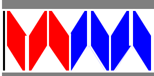
SpectruMedix Application Notes



Mutation Screening by Temperature Gradient Capillary Electrophoresis

Introduction

With more DNA sequence information available, genome-wide single nucleotide polymorphism (SNP) discovery and scanning becomes possible. SNPs are of great interest in genetic illness diagnosis and as genetic markers in linkage and association studies. Although many methods have been reported for mutation detection, low-cost, high-throughput and high-detection-rate methods are still in demand. We have developed a fast and reliable mutation detection system based on temporal temperature-gradient capillary electrophoresis (TGCE) in a massively parallel format. At a temperature close to the Tm of the mutant amplicon, the randomly reannealed combinations of a mutation sample can be separated. By comparing the electropherogram patterns of the wild-type control with mutation samples, mutation positives can be readily identified (Fig. 1).

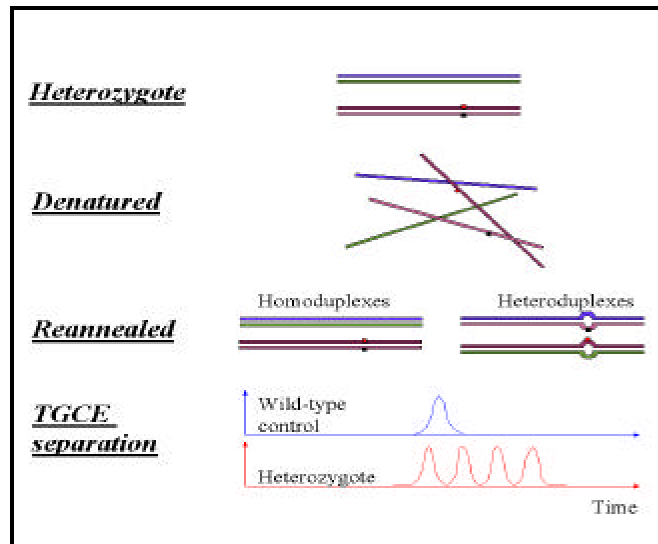


Figure 1. Scheme of Sample preparation and mutation detection by capillary electrophoresis

Using the SCE9610 genetic analyzer, up to 96 samples can be screened for mutations simultaneously with a fraction of the cost of other methods with greater sample throughput. The ability of SCE9610 to analyze large number of mutation samples simultaneously comes from the innovative design that sweeps capillary array through a wide range of temperature during electrophoresis covering all possible melting temperatures of the samples. Modulated capillary cartridge design in the SCE9610 allows precise and fast-response temperature control of the capillary array. Each sample stays at the vicinity of its Tm for a fraction of the total electrophoresis time. Therefore, the method does not require prior knowledge of the type and location of the SNP in a target amplicon (Fig. 2).

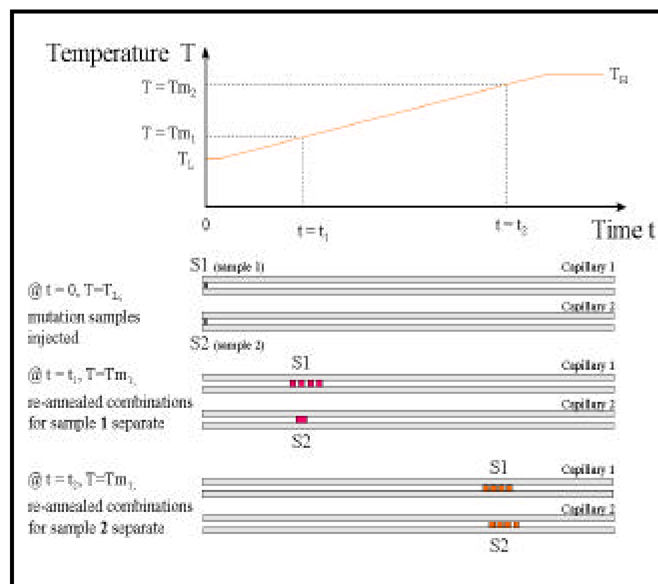


Figure 2. Parallel TGCE for high throughput mutation screening

This novel approach provides 2 distinctive advantages:

1. eliminates the need of calibration runs usually required with other technologies to determine the optimum melting temperature for a particular sample;
2. allows simultaneous screening of large number of samples with different Tm in a single run.

The technique requires laser-induced fluorescence detection and SpectruMedix screening gel. The use of SpectruMedix screening gel eliminates the need of costly fluorescent dye-tagged reagents. It also differentiates double-stranded fragments from single-stranded DNA for clearer pattern recognition.

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Sample preparation: heteroduplex and homoduplex formation

In this procedure, DNA heteroduplexes and homoduplexes are formed from PCR amplified products for mutation screening analysis. It starts with PCR-amplification of DNA templates from homozygous or heterozygous mutants and wild-type controls, which is followed by a denaturing and annealing step to produce heteroduplex and homoduplex species for temperature gradient capillary electrophoresis.

Materials:

1. DNA templates (25 to 40 ng/ì l): homozygous or heterozygous mutants, and wild type control.
2. A thermal cycler and PCR-related reagents with a proofreading polymerase (*e.g. Pfu* DNA polymerase from Stratagene, La Jolla, CA).
3. 1 X TE buffer: 10 mM Tris-Cl, 1 mM EDTA.

Procedure outline:

1. Perform PCR reactions as described by the thermocycler manufacturer.
   The following is a typical thermal cycle profile:
   a. Hold 2 min at 94 C.
   b. 30 cycles of 94 C 30 sec, 56 C 20 sec, 72 C 40 sec.
   c. Hold at 72 C 10 min.
   d. Hold at 4 C indefinitely.
2. Analyze PCR products to validate purity and concentration.
3. Determine dilution factors for heteroduplex analysis. Crude PCR products can be used without purification. Dilution buffer is recommended for dilution if necessary. Typically, 1:10-1:50 dilutions are recommended. The minimum detectable concentration for dsDNA using SpectruMedix screening gel is ~200 pg/ì l.
4. Prepare heteroduplex samples:
   a. For mutation heterozygotes, use PCR reactions directly.
   b. For mutation homozygotes, mix the mutant DNA with the wild type at a 1:1 ratio.
   c. Add a minimum of 4 ì l of the diluted PCR product into each well in a 96-well tray. Overlay each sample with ~10 ì l of mineral oil.
   d. Prepare heteroduplex samples in a thermal cycler. A typical temperature profile is described as follows:
      Hold 3 min at 95 C.
      Decrease from 95 C to 80 C at 3 C/min.
      Decrease from 80 C to 55 C at 1 C/min.
      Hold 20 min at 55 C.
      Decrease from 55 C to 45 C at 1 C/min.
      Decrease from 45 C to 25 C at 2 C/min.

Samples can be stored at 4°C for a week, or at room temperature for 24 hours. Samples can be injected up to five times.

Mutation screening using the Genetic Analysis System

In this procedure, samples prepared as above will be analyzed by SCE 9610. It includes several major steps: 1) filling capillaries with the mutation screening gel, 2) performing a prerun for 25 min to allow screening dye to mix with the gel matrix, 3) injecting samples for 30-60 sec at 5 kV, 4) running samples with a temperature gradient for 60-90 min, depending on the sizes of the DNA fragment tested.

Materials:

1. DNA sequencing gel matrix.
2. Heteroduplex and wild-type DNA samples
3. Mutation screening buffer.
4. Mutation standards.

Operation parameters (provided in method file):

1. Prerun: 25 min
2. Injection voltage: 5Kv.
3. Injection time: 60 sec.
4. Running voltage: 10Kv.
5. Electrophoresis time: 60 min (separation up to 600 bp). The time includes current monitoring time and data acquisition time.
6. Temperature ramp: 55-65 ^{0}C in 21 min.

SCE9610 operation procedure for mutation screening:

1. The power line to the front heater in the capillary cartridge has to be turned off or disconnected.
2. The running buffer trays at the sample injection end and the buffer reservoir at the gel injection end of the capillary array must contain mutation screening buffer.
3. The rest of the operation procedures are the same as DNA sequencing. Refer to the SCE9610 manual for details.

Establishing a new temperature ramping profile:

The standard temperature ramping and electrophoresis parameters are provided in the method file. If a user needs to change the running voltage, or temperature ramp, a new set of operation parameters should be established and a new running method file should be created. In order to generate reasonably accurate temperature ramping range and speed for all of the fragments in a sample tray, the configuration of the capillary cartridge layout has to be considered.

Fig. 3 depicts the layout of capillaries in a temperature-regulated cartridge. There are two sections of a capillary that contribute to the separation of samples prior to detector but are not temperature regulated, namely L_{inj} and L_{det} in Fig. 3. The temperature of these two sections of capillaries is in the vicinity of 35°C. By controlling the temperature of the heating box at 35°C and performing a constant temperature run for a set of different lengths of DNA fragments such as molecular ladders, one can calculate the time required for certain length of fragment to migrate through these two sections of capillaries. The data can then be used to calculate the time for a fragment to migrate through the temperature-controlled zone.

A temperature ramping profile is defined by the following parameters:

1. Electrophoresis running voltage,
2. Temperature ramping range (low temperature T_L to high temperature T_H),
3. The time to start ramping from T_L,
4. The rate to ramp to T_H.

Procedures to determine a temperature ramping profile:

1. Decide the running voltage.
2. Select a sample standard that includes fragments covering the size range of the samples to be analyzed. The sample standard can be molecular ladder, and/or SpectruMedix mutation standards. The fragment size envelope is from the smallest fragment F_S to the largest fragment F_L.

Figure 3

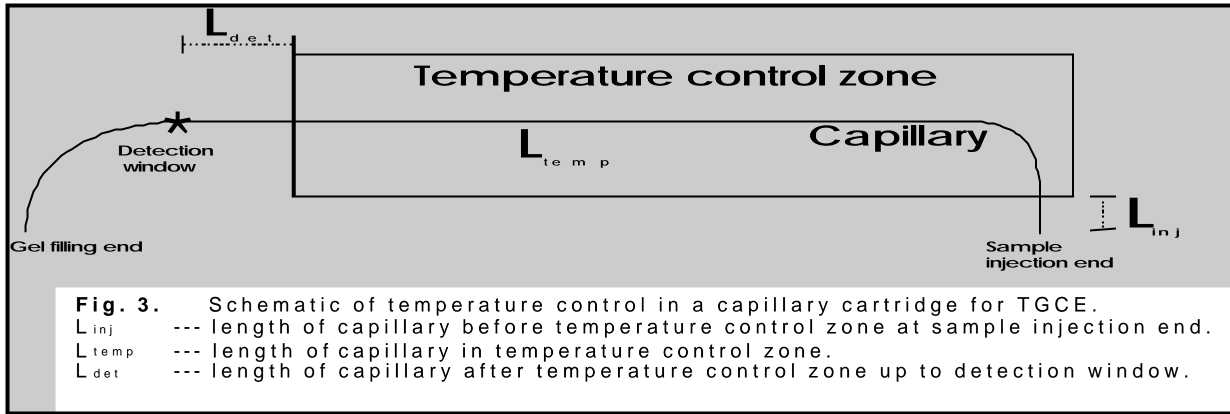

Fig. 3. Schematic of temperature control in a capillary cartridge for TGCE.
L_{inj} --- length of capillary before temperature control zone at sample injection end.
L_{temp} --- length of capillary in temperature control zone.
L_{det} --- length of capillary after temperature control zone up to detection window.

Procedure:

1. Run mutation standard or molecular ladder at 35°C. Obtain the migration time $t_{35C,\,FL}$ for the high end of the size envelop F_L fragment. The time to start the temperature ramping after sample injection, t_L, can be calculated as:

$$t_L = \frac{L_{inj}}{L} t_{35C,\,FL}$$

Where $L = L_{inj} + L_{temp} + L_{det}$. At this time, the largest fragment in the size envelope has entered the temperature-controlled zone.

2. Run mutation standard and/or molecular ladder at the high end of temperature ramp, T_H. Obtain the migration time for the low end of the size envelope F_S fragment. The latest time for the smallest fragment to exit the temperature controlled region during a temperature ramp t_H can be estimated as:

$$t_H = t_{TH,\,FL} - \frac{L_{det}}{L} t_{35C,\,FL}$$

This will be the time for the temperature to reach the high end of the temperature ramp, T_H.

3. The temperature ramping rate is then:

$$r = \frac{T_H - T_L}{t_H - t_L}$$

4. Since temperature ramping starts right after injection or before DNA samples enter the controlled-temperature zone, a broader ramp is required to compensate for the temperature ramping occurring in the zone of L_{inj} and ensure the estimated for the L_{temp} zone. Thus, the starting low end of the temperature ramp, , can be estimated as

$$T_L' = T_L - rt_L$$

where is the temperature increment required along L_{inj}.

Example

Assume that the sizes of the DNA samples range from 200 to 500 bp. Total length of the capillary for the Version 4 cartridge used on SCE 9610 is:

$$L = L_{inj} + L_{temp} + L_{det} = 4.5\ \text{cm} + 40.5\ \text{cm} + 10.0\ \text{cm} = 55.0\ \text{cm}$$

When run at 10 kV and 35 ^{0}C constant temperature (see above for other conditions), the migration time for 200- and 500-bp is 36 and 55 min, respectively. *The migration time is the time for a fragment to migrate to the detection window after electrophoresis starts. It includes the time for current monitoring, if any, and the time for data acquisition.* The time to start the temperature ramping for the controlled-temperature zone, t_L, can be estimated as:

$$t_L = \frac{L_{inj}}{L} t_{35C,\ FL} = \frac{4.5\ \text{cm}}{55.0\ \text{cm}} \text{ x } 55\ \text{min} = 4.5\ \text{min}$$

When run at 10 kV and 60 ^{0}C, the migration time for the 200-bp DNA fragment is 27 min. The time for the 200-bp fragment to exit the controlled-temperature zone can then be determined as:

$$t_H = t_{TH,\ FL} - \frac{L_{det}}{L} t_{35C,\ FL} = 27\text{min} - \frac{10.0\text{cm}}{55.0\text{cm}} \times 36\text{min} = 21\text{min}$$

The rate for temperature ramping from 57 to 65 ^{0}C is then estimated as:

$$r = \frac{T_H - T_L}{t_H - t_L} = \frac{65\ C - 57\ C}{21\ \text{min} - 4.5\ \text{min}} = 0.49\ C\ /\ \text{min}$$

Because the SCE9610 begins temperature ramping at sample injection stage, the actual starting temperature in the SCE9610 should be adjusted as

$$T_L' = T_L - rt_L = 57C - 0.49C/\text{min} \times 4.5\text{min} = 55C$$

In summary, the temperature ramping profile should be 55 ^{0}C to 65 ^{0}C ramp in 21 min.

SpectruMedix Parts and Supplies:

Part Number	**Description**
	Mutation Gel Syringe, 120 Ml mutation screening gel
	Mutation Gel Refill, 120 ml mutation screening gel refilled in syringe
	Mutation Capillary Array
	Mutation Capillary Refurbished, requires return of original array
	Mutation Screening Running Buffer, 500ml
	Mutation Standards:

This application note is provided for informational purposes only. Users should develop and test own methodology.