling, each of the four dideoxy terminators (ddNTPs) is tagged with a different fluorescent dye. Thus, the growing chain is simultaneously terminated and labeled with the dye that corresponds to that base (Figure 1A).

<u>Advantages of dye-labeled terminators method</u>: 1) an unlabeled primer can be used, 2) four sequencing reactions are performed simultaneously in one tube, 3) false stops caused by the enzyme prematurely falling off the template go undetected since no dye is attached, 4) dye terminators require fewer pipetting steps than dye primers since reactions are performed in a single tube.

Dye Primer Labeling: With dye primer labeling, extension products are identified by using primers tagged with four different fluorescent dyes in four separate base-specific reactions. The products from these four reactions are then combined and loaded into a single gel lane (Figure 1B).

<u>Advantages of dye-labeled primers method:</u> 1) labeled primers are available for common priming sites, 2) dye primers generally produce more even signal intensities among bases than dye terminator chemistries.

Cycle Sequencing: Template DNA, dNTP, ddNTPs and DNA polymerase are pipetted into a tube and place in a thermal cycler. Successive rounds of denaturation, annealing, and extension result in linear amplification of extension product (Figure 1C). Both dye primers and dye terminators can be used with cycle sequencing.

<u>Advantage of cycle sequencing method</u>: 1) A broader range of starting DNA quantity is acceptable, when compared with Sequenase, 2) Protocols are robust and easy to perform, 3) High temperatures reduce secondary structure, allowing for more complete extension, 3) Works well for double-stranded PCR direct sequencing.

Sequence Analysis

After DNA fragments are labeled, the fragments are analyzed by gel or capillary electrophoresis. ABI PRISM 310 and 377 Genetic Analyzers (from Perkin Elmer) are used in our laboratory. In this paper, we explain several points for DNA sequencing by ABI PRISM 310 Genetic Analyzer.

ABI PRISM 310 Genetic Analyzer

The ABI PRISM 310 Genetic Analyzer is a laser-induced fluorescence capillary electrophoresis system. DNA samples labeled with four different fluorescent dyes, that are used to identify the A, G, C, and T extension reactions, load onto the autosampler (up to 96 samples). The sequencer automatically introduces the samples into a polymer-filled capillary for electrophoresis. The dye-labeled DNA fragments electrophorese through the polymer, and DNA fragments separate according to size. As the labeled samples travel through the capillary and into the window, they are illuminated. The fluorescent dyes attached to the fragments are excited by the laser and emit light at a

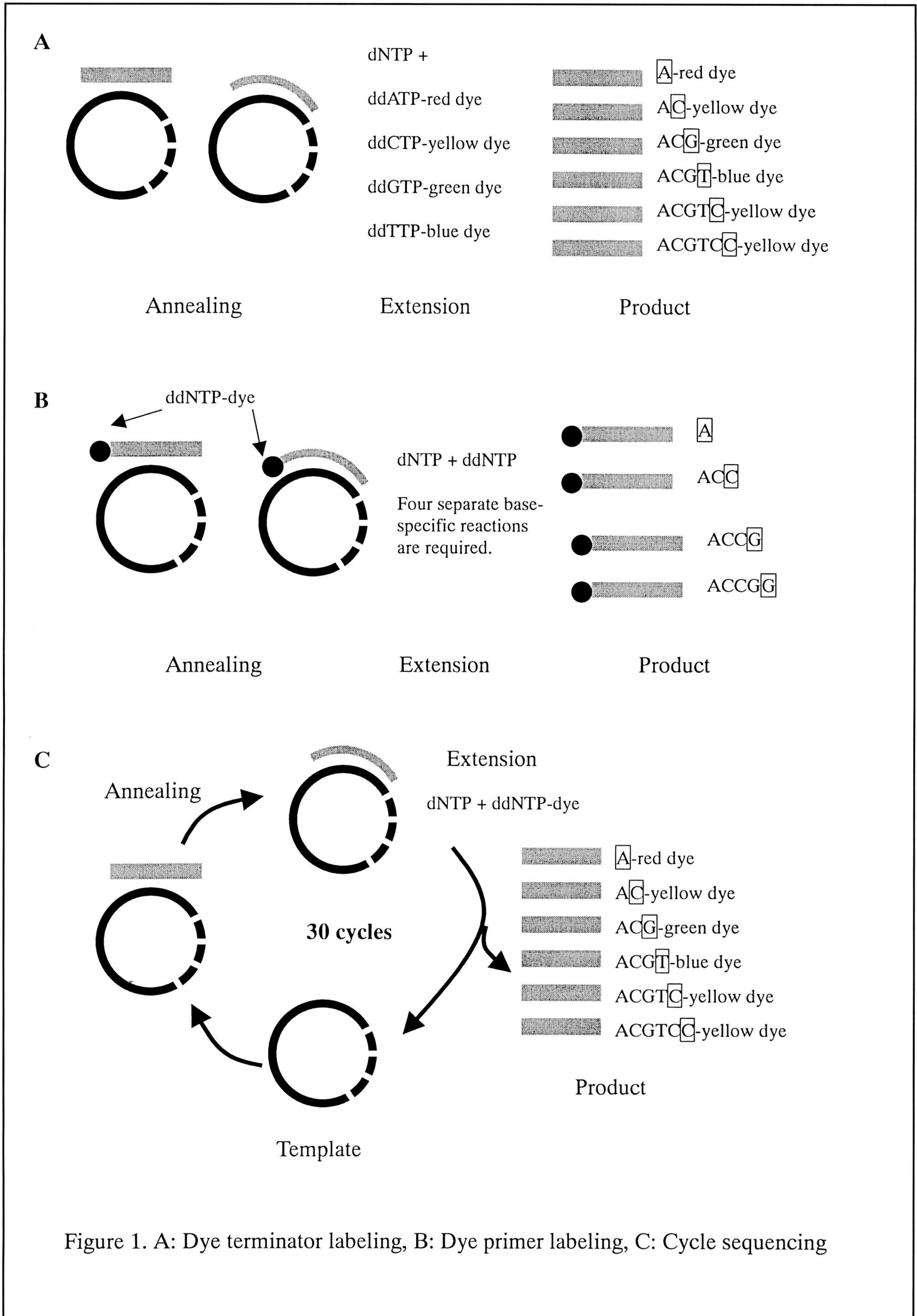

Figure 1. A: Dye terminator labeling, B: Dye primer labeling, C: Cycle sequencing

specific wavelength for each dye. The light is collected and separated by a spectrograph according to wavelength. It is collected onto a charge-coupled device (CCD) camera, so all four types of fluorescent emissions can be detected simultaneously (Figure 2).

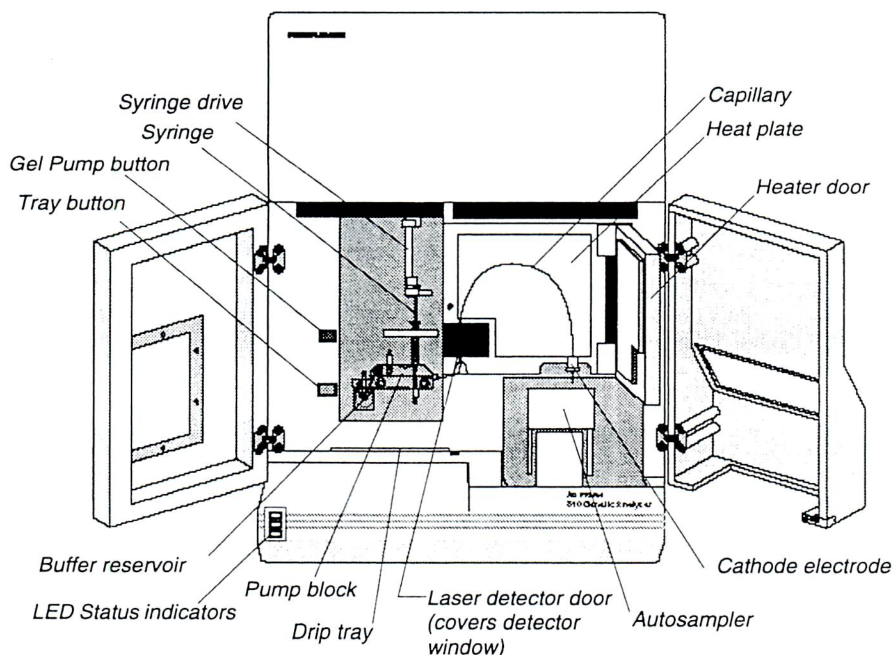

Figure 2. The instrument of ABI PRISM310 Genetic Analyzer

All four colors can be detected and distinguished in a single gel lane This strategy improves sequencing accuracy because it eliminates problems caused by variations in electrophoretic mobility from lane to lane. It also increases the number of templates that can be analyzed on a single gel by a factor of four.

The data collection software collects the light intensities using software selectable filters (spectral or wavelength) and stores them as electrical signals for eventual processing.

The hard disk of the Macintosh computer stores the digitized output signal. As data is collected for one sample, another sample can be analyzed. The computer screen displays the newest information received from the ABI PRISM 310 as it is generated. At the end of a run, the computer can automatically analyze the collected data and print electropherograms on a color printer (Figure 3).

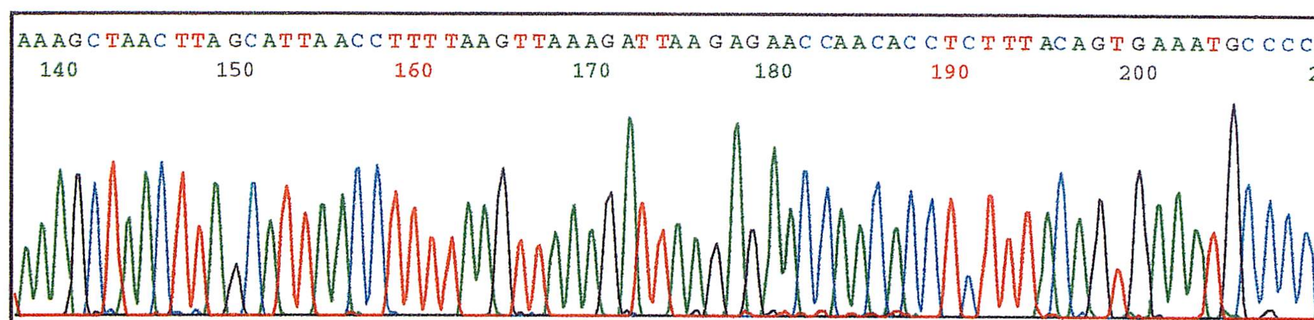

Figure 3. Electropherograms of DNA sequence

Capillary: The capillary has a nominal life of 100 injections for GeneScan analysis and 100 injections for sequencing. For sequencing analysis, the capillary is 47 cm long with a 75 um i.d. A window in the polyimide coating is located 36 cm from the sample injection end. For GeneScan™ analysis, the capillary is 61 cm long with a 75 um i.d. A window in the polyimide coating is located 50 cm from the sample injection end.

Temperature Control: The capillary passes through a temperature-controlled region of the instrument. This region contains a resistively-heated, thermostatically-controlled hot plate. Temperature settings range from 30℃ to 60℃, with an accuracy of ±3℃.

How to get the Best Sequencing Data

With conscientious template preparation and sequencing technique, both dye primer and dye terminator protocols yield reliable base calling that often meet or exceed published specifications. Nevertheless, any one component in the system whether it be the operator, the gel, the instrument, the template, or the reagents can contribute to disappointing results if not performing optimally! Many factors that affect the quality of sequencing data are known and it is important to review those things that can go wrong and the ways in which they can be corrected.

In our experience, sequencing results are best when we use Perkin-Elmer chemistry kits, for example BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, and follow the recommended protocols carefully. We occasionally make a small modification to the recommended protocols. No single chemistry always works well on every possible sequence.

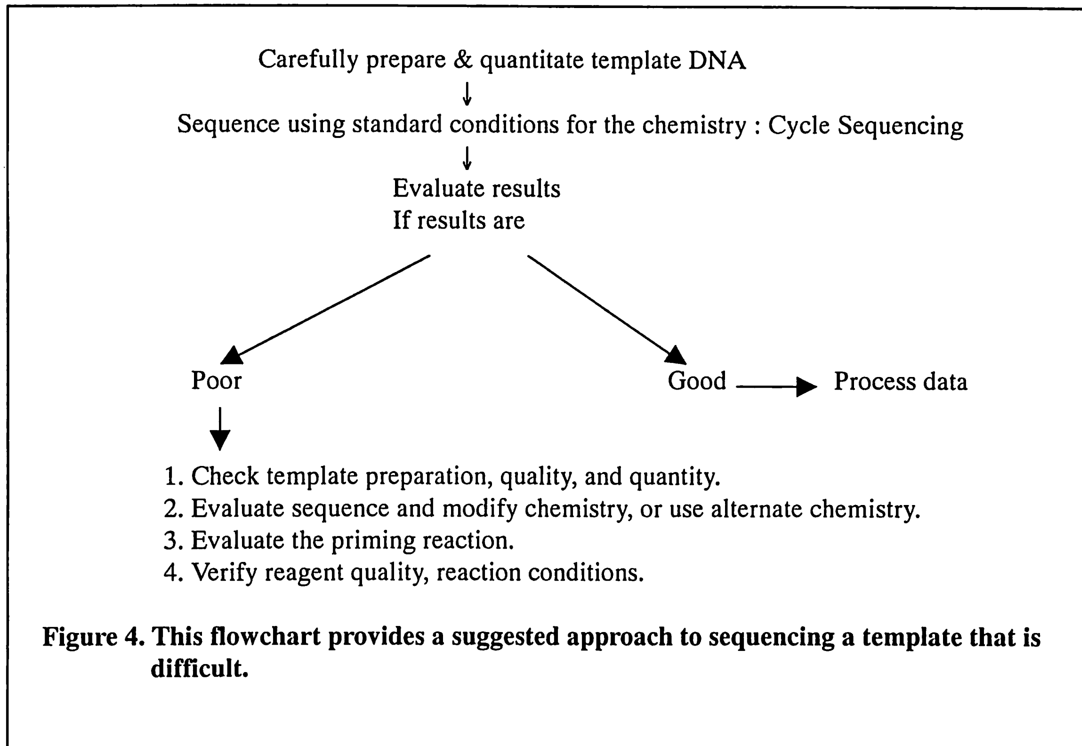

Figure 4. This flowchart provides a suggested approach to sequencing a template that is difficult.

Factors that affected the Quality of Data

The following list summarizes the most critical factors in producing good quality sequencing data. They are listed in the order of importance and each is discussed in detail in this section.

- DNA template quality and quantity, and sequence composition
- Gel quality and glass plate cleanliness
- Primer design
- Reagent age, purity, and storage conditions (both for kit reagents and user-supplied reagents)
- Post-reaction clean-up procedures
- Equipment: calibration of thermal cycler.
- Protocol modifications (not so recommended)

Template Preparation

The quality of the template DNA can have a major impact on the quality of the sequence data obtained for both dye terminator and dye primer reactions. In fact, inadequate template preparation is the most common cause of sequencing problems. For all template preparation methods, be sure to follow the recommended procedures.

Characteristics of Data from Poor Template Preparations

- Top-heavy data with a short length of read
- Acceptable signal with some noise peaks (Ns) or peaks under peaks
- Weak signal and overall noisy data
- Flat lines (no signal) in the raw data

Both the method of purification and host strain variability can impact the quality of template preparation. Potential problems include protein contamination, quantitation problems due to contaminants such as RNA or chromosomal DNA, and residual salts and other contaminants (from reagents used in the preparation, from the media, or from the cells).

Consistent Template Purification Methods

QIAGEN DNA Isolation products and alkaline lysis with PEG precipitation have proven to be the most reliable mini- or midi-preparations for sequencing DNA. These two methods generally provide good yields and clean DNA. Other template purification methods may also be satisfactory.

Cleaning a Failed Template Preparation

It is sometimes possible to clean up a failed template preparation with one of the following methods:

- Purify the DNA by ultrafiltration (for example, with CentriconR-100 Micro-Concentrator columns, P/N N930-21 19)
- Extract the DNA twice with 1 volume of chloroform or chloroform: isoamyl alcohol in a 24:1 (vol:vol) ratio. Precipitate the aqueous layer to remove all traces of chloroform.
- Precipitate with PEG. Add 0.16 volumes of 5M NaCl and 1 total volume of 13% PEG. Incubate on ice for 20 minutes, then centrifuge at 4℃ for 20 minutes. Rinse with 70% ethanol.

DNA Quantity

The amount of DNA template used in a sequencing reaction can affect the quality of the data. Too much template makes data appear top heavy with peaks that are very strong at the beginning of the run and then fade rapidly. Too little template or primer DNA reduces the signal strength and peak height.

Table 1. Quantity of Template DNA to Use for Each Method

	Dye Primer Cycle Sequencing	Dye Terminator Cycle Sequencing	Dye Primer Sequenase	Dye Terminator Sequenase
PCR product	20-200 ng	20-200 ng	with mag. beads*	0.3-1.0 ug
Single-stranded	0.6 ug	0.25-0.5 ug	2.0 ug	2.0 ug
Double-stranded	1.2-1.5 ug	1.0 ug	Not applicable	5.0 ug
Cosmid		0.5-2.0 ug	Not applicable	

* Determined by automated solid phase protocol

DNA Sequence

The types of sequences that cause problems may be different depending on the chemistry used. No single chemistry always works with all sequences. Modification of a particular method or use of an alternative chemistry may be necessary. The following list describes the types of templates that are most difficult to sequence.

- Templates that have an overall GC-rich base composition
- Templates that have an overall AT-rich base composition
- Templates with extremely AT-rich or GC-rich regions of sequence
- Templates with regions of pronounced secondary structure
- Templates with long homopolymer regions
- Templates with long repeats

GC-rich Templates

In all organisms, the GC base composition of DNA varies from 25-75%, with the greatest variation in bacteria. Mammalian genomes typically have a GC content of 45-50%, but any sequence may contain a GC rich region. GC-rich templates with a GC content greater than 62 percent may be difficult to sequence when using the standard reaction conditions of cycle sequencing chemistries. We have examined the requirements for sequencing such templates with Dye Terminator Cycle Sequencing chemistry in detail. Certain modifications are presented here as a method to effectively sequence through GC-rich templates. To determine the best way to handle GC-rich templates with Dye Terminator Cycle Sequencing chemistry, we tried many modiflcations to the standard chemistry. The most successful modifications were the addition of 5% DMSO to the standard reaction or a combination of several other modifications.

We here mentioned some technical aspects of DNA sequencing especially in the case of DNA sequencing with ABI PRISM 310 Genetic Analyzer. We think it is important to analyze immunoglobulin heavy chain gene variable region in Chinese gastric B-cell lymphoma on these technical points.

References

ABI PRISM310™ Genetic Analyzer User's Manual
ABIPRISM™ DNA Sequencing Chemistry Guide