TGGE-STAR: PCR-primer design and melting analysis. Optimization of PCR-Gradient-Gel-Electrophoresis

Christoph Gille and Andreas Gille
Institute of Biochemistry, Charité,
Medical Faculty of the Humboldt University
10117 Berlin
Monbijoustraße 2a
Germany
E. mail: christoph.gille@charite.de
http://www.charite.de/bioinf/tgge

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Abstract

PCR combined with temperature or denaturing gradient gel electrophoresis (TGGE) or (DGGE) are rapid and very sensitive screening method for point mutations. Computer aided design of PCR primers for denaturing gradient gel electrophoresis and the careful choice of a suitable gradient are the most important factors to guarantee the success of the screening. The program TGGE-STAR was written to facilitate the design of PCR primers fulfilling the requirements for a sensitive mutation screening with TGGE or DGGE. It supports the new concept of bipolar clamping which is essential when mono-polar clamping leads to fuzzy bands. The optimal type of clamping can be predicted from the melting profiles produced by the program. TGGE-STAR can be downloaded from http://www.charite.de/bioinf/tgge and requires a MICROSOFT operating system or a MS-DOS-EMULATOR.

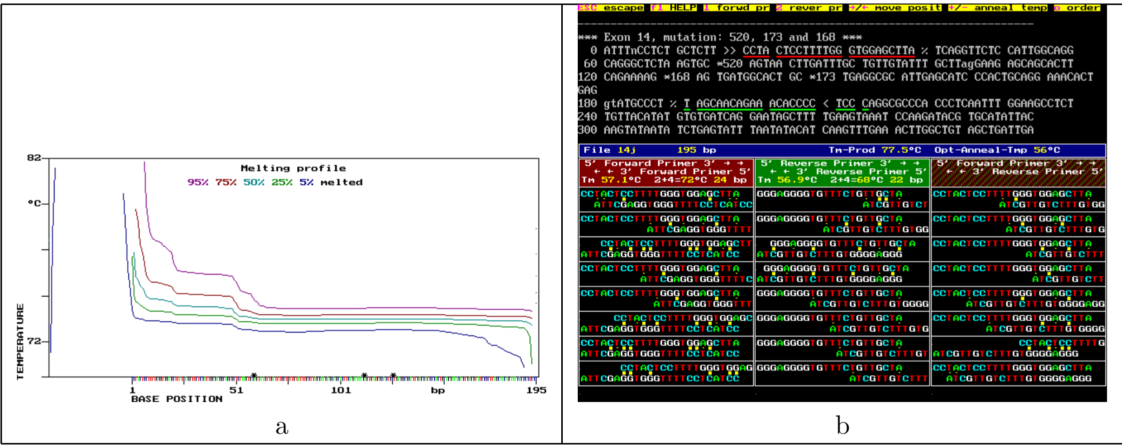

Figure 1: Screenshots. **1a** Melting profile. The DNA-molecule stretches along the x-axis. Each tick on the x-axis represents a base pair. The y-axis shows the melting temperature The middle one of the group of five "curves" describes the temperature at which a base pair is unordered or helical with equal probability of 50%. The other "curves" correspond to higher or lower probabilities. At the 5'-end (left) the thermostability is artificially increased by a clamp and the temperature profile ascends. Apart from the clamp there are two melting domains: from the 5'-terminus to the 50th base pair and from the 50th base pair extending to the 3'-terminus. Generally, the sensitivity in upper melting domains is reduced. However, in this case the temperature step is not that significant and all mutations may still be found. Three asterisks above the x-axis mark positions of mutations which had been detected with this assay. They had been typed into the sequence text in figure 1b using a text editor. **1b** Primerdesign. Primer dimers are calculated on the fly when the primer binding sites (red and green underlining) are moved with the arrow keys. The upper part contains the sequence text. Three asterisks were typed at sequence positions where mutations had been identified. The bottom part shows the primer complentarities. The annealing of identical primers are predicted in the 1st and 2nd column and annealing of forward with reverse primers is given in the 3rd column. Please note, that the binding partners are written in opposite direction. The dimers are sorted according to their significance. The goal is to reduce the hydrogen bonds which are drawn as small and big yellow dots. Especially unfavorable are hydrogen bonds at the 3' termini.

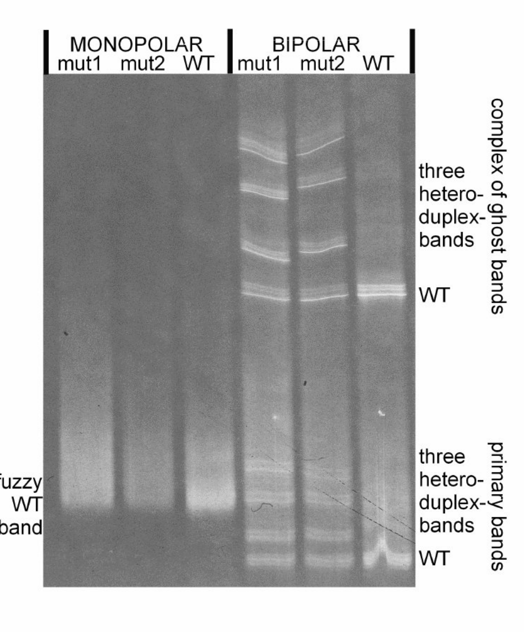

Figure 2: Two mutations which are missed in traditional TGGE with only one clamp. The bands are fuzzy (left) and no hetero-duplex bands appear though the PCR-products are heterozygous for a point mutation. Clamping both ends of the PCR-product renders the bands sharp and both mutations clearly detectable.

Technical Report

Introduction to PCR-TGGE

TGGE is a type of acrylamide gel electrophoresis which is used to detect point mutations and polymorphisms within PCR-products (Fodde and Losekoot, 1994). Electrophoresis of the DNA fragments is performed through an increasing linear temperature gradient from anode to cathode. With increasing temperature the DNA "melts". Hydrogen bonds are disrupted and the number of unordered non-helical molecules rises. In order to prevent both strands from completely falling apart, they are bound by an artificial clamp at one or both ends. Alternatively the denaturing gradient can also be achieved by an increasing urea concentration within the gel (DGGE). In the Transgenomic Wave^{Tm} (Kuklin et al., 1999) the DNA-fragmants also electrophoretically migrate in an increasing temperature environment.

TGGE is very fast and sensitive, detecting virtually all heterozygous sequence variations within the PCR-product. These qualities should make TGGE the screening method of choice.

Problems of PCR-TGGE

However, TGGE is not widely used because the setup is relatively difficult. During our project, the technique exhibited mainly two difficulties reducing its applicability.

- Primarily, the primer design is difficult. Not only the two primers must be compatible with each other, but also the melting property of the PCR-product must have certain features. (Poland, 1974).
- Secondly, the electrophoretic bands are sometimes fuzzy and therefore mutations are not detected (Macek M et al., 1997). This is probably due to an essentially irreversible conformational change and can be predicted with the computed melting profiles.

Previously available software

The program MELT87.EXE (Lerman and Silverstein, 1987) is widely used to calculate melting profiles (figure 1a) and is available for academic institutions. It has no user interface. Instead it requires the manual preparation of input files and the visualization of the text output with graphic programs. For plain PCR-primer design plenty of programs are available. Thus, at the beginning of our project, three programs were involved in the design of primers: MELT87, a graphical plotting program and a primer design package. MELT94 is an updated version of MELT87, which contains a graphical display (http://web.mit.edu/osp/www/melt.html). These three programs were successively run several times in order to find the best compromise between an optimal melting characteristic and an optimal primer pair .

Features of TGGE-STAR

TGGE-STAR was developed in order to streamline primer design for TGGE and DGGE, such that all steps involved in primer design could be performed within one environment. The user interface was optimized so that it allows to design many primers in a short time. In order to optimize its speed it was written in ANSI-C to take advantage of fast pointer arithmetics. Since it is a pure MS-DOS program, it runs very fast on native MICROSOFT-machines and many operating systems which can emulate DOS, such as LINUX or OS/2. The user interface is keystroke driven, and is very comfortable. The program consists of two parts: The melting analysis of PCR-products (figure 1a) and the primer optimization 1b. Both parts are closely linked. The calculation of the melting

characteristics is automatically performed by MELT87.EXE which is embedded into TGGE-STAR. The primer optimization part differs from other programs to some degree. First of all the DNA-sequence file does not require any specific format. Moreover the sequence is displayed in its original format preserving all line breaks and comments. This greatly increases the orientation in a text which otherwise consists only of four different letters. The primer binding sites are underlined red and green and can be moved using the arrow keys. Simultaneously unfavorable primer self binding can be watched in the lower half of the screen. The goal is to minimize primer self priming. The primer binding sites can also be found automatically. Furthermore, four features were added which proved to be extremely helpful for our project.

- Broadened bands are a frequent problem of TGGE and DGGE (Macek M et al., 1997) and were observed in about 20% of all exons of the NF1-gene (Fahsold et al., 2000). It is a particular problem in genomic DNA analysis since the GC-content is lower in introns than in exons. The relative thermic instability of intronic regions can lead to a melting profile which does not decline towards the unclamped end. In our NF1-Project (Fahsold et al., 2000) allmost all melting profiles with this feature are associated with widened bands wheras monotonously slightly declining profiles are associated with sharp bands. The program supports bipolar clamping which can rescue cases of blurred bands. An explanation of these kinetic phenomenons is given by Abrams et al. (1995). It is generally believed that despite the increased band width, sequence variants result in readily detectable band shifts in the gradient. In order to disprove this assumption two missed mutations are shown in figure 2. Both become visible by bipolar clamping.
- The superposition of the curves of cumulative probability ($\bar{P}$-plots) of several fragments can guide the design of multiplex PCR-TGGE essays. This is because the $\bar{P}$-plots give a good estimate for the position of the bands on the gel. In multiplex-TGGE it is important that bands do not obscure each other and that the fragments arrive at their individual melting temperature approximately at the same time.
- The program assists fragmentation of the sequence to be analyzed whenever this is necessary because of an unfavorable melting behavior.
- An interface can be created to other programs such as programs for restriction analysis.

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