

Real-Time PCR*/Melt-Curve Analysis: SNP Detection with FRET

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Introduction

The detection of fluorescence resonance energy transfer (FRET) through real-time polymerase chain reaction (PCR) and melt-curve analysis is a powerful tool for the identification of single nucleotide polymorphisms (SNPs). The frequency of SNPs in association with disease makes the identification of specific sequence differences important. Through the use of real-time PCR and melt-curve analysis, genotyping can be done more rapidly than by the conventional method of restriction enzyme digestion of PCR products.

Several probe designs can be utilized for SNP detection including molecular beacons, TaqMan minor groove binder probes, and single-labeled probes. The technique described below involves the use of a Cy5-labeled reverse primer that is incorporated into the amplified PCR product. A sequence-specific 6-FAM-labeled probe, with the polymorphic nucleotide placed in the middle, hybridizes next to the Cy5 fluorophore in the PCR product. FRET occurs from FAM to Cy5 with excitation of the FAM fluorophore, and the iCycler iQ™ system detects the increase in emission signal of the Cy5 fluorophore.

Following amplification with the Cy5-labeled primer in the presence of a hybridization probe to the wild-type sequence, a melt-curve analysis is performed. Cy5 fluorescence is continuously monitored as the temperature is slowly increased. The Cy5 fluorescence decreases slowly with increasing temperature until the melting temperature of the probe-template hybrid is reached, at which time there is a more rapid decrease in the Cy5 fluorescence. This point at which there is a shift in the rate of decrease of Cy5 fluorescence can be more easily identified by viewing a plot of the first derivative of the fluorescence vs. temperature, i.e., a plot of the rate of change in fluorescence vs. temperature. The first-derivative plot is presented as the negative of the first derivative, so that the peaks have positive values. The point of maximum rate of change is considered the melting temperature of the probe-template hybrid.

Because the probe is an exact complement of the wild-type sequence, wild-type templates will form stronger hybrids with the probe and will consequently melt at a higher temperature than templates that contain a mutation. The distinctly different melting temperatures of the two possible hybrids allow identification of each unknown sample as mutant, wild type, or heterozygote (one allele of mutant and one allele of wild-type sequence).

Methods

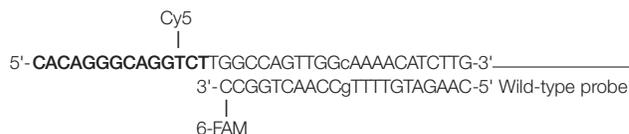
Primer and Probe Design

The forward (sense) and reverse (antisense) primer sequences were:

Forward: 5'-CGTCTGGGCTTCTTG-3'

Reverse: 5'-CACAGGGCAGGTCT-3'

The third nucleotide position from the 3' end of the reverse primer was labeled with Cy5. The probe sequence was 5'-CAAGATGTTTTgCCAAGTGGCC-3', with the single nucleotide polymorphism located in the middle (indicated by lowercase letter) and the 3' end labeled with 6-FAM. The wild-type probe complements the antisense strand and binds as indicated below. (The reverse primer sequence is indicated in heavier type.)



Real-Time PCR and Melt-Curve Analysis

Asymmetric PCR was performed to produce the Cy5 incorporated antisense target strand in excess for the FAM-labeled probe. PCR was performed with 50 µl reactions containing 100 nM forward primer, 500 nM reverse primer, and 200 nM FAM probe, 1 mM dNTPs, 3 mM MgCl₂, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1 µl Clontech KlenTaq LA polymerase. The DNA source was the *p53* dominant-negative vector set (Clontech), which provides vectors containing the wild-type *p53* suppressor gene and the dominant-negative *p53* mutant, differing only by a G/A transition at nucleotide 1,017. The experiments were done

with 5 replicates containing 10^6 copies of wild-type *p53* vector alone, *p53* dominant-negative mutant vector alone, or both in a 1:1 ratio, using the same primer set. A master mix was prepared containing buffer, primers, and probe, then aliquotted into 3 tubes containing the DNA samples. These 3 reaction mixes were then pipetted into the wells of a 96-well PCR plate.

PCR conditions were an initial denaturation step of 95°C for 3 min, followed by 45 repeats of 95°C for 10 sec and 50°C for 40 sec. A melt-curve protocol immediately followed amplification with 95°C for 1 min and 50°C for 1 min, followed by 70 repeats of heating for 10 sec, starting at 50°C with 0.5°C increments.

Results

Negative first-derivative melting curves were produced from the fluorescence vs. temperature plots of the products and probe (Figure 1). Probe binding to the *p53* wild-type target produced a single peak with a T_m of 67.5°C (Figure 2). With the *p53* dominant-negative mutant target, there was a 6°C shift in the T_m of the probe binding to target, giving a T_m of 61.5°C (Figure 3). The 1:1 heterozygous mixture produced 2 distinct peaks with T_m of 61.5°C and 67.5°C (Figure 4).

Discussion

These data demonstrate that melt-curve analysis combined with FRET chemistry can be used for SNP detection in wild-type and mutant sequences. This technique involved performing asymmetric PCR with a Cy5-labeled primer to produce the Cy5-labeled strand in excess. The wild-type, mutant, and heterozygous alleles were then detected with a single-labeled FAM probe that hybridized to the amplified Cy5-labeled target strands. Melt-curve analysis produced distinct T_m peaks for each type of sample. In this FRET experiment, excitation of the FAM fluorophore and detection of Cy5 emission were demonstrated. We have also demonstrated the use of other acceptor fluorophores such as Texas Red (data not shown). The iCycler iQ detection system's versatility allows detection with a wide range of fluorophores, and melt-curve analysis following amplification is an effective tool for SNP discrimination.

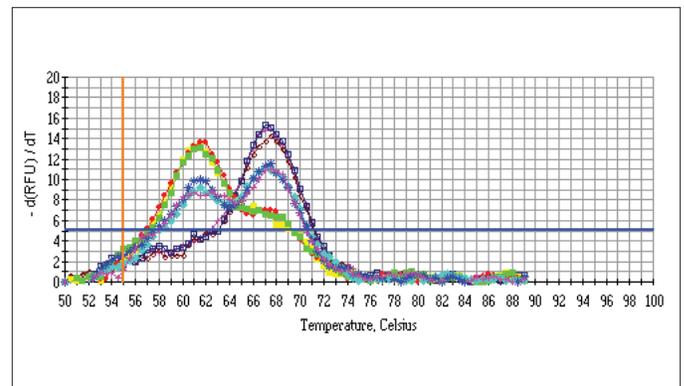


Fig. 1. Melt-curve plot of all samples.

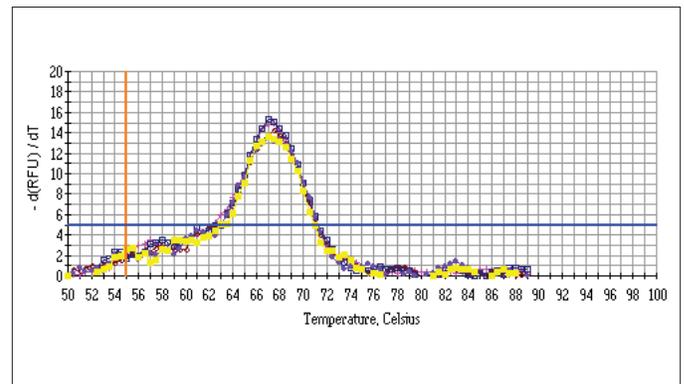


Fig. 2. Melt-curve plot of probe binding to *p53* wild-type target.

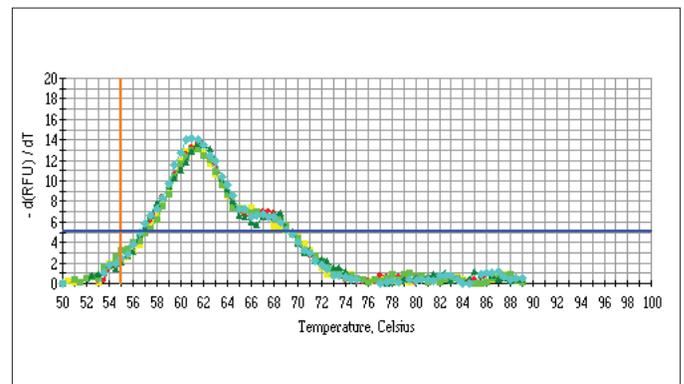


Fig. 3. Melt-curve plot of probe binding to *p53* dominant-negative mutant target.

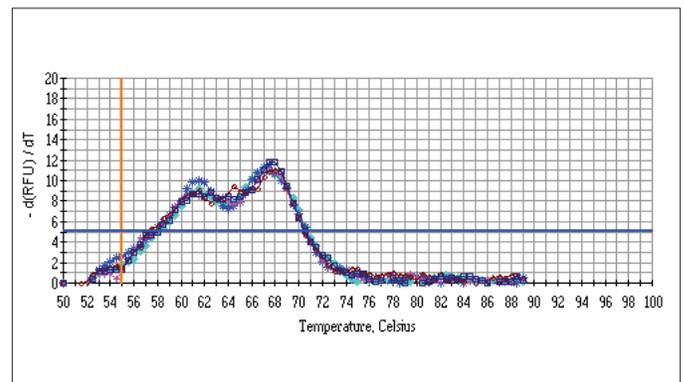


Fig. 4. Melt-curve plot of probe binding to a 1:1 mixture of both wild-type and mutant *p53* alleles.

Appendix: SNP FRET Detection with the iCycler iQ System

Fluorophore Selection

Similar FRET experiments have been demonstrated on the Roche LightCycler system using FAM-LC640 and FAM-LC705 fluorophore combinations. LightCycler Red 640 (LC640) has an excitation maximum at 625 nm and an emission maximum at 640 nm. LightCycler Red 705 (LC705) has an excitation maximum at 685 nm and an emission maximum at 705 nm. In FRET experiments, these fluorophores are excited with the emission signal of 6-FAM, which is excited at 492 nm and emits at 518 nm. These fluorophores may be used on the iCycler iQ system, but we do not recommend them because of their physical properties. They do not typically yield high levels of fluorescence and there are better alternatives. Both FAM-Texas Red and FAM-Cy5 fluorophore combinations can be used instead of the FAM-LightCycler dyes, and both of these combinations produce stronger signals than the LightCycler dyes. Texas Red is excited at 583 nm and emits at 603 nm. Cy5 is excited at 643 nm and emits at 667 nm. These dyes are easily obtainable and are commonly used for dual-labeled probes and molecular beacons.

Filter Positioning for FRET Experiments in the iCycler iQ System

FRET experiments require excitation with a FAM filter (490/20X) and emission with either a Texas Red (620/30M) or Cy5 (680/30M) filter. Home the filter wheels before changing filters.

- FAM-Texas Red filter pair

Remove FAM excitation filter (490/20X) from position 2 and replace with blank.

Move filter wheel to position 5 and replace the Texas Red excitation filter (575/30X) with the FAM excitation filter (490/20X).

- FAM-Cy5 filter pair

Remove FAM excitation filter (490/20X) from position 2 and replace with blank.

Move filter wheel to position 6 and replace Cy5 excitation filter (635/30X) with the FAM excitation filter (490/20X).

Plate Setup for FRET Experiments

In the plate setup, wells should be labeled with the acceptor fluorophore, so that the filters in position 5 will be used to detect the Texas Red signal and the filters in position 6 will be used to detect the Cy5 signal. Although the FAM excitation filter is being used, the data displayed are from the Texas Red or Cy5 emission signal.

Calibrating for FRET

Most FRET experiments require only single-color detection. For example, either the Texas Red or Cy5 signal will be detected. The dye calibration should be done beforehand

for these fluorophores with the filters in the original positions and with the iCycler iQ calibrator solutions. It is not necessary to calibrate for the Texas Red or Cy5 fluorophores with the FAM-Texas Red or FAM-Cy5 filter pair.

Well Factors for FRET Experiments

It is necessary to use external well factors for FRET experiments due to the nature of the probe (labeled with one fluorophore) and the unusual filter combination. The iCycler iQ 10x external well factor solution can be diluted to 1x and used for FAM-Texas Red combinations, but must be supplemented with ethidium bromide for use with FAM-Cy5, FAM-LC640, or FAM-LC705 combinations. For these latter three combinations, it is simpler and as effective to supplement PCR buffer (10 mM Tris, pH 8, 50 mM KCl, 3 mM MgCl₂) with ethidium bromide (see below) in place of the 1x well factor solution. This is necessary because the well factor solution is optimized for the traditional filter set pairs. The combination of a FAM excitation filter and a Cy5 or LightCycler emitter requires a solution that can be excited over a wider range of wavelengths.

Option 1. Using the iCycler iQ external well factor solution:

- For FAM-Cy5 filter combination, add 100 µl of ethidium bromide (10 mg/ml stock) to 0.9 ml of the 10x external well factor solution.

Dilute to 1x for a final ethidium bromide concentration of 100 µg/ml.

- For FAM-Texas Red filter combination, dilute the 10x external well factor solution to 1x as usual.

Option 2. Using PCR buffer:

- Add 15 µl of ethidium bromide (10 mg/ml stock) to 5 ml of PCR buffer for a final concentration of 300 µg/ml.

This mixture can be used for FAM-Texas Red and FAM-Cy5 experiments.

Use gloves when handling ethidium bromide and dispose of both properly.

Bibliography

Lyon E et al., Detection and identification of base alterations within the region of factor V Leiden by fluorescent melting curves, *Mol Diagn* 3, 203–209 (1998)

Neoh SH et al., Rapid detection of the factor V Leiden (1691 G → A) and haemochromatosis (845 G → A) mutation by fluorescence resonance energy transfer (FRET) and real time PCR, *J Clin Pathol* 52, 766–769 (1999)

- * Practice of the patented polymerase chain reaction (PCR) process requires a license. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from Applied Biosystems. Its use with authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may require licenses from other parties.

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